

Mediators and Cytokines in Persistent Allergic Rhinitis and Nonallergic Rhinitis with Eosinophilia Syndrome

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Key Words

Allergic rhinitis · Eosinophilia syndrome · Interleukin-5 · Interleukin-17 · Granulocyte macrophage colony-stimulating factor · Eosinophil cationic protein · Tryptase

Abstract

Background: Patients with nonallergic rhinitis with eosinophilia syndrome (NARES) show typical symptoms of persistent allergic rhinitis (PAR). The aim of the present study was to compare nasal cytokine patterns between NARES and PAR. **Methods:** Nasal secretions of 31 patients suffering from NARES, 20 patients with PAR to house dust mite and 21 healthy controls were collected using the cotton wool method and analyzed for interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 β (MIP-1 β) by BioPlex Cytokine Assay as well as eosinophil cationic protein

(ECP) and tryptase by UniCAP-FEIA. **Results:** NARES and PAR presented elevated levels of tryptase, while ECP was markedly increased solely in NARES compared to both the controls and PAR. Elevated levels of IL-1 β , IL-17, IFN- γ , TNF- α and MCP-1 were found in NARES compared to the controls as well as PAR. MIP-1 β was elevated in NARES and PAR, while IL-4, IL-6 and G-CSF showed increased levels in NARES, and IL-5 was elevated in PAR only. **Conclusions:** In patients with NARES and PAR, eosinophils and mast cells appear to be the pivotal cells of inflammation, reflected by high levels of tryptase and ECP as well as IL-5 and GM-CSF as factors for eosinophil migration and survival. The elevated levels of proinflammatory cytokines in NARES may indicate the chronic, self-perpetuating process of inflammation in NARES which seems to be more pronounced than in PAR. IL-17 might be a factor for neutrophilic infiltration or be responsible for remodeling processes in NARES.

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M.G. and C.K. contributed equally to the study.

Introduction

Rhinitis is defined as an inflammation of the nose and since markers of inflammation are not routinely examined in daily practice, the term rhinitis is mainly used for a complex of symptoms including rhinorrhea, nasal blockage, sneezing and nasal itching [1]. Compared to acute rhinitis that is self-limiting and resolves within 1 or 2 weeks, chronic rhinitis can persist for several weeks and may even be perennially affecting up to 20% of the general population [2].

Allergic rhinitis constitutes the most frequent manifestation of atopic diseases with nearly 80 million affected people in the USA, and thus accounts for about 50% of patients with chronic rhinitis [3]. The classification into 'seasonal' and 'perennial' has been replaced by the terms 'intermittent' and 'persistent', with persistent meaning symptoms occurring >4 days a week and >4 consecutive weeks per year [2]. The pathogenic features of allergic rhinitis have been explored in many studies. In the early phase, the bridging of mast cell-bound allergen-specific IgE with allergens leads to the degranulation of mast cells and the release of products like histamine and leukotrienes. Later, the influx of inflammatory cells like eosinophils and basophils leads to persistent inflammation and associated tissue changes [4, 5].

As a separate entity of chronic rhinitis, nonallergic rhinitis with eosinophilia syndrome (NARES) constitutes a rare nasal condition, although prevalence rates range from 2 to 14% among patients with chronic rhinitis [6]. Patients present typical symptoms of persistent rhinitis, mainly nasal blockage and rhinorrhea, but *in vitro* and *in vivo* tests fail to detect any sensitization [7]. A chronic, unspecific liberation of histamine and a self-perpetuating eosinophilic infiltration have hitherto been supposed to be pathogenic factors of this disease, with a nasal smear showing more than 25% eosinophilia as a diagnostic criterion [7]. However, recent literature has provided evidence that some patients with rhinitis show nasal allergy with no systemic markers of atopy, either by local IgE production or even through an IgE-independent antigen-specific pathway [8, 9]. In addition, it has been suggested that this condition may be prevalent in up to one third of adults with nonallergic rhinitis, and although it usually occurs as an isolated disorder, there is evidence of association with asthma, aspirin sensitivity and nasal polyps [10–13].

Investigating cytokine levels and other mediators in the nasal fluid of patients with rhinitis has been used in a number of studies, either to get more insight of the

pathogenic conditions [14], for diagnostic use [15] or to measure therapeutic effects [16].

The aim of this study was to investigate cytokine levels and other inflammatory mediators in the nasal fluid of patients with persistent allergic rhinitis (PAR) and NARES in comparison with healthy controls. An attempt was made to identify disease-specific cytokine patterns and to identify the role of different cytokines in both diseases.

Methods

Study Population

Seventy-two adult volunteers (46 males, 26 females, median age 32 years, range 15–68 years) participated in this study. Clinical history was taken by one of the investigators. All volunteers completed a questionnaire referring to nasal obstruction, rhinorrhea, sneezing and nasal itching. Symptoms were rated on a scale from 0 to 3 (0 = no symptoms, 1 = minor symptoms, 2 = moderate symptoms and 3 = severe symptoms). Patients presenting with sensitization to seasonal allergens, a history of bronchial asthma and/or bronchial hyperreactivity, chronic rhinosinusitis, nasal polyposis or aspirin sensitivity were excluded from the study. Any medication taken concerning the nasal disease during or 6 weeks prior to the examination also constituted an exclusion criterion, especially anti-inflammatory medication such as nasal steroids or antihistamines.

Nasal endoscopy was performed in all participants in order to assess clinical signs of rhinitis and to exclude patients with signs of purulent rhinitis or polyposis. Allergic rhinitis was determined by the patient's history and by a positive skin prick test (ALK-Abelló, Wedel, Germany) for the following allergens: timothy grass, rye, birch, hazel, alder, beech, mugwort, ribwort, nettle, dandelion, house dust mite, storage mite, dog, cat and horse epithelial dander, *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*. Histamine dihydrochloride solution at 1 mg/ml as a positive control and allergen-free saline solution as a negative control were used. Thereafter specific IgE to the allergens tested positive in skin prick tests was measured in serum (UniCAP-FEIA, Phadia, Freiburg, Germany).

NARES (n = 31; 5 female, 26 male; median age 31 years, range 15–68 years) was determined by typical nasal symptoms and negative skin prick test to all the tested allergens, but with excessive levels of eosinophil cationic protein (ECP) in nasal secretions (>200 ng/ml) in the absence of any other sinonasal disease such as polyposis.

PAR (n = 20; 7 female, 13 male; median age 31 years, range 17–62 years) due to house dust mites was determined by the patient's history, a sensitization to house dust mites with a positive skin prick test and a house dust mite-specific IgE (CAP class ≥ 2), and a positive intranasal allergen challenge.

Healthy controls (n = 21; 14 female, 7 male; median age 36 years, range 15–66 years) presented no history of nasal complaints, normal ECP levels in nasal secretions and a negative *in vitro* allergy screening test Sx1 (Phadia).

The study was approved by the local ethics committee and written informed consent was obtained from all participants.

Biochemical and Immunological Methods

Nasal secretions were gained using small cone-shaped cotton wool pieces (absorbent cotton, Hartmann Inc., Heidenheim/Brenz, Germany) with a length of about 3 cm and a diameter of about 6 mm. Introduced into the middle meatus of the nose, the cotton wool pieces were left in place for 20 min and were subsequently centrifuged (+4°C, 2,000 g) on a sieve for 10 min [15]. Control experiments were performed with diluted nasal secretions tested at 1:2, 1:5 and 1:10 for reproducibility and recovery rates, which were best at a dilution of 1:5.

All samples were analyzed for Interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 β (MIP-1 β) using a human cytokine 17-plex panel (Bio-Plex Cytokine Assay, Bio-Rad Laboratories, Hercules, Calif., USA). The cytokine assay uses fluorescently addressed polystyrene beads with conjugated capture antibodies directed to the above-mentioned cytokines. After washing, a fluorescently marked detection antibody builds an immunoassay with the cytokine. For analysis, two lasers excite the fluorochromes: one for classifying each bead, the other for quantifying the amount of bound analyte [10]. Detection levels were 0.5 pg/ml.

Total IgE was measured in nasal secretion diluted 1:2 (UniCAP-FEIA, Phadia). ECP and tryptase were measured by ELISA (UniCAP-FEIA, Phadia). Thresholds for detection were 10 ng/ml for ECP and 5 ng/ml for tryptase.

Statistics

SPSS 10.0 software was used for statistical evaluation. Significances were obtained by the Kruskal-Wallis one way analysis of variance on ranks. When significant differences were indicated, the Mann-Whitney U test was also performed. *p* values <0.05 were regarded as significant. As values were not normally distributed, data are given as median and range. Data are shown as box plots for graphic presentation of the results with significances graphically represented between the corresponding scatter plots.

Results

The clinical characteristics of all patients are given in table 1. No relevant differences in the clinical appearance of PAR and NARES were detectable. As shown in figure 1, a significant difference among the groups could be seen in the total IgE in nasal secretion. In PAR total nasal IgE (median 4 ng/ml, range 0–145 ng/ml) was significantly higher in comparison to the controls (median 0 ng/ml, range 0–0 ng/ml, *p* < 0.001) as well as to NARES (median 0 ng/ml, range 0–83 ng/ml, *p* < 0.05). IgE levels in NARES were not significantly elevated.

Concerning the nasal fluid specimens, ECP showed significantly higher levels in NARES (median 472 ng/ml, range 241–1,000 ng/ml) compared to PAR (median 72 ng/

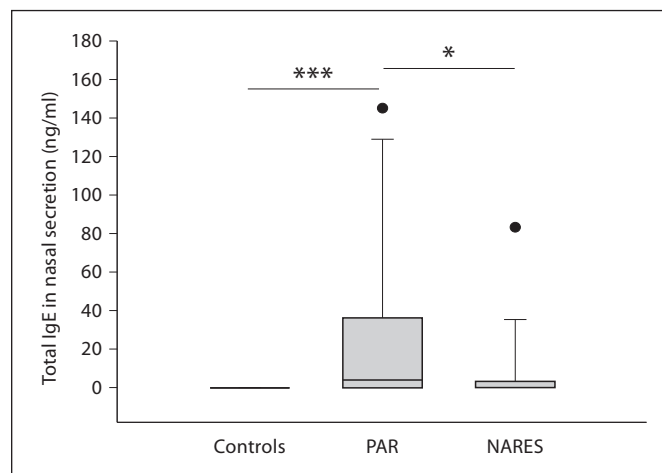


Fig. 1. Box plot of levels of total IgE in nasal secretions of the three patient groups. Total IgE is significantly elevated in PAR versus controls (***) *p* < 0.001) as well as versus NARES (* *p* < 0.05).

Table 1. Mean symptom scores

Symptom score	Controls	PAR	NARES
Obstruction	0	1.94	1.45
Secretion	0.24	1	1.33
Sneezing	0.38	1	0.81
Itchy nose	0.24	0.8	0.55

ml, range 10–1,000 ng/ml, *p* < 0.001) as well as to healthy controls (median 23 ng/ml, range 10–90 ng/ml, *p* < 0.001). As shown in figure 2, tryptase was significantly elevated in PAR (median 14 ng/ml, range 5–1,000 ng/ml, *p* < 0.01) compared to the controls (median 5 ng/ml, range 5–16 ng/ml), and also showed significantly higher levels in NARES (median 5 ng/ml, range 5–207 ng/ml, *p* < 0.01).

For IL-2 (controls: median 0 pg/ml, range 0–12 pg/ml; PAR: median 0 pg/ml, range 0–15 pg/ml; NARES: median 0 pg/ml, range 0–94 pg/ml) and IL-12 (controls: median 0 pg/ml, range 0–15 pg/ml; PAR: median 0 pg/ml, range 0–15 pg/ml; NARES: median 0 pg/ml, range 0–17 pg/ml), similar levels could be found in the nasal fluids of all three groups.

We detected significantly higher levels of the proinflammatory cytokines IL-1 β (controls: median 30 pg/ml, range 0–412 pg/ml; PAR: median 18 pg/ml, range 3–996 pg/ml; NARES: median 285 pg/ml, range 41–2,618 pg/ml; *p* < 0.001; fig. 3), IFN- γ (*p* < 0.001; table 2) and TNF- α

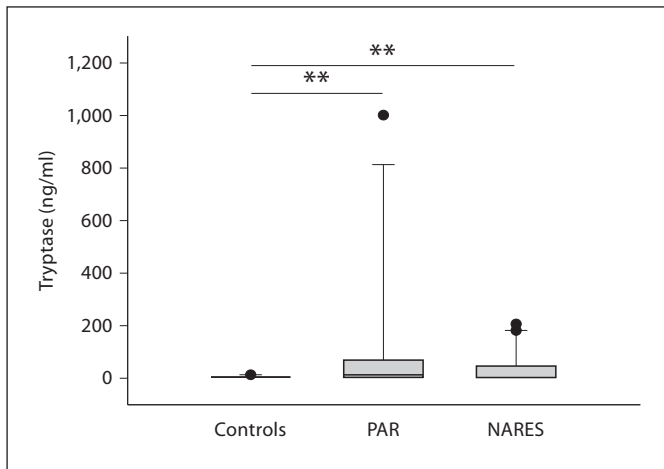


Fig. 2. Box plot of levels of tryptase in sera of the three patient groups. Tryptase is significantly elevated in PAR and NARES versus controls (** $p < 0.01$).

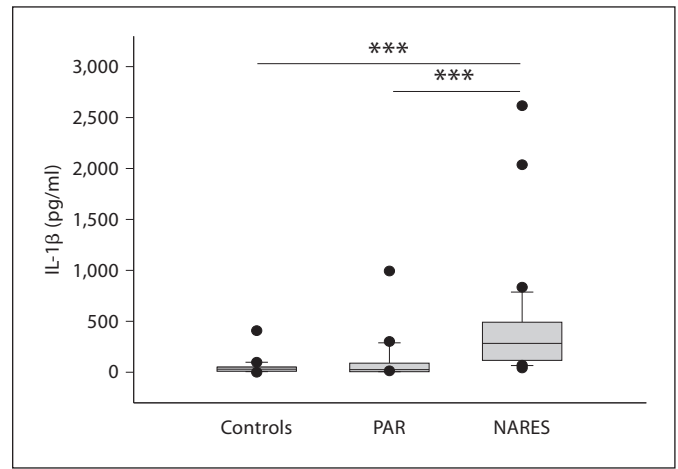


Fig. 3. Box plot of levels of IL-1 β in sera of the three patient groups. IL-1 β is significantly elevated in NARES versus controls as well as versus PAR (*** $p < 0.001$).

Table 2. Cytokine levels

	IFN- γ	TNF- α	MCP-1	MIP-1 β
Controls	26 (0–253)	9 (0–71)	246 (118–388)	136 (18–455)
PAR	49 (0–502)	10 (0–171)	218 (87–523)	259 (73–1,671)
NARES	203 (30–1,129)	62 (11–348)	388 (159–1,022)	378 (121–2,511)
p values				
Controls vs. PAR	n.s.	n.s.	n.s.	<0.01
Controls vs. NARES	<0.001	<0.001	<0.01	<0.001
PAR vs. NARES	<0.001	<0.001	<0.01	n.s.

Data are measured as pg/ml and presented as median values with ranges in parentheses. n.s. = Not significant.

($p < 0.001$; table 2) in NARES compared to the controls and PAR, while there were no significant differences between levels in PAR and the controls. IL-6 ($p < 0.001$; table 3) was significantly elevated in NARES solely in comparison to the controls.

Levels of IL-7 were quite similar in all the groups (controls: median 22 pg/ml, range 7–51 pg/ml; PAR: median 29 pg/ml, range 12–99 pg/ml; NARES: median 39 pg/ml, range 6–196 pg/ml). The magnitude of IL-17 in NARES (median 8 pg/ml, range 0–35 pg/ml) was significantly higher compared to the controls (median 0 pg/ml, range 0–22 pg/ml, $p < 0.01$) as well as to PAR (median 0 pg/ml, range 0–13 pg/ml, $p < 0.01$; fig. 4).

IL-8 showed no elevated levels in inter-group comparison, while significantly higher levels of the chemokine

MCP-1 could be detected in NARES ($p < 0.01$; table 2 and 3). MIP-1 β was significantly elevated in NARES as well as in PAR ($p < 0.001$ and $p < 0.01$, respectively; table 2).

As shown in figure 5, IL-4 was significantly elevated in NARES (median 106 pg/ml, range 0–500 pg/ml, $p < 0.001$) in comparison with the controls (median 0 pg/ml, range 0–206 pg/ml) but not versus PAR (median 0 pg/ml, range 0–206 pg/ml). The levels of IL-5 in PAR were significantly higher compared to the controls ($p < 0.05$), while there were no significant differences in levels of IL-13 in the nasal secretions from all three groups (table 3).

G-CSF showed significantly elevated levels in NARES (median 375 pg/ml, range 0–5,856 pg/ml, $p < 0.01$) compared to the controls (median 53 pg/ml, range 0–2,086 pg/ml) but not to PAR (median 214 pg/ml, range 0–5,688

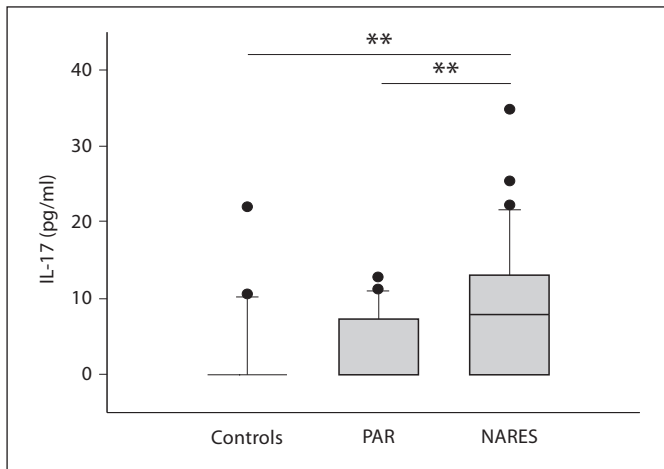


Fig. 4. Box plot of levels of IL-17 in sera of the three patient groups. IL-17 is significantly elevated in NARES versus controls as well as versus PAR (** $p < 0.01$).

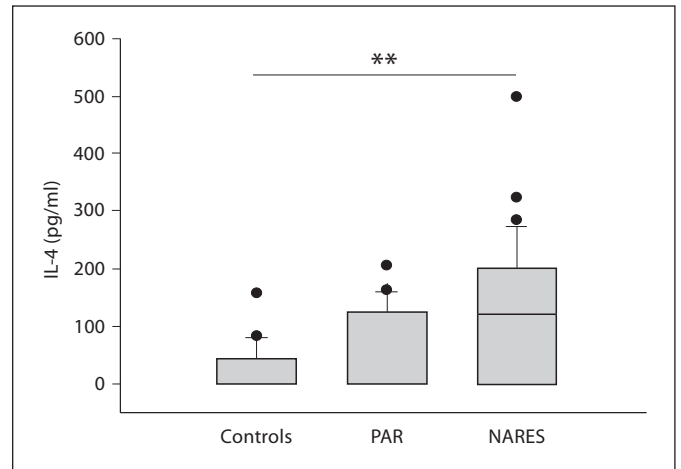


Fig. 5. Box plot of levels of IL-4 in sera of the three patient groups. IL-4 is significantly elevated in NARES versus controls (** $p < 0.01$).

Table 3. Interleukin levels

	IL-5	IL-6	IL-8	IL-13
Controls	0 (0–20)	93 (13–1,594)	3,573 (467–23,277)	0 (0–61)
PAR	5 (0–188)	202 (25–750)	4,205 (524–11,759)	0 (0–58)
NARES	0 (0–320)	309 (70–8,168)	3,485 (0–33,932)	13 (0–62)
p values				
Controls vs. PAR	<0.05	n.s.	n.s.	n.s.
Controls vs. NARES	n.s.	<0.001	n.s.	n.s.
PAR vs. NARES	n.s.	n.s.	n.s.	n.s.

Data are measured as pg/ml and presented as median values with ranges in parentheses. n.s. = Not significant.

pg/ml). The quantity of GM-CSF was not significantly different among the three groups (controls: median 0 pg/ml, range 0–905 pg/ml; PAR: median 0 pg/ml, range 0–582 pg/ml; NARES: median 90 pg/ml, range 0–1,176 pg/ml). IL-10 could not be detected in the controls or PAR, but was measurable in a few patients with NARES (median 0 pg/ml, range 0–125 pg/ml).

Discussion

Both chronic allergic as well as chronic nonallergic rhinitis constitute frequent inflammatory diseases of the upper-airway mucosa. Patients suffering from NARES, a nonallergic rhinitis defined by nasal mucosa eosinophil-

ia, show symptoms of perennial allergic rhinitis without systemic sensitization. Recently, new insights into inflammatory processes have become possible by studying the release of cytokines and chemokines involved in pathogenic pathways. Detecting these mediators in nasal fluid has become a standardized and well-evaluated technique [11, 15]. However, the results from different studies remain inconclusive, particularly regarding the technique of specimen collection. We used the cotton wool method for its accessibility and for prevention of predilution, which occurs in nasal lavage [12].

Eosinophils are the pivotal cell type in NARES, with eosinophilia in the nasal smear as a diagnostic criterion. Accordingly, the highest levels of nasal ECP were found in patients with NARES. This is in accordance with oth-

er studies comparing different types of chronic rhinitis [15]. We found even higher levels of ECP in patients with sensitization to the perennial allergen house dust mite than we had shown in patients with seasonal allergic rhinitis in a previous study (210.2 vs. 115.5 ng/ml) [14]. This might be due to the fact that the nasal fluid of seasonal allergic patients was not collected during assured allergen exposure. The importance of eosinophils in mucosa inflammation in allergic rhinitis has also been highlighted in other studies [13, 17].

Tryptase could clearly discriminate the nasal secretions of allergic patients from those of the controls. Mast cell activation is one of the main mechanisms in allergic rhinitis, which has been proven in former studies [14, 15]. Interestingly, high levels of tryptase were detected in the nasal secretions of patients with NARES, which is in accordance with previous studies showing increased mast cells and mast cell activation in different forms of nonallergic rhinitis [9, 15]. This might also implicate a kind of allergen-dependent mast cell activation, either IgE-dependent as suggested in recent challenge studies for local allergies [18], or IgE-independent as indicated by finding free light-chain expression in patients with nonatopic rhinitis [8].

For IL-12, which, among other cytokines, is supposed to be important in mounting a Th1-helper cell response, only low levels were found in all the three groups, suggesting an inferior role of Th1-helper cells in NARES and PAR. IL-2, which plays a pivotal role in inflammation and stimulates the synthesis of other proinflammatory cytokines like IL-1 or IL-6, showed higher but not significantly elevated level in NARES. Like IL-12, it could not be detected in all the samples. Whether this is because of its short half-life or the time course in cytokine expression cannot be answered conclusively.

We found a remarkable upregulation of the proinflammatory cytokines IL-1 β , IL-6, TNF- α and IFN- γ in NARES in comparison to the controls. Levels of IL-1 β , TNF- α and IFN- γ were also significantly higher compared to PAR. Whilst in PAR IL-1 β was 2-fold higher than in healthy subjects, a significant increase in comparison with the controls was not detected in any of these proinflammatory cytokines. Th1-derived, proinflammatory cytokines have often been studied in the nasal secretions of patients with allergic rhinitis, mostly in challenge studies [19] or in seasonal allergic rhinitis [20, 21], where high levels of IL-1 β could mostly be found after challenge or during the pollen season. This secretion of IL-1 β , which activates T lymphocytes and endothelial cells and leads to a release of further cytokines, was also detected

in PAR [21], which is in accordance with our findings. Measuring proinflammatory cytokines could also be interesting for the detection of a subclinical state of inflammation in patients with seasonal [21, 22] as well as perennial [23] allergic rhinitis. In this so called 'minimal persistent inflammation' [24] infiltration of neutrophils and eosinophils is found without overt allergic symptoms. Detecting and treating this state is important, as data show a priming effect of the minimally inflamed mucosa leading to hyperreactivity of upper and lower airways and an increased allergic response [25].

Another interesting finding was the significantly elevated level of IL-17 in NARES. The recent identification of an IL-17-producing T helper cell line, Th17 cells, provided new insight into the development of infectious and autoimmune diseases as well as immune responses [26]. IL-17 can enhance human bronchial fibroblast production of IL-6, IL-8, and chemokine (C-X-C motif) ligand 1 (CXCL1) as well as human bronchial epithelial cell expression of IL-8, CXCL1, chemokine (C-C motif) ligand 20 (CCL20), intercellular adhesion molecule-1 and G-CSF [27, 28]. IL-8 and CXCL1 are potent neutrophil chemoattractants, whereas IL-6 and G-CSF are important in neutrophil maturation. Thus, IL-17 seems to play an important role in neutrophil activation and proliferation in forms of noneosinophilic asthma. Whether or not these findings can be transferred to inflammatory processes of the nose has not been studied yet. While in our study IL-6 and G-CSF were significantly elevated in NARES, IL-8 showed slightly lower levels. On the other hand, MIP-1 β , a chemoattractant for neutrophil granulocytes as well as for eosinophils and basophils, and MCP-1, a chemokine for monocytes, were detected in high levels in NARES, indicating that not only eosinophils play a role in this inflammatory disease. The etiology of neutrophilic infiltration in nonallergic rhinitis without infection is multifactorial, including pollutants and tobacco smoke [29, 30]. As we did not exclude smokers, this cause of neutrophilic infiltration cannot be ruled out. In further studies, nasal cytology should be performed to measure different cellular patterns, as a high presence of neutrophils could lead to resistance to nasal steroids as already shown for the lower airways [31]. Not only Th17 cells can be the source of IL-17 as neutrophils, eosinophils, plasma cells and serous glands have also been identified [32, 33]. A recent study by Saitoh et al. [34] detected IL-17 in the nasal polyps of patients with asthma and, besides CD4 T cells, could clearly determine eosinophils as the major source of IL-17. Moreover, the authors found correlations between IL-17-positive cells and the eosinophil recruitment

into the polyps, epithelial damage and the thickness of the basal membrane. This indicates that IL-17 is a determining factor for mucosal remodeling of nasal polyps being related to cytotoxic mediators derived from eosinophils and/or having a profibrotic capability itself. Some investigators even suggest NARES as being a precursor of nasal polyps, subsequent asthma or aspirin sensitivity [35].

As for Th2-cytokines, we found significantly higher levels of IL-4 in NARES compared to the controls, while IL-13 showed similar levels in the nasal secretions of all groups. IL-4 was insignificantly elevated in patients with PAR. Although both cytokines are key mediators in allergic inflammation [36] and should therefore be elevated in PAR, these results are in accordance with previous findings showing elevated levels of IL-4 in the nasal secretions of patients with allergic rhinitis [37, 38] but no elevation of IL-13 [13]. IL-5 was significantly elevated in PAR and to a similar extent also in NARES. It activates

the selective migration of eosinophils from the peripheral circulation into tissues and constitutes a major survival factor for eosinophils [39] being the only human leukocytes expressing a specific IL-5 receptor [40]. The interesting finding of high amounts of Th2 cytokines IL-4 and IL-5 in NARES cannot be explained yet. As we could detect IgE in the nasal secretions of about 20% of NARES subjects, it cannot be ruled out that in a small subgroup of NARES patients a local nasal allergic disease pathway may be present. However, this cannot sufficiently explain the more general characteristics of our findings. Therefore, further work is necessary to confirm these results.

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