Mechanisms of IL-6, IL-8, and GM-CSF release in nasal secretions of allergic patients after nasal challenge*

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SUMMARY

Cytokines are potentially active biological peptides that are known to play an important role in several immune responses. Several studies have reported the existence of a variety of cytokines in the nasal mucosa of patients with allergic rhinitis. However, there are few reports on cytokines released into the nasal secretion. In the present study, we investigated the sources, and levels of three key proinflammatory cytokines namely, IL-6, IL-8, and GM-CSF in the nasal secretion, as well the mechanisms of their release, by ELISA and immunohistochemistry. Firstly, we examined the levels of IL-6, IL-8, and GM-CSF in the nasal secretion after in vivo nasal challenge with methacholine (MC), histamine (HI) and allergen (Ag) in patients with nasal allergy to house dust mite (HDMAR). Next, we examined the levels of IL-6, IL-8, and GM-CSF released, in vitro, after Ag challenge of nasal scrapings from patients with HDMAR. Finally, we examined the sources of these cytokines in the nasal mucosa, by immunohistochemistry. After MC challenge in patients with HDMAR, the concentration of IL-6, but not IL-8, and GM-CSF, was significantly greater on the challenged side than on the contralateral side. Ag and HI provocation induced significantly greater levels of IL-6 and IL-8 secretion in patients with HDMAR, on the challenged side than on the contralateral side. GM-CSF was only detected in the nasal secretion after Ag challenge. Immunoreactivity for IL-6 and IL-8 was very similar in that it was predominantly localised to the apical portion of epithelial cells, the superficial lamina propria, gland cells, and migrating cells. The immunoreactivity for GM-CSF varied slightly from that of IL-6 and IL-8: strong immunoreactivity was detected in the basal part of epithelial cells, basement membrane, glandular ducts, and migrating cells. These results suggest that the levels, sources, and mechanisms of release of IL-6, IL-8, and GM-CSF in the nasal secretion of patients with HDMAR do vary, but are important in the manifestation of the allergic reaction.

Key words: allergic rhinitis, proinflammatory cytokines, IL-6, IL-8, GM-CSF, nasal secretion.

INTRODUCTION

The allergic inflammatory reaction occurs due to the activation and interaction of a variety of migrating cells like T cells, B cells, mast cells, eosinophils, as well as resident/structural cells such as epithelial cells (Okuda, 1989a). It is well-known that many cytokines play a role in the manifestation of nasal allergic reaction through the activation and proliferation of migrating cells, such as mast cells, eosinophils, and lymphocytes, as well as epithelial cells. These cells produce a variety of cytokines that in turn regulate the immunological reaction (Haak-Frendscho et al., 1988; Broide et al., 1992; Brorish and Rosenwasser, 1993). Recently, several investigators have reported about the cytokine profile in the nasal mucosa of patients with nasal allergy (Bachert et al., 1992; Durham et al., 1992; Pawankar et al., 1995; Pawankar and Ra, 1996; Kuna et al., 1996) including those from cultured nasal epithelial cells (Ohnishi et al., 1989). However, these studies have primarily focused on the detection of cytokines in the allergic nasal mucosa. Since the nasal secretion and mucous layer form the first barriers to external antigens, it is essential to know the amount and type of cytokines that are released into the nasal secretion after allergen (Ag) challenge. There are but few reports on the cytokines released into the nasal secretion, in different inflammatory diseases of the nose like allergic (Sim et al.,1994; Ohkubo and Okuda, 1994) and

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infectious rhinitis (Baggiolini et al., 1989). Studies on the chemical mediators in nasal allergic reaction have demonstrated that almost all chemical mediators that are secreted after allergen provocation can be detected in the nasal secretion. Furthermore, lymphocytes, mast cells, eosinophils, and epithelial cells that infiltrate from the main source of the cytokines and reside in the epithelial compartment of the nasal mucosa (Pawankar and Ra, 1996).

The cascade of events leading to an allergic reaction includes the production of IgE, the acquisition of IgE by mast cells and basophils via the high affinity IgE receptor (FcεRI), and the subsequent cross- linking of bound IgE-FcεRI complex with multivalent allergen, resulting in the release of inflammatory mediators. The nasal allergic reaction therefore occurs due to the release and effect of such chemical mediators like histamine, PGD2 or leukotrienes, that are released from the activated mast cells in the nasal surface. Sneezing and nasal discharge occur due to histamine-induced stimulation of the sensory nerve endings. Nasal blockage occurs due to the release of mediators that stimulate the blood vessels directly and also due to indirect neuronal stimulation.

IL-6, IL-8, and GM-CSF are three key proinflammatory cytokines that are known to exist at various inflammatory sites. IL-6 is an important Th2 type cytokine involved in the induction of IgE synthesis, as well as in mast cell proliferation and maturation. IL-8 is one of the several chemokines (Leonard et al., 1990), which is known to induce leukocyte migration (Dahinden CA et al., 1990), and the production of leukotriene B4 (Bischoff et al., 1990). IL-8 is reported to play an important role in the late phase allergic reaction, especially in inducing histamine release, as against the acute phase reaction (Bischoff et al., 1990). GM-CSF may play a role as an eosinophil colony stimulating factor (Soloperto et al., 1991). Eosinophil accumulation in allergic inflammation, asthma (Frigas and Gleich, 1986), and nasal allergy (Bascom et al., 1988), is well documented.

In the present study, we investigated the sources, levels, as well the mechanisms of release of IL-6, IL-8, and GM-CSF into the nasal secretion by *in vivo* and *in vitro* experiments. We detected IL-6, IL-8, and GM-CSF release into the nasal secretion in the early phase, after Ag challenge. The levels, sources and mechanisms of IL-6, IL-8 and GM-CSF release into the nasal secretion were varied.

MATERIALS AND METHODS

Subjects

Twenty untreated patients with nasal allergy to house-dust mite (HDMAR), were enrolled in this study. They were symptomatic based on a positive history of typical symptoms of allergic rhinitis, and positive allergy tests, such as anterior rhinoscopic examination, test for nasal eosinophilia, skin test, nasal provocation test, and RAST (Radio-allergosorbent test). None of these patients had any associated sinusitis or acute rhinitis. All drugs were prohibited during and for one month before the study. The study was approved by the local institutional review board and informed consent was obtained.

In vivo challenge

Nasal secretion was separately collected from both nostrils of patients with HDMAR, by suction at 5 min post unilateral challenge with Ag, using the paper disk method (Okuda, 1989b). Nasal secretions were also obtained from both nostrils of patients with HDMAR, by unilateral methacholine (MC; 24 mg/ml) challenge, and unilateral histamine (HI; 10 mg/ml) challenge for 0.5 ml (total volume), 10 min. after challenge.

In vitro challenge

In vitro Ag challenge was performed using nasal scrapings from patients with HDMAR. Nasal scrapings were obtained by gentle curetting of the nasal inferior turbinates (bilaterally) without any anaesthesia. One mg of nasal scrapings from one side was stimulated with 1:100 diluted Ag, whereas 1 mg of nasal scrapings from the contralateral side was incubated with PBS (0.01M, pH 7.4) as control. Samples were collected 5 min. after in vitro incubation in Ag/PBS, and then centrifuged. The supernatants were stored at –80°C before use.

Collection and storage of nasal secretion

The volume of each sample was measured. One ml of 0.01M PBS supplemented with 0.05% dithio-erythritol was added to each sample to dissolve the mucin and the samples were vortexed without cell destruction. Conditioned samples were then centrifuged at 400 *g* for 10 min and the cell free supernatant was collected and stored at –20°C until further analysis of cytokines by ELISA.

ELISA assays

IL-6, IL-8 and GM-CSF were measured in triplicate by quantitative sandwich ELISA using IL-6, IL-8, and GM-CSF specific ELISA kits (Quantikine Kit, R&D systems). The sensitivities of the assay systems for IL-6, IL-8 and GM-CSF ranged between 3.13 and 100 pg/ml, 93.8 and 3000 pg/ml, and 7.8 and 250 pg/ml, respectively. We calculated the exact concentration of cytokines in the collected volume of nasal secretion (without diluting the nasal secretion).

Statistical Analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed by the Student t-test.

Indirect Immunohistochemistry

Biopsy specimens from the nasal inferior turbinate mucosa (3 x 4 mm in size) were obtained from patients with HDMAR at the time of surgery (conchotomy). Tissues were fixed in PLP (periodate poly lysine paraformaldehyde), snap frozen in liquid nitrogen and stored at –80°C, until use. Five μm thick sections were cut, rehydrated in 0.05M TBS (pH 7.6) and incubated with the relevant primary antibody (anti-IL-6, anti-IL-8, or anti-GM-CSF mAbs) for one hour, followed by 30 min. incubation with rabbit anti-mouse IgG, and 30 min. incubation in the alkaline phosphatase anti-alkaline phosphatase reagent (APAAP).

Subsequently, the reaction was developed with the substrate Naphtol AS MX and Fast red TR. For IL-8 staining an addi-

tional step of incubation with a mouse anti-rabbit IgG was introduced between the first Ab and rabbit anti-mouse IgG, after appropriate blocking with normal mouse serum. Negative controls were performed by substituting the primary Abs with isotype matched Abs.

RESULTS

Levels of IL-6, IL-8, and GM-CSF in the nasal secretion of patients with HDMAR (in vivo and in vitro challenge)

The amount of collected secretion ranged from 0.25 ml to 1.23 ml with mucin. The level of IL-6 in the nasal secretion of patients with HDMAR after MC challenge was significantly higher on the challenged side (7.30±2.58 pg/ml) than on the contralateral side (3.72±1.69 pg/ml) ($p < 0.05$) (Table 1). The level of IL-6 in the nasal secretion after Ag challenge was also significantly higher on the challenged side (6.49 pg/ml) than on the contralateral side (3.92 pg/ml) ($p<0.05$, Table 1). Again, the level of IL-6 in the nasal secretion of patients with HDMAR after HI challenge was significantly higher on the challenged side (challenge side : 11.80±3.53 pg/ml, contralateral side : 7.50±2.27 pg/ml; $p<0.05$) (Table 1). Even when we stimulated nasal scrapings *in vitro* with Ag, we detected increased levels of IL-6 in Ag stimulated supernatants as compared to controls (Ag challenged: 4.67±1.08, PBS challenged: 2.20±0.64 pg/ml).

Table 1. IL-6, IL-8, and GM-CSF release in nasal secretion after challenge. Upper panel shows data of challenge side. Lower panel shows data of contralateral side.
*: $p < 0.05$ compared to contralateral side.

	MC	HI	Ag	*in vitro* Ag
IL-6	7.30±2.58*	11.05±4.07*	6.49±2.21*	4.67±1.08
	3.72±1.69	5.87±2.29	3.29±1.42	2.20±0.64
IL-8	781.0±210.8	951.0±316.1*	1284.5±233.0*	397.9±74.8
	528.3±135.8	657.1±233.0	702.6±247.8	277.9±82.0
GM-CSF	NS	NS	6.80±4.75	0.63±0.38
	NS	NS	1.40±0.88	0.63±0.30

The level of IL-8 in the nasal secretion of patients with HDMAR after MC challenge was not different on the challenged side (781.0±210.8 pg/ml) as compared to the one on the contralateral side (528.3±135.8 pg/ml) (Table 1). The level of IL-8 in the nasal secretion of patients with HDMAR on the challenged side after HI challenge (951.0±316.1 pg/ml), was significantly higher than one the contralateral side (657.1±233.0 pg/ml) (Table1). The level of IL-8 in the nasal secretion of patients with HDMAR after *in vivo* Ag challenge was 1284.5±396.8 pg/ml, which was significantly higher than on on the contralateral side (702.6±247.8 pg/ml) ($p<0.05$) (Table1). The level of IL-8 in the

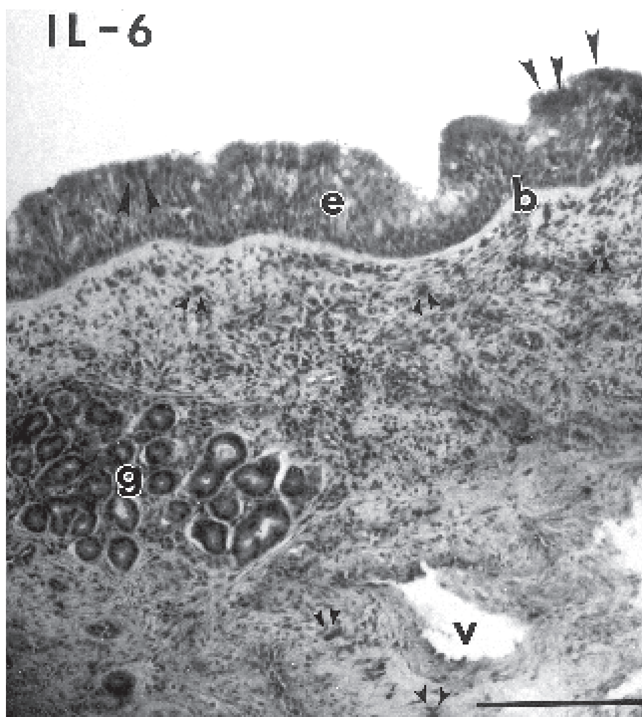

Figure 1. Immunoreactivity for IL-6 in the human nasal mucosa of allergic subjects, by immunohistochemistry. Immunoreactivity for IL-6 was seen in the superficial part of the epithelium (e; positive cells are indicated by big arrow heads) and lamina propria (positive cells are indicated by small arrow heads). Represented characters are (b) as the basement membrane, (g) as the glands, and (v) as the vessels. Bar represent 100 μm.

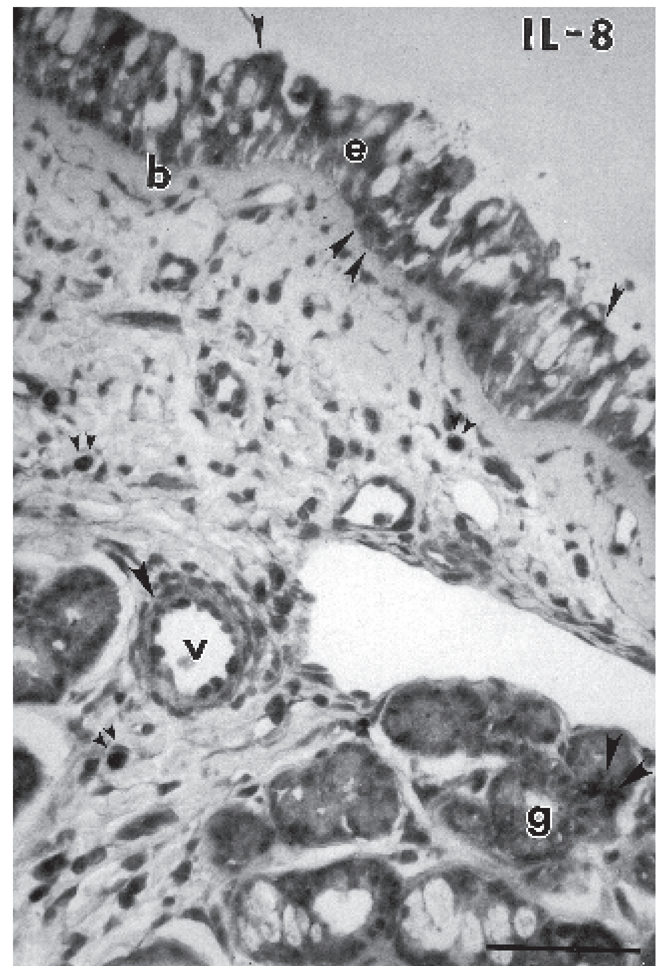

Figure 2. Immunoreactivity for IL-8 in the human nasal mucosa from allergic subjects by immunohistochemistry. Immunoreactivity for IL-8 is seen in the epithelium (e; positive cells are indicated by big arrow heads). In the lamina propria positive cells (are as indicated by small arrow heads) and are seen near the vessels (v) and between the gland (g) cells only in superficial layer. Bars represent 25 μm.

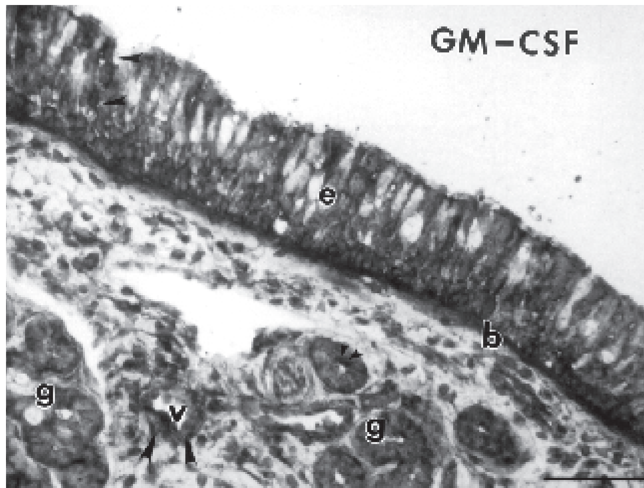

Figure 3. Immunoreactivity for GM-CSF in the human nasal mucosa from allergic subjects by immunohistochemistry. Intense staining was seen in the epithelium (e), especially around the basement membrane (b). In the epithelium immunoreactivity was detected in migrating cells (indicated by big arrow heads). In the lamina propria, positive cells are seen near the vessels (v; positive cells are indicated by big arrow heads) and between the gland(g) cells (indicated by small arrow heads). Bars represented 25 μm.

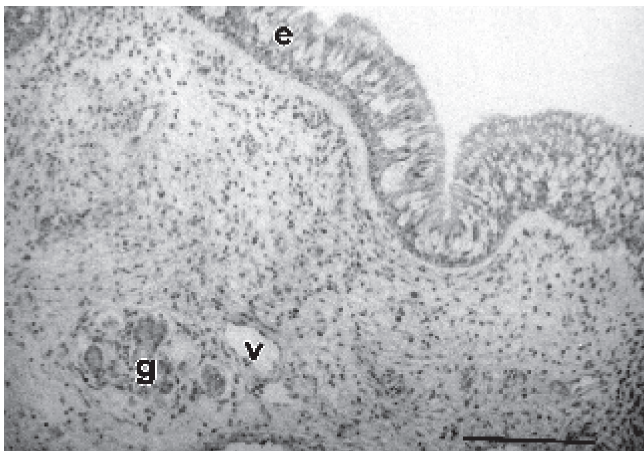

Figure 4. Negative control staining was obtained using isotype matched control mAb instead of the primary antibody. No staining was seen in the gland(g), vessels(v), and epithelium(e) of allergic nasal mucosa. Bars represented 100 μm.

nasal secretion after *in vitro* Ag challenge, however, showed no significant difference from the controls (PBS challenge) (Ag challenge: 397.9±74.8 pg/ml, PBS challenge: 277.9±82.0 pg/ml) (Table 1).

The level of GM-CSF in nasal secretion of patients with HDMAR after Ag challenge was four times more on the challenged side (6.62±2.76 pg/ml, range 0 - 16.5 pg/ml) than on the contralateral side (1.56±0.68 pg/ml, range 0 - 7.8 pg/ml), but with no statistically significant difference (Table 1).With *in vitro* Ag challenge, no significant difference was detected in the level of GM-CSF between Ag challenge and PBS challenge (Ag challenge : 0.63±0.38 pg/ml, PBS challenge: 0.63±0.30 pg/ml) (Table 1). Neither MC challenge nor HI challenge induced GM-CSF release into the nasal secretion.

Immunohistochemistry

IL-6 in the allergic nasal mucosa was localised to the epithelial cells, especially the superficial (apical) portion of the epithelial cells, gland cells in the superficial lamina propria, as well as near the sinusoids in the deep lamina propria. IL-6 immunoreactivity in the epithelial compartment was detected in the ciliated epithelial cells and probably migrating lymphocytes. There was weak staining of the basement membrane (Fig.1).

IL-8 was localised to the migrating and resident cells in the allergic nasal mucosa in a pattern almost similar to that of IL-6, and to the epithelial cells, as well as cells in the gland cells of superficial lamina propria, near the sinusoids and near the vessels of deep lamina propria (Fig. 2).

GM-CSF immunoreactivity was very strong in the epithelium and was mostly concentrated in the basement membrane. We could observe positive staining in the gland cells and vessels, also. Moreover, the positive area was seen at the inner lumen of the glandular ducts. This indicated that GM-CSF may be released by the gland cells (Fig. 3). Negative controls demonstrated no staining for the respective cytokines in the nasal mucosa (Fig. 4).

DISCUSSION

Recently, we reported that IL-6, IL-8, GM-CSF and TNF-α were released from cultured nasal epithelial cells (Ohnishi et al., 1994). The existence of IL-6, IL-8, GM-CSF, and TNF-α in the nasal secretion have also been well documented. In the present study, we examined the levels and sources of IL-6, IL-8, GM-CSF, as well as the mechanism of release of these cytokines into the nasal secretion after unilateral provocation with HI, MC and Ag. Th2 type cytokines such as IL-4, IL-5, IL-6, and IL-13 are predominant in the allergic nasal mucosa (Bachert et al., 1992; Durham et al., 1992; Pawankar et al., 1995; Pawankar and Ra, 1996; Kuna et al., 1996; Pawankar et al., 1997). In infectious diseases Th1 type cytokines are predominant (Shirakawa et al., 1996). Here we demonstrated an increase in IL-6, IL-8 and GM-CSF in the nasal secretion of patients with allergic rhinitis, the different mechanisms of their release, and their sources in the allergic nasal mucosa.

Three kinds of stimuli were used for nasal challenges in order to clarify the mechanism of cytokine release in this study. MC challenge induces cholinergic stimulation resulting in ipsilateral glandular secretion. However, the contralateral side is not stimulated cholinergically. Therefore, the release of cytokines in the contralateral side was only spontaneous. The concentration of cytokines from pure glandular secretion resulting from MC challenge was expressed as [(those of challenged side) - (those of contralateral side)]. HI challenge induces nasal secretion through the action on H1 receptor and weak cholinergic stimulation resulting in ipsilateral glandular secretion similar to that induced by saline (Raphael et al., 1989). Cytokines released in the contralateral side were induced only by cholinergic stimulation. So the concentration of cytokines from pure vascular permeability through H1 receptor was expressed as (those of challenged side) – (those of contralateral side). Ag stimulation and HI stimulation induced a similar nasal reaction, immediately following the challenge.

In patients with house dust mite allergic rhinitis, IL-6 was released from the nasal mucosa by MC and/or HI challenge, as well as by Ag challenge. IL-6 was localised to the epithelial cells and migrating cells in the epithelial and lamina propria compartments of the allergic nasal mucosa. It has been previously reported that mast cells and T lymphocytes form the main source of IL-6 in the lamina propria. These results suggest that IL-6 is released from migrating cells or epithelial cells into nasal mucosa by natural exposure of allergens and go into the nasal secretion of allergic rhinitis by cholinergic stimulation and direct action as well as by HI and Ag stimulation. MC mainly stimulates glandular secretion and to a lesser extent innervates the vascular permeability and HI stimulates mainly vascular permeability and to a lesser extent it innervates glandular secretion on the challenged side previously noted (Raphael et al., 1989). From these results, cholinergic stimulation induces about 4 pg/ml of IL-6 release into the nasal secretion. IL-6 content in nasal secretion by HI stimulation via H1 receptor was about 3-4 pg/ml. Allergic subjects released in nasal secretion about 5 times more IL-6 than normal volunteers by any kind of challenge, even at baseline levels (data not shown). Normal subjects may have different mechanisms of release of IL-6 as compared to allergic subjects, because the former secrete cytokines without the contribution of cytokines from migrating cells. So we conclude that IL-6 was released to the nasal mucosa mainly from the migrating cells and epithelial cells as a result of the allergic reaction, and released to nasal secretion through cholinergic control and from epithelial cells by direct action of HI.

IL-8 release in the allergic nasal secretion by MC challenge did not increase significantly as compared to the one on the contralateral side. This means that cholinergic stimulation does not play a role in the release of IL-8 to nasal secretion. Direct action of HI, induced IL-8 release into the nasal secretion, probably by stimulating the epithelial and/or migrating cells. Recently, it was demonstrated that epithelial cells express the mRNA for H1 receptors by RT-PCR (Iriyoshi et al., 1996). Migrating cells, however, do not have H1 receptors. Ag stimulates migrating cells directly and/or epithelial cells indirectly. In the allergic reaction, Ag usually stimulates migrating cells which may release IL-6 and IL-8. Thereafter, mast cells and basophils release HI which can then stimulate the structural cells like epithelial cells. The increase in IL-8 in the allergic nasal secretion may be supported by the distribution of IL-8 in the allergic nasal mucosa, by immunohistochemistry. However, *in vitro* Ag challenge resulted in spontaneous and abundant release, but there was no increase in IL-8 release. This spontaneous secretion of IL-8 in allergic subjects may result in the infiltration of neutrophils through the action of chemokines and eosinophil infiltration by production of LTB4, and a surface defence mechanism by release of lysozymes in allergic inflammation of the nasal mucosa. Moreover, our previous *in vitro* studies showed a very high level of IL-8 secretion from cultured nasal epithelial cells (Ohnishi et al., 1994) suggesting some additional role of IL-8 in the inflammatory reaction. These results suggest that a major part of IL-8 in the nasal secretion comes from the epithelial cells by the direct action of HI, and the remaining part may be in response to Ag provocation; migrating cells react to the Ag and release IL-8 from cells to the interstitium of the nasal mucosa. Interstitial IL-8 in the nasal mucosa seems to be secreted to the mucous layer via vascular permeability by allergic reaction.

This experiment shows the importance of GM-CSF even in the nasal secretion. GM-CSF release from the allergic nasal surface tissue by Ag challenge *in vivo* was more than without Ag provocation. *In vitro* provocation resulted in hardly any difference between the challenged and unchallenged nasal secretion. These two results suggest that spontaneous release of GM-CSF from the allergic nasal epithelial cells would be detected in an unstimulated state, however the allergic reaction induces active GM-CSF release through the lamina propria and epithelial layer from migrating cells in the nasal mucosa. Immunohistochemistry of GM-CSF of the allergic nasal mucosa supports this hypothesis because GM-CSF appeared to be most intense just beneath the basement membrane and the glandular area. However, normal nasal mucosa showed very weak immunoreactivity of GM-CSF (data not shown). GM-CSF secretion in normal volunteers also was below the detectable levels by any stimulation (data not shown). So GM-CSF in the nasal secretion comes mainly from the epithelial cells, part of that coming from migrating cells by allergic reaction.

Altogether, these results show that the migrating cells and the epithelial cells are the main source of IL-6, IL-8, and GM-CSF, and this release may be induced by Ag stimulation and by direct action of chemical mediators in allergy nasal mucosa. Other studies mentioned that IL-2, IL-6 and GM-CSF in the nasal secretion were increased 4-5 hours after Ag provocation, not in the immediate phase of the allergic reaction (Sim et al., 1994). Our study focused on the cytokine release of early phase reaction to Ag challenge. Furthermore, the method of collection of the nasal secretion was also different. We performed Ag challenge to patients with perennial allergic rhinitis, under natural allergen exposure in which the expression of each cytokine mRNA had already been increased before challenge. This is the reason that we could detect IL-6, IL-8, and GM-CSF in the nasal secretion in the early stage of the allergic reaction. Some studies of the cytokines in nasal secretion were published, but those did not focus on the source of cytokine increases or on the mechanism of secretion (Bachert et al., 1992; Sim et al, 1994; Kametani et al., 1995).

In this study, we have demonstrated for the first time that IL-6, IL-8, and GM-CSF from migrating cells or epithelial cells are released into the nasal secretion by the direct action of HI, reflex action of MC, and stimulation of Ag. These results suggest that the major source of IL-6 in the nasal secretion of patients with HDMAR are migrating cells activated by natural exposure to Ag, and released into the nasal secretion by cholinergic stimulation. The epithelial cells were also the major source of IL-6 in nasal secretion, released by direct action of HI, as well as by Ag stimulation. The major source of IL-8 in the nasal secretion of patients with HDMAR were epithelial cells due to direct action, and the migrating cells due to allergic reaction. The major source of GM-CSF in the nasal secretion were epithelial cells and migrating cells due to allergic reaction.

Taken together, these results suggest that the levels, sources, and mechanisms of release of IL-6, IL-8 and GM-CSF in the nasal secretion of patients with HDMAR are varied, but are important in the manifestation of the allergic reaction. Further studies are necessary to analyse the precise mechanism and role of these cytokines in the nasal secretion.

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