

# Nasal challenge test in the diagnosis of allergic respiratory diseases in subjects occupationally exposed to a high molecular allergen (flour)

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The objective of this study was the evaluation of the usefulness of the nasal challenge test in the diagnosis of allergic respiratory diseases in subjects occupationally exposed to flour. A single-blind, placebo controlled study was conducted in 100 subjects with occupational atopic asthma with rhinitis. The control groups consisted of 20 atopic subjects not sensitized to investigated allergens and 20 healthy subjects. A 'nasal pool' technique was used to evaluate the changes of the cellular response and protein level in nasal washings after topical provocation with allergen or placebo. The concentrations of eosinophil cationic protein and mast cell-derived tryptase in nasal fluid were evaluated in 60 cases. There were significant increases in eosinophil and basophils number, albumin/ total protein ratio, eosinophil cationic protein and tryptase levels in occupationally sensitized patients challenged with specific allergens. There were neither severe bronchial reactions or an increase of bronchial hyperreactivity in occupationally sensitized patients after the nasal provocation with flour. The nasal challenge test appears to be a very useful and safe tool for diagnosing occupational allergy.

**Key words:** Allergic inflammation; mediators; nasal challenge; occupational allergy.

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## INTRODUCTION

In diagnosis of occupational airway sensitizations, the differentiation between specific-allergic and nonspecific-irritant reactions may be very difficult. The clinical response to acute antigen challenge in some allergic patients is biphasic. The early response (ER) follows within minutes of antigen challenge and is characterized by bronchoconstriction in the lung and by rhinorrhea, congestion and sneezing. In the bronchi,

the ER fades out after a few hours and is often followed by a late response (LR), which is characterized by reappearance of symptoms after 4–8 h. Using the nasal challenge test we have previously demonstrated that clinical early and late-phase response occurring after challenge with a high molecular allergen (flour) is associated with mediators released presumably from the mast cells and basophils (*e.g.*, histamine and tryptase) and from the eosinophils (Eosinophil-derived Cationic Protein, ECP and Major Basic Protein, MBP).<sup>1</sup> Tryptase, a tetrameric neutral protease is preferentially found in mast cell secretory granules;<sup>2,3</sup> in basophils its levels are about 0.2–0.4% of levels found in mast cells.<sup>4,5</sup> Studies of mediator release during nasal-allergen challenge indicate that increase of tryptase concentration is the most specific marker of mast cell activation during the immediate allergic response.<sup>6</sup>

Cell counts in nasal lavage have confirmed an

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increase in eosinophils, particularly during LR.<sup>7,8</sup> In severe asthma, there is an increased level of eosinophils in the peripheral blood which is accompanied by a rise in the level of ECP<sup>9</sup> — occasionally reaching 30 µg/l. Nasal antigen provocation leads to a local rise in ECP, especially between the 6th and 24th hour.<sup>10</sup>

The present study was initiated to follow the cellular changes, mucosal/vascular permeability and mediator levels induced by specific and nonspecific nasal provocation. Overall, we intended to evaluate the usefulness of these parameters for a differential diagnosis of occupational allergy.

## MATERIAL AND METHODS

### Subjects

Our study groups consisted of patients admitted to the Clinic of Occupational Diseases in 1993–96. A total of 100 patients (46 males, 54 females) with occupational atopic asthma and rhinitis participated in the study. The mean age of patients was 32.5–11.2 yrs. The diagnosis of occupational allergy was based on a positive history and a significant (> 20%) fall of PEFR (Peak Expiratory Flow Rate) induced by occupational exposure. In 67 patients positive skin prick tests (SPT) with occupational allergens and/or presence of specific serum IgE were also found.

The control group consisted of 20 subjects suffering from a non-occupational bronchial asthma and rhinitis, who were sensitized to house dust mites and 20 healthy volunteers. Sensitization was confirmed by SPT and high level of specific IgE. The group of healthy subjects was recruited from physicians and medical students, and had a mean age of 25.8–3.2 years, with a negative SPT to common allergens and a level of total serum IgE below 60 kU/L. The study was approved by the Medical Ethical Committee and all participating subjects gave their written consent.

### Study protocol

The study was designed as a two-stage, crossover, single blind trial. In the first stage, the patients were challenged with placebo. At least seven days later, during the second stage, participants were challenged with allergen. At least two weeks later, 15 patients with occupational asthma and nine healthy subjects were also challenged intranasally with histamine.

### Nasal lavage and provocation challenge procedure

All procedures were performed by the 'nasal pool' method. Before the provocation each nostril was washed 10 times with 6 ml of saline using the 'nasal pool' device (5-ml syringe closely fitting the nostril). Nasal washings were collected immediately before the provocation and 30 min, 4 and 24 h after the provocation. The allergen (100 mg of flour) was suspended

in 10 ml of phosphate-buffered saline solution (PBS) without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Dulbecco, Sigma, USA). PBS was used as placebo. Saline in the volume of 6 ml was inserted into the nasal cavity for 5 min and then recovered. Then the allergen was applied to the nasal mucosa following the same procedure. This technique permitted the application the agents at predetermined concentrations over a large and well-defined area of the nasal mucosa for extended periods of time. All washings were always performed on the same side of the nasal cavity.

To induce nonspecific nasal irritation, we performed provocation with histamine dihydrochloride (BDH Ltd., Poole UK) at a dose of 50 mg/ml.

### Symptom score

The number of sneezes and the degree of mucosal oedema, rhinorrhea and itching were evaluated. Total symptom scores (SS) ranged from 0–7 and represented the sum of the scores for sneezing (0 sneezes: 0 points; 1–4 sneezes: 1 point; > 4 sneezes: 2 points); rhinorrhea (none: 0 points; mild: 1 point; abundant: 2 points); mucosal oedema (none: 0 points; mild: 1 point; nasal block: 2 points) and itching (none: 0 points; itchy eyes: 1 point). Positive clinical challenge was defined as > 3 points.

### Nasal washings processing

Centrifugation (10 min at 1,000 rpm) of the nasal washings was performed to isolate the cells pellet and the supernatant. The obtained sediment was washed with sterile phosphate buffered saline (Dulbecco, Sigma) and 0.1% human serum albumin (HSA, Behringwerke AG) and then suspended in 1 ml buffer with HSA. Subsequently, the cells were stained using: (1) the Turk method for leukocytes; (2) the Dunger method for eosinophils and (3) 0.06% toluidine blue in 30% ethanol for basophils (metachromatic cells). The cells were counted in a Fuchs-Rosenthal chamber. The number of cells in 1 ml of the recovered fluid was determined.

The samples were further centrifuged at 2,000 rpm for 5 min, transferred onto a slide and air-dried. The slides were stained following the Giemsa method. On each slide the first 200 cells were classified into epithelial cells, eosinophils, neutrophils, basophils and mononuclear cells — a category including lymphocytes and monocytes.

The supernatant total protein content was evaluated with the Lowry method.<sup>11</sup> Albumin concentration was measured using the 'rocket' Laurell method<sup>12</sup> (the assay ranged between 20 and 200 µg/ml). The permeability index, *i.e.*, albumin to total protein ratio was counted.

### Mediator levels

Nasal concentrations of ECP and tryptase were measured with the use of radioimmunoassay (RIA kits, Pharmacia, Sweden) according to the manufacturer

protocol. The samples for these assays were collected before, 30 min, 4 h and 24 h after the provocation challenge.

Skin prick tests (SPT) were performed using common and occupational allergens such as *Dermatophagoides pteronyssinus*, pollens, moulds, trees, house dust flour (Allergopharma, Germany). A negative control was made with the allergen diluent and a positive control with histamine solution. All the sites were examined after 20 min: the grading of the wheal (4 mm > control was considered positive) and flare (5 mm > control — positive) reaction was conducted following standard methods.

Total serum IgE level and presence of specific IgE (RAST) were evaluated (Pharmacia). The threshold of positivity was set at 0.35 kU/l for RAST.

### Pulmonary function and histamine challenge testing

Bronchial response was measured by serial monitoring of Forced Expiratory Volume in 1 sec (FEV1) by a spirometer (Vicatest 2A, Mijhardt, Holland) — before, and then 5 min, 5 h and 24 h after the provocation. Prior to histamine challenge all the patients with occupational allergy presented baseline FEV1 above 70% of the Forced Vital Capacity (FVC). Histamine dihydrochloride obtained from Sigma Chemical Company was prepared in normal saline immediately before the inhalation and delivered through the DeVilbiss nebulizer No 646. The histamine bronchial challenge was performed immediately, 5 and 24 h after the nasal challenge with an allergen or placebo. The histamine concentrations were as follows: 0.03; 0.06; 0.125; 0.250; 0.5; 1; 2; 4; 8 and 16 mg/ml. Histamine PC20H FEV1 is defined as a provocative concentration of histamine causing a 20% fall in FEV1.

### Statistics

Correlation between nonparametric data (such as SS values) and parametric data were analyzed by the Spearman's rank correlation test. The Wilcoxon matched pairs, signed rank test was used to determine the significance of the increase in albumin, cell proportion and total number of cells and mediators levels. The data were expressed as the mean  $\pm$  SEM. The results obtained after the nasal challenge in occupational allergics were compared with those in the healthy subjects and atopic nonoccupational patients using the Mann-Whitney U test. The differences were regarded as significant at  $p < 0.05$ .

## RESULTS

### Symptom score

Nasal challenge with allergen produced the symptoms of rhinitis in 70 out of 100 patients with occupational allergy. The symptoms included sneezing, mucosal

oedema, rhinorrhea and itching with the mean score amounting to  $6.9 \pm 1.0$ . In 55 patients these symptoms appeared immediately after the provocation and lasted for 4 h ( $6.7 \pm 1.1$  points). In 15 patients the symptoms occurred 4 h after the provocation ( $6.4 \pm 1.0$  points) and were still observed 24 h after the provocation ( $4.4 \pm 1.3$  points).

In 17 out of 100 patients with occupational airway allergy, placebo was also found to induce the symptoms of rhinitis. They occurred only immediately after the challenge ( $2.9 \pm 2.3$ ). None of the subjects suffered from symptoms of rhinitis 4 and 24 h later.

After the allergen and placebo challenges none of the healthy and atopic subjects from the control groups presented symptoms of rhinitis.

Nasal challenge with histamine induced symptoms of rhinitis in all of the 15 patients with occupational allergy and in all of the nine healthy subjects. In allergic subjects the symptoms were more severe ( $6.8 \pm 0.4$  points) than in the healthy ones ( $5.7 \pm 1.1$  points). In both the groups, the reaction to histamine was observed only during and immediately after the challenge.

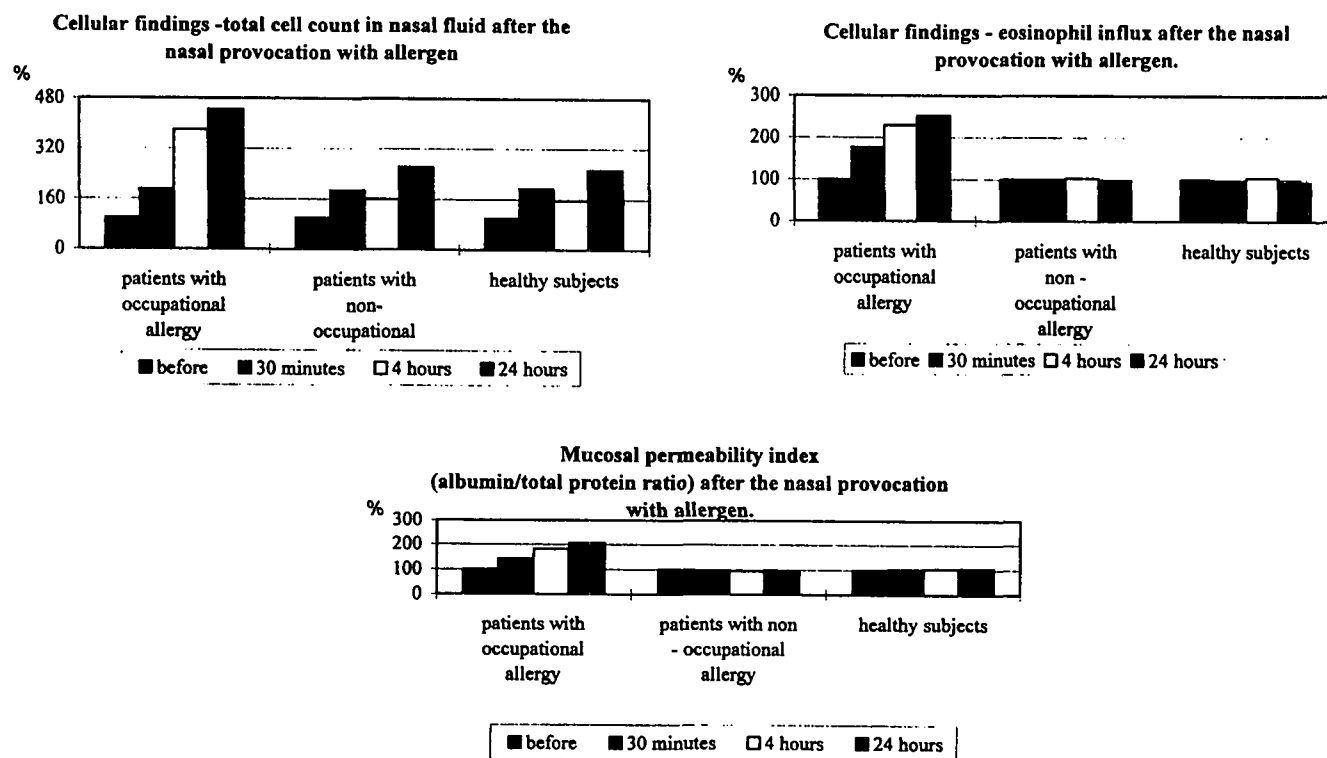
### Cellular and biochemical findings

The provocation with specific allergen caused a significant increase in the number of leukocytes, including eosinophils and basophils in the nasal washings obtained from the patients with occupational allergy. Immediately after allergen provocation, occupational allergic subjects presented a 190% increase in number of leukocytes in the nasal lavage fluid (from  $21 \pm 13.4 \times 10^3$  /ml to  $40 \pm 15.8 \times 10^3$  /ml,  $p < 0.05$ ). The leukocyte count continued to be elevated 4 and 24 h after provocation (counts  $\pm$  SEM at 4 h:  $80 \pm 20.6 \times 10^3$  /ml; 380% increase and at 24 h:  $93.5 \pm 20.7 \times 10^3$  /ml; 445% increase),  $p < 0.05$  vs. baseline value (Figure 1). Immediately after allergen provocation the patients with occupational allergy presented a significant influx of eosinophils into the nasal washings from  $39 \times 10^3$  /ml (SEM  $\pm 11 \times 10^3$  /ml) to  $69 \times 10^3$  /ml (SEM  $\pm 20 \times 10^3$  /ml,  $p < 0.05$ ) (increase to 177% and the eosinophil proportion increased from 8–38%. The eosinophil count was significantly increased even 4 and 24 h after the provocation nasal challenge (counts  $\pm$  SEM at 4 h:  $90 \pm 14 \times 10^3$  /ml; 230% increase and at 24 h:  $99 \pm 10 \times 10^3$  /ml; 253% increase),  $p < 0.05$  vs. baseline value (Figure 1).

A two-fold increase in the eosinophil count was observed 4 and 24 h after the challenge.

A significant increase in the proportion of basophils in occupational allergics was observed immediately and 4 h after the provocation test with occupational allergens (from 2.3–5.4% immediately after provocation,  $p < 0.05$ ).

Biochemical analysis of the nasal washings showed an increase in the albumin level at all time points after provocation. The albumin level increased from  $90 \pm 25$   $\mu$ g/ml to  $169 \pm 32$   $\mu$ g/ml immediately after provocation (143% increase) and was significantly increased

**Figure 1.** Cellular and biochemical findings (mucosal permeability index) in nasal fluid after nasal provocation with allergen.

4 and 24 h after the challenge: 221 µg/ml (181%) and 210 µg/ml (206%),  $p < 0.05$  vs. baseline value. In occupational allergics the nasal provocation with allergen induced an increase in the mucosal/vascular permeability index which persisted till 24 h after provocation from 28–52% immediately after, to 64% and 60%, 4 and 24 h after the challenge, respectively,  $p < 0.05$  (Figure 1). No statistically significant changes in the morphologic and biochemical responses were observed in patients with occupational allergy, with non-occupational allergy and in healthy subjects after the placebo challenge (Figure 2).

Nonspecific challenge with histamine induced cellular influx only immediately after the test. A significant increase in the number of leukocytes in occupational allergic subjects from  $98 \pm 34 \times 10^3/\text{ml}$  to  $176 \pm 70 \times 10^3/\text{ml}$  (200% increase)  $p < 0.05$  and in healthy subjects from  $72 \pm 59 \times 10^3/\text{ml}$  to  $180 \pm 70 \times 10^3/\text{ml}$ , (250% increase),  $p < 0.05$  (Figure 3). The number of eosinophils was also significantly increased in occupational allergic subjects from  $24 \times 10^3/\text{ml}$  to  $64 \times 10^3/\text{ml}$ , from 8–16% of the total cell count (Figure 3) and in healthy subjects from  $19 \times 10^3/\text{ml}$  to  $72 \times 10^3/\text{ml}$ , from 8–15% of the total cell count observed immediately after the challenge. No significant changes between these two groups were found (Mann–Whitney U Test).

Thus, since histamine, a nonspecific irritant, and placebo induce changes in the number of cells and mucosal permeability rate, we considered the test as

positive when the increase in the cell number (especially eosinophils and basophils and in the permeability index) which appeared immediately after the provocation persisted for up to 24 h.

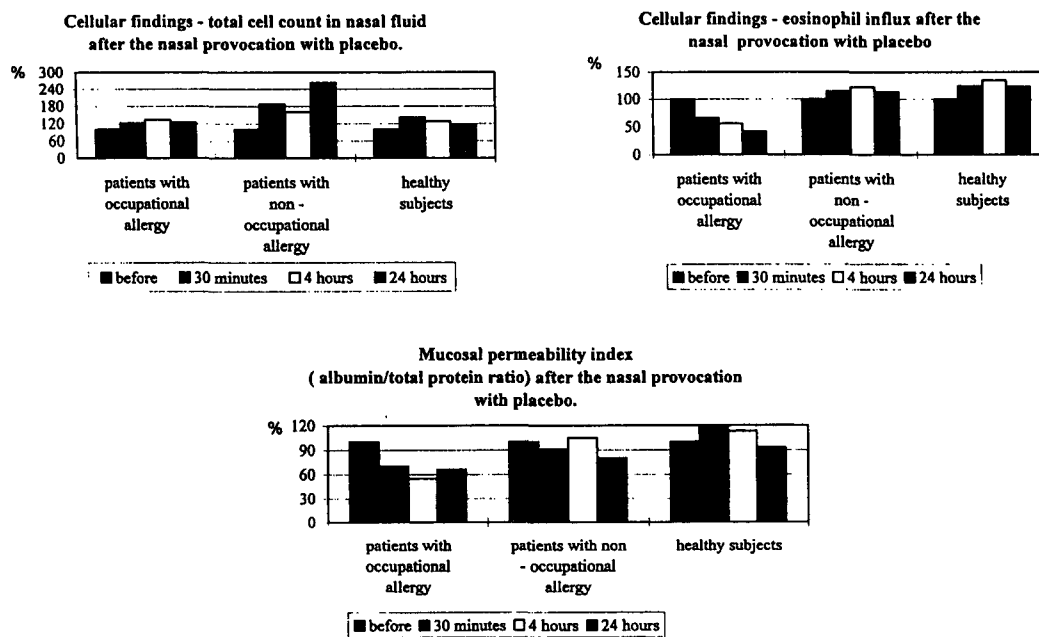
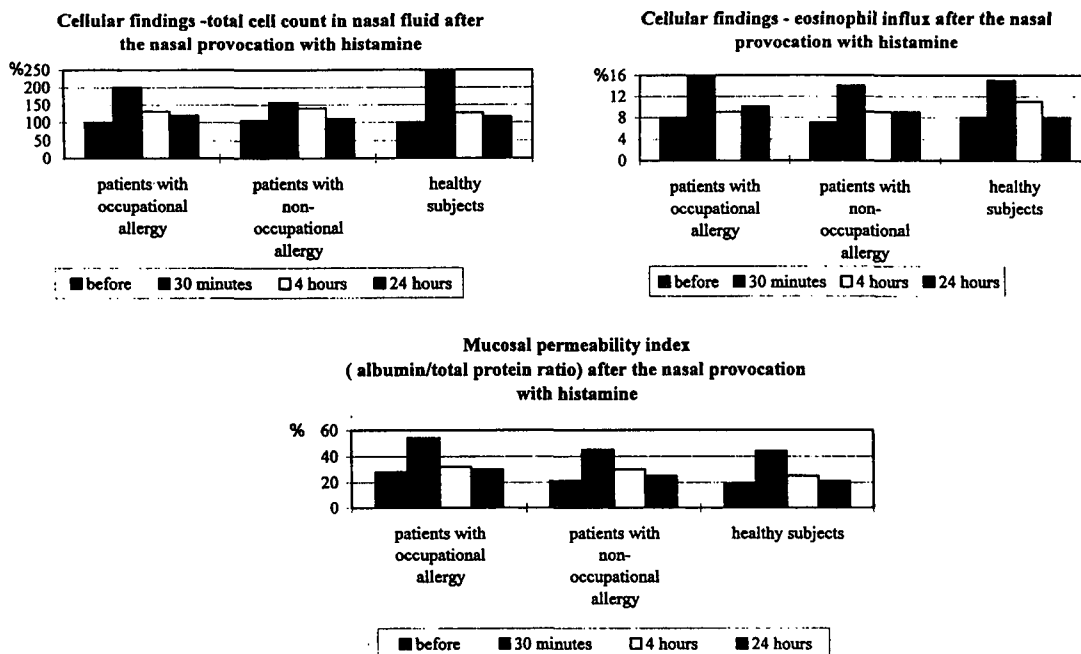
In patients with occupational allergy, histamine induced a higher increase in the albumin concentration (from  $88 \pm 34$  µg/ml to  $184 \pm 56$  µg/ml) and permeability index (from 28–54%) as compared to healthy subjects. These changes could be observed 30 min after the challenge, but 4 and 24 h since the provocation albumin concentration and permeability index did not differ from the initial values (Figure 3).

Using such criteria we looked for a correlation between history and the results of the nasal challenge test, SPT and RAST. We found a significant positive correlation between the history and the challenge test ( $r = 0.78$ ). There was no correlation between positive results of nasal provocation test and SPT or RAST.

### Mediator levels

In occupational allergics the nasal challenge with allergen induced a statistically significant increase in the tryptase concentration only immediately after the test (from  $1.2 \pm 0.9$  to  $3.3 \pm 0.6$  U/l,  $p < 0.05$ ) (Table 1).

Furthermore, allergics were found to have a significantly higher post-challenge levels of ECP in the nasal secretions at all times after the provocation compared with the pre-challenge levels ( $1.9 \pm 0.9$  µg/litres before

**Figure 2.** Cellular and biochemical findings (mucosal permeability index) in nasal fluid after nasal provocation with placebo.**Figure 3.** Cellular and biochemical findings (mucosal permeability index) in nasal fluid after nasal provocation with histamine.

the test and  $4.1 \pm 1.2 \mu\text{g/litres}$  immediately after the challenge;  $19.1 \pm 7.6 \mu\text{g/litres}$  4 h after the test and  $55 \pm 10.3 \mu\text{g/litres}$  24 h after the test,  $p < 0.05$ ) (Table 1). No statistically significant changes were observed in ECP and tryptase levels at any time after the provocation with occupational allergen and placebo in patients with non-occupational allergy and in healthy subjects (Mann–Whitney U Test).

#### The influence of nasal challenge with allergen and placebo on pulmonary function and airway hyperreactivity in patients with occupational allergy

The measurements of the pulmonary function including FEV1 were performed before, 5 min, 5 h and 24 h after the nasal provocation with allergen and placebo.

**Figure 4.** FEV1 and bronchial reactivity to histamine (PC20H) before and after nasal provocation with allergen or placebo in 100 patients with occupational allergy.

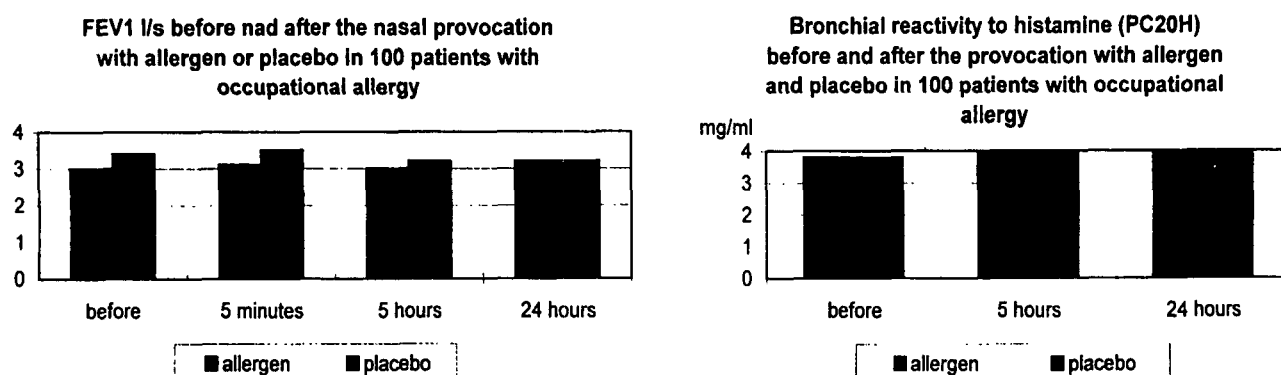


**Table 1.** Mediator levels in the nasal fluid before and after provocation with allergen in 60 patients with occupational allergy

| Time point   | Pre-challenge | 30 min    | 4 h        | 24 h      |
|--------------|---------------|-----------|------------|-----------|
| Tryptase U/l | 1.2 ± 0.9     | 3.3 ± 0.6 | 1.9 ± 1.1  | 1.1 ± 0.9 |
| ECP µg/l     | 1.9 ± 0.9     | 4.1 ± 1.2 | 19.1 ± 7.6 | 55 ± 10.3 |

In patients with occupational allergy the FEV1 value before the nasal provocation =  $3.0 \pm 0.19$  l; immediately after the provocation =  $3.1 \pm 0.2$  l; 5 h after the nasal challenge =  $3.0 \pm 0.16$  l and 24 h after the challenge =  $3.2 \pm 0.3$  l (Figure 4).

Placebo provocation in patients with occupational allergy did not induce significant changes in FEV1 during the time of observation (FEV1 before the provocation =  $3.4 \pm 0.14$  l; immediately after =  $3.5 \pm 0.18$ ; 5 hr after =  $3.2 \pm 0.28$  and 24 hr after =  $3.2 \pm 0.30$  litres,  $p < 0.05$ ).

No statistically significant differences in PC20 were found in patients with occupational allergy after the provocation with allergen (PC20 before = 3.8 mg/ml; 5 hr after the challenge = 3.96 mg/ml and 24 h after the challenge = 4 mg/ml) (Figure 4).

No remarkable changes could be noted after the provocation with placebo in these patients (PC20 before = 3.8 mg/ml; 5 hr after = 4.0 mg/ml and 24 hr after = 3.9 mg/ml).

## Discussion

Occupational airway allergies such as asthma and rhinitis are diseases characterized by bronchial or nasal reactions caused by factors attributed to a particular occupational environment but not to a stimulus encountered outside the workplace. Many agents derived from the occupational environment may cause nonspecific bronchial or nasal reactions, clinically similar to the allergic ones.<sup>13</sup> In the diagnosis of occupational allergy the provocation tests are used more often than in the diagnosis of nonoccupational

diseases. Specific inhalation challenge tests either in the laboratory or in the workplace are used to confirm the relationship between the workplace and the symptoms, to relate the symptoms to a specific agent and to reproduce the temporal relationship between exposure and the onset of symptoms. As bronchial challenge may be sometimes troublesome and dangerous we adopted a nasal pool provocation as a useful and safe method.<sup>14</sup> Typically, the nasal allergen challenge induces a prolonged increase in the total cell count, eosinophil number and a less pronounced but very characteristic increase in metachromatic cell number.<sup>15,16</sup>

Previous studies have revealed that a prolonged increase in albumin/protein ratio as an index of mucosal permeability is more specific for the allergic response than the morphologic changes of nasal fluid.<sup>17,18</sup> The observed similar clinical reaction (itching, swelling, mucosal edema) as well as an increase in the eosinophil count and in the vascular permeability at 4 h after the irritant or allergen challenge suggests that these parameters are not good enough for a differentiation of the cause of ER in the nasal mucosa. The close correspondence of symptoms scores with tryptase activity in all 60 patients with occupational allergy suggests that activation of metachromatic cells in the nasal tissue with releasing of their mediators is the major factor in production of clinical symptoms during the ER in the nasal tissue. We have confirmed that tryptase is a good marker for monitoring mast cell degranulation.<sup>19</sup> In almost all patients with rhinitis we observed prolonged influx of eosinophils and an increase in the mucosal permeability index after the specific allergic provocation. These changes were not observed after placebo or irritant provocation. Similarly Prat *et al.*<sup>17</sup> reported an increased percentage of eosinophils in the nasal washings from rhinitic patients. As described in the present study, the influx of eosinophils and increase in albumin/protein ratio up till 24 h after exposure suggests that these cells are involved in the active inflammatory process present in the nose of rhinitic patients resulting in increased vascular permeability. Thus, since histamine and placebo can induce changes in the eosinophil count and

mucosal permeability we considered the nasal challenge test as positive when the increase of eosinophils and the permeability index persisted for up to 24 h after the challenge and when a two-fold increase of these parameters was observed. In some healthy subjects and patients treated with placebo an increase in the eosinophil number occurred on a very small level of 1–3 cells. Thus, we estimate a cut-off point for the test as at least five cells. If fewer than five eosinophil cells were found in the nasal fluid the test should be considered neither as positive nor negative. Other events apart from eosinophil influx found after allergen challenge were a prolonged increase in the level of eosinophil mediator (ECP).<sup>20</sup> It is interesting to note that the first increase of ECP concentration was observed 4 h after the nasal challenge, which paralleled the significant increase of the eosinophil count and index permeability. This finding supports our hypothesis, that only prolonged increases in the number of these cells, albumin levels and ECP concentration confirm the presence of prolonged nasal inflammation. Wang *et al.*<sup>21</sup> noticed that the combined measurement of the percentage of eosinophils together with the ECP concentration in the nasal secretions seems to be a very useful model in monitoring and assessing the condition of chronic nasal inflammation in patients with allergic rhinitis.

In our experience, the nasal allergen challenge appeared to be very safe method, as neither severe bronchial reactions nor any increase of bronchial hyperreactivity were observed after the provocation. Iliopoulos *et al.*<sup>22</sup> reported that after nasal allergen challenge more allergic subjects showed an increase in cell number (*i.e.*, eosinophils and neutrophils) than developed a LR. A significant correlation between the nasal provocation test and history confirmed by PEFR variability at the workplace is the best evidence of the sensitivity of this test. Lack of correlation between SPT or RAST and the nasal test is not surprising, as purified commercially available antigens or antibodies for skin test and specific IgE test were used. Workplaces are contaminated with mixtures of allergens and that is the reason why purified commercially produced antigens are usually insufficient for the diagnosis of occupational allergies.

In summary, the nasal provocation combined with lavage technique and subsequent morphologic and biochemical analysis of the nasal washings appears to be a very useful and safe tool for diagnosing and differentiating occupational airway sensitizations.

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