Novel C5a Receptor Antagonists Regulate Neutrophil Functions In Vitro and In Vivo

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Novel recombinant human C5a receptor antagonists were discovered through modification of the C terminus of C5a. The $C5a_{1-71}T_1M,C_{27}S,Q_{71}C$ monomer, (C5aRAM; CGS 27913), was a pure and potent functional antagonist. The importance of a C-terminal cysteine at position 71 to antagonist properties of C5aRAM was confirmed by studying $C5a_{1-71}$ derivatives with replacements of Q_{71}, C5a derivatives of various lengths (70–74) with C-terminal cysteines, and C5a derivatives of various lengths (71–74) with $Q_{71}C$ replacements. The majority of $C5a_{1-71}Q_{71}$ derivatives were agonists (C5a-like) in the human neutrophil C5a-induced intracellular calcium mobilization assay. The $C5a_{1-71}Q_{71}C$ derivative was an antagonist. C5a derivatives of lengths 73 and 74 with C-terminal cysteines were agonists, while lengths 70 to 72 were antagonists. C5a derivatives of lengths 72, 73, and 74 with $Q_{71}C$ replacements were agonists, while, again, $C5a_{1-71}Q_{71}C$ was an antagonist. C5aRAM and its adducts, including its dimer, C5aRAD (CGS 32359), were pure antagonists. Additionally, C5aRAM and C5aRAD inhibited binding of ^{125}I-labeled recombinant human C5a to neutrophil membranes (K_i = 79 and 2 pM, respectively), C5a-stimulated neutrophil intracellular calcium mobilization (8 and 13 nM), CD11b integrin up-regulation (10 and 1 nM), superoxide generation (182 and 282 nM), lysozyme release (1 and 2 μM), and chemotaxis (11 and 7 μM). In vivo, intradermal injection of C5aRAM inhibited C5a-induced dermal edema in rabbits. Furthermore, a 5-mg/kg i.v. bolus of C5aRAD significantly inhibited C5a-induced neutropenia in micropigs when challenged with C5a 30 min after C5aRAD administration. C5aRAM and C5aRAD are novel, potent C5a receptor antagonists devoid of agonist or proinflammatory activity with demonstrated efficacy in vitro and in vivo. ***The Journal of Immunology*****, 1998, 160: 5616–5621.**

The anaphylatoxin, C5a (Fig. 1), is a 74-amino acid, 4-helix bundle glycoprotein generated by the proteolytic cleavage of the fifth component of complement (C5) by C5 convertase (1). C5a is responsible for generating a number of diverse functions on cellular systems, especially on neutrophils. These include an increase in intracellular calcium mobilization (Ca^{2+} rise),[2] stimulation of chemotaxis, aggregation, degranulation, and production of superoxide anion (2–5). C5a has been implicated in rheumatoid arthritis, psoriasis, adult respiratory distress syndrome, and reperfusion injury (6–9). Inhibition of C5a-induced responses with a C5a receptor antagonist should prevent cell damage and death due to acute inflammatory injury via C5a without affecting other activities of the complement system.

C5a receptor antagonists described previously include short peptides (10, 11) and anti-C5a receptor Abs (12). We report here an alternative, with derivatives of C5a that lack C5a effects. Previous structural and functional studies with C5a and its receptor suggested a two-site binding hypothesis in which the extracellular N terminus of the receptor binds the disulfide-linked core segment of C5a (residues 1–63) (13–16). The C-terminal tail (residues 64–74) of C5a presumably interacts with another, as yet undefined, site within the receptor. The present approach utilized the native core region of C5a with various C-terminal modifications. C-terminal truncated, cysteine-terminated C5a derivatives were found to be potent and effective C5a receptor antagonists. We report the effects of our recombinant C5a receptor antagonists, C5aRAM[3] (monomer) and its dimer, C5aRAD, on neutrophil responses to C5a in vitro and in vivo, and we show functional receptor antagonism without agonist activity.

Materials and Methods

Strains, plasmids, and materials

Escherichia coli strain LCIQ was used for the expression of all recombinant C5a proteins (16). Reagents and vendors: DH5αF′IQ for cloning and DNA manipulations (Life Technologies, Gaithersburg, MD); oligonucleotide-directed in vitro mutagenesis system and [α-^{32}P]dATP (Amersham, Arlington Heights, IL); plasmids pTZ19R and pKK223-3 (Pharmacia, Piscataway, NJ); enzymes (Boehringer Mannheim, Indianapolis, IN, or New England Biolabs, Beverly, MA); acetonitrile (Baker, Phillipsburg, NJ); DNA synthesis reagents (Applied Biosystems, Foster City, CA); oligonucleotides were made with an Applied Biosystems 381A DNA synthesizer. All other reagents and chemicals were from Sigma (St. Louis, MO).

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[2] Abbreviations used in this paper: Ca^{2+} rise, increase in free intracellular calcium; rhC5a, recombinant human C5a; C5aRA, C5a receptor antagonist; C5aRAM, $C5a_{1-71}T_1M,C_{27}S,Q_{71}C$ monomer, C5aRAD, $C5a_{1-71}T_1M,C_{27}S,Q_{71}C$ dimer; EC_{50}, effective concentration necessary to achieve half-maximal (agonist) response; AcC, acetylated cysteine; Cha, cyclohexylalanine; dR, D-isomer of arginine; NMeF, *N*-methylphenylalanine; LTB_4, leukotriene B_4.

[3] For reference purposes, the C5a receptor antagonists CGS 27913 and CGS 32359 (Novartis Pharmaceuticals, formerly Ciba Pharmaceuticals) are designated "C5aRAM" and "C5aRAD," respectively.

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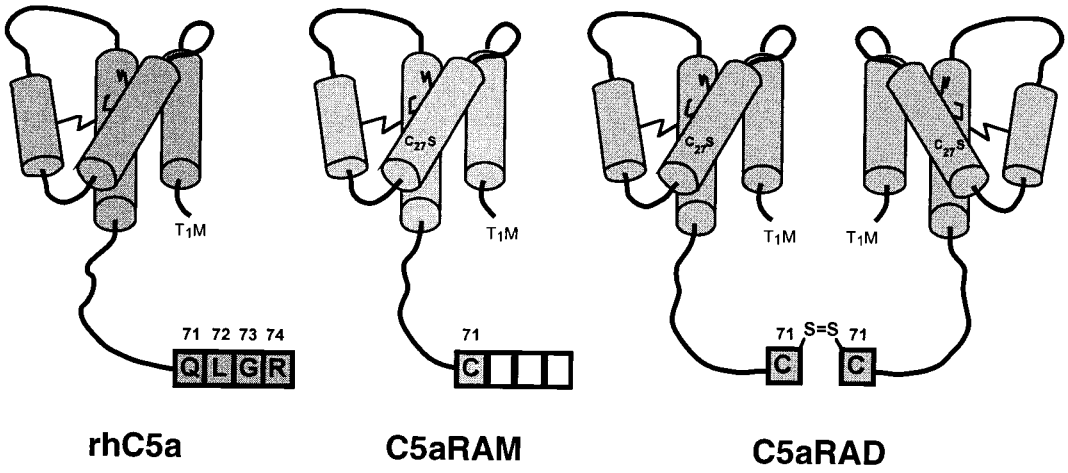

FIGURE 1. Schematic representations of recombinant human C5a (rhC5a) and C5a receptor antagonists, C. C5a and the antagonist derivatives are 4-helix bundle proteins (α-helices represented by cylinders). The antagonist C5aRAM is the C5a derivative $C5a_{1-71}T_1M,C_{27}S,Q_{71}C$ monomer, and C5aRAD is a dimer linked via a disulfide bond between carboxyl-terminal cysteines.

Plasmids containing C5a derivatives

Standard cloning techniques and manipulations were used (17). A synthetic gene encoding the 74-amino acid human C5a protein (15) was used with changes made at T_1M for proper translation initiation in *E. coli* and $C_{27}S$ to avoid potential covalent reactions with carboxyl-terminal Cys derivatives. All the C5a derivative proteins were prepared from a pKK223-3 plasmid using cassette mutagenesis with synthetic linkers. Standard ds DNA sequencing methods were used to confirm derivative C5a sequences.

Protein expression and purification

Strain LCIQ was transformed with the mutated pWCB plasmids and grown in Luria broth at 30°C with ampicillin and kanamycin. Cultures were frozen at −80°C before protein purification. Pellets were resuspended in a 10:1 ratio of 6 M guanidine-HCl to cell paste (v/w), sonicated (Branson Ultrasonic, model 250; Danbury, CT) for 3 × 30 s at a setting of 3 using the microtip, and dialyzed overnight (3500 m.w. cutoff; Spectrum, Houston, TX) against renaturation buffer (1 mM reduced/oxidized glutathione, 50 mM Tris-HCl, pH 8.0) at a ratio of 1:20. The solution was acidified to pH 3.0 with 1 N HCl, centrifuged, and filtered (0.2-μm pore size) before HPLC purification. Protein was initially purified on a C_4, 10- × 200-mm, 15-μm HPLC preparative column (Waters, Milford, MA) using a 20 to 35% acetonitrile gradient with 0.09% trifluoroacetic acid. Further purification was conducted with a C_4, 4.6- × 250-mm, 5-μm HPLC analytical column (Alltech, Deerfield, IL) and a similar gradient. The final yield of protein was usually 1 to 2 mg/g of cell paste. Product was >90% pure by polyacrylamide gel electrophoresis, and m.w. was verified with electrospray-mass spectroscopy.

Synthesis of adducts and free thiols

A cysteine adduct of $C5a_{1-71}T_1M,C_{27}S,Q_{71}C$ was prepared by replacing the redox couple of reduced/oxidized glutathione with 1.5 mM cysteine/cystine. The mercaptoethanol adduct was formed by adding β-mercaptoethanol directly to the 6 M guanidine-HCl solution and dialyzing against 50 mM Tris-HCl, pH 8.0. The iodoacetamide derivative of $C5a_{1-71}T_1M,C_{27}S,Q_{71}C$ was prepared by dissolving the free thiol in 0.2 M borate buffer (pH 8.2), adding 100-fold excess iodoacetamide in buffer, and stirring the reaction at room temperature for 2 h followed by reverse phase HPLC purification. Carboxyl-terminal free thiols were made by reducing either the glutathione or the cysteine adduct with 3-fold excess DTT in 100 mM Tris-HCl, pH 7.6, for 4 h. The reaction was quenched by adjusting the solution to pH 4.0 with 1 N HCl and was followed by reverse phase HPLC purification. Conversion of starting material to product was usually 70 to 80%. The m.w.s were verified with electrospray-mass spectroscopy.

^{125}I-Labeled rhC5a receptor binding studies

Measurement of inhibition of ^{125}I-labeled C5a binding to isolated human neutrophil membranes was as described previously (18).

Ca^{2+} rise assay

Measurements of C5a-stimulated changes in intracellular calcium in human neutrophils were made using the fura-2 AM fluorescent dye as described previously (19). Neutrophils were purified from human peripheral blood by sedimentation in 6% hetastarch (Hespan, DuPont, Waukegan, IL) followed by counter flow elutriation (20). Neutrophils were loaded with the calcium-binding fluorescent dye fura-2 AM, mixed for 30 min, and washed once. Neutrophils were then exposed to various concentrations of protein, and the fluorescence signal was measured on a SLM 8000 spectrofluorometer (SLM-Aminco Instruments, Urbana, IL). C5a antagonists were added, and any change in fluorescence excitation ratio of 343 nm:393 nm (emission of 510 nm) was measured. Forty seconds after antagonist addition, a challenge dose of C5a (100 pM) was added, and the resulting change in excitation ratio was measured. EC_{50} and K_i values were used as measures of agonist and antagonist potencies, respectively. K_i values were determined according to the method of Cheng and Prusoff (21) and expressed as geometric means ± SE.

Chemotaxis assay

C5a-induced chemotactic responses were measured in isolated human neutrophils by a thick filter assay (22), using Hydrophilic Durapore filters following a 90-min incubation at 37°C with 5 nM rhC5a (EC_{90}). K_i values were determined according to the method of Cheng and Prusoff (21) and expressed as geometric means ± SE.

Degranulation assay

C5a-induced lysozyme release was measured in isolated, cytochalasin B-treated, human neutrophils using a microtiter assay (23), in triplicate following a 20-min incubation at 37°C with 1.5 nM rhC5a (EC_{65}). K_i values were determined according to the method of Cheng and Prusoff (21) and expressed as geometric means ± SE.

Superoxide generation assay

Superoxide anion (O_2^-) generation was measured in isolated human neutrophils (24) by luminol-enhanced detection of released superoxide during a 30-min incubation at 37°C using 50 nM rhC5a (EC_{70}). K_i values were determined according to the method of Cheng and Prusoff (21) and expressed as geometric means ± SE.

CD11b integrin up-regulation assay

C5a-induced CD11b integrin up-regulation (cell surface expression) was measured in neutrophils in human whole blood (25, 26) by anti-CD11b mAb (Becton Dickinson, San Jose, CA) and FACScan flow cytometer (Becton Dickinson, San Jose, CA) analysis following a 15-min preincubation with vehicle or antagonist and a 20-min incubation at 37°C with 2.6 nM rhC5a (EC_{70}). K_i values were determined according to the method of Cheng and Prusoff (21) and expressed as geometric means ± SE.

Rabbit dermal edema assay

C5a-induced dermal edema was measured in rabbits. Briefly, 50 ng/site rhC5a, with or without antagonist, were injected intradermally into sites on the back of the rabbit. After 25 min, ^{125}I-BSA was administered i.v.; 20 min later (45 min post-rhC5a), the animal was killed, the skin was removed, and the injected sites were counted for radioactivity, reflective of the degree of C5a-induced dermal edema (27).

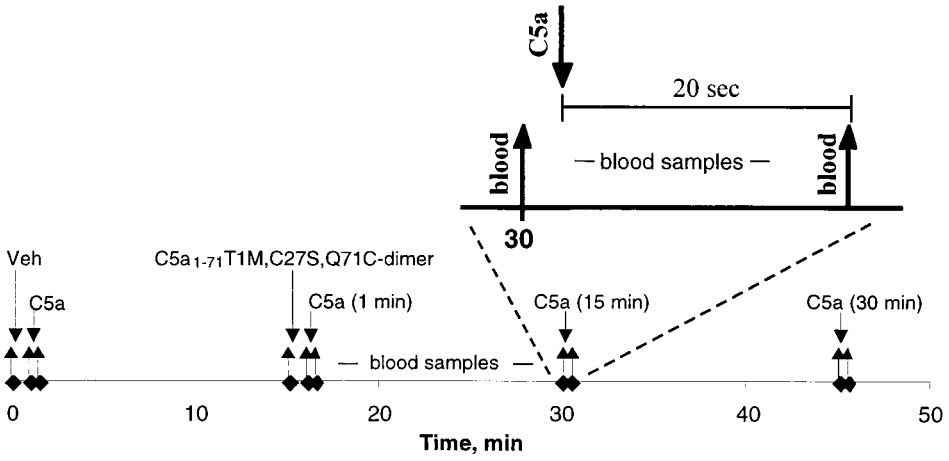

FIGURE 2. Experimental regimen used to determine inhibition by C5aRAD of rhC5a-induced neutropenia in micropigs. Micropigs were challenged with C5a at intervals after vehicle (Veh) and C5aRAD ($C5a_{1-71}T_1M,C_{27}S,Q_{71}C$-dimer) administration. Each C5a challenge included a baseline blood sample, a C5a i.v. bolus injection, and a subsequent blood sample 20 s after C5a.

Micropig neutropenia assay

The C5a-induced neutropenia model was based on the observation that neutrophils transiently disappear from the circulation (marginate) after systemic infusion of chemoattractants such as C5a or leukotriene B_4 (LTB_4) (28). To determine levels of C5a-induced neutropenia, neutrophil counts were compared in blood samples obtained just before and 30 s after i.v. bolus of rhC5a. The experimental regimen is presented in Figure 2. Anesthetized micropigs (Panepinto & Associates, Masonville, CO) were pretreated with vehicle (Dulbecco's PBS) and challenged 1 min later with rhC5a (50 ng/kg) i.v. to determine baseline C5a-induced neutropenia. Micropigs were then treated with C5aRAD i.v. bolus (1, 3, or 5 mg/kg) 15 min after the initial C5a series. Additional C5a series (consisting of baseline blood sample, C5a injection, 30-s blood sample) are performed at 1, 15, and 30 min post-C5aRAD administration. It was determined previously that the magnitude of neutropenia was not affected by multiple C5a challenges if separated by at least 5 min. Inhibition of neutropenia was determined for C5aRAD at each C5a challenge interval by comparing each to the level of neutropenia determined after vehicle treatment; i.e., each micropig served as its own control.

Results

$C5a_{1-71}T_1M,C_{27}S,Q_{71}X$ derivatives

C5a-induced free intracellular Ca^{2+} rise in human neutrophils was the primary functional screen for determining agonist/antagonist activity of C5a derivatives. Using this assay, the importance of a C-terminal cysteine was determined by making $C5a_{1-71}T_1M,C_{27}S$ derivatives in which Q_{71} was replaced with different amino acids (Table I). Derivatives were tested either alone, to measure agonist activity, or in the presence of rhC5a, to measure antagonistic properties. The majority of $Q_{71}X$ derivatives (X = Q, R, D, S, M, H, or L) were agonists. Antagonist activity was achieved by replacement of Q_{71} with cysteine. The free thiol, $C5a_{1-71}T_1M,C_{27}S,Q_{71}C$ (C5aRAM; Fig. 1), was a potent C5a receptor antagonist with a K_i of 8 nM. Additionally, a similar derivative with an additional internal change, $C5aRAM,H_{67}F$, was a pure antagonist with a K_i of 1 nM. For comparison, native C5a ($C5a_{1-74}$) had an EC_{50} of 0.06 nM and $C5a_{desArg}$ ($C5a_{1-73}$) had an EC_{50} of 6 nM. Also, the reported C5a receptor antagonist, a modified C5a "tail" peptide NMeF-K-P-dCha-W-dR (10), had a K_i of 206 nM in our human neutrophil Ca^{2+} rise assay.

Table I. *Agonist (EC_{50}) or antagonist (K_i) activities of $C5a_{1-71}$ carboxyl-terminal derivatives in the human neutrophil Ca^{2+} rise assay*

$Q_{71}X$[a]	*n*	Agonist EC_{50} (nM)	Antagonist K_i (nM)	Classification
S	7	1820		Agonist
R	5	680		Agonist
M	7	1150		Agonist
Q	8	2180		Agonist
D	5	2710		Agonist
L	6	1060		Agonist
H	5	1640		Agonist
C (C5aRAM)	37		8	Antagonist
C ($H_{67}F$)	6		1	Antagonist

[a] X denotes the amino acid to which Q_{71} was mutated. The agonist EC_{50} of rhC5a was 0.06 nM and that of $C5a_{1-73}$ ($C5a_{desArg}$) was 6 nM in the Ca^{2+} rise assay.

C5a of differing lengths

The effect of varying the length of a Cys-terminal C5a molecule was measured in the Ca^{2+} rise assay (Table II). Interestingly, the carboxyl-terminal cysteine derivatives with lengths of 73 and 74 amino acids had agonist properties, while derivatives of 72 residues or less possessed antagonistic activities. As before, the most potent antagonist was C5aRAM. Similarly, the effect of varying the length of the C5a molecule beyond residue 71 (72, 73, or 74) while also having an internal mutation of $Q_{71}C$ was measured (Table III). Derivatives with extensions past C_{71} all showed agonistic activities in the Ca^{2+} rise assay.

$C5_{1-71}(Q_{71}C)$ adducts

The importance of a free thiol on the terminal cysteine of C5aRAM was assessed by testing various adducts (mercaptoethanol, cysteine, glutathione, dimer) in the calcium rise assay on human neutrophils. All of the adducts tested (mercaptoethanol adduct (K_i 133 nM), cysteine adduct (K_i 41 nM), glutathione adduct (K_i 397 nM)) were antagonists without agonist activity. All were less potent antagonists than the free thiol C5aRAM. The dimer of C5aRAM, named C5aRAD (Fig. 1), was the most potent of the adducts tested, with a K_i of 13 nM.

Two of the more favored antagonists, C5aRAM and C5aRAD, were further characterized in neutrophil-based assays in vitro and in mechanistic (C5a-induced) models in vivo.

Table II. *Agonist (EC_{50}) or antagonist (K_i) responses of C5a derivatives of differing lengths, carboxyl-terminal cysteine (free thiols) in the human neutrophil Ca^{2+} rise assay*

$C5a_{1-xx}$[a]	–70	–71	–72	–73	–74	*n*	Agonist EC_{50} (nM)	Antagonist K_i (nM)
1–70	–C					4		32
1–71	–M	C	(C5aRAM)			37		8
1–72	–M	Q	C			3		39
1–73	–M	Q	L	C		3	2	
1–74	–M	Q	L	G	C	1	48	

[a] xx denotes length.

Table III. *Agonist (EC_{50}) or antagonist (K_i) responses of C5a derivatives of differing lengths, internal $Q_{71}C$ in the human neutrophil Ca^{2+} rise assay*[a]

$C5a_{1-xx}$[b]	–71	–72	–73	–74	*n*	Agonist EC_{50} (nM)	Antagonist K_i (nM)
1–71	–C	(C5aRAM)			37		8
1–72	–C	L			2	5	
1–73	–C	L	G		3	1	
1–74	–C	L	G	R	1	0.3	

[a] All derivatives were glutathione adducts of internal cysteine.
[b] xx denotes length.

C5aRAM and C5aRAD in vitro

Effects on ^{125}I-labeled C5a binding to isolated human neutrophil membranes, rhC5a-induced Ca^{2+} rise, CD11b up-regulation, superoxide generation, lysozyme release, and chemotaxis in human neutrophils are presented in Table IV. Both C5aRAM and C5aRAD inhibited C5a-induced functional responses, and in no case was agonist activity observed when monomer or dimer antagonist were tested alone in the functional assays.

Also, to assess differences in the behavior of the antagonists between species, C5aRAM was evaluated in the rhC5a-induced Ca^{2+} rise assay using isolated rabbit and pig blood neutrophils. On rabbit neutrophils, C5aRAM was an antagonist with a K_i of 195 nM; on domestic Yorkshire pig blood neutrophils, C5aRAM was an antagonist with a K_i of 22 nM.

Inhibition of rhC5a-induced dermal edema in rabbits with C5aRAM

C5aRAM was shown to be a functional antagonist in the C5a-induced dermal edema model in the rabbit (Fig. 3). C5aRAM, when coinjected intradermally with rhC5a, inhibited dermal edema formation with an ID_{50} of 50 μg/site. No proinflammatory activity was noted when C5aRAM was injected alone at the highest dose tested, 175 μg/site.

Inhibition of C5a-induced neutropenia in micropigs with C5aRAD

C5aRAD demonstrated antagonist activity in C5a-induced blood neutropenia in micropigs. The rhC5a when given i.v. at 50 ng/kg induced a 32 ± 3% neutropenia in 20 s. This level of neutropenia was somewhat lower than that observed in rabbits with rhC5a (our unpublished observations) but was adequately reproducible for measuring antagonism. Also, C5aRAD administered alone did not induce changes in circulating neutrophil counts (data not shown). A 5-mg/kg i.v. bolus of C5aRAD significantly inhibited neutropenia in micropigs when challenged with C5a 30 min after C5aRAD administration (Table V). Lower doses of C5aRAD revealed significant inhibition with C5a challenges at shorter intervals.

Table IV. *Agonist (EC_{50}) responses of rhC5a and antagonist (K_i) responses (mean ± SEM) of C5aRAM and C5aRAD on rhC5a-induced functional responses in human neutrophils in vitro*

Assay[a]	C5a Agonist EC_{50}	C5aRAM Antagonist K_i	C5aRAD Antagonist K_i
Receptor binding	3.0 pM	79.4 ± 4.5 pM	1.7 ± 0.2 pM
Calcium rise	0.06 nM	8.4 ± 3.3 nM	12.6 ± 4.0 nM
CD11b up-regulation	0.9 nM	10.1 ± 5.2 nM	1.3 ± 0.2 nM
Superoxide release	4.9 nM	182 nM	282 nM
Lysozyme release	1.0 nM	1.2 ± 0.3 μM	1.7 ± 0.8 μM
Chemotaxis	5.0 nM	10.7 ± 1.2 μM	7.3 ± 5.6 μM

[a] All results are from at least three experiments, except for superoxide release where $n = 2$.

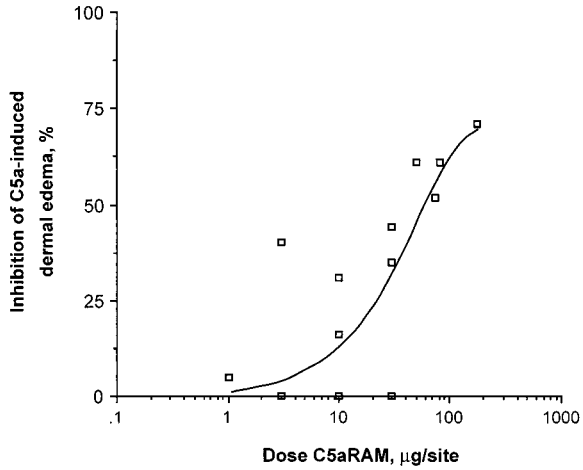

FIGURE 3. Inhibitory effects of C5aRAM on rhC5a-induced dermal edema in the rabbit. Results are included from four experiments.

Discussion

C5a elicits a variety of responses on neutrophils in vitro and in vivo. We used a C5a-induced intracellular calcium mobilization (Ca^{2+} rise) assay to identify functional C5a receptor agonists and antagonists among derivatives of C5a. The C5a receptor antagonists reported herein, C5aRAM and C5aRAD, behaved like pure antagonists without agonist activity in a variety of neutrophil assays in vitro, and, consistent with those responses, they were active in vivo in rabbits and micropigs, again without C5a-like (proinflammatory) activity.

Structural and functional studies using C5a suggested that the "core" segment (amino acids 1–63) was responsible for supplying a large portion of the binding energy for receptor interaction (13–16). The C-terminal "tail" (amino acids 64–74) was found to be important for the functional response and for additional binding. Short peptide derivatives of the C5a C terminus possess agonist properties (with the exception of NMeF-K-P-dCha-W-dR) and in general lack the high binding affinity of the entire molecule (4 pM) (10, 18, 29–33). Our approach toward a potent functional antagonist molecule utilizes the native core region and a modified C terminus.

The functional antagonists reported herein contain a large portion of the native C5a molecule to retain natural binding properties. The C terminus was systematically changed to alter the functional response. Our studies revealed that C5aRAM is a potent and pure C5a receptor antagonist. This observation led to experiments that tested alternative C-terminal amino acids at position 71. Of the various amino acids substituted at position 71, only C-terminal cysteine gave pure antagonism. C5a molecules of differing lengths

Table V. *Inhibition of rhC5a-induced neutropenia in micropigs at intervals after various doses of C5aRAD i.v. bolus administration*

Dose of C5aRAD (mg/kg)	Inhibition of C5a-Induced Neutropenia (%) at Following Time of C5a Challenge After C5aRAD i.v. Bolus		
	1 min	15 min	30 min
1	79	35	33
3	96	78	31
5	93	94	95

and having carboxyl-terminal cysteines (free thiols) showed an interesting set of activities in Ca^{2+} rise. C5a molecules of lengths 70, 71, and 72 (carboxyl-terminal cysteines) were antagonists, while lengths 73 and 74 were agonists. Again, the most potent antagonist was C5aRAM. Changes in length in either direction (shorter or longer) reduced antagonist potency, and, in the case of longer molecules, switched antagonism to agonism. Additional experiments using C5a derivatives of native sequences of lengths 72, 73, or 74 with an internal cysteine at position 71 showed that all three molecules were agonists in Ca^{2+} rise. We conclude from these experiments that antagonism in C5a-induced Ca^{2+} rise is not due exclusively to a 71-residue length or a cysteine in position 71 but to the stringent combination of a carboxyl-terminal cysteine in position 71.

Also, an interesting derivative included a $H_{67}F$ change in the context of the C5aRAM molecule. As reported in the literature (31, 32), the $H_{67}F$ modification of short peptides derived from the C terminus of C5a resulted in increased binding affinity to the receptor and a concomitant increase in agonist potency. Conversely, the $H_{67}F$ change in the antagonist C5aRAM produced an eightfold increase in antagonist potency, presumably as a result of increasing the binding affinity of the molecule.

The importance of a free thiol on the terminal cysteine of C5aRAM was assessed with adducts. All adducts tested were antagonists; hence, a free thiol group is not necessary for antagonism. The more potent adducts had a cysteine-cysteine linkage. Finally, an important adduct was formed when C5aRAM molecules dimerized through their terminal cysteines into C5aRAD. C5aRAD showed an antagonist potency in the Ca^{2+} rise assay that was similar to that of C5aRAM.

At this point, our investigations focused on the two antagonists, C5aRAM and C5aRAD, in neutrophil-based assays in vitro and in mechanistic (C5a-induced) models in vivo. C5aRAM and C5aRAD were compared in a variety of in vitro assays, including neutrophil membrane receptor binding, neutrophil chemotaxis, degranulation (enzyme release), oxidative burst (superoxide anion generation), and integrin adhesion molecule (CD11b) up-regulation. C5aRAD was significantly more potent (40-fold) than C5aRAM in the competitive binding assay, with C5aRAD showing the same affinity for the receptor as C5a itself (2–3 pM). Several explanations could account for these observations. The binding assay was performed on neutrophil membranes, and it is possible that receptor clustering occurred during membrane preparation, which, in turn, favored binding of the dimeric antagonist C5aRAD. Alternatively, the free sulfhydryl of C5aRAM could possibly have been partially oxidized in this system, yielding lower potency derivatives. For the most part, the antagonists C5aRAM and C5aRAD were roughly equipotent in the functional assays, with the exception of CD11b up-regulation where C5aRAD was an order of magnitude more potent than C5aRAM. Curiously, CD11b up-regulation was the only whole blood assay of the panel; the other assays used isolated neutrophils.

In different assays, the EC_{50} values of the agonist C5a varied from 0.06 nM in calcium rise to 5 nM in superoxide release and chemotaxis assays. Furthermore, the ratios of the EC_{50} of agonist C5a to the K_i of antagonists (C5aRAM or C5aRAD) varied; the smallest EC_{50}:K_i ratio was observed in the CD11 up-regulation assay (1.4, with C5aRAD), while the largest ratio was observed in the chemotaxis assay (2140, with C5aRAM). Discrepancies of this type are frequently found when comparing different assays, and several explanations could account for this observation. Receptor recruitment might explain some of the differences, with a higher fraction of receptor occupancy required for responses with higher C5a EC_{50}s. Furthermore, considering the complex number of G proteins and signal transduction pathways possible, multiple receptor:G protein states could exist, having different agonist/antagonist affinities. Finally, it has been demonstrated that other receptors, although not of this family, exist or can be induced to exist as dimers; thus, the potential for allosteric influences could add a further level of complexity.

Our next major challenge was to demonstrate that our antagonists were functional in vivo. A rabbit rhC5a-induced dermal edema model was used to show that C5aRAM was active in vivo following local (intradermal) administration. Reinforcing our results in vitro that C5aRAM had no agonist (C5a-like) activity, the antagonist when injected alone intradermally elicited no proinflammatory response; i.e., there was no increase in inflammatory exudate. Furthermore, C5aRAM when coinjected with C5a significantly suppressed the normal C5a-induced exudate.

Functional C5a receptor antagonism was also demonstrated in vivo following systemic (i.v.) administration of C5aRAD to micropigs in a C5a-induced neutropenia model. As was the case previously both in vitro and in vivo, C5aRAD when given i.v. alone had no direct C5a-like effect, as no changes in circulating neutrophil counts were observed. When micropigs were given neutropenia-inducing challenge doses of C5a at intervals after C5aRAD administration, the antagonist inhibited C5a-induced neutropenia in a dose-dependent manner. Increasing the i.v. dose of C5aRAD from 1 to 5 mg/kg increased the duration of action, reflected as significant inhibition of neutropenia with C5a challenges at longer intervals after compound administration. Thus, the recombinant C5a receptor antagonist C5a RAD, was shown to be effective in vivo following systemic administration.

In summary, we have identified, through systematic variation of the rhC5a molecule, pure human C5a receptor antagonists. These molecules are derivatives of C5a but are antagonistic to a variety of C5a-induced responses both in vitro and in vivo. We currently are testing the in vivo efficacy of our antagonists in disease models to determine the clinical efficacy of these compounds and finally to elucidate the specific role C5a plays in these important diseases.

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