

Original article

The effect of recombinant interleukin-8 on eosinophils' and neutrophils' migration *in vivo* and *in vitro*

Background: Interleukin-8 (IL-8) is a chemokine that causes chemotaxis of neutrophils, eosinophils and lymphocytes *in vitro*; however, its role as a chemotactant in allergic inflammation is unclear. The objective of this study was to investigate the effect of nasal instillation of IL-8 on the influx of inflammatory cells.

Methods: Twelve patients suffering from seasonal allergic rhinitis hypersensitive to grass pollens, with average age 30.1 ± 2.67 years were challenged both with diluent for IL-8 and IL-8 on a subsequent day, in two phases: before the pollen season (unprimed mucosa) and during the season (primed mucosa). The number of neutrophils, eosinophils and myeloperoxidase (MPO) levels in the nasal fluid collected after IL-8 or placebo challenge were determined.

Results: Challenge with IL-8 of primed nasal mucosa induced a significant influx of neutrophils (29×10^4 cells/ml at 0.5 h, 251×10^4 at 2 h and 334×10^4 at 3 h). Number of eosinophils in comparison with diluent challenge was not significant. There was no difference in MPO levels in the nasal lavage between IL-8 and diluent challenge of unprimed and primed mucosa. We did not find the relationship between MPO levels and the neutrophils number in the lavage (rank Spearman factor, $R_s = 0.258$, $P = 0.42$).

Conclusion: We have demonstrated that IL-8 causes influx of neutrophils but not eosinophils into nasal mucosa *in vivo*. MPO level seems to be of little value as a marker of neutrophil influx into nasal mucosa.

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Chemokines represent a new family of small molecular weight (8–10 kDa) cytokines that are predominantly chemotactic for inflammatory cells (1). Interleukin-8 (IL-8), a member of CXC subfamily, is produced by several immunocompetent cell types including epithelial cells, alveolar macrophages, lymphocytes, fibroblasts, endothelial cells and eosinophils (2). Subsequently, phagocytosing neutrophils have been shown to express high level of IL-8, and release considerable amounts of the mature peptide, suggesting that they may, in this way, promote the influx of new defense cells (3). It has been proven that neutrophils play an important role in allergic inflammation. The persistent bronchial neutrophilia is associated with asthma exacerbations (4) and the number of activated neutrophils is significantly higher in the airways of patient with severe or occupational asthma (5, 6). The *in vitro* effects of IL-8 on neutrophils have been studied in detail and include directional migration, intracellular calcium mobilization, morphological polarization, LTB₄ release as a result of increased arachid onate 5-lipoxygenase activity, and exocytosis of specific granules in the presence of cytochalasin B (7–10). IL-8

also modulates CD11b/CD18 dependent adhesion and transmigration of neutrophils across endothelial cell monolayers (11, 12). The activity of IL-8 *in vivo* in humans and its role in the pathogenesis of allergic inflammation is controversial. The objective of this study was to investigate the effect of IL-8 on inflammatory cell migration in the nasal mucosa of allergic patients, as a model of allergic inflammation. Myeloperoxidase (MPO) level, as a marker of neutrophil influx into nasal mucosa, was determined.

Materials and methods

Patients

Twelve allergic patients – five women and seven men 21–51 years old, average 30.1 ± 2.67 years – were recruited from the outpatient clinic of the Department of Pneumology and Allergology, Lodz, Poland. Allergic status was evaluated by skin prick testing with a panel of 10 allergens including grass pollens, rye pollen, trees pollens, Dermatophagoides pteronyssinus, feather, cat dander, dog dander and molds: *Cladosporium*, *Aspergillus fumigatus* and *Alternaria alternata* (Allergopharma Joachim Ganzer KG, Hamburg,

Germany). Eight patients were allergic to grass pollens, five to trees pollens and two both to grass and trees pollens. Total IgE level was determined in all patients (mean 205.7 ± 45.7 IU). All subjects had a history of allergic rhinitis occurring during the grass and/or tree season. They were prescreened for the development of allergen-induced rhinitis symptoms (sneezing, rhinorrhea and nasal congestion). All patients refrained from taking oral antihistamines and topical nasal medication for a period of 14 days before the study and throughout the study. Because of the long half-life of astemizole, volunteers receiving this drug were excluded from the study. Patients with concurrent asthma, pregnancy, severe systemic illness and recurrent nasal bleeding were excluded. All subjects gave their written consent to participate in the study, and the study protocol was reviewed and approved by the Regional Ethics Committee.

Study design and nasal challenge technique

The study was performed in two phases, before the pollen season and during the season (Fig. 1). In both phases, each patient was challenged with diluent for IL-8 [phosphate buffered saline (PBS) buffer containing 0.3% HAS] first and with IL-8 on a subsequent day. IL-8 was purchased from Pepro Tech Inc., Rocky Hill, NJ, USA. On the study day, patients underwent a baseline nasal lavage with saline. After 10 min nasal challenge was performed. The technique of nasal challenge with IL-8 has been described elsewhere (13, 14). In brief, 5 ml PBS/0.3%HAS (diluent) or IL-8 (5 µg in diluent) was instilled into the nasal cavity. Patients were asked to record symptom scores according to the following protocol: rhinorrhea (none – 0 scores, mild – 1 score, moderate – 2 scores, severe – 3 scores), sneezes (none – 0 scores, 1–3 during 10 min – 1 score, 3–6/10 min – 2 scores, more than 6/10 min – 3 scores), nasal congestion

(none – 0 scores, mild – 1 score, moderate – 2 scores, severe – 3 scores), postnasal drip (none – 0 scores, mild – 1 score, moderate – 2 scores, severe – 3 scores), frontal headache (none – 0 scores, mild – 1 score, moderate – 2 scores, severe – 3 scores). A clinically positive challenge was defined as more than 3 scores.

Nasal lavage

Nasal lavage with saline was performed by means of the 'nasal pool' technique described by Greiff et al. (15), 10 min before and 0.5, 2 and 3 h after challenge. The 'nasal pool' device (10-ml syringe with closely fitting nostril), filled with 6 ml saline, was inserted in the nasal cavity for 5 min and then recovered. Centrifugation (10 min at 400 g) of saline washings separated the cell pellet and the supernatant. The obtained sediment was washed in sterile PBS (Sigma Chemical Co., St Louis, MO) and then suspended in 1.0 ml of RPMI 1640 (Sigma Chemical Co.). After staining the cells (Hansel's method) the total number of eosinophils and basophils (metachromatic cells) was counted with the use of Fuchs–Rosenthal chamber allowing determination of the number of cells in 1.0 ml of recovered fluid (9). The sample was further centrifuged at 200 g and transferred onto a slide stained by the Giemsa method. A minimum of 400 cells was counted per smear to enable differential cell count to be made for each specimen. Cells were classified as neutrophils, lymphocytes, epithelial cells and eosinophils.

Isolation of eosinophils and neutrophils

Cells were isolated from peripheral blood by dextran sedimentation followed by Percoll® (Sigma Chemical Co.) density gradient centrifugation according to Gartner (16), and negative (eosinophils) or

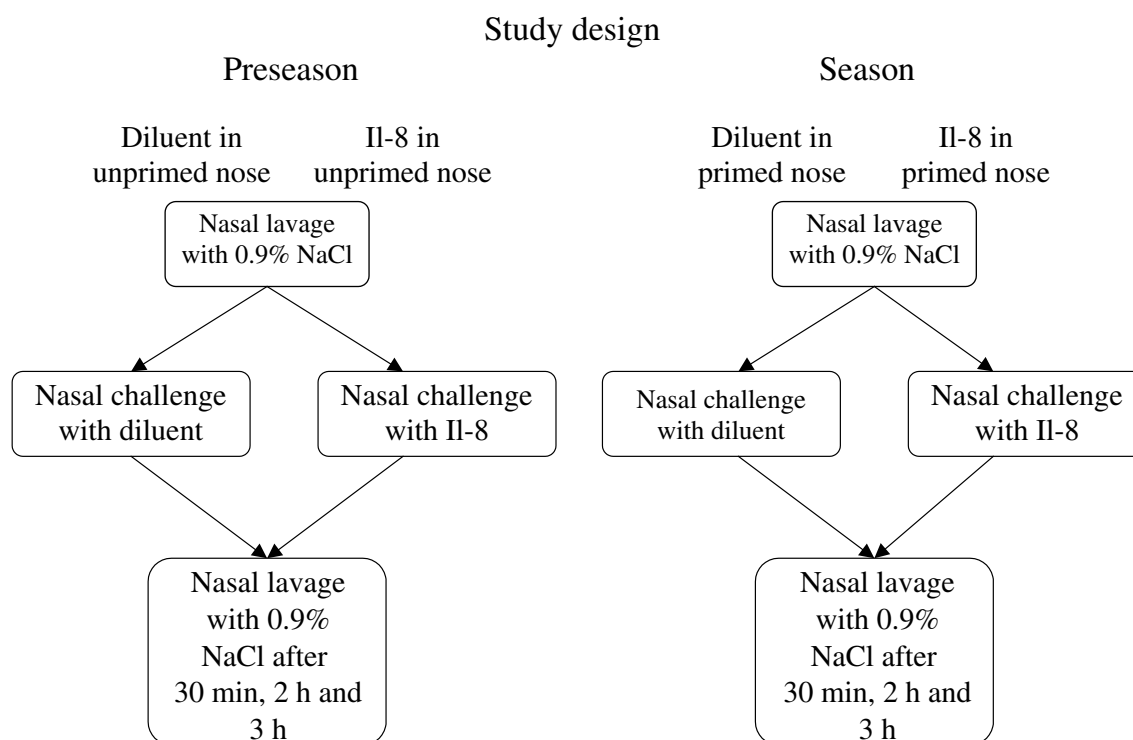


Figure 1. The study design scheme. IL-8 or diluent (placebo) challenge of allergen primed or not primed nasal mucosa.

positive (neutrophils) selection with monoclonal mouse anti-human CD16 antibodies (CD16 MicroBeads®, Miltenyi Biotec MACS, Bergisch Gladbach, Germany) as previously described by Kuna et al. (13). The purity of eosinophils was 90–98%, as judged by Wright's stain. The contaminating cells were neutrophils.

Chemotaxis assays

Chemotaxis assays were performed in a 48-well chemotactic chamber. Twenty-eight microlitres of IL-8, Rantes, C5a or FMLP were placed in the lower chamber. Aliquots of 25 µl of cells at 2×10^6 /ml were placed in the top wells of the chamber, which were separated from the bottom wells containing buffer or proteins by a polycarbonate filter without PVP with 5-µm pores. The chambers were incubated for 1 h at 37° C, in 5% CO₂. Each experiment was conducted in duplicate. The cells that migrated across the filter and were adherent to the bottom side were stained with Quick III Stain (Wright/Giemsa) and counted in 20 high-power randomly chosen fields. The positive chemotactic response was defined as chemotactic index higher than for the control solution. Chemotactic index was defined as: cells migrating towards the chemoattractant/cells migrating towards the control solution (PBS).

Myeloperoxidase levels

Myeloperoxidase level, as a marker of neutrophil influx into unprimed and primed nasal mucosa, was determined in the supernatant from the nasal challenge fluid using the 'sandwich' Elisa method (Bioxytech® MPO Enzyme Immunoassay, R&D Systems Inc., Minneapolis, MN).

Statistical analyses

Paired data were analyzed by Wilcoxon's signed rank test using the Statistica program (StatSoft. Inc., Krakow, Poland). Results of this study are presented as mean $\pm$ SEM. All statistical analyses were considered significant at $P < 0.05$.

Results

Analyses of symptom scores

Analysis of the individual symptom scores of all subjects did not reveal any major symptoms of nasal congestion, sneeze counts, frontal headache or rhinorrhea after challenge with IL-8 or diluent (PBS/HAS) of unprimed mucosa. However, in three subjects after IL-8 challenge in the pollen season we have observed mild symptoms of rhinitis, but it never exceeded 3 points and did not require any medication. The mean volume of nasal fluid recovered after nasal lavage with saline was 4.62 ± 0.19 ml (mean $\pm$ SEM).

Chemotaxis of neutrophils and eosinophils

In order to assess the effect of IL-8 upon neutrophil and eosinophil migration, we first assessed chemotaxis at a dose range between 10^{-7} and 10^{-10} M. Blood was taken from our donors during the allergy season and all of them were symptomatic at this time. The positive chemotactic

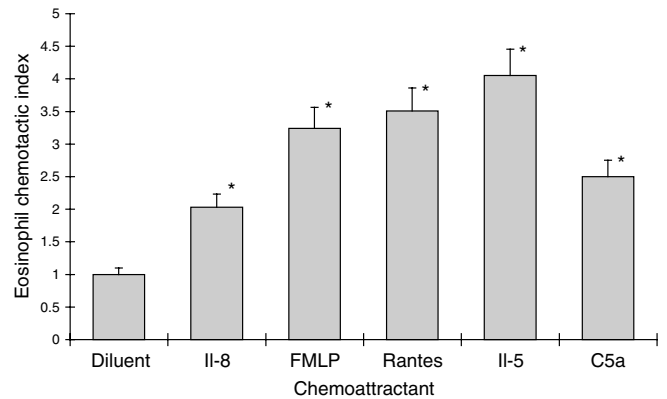


Figure 2. Comparison of chemotactic response of eosinophils isolated from allergic donors during the season to IL-8, FMLP, Rantes, IL-5 and C5a. The differences are statistically significant as compared to diluent (* $P < 0.05$).

response was seen at the concentration of IL-8 from 10^{-10} to 10^{-8} M (data not shown). The optimal concentration of IL-8 for eosinophil chemotaxis was 10^{-9} M and the chemotactic index was 2.73 ± 0.6 . The optimal concentration for neutrophil chemotaxis was 10^{-8} M and the chemotactic index was 4.58 ± 0.94 . Then we compared the activity of optimal concentration of IL-8 to that of C5a (10^{-8} M), FMLP (10^{-7} M), Rantes (10^{-8} M), IL-5 (10^{-8} M) and control buffer on eosinophil chemotaxis. The chemotactic activity of IL-8 was comparable to that of C5a, but was significantly lower to that of FMLP, IL5 or Rantes, as shown in Fig. 2. When we tested IL-8 for chemokinesis using equal but increasing concentrations on both sides of the membrane, a concentration-dependent increase in migration was seen, which varied from 30 to 60% of the chemotactic response depending on the donor tested; the data for C5a was similar (data not shown).

Analyses of cellular influx into the unprimed nasal mucosa

After the challenge with the diluent no significant increase in the count of neutrophils, eosinophils or lymphocytes was observed at any time point. After the challenge with IL-8 of unprimed nasal mucosa the number of neutrophils increased from $28 \pm 7 \times 10^4$ cells/ml at the baseline to $76 \pm 29 \times 10^4$, $172 \pm 33 \times 10^4$ and $134.5 \pm 38 \times 10^4$ cells/ml at 0.5, 2 and 3 h, respectively (Fig. 3). The number of lymphocytes and eosinophils did not increase significantly over the baseline recorded before the challenge (Fig. 3). The differences in the number of cells between diluent and IL-8 were not significantly different except for neutrophils at 0.5, 2 and 3 h time points.

Cellular influx into the primed nasal mucosa

After the challenge with the diluent of the primed mucosa significant increase in the count of neutrophils

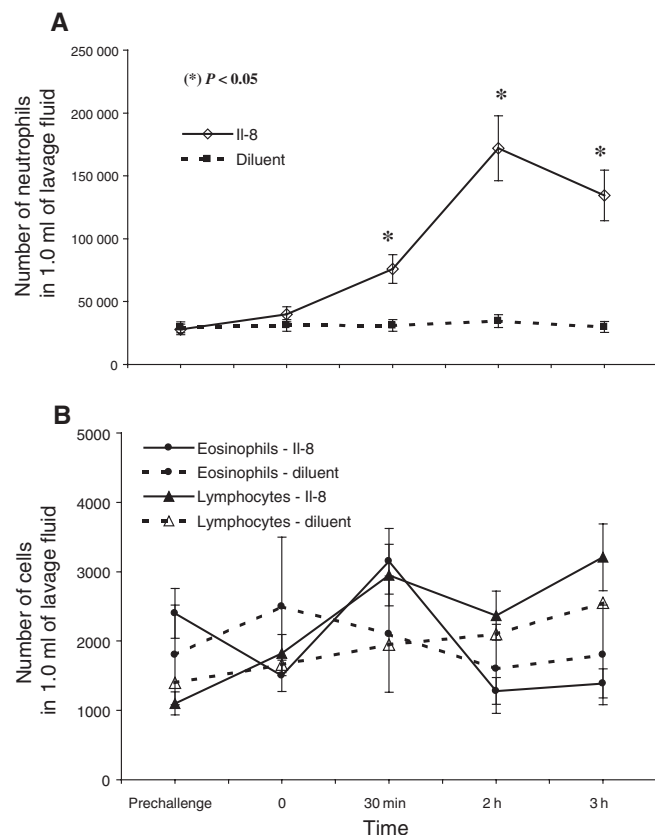


Figure 3. The number of neutrophils (A), lymphocytes and eosinophils (B) in the nasal lavage fluid obtained at baseline, 0.5, 2 and 3 h after the challenge with IL-8 of unprimed nasal mucosa. The IL-8 induced neutrophil count differed significantly ($*P < 0.05$) from the baseline value recorded before challenge. The differences for lymphocytes and eosinophils are not statistically significant. Challenge with diluent did not induce significant influx of cells.

as compared to the baseline was observed at 0.5, 2 and 3 h. The number of neutrophils increased from $31 \pm 8 \times 10^4$ cells/ml at the baseline to $88.5 \pm 19 \times 10^4$, $79 \pm 19 \times 10^4$ and $90.5 \pm 20 \times 10^4$ cells/ml after 0.5, 2 and 3 h, and the differences are statistically significant ($P < 0.05$) (Fig. 4). We also observed noticeable influxes of eosinophils and lymphocytes at 2 and 3 h time points after the diluent challenge (Fig. 4). After challenge with IL-8 of primed nasal mucosa the number of neutrophils increased significantly over the baseline from $28 \pm 6 \times 10^4$ to $288 \pm 16 \times 10^4$ cells/ml after 0.5 h, to $251 \pm 27 \times 10^4$ cells/ml after 2 h and $334 \pm 50.5 \times 10^4$ cells/ml after 3 h, and the differences are statistically significant ($P < 0.05$) (Fig. 4). The peak of the inflammatory cells influx was observed at 3 h time point after the challenge. The comparison between neutrophils influx into the primed nasal mucosa after challenge with diluent and IL-8 showed that the number

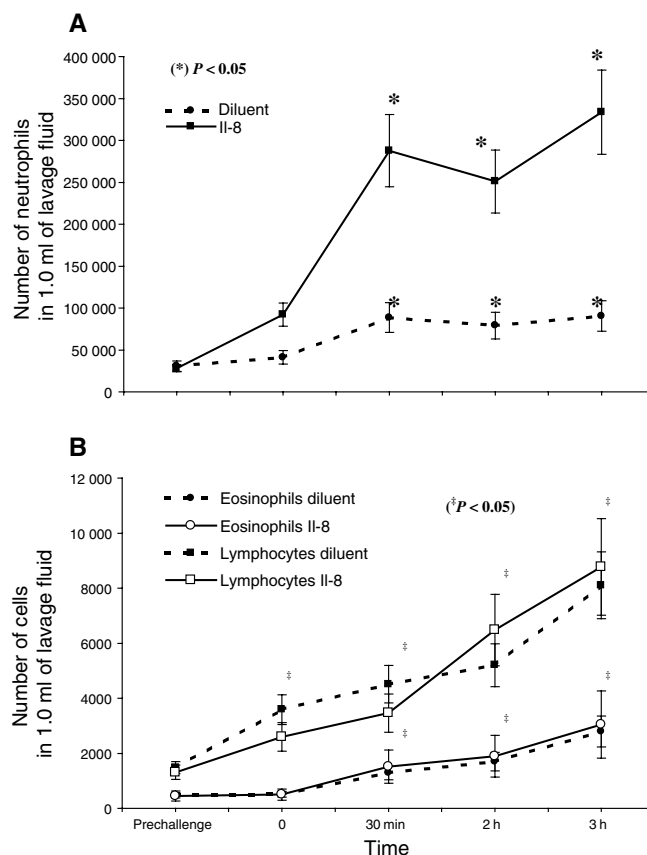


Figure 4. The number of neutrophils (A), lymphocytes and eosinophils (B) in the nasal lavage fluid obtained at baseline, 0.5, 2 and 3 h after the challenge with IL-8 of primed nasal mucosa. We can see spontaneous influx of neutrophils, lymphocytes and eosinophils after nasal challenge with diluent, the differences are significant as compared to the baseline value ($\dagger P < 0.05$). The IL-8 induced neutrophil count differed significantly from the baseline value, recorded before challenge and from the cell influx induced by diluent ($*P < 0.05$). For lymphocytes and eosinophils the differences are statistically significant as compared to the baseline value recorded before challenge, but are not statistically different in comparison with the number of cells after challenge with diluent.

of cells was significantly higher at all time points after IL-8 instillation, with a peak of influx at 3 h after the challenge. The number of eosinophils after IL-8 challenge of primed nasal mucosa in comparison with diluent challenge was not significantly higher (Fig. 4). The peak of eosinophils influx was at 3 h after the challenge, reaching the number of $3 \pm 0.8 \times 10^4$ cells/ml. The number of lymphocytes after challenge with IL-8 in comparison with diluent challenge was also not significantly higher (Fig. 4). The peak of lymphocytes influx was at 3 h after the challenge reaching the number of $9 \pm 3 \times 10^4$ cells/ml.

Comparison of cellular influx between the primed and unprimed mucosa

We compared the number of neutrophils, eosinophils and lymphocytes in nasal lavages obtained after IL-8 challenge of primed nasal mucosa with the number of cells found after challenge of unprimed nasal mucosa with IL-8 and with the diluent. The number of neutrophils in the nasal lavage of primed mucosa after the IL-8 challenge was significantly higher at all time points in comparison with the diluent and IL-8 challenges in the unprimed mucosa (Figs 3 and 4). The number of eosinophils and lymphocytes in the nasal lavage of primed mucosa after the IL-8 challenge was significantly higher than the number of cells found after challenge of unprimed mucosa with IL-8 at each time point, but there was no significant difference between influxes of these cells after IL-8 and diluent challenges of primed mucosa. If we compared the results after diluent challenge of primed mucosa with that of IL-8 challenge of unprimed mucosa, the number of eosinophils and lymphocytes were significantly higher at 0.5, 2 and 3 h.

Myeloperoxidase levels

There was no difference ($P > 0.05$) in MPO levels in the nasal lavage between IL-8 (mean 17.43 ± 10.98 ng/ml) and diluent challenge (20.98 ± 11.89 ng/ml) of unprimed mucosa. In the primed mucosa fluid we observed a peak of MPO level (50.84 ± 53.23 ng/ml) at 2 h time point; however, this was not significant as compared to diluent challenge ($P = 0.465$) (data not shown). We did not find the relationship between MPO levels and the neutrophils number in the lavage (rank Spearman factor, $R_s = 0.258$, $P = 0.42$).

Discussion

The *in vitro* part of our study confirmed previous observations that IL-8 has a chemotactic properties both for eosinophils and neutrophils. IL-8 is produced by epithelial cells, alveolar macrophages, lymphocytes, fibroblasts, endothelial cells and eosinophils. IL-8 along with neutrophil activatin protein-2 (NAP-2) has neutrophilotactic property. IL-8 is a potent mediator of eosinophil chemotaxis and induces transendothelial migration of eosinophils *in vitro* (17, 18). One of the most common eosinophilic inflammatory diseases is allergic rhinitis and one of the most common neutrophilic inflammatory diseases is chronic, nonallergic rhinitis. Recently, it has been proven that neutrophils also play an important role in the pathogenesis of severe and fatal asthma (5, 19). Neutrophil elastase (NE) release may result in tissue damage inducing airway wall remodeling and progressing, irreversible deterioration of lung function (20). Prominent airways neutrophilia

remains apparent even after treatment with high doses of corticosteroids (CS). Corticosteroids paradoxically enhance neutrophil survival by reducing apoptosis, which can explain poor response to CS in some asthmatic patients (21). IL-8 in these conditions is a possible therapeutical intervention target; however, the mechanism of influx of eosinophils and neutrophils and the role of IL-8 in allergic disorders are not completely characterized.

IL-8 is a potential mediator of allergic inflammation. For this reason it is important to determine the inflammatory activity of IL-8 in the nasal mucosa. The results of our study clearly establish that IL-8 is capable of eliciting neutrophilic but not eosinophilic inflammation in the upper airways. In the unprimed mucosa IL-8 caused a significant influx of neutrophils at 0.5, 2 and 3 h. This is one of the first demonstrations of induction of neutrophilic inflammation by IL-8 in human upper airways. As there are developmental and pathophysiological similarities between upper and lower airways, our results may also be relevant to neutrophilic inflammation in the pulmonary airways.

We have instilled 5 µg of IL-8 into one nostril of the study subjects. Although the tissue level of IL-8 in acute inflammation is unknown at this time, IL-8 is active in this concentration *in vivo*. If one assumes that 5 µg of IL-8 that is instilled over the nasal mucosa is distributed in 1–5 cc of mucosal and extracellular fluid, it is likely to achieve a concentration range of $0.5\text{--}1.0 \times 10^{-7}$ M. We have shown that the concentration of IL-8 in the nasal lavage fluid from patients with allergic rhinitis is 1–2 µg/ml (14). Thus, the concentration of 5 µg of IL-8 is biologically relevant. Nonetheless, a dose–response study will be necessary to determine the optimal dose of IL-8 for biologic activity *in vivo*.

We have shown that IL-8 has a significant but moderate activity on unprimed nasal mucosa and causes the influx of neutrophils. A more profound inflammatory activity of IL-8 is observed in the allergen primed mucosa. This is not a surprising observation. The penetration of IL-8 into the nasal mucosa is unknown; however, the allergen-challenged mucosa may have increased permeability for IL-8 because of the action of histamine or leukotrienes. Further, there is evidence of increased production of growth factors and cytokines (e.g. granulocyte macrophage colony stimulating factor (GM-CSF) and proinflammatory cells for increased chemotactic response to IL-8. Interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) may upregulate adhesion molecules on endothelium. The importance of the sequential effect of various cytokines has recently been shown by a study with eotaxin. The injection of eotaxin into the guinea pig skin does not cause an eosinophilic inflammation. However, if the animals first receive IL-5 intravenously followed by eotaxin locally, there is a significant eosinophilic infiltration at the site of the injection (22).

The response of the human airways to allergens is usually biphasic. The immediate response is followed by a late-phase response that is characterized by the presence of eosinophils, neutrophils and lymphocytes. The mechanism of influx of the forgoing inflammatory cells in the late-phase reaction is unclear. The challenge with allergens causes the secretion of various cytokines into the nasal secretion in allergic patients. The cytokines include IL-1, GM-CSF, IL-8, RANTES and MIP-1 α (14, 23). The peak appearance of IL-8 occurs at 1 h postchallenge, which precedes the development of the late-phase allergic reaction. We have shown that IL-8 induces nasal inflammation that comprised neutrophils. On the basis of these data, we believe that IL-8 could play a role in the pathogenesis of the late-phase allergic reaction. However, studies involving antibodies against IL-8 or specific antagonists are necessary to establish the causal association between IL-8 and neutrophilic inflammation.

Myeloperoxidase has been proposed to mirror the degree of neutrophil activation; however, its role as a marker of the participation of neutrophils in allergic inflammation is still unclear as the literature is controversial (24, 25). We compared the MPO levels and neutrophil influx in nasal lavage after recombinant IL-8 challenge. After the challenge with IL-8 of primed mucosa there was a significant influx of neutrophils with the peak at 3 h after the challenge. Although we observed

a peak of MPO level (50.84 ± 53.23 ng/ml) at 2 h time point, it was not significant as compared to the diluent challenge. We did not find the relationship between MPO levels and the neutrophils number in the lavage. We conclude that because of the lack of statistically confirmed relationship between MPO level and the number of neutrophils, MPO seems to be of little value as a marker of neutrophil influx into nasal mucosa. The possible explanation could be that in our study model IL-8 induced chemotaxis but was not so potent in induction of degranulation of neutrophils. At the site of allergic inflammation with possible exacerbation after viral or bacterial infection, activated neutrophils can degranulate and release detectable and significant levels of MPO.

In conclusion, our study shows that IL-8, which is a potent chemoattractant for both eosinophils and neutrophils *in vitro*, is capable of eliciting neutrophilic but not eosinophilic inflammation in the human upper airways. It is possible that a combination of various cytokines acting together or in a sequence may determine the nature of inflammation. For example, the presence of GM-CSF, TNF α or IL-1 may affect the subsequent response of eosinophils to IL-8. Further studies are necessary to understand complex mechanisms underlying allergic inflammation and to propose possible therapeutic intervention targets.

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