

A New Model of Experimental Allergic Rhinitis Using Japanese Cedar Pollen in Guinea Pigs

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ABSTRACT -In the majority of the models of experimental allergic rhinitis, antigen challenge has been performed by single topical instillation or perfusion with the solution. The present study was performed to establish a good model using Japanese cedar pollen, which is able to repeatedly induce allergy restricted to the upper airway. Guinea pigs sensitized with the pollen extracts were subjected to quantitative and repeated inhaling of the pollen with a devised apparatus. Following the respective challenges, the nasal cavity was washed with a new technique: Washing with physiologic saline was performed from one nostril to the other one, the latter of which was kept under slightly reduced pressure. When the animal was subjected to cedar pollen inhalation, almost all the pollens inhaled were located in the upper airway. At the 5th inhalation, nasal cavity lavage revealed that both albumin leakage and histamine release into the nasal cavity were increased at maximum levels in 1 hr (respectively 2 mg and 3 ng/animal); and at the same time, a considerable number of leukocytes, especially eosinophils, were found migrating into the nasal cavity for at least 10 hr. The present methods can permit various analyses of allergic rhinitis and the assessment of drugs without sacrificing the animal over the long term.

Keywords: Allergic rhinitis, Airway, Nasal cavity lavage, Histamine, Pollen

Allergic rhinitis is one of the typical atopic diseases. So far, there have been no extensive studies to develop diverse models of allergic rhinitis in experimental animals, although the prevalence of the disease seems to be accelerated worldwide. Clinical studies are easy to perform in the nose because it is readily accessible for biopsies, lavages and recovery of the nasal secretion with little risk and discomfort to the patient. However, considering the difficulties for performing investigations in patients under certain conditions and of evaluating therapeutic drugs, newly developed or presently used, we believe that the development of experimental animal models for allergic rhinitis is valuable.

There have been several reports on experimental allergic or non-allergic rhinitis using mainly guinea pigs. However, in the majority of these papers, the results were either from the experiments by single challenge by the topical application of intranasal instillation with antigen solution (1, 2) or from the method of antigen perfusion of the nasal cavity from the pharynx to the nostrils (3, 4). To resolve such drawbacks in experimental allergic rhinitis,

we attempted to establish a good experimental model for research on allergic rhinitis using the guinea pig.

Japanese cedar (*Cryptomeria japonica*) pollen was used for a number of reasons. It is the prevailing allergen in Japan, which mainly induces allergic rhinitis and conjunctivitis. In addition, its large diameter of 30–40 μm makes it a particularly suitable antigen for inducing experimental allergic rhinitis.

In the present paper, we will describe a new method for producing experimental allergic rhinitis with Japanese cedar pollen in guinea pigs, in which allergy restricted to the upper airway can be repeatedly induced by quantitative pollen inhalation with a simple hand-made apparatus; in this model, the nasal cavity lavage fluid (NCLF) for analyses can be effectively recovered without sacrificing the animal.

MATERIALS AND METHODS

Animals

Male, 3-week-old Hartley guinea pigs weighing 200–

250 g were purchased from Japan SLC, Hamamatsu.

Materials

Sodium pentobarbital (Abbott Lab., North Chicago, IL, USA); guinea pig albumin and anti-guinea pig albumin antibody rabbit serum (Organon Teknika, West Chester, PA, USA); biotin-*N*-hydroxysuccinimide and streptavidin-horseradish peroxidase conjugate (Gibco BRL, Gaithersburg, MD, USA); ovalbumin, *o*-phenylenediamine, histamine dihydrochloride and *o*-phthaldialdehyde (Wako Pure Chem., Osaka); and Evans' blue (Merck, Darmstadt, Germany). The other reagents used were the highest grade of commercial products available.

Japanese cedar pollens were donated from the laboratory of Torii Pharm. Co., Ltd., Chiba.

Preparation of Japanese cedar pollen extracts

The cedar pollen extracts used for the sensitization were prepared as follows: The pollens were suspended in physiologic saline at 50 mg/ml and allowed to stand at 4°C for 18 hr under mild stirring. After centrifugation at $1,700 \times g$ for 15 min at 4°C, the resultant supernatant was used as the sensitization antigen; it was stored at -80°C until use. The protein concentration in the solution was quantified according to the method of Bensadoun and Weinstein (5); it was estimated to be 110 µg protein/ml.

Preparation of aluminum hydroxide gel [$Al(OH)_3$]

$Al(OH)_3$ was prepared by modifying the method of Levine and Vaz (6). In brief, 0.5 N NaOH was instilled into 0.5 N $Al_2(SO_4)_3$ under vigorous stirring. After washing 3 times with purified water, the gel was stored at 60 mg/ml in physiologic saline in an air-tight glass bottle at 4°C.

Inhalation apparatus and inhalation

An apparatus was devised for the challenge/boost by inhaling a quantitative amount of the pollen. As shown in Fig. 1, one side of the tubing was netted with a nylon gauze (25 µm), and 3 mg of the pollen was poured into the tube. The apparatus was properly positioned in the left nostril of either an anesthetized or unanesthetized guinea pig for 1 min so that it could inhale the pollen under spontaneous breathing and, thereafter, in some cases the pollen was also put in the right nostril. During the inhalation, the other nostril was plugged with a finger.

Counting the number of inhaled pollen particles distributed to the airways

At 10 and 60 min after the pollen inhalations (3 mg each) through both nostrils, the guinea pig was sacrificed under pentobarbital anesthesia (40 mg/kg, i.v.). After tracheothoracotomy and plugging the oral cavity with glycerinated cotton, the upper airway was washed with 100 ml physiologic saline through the larynx. The number of cracked but not split pollen ghosts in the recovered fluid was counted under a microscope following centrifugation at $1,700 \times g$ for 10 min at room temperature. The number of pollen particles inhaled into the lower airway was also counted microscopically after the following procedures: The lung was incised and placed under reduced pressure to eliminate the air in alveoli. The lung then was immersed into 1.2 N KOH (15 ml/animal), with which the internal surface of the trachea had been washed to recover the pollen (ghost). After allowing the immersed tissue to stand at 37°C for 18 hr to solubilize the tissue, the suspension was centrifuged at $1,700 \times g$ for 10 min at room temperature. The precipitate was isolated by filtration over nylon gauze (70 µm) and then suspended in purified water (50 ml). This suspension was further

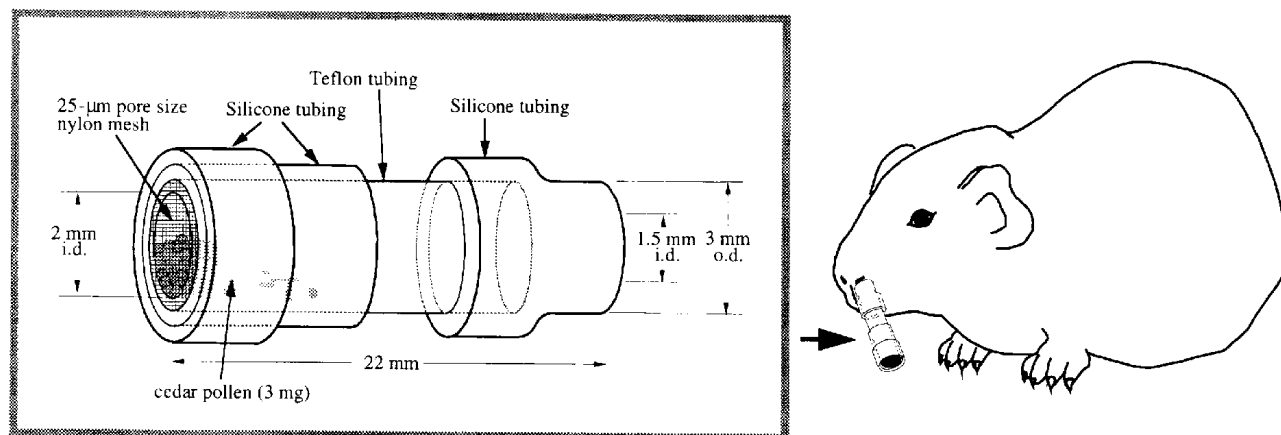


Fig. 1. Experimental apparatus for the inhalation of Japanese cedar pollen by the guinea pig. The inhalation apparatus containing cedar pollen is properly positioned in the left nostril and the other was plugged during the inhalation.

filtered over 5- μ m nylon gauze. The number of ghosts in the precipitate was counted after centrifugation ($1,700 \times g$, 10 min, room temperature) and resuspension in purified water (50 μ l/animal). Through the procedures described above, the ghosts were not destroyed even under the strong alkaline condition (1.2 N KOH for 48 hr). Under these extraction conditions, with 3 mg Japanese cedar pollen and the normal lung, the recovery of pollen ghosts was $95 \pm 3.5\%$ ($n=3$, mean $\pm$ S.E.).

Casting of the nasal cavity

For the knowledge of the structure and the measurement of the volume, the nasal cavity of the guinea pig was casted in silicone by the following procedures: Following killing by bleeding under pentobarbital anesthesia (40 mg/kg, i.v.) and sealing of the mouth with a binding agent, the nasal cavity including the nasopharynx was filled with a gel silicone compound (Silicone Sealant Semedain 8060[®]; Semedain Co., Tokyo) through the nostrils. On the next day, the solidified silicone was isolated from the nasal tissue. Both the volume and area of the mucosal surface of the nasal cavity were measured. The specimen was then cut vertically in approx. 100- μ m-thick sections with a microtome.

Sensitization and challenge

The guinea pig was sensitized by i.p. injection of cedar pollen extracts (10 μ g protein) adsorbed on 10 mg $\text{Al}(\text{OH})_3$ /ml/animal/time twice within a week. After the last sensi-

zation, the animal was repeatedly challenged/boosted by inhalation with the cedar pollen in the left nostril using the inhalation apparatus described above, once every 2 weeks.

Nasal cavity lavage (NCL)

Sensitized and non-sensitized guinea pigs were anesthetized by i.p. injection with sodium pentobarbital (40 mg/kg), and the drug then was i.p. infused continuously to maintain the anesthetized state during the NCL experiment at a rate of 5.5 mg/500 μ l/kg/hr.

The NCL was carried out as follows: One end of the silicone tubing (outside diameter of 3 mm, inside diameter of 1.5 mm), the other end of which was connected to an air pump, was properly positioned in the right nostril and under reduced pressure (-0.19 atm. at complete plugging) by the air pump; then 1 ml saline prewarmed at 37°C was aspirated from the left nostril by a tube (Fig. 2). In the pollen-challenging experiment, the NCL was conducted every hour from 2 hr before to 10 hr after the pollen inhalation.

Measurement of total leukocytes and their classification in the NCLF

The NCLF recovered was centrifuged at $120 \times g$ for 5 min at 4°C . The resultant supernatant was used for histamine and albumin assays. The cell pellet was suspended with a defined volume (200–800 μ l/sample) of physiologic saline. For determining the number of total leukocytes, a portion of the cell suspension was stained with

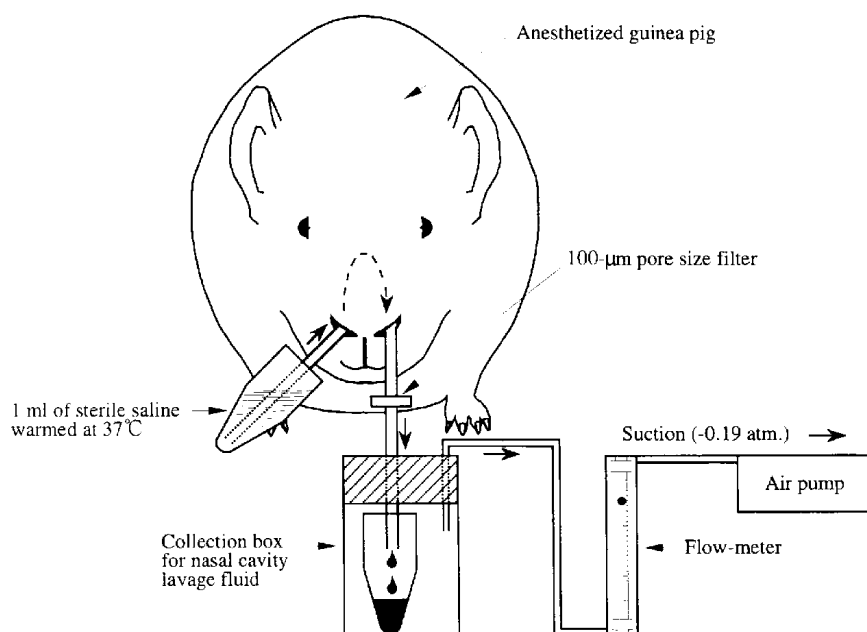


Fig. 2. Nasal cavity lavage (NCL) system in the anesthetized guinea pig. The nasal cavity is washed with 1 ml of sterile saline by the method described in the text. Nasal cavity lavage fluid is recovered into a polystyrene tube in the collection box.

Turk's solution (Nacalai Tesque, Kyoto) followed by counting under a microscope. A 50- μ l aliquot of the cell suspension was used for classification of leukocytes, 400 cells of which were microscopically counted in total; the sample was prepared by centrifugation on the Settling chamber (Neuro Probe, Cabin John, MD, USA) at $50 \times g$ for 30 sec at 4°C and staining with Diff-Quik[®] solution (International Reagent, Kobe).

Measurement of histamine and albumin in the NCLF

Following the centrifugation of the NCLF described above, histamine and albumin in the supernatant was assayed. Histamine was automatically assayed fluorometrically according to the method of Yamatodani et al. (7). In brief, the specimen deproteinized with 3% perchloric acid was applied to a high performance liquid chromatography (HPLC; Tosoh, Tokyo) histamine analytical system, which employed a cation exchange column (TSK gel SP-2SW, 6.0 $\phi \times 150$ mm; Tosoh).

Albumin in the NCLF was measured by enzyme-linked immunosorbent assay according to the method of Gawin et al. (8).

Passive cutaneous anaphylaxis (PCA)

For the estimation of specific γ 1 and IgE antibody titers of the serum in the sensitized guinea pig, which had been repeatedly challenged/boosted by pollen inhalation, 4-hr and 7-day PCAs were performed according to the method of Ovary (9) and Levine et al. (10), respectively. The serum for the PCAs was obtained on day 6 after the respective 1st and 4th inhalations.

For the estimation of PCA titer, the standard anti-serum was prepared by the method of Levine et al. (10): The guinea pig was i.p. sensitized with 10 μ g ovalbumin adsorbed on 1 mg Al(OH)₃/time/animal once every 2 weeks. On day 14 after the 10th sensitization, the serum that possessed titers of 500 $\times$ or more in both 4-hr and 7-day PCAs was obtained and stored at -80°C until use.

Statistical analyses

The results were expressed as the mean $\pm$ S.E of the experiments. Statistical analysis was performed by using one-way analysis of variance (ANOVA). If a significant difference was detected, the individual group difference was determined by Bonferroni's multiple test. A probability value (P) of less than 0.05 was considered to be statistically significant.

RESULTS

Distribution of the pollens into the airway

When 3 mg Japanese cedar pollen (1.07×10^5 pollens/mg) was applied to the respective nostrils of the spontaneously breathing guinea pig, 59% on the average was inhaled without great variance. Almost all of the inhaled pollens were trapped in the upper airways; 10 and 60 min after the inhalation, 81% and 55% of them, respectively, were found in the upper airway, but less than 0.001% reached the lower airways (Table 1). Low numbers of pollen particles in the upper airways with increasing time after the inhalation were mostly found in the esophagus and the stomach as the ghosts.

Shape, volume and area of the mucosal surface of the nasal cavity

Figure 3 shows the shape of the nasal cavity and vertical sections of it. Both the space volume of the cavity and area of the mucosal surface were increased with the body weight of the animal. When the animals weighing 400–900 g were employed, they were estimated to be 0.35–0.5 cm³ and 5.3–8.5 cm², respectively. The cavity is indicated to be quite narrow because the area is extremely large as compared with the volume.

Recovery of NCLF

In the present NCL method, it was revealed that almost the whole nasal cavity was washed when Evans' blue solution was used in place of physiologic saline to monitor

Table 1. Amount of the inhaled cedar pollens and their distribution to the upper and lower airways in the guinea pig

Amounts of inhaled cedar pollens (%)	Time after the inhalation	Distribution of inhaled cedar pollen (%)	
		Upper airway (nasal cavity and pharynx)	Lower airway (trachea and lung)
59.3 $\pm$ 4.31 (N=8)	10 min	81.0 $\pm$ 3.8	0.0008 $\pm$ 0.0002 (N=4)
	60 min	54.5 $\pm$ 3.3	0.0009 $\pm$ 0.0003 (N=4)

Cedar pollens (6 mg/animal) were inhaled. Distribution of the inhaled cedar pollens to the airways was counted following the procedures described in the text. Each value represents the mean $\pm$ S.E. of number of animals shown in parenthesis.

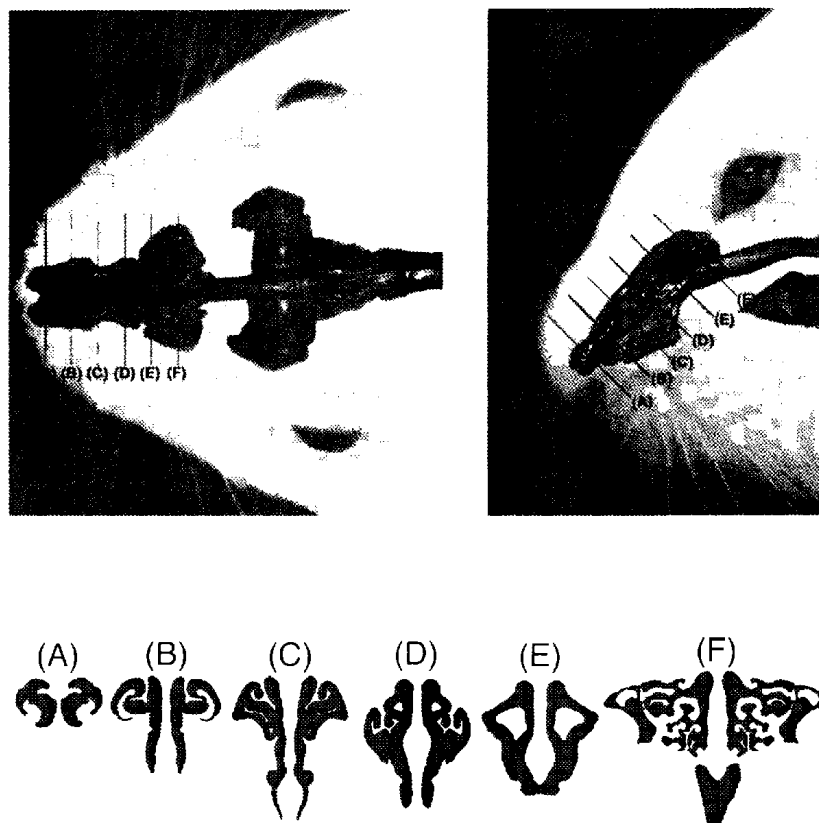


Fig. 3. Shape of the nasal cavity of the guinea pig and vertical sections of it. The nasal cavity was casted in silicone. Vertical sections were prepared by cutting the specimen in approx. 100- μ m-thick sections with a microtome.

the washed area. The time required from the beginning to the completion of the NCL was 10 sec or less on the average, and the recovery was approximately 75% ($75 \pm 3.2\%$, mean $\pm$ S.E., $N=8$); the rest of the NCLF was detected in the nasal cavity in a small volume, in the mouth and esophagus in a large volume, and seldom in the tracheobronchus when assessed with Evans' blue solution in place of physiologic saline.

Time course of the changes in amount of histamine and albumin in the NCLF

Following the sensitization with the pollen extracts + $\text{Al}(\text{OH})_3$ twice within a week, the animal was challenged /boosted by inhalation through one (left) nostril with 1.8 mg pollen once every 2 weeks.

At the 5th inhalation, NCL was carried out. Figure 4 shows the time course of the changes in amounts of histamine and albumin in the NCLF. Histamine was detected in the NCLFs of the sensitized – challenged group at every time after the challenge with the maximum level within 1 hr. The increased level of albumin was also recognized in NCLFs with the peak within 1 hr after the inhalation challenge. On the other hand, changes in these

amounts were hardly observed in NCLFs collected from both non-sensitized – non-challenged and sensitized – non-challenged groups.

Time course of the changes in number of leukocytes in the NCLF

Figure 5 shows the time course of the changes in total number of leukocytes, and the respective numbers of mononuclear cells, eosinophils and neutrophils in the NCLF recovered from the cavity following the 5th inhalation. Leukocytes were hardly observed in the nasal cavity of both sensitized and non-sensitized guinea pigs before the inhalation. Following the inhalation, the number in the sensitized guinea pig was time-dependently increased for the first 3 hr, maintained at a similar level for the next 3 hr and then further increased for another 4 hr, with considerable variation. On the other hand, in both non-sensitized – non-challenged and non-sensitized – challenged controls, the number of total leukocytes also showed gradually increased with time, to which neutrophils are greatly contributed. The time course of the differences in the number of eosinophils between the challenged group and control groups greatly reflected

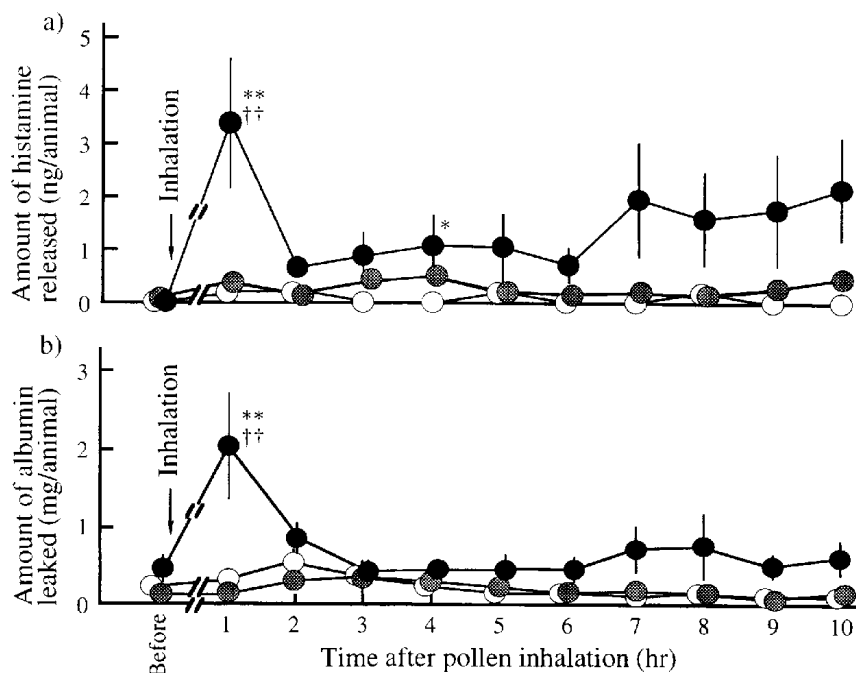


Fig. 4. Time course of changes in amounts of histamine released and albumin leaked into the nasal cavity by the 5th inhalation challenge with Japanese cedar pollen in the guinea pig. The procedures for sensitization, inhalation challenge and nasal cavity lavage, and histamine and albumin assays were described in the text. Each point represents the mean $\pm$ S.E. of 10 animals. a: histamine, b: albumin. ○: Non-sensitized - non-challenged, ◐: Non-sensitized - challenged, ●: Sensitized - challenged. Significantly different from the non-sensitized - non-challenged group (* $P < 0.05$, ** $P < 0.01$) or from the non-sensitized - challenged group († $P < 0.01$).

those of the total leukocytes: the number of eosinophils in the sensitized - challenged group was significantly larger than those in the non-sensitized groups at the 2nd and 4th hr. Mononuclear cells were detected at modestly increased levels in NCLFs at any time in all groups.

The 4-hr and 7-day PCA titers during the challenges /boosters by the pollen inhalation

On day 6 after the 1st and 4th pollen inhalations, the serum was obtained for estimation of $\gamma 1$ and IgE antibody titers using 4-hr and 7-day PCAs, respectively. The results obtained are shown in Table 2. After the 1st inhalation, 7 out of 12 animals were positive to 4-hr PCA with a titer of $64\times$, and 8 out of 12 were positive for 7-day PCA with a titer of $1-64\times$. After the 4th inhalation, all of the animals became positive for both PCAs: Not only the animals negative for either PCA were forming detectable amounts of specific $\gamma 1$ and IgE antibodies in the serum, but also the positive animals showed further increases in the formation of these antibodies.

DISCUSSION

Prior to the sensitization/challenge experiments, we devised a new apparatus that allows animals to effectively

and quantitatively inhale Japanese cedar pollens. Then, we established the new method of NCL that enabled through washing of the nasal cavity which can be used repeatedly without sacrificing the animal. Intraperitoneal injection of Japanese cedar pollen extracts with $Al(OH)_3$ and subsequent repetitive inhalations of the pollens themselves induced substantial increases in the amounts of anaphylactic $\gamma 1$ and IgE antibodies in the serum. At the 5th pollen inhalation, the analysis by NCL revealed that histamine release and albumin leakage into the nasal cavity were induced, suggesting that edema is induced in the nasal mucosa and that leukocytes, especially eosinophils, migrated into the cavity, which lasted at least several hr as seen in humans (11-13).

We assessed where Japanese cedar pollens are located in the airways when the spontaneously breathing animal was subjected to pollen inhalation using the apparatus. Fortunately, the ghost of the pollen was resistant to the strong alkaline condition, and did not split, in which bronchi and lung are almost completely decomposed; this allows easy counting of the exact number of the pollen particles (ghost) located in the tissue following extraction. Consequently, almost all pollen particles inhaled were found to be quantitatively trapped in the upper airways under both conscious and anesthetized conditions.

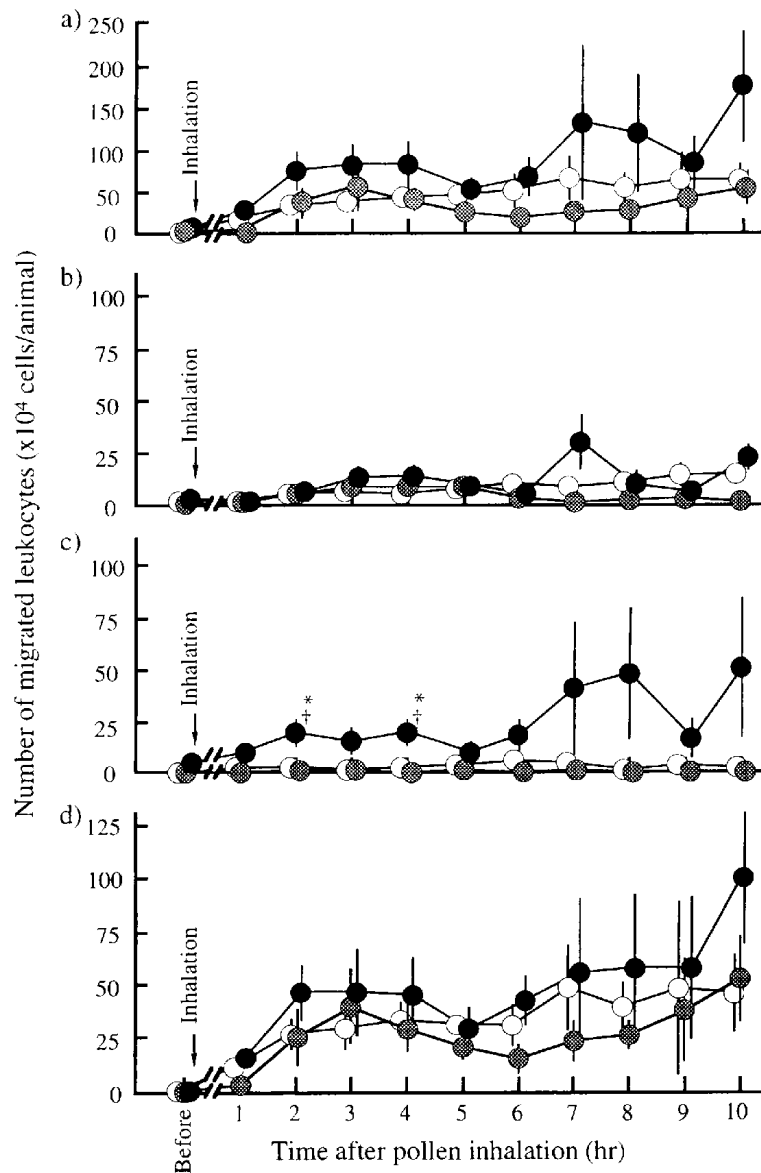


Fig. 5. Time course of changes in number of leukocytes that migrated into the nasal cavity induced by the 5th inhalation challenge with Japanese cedar pollen in the guinea pig. The procedures for sensitization, inhalation challenge and nasal cavity lavage and the number and classification of leukocytes were described in the text. Each point represents the mean $\pm$ S.E. of 10 animals. a: total cells, b: mononuclear cells, c: eosinophils, d: neutrophils. $\circ$: Non-sensitized - non-challenged, $\bullet$: Non-sensitized - challenged, $\square$: Sensitized - non-challenged, $\blacksquare$: Sensitized - challenged. Significantly different from the non-sensitized - non-challenged group (* $P < 0.05$) or from the non-sensitized - challenged group ($^{\dagger}P < 0.05$).

Table 2. The 4-hr and 7-day passive anaphylaxis (PCA) titers of sera from the sensitized guinea pig

Time of cedar pollen inhalation	N		No. of sera showing the indicated PCA titer					
			0	1-8	16	32	64	128 <
1st	12	4-hr PCA	5/12	0/12	0/12	0/12	7/12	0/12
		7-day PCA	4/12	1/12	0/12	1/12	6/12	0/12
4th	12	4-hr PCA	0/12	3/12	1/12	1/12	1/12	6/12
		7-day PCA	0/12	3/12	0/12	1/12	2/12	6/12

The serum was obtained on day 6 after the time of the indicated inhalation.

The high distribution to the upper airway of the inhaled pollen agrees with clinical observations: Inhalation of Japanese cedar pollen as well as possibly other allergic pollens of grasses and trees through the nose does not induce allergic bronchial asthma because of the large granule size (30–40 μm in diameter) of the pollen. It is also generally stated that even if a patient with severe allergic rhinostenosis to Japanese cedar pollen inhales the pollen through the mouth, allergic bronchial asthma is never provoked, while either allergic laryngitis or tracheobronchitis with cough is induced. From the present result and these clinical observations, it appears that cedar pollen is a very useful antigen for developing an experimental rhinitis.

Guinea pigs have a critical drawback as experimental animals because they are poorly documented genetically unlike mice and rats. However, as the airway of the guinea pig is known to be very sensitive in anaphylaxis, similar to the humans (14), the species have been frequently used for experimental asthmatic models. The present results show that the guinea pig is a high responder to antigen proteins of pollen, including *Cry j* I and II (15, 16), for producing cytophilic antibodies and that anaphylactic reactions can be induced by them in the upper airways, although repetitive inhalations with the pollen alone without $\text{Al}(\text{OH})_3$ in a similar sensitization/challenge schedule induced a minimal allergic rhinitis as assessed by the increased number of leukocytes migrating into the nasal cavity and amounts of specific γI or IgE antibody in the serum (data not shown).

In 1993, there was a report on the NCL in the guinea pig in non-allergic experiments that were done by pipetting a small volume of physiologic solution into both nostrils (17); this method had the disadvantages of not only incomplete recovery but also provided unreliable data on the NCLF, if it was applied to actual experiments. The new improved NCL technique in the present experiments has made it possible to obtain more detailed analyses of the pathogenesis of allergic rhinitis because the various released mediators and cell types that migrated into the nasal cavity could be determined. Different from the perfusion method of the nasal cavity through the trachea (3, 4), the present NCL has a great advantage because it permits a continual investigation of developing or chronic allergic rhinitis induced by repetitive antigen inhalations in the same guinea pig over a long term. Unfavorably, the numbers of neutrophils in the non-sensitized groups were increased by NCL. The results indicate the possibility that the operation locally induces an irritability to cause neutrophil infiltration. The increases of histamine and eosinophils, the reason of which was unclear, were insignificant in the NCLF at the late time (7–10 hr) after the challenge with relatively large variations in the sensitized group.

Japanese cedar pollen has a thick pollen wall consisting of outside (exine) and inside (intine) cell wall layers in the outer side. In general, it is described that proteins and glycoproteins including *Cry j* I and II are easily released from the pollen wall that is concomitantly cracked by swelling when the pollen was immersed in aqueous solution. In the present study, since immediate anaphylactic reactions were evoked after the pollen inhalation challenge, it is considered that these antigen proteins were released from the pollen on the mucosal surface and bound to IgE on mast cells. Consequently, various chemical mediators including histamine were released, followed by albumin leakage and leukocyte migration. The observation that a single inhalation with the pollen by non-sensitized guinea pigs did not induce any parameter changes strongly suggests that the changes in the sensitized – challenged animals were not induced by some irritabilities in response to the inhaled pollen.

These results indicate that the present experimental method using pollen extracts with $\text{Al}(\text{OH})_3$ for sensitization and challenge/booster with pollen inhalation is capable of inducing typical allergic rhinitis, and it is quite advantageous for analyzing the disease and evaluation of drugs being developed or already in clinical use for the treatment. It seems that the present experiment is applicable to other grass and tree pollens with fairly large diameters.

We are currently studying the time course of changes in migrating leukocytes for further analysis and investigating nasal airway resistance at the respective pollen inhalations, hyperresponsiveness to some chemical mediators, role of proinflammatory cytokines, effects of various drugs including antihistaminics, antagonists of peptide leukotrienes, antiinflammatory steroids, and so forth.

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