Decapeptide Agonists of Human C5a: The Relationship between Conformation and Neutrophil Response[†,‡]

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Received December 23, 1994[⊗]

A series of decapeptide analogues corresponding to the C-terminal region of the human C5a anaphylatoxin ($C5a_{65-74}$) was synthesized with residue substitutions to restrict conformational flexibility in the C-terminal region (residues 71–74). These analogues behaved as full agonists of natural C5a in their ability to induce shape change (polarization) and the release of enzyme (β-glucuronidase) from human neutrophils (PMNs). There was a significant pharmacological correlation between the polarization and enzyme-release assays, suggesting similarities in PMN responsiveness toward these constrained peptides. Good correlations were also observed between these two PMN responses and spasmogenic activity (smooth muscle contraction of human fetal artery). A structure–function analysis for PMN polarization and enzyme release led to the identification of the following preferred backbone conformations: a twisted, helix-like conformation for residues 65–69, an extended conformation for residues 70–71, and a β-turn of type V for residues (71)72–74. The existence of a C-terminal, type V β-turn is supported by the NOE (nuclear Overhauser effect) results of two peptides from this series. These conformational features are reminiscent of those that were shown to correlate with the expression of spasmogenic and platelet aggregatory activities in an earlier investigation (Sanderson, S. D.; et al. *J. Med. Chem.* **1994**, *37*, 3171). These results suggest that PMNs and the cells responsible for smooth muscle contraction possess C5a receptors that respond to similar topochemical features presented by the agonist peptide ligand.

Introduction

The human anaphylatoxin C5a is the pharmacologically active fragment of the fifth component of the plasma complement system (C5) and is released as a byproduct of complement activation upon the proteolytic cleavage of C5. C5a is a 74-residue glycopolypeptide that expresses proinflammatory activities that can be classified into two broad categories.[1] The first is the C5a-induced release of secondary mediators from a variety of C5a receptor-bearing cells. Examples of this class of activity include the C5a-induced release of histamine and/or lysosomal enzymes from mast cells, neutrophils, and macrophages;[2–4] vasoactive eicosanoids from numerous inflammatory cells;[1,5] and various cytokines from monocytes.[6–9] The second category relates to the ability of C5a to directly activate circulating polymorphonuclear leukocytes (PMNs) and recruit them to sites of tissue injury and infection.[10,11]

The human C5a molecule is composed of a highly ordered N-terminal domain (residues 1–63) that consists of a tightly packed, 4-helix bundle and is primarily involved in receptor recognition and binding.[12,13] The C-terminal region (residues 64–74), which protrudes from the N-terminal domain, is a region of considerable flexibility and poorly defined structure yet alone possesses the requisite information for transducing a biological signal.[12,13] This is demonstrated by the fact that peptides synthesized with sequence homology to the C-terminal 8–10 residues of C5a behave as full agonists relative to the parent polypeptide but at markedly reduced potencies.[8,14,15]

Since these C-terminal agonist peptides appear to interact with the C5a receptor and invoke the same signal transduction pathway(s) as that of natural C5a, there is considerable interest in their use as molecular/mechanistic probes of C5a-mediated signal transduction and as synthons in the development of a C5a receptor antagonist for the treatment of C5a-associated inflammatory disorders. However, the full utility of these agonist peptides as the mechanistic and synthetic tools envisioned is dependent upon an understanding of how specific conformational features expressed by the peptides relate to their observed biological activity. Achieving this level of understanding has been particularly difficult due, in part, to the high degree of conformational flexibility inherent in these C-terminal peptides.

Recently, we reported on a structure–function investigation of a series of C-terminal decapeptide agonists of human C5a ($C5a_{65-74}$Y65,F67) in which conformational flexibility within the C-terminal region was

[†] This work is supported by grants from the Nebraska Cancer and Smoking Disease Research Program #94-90 to S.A.S. and #93-42 to S.D.S., and NCI CA3G727, ACS, SIG-16, NSF Grant MCB-9306501 to J.A.E., and an NSRG grant to S.M.T. from the University of Queensland.

[‡] Except where noted, the single letter designation for the amino acid residues is used as follows: A, alanine; C, cysteine; D, aspartic acid; F, phenylalanine, G, glycine; H, histidine, I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; V, valine; Y, tyrosine. Uppercase letters represent the L-amino acid isomer and lowercase the D-isomer.

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[⊗] Abstract published in *Advance ACS Abstracts*, August 15, 1995.

0022-2623/95/1838-3669$09.00/0

restricted.[16] A structure–function analysis suggested that a C-terminal β-turn of either type II or type V was responsible for the expression of activity in this class of assays. Moreover, those peptides that expressed a structural propensity toward these β-turns appeared particularly potent.

In this paper, we report the results of a similar structure–function study of conformationally constrained analogues of $C5a_{65-74}$Y65,F67 for their ability to induce polarization (shape change) and release of β-glucuronidase from human PMNs. On the basis of this investigation, evidence is presented suggesting that a C-terminal β-turn of type V appears responsible for the expression of these PMN activities and that this C-terminal structural motif is similar to that which correlated with the induction of spasmogenic and platelet aggregatory responses.[16]

Results and Discussion

Conformational Characterization. The most probable backbone conformations within the flexible region of human C5a, represented by the C-terminal 10 residues ($C5a_{65-74}$), was determined by a probabilistic approach[17] that utilized the FISINOE program[18] and input data from the presence and/or absence or sequential *d*-connectivities from the original ^{1}H-NMR study of human C5a.[12] From this analysis, it was determined that an NMR-matched backbone conformation of $C5a_{65-74}$ likely consists of a twisted, helix-like conformation for residues 65–69, an extended conformation for residues 70–71, and a turnlike conformation of residues (71)-72–74.[16] A detailed structure–function analysis of a panel of $C5a_{65-74}$Y65,F67 analogues in which flexibility within the C-terminal region was restricted indicated that the expression of spasmogenic and platelet aggregatory activities correlated with a C-terminal (residues 71–74) β-turn of either type II or type V.[16] This same panel of constrained $C5a_{65-74}$Y65,F67 analogues was used in the present structure–function study in order to determine whether the same conformational features correlate with the expression of PMN activities (polarization and enzyme release).

We assumed that the expression of the twisted, helix-like conformation (residues 65–69) and the extended backbone conformation (residues 70–71), regions of the decapeptide thought to be more involved in receptor binding, would remain the same for both the spasmogenic/platelet aggregatory and PMN activities. Should differences in biological response arise, they would likely reflect subtle changes in the conformation of the C-terminal β-turn (residues 71–74). This notion is supported by the dramatic changes observed in biological activity upon restricting flexibility and altering topography within the C-terminal region of $C5a_{56-74}$ and $C5a_{65-74}$.[15,16,19,20]

Peptide Characterization. The panel of peptides used in this investigation was identical to that used in an earlier study of the relationship between conformation and spasmogenic and platelet aggregatory activities.[16] All peptides were based on the C-terminal 10 residues of human C5a ($C5a_{65-74}$ or ISHKDMQLGR) and on a more potent analogue ($C5a_{65-74}$Y65,F67 or YSFKDMQLGR). The replacement of His at position 67 with the aromatic residue Phe (peptide 1, Table 1) afforded ca. 2 orders of magnitude increase in potency relative to $C5a_{65-74}$.[21,22] Tyr was used in place of Ile-65 in order to provide a site for radioactive iodination[23] and to avoid NMR spectral overlap with Leu-72. Substituted residues that differ from those in $C5a_{65-74}$-Y65,F67 are shown in boldface in Table 1. All peptides were homogeneous by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) and gave the expected residue molar ratios by compositional analysis and parent ion molecular mass by fast-atom bombardment mass spectrometry (FAB-MS).[16]

Table 1. Pharmacological Activities of C5a Analogues in the Polarization and Enzyme Release from Human Neutrophils

		EC_{50} (μM)[b]	
no.	peptide[a]	PMN polarization	PMN enzyme release
	C5a	0.0013	0.0123
1	ISFKDMQLGR	0.9	67.7
2	YSFKDMQLGR	4.0	50
3	YSFKDMQL**P**R	500	>1000
4	YSFKDMQ**P**GR	22	42
5	YSFKDM**P**LGR	5.0	80
6	YSFKDM**PP**GR	50	700
7	YSFKDMQL**A**R	48	1000
8	YSFKDMQL**a**R	1.2	32
9	YSFKDMQ**L**GR	37	520
10	YSFKDM**P**L**a**R	2.1	57.7
11	YSFKDM**PA**GR	215	680
12	YSFKDM**PAa**R	5.6	>1000
13	YSFKDM**P**L**f**R	4.0	114
14	YSFKDM**P**L**p**R	2.5	46.5
15	YSFKD**AP**LGR	15.8	207
16	YSFKD**CP**LGR	19.5	>1000
17	YSFKD**CP**L**C**R	224	>1000
18	YSFKD**CP**L**C**R	500	>1000
19	YSFKD**AP**L**a**R	2.3	114
20	YSFKGMLLGR		70
21	YSFKGLLLGR	0.3	27.7
22	YSFKGMPLGR	2.0	49.6

[a] Residue substitutions to peptide 2 shown in boldface. [b] Mean EC_{50} values derived from individual experiments ($n = 3$).

Residue substitutions in $C5a_{65-74}$Y65,F67 were chosen to restrict flexibility in the C-terminal region of the decapeptide in order to bias certain features of backbone conformation that would be useful in the search for biologically relevant conformations in the flexible, C-terminal region (residues 71–74). Three principal types of modifications were employed (1) Pro substitutions for restricting local φ angle flexibility and for influencing the allowed conformations of the pre-proline residue, (2) Ala substitutions for evaluating the contribution made by the side chains in the peptide, and (3) D-residue substitutions for influencing local backbone configurations. The changes in biological activity induced by these restrictions in C-terminal flexibility and conformational space alterations were assessed by measuring the polarization (shape change) and release of β-glucuronidase from human PMNs.

Pharmacological Activity. Table 1 summarizes the pharmacological activities of human C5a, $C5a_{65-74}$-Y65,F67, and its analogues in the PMN polarization and enzyme release assays. All analogues induced responses in a dose-dependent manner and were shown to be full agonists compared to natural C5a in these assays.

Restricting backbone flexibility at position 73 by the substitution of a Pro residue (peptide 3) was detrimental to polarization and enzyme release activities compared to standard $C5a_{65-74}$Y65,F67 (peptide 2). A Pro substitution in the adjacent Leu-72 position (peptide 4) had

little effect in either PMN assay. This was somewhat surprising because the presence of the Leu-72 side chain had been shown to be crucial for the expression of spasmogenic/platelet aggregatory responses.[16] A Pro substitution for Gln-71 (peptide 5) exhibited minimal effects in either PMN assay compared to $C5a_{65-74}$-Y65,F67. Again, this was an unexpected result in light of the favorable effect the same substitution had on the expression of spasmogenic and platelet aggregatory activities.[16] The double-Pro substitution for residues 71 and 72 (peptide 6), however, adversely affected both polarization and enzyme release. It is likely that the presence of the two Pro residues restricts flexibility to the point that the C5a receptor is no longer capable of inducing the proper C-terminal conformation in the ligand necessary for effective signal transduction.

The introduction of a methyl side chain at position 73 (i.e., Ala for Gly, peptide 7) had an adverse effect on both polarization and enzyme release activities. However, activity in both assays was restored to the levels of $C5a_{65-74}$Y65,F67 (peptide 2) when L-Ala-73 was replaced by D-Ala (peptide 8), a trend that was also observed with the spasmogenic/platelet aggregatory assays.[16] Peptide 10, having both D-Ala-73 and Pro-71, however, was not much more potent than $C5a_{65-74}$-Y65,F67 in the PMN assays and appeared to be less potent than peptide 8, having the D-Ala-73 substitution alone. Interestingly, the presence of both Pro-71 and D-Ala-73 (peptide 10) afforded a 2 orders of magnitude increase in potency relative to $C5a_{65-74}$Y65,F67 in the spasmogenic/platelet aggregatory assays.[16]

The presence of Pro at position 71 and Ala at position 72 (peptide 11) was detrimental to both PMN assays as well as to the spasmogenic/aggregatory activities from our earlier study. In the case of the spasmogenic/platelet aggregatory responses, this decrease in activity was attributed to the loss of the favorable contribution made by the side chain of Leu-72 because the presence of Pro-71 alone (peptide 5) had been shown to increase potency in these assays.[16] In the case of the PMN activities, however, the decrease in activity observed with peptide 11 appeared to be influenced by both the decrease in hydrophobicity of the residue at position 72 and the less than favorable backbone conformation induced by the presence of Pro at position 71. Despite the adverse effects of Pro-71 and Ala-72, polarization activity was almost completely restored by the presence of D-Ala at position 73 (peptide 12).

Increasing the bulk of the side chain at position 73 with a D-Phe residue (peptide 13) had no effect on polarization but appeared to have a detrimental effect on enzyme release. Peptide 14, which contained L-Pro at position 71 and D-Pro at position 73, was reasonably potent in both PMN assays. This result was not unexpected, given the favorable effects that D-residue substitutions at position 73 appear to have on biological activity.[16]

Unlike the expression of spasmogenic/platelet aggregatory activities, substituting an Ala for the oxidizable Met at position 70 (peptide 15) had an adverse effect on PMN polarization and enzyme-release activities compared to its Met-containing counterpart (peptide 5). However, the presence of both L-Ala at position 70 and D-Ala at position 73 (peptide 19) afforded a reasonable recovery of activity in the enzyme release assay. The

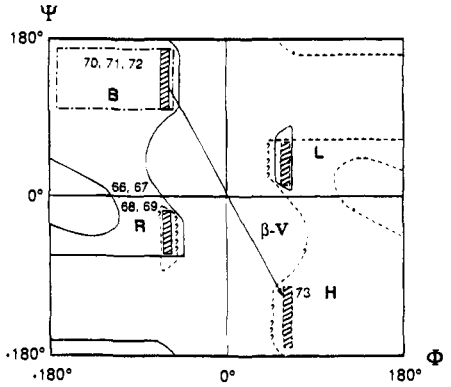

Figure 1. Ramachandran plot showing the sterically allowed conformational space occupied by the amino acid residues in the decapeptide $C5a_{65-74}$. (The terminal residues Ile-65 and Arg-74 are omitted.) Sterically allowed space for L- and D-residues is contained within the solid and dashed lines, respectively. The narrow, vertical regions depict sterically allowed space for L-Pro (left-hand quadrants outlined with solid lines) and D-Pro (right-hand quadrants outlined with dashed lines). The boxed region in quadrant B is the sterically allowed conformational space for the pre-proline residues. B, R, L, and H refer to the ϕ, ψ regions that correspond to β-conformations, right-handed helices, left-handed helices, and high-energy conformation, respectively.

presence of Cys at position 70 (peptide 16), Cys at positions 70 and 73 (peptide 17), and a restrictive disulfide bridged formed between Cys-70 and Cys-73 (peptide 18) did not appear to contribute to a biologically favorable C-terminal conformation.

Peptides 20–22 are the rat[24] analogues of peptides 2 and 5. As with the human sequences, the rat decapeptides were based on the Tyr for Ile and Phe for His substitutions at positions 65 and 67, respectively. Rat $C5a_{65-74}$Y65,F67 (peptide 20) was about equipotent with the human analogue (peptide 2) in enzyme release. However, the substitution of Met at position 70 with Leu (peptide 21) increased hydrophobicity that appeared favorable for the expression of both polarization and enzyme release. Replacement of Leu-71 with Pro (peptide 22) appeared to contribute to an increase in potency relative to its control analogue (peptide 20), but the presence of Pro-71 in the human analogue (peptide 5) appeared to be less favorable for PMN activity.

Conformational Analysis. An analysis of the sequential *d*-connectivities of human C5a by the FISINOE program suggested that the C-terminal decapeptide $C5a_{65-74}$ possessed twisted, helix-like conformation for the region comprised of residues (65)66–69. This backbone conformational pattern indicated that the sterically allowed ϕ,ψ angular rotations for these residues lie at the interface of the B and R quadrants (i.e., $\phi = -90°$ and $\psi = 0°$) of Ramachandran space (Figure 1). The extended backbone conformation suggested for residues 70–71 places them in the boxed region within B quadrant of allowed Ramachandran space. Finally, on the basis of a detailed analysis of the conformational relationship between the peptides listed in Table 1 and the expression of spasmogenic and platelet aggregatory activities, it was shown that the C-terminal residues 71–74 occupied the sterically allowed angular space within the B, B, L or H, and B quadrants of the Ramachandran plot, respectively.[16] Thus, the set of sterically allowed ϕ,ψ angles for the C-terminal residues 71–74 existed in either of two combinations: B, B, L,

B or B, B, H, B, dihedral combinations consistent with β-turns of either type II or type V, respectively.[25]

In the present study, the same peptides were analyzed in two independent PMN assays: polarization and enzyme release. It was assumed that the twisted, helix-like conformation for residues 65−69 and the elongated conformation for residues 70−71, regions that appeared to play a role in receptor binding in the spasmogenic/platelet aggregatory assays, would be retained for receptor-binding purposes in the induction of the PMN responses. Therefore, any differences observed in biological responses between PMNs and smooth muscle/platelets would likely result from subtle differences in the expression of conformational features within the flexible, C-terminal region (residues 71−74), which is thought to be involved in signal transduction. Consequently, residues (65)66−69 were assigned to the interface of the B and R quadrants of sterically allowed Ramachandran space and residues 70−71 to the boxed region within the B quadrant.[16,25] Residues (71)72−74 were assigned to sterically allowed region of angular space according to the observed patterns of PMN responsiveness toward the peptides in Table 1.

The assignment of Gln-71 to the boxed region within the B quadrant of Ramachandran space came from the analysis of the NMR data from human C5a[12] and from the biological behavior of peptides 4 and 5. The incorporation of a Pro in both peptides fixes the pre-proline residue in an extended conformation by restricting ψ angle rotations within the narrow interval of ca. 100−160°, corresponding to the boxed region of the B quadrant in Figure 1. Thus, in peptide 4, the presence of Pro at position 72 forces the backbone of the pre-proline residue Gln-71 into an extended conformation. The fact that the presence of Pro-72 had very little effect on either PMN assay supports the assignment of Gln-71 to the boxed region within the B quadrant of Ramachandran space. Likewise, the presence of Pro at position 71 (peptide 5) had very little effect on either PMN assay and supports residue 71 occupying the narrow strips of allowed space within the B or R quadrant. Since the B quadrant of Ramachandran space is common to both of these scenarios, the biological and NMR results support residue 71 occupying the boxed region within the B quadrant.

Leu-72 was assigned to the same boxed region within the B quadrant of Figure 1 on the basis of the biological responses expressed by peptides 4, 9, and 14. Peptide 4, in which a Pro residue occupied position 72, had very little effect on PMN activities. This observation argues in favor of Leu-72 occupying the narrow strips of allowed space in the B or R quadrant of the Ramachandran plot (Figure 1). The presence of D-Leu at position 72 (peptide 9) contributed to observed decreases in polarization and enzyme release, supporting the notion that Leu-72 likely resides in Ramachandran space other than the L, H, or R quadrant. Finally, the presence of D-Pro at position 72 (peptide 14) gave rise to a slight increase in the PMN polarization response and had no effect on enzyme release. Given the tendency of a proline residue to elongate the backbone conformation of the adjacent residue, the biological results of peptide 14 support the assignment of Leu-72 to the boxed region within the B quadrant of Figure 1.

The assignment of residue 73 to the H quadrant of Ramachandran space came from the PMN responses to peptide 3, 7, 8, 10, and 14. In peptide 3, the Pro residue at position 73 was detrimental to both PMN responses and argues that residue 73 does not occupy the B or R quadrant, leaving only the L and H quadrants as possibilities. In peptide 7, the Ala at position 73 is also detrimental to the expression of both PMN activities. Thus, the B and R quadrants, along with the narrow region within the L quadrant, can be eliminated as possibilities for sterically allowed regions that residue 73 can occupy, and this leaves only the H quadrant as a possibility. The presence of D-Ala at position 73 (peptide 8) contributed to an enhancement of polarization and enzyme-release responses and supports Gly-73 occupying the right-hand side of the Ramachandran plot (quadrants L and H) and the narrow region within the R quadrant. This is supported by the fact that the presence of D-Ala-73 in peptide 10 was capable of offsetting the rather unfavorable affect the presence of Pro-71 alone (peptide 5) had no the expression of PMN activity. Finally, the favorable expression of PMN activities observed when D-Pro occupied position 73 (peptide 14) supports the residue at position 73 occupying the narrow strip of allowed angular space within the L or H quadrant of the Ramachandran plot (Figure 1). Of the above scenarios, the H quadrant is the only assignment common to all possibilities.

From the data reported in Table 1, it is not possible to determine the probable regions of angular space occupied by Arg-74. However, since Arg-74 occupies the C-terminal position, the B and L quadrants are the only two conformational possibilities. Also, it was shown that the presence of D-Arg at position 74 had a detrimental effect on the expression of spasmogenic/platelet aggregatory responses,[16] supporting the likelihood of Arg-74 occupying the left-hand side of the Ramachandran plot (i.e., the B quadrant). Since the C-terminal Arg residue has been shown to be critical for the expression of C5a activity, it is assumed that this same angular space is occupied by Arg-74 for the expression of PMN activities.

Thus, for the expression of PMN polarization and enzyme release, the regions of sterically allowed angular space occupied by the C-terminal four residues (71−74) can be assigned, respectively, to the B, B, H, and B quadrants of the Ramachandran plot, a dihedral combination that is consistent with a β-turn of type V.[25]

This type of V β-turn is further supported by nuclear magnetic resonance (NMR) analysis of two peptides from Table 1: the standard peptide YSFKDMQLGR (peptide 2) and one of the most potent analogues in PMN responses, YSFKDMPLaR (peptide 10). Sequence specific proton resonance assignments for these peptides were made using standard two-dimensional (2D) NMR methods on the basis of through-bond and through-space connectivities. The through-bond connectivities were made via double-quantum-filtered correlation spectroscopy (DQF-COSY)[26,27] and total correlation spectroscopy (TOCSY) experiments.[28] Through-space connectivities were obtained by nuclear Overhauser enhancement spectroscopy (NOESY)[29] and rotating frame nuclear Overhauser enhancement spectroscopy (ROESY)[30,31] experiments performed with different mixing times. For residues with short side chains (Ala, Asp, Gly and Ser, etc.), assignment of through-bond connec-

tivities was straightforward. For residues with long side chains (Arg, Leu, Lys, Met and Pro, etc.), assignment of through-bond connectivities was achieved in a progressive manner. (Detailed sequential proton assignments and the three-dimensional solution structure of these peptides will be presented elsewhere.)

The NMR results suggest that the region of the peptide represented by residues Tyr-65–Asp-69 adopt a twisted, helix-like conformation due to the presence of a medium NOE, $d_{\alpha N}(i,i+3)$, between the α-proton of Ser-66 and the amide proton of Asp-69 and NOE cross peaks, $d_{NN}(i,i+1)$, between the amide protons of Ser-66 and Phe-67 as well as between Lys-68 and Asp-69. NOEs characteristic of well-defined secondary structural features were absent for the region of the peptide made up of residues Met-70 and Gln/Pro-71, suggesting the presence of extended backbone conformation in this region. Finally, the NMR data support the existence of a type V β-turn for the C-terminal four residues (Gln/Pro-71, Leu-72, Gly/D-Ala-73, and Arg-74). This conclusion is based on the following NOE cross peaks observed in this region of the peptides: (1) a weak NOE cross peak at $d_{NN}(i+2,i+3)$, (2) a weak NOE cross peak at $d_{\alpha N}(i+2,i+3)$, and (3) a medium NOE cross peak at $d_{\beta N}(i+2,i+3)$. The magnitude and pattern of these NOE cross peaks are consistent with a β-turn of type V.[32] However, on the basis of these data alone, we cannot totally rule out the possibility of a type II β-turn. Nevertheless, the NMR data in this study support the three structural motifs identified by our conformational analysis based on the biological data, i.e., the presence of a twisted, helix-like conformation for residues 66–69, an extended backbone conformation for residues 70–71, and a β-turn of type V for residues (71)72–74. These structural motifs are reminiscent of those identified as being important for the expression of spasmogenic and platelet aggregatory responses.[16] Therefore, it appears that the C5a receptors expressed on human PMNs, the cells associated with smooth muscle tissue, and guinea pig platelets recognize similar topochemical features presented by the agonist peptides.

Pharmacological Correlations. Two different PMN assays were used to assess the activity of the decapeptides shown in Table 1. The polarization assay was more sensitive to C5a than was the enzyme-release assay, and in general, this difference in sensitivity was seen with the peptides. Moreover, there was a significant ($p < 0.05$) correlation between the EC_{50} (pD_2) values for the polarization and enzyme release assays (Figure 2). This good correlation of potencies between two independent PMN assays suggests that a common, C5a receptor-mediated pathway is involved in the expression of these PMN responses. This notion is also supported by several other studies that have demonstrated the presence of a single class of high-affinity binding sites on PMN membranes.[21,22,24,33] Thus, it appears that the end points of these PMN bioassays (shape change and enzyme release) are mediated via a common receptor-mediated pathway.

The PMN correlation shown in Figure 2 is reminiscent of a similar relationship that we recently described between the activity of this same panel of peptides in the expression of spasmogenic and platelet aggregatory activities.[16] The spasmogenic effects of these peptides are due to the release of secondary mediators such as

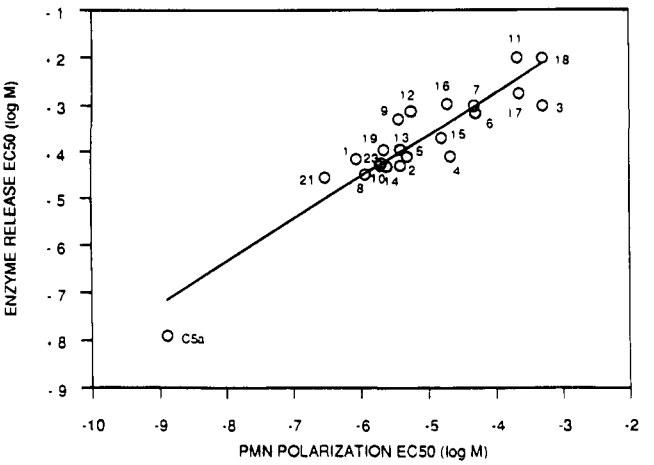

Figure 2. Correlation between the effective peptide concentrations (EC_{50}, log M) for enzyme release (β-glucuronidase) from human PMNs and the polarization of human PMNs. Data are derived from Table 1. Regression coefficient $r = 0.93$.

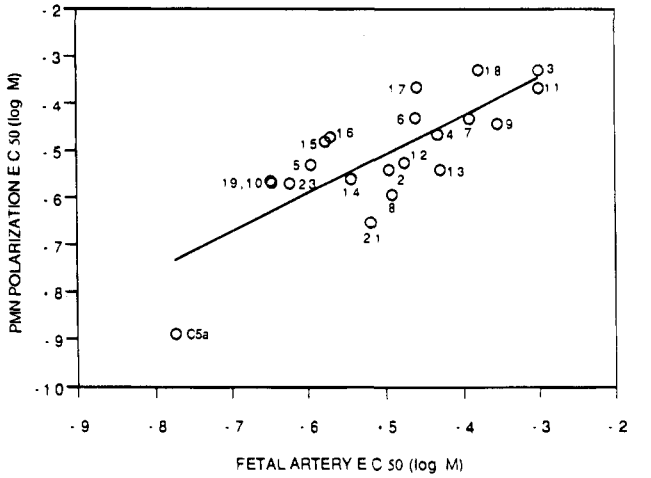

Figure 3. Correlation between the effective peptide concentrations (EC_{50}, log M) for the polarization of human PMNs and smooth muscle contraction of human fetal artery, $r = 0.77$. Data are derived from Table 1 (PMN polarization) and from Table 2 of ref 16 (fetal artery contraction).

histamine and eicosanoids from inflammatory cells. Mast cells are not present in human fetal artery; however, there is evidence that macrophages may be the cellular source of the eicosanoids released in this tissue.[34] A comparative analysis revealed significant ($p < 0.05$) correlations between spasmogenic activity in human fetal artery and PMN polarization ($r = 0.77$) as well as with enzyme release from PMNs ($r = 0.76$) (Figures 3 and 4). Also, the correlation lines were virtually superimposable (Figure 5). These observations suggest that the C5a receptor(s) of PMNs and macrophages in smooth muscle recognize similar topochemical features presented by the peptide ligand.

Despite similarities in the ability of the C5a receptor to recognize certain topochemical features of the peptide ligand, there remains an apparent biological difference in the way PMNs and smooth muscle (via macrophages) respond toward these peptides. In the spasmogenic and platelet aggregatory assays, the most potent analogues (peptides 10 and 19) were about 4–5% of the potency of natural C5a.[16] In the PMN assays, these same peptides expressed only 0.02–0.06% of the potency of C5a. On the other hand, the most potent analogues in the PMN assays were peptide 21 in the polarization

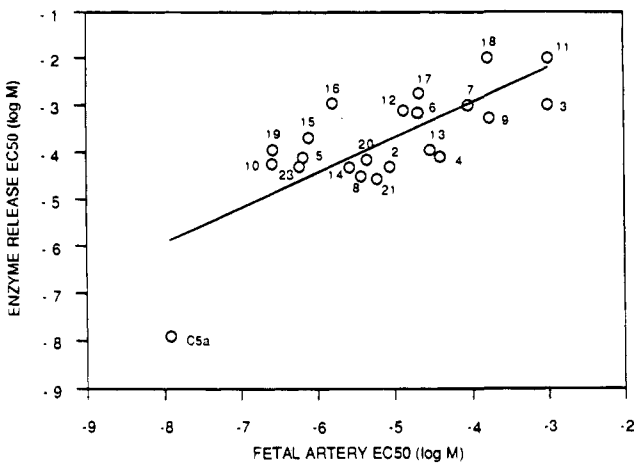

Figure 4. Correlation between the effective peptide concentrations (EC_{50}, log M) for β-glucuronidase release from human PMNs and smooth muscle contraction of human fetal artery, $r = 0.76$. Data are derived from Table 1 (enzyme release) and from Table 2 of ref 16 (fetal artery contraction).

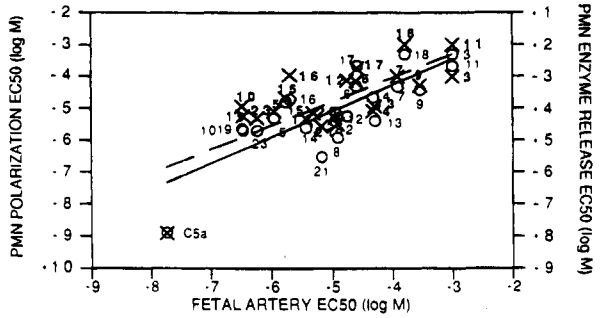

Figure 5. Comparison of the correlations between effective peptide concentrations (EC_{50}, log M) for human PMN polarization (O), enzyme release from human PMNs (×), and smooth muscle contraction of human fetal artery.

response (0.4% of the potency of C5a) and peptide 8 in enzyme release (0.04% of the potency of C5a). In the spasmogenic/platelet aggregatory responses, however, these peptides expressed only 0.1–0.3% (peptide 21) and 0.05–0.1% (peptide 8) of the potency of C5a.[16] These different patterns of responsiveness might be explained by the restriction of conformational flexibility within the signal-transducing C-terminal region, thereby imposing some limitations on the receptor's ability to effectively induce the conformation most favored for efficient signal transduction. Studies are currently underway to evaluate this hypothesis.

Summary and Conclusions

In this paper we have characterized a common, preferred backbone conformation in a series of C-terminal decapeptide agonists of human C5a that correlate with the polarization and enzyme release assays from human PMNs. These structural features appear to be a twisted, helix-like backbone conformation for residues 65–69, an extended backbone conformation for residues 70–71, and a β-turn of what appears to be type V for the region comprised of residues (71)72–74. These conformational motifs are similar to those that were shown to correlate with the expression of spasmogenic and platelet aggregatory responses.[16] These results suggest that the C5a receptor(s) expressed on PMNs, the inflammatory cells responsible for smooth muscle contraction, and guinea pig platelets appear to recognize similar topochemical features presented by these C-terminal peptide agonists.

Experimental Section

Structural Interpretation of NMR and Biological Data. The preliminary estimation of the backbone conformational features of $C5a_{65-74}$ and $C5a_{65-74}$Y65,F67 analogues was determined by a probabilistic approach utilizing the FISINOE program.[17,18] FISINOE determines the backbone conformation of a peptide or protein in solution by combining NMR data with two-dimensional distribution functions of ϕ and ψ angles derived from the Protein Data Bank (PDB). For this study, the sequential d-connectivities (SDC) ($d_{\alpha N}$, d_{NN}, and $d_{\beta N}$) observed in the C-terminal region ($C5a_{65-74}$) for human C5a were used as input data to the FISINOE program. For each residue within $C5a_{65-74}$, FISINOE calculated the most probable values for ϕ and ψ angles and their standard deviations that correspond to the given set of SDC.

Further identification and characterization of unique conformational features in the backbone of the C-terminal decapeptides used in this study were made by a comprehensive analysis of the structure–function relationships with the 23 analogues listed in Table 1. This analysis was made using an approach described by Hruby and Nikiforovich.[25]

Peptide Synthesis, Purification, and Characterization. All peptides were synthesized by standard solid-phase methodologies on an Applied Biosystems (Foster City, CA) Model 430A synthesizer, purified by analytical and preparative HPLC on columns packed with C_{18}-bonded silicagel, and characterized by amino acid compositional analysis and mass spectrometry. The details of these methods have been previously reported.[16]

Biological Assays. PMN Polarization. The assay for measuring the effect of the peptides in Table 1 on PMN polarization was described by Haston and Shields.[35] Briefly, PMNs (1×10^6 cells/mL) were suspended in 10 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS; Sigma) containing Earle's balanced salt solution (EBSS). The cells were incubated with the chemoattractant for 30 min at 37 °C and then fixed with 2.5% glutaraldehyde. After 10 min of incubation at room temperature, the cells were washed twice and stored until microscopic examination. Cells deviating from the typical spherical shape were visually scored as polarized. Results were expressed as a percent of polarized cell/total cells counted (300 cells counted/sample).

Enzyme Release. β-Glucuronidase release from human PMNs was determined as described by Schroder et al.[36] Briefly, human PMNs were pretreated with cytochalasin B (5 μg/mL, 10 min, 37 °C). The cells (10^6) were incubated in the presence of stimulants for 60 min at 37 °C in a final volume of 200 μL and then centrifuged. The supernatant was collected, and 50 μL was incubated with 50 μL of 0.01 M *p*-nitrophenyl β-D-glucuronide (in 0.1 M sodium acetate at pH 4 for 18 h) as triplicate determinations in 96-well microtiter plates (Corning). The reaction was stopped by the addition of 100 μL of 0.4 M glycine buffer at pH 10. The reaction mixture developed color that was read at 405 nm in a Titertec Multiscan (MCC/340) ELISA reader. The β-glucuronidase released was expressed as the percent of total enzyme in the cell that could be released by the addition of 0.2% Triton X-100 (Pierce).

NMR Spectroscopy. 2D NMR experiments were performed with a 11.5T Varian UNITY plus spectrometer, operating at 499.96 MHz for ^{1}H, with a 5 mm triple-resonance inverse detection probe. NMR data sets were acquired at both 30 and 50 °C in DMSO-d_6 and were processed and analyzed using Varian NMR software VNMR 4.3b on a Silicon Graphics workstation. Sequence specific proton resonance assignments were established from phase sensitive, 2D NOESY.[29] Proton resonance assignments were confirmed by a comparison of cross peaks in a NOESY spectrum with those in a TOCSY[28] spectrum acquired for the peptides under similar experimental conditions. A total of 512 increments of 4K data points were recorded for DQF-COSY[26,27] experiments, and a total of 256 increments of 2K data points were recorded for all other

experiments. All data sets were collected in hypercomplex phase sensitive mode. Inter- and intraresidue NOE intensities were measured in 200, 300, 400, and 500 ms mixing time NOESY and ROESY[30,31] experiments. Before processing, the t_1 dimension of DQF-COSY data sets were zero-filled to 4K and t_1 dimensions of data sets of all other experiments were zero-filled to 2K. When necessary, spectral resolution was enhanced by Lorenzian–Gaussian apodization.

Acknowledgment. We wish to acknowledge the expert technical assistance of Jan Williamson and Dr. Fulvio Perini for amino acid compositional analyses, the Midwest Regional Center for Mass Spectrometry at the University of Nebraska at Lincoln, the Eppley Cancer Institute's Molecular Modeling Core Facility, and the theater staff of the Mater Misericordiae Hospital, New Life Centre.

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JM940844R