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Biochemistry

Synthetic peptides with the biological activities and specificity of human C3a anaphylatoxin

(solid-phase peptide synthesis/C3a-(70-77)/complement fragments/histamine release/vascular permeability)

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ABSTRACT **Two peptides identical to the COOH-terminal sequence of human C3a anaphylatoxin and two analogs were synthesized by the solid-phase method and tested for biological activity. The synthetic COOH-terminal octapeptide, C3a-(70-77) or Ala-Ser-His-Leu-Gly-Leu-Ala-Arg, caused contraction of guinea pig ileum and uterus, release of vasoactive amines from rat mast cells, and increased vascular permeability in guinea pig and human skin. On a molar basis, the synthetic octapeptide possessed 1–2% of the biological activities of C3a and specifically desensitized smooth muscle to stimulation by C3a. Like natural C3a, the synthetic C3a-(70-77) was inactivated by digestion with carboxypeptidase B [peptidyl-L-lysine(-L-arginine) hydrolase, EC 3.4.12.3], which removed the essential COOH-terminal arginine. A synthetic nonapeptide [C3a-(70-77)-Gly], containing a glycyl instead of an arginyl COOH terminus, was approximately 1% as active as the octapeptide when assayed with smooth muscle. The COOH-terminal 13-residue peptide of C3a, C3a-(65-77), was equal in activity to C3a-(70-77); similarly, C3a-(65-77)-Gly expressed the same activity as C3a-(70-77)-Gly. It is concluded that both the biological specificity and the activity of human C3a anaphylatoxin are determined by eight or fewer residues located at the COOH terminus of the natural protein. However, expression of full activity requires additional groups and the secondary conformational integrity of the C3a molecule.**

Fragments released during activation of serum complement (C) proteins exhibit potent, noncytolytic biological activities (1). Enzymatic cleavage of the α chains of C3 and C5 near their NH_2 termini generate the activation peptides C3a and C5a, respectively. The activation peptides are designated "anaphylatoxins" for historical reasons and because of their ability to elicit a systemic reaction in guinea pigs that closely resembles acute anaphylactic shock. These fragments also cause the contraction of smooth muscle, the cellular release of histamine, other vasoactive amines, and lysosomal enzymes, enhancement of vascular permeability, and, in the case of C5a, chemotactic attraction of polymorphonuclear leukocytes. These biological activities implicate the anaphylatoxins as mediators in the inflammatory process and of tissue injury (1, 2).

Detailed chemical descriptions of human C3a and C5a have recently been reported (3, 4). Peptide C3a is the most abundant of the C activation peptides of human serum: its concentration can reach 60 μg/ml during maximal C activation. It is also the most thoroughly characterized of the C fragments. The complete primary structures of human C3a and porcine C3a have recently been determined (5, 6). C3a is rendered biologically inactive by removal of just 1 of its 77 amino acid residues, the COOH-terminal arginine-77 (7). In order to explore the role of the COOH-terminal structure in the generation of anaphylatoxin activity, peptide analogs of the COOH terminus of human C3a were synthesized by the solid-phase method (8, 9) and tested for biological activity. Four synthetic C3a oligopeptides were examined, and each expressed not only the qualitative activities but also the biological specificity of C3a anaphylatoxin (10).

Abbreviations: C, complement; C3, third component of serum C system; C3a, 77-residue peptide cleaved enzymatically from NH_2 terminus of C3 α chain during activation.

MATERIALS AND METHODS

Preparation of Human C3a, C5a, and C3a Derivatives. Human C3a was isolated by the procedure of Hugli *et al.* (3), and human C5a was purified by the method of Fernandez and Hugli (4). Human C3a-(1-76) was formed by cleavage of arginine-77 from natural C3a with 1% (wt/wt) carboxypeptidase B [peptidyl-L-lysine(-L-arginine) hydrolase, EC 3.4.12.3] (Worthington) for 10 min at 37° and pH 7.5. Human C3a-(1-69) was prepared by digestion of C3a with 1% (wt/wt) carboxypeptidase Y (gift from R. Hayashi) for 1 hr at 25° in 0.15 M sodium acetate (pH 5.5).

Parallel Synthesis of C3a Oligopeptides on Two Resin Supports. Four oligopeptides (Table 1) related to the COOH terminus of human C3a were synthesized by the solid-phase method of Merrifield (8, 9) with the double-resin strategy (11, 12). Resin R (8) consisted of beads of styrene-*copoly*-1% divinylbenzene (Bio-Rad). Resin K (13) (gift from ICI Australia, Ltd.) consisted of beads of linear, non-cross-linked polystyrene graft copolymerized onto *poly*-chlorotrifluoroethylene (Kel-F) spheres. Both solid supports were functionalized by chloromethylation of the aromatic rings (8). Boc-[^{14}C]Leu-Ala-Arg-(Tos)-OCH_2-resin R (1.9 g; 0.17 mmol of Ala; 3.4×10^8 cpm) and Boc-Leu-[^{3}H]Ala-Arg(Tos)-Gly-OCH_2-resin K (5.0 g; 0.28 mmol of Ala; 5.5×10^8 cpm) were prepared separately and then combined in the 100-ml reaction vessel of a Schwarz/Mann automated peptide synthesizer. Then, during five cycles of solid-phase synthesis, Boc-Gly, Boc-Leu, Boc-His(Tos), Boc-Ser(Bzl), and Boc-Ala were successively added to the growing peptide chains attached to both resins (Fig. 1). The chemicals, solvents, and general synthetic procedures have been described previously (14, 15). After parallel assembly of the peptide-resins was complete, the mixture of peptide-resins was allowed to stand in dichloromethane. The [^{14}C]octapeptide-resin R floating at the surface was selectively sucked off and the [^{3}H]nonapeptide-resin K, which sank, was left behind.

[^{14}C]Leu^{75}-C3a-(70-77). Part of the octapeptide-resin R (0.26 g) was treated for 1.0 hr at 0° with anisole (0.6 ml) and liquid HF (5.4 ml). After evaporation of the HF, the resin was washed with ether (three 2-ml portions) to remove anisole and with trifluoroacetic acid (five 2-ml portions) to remove the peptide. After evaporation of the trifluoroacetic acid, the residue was dissolved in 5% (vol/vol) aqueous acetic acid and lyophilized to afford the crude octapeptide (48 mg), which was dissolved in 1% acetic acid (2 ml) and gel filtered on a 2.5 × 100-cm column of Bio-Gel P-2 (100-200 mesh, Bio-Rad) eluted with 1%

Table 1. Four synthetic peptides based on the COOH terminus of human C3a

Peptide	Amino acid sequence
	65 70 75 77 77a
C3a-(70-77)	H-Ala-Ser-His-Leu-Gly-Leu-Ala-Arg-OH
C3a-(70-77)-Gly	H-Ala-Ser-His-Leu-Gly-Leu-Ala-Arg-Gly-OH
C3a-(65-77)	H-Arg-Gly-His-Ala-Arg-Ala-Ser-His-Leu-Gly-Leu-Ala-Arg-OH
C3a-(65-77)-Gly	H-Arg-Gly-His-Ala-Arg-Ala-Ser-His-Leu-Gly-Leu-Ala-Arg-Gly-OH

acetic acid at 23 ml/hr. The major radioactive peak (175–300 ml) was pooled and lyophilized. Part of the desalted octapeptide (19 mg) was dissolved in 0.01 M NaCl/0.01 M NaH_2PO_4, pH 4.5 (1.5 ml) and applied to a 1 × 50 cm column of carboxymethyl-cellulose (Cellex CM, Bio-Rad), which was eluted at 30 ml/hr with a linear salt gradient from 0.01 to 0.20 M NaCl (both solutions 100 ml, 0.01 M NaH_2PO_4, pH 4.5) and continuously monitored at 206 nm. The major peak (230–170 ml) was pooled and lyophilized. This mixture was dissolved in 1% acetic acid (2 ml) and desalted on a 1.5 × 85-cm column of Bio-Gel P-2 eluted with 1% acetic acid at 15 ml/hr and monitored at 206 nm. The major UV-absorbing and radioactive peak (50–75 ml) was pooled and lyophilized to provide pure Ala-Ser-His-Leu-Gly-[^{14}C]Leu-Ala-Arg (10 mg). Amino acid analysis gave (relative values): Ala, 1.94; Ser, 0.97; His 0.95; Leu, 1.94; Gly 1.13; and Arg, 1.00. It also showed a single ninhydrin-positive and Pauly-positive spot by thin-layer chromatography [R_F = 0.73; for Val, R_F = 0.64 in 1-butanol/water/pyridine/acetic acid, 15:12:10:3 (vol/vol)] and by high-voltage paper electrophoresis (R_{Arg} = 0.76 at pH 5.0, 0.58 at pH 6.4); R_{Arg} is the distance between the centers of the sample and valine spots divided by the distance between the centers of the arginine and valine spots. Part (180 nmol) of the C3a-(70-77) was sequenced by automated Edman degradation on a Beckman Model 890B Sequenator.

[^{3}H]Ala^{76}-C3a-(70-77)-Gly. The nonapeptide was cleaved and purified similarly to C3a-(70-77). Part of the nonapeptide-resin K (0.88 g) was cleaved with HF/anisole, 9:1 (vol/vol), for 1.0 hr at 0° to finish the crude peptide (44 mg). The pure nonapeptide (9 mg) was isolated by successive gel filtration on Bio-Gel P-2, ion-exchange chromatography on Cellex CM, and desalting on Bio-Gel P-2. On amino acid analysis, the relative values were: Ala, 1.92; Ser, 0.97; His, 0.90; Leu, 2.09; Gly, 2.20; and Arg, 1.00. This material showed a single ninhydrin-positive and Pauly-positive spot by thin-layer chromatography (R_F = 0.73; for Val, R_F = 0.64 as above) and by high-voltage paper electrophoresis (R_{Arg} = 0.73 at pH 5.0, 0.55 at pH 6.4).

[^{14}C]Leu^{75}-C3a-(65-77) and [^{3}H]Ala^{76}-C3a-(65-77)-Gly were also prepared by this double-resin variation of the solid-phase method. Amino acid analysis of C3a-(65-77) gave (relative values): Arg, 3.00; Glu, 1.10; His, 1.97; Ala, 3.30; Ser, 1.04; Leu, 2.18; Gly 1.11. For C3a-(65-77)-Gly, the values were: Arg, 3.00; Glu, 0.96; His, 1.90; Ala, 3.09; Ser, 0.94; Leu 2.20; and Gly 2.30.

Bioassays for Anaphylatoxin Activity. Smooth muscle contraction was measured on guinea pig ileum and uterine strips by the methods of Cochrane and Müller-Eberhard (16). Responses to intracutaneous injections of native C3a and of the synthetic peptides were graded and documented after Wuepper *et al.* (17). The release of vasoactive agents from isolated rat mast cells was measured by the procedure of Morrison *et al.* (18). Changes in vascular permeability of guinea pig skin were followed by the procedure of Cochrane and Müller-Eberhard (16).

RESULTS

Synthesis of C3a Peptides. Two oligopeptides based on the COOH terminus of human C3a anaphylatoxin and their glycine-77a analogs (Table 1) were synthesized by the solid-phase method using the double resin strategy. This technique permits two analogous peptides to be assembled simultaneously on different solid supports under essentially identical chemical environments as outlined in Fig. 1. This approach ensures that differences in biological activity for the individual peptides of each synthetic pair are due only to designed chemical differences and not to dissimilarities arising in the course of the synthesis. The efficiency of separation was greater than 99% as measured by double-label scintillation counting of the peptide-resins.

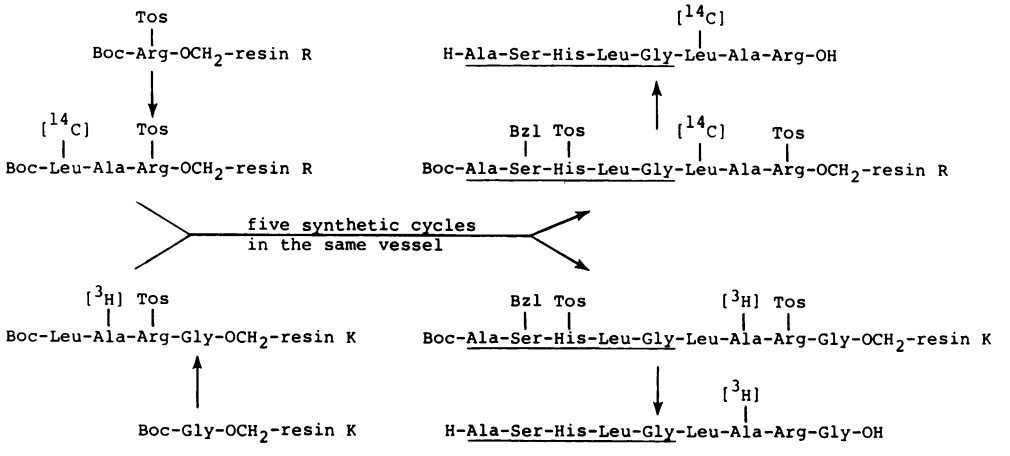

FIG. 1. Solid-phase assembly of the peptides [^{14}C]C3a-(70-77) and [^{3}H]C3a-(70-77)-Gly on solid supports of different density. The five amino acid residues underlined were added to both peptides under essentially identical conditions.

Table 2. Contraction of smooth muscle by natural human C3a and synthetic C3a peptides

Peptide	Minimal amount for full contraction*		Relative molar activity, %
	Weight, μg	Concentration, M	
C3a-(1-77)	1.0–1.2	$1.1–1.2 \times 10^{-8}$	100
C3a-(70-77)	4.0–6.0	$5.0–7.3 \times 10^{-7}$	2
C3a-(65-77)	5.8–8.0	$4.0–5.5 \times 10^{-7}$	2.5
C3a-(70-77)-Gly	250–375	$2.8–4.2 \times 10^{-5}$	0.03
C3a-(65-77)-Gly	366–550	$2.4–3.6 \times 10^{-5}$	0.04

* Strips of guinea pig ileum 2–3 cm in length were suspended in a 10-ml bath containing Tyrode's solution at 37° and tested according to Cochrane and Müller-Eberhard (16). Minimal amounts of each peptide required to give a full contraction in two or more separate ileum preparations were determined.

Each peptide was separately cleaved from the resin and freed of protecting groups by HF treatment and was then purified by gel filtration and ion-exchange chromatography. The synthetic peptides were homogeneous by thin-layer chromatography and paper electrophoresis and gave the expected amino acid analyses. Synthetic C3a-(70-77)-octapeptide was also shown to have the correct sequence of amino acids by sequential Edman degradation.

Contraction of Smooth Muscle. A reliable and sensitive measure of anaphylatoxin activity is the minimum effective dose required to evoke a contraction of isolated smooth muscle strips from the guinea pig ileum. When the ileum receptors specific for C3a become saturated, the muscle becomes insensitive (tachyphylactic) to repeated applications of the peptide. The C3a receptors are distinct from the C5a and bradykinin receptors because these three agents show no cross-desensitization.

Each of the four synthetic C3a peptides induced smooth muscle contraction at relatively low concentrations (Table 2). The effective concentrations of C3a-(70-77)-octapeptide and C3a-(65-77)-tridecapeptide were nearly equivalent, but each was only about 2% as active as the native C3a on a molar basis. Addition of a glycyl residue to the COOH terminus of either C3a-(65-77) or C3a-(70-77) decreased the specific activity about 65-fold. The smooth muscle activity of C3a-(70-77)-Gly was not due to trace amounts of C3a-(70-77) because short treatment with carboxypeptidase B produced no change in activity. Identical digestion of C3a-(70-77) resulted in removal of the COOH-terminal arginyl residue and complete loss of activity (Fig. 2*A*). The latter result was previously observed after carboxypeptidase digestion of natural C3a (7).

The synthetic C3a peptides presumably induced smooth muscle contraction by specific stimulation through interaction with C3a receptors. Application of any of the four synthetic C3a oligopeptides at the level shown in Table 2 rendered the muscle strip insensitive to a second challenge from either the oligopeptide or human C3a. Fig. 2*B* illustrates that C3a-(70-77) was specific for blocking C3a activity and vice versa. Ileum strips previously challenged with peptide C3a-(70-77), however, were fully responsive to human C5a, bradykinin, and histamine. The antihistaminic drug chlorpheniramine (Chlortrimeton) prevented contraction by the synthetic C3a oligopeptides (Fig. 2*C*) at the same concentration needed to block contraction by human C3a (8).

Attempts were made to enhance or restore full smooth muscle activity by combining the synthetic oligopeptides with a selectively degraded and inactive C3a derivative. Short-term carboxypeptidase Y digestion of C3a removed the eight residues corresponding to the COOH-terminal octapeptide. Amino acid analysis of this hydrolysate and of the product after dialysis showed that the C3a derivative obtained by the digestion was C3a-(1-69). Ten equivalents of synthetic C3a-(70-77) or C3a-(65-77) was added to 1 equivalent of C3a-(1-69) in 0.05 M Tris·HCl (pH 7.5), the mixture was kept at 25° for 30 min, and the solution was tested for activity. Because smooth muscle contracting activity for these mixtures was the same as that expected for the synthetic oligopeptides alone, a noncovalent complex between C3a-(1-69) and C3a-(70-77) or C3a-(65-77) either failed to form or was no more active than the synthetic oligopeptides.

The effect of peptide C3a-(70-77) was tested on a strip of guinea pig uterine muscle. A rapid and intense contraction was induced when 40 μg (about 50 nmol) of C3a-(70-77) was added to the 10-ml bath. Because comparable uterine contraction was observed with 2.5–5.0 μg (0.3–0.6 nmol) of human C3a, the synthetic octapeptide was about 1% as effective as C3a on a molar basis.

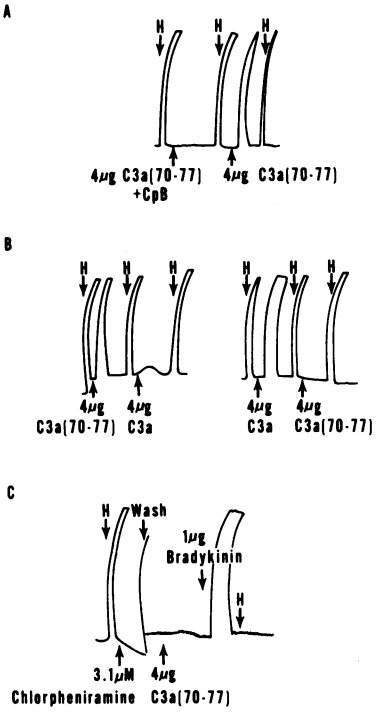

FIG. 2. Contraction of guinea pig ileum by synthetic C3a-(70-77). H = 0.5 μg of histamine added. The recorder chart speed was 3 cm/min and each experiment illustrated took approximately 3–4 min to complete. (*A*) *Left*, no contraction after digestion of C3a-(70-77) with 1% (wt/wt) carboxypeptidase B for 10 min at 37° and pH 8.5; *Right*, full contraction induced by C3a-(70-77). (*B*) Ileum strip was densensitized to contraction with C3a after contraction by C3a-(70-77) and vice versa. (*C*) Antihistaminic chloropheniramine prevented subsequent contraction by C3a-(70-77) but not by bradykinin.

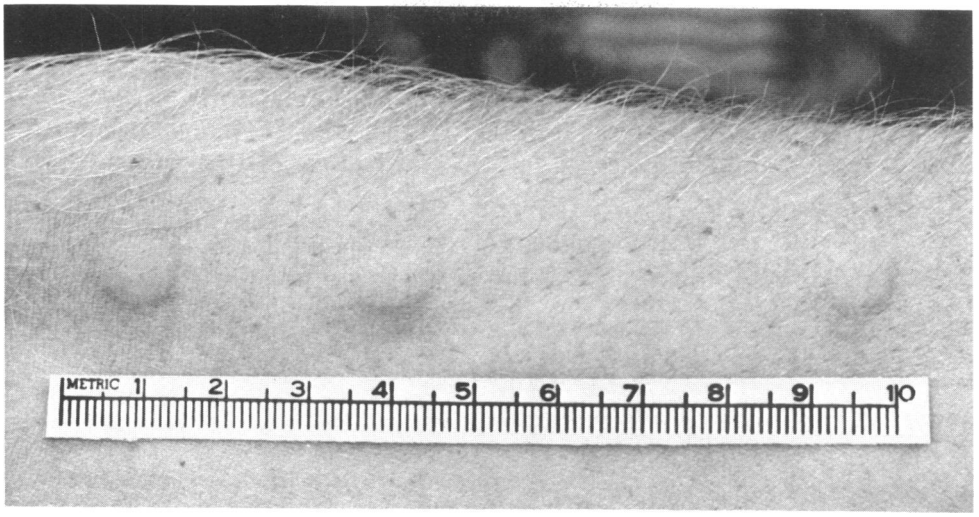

FIG. 3. Wheal and flare of human skin produced by synthetic C3a peptides. Peptide was dissolved in isotonic saline (50 μl) and injected into forearm skin. Left to right: nmol of C3a-(70-77), 10 nmol of C3a-(70-77)-Gly, saline control, and 10 pmol of human C3a. Each peptide gave a wheal of 9–10 cm but the saline control (above the 7-cm mark) gave little response.

Increase in Vascular Permeability. Intradermal injection of anaphylatoxin causes an immediate edema and erythema (wheal and flare) reaction that can be semiquantitatively measured to obtain a dose-response curve. Intradermal injection of synthetic C3a-(70-77)-octapeptide into the human forearm produced the wheal shown in Fig. 3. A visible response to human C3a was observed with 0.01 nmol but 1 nmol of C3a-(70-77) was required to induce a reaction equivalent to natural C3a *in vivo*. Peptide C3a-(70-77)-Gly producd a wheal and flare at a dose of 10 nmol, which was at least 10^3 times greater than necessary to evoke an equivalent skin reaction with C3a. The relative dose-responses for human C3a-(70-77), C3a-(70-77)-Gly, and C3a in human skin are shown in Fig. 4. As found *in vitro* by the guinea pig ileum assay, the relative potency of these peptides *in vivo* was C3a ≫ C3a-(70-77) ≫ C3a-(70-77)-Gly.

Vascular permeability changes were measured by injecting a solution of the peptide in sterile saline into guinea pig skin containing circulating Evans blue dye and then observing the area of tissue bluing. Saline alone produced a faint blue region, 3 mm in diameter, whereas 3 nmol of C3a-(70-77) gave a deep blue area 7–8 mm diameter. Because 0.01–0.02 nmol of human C3a is required to produce a similar response (10), the synthetic octapeptide was also about 1% as active as C3a in this assay.

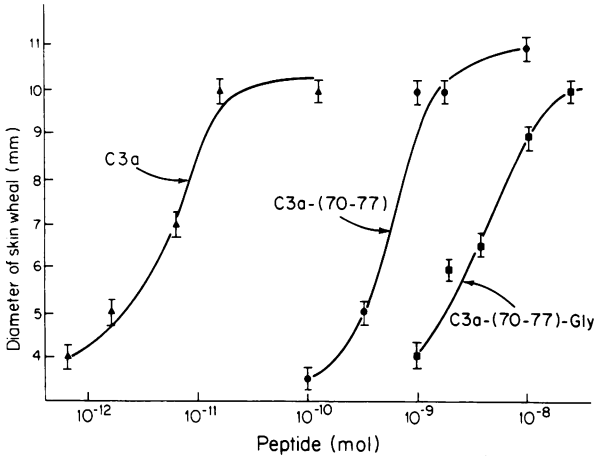

FIG. 4. Cutaneous response versus peptide dose. Diameter of the maximal wheal was measured 10–20 min after injection of peptide in saline (50 μl) into human forearm skin.

Degranulation of Mast Cells. Isolated rat mast cells containing [^{3}H]serotonin were degranulated on treatment with C3a or synthetic C3a-(70-77)-optapeptide. Human C3a and C3a-(70-77) induced the release of 20% of the stored serotonin at concentrations of 20 μM and 4.7 mM, respectively. Thus, in degranulation of mast cells with associated release of serotonin and histamine, the octapeptide was about 1% as active as human C3a, which is consistent with its relative potency in the previously described anaphylatoxin assays.

DISCUSSION

Recent elucidation of the primary structure of human C3a anaphylatoxin has permitted the initiation of synthetic studies to determine if peptides smaller than C3a exhibit biological activity. The synthesis of four oligopeptides was based on the COOH-terminal sequence of C3a. Both C3a-(70-77)-octapeptide and C3a-(65-77)-tridecapeptide effectively caused contaction of guinea pig ileal and uterine smooth muscle at micromolar concentrations and increased vascular permeability in human and guinea pig skin in nanomole quantities. The octapeptide also induced the selective release of vasoactive amines from isolated rat mast cells. These synthetic peptides were about 1–2% as active as human C3a on a molar basis and about 10% as active on a weight basis. Thus, COOH-terminal fragments of human C3a containing 8 or 13 residues elicited the complete range of physiological responses observed with the intact 77-residue polypeptide.

Both the octapeptide and the tridecapeptide exhibited not only the activities but also the specificity of C3a. After treatment with either synthetic peptide, smooth muscle strips from guinea pig ileum were selectively desensitized to contraction induced by human C3a. Neither oligopeptide, however, blocked the action of human C5a or bradykinin on guinea pig ileum. Thus, the synthetic C3a oligopeptides appear to act through cellular receptors that are specific for C3a. This combination of potency and specificity suggests that C3a-(70-77)-octapeptide and related synthetic oligopeptides will be useful pharmacological factors for studying the pathophysiology of C3a anaphylatoxin.

One functionally essential structural feature of the C3a molecule is the COOH-terminal arginyl residue. Previous work

(7) has shown that specific enzymatic removal of arginine-77 by carboxypeptidase B furnishes C3a-(1-76) which neither exhibits anaphylatoxin activity nor blocks smooth muscle contraction induced by intact C3a (1-77). The latter result indicates that the absence of arginine-77 either prevents effective binding, or some other interaction, of C3a-(1-76) with the cellular C3a receptor site. Synthetic C3a-(70-77)-octapeptide, however, exhibits both the activities and the specificity of C3a. Thus, both the binding and stimulatory functions of C3a can be structurally assigned to eight or fewer of its COOH-terminal residues.

The significance of having an arginyl residue located at the COOH-terminal position in C3a was studied with synthetic peptides terminating in a glycyl residue. C3a-(70-77)-Gly and C3a-(65-77)-Gly were only about 2% as active, on a molar basis, as C3a-(70-77) and C3a-(65-77) in stimulating contraction of ileal smooth muscle. Thus, locating arginine-77 in a penultimate rather than terminal position of the synthetic peptide maintained C3a-like activity and specificity but diminished potency by 50-fold in comparison to the octapeptide C3a-(70-77). These results suggest that the carboxyl group of arginine-77 plays a specific functional role that is less adequately fulfilled by the carboxyl group of glycine-77a.

The biological activity of C3a anaphylatoxin is controlled *in vivo* by serum carboxypeptidase B (7). This endogenous enzyme inactivates C3a, C5a, and the kinins by removing their essential COOH-terminal arginyl residues. C3a is inactivated so rapidly by serum carboxypeptidase B that it is difficult to assess its true potency *in vivo*. An advantage of using synthetic peptides in biological studies is that active agents can be structurally modified or designed to prevent or minimize immediate metabolic degradation. One modification that diminishes digestion by carboxypeptidase B is the attachment of a glycyl residue at the COOH terminus of the peptide. For example, carboxypeptidase B attacks the arginyl–glycine peptide bond of Gly-Ala-Leu-Arg-Gly at least 300 times less rapidly than it cleaves the leucyl–arginine bond of Gly-Ala-Leu-Arg (B. W. Erickson and J. J. Hemperly, unpublished data). C3a-(70-77)-Gly was about 10% as active as C3a-(70-77) in increasing the vascular permeability of human skin *in vivo* (Fig. 4). Comparatively, C3a-(70-77)-Gly was only 1–2% as active as C3a-(70-77) for contracting smooth muscle *in vitro*. Although C3a-(70-77)-Gly is inherently 50-fold less active than C3a-(70-77) *in vitro*, its 5-fold greater relative activity *in vivo* is most readily explained by a greater metabolic stability of C3a-(70-77)-Gly.

Previous studies (19) have shown that more than 90% of the potency of natural C3a is lost upon disruption of its secondary structure. The biological activity of native C3a is about 50-fold greater than that of the synthetic octa- or tridecapeptide and this difference is obviously due to the properties that residues 1–64 confer on the terminal portion of the molecule. Residues 1–64 may introduce constraints to residues 70–77, enabling a more favorable conformation for interaction with cellular receptors than exists in the octapeptide alone. However, the anaphylatoxic activity of C3a-(70-77) was not enhanced in the presence of C3a-(1-69). Thus, no evidence could be obtained for the formation of a more active noncovalent complex simply by combining the two C3a fragments. Alternatively, the greater activity of native C3a may be due to additional direct interactions between side chains of residues 1–64 and the cellular receptors. Although the specific interactions contributed by residues 1–64 are uncertain at present, it is clear that this region of C3a quantitatively enhances, but does not qualitatively alter, the anaphylatoxin activity inherent in the COOH-terminal octapeptide.

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