Identification and Synthesis of a Receptor Binding Site of Human Anaphylatoxin C5a

Megumi Kawai,* David A. Quincy, Benjamin Lane, Karl W. Mollison, Jay R. Luly, and George W. Carter

Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60064. *Received July 5, 1990*

C5a is a 74 amino acid polypeptide that likely plays an important role in the pathogenesis of a number of inflammatory diseases. Therefore, the discovery of a C5a antagonist is of considerable therapeutic interest. A series of peptides designed to survey various regions of the molecule was synthesized by solid-phase peptide synthesis and evaluated for receptor-binding activity with polymorphonuclear leukocyte membranes. The C-terminal octapeptide (Ac-His-Lys-Asp-Met-Gln-Leu-Gly-Arg-OH) was identified as the smallest fragment which possessed reasonable C5a receptor binding activity.

Introduction

Human C5a is a single polypeptide chain that is liberated from the fifth component of complement (C5) during complement activation. It consists of 74 amino acids with an N-linked carbohydrate moiety attached at Asn 64, which contributes 2700 g/mol to the overall molecular weight of ca. 11 000 g/mol.[1,2] C5a, the most potent of the complement-derived anaphylatoxins, expresses several important biological activities[2] including secretion of granular enzymes from polymorphonuclear leukocyte (PMNL), contraction of smooth muscle, degranulation of mast cells, chemotaxis of PMNL and monocytes, and generation of cytotoxic oxygen radicals from both PMNL and macrophages. Although C5a affects the function of several different cell types, its most important target appears to be the PMNL, a cell that plays a central role in a number of inflammatory processes. Available evidence suggests that a C5a antagonist would have significant inhibitory effects on these events, by preventing the deleterious effects of C5a without affecting other activities of the complement system. Therefore, the goal of the present studies was to identify a small fragment of C5a that was capable of binding to the PMNL receptors and then to use this information to develop a specific antagonist of human C5a. The proposed secondary structure of human C5a and its primary sequence are shown in Figures 1 and 2, respectively. The biological properties of human C5a have recently been reviewed by Yancey[3] and Goldstein.[4] Unlike other peptide hormones, C5a is a relatively large molecule with an attached carbohydrate moiety. Experimental data suggest that the carbohydrate moiety is not necessary to maintain full biological activity.[5] However, the carbohydrate moiety has been reported to act as an important factor to maintain full activity when C5a is converted to C5a des-Arg[74] by carboxypeptidase N or arginine specific carboxypeptidase.[6,7]

The interaction of C5a and C5a fragments with PMNL receptors has been reported by several groups. With enzymatic degradation, it was speculated that N-terminal residues play an important role in the receptor binding of C5a, possibly by stabilizing the conformation of the C-terminus.[8] The disulfide-linked core region as well as the C-terminal region have also been implicated in receptor binding by studies of site-directed mutagenesis,[9] enzymatic degradation, and by using anti-human C5a monoclonal antibodies.[10] Early structure-activity work also demonstrated that C5a potency declines with enzymatic removal of the carboxy-terminal arginine and to an even greater extent with truncation of the last five amino acid residues.[11] This might suggest that there is a receptor recognition site at the carboxyl terminal area. However, when 11 amino acids were deleted by enzymatic degradation, reasonable binding potency was observed with C5a (1–63) (K_i = 1 μM), suggestting that elements important for receptor binding could be in the core and/or N-terminal regions.[12] This speculation was supported by molecular modeling studies of C5a, which indicated that possible receptor binding sites involve residues 3–5 and 7–13 of the N-terminal region and 19–25 of the disulfide-linked core.[13] As yet, however, the receptor binding domains on C5a are not well understood.

C3a is another anaphylatoxin that has been investigated in detail. It has been shown that a carboxyl terminal peptide consisting of the last 21 residues of C3a, when compared on a molar basis, can essentially account for the spasmogenic activity of C3a.[15] Recently, smaller peptides

(1) Abbreviations follow IUPAC–IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides: *Eur. J. Biochem.* **1984**, *158*, 9. Additional abbreviations used herein are as follows: Acm, acetamidomethyl; Ac, acetyl.

(2) (a) Fernandez, H. N.; Hugli, T. E. *J. Biol. Chem.* 1978, *253*, 6955. (b) Fernandez, H. N.; Hugli, T. E. *J. Immunol.* 1976, *117*, 1688. (c) Hugli, T. E.; Muller-Eberhard, H. J. *Adv. Immunol.* 1978, *26*, 1. (d) Hugli, T. E. *Crit. Rev. Immunol.* 1981, *1*, 321. (e) Weigle, W. O.; Goodman, M. G.; Morgan, E. L.; Hugli, T. E. *Springer Semin. Immunopathol.* 1983, *6*, 173.

(3) Yancey, K. B. *Clin. Exp. Immunol.* 1988, *71*, 207.

(4) Goldstein, I. M. *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., Goldstein, I. M., and Snyderman, R., Eds.; Raven Press Ltd.: New York, 1988; p 55.

(5) (a) Gerard, C.; Hugli, T. E. *Proc. Natl. Acad. Sci., U.S.A.* 1981, *78*, 1833. (b) Gerard, C.; Chenoweth, D. E.; Hugli, T. E. *J. Immunol.* 1981, *127*, 1978.

(6) Perez, H. D.; Chenoweth, D. E.; Goldstein, I. M. *J. Clin. Invest.* 1986, *78*, 1589.

(7) Campbell, W.; Okada, H. *Biochem. Biophys. Res. Commun.* 1989, *162*, 933.

(8) (a) Damerau, B.; Zimmerman, B.; Czarniak, K.; Wustefeld, H.; Vogt, W. *Mol. Immunol.* 1986, *23*, 433. (b) Gerard, C.; Showell, H. J.; Hoeprich, P. D., Jr.; Hugli, T. E.; Stimler, N. P. *J. Biol. Chem.* 1985, *260*, 2613.

(9) Mollison, K. W.; Mandecki, W.; Zuiderweg, E.; Fayer, L.; Fey, T. A.; Krause, R. A.; Conway, R. G.; Miller, L.; Edalji, R. P.; Shallcross, M. A.; Lane, B.; Fox, J. L.; Greer, J.; Carter, G. W. *Proc. Natl. Acad. Sci. U.S.A.* 1989, *86*, 292.

(10) (a) Johnson, R. J.; Tomerius, J. D.; Chenoweth, D. E. *J. Immunol.* 1987, *138*, 3856. (b) Hahn, G. S. *U.S. Patent* 4,692,511, 1987.

(11) Gerard, C.; Chenoweth, D. E.; Hugli, T. E. *J. Reticuleoendothel. Soc.* 1979, *26*, 711.

(12) Edalji, R. P.; Mollison, K. W.; Zuiderweg, E. R. P.; Fey, T. A.; Krause, R. A.; Conway, R. G.; Miller, L.; Lane, B.; Henkin, J.; Greer, J.; Carter, G. W. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 1987, *46*, 980.

(13) Greer, J. *Science* 1985, *228*, 1055.

(14) (a) Zuiderweg, E. R. P.; Mollison, K. W.; Henkin, J.; Carter, G. W. *Biochemistry* 1988, *27*, 3568. (b) Zuiderweg, E. R. P.; Henkin, J.; Mollison, K. W.; Carter, G. W.; Greer, J. *Proteins Struct. Funct. Genet.* 1988, *3*, 139. (c) Zuiderweg, E. R. P.; Nettesheim, D. G.; Mollison, K. W.; Carter, G. W. *Biochemistry* 1989, *28*, 172.

0022-2623/91/1834-2068$02.50/0

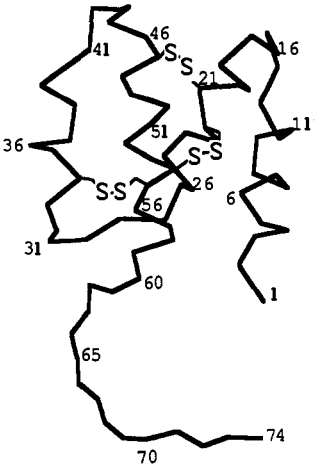

Figure 1. Model structure for C5a.[13]

5 10

Thr- Leu- Gln- Lys- Lys- Ile- Glu- Glu- Ile- Ala-

Ala- Lys- Tyr- Lys- His- Ser- Val- Val- Lys- Lys-

Cys- Cys- Tyr- Asp- Gly- Ala- Cys- Val- Asn- Asn-

Asp- Glu- Thr- Cys- Glu- Gln- Arg- Ala- Ala- Arg-

Ile- Ser- Leu- Gly- Pro- Arg- Cys- Ile- Lys- Ala-

Phe- Thr- Glu- Cys- Cys- Val- Val- Ala- Ser- Gln-

Leu- Arg- Ala- Asn- Ile- Ser- His- Lys- Asp- Met-

Gln- Leu- Gly- Arg[74]

Figure 2. Human C5a primary sequence.

corresponding to the C-terminal region of C3a, although having slightly weaker binding potencies than C3a, have been shown to possess full biological activity.[16]

Synthetic approaches to identify a receptor binding site on C5a have been initiated. It has been reported that the C-terminal pentapeptide of human C5a (H-Met-Gln-Leu-Gly-Arg-OH) has no chemotactic activity for human neutrophils, but that N-formylated analogues display such activity.[17] Although designed from the C-terminal structure of the human C5a molecule, these active peptides interact with the *N*-formyl peptide receptor rather than the C5a receptor. However, Tyr-C5a(55–74), a 21-residue peptide which corresponds to the C-terminal segment of human C5a (20 residues) proceded by a Tyr residue, was shown to exhibit spasmogenic activity against the guinea pig ileum at concentrations as low as 4–6 μM and aggregated guinea pig platelet at 130–260 μM.[18] It was also noted that this synthetic peptide did not induce a chemotactic response in human neutrophils. Thus, it was speculated that the spasmogenic and chemotactic activities of C5a may be separate and distinct functions of this complement factor.[18]

Table I. Inhibition of [^{125}I]C5a Receptor Binding by Synthetic Derivatives Representing Various Regions of C5a

compd	segment	modifications	PMNL membranes	intact PMNL
1	1–14	none	inactive	inactive
2	1–26	Cys^{6}-S-S-Cys^{22},Ala^{21}	inactive	inactive
3	10–20	*N*-Ac	inactive	inactive
4	10–19	Cys^{10}-S-S-Cys^{19}	inactive	inactive
5	14–20	none	inactive	inactive
6	16–26	Ala^{21},Cys(Acm)^{22}	inactive	inactive
7	15–32	Ala^{21,22,27}	inactive	inactive
8	25–35	Cys(Acm)^{25,34},Met^{27}	inactive	inactive
9	33–43	*N*-Ac,Cys(Acm)^{34}	inactive	inactive
10	46–62	Cys(Acm)^{47,54}Ala^{55}	inactive	inactive
11	38–49	Cys(Acm)^{38,48},Met^{47}	inactive	inactive
12	55–62	Ala^{55}	inactive	inactive
13	59–74	none	active[a]	inactive

[a] Greater than 20% inhibition of binding at concentrations of 0.1–1 mM.

The secondary structure of human C5a, determined by comparative computer modeling on the basis of the crystal structure of C3a and by proton NMR has been reported.[13,14] Parallel to these spectroscopic and computer modeling approaches to evaluate its structure, we developed a synthetic approach to identify receptor binding sites of human C5a. We report here the synthesis and biological results of a series of peptides which was prepared by solid-phase peptide synthesis and designed to survey various regions of C5a in order to identify a fragment with reasonable affinity for the C5a receptor.

Results and Discussion

Synthesis of C5a Fragments. The C5a analogues shown in Tables I and II were synthesized by solid-phase peptide synthesis. The substitution of Boc-amino acid resins used herein was in the range of 0.2–0.6 mequiv/mol. The Kaiser test was used to monitor coupling reactions.[19] Peptides were constructed on the resin, treated with anhydrous hydrogen fluoride, and then lyophilized. The crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC).

Competitive Binding Results. A series of peptides corresponding to various regions of C5a were tested for C5a receptor binding activity and the results are summarized in Table I. It has been proposed that the regions which have some loop structure, stabilized by either a disulfide bridge, a helix–helix interaction, or by an antiparallel sheet interaction, could be a receptor recognition site.[20] Important functional groups and/or hydrophobic residues are likely to be on the surface of the C5a molecule, since these residues would be expected to be able to interact with a receptor without causing a vigorous conformational change of the ligand. With the proposed modeling structure,[13] shown in Figure 1, which is supported by NMR data,[14] we focused on regions which might be exposed. The locations of these peptides within the predicted secondary structure of human C5a are residues 10–20, 25–35, and 38–49. Thus, compounds 3, 8, and 11 were prepared. As shown in Table I, none showed inhibition of C5a binding to PMNL membranes or intact cells. Next, we considered that an α-helical

(15) Hoeprich, P. D., Jr.; Hugli, T. E. *Biochemistry* **1986**, *25*, 1945.
(16) (a) Gier, M.; Ambrosius, D.; Casaretto, M.; Gilge, G.; Saunders, D.; Brandenburg, D. *11th Am. Pept. Sympos.* **1989**, 120. (b) Gerardy-Schahn, R.; Ambrosius, D.; Casaretto, M.; Grotzinger, J.; Saunders, D.; Wollmer, A.; Brandenburg, D.; Bitter-Suermann, D. *Biochem. J.* **1988**, *255*, 209. (c) Ambrosius, D.; Casaretto, M.; Gerardy-Schahn, R.; Saunders, D.; Brandenburg, D.; Zahn, H. *Biol. Chem. Hoppe-Seyler* **1989**, *370*, 217.
(17) Chenoweth, D. E.; Erickson, B. W.; Hugli, T. E. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 227.
(18) Kahn, S. A.; Erickson, B. W.; Kawahara, M. S.; Hugli, T. E. *Complement* **1985**, *2*, 42.
(19) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
(20) For examples, see: (a) Atassi, M. Z. *Eur. J. Biochem.* **1984**, *145*, 1. (b) Schulz, G. E. *Angew. Chem. Int. Ed. Engl.* **1977**, *16*, 23.

Table II. Inhibitions of [^{125}I]C5a Receptor Binding by Synthetic C5a Derivatives of the Carboxyl-Terminal Region

		inhibn of [^{125}I]C5a binding	
		PMNL membranes[a]	intact PMNL
14	AcMIKAFTEMMVVASQLRANISHKDMQLGR	0.1[b]	active[c]
15	FTEMMVVASQLRANISHKDMQLGR	0.1[b]	active[c]
16	Ac-MVVASQLRANISHKDMQLGR	0.05	inactive
13	SQLRANISHKDMQLGR	0.3	inactive
17	Ac-ISHKDMQLRGR	0.1	inactive
18	ISHKDMQLGR	0.3	inactive
19	Ac-HKDMQLGR	0.15	inactive
20	HKDMQLGR	0.3	inactive
21	Ac-KDMQLGR	30% a 1 mM	inactive
22	Ac-DMQLGR	inactive	inactive

[a] Apparent K_i in mM. [b] Peptides were poorly soluble in aqueous solutions. [c] Greater than 20% inhibition of binding at concentrations of 0.1–1 mM.

region could be a receptor recognition site, since a number of amphiphilic helical structures have been reported to have a high affinity for their receptors.[21] In fact, an enhancement of the α-helicity of a C3a fragment dramatically increased its affinity for the C3a receptor.[15] Computer modeling and NMR studies[13,14] suggest four α-helical structures in C5a, i.e. residues 3–10 (tested with compounds 1 and 2), 16–26 (6 and 7), 33–43 (9), and 59–74 (13). On the basis of NMR experiments, it must be emphasized that the carboxyl-terminal eight amino acids are disordered in solution.[14] Although none of the analogues in Table I possess measurable binding potency in whole cells, the 16 amino acid peptide analogue of the C-terminus 13 displayed weak binding to PMNL membranes. Consequently, we turned our attention to C-terminal analogues (Table II). Since the observed K_i for the C-terminal 16-mer 13 was in the range of 0.2–0.6 mM, larger peptides 14–16 were synthesized to see whether additional amino acids or peptide fragments might increase binding potency. As shown in Table II, these larger analogues were only slightly more potent.

To minimize the size of the peptide, analogues equal to or greater than seven residues in length were synthesized, tested, and found to have weak but measurable inhibitory activities against C5a binding to human neutrophil membranes. It should be noted that the two different binding assays differ in sensitivity. Only two larger peptides (14 and 15) significantly inhibited binding of [I^{125}]C5a to intact PMNL at 1×10^{-4} M, while the rest of the peptides are inactive in this assay even though many have inhibitory activity in the membrane assay. The difference in activity of peptides in the membrane and intact cell binding assays was likely due to the difference in the binding affinity of C5a for these tissues. C5a bound to the PMNL membranes and intact cells with average dissociation constants of 0.08 and 0.5 nM, respectively. It is likely that competitive binders for the C5a receptor would demonstrate similar relative potencies in competition for C5a binding to the membranes and cells. Thus, the absolute peptide concentration needed for inhibiton of C5a binding to intact cells would likely be higher than the concentrations needed to inhibit C5a binding to the membranes. A similar absolute difference in potency has been previously reported for binding inhibitory activity of mutant C5a molecules.

Generally, to identify a receptor binding domain in a large molecule, such as C5a, is quite a challenge. Moreover, previous data have suggested that C5a may have several discontinuous binding sites, potentially involving not only the carboxyl terminus[11,23] but also the disulfide-linked core[9,13,24] and the amino terminus as well.[8] The fact that a C5a C-terminal octapeptide (residues 67–74, 19 or 20) was found to have binding activity and the observation that significant activity was retained in the C-terminus truncated C5a molecule (residues 1–63)[12] are both consistent with the existence of two or more receptor binding domains. Although several other regions of C5a have been proposed to interact with the receptor, analogues spanning these regions were inactive. If binding regions are indeed discontinuous and scattered throughout C5a, it could be very difficult to identify an active fragment that is a much smaller molecule than 19.

In summary, several peptides corresponding to various C5a regions have been synthesized in order to identify a small compound capable of binding with the receptor. In this investigation, the carboxyl-terminal octapeptides 19/20 were identified as a minimal active sequence. Extensive studies are in progress to further assess the structure–activity relationships of these octapeptides.

Experimental Section

Synthesis. The synthesis of C5a fragments was performed on a Biosearch 9500ex and/or SAM 2 peptide synthesizers using Merrifield resin. The TFA (trifluoroacetic acid)–Boc (*tert*-butyloxycarbonyl) protecting scheme was used. Side-chain protection was accomplished with benzyl-type protecting groups (Ser, Thr, Tyr, Cys, Asp, Glu), acetamido (Cys), 2-chlorobenzyloxycarbonyl (Lys), or tosyl (His, Arg). For chain elongation, DIPCDI (*N*,*N*′-diisopropylcarbodiimide) was employed, except DIPCDI-HOBt (*N*-hydroxybenzotriazole monohydrade) was used during Bos-Asn-OH and Boc-Gln-OH couplings. Some peptides were N-acylated with acetic anhydride or acetyl chloride and a catalytic amount of DMAP [1,4-(*N*,*N*-dimethylamino)pyridine]. The peptide was isolated from the resin by treatment with liquid HF (0 °C, 45–60 min, 10% anisole with or without ethanedithiol), extraction with 20% aqueous acetic acid solution, lyophilization, and purification by RP-HPLC (21.4 mm i.d. × 25 cm, C18, 8 μm silica, Dynamax preparative HPLC, Rainin). The peptides were typically eluted with a gradient from 20% solution A (solution A, water with 0.1% TFA) to 60% solution B (solution B, acetonitrile/water/TFA = 80:20:0.1%) during a 30-min period. Typical flow rate used were 12.8–15 mL/min. The cyclization of the peptides containing free SH groups was achieved by the following procedure. The crude peptide, isolated from resins by treatment with liquid HF, was extracted with degassed 20% aqueous acetic acid (20–40 mL) and was then diluted with degassed distilled water (peptide concentrations were in the range of $2–5 \times 10^{-5}$ M). The

(21) For examples, see: Kaiser, E. T. *Biochem. Pharmacol.* 1987, *36*, 783 and the references herein.
(22) Lane, B.; et al. Unpublished result.
(23) (a) Fernandez, H. N.; Henson, P. M.; Otani, O.; Hugli, T. E. *J. Immunol.* 1978, *120*, 109. (b) Chenoweth, D. E.; Hugli, T. E. *Proc. Natl. Acad. Sci. U.S.A.* 1978, *75*, 3943.
(24) (a) Muto, Y.; Fukumoto, Y.; Arata, Y. *Biochemistry* 1985, *24*, 6659. (b) Huber, R.; Scholze, H.; Paques, E. P.; Deisenhofer, J. *Hoppe-Seyler's Z. Physiol. Chem.* 1980, *361*, 1389. (c) Harting, H. P.; Bitter-Suermann, D.; Hadding, U. *Agents Actions* 1984, *15*, 14.

Table III. Characterization Data of the C5a Fragments

no.	FAB-MS calcd	FAB-MS found	amino acid analysis
1	1661	1663[b]	Ala 2.1 (2), Glx 3.4 (3), Ile 1.9 (2), Leu 1.1 (1), Lys 4.0 (4), Thr 1.0 (1), Tyr 1.0 (1)
2	2910	a	Ala 4.0 (4), Asx 1.0 (1), Cys 1.4 (2), Gly 1.0 (1), Glx 3.1 (3), His 1.0 (1), Ile 1.0 (1), Leu 1.0 (1), Lys 5.7 (6), Ser 1.0 (1), Thr 1.0 (1), Tyr 1.8 (2), Val 1.6 (2)
3	1299	1300	Ala 2.0 (2), His 1.0 (1), Lys 4.3 (4), Ser 0.9 (1), Tyr 1.0 (1), Val 1.8 (2)
4	1134	1135	Ala 1.0 (1), Cys 1.3 (2), His 1.0 (1), Lys 2.0 (2), Ser 0.5 (1), Tyr 0.9 (1), Val 2.1 (2)
5	824	825	His 1.0 (1), Lys 3.0 (3), Ser 0.8 (1), Val 1.9 (2)
6	1210	1211	Ala 2.0 (2), Asp 1.0 (1), Gly 0.9 (1), Lys 2.1 (2), Ser 0.9 (1), Tyr 1.2 (1), Val 1.7 (2), Cys[c]
7	1792	1793	Ala 4.1 (4), Asx 3.9 (4), Glx 1.1 (1), Gly 1.0 (1), His 0.9 (1), Lys 1.9 (2), Ser 0.7 (1), Tyr 1.0 (1), Val 2.7 (3)
8	1369	1370	Ala 1.0 (1), Asx 3.0 (3), Glx 2.0 (2), Met 1.0 (1), Thr 0.8 (1), Val 1.0 (1), Cys[c]
9	1359	1360	Ala 2.0 (2), Arg 2.1 (2), Glx 2.1 (2), Ile 1.1 (1), Leu 1.2 (1), Ser 0.9 (1), Thr 0.7 (1), Cys[c]
10	2036	2037	Ala 2.9 (3), Arg 2.0 (2), Glx 2.1 (2), Ile 0.9 (1), Leu 1.1 (1), Lys 1.0 (1), Phe 0.9 (1), Ser 0.9 (1), Thr 1.0 (1), Val 1.6 (2), Cys[c]
11	1475	1476	Ala 1.0 (1), Arg 2.0 (2), Gly 1.0 (1), Ile 1.0 (1), Leu 1.0 (1), Lys 1.0 (1), Met 1.1 (1), Pro 1.0 (1), Ser 0.6 (1), Cys[c]
12	842	843	Ala 1.8 (2), Arg 1.0 (1), Glx 1.0 (1), Leu 1.0 (1), Ser 0.9 (1), Val 1.4 (2)
13	1854	1855	Ala 1.0 (1), Arg 2.1 (2), Asx 2.2 (2), Glx 2.0 (2), Gly 1.0 (1), His 1.0 (1), Ile 0.9 (1), Leu 1.9 (2), Lys 0.9 (1), Met 1.1 (1), Ser 1.7 (2)
14	3247	a	Ala 3.2 (3), Asx 1.9 (2), Arg 2.2 (2), Glx 3.1 (3), Gly 1.0 (1), His 0.9 (1), Ile 2.1 (2), Leu 2.2 (2), Lys 2.2 (2), Met 3.2 (3), Phe 1.1 (1), Ser 1.6 (2), Thr 0.9 (1), Val 1.9 (2)
15	2762	2763	Ala 1.9 (2), Arg 2.0 (2), Asx 1.7 (2), Glx 2.8 (3), Gly 0.9 (1), His 0.8 (1), Ile 1.1 (1), Leu 2.0 (2), Lys 1.0 (1), Met 2.9 (3), Phe 1.0 (1), Ser 1.7 (2), Thr 0.8 (1), Val 1.7 (2)
16	2295	2297[b]	Ala 2.0 (2), Arg 2.0 (2), Asx 1.9 (2), His 1.0 (1), Glx 1.7 (2), Gly 0.9 (1), Ile 1.0 (1), Leu 2.1 (2), Lys 1.0 (1), Met 1.8 (2), Ser 1.0 (1), Val 1.8 (2)
17	1225	1226	Asx 1.0 (1), Arg 1.0 (1), Glx 1.0 (1), Gly 1.0 (1), Ile 0.9 (1), Leu 1.0 (1), His 1.0 (1), Lys 1.0 (1), Met 1.0 (1), Ser 0.7 (1)
18	1183	1184	Asx 1.0 (1), Arg 1.0 (1), Glx 1.0 (1), Gly 1.0 (1), Ile 0.9 (1), Leu 1.1 (1), His 1.0 (1), Lys 1.0 (1), Met 1.0 (1), Ser 0.6 (1)
19	1025	1026	Asx 1.0 (1), Glx 1.0 (1), Gly 0.9 (1), Met 1.1 (1), Leu 1.0 (1), His 1.0 (1), Lys 0.9 (1), Arg 1.0 (1)
20	983	984	Asx 1.0 (1), Glx 1.1 (1), Gly 0.9 (1), Met 1.0 (1), Leu 1.1 (1), His 1.0 (1), Lys 0.8 (1), Arg 1.0 (1)
21	888	889	Asx 1.0 (1), Glx 1.0 (1), Gly 0.9 (1), Met 1.0 (1), Leu 1.0 (1), Lys 1.1 (1), Arg 1.0 (1)
22	760	761	Asx 1.0 (1), Glx 1.0 (1), Gly 1.0 (1), Met 1.1 (1), Leu 1.0 (1), Arg 1.0 (1)

[a] Calculated FAB-MS relatively high, so observed M + 1 ions were not clearly detected. [b] Observed numbers are consistent for the A + 1 isotope for the $[M + H]^+$ of the desired product. [c] Cys residues were not well determined due to the protecting group (Acm).

pH of the solution was adjusted to 8.0 by the addition of concentrated ammonium hydroxide. Potassium ferricyanide aqueous solution (0.01 N) was added dropwise to the stirred solution until a yellow color appeared. The resultant solution was stirred for an additional 30 min at room temperature, and the pH was adjusted to 5.0 with glacial acetic acid. Packed Bio-Rad anion-exchange resin AGX-4 (Cl form) was added, stirred for 30 min, and filtered. The filtrate was directly applied to a column containing XAD-16 molecular adsorbent resin. The sample was desalted by first washing the column with distilled water and then eluting from the column with 50% aqueous ethanol. The ethanol fractions were concentrated to ca. 100 mL in vacuo and lyophilized to a dried powder. The purified peptides were recovered by lyophilization of the RP-HPLC fractions. All products were at least 95% pure and gave NMR and mass spectra consistent with the proposed structure. Characterization data are summarized in Table III.

Receptor Binding Assay. PMNL Membranes. C5a receptor binding affinity of peptides was determined by competing for binding of radioiodinated C5a to human PMNL membranes. Human PMNL membranes were isolated following cell lysis by nitrogen cavitation and Percoll density-gradient centrifugation.[25] Radiolabeled C5a was prepared by glucose oxidase-lactoperoxidase catalyzed radioiodination[26] and the product was affinity purified with a goat anti-human C5a resin.[27] Binding was performed in buffered balanced salts solution (pH 7.0) containing 0.25% gelatin, a cocktail of protease inhibitors, 50–250 pM [^{125}I]C5a, and 5–20 μg/mL PMNL membranes. Peptides were initially solubilized in binding buffer at concentrations up to 4 mM. Dilutions of peptides in buffer which demonstrated visible signs of insolubility were tested without further treatment. Thus, the activity of these poorly soluble peptides may have been underestimated. Samples were incubated 60 min at ambient temperature and membrane-bound C5a collected by filtration onto Millipore HVLP filters. The inhibitor concentration that displaced [^{125}I]C5a binding by 50% was determined by linear-regression analysis of the data and an apparent inhibition constant (K_i) was calculated by the method of Cheng and Prusoff.[28] The data represents the average of the results from at least two separate tests performed in triplicate.

Intact PMNL. For measurement of C5a radioligand binding to intact cells, human PMNL were purified from peripheral blood samples by a one-step density-centrifugation technique using Mono-Poly Resolving Medium (Flow Laboratories). Radioiodonated C5a was prepared as above. To determine the ability of synthetic peptides to compete for C5a binding, PMNL were incubated for 20 min at 23 °C with [^{125}I]C5a (1×10^{-9} M) and the test agent. The reaction mixture included a cocktail of protease inhibitors to minimize possible degradation of the test substances. Following the addition of silicone oil, cells were separated from free ligand by centrifugation at 10000*g* and the pellets recovered for counting of γ emissions.[29]

Acknowledgment. We thank Mr. Richard Conway, Mr. Thomas Fey, and Ms. Ruth Krause for collection of biological data, and thank analytical services at Abbott Laboratories for spectroscopic determinations.

Registry No. 1, 133009-77-5; 2, 133039-94-8; 3, 133009-78-6; 4, 133009-79-7; 5, 133009-80-0; 6, 133009-81-1; 7, 133039-95-9; 8, 133009-82-2; 9, 133009-83-3; 10, 133009-84-4; 11, 133009-85-5; 12, 133009-86-6; 13, 133009-87-7; 14, 133009-88-8; 15, 133009-89-9; 16, 133009-90-2; 17, 133009-91-3; 18, 133009-92-4; 19, 133009-93-5; 20, 133009-94-6; 21, 133009-95-7; 22, 133009-96-8; human anaphylatoxin C5a, 68190-48-7.

(25) Borregard, N.; Heiple, J. M.; Simon, E. R.; Clark, R. A. *J. Cell. Biol.* **1983**, *97*, 52.
(26) Gerard, C.; Chenoweth, D. E.; Hugli, T. E. *J. Immunol.* **1981**, *127*, 1978.
(27) Chenoweth, D. E.; Goodman, M. G.; Weigle, W. O. *J. Exp. Med.* **1982**, *156*, 68.
(28) Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* **1983**, *22*, 3099.
(29) Chenoweth, D. E.; Hugli, T. E. *J. Immunol.* **1980**, *124*, 1517.