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SPECIAL ARTICLE

The Significance and Technical Aspects of Quantitative Measurements of Inflammatory Mediators in Allergic Rhinitis

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SUMMARY **The pathophysiology of allergic rhinitis induced by various inhalant allergens through an IgE mediated mechanism, has been well demonstrated. The participation of many important inflammatory cells and mediators released by these cells in the human nasal allergic reaction provides insight into the relationship between the responsiveness to allergen exposure and nasal symptoms of allergic rhinitis. This paper summarizes our previous studies on some important mediators in the nasal secretions of atopic patients during different phases after nasal allergen challenge and during natural allergen exposure. The microsuction technique proves to be an especially useful and reliable nasal sampling method permitting quantitative analysis of important mediators such as histamine, tryptase, leukotriene C4 and eosinophil cationic protein in nasal secretions. The measurement of these mediators during allergic reactions provides accurate data on the activity of some important inflammatory cells (i.e., mast cells, basophils, and eosinophils) and their responses to therapy.**

In the past 10 years, there has been extensive research directed towards the basic biology and immunological functions of the components that constitute the framework of the human immune system and the pathogenic mechanisms involved in allergic diseases (Fig 1). We have learned about the role of different inflammatory cells and mediators in mucosal inflammation. At the cellular level, the mechanism of allergic reactions usually consists of the following stages:

(a) Airborne allergen deposit in the nose producing an interaction with cell-bound IgE attached to high affinity receptors on mast cells and low-affinity receptors on T-lymphocytes, Langerhans' cells, and eosinophils.

(b) Allergen binding causes IgE molecules to cross-link which triggers a membrane and cytoplasmic 'cascade' leading to mast cell degranulation and release of inflammatory or immunological mediators (Fig 2). These include preformed mediators, ie. histamine, heparin, tryptase, and other chemotactic factors for neutrophils and eosinophils. Newly generated membrane derived lipid mediators such as arachidonic acid metabolites (ie. prostaglandins, leukotrienes) and platelet activating factor also participate in the allergic inflammation.

(c) Release of mediators, cytokines and granular constituents from mast cells and other inflammatory cells (ie. lymphocytes and macrophages) can directly cause inflammation or activate the local vascular endothelium to further enhance the recruitment of leukocytes through the expression and function of adhesion molecules. The increased mucosal accumulation and activation of leukocytes and the formation of oedema and vasodilatation results in the so called "late-phase reaction" which may occur several hours after allergen exposure.

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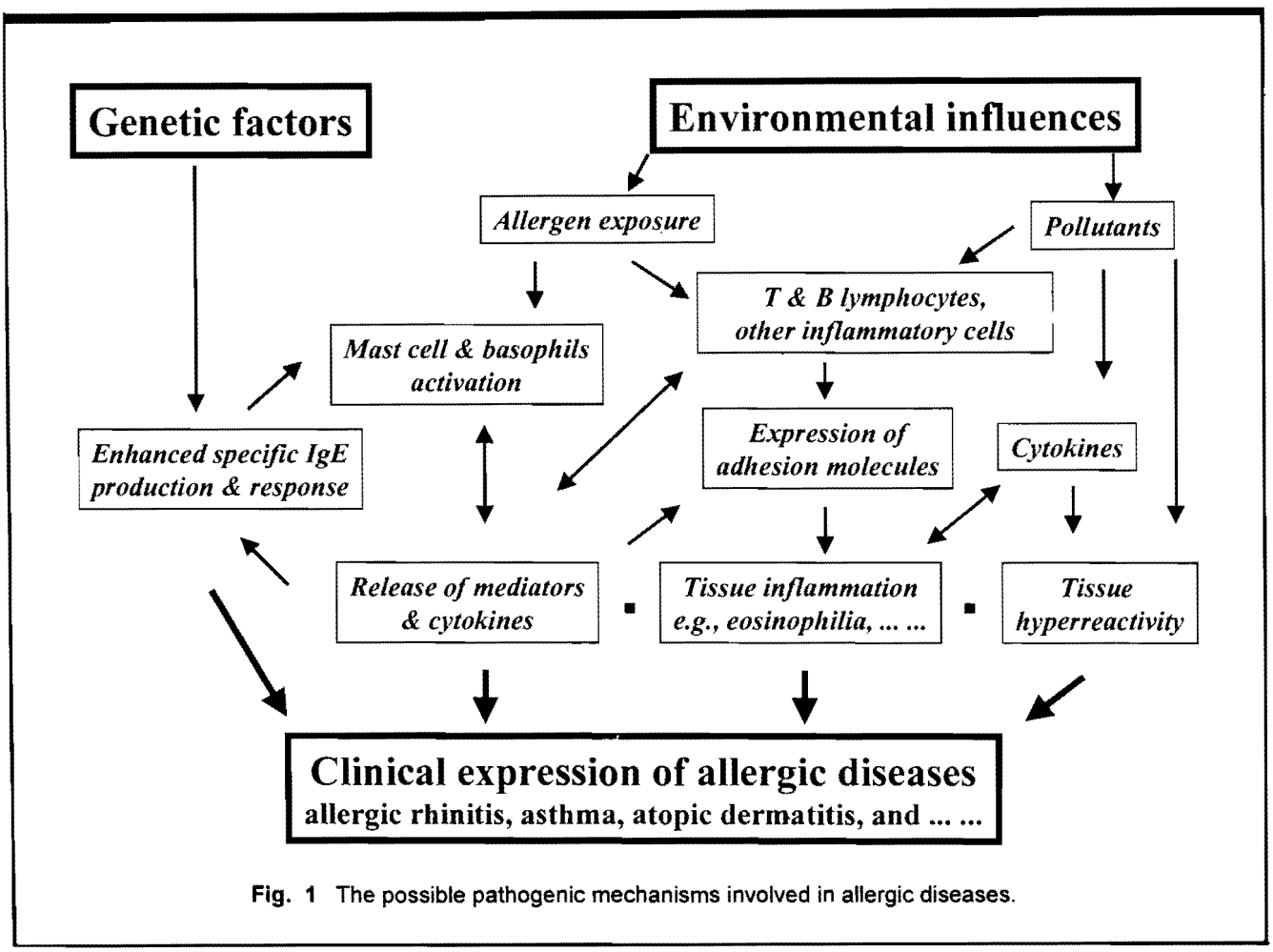

Fig. 1 The possible pathogenic mechanisms involved in allergic diseases.

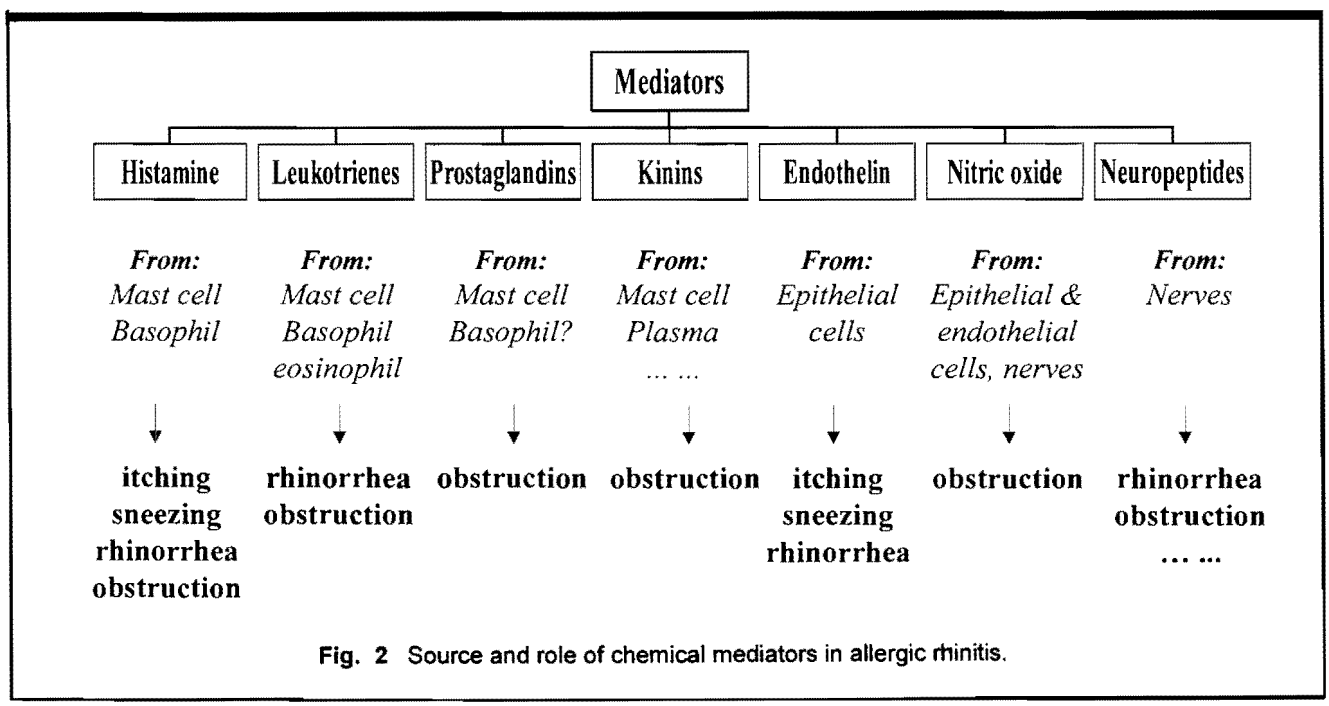

Fig. 2 Source and role of chemical mediators in allergic rhinitis.

Recently, there has been a better understanding of the exquisite respiratory defense mechanisms regulated by cytokines, chemokines and adhesion receptors, and how these factors orchestrate the inflammatory reactions.[1] The respiratory epithelium, together with subepithelial cells such as lymphocytes, macrophages, eosinophils and mast cells, are major contributors to cytokine production and can be stimulated to increase the synthesis and the release of mediators due to pollutants, allergens or viral infection.

A widely used technique of nasal allergen challenge (NAC) provides a novel study model for the direct evaluation of the response of nasal mucous membrane to specific allergens. The use of bio-immunological techniques to determine the inflammatory mediators in nasal secretions after NAC and during natural allergen exposure has greatly enhanced our understanding of the pathophysiology of allergic rhinitis. From a clinical viewpoint, it is important to interpret the ongoing nasal allergic condition on the basis of observing these inflammatory mediators after experimental NAC. To reach this goal, one must have reliable and valuable quantitative data on these inflammatory cells and mediators in the nasal secretions.

This paper reviews the significance of the quantitative measurements of inflammatory mediators in nasal secretions of atopic patients after NAC and during natural allergen challenge, as well as how these measurements can be achieved.

Techniques involved in the study of mediators in nasal secretions

Nasal sampling technique

Collecting and quantitatively analysing nasal secretions constitute an important method of assessing mucosal response to local allergen provocation in atopic patients. The accuracy of this analysis is very dependent on the sampling technique.

Various nasal sampling methods have been employed, such as nasal washes or lavages, filter paper, forceful blowing, microsuction, nasal smear, brush, or scraping and biopsy. Each technique has its advantages and disadvantages. Among these techniques, the most commonly used sampling technique, nasal lavage, has been able to give only qualitative data because of an unknown dilution factor of the collected secretions. When performing a nasal lavage with 10 ml of saline, the volume of lavage fluids recovered was only 8.3 ml.[2] This unknown dilution factor has also contributed to the inaccuracy of the measurement of the mediators which are already present in small amounts. Furthermore, all patients must be pretreated with a sympathomimetic drug (0.05% oxymetazoline hydrochloride) that will interfere with the observations of nasal obstruction, which is one of the main symptoms reflecting nasal allergic reactions.

Nasal microsuction has been used by several investigators.[3-5] The major advantage of this technique is that it permits a quantitative measurement of the mediators in nasal secretions. It is possible to obtain nasal secretions with an exactly known and minimally diluted volume. The amount of secretion obtained from the normal volunteers varied between 50 and 540 mg (median 285 mg), while that from allergic patients varied from 80 to 870 mg (median 300 mg).[4] Secretions were easier to obtain after NAC. This sampling technique is easy to perform and atraumatic. Only a minor local irritation is induced and is generally well tolerated by volunteers and allergic patients.

Measurement of mediators

There are several methods for measuring the mediators in nasal fluid samples, ie. high-performance liquid chromatography (HPLC),[6-8] radioimmunoassay (RIA),[7-11] immunoradiometric assay (IRMA),[11-13] spectrofluorometric assay,[2,14] enzyme-linked immunoassay,[11] enzymatic isotopic assay,[7] and the Pharmacia CAP system.[15] The technique of HPLC is the most specific method. It is, however, not adapted to the analysis of a large series of samples because it is labour intensive and expensive.

It is known that most inflammatory mediators such as histamine, prostaglandin D2 (PGD2) and leukotriene C4 (LTC4) are labile compounds. In the extracellular environment and as well as in the circulation, histamine has a half-life of less than one minute because of its rapid metabolization into inactive products.[16] Histamine is metabolized via two pathways: a diaminooxidase pathway leading to imidazole acetic acid and a methyltransferase pathway leading to N-methylhistamine and N-methylimidazoleacetic acid.[17-18]

PGD2 has been described as the most unstable of all prostaglandins due to the presence of a β-hydroxy-ketone group.[19] Once released into the extracellular environment, PGD2 is rapidly metabolized by 11-ketoreductase into a product similar to PGF2α, except

that the hydroxyl groups of the cyclopentane ring are in an αβ configuration rather than in a coplanar α-geometry. Therefore, an approach to PGD2 stabilization using a derivation of the PGD2 to PGD2-Methoxamine prior to specific enzyme immunoassay has been introduced.[11,20,21]

The biosynthesis of LTC4 is via the second arm of the arachidonate metabolism pathway (so-called lipoxygenase pathway).[16] In the extracellular condition, it is bioconverted sequentially to LTD4 and LTE4. In order to increase the specificity of the measurement of LTC4, special precautions were taken in the methodology to decrease the possible interference from LTD4, LTE4, and LTB4.[11] In any case LTD4 and LTE4 are derived from LTC4 so other investigators did not find a clear advantage in separating the leukotrienes with HPLC instead of performing immunoassays of unfractionated nasal fluid samples.[6]

Reference values of inflammatory cells and mediators in nasal secretions

In the literature, there is no quantitative data on the normal range of inflammatory cells and chemical mediators in nasal secretions. In a group of ten non-allergic healthy volunteers, the concentrations of the biochemical mediators and cytogram of nasal secretions were measured in our previous study (Table 1).[22] In atopic patients (n = 17) outside the allergy season, there existed a significant higher baseline concentration of histamine (median 36 ng/g) than in normals. There was no significant difference between males and females for these mediators and cells in nasal secretions.

Significance of quantitative measurement of mediators in the evaluation of nasal allergic reactions

Mediators in the early phase reaction

Many authors performed intranasal challenges with different allergens in atopic patients and showed the occurrence of nasal symptoms (sneezing, nasal obstruction, and rhinorrhea) within minutes. This response has been described as the early phase reaction (EPR). Many investigators have observed that a significant increase in mediators such as histamine,[2,14] tryptase,[12,13] PGD2,[2,14] LTC4,[6,7] and kinins[2,14] was associated with the nasal symptoms several minutes after the challenge.[2,6,7,12-14]

We have previously demonstrated that a significant increase in the concentrations of histamine, tryptase, and LTC4, but not for PGD2 in nasal secretions occurred five minutes after NAC (Fig 3), and this was accompanied by nasal symptoms such as sneezing (median: 8 times) and unilateral and/or bilateral nasal obstruction (more than 100% increase of nasal airway resistance).[4,23,24] Meanwhile, the percentage of eosinophils and the eosinophil cationic protein (ECP) concentration did not increase. This data strongly supports the important role of mast cell activation during EPR. Furthermore, we demonstrated that the onset of itching and sneezing may occur as early as approximately 30 seconds after NAC and they were the predominant symptoms within 5 minutes.[25] Rhinorrhea and nasal obstruction started a few minutes after NAC and lasted more than 1 hour after NAC. Meanwhile, a maximal mediator concentration in nasal secretions was reached after 1 minute for histamine, 5 minutes for tryptase, and 5-10 minutes for LTC4 after NAC.

When evaluating symptoms during the EPR, one must not consider only the severity of these symptoms, but also the time period within which these symptoms oc-

Table 1 Concentrations of biochemical mediators and cytogram of nasal secretions of non-allergic healthy volunteers

Mediators and cells (units)	**Median value**	**Range**
Mediators		
Histamine (ng/g)	19	7.5-32
Tryptase (μU/g)	< 0.5	< 0.5-11
Leukotriene C4 (ng/g)	5.7	3.6-13
Prostaglandin D2 (pg/g)	477	220-788
ECP (ng/g)	105	2-281
Myeloperoxidase (μg/g)	2.0	0.08-13.9
Cells		
Neutrophils (%)	99.7	99-99.7
Eosinophils (%)	0	0-0.6
Lymphocytes (%)	0.3	0-0.6
Mast cells (%)	0	0

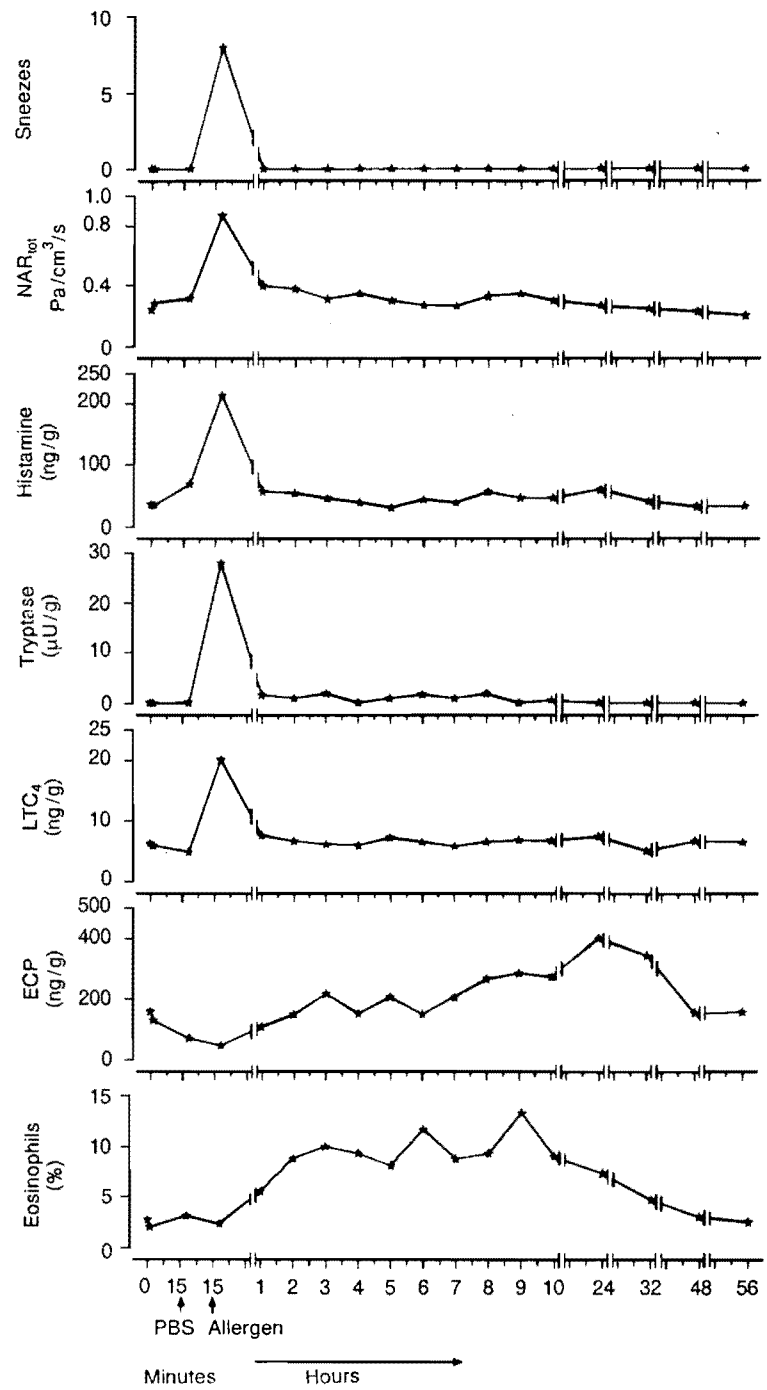

Fig. 3 Median number of sneezes, total nasal airway resistance (NAR_{tot}), concentrations of the mediators, and percentage of eosinophils in nasal secretions before (time zero) and after PBS and allergen challenge in 17 atopic patients (Adapted from Wang *et al.* Int Arch Allergy Immunol 1995; 106: 278-85, with permission of Int Arch Allergy Immunol).

cur. For the symptoms of itching and sneezing, EPR may start as early as seconds and often last for only a few minutes after NAC. However, when one considers nasal obstruction and rhinorrhea, the EPR may last more than 1 hour.

Mediators in the late phase reaction

In comparison to the EPR, the late phase reaction (LPR) has been described to occur hours or days after NAC. In one study these recurrent nasal symptoms were manifested primarily by sneezing and rhinorrhea 3-11 hours after NAC, since nasal congestion was diminished by a pretreatment of intranasal oxymetazoline.[2] These symptoms were accompanied by a second increase of histamine, TAME-esterase and kinin in nasal lavage fluids over baseline values. This study suggested that these increases of mediators were induced by the participation of the basophils.

More studies demonstrated that the late phase nasal reaction to NAC was accompanied by increases in the number of eosinophils and neutrophils in nasal secretions.[26,27] However, more subjects showed an increase in cells (ie. eosinophils and neutrophils) than an actual developed signs and symptoms of LPR. This suggests that the interaction of additional factors explains the activation of incoming cells and the development of LPR.[28] Later eosinophils and their secretory products (such as ECP) have been reported to increase during LPR.[29] Venge *et al.*[30] were able to show a highly significant correlation between serum ECP levels and histamine sensitivity in atopic asthmatics during the pollen season. This data suggests a role of the eosinophil granulocytes in LPR. However, the relationship between eosinophil dominated nasal allergic inflammation and mucosal hypersensitivity to specific allergens or non-allergic stimuli remains to be explored objectively and quantitatively.

The LPR in the nose is clinically characterized by nasal obstruction with little sneezing and rhinorrhea. In this respect passive anterior rhinomanometry is a qualitative as well as a quantitative method for the objective evaluation of the degree of nasal obstruction. When nasal obstruction is defined as a 100% increase in total nasal airway resistance, a clinical LPR occurred in only 41% of the patients, eighty-two percent had a response defined as a 100% increase of unilateral nasal airway resistance. Alternating unilateral nasal obstruction (47%) appeared to be the most common.[23]

When assessing cell count and mediator concentrations we found that one hour after NAC, a simultaneous increase was seen both in the percentage of eosinophils and in the ECP concentrations of the nasal secretions (Fig 3). Both parameters clearly differed in the time at which the maximum value was reached and the duration of their peak value.[4] The eosinophil count reached a peak 2 hours after a nasal allergen challenge and lasted for 8 hours (median percentage of eosinophils: 8.8-13.3%), while the highest ECP level (median: 401 ng/g, range: 32-2298 ng/g) was reached after 24 hours with no clear-cut plateau. Our findings again confirmed the role of migration and activation of eosinophils in the pathogenesis of nasal allergic reactions, especially during LPR. The other mediators (ie. histamine, tryptase and LTC4) were significantly increased immediately after NAC and returned to the baseline levels one hour later. A recurrent peak concentration of histamine (in 53% of the patients), tryptase (in 41% of the patients) and LTC4 (in 65% of the patients) was also observed during LPR compared to baseline values. However, the pattern of the recurrent increases of these mediators during LPR varied with respect to the occurrence in time of the peaks and the concentrations of these mediators were far lower than the concentrations occurring during the EPR.

The term "late phase reaction" in fact covers a phenomenon of chronic inflammation in the nasal mucosa of an allergic individual following NAC. For hours or sometimes days after the challenge, nasal obstruction is the predominant clinical symptom. When assessing the inflammatory cells and mediators in nasal secretions, the infiltration and activation of eosinophils were found to be the predominant mechanism in the pathogenesis of this inflammatory condition.

Comparison of mediators after nasal allergen challenge in seasonal allergic rhinitis patients in and outside the pollen season

It is known that infiltration of the nasal mucosa by migratory cells and consequent release of a large number of inflammatory and immunological mediators are important pathophysiologic conditions of allergic rhinitis. After experimental or natural allergen exposure, an increase in the number of various cell populations can be observed within the mucosa and on the mucosal surface. This includes mast cells, basophils, eosinophils,

IgE-positive cells, macrophages, monocyte-like cells, Langerhans cells and T- cells. Eosinophils and basophils have been shown to migrate into the nasal mucosa within hours after an allergen challenge and have been linked to the release of different specific mediators into nasal secretions.[31] It is, however, not clear whether this inflammatory condition will affect the responsiveness of nasal mucosa to a specific allergen.

In the literature, most of the NAC studies were performed in seasonal allergic rhinitis patients outside the pollen season when the patients were totally symptom free. The subjective and objective nasal symptoms and the changes in inflammatory components in the nasal mucosa were measured. However, the baseline condition of patients with ongoing allergic rhinitis during the natural allergen exposure is quite different. Normally during the allergic season a hay fever patient is in a continuous late phase reaction characterized by nasal eosinophilia and complaints mainly of nasal obstruction.[22] Intermittent exposure to grass pollen results in attacks of sneezing, rhinorrhea and increased nasal blockage. It seems that NAC performed in a particular season should mimic the phenomenon that occurs in allergic patients during the season after natural allergen exposure.

In order to investigate the difference of nasal response to a specific allergen with or without the presence of "priming" conditions, we performed NACs in 12 patients with seasonal allergic rhinitis both in and outside the pollen season.[32] In these patients a nasal mucosal inflammation characterized by eosinophilia and significantly increased ECP production was found during the season, but not outside the season. However, the number of sneezes and the increase of the total nasal airway resistance did not differed at 5 minutes after NAC between these two challenges. There was a significantly higher histamine concentration after NAC in season, but this was not measured for tryptase and LTC4 when performed outside the season. Our results confirm the increased basophil infiltration in the nasal mucosa of these patients during the pollen season.

Mediators in atopic patients with ongoing seasonal and perennial allergic rhinitis

In the literature many studies have been performed comparing the concentrations of the mediators in nasal lavage fluids before and after experimental NAC. There is no reference concerning the concentration of these inflammatory mediators during natural allergen exposure. In addition, methodological flaws such as the existence of an unknown dilution factor and the use of a topical decongestant make it less reliable for diagnostic use in patients with allergic rhinitis. Therefore an assessment of the normal and pathological concentrations of mediators in nasal secretions seemed to be necessary.

We have compared the biochemical markers in nasal secretions of patients with seasonal allergic rhinitis before and after an allergen challenge outside the pollen season[22] and in patients with ongoing allergic rhinitis in season (Table 2). All data are the median values summarized from our previous studies of patients with ongoing seasonal allergic rhinitis during the pollen season and with perennial allergic rhinitis (mite allergy) during the autumn.[22,25,32-35]

From the data the eosinophil count and mediator profile during the pollen season is similar to the late-phase response after NAC. ECP was predominantly found in a high concentration and was accompanied by a high percentage of eosinophils in the nasal secretions of most patients with seasonal allergic rhinitis. Evidence has been accumulating emphasizing the role of eosinophils in response to an ongoing allergic inflammation in the nasal mucosa during the pollen season. The infiltration and activation of eosinophils in chronic inflammatory conditions during natural allergen exposure provides important diagnostic and therapeutic information. It seems that the combination of measuring the percentage of eosinophils and the ECP concentration in nasal secretions is a very useful model for monitoring and assessing chronic nasal inflammation in patients with allergic rhinitis.

The mast cell remains a focal point of interest in the study of the acute allergic reaction. Regardless of the site of challenge, there is considerable evidence to implicate mast cells in the mediation of the immediate response generated by the allergen. For this reason, one study suggested that the immediate response of nasal mucosa to antigen reflects only a minor part of the allergic diathesis.[2] It was previously reported that an increase in histamine levels failed to be demonstrated in nasal lavage fluids during natural allergen challenge.[10] From our data the median histamine concentrations measured in the patients with ongoing seasonal and perennial allergic rhinitis are far lower than the concentration

Table 2 Comparison of the biochemical markers in nasal secretions of patients with seasonal allergic rhinitis before and after an allergen challenge outside the pollen season and in patients with ongoing allergic rhinitis in season. All data are the median values summarized from our previous studies: 5 groups of patients with seasonal allergic rhinitis during (in 4 groups) and at the end (in 1 group) of the pollen season, and 1 group of patients with perennial allergic rhinitis (mite allergy) during the autumn.[4,22,25,32,35,37]

	Histamine (ng/g)	Tryptase (μU/g)	LTC4 (ng/g)	ECP (ng/g)
Outside season after NAC (n = 17)				
Baseline	36	< 0.5	6.4	160
Early-phase response	214	28	20	47
Late-phase response (8 hours)	56	1.9	6.6	267
Late-phase response (24 hours)	60	< 0.5	7.5	401
Seasonal allergic rhinitis in season				
Group 1 (n = 40)	51.5	< 0.5	23	410
Group 2 (n = 14)	41.5	< 0.5	16.5	684
Group 3 (n = 16)	30	< 0.5	26.5	408
Group 4 (n = 31)	33.7	< 0.5	25.8	515
Group 5* (n = 28)	23.3	< 0.5	1.44	203
Perennial allergic rhinitis	39	< 0.5	14.8	182

* At the end of pollen season when patients were symptom free.

occurring during the EPR and some of them are similar to the concentration during the LPR. Tryptase is also found in a very low concentration in most of these patients. This leads us to speculate that massive mast cell activation is rarely observed at the time when patients are referred to the outpatient clinic. On the other hand, it must be said that when patients were inside the clinic they did not sneeze, because they were not directly exposed to allergens at that time.

LTC4 is one of the major lipoxygenase products of arachidonic acid metabolism involved predominantly in the pathogenesis of allergic reactions. Several cell types are capable of synthesizing leukotrienes in response to specific or nonspecific stimuli: mast cells, eosinophils, basophil and neutrophil granulocytes, monocytes, and macrophages.[36] Our data shows that LTC4 was measured in high concentrations and correlated very well with the increases of ECP concentrations and the percentage of eosinophils in the nasal secretions of the patients with ongoing seasonal and perennial allergic rhinitis. These findings clearly demonstrate that LTC4 production *in vivo* is not derived exclusively from mast cells, but also from other inflammatory cells such as eosinophils, especially during ongoing mucosal inflammation.

The other cells such as neutrophils, macrophages, epithelial and endothelial cells are certainly playing an important role in nasal allergic inflammation. However, in our study the measurements of myeloperoxidase (MPO), a protein specific for primary granules of neutrophils,[9] did not reveal changes in atopic patients compared to nonallergic controls both after nasal allergen challenge and during natural allergen exposure.[37] When performing an immunohistological study of the nasal mucosa in patients with seasonal allergic rhinitis, Bentley *et al.*[38] demonstrated increases in the number of activated eosinophils and epithelial mast cells, but not of neutrophils within the submucosa and epithelium. Hence, further studies are needed to elucidate the role of neutrophils in the pathophysiological processes of allergic rhinitis.

Conclusion

The study of biochemical mediators in nasal secretions has contributed to the understanding of the pathogenesis and the role of inflammatory cells and mediators in IgE-mediated allergic rhinitis. The presence of some important mediators such as ECP, tryptase, histamine and LTC4 during the different phases of an allergic reaction suggests that they are important markers for mucosal inflammation. The quantitative measurements of inflammatory mediators in clinical trials can be of help in a better understanding of drug effects. Inclusion of these objective quantitative parameters in drug studies can reduce the sample size of patients studied and statistical relevance can be reached with smaller numbers.

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