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Immunology

C4a: The third anaphylatoxin of the human complement system

(phlogogenic peptides/C3a and C5a anaphylatoxins/structural homology/cell surface receptors)

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ABSTRACT **The activation peptide C4a was isolated from C1s̄-cleaved C4, the fourth component of complement. The peptide appeared to be homogeneous by electrophoresis on cellulose acetate and by polyacrylamide gel electrophoresis. C4a has a molecular weight of 8650 and an electrophoretic mobility at pH 8.6 of $+2.1 \times 10^{-5}$ cm^2 V^{-1} sec^{-1}. Carboxypeptidase B released approximately 1 mol of arginine per mol of C4a. The partial COOH-terminal sequence was determined to be Leu-Gln-Arg-COOH. The isolated C4a was spasmogenic for guinea pig ileum at a concentration of 1 μM and it desensitized the muscle (i.e., produced tachyphylaxis) with respect to human C3a anaphylatoxin (at 0.33 μM) but not with respect to human C5a anaphylatoxin. Increased vascular permeability was observed in human skin after intradermal injection of 1 nmol of C4a, as evidenced by immediate erythema and edema formation. The spasmogenic, tachyphylactic, and vascular activities of C4a were abrogated by removal of the COOH-terminal arginine, a property that is characteristic also of the C3a and C5a anaphylatoxins. Contamination of C4a with either C3a or C5a has been ruled out by using radioimmunoassays for these peptides. Although C4a is considerably less active than are C3a and C5a, the present observations suggest that C4a constitutes a heretofore unrecognized anaphylatoxin that is related biologically and chemically to the activation peptides of C3 and C5.**

The two known anaphylatoxins, C3a and C5a, are activation peptides of the complement proteins C3 and C5 (1). In the course of complement activation, the peptides are released from their respective precursors by specific complement enzymes. Both peptides induce histamine release from mast cells (2), release hydrolytic enzymes from neutrophils (3), and cause contraction of smooth muscle and increase vascular permeability (1). C5a, but not C3a, also effects directed migration of polymorphonuclear leukocytes (4) and monocytes (5). The complete amino acid sequences of human C3a and C5a have recently been determined (6, 7) and 36% homology in primary structure has been noted (8). Except for chemotactic activity, expression of the various biological activities depends on the presence of the COOH-terminal arginine residue of both peptides.

The NH_2-terminal sequence of C4a (9) suggests that a limited structural homology exists between C4a and both C3a and C5a. In view of these apparent structural similarities, a search was conducted for possible biological activities of C4a. The results show that C4a possesses activities that qualitatively resemble those of the two known anaphylatoxins.

MATERIALS AND METHODS

Purification of C4a. The method of purification will be described in detail elsewhere (10). Briefly, 450 mg of C4, isolated as described (11), was subjected to treatment with 4.5 mg of C1s̄ (12) for 1 hr at 37°C at pH 7 in 0.1 M Tris acetate buffer containing 0.15 M NaCl and 0.01 M EDTA. The pH was decreased to 4.5 with glacial acetic acid and the protein solution was held for 2 hr at 4°C to facilitate dissociation of C4a from cleaved C4. The pH was then adjusted to 6.5 and the material was applied to a CM-Sephadex A-50 column equilibrated with 0.05 M sodium acetate, pH 6.5/0.01 M EDTA/15 mM benzamidine·HCl/0.05 M ε-amino-*n*-caproic acid/0.02% NaN_3. The column was thoroughly washed with the same buffer to remove C4b and C1s̄, and C4a was eluted with a concentration gradient of NaCl to a limiting concentration of 0.3 M. The material eluting at a conductivity of 22 mS was concentrated by lyophilization and subsequently applied to a column of Sephadex G-50 that was equilibrated with 0.15 M Tris acetate pH 8 buffer.

Preparation of Human C3a and C5a. Human C3a was isolated by the procedure of Hugli *et al.* (13) and human C5a was isolated by that of Fernandez and Hugli (14).

COOH-Terminal Analysis. Approximately 50 μg of C4a (6 nmol) was boiled in 0.1 M *N*-ethylmorpholine buffer (pH 8.5) for 10 min. Carboxypeptidase B, 1% (wt/wt), was added and the mixture was incubated for 1 hr at 37°C. The reaction was terminated by boiling for 10 min, and this mixture was analyzed for free amino acids in a Beckman 121 M AutoAnalyzer. Seventeen nanomoles of C4a from which the COOH-terminal arginine was removed (des-Arg-C4a) in 0.1 M pyridine acetate buffer at pH 5.5 was boiled for 10 min and then subjected to digestion with carboxypeptidase Y (a gift from R. Hayashi, Kyoto University, Japan) at 1 or 0.15% (wt/wt) at 37°C. Samples were withdrawn at selected intervals and treated as described above.

Analytical Zone Electrophoresis. Electrophoresis was performed on cellulose acetate strips in a Beckman microzone electrophoresis apparatus, model R-101, with barbital buffer (pH 8.6; ionic strength, 0.075) at 250 V for 20 min at 20°C.

Bioassays. Smooth muscle contraction was measured on segments of isolated guinea pig ileum (1). Erythema and edema formation in the human skin was tested and quantitated as described (15). Chemotactic activity was tested by using the chemotaxis under agarose method (16) with human peripheral blood polymorphonuclear neutrophils.

RESULTS

Comparative Electrophoretic Analysis of Isolated C4a. Fig. 1 shows the electrophoretic behavior of isolated C4a on cellulose acetate at pH 8.6; no impurities were detectable. Its electrophoretic mobility was estimated to be $+2.1 \times 10^{-5}$ cm^2 V^{-1} sec^{-1}, which is identical with that of human C3a. C5a is less cationic than either C4a or C3a. Polyacrylamide gel elec-

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Abbreviations: C3a and C5a, activation peptides derived from the NH_2 terminus of the α-chains of C3 and C5, respectively; des-Arg-C4a C4a peptide from which the COOH-terminal arginine residue was removed.

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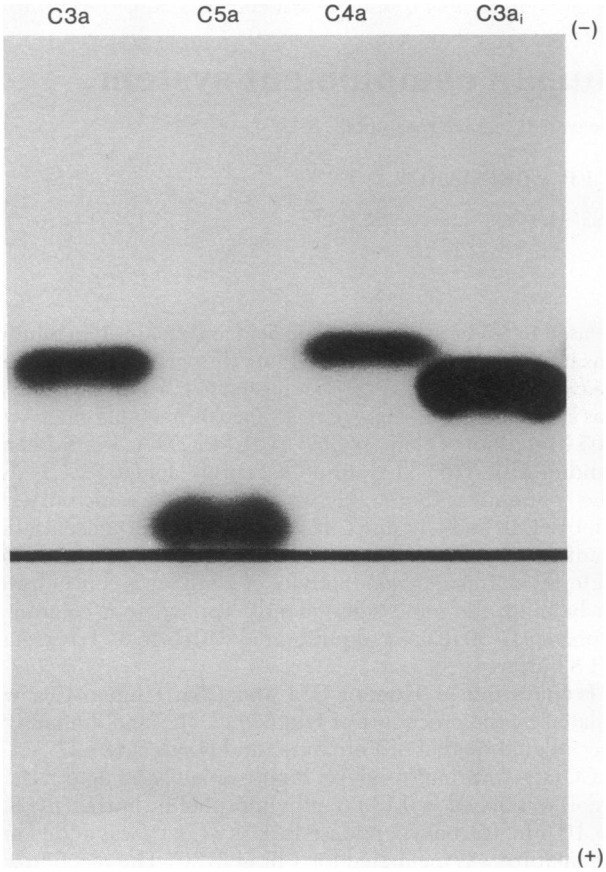

FIG. 1. Comparative zone electrophoretic analysis of C4a with C3a and C5a. Electrophoresis was performed on cellulose acetate strips at pH 8.6. The line indicates the origin. The electrophoretic mobilities of C3a and C4a are indistinguishable. The des-Arg^{77} form of C3a (C3a$_i$) migrated anodally to C3a and C4a, illustrating the effect of a difference of 1 net charge on electrophoretic behavior.

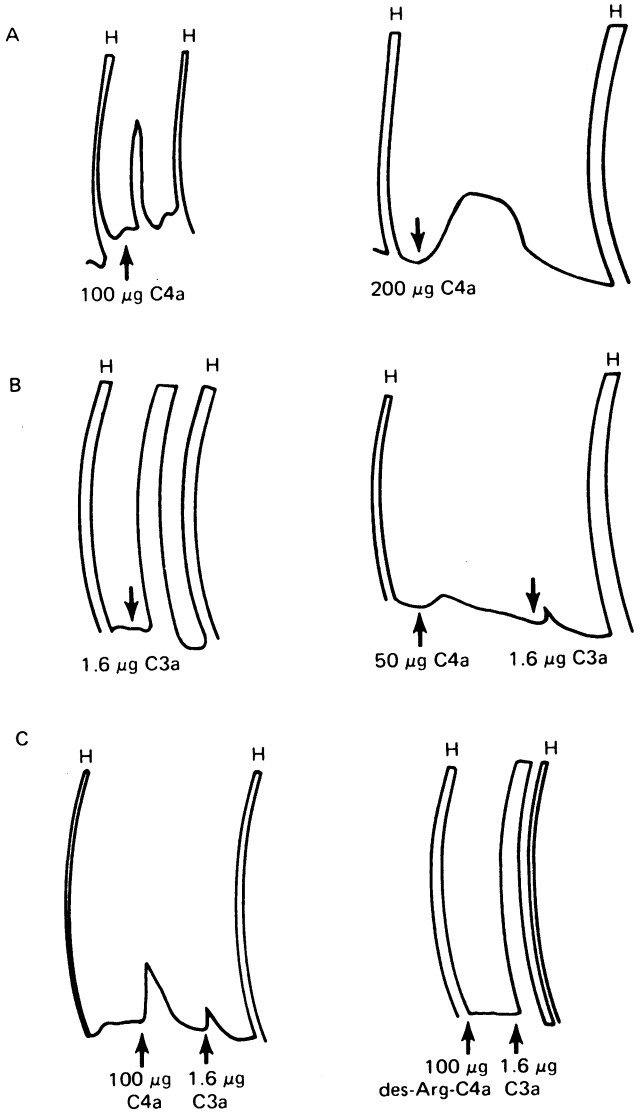

FIG. 2. Demonstration of smooth muscle contracting activity of C4a. (*A*) Contraction of guinea pig ileum by C4a at 100 and 200 μg. (*B*) Desensitization of the ileum to C3a by 50 μg of C4a which did not cause a contraction. (*C*) Desensitization of the ileum to C3a by 100 μg of C4a which did cause a contraction and elimination of C4a activity by removal of the COOH-terminal arginine residue. H, histamine control.

trophoresis in sodium dodecyl sulfate (not shown) revealed a similar degree of homogeneity.

Contraction of Smooth Muscle by C4a. C4a in doses of 20–200 μg was tested for the ability to elicit smooth muscle contraction. Under the experimental conditions used, 100 μg of C4a was required for a positive response (Fig. 2*A*). This amount of C4a corresponds to a concentration of 1 μM in the organ bath. Application of 200 μg of C4a did not significantly increase the magnitude of the contractile response; however, the contraction was prolonged. Spontaneous relaxation of the muscle was observed after each contraction induced by C4a, but relaxation occurred more slowly than with C3a or C5a. As shown in Figure 2*B*, 50 μg of C4a, corresponding to a concentration of 0.5 μM, caused tachyphylaxis of the ileum toward C3a. The dose of C3a applied was 1.6 μg, which, in the absence of C4a, would have induced a full contraction. The minimal dose of C4a necessary to achieve complete desensitization of ileum to C3a was 33 μg. Whether C4a influences the ileal contraction induced by C5a is uncertain. Prior challenge of the muscle with C4a often diminished but never abrogated the response induced by C5a.

In view of the quantities of C4a used in these experiments, contamination with C3a or C5a had to be excluded. C4a was examined by using radioimmunoassays designed for the detection and quantitation of the two peptides. The results showed that the C4a preparations tested contained less than 0.002% of C3a and 0.006% of C5a. Such values represent the lower limits of detection by the methods used, and the corresponding quantities cannot account for the activity observed with C4a preparations.

Removal of the COOH-terminal arginine residue of C4a by carboxypeptidase B abolished the spasmogenic activity of C4a and its ability to render the ileum tachyphylactic toward C3a (Fig. 2*C*). Amino acid analysis showed that 0.9 mol of arginine per mol of C4a had been released by this enzyme.

Lack of C4a Chemotactic Activity. C4a was tested in the chemotaxis under agarose assay at doses ranging from 0.5 to 50 μg/ml. No chemotactic activity was detectable. Under identical conditions, C5a was maximally active at 50 ng/ml.

Erythema and Edema Formation by C4a in Human Skin. C4a was injected intradermally into the forearm of a volunteer in amounts ranging from 0.2 to 3.1 nmol. An immediate erythema and edema reaction was observed; it was measured after 15 min (Fig. 3). An edematous area 10 mm in diameter was

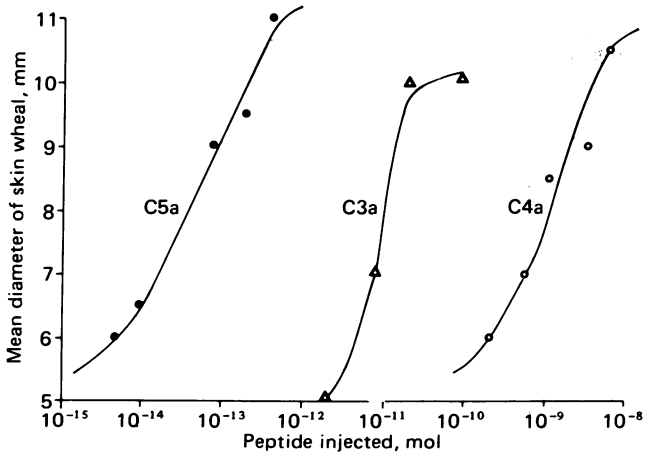

FIG. 3. Erythema and edema formation by C4a in the human skin: comparison with the effects of C3a and C5a. The diameter of the edema (wheal) was measured 10–15 min after intracutaneous injection.

obtained with 1 nmol of C4a. When C3a and C5a were injected at the same time for comparison, the equivalent doses were 10 pmol and 40 fmol, respectively.

Partial COOH-Terminal Sequence of C4a. Because C4a exhibited biological activity that was qualitatively identical to that of C3a and because the COOH-terminal sequence of C3a is known to be primarily responsible for its activity (17), the partial COOH-terminal sequence of C4a was determined. Fig. 4 shows a kinetic analysis of the release of amino acid residues by carboxypeptidase Y. The data indicate the following sequence: Leu-Gln-Arg-COOH.

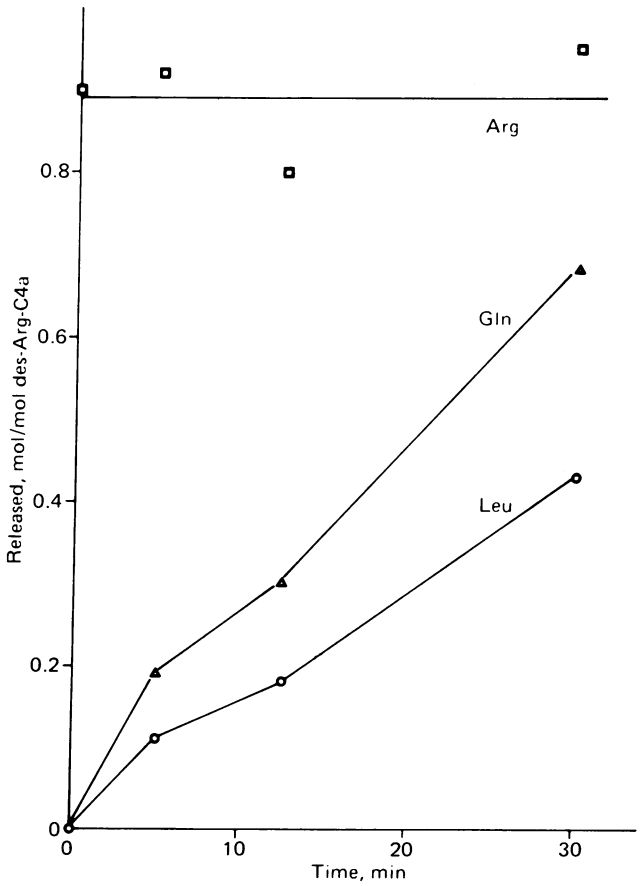

FIG. 4. Kinetics of release of amino acids from des-Arg-C4a by carboxypeptidase Y. The des-Arg-C4a was obtained by prior digestion with carboxypeptidase B. During subsequent digestion with carboxypeptidase Y, no further arginine was released. The COOH-terminal sequence accordingly was established as Leu-Gln-Arg-COOH.

Table 1. COOH-Terminal sequences of C4a, C3a, and C5a

C4a	-Leu-Gln-Arg-COOH
C3a	-Leu-Ala-Arg-COOH
C5a	-Leu-Gly-Arg-COOH

DISCUSSION

We have shown that C4a expresses several biological acitivities: it induces contraction of guinea pig ileum, desensitizes this muscle selectively to the action of C3a anaphylatoxin, and produces an immediate erythema and edema when injected into human skin. The known activities of C4a are dependent on the presence of its COOH-terminal arginine residue. According to its biological and chemical properties, C4a may be regarded as a representative of the family of activation peptides called analphylatoxins. The data presented here indicate that a similarity in structure exists between the COOH-terminal region of C4a and the COOH-terminal sequences of C3a and C5a (6, 7) (Table 1). The COOH-terminal residue of all three peptides is arginine and the third residue from this terminus is leucine. In studies with active synthetic C3a pentapeptides, it was shown that the leucine corresponding to position 75 of native C3a is functionally very important and that replacement of this residue by an alanyl residue greatly diminished its activity (18). Because of its anaphylatoxin-like functional properties and its COOH-terminal sequence, C4a was also subjected to NH_2-terminal sequence analysis (9). Approximately 38% homology in primary structure was found between C4a and C3a or C5a after a manual alignment of corresponding NH_2-terminal regions. The similarity among the three complement activation peptides extends to their conformations (19, 20); all three peptides possess a high content of α-helical structure according to their circular dichroism spectra (10).

The quantitative differences between C4a activity and the activity of C3a or C5a are considerable. C4a expresses only 1% of the spasmogenic activity of C3a and only 0.05% of that of C5a. In the human skin, C3a is 100 times more active and C5a about 25,000 times more active than C4a. However, 1 nmol of C4a, the half-maximal dose for erythema and edema formation, is nonetheless a small quantity of material by comparison with many other biologically active substances. Two to 3 times this amount of C4a is potentially available in 1 ml of human serum. Like C3a, the C4a peptide was found to be devoid of chemotactic activity, whereas C5a is known to be a potent chemotactic factor. It therefore appears on the basis of functional properties that C3a and C4a are most closely related and act on common receptors of smooth muscle. In contrast, C4a and C5a appear to be less related functionally because they differ in chemotactic activity and act on distinct receptors of smooth muscle.

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1. Cochrane, C. G. & Müller-Eberhard, H. J. (1968) *J. Immunol.* **127**, 371–386.
2. Chenoweth, D. E. & Hugli, T. E. (1979) *Mol. Immunol.*, in press.

3. Johnson, A. R., Hugli, T. E. & Müller-Eberhard, H. J. (1975) *Immunology* **28,** 1067–1080.
4. Fernandez, H. N., Henson, P. M., Otani, A. & Hugli, T. E. (1978) *J. Immunol.* **120,** 109–115.
5. Snyderman, R., Shin, H. S. & Hausman, M. H. (1971) *Proc. Soc. Exp. Biol. Med.* **138,** 387–390.
6. Hugli, T. E. (1975) *J. Biol. Chem.* **250,** 8293–9301.
7. Fernandez, H. N. & Hugli, T. E. (1978) *J. Biol. Chem.* **253,** 6955–6964.
8. Fernandez, H. N. & Hugli, T. E. (1977) *J. Biol. Chem.* **252,** 1826–1828.
9. Gorski, J. P., Hugli, T. E. & Müller-Eberhard, H. J. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 1010.
10. Gorski, J. P., Hugli, T. E. & Müller-Eberhard, H. J. (1979) *Biochemistry,* in press.
11. Bolotin, C., Morris, S., Tack, B. & Prahl, J. (1977) *Biochemistry* **16,** 2008–2015.
12. Valet, G. & Cooper, N. R. (1974) *J. Immunol.* **112,** 339–350.
13. Hugli, T. E., Vallota, E. H. & Müller-Eberhard, H. J. (1975) *J. Biol. Chem.* **250,** 1472–1478.
14. Fernandez, H. N. & Hugli, T. E. (1976) *J. Immunol.* **117,** 1688–1694.
15. Wuepper, K. D., Bokisch, V. A., Müller-Eberhard, H. J. & Stoughton, R. B. (1972) *Clin. Exp. Immunol.* **11,** 13–20.
16. Chenoweth, D. E., Rowe, J. G. & Hugli, T. E. (1979) *J. Immunol. Meth.* **25,** 337–353.
17. Hugli, T. E. & Erickson, B. W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1826–1830.
18. Unson, C. G., Erickson, B. W. & Hugli, T. E. (1979) *Peptides: Structure and Biological Function,* Proceedings of the Sixth American Peptide Symposium, eds. Gross, E. & Meienhofer, J. (Pierce Chem. Co., Rockford, IL), in press..
19. Hugli, T. E., Morgan, W. T. & Müller-Eberhard, H. J. (1975) *J. Biol. Chem.* **250,** 1479–1483.
20. Morgan, W. T., Vallota, E. H. & Müller-Eberhard, H. J. (1974) *Biochem. Biophys. Res. Commun.* **57,** 572–577.