Selection of a C5a Receptor Antagonist from Phage Libraries Attenuating the Inflammatory Response in Immune Complex Disease and Ischemia/Reperfusion Injury

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Selection of a C5a Receptor Antagonist from Phage Libraries
Attenuating the Inflammatory Response in Immune Complex Disease and Ischemia/Reperfusion Injury

Tanja Heller,* Meike Hennecke,* Ulrich Baumann,† J. Engelbert Gessner,‡ Andreas Meyer zu Vilsedorf,‡ Melanie Baensch,* Francois Boulay,§ Axel Kola,* Andreas Klos,* Wilfried Bautsch,* and Jörg Köhl*‡*‡

A C5a-receptor antagonist was selected from human C5a phage display libraries in which the C terminus of des-Arg⁷⁴-hC5a was mutated. The selected molecule is a competitive C5a receptor antagonist in vitro and in vivo. Signal transduction is interrupted at the level of G-protein activation. In addition, the antagonist does not cause any C5a receptor phosphorylation. Proinflammatory properties such as chemotaxis or lysosomal enzyme release of differentiated U937 cells, as well as C5a-induced changes in intra-cellular Ca²⁺ concentration of murine peritoneal macrophages, are inhibited. The in vivo efficacy was evaluated in three different animal models of immune complex diseases in mice, i.e., the reverse passive Arthus reaction in the peritoneum, skin, and lung. The i.v. application of the C5a receptor antagonist abrogated polymorphonuclear neutrophil accumulation in peritoneum and markedly attenuated polymorphonuclear neutrophil migration into the skin and the lung. In a model of intestinal ischemia/reperfusion injury, i.e., administration of the C5a receptor antagonist decreased local and remote tissue injury: bowel edema and hemorrhage as well as pulmonary microvascular dysfunction. These data give evidence that C5a is an important mediator triggering the inflammatory sequelae seen in immune complex diseases and ischemia/reperfusion injury. The selected C5a receptor antagonist may prove useful to attenuate the inflammatory response in these disorders. The Journal of Immunology, 1999, 163: 985–994.

In the last few years, complement inhibitors have been developed, which block the complement cascade at different points. The soluble version of the human complement receptor 1, i.e., sCR1 (1), inhibits classical and alternative pathway activation at the level of C3 and C4 and has been extensively analyzed in vitro and in vivo (for review, see Refs. 2 and 3). The C1 inhibitor (C1-INH), belonging to the family of serine-proteinase inhibitors, blocks C1r and C1s of the classical pathway. In addition, it is the major inhibitor of factor XII and prekallikrein of the contact system. The efficacy of both molecules has been demonstrated in animal studies and they are now assessed in clinical trials (for review, see Ref. 2). However, little is currently known of whether the inhibition of whole complement pathways is really needed to attenuate complement-mediated inflammation, in particular in immune complex (IC) diseases and ischemia/reperfusion (I/R) injury.

Recently, a mAb has been described (4) that inhibits cleavage of C5 and the subsequent generation of C5a and the membrane attack complex (MAC). Surprisingly, this mAb prevented the onset of arthritis and ameliorated established disease in a murine model of collagen-induced arthritis (5). In addition, this mAb attenuated glomerulonephritis and increased survival in a systemic lupus erythematosus-prone mouse strain (6). These data address the question of how much tissue damage in IC disease is produced by C3b and C4b deposition and subsequent phagocytosis and how much is due to generation of C5a and MAC. A critical role for C5a in IC diseases has recently been demonstrated in the reverse passive Arthus reaction using C5a receptor (C5aR)-deficient mice (7, 8).

C5a is a small polypeptide released from the α-chain of C5 during complement activation. It mediates a variety of proinflammatory effector functions such as recruitment of polymorphonuclear neutrophils (PMN) and macrophages to inflammatory sites and the activation of these cells to release increased levels of cytokines, chemokines, lysosomal enzymes, products of the arachidonic acid metabolism and histamine (for review, see Refs. 9 and 10). In addition, endothelial cells are stimulated to increase P-selectin (11).

The fact, that C5a appears very early in the inflammatory cascade makes this molecule a promising target for therapy. In addition, a specific inhibition of C5a does not impair C3b-mediated...
opsonization. Up to now, C5a and C5aR Abs, nonpeptidic and peptidic C5aR antagonists (C5aRA) have been developed (for review, see Ref. 2). Although many of these compounds have been proven to be effective in vitro, only rare examples exist for their potential use in vivo (12–15).

Recently, we described a method to display C5a on the tip of a filamentous phage using the pJuFo vector (16). C5a is fused to the leucine zipper part of Fos and is expressed with a free C terminus previously demonstrated to be essential for both receptor binding and functional activity (2). Panning C5a C-terminal libraries directly on a C5aR-expressing cell line, we now isolated a potent and specific C5aRA. Using this C5aRA, we were able to address an important role to C5a in IC disease and in intestinal I/R injury.

**Materials and Methods**

**Construction of C5a C-terminal libraries**

All of the constructed libraries are based on pJuFo plus [Ala27]C5a vector including Cys27Ala replacement in the C5a molecule (16). Altogether, five different libraries with modifications at the C5a C-terminus were constructed (Table I). For all libraries, primer MH40 (5’ end of C5a) was combined with five different mutagenesis primers (3’ end of C5a). Sequences of all primers are given below. All mutagenesis primers introduced a His6Phe replacement (Table I). C5a mutants from libraries I–III had 73 aa. C5a mutants from libraries IVa and IVb had 75 aa. For library I, mutagenesis primer MH44 was used, resulting in Asp69 Phe, Met70 Lys, and Gln71 Pro replacements were introduced by primer MH46 created random mutagenesis at positions 69–73. In library III, positions 70–73 were randomly mutated. At position 74, the VDN codon was used encoding all of the 20 naturally occurring amino acids except Arg, Gln, or Cys. Primer MH48, introduced the same naturally occurring amino acids except Arg, Gly, or Cys cannot occur (for details see Materials and Methods).

**Affinity enrichment of C5a mutants**

Selection of C5a receptor binding clones was performed on U937 cells, which had been differentiated with 1 mM N3,2′-O-dibutyrylcytidine 3′,5′-cyclic monophosphate (Bt2cAMP; Boehringer Mannheim, Mannheim, Germany) (dU937 cells) for 3 days as described (16). For the first round of panning, 2.5 x 10^10 cells grown in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FCS and penicillin (50 IU/ml)/streptomycin (50 μg/ml) at 37°C in a humidified atmosphere with 5% CO2 were harvested and resuspended in 500 μl medium containing 100 μl phage (5 x 10^10 pfu/ml). This mixture was incubated at room temperature for 30 min with gentle agitation. Cells were pelleted (3 min, 800 x g) and washed five times with 1 ml medium. Specifically bound phage were eluted with 200 μl 0.1 M HCl/glycine, pH 2.2, supplemented with 0.1% BSA at room temperature for 10 min. Cells were pelleted by centrifugation and the supernatant was immediately neutralized with 37.5 μl 1 M Tris/HCl, pH 9.1. Then, 100 μl of neutralized eluate were used to infect Escherichia coli TG1 cells. Numbers of phage before and after panning were determined by plating infected E. coli TG1 cells onto TYE+amp+gluc (18).

Altogether, three panning cycles were performed. The washing steps were increased from five times to 10 times in rounds two and three. In panning round three, 2.5 x 10^5 cells were used. All other steps were performed as described above.

**Generation of the ΔpIII-A8 mutant**

To obtain the ΔpIII-A8 mutant, gene III, encoding the viral coat protein pIII, was removed by digestion of pJuFo plus [Ala27]C5a-[A8] with SpeI and XhoI. SpeI and XhoI produce compatible cohesive ends. The digested vector was religated yielding ΔpIII-A8, in which the pH moiety of the Jun-pIII fusion protein was deleted (see Fig. 1). The PCR reactions, DNA fragment digestion, ligation, transformation, growth of the libraries, helper phage infection, phage purification, and DNA sequencing of the mutants was performed as described (18).

**Purification of C5a mutants by affinity chromatography**

For all in vitro and in vivo experiments, purified soluble C5a mutants were used. For protein expression TG1 bacteria were grown in 2x TY + amp medium containing 100 μg/ml ampicillin and 0.1% glucose. At an OD600 of 0.9, isopropyl β-D-thiogalactoside was added to give a final concentration of 0.5 mM. Bacteria were grown overnight with shaking at room temperature. The next day, the periplasmic fraction was prepared by freezing and thawing. Periplasmic fractions from the selected C5a mutants selected were applied to Sepharose columns coupled with C5a-specific mAb 561 as described (16). Highly purified C5a mutants were obtained as determined by SDS-PAGE and subsequent silver staining.

**Competitive 125I-labeled recombinant human C5a (125I-rhC5a) binding studies**

125I-rhC5a binding assays were conducted on dU937 cells and naïve murine peritoneal cells obtained by peritoneal lavage essentially as described (19). Briefly, increasing concentrations of unlabeled C5a or a C5a mutant were tested against a constant amount of 125I-rhC5a as tracer (17,000 cpm). After separation of the free 125I-rhC5a by filtration, the cell bound 125I-rhC5a was determined in a γ-counter (Packard, Canberra, Australia). The plot of cell-bound 125I-rhC5a vs the concentration of unlabeled competitor yielded the half-maximal inhibitory concentrations (ID50).
FIGURE 1. Cartoon representation of a filamentous phage expressing the selected C5aRA A8. The leucine zipper of transcription factor Jun is fused to the minor coat protein (pII) of the filamentous M13 phage. The C5a mutant A8 is fused to the leucine zipper of Fos. Jun and Fos form a heterodimer that is stabilized by two disulfide bonds at the N and C terminus of Jun and Fos as described (53).

Degranulation assay
As an example for the C5a-induced degranulation of phagocytic cells, the N-acetyl-β-D-glucosaminidase release from dU937 cells was determined. The assay was performed exactly as described (20). The ED_{50} of C5a or C5a mutants was determined as the concentration leading to half-maximal enzyme release.

Chemotaxis assay
Chemotactic activity of dU937 cells was assessed as described by Schwenk et al. (21). Modified Boyden chambers (Nucleopore, Tübingen, Germany) were filled with increasing concentrations of rhC5a (Sigma, Diesenhofen, Germany) or, in case of inhibition experiments, with a mixture of increasing concentrations of rhC5a and ΔpIII-A8 at a constant concentration of 10^{-10} M. Subsequently, chambers were covered with polycarbonate filters (pore size, 3 μm; Nucleopore). Then, 100 μl of dU937 cells at a concentration of 5 × 10^{7}/ml were applied to each chamber. Cells were allowed to migrate for 3 h at 37°C. Migrated cells were lysed by adding 0.1% Triton X-100 (Boehringer Mannheim), and β-glucuronidase activity in the lysates was determined photometrically using p-nitrophenol-β-D-glucuronic acid as substrate (Sigma). Chemotactic activity was expressed as chemotactic index, which is defined as the ratio of the number of migrating cells in presence of stimuli vs the number of migrating cells in presence of medium.

GTPase assay
To determine the GTPase activity of C5a or C5a mutants, the method of Gierschik (22) was used. Different concentrations of C5a or the C5a mutants were suspended in 50 μl of 50 mM triethanolamine-HCl, 5 mM MES pH 7.3 containing 5 mM MgCl2, 143 mM NaCl, 1 mM EDTA, 0.16% BSA, 0.8 mM 5'-adenylylimidodiphosphate (App(NH)p), 0.1 mM ATP, 5 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase, 0.1 μM GTP, and 0.2 μl [γ-^{32}P]GTP (10.8 μCi/μl; 3000 Ci/mmol). The reaction was started by adding 50 μl of dU937 membranes at a concentration of 3 μg dissolved in the same buffer. Cell membranes were prepared as described by Laugwitz et al. (23). After 15 min incubation at 30°C, 700 μl of dU937 cells at a concentration of 3 × 10^{7}/ml were added and the samples were centrifuged at 12,000 × g for 30 min at 4°C. Then, 500 μl of the supernatant were counted in a Beckmann LS 6000 SE counter (Beckman Coulter, Fullerton, CA).

Metabolic labeling and immunoprecipitation of C5aR
A stable cell line of C5aR-transfected RINm5F cells was used for the phosphorylation experiments (24). Cells were metabolically labeled with [γ-^{32}P]orthophosphoric acid (0.3–0.5 μCi/ml) for 3 h at 37°C, as described (25). Briefly, phosphorylation of C5aR was initiated by adding a saturating concentration of C5a (5 × 10^{-8} M) or ΔpIII-A8 (8 × 10^{-7} M). After 10 min incubation, the monolayers were lysed and C5aR was immunoprecipitated as described (26). Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions (27) and autoradiography.

Detection of C5aR by immunoblotting
Immunoblotting of C5aR-transfected RINm5F cells was performed as described (24). In brief, cells were incubated with either rhC5a (5 × 10^{-8} M) or ΔpIII-A8 (8 × 10^{-7} M) and subsequently lysed in Laemmli buffer (27) supplemented with DTT, separated by SDS-PAGE, and transferred to a 0.22-μm nitrocellulose filter (Schleicher & Schuell, Keene, NH). Filters were blocked with PBS, 0.1% Tween 10, 3% BSA for 1 h at room temperature. Subsequently, filters were incubated with affinity-purified anti-C5aR rabbit IgGs diluted 1:400 in blocking buffer. The filter was washed with PBS, 0.1% Tween 20 and incubated with 125I-labeled protein A (1 μCi/ml of blocking buffer) for 1 h at room temperature. Bound radioactivity was detected by autoradiography using Fuji RX film (Tokyo, Japan) at ~80°C.

Measurement of intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) of murine peritoneal cells
The peritoneal cavity of BALB/c mice was lavaged with 2 ml of ice-cold PBS. Cell staining using Diff-Quick (Baxter Merz & Dade, Dudingen, Switzerland) revealed 95–98% macrophages and 2–5% mast cells. The cells were centrifuged, resuspended in HBSS buffer without Ca^{2+}, and subsequently loaded with qu-area/2AM (Calbiochem, Bad Soden, Germany) as described (20). After reconstitution of the HBSS buffer with Ca^{2+}, the C5a-induced rise in [Ca^{2+}]_{i} was determined using the Luminescence Spectrometer LS 50 B (Perkin-Elmer, Beaconsfield, U.K.).

Animals
Female BALB/c mice (8- to 10-wk old) were purchased from Charles River Laboratory (Wilmington, MA) and were maintained under pathogen-free conditions.

Reverse passive Arthus reaction in the peritoneum
To induce the reverse passive Arthus reaction in the peritoneum, chicken egg albumin (20 mg/kg body weight; Sigma) was injected i.v., subsequently followed by an i.p. injection of IgG rich in Ab to chicken egg albumin (800 μg/mouse; ICN, Eschwege, Germany) exactly as described (7). Mice were killed 6 h after injury, and the peritoneal cavity was lavaged with 2 ml PBS, 0.1% ice-cold BSA. Peritoneal cells were stained using Diff-Quick (Baxter Merz & Dade) and subsequently assessed for differential cell count. Where indicated, mice were injected i.v. with 8 μg cobra venom factor (CVF) the day before IC challenge and again with 8 μg CVF 4 h before IC application. CVF was purified from Naja naja venom (Miami Serpentarium Laboratories, Miami, FL) according to a previously described procedure (28). In inhibition experiments, 200 μl (10^{-5} M) of the C5aRA ΔpIII-A8 were applied i.p. either at the time of IC challenge (T_{0}) or 1 h and 2 h after IC application. In a further set of experiments, 100 μl of ΔpIII-A8 at a concentration of 10^{-5} M were given i.v. at T_{0} or 1 h and 2 h after IC application. In a third set of experiments, 200 μl ΔpIII-A8 were injected i.p. and 100 μl i.v., both at T_{0} and 1 h and 2 h later. Serum complement levels were determined as described (29).

Reverse passive Arthus reaction in skin and lung
Mice were anesthetized with ketamine and xylazine and shaved at their basolateral sides. The trachea was cannulated and 150 μg rabbit anti chicken egg albumin Ab (Sigma) was applied. In addition, 30 μg Ab were injected intradermally. Ag (chicken egg albumin, 20 mg/kg) was subsequently given i.v. Where indicated, 200 μl ΔpIII-A8 at a concentration of 7.3 × 10^{-5} M were given i.v. before the Arthus reaction, or, additionally, 100 μl ΔpIII-A8 (7.3 × 10^{-6} M) were applied 60 and 120 min after IC challenge. After 4 h, mice were killed and PMN accumulation in the skin...
was assessed by quantitation of myeloperoxidase (MPO). In the lung, the number of PMN was determined by bronchoalveolar lavage.

Bronchoalveolar lavage

The lungs were lavaged with 5 × 1 ml PBS following cannulation of the trachea. Lavaged cells were stained using May–Gr ü nwald and Giemsa stain. The differential cell count of the bronchoalveolar lavage fluid was assessed with a hemocytometer (Neubauer Zählkammer Jürgens, Germany).

MPO assay

Skin punches (1 cm²) of the injection sites were assayed for MPO activity. MPO was extracted and determined as described (30). Briefly, homogenized tissue was suspended in 50 mM potassium phosphate buffer, pH 6.0, 0.5% hexadecyltrimethylammoniumbromide. Cells were broken by three cycles of freezing and thawing and subsequent sonification. The supernatant was mixed with 0.167 mg/ml O-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide. The MPO release was calculated by assessing the absorbance at 450 nm. A serial dilution of MPO from human PMN (Calbiochem-Novabiochem, Bad Soden, Germany) served as standard. Samples were run in duplicate.

Model of intestinal I/R injury

Mice were anesthetized with ether and were kept under a heating lamp. A laparotomy was performed, and I/R was induced by occluding the superior mesenteric artery with a microbulldog clamp. The abdomen was covered with warm, moist gauze during this period. Weight-matched control mice underwent sham operation in which the superior mesenteric artery was exposed but not occluded. After 60 min ischemia, the clamp was removed, the incision was closed with a silk suture, and the mice were returned to their cages. The bowel was reperused for 60 min or until the animals died (50–55 min after starting the reperfusion). Bowel injury (hemorrhage, bowel wall edema, and purple black discoloration) was assessed macroscopically at the end of the reperfusion period or when animals died. Where indicated, mice were treated with CVF before I/R as described for the reverse passive Arthus reaction. The C5aRA was given twice: 200 µl (7.3 × 10⁻⁶ M) were applied i.v. before starting ischemia; another 100 µl were given i.v. directly before removing the clamp.

Quantification of I/R-induced microvascular dysfunction

Intestinal reperfusion-induced dysfunction was quantitated by measuring the extravasation of plasma proteins into the lung as described (31). Evans blue dye binds avidly to albumin and was used as a marker of protein extravasation. This technology compares favorably with the methodology involving radiolabeled albumin (31). After 60 min of reperfusion, animals were killed and lung tissue was harvested. Pulmonary vessels were emptied by flushing saline into the right ventricle. Subsequently, the lungs were excised, weighed, and the right lung lobe was placed in 1 ml of formamide and homogenized. After incubation in formamide at 37°C for 16 h, the concentration of dye within the eluate was measured spectrophotometrically at 620 nm and expressed as ng dye/mg wet lung weight.

Statistical analysis

Statistical analysis was performed using the SigmaStat version 2.0 statistical package (Jandel, Erkrath, Germany). All data are given as mean ± SEM. First, we tested for a normal distributed population using the Kolgomoroff-Smirnov test. To analyze differences between two normally distributed groups, an unpaired t test was used. Comparison of the means of more than two groups were done by one-way ANOVA. When the differences in the mean values of the groups showed a significant difference, pairwise comparison was performed using the Tukey test. A p value < 0.05 was considered to be significant, and a p value < 0.001 was considered to be highly significant.

Results

Panning of the C5a phage libraries on differentiated U937 cells

We constructed 5 C5a-C-terminal libraries, i.e., libraries I, II, III, IVa, and IVb. In libraries I, II, and III, the functionally important Arg⁷⁴ residue was deleted. In libraries IVa and IVb, C5a mutants with a length of 75 aa were constructed, in which no Arg⁷⁴ residue was allowed. In library III, positions 69–73 were randomly mutated (Table I).

For the first panning cycle, 3.0 × 10¹² phage of a mixture of all five libraries were used, which is 6 × 10¹¹ phage from each library. Panning was performed in solution using C5aR-expressing dU937 cells. Next, 2.1 × 10⁷ phage were eluted from dU937 cells giving an output/input ratio of 7.2 × 10⁻³%. During the panning cycles, the number of eluted phage increased by a factor of ~200, giving a final output/input ratio of 1.1% after three panning rounds. After the third pan, 50 clones were randomly picked and screened for consensus motifs. All clones selected belonged to library III, in which positions 69–73 were randomly mutated. The Phε⁶⁹Lys⁷⁰Pro⁷¹ motif present in library I, II, IVa, and IVb was not found. The motif most often selected included a Leu residue at positions 70, 71, or 72 and a Tyr or an Arg residue at position 73, while no particular amino acid was enriched at position 69, as we have described recently (19).

Inhibition of C5a binding to dU937 cells by the selected C5a mutants

Selected C5a mutants with C-terminal consensus motifs Ser⁶⁹Leu⁷¹Leu⁷²Arg⁷³, i.e., A10, B4, and B8, were expressed as soluble proteins, purified by affinity chromatography, and tested in competitive binding studies using dU937 cells (Table II). All clones showed ID₅₀ values in the range of rhC5a, while the ID₅₀ of one mutant, A8, the structure of which is Jun-III(Fos-C5a-[1–66])-Phε⁶⁹Lys⁶⁸Arg⁶⁰Ser⁷⁰Leu⁷¹Leu⁷²Arg⁷³, was about 15-fold higher as compared with rhC5a (Table II). Mutants A10, B4, B8, and A8 share the same C-terminal sequence except position 69, which is Arg in A8 and Ala, Glu, or His in A10, B4, and B8, respectively. In clone A8, a cartoon representation of which is depicted in Fig. 1, the minor coat protein pIII was deleted, resulting in ΔpIII-A8. This deletion did not change binding affinity (Table II). Mutant ΔpIII-A8 was used throughout additional experiments.

The ΔpIII-A8 mutant does not induce degranulation of dU937 cells

Mutants A10, B4, and B8 induced degranulation of dU937 cells and had ID₅₀ values quite similar to C5a (Table II), whereas
DpIII-A8 did not induce degranulation up to the highest concentration tested, which was $3 \times 10^{-6}$ M. At that concentration, rhC5a was completely displaced from the receptor. DpIII-A8 was also devoid of any chemotactic activity (data not shown). To check whether signal transduction was blocked at the level of G-protein coupling, hydrolysis of [$\gamma$-32P]GTP was determined. A dose-dependent increase in GTPase activity was observed for C5a and B4 but not for DpIII-A8, even at $2 \times 10^{-6}$ M (Fig. 2). Next, we tested the ability of DpIII-A8 to induce the phosphorylation of the C5aR. As shown in Fig. 3, C5a caused a strong phosphorylation of the C5aR, whereas no ligand specific phosphorylation occurred for DpIII-A8.

Inhibition of C5a induced enzyme release and chemotaxis

To assess the potency of DpIII-A8 to inhibit C5a-induced degranulation of dU937 cells, increasing concentrations of DpIII-A8 were incubated with rhC5a at a concentration of $8.6 \times 10^{-10}$ M (ED$_{90}$). The C5a mutant inhibited cell degranulation with an ID$_{50}$ of $7.85 \pm 1.57 \times 10^{-8}$ M. Increasing concentrations of C5a were applied to dU937 cells in the absence or the presence of DpIII-A8 ($10^{-8}$ M). Maximal cell migration induced by C5a at a concentration of $5 \times 10^{-9}$ M was inhibited by 77%. Lower concentrations of C5a, i.e., $10^{-9}$ M (ED$_{90}$) or $5 \times 10^{-10}$ M (ED$_{50}$) were inhibited by 92% and 100%, respectively. Data from one typical experiment of five are given. **, p < 0.001 (t test).
From the murine C5aR with an ID 50 of 1.68 in a 70% inhibition (data not shown). Simultaneous application of ΔpIII-A8 at a concentration of 10⁻⁶ M ΔpIII-A8 was applied 10 min before the C5a stimulus. The C5a-induced rise in [Ca²⁺], was completely inhibited when C5a was applied at a concentration of 10⁻⁶ M or 2 × 10⁻⁸ M. At higher concentrations, the inhibition was between 50 and 95%. Data from one typical experiment of three are depicted. **, p < 0.001; *, p < 0.05 (t test).

**FIGURE 5.** ΔpIII-A8 inhibits C5a-induced [Ca²⁺], increase in murine peritoneal macrophage. Naive peritoneal macrophage were challenged with increasing concentrations of rhC5a in the absence or the presence of ΔpIII-A8 at a concentration of 10⁻⁶ M. ΔpIII-A8 was applied 10 min before the C5a stimulus. The C5a-induced rise in [Ca²⁺], was completely inhibited when C5a was applied at a concentration of 10⁻⁶ M or 2 × 10⁻⁸ M. At higher concentrations, the inhibition was between 50 and 95%. Data from one typical experiment of three are depicted. **, p < 0.001; *, p < 0.05 (t test).

ΔpIII-A8 is a C5aRA on murine peritoneal macrophages

The A8 mutant was selected using the human histiocyte cell line U937. To test whether this mutant also binds to the murine C5αR and, moreover, whether it is able to displace C5a from the murine C5αR, we performed competitive binding studies with naive murine peritoneal macrophages. Indeed, ¹²⁵I-rhC5a was displaced by pIII-A8 completely inhibited Ca-mobilization mediated by 10⁻⁶ M (ED₅₀) and 2 × 10⁻⁸ M (ED₂₅) C5a, when applied at a constant concentration of 10⁻⁶ M. Higher concentrations of C5a, i.e., 10⁻⁸ M or 2 × 10⁻⁸ M, were inhibited by 50–75%.

Inhibition of neutrophil recruitment in IC peritonitis

To analyze the in vivo potency of ΔpIII-A8, it was tested in a murine model of IC peritonitis, i.e., the reverse passive Arthus reaction, BALB/c mice were challenged i.v. with chicken egg albumin and i.p. with anti-chicken egg albumin Ab. This treatment resulted in the influx of 3.8 ± 0.8 neutrophils × 10⁶/mouse, corresponding to 46.5 ± 7.3% of cells in the peritoneum 6 h after IC challenge (Fig. 6). Administration of 200 μl (10⁻⁵ M) of ΔpIII-A8 i.p. at the time of IC challenge or 1 h and 2 h after IC challenge did not alter the PMN influx 6 h after initiation of IC peritonitis. A significant reduction in PMN recruitment was found when 100 μl (10⁻⁵ M) of ΔpIII-A8 were applied i.v., independent of whether the molecule was given at the time of IC challenge or 1 h and 2 h after the initiation of IC peritonitis (PMN, 20.1 ± 5.9% or 20.1 ± 7.4%; p < 0.05). When 200 μl ΔpIII-A8 were injected i.p. and 100 μl i.v. (both 10⁻⁵ M) at the time of Ag/Ab application, and again 1 h and 2 h after IC application, PMN influx was abolished (9.5 ± 3.1%) (p < 0.001; ANOVA). An identical reduction in neutrophil recruitment was observed after complement depletion with CVF (10.1 ± 4.8%). Neutrophil accumulation in Ag and Ab controls was 21.2 ± 0.8 or 13.6 ± 6.4% of peritoneal cells, respectively.

**FIGURE 6.** ΔpIII-A8 impairs PMN recruitment in IC peritonitis. Intra-peritoneal challenge of BALB/c mice with IC resulted in an increase in the portion of PMN in the peritoneal cavity from 1 to 2% in untreated animals to 46.5 ± 7.3% 6 h after IC application. A combination of i.p. and i.v. application of ΔpIII-A8 1 h and 2 h after IC challenge abrogated PMN migration (46.5% ± 7.3% (n = 26) vs 9.5% ± 3.1% (n = 6); p < 0.001, ANOVA and Tukey test). An identical reduction was found for CVF-treated animals (10.1% ± 4.8% (n = 8); p < 0.001, ANOVA and Tukey test). PMN recruitment was also significantly blocked when ΔpIII-A8 was injected solely i.v. 1 h and 2 h after IC challenge (PMN, 20.1% ± 5.9% (n = 12); p < 0.05, ANOVA and Tukey test) or at the time of IC application (T₂) (PMN, 20.1 ± 7.4% (n = 8); p < 0.05, ANOVA and Tukey test). Intrapерitoneal application of ΔpIII-A8 did not influence PMN migration. **, p < 0.001.

**Attenuation of the PMN influx in the reverse passive Arthus reaction in skin and lung**

The properties of ΔpIII-A8 to serve as a C5αRA were further evaluated in two other models of IC diseases, i.e., the reverse passive Arthus reaction in the skin and in the lung. After i.v. application of OVA and a combination of intratracheal and subcutaneous application of anti-OVA Abs, a tremendous accumulation of neutrophils was observed, both in the skin and in the lung (Fig. 7). The MPO activity in the skin, 4 h after challenging mice with IC, was 241.6 ± 51.6 μg/cm² as compared with 44.1 ± 7.1 μg/cm² or 57.3 ± 8.7 μg/cm² in mice receiving only the Ag or the Ab. When mice were treated with a single dose of 200 μl i.v. ΔpIII-A8 at a concentration of 7.3 × 10⁻⁶ M or, additionally, with 100 μl C5αRA 1 h and 2 h after initiation of the Arthus reaction, the MPO activity decreased to 101.2 ± 33.4 or 80.5 ± 23.7 μg/cm² (Fig. 7A).

In the lung of mice receiving only OVA or anti-OVA Ab, a maximum of 8 × 10⁶ PMN was found in the pulmonal lavage fluid taken 4 h after the onset of the Arthus reaction (Fig. 7B). In mice challenged with ICs, the neutrophil number increased to 324 ± 86 × 10⁵. In mice receiving a single dose of 200 μl i.v. of the C5αR (7.3 × 10⁻⁶ M), the neutrophil number decreased to 264 ± 91 × 10⁵. This reduction was statistically not significant. However, the additional application of 100 μl ΔpIII-A8 i.v. 60 and 120 min after initiation of the Arthus reaction resulted in a significant reduction of the neutrophil number to 73 ± 22 × 10⁵ (p = 0.003).
Effects of the C5aRA on I/R-induced local and remote organ injury

Occluding of the superior mesenteric artery for 60 min and subsequent reperfusion for 1 h resulted in severe local and remote tissue injury, i.e., in the bowel and the lung. In the group of untreated animals, 5 of 10 animals died 50–55 min after reperfusion. All animals suffered from heavy bowel injury (hemorrhage, bowel wall edema, and purple black discoloration located at different sites) (Table III). In contrast, all animals receiving CVF survived and showed no signs of bowel injury at all. When mice were treated with 200 μl ΔIII-A8 i.v. at a concentration of 7.3 × 10^{-6} M before arterial occlusion and another 100 μl of the C5aRA just before reperfusion, four of six animals survived. None of the surviving animals showed intestinal injury, while the two nonsurviving animals exhibited intestinal hemorrhage and bowel edema. Sham-operated controls had completely normal bowel appearance and survived.

To assess remote tissue injury, change in lung vascular permeability was assessed. I/R in untreated animals was associated with a significantly greater extravasation of plasma proteins into the pulmonary interstitium as compared with sham-operated controls or CVF- or C5aRA-treated animals (Table III; p < 0.001; ANOVA). In fact, the concentration of Evans blue dye within the lungs of CVF- and C5aRA-treated animals was indistinguishable from sham-operated controls, suggesting that C5a is a key mediator of the intestinal I/R-induced increase in lung vascular permeability.

**Discussion**

The interaction of C5a and its receptor is complex. A minimum of two binding domains has been described, the first of which is located in the core region while the second one is located at the C terminus of C5a. Using C-terminal-derived peptides and site-directed mutagenesis, this domain and in particular residues Lys^{68}, Leu^{72}, and Arg^{74} have been demonstrated to be crucial for all effector functions of C5a (18, 32–36). In addition, synthetic C-terminal peptide analogues have been reported, which are C5aRA. The peptide NMePhe-Lys-Pro-dCha-Trp-dArg was described as the most potent antagonist (32). Thus, targeting the C5a C-terminus seemed a reasonable approach to modulate C5a agonist properties.

We constructed C5a libraries, in which either the whole C-terminal domain from positions 69–73 or only particular residues were randomly mutated. These libraries were cloned into the pluFo vector and were expressed on the tip of a filamentous phage. After a three-round pan on a C5aR-expressing cell line, all of the selected clones were from library III, in which positions 69–73 were randomly mutated. None of the selected clones had the Phe-Lys-Pro motif present in three of the five libraries, although this motif was demonstrated to confer binding affinity in the C5a analogue peptide NMePhe-Lys-Pro-dCha-Trp-dArg (32). In contrast to the native C5a sequence or the sequences selected from the libraries, D-amino acids have been introduced in this hexapeptide resulting in changes in chirality. This in turn may heavily affect the binding of the Phe-Lys-Pro motif to the C5aR. All of the selected clones were pure C5a agonists except clone A8 (see Fig. 1), which bound to the C5aR with an ID_{50} in the low nanomolar range, i.e., 1.6 × 10^{-8} M, and, however, which was

### Table III. Effects of ΔIII-A8 on pulmonary microvascular dysfunction, bowel injury, and survival

<table>
<thead>
<tr>
<th>Pulmonary Microvascular Dysfunction (Evans blue dye [ng/mg tissue])</th>
<th>Survival</th>
<th>Intestinal Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham animals (n = 5)</td>
<td>305 ± 46[^*a^]</td>
<td>5/5</td>
</tr>
<tr>
<td>I/R (n = 10)</td>
<td>471 ± 34</td>
<td>5/10</td>
</tr>
<tr>
<td>I/R + CVF (n = 5)</td>
<td>323 ± 40[^*a^]</td>
<td>5/5</td>
</tr>
<tr>
<td>I/R + ΔIII-A8 (n = 6)</td>
<td>265 ± 33[^*a^]</td>
<td>4/6</td>
</tr>
</tbody>
</table>

[^*a^] Hemorrhage, bowel wall edema, and purple black discoloration.
[^*b^] Values are compared with the I/R group.
[^*c^], p < 0.001 (ANOVA and Tukey-test).
devoid of any signaling activity as determined in different in vitro assays such as cell degranulation, chemotaxis, and increase in [Ca^{2+}]. With an ID_{50} only 16-fold lower as compared with the natural ligand C5a, we tested this molecule for its ability to antagonize C5a effects in vitro. We found, that C5a effector functions could be blocked to nearly 100% when ΔPIII-A8 was used at a concentration of 10^{-6} M. This concentration was chosen, because it results in a complete displacement of C5a from its receptor, which is a “condito sine qua non” for a complete and successful inhibition of C5a-mediated effects. In fact, from all C5αRA reported, ΔPIII-A8 is the one with the highest in vitro potency (2).

Common to all effector functions of C5a is the activation of a heterotrimeric G-protein as the first step in signal transduction. C5αR have previously been shown to couple to either PTX-sensitive G-proteins or to the PTX-insensitive Grα subunit subsequently activating phospholipase C (37, 38). The selected antagonist did not induce any measurable GTPase activity, demonstrating that signal transduction is interrupted at the level of G-protein activation (Fig. 2).

After C5αR binding, cells become rapidly refractory to further stimulation, a phenomenon termed homologous desensitization. The receptors are uncoupled from the G-protein, internalized in endosomes, and subsequently recycled to the cell-surface membrane. Recent data suggest that receptor phosphorylation is the key mechanism for C5αR desensitization (24, 39). In contrast to C5a, ΔPIII-A8 did not induce any phosphorylation of the C5αR (Fig. 3), suggesting that ΔP III-A8 inhibits C5a effector functions by displacement of C5a from its receptor but not by receptor desensitization.

To check whether ΔPIII-A8 might prove useful to analyze C5a effects in murine animal models, we first tested its ability to antagonize C5a-induced increases in [Ca^{2+}], in murine peritoneal macrophages. In fact, binding and antagonistic properties of ΔPIII-A8 on murine macrophages were indistinguishable from dU937 cells. Then, ΔPIII-A8 was analyzed in a murine model of IC peritonitis. PMN migration into the peritoneal cavity was significantly impaired 6 h after initiation of the IC disease, when ΔPIII-A8 was applied i.v. Moreover, it was completely abrogated when the C5αRA was injected i.v. and i.p. at the time of IC challenge and 1 h and 2 h after initiation of the Arthus reaction. Because a mere intraperitoneal application of ΔP III-A8 did not affect the inflammatory response, we draw the conclusion that C5αR on PMN must be already blocked in the circulation to prevent neutrophil migration into the inflammatory focus.

As the antagonist is based on the natural ligand, pharmacokinetics are quite similar (T. Heller et al., manuscript in preparation). This is an important point, because the low molecular mass of ΔPIII-A8, i.e., 18 kDa, allows easy tissue penetration, as is the case for the natural ligand (12 kDa).

At the moment, the most potent and most commonly used inhibitor of complement activation is the soluble complement receptor 1 (sCR1). It has been demonstrated by Weismann et al. (1) that sCR1 blocks complement activation in vitro with an ID_{50} of ~1.5 × 10^{-10} M. However, the effective concentration of sCR1 to inhibit complement activation in vivo is 1.3 × 10^{-6} M, which is nearly 10,000 times the concentration required for blocking 50% of complement in vitro. In fact, doses of 15–20 mg/kg body weight are commonly used in animal models resulting in micromolar concentrations of sCR1 within the circulation. In accordance to the in vitro data, the C5αRA was applied at concentrations from 3.65 × 10^{-7} M to 10^{-6} M, given that the injected 200 or 100 μl of a 7.3 × 10^{-6} M to 10^{-5} concentration were 10-fold diluted in the 2 ml blood volume of the mouse. These concentrations are similar to or even below the molar concentrations commonly used for the sCR1.

Recently, we described that the PMN recruitment into the peritoneal cavity in IC peritonitis is predominantly mediated by complement or the high-affinity Fc receptor type I for IgG (FcγRI) (CD64) on macrophages, depending on the mouse strain used (40). In BALB/c mice, complement mainly contributes to PMN migration, whereas in C57BL/6 mice it is the interaction of IC with FcγRI. The results obtained in the current manuscript give evidence that the complement-mediated effect in BALB/c mice can be mainly attributed to C5a. In addition, the data give evidence that the antagonist has the potential to protect mice from C5α-induced PMN accumulation in IC peritonitis.

In two further models of IC disease, i.e., the reverse passive Arthus reaction in the skin and in the lung, we found a substantial reduction of the neutrophil influx of about 70–80% when the antagonist was applied i.v. These data demonstrate that the C5a-inhibitory effect is not only restricted to the peritoneum but is also valid for the protection of other tissues, such as the lung or the skin. In addition, these data imply an important general role for C5α in the initiation of the inflammatory sequelae seen in IC disease.

An important role for C5a in IC-triggered inflammation in peritoneum, skin, and lung has recently also been addressed by the use of C5αR-deficient mice (7, 8). These authors found that PMN migration was substantially impaired in C5αR-deficient mice as compared with their normal littermates. They observed a clear tissue dependency with a key role of C5a in the lung and a synergistic role in peritoneum or skin. While we also found a clear contribution of C5α to the IC-mediated inflammation, the most prominent effect of C5a inhibition by ΔPIII-A8 occurred in IC-mediated peritonitis. A possible explanation for the observed differences is that different mouse strains were used for the experiments. The C5αR-deficient mice have a mixed genetic background comprising two strains, 129 and C57BL/6, whereas we used BALB/c mice throughout the experiments.

The overall contribution of complement proteins and FcγRs in the initiation of the inflammatory response in IC disease is puzzling. Data obtained with mice lacking the Fcγ-chain or the FcγRII strongly support the data by Bozic et al and identify C5α as an important mediator in IC alveolitis. In addition, our results argue against a concept in which only FcγRs play a role in the pathogenesis of IC disease but favor the view that both complement, in particular C5α, and FcγRs have to be considered as mediators in the inflammatory concert of IC disease.

Several experimental reports suggest that complement activation is critically involved in IC trigger in the heart (1, 46), lung (47), skeletal muscle (48), and intestine (49, 50). Complement activation occurs by either interstitial or intravascular activation of the classical (48) or the alternative pathway (46), leading to the release of the anaphylatoxins C3a and C5a and the deposition of the MAC on injured endothelial cells. Evidence for the contribution of both C5α and the MAC to the inflammatory sequelae, such as neutrophil accumulation, increased vascular permeability, or cellular apoptosis, has been provided by specific inhibition of C5 cleavage with a mAb (51) or by means of the sCR1 during myocardial infarction in rats (1) or pigs (46).
In the model of intestinal I/R, local and remote injury occurs. Local injury is characterized by gross bowel injury including bowel hemorrhage, bowel wall edema, and purple black discoloration. Remote injury is manifested by pulmonary accumulation of neutrophils as well as microvascular dysfunction. The application of ΔIII–A8 improved both local and remote inflammation. All untreated animals suffered from heavy local bowel injury, while only 33% of the C5aRA-treated group showed signs of bowel damage, demonstrating a bowel protective effect of ΔIII–A8. The fact that not all animals were protected from local injury, as in the CVF-treated group, suggests that additional mediators from the complement system may be involved. The most likely candidate is the C5b-9 complex, because ΔIII–A8 has no effect on the formation or the deposition of the MAC on injured endothelial cells. Similar to C5a, the MAC up-regulates CD62p on endothelial cells, resulting in the adherence of neutrophils (52). In addition, it is able to induce apoptosis by Ras activation in ischemic tissue, at least in myocardial tissue (51).

Pulmonary vascular permeability was significantly decreased in the C5aRA-treated group as compared with untreated animals. In fact, the pulmonary microvascular dysfunction was as low as in sham controls or CVF-treated mice. These data provide evidence that C5a is crucial for the formation of the pulmonary edema following intestinal ischemia. C5a may affect the integrity of the endothelial cell layer by a direct activation of endothelial cells or alveolar macrophage. In addition, C5a can initiate and augment PMN adherence, transmigration, and activation (10).

Whatever the most important mechanism is, intestinal ischemia is a common clinical problem, either as a primary event, e.g. after superior mesenteric artery embolism, or associated with the systemic inflammatory response syndrome. Targeting the C5a-C5aR interaction by ΔIII–A8 has major potential as a clinical tool to reduce the extent and severity of intestinal I/R injury.

In summary, we have selected a potent C5aRA from phage display libraries, which allowed us to identify C5a as an important mediator in IC disease and I/R injury. This novel C5aRA may prove useful as a specific therapeutic agent targeting selectively a particular effector function of the complement system, the C5a-C5aR interaction. The beneficial and necessary functions, such as bacterial opsonization by C3 cleavage products or the clearance of ICs, are not affected, as is the case when “broad spectrum” complement inhibitors such as sCR1 or C1-INH are used.

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References


