

## Aspirin-sensitive rhinitis-associated changes in upper airway innervation

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**ABSTRACT:** Aspirin-sensitive rhinitis is the manifestation of aspirin intolerance in the upper respiratory tract. The disease represents a pseudoallergy against aspirin or related nonsteroidal anti-inflammatory drugs. As a major immunomodulatory role for airway innervation has been proposed in airway inflammatory diseases, the present study assessed changes in human nasal mucosa innervation in patients with aspirin-sensitive rhinitis in comparison to a control group.

Immunohistochemistry for protein gene product 9.5, tachykinins, calcitonin gene-related peptide, vasoactive intestinal peptide (VIP) and neuropeptide tyrosine was performed on cryostats sections of nasal mucosa and neuropeptide containing nerves were examined independently using a score grading.

In comparison to the control, the aspirin-sensitive rhinitis group had a significant increase of VIP-like immunoreactivity in mucosal nerve fibres. In contrast to constant numbers of mast cells, highly significant increases in the numbers of eosinophils were found in the group of aspirin-sensitive rhinitis patients.

In summary, the present quantification of neuropeptide-immunoreactivity of mucosal nerves demonstrated differences in the human nasal mucosa innervation between nonrhinitic and aspirin-sensitive rhinitic individuals. These differences may reflect a pathophysiological role of upper airway innervation in pseudoallergic reactions.

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Aspirin (acetylsalicylic acid) is still one of the most frequently used drugs of all times. In addition to its role as an analgetic drug, aspirin is also increasingly used in the prophylaxis of vascular diseases. The prevalence of aspirin intolerance varies from 5–6% [1] and an association of nasal polyposis, asthma and sensitivity towards aspirin has been known for ~80 yrs [2]. The association of asthma, nasal polyposis and aspirin sensitivity is known as the aspirin triad [3].

Aspirin-induced asthma (AIA) represents the development of a severe drop in forced expiratory volume in one second (FEV<sub>1</sub>) in patients following the administration of aspirin. In patients with AIA, acute symptoms are often superimposed on chronic severe asthma. The attacks may be precipitated following the ingestion of small amounts of aspirin or other nonsteroidal anti-inflammatory drugs (NSAID)s. HEDMAN *et al.* [4] showed an overall aspirin intolerance of 5.7% and the prevalence of AIA to be 1.2%. Over the last few years, there has been an increased understanding of the pathogenesis and management of AIA. Aspirin-induced bronchoconstriction is thought to be caused by the shunting of the arachidonic acid metabolism away from the cyclooxygenase pathway toward the lipoxygenase pathway. This results in an increased production of leukotrienes (LT)s with resultant bronchoconstriction [2]. Consistent with this finding, a severe drop in FEV<sub>1</sub> in

patients with AIA can be inhibited by LT receptor antagonists [5] and an abnormal release of LTs is found in aspirin-sensitive asthmatic patients, which contributes to nasal and bronchial adverse reactions [6].

It is known that rhinosinusitis in aspirin-sensitive asthmatic patients is more severe than in aspirin-tolerant patients [7]. Also, the extent and severity of the mucosal inflammation has been suggested to be higher in aspirin-sensitive patients than that in aspirin-tolerant patients. Using computed tomographic scanning to assess the extent of sinus involvement and measurement of mucosal thickening in the paranasal sinuses, a semiquantitative scoring system was developed and revealed that the extent of hyperplastic rhinosinusitis is greater in aspirin sensitive than in aspirin-tolerant asthmatic subjects with rhinosinusitis of similar clinical severity [3].

It is not clear if the basic mechanism of chronic airway inflammation in aspirin-sensitive patients is different from that in aspirin-tolerant patients with chronic rhinosinusitis and nasal polyps. As the autonomic airway nervous system has been reported to play a major role in the development of inflammatory airways diseases of the upper and lower respiratory tract [8, 9], it was presently hypothesised that aspirin-sensitive patients have an altered pattern of neuropeptide-immunoreactive nerve fibres in their nasal mucosa if compared to subjects with no rhinitis. Therefore, the present project investigated the

composition of neuropeptide-containing nasal mucosal nerves in a group of aspirin-sensitive rhinitis patients and nonrhinitic individuals and assessed numbers of eosinophils and mast cells.

## Materials and methods

### Subjects

After approval by the ethics committee and informed consent from each individual, a total of 24 male and female individuals (12 with aspirin sensitivity and 12 controls) were included in the present study (table 1). For the identification of aspirin-sensitive rhinitis, a positive medical history for aspirin-sensitivity and positive reaction to a nasal provocation was obligatory, which was performed with the previously described specific lysine-aspirin provocation test [10]. All patients (n=12) included to the aspirin-sensitivity group also had a history of aspirin-sensitive mild asthma and nasal polyposis next to the rhinitis. The patients did not exhibit any signs of atopic dermatitis or other types of allergies as revealed by medical history and serum immunoglobulin E (IgE) measurement by radio immunosorbens. The patients received nasal decongestants but all nasal or oral steroids and nasal cromoglycate were withdrawn 2 weeks prior to surgery.

To exclude other rhinitis types, skin-prick test, nasal examination, rhinomanometric test, nasal smear, and saccharin tests and nasal provocation or serum IgE counts by radio immunosorbens assay were performed. These tests were also performed with the controls to rule out aspirin sensitivity, allergic rhinitis, vasomotoric rhinitis or toxic rhinitis.

### Tissue sampling

Surgical biopsies of the lower nasal conchae were obtained during rhinosurgical interventions due to airflow limitations with causes such as deviation of the septum or conchal hyperplasia in the control group. Also, in the control group, a normal cytology and histology was obligate to rule out possible interfering inflammatory processes, such as bacterial or viral inflammation, whereas in the aspirin-sensitive rhinitis

group, inflammatory changes were found, as described previously [3, 7, 11]. The biopsies were then processed to cryostat sections using a routine protocol [12]. Immersion-fixation in Zamboni-solution [13] for 4 h was followed by washing the tissues in phosphate-buffered solution (PBS). Cryoprotection with 18% saccharose (1604; Riedel-de Haen AG, Seelze, Germany) was performed overnight before the biopsies were frozen in liquid nitrogen-cooled isopentane. After storage at -80°C the tissues were cut to 8–10 µm sections using a cryostat.

### Eosinophil and mast cell quantification

Histomorphological examination of tissue sections was performed using haematoxylin-eosin staining [14]. Differentiation of mast cells and eosinophils was carried out using either conventional histochemistry or immunohistochemistry. Histochemically, mast cells and eosinophils were detected with Toluidine or Giemsa staining. For immunohistochemical detection of the two cell types, specific markers were employed. For eosinophil detection, the sections were subjected to incubation with antibodies against human eosinophil cationic protein (clone EG1 or EG2). For mast cell staining, antihuman mast cell tryptase and anti-IgE antibodies were employed (table 2). For the detection of the primary antibody reaction, biotinylated secondary antibodies and streptavidin-biotin-peroxydase complex were used. Unspecific reactions caused by endogenous tissue peroxidase were excluded by blocking with 0.5% H<sub>2</sub>O<sub>2</sub>-methanol. Negative controls included omitting of primary or secondary antibodies. For quantification, randomised sections were examined by two independent investigators using a 250-fold magnification and a grating (035025, Olympus optical, Hamburg, Germany). The number of marker-positive cells was counted per 1,000 lamina propria associated nucleated, nonorgan related cells. The normal distribution was confirmed by the Kolmogorov-Smirnov test and the groups were tested for significant differences (p<0.05) using a paired t-test. The results were verified using the Bonferroni-Holm procedure, which confirmed significant differences.

### Immunohistochemistry for neuropeptides

Immunohistochemistry was carried out as described elsewhere [15], using a modified immunohistochemical method [16]. In brief, 8 µm cryostat sections were mounted onto chromalau pre-coated glass slides, air dried for 1 h and washed several times in PBS. To exclude unspecific background labeling, the slides were then preincubated for 1 h at room temperature with 0.1 M phosphate buffer (PBS) containing 1% bovine serum albumin and 10% normal swine

Table 1. – Patient characteristics

Patient collective	Total	Male/ female	Age yrs	Allergies	Smokers
Reference collective	12	6/6	17–66	0	0
Aspirin-sensitive rhinitis	12	5/7	13–72	0	0

Data are presented as n.

Table 2. – Primary antibodies

Antibody	Dilution	Order number	Company	Host
Eosinophil cationic protein clone EG1	1:100	10–9195–01	Kabi Pharmacia AB, Sweden	Monoclonal mouse
Eosinophil cationic protein clone EG2	1:100	10–9196–01	Kabi Pharmacia Diagnostics AB	Monoclonal mouse
Mast cell tryptase	1:1500	MAB 1222	Chemicon International Inc., USA	Monoclonal mouse
Immunoglobulin E	1:30	010195 A	Bio-Science Products AG, Switzerland	Monoclonal mouse
Protein gene-product 9.5	1:800	RA95101	Ultraclone, UK	Polyclonal rabbit
Substance P	1:40	YMC1021	Accurate Chemical & Scientific Corp., USA	Polyclonal rabbit
Calcitonin gene-related peptide	1:400	RAS6009N	Peninsula, USA	Polyclonal rabbit
Vasoactive intestinal peptide	1:400	RAS7161N	Peninsula, USA	Polyclonal rabbit
Neuropeptide tyrosine	1:400	CA08295	CRB, UK	Polyclonal rabbit

serum. Sections were then rinsed in PBS and subjected to overnight incubation with markers for protein gene product (PGP) 9.5 and different neuropeptides (table 2). Of these neuropeptide markers, the substance P antiserum may exhibit cross-reactivity to other tachykinins (SP), such as neurokinin A. For the detection of the primary antibodies, different secondary antibodies were employed (table 3). For controls, incubations were performed without the addition of the primary or secondary antibodies. The sections were finally mounted in carbonate-buffered glycerol (pH 8.6) and examined with fluorescence microscopy. For semi-quantitative evaluation of the neuropeptide-immunoreactivity, two independent investigators scored the staining intensity using a 90-fold magnification and a previously established scoring system. The innervation density was graded in steps from 0–4. Grade 0 represents no detectable staining, grade 1 represents occasional (focal) staining, grade 2 represents few nerve fibre profiles (diffuse weak) detectable, grade 3 represents many nerve fibre profiles, and grade 4, densely innervated mucosa. Similar staining protocols were published previously [17]. Samples from both groups were examined on the same day to exclude day-to-day variability influencing intergroup differences.

## Results

### *Distribution of inflammatory cells*

Routine histology demonstrated inflammatory changes in the aspirin-sensitive rhinitis group in comparison to the control group. Apart from inflammatory cell influx with a high degree of eosinophilia, goblet cell hyperplasia was also found. Giemsa-staining demonstrated that eosinophils were predominantly present in the subepithelial layer, around glands and around small blood vessels. Immunohistochemistry with the eosinophil-specific EG1 and EG2 antibodies confirmed the results of Giemsa-staining.

Toluidine-staining demonstrated the presence of mast cells. They were also predominantly present around glands and small blood vessels and in the subepithelial layer. Tryptase/IgE-immunohistochemistry on serial sections led to a similar result (fig. 1). Both the eosinophils and mast cells were not only localised around vessels, indicating a recent diapedesis, but also found in the superficial and deeper layers of the biopsies.

### *Quantification of mast cell and eosinophil numbers*

Quantitative histochemistry using Toluidine and Giemsa and immunohistochemistry with different mast cell and eosinophil-specific markers was employed to assess changes in the cellular influx in aspirin-sensitive rhinitis in comparison to the control group. In the aspirin-sensitive rhinitis group, highly significant differences of the eosinophil count were found, with a value of  $18.7 \pm 4.9\%$  (control group:  $2.6 \pm 1.3\%$ ,  $p < 0.001$ ) after EG1-staining. EG2-staining ( $21.1 \pm 6.9\%$ , control

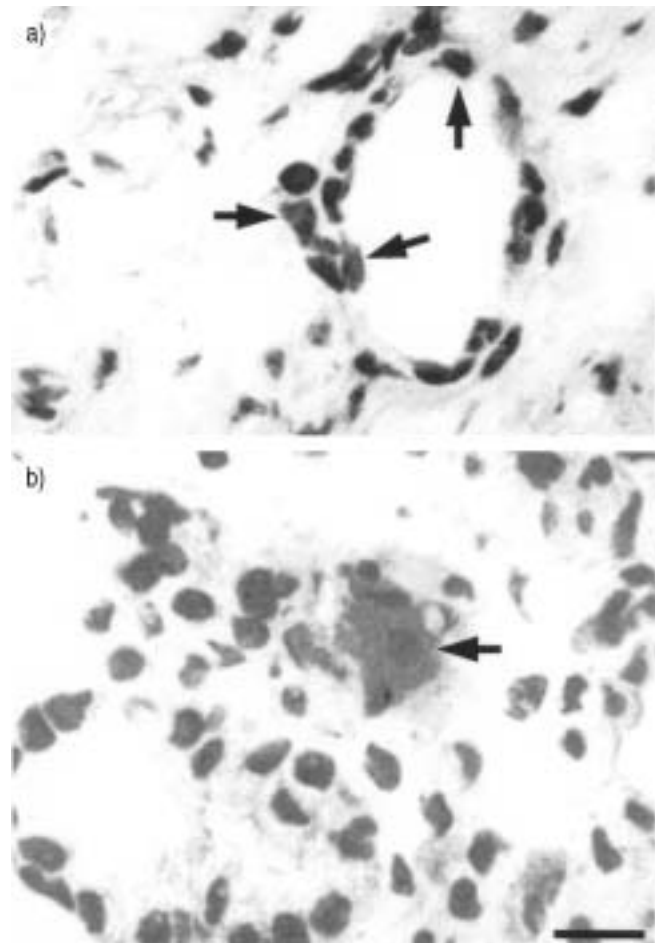


Fig. 1.—Mast cell and eosinophil distribution showing an abundant presence of eosinophils in the mucosa around small blood vessels. Arrows show mast cells. Internal scale bar=40  $\mu\text{m}$ .

group:  $2.7 \pm 1.2\%$ ,  $p < 0.001$ ) confirmed this finding. The mast cells were not significantly increased in the aspirin-sensitive group when compared to the control group (toluidin:  $6.6 \pm 0.9\%$  versus  $6.5 \pm 0.7\%$ ,  $p = 0.921$ ; tryptase:  $6.8 \pm 1.0\%$  versus  $7.9 \pm 1.0\%$ ,  $p = 0.309$ ).

### *Cellular expression of neuropeptides*

Biopsies of turbinate mucosa from nonrhinitic and aspirin-sensitive rhinitic patients contained abundant nerve fibres. Immunohistochemistry using the general neuronal marker PGP 9.5 resulted in staining of mucosal nerve fibres. A dense innervation was found underneath the respiratory epithelium. PGP 9.5-like immunoreactivity was also found within glandular structures underneath the surface epithelium. Immunohistochemistry using a neuropeptide tyrosine (NPY)-antibody lead to staining of nerve fibres that were predominantly

Table 3.—Secondary reagents

Antibody	Dilution	Order number	Company	Target
Anti-rabbit IgG, FITC-conjugated	1:25	4271-1	BioMakor, Israel	Rabbit
Anti-rabbit IgG, biotin-conjugated	1:50	1004	Amersham, UK	Rabbit
Anti-streptavidin texas-red	1:50	RPN1233	Amersham, UK	Rabbit

IgG: immunoglobulin G.

distributed around blood vessels. Incubations of sections for calcitonin gene-related peptide (CGRP)-like immunoreactivity resulted in staining of CGRP-like positive fibres that were predominantly distributed close to blood vessels, glands and sinusoids. Also, tachykinin-like immunoreactivity was found in a subpopulation of nerve fibres that was present below the epithelium and around glandular structures. Immunostaining for the neuropeptide vasoactive intestinal peptide (VIP) revealed a subpopulation of VIP-like positive nerve fibres that was found around blood vessels and submucosal glands (fig. 2). Next to their expression in nerve fibres, neuroendocrine cells also revealed neuropeptide-like immunoreactivity.

#### Quantification of neuropeptide-containing nerve fibres

Using a previously standardised scoring protocol for nerve fibre presence in human nasal mucosa, different scores for neuropeptide-like immunoreactivity were found (fig. 3). In the control group of nonrhinitis subjects, the score was SP  $1.83 \pm 0.39$ , CGRP  $1.58 \pm 0.36$ , VIP  $1.42 \pm 0.45$ , and NPY  $0.83 \pm 0.34$ , respectively (fig. 4).

In the group of aspirin-sensitive rhinitis the neuropeptide-like immunoreactivity was significantly raised for VIP ( $2.50 \pm 0.26$ ,  $p < 0.05$ ), whereas the other values did not differ

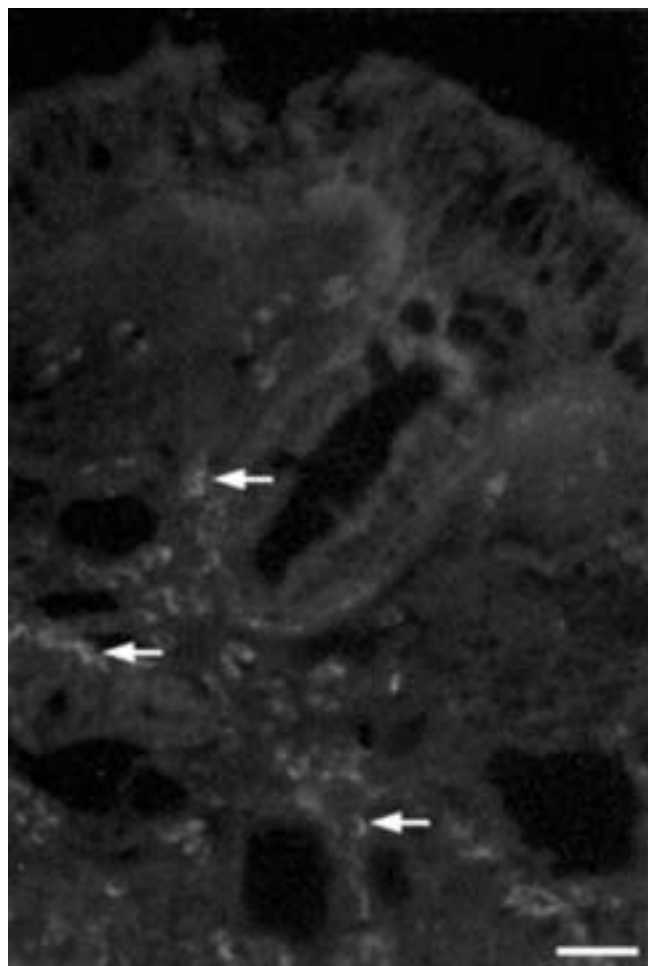


Fig. 2.—Immunohistochemistry for neuropeptides in nasal mucosal nerves. Vasoactive intestinal peptide-immunoreactive nerve fibres (arrows) are present as branching networks in the subepithelial and epithelial layer around submucosal glands and vessels in a rhinitis biopsy. Internal scale bar=40  $\mu\text{m}$ .

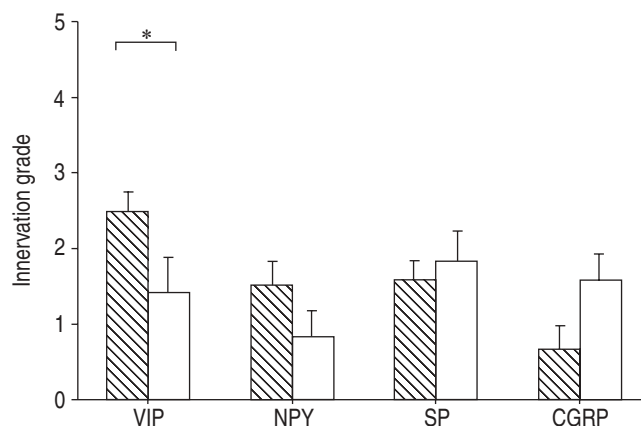


Fig. 3.—Semiquantitative assessment of neuropeptide immunoreactivity in nerve fibres in aspirin-sensitive rhinitis (▨) and control tissues (□). VIP: vasoactive intestinal peptide; NPY: neuropeptide tyrosine; SP: tachykinins; CGRP: calcitonin gene-related peptide. \*:  $p < 0.05$ .

significantly (CGRP:  $0.67 \pm 0.31$ ,  $p = 0.066$ ; SP:  $1.58 \pm 0.26$ ,  $p = 0.596$ ; NPY:  $1.50 \pm 0.31$ ,  $p = 0.167$ ; fig. 3). Also, the immunoreactivity for PGP 9.5 did not change significantly (rhinitis:  $2.91 \pm 0.21$  versus control:  $2.30 \pm 0.56$ ,  $p = 0.303$ ).

#### Discussion

Aspirin-sensitive rhinitis displays the nasal manifestation of aspirin-intolerance, a pseudoallergy against aspirin and/or related NSAIDs, such as indomethacin, diclofenac or ibuprofen [18]. Clinically, aspirin-sensitive rhinitis is often associated to hyperplastic rhinosinusitis with clear secretion, nasal obstruction, sneezing and itching. Rhinoscopy often reveals a swelling of the turbinal mucosa, a massive yellow secretion that can be due to a high content of eosinophils, and a polyposis nasi [11]. Similar features were also found in the present population of aspirin-sensitive rhinitis patients. The present cases were diagnosed according to the generally accepted diagnosis criteria including nasal provocation tests with aspirin [19, 20]. All subjects included to the study had negative radio-allergosorbent test and skin tests. As most asthmatics have atopy and positive test results, the present results can clearly not be generalised to atopic asthmatics or atopic rhinitis.

The pathomechanisms of aspirin-sensitive rhinitis have not been fully elucidated so far. One theory proposes that aspirin leads directly to an unspecific, IgE-independent activation of mast cells and eosinophils [21–23]. Studies that reported an increase in the blood levels of histamine and tryptase [24], and an increase of histamine and leukotriene C4 in nasal lavage [25] after aspirin-provocation supported this theory. A second theory is based on the mode of action of NSAIDs that inhibit the cyclooxygenase, leading to an increased degradation of arachidonic acid by 5-lipoxygenase and an increased production of LTs [26, 27] and numerous studies correspond to this theory [28–31]. Apart from these pathogenetic mechanisms, a modulatory role may be attributed to the airway nervous system.

The current study assessed changes in the innervation of 12 aspirin-sensitive rhinitis patients. Using a previously standardised scoring protocol for nerve fibre presence in human nasal mucosa, a significant increase of neuropeptide VIP-like immunoreactivity was found. With the regard to the difficulties that can be encountered in quantification and semiquantification of nerve fibre profiles, the staining procedures and microscope settings have to be very stringent and reproducible. In this respect, the present study was carried out cautiously to reach the highest standards of reproducibility and stringency.

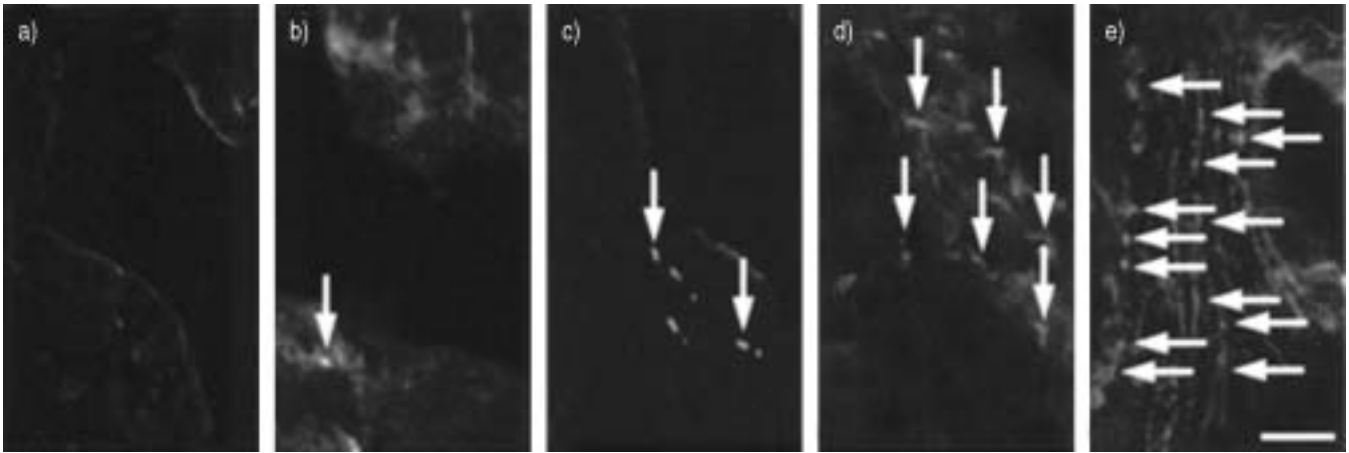


Fig. 4. – Semiquantitative assessment of neuropeptide-immunoreactivity in nerve fibres. a) Staining recorded for calcitonin gene-related peptide-like immunoreactivity in a rhinitis biopsy (grade 0). b) Arrow shows occasional (focal) staining for neuropeptide tyrosine-like immunoreactivity in a control tissue (grade 1). c) Arrows show staining for tachykinin-like immunoreactivity in a control tissue, representing few nerve fibre profiles (diffuse weak (grade 2)). d) Arrows show staining recorded for vasoactive intestinal peptide (VIP)-like immunoreactivity in a rhinitis tissue, representing many nerve fibre profiles (grade 3). e) Arrows show staining recorded for VIP-like immunoreactivity in a rhinitis tissue, representing densely innervated mucosa (grade 4). Internal scale bar=40  $\mu$ m.

The 28-amino acid peptide VIP was first isolated from small intestine and lung as a vasodilatory peptide [32] and is one of the most abundant neuropeptides found in human nasal mucosa [33, 34]. Although there are no reports on the effects of nasally-administered VIP in humans, it is generally accepted that VIP plays a major role in the upper respiratory tract and influences many aspects of airway function [35] due to its role as a neuromodulator of the inhibitory nonadrenergic noncholinergic nervous system [36]. An increase and upregulation of VIP expression in nerve fibres with a resulting vasodilation may be reflected by the increased blood flow and dilated vessels observed in an inflammatory setting as found in the nasal mucosa of aspirin-sensitive rhinitis patients.

VIP-like immunoreactive nerves are widely distributed throughout the respiratory tract in humans and in animals [37] and an increase of VIP has been reported in cases of vasomotor rhinitis as compared to normal controls [38, 39]. Several reports have since confirmed an up-regulation of VIP in allergic rhinitis [17, 40].

Next to their expression in nerve fibres, a number of studies have reported that cells of the immune system may also produce neuropeptides, such as VIP [41]. Hence, the biological significance of inflammatory cell-derived neuropeptides is discussed [42]. As the present study only focused on neuropeptide expression in nerve fibres, which can be visualised by staining with a panneuronal marker, such as PGP 9.5, an involvement of neuropeptide-derived non-neuronal sources, such as inflammatory cells, cannot be ruled out. In this respect, neuropeptide-like immunoreactivity was also found in neuroendocrine cells as reported earlier for allergic rhinitis [40, 43].

The authors have previously demonstrated that neuropeptide-like immunoreactivity also changes in biopsies of hyperreflective rhinitic subjects. In relation to the present study, these changes were not only restricted to VIP-like immunoreactivity but also to SP, indicating that there are differences in the innervation between these two rhinitis types [39].

In the present study, no significant change for was CGRP found. As there was a nonsignificant tendency, studies with higher numbers of tissues and a higher statistical power may reveal a significant difference for this neuropeptide, which was reported to be involved in the regulation of many airway functions [44]. Therefore, future multicenter studies with larger populations should be carried out and may also point to a role for CGRP in aspirin-sensitive rhinitis.

Apart from the well known combination of symptoms of aspirin-intolerance, asthma and polyposis nasi, previous studies reported elevated levels of eosinophils in aspirin-sensitive rhinitis. The results of the current study using three methods to assess eosinophils are consistent with these studies demonstrating a highly significant elevation of the eosinophil concentration. In this respect, the numbers of eosinophils were up to 20% of lamina propria cells in the aspirin-sensitive group, compared to only 2% of lamina propria-associated cells in the nonrhinitic reference population.

A direct participation of mast cells has not been demonstrated in the pathogenesis of aspirin-sensitive rhinitis and the only studies suggesting a mast cell contribution reported elevated levels of tryptase and histamine in nasal fluid lavages of aspirin-sensitive rhinitis patients. There was no significant difference in the levels of mast cells in the present study. However, a direct participation of these cells cannot be ruled out as they may participate *via* a change of their mediator profile while their total numbers remain constant.

A large body of evidence points to a direct interaction between eosinophils and/or mast cells and airway nerves, as demonstrated earlier by numerous reports [45]. As the present study did not include double staining procedures for simultaneous mast cell/eosinophil and airway nerve detection, these findings were not repeated. However, due to the high numbers of eosinophils observed in the aspirin-sensitive group, communication between eosinophils and nerve fibres is possible.

In summary, the results of the present study indicate a change of neuropeptide-like immunoreactivity in mucosal nerves of patients with aspirin-rhinitis. Together, the present data suggest a modulatory role of the autonomous mucosal innervation in aspirin-sensitive rhinitis.

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