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Interleukin 3 and granulocyte/macrophage-colony-stimulating factor render human basophils responsive to low concentrations of complement component C3a

(histamine/leukotriene/inflammation/hypersensitivity)

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ABSTRACT **Complement component C3a is an anaphylatoxin known to induce plasma exudation and smooth muscle contraction in tissues. The effects on inflammatory effector leukocytes, however, are poorly defined and controversial, being at best weak and occurring at very high C3a concentrations. Here, we examined the effect of C3a upon mediator release from human basophils, with and without pretreatment with interleukin 3 (IL-3), a hematopoietic growth factor recently found to profoundly modify the basophil response to various cell agonists. In the absence of cytokines, C3a, even at a concentration of 1 μM, was ineffective or only weakly stimulatory for basophil mediator release. However, when basophils were pretreated with IL-3 at concentrations of only 0.01–1 unit/ml, they became responsive to C3a, releasing large amounts of histamine and also generating leukotrienes. Surprisingly, almost optimal effects occurred with even very low C3a concentrations (1 nM). Another hematopoietic growth factor, granulocyte/macrophage-colony-stimulating factor (GM-CSF), was also found to render basophils capable of responding to C3a, but the effect was weaker than that of IL-3. C3a-induced histamine release and leukotriene generation occurred rapidly in IL-3-primed cells, being complete after 0.5 and 2 min, respectively. The rapid and strong degranulation response, occurring at very low concentrations of C3a, suggests the presence of a high-affinity C3a receptor on basophils, which might be inducible by cytokines. Our results demonstrate that, depending on the presence of IL-3 or GM-CSF, C3a is a potent basophil activator, and such a phenomenon could be of relevance in various inflammatory processes, especially hypersensitivity reactions.**

Human C3a is a 77-amino acid peptide cleaved from C3 by the alternative or classic C3 convertase during complement activation (1–3) but may also be generated by the action of certain proteases, such as mast cell tryptase (4). A well established physiological function of the anaphylatoxin C3a is the induction of an increase in vascular permeability and smooth muscle contraction, probably due to mast cell degranulation (3, 5). Although C3a was isolated in 1973 (6) and its primary structure has been known since 1975 (2), its effects on leukocyte functions are poorly defined and controversial. For example, earlier studies reported that C3a induced granule release, chemotaxis, and aggregation of neutrophils (7, 8) and promoted basophil degranulation (9, 10). However, many effects of C3a reported earlier occurred only at exceedingly high concentrations (1–100 μM) and may have been due to contamination with traces of C5a (11), which possesses potent activation properties on different leukocyte types (3). For example, we have found that all established neutrophil agonists [C5a, des-74-Arg-C5a, formyl-Met-Leu-Phe, platelet-activating factor, leukotriene B_4, 20-hydroxyleukotriene B_4, neutrophil-activating peptide 1 (NAP-1)] at pico- to nanomolar concentration induce a transient rise in intracellular calcium concentration in neutrophils, but pure C3a is ineffective even at 10 μM (unpublished results). Also striking is the paucity of published information, particularly in the last decade, regarding C3a effects on inflammatory effector cell functions, a further indication of its ineffectiveness?

Although basophils represent the smallest leukocyte population in peripheral blood, they presumably play a key role in the pathogenesis of events such as allergic late-phase reactions and delayed-type hypersensitivity (12, 13). They are also the only leukocyte type containing large amounts of histamine and have a particularly high capacity for generating leukotriene C_4, a potent bioactive lipid. Basophils can be activated to release mediators in response to IgE-dependent and IgE-independent stimuli (13). While it is well established that human C5a is a potent inducer of basophil degranulation (14), the effectiveness of C3a as a basophil agonist remains equivocal. In fact, only one group reported weak histamine-releasing activity for C3a (9, 10), and the induction of sulfidoleukotriene (sLT) generation was not examined.

Recently, it became apparent that hematopoietic growth factors, in particular the T-cell product interleukin 3 (IL-3), profoundly modify the effector function of mature basophils (14–18). We found that IL-3 not only enhanced basophil degranulation in response to diverse stimuli but also enabled basophils to produce large quantities of sLT in response to C5a, which by itself is unable to trigger lipid mediator release (14). Furthermore, IL-3 renders basophils responsive to NAP-1, a factor that is otherwise devoid of histamine-releasing activity (15). Thus, IL-3 must be regarded as a response modifier of human basophils. We therefore reinvestigated the effect of C3a on mediator release by basophils, with and without preincubation with IL-3. Our results demonstrate that C3a does not induce basophil degranulation in most donors, even at a concentration of up to 1 μM. Surprisingly, however, C3a at very low concentrations is a potent degranulating agent for basophils preincubated with IL-3. A maximal effect occurs at a C3a concentration of 1 nM, suggesting that, depending on the presence of IL-3, the generation of small amounts of C3a may lead to major pathophysiological consequences *in vivo*.

MATERIALS AND METHODS

Cell Preparation. Blood from unselected volunteers was anticoagulated with 10 mM EDTA and mixed with 0.25

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Abbreviations: IL, interleukin; GM-CSF, granulocyte/macrophage-colony-stimulating factor; sLT, sulfidoleukotriene; NAP-1, neutrophil-activating peptide 1.

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volume of 6% dextran (Pharmacia), and erythrocytes were allowed to sediment at room temperature. Leukocytes were pelleted by centrifugation (150 × g, 20 min, room temperature) and suspended in Hepes/albumin buffer [20 mM Hepes/125 mM NaCl/5 mM KCl/0.5 mM glucose/0.025% bovine serum albumin (fatty acid-free, Boehringer Mannheim)]. The cells were fractionated by Ficoll/Hypaque (Pharmacia) density centrifugation (400 × g, 40 min, room temperature). The basophil-rich mononuclear cell layer was harvested, washed three times (400 × g, 10 min, 4°C) in Hepes/albumin buffer, and finally suspended at a density of at least 2.5×10^6 cells per ml in Hepes/albumin buffer supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

In some experiments, whole leukocytes obtained after dextran sedimentation of erythrocytes were fractionated on discontinuous Percoll gradients (Pharmacia) exactly as described (14). Each cell fraction of different density was analyzed for leukocyte population by flow cytometry and morphologically in stained cytocentrifuge slides (14). This procedure allows the separation of basophils from polymorphonuclear cells (highest density) and monocytes (lowest density). The fraction containing a cell population highly enriched in basophils (30–50%) and contaminated exclusively by small lymphocytes (mainly T cells) was used for the mediator-release experiments shown. For comparison, mediator release was also evaluated in the other Percoll fractions containing variable proportions of monocytes, lymphocytes, basophils, neutrophils, and eosinophils.

Stimuli. The complement components C3a, des-77-Arg-C3a [C3a lacking the C-terminal arginine after cleavage by carboxypeptidase N (3, 6)], and C5a were purified from yeast-activated human serum as described (19) and were homogeneous as determined by amino acid analysis, SDS/PAGE, and microzone electrophoresis at pH 8.6. Tachyphylaxis experiments using the guinea pig ileum assay showed that preexposure of the tissue to 1 mg of C3a did not inhibit the contractile response to 20 ng of C5a, whereas 20 ng of C5a completely abolished the response to a second challenge with 20 ng of C5a, indicating that the C3a preparation used <0.002% C5a. Human recombinant IL-3 (biological activity, 1.5 ng/ml = 1 CML unit) and granulocyte/macrophage-colony-stimulating factor (GM-CSF; 0.3 ng/ml = 1 CML unit) were kindly provided by Sandoz Pharmaceutical. One CML unit is defined as the concentration giving half-maximal incorporation of [^{3}H]thymidine in leukocyte cultures from chronic myeloid leukemia patients. The mouse monoclonal anti-IgE LE 27 was purified as described (20) and dissolved in phosphate-buffered saline. All compounds were stored in small aliquots at −70°C and thawed just before use.

Mediator Release. The experiments were performed in a thermostat-regulated (37°C) shaking water bath, exactly as described (14). In most assays, C3a was added 10 min after the cytokine (IL-3 or GM-CSF) and the reaction was stopped 20 min after stimulus addition by placing the tubes in ice water. In kinetic experiments, the cells were rapidly cooled by the addition of 1 volume of ice-cold buffer at the time indicated. Histamine and sLT were measured in the supernatants obtained by centrifugation as described (14, 21). Histamine release is expressed as percent of total cellular histamine content or as "specific histamine release" (= histamine release in % minus spontaneous histamine release). The leukotriene data are presented as pg of leukotriene per ng of cellular histamine as described (14), in order to correct for different basophil numbers.

Statistics. All experiments were performed in triplicate except for the kinetic studies. Data are generally presented as mean ± SEM when the results from different donors were combined, or as mean ± SD for data obtained from a single cell donor. Correlations between the effects of two agonists were determined by regression analysis.

RESULTS

Basophil Mediator Release Induced by C3a. Histamine release by human basophils isolated from 24 unselected donors is shown in Fig. 1. In most experiments, C3a alone, even at a concentration of 1 μM was ineffective or induced only minimal histamine release as compared to the buffer control. A moderate release of histamine was observed in cell preparations of a few donors only. As reported earlier, basophil degranulation after IL-3 alone at 10 units/ml lies in most cases in the same range as the spontaneous histamine release (14–18). However, when basophils were first exposed to IL-3 for 10 min and then stimulated with C3a, a pronounced degranulation response was evident in all the donors tested. For comparison, in 13 of the 24 cell preparations examined, the cells were also challenged with an optimal dose of monoclonal anti-IgE antibody. As can be seen in Fig. 1, the response of IL-3-primed basophils to C3a was at least as pronounced as that due to the well established IgE-dependent trigger. Also evident from Fig. 1 is the relatively large variability of the basophil responses obtained from cells of different donors, a phenomenon that is known to occur as well when agonists such as formyl-Met-Leu-Phe and C5a are used (14, 21). However, when the release reaction in response to anti-IgE was compared to that induced by IL-3 plus C3a by regression analysis, no correlation was found ($r = 0.14$, $P > 0.05$). To determine the participation of other leukocytes, in particular monocytes, upon C3a-induced histamine release, three experiments were performed with basophils purified by Percoll gradients. Stimulation of the cell fraction containing 30–50% basophils (contaminated exclusively with small lymphocytes) yielded identical results (Fig. 1, solid symbols). Furthermore, when the other Percoll cell fractions were used for mediator release studies, the presence of variable proportions of lymphocytes, monocytes, neutrophils, or eosinophils did not affect the pattern of the release reaction (data not shown).

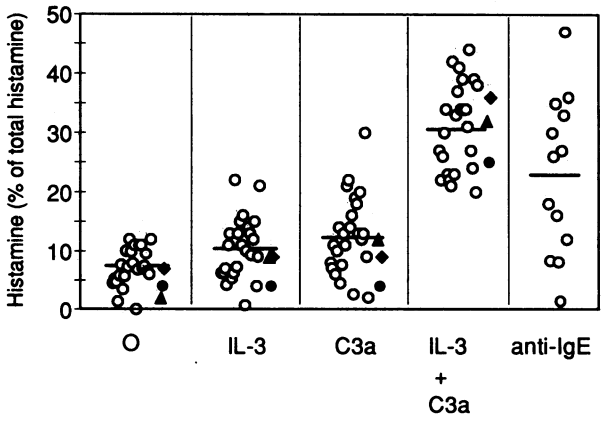

FIG. 1. Histamine release by human basophils from different donors. After a warming-up period of 10 min (37°C), the cells were incubated as follows: 30 min in buffer (O); IL-3 at 10 units/ml for 30 min (IL-3); 10 min in buffer, then C3a at 1 μM for 20 min (C3a); IL-3 at 10 units/ml for 10 min, then C3a at 1 μM for 20 min (IL-3 + C3a); 10 min buffer, then anti-IgE at 100 ng/ml for 20 min (anti-IgE). Histamine release in the supernatants is shown as percent of total cellular histamine content, without correction. Each open symbol represents the mean of triplicates from separate experiments performed with mononuclear cell preparations from 24 donors, except for anti-IgE stimulation (13 donors). Horizontal bars indicate the means of all results obtained from mononuclear cell preparations. Each solid symbol represents the mean of duplicates from three separate experiments performed with leukocyte preparations depleted of monocytes, neutrophils, and eosinophils and containing 30–50% basophils contaminated with small lymphocytes.

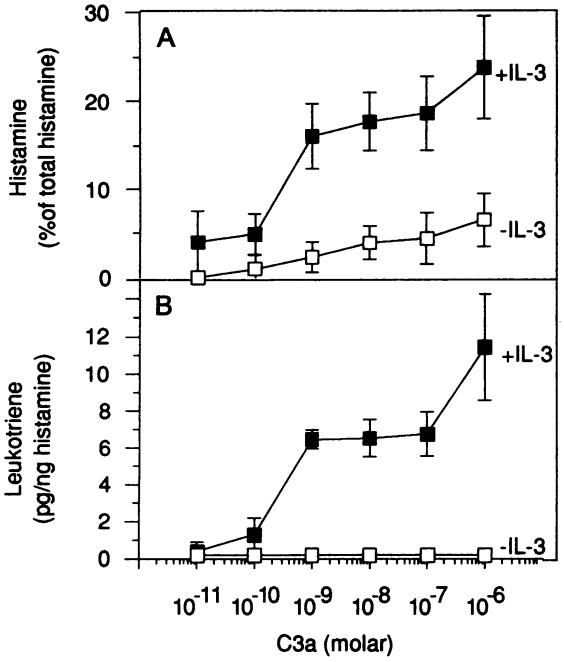

FIG. 2. C3a dose–response curve. Cells were preincubated with (■) or without (□) IL-3 (10 units/ml) for 10 min and then exposed to C3a for 20 min at the concentrations indicated. (*A*) Specific histamine release (percent of total histamine minus spontaneous release; mean ± SEM; n = 7 donors). (*B*) Leukotriene generation (pg of sLT per ng of cellular histamine), performed with cells of a donor responding well with sLT production (mean ± SD; n = 3 experiments).

In 14 out of 24 experiments, sLT generation could be demonstrated after IL-3-primed cells were stimulated with C3a (median, 4.6 pg of sLT per ng of cellular histamine; range, 1–16 pg of sLT; n = 14). Leukotrienes were never

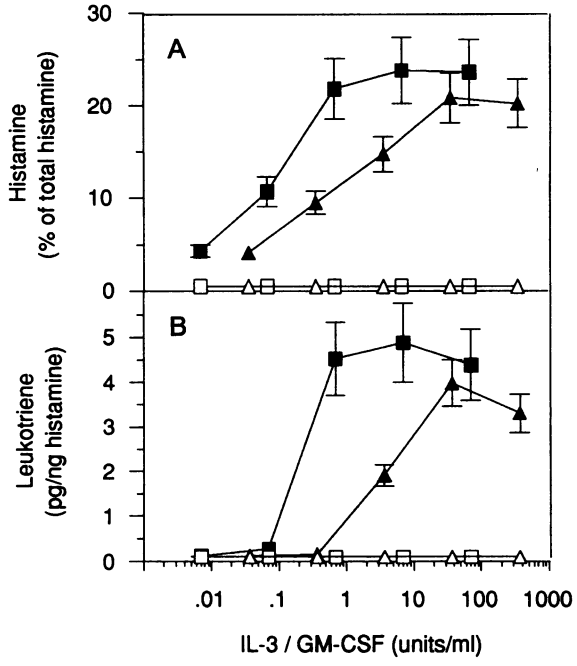

FIG. 3. Effect of IL-3 and GM-CSF concentration on C3a-induced basophil mediator release. Cells were exposed to IL-3 (squares) or GM-CSF (triangles). After 10 min, C3a at 1 μM (solid symbols) or buffer control (open symbols) was added for 20 min. (*A*) Specific histamine release. (*B*) sLT generation. Data were obtained from an individual whose cells did not respond to C3a alone (mean ± SD; n = 3 experiments).

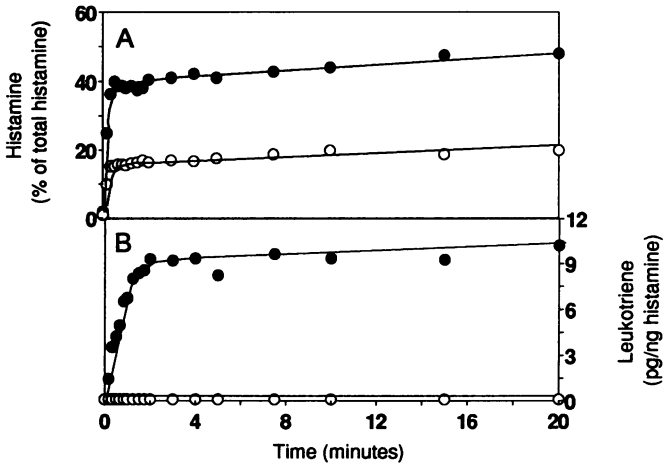

FIG. 4. Time course of C3a-induced mediator release. Cells were preincubated with (●) or without (○) IL-3 (10 units/ml) for 10 min and then stimulated with C3a (1 μM). Histamine release (*A*) and sLT generation (*B*) were determined at various times after addition of C3a.

detected in supernatants from cells exposed to either IL-3 or C3a alone. The positive values lay mostly below the sLT production induced by anti-IgE (median, 7.1 pg of sLT; range, 2–21 pg of sLT; n = 13). As for histamine release, no correlation between the two modes of activation was found. sLT was also produced by highly purified (30–50%) IL-3-primed basophils in response to C3a, and the amount of sLT correlated with the total cellular histamine content of the various Percoll gradient cell fractions (data not shown).

In a few experiments, des-77-Arg-C3a at up to 1 μM concentration was ineffective with or without IL-3, as was C3a in unprimed cells. Cell preparations that were preincubated with IL-3 and exposed to des-77-Arg-C3a did not lose their ability to undergo subsequent mediator release in response to C3a, indicating that des-77-Arg-C3a has no antagonistic effect toward C3a (data not shown).

Dose Dependency of C3a-Induced Mediator Release. Histamine release of basophils in response to a wide range of C3a concentrations (10 pM to 1 μM) was examined with or without IL-3 pretreatment in cell preparations from seven donors. Surprisingly, an almost maximal release reaction occurred at a C3a concentration of 1 nM in IL-3-primed cells, with threshold effects observed even at 10–100 pM (Fig. 2*A*). By contrast, histamine release induced by C3a without IL-3 was marginal but increased steadily over the whole concentration range. In the seven experiments performed with cells of different donors, all dose–response curves were parallel to one another. The interindividual differences in the degree of the degranulation response led to the relatively large SEM shown in Fig. 2*A*. Similar results were observed when sLT production was examined. No sLT generation was induced without IL-3 pretreatment even at the highest C3a concentration (Fig. 2*B*).

Dose Dependency of Basophil Priming for C3a Responsiveness. Cellular responsiveness to C3a for histamine release occurred at extremely low concentrations of IL-3, starting at 0.01 unit/ml and becoming optimal between 1 and 10 units/ml (Fig. 3). Priming of basophils with IL-3 for C3a-induced sLT generation occurred in a narrower concentration range (0.1–1.0 unit/ml). The priming effect of GM-CSF for human basophil mediator release in response to various agonists has been shown to be similar to that of IL-3, albeit somewhat weaker (ref. 16 and unpublished results). In this study, GM-CSF also had the capacity to render the basophils responsive to C3a. The maximal effect was weaker than that of IL-3 and higher doses of GM-CSF were needed for optimal

priming. Priming for C3a responsiveness exerted by IL-3 was a rapid process, becoming optimal within 5 min after addition of IL-3, but the cells remained responsive for C3a over at least 30 min (data not shown), as demonstrated previously for the modification of the basophil response to other agonists, such as C5a and NAP-1 (14, 15).

Kinetics of Mediator Release. The time course of the mediator release reaction after triggering with C3a is shown in Fig. 4. The response of cells from a donor whose basophils reacted particularly well to C3a alone is shown as an example. After 30 sec, the histamine release reaction was completed with or without pretreatment with IL-3, whereas sLT generation proceeded somewhat more slowly during the first 2 min. An identical release reaction was observed after stimulation of IL-3-primed cells from donors whose basophils did not respond to C3a alone.

DISCUSSION

Our results show that among 24 unselected donors, highly purified human C3a, even at 1 μM, induced little or no histamine release from basophils. Only two reports, from Glovsky and coworkers (9, 10), have demonstrated a capacity of C3a to degranulate basophils. However, the concentrations used were extremely high (1–10 μM), and some experiments were performed with selected donors. We also observed a moderate histamine release in many donors when the C3a concentration was raised to 10 μM (unpublished results). However, it remains uncertain whether this effect is receptor-mediated. Indeed, preliminary results indicate that at least part of this high-dose effect may be due to the cationic charge of the C3a peptide rather than to a specific mechanism. Furthermore, at these high C3a concentrations, it becomes difficult to exclude an effect of contaminating C5a, which shows potent histamine-releasing activity, starting at 0.1 nM and reaching maximal effect between 1 and 10 nM (EC_{50} for C5a-induced histamine release: $\approx$0.4 nM without and $\approx$0.06 nM with IL-3 priming) (ref. 14, and Y. Kurimoto, A.L. de W., and C.A.D., unpublished data). Whatever the cause of this high-dose effect, it is certainly without relevance to pathophysiological processes, because such concentrations even exceed that of native C3 in plasma on a molar basis.

By contrast, IL-3 renders basophils responsive to extremely low concentrations of C3a. Cells from all individuals tested so far have become able to respond to C3a, with an almost optimal effect occurring already at 1 nM concentration. Thus, depending on the presence of IL-3, conversion of only 0.02% of plasmatic C3 to C3a would result in basophil activation, suggesting a major pathophysiological role of C3a for inflammation and allergic reactions *in vivo*. Some rare individuals respond moderately to relatively low concentrations of C3a without prior exposure of the cells to IL-3 *in vitro*. The reason for this phenomenon is unclear but could be due to some *in vivo* priming process. In support of this hypothesis is the observation that one "C3a responder" was retrospectively found to have suffered from a viral infection at the time of blood collection.

Only very low concentrations of IL-3, substantially below the concentrations required for induction of progenitor cell proliferation, are needed to render basophils capable of responding to C3a (14). This effect occurs in the same concentration range (0.01–0.1 unit/ml) previously shown to be needed for the enhancement of basophil degranulation triggered by various agonists (14–17). Cytokine priming is strictly required for C3a-induced sLT generation, as demonstrated for C5a, but in contrast to C3a, the anaphylatoxin C5a is by itself a potent histamine-releasing agent. This priming effect for sLT generation occurs between 0.1 and 1.0 unit of IL-3 per ml for both anaphylatoxins (14).

Since most of the experiments were performed with mononuclear cell preparations, the contribution of other leukocyte types to the C3a-induced basophil mediator release reaction has to be considered. Our previous studies (14) and the recent demonstration of high-affinity IL-3 receptors on basophils from chronic myeloid leukemia patients (17) indicate that IL-3 acts directly on basophils. The extremely rapid kinetics of C3a-induced histamine release (Fig. 4) suggests that cellular products from other leukocyte types are not involved in the basophil degranulation response triggered by C3a. Furthermore, an identical pattern of histamine release after C3a stimulation is obtained from highly purified basophils depleted of monocytes, indicating that C3a activates IL-3-primed basophils directly. However, until a method can be worked out that allows the purification of basophils to homogeneity, the improbable involvement of lymphocytes cannot be ruled out definitively.

We previously postulated that IL-3 affects the signal-transduction process for basophil mediator release, especially sLT generation, since IL-3 affects the basophil response to diverse agonists known to act through different receptors (14–17). Similarly to C3a, another recently identified inflammatory mediator, NAP-1/IL-8, is by itself devoid of histamine-releasing activity but induces basophil histamine release after priming with IL-3 (15, 21). However, relatively high concentrations of NAP-1 are needed for optimal effects on basophils. On the other hand, C3a can induce a rapid and pronounced degranulation response at 1 nM concentration, indicating that this anaphylatoxin acts through high-affinity binding sites on IL-3-primed basophils. It is therefore tempting to speculate that the cytokines IL-3 and GM-CSF, in addition to their general effect upon mediator release, induce a high-affinity state of an as yet undefined C3a-receptor. For example, several receptors have been shown to change affinity depending on phosphorylation/dephosphorylation events, and many growth factor receptors are tyrosine kinases (22, 23).

The anaphylatoxins, in particular C5a, have been implicated as a major effector system in a variety of pathophysiological processes, such as inflammation, hypersensitivity reactions, and shock syndromes (3). The potent activating properties of C5a for various inflammatory effector cells are well documented. The present study shows that C3a also has the capacity to activate at least one effector cell type, the basophil. A major difference between the two anaphylatoxins is that C3a acts efficiently only after prior exposure of the cells to IL-3 or GM-CSF.

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