Structure-Function Relationships of Human C5a and C5aR


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Structure-Function Relationships of Human C5a and C5aR


Using peptides that represent linear regions of the powerful complement activation product, C5a, or loops that connect the four α helices of C5a, we have defined the ability of these peptides to reduce binding of 125I-C5a to human neutrophils, inhibit chemotactic responses of neutrophils to C5a, and reduce H2O2 production in neutrophils stimulated with PMA. The data have defined likely sites of interaction of C5a with C5aR. The peptides had no functional activity per se on neutrophils and did not interfere with neutrophil responses to the unrelated chemotactic peptide, N-formyl-Met-Leu-Phe. Although previous data have suggested that there are two separate sites on C5a reactive with C5aR, the current data suggest that C5a interacts with C5aR in a manner that engages three discontinuous regions of C5a. The Journal of Immunology, 2003, 170: 6115–6124.

Human C5a (C5a) is a 74-aa glycoprotein generated upon activation of the complement system via the classical, alternative, or lectin-binding pathways (1). In concert with C4a, C3a and the membrane attack complex (C5b-9), C5a plays a central role in host defenses. Excessive complement activation leading to elevated plasma levels of C5a is known to be associated with many clinical conditions, including sepsis (2), adult respiratory distress syndrome (3), rheumatoid arthritis (4), Alzheimer’s disease (5), and ischemic heart disease (6), to name just a few conditions. Biochemical and pharmacological studies indicate that both C3a and C5a bind and interact with receptors that belong to the G protein-coupled superfamily of rhodopsin-like receptors (7–10). The C5aR (CD88) is expressed widely on inflammatory (9–12) and noninflammatory cells (13–18). For C5a-C5aR interaction, multiple discontinuous residues in two major structural elements within the C5a molecule have been reported to be required, as identified by nuclear magnetic resonance (NMR) structure analysis and site-directed mutagenesis experiments (19, 20). The first site of interaction is the highly ordered disulfide bridge-stabilized core of C5a (aa 1–63) with its four α helices juxtaposed in an antiparallel topology and connected by three peptide loops. The second area of C5a implicated in interaction with C5aR is the relatively disordered and flexible carboxyl-terminal tail of C5a (aa 64–74) (20). To understand the interplay between C5a and C5aR that trigger signal pathways, a “two-site binding” model of C5a-C5aR interaction has been proposed (21). Whereas the disulfide-linked core region of C5a may be recognized and have binding interactions with both the N-terminal segment as well as the third extracellular loop of C5aR (“recognition site”), the C-terminal region of C5a may fit into a binding pocket formed around the fifth transmembrane domain (“effector site”), leading via G protein activation to signal transduction (21). In recent studies, a genetic screen of C5aR mutants has identified in C5aR two distinct clusters of residues that possibly serve as a C5a-activated “molecular switch” (22). One cluster, located at the extracellular face of the receptor (binding area), may be required for interaction with the C5a molecule and the second cluster, at the core of the transmembranous helix bundle, may transmit the signal (via conformational changes) from the extracellular C5a ligand to the G protein (22–24).

For the human C3aR, direct agonist binding and calcium mobilization assays have shown that the large second extracellular loop (between the fourth and fifth transmembrane domain) is necessary not only for high-affinity C3a binding but also for the subsequent functional responses (25). In contrast, for the C5aR and IL-8 receptors, the N terminus and the third extracellular loop have been suggested to contain structures critical for binding (26) but redundant for activation. With regard to the ligands, all binding energy is contributed by the C terminus of C3a, in contrast to C5a where the central portion contributes a considerable amount of receptor interaction energy (27), suggesting, that different regions of the C5a molecule have to act in concert to achieve full potency. Whereas many studies have focused on the C terminus of C5a (which interacts with the “effector site” of the C5aR), little is known about different regions within the middle of C5a (and their interactions with C5aR), especially the inter-α helical peptide loops. Therefore, in the present study the structure-function relationships of different selected peptide regions of C5a were investigated. Loop peptide homologues, which are chemically conserved within the family of C5a species and are structurally different from the corresponding residues in the family of C3a species (20), were investigated as candidates for additional interaction of C5a with its receptor. Based on present data, a “three-site” interaction of C5a with C5aR is proposed.

Materials and Methods

Reagents

Human rC5a was purchased from Sigma-Aldrich (St. Louis, MO). All materials were obtained from Sigma-Aldrich unless otherwise indicated.

Selection and preparation of peptides of human C5a

Based on structure-function studies (19, 20) of human complement C5a (C5a), different regions of the molecule were chosen and synthesized for
evaluation of their ability to interact with the C5aR and affect the biological responses of neutrophils. The amino-terminal “A” region with the sequence MLKQKEEAIAKKSIVKVK (corresponding to aa residues 1–20), the middle “M” region CCYDGAAGVNNDECTEQAAR (corresponding to aa residues 21–40), and the carboxy-terminal “C” region CVVQILRAN IHKSHLGLQR (corresponding to residues 55–74) were selected as peptides of C5a. A scrambled version of the C peptide was also synthesized (C scrambled: CRVGLAQSMQDLKRHASIN). Additionally, based on the proposed three-dimensional (3D) structure (20), smaller peptide regions, representing the first, second, and third inter-α helical loop regions of C5a molecule were synthesized as follows: D1 (KYKSHSVKVK, aa residues 12–20), D2 (VNNDET, aa residues 28–33), and D3 (AAARSLGPR, aa residues 38–46) (Fig. 1A). In addition, scrambled versions of D2 peptide were also synthesized (D2-scrambled: I-TENFNY, D2-scrambled II: EN-TNDV). These peptides were synthesized and purified by HPLC (Research Genetics, Huntsville, AL and Open Biosystems, Huntsville, AL).

Isolation of human neutrophils

Whole blood from healthy donors was drawn into syringes containing 0.1 ml/mill blood of anticoagulant ACD (Baxter, Health Care, Deerfield, IL). Neutrophils were isolated using Ficol-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation (1500 rpm, room temperature, 30 min). The cell pellet was resuspended in dextran (final concentration, 1%) followed by a 45-min sedimentation step of erythrocytes. Supernatant fluids containing neutrophils were centrifuged (1500 rpm, room temperature, 5 min) and residual RBCs were removed by a hypotonic lysis step. After washing, neutrophils were resuspended in assay buffer and evaluated in binding studies, chemotaxis assays, and oxidative burst measurements.

125I-C5a binding studies

For binding studies, commercially available recombinant human C5a (C5a) was labeled with 125I using the chloramine-T-based protocol (28), which allows gentle oxidation and preserves the chemotactic activity of C5a for neutrophils (data not shown). To avoid receptor internalization, all of the following steps were performed at 4°C. To block nonspecific surface binding sites, isolated neutrophils were incubated for 1 h in binding buffer (HBSS, without Ca²⁺, containing 1% BSA). After a gentle washing step, neutrophils (2 × 10⁶ cells) were incubated in binding buffer (HBSS, with Ca²⁺, containing 0.1% BSA) in 1.5-ml microcentrifuge tubes (in a final volume of 200 μl) with 100 pM 125I-C5a (sp. act., 33.24 μCi/μg) in the absence or presence of increasing amounts of unlabeled C5a (ranging from 10⁻¹¹ to 10⁻⁴ M). After an incubation interval of 20 min, cell suspensions were layered over 20% sucrose and sedimented by centrifugation at 11,000 × g (Beckman Microfuge B; Beckman Coulter, Palo Alto, CA) for 2 min. The tubes were then frozen at −80°C and the tips, containing the cell pellet, were cut off to determine the cell-bound 125I-C5a using a gamma counter (1217 Multigamma; Wallac, Gaithersburg, MD). Binding affinities (Kd values) of C5a were calculated in the conventional manner (9). The saturation binding curve was generated using increasing amounts of 125I-labeled C5a (1–20 nM) in the absence or presence of 100-fold excess of unlabeled C5a and analyzed by nonlinear regression analysis using the GraphPad Prism program (GraphPad Software, San Diego, CA). Interference of the different synthetic peptides (1–1000 nM) with C5a binding was determined in the absence or presence of 10 nM 125I-labeled C5a using 2 × 10⁶ neutrophils in 200 μl of binding buffer.

Chemotaxis assay

Following neutrophil isolation, cells were fluorescent-labeled with 2',7'-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes, Eugene, OR) for 30 min at 37°C. After a washing step, labeled neutrophils (5 × 10⁵ cells/ml) were loaded into the upper chamber of a 96-well mini chamber (NeuroProbe, Gaithersburg, MD), separated by a polycarbonate filter with a porosity of 3 μm (NeuroProbe). The lower chambers were loaded with different concentrations of C5a or FMLP in the absence or presence of different concentrations of synthetic peptides of C5a (ranging from 10 to 1000 nM). Cells were then incubated for 30 min at 37°C. The number of cells that migrated through the polycarbonate membrane to the lower surface was determined by cytofluorometry (Cytofluor II; PerSeptive Biosystems, Framingham, MA). For each measurement, samples were done at least in quadruplicate.

Generation of H₂O₂ by neutrophils

H₂O₂ production was determined by an established assay (29). Briefly, isolated neutrophils (2 × 10⁶ cells/ml) were pretreated for 1 h at 37°C with either C5a (10 nM) or the helical peptide regions (described above) A, M, C (each at 1–1000 nM), or the loop peptides D₁, D₂, and D₃ (each at 10–1000 nM), or HBSS (control) alone in the presence of 1 mM sodium azide (to prevent endogenous catalases from destroying H₂O₂). To stimulate neutrophils, PMA (25 ng/ml) was added at the end of the incubation and the cell suspensions were then incubated at 37°C for 15 min. The reaction was then terminated by addition of 0.1 ml of trichloroacetic acid (50% w/v). The samples were centrifuged for 10 min at 500 × g and ferrous ammonium sulfate and potassium thiochyanate were added to the supernatant fluid at final concentrations of 1.5 mM and 0.25 M, respectively. The absorbance of the ferrithiocyanate complex was measured at 480 nm and compared with a standard curve generated from dilutions of reference solutions of H₂O₂.

Analysis of the molecular structure and determination of possible interaction sites of C5a and C5aR

To analyze the molecular structures of C5a and C5aR and to localize possible C5a-C5aR interaction sites, the published sequences of C5a and C5aR were molecular graphics in conjunction with examined using the PROSITE database (30) and PHD coil prediction (31), and based on our current in vitro data a three-site binding model was designed.

Statistical analyses

All values were expressed as mean ± SEM. Data sets of binding, chemotaxis, and H₂O₂ assays were analyzed with one-way ANOVA; differences in the mean values among experimental groups were then compared using the Tukey multiple comparison test. Results were considered statistically significant where p < 0.05.

Results

C5a peptide regions selected for study

Based on the sequence and the NMR structure of C5a, different regions of C5a were chosen for synthesis of peptides. For reference purposes, Fig. 1A depicts the NMR structure indicating positions of the designated inter-α-helical loop peptides (D₁, D₂, D₃). This figure was generated using MOLSCRIPT (32) and PovRay. For reference purposes, the structure of C5a and its proposed interactions with C5aR (shown by the red shaded regions) are shown in Fig. 1B.

Antagonization of 125I-C5a binding to neutrophils by C5a peptides

These studies were undertaken to assess the binding of 125I-C5a to blood neutrophils in the presence or absence of the different selected peptide regions of C5a. Using 100 pM 125I-C5a, competition experiments with unlabeled C5a revealed a Kd value of 1 nM (data not shown), which is consistent with published data (10). The saturation binding curve indicating a plateau of binding beyond 10 nM C5a is shown in the inset of Fig. 2A. Six different peptides termed A, M, and C (aa residues 1–20, 21–40, and 55–74, respectively), and loop peptides D₁, D₂, and D₃ (described in Fig. 1) were evaluated for their ability to inhibit the binding of 10 nM 125I-C5a

FIGURE 1. A, Ribbon diagram of the human C5a molecule with the α helices 1–4 (gray bands) and the less well-defined interconnecting interhelical loop regions (narrow regions). According to the 3D structure of human C5a, the selected inter-α helical regions are surface residues of interest for possible C5a-C5aR interaction (D₁ representing acid residues 12–20, D₂ acid residues 28–33, and D₃ acid residues 38–46, respectively). The core is stabilized by disulfide linkages among residues 21–47, 22–54, and 34–55 (yellow). B, Model for the interaction of C5a with C5aR. The seven transmembrane helices (cytoplasmic and extracellular) contain different charged loop regions; especially the amino-terminal and extracellular loop II with possible interaction sites for the D₁ (acid residues 12–20) and D₂ (acid residues 28–33) region of C5a. Electrostatic interactions with a possible generation of salt bridges (darker gray areas) in extracellular loops of C5aR are shown based on computer-assisted structure analysis of the molecule (models adapted from Refs. 20 and 47).
Figure 1.
were used to antagonize the saturated binding of \(^{125}\text{I}-\text{C5a}\) (10 nM) to neutrophils.

Antagonization of three small loop peptides D\(_1\) (representing residues 12–40), D\(_2\) (residues 21–40 and 55–74, respectively) resulted in a significant reduction (>60%) in the chemotactic responses. In separate and independent experiments, the C peptide (1 \(\mu\)M) reduced the neutrophil chemotactic response to C5a by 87%, whereas the scrambled C peptide (1 \(\mu\)M) reduced the response by only 8.5% (Table I). The N-terminal helix peptide A failed to significantly alter C5a-induced migration of neutrophils, albeit interference with C5a binding to neutrophils was found with the same peptide (Fig. 2A), indicating some differences in binding ability and subsequent effects on functional responses to C5a. Whereas loop peptide D\(_3\) did not significantly alter chemotactic responsiveness of neutrophils to C5a, significant reductions (>60%) by synthetic loop peptide D\(_2\) occurred, while nearly total inhibition by peptide D\(_1\) was found (Fig. 3B). D\(_3\) peptide failed to show any inhibitory effect on the chemotactic response to C5a, consistent with the relatively poor reduction in binding of \(^{125}\text{I}-\text{C5a}\) (Fig. 2B). In contrast to the inhibitory effects of D\(_2\) peptide on chemotactic responses to C5a (85% reduction, Fig. 3B and Table I), the scrambled D\(_2\) peptides (D\(_2\)-scrambled I and D\(_2\)-scrambled II) had no effects on the chemotactic responsiveness of neutrophils to C5a (<7% reductions, Table I). Complete dose responses for the inhibitory activity of each D peptide, normalized to the 10 nM C5a response, are shown in the right-hand inset of Fig. 3B. These data suggest that peptides from the middle (M) and carboxyl (C)-terminal regions as well as from the loop regions (D\(_1\) and D\(_2\), but not D\(_3\)) have the ability to compete functionally with intact C5a, suggesting multiple sites for the binding of C5a to C5aR.

**Specificity of C5a peptides for interference with chemotaxis of human neutrophils to C5a but not to fMLP**

As shown in Figs. 4A and 5A, the dose-dependent chemotactic response of neutrophils to 10 nM C5a was examined in the absence and presence of A, M, and C peptides as well as D\(_1\), D\(_2\), and D\(_3\) peptides (all at 1 \(\mu\)M). As expected, both the helical A peptide and the loop D\(_3\) peptide failed to significantly alter the dose-dependent C5a-induced chemotactic response of neutrophils over a wide dose range of C5a (0.1 pM to 100 nM of C5a). In contrast, the presence of M and C peptides as well as the D\(_1\) and D\(_2\) peptides significantly suppressed the C5a-induced dose-dependent chemotactic responses (Figs. 4A and 5A). Companion experiments were conducted to see whether any of the six peptides (all at 1 \(\mu\)M) would affect the chemotactic responses of human neutrophils to a variety of concentrations of fMLP, another potent chemotactic agonist for human neutrophils. As shown in Figs. 4B and 5B, none of the peptides affected the chemotactic responses of human neutrophils to fMLP. These results demonstrate the specificity of these peptides to C5a-induced responses of human neutrophils.

**Ability of C5a and C5a peptides to inhibit the \(\text{H}_2\text{O}_2\) responses of PMA-stimulated neutrophils**

We have recently shown that in vitro exposure of human neutrophils to human C5a in a dose- and time-dependent manner reduces...
FIGURE 3.  A, Chemotactic response of neutrophils to C5a (left inset) and C5a (at 10 nM) in the presence or absence of helical peptides (A, M, and C, each at 1 µM). Right upper inset, Dose-response inhibition of A, M, and C peptides in chemotactic responses of neutrophils to 10 nM C5a. B, Ability of different C5a loop peptides (D1, D2, and D3, each at 1 µM) to block chemotactic activity of human neutrophils to the whole C5a molecule. Inset, Dose dependency of the peptide-induced interference with chemotaxis of neutrophils to 10 nM C5a. For each experimental end point, n = 6–12. ctrl, Control.
the ability of human neutrophils to generate H$_2$O$_2$ following stimulation with PMA (2, 33). Human neutrophils in suspension were incubated for 1 h at 37°C in the presence or absence of peptides A, M, and C or D$_1$, D$_2$, and D$_3$ (in doses ranging from 1 to 1000 nM). As indicated, cells were then exposed to PMA (25 ng/ml) and the H$_2$O$_2$ response was assessed as described elsewhere (29). The PMA concentration chosen was according to our recently published study, in which the 25-ng/ml dose of PMA exhibited a half-maximal oxidative burst response of neutrophils (33). As shown in Fig. 6, in the absence of PMA, C5a and the six different peptides failed to induce significant H$_2$O$_2$ responses (gray bars). The scrambled C and D$_2$ peptides by themselves also failed to induce H$_2$O$_2$ responses (data not shown). When neutrophils were incubated for 1 h at 37°C in buffer and then exposed to PMA in the absence of peptides (none), robust H$_2$O$_2$ responses were found (Fig. 6). In both sets of experiments, pre-exposure of neutrophils to 10 nM C5a for 1 h at 37°C almost totally abolished the H$_2$O$_2$ response (Fig. 6), which has recently been shown to be caused by a C5a-induced inhibition of the NADPH oxidase assembly (33). Pre-exposure of neutrophils to 1 µM of peptides A, M, or C significantly reduced PMA-induced generation of H$_2$O$_2$ (Fig. 6A). In separate and independent experiments, pre-exposure of neutrophils to 1 µM native C peptide reduced the H$_2$O$_2$ response by 85%, whereas the scrambled C peptide (1 µM) reduced the response by only 35% (Table I). Dose responses for all peptides are shown in the insets in Fig. 6. Pre-exposure of neutrophils with 1 µM of loop peptides D$_1$ or D$_2$ resulted in nearly total abolition of the H$_2$O$_2$ response to PMA, whereas exposure to the D$_3$ peptide did not significantly alter the H$_2$O$_2$ response to PMA (Fig. 6B). In separate and independent experiments, 1 µM D$_2$ peptide reduced the H$_2$O$_2$ response by 78.5%, while 1 µM scrambled D$_2$I and D$_2$II peptides reduced the H$_2$O$_2$ responses by only 1.5% and 15.7%, respectively, (Table I). These data are consistent with the rank order of effects of peptides described in Figs. 2–4, as demonstrated by their ability to

Table I. Ability of C5a peptides to reduce chemotactic and H$_2$O$_2$ responses of neutrophils

<table>
<thead>
<tr>
<th>Incubation with Peptides (1 µM)</th>
<th>Amino Acid Sequence</th>
<th>Chemotaxis in the Presence of C5a (10 nM) (% response to C5a)</th>
<th>H$_2$O$_2$ Response after PMA (25 ng/ml) Stimulation (% response to PMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Not applicable</td>
<td>100.0 ± 2.4</td>
<td>100.0 ± 0.1</td>
</tr>
<tr>
<td>C peptide</td>
<td>CVVSQLRANISHKDMQLGR</td>
<td>13.2 ± 3.4</td>
<td>15.4 ± 0.4</td>
</tr>
<tr>
<td>C scrambled</td>
<td>CRVGLAQSMQDRLKHAASN</td>
<td>91.5 ± 3.4</td>
<td>66.8 ± 0.6</td>
</tr>
<tr>
<td>D$_1$ peptide</td>
<td>VNNDET</td>
<td>15.2 ± 2.2</td>
<td>21.5 ± 0.4</td>
</tr>
<tr>
<td>D$_1$ scrambled I</td>
<td>TENDNV</td>
<td>93.2 ± 7.0</td>
<td>98.5 ± 0.4</td>
</tr>
<tr>
<td>D$_1$ scrambled II</td>
<td>ENTNDV</td>
<td>107 ± 10.1</td>
<td>84.3 ± 0.2</td>
</tr>
</tbody>
</table>

*Chemotaxis and H$_2$O$_2$ assays were performed essentially as described in Materials and Methods. The data are representative of three or more separate and independent experiments performed in triplicate for each condition.

FIGURE 4. A. Dose dependency of the chemotactic responses of neutrophils to C5a, ranging from 0.1 pM to 100 nM, or B, fMLP, ranging from 0.1 nM to 1 µM in the presence or absence of peptides A, M, and C (each at 1 µM). Number of experiments, 6–10. ctrl, Control.

FIGURE 5. A. Dose dependency of the chemotactic responses of neutrophils to C5a or B, fMLP in the presence or absence of peptides D$_1$, D$_2$, and D$_3$ (each at 1 µM). For each end point, n = 6–10. ctrl, Control.
FIGURE 6. A. Blockade of H$_2$O$_2$ responses in PMA-stimulated neutrophils by C5a and different helical peptides A, M, and C (each at 1 μM), or B, and loop peptides D$_1$, D$_2$, and D$_3$, each at 1 μM. Upper inset. Dose responses for A, M, and C peptides in suppressing PMA (25 ng/ml)-induced H$_2$O$_2$ production in neutrophils. Lower inset. Dose-response inhibition for D$_1$, D$_2$, and D$_3$ peptides in PMA-induced H$_2$O$_2$ responses of neutrophils. Number of each experiment: n = 6–10. ctrl, Control.
interfere with C5a binding to human neutrophils and to inhibit C5a-induced chemotactic responses.

Discussion

The complement system is a main buttress of the host defense system. The complement activation product, C5a, is known to be a potent mediator of the monocyte-phagocytic cell system and binds to the seven-transmembrane spanning receptor, C5aR (10). The parts of the C5a molecule mainly involved in binding to C5aR and in subsequent biological responses are still matters of debate (34–36). This is in striking contrast to C3a in which the C-terminal peptide (residues 69–77) contains virtually all of the biological function of intact C3a (37), while C-terminal peptides of C5a contain some biological activity (38–40), albeit this is extremely limited. Only at very high concentrations do these peptides show weak agonist activity when compared with intact C5a. This is consistent with the concept that interaction of C5a with C5aR involves several discontinuous regions of C5a and C5aR (22–24, 32, 41–50). Site-directed mutagenesis studies of C5a have suggested that regions in the middle of the C5a molecule as well as in the C and N terminus ends facilitate C5a binding to C5aR on neutrophil membranes (19). Our findings of substantial inhibition of 125I-C5a binding to intact human neutrophils in the presence of synthetic peptides representing the amino-terminal, middle, and carboxy-terminal regions of C5a implicate an involvement of all three regions in binding of C5a to C5aR (Fig. 2). Although many studies have focused on the amino- or C-terminal regions, little is known about the binding properties of the middle peptide region of C5a or the loop regions of C5a. In one report, a disulfide-linked core peptide including residues 20–37 obtained by tryptic digestion of C5a, was shown to exhibit weak competitive binding of C5a to C5aR (47), which would support our findings.

However, not only linear protein segments but also posttranslational changes (51) and assembled topographic sites (i.e., arrays of amino acids juxtaposed by the 3D folding of the helical region of C5a) are possible targets for binding interactions between C5a and C5aR (51, 52). Therefore, based on 3D NMR structure studies, which showed closely conserved internal residues of the C3a and C5a molecules but completely different surface residues (43, 44), we chose different inter-α helical loop peptide regions containing residues of interest (K-14, H-15, V-17, K-19, E-32, T-33, A-39, R-40, and L-43) as candidates for additional interactions of C5a with C5aR (20) (Fig. 1). In competitive binding assays, peptides representing inter-α helical loop peptides (D1 and D2) significantly reduced C5a binding, but blocking activity was not found with the loop D3 peptide (Figs. 1 and 2). The efficacy of the D1 peptide is supported by other data indicating that a double replacement of K-19 and K-20 in this C5a region reduced binding of C5a to C5aR (45). Further evidence for a possible receptor-interaction site in the C5a peptide loop D1 has been suggested by a greatly increased affinity of a conformationally biased C-terminal decapetide when linked to C5a (12–20) (39). Loop D2, which represents a small peptide region within the M peptide of C5a, revealed equal effects on blocking the binding of C5a to C5aR in contrast to the M peptide, suggesting that the hexapeptide D2 represents a receptor interaction domain of the middle region. In support of this conclusion, C5a (20–37) residues that contain the D2 region have been shown to represent an important receptor recognition domain (47). Inconsistent results have been reported regarding the interaction of the C5a peptide loop D1 (including residues R-40 and R-46) with C5aR. Although substitution of R-40 by G changed C5a binding behavior to neutrophil membranes (19), no participation of this loop inclusive of R-40 and P-45 in receptor binding was found (38, 45) and no functional activity (39, 44) was demonstrated, in confirmation of our findings.

In the current studies, neutrophils were exposed to C5a, resulting in a defective H2O2 response of stimulated neutrophils. When human neutrophils were first exposed to the various peptides representing regions of C5a, all designated peptides except of D1 were capable of profoundly compromising H2O2 responses in a dose-dependent manner (Fig. 5). The D1, M, and D2 peptides, but not the scrambled D2 peptides, were potent molecules for inhibiting H2O2 generation, suggesting that important biological interaction sites may exist in the middle region of C5a. Mutations in the D1 region ((A-19, A-20)-rhC5a), but not in the D2 region, have been shown to influence myeloperoxidase release from human neutrophils (45). In addition, the amino-terminal peptides 1–17 of porcine C5a (des-arg) have been reported to cause neutrophil degranulation in guinea pigs and to contribute to the biological potency of the intact molecule (43). Other studies, which have investigated enzyme release from human neutrophils in the presence of cytochalasin B, indicated an effector site in the C-terminal decapeptide C5a 95–74, with residues 65–69 contributing to biological activity (41, 53). This is consistent with our findings suggesting the biological efficacy of the C peptide region of C5a, in addition to the central (middle) region and D1 loop peptide region.

Almost the same pattern of sites in the C5a molecule interactive with C5aR could be identified in chemotaxis assays using different peptides in the presence of C5a. Neutrophil exposure to these peptides did not affect the chemotactic responses of neutrophils to FMLP (Figs. 3 and 4), suggesting specificity of the peptides. Similarly, the scrambled peptides had no effects on chemotactic and H2O2 responses of neutrophils when compared with the non-scrambled C and D2 peptides. Mutation of the N terminus of C5a did not alter chemotactic responses to C5a, whereas chemotactic responses of neutrophils to R-74, G-73, L-72, K-68, or R-40-substituted mutants of C5a were significantly reduced (19). Additionally, substitution in C5a at A-26 appeared from NMR studies to reduce chemotactic potency of neutrophils by carrying a long-distance conformational change in D1 around H-15, again suggesting discontinuous central regions and the C terminus to be required to achieve full biological response (19, 41).

In an attempt to localize sites within C5aR (9, 10) that could interact with the C5a molecule, the secondary structure and potential interactions of C5a and C5aR were evaluated using the PROSITE database (30) and PHD coil analysis (31). Based on our in vitro data, we propose a three-site binding model (Fig. 1B). There is much evidence that a first binding site is located between the negatively charged amino residues in region 21–30 of the C5aR (21, 34, 54) and the four positively charged K-12/14/19/20 residues within the D1 region. The negatively charged N-terminal residues 10–18 of C5aR were reported not to be directly involved in binding of C5a but it was suggested that they interact with the positively charged and hydrophobic stretch of residues within extracellular C5aR loop II, which is close to the fifth transmembrane helix, presumably forming a stable C5a binding pocket (34). On the other end of the extracellular C5aR loop II (close to the fourth transmembrane helix), there is an additional positively charged region that could interact with the negatively charged D2 region of C5a which therefore might be considered as a second, newly recognized binding site. The disulfide bond between C-109 in the extracellular loop I and C-189 in the extracellular loop II (34) could stabilize this proposed second binding site. There is also fairly good evidence for the existence of a third C5a/C5aR interaction site (21, 34, 35, 54, 55) in which R-206 in the fifth transmembrane domain has been suggested to play an important role in binding the C terminus by acting as a counterion for R-74 of C5a.
(35). A recent report has suggested in neutrophils that further C-terminal interactions also may occur between K-68 of C5a and E-199 of the C5aR, close to the fifth transmembrane helix (56). Another study has shown that substitution of H-67 by F resulted in a significant increase in potency of C5a, suggesting an advantage in receptor binding by adding more hydrophobic residues to the ligand (41), facilitating interactions between the Cα C terminus and transmembrane helices of C5aR (21). Furthermore, in comparison to C5aR, the recently published orphan receptor C5L2 (57) (which binds C5a with high affinity but couples poorly to G protein-mediated signaling pathways) exhibit almost identical electrical charge patterns of the three above suggested interaction sites (Fig. 1B), even if there is only a 35% amino acid identity with C5aR.

These data imply that within the C5a molecule at least three different discontinuous regions exist and interact with C5aR and suggest possible targets for the development of effective C5aR antagonists for intervention in the inflammatory response.

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References


