(Short Communication)

Study on the Inhalation Exposure Conditions of Pollen for Inducing the Production of Japanese Cedar (*Cryptomeria japonica*) Pollen-specific IgE Antibody in Mice

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The objective of this study was to establish the inhalation exposure conditions of pollen under which production of Japanese cedar (*Cryptomeria japonica*) pollen allergens (Sugi major allergens, SMAs)-specific IgE antibodies could be induced in mice. In experiment 1, the four pollen exposure groups of each 10 female BDF_1 mice were exposed to, approximately, either 500,000 or 3,000,000 grains of Japanese cedar pollen (JCP) per cubic meter of air for 2 or 4 h / d, 2 d / wk for 30 wk. No elevation of SMAs-specific IgE antibody titers measured by enzyme-linked immunosorbent assay (ELISA) were recognized in any of the exposure conditions. In experiment 2, 10 female BDF_1 mice were exposed to concentration of about 500,000 grains of JCP / m^3 for 16 h / d, 2 d / wk for 20 wk. SMAs-specific IgE antibody titers clearly increased after 20 wk exposure in 4 of 10 mice, along with the proliferative response of cervical lymph node cells obtained from the 10 mice to SMAs. The results of this study indicate that the quantity of pollen grains taken into the nasal cavity to induce the production of SMAs-specific IgE antibody in mice is more closely to exposure time per day than to pollen concentration.

Key Words : Enzyme-linked immunosorbent assay (ELISA), Immunoglobulin E (IgE) antibody, Inhalation exposure, Japanese cedar pollen, mouse

Japanese cedar (*Cryptomeria japonica*) pollinosis, reported for the first time in 1964 by Horiguchi & Saito [1], has attracted concern as a serious health problem in Japan, because of the rapid increase in patient numbers. The recent incidence of Japanese cedar pollinosis (JCPsis) is presumed to be 10 - 20% in adults and 5 - 10% in children [2]. The JCPsis is of typical immediate-type allergic disease, and Japanese cedar pollen

allergens (Sugi major allergens, SMAs)-specific IgE antibodies are important for its onset. A relation with air pollutants has attracted attention as the cause of the rapid rise in JCPsis, and many studies of this area have been reported [3-6]. In animal experiments, combinations of Japanese cedar pollen allergens or ovalbumin and air pollutants administered to the nasal cavity or the peritoneal cavity result in antigen-specific IgE antibody production. Animal model contracted by the inhalation of the pollen are necessary in order to examine the effect of the air pollutants on crisis and exacerbation of pollinosis. However, there has been no report, as yet, of generation of SMAs-specific IgE antibodies in the blood of experimental animals by more realistic inhalation exposure (whole body exposure). In the present study, we therefore established a new mouse protocol with inhalation exposure to Japanese cedar pollen (JCP), this species being reported to have serum SMAs-specific IgE antibodies [7-8].

Materials and Methods

JCP collected in Yamanashi prefecture from 1995 to 1997 was mixed and used for the experiment. Lots of male flowers of Japanese cedar collected in vinyl bags were dried and taped ; then JCP were refined with a fine mesh to remove the impurities and stored at −80℃ until use. SMAs extracted from JCP, two major allergens have been isolated and characterized as Cry j 1 and Cry j 2 [9, 10]. SMAs (75μg of Cry j 1 and 25μg of Cry j 2 / 100μg of SMAs) was provided kindly by Hayashibara Biochemical Laboratories, Okayama, Japan, and used for the measurements of SMAs-specific IgE and IgG antibodies in sera, and proliferative response of cervical lymph node cells to SMAs.

A schematic illustration of the inhalation exposure apparatus is shown in Fig. 1. Pollen generated by a dust feeder (Sibata, model DF-3, Japan) was fed into the vertical flow-type inhalation chamber with an approximate capacity of 0.9cm^3, and the mice were exposed to the pollen grains with 10 ventilation changes per hour. Pollen concentrations and their grain size distributions in the inhalation chamber were measured hourly by a particle counter (Rion, model KC-2, Japan).

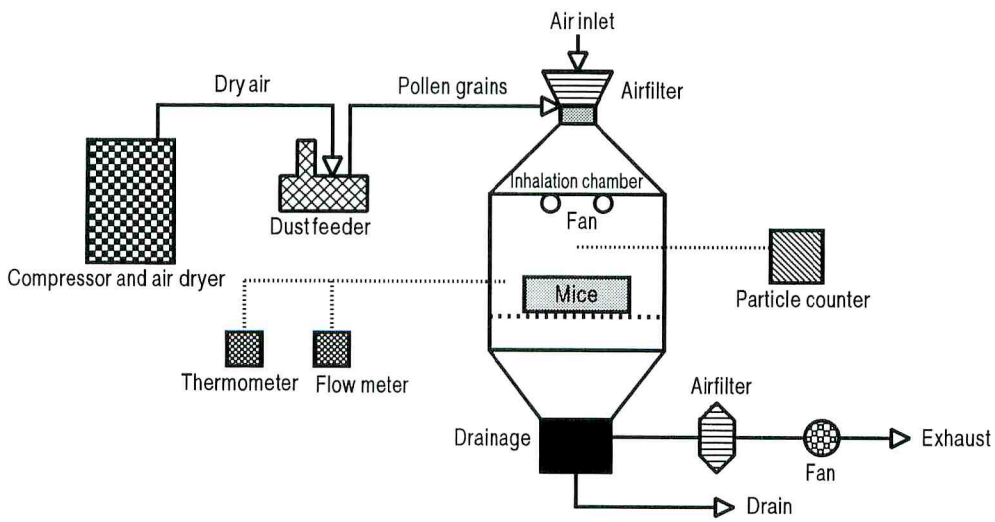

Fig. 1. Inhalation exposure system. During the exposure time, the air including the pollen of 500,000 / m^3 or 3,000,000 / m^3 is flowing from the top to the bottom at 150L / min.

In experiment 1, female BDF_1 mice aged 5 wk (15 − 17g body weight) were obtained from CLEA Japan, Inc., and divided into 5 experimental groups of 10 mice each. Mice were exposed to, approximately, either 500,000 or 3,000,000 grains of JCP per cubic meter of air for 2 or 4 h / d, 2 d / wk for 30 wk. Mean pollen grain numbers per 10L of exposure air in the inhalation chamber during the exposure period were 4,873 and 29,075, respectively. The mice of the four pollen exposure groups were housed in stainless-steel cages in the inhalation chamber maintained at 23 ± 1 ℃ and 65 ± 10% relative humidity with artificial lighting for 12 h / d during exposure times, and in plastic cages with wood shavings in an air-conditioned room at other exposure times, as the mice of the control group. All animals were provided with a normal diet and tap water *ad libitum* throughout the experiment. Mice in each group were bled from the tail vein every 4 wk. At the end of the exposure period, blood was drawn from the hearts of mice under ether anesthesia.

In experiment 2, the pollen exposure group of 10 mice were exposed to about 500,000 pollen grains / m^3 (5,047 grains / 10L) for 16 h / d, 2 d / wk for 20 wk, and other inhalation exposure procedures were the same as those in experiment 1. The control group of 10 mice was also set as well as the experiment 1. In both experiments, pollen grain sizes of 10 − 20μm, 20 − 30μm, 30 − 50μm, and more than 50μm were 2.5%, 80%, 17%, and 0.5%, respectively. Since the diameter of JCP is in the range of 20 − 40μm, that more than 50μm measured in this experiment was presumably due to foreign matter introduced during the pollen collection.

Enzyme-linked immunosorbent assay (ELISA) of SMAs-specific IgE antibodies were carried out using a modification of the method of Sakaguchi et al. [11]. The wells of microtiter plates (Dynatech, USA) were coated with 100μL of a commercial anti-mouse IgE rat monoclonal antibody (Yamasa, Japan) diluted to 20 μg / mL with 0.1M $NaHCO_3$ (pH 8.2) at 37℃ for 3 h, and the plates were then washed 5 times with phosphate buffered saline containing 0.05% Tween 20 using a microplate washer (Bio-Rad Laboratories, model 1550, USA). The wells were then successively incubated at room temperature with the following reagents, washing 5 times between each pair of steps : 250μL of lactoprotein liquid (Dainihon Seiyaku, Japan) for blocking at 4℃ overnight ; 100μL of 10-fold dilutions of exposed or control mice sera; 100μL biotinylated SMAs (1μg / mL) ; 100μL of streptavidin-peroxidase (Zymed, USA) diluted 1 : 1,000 ; 100μL of substrate solution containing 16mg of *o*-phenylenediamine-2HCL (Wako Junyaku, Japan) and 0.005% hydrogen peroxide in 100 mL of substrate buffer (4.7g citric acid and 7.3g Na_2HPO_4 / L distilled water, pH5.0). Biotinylated SMAS was prepared by the method of Sawatani et al. [12]. The enzyme reaction was stopped with 100μL of 4N H_2SO_4, and absorbance was read at 490 − 650nm with a microplate reader (Bio-Rad Laboratories, model 3550, USA). Details of ELISA of SMAs-specific IgG antibodies have been described [8]. The 20-fold dilution of the serum pooled from the 10 mice was used as a sample. IgE and IgG ELISA titers were expressed as the absorbance obtained by subtracting the absorbance of the control serum from that of exposed serum.

The antigen specific proliferative response of the lymphocyte in the lymph node of the nasal cavity vicinity was examined. Assay of proliferative response of cervical lymph node cells to SMAs was carried out by the method of Fujimaki et al. [13]. For the proliferative response to SMAs by cervical lymph node cells (CLNCs), cervical lymph nodes of 10 mice in each group were pooled and CLNCs were obtained by passage through a stainless steel mesh, counted and 6×10^5 cells / well were cultured for 3 days in a CO_2 incubator (5% CO_2, 37℃, air humidity 98%) with various doses of SMAs (0, 0.4, 2, 10μg / mL) in the presence of syngeneic antigen-presenting cells (APCs, 6×10^5 cells / well) in 96-well flat-bottomed plates in RPMI-1640 culture medium containing 10% fetal bovine serum, 5mM Hepes, 2mM L-glutamine, 0.1mM nonessential amino acids, 100U / mL penicillin and 100μg / mL streptomycin. APCs were prepared with 40μg / mL mitomycin C treatment of spleen cells from normal mice for 30 min at 37℃. After 3 days of cell culturing, the CLNCs were subsequently radiolabeled with ^{3}H-thymidine (1μCi / mL : ICN Biochemical Inc., CA) for 16h. The uptake of radiolabeled thymidine was counted by a β-scintillation counter (Beckman Instruments, Inc., CA), and the results of ^{3}H-labeled thymidine incorporation were expressed as a count per minute (CPM). The mean values

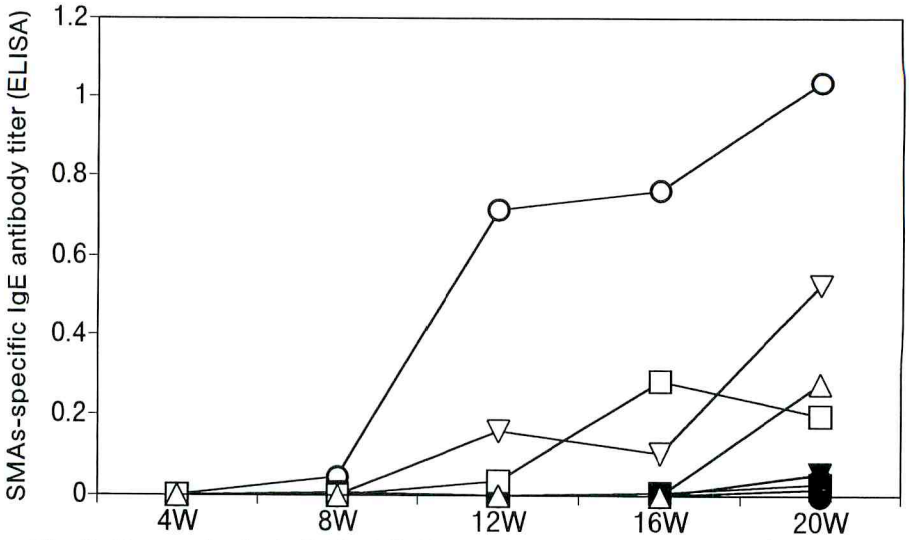

Fig. 2. Changes in the individual SMAs-specific IgE antibody titers (ELISA) of mice exposed to pollen. The symbol shows the individual number of the mouse ; ○ (No. 1), ◇ (No. 2), ▼ (No. 3), ● (No. 4), ◆ (No. 5), ■ (No. 6), ▲ (No. 7), ▽ (No. 8), □ (No. 9), △ (No. 10).

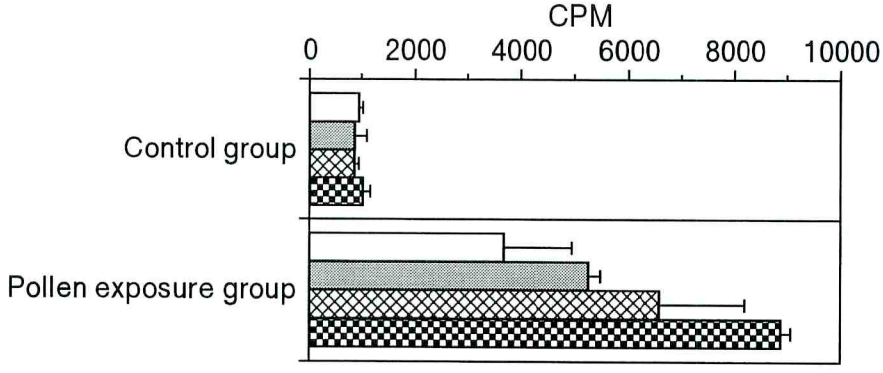

Fig. 3. Proliferative responses to SMAs by cervical lymph node cells obtained from 10 mice in each group (□: SMAs 0μg / mL, ▨: SMAs 0.4μg / mL, ▩: SMAs 2.0μg / mL, ▩: SMAs 10.0μg / mL). Columns and bars represent means ± SD. Means and SD were calculated by values of 3 wells of each microtiter plate.

and the fluctuations of CPM were calculated by values of 3 wells of each microtiter plate.

Results and Discussion

No significant differences in the mean body weights of mice between unexposed and exposed to pollen were found throughout the exposure period in both experiments. In experiment 1, not only no elevation of

SMAs-specific IgE antibody titers but also no proliferative responses to SMAs in mice exposed to pollen were recognized in any of the exposure conditions. Changes of the SMAs-specific IgE antibody titers in each mouse (Nos.1 − 10) of the pollen exposure group in experiment 2 are shown in Fig. 2. No elevation of IgE antibody titer was recognized until 8 wk after the start of exposure. At 12, 16, and 20 wk, clear elevation of titers was apparent in 2, 3, and 4 of the 10 mice, respectively. SMAs-specific IgG antibody titers of pooled serum also increased time-dependently. Proliferative responses to SMAs of the CLNCs from the exposed and the control mice are shown in Fig. 3. A dose-dependent increase of the proliferative responses to SMAs in the pollen exposure group was observed.

In the present study, JCP was given to mice by whole body exposure form. There is an argument that whole body exposure may accompany oral administration of JCP when animals lick their fur. However, licking the fur may occur also in the case of Japanese monkeys (*Macaca fuscata*) and dogs kept out-of-door as well as humans for which natural occurrence of JCPsis has been reported [14, 15]. Although the chances of people taking in pollen orally are very low as compared with animals, whole body exposure which includes oral administration was considered with the most practical method of sensitization.

In the previous study [8], we confirmed that the SMAs-specific IgE antibody titers of mice were clearly increased when around 160,000 grains of JCP (native dry pollen) were dropped onto the tip of the nose of mice twice a week for 6 wk. However, there was no performance on estimating the appropriate inhalation exposure conditions from this experimental result. Then, we had to examine the inhalation exposure conditions which induced the production of the IgE antibody in mice. In experiment 2 of the present study, the SMAs-specific IgE antibody titers in sera clearly increased with the prolongation of exposure time per day to 4 − 8 fold of those used in experiment 1. The results of the study indicate that the quantity of pollen grains taken into the nasal cavity to induce the production of SMAs-specific IgE antibody in mice was related more closely to exposure time per day than to pollen concentration. These results indicated that sensitization force of intranasal administration was stronger than the inhalation exposure, because the formation of the sensitization by the inhalation exposure needed several months.

In the present study, the population which possessed the SMAs-specific IgE antibody increased time-dependently. Though the SMAs-specific IgG antibody titers of pooled serum was also increased time-dependently, it has not reached the plateau. Therefore, it is considered that animal number which possesses the SMAs-specific IgE antibody increases further by extending the exposure duration.

In Japan, JCP counts in atmosphere are commonly calculated as the number of pollens deposited on a glass slide in Durham type collector (gravitation method) and expressed to pollen grains / day / cm^2. Although it is difficult to estimate the relation between the pollen concentration exposed to mice in the present study (pollen gains / m^3) and the actual pollen concentration in the air around the suburbs of typical Japanese cities (pollen grains / day / cm^2), the pollen concentration used in this experiment (about 500,000 pollen grains / m^3) is estimated to be about 1,000 times the concentration in the atmosphere on maximum scattering days in general Japanese city neighborhood [16−18]. People will be suffered from pollinosis, when it was exposed in the pollen of the concentration which is very lower than pollen concentration used in this experiment throughout the long term. Furthermore the inhalation exposure frequency used in the present study was also different from the human case. Therefore, it should be understood that the inhalation exposure conditions used in the present study were a condition for the experiment using small rodents. In the future, lower concentration and more long-term experiments will be necessary.

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マウスのスギ花粉特異 IgE 抗体産生を誘導する花粉の吸入暴露条件の検討

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マウスのスギ花粉特異IgE抗体の産生を誘導する花粉の吸入暴露条件を検討した．実験 1 では，40 匹の BDF_1 マウス（雌）を 10 匹ずつ 4 群に分け，各群のマウスに約 50 万個または 300 万個 / m^3 の花粉を 2 または4時間 / 日，2日 / 週の頻度で 30 週間吸入暴露したが，全ての個体でスギ花粉の主要アレルゲン（Sugi major allergens, SMAs）に対する特異 IgE 抗体価の上昇は認められなかった．実験 2 では，10 匹の BDF_1 マウス（雌）に約 50 万個 / m^3 の花粉を 16 時間 / 日，2日 / 週の頻度で 20 週間吸入暴露した結果，10 例中 4 例に SMAs 特異 IgE 抗体価の明らかな上昇を認めた．また，花粉暴露した 10 匹のマウスから採取した頚部リンパ節細胞の SMAs に対する増殖反応も，添加した SMAs の量に依存して上昇した．これらの結果から，花粉の吸入暴露によるマウスのスギ花粉特異 IgE 抗体産生の誘導には，花粉の暴露濃度よりも 1 日の暴露時間に影響されることが示唆された．