

Preventive treatment of intranasal fluticasone propionate reduces cytokine mRNA expressing cells before and during a single nasal allergen provocation

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Summary

Background The local production and release of a number of cytokines regulate allergic upper airway inflammation. Medication is usually used at the presentation of the first symptoms. There are, however, clues that it is advisable to start taking the corticosteroid before the grass pollen season begins.

Methods This single allergen provocation study was conducted in autumn, out of the hay fever season. Nasal mucosa biopsies were taken twice before provocation (before and after 4 weeks of preventive treatment) and three times after allergen provocation (1 h, 24 h and 1 week). The preventive treatment used was fluticasone propionate aqueous nasal spray (FPANS) ($n = 10$) or a placebo ($n = 9$). Eosinophils and mRNA positive cells (*in situ* hybridization for IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFN γ , RANTES and TNF α) were counted in the biopsies.

Results Preventive treatment with FPANS out of season resulted in a decrease in eosinophils and mRNA positive cells for IL-5 and IL-6. After allergen provocation, levels of most of the measured cytokines (IL-3, IL-5, IL-6, IL-13, IFN γ , RANTES and TNF α) and eosinophils were reduced using corticosteroids. The numbers of cells (eosinophils, IL-3, IL-6 and IL-8) correlated with nasal symptoms. Significant correlations in the early and late allergic phase were found between eosinophils and cytokines (IL-3, IL-10 and IL-13).

Conclusion These results indicate that preventive treatment with FPANS prior to contact with grass pollen is effective in reducing the increase of cytokine mRNA positive cells in reaction to grass pollen contact.

Keywords: allergen provocation, allergic rhinitis, biopsies, corticosteroids, cytokines, eosinophils, hay fever, ISH, nasal mucosa

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Introduction

Topical corticosteroids relieve allergic symptoms by reducing the numbers of inflammatory cells and chemical mediators [1,2]. Allergic patients usually start taking their

medication at the presentation of the first symptoms, but there are indications that it is advisable to start taking corticosteroids before the grass pollen season begins [3–5]. Allergen challenge results in mast cell degranulation, migration of mast cells to the surface of the upper airway mucosa [6], and an influx of inflammatory cells [5,7,8]. This allergic mucosal inflammation is regulated by the local production and release of a number of cytokines [9,10]. The pro-inflammatory cytokines IL-6 and TNF α

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were released within the first hours after allergen exposure and seems to play an important role in the early phase. A major role was originally assigned to the cytokines produced by Th2 lymphocytes, such as IL-4, IL-5 and IL-10 [10–12]. In addition to the well-known Th2 cytokines, chemokines (IL-8 as the CXC chemokines and RANTES as a CC chemokine) were found to play an important role in tissue eosinophilia in allergic inflammation [9,13]. Accumulating data now points to a large network of interacting cytokines produced by a number of cells that regulate mucosal allergic inflammation [14].

A number of studies have been performed which assess the effect of local corticosteroid therapy on cytokines at the mRNA and protein levels in the airway mucosa and in lavages.

Allergen challenge studies have found reductions in mRNA and protein for IL-4 and IL-13 after steroid treatment [10–12]. No steroid therapy effect was observed for IL-5 and IFN γ in the nasal mucosa after allergen provocation [10–12]. However, studies in the lung and in *in vitro* experiments showed that corticosteroids inhibit IL-5 and IFN γ mRNA expression and protein production [15–19]. There were no differences in IL-6 on the protein level (protein-positive cells) in the upper airways between pre-season and in season for allergic patients with or without corticosteroid treatment [10]. However, Weido *et al.* observed in nasal lavage fluids, IL-6 protein levels during the late-phase reaction, which was inhibited by pretreatment with FPANS [20]. Local corticosteroid treatment was found to inhibit allergen challenge-dependent secretion of IL-8 and RANTES protein in the nasal lavage [9,20].

To our knowledge, no data is available *in vivo* about the dynamics of cells expressing mRNA for IL-6, IL-8, IL-10, RANTES and TNF α in the upper airway mucosa after allergen provocation. For other cytokines, details of time-related changes after allergen provocation and corticosteroid preventive treatment are incomplete.

On the basis of earlier studies performed by our group, we hypothesized that 4 weeks of local corticosteroid treatment reduces the number of inflammatory cells irrespective of the presence of symptomatology [21]. In this out-of-season biopsy study, we investigated the effect of preventive treatment with a local corticosteroid, fluticasone propionate aqueous nasal spray (FPANS) and the effect of preventive treatment on a single provocation with grass pollen in patients with an isolated grass-pollen allergy. Biopsies were taken before and during treatment, before provocation and at several points after the provocation. Eosinophils and mRNA-positive cells (*in situ* hybridization) for IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFN γ , RANTES and TNF α were determined in mucosal biopsies. Furthermore, correlations will be evaluated between the

cells and nasal symptoms and also between eosinophils (as a marker of allergic inflammation) and cytokines.

Methods

Patients

Nineteen patients with a median age of 22 (range 18–51) participated in this study (11 female and eight male). They all had a history of seasonal allergic rhinitis for grass pollen of at least 2 years. Allergy to grass pollen was confirmed by a positive skin prick test (3+) with Alutard Soluprick extract of 1 HEP/mL, with no skin prick reactions to other relevant allergens. All patients were symptom-free at the start of the study. None of the patients used any medication during the study or had undergone immunotherapy in the 3 years before this study. No relevant abnormalities were found in ENT examination at the start of the study. All patients gave their written informed consent and the medical ethics committee approved the study.

Study design

This double-blind, randomized, placebo-controlled, parallel-group study was conducted between October and December 1995, well outside the hay fever season in The Netherlands. After randomization, 10 patients (six female and four male) aged 21 (18–51) used 200 μ g fluticasone propionate aqueous nasal spray (FPANS) twice daily and nine patients (five female and four male) aged 23 (19–31) used a placebo for 6 weeks. After this treatment period, on the following day in the morning provocation took place and the use of FPANS or placebo was stopped. Biopsies were taken from the inferior turbinate using a Gerritsma forceps [7]. Each patient underwent five nasal biopsies: the first at baseline, the second after 4 weeks of treatment (before provocation), the third 1 h after provocation with grass pollen, the fourth 24 h after provocation and the fifth 1 week after provocation.

Provocation

The patients acclimatized to the room for at least 15 min. After that, PBS (phosphate-buffered saline), an inert water-rich solution, was administered to rule out nonspecific hyperreactivity. After 10 min, patients were provoked with 50 μ L of an aqueous nasal spray containing grass pollen (10 000 BU/mL, ALK, Groningen, Netherlands) and another 10 min later with 100 000 BU/mL (ALK, Groningen, Netherlands). After both provocations, symptoms like sneezing, rhinorrhea, itching and nasal blockage were recorded on a four-point (0–3) scale. Patients were

also asked to rate their symptoms during the day following provocation after 3, 6, 9, 12 and 24 h.

Immunohistochemical staining of eosinophils

Eosinophil staining was performed in an alkaline phosphatase procedure using monoclonal antibody against Major Basic Protein (BMK 13) (Sanbio, Uden, Netherlands) as previously described by Godthelp *et al.* [7].

In situ hybridization

In situ hybridization was performed as previous described in detail by ourselves [14] and others [22,23]. In short, frozen sections were fixed with formaline 4%, treated with proteinase K and treated with Triton X100. Slides were dehydrated with ethanol up to 100% and air-dried. Sections were hybridized with denaturated digoxigenin-DNA probes overnight [14]. After hybridization, the sections were washed with 30% formamide/ $2 \times$ SSC at room temperature, 30% formamide/ $0.2 \times$ SSC at 42°C for 15 min and washed with PBS for 5 min. The slides were then placed in a semi-automatic stainer (Sequenza, Shandon). Following this, the sections were incubated for 10 min with BSA 0.5–1% in PBS, incubated with normal sheep serum (CLB, Netherlands) for 10 min and subsequently incubated with 1:500 alkaline phosphate-conjugated-sheep-digoxigenin F (ab)2 fragments, diluted in PBS BSA 0.5–1% and normal human serum (10% NHS). They were rinsed again with PBS for 5 min, rinsed with Tris buffer (0.1 M pH 9.5) for 5 min and incubated with NBT BCIP substrate (Sigma). The incubation time is probe-dependent. Substrate development was checked with the microscope. Finally, the sections were rinsed in distilled water and mounted in glycerin-gelatine. Optimal probe concentration was obtained by dilution titration.

Quantification

The biopsies were coded and two sections of each biopsy were counted in a blinded fashion at a magnification of 400 $\times$. The surface area of the epithelium and the lamina propria of two sections were determined by computer image analysis (Kontrons Image Analysis System Videoplan, Zeiss Weesp, The Netherlands). The cell numbers for eosinophils in the epithelium and lamina propria and the number for mRNA positive cells only in the lamina propria were calculated per square millimetre of the section area.

Statistical analysis

The ANOVA repeat measurement (area under the curve) was used for statistical analysis of treatment effects between

the groups. If significant differences were found using ANOVA, further comparisons were made using the Mann–Whitney *U*-test per time-point. A *P*-value <0.05 was considered to indicate a significant difference between groups. The Wilcoxon-signed rank test was used for a comparison between baseline and during treatment and between baseline and one week after provocation.

Correlation coefficients (*rs*) were obtained using Spearman's rank method for cells from each biopsy, for symptoms (baseline, after four weeks treatment, early phase G2 (10 000 BU provocation) and late phase 3–24 h after provocation) correlated with cells (biopsies baseline, after 4 weeks treatment, 1 h and 24 h after provocation). Correlation coefficients were obtained for each biopsy. Because of multiple testing a *P*-value of <0.01 was considered to indicate a significant correlation.

Results

Microscopic evaluation

The cryostat sections of the nasal mucosa had an average surface area of 2 mm² and an intact epithelium. Staining with mAb for eosinophil major basic protein (BMK-13) resulted in easy identification with the red cytoplasmic granules and dark violet nucleus. Eosinophil numbers were determined in the lamina propria. The mRNA *in situ* hybridization staining pattern for cytokines varied from a dark purple circle to a large dark purple dot. Messenger RNA-positive cells were located in the inflammatory cell infiltrate in the lamina propria. The majority of cells showing hybridization signals for cytokines were present in the subepithelial layer. The number of positive cells was only determined in the lamina propria because the number of positive cells in the epithelium was too small for statistical analysis. Figure 1 shows sections from nasal mucosal biopsies expressing mRNA for IL-5 (a) and its Rnase-treated control (b) (magnification $\times 140$) for IL-8, (c) and its Rnase-treated control (d) (magnification $\times 550$) IL-3 (e) and IFN γ (f) (magnification $\times 275$) mRNA for IL-5 (a) and its Rnase-treated control (b) for IL-8, (c) and its Rnase-treated control (d) IL-3 (e) and IFN γ (f).

Effect of preventive treatment (before provocation)

No allergic symptoms were observed by the patients at baseline during treatment or before provocation or provocation with saline Fig. 2.

In the biopsies, the number of eosinophils showed no significant difference between the groups at baseline. After 4 weeks of preventive treatment, before allergen provocation, a significant lower number of eosinophils was observed in the epithelium (*P* = 0.002) and in the lamina propria

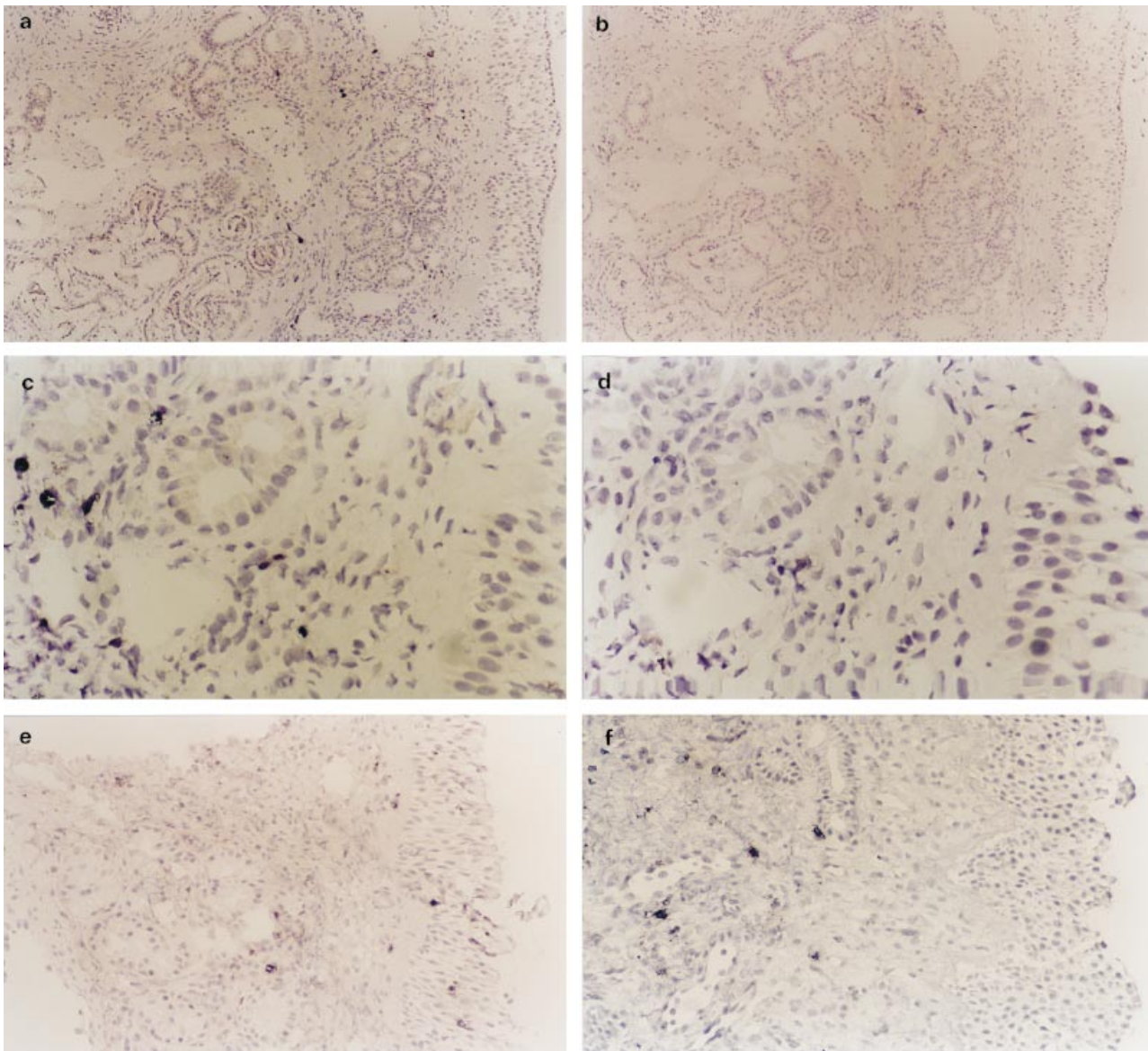


Fig. 1. Photomicrographs showing examples of *in situ* hybridization of nasal biopsies of allergic rhinitis patients following allergen challenge, using digoxigenin-labelled DNA probes coding for IL-5 (a) and its Rnase-treated control (b) (magnification $\times 140$) for IL-8 (c) and its Rnase-treated control (d) (magnification $\times 550$), IL-3 (e) and IFN γ (f) (magnification $\times 275$). Messenger RNA-positive cells exhibited a dark purple staining and give optimal cellular and subcellular resolution, nuclear staining with gill's heamatoxin.

($P = 0.004$) of the FPANS group compared to the placebo group.

At baseline, no significant differences were found in the number of cytokine mRNA-positive cells between the two groups. After 4 weeks of preventive treatment, a significantly lower number of IL-5 and IL-6 mRNA-positive cells were found in the FPANS group compared with the placebo group ($P = 0.03$ and $P = 0.05$), and no effect of preventive treatment within the placebo group or FPANS groups were observed.

Before allergen provocation, no significant correlation was observed between cells expressing mRNA for cytokines and eosinophils.

Treatment effects after allergen provocation

Nasal provocation with allergen resulted in a significant increase in immediate (placebo, nine out of nine; FPANS, 10 out of 10) and late nasal symptoms (placebo, nine out of nine; FPANS, seven out of 10) in both groups with

increases in sneezes, itching, nasal blockage and rhinorrhea. A significant reduction in total symptomatology (in the early phase $P=0.01$, and in the late phase $P=0.009$) was found in the FPANS group compared with the placebo group (Fig. 3).

During the whole study period, in the biopsies fewer eosinophils were present in the epithelium ($P<0.0001$) of the FPANS group compared to the placebo group. The same trend was found in the lamina propria ($P=0.07$).

The numbers of IL-3 mRNA-positive cells showed a significant ($P=0.02$) treatment effect during provocation. The rise in IL-3-positive cells was lower in numbers and shorter in time in the FPANS group than in the placebo group after allergen provocation. This result lasted until a week after provocation ($P=0.0006$).

For the whole study period, significantly ($P<0.0001$) fewer IL-5 mRNA-positive cells in the FPANS group were found compared to the placebo group. Lower IL-5 mRNA-positive cell numbers were observed in the FPANS group compared to the placebo group 24 h and 1 week ($P=0.01$ and $P=0.04$) after allergen provocation.

IL-6 mRNA-positive cells were significantly lower in numbers in the FPANS group compared to the placebo group ($P=0.03$) for the whole study period.

Fewer IFN γ mRNA-positive cells ($P=0.0001$) were observed in the FPANS group than in the placebo group during the whole study period. Numbers of IFN γ mRNA-positive cells were lower in the FPANS group than in the placebo group at 1 h ($P=0.02$), 24 h ($P=0.01$) and 1 week ($P=0.009$) after provocation. Less RANTES mRNA-positive cells were present in the FPANS group than in the placebo group during the whole study period ($P=0.01$). At 1 h after provocation, significantly fewer RANTES mRNA-positive cells were observed in the FPANS group compared to the placebo group ($P=0.05$). Numbers of TNF α mRNA-positive cells were found to be lower in the FPANS group than in placebo group ($P=0.001$) for the whole study period. No significant differences were observed at the different biopsy moments.

Cell numbers for IL-2, IL-4, IL-8, IL-10 mRNA-positive cells showed no significant treatment-dependent differences between the groups. In the placebo group, higher mRNA-positive cell numbers were observed for IL-10 and IL-13 (P -value resp 0.04 and 0.04) at 1 week after provocation in contrast to the FPANS group who dropped back to baseline level within 1 week after allergen provocation.

In the early phase, most cytokines significantly correlate with eosinophil numbers indicating a general influx and activation of inflammatory cells (see Table 1a). However, in the late phase 24 h after provocation, significant correlations were only found between eosinophils and cytokine mRNA of the Th2-type IL-3, IL-5, IL-10 and IL-13.

Discussion

In this study, we investigated the effect on cytokine mRNA expressing cells in nasal mucosal biopsies of corticosteroid (pre)treatment before and after a single provocation with grass pollen and compared these sets of data to tissue eosinophilia and symptomatology.

Cells and cytokines in both allergic and nonallergic inflammation can be reduced by steroid therapy [2,21,24,25]. This study shows, for the first time, that preventive treatment with FPANS, even in the absence of allergic inflammation and symptomatology, results in a decrease in the spontaneous baseline level of inflammatory cells (eosinophils) and cells expressing cytokine mRNA (IL-5 and IL-6).

Allergen provocation induces nasal symptoms and tissue eosinophilia, which clearly correlate with each other (Table 1). Tissue eosinophilia can be used as a marker for allergic inflammation [7,26]. Corticosteroid treatment reduces the number of eosinophils, as shown in this provocation study and other studies [2,25].

Data on IL-4 and IL-13 are conflicting. In this study no treatment effects on IL-4 and IL-13 were found. A possible explanation of the discrepancy between this study and earlier studies of others [11,12] and ourselves [1] could be the method of allergen provocation. Filter disk provocation [11,12] and repeated allergen provocation [1] result in an increase in cytokine expression for IL-4 and IL-13 mRNA. These two methods result in a stronger stimulus compared to a single allergen challenge by spray as performed in the present study.

IL-3 and IL-5, Th2 cytokines that are produced by T-cells and eosinophils, promote their differentiation and activation and prolong the survival of eosinophils. After allergen provocation, the number of IL-3 mRNA and IL-5 mRNA-positive cells was significantly reduced in the FPANS group compared to the placebo group. This is in accordance with data in nasal lavage [27] in the lung [24] and *in vitro* studies [17,28]. A study using filter disk allergen provocation [11] and a seasonal study [10] failed to show effects of FPANS treatment on IL-5, possibly because of the differences in study design mentioned above. The chemokines, RANTES and IL-8, are pro-inflammatory cytokines which are important in, for example, eosinophil chemotaxis. The correlation between IL-8 and nasal symptoms found in this study (Table 2) confirms data from Douglass *et al.* who observed an increase in rhinitis symptomatology after IL-8 challenge in the nasal mucosa [29]. FPANS treatment significantly reduced the rise in RANTES mRNA expressing cells during allergen provocation but failed to show a reduction in IL-8 mRNA expressing cells.

The presence or absence of glucocorticoid response element (GRE) in the promoter region of cytokines could

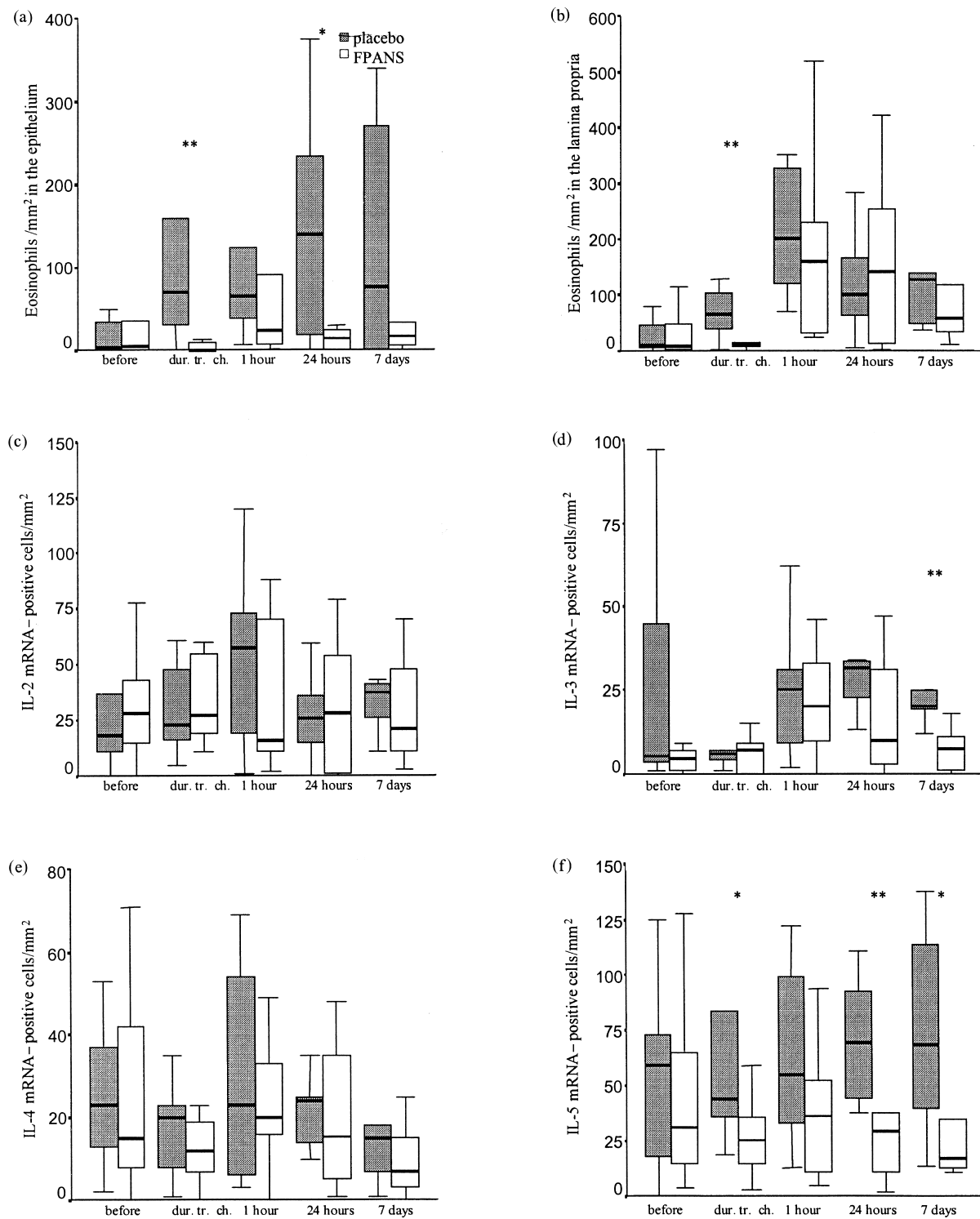


Fig. 2. Box plots showing the number of mRNA-positive cells in nasal mucosa biopsies. Nasal mucosa biopsies were taken before and 4 weeks after starting treatment (dur. tr.), 1 h after provocation, 24 h after provocation and 1 week after provocation. Allergen challenge (ch) with 1000 and 10 000 Bu grass pollen. Treatment with placebo $n = 9$ (grey bar) and FPANS $n = 10$ (white bar). * $P < 0.05$ ** $P < 0.01$ (Mann Whitney U -test).

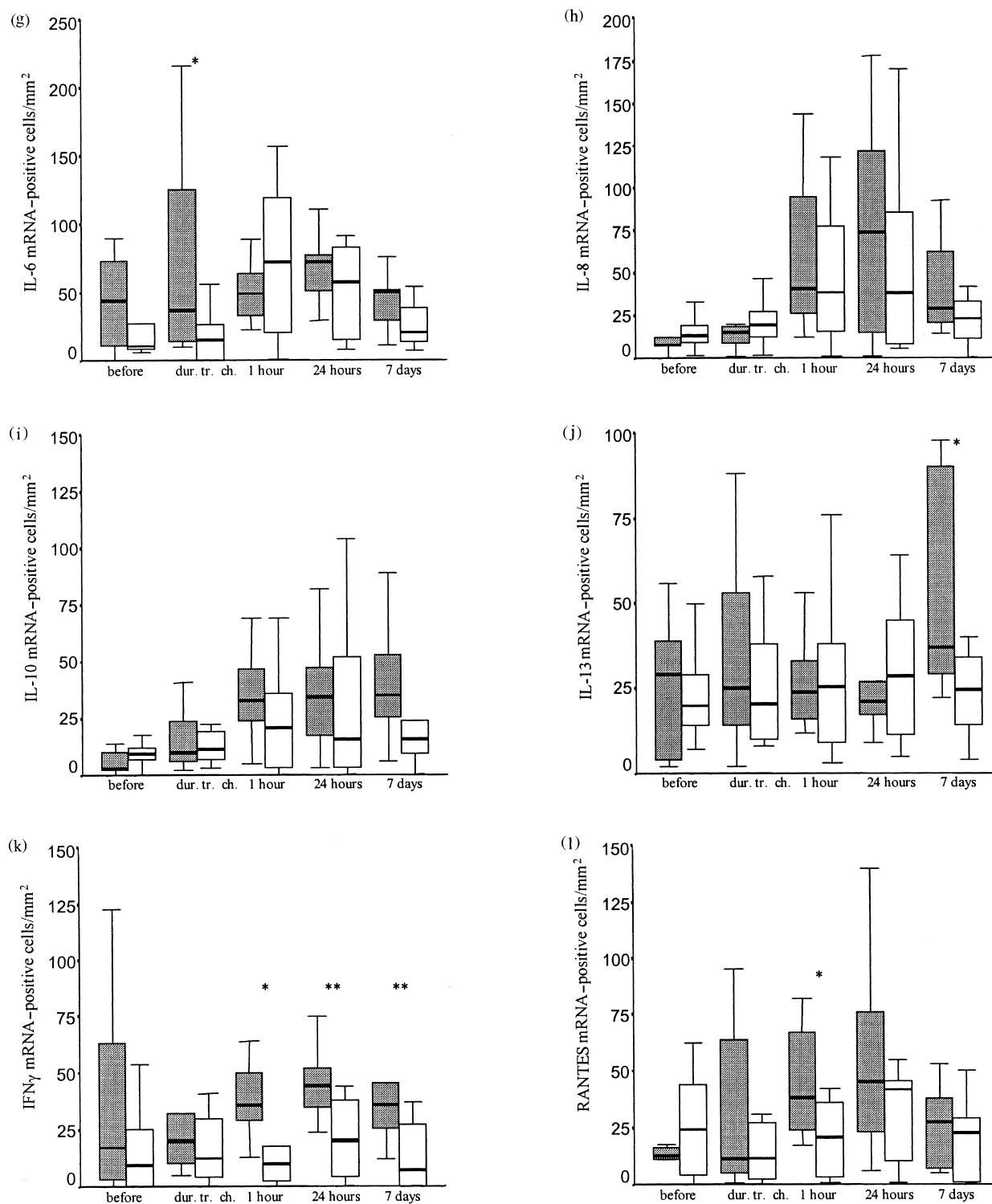


Fig. 2. Continued

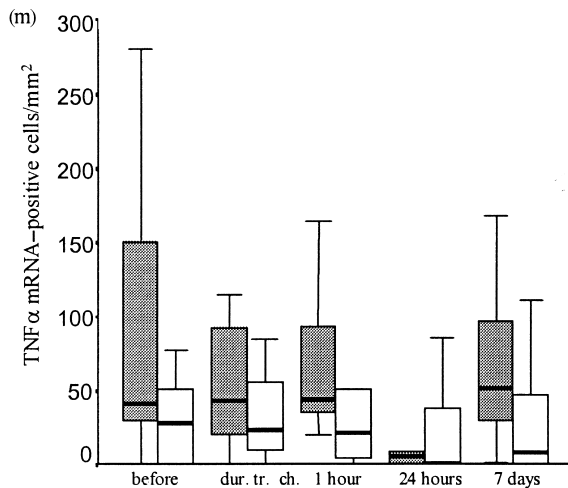


Fig. 2. Continued

explain why corticosteroid treatment has a direct effect, e.g. on IL-5 or RANTES expression but cannot have any direct effect but only an indirect effect, e.g. on IL-2 or IL-8 expression in the allergic inflammation [30].

The reduction in number of TNF α mRNA expressing cells after provocation implies that TNF α was down-regulated in allergic inflammation, irrespective of the treatment. In the FPANS group, we found a reduction in the number of cells expressing TNF α compared to the placebo group. Studies in nasal polyps showing that TNF α was significantly reduced in an allergic group compared to a non-allergic group also indicate that TNF α does not play a specific role in allergic inflammation [31]. However, several sets of data indicate that TNF α has a pivotal role in bacterial infections [32].

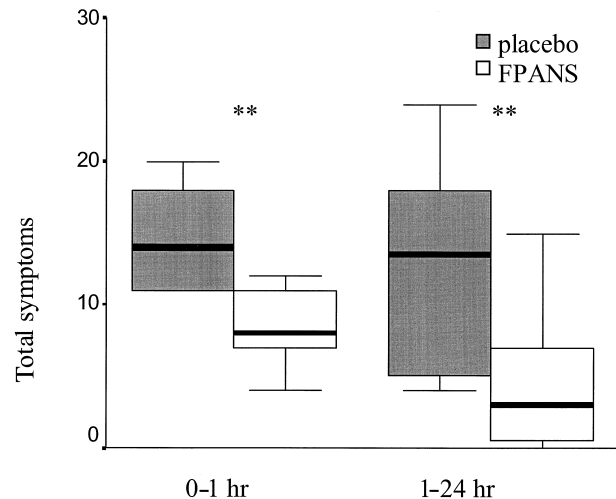


Fig. 3. Total symptom score after allergen provocation. In the first hour and during the following day total symptoms were significantly reduced in the FPANS-treated group compared to placebo, in the early phase ($P = 0.01$) and in the late phase ($P = 0.009$)

IL-6 showed no significant increase after allergen provocation in both groups indicating a minor role in allergic inflammation. Also, on the protein level (protein positive cells), no differences were observed in the upper airways after natural allergen provocation in the season [10]. Numbers of IL-10 mRNA-positive cells increased after provocation and were not reduced by steroid treatment. IL-10, considered to be a Th2 cytokine with anti-inflammatory and immunoregulatory properties, inhibits the release of IFN γ and generally inhibits cytokine synthesis by human monocytes [33]. On the contrary IFN γ , which is considered to be a Th1 cytokine, did not increase significantly after

Table 1. Correlations between the number of eosinophils compared with the number of cells expressing positive hybridization signals at each biopsy moment (for r_s -regression and P -values)

	Eosinophils 1 h after ch r_s (P -value)	Eosinophils 24 h after ch r_s (P -value)	Eosinophils 1 week after ch r_s (P -value)
IL-2	0.56 (0.01)	n.c.	n.c.
IL-3	0.74 (0.0001)	0.59 (0.009)	n.c.
IL-4	0.61 (0.006)	n.c.	n.c.
IL-5	n.c.	0.63 (0.004)	0.59 (0.008)
IL-8	0.75 (0.0001)	n.c.	n.c.
IL-10	0.57 (0.01)	0.70 (0.001)	0.57 (0.01)
IL-13	0.66 (0.002)	0.62 (0.005)	n.c.
IFN γ	n.c.	n.c.	0.68 (0.001)
RANTES	0.63 (0.004)	n.c.	n.c.
TNF α	0.69 (0.001)	n.c.	0.68 (0.001)

n.c., no correlation.

Table 2. Correlations between numbers of eosinophils, IL-3, IL-6 and IL-8 mRNA in biopsy sections and nasal symptom score for blockage, itching, discharge, sneezing and total symptom score (for r-regression and *P*-values)

Cell type	Symptom correlations (regression value and <i>P</i> -value)				
	Blockage	Discharge	Itching	Sneezing	Total
Eosinophil	0.323 (0.005)	0.382 (0.001)	0.405 (0.001)	0.475 (0.001)	0.435 (0.001)
IL-3	n.c.	0.294 (0.01)	n.c.	n.c.	0.297 (0.01)
IL-6	n.c.	0.317 (0.006)	n.c.	n.c.	0.301 (0.009)
IL-8	0.320 (0.006)	0.354 (0.002)	n.c.	n.c.	0.366 (0.001)

n.c., no correlation.

allergen provocation but was significantly reduced in the FPANS group compared to the placebo group. Other studies found no significant reduction in IFN γ after FPANS treatment of allergic rhinitis patients [11].

Almost all cytokine mRNA expressing cells increase in the early phase symptomatology, but only the Th2 cytokines known to be relevant for allergic disease are correlated to eosinophils in the late phase symptomatology (see Table 1), again emphasizing the role of these cytokines in allergic disease. Local corticosteroid treatment results in a significant decrease of a number of cytokines in the early and late phase. The effect of FPANS on eosinophils and cytokines is still visible a week after one single allergen provocation.

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