In Vivo Priming of Platelet-Activating Factor-Induced Eosinophil Chemotaxis in Allergic Asthmatic Individuals


The cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5 are important modulators of eosinophilia and eosinophil function. Eosinophil chemotaxis is known to be particularly sensitive for cytokine priming. In the present study, we compared chemotactic responses of eosinophils derived from peripheral blood of allergic asthmatic individuals. Eosinophils from allergic asthmatics exhibited a markedly increased sensitivity in their chemotactic response toward platelet-activating factor (PAF) compared with eosinophils from normal donors. In contrast, C5a-induced eosinophil chemotaxis between both groups was similar. This in vivo-primed phenotype could be mimicked in vitro, by preincubating eosinophils from peripheral blood of healthy individuals with picomolar concentrations of either GM-CSF, IL-3, or IL-5. The chemotactic response of eosinophils derived from the circulation of allergic asthmatic patients toward GM-CSF was significantly lower compared with the response of eosinophils of healthy individuals. Our data strongly suggest that release of cytokines may be an important in vivo priming mechanism for eosinophils in the circulation of allergic asthmatic patients. Such an in vivo priming can subsequently result in selective upregulation and downregulation of chemotactic responses toward various chemoattractants released in the lung tissue.

IN RECENT YEARS, it has become evident that eosinophils are powerful effector cells in the pathogenesis of asthma, particularly during the late-phase asthmatic response. Eosinophil counts in peripheral blood, bronchial tissue, sputum, and bronchoalveolar fluid in asthma correlate to the severity of the disease, and to bronchial hyperresponsiveness for histamine and metacholine. Moreover, infiltrated eosinophils exhibit an activated phenotype as monitored by the EG-2 monoclonal antibody (MoAb). However, the mechanisms leading to local tissue eosinophilia remain largely unresolved. The general concept that release of chemotactic factors such as platelet-activating factor (PAF), leukotriene B4 (LTB4), and human complement factor C5a solely explain the local accumulation of various cell types in inflammatory lesions remains uncertain, because many of these factors lack cell specificity. Additional mechanisms, such as differential expression of adhesion molecules or cell-specific preactivation (eg, priming), may operate in concert to explain local tissue eosinophilia in allergic asthma and parasitic infections.

Eosinophilia and eosinophil function are known to be regulated by cytokines. Important cytokines in this respect are granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interleukin (IL)-3, IL-5, and most likely interferon gamma (IFN-γ). Elevated levels of GM-CSF, IL-3, and IL-5 are found in the blood of allergic asthmatic individuals. The presence of these cytokines in the peripheral blood of asthmatic individuals may have various implications. IL-5 has been shown to be a selective chemotactic agent for eosinophils, and is also able to selectively enhance in vitro adhesion of eosinophils to endothelial cells, via CD11/CD18 leukocyte integrins. In addition, we recently demonstrated that GM-CSF and IL-3 are capable of inducing potent chemotactic responses in eosinophils from normal individuals toward the well-established neutrophil chemoattractants FMLP and neutrophil-activating factor/IL-8 (NAF/IL-8).

In the present study, we evaluated whether in vivo priming is present in the eosinophil chemotactic responses of cells derived from allergic asthmatic individuals toward PAF, human complement factor C5a, and the cytokine GM-CSF. These responses were compared with those of eosinophils isolated from blood of healthy individuals in the presence or absence of GM-CSF, IL-3, and IL-5. It was observed that chemotactic responses of eosinophils from asthmatic patients resemble chemotactic responses of cytokine-primed eosinophils from normal individuals with regard to PAF- and C5a-induced chemotaxis. Furthermore, we found a marked difference in the chemotactic responsiveness toward GM-CSF between eosinophils from allergic asthmatic versus normal individuals.

MATERIALS AND METHODS

Patients. All allergic asthmatic individuals participating in the current study met the American Thoracic Society definition of asthma, including episodic airway obstruction and hyperresponsiveness to bronchoprovocation with histamine. Seven men and six women aged 20 to 45 years participated in the study. All patients were allergic to house dust mite (HDM) or cat allergens, had positive skin test reactions to these allergens, and had a bronchial hyperreactivity for histamine (PC20 hist ≥ 4 mg/mL). In all patients anti-asthma medication was stopped 10 days before the study except for β-sympathomimetics (salbutamol-inhaler), which were stopped 24 hours before patients entered the study. At the start of the study, values for forced expiratory volume in 1 second (FEV1) ranged from 54% to 100% of the predicted value, and peripheral blood eosinophil counts ranged from 2% to 16%. All studied individuals gave their informed consent, and the study was approved by the hospital's ethical committee.

Reagents. PAF(1-0-hexadecyl-2-acetyI-sn-glycero-3-phosphorylcholine), N-formyl-methionyl-leucyl-phenylalanine (FMLP), and human recombinant complement factor C5a were purchased from Sigma (St Louis, MO). Recombinant human complement C5a was stored at −70°C until use. Ficoll-Paque and Percoll were obtained from Pharmacia (Uppsala, Sweden).
from Pharmacia (Uppsala, Sweden). All other materials were reagent grade. All experiments were performed in Geys' balanced salt solution supplemented with 1.5 mmol/L CaCl₂, 1 mmol/L MgCl₂, heparin (10 IU/mL), 5 mmol/L glucose, and 1.0% human serum albumin (HSA) (wt/vol).

Cytokines. Recombinant human GM-CSF (2.5 x 10⁵ U/mg) and IL-3 (10⁴ U/mg) were purchased from Genzyme (Boston, MA). Recombinant human IL-5 (10⁴ U/mg) was purchased from Amersham (Buckinghamshire, UK). Stock solutions of the cytokines were prepared in phosphate-buffered salt solution, supplemented with 0.5% purified HSA, and were stored at -70°C until use.

Monoclonal antibodies. Anti-human GM-CSF MoAb (mouse IgG₁) neutralized 15 U/mL recombinant human (rh) GM-CSF to 90% to 95% at a concentration of 30 µg/µL; anti-human IL-3 MoAb (mouse IgG₁) neutralized 15 U/mL rhIL-3 at 6.5 µg/µL; and anti-human rhIL-5 MoAb (mouse IgG₁) neutralized 400 U/mL rhIL-5 at 16 µg/µL. Antibodies against GM-CSF and IL-3 were a kind gift from Dr G. Zenke (Preeclinical Research, Sandoz, Basel, Switzerland). The MoAb against IL-5 was a kind gift from Dr J. Tavernier (Roche Research, Gent, Belgium). CLB-FcR-gran 1 was purchased from the Central Laboratory of the Dutch Red Cross Bloodtransfusion Service (CLB, Amsterdam, The Netherlands).

Cell isolation. Blood was obtained from healthy volunteers or from allergic asthmatic individuals. Mixed granulocytes were isolated from 100 mL of blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) as described previously. Eosinophils were subsequently isolated by the method described by Hansel et al. This isolation method makes use of the fact that, in marked contrast to neutrophils, eosinophils lack the epitope on FcyR₁, recognized by the monoclonal antibody CLB-FcR-gran 1 directed against CD16. As a result, highly purified eosinophils can be isolated by removing neutrophils via immunomagnetic beads coupled to FcR-gran 1. Briefly, immunomagnetic beads (Dynal Beads, Dynal, Oslo, Norway) were coated with an MoAb against CD16 (CLB-FcR-gran 1). Coated beads were coincubated at 4°C for 20 minutes with granulocytes (10⁵ cells/mL) at a ratio of 1:4 (cells/beads). Neutrophils were subsequently removed by a magnetic particle concentrator (MPC™, Dynal).

Chemotactic assay. Eosinophil chemotaxis was measured by a modification of the method of Boyden using a 48-well microchemotaxis chamber (Neuprobe, Cabin John, MD). Chemotaxis of eosinophils' buffer (30 µL) were added to the lower and upper compartments. Two filters (cellulose nitrate) were placed between lower and upper compartments. The lower filter had a pore width of 0.45 µm (Millipore, Bedford, MA; type HA) and the upper filter a pore width of 8 µm (thickness, 100 µm; Sartorius AG, Göttingen, Germany; SM 113). Before use, the filters had been soaked in Geys' buffer. Purified eosinophils preincubated with different cytokines or with Geys' were placed in the upper compartments (25 µL of 5 x 10⁶ cells/mL). Concentration ranges of the chemotactic agents PAF and C5a were used as a control. Eosinophils isolated from normal individuals were preincubated for 30 minutes at 37°C (optimal time for GM-CSF and IL-3 priming, Werringa et al; and IL-5 priming, not shown) with the tested cytokines before analysis of chemotactic activity was performed. The chemotaxis chambers were subsequently incubated for 2.5 hours at 37°C. Thereafter, the upper filters were removed, fixed in butanol/ethanol (20/80%, vol/vol) for 10 minutes, and stained with Weigert solution (composition, 1% [vol/vol] hematoxylin in ethanol mixed with a 70-mmol/L acidic FeCl₃ solution at a 1:1 ratio). The filters were dehydrated with ethanol, made transparent with xylene, and fixed upside down. The number of cells per 10 high-power fields (HPF) was determined by light microscopy (magnification 400x). In this way, the number of cells that passed the upper filter (and migrated 100 µm) was determined.

Statistical analysis. Statistical analysis of data was performed using the Complete Statistical System (CSS) by Statsoft (Tulsa, OK). The Students' t test for paired or unpaired observations was used.

RESULTS

Chemotactic responses of eosinophils isolated from the circulation of normal and asthmatic individuals toward PAF and C5a. Figure 1 shows dose-response curves of PAF-induced chemotaxis for eosinophils from the circulation of healthy and allergic asthmatic individuals. Eosinophils derived from the circulation of normal individuals show an optimal chemotactic response at a PAF concentration between 0.1 and 1 µmol/L, which corresponds with previous findings. Eosinophils from allergic asthmatic individuals showed a significant chemotactic response toward
PAF concentrations of $10^{-11}$ mol/L, reaching a plateau between $10^{-10}$ and $10^{-8}$ mol/L. At these plateau levels, the maximal chemotactic response toward PAF was similar to that measured for eosinophils from healthy individuals at $10^{-6}$ mol/L PAF. A scatter plot of the individual eosinophil migration responses in normal individuals versus allergic asthmatics is presented for PAF (0.1 nmol/L)-induced chemotaxis (Fig 1B) and demonstrates that the mass trend is applicable to the majority of cases.

In marked contrast, the human complement C5a-induced chemotaxis was found to be identical between eosinophils from both groups (Fig 2). The optimal chemotactic concentration of complement factor C5a for both eosinophil preparations was 10 nmol/L, which is in agreement with previous findings.23

**Effect of priming with GM-CSF, IL-3, and IL-5 on the chemotactic response of eosinophils toward PAF and C5a.** Human eosinophils isolated from normal individuals were preincubated with optimal priming concentrations of GM-CSF, IL-3 (see also Warringa et al23), and IL-5 (not shown) or in Geys’ buffer (used as a control) for 30 minutes at 37°C, before transfer to the Boyden chamber upper compartment. Subsequently, dose-response curves of eosinophil chemotaxis in response to PAF and C5a were constructed (Figs 3 and 4, respectively).

Figure 3 clearly shows the changes in the chemotactic response of eosinophils from normal individuals toward PAF after priming with the different cytokines. The tested cytokines (at picomolar concentrations) caused a marked leftward shift in the dose-response curve of the chemotactic responses toward PAF. All tested cytokines were capable of inducing significant chemotaxis at very low PAF concentrations ($\sim 10^{-11}$ mol/L).

In marked contrast to this PAF response, the C5a-induced chemotaxis was not influenced by priming with the different cytokines. No statistically significant effect of cytokine priming was observed in either the extent or the sensitivity of the C5a-induced chemotactic response, compared with normal control values (Fig 2).

**Comparison of GM-CSF-induced chemotactic responses of eosinophils derived from normal and allergic asthmatic individuals.** Figure 5 shows the dose-response pattern of eosinophil chemotaxis toward GM-CSF. Eosinophils derived from the circulation of normal donors hardly showed a chemotactic response toward low (picomolar) concentrations of this cytokine. On the other hand, at higher concentrations (nanomolar range), GM-CSF was a potent chemotaxon for these eosinophils.23

The maximal chemotactic response induced by GM-CSF was significantly lower in eosinophils from allergic asthmatic patients. Although some decrease in the chemotactic response toward IL-3 and IL-5 was seen in eosinophils derived from allergic asthmatic individuals, this proved not to be statistically significant (results not shown).
The eosinophil purity in these experiments was 97% ± 2% for both eosinophil preparations. Values indicated with * differed significantly from control values (P < .05, paired Student’s t test). Values indicated with ** differed significantly from the chemotactic response of eosinophils derived from normal donors (P < .05, unpaired Student’s t test).

To demonstrate that the observed effects were specific and not due to a contaminant in our cytokine preparation, control experiments were performed with neutralizing MoAbs against the tested cytokines. The results of these experiments showed that both priming and chemotactic effect of the cytokines could be completely reversed when the eosinophils were incubated with a mixture of the cytokine tested and a corresponding neutralizing antibody (Table 1).25

### DISCUSSION

Influx of eosinophils into the bronchial tissue of humans is considered an important process in the pathogenesis of allergic asthma.1 Despite the importance of this phenomenon, relatively little is known about the underlying mechanisms responsible for local tissue eosinophilia. Until now, several processes were considered of importance for eosinophil extravasation. Mediators such as PAF and LTβ, able to attract eosinophils from the peripheral bloodstream, are released in the lung after an allergen challenge.29 Various cytokines such as GM-CSF, IL-3, IL-5, TNF-α, and IFN-γ, produced by a number of inflammatory and bystander cells, are important modulators of eosinophil function.12-17 GM-CSF, IL-3, and IL-5 are potent growth and differentiation factors for eosinophils.12 These cytokines enhance cytotoxicity,31 migratory responses,29 and respiratory burst activation,16,17 and prolong the survival of eosinophils in vitro.16,32 Furthermore, IL-5 promotes preferential eosinophil adherence to endothelium via CD11/CD18 integrins.33 Recently, GM-CSF, IL-3, and IL-5 were demonstrated in the circulation of patients with allergic asthma.19 Lymphocytes from these patients secreted these cytokines both spontaneously and after activation.19,34 Interestingly, it has been demonstrated that during an allergen-induced late phase cutaneous reaction, messenger RNA for IL-3, IL-4, IL-5, and GM-CSF can be detected in the skin. This finding might be an indication for an infiltration (or differentiation) of T cells of the Th2 phenotype at the site of inflammation.44

In the present study, we have evaluated chemotactic responses of human eosinophils isolated from the peripheral blood of healthy and allergic asthmatic individuals as a model to monitor a possible change in the capacity of eosinophils to extravasate into the lungs of asthmatic patients. Chemotaxins used in this study were PAF, recombinant human complement factor C5a, and the cytokine GM-CSF. In the case of PAF and C5a, we compared chemotactic responses of eosinophils isolated from normal individuals before and after preincubation with GM-CSF, IL-3, and IL-5.

PAF and complement fragment C5a are potent chemotaxins for eosinophils isolated from the blood of healthy individuals, although relatively high concentrations of PAF (1 μmol/L) are required for an optimal response.9,11,28,29 Besides being a potent chemotaxin for eosinophils, PAF is an important inflammatory mediator that can cause bronchial smooth muscle contraction, and increased airway reactivity in normal subjects, as well as in patients with mild asthma.28,35 For these reasons, PAF has been implicated in allergic diseases accompanied by eosinophil infiltration.7,36 Recently, evidence has been obtained for the release of PAF in the circulation during an allergen-induced bronchoconstrictive reaction in allergic asthmatics.30 Complement fragment C5a appears to play a central role in the pathogenesis of bacterial lung infections,30 but no convincing evidence has been presented up to now for a role in allergic asthma.

The experiments described here demonstrate that chemotaxis of eosinophils isolated from blood of allergic asthmatic individuals showed a marked leftward shift in the dose-response curve toward PAF. This results in a broad response range (10^{-11} to 10^{-8} mol/L) for this chemotaxin. In marked contrast, the chemotactic response toward complement fragment C5a is unchanged, and a significant reduction of the chemotactic response toward GM-CSF is observed as compared with eosinophils from normal individuals. This in vivo priming of the PAF-induced chemotaxis could be mimicked by preincubation of eosinophils, isolated from the blood of normal individuals, with low concentrations of GM-CSF, IL-3, or IL-5 (10^{-11} to 10^{-10} mol/L). Therefore, these priming phenomena may play an

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**Table 1. Effects of Neutralizing MoAbs Against GM-CSF-induced Chemotaxis**

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<thead>
<tr>
<th>Chemotaxin</th>
<th>Chemotactic Response (% of control)</th>
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<tr>
<td>Buffer</td>
<td>18 ± 3</td>
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<tr>
<td>GM-CSF (1 nmol/L)</td>
<td>100</td>
</tr>
<tr>
<td>GM-CSF (1 nmol/L) + MoAb</td>
<td>45 ± 10*</td>
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Chemotactic responses of eosinophils towards GM-CSF were measured in the presence or absence of a neutralizing MoAb. The response is presented as the percentage of the control value: 97 ± 10 cells per 10 HPF. Values are mean ± SE of three different experiments in which eosinophil purity was 94% ± 2%.

*P < .05 was considered significantly different from controls.
important role in the in vivo situation. The dramatic increase in sensitivity for PAF causes the cells to be responsive to physiological concentrations of PAF, present at inflammatory sites. In contrast, chemotactic responses toward complement fragment C5a are not influenced by cytokine priming.

These data indicate that eosinophils isolated from allergic asthmatics (1) exhibit a marked increase in the sensitivity for PAF, (2) have an identical chemotactic pattern toward C5a, and (3) have reduced chemotactic responses toward GM-CSF. This in vivo-primed phenotype can be mimicked in vitro by preincubation of eosinophils from normal individuals with GM-CSF, IL-3, or IL-5. A possible explanation for the observed enhanced chemotactic response toward PAF is induction of expression of high-affinity binding sites for PAF on the plasma membrane. Changes on affinity have also been reported for the FMLP receptor in neutrophils, after priming with GM-CSF.30

Our results strongly suggest that an in vivo cytokine-driven priming of eosinophils is present in the peripheral blood of allergic asthmatic individuals. This can probably also account for the reduced chemotaxis found toward GM-CSF, because interactions with cytokines can cause heterologous desensitization.49 A primed state of eosinophils in the blood of allergic asthmatic individuals was previously suggested from chemiluminescence studies showing an increased sensitivity of such eosinophils for FMLP and PAF.42

An earlier study by Håkansson et al on the migratory responses of eosinophils and neutrophils from patients with asthma showed specific increased chemotactic eosinophil responses toward FMLP and zymosan-activated serum (ZAS).3 The results of our study seem in partial contrast with these observations with respect to the changes in C5a (the active component of ZAS)-induced chemotaxis. We think that this difference may be due to the fact that ZAS cannot be considered identical to a pure C5a preparation. Furthermore, mixed granulocyte preparations were used by Håkansson et al instead of pure eosinophil preparations in which neutrophil/eosinophil interactions can be excluded. Another study is in agreement with our results.44 In this last report, circulating eosinophils from patients with allergic skin disease showed increased responsiveness toward PAF, whereas C5a-induced chemotaxis was also found to be unaffected.

In the present study, no correlation was found between the modulation of eosinophil chemotaxis and eosinophil counts in the peripheral blood (not shown). This is in agreement with a report on eosinophil chemotaxis in allergic skin disease.44 The reason for this is unclear. It is possible that phenomena such as altered margination and extravasation and the presence of inhibitory cytokines can complicate simple correlations.

Together with our previous results on cytokine-primed chemotaxis in eosinophils from normal individuals,32 the data presented here strongly suggest that release of cytokines is a priming mechanism for eosinophils in the peripheral blood in allergic asthma. This may result in an increased responsiveness to migratory stimuli in vivo. In vitro experiments demonstrate that GM-CSF, IL-3, or IL-5 are possible candidates responsible for this in vivo eosinophil priming. The fact that these cytokines are indeed present in the circulation of allergic asthmatics19 supports this hypothesis. Nevertheless, these data can only partially explain the increased influx of eosinophils into the lung tissue in asthma. Perhaps a combination of cytokine-driven priming of chemotactic responses and induction of specific adhesion molecules by cytokines may account for specific eosinophil infiltration into lungs.

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