

Allergens, IgE, mediators, inflammatory mechanisms

Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma

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Background: The cytokine granulocyte-macrophage colony-stimulating factors, interleukin-3 and interleukin-5, are important modulators of eosinophilia and eosinophil function. In particular, eosinophil chemotaxis is very sensitive to cytokine priming.

Methods: We evaluated chemotactic responses of eosinophils from patients with allergic asthma. These cells exhibited a primed phenotype as deduced from enhanced responses toward formyl-methionyl-leucyl-phenylalanine and platelet-activating factor and a decreased responsiveness toward granulocyte-macrophage colony-stimulating factor. Bronchoprovocation of patients with allergic asthma with allergen was performed as a possible means to enhance *in vivo* priming.

Results: Indeed, eosinophils isolated 3 hours after allergen challenge exhibited a more pronounced primed phenotype, which was reflected by an induction of responsiveness towards interleukin-8. Eosinophil responses induced by platelet-activating factor, formyl-methionyl-leucyl-phenylalanine, complement fragment C5a, interleukin-3, interleukin-5, and granulocyte-macrophage colony-stimulating factor were not significantly altered after allergen challenge.

Conclusion: These data provide further evidence that eosinophils are already primed in the peripheral blood of individuals with allergic asthma. This is most likely due to the presence of circulating cytokines in the peripheral blood of those individuals. This *in vivo* priming results in selective upregulation and downregulation of responses toward various chemotaxins, which may be released in the lungs during allergic inflammation. (*J ALLERGY CLIN IMMUNOL* 1993;91:1198-1205.)

Key words: Asthma, allergen provocation, chemotaxis, human eosinophils

Asthma, particularly the allergen-induced late-phase reaction, is characterized by infiltration of eosinophils in lung tissue.¹⁻³ The presence of EG-2-positive eosinophils and eosinophil-derived

proteins, such as major basic protein and eosinophilic cationic protein, in the lungs and bronchoalveolar lavage fluid are reflections of the presence of activated eosinophils.⁴⁻⁶ The mechanisms that lead to eosinophil influx are largely unexplained. The general theory, which favors adhesion, followed by diapedesis and migration as a consequence of the release of chemotactic factors at inflammatory lesions, remains uncertain because many chemotaxins lack cell specificity.⁷⁻¹⁰ Therefore additional mechanisms such as priming of eosinophils in combination with the expression of specific adhesion molecules such as vascular cell adhesion molecule-1 and endothelial-leukocyte adhesion molecule-1^{11, 12} on activated lung endothelium must be present to explain local infiltration of eosinophils into sites of allergic reactions.

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Abbreviations used

FMLP:	Formyl-methionyl-leucyl-phenylalanine
PAF:	Platelet-activating factor
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
IL:	Interleukin
IL-8:	Interleukin-8 (identical to neutrophil-activating factor)
C5a:	Complement fragment C5a
BHR:	Bronchial hyperreactivity
FEV ₁ :	Forced expiratory volume in 1 second
HPF:	High power fields
MANOVA:	Multivariate analysis of variance

Recent studies have focused on the role of T lymphocytes in the pathogenesis of allergic asthma.^{5, 13} There is growing evidence that besides eosinophils, specific CD4⁺-lymphocytes of the T_{H2} phenotype infiltrate or differentiate in the lung tissue.¹⁴ This situation is comparable to late-phase cutaneous reactions.¹⁵

Levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and interleukin-5 (IL-5) are elevated in the circulation of individuals with allergic asthma.¹⁶ These T-cell-derived cytokines play an important role in modulation of eosinophilia and eosinophil function.¹⁷ They prolong eosinophil survival *in vitro*, augment antibody-dependent cytotoxicity, induce chemotaxis and adhesion, prime the activation of respiratory burst, and induce degranulation and production of bioactive lipids.¹⁸⁻²² Moreover, IL-5 is demonstrated to be a selective chemoattractant for eosinophils and promotes selective eosinophil adhesion to endothelium.^{21, 22} Further evidence for an interaction between CD4⁺ T cells and eosinophils was recently obtained from a study on migratory responses of eosinophils toward CD4-like molecules.²³

Other cytokines synthesized predominantly by activated macrophages such as interleukin-1 and tumor necrosis factor- α can induce the synthesis and expression of specific adhesion molecules on endothelial cells that promote attachment of leukocytes.²⁴

Recently, we have demonstrated that priming with IL-3 or GM-CSF is essential to elicit migratory responses of eosinophils of normal donors toward well-known neutrophil chemoattractants.²⁵ Eosinophils isolated from peripheral blood of subjects with allergic asthma exhibit a primed phenotype with regard to platelet-activating fac-

tor (PAF)-induced chemotaxis.²⁶ This *in vivo* primed phenotype for PAF can be mimicked by preincubating eosinophils from normal donors with GM-CSF, IL-3, or IL-5.²⁶

In the present study we show that in contrast to eosinophils isolated from normal donors, cells isolated from the peripheral blood of patients with mild asthma show an increased responsiveness toward formyl-methionyl-leucyl-phenylalanine (FMLP) and PAF. Moreover, provocation of an asthmatic response in patients with allergic asthma with an appropriate allergen causes an induction of eosinophil chemotaxis toward IL-8. This may reflect an *in vivo* priming mechanism for eosinophils in the peripheral blood of these patients.

METHODS

Patients

All individuals with allergic asthma who participated in this study met the criteria for The American Thoracic Society definition of asthma, including episodic airway obstruction and hyperresponsiveness to bronchoprovocation with histamine. Five men and four women who ranged in age from 20 to 45 years participated in the study. All patients had positive skin test reactions to allergens and a bronchial hyperreactivity for histamine. (The provocative concentration causing a 20% fall in forced expiratory volume in 1 second [FEV₁] was less than or equal to 4 mg/ml.) Medication was stopped 10 days before the study except for β -sympathomimetics (salbutamol inhaler), which were stopped 24 hours before the patients entered the study. At the start of the study, FEV₁ values ranged from 54% to 100% of the predicted value, and peripheral blood eosinophil counts ranged from 2% to 16% (for individual values see Table I). All participating individuals gave their informed consent. The study was approved by the hospital's ethical committee. In some experiments eosinophils from normal healthy volunteers without any history of allergy were used.

Bronchial provocation test

Airway reactivity to histamine was determined by a standard bronchoprovocation technique as described by Juniper et al.²⁷ The bronchial provocation with allergen was performed by 2-minute inhalations (tidal breathing) in 10-minute intervals. The allergen provocation was preceded by an initial aerosol dose of buffer (2.5 mg/ml NaHCO₃ + 5 mg/ml NaCl with 0.03% human serum albumin and 0.5% phenol wt/vol) in re-

TABLE 1. Lung parameters and reaction pattern after broncho provocation of patients with allergic asthma analyzed in this study

Patient No.	EAR	Δ FEV ₁ (%)	EO (0)	EO (3)	LAR
1	+	33	8.5	8.4	+
2	+	38	15.6	12.1	—
3	+	43	5.2	2.9	+
4	+	31	5.0	3.2	+
5	+	42	1.9	2.6	+
6	+	39	4.3	2.1	+
7	+	39	3.4	1.8	+
8	+	32	5.1	3.5	—
9	+	30	9.0	8.9	—

Changes in FEV₁ were measured and correlated with the occurrence of an early asthmatic reaction. Eosinophil counts (percent of white blood cells in the peripheral blood) were carried out before (0) and 3 hours after provocation (3). The occurrence of a late-phase reaction was measured by symptom scores and change in FEV₁ in which a minimum fall of 15% at 6 hours after provocation was accepted as a criterion for a late-phase reaction.

EAR, Early asthmatic reaction; EO, eosinophil count; LAR, late asthmatic reaction.

sponse to which none of the patients showed a fall in FEV₁ or peak expiratory flow. Increasing concentrations of antigen aerosol were delivered through a nebulizer (model 646, De Vilbiss Co, Somerset, Pa.). Antigen was diluted from stock solutions (10,000 biological units/ml). The dilution of allergen used for the first inhalation was calculated from the skin prick titration test, and the preantigen provocative concentration causing a 20% fall in FEV₁ for histamine was calculated according to the method of Cockcroft et al.²⁸ Inhalations were performed by stepwise doubling of the dose of antigen until the FEV₁ fell at least 20% from baseline values.

Reagents

PAF (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphoryl-choline), N-formyl-methionyl-leucyl-phenylalanine (FMLP), and human recombinant complement factor C5a were purchased from Sigma Chemical Company (St. Louis, Mo.). Recombinant human complement C5a was stored at -70°C until use. The allergen used was house dust mite (10,000 U/ml, Diephuis, Gröningen, The Netherlands). Ficoll-Paque and Percoll were obtained from Pharmacia (Uppsala, Sweden). All other materials were reagent grade. All experiments were carried out 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 (wt/vol).

Cytokines

Recombinant human GM-CSF (2.5×10^8 U/mg) and IL-3 (10^8 U/mg) were purchased from

Genzyme (Boston, Mass.). Recombinant human IL-5 (10^6 U/mg) was purchased from Amersham (Buckinghamshire, England). IL-8 ($10 \mu\text{g/ml}$) was from British Biotechnology (Oxford, England). Stock solutions of the cytokines were prepared in phosphate-buffered saline supplemented with 0.5% purified human serum albumin and were stored at -70°C until use.

Cell isolation

Blood was obtained from individuals with allergic asthma or normal nonatopic individuals. Mixed granulocytes were isolated from 50 ml of blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) as described previously.¹⁰ Eosinophils were subsequently isolated by the method described by Hansel et al.²⁹ This isolation makes use of the fact that, in marked contrast to neutrophils, eosinophils lack the epitope on Fc γ receptor III recognized by the monoclonal antibody CLB-FcR-gran 1 directed against CD16.³⁰ As a result, highly purified eosinophils can be isolated by removing neutrophils via immunomagnetic beads coupled to CLB-FcR-gran 1. In brief, immunomagnetic beads were coated with a monoclonal antibody directed against CD16 (CLB-FcR-gran 1). Coated beads were coincubated at 4°C for 20 minutes with the mixed granulocytes (10^7 cells/ml) at a ratio of 1:4 (cells/beads). Neutrophils were subsequently removed by a magnetic particle concentrator (MPCTH-1, Dynal A.S., Oslo, Norway). The purity of the eosinophils was always over 92%, with some contaminating lymphocytes but no neutrophils. The viability as measured by Trypan blue exclusion was greater than 98%, and the recovery ranged from 75% to 100%.

Chemotaxis assay

Eosinophil chemotaxis was measured by a modification of the method according to Boyden³¹ with a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, Md.). Chemotaxins or Geys' buffer (30 μ l) were placed in the lower compartments. Two filters (cellulose nitrate) were placed between the lower and upper compartments. The lower filter had a pore width of 0.45 μ m (01730, type HA, Millipore Corp., Bedford, Mass.) and the upper filter had a pore width of 8 μ m and a thickness of 100 μ m (SM 113, Sartorius AG, Göttingen, Germany). Before use filters were pre-soaked in Geys' buffer. Purified eosinophils pre-incubated with the tested cytokines or with Geys' buffer were placed in the upper compartments (25 μ l of 5×10^6 cells/ml). Concentration ranges of the chemotactic agents PAF, C5a, IL-8, FMLP and the cytokines GM-CSF, IL-3, and IL-5 were tested with buffer alone as a control.²⁵ In the priming experiments, eosinophils isolated from individuals with allergic asthma were preincubated for 30 minutes at 37° C with 10 picomolar GM-CSF before the chemotaxis assay was carried out. 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's iron hematoxylin stain (composition: 1% vol/vol) hematoxylin in ethanol mixed with an 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 under light microscopy (magnification $\times$ 400). In this way the number of cells that had passed the upper filter (and migrated 100 μ m) was determined.

Statistical analysis

All data concerning dose ranges of different chemotaxins were statistically evaluated with multivariate multiple analysis of variance (MANOVA) of repeated measures. When the overall response was significantly different from that of the buffer control, values at individual concentrations were orthogonally tested against the buffer control and corrected according Bonferroni's correction. Possible differences in chemotactic response at one particular concentration between normal donors and patients with allergic asthma were statistically evaluated with Student's *t* test for unpaired observations. Differences with *p* values of less than 0.05 were accepted as significantly different.

RESULTS

FMLP- and IL-8-induced chemotaxis of eosinophils derived from patients with allergic asthma before and after bronchoprovocation

We studied FMLP- and IL-8-induced chemotaxis of eosinophils from patients with allergic asthma, because these mediators are chemotaxins for eosinophils from normal subjects only after priming in vitro with cytokines.²⁵ Fig. 1, *A* demonstrates that eosinophils derived from the circulation of patients with asthma showed a significant initial chemotactic response to a dose range of FMLP. The optimal concentration was around 10 nmol/L FMLP, which is in the same concentration range as found for FMLP responses in cytokine-primed eosinophils.²⁵ In order to try to enhance this eosinophil response to FMLP in vivo, patients with allergic asthma were challenged with allergen (see Methods section). Eosinophils were isolated 3 hours after bronchoprovocation because bronchial hyperreactivity (BHR) starts to increase at this time point.³² An increase in BHR after bronchoprovocation has been correlated with influx of eosinophils in the lungs in a primate model.³³ We reasoned that possible priming of eosinophils in the peripheral blood should be optimal at the onset of extravasation into the lungs. The chemotactic response of eosinophils toward FMLP isolated after bronchoprovocation (Fig. 1, *A*) was again increased compared with initial buffer values, although not significantly enhanced compared with responses before allergen provocation.

In the same set of experiments IL-8 was used as a chemotaxin. This cytokine proved to be a poor chemotaxin for eosinophils isolated from the blood of patients with allergic asthma (Fig. 1, *B*). However, eosinophils isolated 3 hours after bronchoprovocation responded with a significant chemotactic response toward a dose range of IL-8. The optimal chemotactic concentration was in the same range as that found for IL-8 responses of cytokine-primed eosinophils.²⁵

Effect of bronchoprovocation on the chemotactic responses toward PAF and C5a of eosinophils isolated from the blood of patients with allergic asthma

In the next set of experiments allergen challenge was performed to augment in vivo priming of PAF-induced chemotaxis. Fig. 2, *A* shows chemotactic responses toward dose ranges of PAF of eosinophils derived from the circulation of patients with allergic asthma, which were isolated

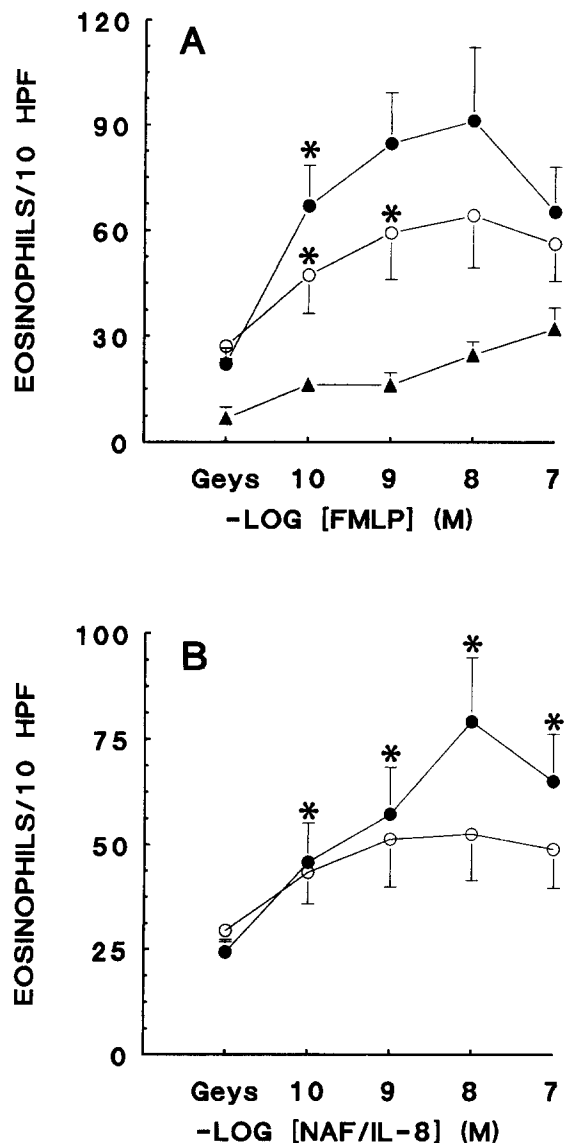


FIG. 1. Dose-response curve of FMLP-induced (A) and IL-8-induced (B) chemotaxis of eosinophils isolated from individuals with allergic asthma before (open circles) and 3 hours after allergen provocation (filled circles). In addition, FMLP responses of eosinophils of normal nonatopic donors are depicted in A (filled triangles). Eosinophil chemotaxis toward different concentrations of FMLP or IL-8 (10^{-10} to 10^{-7} mol/L) were determined with Geys' buffer as a control. The number of cells per 10 high power fields (HPF) was counted. Mean values $\pm$ SEM of nine experiments are presented. The purity of the eosinophils derived from the donors with allergic asthma was $98\% \pm 2\%$ and $92\% \pm 3\%$ before and after allergen provocation, respectively. The purity of eosinophils from the nonatopic control subjects was 97 ± 2 ($n = 6$). Contaminating cells were mononuclear cells. Both dose ranges of FMLP and the dose range of neutrophil-activating factor/IL-8 after allergen challenge were significantly different from the buffer control (MANOVA, $p < 0.01$). Individual values indicated with an asterisk were significantly different according to Bonferroni's correction from the buffer control, $p < 0.05$ (FMLP data) and $p < 0.01$ (neutrophil-activating factor/IL-8 data).

before and 3 hours after bronchial allergen provocation. Eosinophils isolated from the blood of patients with asthma responded with a significant chemotactic response toward PAF concentrations that started at 10^{-11} mol/L and reached a plateau and optimal response between 10^{-10} mol/L and 10^{-8} mol/L, which is markedly lower than the optimal concentration (0.1 to 1 μ mol/L) for eosinophils from normal donors.²⁶ Although values tended to be increased, this proved not to be significant.

This was in contrast to the rh-C5a response, which was similar in eosinophils from patients with allergic asthma, compared with those from healthy donors.²⁶ Interestingly, this response turned out to be insensitive to cytokine priming²⁶ and was not altered by bronchoprovocation (Fig. 2, B).

Effect of allergen challenge on cytokine-induced eosinophil chemotaxis

In a previous report²⁶ we have shown that in contrast to chemotaxis toward IL-3 and IL-5, chemotaxis toward GM-CSF is decreased in eosinophils from patients with allergic asthma. Therefore we have tested the possibility that allergen provocation caused a more pronounced downregulation of cytokine-induced chemotaxis. As shown in Fig. 3, allergen challenge did not significantly influence chemotactic responses of eosinophils toward IL-3, IL-5, and GM-CSF at optimal concentrations.

DISCUSSION

Cellular events that take place during the late-phase asthmatic reaction are considered to be important in the pathogenesis of allergic asthma and other allergic diseases.¹⁻³ The late-phase asthmatic reaction is characterized by the presence of activated T lymphocytes in the lungs and influx of eosinophils from peripheral blood into lung tissue.³ The present study focused on chemotaxis of eosinophils, which is important in extravasation of these cells.

Eosinophils isolated from the blood of normal individuals are unresponsive to FMLP and IL-8 and need high concentrations (1 μ mol/L) of PAF for optimal responses.^{9, 25, 34} After priming of these cells with picomolar concentrations of GM-CSF, IL-3, these cells become responsive toward dose ranges of FMLP and IL-8.²⁵ Moreover, cytokine priming causes a dramatic increase in the sensitivity for PAF.²⁶

Here it is shown that eosinophils isolated from the blood of patients with allergic asthma are

already responsive to FMLP. The dose-response curve is in the same concentration range as that found for cytokine-primed cells *in vitro*.²⁵ Eosinophils from patients with asthma are also very sensitive for PAF (Fig. 2, A).²⁶ On the basis of these studies, eosinophils isolated from the blood of patients with allergic asthma seem to resemble the phenotype of eosinophils that are primed with cytokines *in vitro*.

We tried to augment initial priming of eosinophils *in vivo* by challenging patients with allergic asthma with allergen. Blood was drawn from the patients 3 hours after allergen challenge. This time point was chosen because BHR starts to increase at this time.³² Increase in BHR has been associated with extravasation of eosinophils in the lungs in a primate model.³³ In human beings, this association has not yet been established. Bronchoprovocation leads to an induction of eosinophil chemotaxis for IL-8 and tends to augment FMLP-induced chemotaxis. The enhancement of *in vivo* priming by bronchoprovocation was not always identical, because eosinophils from patients with mild asthma who participated in our study often exhibited an already primed phenotype. This is probably a reflection of the ongoing subclinical allergic inflammation.

Chemotaxis induced by cytokines was not altered after bronchoprovocation. The marked decrease in responsiveness towards GM-CSF persisted, whereas the responses to IL-3 and IL-5 were not significantly different.

The specific increase in the chemotactic response toward PAF was previously also observed in the chemotactic response of eosinophils from patients with severe cutaneous inflammation.³⁵ Furthermore, intradermally injected PAF in human skin caused acute allergic reactions and cellular infiltrates with increased numbers of eosinophils in atopic subjects compared with control subjects.³⁶ It has been shown that PAF is released into the peripheral blood during the late asthmatic response and that the amount correlates with changes of BHR.³⁷

IL-8 is a chemoattractant and activator of degranulation for neutrophils but not for monocytes or eosinophils.^{38, 39} Here we show that IL-8 is a chemotaxin for eosinophils isolated from patients with allergic asthma only after challenge with allergen. This chemotaxin is released by endothelium activated with IL-1 or tumor necrosis factor- α .⁴⁰ These findings might implicate a role for IL-8 in the extravasation of eosinophils into the lungs during a period of allergic inflammation. In contrast to responses to PAF, FMLP, and IL-8, C5a-

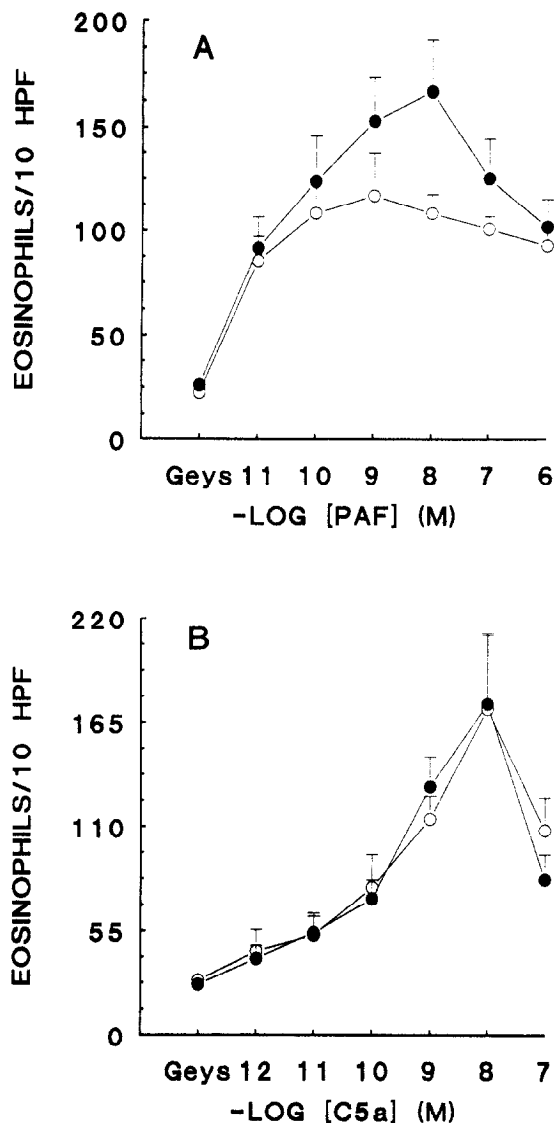


FIG. 2. PAF and human complement factor C5a-induced chemotaxis of eosinophils derived from individuals with allergic asthma before (open circles) and after (filled circles) allergen provocation. Eosinophil chemotactic responses were determined toward dose ranges of PAF (10^{-11} to 10^{-6}) (A) or C5a (10^{-12} to 10^{-7} mol/L) (B) with Geys' buffer as a control. The number of cells per 10 HPF was determined. Mean values $\pm$ SEM of nine experiments are presented. Eosinophil purity was $98\% \pm 2\%$ and $92\% \pm 3\%$ before and after allergen provocation, respectively. Contaminating cells were mononuclear cells. Both dose ranges of PAF and C5a were significantly different from that of the buffer control (MANOVA, $p < 0.001$). There was no statistically significant difference between prechallenge and postchallenge dose ranges.

induced chemotaxis is insensitive to priming both *in vivo* and *in vitro*. This implies that priming of eosinophil chemotaxis is a selective effect and not the consequence of an overall effect on the migratory machinery of eosinophils.

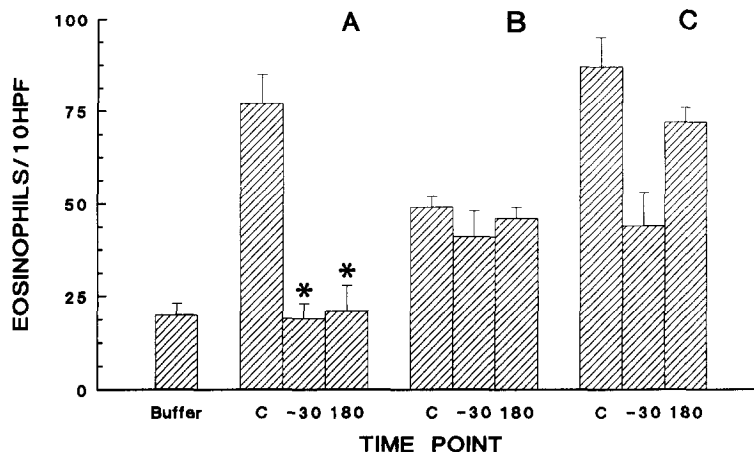


FIG. 3. Chemotactic responses toward optimal chemotactic concentrations of GM-CSF (A), IL-3 (B), or IL-5 (C) with Geys' buffer as a control. The chemotaxis of eosinophils derived from normal individuals (control: C) is compared with that of eosinophils from individuals with allergic asthma isolated before (-30) and 3 hours after (180) allergen provocation. Chemotaxis is expressed as the number of cells per 10 HPF. Mean values of eight different experiments are presented. The eosinophil purity in these experiments was $97\% \pm 2\%$ in the case of normal donors and $98\% \pm 2\%$ and $92\% \pm 3\%$, before and after provocation, respectively. Values indicated by an asterisk are significantly lower compared with values of cells from normal donors ($p < 0.05$, unpaired Student's *t* test).

Our results suggest that differential priming may take place in peripheral blood of patients with allergic asthma. Chemotaxis toward PAF seems particularly sensitive to priming, and eosinophils are potently primed in peripheral blood of patients with allergic asthma before bronchoprovocation (Fig. 2).²⁶ Responses toward FMLP, and more clearly responses toward IL-8, need allergen challenge for a clear activation. It is very likely that a cytokine-driven activation of eosinophils is responsible for the enhancement of chemotaxis toward IL-8, PAF, and FMLP in patients with allergic asthma. These cytokines might be responsible for the induction of high-affinity receptor binding sites for IL-8 and PAF or could activate differential signal transduction pathways involved in eosinophil chemotaxis.

Taken together, our data suggest a possible role for both PAF and IL-8 in the inflammatory response of eosinophils in allergic asthma. The chemotactic response toward these compounds seems controlled by the release of cytokines, which may cause an *in vivo* primed state of peripheral blood eosinophils.

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