Modulation and Induction of Eosinophil Chemotaxis by Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-3

By Ruud A.J. Warringa, Leo Koenderman, Paul T.M. Kok, Johannes Kreukniet, and Piet L.B. Bruijnzeel

Eosinophilia and eosinophil function are regulated by cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5. We have investigated the modulatory role of GM-CSF and IL-3 on the platelet-activating factor (PAF), neutrophil-activating factor (NAF/IL-8), leukotriene B4 (LTB4), N-formyl-methionyl-leucyl-phenylalanine (FMLP), and human complement factor C5a-induced chemotaxis of eosinophils from normal individuals. These eosinophils show a chemotactic response toward PAF, LTB4, and C5a, but not to NAF/IL-8 and FMLP. Preincubation of the eosinophils with picomolar concentrations of GM-CSF caused a significant increase in the response toward LTB4 and induced a significant chemotactic response toward NAF/IL-8 and FMLP. Preincubation of the eosinophils with picomolar concentrations of IL-3 also induced a chemotactic response toward NAF/IL-8 and FMLP, and enhanced the PAF-induced chemotaxis. The chemotactic response toward C5a was not influenced by both cytokines. Nanomolar concentrations of GM-CSF or IL-3 caused a significant inhibition of the C5a-induced chemotaxis. The LTB4-induced chemotaxis was also significantly inhibited in case of GM-CSF. At these concentrations both GM-CSF and IL-3 acted as chemotaxins for eosinophils, with GM-CSF being more powerful than IL-3. When the eosinophils were washed after pretreatment with GM-CSF and IL-3 the potentiation of the chemotactic response remained, whereas the inhibitory mode of action disappeared. Our data indicate that at picomolar concentrations the cytokines GM-CSF and IL-3 can modulate eosinophil chemotaxis and at nanomolar concentrations these cytokines can act as chemotaxins for eosinophils.

EXPOSURE OF ALLERGIC asthma patients to aeroallergens by means of bronchial challenge may give rise to the development of an early- and late-phase asthmatic reaction. The early-phase asthmatic reaction takes place within 30 minutes after allergen challenge and returns to normal within 1 hour. A late-phase asthmatic reaction may develop 4 to 6 hours after allergen challenge without renewed contact with the allergen. The cellular events taking place during this late-phase asthmatic reaction are considered highly relevant to the pathogenesis of asthma. Studies on bronchoalveolar lavage fluids, collected at the beginning of this late-phase asthmatic reaction, have demonstrated that increased numbers of inflammatory cells, particularly eosinophils, infiltrate into the bronchiol. Moreover, a direct relationship between the occurrence of an antigen-induced late-phase bronchoconstrictive reaction and the appearance of extravasated eosinophils in the lung has recently been demonstrated in a primate model. Pretreatment of the sensitized primates, before antigen challenge, with a monoclonal antibody (MoAb) against intercellular adhesion molecule-1 (ICAM-1) not only prevented the eosinophil extravasation but also blunted the antigen-induced late-phase bronchoconstrictive reaction.

Directed locomotion or chemotaxis is essential for extravasation of eosinophils. Both lipids, such as leukotriene B4 (LTB4) and platelet-activating factor (PAF), and proteins, such as complement fragment C5a, are potent chemotaxins for eosinophils. N-formyl-methionyl-leucyl-phenylalanine (FMLP), on the contrary, is not a chemotaxin for eosinophils isolated from the blood of normal individuals.

Recently it has been demonstrated that eosinophilia and eosinophil function are regulated by cytokines. In particular, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), IL-5, tumor necrosis factor-α and eosinophil-activating factor are well-known modulators of eosinophil function. Activated lymphocytes isolated from the blood of asthmatic donors secrete these cytokines.

From the Department of Pulmonary Diseases, University Hospital Utrecht, The Netherlands; and Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland.

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Address reprint requests to P.L.B. Bruijnzeel, PhD, MD, Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH 7270 Davos-Platz, Switzerland or L. Koenderman, PhD, Department of Pulmonary Diseases, University Hospital Utrecht, PO Box 85500, NL 3500 GA Utrecht, The Netherlands.

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MATERIALS AND METHODS

Reagents. PAF (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine), human recombinant complement factor C5a, FMLP, and...
LTB₄ were purchased from Sigma (St Louis, MO). The purity of LTB₄ was controlled by means of reversed-phase high performance liquid chromatography as described by Walstra et al.¹² and proved to be greater than 98% pure. Human recombinant complement C5a was stored at −70°C until use. Ficoll-Paque and Percoll were obtained 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 (rhGM-CSF) (11.5 × 10⁶ U/mg) and IL-3 (3 × 10⁷ U/mg) were kind gifts from Dr G. Zenke (Predclinical Research, Sandoz Ltd, Basel, Switzerland). Neutrophil activating factor (NAF/IL-8; 0.4 mg/mL; identical to neutrophil activation protein-1 (NAP-1)) was a kind gift of Dr H. Aschauer (Sandoz Forschungsinstitut GmbH, Vienna, Austria). Stock solutions of the cytokines were prepared in phosphate-buffered salt supplemented with 0.1% purified HSA and were stored at −70°C until use.

MoAbs. Antihuman GM-CSF MoAb (mouse IgG1) neutralized 15 U/mL of rhGM-CSF to 90% to 95% at a concentration of 30 μg/mL and antihuman IL-3 MoAb (mouse IgG1) neutralized 15 U/mL of rIL-3 at 6.5 μg/mL. Both antibodies were a kind gift from Dr G. Zenke.

Cell isolation. Blood was obtained from healthy volunteers. Eosinophils were isolated from the buffy coat of 500 mL of blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) as described before.¹³ In short, the mononuclear cells were removed via separation of blood over isotonic Ficoll-Paque (1.077 g/mL, pH 7.4). After isotonic lysis of the erythrocytes in an ice-cold solution containing 155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA (pH 7.2), the granulocytes were washed and suspended in Hanks medium supplemented with HSA (1.0%, wt/vol). Subsequently, the granulocytes were incubated for 30 minutes at 37°C to restore initial densities of the cells. After this incubation period, the cells were washed and resuspended in phosphate-buffered saline supplemented with HSA (1.0% wt/vol) and trisodium citrate (0.4% wt/vol). After precoculation of the cells for 5 minutes at 37°C, FMLP (10 nmol/L) was added to the cell suspension and the incubation was continued for 10 minutes at 37°C before 1 mL of cell suspension was layered on 4 mL of an isotonic Percoll solution (density: 1.073 g/mL). To prevent contamination of the eosinophils with cell debris and remaining erythrocytes, 1 mL of Percoll (density: 1.100 g/mL) was brought under the isotonic Percoll solution (density: 1.082 g/mL). After centrifugation (20 minutes, 1,000g, room temperature), the eosinophils were collected from the interface. The eosinophils were washed and resuspended in Geys’ buffer and kept at room temperature until use. The FMLP treatment does not influence eosinophil function, as is extensively documented elsewhere.¹³ The purity of eosinophils was always over 85% and the recovery ranged from 40% to 60%. The viability of the eosinophils was always over 98%. Control experiments were performed with highly purified eosinophils isolated from a granulocyte preparation containing neutrophils and eosinophils as described by Hansel et al.¹⁴ In short, immunomagnetic beads (Dynal Beads; Dynal A.S., Oslo, Norway) were coated with an MoAb against CD16 (CLB FcR gran 1). These beads were coincubated at 4°C for 20 minutes with the granulocyte preparation (10⁷ cells/mL) in a ratio of 1:4 (cell:beads). The neutrophils were subsequently removed by a magnetic field.

Chemotaxis assay. Chemotaxis was measured with a modified Boyden chamber assay using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD). Chemotaxis or Geys’ buffer (30 μL) were placed in the lower compartments. Two filters (cellulose nitrate) were placed between the lower and the upper compartment. The lower filter had a pore width of 0.45 μm (Millipore type HA; Millipore Corporation, Bedford, MA) and the upper filter had a pore width of 8 μm (Sartorius, SM 113; Sartorius AG, Götttingen, Germany). Before use the filters had been soaked in Geys’ buffer. Purified eosinophils were placed in the upper compartments (25 μL of 5 × 10⁶ cells/mL). Concentration ranges of the chemotactic agents PAF, NAF/IL-8, LTB₄, FMLP, and C5a were tested using the buffer as a control (see also reference 11). At optimal concentrations of these chemotactic agents, dose-response curves for the tested cytokines were performed. The cells were preincubated for 30 minutes at 37°C with the tested cytokines before the chemotaxis assay was performed. The chemotaxis chambers were subsequently incubated for 2.5 hours at 37°C. Hereafter, the filters were removed, fixed in butanol/ethanol (20%/80%, vol/vol) for 10 minutes, and stained with Weigert solution (composition: 1% [vol/vol] haematoxylin in ethanol mixed with a 70 mmol/L acidic FeCl₃ solution in 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 with light microscopy (magnification ×400). In this way the number of cells that had passed the upper filter was determined.

Statistical analysis. All data are presented as means ± SE. Statistical analysis was performed using the Student’s t-test for paired observations. P values < .05 were considered significant.

RESULTS

Chemotactic response of eosinophils induced by various chemotaxis. Figure 1 shows the responsiveness of isolated eosinophils to optimal chemotactic concentrations of
PAF, NAF/IL-8, LTB₄, FMLP, and C5a. PAF, LTB₄, and C5a proved to be potent chemotaxins for eosinophils, whereas FMLP and NAF/IL-8 had no chemotactic activity. The optimal concentrations were 1 μmol/L for PAF or LTB₄ and 10 nmol/L for C5a (see also reference 11). The observed chemotactic activity of FMLP was carefully evaluated. Staining the filters with May-Grünwald/Giemsa or control experiments with eosinophils completely devoid of contaminating neutrophils demonstrated that only neutrophils had responded to FMLP. Therefore, FMLP is not a chemotaxin for eosinophils from normal individuals. In our experiments with NAF/IL-8 this problem did not exist because these experiments were performed only with very pure (~98%) eosinophil cell preparations.

Effects of GM-CSF and IL-3 on the chemotactic response of eosinophils induced by optimal concentrations of PAF, NAF/IL-8, LTB₄, FMLP, and C5a. Human eosinophils were preincubated with different concentrations of GM-CSF and IL-3 in Geys' buffer for 30 minutes (optimal time) at 37°C before transfer to the Boyden chamber upper compartment (see Figs 2, 3, and 4). Subsequently, the chemotactic response was measured in response to optimal concentrations of the chemotaxins mentioned above. The LTB₄ response is potentiated by both cytokines, whereas the PAF response is only potentiated by IL-3 (Figs 2 and 3). The C5a-induced chemotaxis is hardly sensitive for low concentrations of both cytokines (Figs 2 and 3). More interestingly, preincubation with GM-CSF or IL-3 at picomolar concentrations caused a marked induction of the chemotactic response toward NAF/IL-8 and FMLP (see Fig 4).

After preincubation of eosinophils with nanomolar concentrations of the GM-CSF or IL-3, both cytokines caused a marked inhibition of the C5a-induced chemotaxis. GM-CSF also inhibited LTB₄-induced chemotaxis significantly (see Fig 2). It is noteworthy that the cytokines remain with the eosinophils during the chemotaxis procedure (see also below).

To exclude the possibility that the observed effects of the cytokines were caused by some contaminating neutrophils, control experiments were performed with filters that were stained with May-Grünwald/Giemsa. With this eosinophil staining the migration of eosinophils toward the tested chemotaxins was clearly shown. To further rule out a possible interaction of the cytokines with contaminating neutrophils in our eosinophil cell preparations, we have prepared eosinophils completely devoid of neutrophils as described in the Materials and Methods section and in detail by Hansel et al.²² Eosinophils isolated in this way had a purity of 95% to 99%, sometimes with a few contaminating lymphocytes, but no neutrophils were present. Results with these pure eosinophils were identical to the above mentioned experiments (data not shown).

To demonstrate that the action of the tested cytokines was specific and not due to a contaminant present in the cytokine preparations, parallel incubations were performed in the presence of neutralizing MoAbs against GM-CSF and IL-3. Table 1 shows that the stimulating effects of the cytokines on the chemotactic responses were completely reversed when eosinophils were preincubated in the presence of a neutralizing antibody against the cytokine used. Also, the inhibitory effect of GM-CSF or IL-3, observed at nanomolar concentrations, was completely reversed by cytokine-neutralizing MoAbs (Table 2). In fact, in some experiments even a potentiation of the chemotactic re-

![Image](https://example.com/image.png)
MODULATION OF EOSINOPHIL CHEMOTAXIS

Fig 3. Effect of concentration ranges of IL-3 on the (A) PAF- (1 μmol/L), (B) LTB₄ (1 μmol/L), and (C) C5a-induced (10 nmol/L) chemotactic response of eosinophils from normal individuals. For experimental details see the legend to Fig 2.

The induction of the NAF/IL-8 and FMLP-induced chemotaxis by GM-CSF and IL-3 is irreversible because washing of the cells with cytokine-free medium did not abrogate the priming (Table 3). In contrast, the inhibitory effect of GM-CSF and IL-3 is only visible when the cytokines are present in the upper chamber compartment during the chemotaxis assay (Tables 2 and 3).

GM-CSF and IL-3-induced chemotaxis of human eosinophils. In a separate series of experiments, the chemotactic response toward different concentrations of cytokines was

Fig 4. Effect of GM-CSF and IL-3 on the FMLP- (10 nmol/L) and NAF/IL-8-induced (10 nmol/L) chemotactic response of eosinophils from normal individuals. The effect of GM-CSF on the FMLP- and NAF/IL-8-induced chemotaxis is shown in (A) and (B), respectively; the effect of IL-3 on the FMLP- and NAF/IL-8-induced chemotaxis is shown in (C) and (D), respectively. For experimental details see the legend to Fig 2.
Table 1. Effects of Neutralizing MoAbs Against GM-CSF and IL-3 on the Cytokine-Enhanced Eosinophil Chemotaxis Induced by NAF/IL-8 and FMLP

<table>
<thead>
<tr>
<th>Chemotaxin</th>
<th>NAF/IL-8 (10 nmol/L)</th>
<th>FMLP (10 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>10 ± 4</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>GM-CSF (10 pmol/L)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GM-CSF (10 pmol/L) + MoAb</td>
<td>14 ± 5*</td>
<td>22 ± 2*</td>
</tr>
<tr>
<td>Buffer</td>
<td>24 ± 4</td>
<td>27 ± 7</td>
</tr>
<tr>
<td>IL-3 (100 pmol/L)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IL-3 (100 pmol/L) + MoAb</td>
<td>22 ± 4*</td>
<td>31 ± 7*</td>
</tr>
</tbody>
</table>

Eosinophils (5 × 10^6/mL) were preincubated for 30 minutes at 37°C with GM-CSF (10 pmol/L), IL-3 (100 pmol/L), or Geys' medium in the absence or presence of neutralizing MoAbs directed against GM-CSF (4 µg/mL) or IL-3 (0.2 µg/mL). The chemotactic responses are expressed as the percentage of the maximal response obtained after cytokine priming (84 ± 13 cells/10 hpf and 196 ± 67 cells/10 hpf in the case of GM-CSF–primed cells activated with NAF/IL-8 and FMLP, respectively, and 72 ± 12 cells/10 hpf and 59 ± 3 cells/10 hpf in the case of IL-3–primed cells activated with NAF/IL-8 and FMLP, respectively). Mean values ± SE are presented of three different experiments. The purity of the eosinophil preparation was 94% ± 2%.

*P < .05; values are considered to differ significantly from the 100% values.

Table 2. Effect of Neutralizing MoAbs Against GM-CSF and IL-3 on the Inhibition by GM-CSF and IL-3 of the Chemotactic Response Induced by LTβ, and C5a

<table>
<thead>
<tr>
<th>Chemotaxin</th>
<th>LTβ (1 µmol/L)</th>
<th>C5a (10 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GM-CSF (1 nmol/L)</td>
<td>51 ± 5*</td>
<td>60 ± 22*</td>
</tr>
<tr>
<td>GM-CSF (1 nmol/L) + MoAb</td>
<td>225 ± 80*</td>
<td>81 ± 31†</td>
</tr>
<tr>
<td>IL-3 (10 nmol/L)</td>
<td>70 ± 21*</td>
<td>ND</td>
</tr>
<tr>
<td>IL-3 (10 nmol/L) + MoAb</td>
<td>109 ± 29†</td>
<td>ND</td>
</tr>
</tbody>
</table>

Eosinophils were preincubated for 30 minutes at 37°C with GM-CSF (1 nmol/L) or IL-3 (10 nmol/L) in the presence or absence of the neutralizing MoAb (0.5 mg/mL). Chemotactic responses are expressed as percentages of control values (56 ± 16 cells/10 hpf in case of LTβ, and 89 ± 19 cells/10 hpf in case of C5a). Mean values ± SE are presented of four different experiments. The purity of the eosinophil preparation was 96% ± 2%.

Abbreviation: ND, not done.

*P < .05; values are considered to differ significantly from the buffer value.

†Values are considered not to differ significantly from the buffer value.

DISCUSSION

Infiltration of human eosinophils into the airways is an essential process for the sequestration of human eosinophils in the lung during the allergen-induced late-phase asthmatic reaction. Little is known about the processes that control this infiltration in vivo. Recently much attention has been paid to the role of different cytokines in the modulation of eosinophil functions. GM-CSF, IL-3, and IL-5 are potent differentiation and growth factors for human eosinophils, and are essential to culture eosinophils in vitro. These cytokines enhance eosinophil function with regard to cytotoxicity, respiratory burst, and degranulation. Moreover, it has been shown that IL-5 is a chemotaxin for eosinophils.

In the present study we have evaluated the possible induction and modulation of eosinophil chemotaxis by GM-CSF and IL-3. It is essential to perform these experiments with eosinophils isolated from peripheral blood of normal individuals, because the cytokines GM-CSF, IL-3, and IL-5 were demonstrated in the circulation of patients with asthma and with moderate peripheral blood eosinophilia. Indeed, it has been shown that eosinophils isolated from the blood of normal individuals are metabolically different compared with eosinophils isolated from the blood of patients with mild or severe eosinophilia. The differences between these types of eosinophils have led to several discrepancies in the literature. In particular, the issue of eosinophil chemotaxis in response to formylpeptides is controversial. It has been shown that FMLP is a chemotaxin for human eosinophils derived from the blood of patients with mild eosinophilia. However, FMLP is not a chemotaxin for eosinophils isolated from the blood of normal individuals (see also below).

PAF, LTβ, and complement fragment C5a are potent chemotaxins for eosinophils isolated from the blood of normal individuals (Fig 1) and (see also below). Here we show that preincubation of eosinophils with low doses of GM-CSF or IL-3 (10^-12 to 10^-10 mol/L) leads to a marked chemotactic response of eosinophils toward the neutrophil chemotaxins NAF/IL-8 and FMLP, and modulates the responses toward PAF, LTβ, and C5a. Our data indicate that the priming of eosinophils from normal individuals with GM-CSF and IL-3: (1) is essential for eosinophil chemotaxis toward NAF/IL-8 and FMLP; (2) enhances PAF– (only in case of IL-3) and LTβ-induced chemotaxis; and (3) does not influence C5a-induced chemotaxis. The different effects of the cytokines on the response of various chemotaxins for eosinophils reflect the complex regulation of eosinophil chemotaxis.

The priming by GM-CSF and IL-3 is irreversible because washing of the cells with cytokine-free medium does not influence the priming response. A possible explanation for this priming effect is the induction of expression of high-affinity binding sites for NAF/IL-8 and FMLP on the plasma membrane. These high-affinity receptors, which do
not seem to be present on resting human eosinophils, are thought to be necessary for the induction of chemotaxis. It has been reported that changes in affinity of FMLP receptors are induced during priming with GM-CSF in human neutrophils.21

Interestingly, inhibition of eosinophil chemotaxis induced by C5a is observed when nanomolar concentrations of GM-CSF and IL-3 are present. Furthermore, a pronounced inhibition of the chemotactic response toward LTB₄ occurs in eosinophils incubated with GM-CSF (Figs 2 and 3). This inhibition, which is blocked by specific MoAbs against both cytokines, is presumably caused by the fact that both cytokines are chemotaxins for eosinophils at these concentrations (Fig 4). Another explanation might be an altered expression of adhesive glycoproteins or alteration of membrane fluidity.22,23

Taken together, this study demonstrates that the cytokines GM-CSF and IL-3 are important modulators of eosinophil chemotaxis. At high concentrations (produced locally during inflammation in, eg, the asthmatic lung) these cytokines can act as true chemotaxins for eosinophils and can directly mediate eosinophil inflammation. On the other hand, at low concentrations these cytokines can enhance the chemotactic response to other inflammatory chemotaxins like PAF and LTB₄, which have been implicated in various diseases where eosinophils infiltrate.24,25 More importantly, priming by these cytokines is essential to make the eosinophil responsive toward NAF/IL-8 and formylpeptides. Thus, production of low concentrations of GM-CSF and IL-3 can augment eosinophil inflammation by potentiation of eosinophil chemotaxis. This low production of cytokines is not necessarily derived from the site of inflammation but can also be produced in peripheral tissues (eg, circulation, bone marrow). Furthermore, these findings may well explain the differences observed between FMLP responses of eosinophils from normal donors and from patients with eosinophilia both in vitro26-28 and in vivo.29 Therefore, priming of human eosinophils by cytokines such as GM-CSF and IL-3 might be a key event in the induction of eosinophil inflammation seen in diseases such as asthma.
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Modulation and induction of eosinophil chemotaxis by granulocyte-macrophage colony-stimulating factor and interleukin-3

RA Warringa, L Koenderman, PT Kok, J Kreukniet and PL Bruijnzeel