

Original article

Increased expression of lipoxygenase enzymes during pollen season in nasal biopsies of pollen-allergic patients

Background: Exposure of patients sensitized to pollen triggers development of seasonal allergic rhinitis symptoms (SAR). Eicosanoids are a group of arachidonic acid metabolites contributing to the symptoms of SAR. The aim of this study was to investigate seasonal changes in the expression of enzymes of the eicosanoid pathway in the nasal mucosa of patients with SAR.

Methods: Twenty SAR patients allergic to birch or grass and eight healthy subjects were included in the study. Patients registered rhinoconjunctivitis symptoms and use of rescue medication before and during the pollen season. Nasal biopsies were obtained before and around the peak of the season, sectioned and stained using markers for eosinophils, mast cells, T cells and neutrophils. Antibodies against the following enzymes were also used: cyclooxygenase (COX-1, COX-2), 5-lipoxygenase (5-LO), 5-lipoxygenase-activating factor (FLAP), LTA₄ hydrolase (LTA₄h) and LTC₄ synthase (LTC₄s).

Results: During the pollen season symptoms of rhinoconjunctivitis and medication score increased significantly ($P = 0.001$; $P = 0.001$ respectively). During the pollen season numbers of eosinophils ($P = 0.02$) and cell positive 5-LO ($P = 0.02$), LTC₄s ($P = 0.04$) and LTA₄h ($P = 0.02$) increased significantly. During season number of mast cells and cells expressing 5-LO and LTA₄h were higher in SAR than in healthy controls group ($P = 0.02$; $P = 0.01$; $P = 0.03$ respectively).

Conclusion: In sensitized patients exposure to pollen allergen results in increased expression of enzymes of the eicosanoid pathway.

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In sensitized patients, seasonal exposure to pollen allergens and their interaction with specific immunoglobulin E (IgE) antibodies on mast cells and basophils triggers a cascade of immunological reactions that results in symptoms of seasonal allergic rhinitis (SAR; 1).

Early symptoms that occur following allergen exposure such as itching, sneezing and runny nose are predominantly evoked by histamine released from the granules of mast cells. At the same time cellular activation results in *de novo* synthesis of other groups of inflammatory mediators: eicosanoids [arachidonic acid (AA) metabolites] contributing to the development of clinical symptoms such as prostaglandins (PG) and leukotrienes (2).

Increased synthesis of eicosanoids has also been detected after nasal allergen provocation (3, 4) and during the natural pollen season (5).

Eicosanoids are produced by enzymatic transformations of AA that is released from the nuclear membrane by phospholipase A₂ action. The AA is metabolized by either of the cyclo-oxygenase isoforms COX-1 or COX-2 or by 5-lipoxygenase (5-LO). The COX-1 is produced constitutively in many tissues while COX-2 is mainly induced at sites of inflammation (6).

The 5-LO translocates to a nuclear membrane and associates with 5-LO-activating protein termed FLAP (7). Activated 5-LO converts the AA to the unstable product LTA₄, which is further processed to LTB₄ by LTA₄ hydrolase (LTA₄h) (8) and/or to LTC₄ by LTC₄ synthase (LTC₄s) (9). LTC₄s is responsible for the addition of glutathione to LTA₄ resulting in the production of LTC₄. LTC₄ is further transformed to LTD₄ and LTE₄ – the end product excreted in the urine (10).

It has recently been shown that the concentration of LTE₄ in urine is significantly higher in SAR patients with severe nasal blockage compared to those with mild disease or without nasal blockage or healthy controls (11).

A number of studies have demonstrated that eicosanoids play an important role in the pathogenesis of asthma (12, 13). We have previously examined the expression of eicosanoid pathway enzymes in bronchial biopsies from patients with seasonal asthma (13) and found increase during the pollen season clearly indicating the importance of AA metabolites in the development of allergic inflammation in the lower airways. In this study we examined changes in expression of eicosanoid enzymes in nasal mucosa biopsies obtained before and during the pollen season from patients with SAR.

Table 1. Characteristics of patients – data from SAR patients and control subjects: age [mean (minimum–maximum)], SPT and specific IgE test [median (minimum–maximum)]

Patients	SAR	Control
Number	20	8
Age (years)	34 (20–55)	26 (22–30)
Sex (M/F)	11/9	5/3
SPT diameter (mm)	5.75 (2–9.5)	Negative
Specific IgE (kU/l)	15.15 (1.97–100)	<0.35

SAR, seasonal allergic rhinitis; SPT, skin prick test; IgE, immunoglobulin E.

Patients

Twenty patients with a clinical history of seasonal rhinitis (14), induced by birch or grass pollen, were included in the study. Allergy was confirmed by positive skin prick test (SPT: ≥ 3 mm) and the presence of specific serum IgE antibodies (class: ≥ 2) against birch or grass (CAP-RAST, Pharmacia Diagnostics, Uppsala, Sweden). Ten of the patients had also positive SPT for cat or dog allergen, but they did not own pets and were not exposed to them on a daily basis. Eight matched healthy individuals, with no history of rhinitis, negative SPT and IgE *in vitro* test were also included and served as a control group (Table 1).

Patients were not allergic to house dust mite or moulds (had no symptoms of perennial allergy and had negative SPT test) and did not suffer from lower airways symptoms during pollen season.

They were never treated with specific immunotherapy and refrained from taking steroids during the study. All patients provided written informed consent. The Ethics Committee of Sahlgrenska University Hospital approved the study.

Methods

Clinical symptoms

Allergic patients recorded eye and nose symptoms and the use of rescue medication daily during 1 week in winter (outside the pollen season) and over 2 weeks around the peak of the pollen season (turn of April and May for birch pollen, and turn of June and July for grass pollen).

Symptoms were recorded on a 4-point scale (0 – no symptoms; 1 – mild; 2 – moderate; 3 – severe). The total rhinoconjunctivitis symptom score was calculated from the average of daily ocular and nasal symptom scores.

During the pollen season patients were supplied with rescue medication to alleviate their symptoms. Each drug was scored for the duration of the dosing according to the recommendation of the manufacturer. No drugs other than those prescribed were permitted. Medication was scored as follows: oral antihistamine Semprex® (acrivastine; GlaxoSmithKline, Solna, Sweden), 8 mg, 1 point/tablet; nasal spray: local antihistamine Livostin® (levocabastine; Janssen-Cilag, Sollentuna, Sweden), 50 µg/dose, 1.5 points/puff and decongestant Otrivin® (xylometazolin; Novartis, Taby, Sweden), 1 mg/ml, 1 point/puff; eye drops: Lomudal® (disodium chromoglycate; Aventis Pharma, Stockholm, Sweden), 40 mg/ml, 2 points/drop and Antasten Privin (antazoline, naphazolin), 5 mg/ml,

1 point/drop. Patients were asked to refrain from taking any medication on the day when biopsy was taken.

Total symptom and medication scores during 1 week outside the pollen season and 2 weeks during the season were counted and differences evaluated statistically.

Nasal biopsy sampling

Nasal mucosa specimens were obtained from inferior turbinate, twice during the study, before and around the peak of the pollen season. Biopsies from control group were taken out of the season. Local anaesthesia of the inferior nasal turbinate was achieved by topical application of cotton wool carrier presoaked in 1 ml of a solution of 4% cocaine and 0.02% adrenaline. After 15 min, a submucosal injection of Xylocain™ (10 mg/ml lidocaine with 5 µg/ml adrenaline; Astra, Södertälje, Sweden) was given and a biopsy was obtained using Gerritsma forceps (Enter-Med™, Woerden, Holland).

The biopsy was immediately embedded in Tragicanth™ gum (Acros Organics, Fairlawn, NJ, USA) and snap-frozen in isopentane cooled with liquid nitrogen. Biopsies were stored at -70°C .

Immunocytochemistry

Biopsy sections 4 µm thick were cut using a cryostat (Leica CM1800, Nustoch, Germany), placed on a slide and air-dried at room temperature.

Mouse monoclonal antibodies were specific for immunostaining of eosinophils (EPO, 1 : 400, a gift from Dr C. Petersson, Pharmacia Diagnostics), mast cells (AA1, 1 : 100; Dakopatts, Glostrup, Denmark), neutrophils (HNL, 1 : 100, a gift from Dr C. Petersson, Pharmacia Diagnostics), T cells (CD4, 1 : 50; Dakopatts), COX-1 (1 : 25; Cayman Chemical Company, Ann Arbor, MI, USA) and COX-2 (1 : 10; Pharmingen, San Diego, CA, USA).

Polyclonal rabbit sera were used to detect 5-LO (1 : 50; Cayman Chemical Company); LTC₄s (1 : 1000), 5-lipoxygenase-activating factor (FLAP; 1 : 300) and LTA₄h (1 : 100; gift from J. Evans, Merck Research Laboratories, Rahway, NJ, USA).

The avidin-biotin (ABC) method was used to detect expression of 5-LO, FLAP, LTA₄h, LTC₄s and COX-1.

Briefly, nasal biopsy sections were fixed and incubated successively with: (i) primary antibody, (ii) secondary biotinylated antibody (Dako Corporation, Carpinteria, CA, USA) for 30 min and (iii) biotinylated peroxidase or alkaline phosphatase conjugated to avidin (ABC/HRP or ABC/AP complex) for 30 min (Vector Laboratories, Burlingame, CA, USA). The reaction was visualized using 3-amino-9-ethylcarbazole (AEC) or Fast Red substrate.

The alkaline phosphatase antialkaline phosphatase (APAAP) method was used as previously described to stain sections with antibodies specific for eosinophils, mast cells, neutrophils, T cells and COX-2. Briefly, sections were incubated successively with: (i) primary mouse antibody, (ii) rabbit antimouse antibody for 30 min and (iii) mouse APAAP complex for 30 min. Fast Red substrate was used to develop the staining.

Double staining

Double staining (using antibody against the eosinophil marker EPO and against LTC₄s) was performed to determine the number of eosinophils producing LTC₄s. Sections were first incubated with a mixture of primary antibodies against EPO and LTC₄s for 1 h, then with secondary rabbit antimouse antibody (Dako Corporation) and finally with APAAP complex. Staining to detect eosinophils was developed with Fast Blue substrate.

To stain for LTC₄s secondary biotin-conjugated, goat antirabbit antibody (Dako Corporation), followed by ABC complex (biotinylated peroxidase conjugated to avidin, ABC/HRP) was used. Staining was developed with AEC (Dako Corporation).

Quantification

Cells were counted using a Nikon Eclipse E600 microscope at 400× magnification (Nikon Corp., Tokyo, Japan). Easy Image 2000 Measuring System (Bergström Inst AB, Solna, Sweden) software was used to capture images and perform counts. Cells were counted in the epithelium and in all accessible fields under the basal membrane. Positively stained cells were expressed as numbers per 1 mm².

Statistical methods

Average of total symptoms score registered each day before (1 week) and during season (2 weeks) was counted. Statistical difference between average total symptoms and medication scores before and during the pollen season were evaluated using the Wilcoxon (signed rank) test for matched pairs.

The difference in the number of cells stained positively for each marker before and during the season was also assessed using the Wilcoxon (signed rank) test.

Comparisons between the number of cells staining positively for each marker for patients with SAR and healthy controls were made using the Mann-Whitney *U*-test. A *P*-value of ≤ 0.05 was considered statistically significant. All tests were performed using the STATSOFT software package (StatSoft, Tulsa, OK, USA).

Results

Clinical results

During the pollen season rhinoconjunctivitis symptoms increased significantly ($P = 0.001$) in SAR patients from 0 ± 0.0 to 1.03 ± 0.15 (mean $\pm$ SEM).

Average medication score also increased significantly ($P = 0.001$) during the season from 0 ± 0.0 to 2.17 ± 2.15 . Symptoms and medication scores paralleled the amount of pollen allergen in the air (Fig. 1).

Immunocytochemistry results

In nasal biopsies from SAR patients significant increases in the number of eosinophils ($P = 0.02$) were observed during the pollen season. The number of mast cells, neutrophils and T cells remained unchanged (Table 2).

However, mast cell numbers were significantly higher in SAR patients during the season than in healthy controls ($P = 0.02$).

Significantly higher number of cells positive for 5-LO ($P = 0.02$), LTC₄s ($P = 0.03$) and LTA₄h ($P = 0.02$) were found during the pollen season (Fig. 2A,C,D). Unaltered number of cells positive for FLAP (Fig. 2B), COX-1 and COX-2 were observed.

During the pollen season the number of cells positive for 5-LO, LTA₄h and LTC₄s were higher in SAR patients compared with the healthy controls. This difference was

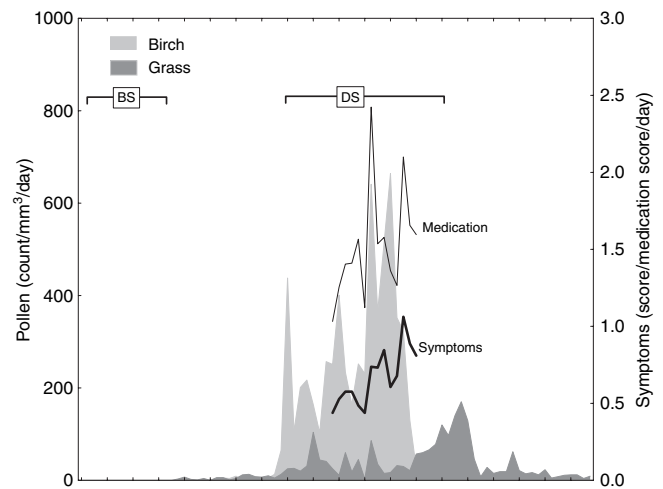


Figure 1. Symptom scores (bold line) and use of medication (fine line) registered by seasonal allergic rhinitis (SAR) patients during 2 weeks around the peak of the pollen season. Significant increases were seen during the season (DS) compared with before the season (BS) for both symptom scores ($P = 0.001$) and the use of medication ($P = 0.001$). Pollen counts are shown for the period of 6 weeks covering the birch and grass pollen seasons.

Table 2. Results of immunocytochemical staining of sectioned nasal mucosa biopsies obtained from SAR patients before (BS) and during (DS) and from HC

Marker	BS	DS	HC
SAR patients			
EPO (eosinophils)	22 (11–38)	49 (7–101)	16 (6–41)
CD4 (T cells)	105 (43–214)	141 (53–230)	123 (79–153)
AA (mast cells)	42 (32–76)	55 (37–69)	27 (9–52)
HNL (neutrophils)	38 (25–56)	56 (30–74)	41 (20–81)
COX-1	69 (24–98)	88 (50–105)	39 (10–92)
COX-2	42 (37–79)	47 (31–69)	42 (34–61)
5-LO	48 (39–99)	77 (67–114)	49 (27–58)
LTC ₄ synthase	81 (44–113)	96 (84–119)	64 (48–101)
FLAP	124 (60–194)	108 (51–152)	85 (60–112)
LTA ₄ hydrolase	42 (22–67)	50 (33–77)	32 (21–419)

All results are given as medians (quartile range).

SAR, seasonal allergic rhinitis; 5-LO, 5-lipoxygenase; FLAP, five lipoxygenase-activating factor; HC, healthy control; AA, arachidonic acid; COX, cyclo-oxygenase.

significant for 5-LO and LTA₄h ($P = 0.01$ and $P = 0.03$, respectively) but the tendency towards significance was also clear for LTC₄s ($P = 0.056$). The majority of eosinophils (68%) during the pollen season were also positive for LTC₄s, as was shown using the double-staining method (Fig. 3).

Discussion

In patients with a clinical history of pollen allergy, we found that symptoms of rhinoconjunctivitis and the use of rescue medication increased significantly during the pollen season. Symptom and medication scores paralleled the amount of pollen grains in the air.

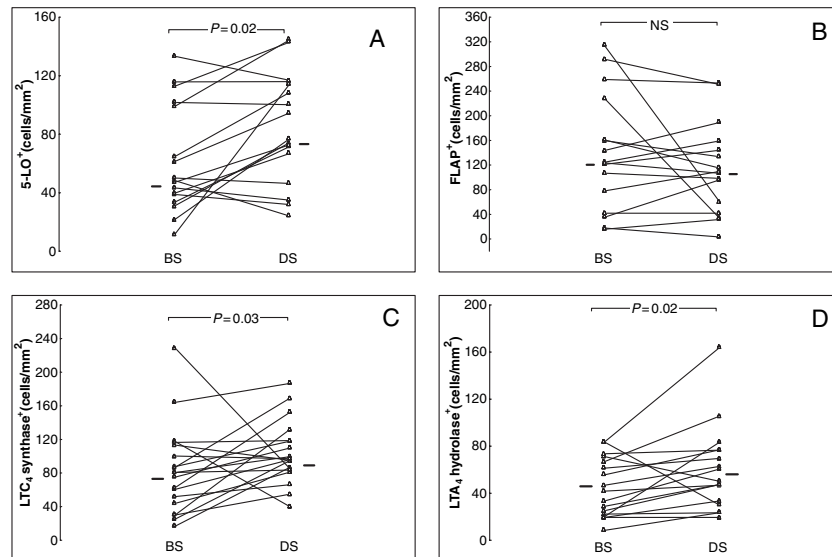


Figure 2. Number of cells stained positively for 5-lipoxygenase (5-LO; A); 5-lipoxygenase-activating factor (FLAP; B); LTC₄ synthase (C) and LTA₄ hydrolase (D) in 1 mm² of nasal tissue biopsies (epithelium and lamina propria) from individual seasonal allergic rhinitis (SAR) patients. Horizontal bars depict median values for before (BS) and during the pollen season [DS; nonsignificant results (NS)].

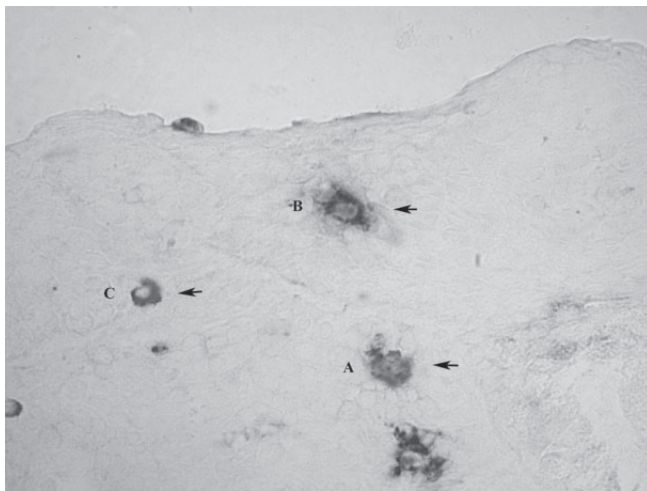


Figure 3. Double staining with antibodies against EPO and LTC₄ synthase at magnification 400x. (A) Cell positive for EPO (eosinophil); (B) cell positive for EPO and LTC₄ synthase (eosinophil-positive for LTC₄); (C) cell positive for LTC₄ synthase.

In nasal biopsies we observed significant increase in the number of eosinophils during the pollen season compared with the baseline; however, the numbers of mast cells, T cells and neutrophils remained unchanged. Compared with healthy control number of mast cells during the season were significantly higher in SAR patients. Significant increases in cells positive for 5-LO, LTC₄s and LTA₄h were also seen during the season.

No significant seasonal changes in the number of cells positive for enzymes of the prostanoid pathway, COX-1

and COX-2, were found. In sensitized individuals seasonal exposure to allergens leads to chronic inflammation of the nasal mucosa, characterized by infiltration of different cell populations.

In some previous studies of patients with SAR, an influx of eosinophils, mast cells (15) and T cells (16) into the nasal mucosa during the pollen season has been demonstrated. There are a number of reasons that could explain the lack of seasonal increase in mast cells in the nasal mucosa seen in our study. One could be the relatively moderate pollen season reflected in corresponding clinical symptoms. The other the tendency in SAR patients to display higher number of mast cells in nasal tissue outside the pollen season compared with the control subjects.

As 50% of SAR patients were also allergic to pets, certain involuntary exposure to cat and dog dander cannot be excluded (17). Although such a weak exposure would not be manifested clinically it could still contribute to the increased number of mast cells found outside the pollen season.

Finally, the lack of increase in number of mast cells could at least partly be explained by their degranulation as we used antibody directed against tryptase as a marker for mast cells (15).

Different populations of inflammatory cells present in the nasal mucosa during allergic inflammation are able to produce eicosanoids. Eosinophils (18) and mast cells (19) are considered the major sources of LTC₄, while monocytes and macrophages produce both LTB₄ and LTC₄ (20) and neutrophils mainly LTB₄ (21).

Neutrophils, monocytes and lymphocytes are all rich sources of LTA₄h, which is an important enzyme in the

production of LTB₄, while eosinophils and mast cells have very low levels of this enzyme (8). LTB₄, produced from LTA₄ by the action of LTA₄h, has chemoattractive properties for both eosinophils (22) and neutrophils (4). Following local allergen challenge of patients with SAR, significantly increased levels of LTB₄ in nasal lavages were demonstrated. LTB₄ levels correlated significantly with the number of neutrophils (4).

Other leukotrienes produced from LTA₄ by the action of LTC₄s (LTC₄, LTD₄, LTE₄) have also been shown to possess chemoattractive properties. In asthmatic patients the number of eosinophils in sputum increased following LTD₄ inhalation (23). Influx of neutrophils and eosinophils into the bronchial mucosa was also observed in biopsies after LTE₄ inhalation (24).

Eosinophils attracted to the site of inflammation are also capable of elaborating lipid mediators themselves thus amplifying and maintaining chronic allergic inflammation.

We have shown that the majority of eosinophils have the ability to produce LTC₄s. This confirms the findings of a study performed on cells from nasal lavages of SAR patients obtained during the pollen season, demonstrating that most eosinophils produced LTC₄s, 5-LO and FLAP (25).

In the present study, numbers of cells positive for 5-LO increased significantly but no changes have been found for FLAP-positive cell numbers. However, our previous results obtained from bronchial biopsies of patients with mild asthma in the same seasonal model showed significantly more FLAP-positive cells during the season (13).

Moreover, Coffey et al. compared expression of 5-LO and FLAP in peripheral blood monocytes and alveolar macrophages using immunoblot and demonstrated that alveolar macrophages produced significantly more FLAP than blood monocytes, while production of 5-LO was comparable between these two cell populations (26).

Although we cannot draw conclusion about the amount of the product based on a qualitative method used in our study, considering findings from bronchial biopsies and alveolar macrophages we can postulate that the pattern of FLAP production in nasal mucosa of SAR patients could be different from that in bronchial mucosa of allergic asthmatics. This suggests that FLAP expres-

sion could vary widely between different cell populations and different organs.

We have also examined the number of cells expressing COX-1 and COX-2 that catalyse the production of PGs. PGD₂ is the predominant prostaglandin released following mast cell activation in allergic rhinitis (2). Significantly higher concentrations of PGD₂ in nasal lavages have been found in patients with allergic rhinitis compared with healthy individuals (27).

We have not found any changes in number of cells positive for COX-1 or COX-2. This confirms our earlier findings from bronchial biopsies obtained during the birch pollen season (13).

Two other previous studies have examined COX-1 and COX-2 expression in allergic patients using immunocytochemical methods. No differences in the number of cells positive for COX isoforms between patients with chronic allergic rhinitis and healthy control subjects were found in nasal mucosa biopsies (28). However, Church et al. found significant increase in number of cells expressing COX-2 in skin biopsies obtained 8 h after allergen provocation (29). It is possible that in a challenge model, high doses of allergen, applied intradermally evokes more vigorous inflammation, which may be necessary to permit changes in COX-2 expression to be seen. It is also possible that there are differences in the pattern of expression of COX-2 between organs-like variation between bronchial and nasal biopsies observed in case of FLAP expression.

In conclusion, we have confirmed that patients with allergic rhinitis induced by seasonal exposure to birch and grass pollens have increased number of eosinophils in the nasal mucosa. The finding of increased expression of enzymes metabolizing AA to leukotrienes, highlights the importance of this group of mediators in the symptomatology of SAR.

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