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Persistent nasal symptoms and mediator release after continuous pollen exposure in an environmental challenge chamber

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ABSTRACT

Background: Immediate- and late-phase reactions are associated with nasal symptoms of patients with allergic rhinitis.

Objective: To examine the symptoms and mediators released after continuous allergen exposure in an environmental challenge chamber (ECC).

Methods: Fifteen patients with Japanese cedar pollinosis were enrolled in this study and continuously exposed to cedar pollen at a concentration of 8,000 grains/m³ for 3 hours in an ECC. Nasal function tests were performed, and nasal secretions were collected before pollen exposure (0 hour), immediately after exiting the ECC (3 hours), and 6 hours after exiting the ECC (9 hours). Symptom scores were recorded every 30 minutes in the ECC and every 3 hours after exiting the ECC. The frequency of sneezing and nose blowing also was monitored.

Results: The severity of symptoms in the ECC peaked approximately 2 hours after the beginning of pollen exposure and continued more than 6 hours after leaving the ECC. Concentrations of histamine, tryptase, interleukins 5, 3, 33, and 31, and substance P increased over time, whereas that of nasal fractional exhaled nitric oxide decreased.

Conclusion: Various mediators are released during continuous allergen exposure, which subsequently induce persistent nasal symptoms. Effective treatment is required to control the intense inflammation observed after allergen exposure.

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Introduction

Nasal symptoms are observed in patients with allergic rhinitis (AR) after nasal provocation with allergen-coated disks or nasal spray and comprise the immediate and late phases of AR.^{1–5} Histamine released from mast cells plays an important role in the immediate-phase reaction and induces sneezing, rhinorrhea, nasal itch, and some degree of nasal congestion. This reaction occurs within 15 minutes after antigen challenge and persists for only a couple of hours.¹ Leukotrienes released from infiltrating

eosinophils are primarily involved in the late-phase reaction, which occurs several hours after antigen exposure and induces nasal congestion.⁴ However, it is difficult to evaluate the 2 phases separately in patients under natural exposure to allergens in daily life, and different pathophysiologic changes can be observed.

Environmental challenge chambers (ECCs) are used to expose subjects to the same amount of pollen in a stable atmosphere and thus could be useful for evaluating the pathophysiology of AR.⁶ As previously described, the ECC has an area of 71.8 m² and can accommodate up to 50 participants. Pollen is supplied from an exposure room above the ceiling through holes where 9 fans agitate the pollen particles. The pollen is collected through the mesh floor of the ECC to avoid accumulation. The pollen level is monitored at 56 points within the ECC using laser particle technology.⁶ Continuous pollen exposure in the ECC induces symptoms mimicking those observed naturally during the pollen season. In particular, Japanese cedar (*Cryptomeria japonica*) pollen is the most common allergen associated with seasonal AR in Japan.⁷

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In the present study, patients with cedar pollen-induced AR were exposed for 3 continuous hours to the pollen in an ECC, and subsequent symptoms, physiologic changes, and various mediators in nasal secretions were carefully examined.

Methods

Subjects

We recruited 15 subjects (mean age 37.8 ± 10.3 years) with a history of Japanese cedar pollen-induced AR for at least 2 consecutive cedar pollen seasons and who met the following inclusion criteria: a positive allergen-specific skin test result (wheal diameter ≥ 5 mm and twice that of the negative control) to standardized cedar pollen extract (Torii Pharmaceutical Co, Ltd, Tokyo, Japan) and a serum cedar pollen-specific immunoglobulin E (IgE) level of at least 0.71 kU/L (class 2) by an ImmunoCAP test (SRL Hachioji Laboratories, Tokyo, Japan). The exclusion criteria were AR caused by other allergens that required treatment, severe asthma, use of antiallergic drugs (antihistamines, leukotriene receptor antagonists, chemical mediator receptor antagonists, and systemic or topical steroids) within 4 weeks of the study, pregnancy, the possibility of pregnancy, and women currently lactating. We also excluded participants with non-AR, including upper respiratory tract infection, rhinosinusitis, and other diseases that appeared to influence the results. The study protocol was approved by the ethics committee of Chiba University, Graduate School of Medicine (Chiba, Japan; approval number 1412), and written informed consent was obtained from all subjects.

Environmental Challenge Chamber

The examinations were conducted in the ECC at Chiba University, which can accommodate up to 50 subjects. As we reported previously,⁶ the concentration of Japanese cedar pollen was set to 8,000 grains/m³. The pollen concentration in the air was monitored every 5 minutes using an automatic pollen measuring instrument (Shinyei Kaisha, Kobe, Japan); we previously confirmed that the symptoms of Japanese cedar pollen-induced AR peak within 3 hours at this pollen concentration.⁶ The symptoms of all subjects in the ECC were recorded using an e-diary device (Willcom, Inc, Tokyo, Japan).

Study Protocol

A schematic representation of the study protocol is shown in Figure 1. This study was planned to avoid the pollen dispersal season and was carried out in 2012. All 15 subjects were exposed to cedar pollen at a concentration of 8,000 grains/m³ for 3 hours in the ECC. Nasal symptoms were evaluated during active pollen exposure and after exiting the ECC. The frequency of sneezing and nose blowing and the severity of nasal symptoms (eg, sneezing, rhinorrhea, and nasal congestion) were recorded every 30 minutes in the ECC using an e-diary device. In addition, the subjects were required to self-record their nasal symptoms at 3, 6 and 9 hours after exiting the ECC using an allergy diary. The frequency of sneezing and nose blowing also was recorded every 3 hours. Furthermore, we measured the levels of nasal fractional exhaled nitric oxide (FeNO) and nasal airway resistance and obtained nasal secretions and nasal lavage measurements before the subjects entered the ECC, immediately after they exited the ECC, and 6 hours after they exited the ECC.

Scoring of Nasal Symptoms

The severity of the 3 surveyed symptoms was evaluated on a 4-point scale (range 0–3) and defined as none (score 0), mild (score 1), moderate (score 2), or severe (score 3). The total nasal symptom score was defined as the sum of these scores (range 0–9).

Nasal FeNO

The nasal FeNO level was measured according to the American Thoracic Society and European Respiratory Society guidelines⁸ using a handheld electrochemical analyzer (NObreath; Bedfont Scientific Ltd, Kent, United Kingdom). The subjects were instructed to inhale ambient air to near total lung capacity and then exhale transnasally through a nose piece connected to the device. The flow rate was set to 50 mL/s, and the duration was 16 seconds. Each measurement was obtained twice, and the mean value was used for the analysis.⁹

Nasal Airway Resistance

Rhinomanometry was performed to examine the degree of nasal airway resistance. The extent of bilateral nasal resistance at $\Delta P100$

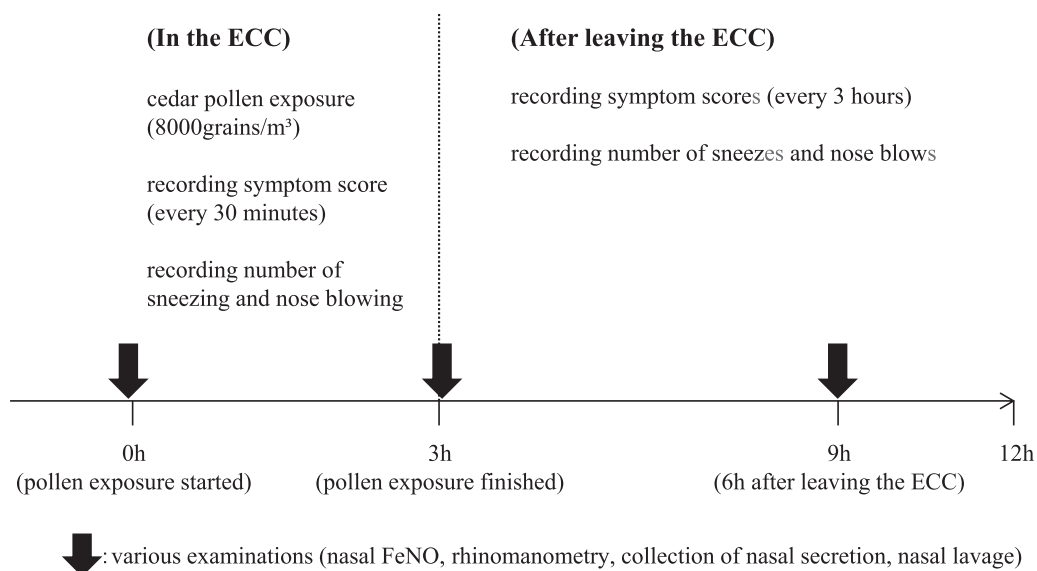


Figure 1. Study protocol. Various examinations (rhinomanometry, measurement of nasal fractional exhaled nitric oxide [FeNO], nasal secretion collection, and nasal lavage) were performed before the subjects entered, immediately after they exited, and 6 hours after they exited the environmental challenge chamber (ECC). Symptom scores were recorded every 30 minutes in the ECC and every 3 hours after exiting the ECC. The frequency of sneezing and nose blowing was recorded every 3 hours.

Pa was measured using active anterior rhinomanometry with a nasal nozzle attached to a rhino rheograph (MPR-3100; Nihon-Kohden, Tokyo, Japan), and the total nasal resistance was calculated as the sum of the bilateral rhinomanometry recordings.^{10,11}

Measurement of Mediators in Nasal Secretions

The IVALON 4000 plus surgical sinus pack ($80 \times 15 \times 20$ mm; First Aid Bandage Co, New London, Connecticut) was gently inserted into each common nasal meatus, removed after 10 minutes, and placed into the shaft of a 5-mL syringe (Terumo Corp, Tokyo, Japan), after which the syringe was inserted into a 15-mL Falcon tube (BD Biosciences, Franklin Lakes, New Jersey). The mass of the collected secretions was calculated by comparing the weight of the sinus pack with that of the Falcon tube and syringe shaft before and after insertion using the same balance (Mettler Toledo, Columbus, Ohio); 1 g of nasal secretions was considered equal to 1 mL. After weighing the samples, 3 mL of sterile physiological saline solution was placed in the sinus pack. Then, the Falcon tube containing the shaft with the sinus pack was centrifuged at $1,500 \times g$ for 10 minutes at 4°C to extract the nasal secretions.¹² Aliquots of 500 μL were prepared and stored at -80°C until further analysis.

Levels of interleukin (IL)-4, IL-5, IL-13 and interferon- γ were measured using the Cytometric Bead Array kit (BD Biosciences), according to the manufacturer's instructions. The IL-31 and IL-33 concentrations were measured using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (PeproTech, Rocky Hill, New Jersey). The histamine, tryptase, leukotriene, and eosinophilic cationic protein concentrations were

measured by SRL Hachioji Laboratories, and the substance P and prostaglandin D_2 levels were measured by Kyowa Hakko Kirin Co, Ltd (Tokyo, Japan) and Shionogi Co, Ltd (Osaka, Japan), respectively.

Immunohistochemical Staining of Cells Infiltrating the Nasal Lavage Fluid

Nasal lavage was performed by irrigating 5 mL of physiologic saline into each nasal cavity for 10 seconds while the subject was in the hanging-head position repeating the "k-k-k" sound multiple times to close the soft palate and minimize fluid running into the throat.¹³ When appropriate, the nasal lavage fluid was collected using a suction kit (MD-33030, Sumitomo Bakelite Co, Ltd, Tokyo, Japan). Mucin was removed through a $70\text{-}\mu\text{m}$ cell strainer (BD Biosciences) and centrifuged at 1,500 rpm for 5 minutes at 4°C . The samples were centrifuged again at 3,000 rpm for 5 minutes at 4°C to obtain single-cell suspensions (5×10^5 cells/mL). The single-cell suspensions were mounted on Cytospin slides and stored at -80°C for future immunohistochemical staining.

The Cytospin slides were fixed in cold acetone for 10 minutes, washed with phosphate buffered saline, and stained with the anti-basophil antibody 2D7 (Abcam Plc, Cambridge, Massachusetts) followed by anaphase-promoting complex-coupled anti-mouse antibody (BioLegend, Inc, San Diego, California). The nuclei were stained with TO-PRO-3 Iodide (Invitrogen, Carlsbad, California). All slides were assessed using a LSM700 scanning laser confocal microscope and an imaging software program (Zen 2011; Carl Zeiss MicroImaging GmbH, Jena, Germany).

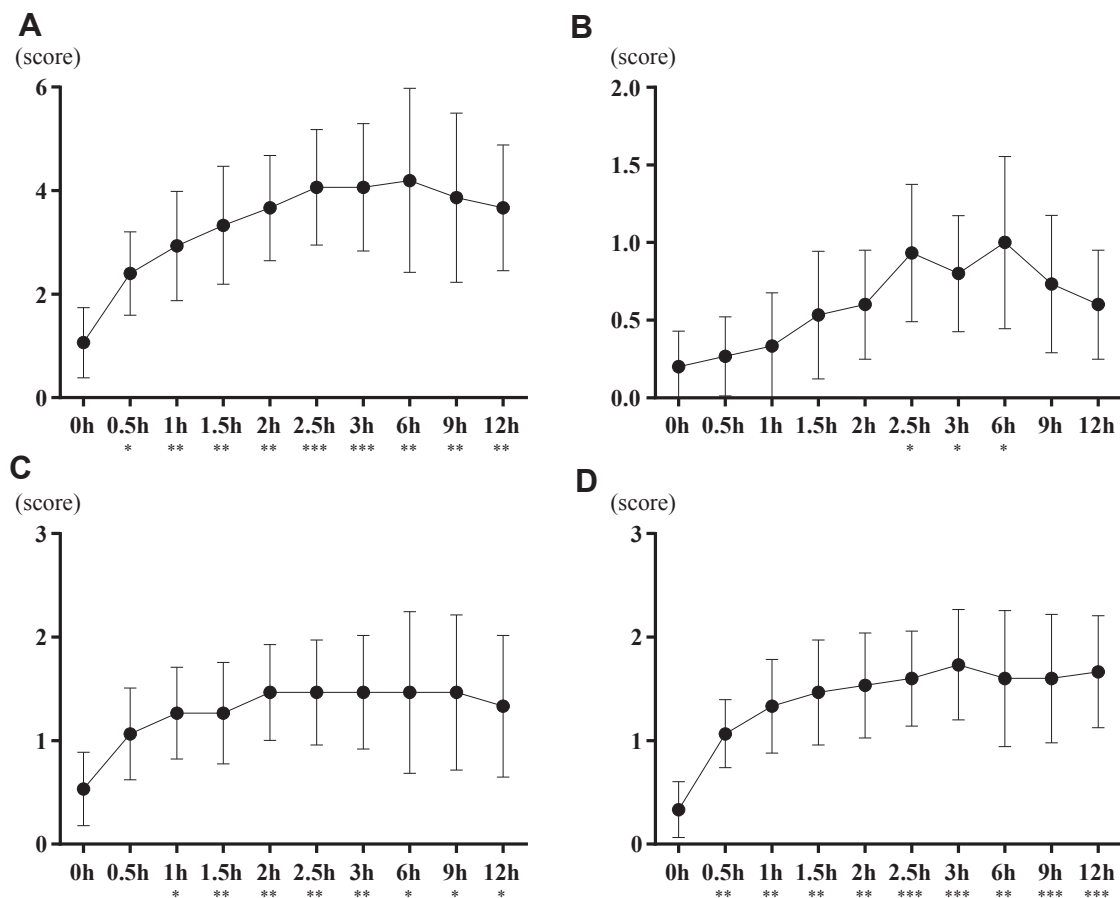


Figure 2. Changes in nasal symptoms: (A) total nasal symptoms, (B) sneezing, (C) rhinorrhea, (D) nasal congestion. Values are presented as mean $\pm$ 95% confidence interval (* $P < .05$, ** $P < .01$, *** $P < .001$ vs 0 hour).

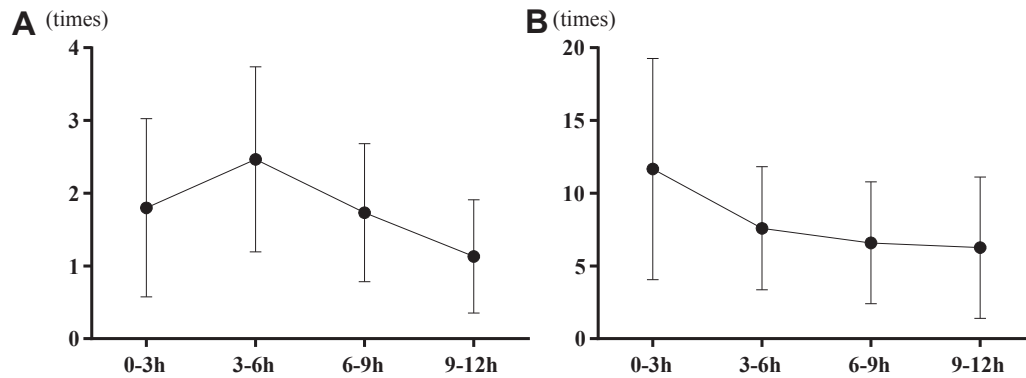


Figure 3. Frequencies of sneezing and nose blowing: (A) sneezing frequency, (B) nose blowing frequency. Values are presented as mean $\pm$ 95% confidence interval.

Statistical Analysis

All statistical analyses were performed using the 2-tailed Wilcoxon signed-rank test. The data are presented as mean $\pm$ 95% confidence interval. A correlation analysis was performed using the Spearman rank correlation coefficient. A probability (*P*) value less than .05 was considered statistically significant.

Results

Changes in Nasal Symptoms

The severity of each symptom and total nasal symptom score are shown in Figure 2. All symptom scores increased gradually and plateaued approximately 2 hours after initial pollen exposure. The total nasal symptom score significantly increased from baseline at all time points and peaked (approximately 4 times that at baseline) at 6 hours after initial pollen exposure. Sneezing, rhinorrhea, and congestion persisted after the subjects exited the ECC. The frequencies of sneezing and nose blowing are shown in Figure 3. Notably, sneezing and rhinorrhea persisted after the subjects exited the ECC.

Nasal Physiologic Function Tests

The 0-, 3- and 9-hour time points in the figures represent the times before pollen exposure, immediately after exiting the ECC, and 6 hours after exiting the ECC, respectively. The degree of nasal airway resistance at 3 and 9 hours was significantly higher than that observed at 0 hour. No significant differences were observed between the 3- and 9-hour measurements (Fig 4A). As shown in

Figure 4B, the nasal FeNO values at 3 and 9 hours were significantly lower than those obtained at 0 hour, whereas no significant differences were observed between the values recorded at 3 and 9 hours.

Mediators of Nasal Secretion

As shown in Figure 5B to E, the IL-5, IL-13, IL-31, and IL-33 levels increased significantly over time. The IL-4 levels were significantly higher at 9 hours than at 0 hour, although no significant differences were observed among the other time points (Fig 5A), and no significant differences were detected in the interferon- γ levels (Fig 5F). The leukotriene, eosinophilic cationic protein, histamine, and substance P levels also increased significantly over time (Fig 5G–I, K). The tryptase levels were significantly increased at 3 and 9 hours compared with those noted at 0 hour, whereas no significant differences were observed between the values obtained at 3 and 9 hours (Fig 5J). The concentrations of prostaglandin D₂ were very low in all samples and did not increase after pollen exposure.

Immunohistochemical Staining of Cells Infiltrating the Nasal Lavage Fluid

Although it was difficult to identify basophils in the nasal lavage fluid collected from the subjects immediately after exiting the ECC (Fig 6A), basophil (2D7-positive cells) infiltration into the nasal lavage fluid was confirmed 6 hours after exiting the ECC (Fig 6B). This tendency was found in most participants.

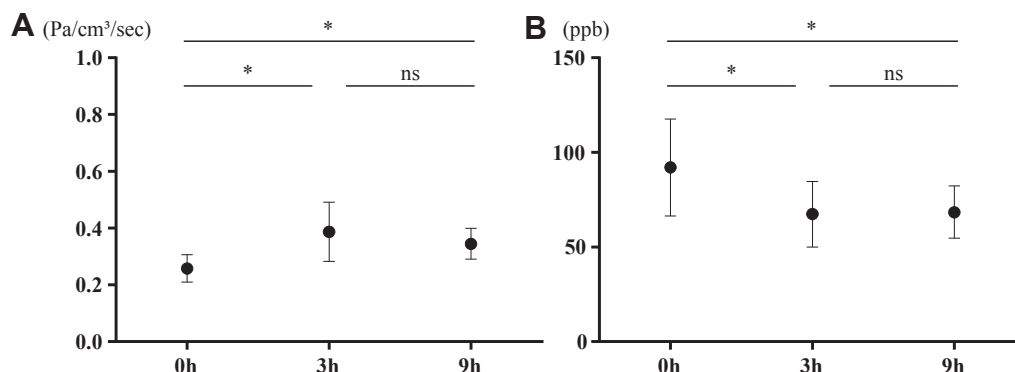


Figure 4. Nasal physiologic function tests: (A) nasal airway resistance, (B) nasal fractional exhaled nitric oxide. Values are presented as mean $\pm$ 95% confidence interval (**P* < .05, ***P* < .01, ****P* < .001).

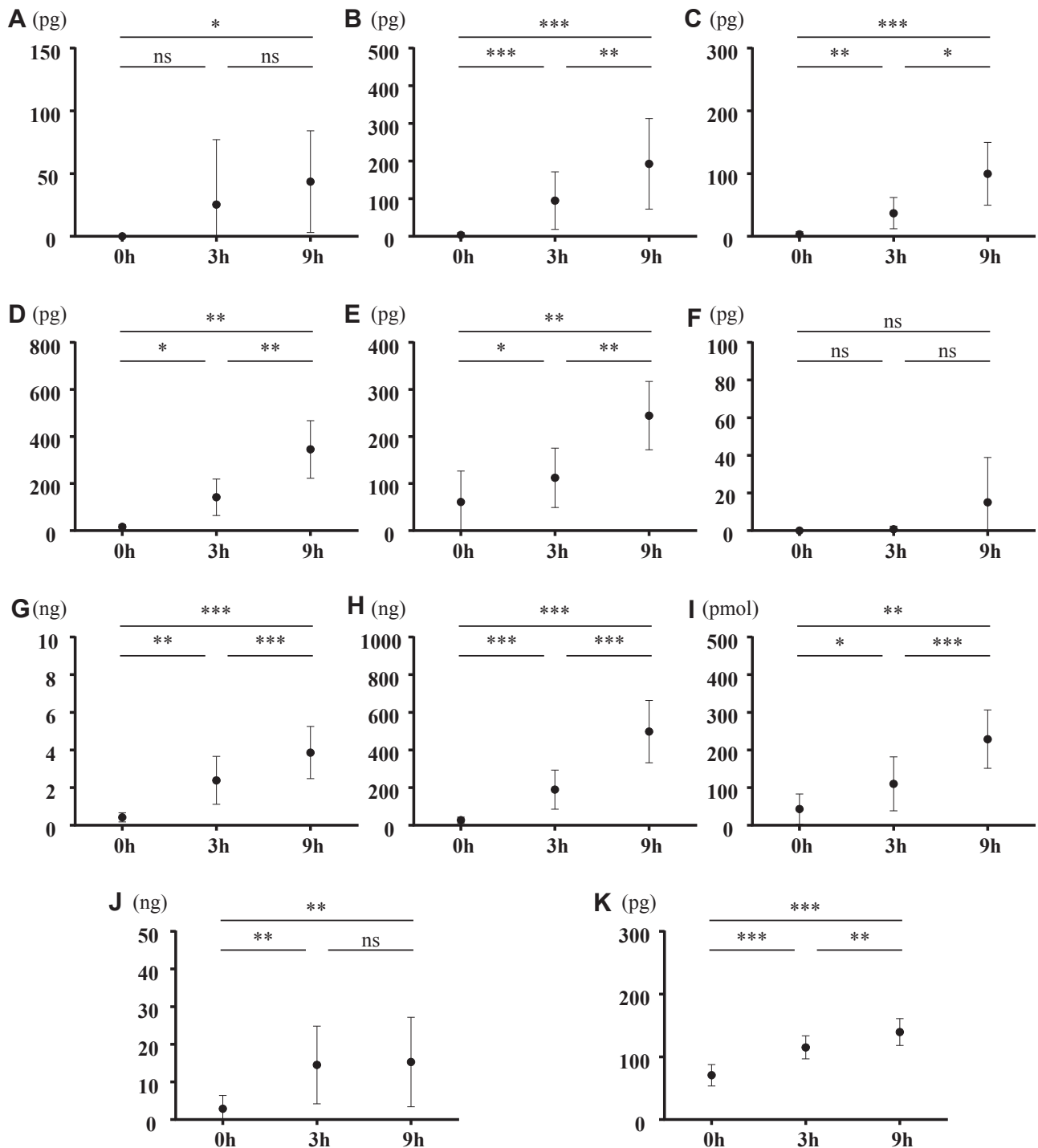


Figure 5. Mediators in the nasal secretions: (A) interleukin-4, (B) interleukin-5, (C) interleukin-13, (D) interleukin-31, (E) interleukin-33, (F) interferon- γ , (G) leukotrienes, (H) eosinophilic cationic protein, (I) histamine, (J) tryptase, (K) substance P. Values are presented as mean $\pm$ 95% confidence interval (* P < .05, ** P < .01, *** P < .001).

Correlations Between Nasal Symptoms and Chemical Mediators in the Nasal Secretions

The histamine, substance P, and IL-31 concentrations at 9 hours significantly correlated with the total number of sneezing and nose blowing episodes from 6 to 12 hours (Fig 7A–C).

Discussion

In the 1990s, some worthwhile studies characterized the early- and late-phase responses of AR by exposing the nasal mucosa to

allergen extract-coated disks or sprays. Approximately half the patients in these studies showed late-phase responses after such provocation.^{1–5} The number of eosinophils and neutrophils in the nasal lavages increased in the late phase but did not always correlate with the development of the symptoms in the late phase. Other unknown factors were expected to contribute the phenomenon.⁵

In the present study, we found that nasal obstruction, sneezing, and rhinorrhea persisted after the 3-hour pollen exposure period in the ECC. The severity of these symptoms observed at 6 hours after

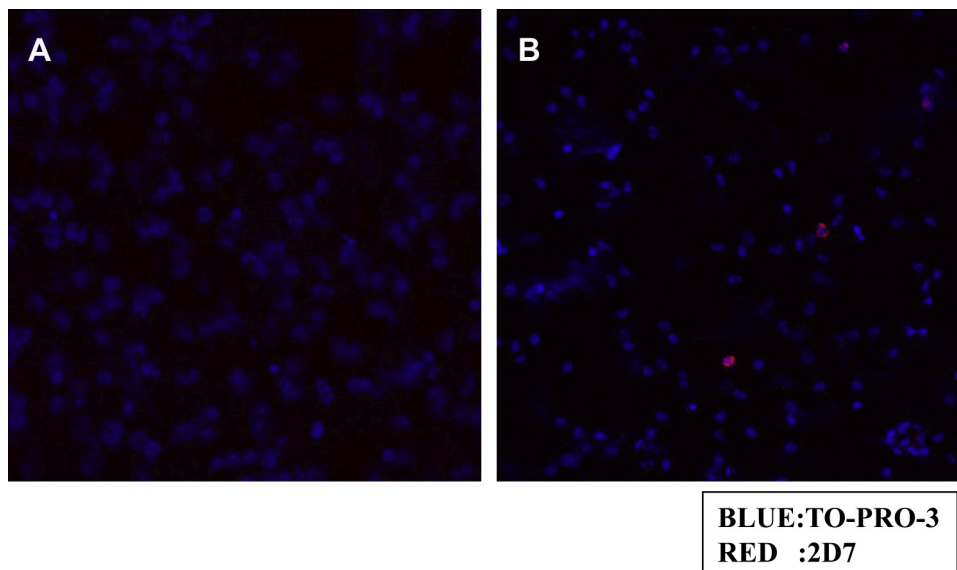


Figure 6. Immunohistochemical staining of cells infiltrating the nasal lavage fluid: (A) immediately after exiting the environmental challenge chamber (3 hours) and (B) 6 hours after exiting the environmental challenge chamber (9 hours). Infiltration of basophils (2D7-positive cells; red) was confirmed 6 hours after exiting the environmental challenge chamber. Cell nuclei were nonspecifically stained using TO-PRO-3 (blue).

exiting the ECC was almost the same as that observed immediately after the 3-hour pollen exposure. In the nasal secretions, various increased mediators were detected. In particular, the histamine and tryptase concentrations in the nasal secretions increased during pollen exposure and remained elevated after exiting the chamber. Histamine is released from mast cells or by basophil degranulation and acts on H_1 receptors to induce vascular permeability and stimulate nociceptive nerves, which subsequently recruit parasympathetic reflexes. Tryptase is a serine protease component of mast cell granules. The quantity of tryptase in basophils is much smaller than that noted in mast cells, although basophils also release tryptase during degranulation.¹⁴ Although performing quantitative analyses of inflammatory cells in nasal secretions is difficult, a significant number of basophils was observed in the nasal secretions in all subjects collected 6 hours after exiting the ECC in this study. Basophils and mast cells present with the high-affinity IgE receptor and are activated by cross-linking with antigens through IgE, followed by consequent histamine release.

Mast cell and basophil degranulation also occurs directly in response to various IgE-independent stimuli, including substance P and IL-33.^{15–17} In the present study, the concentrations of histamine and tryptase increased over time, as did those of IL-33 and substance P. The activation of nociceptive nerves by histamine might induce the axon response-mediated release of neuropeptides, such as substance P,¹⁸ and thereby enhance degranulation and T-helper type 2 cell (T_H2) inflammation. IL-33 is one of the many representative cytokines produced in the airway epithelium that is known to induce allergic inflammation after allergen exposure or by an IgE-independent mechanism.¹⁹

Sneezing and rhinorrhea are induced by histamine and perhaps other mediators, such as substance P and IL-31. IL-31 is markedly released by T_H2 cells and directly stimulates sensory nerve fibers, resulting in a sensation of itching in patients with atopic dermatitis.^{20–23} Although the role of IL-31 in AR is not well defined, it might induce nasal itching and sneezing. Recently, Baumann et al.²⁴ reported that IL-31 levels in nasal secretions become elevated by 5 hours after a single nasal allergen challenge. In the present study, the histamine, substance P, and IL-31 concentrations at 9 hours significantly correlated with the total number of sneezing and nose blowing episodes from 6 to 12 hours. Hence, the

allergen-independent release of mediators, such as substance P and IL-31, and not only histamine, might have contributed to the episodes of sneezing and rhinorrhea observed after leaving the chamber in this study.

Leukotrienes act on leukotriene receptors 1 and 2 present on vessels in the nasal mucosa and subsequently obstruct the nasal passages.^{25,26} In the present study, an increase in the concentrations of leukotrienes was observed, although this trend did not correlate with the severity of nasal obstruction. Many other mediators are expected to participate in nasal obstruction. However, the concentrations of nasal FeNO were not correlated with the severity of nasal symptoms, including nasal obstruction, and even decreased over time under conditions of increased allergic inflammation. NO is produced by inducible NO synthase in inflammatory cells and is a useful inflammatory marker in the lower airways.²⁷ The endothelial NO synthase present in the paranasal sinus mucosa of the upper airway produces a high NO concentration.²⁸ Swelling of the nasal mucosa and the accumulation of nasal secretions induced by pollen exposure obliterate the orifice of the sinus mucosa to the nasal cavity, thus decreasing the concentration of nasal NO.

In addition, nasal airway resistance was not found to be correlated with nasal obstruction or levels of mediators, such as leukotrienes. This lack of correlation might be due to the limited sensitivity and specificity of the rhinomanometry procedure used in this study.

As described previously, IL-33 exists in epithelial cells and is released extracellularly with stimulation or damage caused by various antigens or pathogens, such as pollen, mites, and microbes.^{29–31} Moreover, IL-33 plays an important role in the initiation, amplification, and maintenance of T_H2 -skewed allergic inflammation. We recently found pathogenic T_H2 cells under conditions of eosinophilic inflammation in the respiratory tract in mice and the pathogenic sinonasal mucosa in patients with rhinosinusitis.³² Pathogenic T_H2 cells produce large quantities of T_H2 cytokines compared with conventional memory T_H2 cells after synergistic stimulation of IL-33 with specific antigens.³² Although pathogenic T_H2 cells might be involved in the development and progression of AR, further studies are needed to clarify the role in greater detail.

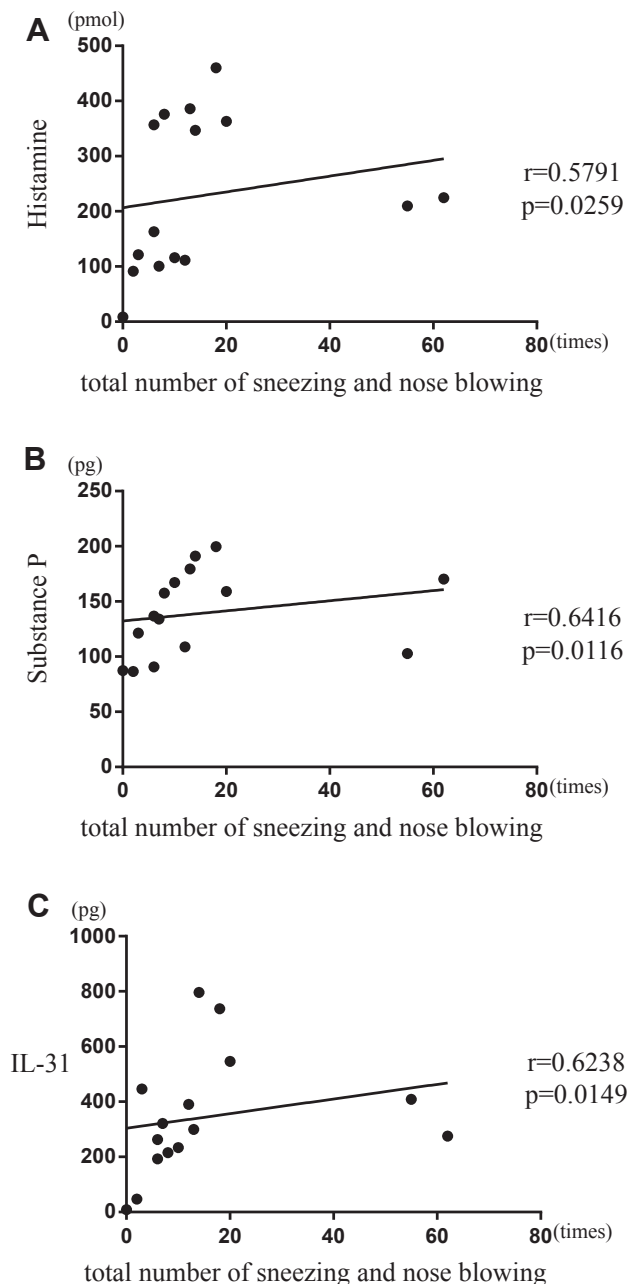


Figure 7. Correlations between nasal symptoms and chemical mediators in the nasal secretions. (A) Histamine, (B) substance P, and (C) interleukin-31 (IL-31) concentrations at 9 hours were significantly correlated with the total number of sneezing and nose blowing episodes from 6 to 12 hours.

During the pollen dispersal season, affected patients can develop indoor nasal symptoms, including sneezing and rhinorrhea, and sleep disturbances, although the number of pollen grains is minimal. In addition, patients often develop severe symptoms on rainy days. Such symptoms could result from minimal persistent inflammation of the nasal mucosa. Furthermore, the allergic inflammation induced by the extensive allergic reaction resulting from outdoor pollen exposure often persists for an extended period. In the present study, intensive inflammation was induced in the nasal mucosa during continuous pollen exposure in the ECC, and the nasal symptoms, including sneezing, persisted after leaving the chamber. This study was conducted outside the natural pollen dispersal season, and the subjects used disposable overalls, hair caps, and shoes

in the ECC, which they subsequently took off when they left the ECC to not carry the pollen outside. Moreover, any pollen would have exited the nasal cavity after the subjects left the ECC as a result of nasal lavage. In previous similar studies conducted using an ECC, we found that nasal symptoms persisted for 3 days after 3 hours of pollen exposure.³³ More severe changes can occur under natural continuous allergen exposure. Therefore, developing an effective treatment for intense inflammation observed after continuous allergen exposure in the nasal mucosa is of critical importance.

We found that a significant degree of nasal symptoms, including sneezing and rhinorrhea, persisted in the late-phase reaction and that levels of leukotriene, histamine, and tryptase were increased in the nasal lavage at that time. However, we could not clearly determine the detailed mechanisms of the nasal symptom in the late phase. Thus, detailed evaluations of the mediators and the infiltrated immune cells in the nasal mucosa are necessary.

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