Binding of Fluorescein-Labeled Anaphylatoxin C5a to Human Peripheral Blood, Spleen, and Bone Marrow Leukocytes

By Thomas Werfel, Martin Oppermann, Matthias Schulze, Gerhard Krieger, Michael Weber, and Otto Götte

The expression of C5a receptors (C5aR) on human leukocytes was evaluated by flow cytometry using fluorescein-labeled human C5a (C5a-F). Granulocytes and CD14⁺ mononuclear cells (MNL) but not CD³⁺, CD20⁺, CD16⁺, CD56⁺, or CD11b⁺ lymphocytes in peripheral blood and spleen bound C5a-F. C5a-F binding was saturable and inhibitable by anti-C5a monoclonal antibody (MoAb) C17/5 or unlabeled C5a. During hemodialysis, which led to the generation of C5a, only granulocytes and monocytes increased their expression of the adhesion molecule CD11b (CR3). In vitro, C5a induced an increase of CD3⁺, CD20⁺, CD16⁺, CD56⁺, or CD11b⁺ lymphocytes in peripheral blood and spleen bound C5a-F. The expression of C5aR on low-density bone marrow cells was analyzed by setting appropriate gates during flow cytometry. Cells that bound C5a-F were found in all populations that contained granulocyte and monocyte precursors, but not in lymphocyte precursor populations. All C5aR⁺ bone marrow cells were CD34⁺ and expressed high levels of CR3, which suggests a late appearance of C5aR during myeloid cell maturation. Our results indicate that C5aR is exclusively expressed on myeloid cells within the hematopoietic cell population.

Flow cytometry has been applied to identify C5a-binding cells in peripheral blood with biologically active C5a-F. In addition to myeloid cells, some lymphocytes bound C5a-F in these studies. Moreover, lymphocyte subpopulations were shown to respond to C5a in functional in vitro assays.

In this study we used highly purified human C5a-F to evaluate the expression of C5aR on unstimulated and stimulated peripheral blood leukocytes, on spleen cells, and on low-density bone marrow cells. The data obtained with peripheral blood leukocytes were consistent with the responsiveness of leukocyte populations to C5a in vitro and in vivo.

MATERIALS AND METHODS

Reagents. Phorbol 12-myristate 13 acetate (PMA) and db-cAMP were from Sigma (Deisenhofen, Germany). Phospholipase C (specific for the cleavage of phosphoinositol-anchored membrane proteins [PI-PLC]) and Iscove’s medium were from Boehringer (Mannheim, Germany). MERGETPA was from Calbiochem (Frankfurt, Germany). Polymyxin B was from Sigma. Mononuclear leukocyte conditioned medium (MNL-CM) was produced as described. Briefly, MNLs were stimulated for 4 hours with phytohemagglutinin (PHA) and PMA, washed, and incubated with the Epstein-Barr virus-transformed B-cell line Laza09 in RPMI-medium (GIBCO, Eggenstein, Germany) with 5% human AB serum for 2 days. Recombinant interferon-gamma (IFN-γ) was kindly donated by Dr G.R. Adolf (Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria). C3a was isolated from human serum to homogeneity as described. C5a was purified as described. Briefly, human serum was activated with yeast cell walls in the presence of the carboxypeptidase N inhibitor e-aminocaproic acid. Column chromatography was performed, in sequence, on Amberlite CG50 (Serva, Heidelberg, Germany), CM-Sepharose CL6B, Sephadex G-75, ConA-Sepharose, and Sephadex G-50 (all from Pharmacia, Freiburg, Germany). The obtained C5a preparation was homogeneous as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Monoclonal antibodies. MoAb C17/5 and M4/1 (mouse IgG1,k) were generated as described. C17/5 is specific for a neopitope on C5a; M4/1 recognizes C5a and intact C5. In functional tests, C17/5, but not M4/1, inhibited C5a-induced release of primary granule contents of granulocytes effectively. Phycoerythrin (PE)-labeled MoAb B73.1 (CD16), Leu4 (CD3), Leu15 (CD11b), Leu3 (CD14), My10 (CD34), anti-HLA-DR, and rat anti-mouse were purchased from Becton Dickinson (Heidelberg, Germany).

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CD56 MoAb NKH1<sup>BD</sup> and CD20 MoAb B1<sup>BD</sup> were from Coulter (Krefeld, Germany). MoAb 63D3 (CD14), MMA (CD15), and OKM1 (CD11c) were obtained from ATCC (Rockville, MD). The following MoAbs were kindly donated as indicated: 2F12 (CD11a) (R.E. Schmidt, Hannover, Germany), KiM1 (CD11c) (J. Radzun, Kiel, Germany), CLB/Fe Gran1 (CD16) (T. Huizinga, Amsterdam, The Netherlands), GZ-4 (anti-IFN-γ) (G. Zahn, Dr Karl Thomae GmbH, Biberach, Germany). MoAbs CLB/Fe Gran1, 63D3, 2F12, MMA, KiM1, and OKM1 were purified and labeled with fluorescein as described.<sup>23</sup>

**Cells.** Granulocytes were isolated by density gradient centrifugation on neutrophil isolation medium (NIM; Packard, Frankfurt, Germany) and were separated by density centrifugation on Lymphoprep (d = 1.077 g/cm<sup>3</sup>) (Nycomed Pharma, Oslo, Norway). Spleen cells were obtained after splenectomy from polytrauma patients without undergoing systemic diseases. Cells were suspended after passing splenic tissue through a steel sieve in Iscove’s medium and were separated by density centrifugation on Lymphoprep (d = 1.077 g/cm<sup>3</sup>). Bone marrow aspirates were obtained from patients who were examined in the Department of Hematology, University Hospital of Göttingen, Germany, for various reasons. Data were only evaluated in this study after exclusion of systemic diseases involving the hematopoietic system. The low-density leukocytes were washed twice in Iscove’s medium after centrifugation on Lymphoprep (d = 1.077 g/cm<sup>3</sup>). The myeloid cell lines KG-1, HL-60, U937, and THP1 were cultured as described.<sup>23</sup> For the stimulation of MNL with MNL-serum. The natural killer (NK) cell clone NKCl was generated and characterized. The density of these cells could not be down-modulated by preincubation with PI-PLC at 37°C. Controls contained only EDTA and MERGETPA. The incubation was stopped by washing the cell suspensions twice with ice-cold PBS-gelatin (0.2%), containing 20 mmol/L sodium azide. Erythrocytes were removed by lysis with a buffer according to Gey.<sup>23</sup> Lysis was followed by centrifuging the cell suspension (10 minutes, 300g) through a 2 cm layer of heat-inactivated (60 minutes, 56°C) human plasma.

**Flow cytometry and analysis.** Flow cytometry was performed with a FACStar Plus flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with an argon laser. The excitation wave length was 488 nm. Granulocytes, monocytes, and lymphocytes were separated by setting appropriate gates. The major leucocyte populations could be distinguished in most experiments by their characteristic light scatter. Results were verified through the use of C5a-F in combination with PE-labeled CD14 MoAb LeuM3. In these experiments an optimal separation of the cells was achieved by the correlation of side scatter with red fluorescence. Results are expressed as the specific mean channel of fluorescence (MCF) (MCF of test MoAb — MCF of negative control) or as the stimulation index (SI) according to the following formula:

\[
SI = \frac{(Ts - TsN)}{(To - TsN)}
\]

where Ts = MCF of test MoAb after C5a stimulation, TsN = MCF of negative control after C5a stimulation, To = MCF of test MoAb without stimulation, and No = MCF of negative control without stimulation.

In initial experiments we excluded systematic errors due to properties of the different fluorochromes FITC and PE (eg, quenching due to lower pH during cell activation). The increase in signals after staining with PE-labeled CD11b MoAb Leu15 or FITC-labeled CD11b MoAb OKM1 were similar upon stimulation in vitro or during the course of hemodialysis.

**Hemodialysis.** Blood was obtained from patients with end-stage renal disease (ESRD) before, during, and after the course of hemodialysis. Informed consent was obtained from patients with ESRD before 1 to 3 mL EDTA-blood was taken at five different times during one dialysis session. Blood was taken from the efferent blood lines between the patients’ veins and the dialyzers. The blood was kept on ice. Cells were stained with FITC- or PE-conjugated MoAb, usually within 20 minutes. Erythrocytes were lysed after staining before analysis of the leukocytes as described above.

**RESULTS**

**Binding of C5a-F to peripheral blood and spleen cells.** C5a-F was added to isolated granulocytes or MNL. In both instances, binding to C5aR-positive cells was saturable at 12.5 ng C5a-F after 60 minutes incubation on ice when 10<sup>6</sup> cells were used. As was described previously<sup>1,15</sup> the density of C5aR on monocytes varied more than on granulocytes (Fig 1). Granulocytes bound two to three times more C5a-F than monocytes as calculated by MCF. C5a-F binding to granulocytes could not be down-modulated by preincubation of the cells with 500 mU/mL phospho-inositol specific phospholipase C (PI-PLC) at 37°C for 30 minutes. The same dose of PI-PLC was sufficient to reduce the membrane expression of the PI-linked molecule FcγRII (CD16) by 50% as detected by a reduction in binding of CD16<sup>+</sup> MoAb CLB/Fe Gran1 to PI-PLC-treated granulocytes.

CD16<sup>+</sup> granulocytes, which consist mainly of eosinophils<sup>24</sup> bound markedly less C5a-F than when compared with CD16<sup>+</sup> granulocytes (Table 1). C5a-F binding to both
Figure 2 shows one of 10 separate experiments. Binding of C5a-F to MNL was specific because it was inhibitable by an excess of unlabeled C5a or by the neoeptope-specific anti-C5a MoAb C17/5 but not by C3a or anti-CS MoAb M4/1 (Fig 3). The NK clone NKC1, which was derived from normal peripheral blood lymphocytes, did not bind any C5a-F (not shown). In experiments performed with cells of two different donors, C5a-F reacted with splenic CD14+ cells and granulocytes but not with CD3+, CD11b+, CD16+, CD20+, or CD56+ splenic lymphocytes (not shown).

Response of peripheral blood leukocytes to C5a. Leukocytes were investigated during hemodialysis with cuprophane membranes, which induced the generation of C5a (Fig 4a). We observed a reduced binding of C5a-F to granulocytes after the start of hemodialysis (Fig 4b) which may reflect the occupancy or, alternatively, the internalization of C5aR-C5a complexes that had been formed in vivo. The transient decrease of granulocytes in peripheral blood during hemodialysis (Fig 4c) may be associated with the increase of the adhesion molecules on granulocytes (Fig 4e) as discussed previously for CR3.27 Monocytes reacted with transient increases in CR3 as well (Fig 4f), whereas virtually no changes were detectable on CD11b+ lymphocytes (Fig 4d).

The stimulation of peripheral blood leukocytes in vitro with C5a led to cellular changes that confirmed the binding experiments with C5a-F and the observations made during hemodialysis. Upon C5a stimulation, increases of CR3 and p150,95 (CR4) were detected on myeloid cells but not on lymphocytes (Fig 5). These increases could be prevented by preincubating C5a with a 100-fold molar excess of anti-C5a MoAb C17/5 (Fab) (not shown). When peripheral blood cells were stimulated with PMA, we observed increases of CR3 and p150,95 not only on myeloid cells but also on lymphocyte subpopulations (Fig 5). This shows that under the conditions used these lymphocytes are able to respond to adequate stimuli, but not to C5a.

Binding of C5a to stimulated MNL. Stimulation of MNL under serum-free conditions with MNL-CM, IFN-γ, or in MLCs for 24 to 72 hours did not induce C5aR on lymphocytes (not shown). However, these treatments resulted in a marked reduction of C5aR on monocytes (Fig 6 and 7), whereas HLA-DR antigens were increased on monocytes under similar conditions (Fig 7). The reduced binding of C5a-F was also detectable when 1 × 10⁸ CD14+ sorted monocytes (purity >90%) were stimulated with 200 U/mL IFN-γ in Iscove’s medium. The effect of IFN-γ on C5aR

Table 1. Binding of FITC-Labeled C5a to CD16 and to CD16+ Granulocytes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CD16+</th>
<th>CD16+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.6f</td>
<td>29.4</td>
</tr>
<tr>
<td>Experiment</td>
<td>3.4</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Granulocytes were isolated and stained with CD16 mAb CLB/FcGranl plus PE-labeled rat anti-mouse MoAb and C5a-F. Two experiments of a total of six are shown.

*Autofluorescence.
†MCF.
expression was inhibitable by anti-IFN-γ MoAb GZ-4, but not by anti-C5a MoAb C17/5 (Table 2). This suggests that down-modulation of C5aR on monocytes was not due to generation of C5a in cultures stimulated with IFN-γ. Moreover, the addition of 50 μg/mL of the LPS-binding reagent polymyxin B did not have any effect on IFN-γ-induced decreases of C5a-F binding (not shown).

PMA leads rapidly to a decreased binding of C5a-F to granulocytes. We observed a reduced binding of C5a-F to PMA-treated monocytes as well (Table 3). IFN-γ or MNL-CM did not have such effects in short time assays (not shown).

**Binding of C5a-F to bone marrow cells and myeloid cell lines.** Low-density bone marrow cells were labeled with C5a-F. The cells were gated by forward and right angle light scattering in accordance with Loken et al as shown in Fig 8A. The GRAN window (1) consists entirely of maturing granulocytes, predominantly myeloblasts, metamyelocytes, and band forms. The LYMPH window (2) contains a mixture of lymphoid cells and maturing erythrocytes. The BLAST window (3) is composed of multipotent and unipotent colony-forming cells, immature and mature monocytes, and immature granulocytes. Before C5a-F was tested, these gates were controlled by staining normal bone marrow cells

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**Fig 2.** Binding of C5a-F to peripheral blood MNL. C5a-F (25 ng) and PE-labeled MoAb in saturating concentrations were incubated for 60 minutes on ice with freshly isolated MNL of a healthy donor, washed, and analyzed by fluorescence-activated flow cytometry for bound fluoresceine (F) and phycoerythrine (PE). Cells that bound both C5a and the respective MoAb are found in the upper right quadrant. Cells that bound only C5a or the respective MoAb are found in the lower right quadrant or the upper left quadrant, respectively. As can be seen in the three frames at the right, only CD14⁺, HLA-DR⁺, and CD11b⁺ cells bound C5a-F. Representative data for 10 separate experiments are shown.

**Fig 3.** FACS analysis of the inhibition of C5a-F binding to monocytes by unlabeled C5a and anti-C5a MoAb C17/5. Experiment 1: 10⁶ MNL were incubated for 60 minutes with 0.1% BSA-PBS (A, B), 1 μg C5a in 0.1% BSA-PBS (C), or 1 μg C5a in 0.1% BSA-PBS (D) before 25 ng C5a-F was added (B-D). Experiment 2: 25 ng C5a-F was incubated with 0.1% BSA-PBS (F), 10 μg anti-C5a MoAb C17/5 Fab (G), or 10 μg M4/1 Fab (H) for 12 hours before 10⁶ MNLs were added to these reagents. (A) and (E) show autofluorescence of monocytes. The MCF is indicated in each frame. Monocytes were gated by light scatter characteristics.
with defined MoAb reactive with lymphocytic or myeloid cells. C5a-F bound to 10% to 40% of cells in the GRAN window and 10% to 30% of cells in the BLAST window. No binding of C5a-F to cells in the LYMPH gate was detectable. Binding to cells in the BLAST and GRAN windows appeared to be specific because it was saturable at 25 ng C5a-F and inhibitable by preincubation of C5a-F with a 500-fold molar excess of anti-C5a MoAb C17/5 (Fab) (not shown). In double-labeling experiments all C5a-F+ cells bound anti-MHC class I MoAb W6/31 (not shown), CD34 MoAb My10 bound to cells in the LYMPH window and BLAST window (Fig 8B) as described. However, CD34+ cells did not bind any C5a-F (Fig 8B). All C5a-F-binding cells in the GRAN window expressed high levels of the CD11b antigen detected by MoAb Leu15-PE (Fig 8C) and the CD15 antigen detected by MoAb MMA (not shown). These antigens are expressed at the promyelocyte stage and increase as the granulocytes mature. FcyRIII (CD16) appears late during neutrophil maturation. C5a-F bound to CD16+ granulocytes or granulocyte precursor cells in the bone marrow but also to CD16+ cells in the GRAN window (Fig 8C).

C5aR+ cells in the blast cell window also expressed high levels of CD11b (Fig 8C) and CD15 (not shown). The myeloid cell lines U937, THP-1, HL-60, and KG-1 did not bind C5a but incubation of U937 cells with 1 mmol/L db-cAMP for 48 hours induced C5aR as described (not shown).

DISCUSSION

Biologically active fluorescein-conjugated C5a was used to evaluate the expression of C5aR on peripheral blood leukocytes and on hematopoietic cells. In previous studies, binding of C5a-F to granulocytes, monocytes, and some lymphocyte subpopulations obtained from normal blood was described. We were able to reproduce binding of C5a-F to granulocytes and monocytes. Granulocytes consistently demonstrated a narrower distribution in their fluores-
Fig 6. Mixed lymphocyte culture conditions reduce the binding of C5a-F to monocytes. MNL (2 x 10^6/mL) were incubated in Iscove’s medium for 48 hours. MLC was performed by incubating 1 x 10^6 MNL from each donor. Histograms in (A) show the autofluorescence. Cells with high autofluorescence were monocytes as shown by binding of PE-labeled MoAb LeuM3 (CD14). Histograms in (B) were obtained after incubation of the cells with C5a-F (25 ng/10^6 cells) for 1 hour on ice.

Donor 1

Donor 2

Donor 1 + Donor 2

Fig 7. Effects of IFN-γ and MNL-CM on the monocyte membrane expression of C5aR and HLA-DR. MNLs were incubated with 200 U/mL IFN-γ (seven experiments, □) or 10% MNL-CM (four experiments, □) in Iscove’s medium for 2 days. Monocytes were distinguished from lymphocytes by different light scatter characteristics, and CD14 or CD64 membrane expression. C5aR was visualized by staining with C5a-F; HLA-DR was detected using FITC- or PE-labeled MoAb.

Table 2. Effects of IFN-γ on the Expression of C5aR and of HLA-DR on Monocytes

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>IFN-γ</th>
<th>Anti-IFN-γ</th>
<th>IFN-γ + Anti-IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5aR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor 1</td>
<td>100.2*</td>
<td>58.1</td>
<td>89.5</td>
<td>49.8</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>743.7</td>
<td>1,285.3</td>
<td>850.0</td>
<td>1,317.1</td>
</tr>
<tr>
<td>Donor 2</td>
<td>101.8</td>
<td>56.3</td>
<td>93.5</td>
<td>62.3</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>663.7</td>
<td>2,184.2</td>
<td>1,126.9</td>
<td>2,093.2</td>
</tr>
</tbody>
</table>

Purified monocytes were stimulated with 200 U/mL IFN-γ for 24 hours in the absence and in the presence of anti-IFN-γ MoAb GZ-4 (10,000 neutralizing units) or anti-C5a MoAb C17/5 Fab (10 μg). Monocytes were analyzed with C5a-F and PE-labeled anti–HLA-DR. For calculation of the MCF see Material and Methods. A total of four experiments with anti-IFNγ and of two with anti-C5a MoAb were performed with similar results.

*MCF.
Table 3. Effect of PMA on the Binding of C5a-F to Granulocytes and Monocytes

<table>
<thead>
<tr>
<th>Incubation With PMA (min)</th>
<th>Granulocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>79.2</td>
<td>35.1</td>
</tr>
<tr>
<td>5</td>
<td>50.9</td>
<td>15.4</td>
</tr>
<tr>
<td>15</td>
<td>24.4</td>
<td>13.7</td>
</tr>
<tr>
<td>30</td>
<td>20.3</td>
<td>15.1</td>
</tr>
</tbody>
</table>

Isolated granulocytes or monocytes in MNL fractions were stimulated with 25 µg/ml PMA in Iscove's medium at 37°C. Cells were washed and incubated with 25 ng C5a-F for 30 min on ice. Monocytes were distinguished from lymphocytes by light scatter characteristics. One experiment of a total of four with similar results is shown.

A subpopulation of C5a-positive granulocyte progenitor cells expresses CD16 (upper right frame). The data presented were obtained from a single bone marrow sample in a single experiment. Two replicate experiments using the same bone marrow sample gave similar results.
study (Fig 7), Fc receptors for IgG, or the IL-2 receptor. A decrease in the binding of the complement fragments C3b and C3bi, of anti-CR1 MoAb, and of CD4 MoAb to IFN-γ-stimulated monocytes has been observed. The decreased binding of C5a-F is not a reflection of a decreased surface area, because IFN-γ increases the volume of monocytes. PMA has recently been shown to induce rapid internalization of C5aR from the membrane of granulocytes, PMA also decreases C5aR on monocytes (Table 3). Down-modulation of C5aR by PMA or IFN-γ might be mediated by similar intracellular events. Both stimuli lead to the translocation of protein kinase C from the cytosol to the plasma membrane fraction of U937 cells, and protein kinase C plays a role in the pathway leading to increased transcription of genes by IFN-γ in these cells after the induction. The decreased expression of C5aR on IFN-γ-treated monocytes may therefore also be due to an increase in the internalization of C5aR mediated by cellular protein kinases. The functional relevance of the down-modulation of this chemotactic peptide receptor by IFN-γ has yet to be addressed. Interestingly, IFN-γ itself has migration-inhibiting activity.

C5aR expression on bone marrow cells was analyzed in binding experiments with C5a-F. We used analysis windows as defined by Loken et al and found no binding of C5a-F to CD34+ early progenitor (colony-forming) cells. Our results indicate that C5aR is expressed later in cellular differentiation than the C3bi-receptor (CR3) on granulocyte progenitors, which is detectable with the beginning of the promyelocyte stage. The expression of CR3 has been correlated in former studies with other antigens because CR3 is known to increase during granulocyte maturation. Both the expression of CR3 and C5aR appear to increase simultaneously during granulocyte maturation. These observations correspond to the findings of Kajita and Hugli, who observed that mainly nonsegmented neutrophils (band forms) were recruited from bone marrow pools in a rabbit model of C5a-induced neutropenia and subsequent neutrophilia, suggesting that C5a receptors are not expressed on earlier maturation stages of granulocytes. The late-appearing Fc-receptor FcyRIII (CD16) was not expressed on all C5a-F-binding granulocytic progenitor cells (Fig 8). Monocytes probably also express C5aR late during maturation because all cells in the BLAST window expressed high levels of CR3. The suggestion of a late appearance of C5aR during myeloid cell maturation was supported by the absence of C5a-F binding to the immature myeloid cell lines U937, HL-60, KG-1, and THP-1.

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