

C3a Is a Chemotaxin for Human Eosinophils but Not for Neutrophils. I. C3a Stimulation of Neutrophils Is Secondary to Eosinophil Activation

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Summary

Inflammatory action of the potent chemotaxin C5a has been well characterized on a variety of human cell types, including neutrophils, monocytes, basophils, and eosinophils. The cellular effects of C3a are less well defined. Contradictory reports have been published for C3a activation of neutrophils. Recent reports that C3a activates both basophils and eosinophils prompted us to reinvestigate the effects of C3a stimulation on eosinophils. We hypothesized that C3a activation of eosinophils, cells that are present in most neutrophil preparations, might lead to neutrophil activation. Using neutrophils of 98% purity, we observed no evidence of cellular activation after stimulation with either C3a, recombinant human C3a (rhC3a), or the synthetic C3a analogue C3a 57-77, Y57. Eosinophils purified to >98% purity displayed concentration-dependent polarization, chemotaxis, and enzyme release by stimulation with C3a, rhC3a, and the synthetic C3a analogue. An inactive form of C3a, C3a_{desArg}, failed to stimulate either eosinophils or neutrophils. Using neutrophil preparations containing 5–9% eosinophils, up to 20% of neutrophils became polarized after exposure to C3a. Likewise, we demonstrated that supernatant from C3a-stimulated eosinophils promotes neutrophil chemotaxis. Eosinophil polarization experiments were repeated in the presence of antibody to the C5a receptor (C5aR) to show that C3a and C5a interact with different receptors. C3a activates eosinophils in the presence of anti-C5aR antibody at concentrations that fully block C5a activation. We conclude that eosinophils are directly activated by either C3a or C5a, whereas C3a failed to activate neutrophils. C3a acts on eosinophils via a receptor that is distinct from C5aR. Since neutrophils are indirectly stimulated by C3a, eosinophils contaminating neutrophil preparations may explain earlier reports that C3a activates human neutrophils.

BBiologic responses to complement-derived anaphylatoxins C3a and C5a have been studied in a variety of human and animal cells. C5a is a potent activator of many human cell types and induces in vivo cellular responses such as neutropenia and leukocytosis (1). C5a is a highly potent chemotaxin, and it stimulates the release of hydrolytic granular enzymes, bioactive lipids, and superoxide anions from neutrophils (2–4). In addition, C5a is one of the most potent chemotactic agents known for eosinophils (5–10). Recently, a C5a receptor has been characterized on eosinophils (11). The eosinophil receptor for C5a appears to be unique from

the neutrophil C5a receptor (C5aR),¹ having different binding characteristics and displaying a greater apparent mass based on cross-linking studies.

In contrast to well-characterized responses to C5a by many types of cells, there are few studies of responses from purified populations of human leukocytes to C3a. Convincing evidence for C3a activation exists primarily for guinea pig platelets (12, 13). However, nonspecific mechanisms of cellular activation appear to account for the stimulatory responses of rat mast cells, and perhaps other cell types, to C3a (14, 15). C3a effects on human granulocytic cell types remain controversial.

Earlier studies reporting C3a-induced degranulation, aggregation, and chemotaxis of human neutrophils (16–19) have been criticized for possible contamination by C5a and for

This is publication 9257-IMM from the Department of Immunology, The Scripps Research Institute. Preliminary data from a portion of this work was presented as an abstract entitled "C3a and a Synthetic C3a Peptide Induces Polarization and Chemotaxis of Eosinophils But Not of Neutrophils" (1994. *FASEB J.* 8:A979).

¹ Abbreviations used in this paper: C3aR, C5aR, C3a or C5a receptor, respectively; EBSS, Earl's balanced salt solution; EPO, eosinophil peroxidase; MOPS, morpholinopropane sulfonic acid; rhC3a, recombinant human C3a.

the nonphysiologic concentrations of C3a required to elicit the response (20, 21). More recently, ^{125}I -labeled C3a was used to demonstrate binding to human neutrophils and C3a-dependent increases in cytosolic calcium (22–24). These findings contradict low level or negligible binding to neutrophils of either radiolabeled or fluorescein-labeled C3a that have been reported by other investigators (25, 26).

However, recent studies report that granulocytes other than neutrophils have functional C3a receptors (C3aRs). Binding of ^{125}I -C3a to basophils has been shown (25). Histamine release occurs after C3a stimulation of mixed leukocytes (27), a response that is augmented by priming with IL-3 or GM-CSF (28). In addition, a basophilic leukemia cell line shows similar C3a-binding and functional responses (29).

There is increasing evidence that eosinophils may bear functional C3aRs. Radiolabeled C3a has been demonstrated to bind to eosinophils and to activate the respiratory burst (30, 31). Both C3a and C5a stimulate release of eosinophil cationic protein and induce increased intracellular calcium levels (32, 33). Based on these reports, we hypothesized that C3a should induce eosinophil polarization, chemotaxis, and granule release. A second hypothesis was that C3a-activated eosinophils may release factors capable of stimulating a neutrophil response. Consequently, the presence of small numbers of eosinophils in neutrophil preparations, rather than direct activation of neutrophils by C3a, could explain previous reports of apparent direct neutrophil activation by C3a.

Materials and Methods

Reagents. Dextran 70 was supplied by Baxter Healthcare Corp. (Deerfield, IL). Ficoll and Percoll were purchased from Pharmacia Inc. (Piscataway, NJ). Hypaque was supplied by Sanofi-Winthrop Pharmaceuticals (New York, NY). Earle's balanced salt solution (EBSS) was supplied by GIBCO BRL (Gaithersburg, MD). BSA, guaiacol, morpholinopropane sulfonic acid (MOPS), cytochalasin B, *p*-nitrophenyl- β -D-glucuronide, and eosin Y were purchased from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse IgG-coated magnetic beads and a magnetic separation unit were purchased from Advanced Magnetics, Inc. (Cambridge, MA). Unconjugated mouse anti-human CD16 monoclonal antibodies were purchased from either Biosource International (Camarillo, CA) or Chemicon International, Inc. (Temecula, CA). 6.5-mm Transwell plates were purchased from Costar Corp. (Cambridge, MA). Triton X-100 was supplied by Pierce Chemical Co. (Rockford, IL). RPMI-1640 was purchased from Bio-Whittaker (Walkersville, MD). A 10-ml pycnometer was purchased from Kimble Science Products (Vineland, NJ). RIA kits for C5a assays were purchased from Amersham Corp. (Arlington Heights, IL). The synthetic C3a analogue C3a 57-77, Y57 (peptide sequence YCNYITELRRQHARASHLGLAR) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Recombinant human C3a (rhC3a) was a gift from Dr. Yoshihiro Fukuoka (Tohoku University, Sendai, Japan) (34). Polyclonal antibody to the C5aR (CD88) was generated as described by Morgan et al. (35).

Purification of C3a and C5a. Human C3a and C5a were purified by the method of Hugli et al. (36). Human C3a prepared in this fashion was shown to contain 1 part per million C5a as measured by RIA. C3a_{deArg} was obtained by treating the highly purified C3a with 1% (wt/wt) carboxypeptidase B for 30 min at 37°C in am-

monium bicarbonate buffer at pH 8.0. Concentrations of C3a, C5a, and rhC3a were determined by amino acid analysis.

Neutrophil Preparation. Neutrophils were isolated from peripheral blood of healthy human donors according to the method of Ferrante and Thong (37, 38). Blood was drawn into EDTA-containing syringes to achieve a final concentration of 10 mM EDTA. 35 ml of whole blood was carefully layered over 15 ml of Ficoll-Hypaque (density of 1.106 g/ml) in a 50-ml conical tube and centrifuged at 700 *g* for 25 min. The granulocyte fraction was recovered from between the mononuclear cell layer and the red cell pellet. The total granulocyte count was obtained through manual counting using Turk stain. The percentage of eosinophils in the granulocyte fraction was determined by staining the cells with 0.1 part of a solution of 0.1% eosin Y in 10% acetone and counting 200 cells in a hemocytometer. The granulocyte fraction obtained in this fashion contained from 2 to 9% eosinophils. Only the top half of the visible neutrophil band was collected for obtaining highly purified neutrophils. This procedure resulted in neutrophil preparations of >98% purity as assessed by eosin staining. The remaining granulocyte fraction was enriched for normodense eosinophils and could be further purified as described below. The neutrophils were washed twice in EBSS containing 10 mM MOPS at pH 7.35 before resuspension in the buffer used for the assays.

Eosinophil Preparation. Enriched preparations of eosinophils were obtained from the blood of normal donors using one of two methods. Eosinophils were obtained by Percoll density gradient separation following a modification of the procedure described by Gartner (39). Briefly, isotonic Percoll was prepared using a 10 \times solution of PBS-BSA-citrate and then diluted with PBS-BSA-citrate solution to achieve specific gravities of 1.094, 1.085, and 1.077 g/ml, respectively (as measured with a pycnometer at 20°C). 3 ml of each Percoll suspension were carefully layered on top of one another in a 15-ml conical polypropylene tube before applying the cell suspension (3 ml of PBS solution containing <200 \times 10⁶ cells). The tubes were spun at 1,000 *g* for 20 min at 20°C. The cells were collected from just below the interface between the two upper Percoll solutions (i.e., specific gravities of 1.077 and 1.085) down to the red cell pellet. This fraction was further purified by negative selection using a modification of the anti-CD16 method described by Hansel et al. (40). The granulocyte mixture was resuspended in RPMI-1640 containing 10% FCS. The neutrophil-specific mouse anti-CD16 antibody was added (10 μ l/10 million neutrophils) and incubated for 30 min at 4°C. Anti-mouse IgG-coated magnetic beads (60 particles per neutrophil) were added to this mixture and incubated at room temperature for 30 min. The beads were then removed with the adherent neutrophils by magnetic separation. The remaining eosinophils were >98% pure as assessed by eosin staining. Alternatively, blood was processed as described above for the neutrophil isolation procedure using Ficoll-Hypaque. The eosinophil-enriched neutrophil fraction was further purified by negative selection of neutrophils as described above.

Polarization Assay. Neutrophil polarization assays were performed according to a modification of the procedure described by Smith et al. (41). Neutrophils were suspended at 2 \times 10⁶ cells/ml in EBSS containing 10 mM MOPS. The cells were incubated at 37°C for 0.5–40 min with an equal volume of the stimulant at varying concentrations. The reaction was terminated by addition of an ice-cold solution of 2.5% glutaraldehyde and 0.04% eosin in 0.9% saline. 200 cells from each aliquot were examined microscopically at a magnification of 400. Cells of atypical shape (i.e., other than spherical) were scored as polarized, and results were expressed as percentage of cells polarized. The percentage of eosinophils in

the granulocyte fraction was further confirmed using this combined staining/fixation technique.

Polyclonal neutralizing antibody to the C5aR was affinity purified and used for C5aR inhibition experiments (35). Cells (4×10^6 /ml) and an equal volume of anti-C5aR antibody (10 μ g/ml) were incubated for 15 min at room temperature. Polarization was assessed after addition of an equal volume of stimulant as described above.

Chemotaxis Assay. Chemotaxis assays were performed in 6.5-mm Transwell plates using a modification of the procedure of Ember et al. (42). The wells were filled with 600 μ l of the desired dilution of chemotactic factor in EBSS-MOPS-1% BSA solution. Cells were suspended at 3 million/ml, 100- μ l aliquots were pipetted onto the filter, and the plates were incubated for 2 h at 37°C. Cells from the lower compartment were collected, pelleted, and fixed in eosin-containing glutaraldehyde solution, and then counted with a hemocytometer.

Eosinophil Peroxidase Assay. Purified eosinophils were resuspended in EBSS-MOPS-1% BSA solution at 5×10^6 cells/ml. The cell suspension was preincubated with 5 μ g/ml of cytochalasin B at 37°C for 10 min and then 100- μ l aliquots were added to an equal volume of stimulant in Eppendorf tubes. After incubation at 37°C for 1 h, the tubes were centrifuged and placed on ice. Eosinophil peroxidase (EPO) activity was measured through guaiacol oxidation (43). Briefly, 1.2 ml of 1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 7.0), 174 μ l of guaiacol, 4.6 μ l of 30% H_2O_2 , and 115 μ l of water were mixed. 50 μ l of eosinophil supernatant was added to 3 ml of this solution in polystyrene cuvettes. The change in extinction (ΔE) at 470 nm is measured over 1 min. With an $\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$, activity (in units per liter) was calculated as

$$A = [\Delta E \times V_t \times 4,000] / [\epsilon_{470} \times V_a \times t]$$

where V_t is the total volume in microliters, V_a is the volume of the analyzed sample in microliters, and t is the time in minutes. EPO activity is expressed as the percentage of total cell peroxidase after cell lysis with 3% acetic acid in 1% Triton X-100.

β -Glucuronidase Release Assay. β -Glucuronidase released from stimulated cells was determined as described by Schröder et al. (44). Neutrophils were suspended in EBSS with 1% BSA at 10×10^6 /ml. After a 10-min preincubation with 5 μ g/ml cytochalasin B, 100 μ l of cells was stimulated for 1 h at 37°C. The supernatant was incubated with an equal volume of 0.01 M *p*-nitrophenyl- β -D-glucuronide for 18 h. The reaction was quenched by addition of an equal volume of 0.4 M glycine buffer, pH 10.0. Color intensity was read at 405 nm in a Titertec Multiscan/340 ELISA reader (Lab-systems, Inc., Marlboro, MA). β -glucuronidase release is expressed as the percentage of total cell enzyme content after cell lysis with 0.2% Triton X-100.

Results

C3a- and rhC3a-induced Polarization of Eosinophils and Neutrophils. Polarization results for highly purified eosinophil and neutrophil preparations stimulated by C3a and rhC3a are shown in Fig. 1. C3a induces polarization of up to 70% of the eosinophils with an ED_{50} of 30 nM C3a. An ED_{50} of <100 pM was observed for rhC3a. Likewise, the synthetic C3a analogue C3a 55-77, Y57 gave similar polarization responses, though with $\sim 10\%$ of the potency of the natural factor (data not shown). In control experiments, up to 70% of the eosinophils and 95% of the neutrophils were polarized after stimulation with 0.5 nM C5a, but concentrations

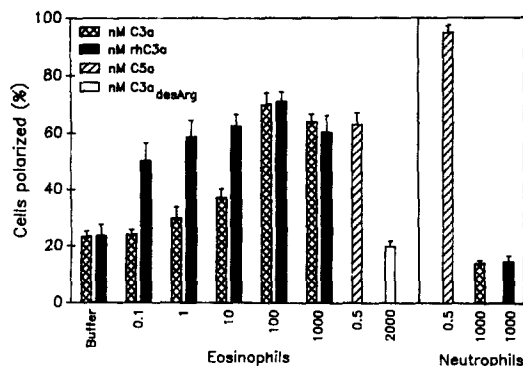


Figure 1. Polarization of highly purified human eosinophils and neutrophils in response to C3a, rhC3a, C3a_{desArg}, and C5a. Highly purified neutrophils or eosinophils were incubated with various concentrations of stimulant (as indicated on the x axis) at 37°C for 30 min. Polarization is expressed as percentage of the total number of cells counted. Mean values \pm SE ($n = 7$). Note that C3a stimulates eosinophils but not neutrophils.

of rhC3a as high as 1 μ M failed to stimulate neutrophils. Neither cell type was activated after incubation with up to 2 μ M C3a_{desArg}.

Time Course of Cell Activation by C3a and C5a. Fig. 2 A depicts the time course for neutrophil polarization at various C5a concentrations. Neutrophils display maximal (>99%) and sustained polarization within 30 s of stimulation at C5a concentrations ≥ 0.5 nM. Only transient responses are seen at lesser concentrations; however, both responses occur within seconds of exposure.

Fig. 2 B depicts eosinophil shape changes using similar concentrations of C5a. Unlike neutrophils, eosinophils do not permanently polarize at C5a concentrations <0.5 nM. However, biphasic responses occur between 2 and 20 min at C5a concentrations between 0.5 and 2 nM. Sustained polarization of $\sim 70\%$ of the eosinophils was achieved at C5a concentrations ≥ 5 nM.

The response of eosinophils to C3a is shown in Fig. 2 C. A markedly delayed pattern of activation is apparent when compared with C5a activation of either eosinophils or neutrophils. A maximum of 70% of the eosinophils are polarized after 20 min by 100 nM C3a.

C3a-induced Chemotaxis of Eosinophils. Chemotactic responses of eosinophils and neutrophils to C3a and C3a 57-77,Y57 are shown in Fig. 3. Concentration-dependent chemotactic responses of eosinophils were observed for C3a and the C3a analogue as depicted in Fig. 3 A. The ED_{50} for chemotaxis to C3a is 100 nM and for the C3a analogue is 1 μ M (data not shown). These results also reveal that highly purified neutrophils were not stimulated by either C3a or the analogue C3a 57-77,Y57 up to the indicated maximal concentrations. In control experiments, the migratory responses of both cell types to C5a was examined. As shown in Fig. 3 B, neutrophils show maximal responses of 55% of the cells migrating to 5 nM C5a, with an ED_{50} of 1 nM C5a. 40% of the eosinophils were induced to migrate by 0.3 nM C5a, a level similar to that which induced detectable polarization. These results suggest that the potency of C5a for

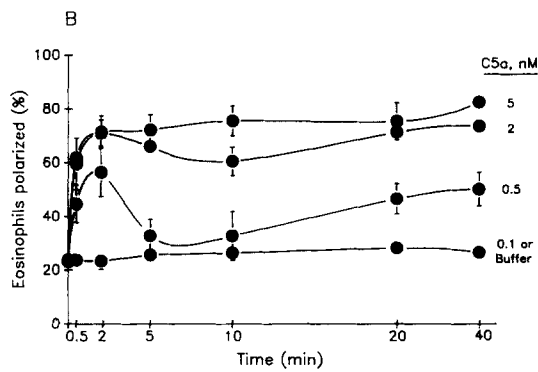
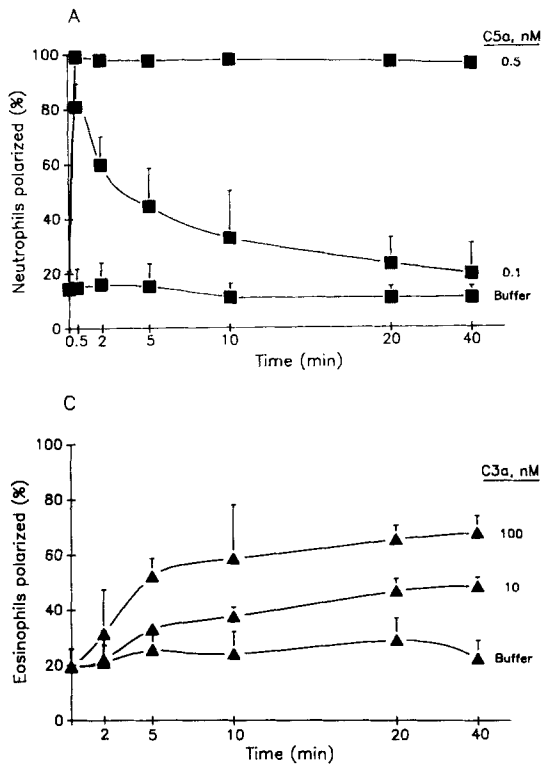


Figure 2. Time course of cell activation by C3a and C5a. Cells were incubated with specified concentrations of stimulant, and aliquots were removed at the indicated times. Experimental conditions are the same as in Fig. 1. (A) Neutrophils polarization induced by C5a versus time (■). (B) Eosinophil polarization by C5a versus time (●). (C) Eosinophil polarization induced by C3a versus time (▲). Results are expressed as mean \pm SE ($n = 9$). Note the differences in rates of activation.

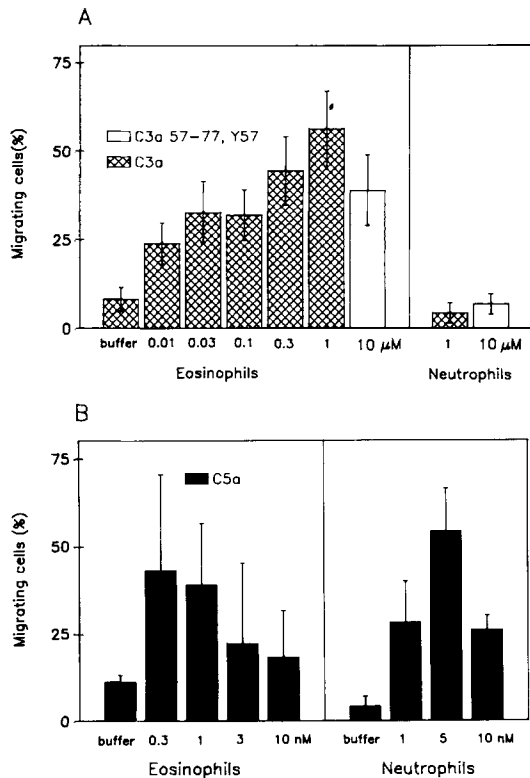


Figure 3. Chemotactic response of eosinophils and neutrophils to C3a and to the C3a analogue 57-77,Y57. Eosinophils and neutrophils were isolated, and 3×10^5 cells were added to the upper chamber. The chemoattractants were added to the lower chamber. Migration is expressed as the percentage of the total number of cells added to the chamber. Mean values

chemotaxis of eosinophils is ~ 10 times greater than for neutrophils.

Enzyme Release by C3a, rhC3a, and C5a. C5a stimulation of either eosinophils or neutrophils leads to release of β -glucuronidase, as shown in Fig. 4 A. Both C3a and rhC3a stimulated concentration-dependent release of β -glucuronidase from eosinophils but not from neutrophils. The corresponding ED₅₀ for C3a and rhC3a is 100 nM, although the recombinant form of C3a appears slightly more potent. The synthetic C3a analogue was also weakly stimulatory for promoting enzyme release from eosinophils (data not shown), while C3a_{desArg} was inactive for both cell types. Fig. 4 B indicates that eosinophil peroxidase was released after stimulation with C3a, rhC3a, or C5a but not with C3a_{desArg}. C5a was 50–100 times more potent than C3a for enzyme release.

Effects of C3a-stimulated Eosinophils on Neutrophil Responses. Studies reporting that C3a activates neutrophils have generally failed to indicate the purity of their preparations. Others report purity of 90–96% neutrophils, and the contaminating cells are stated to be eosinophils. However, differential counts of Wright–Giemsa-stained whole blood or granulocyte preparations are known to be inaccurate and may routinely underestimate eosinophils (45). Using published neutrophil preparation methods, as well as the neutrophil isolation procedure

\pm SD for four experiments are shown. (A) Chemotaxis to C3a and synthetic C3a analogue. (B) Chemotactic response to C5a. Note that C5a induced migration of both cell types, while C3a induced migration of eosinophils only.

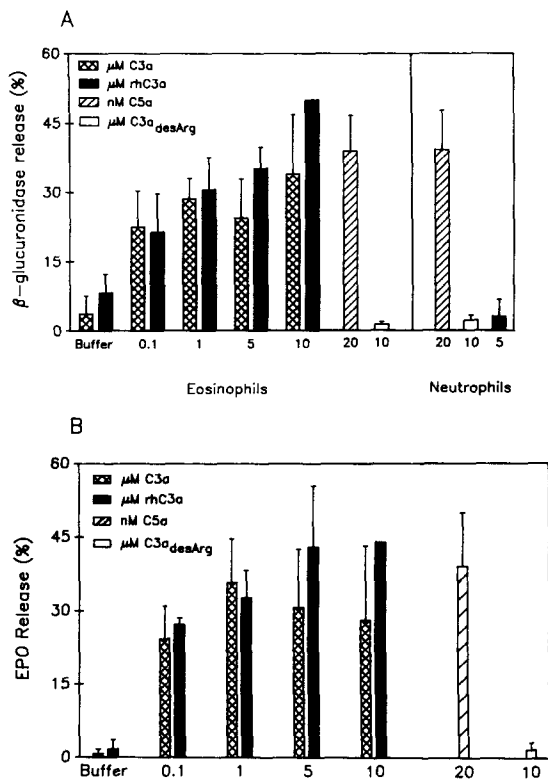


Figure 4. Release of β -glucuronidase and EPO by C3a, rhC3a, C5a, and C3a_{desArg}. Purified neutrophils (10%) or eosinophils (0.5×10^6) were incubated with specified concentrations of the agonist as indicated on the x axis. After 1 h, supernatants were assayed for enzyme content. Mean values \pm SE ($n = 5$) are expressed as percentage of total cell content of enzyme released after cell lysis. (A) β -Glucuronidase release induced by C3a and C5a from neutrophils and eosinophils. (B) EPO release by C3a and C5a from eosinophils.

described in Materials and Methods, the yield of eosinophils in the granulocyte fraction from normal donors is 2–9%, in our hands.

To test the hypothesis that eosinophils are activated by C3a and then secondarily activate neutrophils, the two following studies were performed on granulocyte preparations containing varying percentages of eosinophils. First, we stimulated granulocyte preparations containing indicated percentages of eosinophils for various intervals with a constant concentration of C3a (100 nM). Second, we stimulated granulocyte preparations containing 5–9% eosinophils with increasing concentrations of C3a. Fig. 5 A shows that C3a-induced neutrophil polarization depends on the eosinophil concentration. Using granulocyte preparations that contain >5% eosinophils, the neutrophils undergo C3a-induced polarization. Polarization of neutrophils by C3a follows a delayed time course such as that depicted for eosinophils in Fig. 2 C rather than the rapid response typical of C5a-induced neutrophil activation (Fig. 2 A). Neutrophil preparations containing 3% or less eosinophils were not activated by C3a, even though the eosinophils themselves were polarized. When neutrophil preparations containing >15% eosinophils were stimulated

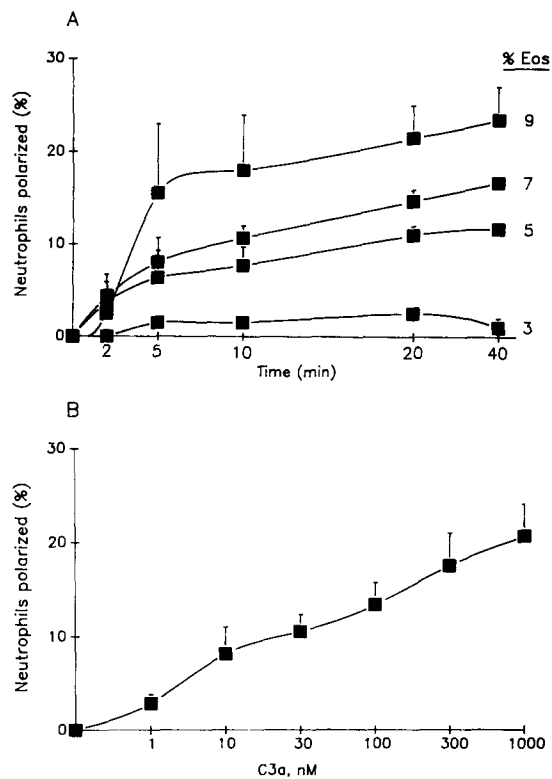


Figure 5. Eosinophil-dependent neutrophil activation by C3a. Neutrophils containing 2–9% eosinophils were collected from normal donors. The cells were suspended without further purification or hypotonic lysis in EBSS/MOPS solution at 2×10^6 /ml, and 125 μ l of cells was added to an equal volume of stimulant. Cells were fixed and stained using a cold solution of eosin and glutaraldehyde to differentiate between neutrophils and eosinophils. Buffer control values were subtracted from the values at the relevant time points. (A) Time course for stimulation of the granulocytes with 100 nM C3a. The granulocyte preparations contain various percentages of eosinophils as marked on the right side of the figure. Mean values \pm SD ($n = 3$) are shown. (B) Increasing concentrations of C3a were incubated for 40 min with neutrophil preparations containing 5–9% eosinophils. Mean values \pm SE ($n = 7$) are shown.

by C3a, $\geq 90\%$ of the neutrophils were polarized (data not shown). Fig. 5 B shows the effect of increasing concentrations of C3a on neutrophil preparations containing 5–9% eosinophils. A concentration-dependent response to C3a stimulation is apparent, with up to 25% of neutrophils activated by 1 μ M C3a after 40 min.

To extend these observations, we examined whether C3a stimulation of eosinophils would induce neutrophil chemotaxis. Neutrophil migration from the upper to the lower chamber was assessed when eosinophils and/or C3a were added to the lower chemotaxis chamber. Neutrophils failed to migrate when either C3a or unstimulated eosinophils alone were placed in the lower chamber, as shown in Fig. 6. However, a concentration-dependent chemotaxis of neutrophils occurred when C3a-stimulated eosinophils were present in the lower chamber. Additionally, neutrophil migration to suboptimal levels of C5a was enhanced by the presence of eosinophils in the lower chamber. The combination of C5a and eosinophils

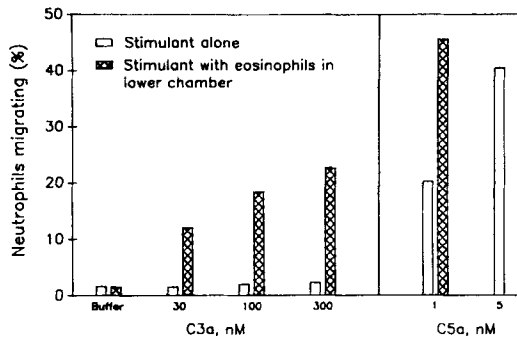


Figure 6. Eosinophil-induced chemotaxis of neutrophils. Highly purified eosinophils or neutrophils were suspended in EBSS with 1% BSA at 10^7 cells/ml. In addition to the chemoattractant, 10^6 eosinophils or equal volume of buffer were added to the lower chamber. Neutrophils (5×10^6) were added to the upper chamber and incubated for 90 min at 37°C . Neutrophils migrating into the lower chamber were then counted as described. C3a- and C5a-induced migration in the presence or absence of eosinophils is shown. A representative experiment (out of four) is shown and indicates that eosinophils enhanced the chemotactic response to C3a and C5a. Note the difference in numbers of eosinophils and neutrophils used.

resulted in migration of 45% of the neutrophils, a migratory response requiring five times higher level of C5a.

Evidence for a C3aR-specific Response. Previous reports indicated cross-desensitization between C3aR and C5aR using COOH-terminal C5a analogues in guinea pig platelets (46). It was suggested that COOH-terminal C5a analogues, but not natural C5a, interact with receptors for both C3a and C5a on these cells (46). Experiments were repeated with rhC3a in both the presence and absence of neutralizing antibody to the C5aR to rule out the possibility that cross-reactivity between C3a and both C3aR and C5aR could have been responsible for C3a-induced activation of eosinophils. Incubation of eosinophils or neutrophils with antibody to C5aR does not activate the cells (Fig. 7). Preincubation of neutrophils or eosinophils with anti-C5aR antibody did inhibit activation by C5a, although eosinophil stimulation by rhC3a was unaffected by the antibody. These results indicate that C3a interacts with a unique receptor on eosinophils, and its effects are not the result of cross-reactivity with the C5aR.

Discussion

We present evidence for activation of eosinophils by C3a, which resulted in polarization, chemotaxis, and degranulation (Fig. 1-3). Our results appear to establish that highly purified neutrophils lack these responses to C3a. The failure of recombinant C3a or synthetic C3a analogues to activate neutrophils suggests that earlier reports of neutrophil activation by C3a can be explained either by trace contamination of the native C3a by C5a (20, 21) or by C3a activation of eosinophils present in neutrophil preparations. Evidence that activation of eosinophils is receptor specific is strengthened by an inability of C3a_{desArg} to activate eosinophils and also by the fact that eosinophils are activated by rhC3a in the presence of neutralizing antibody to C5aR (Fig. 7). Like C3a, C3a_{desArg} is strongly cationic but lacks the COOH-terminal

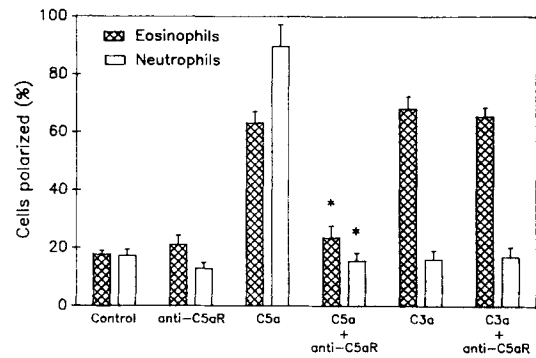


Figure 7. Anti-C5aR antibody inhibits C5a- but not C3a-induced polarization of eosinophils. Either eosinophils or neutrophils were incubated with control IgG or affinity-purified antibody to the C5aR (*anti-C5aR*) for 15 min at 20°C . The cell suspensions were then incubated with 0.5 nM C5a or 100 nM C3a for 30 min at 37°C , and polarization was assessed. Mean values \pm SE for five experiments indicate that anti-C5aR did not inhibit C3a activation of the eosinophils. (Asterisks indicate significant inhibition compared with C5a alone.)

arginyl residue required for binding to the receptor. Therefore, nonspecific polycationic activation, such as that observed with rat mast cells (14, 15), does not account for C3a-induced eosinophil activation.

Guinea pig platelets are the only other cell type for which there is strong evidence for both C3a and C5a receptors and activation (12, 13). While the intact natural factors C3a and C5a do not show cross-desensitization in guinea pig platelets, COOH-terminal analogues of C5a have been reported to interact with both C3aR and C5aR (46). These data indicate that C3a and C5a receptors lack selectivity for the C3a and C5a COOH-terminal effector sequences (46). Using site-directed mutagenesis, a hybrid C5a-C3a molecule was constructed by replacing three of the five COOH-terminal residues in C5a with the corresponding COOH-terminal residues of C3a (i.e., MQLGR is replaced by LGLAR) (47). Binding studies indicated that the hybrid C5a-C3a molecule binds to receptors for both C3a and C5a on guinea pig platelets. Cellular activation studies, on the other hand, indicated that the COOH-terminal residues in the C3a ligand (i.e., LGLAR) determine specificity for interaction with the C3aR, while the C5aR appears more permissive (47). We believe that our data indicate that C3a and C5a each use a unique receptor for eosinophil activation.

The potency of native C3a for eosinophil activation is 20-30-fold less than that for C5a on a molar basis (Fig. 1-4). On the other hand, levels of C3 in plasma are 20-30-fold greater than C5 levels (48). Therefore, in situations where complement is activated, the potential activities of C3a and C5a on eosinophils are nearly equivalent. The enhanced potency of rhC3a compared with the native factor can only be explained by the presence of an additional methionyl group at the NH₂ terminus of rhC3a (34).

The time course for C3a-induced polarization of eosinophils is significantly slower than C5a activation in either eosinophils or neutrophils (Fig. 2, A-C). This suggests that C3a may stimulate eosinophils to produce secondary mediators, which

then act either as autocoids or neutrophil stimulants. It is known that eosinophil products can activate a variety of cell types, including the neutrophil. The time course for neutrophil activation that was observed with C3a suggests that the activating factor(s) is either preformed or rapidly synthesized by the eosinophil. Major basic protein is found in eosinophil granules and has been shown to cause noncytotoxic neutrophil activation (49) and upregulation of CR3 and p150,95 expression (50). Reports have demonstrated the release of both preformed and newly synthesized IL-8 from calcium ionophore-stimulated eosinophils (51, 52), making this cytokine a potential candidate for neutrophil activation. Platelet activating factor, which is rapidly synthesized and released extracellularly from eosinophils (53), is a potent activator of both eosinophils and neutrophils (5, 6, 8–10, 54, 55). Other eicosanoid products made by eosinophils, such as 8,15-dihydroxyeicosatetraenoic acid, have also been reported to have chemotactic activity for neutrophils (56, 57). Hence, there are many possible explanations for the ability of eosinophils to stimulate and enhance neutrophil function.

The *in vitro* effects of this interaction between eosinophils and neutrophils could explain the inconsistencies in previous reports suggesting that C3a can activate neutrophils (16–19, 22–24). Even small numbers of eosinophils in a neutrophil preparation activate a significant number of neutrophils if the C3a concentrations are sufficiently high (>1 μM). However, the potential *in vivo* role of C3a in disease states remains

speculative. Systemic consequences of complement activation may be regulated by the carboxypeptidases in plasma and on cell membranes that cleave COOH-terminal arginine from both C3a and C5a to produce C3a_{desArg} and C5a_{desArg}, respectively (58, 59). While C5a_{desArg} retains up to 50% of its potency as a cell activator, the desArg form of C3a retains little if any residual activity (60). This implies that C3a may be active only at extravascular sites, where there is relatively less exposure to these carboxypeptidases.

Several types of diseases are commonly associated with peripheral blood or tissue eosinophilia. These include allergic disorders, parasitic or other infectious diseases, selected cutaneous and pulmonary diseases, and connective tissue diseases (61). Connective tissue disorders are known to be associated with complement activation and are occasionally accompanied by striking levels of peripheral blood or tissue eosinophilia (62). It is not yet clear how or why the inflammatory response in these diseases favors eosinophilic versus neutrophilic infiltrations. In addition to connective tissue disorders, deposition of C3 with eosinophilic infiltrates has been demonstrated in lesional skin biopsies from patients with the skin diseases pemphigoid and herpes gestationis (63). The role of C3a in either of these disorders has not been previously examined. However, the potential role of both C3a and C5a in mediating eosinophilic inflammation merits further investigation in light of our results suggesting that C3a is able to selectively activate eosinophils.

This work was supported in part by National Institutes of Health (NIH) grants AI-31963 and HL-25658 (T. E. Hugli). P. J. Daffern was supported by NIH training grant 2T32AR04177. J. A. Ember was supported by a Fellowship grant from Zeneca, Inc., and National Science Foundation grant MCB-9306501.

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Received for publication 6 January 1995.

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