Articles

C3a Receptor on Dibutyryl-cAMP-Differentiated U937 Cells and Human Neutrophils: The Human C3a Receptor Characterized by Functional Responses and ^{125}I-C3a Binding

Andreas Klos,* Stephanie Bank, Claudia Gietz, Wilfried Bautsch, Jörg Köhl, Michael Burg, and Titus Kretzschmar

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61, Germany

Received March 5, 1992; Revised Manuscript Received August 7, 1992

ABSTRACT: The anaphylatoxic peptide C3a is part of a basic immunological defense mechanism, the complement system. Research on the human C3a receptor and signal transduction is hampered by the lack of a suitable human cell or cell line. We screened tumor cell lines and human blood cells for a C3a-dependent increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) and analyzed this reaction in a fura-2/AM fluorescence assay for cells in suspension. U937 cells, when differentiated with dibutyryl-cAMP (Bt_2cAMP), and purified human neutrophils reacted in a dose-dependent fashion to C3a and a C3a analogue synthetic peptide. We found complete homologous desensitization of this response and no heterologous desensitization to human C5a. Pertussis toxin totally blocked the increase in $[Ca^{2+}]_i$, indicating the possible involvement of a G-protein. Single-cell analysis by digital imaging fluorescence microscopy indicated that neutrophilic granulocytes responded to C3a. In binding studies with Bt_2cAMP-differentiated U937 cells and human granulocytes, the ^{125}I-C3a binding was displaced by C3a, yielding one class of C3a binding sites with dissociation constants (K_d) in the low nanomolar range. We identified *myo*-inositol 1,4,5-trisphosphate (IP_3) as the second messenger possibly causing the $[Ca^{2+}]_i$ increase and the release of *N*-acetyl-β-D-glucosaminidase as one secretory cell response. By functional and binding studies we demonstrated the expression of the C3a receptor on Bt_2-cAMP-differentiated U937 cells and human neutrophils and characterized parts of the C3a signal pathway. Our data support a physiological concept in which C3a might be more important than presently thought.

The biologically active anaphylatoxic peptides C3a,[1] C4a, C5a, and C5a-desArg are split products of the complement system, one of the basic immunological defense systems. Human C3a is a polypeptide of 77 amino acids. Its C-terminal arginine is crucial for its biological activity. Human C3a causes, e.g., smooth muscle contraction, an increase in vascular permeability, and activation of guinea-pig platelets [for review, see Bitter-Suermann (1988), Hugli (1990), and Fukuoka et al. (1989)]. Yet its physiological and pathophysiological role is not as clearly understood and characterized as for C5a. The C5a receptor was recently cloned, opening access to more detailed studies (Gerard & Gerard, 1991; Boulay et al., 1991). In contrast to the C5a response, the signal transduction pathway for the C3a receptor is unknown, partly because of the lack of a suitable cell line for such studies. Most investigations on C3a use guinea-pig platelets (Zanker, 1982; Meuer et al., 1981; Grossklaus et al., 1976). Recently, Bischoff et al. (1990) have reported that human basophilic granulocytes pretreated with interleukin-3 become sensitive to C3a and release histamine and leukotriene. However, human basophilic granulocytes are difficult to obtain in sufficient number and purity for in-depth studies.

The purpose of our research was to find a human tumor cell line expressing the C3a receptor to overcome these limitations and to characterize this receptor and part of its signal transduction pathway on these cells. Additionally, with a subset of experiments, we wanted to verify the physiological significance of the expression of the C3a receptor on a related physiological cell type—thus providing a clue for the biological importance of this anaphylatoxic peptide.

Because of similar effects of C3a and C5a, e.g., ATP release from guinea-pig platelets (Zanker, 1982; Meuer et al., 1981; Grossklaus et al., 1976), we assumed that the two anaphylatoxic peptides might share part of their signal transduction pathway and regulation of receptor expression. In neutrophilic granulocytes a challenge with C5a causes an increase in $[Ca^{2+}]_i$ (Gennaro et al., 1984). Following our assumptions, we screened different tumor cell lines for a C3a-dependent increase in $[Ca^{2+}]_i$ by a fura-2/AM fluorescence method (Cobbold & Rink, 1987). We investigated, in particular, those cell lines known for expression of the C5a receptor and analyzed in detail this response to C3a on Bt_2cAMP-differentiated U937 cells (Harris & Ralph, 1985; Sundström & Nilsson, 1976) by applying C3a, C3a-desArg, and a C3a analogue synthetic peptide. A subset of experiments was performed with human PMNLs to support the physiological significance of the C3a receptor expression in the human system.

In this paper we have shown functional and binding data suggesting the expression of a specific C3a receptor on Bt_2-cAMP-differentiated U937 cells, a human cell line showing many characteristics of an immature monocyte (Sheth et al., 1988), and on purified human PMNLs. Additionally, we have studied part of the C3a signal transduction pathway in

[1] Abbreviations: $[Ca^{2+}]_i$, concentration of free cytosolic Ca^{2+}; Bt_2-cAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate; C3a and C5a, anaphylatoxic peptides; C3a-desArg, C3a without C-terminal arginine; IP_3, *myo*-inositol 1,4,5-trisphosphate; K_d, dissociation constant; huPMNLs, human polymorphonuclear leukocytes.

0006-2960/92/0431-11274$03.00/0

the cell line.

EXPERIMENTAL PROCEDURES

Materials. HEPES, MERGETPA, and fura-2/AM were obtained from Calbiochem. $Na^{125}I$ (carrier-free) was from Amersham Buchler (Braunschweig, FRG) and Iodogen from Pierce. We obtained BSA and Bt_2cAMP from Boehringer, Mannheim. All chromatography media were from Pharmacia; Zymosan was from Sigma. We used pertussis toxin from List Biological Laboratories (Campell, CA). Cell culture media were obtained from Gibco BRL. We obtained Diff-Quik from Baxter Dade (Düdingen, CH). Polymorphprep was obtained from Nycomed (Oslo, Norway). All other chemicals were purchased from Merck, Darmstadt.

Preparation of the Stimuli. We purified human C3a from zymosan-activated EGTA plasma in the presence of serum carboxypeptidase N inhibitors as described (Kretzschmar et al., 1991).

Biologically active C3a fractions from cation-exchange chromatography were pooled and further purified by reverse-phase FPLC, running a linear gradient from 0% to 60% acetonitrile, pH 4.0, as described for production of des-Arg peptides (Köhl et al., 1990). The peak of biologically active C3a appeared at approximately 30% of acetonitrile. The protein concentration and purity were controlled as described for C5a (Kretzschmar et al., 1991). Additionally, we checked the human C3a for contamination with C5a by the myeloperoxidase assay on human granulocytes (Gerard et al., 1989). Even 4 μM C3a did not cause any enzyme release. Considering the detection limit for C5a on granulocytes, we could exclude a contamination with more than 0.0025% of C5a. We obtained C3a-desArg by essentially the same procedure as described above but omitting addition of any inhibitor of serum carboxypeptidase N. Analysis by ^{252}Cf plasma desorption mass spectroscopy showed that only the C-terminal arginine was removed without any further degradation (Suckau et al., 1990). We did not find any adenosine 5′-triphosphate release from guinea-pig platelets, applying up to 20 μM C3a-desArg (Zanker et al., 1982). Considering the detection limit for C3a in this assay, we could exclude a contamination of the C3a-desArg preparation with more than 0.0025% C3a.

Human recombinant C5a was purified and characterized as previously described (Bautsch et al., 1992). The preparation of a C3a analogue synthetic peptide (YRRGRAAALGLAR) was recently described. In the guinea-pig ATP release assay this peptide rendered 8.3% of C3a activity, based on the ED_{50} value (Gerardy-Schahn et al., 1988).

Preparation of Human PMNLs. We prepared human PMNLs with Polymorphprep following the manufacturer's instructions, using citrate as anticoagulant, and obtained approximately (3–4) $\times 10^7$ cells from 40 mL of blood of a healthy donor. PMNLs were washed once and resuspended in HBSS (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 1.3 mM Ca^{2+}, 0.2% BSA, 10 mM HEPES, pH 7.4). For fura-2/AM loading PMNLs were finally resuspended in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and penicillin (50 units/mL)/streptomycin (50 μg/mL). The purity of the polymorphonuclear cell preparation was checked by Diff-Quik: More than 96% of all cells were neutrophils, almost exclusively contaminated by eosinophils.

Cell Culture Conditions. We used U937 cells obtained from American Tissue Culture Collection. The cells were grown in RPMI 1640 medium supplemented as described above at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded at densities of 1×10^5/mL and harvested every third day at approximately 1×10^6/mL. We diferentiated these cells into macrophage-like cells by supplementing the medium for 3 days with 1 mM Bt_2cAMP (Chenoweth et al., 1984; Barker et al., 1986).

$[Ca^{2+}]_i$ Measurement in Cell Suspension. We centrifuged the tumor cell line at 300g for 10 min, adjusted the cell density with fresh medium to 3×10^7 cells/5 mL, and added fura-2/AM at a final concentration of 5 μM. After incubation of the cell line in the dark for 0.5 h in a slowly shaking water bath at 37 °C, cells were diluted 1 to 10 with HBSS and incubated for further 30 min.

Human PMNLs were not suited for this treatment. Therefore, we resuspended them after preparation in supplemented RPMI 1640 medium at a density of 1×10^7 cells/mL and transferred them directly into a cell culture flask (25 cm^2, Nunc), which we maintained at 37 °C in a humidified atmosphere with 5% CO_2. After 1 h we added fura-2/AM to the flask at a final concentration of 5 μM. After 30 min without any shaking we diluted the cells 1 to 10 with HBSS and kept them in the incubator for another 30 min.

We washed the fura-2 loaded cells once and resuspended them in HBSS at a final density of 1×10^7 cells/mL. The cells were kept at room temperature, and the $[Ca^{2+}]_i$ assay was performed within 4 h.

For each sample 100 μL of these cell suspensions was diluted with HBSS to a final volume of 750 μL. We transferred this volume into a cuvette with a 1-cm light path and placed it in the spectral fluorometer RF-5001PC (Shimadzu) equipped with a stirring apparatus and a heating block adjusted to 37 °C. Controlled by Labtime DWT software (Shimadzu) fura-2 excitation was performed every 2 s at 340 and 380 nm. Fluorescence at 500 nm was recorded. At appropriate times 50 μL of stimulus was added. We converted the fluorescence data into $[Ca^{2+}]_i$ by using the formula (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R)(S_{f2}/S_{b2})K_d$$

We assumed the K_d for the fura-2/Ca^{2+} complex to be 224 nM. For each sample R_{max}, R_{min}, S_{f2}, and S_{b2} were determined by in vivo calibration, applying 50 μL of 10% Triton X-100 or 50 μL of a 500 mM EGTA solution, respectively.

$[Ca^{2+}]_i$ Measurements in Single Cells. Fura-2-loaded cells were diluted with HBSS to 1 mL with 10^6 cells/mL. Cells were allowed to settle down (5 min) on a cover slip. The cover slip was transferred to the stage of an inverted epifluorescence microscope (Diaphot, Nikon) equipped with a high numerical aperature fluorescence lens (63×, Zeiss, NA 1.22) and a heating device set to 37 °C. We applied neutral density filters to decrease excitation to 6% of the incident intensity (75-W xenon lamp) to reduce photobleaching. Every 3 s Videoprobe software (ETM Systems) loaded on an AT-386 personal computer started excitation at 350 and 380 nm and recorded emission at 510 ± 20 nm as detected by an intensified charge-coupled device camera (C 2400-97, Hamamatsu). One hundred microliters of the stimulus was added. Converting fluorescence ratio into $[Ca^{2+}]_i$ occurred as described above, on a mean pixel basis. By in vitro calibration R_{max}, R_{min}, and S_{f2}/S_{b2} values were determined as 8.5, 0.5, and 11.1, respectively.

Radiolabeling of C3a. We performed iodination of C3a with Iodogen analogously to a method described for C5a (Kretzschmar et al., 1991). We recovered approximately 70% of the biologically active peptide as determined in the guinea-pig platelet assay with an average specific activity of 450

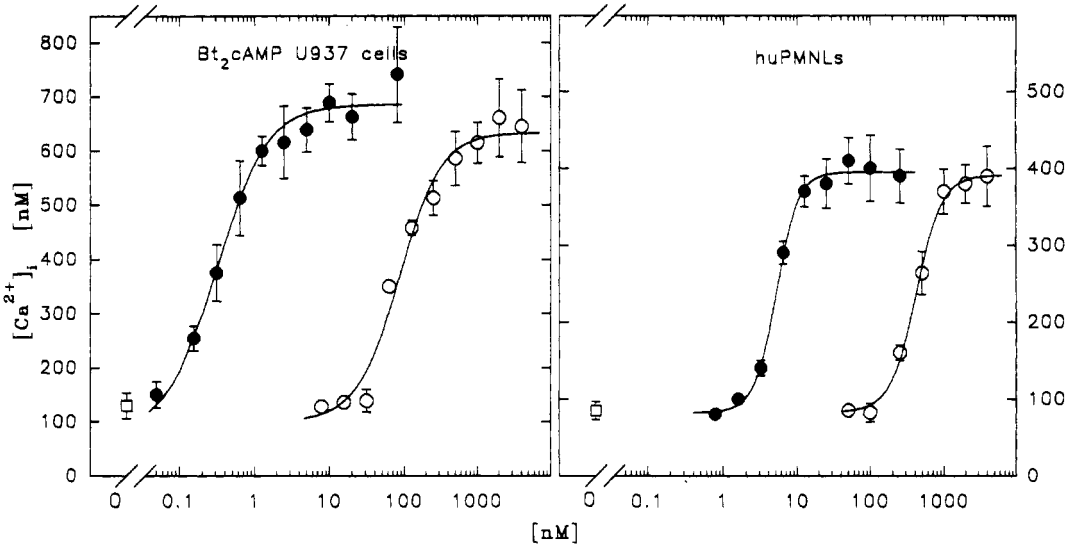

FIGURE 1: $[Ca^{2+}]_i$ dose–response curves of Bt_2cAMP-differentiated U937 cells (left panel) and human PMNLs (right panel) to a stimulus of human C3a (●) or a C3a analogue synthetic peptide (YRRGRAAALGLAR, ○). Plotted are the mean and standard deviation (n = 3) of data from one cell preparation.

Ci/mmol. We verified the purity of ^{125}I-C3a by SDS–PAGE and autoradiography.

Binding Studies. For binding kinetics we prepared for each indicated temperature 1 mL of cell suspension in HBSS buffer. We started by adding ^{125}I-C3a and removed from each tube three aliquots of 45 μL at different time points to determine bound radioactivity as described below.

For competitive binding studies a mixture of 30 μL of 1–2 nM ^{125}I-C3a and 30 μL of various concentrations of unlabeled ligand was mixed with 90 μL of cell suspension. After 15 min at 22 °C for U937 cells, or 30 min at 37 °C for human PMNLs, we removed three aliquots of 45 μL.

To separate bound from free ^{125}I-C3a, we centrifuged the cell aliquots through 200 μL of a 10% (w/v) sucrose cushion in HBSS (6 min, 12000g, 4 °C). We cut off the base of the polyethylene tube (No. 9409325, Jürgens, Hannover, FRG) just above the cell pellet and determined its radioactivity. We performed iterative curve fitting of the binding data on the basis of one- and two-site models (LIGAND; Munson & Rodbard, 1980). All experiments were repeated three times.

Inositol Phosphate Metabolism. After 2 days of differentiation with Bt_2cAMP, U937 cells were washed and resuspended in inositol-free DMEM medium (ICN) supplemented with 10% heat-inactivated, dialyzed fetal calf serum, 2 mM glutamine, and penicillin (50 units/mL)/streptomycin (50 μg/mL). To the cells, adjusted to 8×10^5 cells/mL, was added Bt_2cAMP again and, in addition, *myo*-[2-^{3}H]inositol (TRK.807, Amersham, 10–20 Ci/mmol) at 2 μCi/mL. After overnight incubation U937 cells were washed and resuspended in HBSS without Ca^{2+} and adjusted to 2×10^7 cells/mL. To 0.5 mL of cells prewarmed to 37 °C was added 10 mL of stimulus. The sample was briefly vortexed and incubated at 37 °C. At indicated time points we stopped the reaction by adding 300 μL of ice-cold chloroform/methanol (2:1). Samples were centrifuged at 4000g, and the inositol phosphates in the aqueous phase were separated on Dowex IX8 (formate form) as described (Berridge et al., 1982).

N-Acetyl-β-D-glucosaminidase Assay. Release of *N*-acetyl-β-D-glucosaminidase after 3 min of stimulation at 37 °C was determined as described for C5a (Gerard & Gerard, 1990).

RESULTS

(I) C3a-Dependent Increase of $[Ca^{2+}]_i$ in U937 Cells and Human PMNLs: Dose–Response Curves with Human C3a and a C3a Analogue Synthetic Peptide. We performed fura-2/AM assays with Bt_2cAMP-differentiated U937 cells by applying increasing concentrations of the natural stimulus human C3a (Figure 1, left panel). In a second set of experiments we used a C3a analogue synthetic peptide (Figure 1, left panel) which obviously could not be contaminated with C5a or any other serum component. With buffer as the negative control $[Ca^{2+}]_i$ remained at the resting level of 130 ± 24 nM (mean ± SD). With increasing C3a concentrations the peak value of $[Ca^{2+}]_i$ increased almost 6-fold. This upper plateau was reached with approximately 10 nM C3a. The ED_{50} from four such experiments was 1.3 ± 1.1 nM. Experiments with the synthetic peptide resulted in a similar curve with an ED_{50} of 115 ± 60 nM (n = 3). We compared the plateau values for C3a (40 nM) and the synthetic peptide (1 μM) in parallel and found no significant difference (data not shown).

The maxima of the $[Ca^{2+}]_i$ increase caused by a single application of C3a or C5a at a maximal dose (40 nM each) varied from day to day and might simply be due to slightly varying cell preparation conditions. In more than 20 experiments we found $[Ca^{2+}]_i$ maxima for C3a between 400 and 700 nM. But the standard deviation within one preparation of cells was usually less than 10%.

We got equivalent results with human PMNLs (Figure 1, right panel). Here, the ED_{50} was 4.1 ± 1.9 nM (n = 4) for C3a and 330 ± 170 nM (n = 3) for the synthetic peptide.

$[Ca^{2+}]_i$ Kinetics and Homologous Desensitization of the C3a Response. We sequentially applied within one recording first human C3a-desArg, then human C3a twice, and finally recombinant human C5a as shown for differentiated U937 cells in Figure 2, left panel. As expected, Bt_2cAMP-differentiated U937 cells did not react at all to 40 nM C3a-desArg. Only seconds later the same cells were able to respond to 40 nM C3a with a 3–4-fold increase of $[Ca^{2+}]_i$. Seventy seconds after the first C3a stimulus, when $[Ca^{2+}]_i$ had again reached baseline values, a second stimulus with 40 nM C3a did not cause any detectable response. To check whether that effect was due to homologous desensitization or whether these cells had become unspecifically nonresponsive to other stimuli as well, we added 40 nM C5a, a concentration which had been tested to cause maximal response. The increase of $[Ca^{2+}]_i$ was even higher than the one after the first challenge with

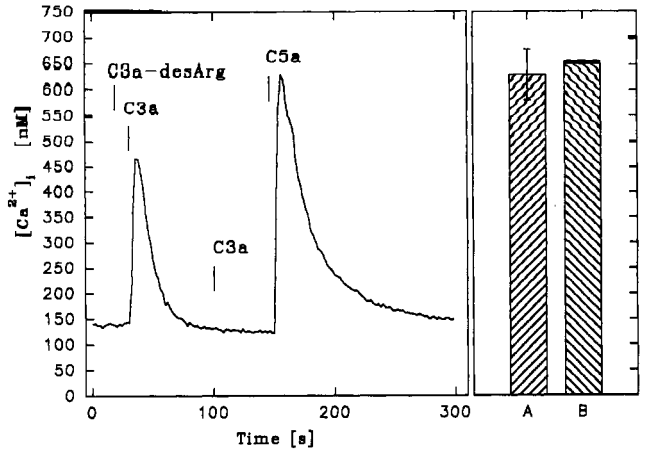

FIGURE 2: $[Ca^{2+}]_i$ kinetics of Bt_2cAMP-differentiated U937 cells challenged with 40 nM human C3a-desArg, twice with 40 nM human C3a, and then with 40 nM human recombinant C5a (left panel). Plotted are the mean values of $n = 4$ readings obtained with different aliquots of cells from one batch. The maxima of the C5a peaks of this experiment (A, right panel) are compared with the maxima of the C5a peaks ($n = 4$) when buffer was applied instead of C3a (B, right panel).

C3a. As a control, buffer was added instead of C3a: The $[Ca^{2+}]_i$ increase to C5a was not altered (Figure 2, right panel), indicating no cross desensitization of the C5a response.

In further experiments we found that even 1 μM C3a-desArg did not cause any $[Ca^{2+}]_i$ increase.

Similar results with no response to C3a-desArg, complete desensitization of the C3a response, and no alterations of the response to C5a when following a challenge with C3a were obtained using human PMNLs (data not shown).

Analysis of the $[Ca^{2+}]_i$ Response to a Maximal Dose of C3a, C5a, or a C3a Analogue Synthetic Peptide. A single stimulation with a maximal dose of C3a or C5a immediately led to a fast rise of $[Ca^{2+}]_i$ at the limit of the time resolution of the fluorometer (indistinguishable from responses as demonstrated in Figure 2). Within the same experiment the change of $[Ca^{2+}]_i$ from baseline to maximum caused by C3a was only 74 ± 5% of the change caused by C5a ($p = 0.99$, $n = 3$). The time for half-maximal decay was 15 ± 3 s for C3a and 25 ± 4 for C5a ($n = 3$). The $[Ca^{2+}]_i$ returned after a stimulus with C3a within 1 min to the original level, whereas the peak caused by C5a showed a much broader base and was still elevated after approximately 3 min.

We found no significant difference between the increase of $[Ca^{2+}]_i$ caused by maximal doses of human C3a or the C3a analogue synthetic peptide.

Cross Desensitization of a C3a Analogue Synthetic Peptide and C3a. If any contaminating serum component would add to and modify the $[Ca^{2+}]_i$ response caused by C3a itself, one could not interpret the C3a dose–response curve correctly. To exclude this possibility within the fura-2/AM assay, we used cross-desensitization experiments between the synthetic peptide and C3a. We stimulated Bt_2cAMP-differentiated U937 cells and human PMNLs with 1 μM synthetic peptide. Thereupon, the cells were completely homologously desensitized, as they failed to react to a second challenge with the synthetic peptide. When we then applied 40 nM C3a, the cells did not react at all, due to complete cross desensitization. As a control we added 40 nM C5a and observed again an increase of $[Ca^{2+}]_i$ higher than the first peak caused by the synthetic peptide. Application of the stimuli in reverse order caused again complete cross desensitization, as expected (data not shown).

Table I: Effect of Pertussis Toxin Treatment on the C3a-Dependent $[Ca^{2+}]_i$ Response of Bt_2cAMP-Differentiated U937 Cells and Human PMNLs

	$[Ca^{2+}]_i$ response (nM) to			
cell type	40 nM C3a (no PTX)	buffer (no PTX)	40 nM C3a (+PTX)	buffer (+PTX)
Bt_2cAMP U937 cells ($n = 4$)	650 ● 53	120 ± 22	110 ± 15	104 ± 5
huPMNLs ($n = 4$)	477 ± 29	88 ● 14	92 ± 7	86 ± 12

Inhibition of the C3a Response by Pertussis Toxin. We pretreated Bt_2cAMP-differentiated U937 cells for 15 h and huPMNLs for 4 h with 0.5 μg/mL pertussis toxin and found no significant difference in the basal $[Ca^{2+}]_i$ level compared to control cells (Table I). The C3a stimulus (40 nM) caused in pertussis toxin treated U937 cells and human PMNLs no elevation from baseline $[Ca^{2+}]_i$ values, whereas untreated control cells responded as usual.

$[Ca^{2+}]_i$ Measurement in Single Cells. By digital imaging fluorescence microscopy (Tsien & Harootunian, 1990), we found in two experiments that 122 of 140 huPMNLs responded to 100 nM C3a (Figure 3) and 122 of 125 huPMNLs responded to 100 nM C5a. All cells responding to C3a also reacted to a subsequent stimulus with C5a, excluding the possibility of two either C3a- or C5a-specific subpopulations within the neutrophilic granulocytes. Seventy-three of 130 differentiated U937 cells showed a $[Ca^{2+}]_i$ increase to 100 nM C3a and 63 of 86 to 100 nM C5a.

Time Dependence of Bt_2cAMP Differentiation. In the fura-2/AM assay we compared the C3a responsiveness of non-differentiated U937 cells as well as U937 cells differentiated with 1 mM Bt_2cAMP for 1, 2, 3, or 4 days. Undifferentiated U937 cells did not react, even to an extremely high stimulus of 1 μM C3a. After 24 h of incubation with Bt_2cAMP, U937 cells became responsive. However, we observed the highest $[Ca^{2+}]_i$ response after 3 days of differentiation (Table II).

(II) Binding Studies: Kinetics and Temperature Dependence of ^{125}I-C3a Binding. The time course and temperature dependence of human ^{125}I-C3a binding to Bt_2cAMP-differentiated U937 cells are demonstrated in Figure 4, upper panel. At 22 °C the binding of ^{125}I-C3a reached a maximum after 15 min. After 30 min we observed a steady decrease. At 37 °C the maximum of ^{125}I-C3a uptake was already reached after 5 min. However, this maximum was smaller than the maximum at 22 °C, and it was followed by a fast reduction in binding. The binding at 4 °C remained at a low level during the period of observation.

Human PMNLs showed a different functional behavior (Figure 4, lower panel). The maximum of binding at 37 °C was higher than the maximum at 22 °C. Maximal binding was reached much later, in particular at 22 °C. Binding of ^{125}I-C3a to huPMNLs at 37 °C decreased more slowly compared to Bt_2cAMP-differentiated U937 cells.

Competitive Binding Studies. We characterized the C3a binding sites by competitive binding studies with ^{125}I-C3a at a constant concentration and increasing concentrations of unlabeled C3a or C3a-desArg, using Bt_2cAMP-differentiated and undifferentiated U937 cells (Figure 5). The binding data were analyzed by iterative curve fitting to one- and two-site models: 67 000 ± 23 000 C3a binding sites per cell were detected on differentiated U937 cells with a K_d of 4.5 ± 0.6 nM ($n = 3$). C3a-desArg did not compete for these binding sites up to a concentration of 5 μM. Undifferentiated U937 cells bound ^{125}I-C3a only unspecifically.

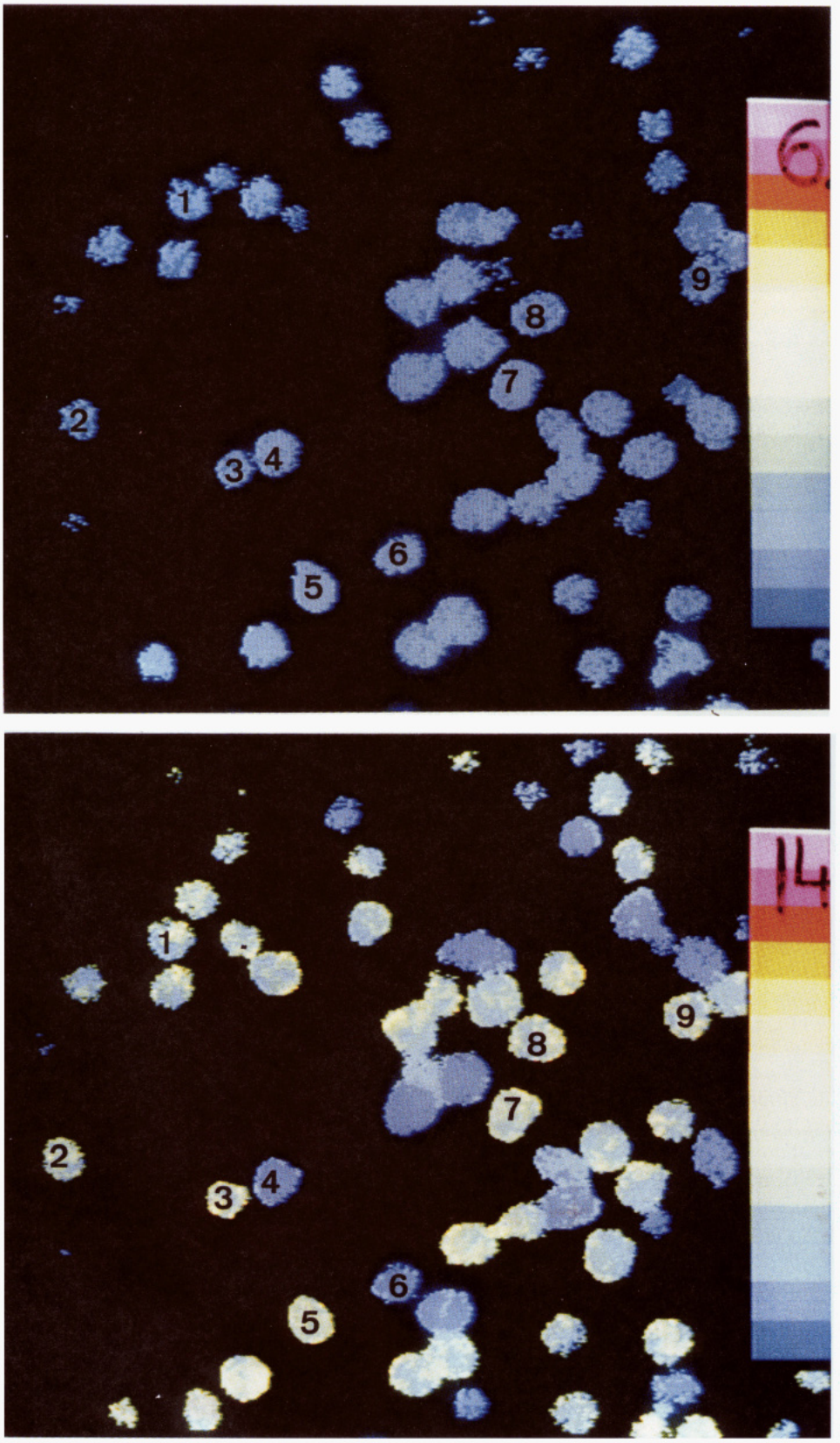

FIGURE 3: Single-cell $[Ca^{2+}]_i$ analysis of purified human PMNLs by digital imaging fluorescence microscopy. Different $[Ca^{2+}]_i$-dependent fluorescence values are presented as different colors. Shown is one typical imaging area when buffer was applied (upper panel) or when 100 nM C3a was added (lower panel). The following $[Ca^{2+}]_i$ (nM) were calculated for the numbered cells (upper/lower panel): 1, 85/330; 2, 87/374; 3, 78/430; 4, 90/86; 5, 78/487; 6, 80/92; 7, 85/470; 8, 78/450; 9, 78/412.

In similar binding studies (Figure 5, lower panel) we found 26 500 ± 8000 receptors per human PMNL. The K_d was calculated to 2.0 ± 0.5 nM (n = 3). C3a-desArg even at a concentration of 5 μM did not decrease the uptake of ^{125}I-C3a.

(III) C3a-Dependent Inositol Phosphate Metabolism in Bt_2cAMP-Differentiated U937 Cells. *myo*-Inositol 1,4,5-trisphosphate is thought to mobilize Ca^{2+} from intracellular stores in different cells (Streb et al., 1983; Volpe et al., 1988). To assess its role in C3a signal transduction, we performed

Table II: Time Dependence of Differentiation of U937 Cells Caused by 1 mM Bt_2cAMP, As Determined by the Fura-2/AM Assay ($n = 3$)

time of differentiation (days)	$[Ca^{2+}]_i$ response (nM) to buffer	40 nM C3a
0	112 ± 20	120 ± 15
1	110 ± 13	160 ± 17
2	131 ± 16	415 ± 35
3	105 ± 19	620 ± 51
4	123 ± 9	395 ± 29

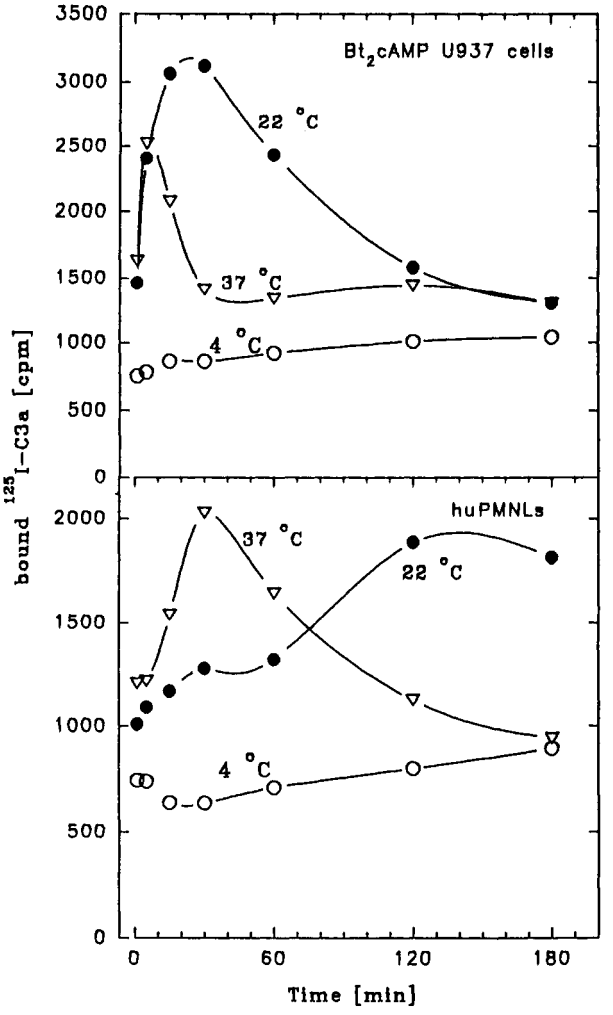

FIGURE 4: Kinetics and temperature dependence of ^{125}I-C3a binding to Bt_2cAMP-differentiated U937 cells (upper panel) and purified human PMNLs (lower panel) (Bt_2cAMP-differentiated U937, 1.1 × 10^7 cells/mL and 0.5 nM ^{125}I-C3a; human PMNLs, 1.0 × 10^7 cells/mL and 0.9 nM ^{125}I-C3a).

IP_3 kinetics with Bt_2cAMP-differentiated U937 cells (Figure 6, left panel). We found a maximal increase at approximately 15 s after C3a stimulation (40 nM). In a second experiment IP_3 values found after 15 s of stimulation with 40 nM C3a were compared with values obtained with buffer, as a negative control (Figure 6, right panel). The observed increase was significant ($n = 6$, $p = 0.99$).

(IV) C3a-Dependent Release of N-Acetyl-β-D-glucosaminidase from Bt_2cAMP-Differentiated U937 Cells. C5a causes the release of the granule enzyme *N*-acetyl-β-D-glucosaminidase from cytochalasin B treated, Bt_2cAMP-differentiated U937 cells (Gerard & Gerard, 1990). We checked whether these cells would also respond to C3a and used C3a-desArg as a negative control to exclude unspecific effects (a representative experiment is displayed in Figure 7). We found an

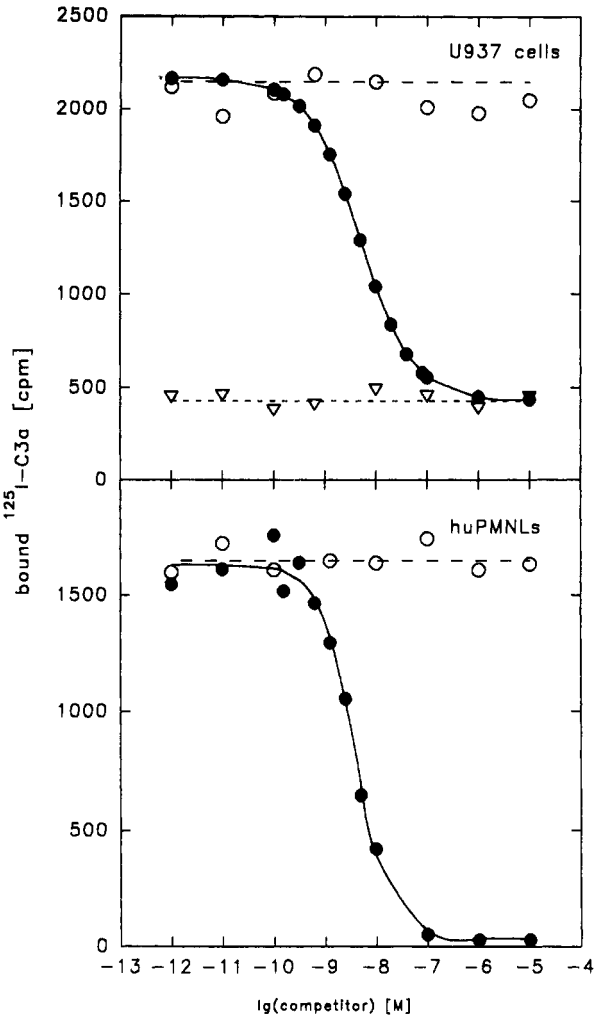

FIGURE 5: One representative competitive binding study of Bt_2cAMP-differentiated (upper panel, ● and ○) and nondifferentiated (upper panel, ▽) U937 cells and human PMNLs (1 × 10^7/mL) (lower panel). A constant concentration of ^{125}I-C3a was displaced by increasing concentrations of human C3a (● and ▽) or C3a-desArg (○), respectively. A total of 9950 cpm for U937 cells or 9790 cpm for huPMNLs per sample was used. Binding parameters were calculated by iterative curve fitting from three such experiments.

ED_{50} of this response of 2.4 ± 1.1 nM ($n = 3$). The cells did not respond to C3a-desArg.

We could not demonstrate any enzyme release from cytochalasin B treated, human PMNLs up to 1 μM C3a. In contrast, the cells were able to respond to 20 nM C5a, but the observed release was much smaller than that from Bt_2cAMP-differentiated U937 cells. When both cell types were lysed with Triton X-100, we found in granulocytes only 5–10% of total *N*-acetyl-β-D-glucosaminidase activity as compared to U937 cells.

DISCUSSION

Investigation of the human receptor and signal transduction for the anaphylatoxic peptide C3a has been hampered by the lack of an appropriate receptor-containing cell line or purified cells. Guinea-pig platelets are only suited for a small set of functional studies (Zanker et al., 1982; Meuer et al., 1981; Grossklaus et al., 1976). Moreover, it remains to be analyzed to which extent data obtained for the guinea-pig C3a receptor can be transferred to the human system. This is emphasized by the fact that C3a receptors cannot be identified on human platelets. Human basophils pretreated with interleukin-3 release histamine and leukotriene, suggesting the expression

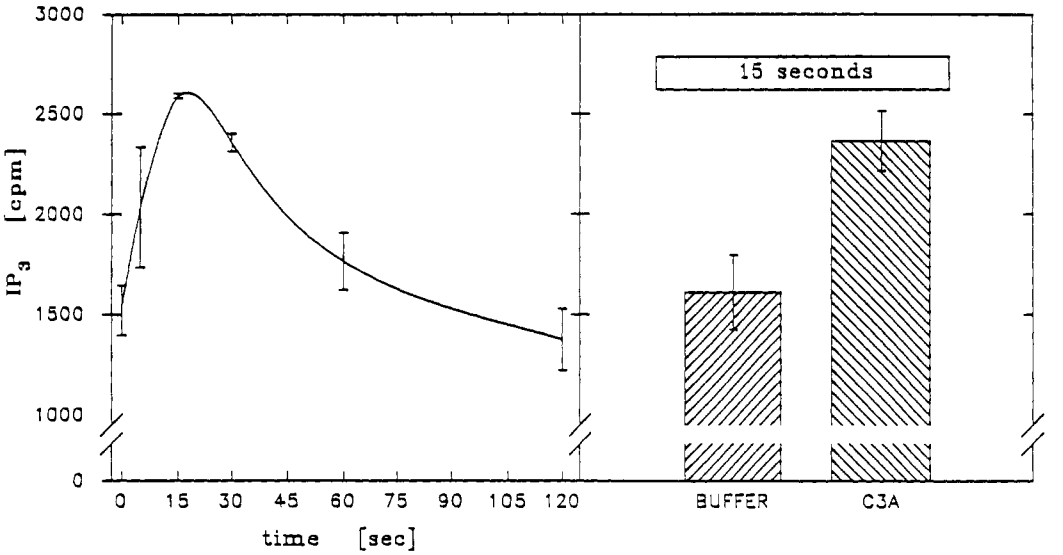

FIGURE 6: IP_3 kinetics of Bt_2cAMP-differentiated U937 cells stimulated with 40 nM human C3a (left panel, $n = 3$) and mean and standard deviation ($n = 6$) of the IP_3 response stopped 15 s after a stimulus (right panel) with buffer versus 40 nM C3a.

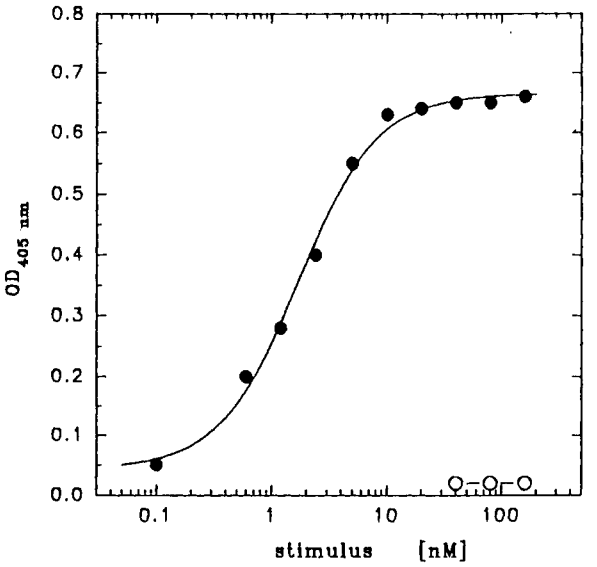

FIGURE 7: *N*-Acetyl-β-D-glucosaminidase release of Bt_2cAMP-differentiated U937 cells caused by various concentrations of human C3a (●) and C3a-desArg (○).

of a human C3a receptor on these cells (Bischoff et al., 1990). Human basophils represent an improvement for research on the receptor for C3a. But, unfortunately, they are difficult to obtain in sufficient number, purity, and homogeneity for in-depth studies. Consequently, it is difficult to show that an observed effect is not caused by any other copurified blood cell subpopulation.

To overcome these limitations, we screened different human tumor cell lines for a C3a-dependent increase in $[Ca^{2+}]_i$ by a fura-2/AM fluorescence assay. We analyzed in detail C3a-responsive Bt_2cAMP-differentiated U937 cells and other granulocyte/monocyte-related cell lines. With a selected set of experiments we verified the expression of the C3a receptor on a related physiological cell type, the human neutrophilic granulocyte. Our functional as well as our binding data clearly showed for the first time that there exists a specific receptor for C3a on Bt_2cAMP-differentiated U937 cells, and our data confirmed the existence of the C3a receptor on human neutrophilic PMNLs. The increase in $[Ca^{2+}]_i$ was C3a dose dependent with ED_{50} values in the low nanomolar range. Until now, there were no studies on the C3a signal transduction with C3a in the physiologically important nanomolar range. We could exclude effects caused by a contaminating copurified serum component, in particular, caused by C5a. C3a-desArg was without any biological effect even at a high concentration of 1 μM. The C3a analogue synthetic peptide is a C3a-specific stimulus without being possibly contaminated with any serum component. Comparing the ED_{50} values, the synthetic 13mer showed a C3a activity of approximately 1.3%. A slightly different activity (8.3%) is described from the ATP release assay with guinea-pig platelets (Gerardy-Schahn et al., 1988). Functional assays and binding studies on Bt_2cAMP-differentiated U937 cells will now allow comparison of other C3a analogue synthetic peptides in a homologous human assay system.

The $[Ca^{2+}]_i$ response to C3a or the C3a analogue synthetic peptide showed complete homologous desensitization in cells which remained able to respond normally to a subsequent stimulus with C5a, pointing to distinct ways of activation by specific receptors for C3a and C5a and distinct ways of desensitization.

The $[Ca^{2+}]_i$ response to a maximal dose of C3a was much smaller and shorter lasting than that to a maximal dose of C5a. (1) These different patterns of reactions to maximal doses of stimuli further support the fact that the observed response to C3a was not caused by contaminating C5a. (2) They point again to distinct ways of activation by specific receptors for C3a and C5a. (3) The different calcium response curves for C3a and C5a potentially indicate that C3a and C5a receptors couple to partly different signal transduction pathways (e.g., C5a but not C3a receptors stimulating calcium influx). Otherwise, the two reaction patterns should have been identical. Additionally, the smaller effect of C3a in increase and duration of $[Ca^{2+}]_i$ could explain the smaller effect of this anaphylatoxic peptide compared to C5a in the release of *N*-acetyl-β-D-glucosaminidase from Bt_2cAMP-differentiated U937 cells.

Pretreatment with pertussis toxin caused a complete block of this signal pathway, indicating that a pertussis toxin sensitive G-protein might be involved, as suggested for the C5a signal transduction (Shirato et al., 1988; Lad et al., 1985; Becker et al., 1985; Offermans et al., 1990; Rollins et al., 1991).

In single-cell analysis by digital imaging fluorescence microscopy with fura-2/AM, approximately 87% of the prepared huPMNLs responded to C3a. In the differential

white blood count 96% of these cells were neutrophils. This indicates that the majority of purified blood cells responded to C3a were human neutrophilic granulocytes.

We analyzed C3a–receptor interaction by binding kinetics and competitive binding studies to verify the expression of a C3a-specific receptor by a second, independent method. Bt_2-cAMP-differentiated U937 cells and purified huPMNLs showed differences in their binding kinetics of ^{125}I-C3a. Because binding at the applied temperatures is the complex result of at least association, dissociation, receptor number modified by receptor internalization or even externalization, and functional status of receptor, we presently can only speculate on which level these differences between U937 cells and huPMNLs were caused. It is important to realize that the biological functions of the two cells are not always identical. Therefore, the physiological significance of data obtained with differentiated U937 cells should be supported using neutrophilic granulocytes or other nontumor cells. The missing displacement of ^{125}I-C3a by C3a-desArg to Bt_2cAMP-differentiated U937 cells and the lacking specific binding of ^{125}I-C3a to undifferentiated cells in competitive binding studies strongly support the specificity of the observed ^{125}I-C3a binding to the differentiated cell line. C3a binding sites are described on guinea-pig platelets and suggested on human granulocytes and, just recently, on Bt_2cAMP-differentiated HL-60 cells. Our calculated K_d values are in the same range as described for the guinea-pig C3a receptor [1.7 nM for high-affinity sites (Gerardy-Schahn et al., 1989) or 0.8 nM (Fukuoka & Hugli, 1988), respectively]. The K_d for differentiated HL-60 cells was described to be also in the nanomolar range (Symon et al., 1991). Our calculated K_d for the receptor of huPMNLs is approximately 15 times lower than already described (2.0 versus 30 nM) (Gerardy-Schahn et al., 1989), with a slightly different receptor number (26500 versus 40000 per cell). The divergent values might be due to methodical differences in cell purification, iodination of C3a, assay design, and calculation. In contrast to former studies on PMNLs and HL-60 cells, we showed for the first time functional data in the low nanomolar range strongly supporting the expression of a C3a-specific receptor and verified by functional single-cell analyses that neutrophilic granulocytes are the receptor-bearing cells within the PMNL preparation.

We could only detect specific C3a binding and biological responses to C3a on U937 cells when they were differentiated with Bt_2cAMP. In these cells C5a-receptor mRNA can only be detected in cells stimulated with Bt_2cAMP (Boulay et al., 1991). Perhaps, the expression of the C3a receptor might be controlled on the same level.

Moreover, we investigated parts of the C3a signal transduction and identified in Bt_2cAMP-differentiated U937 cells IP_3 as a second messenger possibly mediating between a suggested G-protein activated phospholipase C and the observed increase in $[Ca^{2+}]_i$.

Additionally, with the release of *N*-acetyl-β-D-glucosaminidase, we described one secretory function of Bt_2cAMP-differentiated U937 cells, demonstrating that the first observed steps of the C3a response do not lead to a dead end of signal transduction.

In spite of our failure to detect any C3a-dependent release of *N*-acetyl-β-D-glucosaminidase from huPMNLs, we cannot exclude a small release of this enzyme below the detection limit of the assay because of the much smaller content of enzyme of PMNLs compared to U937 cells.

Observing the differences in ^{125}I-C3a-binding kinetics and release of *N*-acetyl-β-D-glucosaminidase, it should be realized that Bt_2cAMP-differentiated U937 cells do not serve as the ideal model for C3a responses in human neutrophils, even if they might give hints as to what to expect there.

The presence of the C3a receptor and its coupling to a signal pathway, not only on tumor cell lines but also on human neutrophils, point to a (patho)physiological role of C3a. C3a might directly activate human neutrophils or modify their reactions to other stimuli in all situations where the complement system is activated. Our data contribute to a better understanding of the biological role of C3a, and support a physiological concept, in which C3a is more important than presently thought. Bt_2cAMP-differentiated U937 cells and human neutrophils will provide powerful tools for further investigations, e.g., of ligand–receptor interaction with C3a analogue synthetic peptides within the homologous species and of the C3a signal transduction. In addition, they may serve as an easy to obtain source of mRNA for cloning of the human C3a receptor.

ACKNOWLEDGMENT

We thank Prof. D. Bitter-Suermann for his strong support and his helpful ideas and Prof. L. Birnbaumer for his useful suggestions. We want to mention the excellent technical assistance of K. Rech and B. Lübbers. We thank Dr. V. Kaever and Prof. K. Resch for use of and advice on the equipment for $[Ca^{2+}]_i$ measurement in cells in suspension and Dr. G. Rechkemmer and A. Jeromin for use of and advice on the digital imaging fluorescence microscope equipment. We are grateful for the help of B. M. Haack, G. F. Böl, and Prof. M. Szamel in the assay for inositol phosphate metabolism, and we thank Dr. D. Suckau for performing ^{252}Cf plasma desorption mass spectroscopy.

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Registry No. C3a, 80295-42-7; IP_3, 88269-39-0.