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Inactivation of C5a Anaphylatoxin by a Peptide That Is Complementary to a Region of C5a

Emiko Fujita,* Imre Farkas,‡ William Campbell,† Lajos Baranyi,† Hidechika Okada,† and Noriko Okada‡

PL37 (RAARISLGPRCIAKAFTE) is an antisense homology box peptide composed of aa 37–53 of C5a-anaphylatoxin and is considered to be the region essential for C5a function. Using a computer program, we designed the complementary peptides AGSAPAGPAGPLRPMF (Pep-A) and ASTAPARAGLPRLPKFF (Pep-B). Pep-A bound to PL37 and to C5a with very slow dissociation as determined by analysis using surface plasmon resonance, whereas Pep-B failed to bind at all. C5a was inactivated by concentrations of 7 nM or more of Pep-A, and this concentration of Pep-A inhibited induction of intracellular Ca2+ influx in neutrophils. Patch clamp electrophysiology experiments also showed the effectiveness of Pep-A in C5aR-expressing neuroblastoma cells. Furthermore, Pep-A administration prevented rats from C5a-mediated rapid lethal shock induced by an Ab to a membrane inhibitor of complement after LPS sensitization. The Journal of Immunology, 2004, 172: 6382–6387.

Complement anaphylatoxin C5a is a 74-aa peptide generated from the fifth component of complement (C5) during complement activation (1, 2). C5a acts efficiently as an anaphylatoxin, stimulating cells such as leukocytes and endothelial cells, and is also a potent chemotactic factor for neutrophils and other inflammatory cells bearing C5aR. Therefore, C5a is considered to be one of the most potent inflammatory mediators (3). Inflammatory cells respond to nanomolar concentrations of C5a anaphylatoxin fragment (aa 37–53) and is an antisense homology box (AHB) peptide of C5a (5, 6). The sequences within the AHBs were based on the molecular recognition theory, which states that peptides that are encoded on opposite strands of DNA in a given reading frame show affinity in binding each other and that this binding occurs as a result of the hydrophobic complementary of the peptides. In addition, such sense-antisense amino acid sequences might represent both intra- and intermolecular interaction sites. Approximately 8- to 15-aa-long regions of this type were found in proteins, which we termed AHBs (5). PL37 is an AHB of C5a; however, it is also antisense to two regions of the C5aR (6). PL37 in multiple antigenic peptide form (PL37-MAP) evoked inward calcium current pulses on human neuroblastoma TGW cells or dibutyryl cAMP-treated U937 cells (6, 7). Therefore, we generated complementary peptides (C-peptides) to PL37, expecting that they could interfere with the function of C5a. To design the C-peptides, we used the software program MIMETIC (8). The algorithm scores several physicochemical parameters of each candidate peptide. C-peptides generated in this manner have already been shown to be inhibitory to HIV-1 reverse transcriptase (8) and thrombomodulin (9).

We synthesized two C-peptides targeting PL-37, and examined their reactivity to PL37-MAP and C5a in various assays such as binding measurements, intracellular calcium mobilization, calcium influx, and in an in vivo C5a-mediated lethal shock rat model (10–12).

Materials and Methods

Design of C-peptides

We used the evolutionary software program MIMETIC (Institute for Protein Science, Nagoya, Japan) to design C-peptide sequences for interaction with PL37 (7). MIMETIC assigns a score using a genetic algorithm based on several physicochemical parameters including hydrophatic complementarity optimization, average structural similarity optimization, minimization of bulky side chain interference, and backbone alignment. MIMETIC uses a genetic algorithm that generates a series of peptides by random alteration and by shuffling segments to optimize fitting to the target. MIMETIC then ranks the C-peptides according to their score. We synthesized the two highest score peptides and tested their activity.

Measurement of binding interactions by surface plasmon resonance (SPR)

Binding interactions between PL37 or C5a with C-peptides were evaluated using SPR technology with the Biacore system (Biacore International, Up sala, Sweden). PL37 and C5a were covalently immobilized on the CM5 sensor chip by amine-coupling methods using N-ethyl-N-(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) according to the manufacturer’s instructions. We activated the surface of the CM5 sensor by guest on April 12, 2017 http://www.jimmunol.org/ Downloaded from
chip with EDC/NHS for 20 min before injection with PL37-MAP (200 μg/ml in 10 mM sodium carbonate buffer, pH 8.5, over flow cell 2) or C5a (100 μg/ml in 10 mM acetic buffer, pH 5, over flow cell 3). Afterward, excess NHS was deactivated for 20 min with 1 M ethanolamine hydrochloride (EA), pH 8.5. The reference flow cell was activated with EDC/NHS and blocked with EA. Coupling was performed at a flow rate of 5 µl/min at 25°C in PBS. Analyte (30 µl of C-peptide A or B) was injected at 10 µl/min at 25°C in PBS. Binding interactions were determined by passing samples simultaneously over both the EA-blocked cell and the flow cell with immobilized PL37 or C5a so as to obtain units of resonance response (RU) by subtraction of the background using Biacore software evaluation.

**Patch clamp measurements**

The measurements were conducted on TGW human neuroblastoma cells bearing C5aR. Cells were voltage clamped at room temperature at a holding potential of −70 mV using a whole cell clamp configuration. The instruments used for electrophysiology were an Axopatch 200-A patch clamp amplifier, a Digidata-1200 data acquisition system; and pCLAMP 6.02 software from Axon Instruments (Foster City, CA). The head stage of the amplifier was fitted to an MHW-3 hydraulic manipulator manufactured by Narishige (Tokyo, Japan), and the cells were visualized using an Olympus IMT-2 invert microscope (Olympus, Melville, NY). Data acquisition and analysis were performed using an IBM-compatible personal computer. Patch electrodes (OD = 1.5 mm; thin wall; Garner Glass, Claremont, CA) were pulled with a PP-83 puller and polished with an MF-83 microforge (Narishige). The resistance of patch electrodes was 8–10 MΩ. The solutions used were as follows: an extracellular solution (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose, pH 7.34); and an intracellular pipette solution (10 mM HEPES, 110 mM KCl, 15 mM NaCl, 0.1 mM CaCl2, 2 mM MgCl2, 1 mM EGTA, pH 7.25). Recordings were conducted on several cells (n = 10 in each experiment) and were initiated simultaneously with the peptide application.

The PL37-MAP peptide or mixture of peptides (PL37-MAP and C-peptides) were incubated in an Eppendorf tube at room temperature for 1 h and were initiated simultaneously with the peptide application. The PL37-MAP peptide or mixture of peptides (PL37-MAP and C-peptides) were incubated in an Eppendorf tube at room temperature for 1 h in the extracellular solution and then applied to the cells via a puff pipette from a distance of 300–500 µm for 2 min.

**Neutrophil isolation**

Neutrophils were isolated from fresh human blood with 0.2% EDTA as an anticoagulant. Whole blood was collected from a healthy volunteer (a collaborator of this study) via venipuncture. A 2.4-ml aliquot of blood was then layered onto 2 ml of Mono-Poly Resolving Medium (Dainihon Seiyaku, Tokyo, Japan) in a centrifuge tube and centrifuged at 400 × g for 20 min at room temperature. The polymorphonuclear leukocytes (neutrophils) were then harvested, and the cell fraction was washed with HBSS.

**Intracellular Ca2+ mobilization measurement**

The isolated neutrophils were loaded with 2 µM fura-2/AM (Molecular Probes, Eugene, OR) mixed with 0.02% Pluronic F-127 (Molecular Probes) and 0.2% DMSO in HBSS for 40 min at 37°C. The suspension was agitated to prevent sedimentation. After loading, the cells were washed with HBSS twice and suspended in HBSS containing 0.3% BSA (HBSS/BSA). Approximately 1 × 106 cells in 900 µl of HBSS/BSA were added to a polystyrene-coated 35-mm petri dish and allowed to attach to the bottom of the dish for 30 min. Changes in intracellular calcium concentrations in response to C5a or a mixture of peptides (C-peptides and C5a) were determined by monitoring the ratio of fluorescence light emission at 510 nm as a result of excitation at 340 and 380 nm at 37°C using an ARGUS HiSCA calcium imaging system (Hamamatsu Photonics, Hamamatsu, Japan). The mixture of peptides and C5a was incubated in an Eppendorf tube on ice for 30 min in the HBSS solution and then applied to the cells after a 2-min baseline recording.

**Rat lethal shock induced by anti-Crry Ab after LPS priming**

Administration of anti-rat Crry mAb (5I2) (10) induces lethal shock in rats primed with a trace amount of LPS (11, 12). With this model, all rats sensitized with 0.05 mg/kg LPS died within 30 min of injection of mAb 5I2.

Male Wistar rats weighing ~250 g were purchased from Chubu Kagaku Shizai (Nagoya, Japan) and were allowed free access to food and water. Each rat was injected with 0.05 mg/kg LPS, prepared from a phenol extraction of Salmonella typhosa (Sigma-Aldrich, St. Louis, MO) in 250 µl of saline. After 20 h, 0.75 mg/kg 5I2 was administered. Ten minutes before the injection of 5I2, saline or C-peptides in saline were injected. All injections were administered i.v. through the tail vein. Animal experiments were conducted according to the Nagoya City University Guideline for the Care and Use of Experimental Animals and approved by the Nagoya City University Graduate School of Medical Sciences Animal Care Committee.

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**FIGURE 1.** Binding of C-peptides to immobilized PL37-MAP and C5a using Biacore equipment. An overlay plot of response curves was obtained from the Biacore instrument when various concentrations of Pep-A and Pep-B were injected. All samples were injected at time 0, and the association was monitored as an increase in RU. a, Various concentrations of Pep-A were injected over the PL37-MAP-coupled flow cell. b, The same concentrations of Pep-B were also injected over the PL37-MAP-coupled flow cell. c, Pep-A was injected over the C5a-coupled flow cell at the same concentrations of Pep-A as in a, d, The same concentrations of Pep-B were also injected over the C5a-coupled flow cell.
Results

C-peptides generated to a target peptide, PL37

The best fit peptide sequences to the target region of C5a (sequence of PL37: RAARISLGPRCIKAFTE) designed by MIMETIC were Peptide A (Pep-A: ASGAPAPGPAGPLRPMF) and Peptide-B (Pep-B: ASTAPARAGLPRLPKFF).

Binding of C-peptides to PL37-MAP and C5a

The Biacore instrument that uses SPR technology measures association and dissociation in real time of an unlabeled analyte with an immobilized ligand. PL37-MAP was covalently coupled to a CM5 sensor chip, resulting in a net increase of 8979 RU. As analytes, Pep-A and Pep-B were injected over the PL37-MAP-coupled flow cell and to the flow cell coupled with EA only. All cycles were performed in PBS at 25°C at a flow rate of 10 μl/min. As shown in Fig. 1a, binding data for the PL37-MAP-coupled flow cell were adjusted by subtraction of the data obtained by injection of the same sample over the EA-blocked flow cell (background). Pep-A bound to PL37-MAP, whereas Pep-B did not (Fig. 1, a and b). Binding of Pep-A was concentration dependent. Pep-A bound to PL37-MAP did not dissociate by increasing the salt concentration or by the addition of DMSO. A 6 M urea treatment was required to dissociate the complex.

Binding of C-peptides to C5a was also examined. C5a was covalently coupled to a CM5 sensor chip resulting in a net increase of 5770 RU. As analytes, Pep-A and Pep-B were injected over both the C5a-coupled and EA-coupled (as the control) flow cells. All cycles were performed in PBS at 25°C at a flow rate of 10 μl/min. In Fig. 1c, the binding data for the C5a-coupled flow cell were adjusted by subtraction of the data obtained by injection of
FIGURE 3. Measurement of intracellular Ca$^{2+}$ mobilization in neutrophils using a calcium imaging system. 

a, 0.7 nM C5a triggered a transient calcium influx in neutrophils. 

b, Incubation with 700 nM Pep-A blocked the effect of C5a completely. 

c, 95% inhibition was found when 350 nM Pep-A was incubated with C5a. 

d and e, Lower concentrations of Pep-A (70 or 7 nM) caused partial inhibition. 

f, The inhibitory effect was not detected when 0.7 nM Pep-A was mixed with C5a. 

g and h, Pep-B (700 or 70 nM) failed to cause inhibition of the calcium influx triggered by C5a. 

The area-under-curve data show the concentration dependency of the inhibitory effect of Pep-A.
Ca\(^{2+}\) influx measurement of TGW cells

As reported previously, inward calcium current pulses detected by a patch clamp assay, were evoked when TGW cells were exposed to 500 nM PL37-MAP (Fig. 2a). However, 50 \(\mu\)M Pep-A inhibited the pulse almost completely (Fig. 2b). After washing out, 500 nM PL37-MAP alone was added to the same cells without incubation with C-peptide, and the PL37-MAP peptide triggered an ion current pulse (data not shown). Pep-B did not block the ion current evoked by PL37-MAP (Fig. 2c). Inhibition by Pep-A was concentration dependent, because lower concentrations of the C-peptide blocked the effect of PL37-MAP only partially (Fig. 2, d and e). When the cells were exposed to Pep-A alone, ion current could not be observed even at the highest concentration used (not shown). Normalized data for amplitudes of the ion current responses are shown in Fig. 2f.

Intracellular Ca\(^{2+}\) mobilization measurement of neutrophils

The effect of C-peptides on C5a function was analyzed by measuring Ca\(^{2+}\) mobilization using an ARUGUS HiSCA calcium imaging system (Hamamatsu, Japan). Administration of 0.7 nM recombinant human C5a induced a transient increase in the level of intracellular Ca\(^{2+}\) of human neutrophils (Fig. 3a). The level of this activation was \(\sim 50\%\) of the maximum achieved with higher concentrations of C5a. A mixture of C-peptide and C5a was incubated in an Eppendorf tube on ice for 30 min in the HBSS solution and then applied to the cells. Incubation of C5a with 700 nM Pep-A inhibited the Ca\(^{2+}\) mobilization almost completely (Fig. 3b). A lower concentration of Pep-A (350 nM) still inhibited \(\sim 95\%\) of the effect of C5a (Fig. 3c). Inhibition was therefore concentration dependent: lowering the concentration of Pep-A resulted in a higher amplitude of calcium influx triggered by C5a (Fig. 3, d and e). The inhibitory effect of Pep-A disappeared at 0.7 nM (Fig. 3f). However, incubation of C5a with Pep-B showed no inhibition at any concentration used (700 and 70 nM Pep-B; Fig. 3, g and h). The results were normalized results using the area-under-curve data of the calcium influx and are described in Fig. 4.

Effect of C-peptides in a rat lethal shock model

Administration of 0.75 mg/kg anti-rat Crry mAb (10) induces rapid lethal shock in rats primed with 0.05 mg/kg LPS 20 h earlier (11, 12). The lethal outcome was C5a mediated (12). To investigate the inhibitory effect of the C-peptides in this model, we injected rats with saline (for the control) or C-peptides in 250 \(\mu\)l of saline 10 min before the 512 injection. All rats injected with saline died within 30 min (Table I). However, all rats injected with 4 mg/kg Pep-A survived. The inhibition was concentration dependent.

Table I. Effect of C-peptides on the rat lethal shock model

<table>
<thead>
<tr>
<th>C-peptides(^a)</th>
<th>Surviving/Total No. of Rats</th>
<th>Survival Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline alone</td>
<td>0/8(^b)</td>
<td>0</td>
</tr>
<tr>
<td>Pep-B, 4 mg/kg</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>Pep-A 4 mg/kg</td>
<td>4/4(^b)</td>
<td>100</td>
</tr>
<tr>
<td>2 mg/kg</td>
<td>2/3</td>
<td>67</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>1/3</td>
<td>33</td>
</tr>
<tr>
<td>0.5 mg/kg</td>
<td>3/4</td>
<td>75</td>
</tr>
</tbody>
</table>

\(^a\) C-peptides in 250 \(\mu\)l of saline were injected i.v. 10 min before the i.v. injection of anti-Crry mAb.

\(^b\) Saline control and Pep-A, 4 mg/kg, are statistically significant (\(p < 0.003\), Fisher’s method).

Lowering the concentration of Pep-A (from 2 mg/kg to 1 mg/kg) resulted in a lower proportion of surviving rats. Some of the surviving rats stopped breathing briefly in the first 1 or 2 min after injection of 512, but were soon breathing again, and \(\sim 20 \sim 40\) min later began moving. When the LPS-sensitized rats were treated with Pep-A without anti-Crry mAb administration, the animals survived with no harmful effect of Pep-A. In contrast, all rats injected with Ab and 4 mg/kg Pep-B died, as did those treated with the saline control.

Discussion

Various C5aR antagonists have already been reported, and some of these interfered with C5a-mediated functions in vitro and in vivo (3, 13–15). However, the only reported inhibitors of C5a were anti-C5a Abs (16–18). In this study, we designed C-peptides expecting them to interact directly with C5a, resulting in abrogation of C5a function. Two peptides, PepA and PepB, were designed by the computer program MIMETIC to target PL37, an AHB region of human C5a (aa 37–53). PepA bound to the target PL37, as determined using SPR technology, and inhibited the inward ion current pulse induced by PL37-MAP in neuroblastoma cells and C5a induction of intracellular Ca\(^{2+}\) mobilization in neutrophils. Furthermore, Pep-A bound to the whole C5a molecule so strongly that the complex could be dissociated only with 6 M urea.

Although Pep-B was designed by the same method as used for Pep-A and although both Pep-A and Pep-B showed maximum best fit values using MIMETIC, Pep-B showed no reactivity with PL37-MAP or C5a in any assay conducted. Careful examination of Pep-A and Pep-B might therefore provide useful information for improvement of the algorithm. Furthermore, the information obtained will contribute to a better understanding of peptide characteristics necessary to ensure interaction with a target amino acid sequence.

Two binding sites in C5a to C5aR have been reported. One is at the core of C5a and is centered around Arg 40 (19). The other is contained in the eight amino acids of the C terminus (20). PL37 is an important region in C5a.

Treatment with Pep-A was effective in our rat lethal shock model. In this model, rats primed with LPS died within 30 min when injected with anti-Crry mAb. In addition, the lethal outcome was mediated by C5a (12). Hence, our data suggest that Pep-A might bind selectively to C5a in vivo. However, one weak point of using peptide drugs is their short half-life in vivo. Therefore, a time delay between triggering shock and administration of the peptide could be crucial. Administration of 4 mg/kg Pep-A 30 min before the injection of anti-Crry mAb was not effective, indicating

FIGURE 4. The area-under-curve graph of the data of Fig. 3 show the concentration dependency of the inhibitory effect of Pep-A.
that PeP-A had been degraded in 30 min in vivo. The variable results at lower concentration of Pep-A (Table I) could be due to the short half-life of the peptide in vivo. In contrast, this short half-life could be advantageous. In endotoxic shock, endotoxin induces transient activation of complement and generation of C5a and C3a fragments, which cause lethal shock. In this form of shock, the short duration of the peptide drug and its rapid clearance would be an advantage in avoiding possible long-lasting side effects. Indeed, rats injected with Pep-A survived without any noticeable deleterious side effects.

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