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SYNTHETIC C5a RECEPTOR AGONISTS

PHARMACOLOGY, METABOLISM AND *IN VIVO* CARDIOVASCULAR AND HEMATOLOGIC EFFECTS

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Abstract—Recent investigations have produced novel compounds that act on the receptor for anaphylatoxin C5a. These products are C-terminal analogues of C5a, some of which are modified extensively. We have measured the receptor affinities of such analogues in a binding assay on human neutrophils (PMNs). We have also characterized their pharmacological profiles *in vitro* on the isolated rabbit portal vein and pulmonary artery, on superoxide release by PMNs as well as *in vivo* in the anesthetized rabbit (acute hypotensive and neutropenic effects). The metabolic resistance of these analogues was also evaluated in the presence of different peptidases. One of these compounds, MePhe-Lys-Pro-D-Cha-Phe-D-Arg, behaved as an antagonist on the release of superoxide by neutrophils while exerting agonist activity in all other assays. Its partial agonist status was documented in a receptor down-regulation experiment on PMNs where its activity was compared with those of recombinant C5a and of protamine which behaves as a competitive antagonist on these cells. Degradation studies indicated that the discrepancy between the affinity of certain analogues *in vitro* and their potency *in vivo* was probably linked to their metabolic stability.

Within the multifunctional complement system, anaphylatoxin C5a plays a prominent role as a pharmacologically active fragment of C5 that activates various leukocyte functions (reviewed by Hugli [1]). Basophils and mast cells release histamine and other secondary mediators in response to C5a [1, 2]. In phagocytic leukocytes, namely neutrophils and monocytes/macrophages, several distinct activation pathways are stimulated by C5a: there is a chemotactic and chemokinetic response, an increased eicosanoid formation, a release of reactive radicals and of granule content [1], an increased adherence to cultured endothelium [3] and a potentiation of the synthesis of interleukins 1 and 6 in monocytes [4–6]. The receptor for C5a has been cloned and sequenced recently from human myeloid-like cell lines [7, 8]. It belongs to the rhodopsin superfamily of signaling proteins, consistent with the role of a G protein in its signal transduction [9].

The *in vivo* pharmacology of C5a is characterized by two broad types of events. First, effects on circulating leukocytes are prominent and depend on the route of administration. Local administration (e.g. intratracheal instillation, intradermal injection) promotes the migration of polymorphonuclear leukocytes (PMNs)† from the blood into the tissues [10, 11]. C5a, given by the intravenous route in the rabbit, produces an acute fall of the circulating neutrophil count, interpreted as transient adherence to the blood vessel walls, followed by leukocytosis [12]. Recent evidence suggests that, under these circumstances, the bone marrow is actively delivering neutrophils to the blood, some of which are immature forms, and that eicosanoids are not involved in the biphasic neutropenia/neutrophilia response [13, 14]. The second type of *in vivo* response to C5a is dependent on secondarily released mediators and may affect many systems. Examples of this phenomenon are bronchospasm in guinea pigs and, in several species, a complex cardiovascular response that includes hypotension with pulmonary hypertension and decreased cardiac output [12, 15]. In the rabbit, the cardiovascular response is mainly dependent on the production of vasoconstrictor and vasodilator eicosanoids, such as thromboxane A_2 and 6-keto-prostaglandin $F_{1\alpha}$, and is independent of circulating, neutrophils [12]. Isolated blood vessels from the rabbit [16, 17], guinea pig [18] and humans [19] exhibit *in vitro* mechanical responses to C5a that are not tachyphylactic and are dependent mainly on eicosanoids; other mediators such as histamine and nitric oxide are possibly involved in minor components of the response [16–18]. There is significant evidence suggesting that responses to C5a in smooth muscle preparations are ultimately explained by the presence of tissue forms of leukocytes (mast cells [1] or resident macrophages [19]).

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† Abbreviations: ACE, angiotensin-converting enzyme; AmM, aminopeptidase M; Ac-Phe, *N*-acetyl-phenylalanine; BSA, bovine serum albumin; Cha, cyclohexylalanine; CPN, carboxypeptidase N; DPN, 1,2-diphenyl-4-[3-(1-naphthyl)-propyl]-3,5-pyrazolidinedione; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; MePhe, *N*-methyl-phenylalanine; mergetpa, DL-mercaptomethyl-3-guanidinoethylthiopropanoic acid; and PMNs, polymorphonuclear leukocytes.

The powerful activities of C5a *in vivo* are regulated by arginine-carboxypeptidases found in blood serum and also at the cell surface: the carboxypeptidases M and N [20, 21]. Other unstable arginine-carboxypeptidases are also formed in plasma during coagulation [22, 23] and may, in some instances, participate in C5a inactivation. C5a-des-Arg, the resulting molecule, exhibits only a small fraction of the potency of the parent molecule in binding and functional assays [1]. There is evidence that C5a internalization by leukocytes is followed by extensive proteolysis [24]. Indeed, the concentration of immunoreactive C5a-des-Arg is very low in biological fluids relative to the expected turn-over of C5 and to C3a-des-Arg and C4a-des-Arg concentrations [25–27].

The recent success of the synthesis of C5a receptor agonists modeled on the C-terminal region of the molecule [28–31] has permitted a new approach to the study of C5a actions and metabolic inactivation. Synthetic agonists with varying degrees of affinity and metabolic resistance have been tested in comparison to C5a in increasingly complex biological assays. Ultimately, the complex integrated cardiovascular and hematologic responses to anaphylatoxin C5a in the rabbit have been reproduced by the metabolically resistant analogues with a potency approaching that of C5a.

MATERIALS AND METHODS

Drugs

Indomethacin, amastatin, bestatin, captopril, salmon protamine (type X), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and human recombinant C5a from *Escherichia coli* [32] were purchased from Sigma (St. Louis, MO). DL-Mercaptomethyl-3-guanidinoethylthiopropanoic acid), (mergetpa) was purchased from Calbiochem (La Jolla, CA). 1,2 - Diphenyl - 4 - [3 - (1 - naphthyl) - propyl] - 3,5 - pyrazolidinedione (DPN), a non-peptide FMLP antagonist [33, 34], was purchased from the Alfred Bader Chemical Library of Rare Chemicals (Milwaukee, WI).

Synthesis of C-terminal analogues of C5a and HPLC analysis

C5a analogues were synthesized by solid phase methodology using equipment and methods previously described [35,*]. HPLC analysis of pure peptides or samples from metabolic studies were performed using a Waters system equipped with a 746 data module and a 486 UV detector set at 214 nm. Separations were achieved with a Vydac 10 μm (3.9 × 300 mm) reverse phase C_{18} column using a linear gradient of 5–65% acetonitrile/0.05% trifluoroacetic acid (TFA)/water at 2 mL/min over a period of 20 min. For degradation studies, 50 μL of each aliquot was injected and rates of peptide metabolism were calculated from the decrease of peptide substrate concentration.

Metabolic stability of C5a analogues

The metabolic resistance of C5a analogues was evaluated in rabbit plasma as a potential source of aminopeptidase M (AmM) and carboxypeptidase N (CPN) activities, with a kidney microvillar membrane preparation (neutral endopeptidase 24.11 activity), with purified angiotensin-converting enzyme (ACE) and in the presence of neutrophils. Rates of hydrolysis, evaluated by HPLC as described above, are expressed as substrate consumption (in nmol) per minute per quantity of enzyme preparation (mg of protein, mL of plasma or number of cells). Integration of peak areas and quantitation of peptide substrate were calculated automatically by the data module. Metabolites, arising from cleavage of substrate by enzyme preparations, were observed in every case, as peaks with retention times different from the peptide substrate, but were not identified. Rates of hydrolysis were directly proportional to the time of incubation and the amount of enzyme preparation.

Rabbit plasma. Blood was obtained from the carotid artery of anesthetized rabbits and drawn into heparinized tubes. The tubes were centrifuged at 500 *g* for 15 min in a refrigerated table top centrifuge. The plasma was aliquoted and kept at −20° until used. For measurement of plasma activity, peptides (50 μM) were placed in a Tris buffer (pH 7.5) containing 300 mM NaCl, 0.1 mM $CoCl_2$ and 10 μM captopril. The sample was incubated for 5 min at 37° before adding 15 μL of plasma (total volume 1000 μL). Aliquots were taken at 0, 15, 30 and 60 min, immersed in boiling water for 5 min and centrifuged before HPLC analysis. Selective evaluation of CPN or AmM activity was obtained, respectively, by adding an AmM inhibitor (amastatin; 10 μM) or a CPN inhibitor (mergetpa; 10 μM) in the sample prior to incubation at 37°.

Crude membrane preparation from rabbit kidneys. Kidneys were perfused *in situ* with saline containing heparin (10 U/mL) and semi-purified neutral endopeptidase was prepared according to Fagny *et al.* [36]. Briefly, the cortex was homogenized in a Hepes buffer (pH 7.5 with 10 mM $MgCl_2$). The homogenate was centrifuged at 1,500 *g* and the supernatant collected and centrifuged for 20 min at 15,000 *g*. The pellet was washed, rehomogenized in the Hepes buffer without $MgCl_2$, aliquoted and stored at −20°. Protein concentration was measured by the method of Lowry *et al.* [37]. Peptides (50 μM) were incubated at 37° with the membrane preparation (final protein concentration 4.3 μg/mL) in a 50 mM Tris–HCl buffer, pH 7.6, containing bestatin (0.1 mM) and captopril (20 μM). The total incubation volume was 1000 μL and aliquots were withdrawn periodically, mixed with 20 μL of 10% TFA/water, and centrifuged.

Purified ACE. Purified ACE (from rabbit lungs; Sigma) was dissolved in the same buffer used for plasma assays but without inhibitors or $CoCl_2$. The incubation conditions were also the same except for the use of 1 μL of ACE (final concentration 1 μg protein/mL) as a source of enzyme instead of plasma.

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Isolated neutrophils. Neutrophils were prepared from heparinized venous blood obtained from healthy volunteers according to Böyum [38]. Peptides (50 μM) were incubated with 6×10^5 neutrophils in Hanks' balanced salt solution (HBSS), total volume 200 μL, for 0, 15, 30 and 60 min. At the end of the incubation the sample was centrifuged at 2000 *g* for 1 min and 120 μL of the supernatant was withdrawn and boiled for 5 min before HPLC analysis.

Binding to human PMNs

Human recombinant C5a (Sigma) was ^{125}I-labeled using Iodogen (Pierce, Rockford, IL). A solution of C5a, 10 μg in 25 μL HBSS, was placed in a 1-mL Iodogen-coated tube; 500 μCi of $Na^{125}I$ (Amersham, Oakville, Ontario) was added and the mixture allowed to react for 15 min. The reaction was stopped by removing the mixture from the reaction tube and mixing it with a solution of 0.25 M KI and Na_2SO_4 in HBSS. The free iodine was separated from the labeled protein using a PD-10 column (Pharmacia, Baie D'Urfé, Québec) equilibrated with HBSS buffer containing 0.1% (BSA) bovine serum albumin. The specific activity range was 220–570 Ci/mmol.

For the binding assay, a suspension of PMNs (5×10^6/mL) in HBSS containing 0.25% BSA was incubated for 60 min at 0° with the appropriate concentration of [^{125}I]C5a. Total volume for binding assays was 200 μL. PMN-bound radioligand was then separated from the unbound fluid phase by centrifugation of a 100-μL sample of the cell suspension through a 3:7 (v/v) mixture of dioctyl phthalate:dibutyl phthalate (Aldrich Chemical Co., Milwaukee, WI) for 1 min at 10,000 *g* in a microfuge (Beckman, Fullerton, CA) [39]. The tubes were then cut in half with scissors for evaluation of cell-bound [^{125}I]C5a in the cell pellet.

Down-regulation of C5a receptors

Additional evidence of the agonist or antagonist behavior of C5a receptor ligands was obtained in the form of receptor down-regulation. Acute exposure to C5a at 37° results in an apparent loss of binding sites, secondary to receptor–ligand internalization, without significant changes in the affinity of the residual receptors [40]. In the present study, a suspension of PMNs (5×10^6/mL) in HBSS containing 0.25% BSA was incubated for 10 min at 37° in the presence of a cold ligand (C5a, a synthetic peptide, protamine or no ligand in controls) at a concentration proven to occupy a large fraction of the binding sites. After this incubation, the cells were washed twice with ice-cold HBSS–BSA and the cells were reincubated as above at 0° to measure the binding of 2 nM [^{125}I]C5a (non-specific binding evaluated under each condition in the presence of a 100 nM concentration of cold C5a).

Superoxide anion release by neutrophils

Superoxide production was monitored as the reduction of cytochrome *c* for 5 min at 37° in tubes containing 2.5×10^6 neutrophils, 62.5 μM cytochrome *c* (type VI, Sigma) and C5a or the desired analogue (total volume of 1 mL). The reactions were stopped by transferring the tubes to an ice-cold bath and the addition of superoxide dismutase (final concentration, 62.5 μg/mL) followed by centrifugation. The absorbance was read at 550 nm and the amount of superoxide produced was calculated by using an extinction coefficient of 21,000 $M^{-1}\,cm^{-1}$. Conditions tested included the direct analogue-induced superoxide release (possible agonist action) and the inhibitory effect of analogues or drugs on C5a- or FMLP-induced effect (antagonist action).

Isolated blood vessels

Rabbit vascular strips were prepared as described [41] and were suspended in 5-mL tissue baths containing Krebs solution [18]. Their responses to agents were recorded using isometric transducers (model 52-9545, Harvard Bioscience, South Natick, MA). The pulmonary artery and portal vein segments were cut helically and subjected to a baseline tension of 1 and 0.5 g, respectively. After an equilibration period of 1 hr, tissues were challenged with one of the chemotactic agents. The response to C5a is predominantly a relaxation. To record this type of response, tissues were always contracted with a submaximal concentration of phenylephrine (500 nM) for 10–30 min, which is the period required to reach a contraction plateau, before injecting the chemotactic peptides [17]. The effects of C5a or

Table 1. Primary structure of the C-terminal portion of human and porcine C5a and of synthetic analogues*

	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
huC5a 60–74	Gln	Leu	Arg	Ala	Asn	Ile	Ser	His	Lys	Asp	Met	Gln	Leu	Gly	Arg-COOH
poC5a 60–74	Gln	Val	Arg	Ala	Glu	Gln	Ser	His	Lys	Asn	Ile	Gln	Leu	Gly	Arg-COOH
I	Tyr	Val	Arg	Ala	Glu	Gln	Ser	Phe	Lys	Asn	Ile	Gln	Leu	Gly	Arg-COOH
II							Ile	Ser	Phe	Lys	Asn	Ile	Gln	Leu	Gly	Arg-COOH
III							Ac-Phe	Lys	Asp	Cha	Cha	Val	D-Ala		Arg-COOH
IV							MePhe	Lys	Pro	D-Cha	Phe	D-Arg			COOH

* Analogues I and II were developed from the porcine sequence and analogues III and IV from the human sequence, which are shown for comparison. Substituted residues are underlined. Abbreviations: Cha, cyclohexylalanine; Ac-Phe, *N*-acetyl-phenylalanine; and MePhe, *N*-methyl-phenylalanine.

Table 2. Rates of hydrolysis of analogues II–IV by different enzyme preparations*

	Metabolic degradation				
	Plasma		Purified	Kidney	Human
Analogue	AmM (nmol/min/mL)	CPN (nmol/min/mL)	ACE (nmol/min/mg)	extract (nmol/min/mg)	neutrophils (nmol/min/10^6 cells)
II	13 ± 5	20 ± 5	500 ± 50	69 ± 8	0.9 ± 0.2
III	NS	NS	NS	6 ± 3	0.3 ± 0.2
IV	NS	NS	NS	NS	0.2 ± 0.1

* Values are the means ± SEM of at least three determinations; NS, not significant. Assays were performed at a final substrate concentration of 50 μM. Total incubation volume was 1 mL in which the following quantities of enzyme preparation were used for individual assays: plasma, 15 μL; ACE, 1 μg; kidney extract, 4.3 μg of protein; and human neutrophils, 6×10^5 cells.

related peptides were tested up to four times at 90-min intervals, with extensive washings between the tests. Results are expressed as the percentage of relaxation of the phenylephrine-induced plateau.

In vivo *evaluation of C5a receptor agonists*

The hemodynamic and hematologic effects of C5a and analogues were evaluated using New Zealand white rabbits of either sex (1.5 to 2.0 kg). The animals were anesthetized with sodium pentobarbital (30 mg/kg, i.v., adjusted individually). Lidocaine (2%) was used in addition at sites of incision. The trachea was intubated and ventilatory assistance was provided with a Harvard respiratory pump. A polyethylene catheter PE-90 was inserted into the right common carotid artery and pushed into the aorta for recording blood pressure with a pressure transducer (Statham P23ID) on a Grass polygraph (model 79). A Grass tachograph (model 7P44C) was coupled electronically to the blood pressure recording system and provided a continuous recording of the heart rate. The carotid artery catheter was also used to obtain small blood samples at specific times for recording hematological responses to C5a. To avoid clotting in the catheter, the animals were given 400 U of heparin at the beginning of the experiment. A second PE-90 catheter was inserted into the left external jugular vein for intravenous injections. This *in vivo* model has been used to perform a comparative evaluation of C5a and of the new agonists synthesized with the following end points: acute blood pressure changes, acute neutropenia. A given animal was not injected more than twice with C5a or a related analogue, with a 45-min interval between doses; under these conditions, the response exhibits little tachyphylaxis [12]. Each hypotensive episode was characterized quantitatively by the maximal response (fall of the mean blood pressure, mm Hg) and by the time course, evaluated by the period starting at the bolus injection and ending when half of the maximal hypotensive response had faded (time for half-recovery).

Arterial blood samples were obtained 5 min before and 5 and 30 min after C5a injection for a determination of the white blood cell counts (in a hemacytometer after lysis of erythrocytes in 1% acetic acid) and a differential leukocyte count (following Wright staining of blood smears). The results of the leukocyte counts are expressed as a percentage of the baseline value (count 5 min before C5a) for neutrophils and lymphocytes; other leukocyte types are infrequent in rabbit peripheral blood [42].

Statistics

Results are expressed as means ± SEM. Student's *t*-test, one-way analysis of variance, Dunnett's test and linear regression were used in the form of a software package [43] to analyze the data.

RESULTS

Peptide synthesis and metabolic studies

The primary structures of the analogues synthesized for this study are listed in Table 1. Analogue I has been reported by Ember *et al.* [28]; it is porcine C5a 60–74 with the Tyr^{60} and Phe^{67} substitutions. Peptide II is a shortened version of analogue I in which we have substituted an Ile residue for the N-terminal Gln^{65}. Analogue III is an extensively modified analogue of the C-terminal octapeptide of human C5a. Its structure is based on a compound (Ac-His-Lys-Asp-Cha-Cha-Val-D-Ala-Arg-OH) reported by Kawai *et al.* [30], that we have modified slightly to include the N-terminal Phe^{67} for His substitution shown to improve affinity for the receptor [28, 29]. Analogue IV is a hexapeptide introduced by Mollison *et al.* [31] in a report where its activity on human neutrophils is described.

Analogues II–IV were incubated with different enzyme preparations in order to examine their metabolic stability. These include plasma, in which AmM and CPN activity were measured separately, purified ACE, a semi-purified neutral endopeptidase preparation and incubation with intact human neutrophils. Although the degradation of peptides was evaluated by decreasing substrate concentration, metabolite peaks, that increased as a function of time, were observed in parallel with substrate decrease. No attempt was made to identify these metabolites. Results presented in Table 2 show that in plasma, where selective CPN or AmM activities were examined, analogue II was metabolized at comparable rates by either enzyme while the other

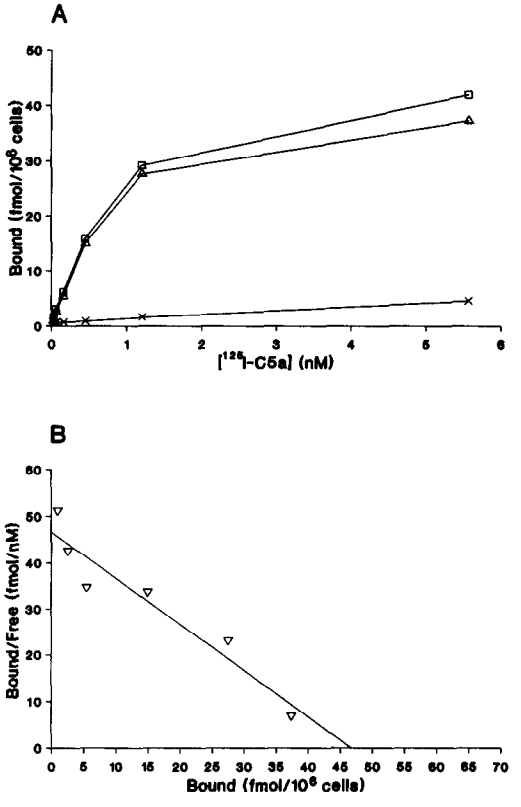

Fig. 1. Binding of [^{125}I]C5a to intact human PMNs. The experiments were performed simultaneously with the cells of a single donor. (A) Total (□), non-specific (×) and specific (△) binding in the 18 pM to 5.6 nM range of radioligand. Non-specific binding was established in the presence of a 100 nM concentration of cold C5a (1 μM for the 5.6 nM concentration level of [^{125}I]C5a). (B) Scatchard plot analysis derived from the same data and assuming a single class of binding sites. The correlation coefficient was 0.958. K_D and B_{max} values derived from the Scatchard analysis were 1.0 nM and 46.7 fmol/10^6 cells, respectively.

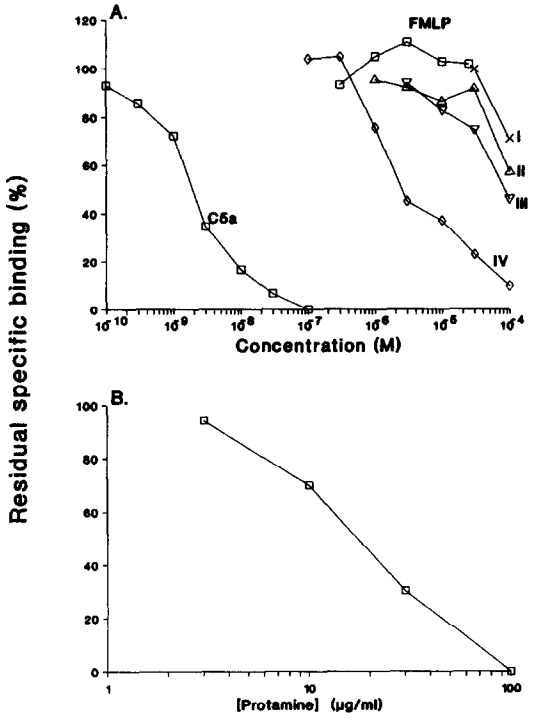

Fig. 2. Competition for [^{125}I]C5a (2 nM) receptor binding to intact PMNs by cold C5a, synthetic analogues, and the unrelated chemotactic peptide FMLP (A) and by protamine (B). Values are the means of triplicate determinations from each one of three separate experiments. The ordinate scale is the residual binding relative to the total binding without competing drug established for each donor. The non-specific binding was subtracted.

two analogues were completely resistant. In fact at substrate concentrations of 50 μM, analogue II was degraded by all preparations tested while, inversely, analogues III and IV were found resistant to all except for some degradation by neutrophils and marginal catabolism of analogue III by the kidney extract. The rate for the catabolism of analogue II by ACE (500 nmol/min/mg protein) was almost ten times higher than the one observed with bradykinin [44], a peptide that also possesses a C-terminal Arg residue, whereas the rate for NEP degradation of this peptide was about half that observed with bradykinin analogues.* Enzymes involved in the degradation of the C5a analogues by intact neutrophils were not identified and, although peaks probably attributable to metabolites were observed by HPLC analysis, part of the decrease in substrate concentration may be due to uptake by neutrophils.

Studies in human neutrophils

We compared the activities of C5a and of its mimetic analogues in a well characterized model, the isolated human neutrophil, to establish their relative potencies and agonist status. The binding of [^{125}I]C5a to intact human PMNs was performed at 0° and it was found to be saturable; the proportion of non-specific binding was relatively small (Fig. 1A). Scatchard plot analysis of the data (Fig. 1B) revealed a single class of receptors with K_D and B_{max} values of, respectively 1.0 nM and 46.7 fmol/10^6 cells (about 28,000 receptors per cell in this particular experiment). These values are similar to those previously reported for the binding of purified C5a to intact human PMNs [40, 45]. The abilities of C5a and its C-terminal analogues to displace the binding of [^{125}I]C5a at 0°, were evaluated (Fig. 2). The unrelated chemotactic peptide, f-Met-Leu-Phe (FMLP), and protamine, a proposed C5a antagonist [46], were also tested. All the C-terminal analogues of C5a were at least 1000-fold less potent than the recombinant cold protein to compete for binding. The order of potency was C5a > IV > III > II > I. FMLP failed to compete for C5a binding sites.

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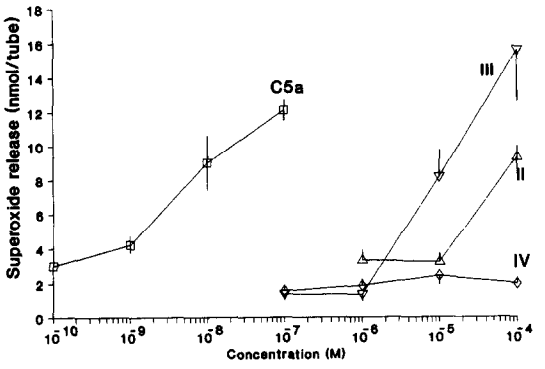

Fig. 3. Superoxide anion release from human PMNs stimulated with C5a or with synthetic analogues. Tubes contained 2.5×10^6 neutrophils. The control absorbance reading (no stimulus) was subtracted from the other readings. Values are the means ± SEM of 3–9 determinations. The baseline release in cells incubated at 37° without stimulus was 4.0 ± 0.03 nmol/tube.

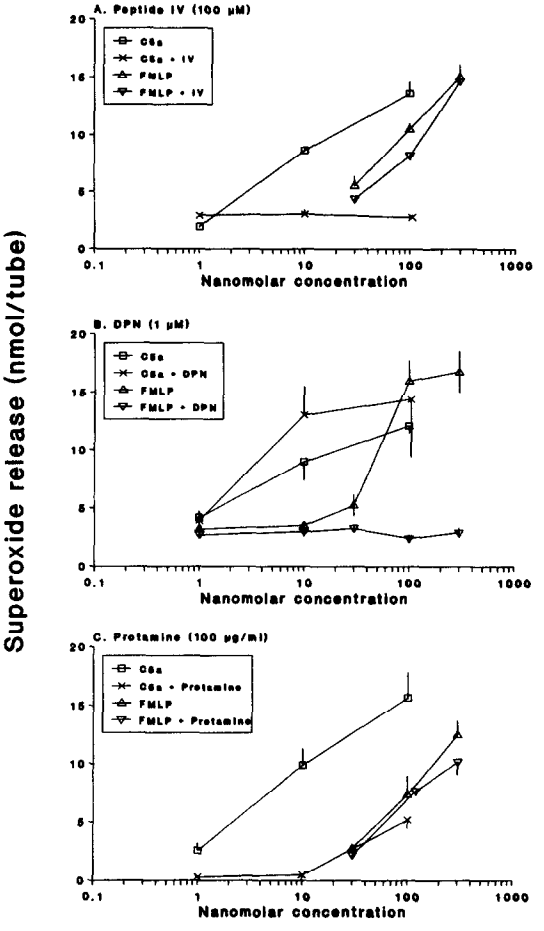

Fig. 4. Selective antagonism of chemotactic peptide-induced superoxide release by peptide IV (A), DPN (B) and protamine (C). The experimental conditions were as in Fig. 3. Values are the means ± SEM of 4 determinations in panel A, of 3–10 determinations in panel B and of 6 determinations in panel C. The baseline release in cells incubated at 37° without stimulus was 2.3 ± 0.3, 3.2 ± 0.5 and 0.9 ± 0.3 nmol/tube in experiments reported in panels A, B and C, respectively (N = 4, 10, and 6, respectively). DPN alone does not release superoxide significantly [42], nor did protamine (2.0 ± 0.8 nmol/tube).

Peptide I exhibited a low relative potency and consequently was not systematically studied in the other assays. Protamine concentration was expressed as weight because it is not necessarily a homogeneous protein; the IC_{50} competition value against [^{125}I]C5a was about 20 μg/mL.

The abilities of C5a and of the synthetic fragments to stimulate functional responses after receptor occupancy were studied. C5a released superoxide anions from human PMNs at the relatively high concentration of 10 nM and above (Fig. 3). Analogue II showed some activity at 100 μM, and peptide III was a significant releaser at 10 μM and above. The relative activities suggest an order of potency similar to the receptor binding assay, with the exception of analogue IV, which was found to be inactive as a superoxide anion releaser up to 100 μM. Claims of antagonist behavior for this peptide [31] prompted us to test analogue IV as an antagonist of C5a since its receptor affinity had been shown to be significant (Fig. 2A). Analogue IV, at 100 μM, suppressed completely and selectively the superoxide release from human PMNs stimulated with C5a, but not with FMLP (Fig. 4A). For comparison, a non-peptide antagonist of FMLP, DPN [33, 34] was shown to suppress superoxide release with the opposite specificity (Fig. 4B). The slight potentiation of C5a-induced superoxide release by DPN has been observed previously [33], although it did not reach statistical significance. Protamine, at 100 μg/mL, behaved as a selective C5a antagonist (Fig. 4C).

The agonist/antagonist status of C5a receptor ligands was further assessed in down-regulation experiments. As shown in Fig. 5, the specific binding of [^{125}I]C5a (2 nM) was variably depressed by a preincubation with the cold form of the C5a receptor ligand under study. The concentration of the ligands used in the 10-min experimental period at 37° was chosen on the basis of competition data presented in Fig. 2; a large proportion of the receptors was expected to be occupied in each case. C5a (10 nM) and analogue III (100 μM) desensitized extensively the human PMNs, as measured by the subsequent binding of [^{125}I]C5a (Fig. 5). Analogues II and IV (100 and 30 μM, respectively) produced only a partial desensitization, whereas protamine (30 μg/mL) had no significant effect, as compared with controls.

Vascular and in vivo *effects of C5a and selected C-terminal analogues*

C5a and the peptides II–IV were found to elicit biphasic mechanical response in rabbit isolated portal veins and pulmonary arteries precontracted with phenylephrine (Fig. 6): a small, brief and inconsistent contraction was followed by a more sustained relaxation that faded away over time. These reactions have been analyzed previously [16, 17]. Only the relaxant response, expressed as a percentage of the

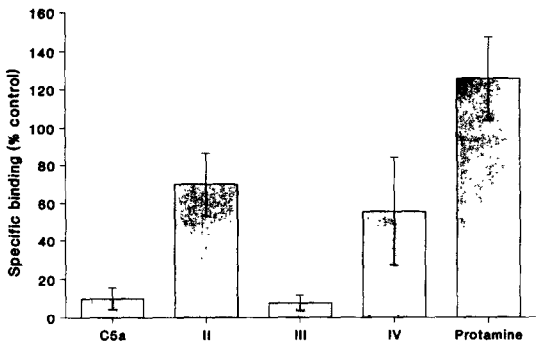

Fig. 5. Down-regulation of the specific binding of [^{125}I]C5a to human PMNs in cells pretreated at 37° with cold ligands of the receptors for C5a. The cells were exposed for 10 min to C5a (10 nM), analogues II (100 μM), III (100 μM), or IV (30 μM) or to protamine (30 μg/mL), and then washed; the binding to [^{125}I]C5a (2 nM) was established at 0°, as explained in the text. Controls were preincubated in HBSS–BSA buffer only. The average value for controls was 570 ± 300 cpm. Values are the means ± SEM of triplicate determinations in three separate experiments with different blood donors and are expressed as a percentage of the controls.

contractile plateau, was used in the quantitative analysis of biological activity in the portal veins (Fig. 7A) and the pulmonary arteries (Fig. 7B). The order of potency of the analogues was conveniently assessed by comparing the mean amplitude observed at 0.1 to 1 μM; this order was C5a > III ≥ IV > II in both tissues. Peptide IV behaved as an agonist in these vascular assays, possessing at least a large fraction of the intrinsic activity of analogue III. The profiles of the relaxant response induced by the analogues, notably the relaxation duration, were similar to the ones recorded with recombinant C5a (Fig. 6).

As reported previously [12], C5a elicited hypotensive responses in anesthetized rabbits (Table 3). C5a-induced hypotension was not accompanied by a significant change of heart rate (results not shown). Compared to the acute neutropenic effect (Table 4), an assay that was approximately one order of magnitude more sensitive, relatively large doses (1.7 nmol, i.v., and above) were needed to produce hypotensive responses.

The acute neutropenic effect of C5a (recorded 5 min after i.v. boluses) was dose-related and only transient, as it disappeared 30 min after C5a injection, except for the 5.6 nmol dose level. There was only a partial recovery 30 min after injection at this intense receptor stimulation level. Lymphocyte

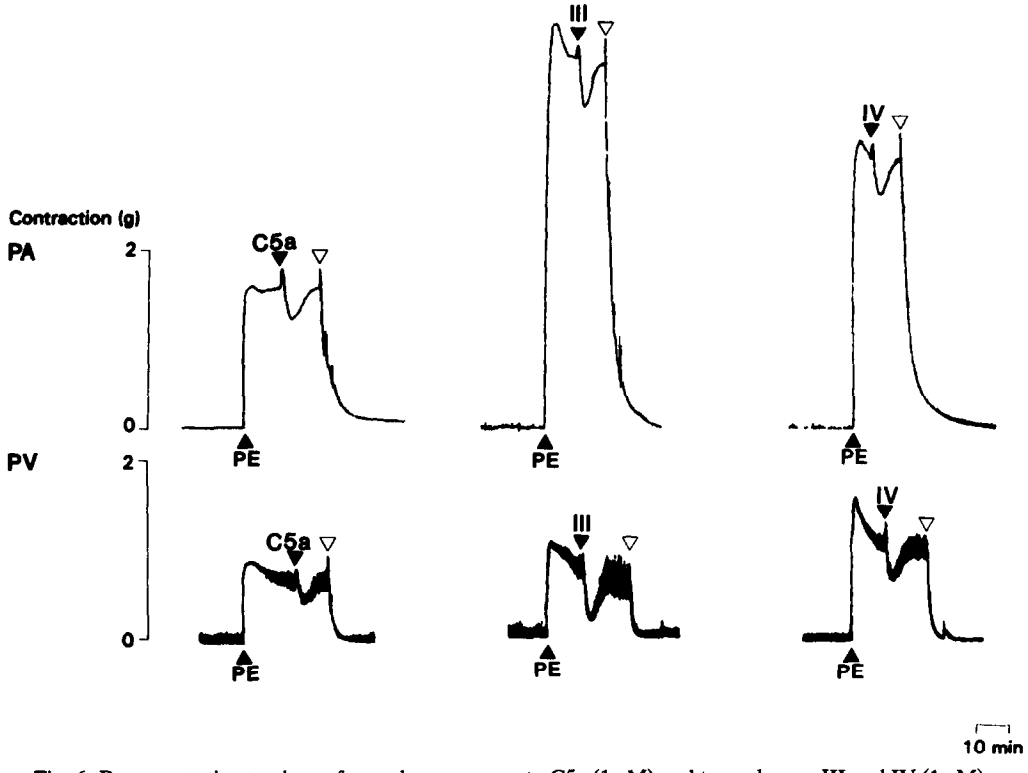

Fig. 6. Representative tracings of vascular responses to C5a (1 nM) and to analogues III and IV (1 μM). C5a or analogues were applied in the bathing fluid of precontracted rabbit vascular strips (PV, portal vein; PA, pulmonary artery). There were 90 min periods between applications of stimulants. The contraction was obtained with phenylephrine (PE, 500 nM). Abscissa scale: time; ordinate scale: isometric contraction. Closed symbols refer to the application of agents and open symbols to washout of stimulants.

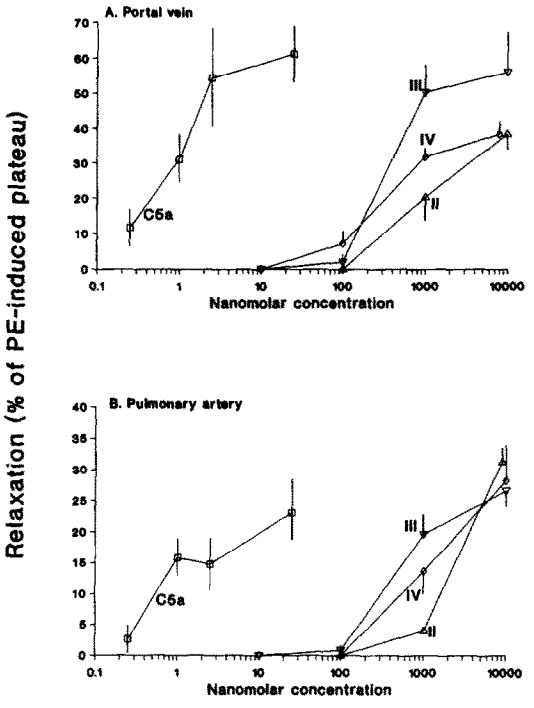

Fig. 7. Relaxant effect of C5a and of analogues on the rabbit isolated portal vein (A) and on the rabbit isolated pulmonary artery (B). Values are the means ± SEM of 4–7 determinations.

counts were not affected significantly by C5a (Table 4). The experimental design excluded the study of the late neutrophil count rebound.

The hypotensive and neutropenic effects of selected C5a synthetic peptides with varying degrees of metabolic resistance and affinity are shown in Tables 3 and 4. To obtain hypotensive responses similar in amplitude and in duration, doses of analogue II equivalent to 450 times that of C5a had to be given. Similar results were obtained with analogue IV at only 20 times that of C5a. The neutropenic effects of peptides II, III and IV 5 min after bolus injection indicated a relative activity of about 0.2, 2 and 16%, respectively, compared to C5a. Therefore, the order of potency for both the hypotensive and neutropenic effect was C5a>IV >III>II.

DISCUSSION

Recently, synthetic peptides that retained a significant fraction of the potency of C5a were synthesized by several groups [28–31; present results]. These analogues exhibited the whole spectrum of C5a effects, such as neutrophil chemotaxis, histamine release [28] and cytokine production in human mononuclear cells [47]. The most active peptides usually included a key substitution, His^{67} for Phe, which was shown to increase the affinity of C-terminal fragments by several orders of magnitude [29]. Other modifications, consisting mainly in hydrophobic substitutions, were designed to increase the affinity [30]. Multiple substitutions with unnatural amino acids, with the resulting constraints on the peptide backbone, yielded peptide IV, claimed to antagonize some of the effects of C5a on human neutrophils [31]. The relative metabolic resistance of these increasingly modified peptides has not been reported. We have observed that some of these peptides, analogues III and IV, are quite resistant to peptidases present in blood or on cell membranes such as AmM, CPN, ACE and neutral endopeptidase 24.11. All of these enzyme preparations have been shown to degrade analogue II, a relatively unmodified C-terminal analogue of C5a. The fragments generated by cleavage of analogue II by these peptidases were not identified. It is suspected, however, that CPN cleaves the C-terminal Arg residue to generate the

Table 3. Hypotensive effects and time for half-recovery (TR_{50}) elicited by i.v. boluses of human anaphylatoxin C5a or synthetic analogues in anesthetized rabbits

Peptide	Doses ($\times 10^{-9}$ mol)	N	Baseline MAP (mm Hg)	ΔMAP (mm Hg)	TR_{50} (sec)
Vehicle*		8	90.4 ± 3.0	−1.4 ± 0.8	
C5a	0.17	3	98.0 ± 7.4	0	
	0.56	4	96.0 ± 2.5	0	
	1.7	6	91.7 ± 4.9	−9.0 ± 3.0	179 ± 32.5
	5.6	4	82.6 ± 7.1	−21.0 ± 1.3	214 ± 48.9
II	850	2	102.5 (95/100)	2.5 (−5/10)	
	2500	2	100.0 (100/100)	−18.5(−13/−24)	195 (180/210)
III	94	4	90.0 ± 6.5	0 ± 3.7	
	280	7	83.1 ± 7.6	−9.2 ± 7.7	
	940	5	92.6 ± 12.6	−29.0 ± 15.8	234 ± 115
IV	12	2	98.5 (90/107)	3.5 (0/7)	
	35	5	76.6 ± 8.3	−1.0 ± 1.6	
	120	4	80.0 ± 8.5	−33.7 ± 14.2	214 ± 31

* Vehicle for C5a: 0.1 mL of 0.25% BSA followed by 0.3 mL saline. Results are means ± SEM or the average of two determinations with values in parentheses. MAP = mean arterial pressure.

Table 4. Changes of polymorphonuclear leukocyte and lymphocyte counts in peripheral blood of anesthetized rabbits at two different times (5 and 30 min) after i.v. boluses of human recombinant C5a or of synthetic analogues

Peptide	Doses ($\times 10^{-9}$ mol)	N	Polymorphonuclear leukocytes Response (% control) 5 min	30 min	Lymphocytes Response (% control) 5 min	30 min
Vehicle*		4	136 ± 24	131 ± 27	94 ± 14	126 ± 15
C5a	0.17	4	90 ± 14	109 ± 31	107 ± 9	108 ± 9
	0.56	4	79 ± 15	126 ± 12	96 ± 17	89 ± 15
	1.7	6	65 ± 12	128 ± 35	100 ± 8	91 ± 9
	5.6	4	15 ± 5	48 ± 16	100 ± 8	103 ± 14
II	850	2	59 (30/89)	86 (68/105)	74 (74/75)	78 (57/100)
	2500	2	23 (15/32)	119 (109/129)	80 (77/83)	119 (92/147)
III	94	4	51 ± 13	124 ± 18	94 ± 20	77 ± 7
	280	7	13 ± 4	133 ± 34	68 ± 4	100 ± 8
	940	5	3 ± 2	61 ± 10	64 ± 7	98 ± 14
IV	12	3	90 ± 10	129 ± 20	101 ± 3	96 ± 8
	35	6	27 ± 6	161 ± 26	81 ± 7	107 ± 10
	120	5	5 ± 3	78 ± 25	66 ± 3	114 ± 13

* Vehicle: 0.1 mL of 0.25% BSA followed by 0.3 mL saline. Results are means ± SEM or the average of two determinations with values in parentheses. Baseline (pre-injection) polymorphonuclear leukocyte and lymphocyte counts for all groups were, respectively, 2464 ± 220 and 1795 ± 88 cells/μL of blood (N = 56).

des-Arg metabolite, ACE would likely liberate the C-terminal Gly-Arg dipeptide, while AmM would sequentially release the N-terminal residues. The relative importance of ACE and neutral-endopeptidase as proteases involved in the regulation of C5a *in vivo* remains to be established. However these enzymes could play a protective role against sub-clinical complement activation. It is of interest to note that clinical treatment of hypertension by ACE inhibitors is sometimes accompanied by side effects that are reminiscent of inflammatory processes.

The competition for [^{125}I]C5a receptor binding showed that analogue IV was the most potent one examined, followed by peptides III and II. *In vitro* biological assays generally confirmed this order of potency with some particular findings for peptide IV. The latter behaved as a selective antagonist of C5a-induced superoxide release from human PMNs, suggesting that receptor binding by this hexapeptide is not followed by functional response.

The partial agonist status of analogue IV is also suggested by experiments designed to show ligand-induced down-regulation (Fig. 5). C5a and analogue III were very effective to acutely down-regulate C5a receptors at 37°, a process presumably linked to ligand–receptor complex internalization [40]. However, the antagonist protamine, at a concentration that occupies a large fraction of the receptors, failed to down-regulate the specific binding of [^{125}I]C5a. Only small down-regulations have been observed in cells pretreated with analogues II or IV, possibly for different reasons. Analogue II exhibits a low affinity and is susceptible to degradation by human PMNs (Table 2), possibly resulting in a smaller effective receptor occupancy during the 10-min desensitization period. Analogue IV, by opposition, has a much higher affinity and stability and was expected to occupy at 30 μM at least as many receptors as analogue III in this series of experiments. The low down-regulation observed must be explained by a low, but measurable efficacy, supporting its intermediate status between a pure antagonist (protamine) and full agonists (C5a, analogue III). In their report Mollison *et al.* [31] showed that analogue IV is an antagonist of myeloperoxidase release in human neutrophils while behaving as a partial agonist on the chemotactic responses observed in these cells.

The discrepant agonist-antagonist behavior of peptide IV may be explained by the hypothesis of an efficacy lower for this peptide than for C5a or the other peptides. The superoxide release assay is not a sensitive one for C5a relative to the degree of receptor occupancy at the corresponding concentrations (compare Fig. 1A with Fig. 3). If a pharmacologic effect requires the stimulation of a very large proportion of the receptors on each cell, ligands with reduced efficacy are more likely to behave as antagonists (discussed by Leslie; [48]). In rabbit isolated vascular tissue, systems that respond to subnanomolar concentrations of C5a, peptide IV behaved as an agonist, with possibly a weaker potency than expected from the binding data, relative to analogues III and II. This type of situation is expected for a low efficacy agonist if there is some "spare receptor" reserve in the responding cells [48]. However, the different experimental conditions in these two assays (i.e. binding vs isolated tissues) and, possibly, the species barrier may also contribute to the potency difference. *In vivo*, peptide IV was a potent C5a mimetic. Therefore, analogue IV does not appear to behave generally like an antagonist of C5a, but is possibly a "lead" compound towards ligand with lower efficacy that could be developed into antagonists. The discrepant agonist-antagonist

behavior of analogue IV does not necessarily suggest the existence of multiple receptor types; binding data from Fig. 1 indicate a single type of sites in the ligand concentration range 18 pM to 5.6 nM.

The three C-terminal analogues of C5a examined for metabolic degradation by various peptidase sources exhibited wide differences of susceptibility. However, multiple metabolic resistance did not result in a different time course for some of the C5a-induced effects, such as the relaxation of rabbit isolated vascular strips (Fig. 6) or the acute neutropenic effect following bolus injections *in vivo* (Table 4). The latter responses were no longer detectable at the 30-min time point, except for extremely intense stimulation levels. Mechanisms different from ligand metabolic disposition, such as receptor desensitization or ligand diffusion and dilution *in vivo*, may account for the limited time course of these effects. The most striking effect of metabolic resistance *in vivo* was the gain in relative potency for analogues III and IV, which approached the molar potency of C5a, despite a wide affinity gap between the analogues and the recombinant protein revealed by the *in vitro* assays. A good comparison term for assessing the effect of metabolic resistance *in vivo* is the susceptible analogue II, which exhibited *in vivo* the typical wide gap of molar potency relative to C5a observed *in vitro*.

The *in vivo* effect of the synthetic analogues can probably be explained entirely by their action on C5a receptors. Notably, their relative lack of effect on lymphocyte counts, a cell type which lacks receptors for C5a [49], supports such a proposition. Hypotension induced by analogue IV was parallel to an increased central venous pressure (results not shown), similar to C5a-induced hypotension [12]. A full characterization of the mechanisms by which these analogues produced their effects will be possible when potent C5a receptor antagonists become available.

The present study suggests that the pharmacology of anaphylatoxin C5a can be approached successfully with the methodologies developed for much shorter peptides and with concepts developed from the study of conventional drugs. The affinity, efficacy and metabolic disposition rate are three distinct parameters that govern the *in vivo* pharmacology of any C5a receptor ligand. The selective antagonist effect of protamine for C5a [46] has been confirmed and studies are underway to define the minimal structure within this natural protein that binds the receptors for C5a.

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