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ORIGINAL ARTICLE

Relationship between Airborne Cry j 1 and the Onset Time of the Symptoms of Japanese Cedar Pollinosis Patients

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ABSTRACT

Background: Some patients with Japanese cedar (JC) pollinosis already show pollinosis symptoms before the first day of the pollen season as determined by microscopic pollen counts.

Methods: Airborne pollen allergen (Cry j 1) levels were measured by electron spin resonance radical immunoassay, a highly-sensitive method for Cry j 1 with a sensitivity 10–100-fold higher than conventional enzyme-linked immunosorbent assay. The symptom data from patients with JC pollinosis were collected from a mobile phone site, "pollen check sheet", and the onset times of the patients' symptoms were analyzed.

Results: The relationship between airborne Cry j 1 levels and the onset time of pollinosis symptoms was investigated. The symptoms of some patients began at the time airborne Cry j 1 levels fluctuated at 1 to 3 pg/m^3 and symptom scores increased at the time of sudden increase in Cry j 1 levels. About 40% of patients began to show symptoms until the first day of the pollen season and the time nearly corresponds to the time of sudden increase in Cry j 1 levels.

Conclusions: Pollinosis symptoms of some patients began at the time airborne Cry j 1 levels fluctuated at 1 to 3 pg/m^3 and symptom scores increased at the time of sudden increase in Cry j 1 levels. The latter time nearly corresponds to the first day of the pollen season.

KEY WORDS

Cry j 1, electron spin resonance (ESR) radical immunoassay, Japanese cedar (JC) pollinosis, pollen information, symptom score

INTRODUCTION

Patients can take prophylactic measures (medication) against the symptoms of pollinosis if it is possible to predict the onset of their symptoms before they are observed.[1-6] In the case of Japanese cedar (JC) pollinosis, the first day of the pollen season is defined as the first day when a certain amount of JC pollen is detected in the air by microscopic pollen counts (one or more pollen grains per cm^2 for not less than 2 days collected with a Durham sampler (Nishiseiki Co. Ltd., Funabashi-City, Japan)).[7] However, it is widely known that some patients experience pollinosis symptoms before the first day of the pollen season.[1,2] If very small amounts of Cry j 1 could be quantified, this could be used to estimate the onset time of symptoms in some patients. For the quantification of airborne Cry j 1, we applied electron spin resonance (ESR) radical immunoassay, which was developed as a highly-sensitive method for detecting hepatitis B virus antigen.[8,9] In our previous study, we clarified that the sensitivity of the immunoassay is 10–100-fold higher than that of conventional enzyme-linked immunosorbent assay (ELISA).[10] By using this analytical method, it might be possible to inform patients of the onset time of pollinosis symptoms before they are provoked.

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METHODS

SAMPLING AND ANTIGEN EXTRACTION

A cyclone sampler, CM 90 from Burkard Co. Ltd (Rickmansworth, UK) was installed in Iwanami in Yamagata-City and airborne pollen antigens were collected during the periods February 23 to April 24, 2003; February 23 to April 24, 2004; January 23 to May 11, 2005; and February 12 to June 26, 2006. Airborne particles greater than 1 μm in diameter were collected in 1 mL tubes. The tubes were changed at 06 : 00, and collected at 24-hour intervals. The antigens in the airborne samples were extracted with 100 μL of 0.125 M ammonium bicarbonate in 0.1% bovine serum albumin for 2 hours at room temperature.

MEASUREMENT OF CRY J 1 WITH ESR RADICAL IMMUNOASSAY METHODS

Anti-Cry j 1 monoclonal antibody (013) (Hayashibara Biochemical Laboratories, Inc. Okayama-City, Japan) was diluted to 10 μg/mL with phosphate buffer (PB; 0.1 M and pH 7.0). The diluted antibody (100 μL) was put in a 96-well Nunc plate (Nunc-immuno Module 469949, Nunc Co. Ltd. Kamstrupvej, Denmark) and reacted for 6 hours at 4℃. After three washes with ultra-pure water, 370 μL of Stabilguard (SurModics, Inc., Minneapolis, MN, USA) was placed in each well and blocked overnight at 4℃ (Cry j 1-coated plate). After washing with pure water, the plates were placed in a desiccator until use, and kept at 4℃. Measurements were done as follows. Seven different concentrations of Cry j 1 standard solution (twofold dilution from 15.6 pg/mL to 1,000 pg/mL with 10% fetal bovine serum (FBS; ThermoTrace Ltd., Melbourne, Australia), 0.1 M PB (pH 7.0)) were prepared each time prior to measurement. A standard solution (100 μL) of 10% FBS and 0.1 M PB (100 μL) were placed in each well of the anti-Cry j 1 monoclonal antibody-coated plate, and then 0.125 M ammonium bicarbonate (30 μL) or an airborne sample (30 μL) was added. Horseradish peroxidase-conjugated anti-Cry j 1 monoclonal antibody (053) (Hayashibara Biochemical Laboratories, Inc., Okayama-City, Japan) diluted with 10% FBS, 0.1 M PB was added to each well and mixed thoroughly. The plate was left for 1 hour at room temperature. After several washings with ELSIA-F washing solution, 150 μL of 4 mM *p*-acetamidophenol and 0.34 mM 1-hydroxy-2, 2, 5, 5-tetramethyl-3-imidazoline 3-oxide and 0.01% hydroperoxide were added and reacted for 1hour at 37℃. The enzyme reaction was stopped with 50 μL sodium azide (100 mM). The amount of nitroxide radical (stable radical) obtained as a result of the enzyme reaction was measured with an electron spin resonance device (ESR: FR30 and JEOL). Details of the ESR measurement were reported elsewhere.[8,9]

SYMPTOM SCORES

Symptom data from anonymous JC pollinosis patients who had access to the mobile phone site "pollen check sheets" supplied by Weathernews Co. Ltd. were used. The sheets are on a personal site which can be accessed with a user I.D. and password by mobile phone. A remarkable feature of the sheets is their easiness in keeping a record of everyday symptoms because the record is managed through mobile phones that users always carry. It is like an electronic version of a pollen diary. The user obtains the right information and proper measures in order to input everyday symptoms by use of a two-way communication system. First, people living in Yamagata prefecture or Tokyo were extracted using point numbers of the Automated Meteorological Data Acquisition System (AMeDAS) station from an enormous amount of data on "pollen check sheets" collected from the whole of Japan (ca. 190,000 accesses in total in 2003). The point number of the nearest AMeDAS station is necessary for information and measures for each user, and therefore all the point numbers were assigned based on reported addresses. People who used the mobile phone site for the first time were asked whether they had been given a "diagnosis of JC pollinosis in a clinic/hospital" and "when they noticed pollinosis symptoms after new year's day". In our study, only patients who replied to have received a "diagnosis of JC pollinosis in a clinic/hospital" were extracted and the extracted patients were regarded as JC pollinosis patients in this paper. The extracted data belonging to Tokyo were classified by age and used as control of an urbanized area for age distribution in Figure 2. The data belonging to Yamagata were arranged in the necessary order: time sequence and age.

The symptom data consisted of four symptom scores. They are self-assessed four-point severity scale subjective symptom scores, namely, sneezing, blowing one's nose, nasal blockage, and itching of the eyes, according to Okuda's criteria.[11] The scores used for the analysis are shown in Table 1. The mean scores for each day were calculated by adding the four subjective symptoms together day by day. The mean scores for each day in Figure 3C were calculated by adding the four subjective symptoms of all accesses together day by day. Then they were divided by the numbers of accesses on each day to obtain the total scores for each access. The mean scores of the four subjective symptoms were finally calculated by dividing the total scores for each access by four. The data used for the analysis comprised 83 patients (41% men and 59% women) in 2003 (Feb 1–Mar 20), 25 patients (36% men and 64% women) in 2004 (Feb 12–Mar 23), 53 patients (38% men and 62% women) in 2005 (Feb 1–Mar 17), and 13 patients (31% men and 69% women) in 2006 (Feb 10–Mar 10).

Table 1 Subjective symptom scores from the pollen check sheets

Sneezing	Score
	How many times did you sneeze today?
	0: None
	1: More than one time
	2: More than 6 times
	3: More than 11 times
	4: More than 21 times
Blowing one's nose	
	How many times did you blow your nose today?
	0: None
	1: More than one time
	2: More than 6 times
	3: More than 11 times
	4: More than 21 times
Nasal blockage	
	What was the degree of your nasal blockage?
	0: My nose wasn't clogged
	1: Breathing through my nose was easy
	2: I could sometimes not breathe through my nose
	3: It was always hard to breathe through my nose
	4: I could not breathe through my nose, and I could not endure it
Itching of the eyes	
	How itchy were your eyes?
	0: No itchiness
	1: Not as much as to rub my eyes
	2: I sometimes rubbed my eyes
	3: I rubbed my eyes extensively
	4: Unbearably itchy

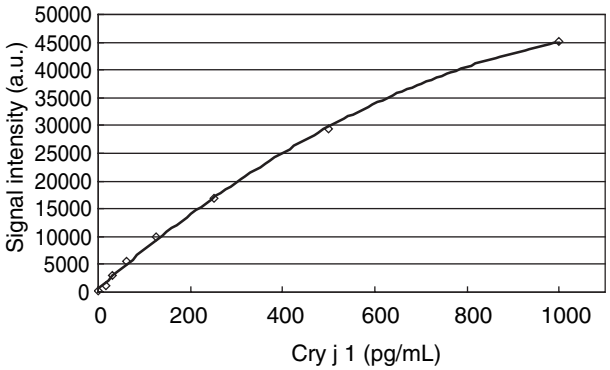

Fig. 1 Standard curve obtained from ESR radical immunoassay. The vertical axis indicates the signal intensity measured by ESR. The intensity is expressed in arbitrary (au) unit measured at 336.1 ± 5 milli Tesla (mT) as a center field. The horizontal axis indicates Cry j 1 concentration expressed in pg/mL.

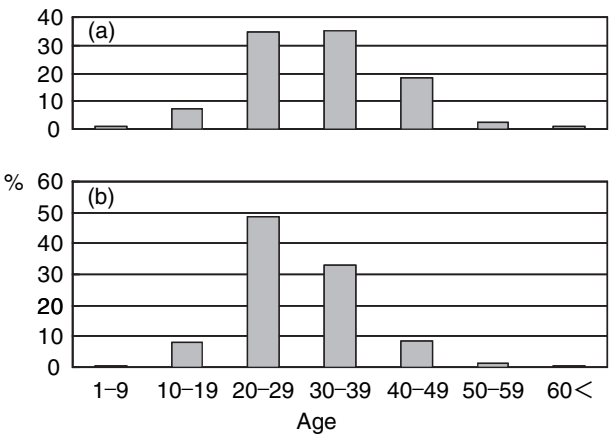

Fig. 2 Age distribution of JC pollinosis patients in Yamagata (**a**) and Tokyo (**b**) in 2003.

RESULTS

A standard curve obtained from ESR radical immunoassay is shown in Figure 1. The vertical axis indicates the signal intensity measured by ESR and the horizontal axis indicates Cry j 1 levels expressed in pg/mL. The detection limit, which was defined as 1.5-fold of blank signal intensity, was estimated to be 3.5 pg/mL and it is possible to measure 0.1 pg Cry j 1 in a sample which needs 30 μL for each measurement.

Figure 2 (a) shows the age distribution of JC pollinosis patients in Yamagata in 2003. Figure 2 (b) shows the age distribution in Tokyo in 2003 as comparison. Most of the data obtained from the "pollen check sheets" were from patients in their 20's to 30's. In Tokyo in particular, 81.2% of patients belonged to these generations. On the other hand, a slightly older age distribution was seen in Yamagata and 88.2% of patients were in their 20's to 40's. Similar age distributions were seen in Yamagata in the other 3 years.

Table 2 summarizes the 4-year results of the first day of reported pollinosis symptoms, the first day of the pollen season, and the first day of airborne Cry j 1 reaching a certain value in 2003 to 2006. About 40% of JC pollinosis patients using the site "pollen check sheets" showed pollinosis symptoms before the first day of the pollen season. Some of the patients began to show symptoms more than 1 month before the first day of the pollen season, on days distributed between late January and early-to-middle February. Cry j 1 levels reached 1 pg/m^3 2–3 weeks before the first day of the pollen season. Figure 3 (a) to Figure 3 (d) indicate the relationship between airborne Cry j 1 levels in pg/m^3 and mean symptom scores of JC pollinosis patients before and after the first day of the pollen season in 2003 and 2006. The symptoms of some patients already began at the time Cry j 1 levels fluctu-

Table 2 Results of the first day of pollinosis symptoms reported, the first day of the pollen season and the first day of airborne Cry j 1 reaching a certain level for each year.

Year	2003	2004	2005	2006
(1) The first day pollinosis symptoms appeared in some patients	2-Feb	12-Feb	26-Jan	26-Jan
(2) The first day of the pollen season	11-Mar	12-Mar	11-Mar	7-Mar
(3) Rates of patients who showed symptoms before the first day	40.5%	42.6%	42.2%	35.1%
(4) The first day of Cry j 1 reaching approximately 5 pg/m^3	15-Mar	17-Mar	11-Mar	7-Mar
(5) The first day of Japanese cedar pollen observed by microscopy	1-Mar	11-Mar	11-Mar	27-Feb
(6) The first day of Cry j 1 reaching approximately 1 pg/m^3	24-Feb	24-Feb	24-Jan	13-Feb

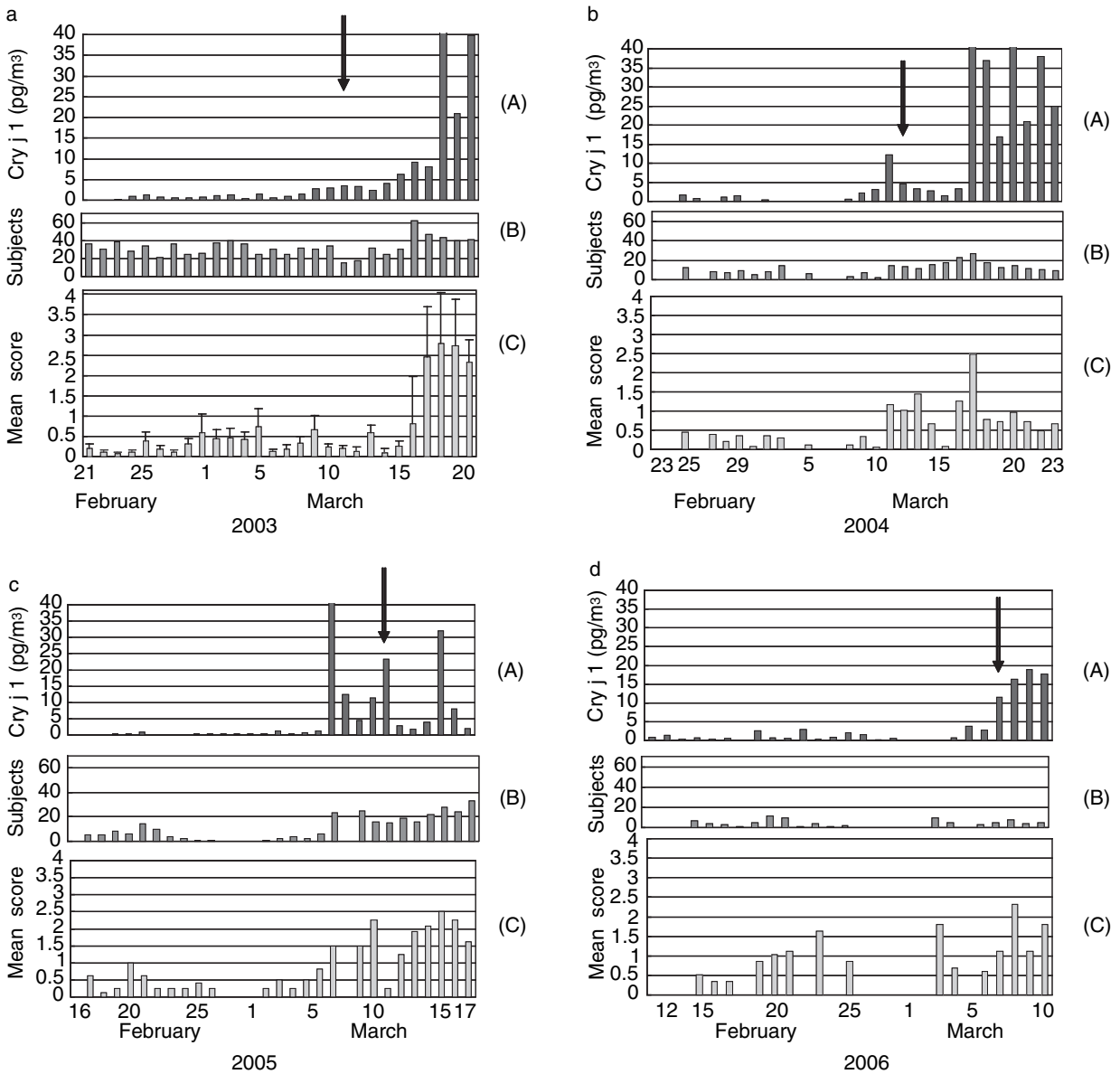

Fig. 3 Relationship between airborne Cry j 1 levels in pg/mL, the numbers of subjects and mean symptom scores of JC pollinosis patients in 2003 to 2006. The vertical arrow indicates the first day of the pollen season by microscopic pollen counts. **A**: Airborne Cry j 1 level each day. **B**: Number of subjects each day. **C**: Mean symptom score each day.

Table 3 The total amounts of Cry j 1 and total JC pollen counts during the pollen season.

Year	2003	2004	2005	2006
(1) The total amounts of Cry j 1 during the pollen season (ng/m^3)	14.3	1.5	55.9	20.3
(2) The total JC pollen counts during the pollen season (grains/cm^2) ＊＊	2280	654	5333	1292
(3) Ratio of (2)/(1)	159.4	436	95.4	63.6

Research periods for each year are as follows; Feb 23 to Apr 24, 2003; Feb 23 to Apr 22, 2004; Jan 23 to May 10, 2005; and Feb 12 to May 9, 2006.

＊＊ Officially announced values counted by Durham sampler

ated at 1 to 3 pg/m^3 in the air. After that, the time of increase in the symptom scores corresponded to that of sudden increase in Cry j 1 levels. The first days of the pollen season in 2003 and 2006 nearly corresponded to the time of sudden increase in Cry j 1 levels. There were 20 to 60 accesses per day in 2003, the first year of the information service. The accesses decreased gradually, and there were some days without any access in 2006.

The total amounts of Cry j 1 during the pollen season are shown in Table 3. There are large yearly differences from 1.5 ng/m^3 (2004) to 55.9 ng/m^3 (2005). It is calculated that 1.5 ng of Cry j 1 is equivalent to 300 JC pollen grains and 55.9 ng to 11,180 JC pollen grains, if the amount of Cry j 1 in a pollen grain is assumed to be 5 pg. A correlation was seen between the total pollen counts by microscopy and the total Cry j 1 amounts during the pollen season investigated over 4 years ($r = 0.953$, $p < 0.05$). However, at most a 7-fold difference was seen among the ratio of these two values.

DISCUSSION

Pollinosis diaries have been widely used to analyse the symptoms of pollinosis patients. However, these allow acquisition of data on symptoms only after the day a patient visits the clinician, unless asked in advance. Investigation using pollinosis diaries was not considered feasible for early-stage symptoms, which were the object of this study, because most patients do not come to visit the clinic/hospital during our target period. The advantages of the method used in this study include the ease of inputting everyday symptoms one after another by mobile phone and the possibility of obtaining data from various patients who do not visit their clinician often. Regarding fixed diagnosis such as with specific IgE antibody examination (RAST test), patients at large hospitals, such as university hospitals, are examined with various reliable tests before being given a diagnosis of JC pollinosis; however, most patients visited clinics or small-scale hospitals where such reliable tests are not conducted to establish a diagnosis of JC pollinosis. Our symptom data do show some bias regarding diagnosis, sex (a majority of women), and age (a majority of young patients; 20's to 40's). We must keep this in mind when analyzing the data. It is considered, though, that our results are useful for mobile phone users, as far as the information supplied through mobile phones is concerned. Recently, Okubo *et al.* investigated the efficacy and safety of Omalizumab in the treatment of JC pollen-induced seasonal allergic rhinitis.[12] In their study, patients needed to be carefully chosen to only include JC pollinosis patients. Our aim is the improvement of pollen information and examination of the validity, so we tried to collect many data from various patients who did not visit their clinician often.

It is well known that JC pollinosis patients who start their medication several weeks before the first day of the pollen season can spend the pollen season without severe symptoms.[1-6] It was indicated from our study that Cry j 1 levels reached 1 pg/m^3 2–3 weeks before the first day of the pollen season. Therefore, a Cry j 1 level of 1 pg/m^3 is the suitable time for medication for patients whose symptoms start early in the pollen season as well as for the start of provision of pollen information. It was shown that about 40% of users of the site "pollen check sheets" suffered pollinosis symptoms before the first day of the pollen season. Cry j 1 levels fluctuated at 1 to 3 pg/m^3 at the time symptoms appeared in some patients. This point became clear for the first time in the present study by using a highly-sensitive method called ESR radical immunoassay. The time of sudden increase in Cry j 1 levels nearly corresponded to the time the symptom scores increased. It could confirm that the time almost corresponded to that of the first day of the pollen season. We think that the definition of the first day of the pollen season, which is publicized widely in Japan, should be verified on the basis of our investigation of airborne Cry j 1 levels.

Lehtimäki *et al.* reported that sensitive birch pollen-allergic patients may experience symptoms when the allergen level reaches about 5 pg/m^3 of Bet v 1.[13] It was reported that the average amounts of Cry j 1 in 1 *g* of JC pollen is about 500 μg[14] and the number of pollen grains in 1 *g* of JC pollen is approximately 10^8. [15] From these values, the amount of Cry j 1 in a pollen grain is calculated as 5 pg. Lehtimäki *et al.*'s results for Bet v 1 coincided with ours for Cry j 1. According to Pehkonen *et al.*'s report about birch pollen antigens,[16] large and very small particles predominate before the pollen season and medium-sized

particles are absent. They concluded that small particles are also allergologically important.

We showed previously that small particles containing Cry j 1 existed in the air during the JC pollen season.[17-19] Sagehashi *et al*. reported that JC pollen releases its allergen in environmental water having a high ion concentration and to hydrophobic surfaces, such as diesel exhaust particles (DEP), which constitute pollen allergen-DEP complex generators.[20] From these results, we think that Cry j 1 particles without pollen in appearance may also contribute to airborne Cry j 1 sources, especially in high-exposure years as indicated in Table 3. The total pollen counts and the total Cry j 1 amounts during the pollen season did not correlate precisely. The reason is considered to be that pollen once fallen on the ground whirls up again and again repeatedly. Some pollen grains collapse morphologically, and they can not be recognized as pollen grains any more. It can be thought that the amounts of Cry j 1 bearing particles differ from year to year. In addition to these, the amounts of Cry j 1 in JC pollen grains differ from tree to tree. These are the causes of the inconsistency between the total pollen counts and the total Cry j 1 amounts.

The ESR method is a new technique developed by the laboratory of the Institute for Life Support Technology (Matsuei, Yamagata-City, Japan).[8-10] Briefly, an antigen-antibody reaction is performed in the same manner as in the usual ELISA method on a 96-well microplate. What is different in the new method is the use of a stable radical substance as the final product of the HRP enzyme reaction instead of a substrate for coloration. The discovery of the stable nitroxide radical enables the measurement. Radicals are usually unstable and instantly disappear, but the nitroxide radical is a stable material living for a day or two. The existence of the stable radical is a strong point of the method, because the stable radical only remains in the solution after the enzyme reaction. No radicals exist in the solution except for the stable radical, which makes an extremely high S/N ratio possible and realizes highly-sensitive measurement. It can be attained with several allergological substances, such as Der f 2, Dac g and IF-γ (unpublished data).

In 2005, we started an allergen information service through the Internet. Pollen allergen information will be provided in the future. At present, however, information is given on the pollen count obtained by microscopy. The ESR method is extremely useful for obtaining information before the first day of the pollen season. There is a wide difference between patients' sensitivity to Cry j 1, though, and by applying the ESR method to the quantification of Cry j 1, proper information about the onset time of individual patients' symptoms may be delivered through a mobile phone site for those whose past symptom data have already been accumulated. Measurement of Cry j 1 by the latex agglutination method[21] which we developed recently will be useful after the first day of the pollen season. The latter method does not need any special apparatus, requiring only anti-Cry j 1 polyclonal antibody-coated latex beads. The procedure is simple and easy and takes only 5 minutes each day. The agglutination reaction requires 2 hours for completion and therefore, we have to wait 2 hours before the information can be released. However, the time lag of 2 hours may be overlooked for information that is issued at 24-hour intervals. The combination of the ESR method[10] and the latex agglutination method[21] will cover the entire pollen season.

It is estimated from Table 3 that we inhale about 15 ng of Cry j 1 during a low-exposure year and 550 ng or more during a high-exposure year during the JC pollen season, if an adult is assumed to respire approximately 10 m^3 of air per day. From our past data of total pollen counts, we speculate that the maximum levels of Cry j 1 inhaled in a single year in the natural environment must not exceed 1 μg. The amounts of Cry j 1 used for animal exposure experiments are extremely high compared with the amounts of Cry j 1 inhaled naturally. For example, Sakurai *et al*. exposed rats to 2 μg Cry j 1 on 5 days per week for 3 months in a low-dose group and to 20 μg Cry j 1 in a high-dose group.[22] The amounts given in a single day exceeded those that people inhale during a whole pollen season even in a high-exposure year, and this is without considering the size of rats. However, these amounts are needed for sensitization of animals in a short period of time (within 30–60 minutes) in these experiments, and not all animals are high responders to the allergens. Pollen exposure units have been constructed in several countries including Japan (in Wakayama-City, Osaka-City and Tokyo).[23,24] Krug *et al*. constructed a pollen exposure unit to study human inhalation.[25] They observed dose-dependent induction of allergic rhinitis symptoms by grass pollen. Vast numbers of pollen grains are used for the experiments, because concentrations of 1,000–8,000 grains/m^3 are reported in dose-dependent induction of allergic symptoms. These concentrations exceed the high-exposure period in the natural environment.

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