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Modulation of C3a Activity: Internalization of the Human C3a Receptor and its Inhibition by C5a

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The C3a receptor (C3aR) is expressed on most human peripheral blood leukocytes with the exception of resting lymphocytes, implying a much higher pathophysiological relevance of the anaphylatoxin C3a as a proinflammatory mediator than previously thought. The response to this complement split product must be tightly regulated in situations with sustained complement activation to avoid deleterious effects caused by overactivated inflammatory cells. Receptor internalization, an important control mechanism described for G protein-coupled receptors, was investigated. Using rabbit polyclonal anti-serum directed against the C3aR second extracellular loop, a flow cytometry-based receptor internalization assay was developed. Within minutes of C3a addition to human granulocytes, C3aR almost completely disappeared from the cell surface. C3aR internalization could also be induced by PMA, an activator of protein kinase C. Similarly, monocytes, the human mast cell line HMC-1, and differentiated monocyte/macrophage-like U937-cells exhibited rapid agonist-dependent receptor internalization. Neither C5a nor FMLP stimulated any cross-internalization of the C3aR. On the contrary, costimulation of granulocytes with C5a, but not FMLP, drastically decreased C3aR internalization. This effect could be blocked by a C5aR-neutralizing mAb. HEK293-cells transfected with the C3aR, with or without Gα16, a pertussis toxin-resistant G protein α subunit required for C3aR signal transduction in these cells, did not exhibit agonist-dependent C3aR internalization. Additionally, preincubation with pertussis toxin had no effect on C3a-induced internalization as one negative feedback mechanism and is influenced by the C5aR pathway.


The anaphylatoxins C3a, C5a, and C5a-desArg are generated during complement activation. Through binding to the C3aR and C5aR on neutrophils, monocytes, basophils, mast cells, and eosinophils, they function as potent proinflammatory mediators (1–9). Agonist binding stimulates a pertussis toxin-sensitive (PTX) increase in free cytosolic [Ca$^{2+}$], (10–12) and initiates a repertoire of host defense actions, from secretory granule release from neutrophils (12, 13) to chemotaxis in mast cells (6; for a review, see Ref. 14). Recent studies on both receptors suggest a much broader tissue distribution than previously surmised. C3aR is expressed during inflammation in the brain and on activated B lymphocytes (15–18); in addition, C5aR is expressed on hepatocytes, lung, smooth muscle, and endothelial cells (19–24). The complement system is strictly regulated by a variety of positive and negative feedback mechanisms to avoid self-destruction of the organism. Similarly, the signaling mediated by the anaphylatoxins must also be tightly regulated. Serum carboxypeptidase N, acting on the anaphylatoxins’ C-terminal arginine residue, rapidly inactivates newly generated C3a and greatly reduces the biologic activity of C5a (25). An additional receptor control mechanism is homologous desensitization. Details of the negative feedback mechanisms of C5aR have been described. The cytosolic C-terminus of the C5aR is phosphorylated within minutes of C5a addition (26–28), and the receptor is rapidly internalized (29, 30). Major and minor phosphorylation sites at the C5aR C-terminus, which seem to be important for internalization and receptor recycling, have been identified (29, 31, 32). Less is known about the regulation of the C3aR, but, as with C5aR, homologous desensitization has been noted at least in the guinea pig system (33).

Several groups have used the β2-adrenergic receptor as a model system to study G protein-coupled receptor internalization; C-terminal Ser and Thr residues are phosphorylated by G protein-coupled receptor kinases (for a review, see Refs. 34 and 35). Kinase activation is enhanced by their βγ subunit-dependent translocation from the cytosol to the membrane (36, 37) and their phosphorylation by protein kinase C (38, 39). A member of the β-arrestin family binds to the phosphorylated receptor, thereby uncoupling it from its G protein, but improving its attachment to the clathrin-coated vesicle-mediated endocytic pathway (40–42). The receptor is then rapidly internalized, and the cells are desensitized. Receptor class desensitization (43) has also been described after stimulation of transfected RBL-2H3 cells by C5a or FMLP, which results in the cross-phosphorylation and desensitization of IL-8R A (44), whereas nonchemotactic receptors are not affected. These pathways are dependent on protein kinases C or A (45–47). Simultaneously, negative feedback mechanisms distal from the G protein exist, such as the phosphorylation of phospholipase Cβ1 (48). The internalized receptors are either degraded or dephosphorylated and recycled to the cell surface (29).

The aim of this study was the detailed characterization of human C3aR internalization as one negative feedback mechanism on granulocytes, as the largest leukocyte population naturally bearing the C3aR. A subset of experiments was performed on the human...
mast cell line HMC-1, human monocytes, and differentiated mono-
cyte-like U937 cells to demonstrate that internalization is not a reg-
ulatory mechanism limited to granulocytes. Transiently transfected HEK293 cells were additionally investigated. In particular, the influ-
ence of C5a and FMLP on C3aR internalization was analyzed.

Materials and Methods

Reagents

Human C3a was purchased from Advanced Research Technologies (San
Diego, CA). The C3a analogue synthetic peptide P117 (LRQQARAS-
ALGLAR; aa 63–77 of human C3a) and the control peptide P252 (YTTD-
DYGHYDD) (49) were prepared by solid phase synthesis. FITC-labeled
 goat anti-rabbit IgG was obtained from Dianova (Hamburg, Germany). All
other materials, including FMLP and recombinant C3a, were obtained from
 Sigma (Deisenhofen, Germany). The polyclonal anti-C3aR serum, specific
 for the second extracellular loop, was a gift from R. Ames (SmithKline Beecham,
Philadelphia, PA); its generation and use in characterization of the C3aR
expression pattern of leukocytes have recently been described (49).

Cell lines and cell culture conditions

The culture conditions of U937 cells (American Type Culture Collection,
Manassas, VA) and C3aR induction by IFN-γ (1000 U/ml for 3 days) have been
described previously (50). HEK293 cells (human embryonic kidney; American
Type Culture Collection) were grown in DMEM/nutrient mix
F-12 (Life Technologies, Egggenstein, Germany) supplemented with 10%
heat-inactivated FCS, penicillin (50 U/ml)/streptomycin (50 μg/ml), 2 mM
L-glutamine, and 1 mM sodium pyruvate at 37°C in a humidified atmos-
phere with 5% CO2. HMC-1 cells (provided by J. H. Butterfield) were
 cultured in RPMI 1640 medium (Life Technologies) supplemented as in-
dicated above.

Transient transfection of HEK293 cells

Using lipofectamine reagent from Life Technologies (Egggenstein,
Germany) according to the manufacturer’s instructions, HEK293 cells (5 × 106
cells in a 75-cm2 culture flask) were transiently cotransfected with
4 μg of C3aR in pCDNA3/neo vector (Invitrogen, De Schelp, The Neth-
erlands) and 2 μg of pCDMS (Invitrogen) encoding Gsα1. As a negative
control, cells were transfected with plasmid encoding C5α or pCDMS
without any insert. The transfected HEK293 cells were harvested on day 3
for additional experiments, at which time they expressed approximately
50,000–150,000 receptors/cell, with a Kd in the range of 1–5 nM (12, 49).

C3aR internalization assay based on flow cytometry

PBLs were prepared from EDTA blood of healthy donors; erythrocytes
were lysed by NHCl. HEK293 cells were harvested using cell dissociation
solution (Sigma). The remaining PBLs and the HEK293 cells as well as
harvested HMC-1 cells, which grow in suspension, were washed twice and
resuspended in PBS (kinetics and dose-response curves) or with RPMI
without phenol red (all other experiments) at 4°C. For all experiments, cells
were resuspended at a density of 1 × 107/ml. Cells and stimuli were pre-
incubated separately at 37°C for 5 min. The internalization was started in
a total volume of 100 μl by adding a cell sample (8 × 107 cells in 80 μl)
 to the agonist (20 μl). The sample was halved when the indicated incuba-
tion time was reached. One part (50 μl) was immediately added to 100 μl
of ice-cold polyclounal rabbit anti-C3aR serum (1/4000), the other to 100
mM NaCl, 5 mM KCl, 0.5 mM glucose, 0.25% BSA, 1 mM
HEPES, 125 mM NaCl, 5 mM KCl, 0.5 mM glucose, 0.25% BSA, 1 mM
CaCl2, and 1 mM MgCl2. Incubation was continued for 10% (w/v) sucrose
at 37°C, internalization was stopped by the addition of 50
mM C3a was
separated from free tracer by centrifugation through a 10% (w/v) sucrose
cushion (12,000 × g, 6 min, 4°C) as previously described (12). The samples
were counted on a gamma counter (Canberra Packard, Dreieich, Germany).
The degree of receptor internalization was calculated from the ratio of specific
125I-labeled ligand bound obtained after the acid wash compared with that
 obtained after a buffer wash. All acid and buffer washes were performed in
quadriplate; non-specific binding (acid wash) was performed in triplicate.

Due to technical limitations, a maximum of four time points can be determined
per assay. For a complete kinetic study, as depicted in Fig. 4, three experiments
with overlapping time points were performed.

Results

C3aR flow cytometric internalization assay

A quantitative flow cytometric assay was used to estimate C3aR
internalization. C3aR-dependent fluorescence signals of granulo-
cytes that had been stimulated were compared with those of controls.
Human granulocytes (in the presence of other leukocyte

As additional controls for the specificity of our flow cytometric assay, a subset of experiments on PMNs was repeated using the mAb 8H1 (IgG1) raised against the identical part of the receptor as the polyclonal rabbit antiserum (W. Bautsch et al., submitted). Essentially the same internalization pattern was noted as that obtained above with the polyclonal antiserum. However, the difference in fluorescence intensity between that obtained with this C3aR-specific mAb and that of a nonrelevant control IgG1, which served as relative measure for the number of C3aR on the cell surface in this experiment, was smaller than the difference obtained with specific polyclonal rabbit anti-C3aR serum and control serum. Therefore, a higher relative error of the calculated C3aR internalization resulted using the mAb, restricting its use to less quantitative control experiments (data not shown). Synthetic peptides were used as stimuli, and internalization after 10-min incubation was determined. Stimulation with 100 nM C3a analogue peptide P117 caused on granulocytes 81 ± 9% C3aR internalization and 100 ± 6% internalization at a concentration of 1 μM compared with negligible internalization of 6.6 ± 7.3 and 3.0 ± 3.3% with the irrelevant peptide P252 (n = 3). Both peptides have been used by us before as controls for C3a specificity (49, 56).

The human mast cell line HMC-1 and human monocytes were also partially analyzed. On HMC-1 cells, the maximal C3aR internalization (∼60–70%) was reached within 5 min (filled diamonds in the right panel of Fig. 2). These cells seemed to be more sensitive than PMN to the manipulations necessary in this assay. In the buffer control (open diamonds), there was a slow decrease in detectable C3aR, corresponding to a slight spontaneous C3aR internalization. Therefore, the experiment was repeated under modified conditions, making it unlikely that the lack of complete C3aR internalization was due to the fact that the HMC-1 cells had been overstressed; C3aR internalization was triggered by the addition of 100 nM C3a (final concentration) directly into the cell culture medium of HMC-1 cells that had been cultured undisturbed for 48 h. By 15 min the maximal internalization was still only approximately 70% (data not shown). The half-maximal response after 3 min of incubation with the stimulus was reached at about 41 ± 18 nM C3a (n = 3; Fig. 1, middle panel). A typical histogram of the fluorescence obtained on HMC-1 cells is depicted in Fig. 3. Stimulation with 100 nM C3a analogue peptide P117 caused 9.1 ± 4.0% C3aR internalization and 51.3 ± 4.8% internalization at a concentration of 1 μM compared with negligible internalization of 6.6 ± 4.7 and 8.0 ± 3.4% with the irrelevant peptide P252 (n = 3).

A similar dose-dependent shift of the C3aR-specific fluorescence was observed on monocytes (Fig. 1, lower panel). The histogram in Fig. 3 (lower panel) demonstrates that the C3aR-dependent fluorescence signal was much smaller on these cells than on neutrophils (Fig. 5) or HMC-1 cells, most likely corresponding to a smaller number of C3aR on native peripheral monocytes. Because of that smaller signal-to-background ratio, the statistical error of the calculated percentage of internalized C3aR in a dose-response curve increased (Fig. 1, lower panel). In monocytes, the half-maximal response in three independent experiments ranged from 15–50 nM C3a. Stimulation with 100 nM C3a analogue peptide P117 caused 17.9 ± 8% C3aR internalization, and there was 66.7 ± 16.5% internalization at a concentration of 1 μM compared with negligible internalization of 3.0 ± 11.5 and 9.0 ± 15.7% with the irrelevant peptide P252 (n = 3). Since monocytes attached rapidly onto the surface of the vials (different plastic surfaces were compared) during the incubation at 37°C, a process that led to a drastic loss of flow cytometrically detectable cells and that might simultaneously lead to C3a-independent cell activation, time points beyond 3 min were not analyzed on these cells.

FIGURE 1. Dose-response curve of C3aR internalization on PMNs, HMC-1 cells, and monocytes. The cells were incubated with increasing concentrations of C3a for 3 min at 37°C. Receptor internalization (mean ± SE calculated from three single samples) was detected antigenically by flow cytometry. A representative experiment (n = 3) is depicted.

FIGURE 2. Kinetics of ligand-dependent C3aR internalization. Human granulocytes (left panel, with curves from three independent experiments) and the human mast cell line HMC-1 (one typical experiment of four; right panel, ○) were incubated at 37°C for an increasing period of time with 13 or 100 nM C3a, respectively. C3aR internalization was detected antigenically by flow cytometry. Values represent the mean ± SE calculated from three single experiments. Control experiments were performed using buffer instead of C3a. For PMNs, the percentage of C3aR internalization increased only slowly to <20% after 1800 s (data not shown). HMC-1-cells were more sensitive to the experimental conditions’ the buffer control increased to almost 40% (right panel, ○).
The values obtained by the flow cytometric assay are usually very exact as long as relatively high numbers of C3aR are present on the surface of the analyzed cell (~24,000 molecules/neutrophil) and as long as a relatively large proportion of receptors disappear from the cell surface. However, this type of assay is insensitive to relatively low agonist concentrations. For this reason and to confirm our data by an independent method, a second assay based on insulin receptor immunoprecipitation was used for analysis of C5aR internalization (31). As depicted in Fig. 4, IFN-γ has been used for analysis of C3aR internalization. A similar system has been used for analysis of C5aR internalization (31). As depicted in Fig. 4, IFN-γ-treated, monocyte-related U937 cells (50) internalized the 125I-labeled ligand-receptor complexes, but relatively slowly. Unfortunately, a very limited number of samples can be processed simultaneously using the acid wash technique, and, as depicted, it is additionally hampered by a relatively high SD, mainly resulting from a relatively high nonspecific 125I-C3a internalization. Therefore, the flow cytometric assay was used for all further analyses. Unfortunately, U937 cells themselves were not suited for flow cytometric analysis; only the related monocytes can be used for comparison.

PTX did not modulate C3aR internalization on PMNs

The data for U937 cells using 0.2 nM C3a indicated that C3aR-dependent signal transduction is not an absolute prerequisite for C3aR internalization. However, these data do not exclude that signal transduction would modify the C3aR internalization, causing the faster C3aR internalization detected on granulocytes with higher concentrations of the ligand (13 vs 100 nM; see Figs. 1 and 2). PTX inhibits C3aR signal transduction (4, 9, 10, 12, 57) and was tested for its effect on C3aR internalization. The efficiency of this treatment was checked in a fura-2/AM assay. The C3a-dependent increase in [Ca2+] was completely blocked by the toxin (data not shown). In parallel, C3aR internalization was determined using 100 nM C3a (Table I). At this concentration, signal transduction (e.g., determined as the increase in [Ca2+]i) is maximal (49). In all experiments performed, C3aR internalization (~90%) was not affected by PTX treatment.

The phorbol ester PMA induced complete C3aR internalization in human PMNs

Phorbol esters such as PMA are known to induce the sequestration of a variety of receptors, e.g., the C5aR (31). Granulocytes were incubated for 30 min at 37°C with increasing concentrations of this protein kinase activator (Fig. 5). At 400 nM PMA, the histograms of the fluorescences obtained with C3aR-specific antiserum and preimmune serum were almost identical, indicating complete C3aR internalization following PMA stimulation.

C5a, but not FMLP, had a dose-dependent, negative effect on C3a-induced C3aR internalization

Neither C5a nor FMLP, whose receptor is also highly expressed (58, 59) and also internalized (30) on human neutrophils, caused any fast cross-internalization of C3aR (Fig. 6, right panel). Even after 15 min of incubation with 100 nM C5a or FMLP (at 37°C), no significant change in the degree of C3aR internalization, induced by 100 nM C3a within 3 min, could be observed. In simultaneously performed control experiments, the C3a-dependent increase in cytosolic Ca2+ of these granulocytes (fura-2/AM assay (12)) was completely inhibited by this treatment (data not shown).

<table>
<thead>
<tr>
<th>Expt.</th>
<th>C3aR Internalization (%)</th>
<th>+PTX</th>
<th>−PTX</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96.9 ± 7.3</td>
<td>90.8 ± 7.4</td>
<td>96.9 ± 7.3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>90.9 ± 1.3</td>
<td>90.9 ± 7.1</td>
<td>90.9 ± 1.3</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>90.0 ± 3.3</td>
<td>89.8 ± 6.4</td>
<td>90.0 ± 3.3</td>
<td>6</td>
</tr>
</tbody>
</table>

*Human granulocytes were pretreated for 3 h with 0.5 μg/ml PTX or buffer. No significant change in the degree of C3aR internalization, induced by 100 nM C3a within 3 min, could be observed. In simultaneously performed control experiments, the C3a-dependent increase in cytosolic Ca2+ of these granulocytes (fura-2/AM assay (12)) was completely inhibited by this treatment (data not shown).
FIGURE 5. Phorbol ester induced C3aR internalization on human granulocytes. PMNs were preincubated for 30 min at 37°C with 0, 50, 100, and 400 nM PMA. The fluorescence caused by the binding of FITC-labeled goat anti-rabbit serum to the anti-C3aR rabbit serum (solid lines) decreased with increasing concentrations of PMA, down to the background fluorescence caused by the binding of rabbit preimmune serum (dotted line). A representative experiment (n = 3) is depicted.

Table II. The negative effect of C5a on C3a-dependent C3aR internalization can be blocked specifically by a mAb inhibiting C5a-binding to its receptor

<table>
<thead>
<tr>
<th>C3a (nM)</th>
<th>C5a (nM)</th>
<th>mAb</th>
<th>IgG2a Control</th>
<th>Anti-C5aR mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.6</td>
<td>0</td>
<td>0</td>
<td>88.0 ± 4.1</td>
<td>88.0 ± 4.1</td>
</tr>
<tr>
<td>31.6</td>
<td>100</td>
<td>0</td>
<td>52.3 ± 7.2</td>
<td>52.3 ± 7.2</td>
</tr>
<tr>
<td>31.6</td>
<td>0</td>
<td>10</td>
<td>82.6 ± 4.0</td>
<td>86.8 ± 1.5</td>
</tr>
<tr>
<td>31.6</td>
<td>100</td>
<td>10</td>
<td>51.1 ± 8.2*</td>
<td>74.4 ± 2.1*</td>
</tr>
<tr>
<td>31.6</td>
<td>0</td>
<td>100</td>
<td>90.5 ± 3.9</td>
<td>88.5 ± 3.0</td>
</tr>
<tr>
<td>31.6</td>
<td>100</td>
<td>100</td>
<td>39.0 ± 1.9*</td>
<td>82.8 ± 2.7*</td>
</tr>
</tbody>
</table>

* Significant difference (p < 0.01) between the effect of mAb S5/1 and control IgG2a.

The negative effect of C5a on C3a-dependent C3aR internalization can be blocked specifically by a mAb inhibiting C5a-binding to its receptor. Human granulocytes were preincubated for 30 min at 4°C with 0, 10, or 100 μg/ml (10 μg/ml to ~60 nM) of the C5aR-specific mAb S5/1 (7) or a control mAb of the same subtype (IgG2a), respectively. Then, still in the presence of the mAbs, the cells were simultaneously stimulated (for 2 min at 37°C) with C3a and/or C5a at the indicated concentrations. Depicted is one typical out of three independent experiments (n = 3 single values, each).

HEK293 cells lack the ability to ligand dependently internalize C3aR

Human embryonic kidney 293 (HEK293) cells have previously been used for functional studies with transiently transfected C5aR (43). These cells show agonist-induced internalization of other transfected receptors, such as the histamine H1 receptor (60) or the human β2-adrenergic receptor (61, 62). However, the C5aR is poorly internalized in these cells after transient or stable transfection (31). As depicted in Fig. 7, after 3 min at 37°C we did not observe any C3a-induced internalization. C3aR internalization was also not seen 15 min after addition of 100 nM ligand (data not shown). Similar results were obtained with HEK293 cells from three different sources. For functional coupling of the C3aR or C5aR in HEK293 cells (as determined by phosphoinositol hydrolysis in a control experiment; Table III) the receptors must be coexpressed with Gr16, a human PTX-resistant, G protein α subunit (56). However, cotransfection of HEK293 cells with human Ga16 did not improve the internalization of C3aR (Fig. 8).

Discussion

Receptor internalization as one putative control mechanisms of the C3aR protecting cells against overstimulation was investigated in detail on human granulocytes using the rapid decrease in antigenically detectable C3aR in a flow cytometric assay. After stimulation of human granulocytes and the human mast cell line HMC-1 with 100 nM C3a, maximal internalization was reached within 5–10 min, resembling the fast internalization of the C5aR on polymorphonuclear leukocytes or monocytes (30, 63). Although different blood donors were used for kinetic studies, the three curves obtained on granulocytes were almost indistinguishable, indicating the high reproducibility of this flow cytometric assay and demonstrating the variability between cells from different donors. The degree of C3aR internalization seemed to be lower on HMC-1 cells than on PMNs. Even under optimized conditions, on HMC-1 cells only approximately 70% of the receptors were internalized.
Compared with almost 100% internalization on granulocytes. The remaining 30% C3aR detected on the cell surface either were simply not internalized or may represent a steady state situation resulting from rapid receptor internalization and recycling. Monocytes internalized in a C3a dose-dependent fashion this receptor as well, demonstrating, just as for the HMC-1 mast cell line, that internalization of the C3aR is a general control mechanism on a variety of cells.

C3aR internalization detected on IFN-γ-induced U937 cells by the $^{125}$I-C3a acid wash technique 1) supported our data obtained by flow cytometry, 2) confirmed that human monocyte/macrophage-like cells internalize the C3aR, and 3) demonstrated that internalization of the C3aR takes place at agonist concentrations at which one can hardly expect any relevant receptor activation and signal transduction (49). However, it is not only the low C3a concentration making it very unlikely that signal transduction is necessary for C3aR internalization. In contrast to cells induced by dibutyryl cAMP, there is no detectable C3a-dependent increase in $[Ca^{2+}]$, in U937 cells induced by IFN-γ (50).

Internalization on granulocytes was dose dependent in the sense that it took longer for smaller concentrations of C3a (13 nM) to reach the same maximum. One possible explanation for the faster C3aR internalization at higher concentrations could have been that even if signal transduction is not a prerequisite for C3aR internalization, it could speed up this process. However, pretreatment with PTX did not alter significantly the fast internalization caused by 100 nM C3a, a concentration at which maximal signal transduction can be expected.

The phorbol ester PMA, a potent activator of protein kinases, in particular of protein kinase C (64–66), caused a dose-dependent internalization of C3aR on granulocytes. Such a rapid phorbol ester-induced internalization has been described for several other receptors (61, 67, 68), including C5aR (31). Although, C5aR is phosphorylated after application of PMA (28, 32), the underlying mechanism among PMA, protein kinases, and receptor internalization seems to be more indirect and complex, since a C5aR mutant in RBL cells lacking any putative protein kinase C phosphorylation motif is still internalized after PMA application, comparable to the wild-type C5aR (31). The activation of signal transduction by maximal doses of either C5a or FMLP did not lead to any cross-internalization of the C3aR, although FMLP, for example, can trans-locate protein kinase C to the plasma membrane of neutrophils (69). Therefore, if there was a physiological correlate to the C3a-independent C3aR internalization caused by PMA in vivo, it should be a more extreme situation, where either desensitization of these signal transduction pathways completely fails or high or long lasting intracellular activation is caused by the simultaneous stimulation of granulocytes by a variety of different mediators.

The inhibitory effect of C5a on C3aR internalization was observed on human granulocytes and not only on a more or less artificial system, such as C3aR-transfected cells or differentiated tumor cell lines. The dose-dependent effect, its relative specificity (FMLP as coactivator had no influence on the amount of determined C3aR), and its inhibition by an anti-C5aR mAb competing with C5a for receptor binding suggest that the inhibitory effect

<table>
<thead>
<tr>
<th>Transfected Plasmids</th>
<th>Buffer (cpm)</th>
<th>100 nM C3a (cpm)</th>
</tr>
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<tbody>
<tr>
<td>C3aR</td>
<td>2050 ± 180</td>
<td>1930 ± 40</td>
</tr>
<tr>
<td>Ga16</td>
<td>2230 ± 210</td>
<td>2350 ± 235</td>
</tr>
<tr>
<td>C3aR + Ga16</td>
<td>1410 ± 90</td>
<td>3750 ± 220</td>
</tr>
</tbody>
</table>

* HEK293 cells were cotransfected with plasmids coding for the human C3aR or Ga16, as indicated. Accumulation of $^{3}H$phosphoinositides upon stimulation with 100 nM C3a or buffer as control, respectively, was determined. Data are presented as mean ± SE ($n = 3$) from one representative experiment. The experiment was performed as described (56).
itself is not an experimental artifact. In vivo one would expect that C3a and C5a are simultaneously present at sites of complement activation. Therefore, the experimental costimulation setting should actually reflect the in vivo situation near the site of complement activation. In general, receptor internalization is considered a negative feedback mechanism, limiting the amount and duration of signaling. Consequently, the decreased C3aR internalization in the presence of C5a could augment the activation of granulocytes and other C3aR-expressing cells, since more noninternalized receptors would be present for longer time periods. Conversely, in the periphery where diverging gradients of the two anaphylatoxins are more likely or if spontaneous generation of only one anaphylatoxin, for example by the direct action by proteinases, occurred, C3a-mediated inflammation would be limited due to fast receptor internalization and cell desensitization. This is difficult to demonstrate experimentally because there is no way to distinguish between the intracellular signaling of the two costimulated receptors.

The cross-inhibitory effect on C3aR internalization was not observed by costimulation with FMLP, indicating a specific component of the C5aR-C3aR cross-talk. The signal transductions of C5aR and FMLP receptor are very similar (70, 71). Ca\(^{2+}\) mobilization by FMLP in U937 cells or granulocytes was even higher and longer lasting than that caused by C5a (data not shown). Therefore, it seems unlikely that signal transduction by C5a was the reason for the interaction between the two anaphylatoxin receptors. Two explanations seem possible. C3aR and C5aR may share a limiting cell component required for receptor internalization, which is not (solely) used by FMLP receptor (58, 59); then, receptor internalization of C5aR and FMLP receptor are very similar (70, 71). Ca\(^{2+}\) mobilization by FMLP receptor (very similar (70, 71)).

Acknowledgments
We thank Dr. Robert Ames from SmithKline Beecham Pharmaceuticals for the critical reading of the manuscript and the C3aR antisera, Dr. Ulrich Martin for the assistance with FACS analysis, and Dr. Lubomir Arseniev and Prof. Dr. Arnold Ganser of the Department of Hematology for their help and use of their FACS equipment. The HMC-1 cells were kindly provided by J. H. Butterfield. We thank the head of our department, Prof. Dr. D. Bitter-Suermann, for his, as always, strong support.

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35. C5αR INTERNALIZATION AND ITS INHIBITION BY C5a


