

Original article

RANTES, eotaxin and eotaxin-2 expression and production in patients with aspirin triad

Background: Polyposis, asthma, aspirin-intolerance and aspirin-triad are mostly accompanied with eosinophilia of mucosal airways. Chemotactic cytokines, the CC-chemokines regulated on activation, normal T-cell expressed (RANTES), eotaxin, and eotaxin-2 activate and attract eosinophilic leukocytes to the site of inflammation. This points to the implication of CC-chemokines in eosinophilia of nasal tissue of these diseases.

Methods: Therefore, nasal polypous tissue specimens of patients suffering from chronic nasal polypous sinusitis (NP), intrinsic asthma (ATA), aspirin-intolerance (AINA), and aspirin-triad (TRIAD) were investigated. The amount of mRNA and protein of CC-chemokines was analyzed using semi-quantitative reverse transcriptase polymerase chain reaction and chemokine-specific enzyme-immuno-assays. The patterns of CC-chemokines were compared.

Results: The mRNA-expression as well as protein synthesis of CC-chemokines was quantified in all tissues investigated. The expression of RANTES-mRNA in NP, ATA, AINA, and TRIAD (averaging 148–324% D-glyceraldehyde-3-phosphate dehydrogenase) and protein synthesis (0.13–0.15 ng/mg tissue weight) did not differ significantly. But the protein synthesis of eotaxin- and eotaxin-2-mRNA was significantly ($P < 0.05$) higher in TRIAD (3.3 pg/mg and 3.4 ng/mg tissue weight) (4 ng/mg tissue weight), than in NP, ATA, or AINA (1.8 pg/mg and 2.1 ng/mg, 2.1 pg/mg and 1.6 ng/mg, or 1.7 pg/mg and 2.2 ng/mg tissue weight, respectively).

Conclusion: Patients suffering from TRIAD in association with tissue eosinophilia were characterized by elevated eotaxin and eotaxin-2 mRNA-expression as well as protein-synthesis. This pointed to the implication of eotaxins and RANTES in eosinophilia-associated diseases. Further studies will have to prove, whether the analysis of these chemokines might improve the diagnosis of eosinophilia associated polyposis and initiate the development of new therapeutic strategies.

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The triad of nasal polyposis, asthma bronchiale, and intolerance to nonsteroidal anti-inflammatory drugs – known as aspirin-intolerance – was described by several authors since first mentioned by Widal in 1922 (1). Chronic rhinosinusitis, especially if combined with recurrent nasal polyposis, is a common problem often seen in patients with symptoms associated with aspirin intolerance. This triad is associated with the presence of bronchial asthma, nasal polyposis as well as aspirin-induced asthmatic symptoms, sometimes along with nasal congestion, rhinorrhea and sneezing after ingestion of aspirin (1, 2). Aspirin-intolerance is defined as a nonallergic and non-IgE-mediated reaction and is rather seen as a pharmacological effect (3, 4). A dysregulation of the arachidonic acid metabolism in aspirin-intolerance with symptoms of asthma is suspected (5–9).

Neither the development of symptoms, nor the pathogenesis of the formation of nasal polyps is well-known. Tos constructed a model of polyp development which blames an increased edema of submucosal tissue associated with rupture of epithelial layer, prolaps into sinus, and following re-epithelization (10). The inflammatory mediators interleukin-5, eotaxin, eosinophilic cationic protein, leukotrienes, transforming growth factor and extracellular matrix components were measured in nasal polyps. Histomorphologic analysis of early stage manifestations of nasal polyposis showed the presence of eosinophilic granulocytes, forming a subepithelial cap over a pseudocyst area which was filled with albumin (11). However, none of these models explain the most striking common finding in all three diseases: the extensive eosinophilia of tissue.

Eosinophilic granulocytes are attracted and activated by chemokines, proteins of the cytokine family. The number of activated eosinophilic granulocytes correlates directly to nasal polyp formation; number and level of activity depend on the concentration of the chemokines in the tissue (12). Eosinophils initiate tissue damage by the release of cytotoxic substances like major basic protein, eosinophil cationic protein (ECP), eosinophil peroxidase, and autocrine production of chemokines which cause a self-sustained inflammatory process and chronic disease. The underlying mechanisms of selective infiltration of eosinophilic granulocytes into the tissue of diseased nasal mucosa are partially still unknown.

Four classes of chemokines, CXC, CC, C, and CX₃C, have been defined by the arrangement of conserved cystein-residues immature proteins. Chemokines are known to have leukocyte subtype-selective properties *in vitro*. In particular, the CC-chemokines regulated on activation, normal T-cell expressed (RANTES), eotaxin, and eotaxin-2 attract eosinophils *in vitro*. Therefore, these chemokines might explain leukocyte-specific infiltration of affected tissue (13).

The RANTES is produced by endothelium cells, fibroblasts, T-lymphocytes, eosinophilic granulocytes and other cells. There is a time- and dose-dependent upregulation after stimulation of fibroblasts from patients with polypous sinusitis with tumor necrosis factor- α or interferon- γ (14). It acts via CC-chemokine receptor (CCR)-1, -3 and -5 on monocytes, T-lymphocytes, eosinophilic and basophilic granulocytes. The RANTES is therefore unspecific for eosinophilic granulocytes expressing only CCR-3. Although eotaxin and eotaxin-2 have little structural homologies, they act exclusively on CCR-3 and show the same efficiency in selective eosinophil chemoattraction, but eotaxin seems to have a higher potency (15). Eotaxin-mRNA-expression is increased in nasal polypous tissue of atopic and nonatopic patients (16). Similar results were found for eotaxin-2 in bronchial biopsies of patients with asthma (17). Eotaxin is expressed by nasal fibroblasts and epithelial cells in cell culture, whereas eotaxin-2 was not detected in native fibroblasts (18, 14).

Eotaxin, eotaxin-2 and RANTES are ligands of the G-protein-coupled CCR-3-receptor, which is mainly found on eosinophils, in less quantities on basophils and activated TH₂-lymphocytes. The CCR-3 receptor also is activated by other chemokines, but eotaxins are the most potent mediators (14). Furthermore the expression of eotaxins is downregulated by glucocorticoids (19, 20).

The aim of this study was to investigate the pattern of RANTES, eotaxin and eotaxin-2 mRNA-expression and protein-synthesis in nasal polypous tissue. This was performed to clarify the implication of these chemokines on diseases associated with nasal polyposis.

Patients and methods

Classification of patients

Patients were classified in to four groups: patients with nasal polyps only (NP), aspirin-tolerant asthmatics (ATA), aspirin-intolerant-non-asthmatics (AINA) and aspirin-intolerant asthmatics (TRIAD). Nasal polyposis was diagnosed by anamnesis, endoscopic investigation and CT-scan. In this study 118 patients were investigated. Only those patients were included with nasal polyps having > 100 eosinophils/high power field (71%) were included. None of the patients were treated with antibiotics, corticosteroids (exception see ATA), anti-histamines, and anti-leukotrienes at least 2 weeks before surgery.

Nasal polypous tissue. Specimens of 41 NP-patients suffering from polypous rhinosinusitis (10 female and 31 male) aged 19–87 years were investigated. These patients had no further complications.

Aspirin-tolerant asthmatics. Polypous tissue specimens of 10 patients (seven females and three males) aged 23–76 years with long-term intrinsic asthma and nasal polyposis were included. The ATA patients were characterized by known asthma but without bronchial symptoms upon oral aspirin-provocation. All of them used inhalative β -sympathomimetics as regular medication, three of them used inhalative corticoids (budesonide 0.2 mg/day b.i.d.) as additional drug.

Aspirin-intolerant nonasthmatics. Polypous tissue specimens of eight aspirin-intolerant nonasthmatics (0 female and eight males) aged 12–46 years were selected. The AINA patients were characterized by sneezing, rhinorrhea, itching attraction and nasal obstruction or urticaria upon oral aspirin-provocation, but without bronchial symptoms at time of inclusion into the study.

Aspirin-triad. Polypous tissue specimens of 15 patients (seven females and eight males) with aspirin-triad (nasal polyposis, intrinsic asthma and aspirin-intolerance) aged 31–67 years were included. The TRIAD patients were characterized by bronchial symptoms upon oral provocation with aspirin and verification by body plethysmography.

Test procedure

The oral provocation test was performed for reviewing the existence of aspirin-intolerance. The test was performed at the department of dermatology of the university hospital of Kiel. Patients of known bronchial asthma were tested by a starting dose of 2 mg aspirin. The starting dose for patients with unknown bronchial asthma was 5 mg. This dose was increased up to 1000 mg in the provocation time of 2 days if no symptoms were observed. Otherwise, when bronchial symptoms occurred, this dose was verified by body plethysmography.

Histological examination, RNA-isolation and cDNA-synthesis

Nasal polypous tissue specimens were gained during endoscopic surgery of the sinus, immediately frozen in liquid nitrogen, and stored at –80°C until further processing. Eosinophilia was identified histologically by the department of Pathology, University Kiel. Only patients with > 100 eosinophils/high power field were included in the study.

Total-RNA was isolated according to the protocol of Chomcynski and Sacchi (21). RNA was quantified photometrically at 260 and 280 nm. Quality of isolated RNA was determined by measuring the absorption ratio at 260/280 nm and using an 1% agarose checking gel. Only RNA with a ratio between 1.7 and 2.0 and two clear bands (18s and 28s) on the gel were selected for further examination.

Semi-quantitative reverse-transcriptase polymerase chain reaction

Intron spanning sets of primers specific for D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to differentiate between genomic and cDNA-templates. The cDNA corresponding to 50 ng RNA served as template in a duplex-PCR-reaction containing 0.8 μ M of primers specific for RANTES, eotaxin or eotaxin-2 and (as internal control for equal amounts of cDNA before PCR) 0.1 μ M of a GAPDH specific primer pair (22). Amplification and analysis of the PCR products were performed as described (23).

To verify the primer-specificity, exemplary one band for every chemokine was extracted from the gel, nucleotide sequence was determined (310 Genetic Analyzer; Perkin Elmer, Weitenstadt, Germany), and compared with data from gene bank on PubMed. The forward primer (F) and backward primer (B) sequences and amplified products were as follows:

GAPDH: F: 5'-CCA GCC GAG CCA CAT CGC- 3'
 B: 5'-ATG AGC CCA GCC TTC ACC AT- 3' (360 bp)
 RANTES: F: 5'-CAT CCT CAT TGC TAC TGC CCT CTG-3'
 B: 5'-CGG GTT CAC GCC ATT CTT CT-3' (618 bp)
 Eotaxin: F: 5'-CCC AAC CAC CTG CTT TAA CCT-3'
 B: 5'-TGG CTT TGG AGT TGG AGA TTT TTG-3' (208 bp)
 Eotaxin-2: F: 5'-CTA CCG GCT CTG TGG TC-3'
 B: 5'-GGT TTG GTT GCC AGG ATA-3' (290 bp)

ELISA

Protein concentration was determined by enzyme linked immunosorbent assay (ELISA), therefore exactly 200 mg of polyp tissue were ground up under liquid nitrogen, dissolved in 2 ml citrat-buffer pH 2.5 and homogenized with ultrasound for 1 min. After centrifugation and lyophilization, phosphate buffer pH 7 was added and the solution stored at -70°C until further processing.

The protein concentration was determined on the one hand by double sandwich ELISA. A 96-well microtiter plate (Nunc, Roskilde, Denmark) was incubated overnight at 4°C with 10 μ g/ml polyclonal mouse-anti-RANTES/ -eotaxin/ -and goat-anti-eotaxin-2 IgG in coating buffer containing 0.1 M Na₂CO₃ and 0.2 M NaHCO₃ (pH 10.5), washed with PBS containing 0.5% (v/v) Tween (Calbiochem, Bad-Godesberg, Germany) and blocked with 1% (w/v) bovine serum albumin in PBS for 1 h at room temperature. Subsequently, a total of 40 samples in pairs each of 100 μ l were incubated for 1 h at room temperature. Then secondary mouse monoclonal antibodies (clones 2D5/G6 and 1D2/A12, produced in the lab of the Dermatology Department, University of Kiel, Germany) for 45 min and tertiary peroxidase labeled anti-mouse gamma globulin (Jackson Immunoresearch Lab., West Grove, PA) for 30 min were added. Finally, the plates were treated with o-phenylenediamine dihydrochloride substrate solution (Sigma, St Louis, MO), according to the manufacturers instruction, for 10–15 min and the reaction was stopped by the addition of 100 μ l of 3 M H₂SO₄ per well. To determine chemokine concentration dilution of recombinant human RANTES/ eotaxin /eotaxin-2 (R & D Systems; Wiesbaden, Germany) from 9 to 5000 pg per 100 μ l was used.

Optical density was measured at 492 nm in a microplate reader (MR-5000 Dynatech Lab., Denkendorf, Germany). A computerized complete calibration curve was used to calculate the concentration of RANTES/eotaxin/eotaxin-2 in ng protein/ml. Results were expressed in ng/mg polypous tissue.

Statistical evaluation

Statistic analysis was performed using a Kruskal–Wallis test for detecting any significant differences between the four groups. Specifying the results a multiple-pair comparison with the control group was performed according to Zar (24). $P < 0.05$ was considered significant and marked with *. Outliers higher or lower than 1.5-fold of the quartile distance are marked with °.

Results

The variance of the expression of RANTES-mRNA did not differ significantly among the groups (Fig. 1A).

The RANTES protein synthesis did not significantly differ between the diseases revealing values between 0.13 and 0.15 ng/mg on average (Fig. 2A).

Expression of eotaxin-mRNA in samples from TRIAD was significantly ($P = 0.019$) elevated when compared with nasal polyps. Samples from patients with ATA or AINA did not show differences to nasal polyps (Fig. 1B).

Eotaxin protein synthesis corresponded with eotaxin-mRNA expression. Samples from patients with ASS-triad contained significantly higher amounts of eotaxin protein (3.3 pg/mg on average) than nasal polyps. Eotaxin protein synthesis did not show significant differences between the other diseases (Fig. 2B).

There was a high variance of the expression of eotaxin-2-mRNA which did not differ significantly among the groups. Expression of eotaxin-2-mRNA in samples from TRIAD was elevated when compared with nasal polyps, but results were not significant. Samples from patients with ATA or AINA did not differ from control (Fig. 1C).

Eotaxin-2 protein synthesis of samples from ASS-triad patients were significantly ($P = 0.001$) increased (3.4 pg/mg) compared with nasal polyps (2.1 pg/mg). Eotaxin-2 protein synthesis did not significantly differ between the other diseases (Fig. 2C).

Discussion

Chronic-polypous sinusitis, asthma, and aspirin-intolerance frequently occur together. As its description of the triad by Widal in 1922 and Samter and Beers in 1968, the acronyms 'Syndrom Widal' (1) and 'Samter's disease' (2) were introduced. The pathogenesis of aspirin-intolerance has not yet been clearly defined. The risk of recurrence seems to be significantly increased in patients with aspirin-intolerance (25). Several studies have shown that aspirin-sensitive individuals can be desensitized by oral administration of aspirin over a longer period of time

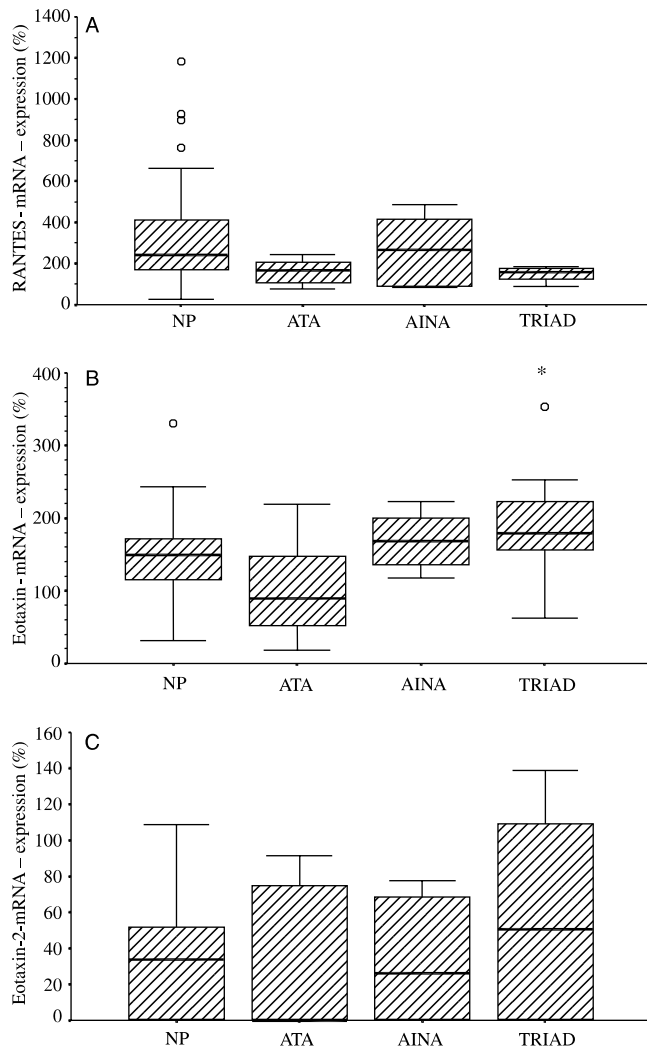


Figure 1. Chemokine mRNA expression of the chemokines RANTES (A), eotaxin (B) and eotaxin-2 (C) in polypous tissue (NP; $n = 41$), polypous tissue from aspirin tolerant asthmatics (ATA; $n = 10$), polypous tissue from aspirin-intolerant non-asthmatics, (AINA; $n = 8$) and aspirin-triad (TRIAD; $n = 15$). Values are expressed as quotient of chemokine expression to D-glyceraldehyde-3-phosphate dehydrogenase-expression. Horizontal lines represent the median, squares the 25th and 75th percentiles. Outliers higher or lower than 1.5-fold of the quartile distance are marked with °, statistical significances (P -value $< 0,05$) are marked with *.

(26), which may result in a decreased risk of recurrence of nasal polyps. Therefore the identification of those individuals with aspirin intolerance is essential, even if they do not represent the full clinical triad. On the one hand, inhibition of cyclooxygenase and lack of metabolites, e.g. prostaglandin E_2 , is assumed to initiate a chain of reaction (6). On the other hand leukotrienes are proposed mediators of aspirin-induced asthma (27).

All three major symptoms of aspirin triad are accompanied by eosinophilia of mucosal airways.

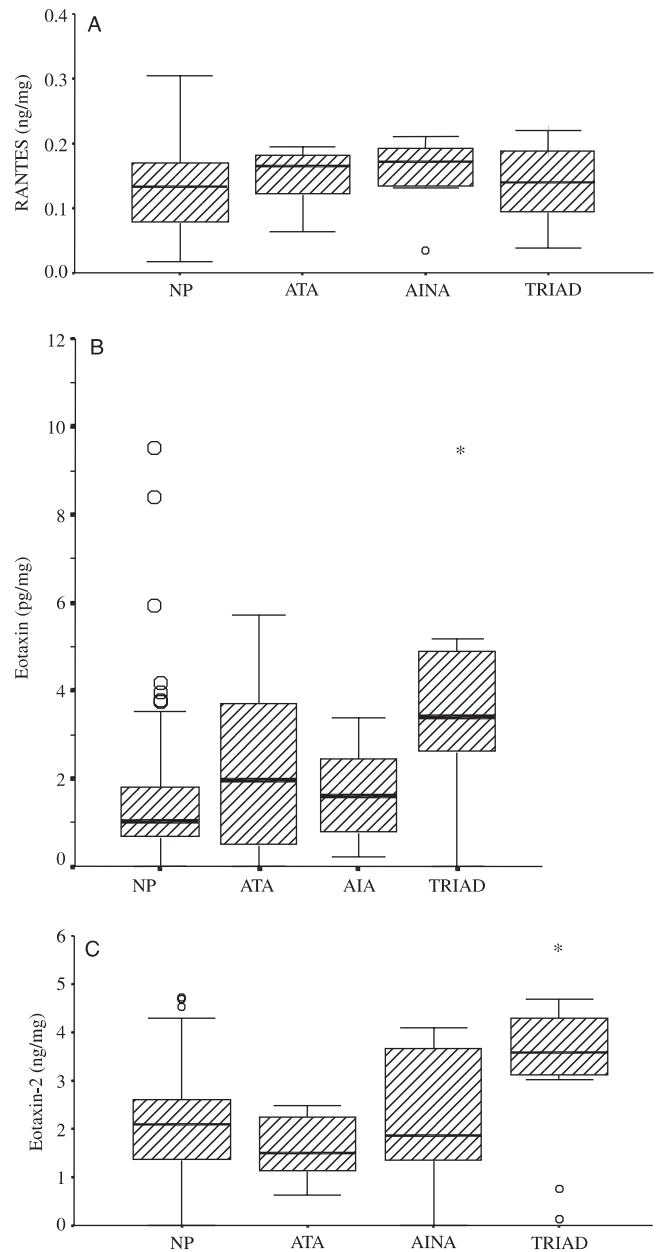


Figure 2. Chemokine protein-levels of the chemokines RANTES (A), eotaxin (B) and eotaxin-2 (C) in polypous tissue (NP; $n = 41$), polypous tissue from aspirin tolerant asthmatics (ATA; $n = 10$), polypous tissue from aspirin-intolerant nonasthmatics, (AINA; $n = 8$) and aspirin-triad (TRIAD; $n = 15$). Values are expressed in ng/mg tissue (RANTES), pg/mg tissue (eotaxin) and ng/mg tissue (eotaxin-2). In (B) three outliers in the TRIAD-group are not displayed.

Aspirin-intolerant patients frequently show a peripheral blood eosinophilia and elevated levels of ECP in nasal secretions after provocation (28, 29). The number of activated eosinophils in nasal polyps is significantly higher than in unaffected nasal tissue (12). This points to the implication of eosinophils in the nasal mucosa of

these diseases, although the underlying chemotactic mechanism causing selective infiltration of eosinophilic granulocytes into the tissue are unknown.

CC-chemokines attract and activate eosinophilic leukocytes to the site of inflammation (13, 30). Immunohistochemical staining of RANTES in nasal polyp tissue demonstrated largely localization to airway and glandular epithelium (31). Furthermore eosinophilic chemotactic activity was extracted from nasal polyps (32). In addition, certain leukocytes as well as respiratory epithelium and human nasal polyps were immunohistochemically stained using anti-eotaxin monoclonal antibodies, and infiltration of eosinophilic granulocytes occurred at sites of eotaxin upregulation (33). Also significantly higher amounts of eotaxin and eotaxin-2 were found in polypous tissue than in middle turbinates of controls (34). The RANTES is supposed to be associated with TH₁ immune response (35), whereas eotaxin and eotaxin-2 are most likely associated with TH₂-immune response (36).

In our study RANTES-, eotaxin-, and eotaxin-2-expression and -production was measured in polypous tissue of all patient groups. Protein synthesis and RANTES mRNA-expression did not differ between the diseases, whereas eotaxin and eotaxin-2 mRNA and protein synthesis was significantly elevated in samples from patients suffering from aspirin-triad.

These results are in accordance with others showing a correlation of eotaxin-, but not RANTES-mRNA expression, with tissue eosinophilia, nasal ECP levels and IL-5 (12, 37).

We conclude from our results that eotaxin and eotaxin-2 are most likely implicated in the underlying mechanisms of eosinophilic attraction in eosinophilia associated diseases investigated in this study. This might be especially true for the aspirin-triad. We hypothesise a disease-specific CC-chemokine based receptor binding and activation mechanism. Further studies were initiated to clarify the implication of the CCR-3-receptor in eosinophilia-associated diseases like aspirin-triad. Clarifying the underlying mechanisms of eosinophilic attraction might improve the diagnosis and initiate the development of new therapeutic drugs for the treatment of eosinophilic associated diseases like aspirin-triad.

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