| | |
|---|---|
| NHS | *N*-hydroxysuccinimide |
| PBS | Phosphate-buffered saline |
| RSI | Relative signal intensity |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SPT | Skin prick test |

## 1 Introduction

The number of airborne pollen grains is typically counted morphologically using microscopy. Pollen information is derived from these data. However, because grass pollen shares similar morphological characteristics, it is difficult to discriminate between species of pollen grains using light-microscopic pollen counts. It is known that species of grass pollen antigenicity differ with each species of grass pollen, and the pollen antigens of some grasses are not cross-reactive with those of other grass species (Esch 1999; Martin et al. 1985; Weber and Nelson 1985). Fortunately, most grasses associated with pollinosis in our region are cross-reactive species belonging Poöideae. In addition, submicron-size particles bearing pollen antigens such as starch granules (Spieksma et al. 1991) exist other than as antigens from the pollen itself. Such submicron-size particles have been isolated from pollen grains under wet conditions (Suphioglu et al. 1992) and are also considered to be the causative agents of pollinosis.

To obtain more information about the pollen, it is desirable to measure antigens that are cross-reactive with grass pollen. We applied an immunochemical technique for the quantification of airborne grass pollen antigens. A major problem in this context is that only small amounts of these antigens are present in air, and a highly sensitive analytical technique is required for such measurements. Recently, an electron spin resonance (ESR) radical immunoassay was developed as a highly sensitive method for detecting hepatitis B surface antigen (Matsuo et al. 1998; Aoki et al. 2002). In our previous study, we have shown that the sensitivity of this immunoassay is 10- to 100-fold higher than that of the conventional enzyme-linked immunosorbent assay (ELISA) (Aoyama and Takahashi 2004; Takahashi et al. 2007).

In this study, we used the ESR radical immunoassay to quantify airborne grass pollen antigen. Orchard grass pollen antigen was chosen as the target, because this species is universally distributed throughout our region and is considered to be the most common species contributing to total regional airborne grass pollen antigens.

## 2 Materials and methods

### 2.1 Sampling and antigen extract

A cyclone sampler, CM 90 (Burkard Manufacturing, Rickmansworth, UK) was installed at Iwanami in Yamagata City, and airborne pollen antigens were collected in 1-ml tubes. No large community of grasses is present in any direction within 500 m of the sampling site. The tubes were replaced and samples were collected at 6:00 daily. The antigens in the airborne samples were extracted with 100 μl of 0.125 M ammonium bicarbonate in 0.1% bovine serum albumin (BSA) for 2 h at room temperature. The scratch extract of *Dactylis glomerata* pollen (B3SFV2) (Torii Pharmaceutical, Tokyo, Japan) was used for the standard solution, and we defined the concentration of the scratch extract as 100,000 arbitrary units/ml. Dac g content in single pollen grains was determined by extraction of a reference pollen provided from International Biological (Piedmont, OK) with 300 μl of 0.125 M ammonium bicarbonate overnight at 4°C. After centrifugation, the aliquot was used to measure Dac g antigen, and the pellets were used to count the number of Dac g pollen grains.

### 2.2 Preparation of horseradish peroxidase (HRP)-conjugated antibody

Antibodies against Dac g antigens were prepared with Japanese white rabbits. Two rabbits were immunized subcutaneously with 400 μg (200 μg each rabbit) Dac g antigen from Greer Laboratories (Lenoir, NC) mixed with Freund's complete adjuvant. Boosts were given at 4, 8 and 12 weeks after the first injection and small bleeds were taken a week after each boost to check the antibody response. A large bleed was taken a week after the final boost (Kane and Banks 2000). Antisera were precipitated with 35% saturated ammonium sulfate, and they were dialyzed with 0.125 M NaCl and 0.05 M phosphate-buffered saline (PBS) at

pH 7.0. The ammonium precipitates were then passed through protein A column to purify IgG fraction. A portion of the IgG fraction was further purified with the antigen-combined column, and the purified samples were eluted with 10 mM glycine-hydrochloric acid at pH 2.0. The eluate was immediately replaced with PBS (pH 7.0). The final protein concentration of the refined antibody against Dac g was 0.5 mg/ml as measured using the BCA protein assay kit (Pierce, Rockford, IL). The antigen-combined column was made using Dac g antigen (Funakoshi, Tokyo, Japan) reacted with CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The refined antibody was conjugated with horseradish peroxidase using peroxidase labeling Kit-SH (Dojindo Molecular Technologies, Tokyo, Japan).

### 2.3 Electron spin resonance (ESR) radical immunoassay for Dac g antigen

Dac g antigen for SPT extract (Torii Pharmaceutical) was diluted 2,000-fold with PBS. The protein concentration of the Dac g antigens measured with BCA protein assay kit (Pierce) was 2.75 mg/ml. One hundred µl of the diluted antigen was put in a 96-well plate (Nunc, Kamstrupvej, Denmark), and the samples were reacted for 6 h at 4°C. After three washes with ultra pure water, 370 µl Stabilguard (SurModics, Eden Prairie, MN) was placed in each well, and the reaction was blocked overnight at 4°C. The plate were washed with ultra-pure water, dried in a desiccator, and kept at 4°C until use. Measurements were carried out as follows. Seven different concentrations of Dac g standard solution (5, 10, 20, 40, 100, 200, and 400 units/ml) were prepared prior for each measurement. One hundred µl of the standard solution or 100 µl of PBS containing 2% BSA was placed in each well of Dac g antigen-coated plate, and then 0.125 M ammonium bicarbonate (30 µl) or an airborne sample (30 µl) was added to each well. Then, 50 µl HRP-conjugated antibody against Dac g diluted with 10% fetal bovine serum (FBS) and 0.1 M PBS was added to each well, and the solutions were mixed thoroughly. Each sample was usually put in a single well, and in some noticeable cases, samples were measured in duplicate. The seven standard solutions were measured each time. The plate was left for 2 h at room temperature or left overnight at 4°C. After several washes in 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 to each well and reacted for 1 h at 37°C. The enzyme reaction was stopped with 50 µl sodium azide (100 mM). The amount of nitroxide radical (stable radical) produced as a result of the enzyme reaction was measured with an ESR device (FR30, JEOL, Tokyo, Japan) at a center field of 336.1 ± 5 mT. Details of the ESR measurement technique have been reported elsewhere (Matsuo et al. 1998; Aoki et al. 2002).

### 2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The pollen protein samples were analyzed by SDS-PAGE (12.5% acryl amide concentration; Bio-Rad, Hercules, CA). Prestained protein molecular weight standards-high (Life Technologies, Tokyo, Japan) was used as a molecular weight standard. Electrophoresis was performed at a constant voltage of 12 V for 1 h. After electrophoresis, the gel was placed on a nitrocellulose membrane, and the antigens were transferred onto the membrane. The gels were stained with Coomassie-blue G-250. The membrane was blocked with PBS in 5% BSA overnight at 4°C, then the samples were reacted with 500-fold diluted biotinylated antibody against Dac g for 2 h. Antibody against Dac g was biotinized with long-arm NHS biotin (Vector Laboratories, Burlingame, CA) (Abdul-Ahad and Brett 2000). After three washes with PBS, the samples were reacted with 500-fold diluted alkaline phosphatase conjugated streptavidine (Vector Laboratories). Finally, the Dac g antigen bands were visualized with BCIP/NBT substrate (KPL, Washington, DC).

## 3 Results

### 3.1 Dac g reacts with the antibody against Dac g

The Dac g antigen from Greer laboratory that was used for immunization showed five clear bands in SDS-PAGE, with approximate molecular weights of

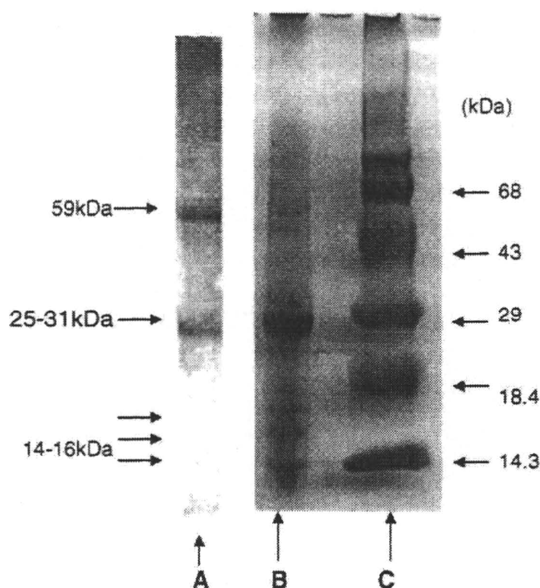


**Fig. 1** Determination of molecular components of Dac g extract analyzed by SDS-PAGE and antibody against Dac g specificity by Western blotting. A: Antibody against Dac g analyzed by Western blotting. B: Molecular components of the Dac g extract analyzed by SDS-PAGE. C: Molecular weight markers: lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), and albumin (68 kDa)

59, 25–31, 16, 15, and 14 kDa. The 25–31 kDa band was broad and dense. The 59 and 25–31 kDa bands reacted with the antibody against Dac g. Three bands of low molecular weights, 14, 15, and 16 kDa, did not react with the antibody against Dac g (Fig. 1). Therefore, the antibody against Dac g contained antibodies against 59 and 25–31 kDa antigens.

### 3.2 Standard curve of ESR radical immunoassay

A standard curve was obtained using ESR radical immunoassay, and the results are shown in Fig. 2. The vertical axis indicates the relative signal intensity (RSI) (%); the horizontal axis indicates Dac g concentration expressed as unit/ml. The RSI (%) was calculated from the following equation.

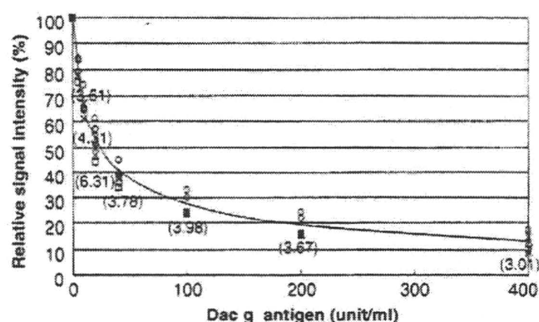


**Fig. 2** Relationship between Dac g levels and relative signal intensity determined by ESR radical immunoassay. Seven concentrations (5, 10, 20, 40, 100, 200, and 400 units/ml) of Dac g were measured five times, and the RSI (%) was plotted on the vertical axis. A regression curve is derived from the mean value of the five measurements. Standard errors are shown in parentheses

The standard error of RSI (%) was in the range of 3.0–6.3% (mean 4.1%, $n = 6$) (Fig. 2), and therefore we defined a 90% intercept value of RSI to the zero standard signal intensity as the detection limit in this study. A zero standard means maximum binding capacity of the system in this case. The 90% intercept value of the signal intensity was approximately 3.5 units/ml. On the other hand, the amount of Dac g in single pollen grains was found to be 1.84 units/grain (1.38–2.24 units/grain, $n = 8$) based on a reference pollen obtained commercially.

### 3.3 Measurement of airborne Dac g antigen

The level of airborne Dac g antigens was examined during the period of 22 March and 30 June in 2006 (Fig. 3). Minor amounts of cross-reactive antigens to Dac g were already detected in late March, and the levels gradually increased thereafter. The levels were found to be 10 units/m$^3$ until the middle of May. The Dac g levels increased after the blooming of orchard grass, and a high level of Dac g antigen was maintained until the middle of June. Symptoms of some grass pollinosis patients had already begun in

$$\mathrm{RSI}(\%) = \frac{\text{Signal intensity of a standard Dac g}}{\text{Signal intensity of a zero standard (without Dac g)}} \times 100$$

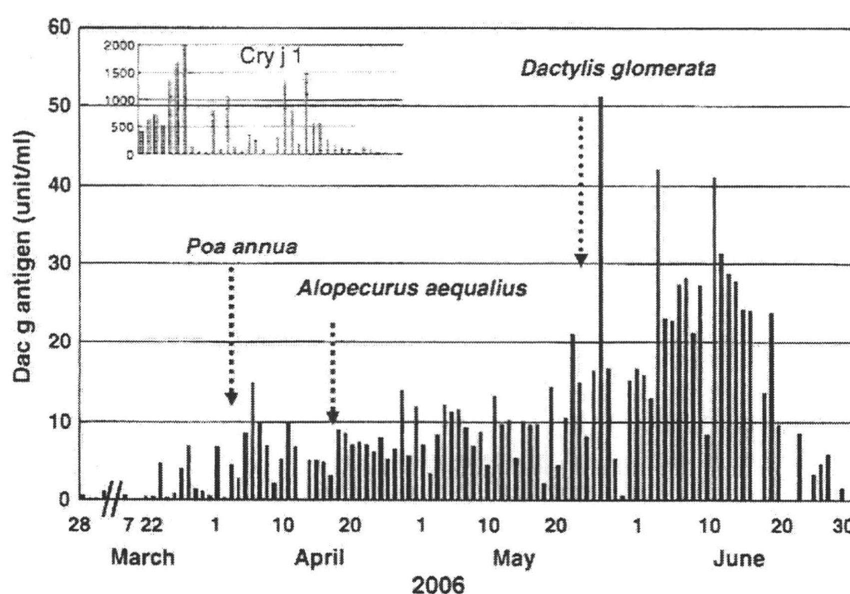


**Fig. 3** Daily fluctuation of airborne Dac g measured by ESR radical immunoassay during the period from 7 March to 30 June 2006. Arrows indicate the flowering time of relevant grasses near the sampling site. Daily fluctuation of airborne Cry j 1 level is shown. The horizontal scale is adjusted for comparison

late April, when the airborne Dac g levels were fluctuating between 5 and 10 units/m$^3$.

The relationship between the daily amount of airborne Cry j 1 (a major pollen allergen from Japanese cedar pollen) and that of Dac g was examined, and no relationship between the two values was observed (between 22 March and 10 May, $r = -0.1828$, $n = 50$).

## 4 Discussion

We have developed a highly sensitive method for measuring airborne Dac g antigens using ESR radical immunoassay. The 90% intercept value was calculated as 3.5 units/ml as described in Sect. 3. Each measurement needs 30 μl extract; therefore, a level of Dac g in excess of 0.1 unit in the sample is detectable. The Dac g content in single pollen grains of *D. glomerata* was determined as 1.84 units. Thus, the amount of Dac g that could be detected was as low as 1/20th of that contained in single grains. Using this method, the Dac g antigen was detectable during the early stage of the grass pollen season. This approach is expected to provide useful information for grass pollinosis patients, especially for those who show symptoms at times when only a low level of antigen is present in air. Data are available 2½ h after sampling, namely, 30 min extraction (Takahashi et al. 2001), 60 min antibody reaction and 60 min radical reaction. We could use the above treatment times without sensitivity loss on the occasion of the pollen allergen information. It is possible to supply the pollen allergen information to local residence through mass media until evening time on the same day.

Also, we have been providing information about Cry j 1 antigen in airborne pollen during the pollen season since 2005. It is well known that some patients displayed symptoms several weeks before airborne Japanese cedar pollen has been detected by microscopy. No airborne Cry j 1 has been detected using the conventional ELISA during this times. The development of the ESR radical immunoassay has now made it possible to conduct such measurements, and it has been reported that some patients show their symptoms during a period in which airborne Cry j 1 levels fluctuating between 1 and 3 pg/m$^3$. Such information could be useful for the patients whose symptoms begin early in the flowering season (Takahashi et al. 2007). Now we have been supplying airborne Cry j 1 information through internet and local TV at the pollen season.

Low levels of Dac g were present during the latter half of the Japanese cedar pollen season. Japanese cedar pollinosis is the most common in Japan, and more than 10% of Japanese suffer from it. Some patients who suffer from the pollinosis during this

season think that Japanese cedar pollen is their causative pollen, but it is clarified from this study that Dac g pollen was also detected in air at that time. It has been reported that some grass pollinosis patients show symptoms in late April and early May in our region (Takahashi et al. 1987), and this time corresponds to the period when early flowering species of grasses start to bloom. It is likely that the symptoms of these patients are provoked not only by Japanese cedar pollen, but also by grass pollen. Interestingly, no positive correlation was observed between the amounts of Cry j 1 and Dac g antigen in air, samples of which were collected during Japanese cedar pollen season. Dac g and Cry j 1 do not have any cross-reactive antigens. The comparison was made because we want to examine whether they appear in air simultaneously or independently controlled under different meteorological conditions, and further whether there are more patient symptoms in connection with the appearance of these antigens.

Airborne samples were taken using Burkard Cyclone sampler in this research. The manufacturer of the sampler announced that the collection efficiency of the sampler is 90% in 1-μl range particles; that is to say, allergens existed as minute particles under 1 μl, and gas-shaped particles could not be sampled. In Europe, the MONALISA (Monitoring Network of Allergen by Immuno-Sampling) project was initiated in 2005. The aim of the project is to characterize pollen allergens such as Poaceae, *Betula, Ambrosia, Artemisia, Cupressus, Parietaria*, and *Olea* pollens for the benefit of pollinosis patients. Several sampling methods were examined in the MONALISA project. According to Rantio-Lehtimäki of Turk University, samplings into the liquid were diluted, and ELISA results were not reliable (personal communication). The advantage of the Cyclone sampler is that allergens in large volumes of air could be collected in a very small quantity of extraction medium (at least 50 μl). Therefore, we chose the cyclone sampler for the study.

In this research, we used an antibody against Dac g antigen, because *D. glomerata* is the most widely distributed species in our region and is considered to be the main species contributing to the total airborne grass pollen antigens in this region. A number of studies have been conducted to analyze *D. glomerata* pollen antigens (Esch and Klapper 1989; Cuerin-Marchand et al. 1996; Roberts et al. 1993; Leduc-Brodard et al. 1996; van Oort et al. 2001). The extent of cross-antigenicity among grass antigens remains difficult to estimate. The antibody used in this study recognized antigens with molecular weights of 59 and 27–35 kDa. It is likely that 59 kDa antigen is Dac g 4, and 27–35 kDa antigen is Dac g 1 and/or Dac g 5. According to Esch (1999), Dac g 5 has a wide molecular weight distribution and is cross-reactive within pollen antigens from Poöideae. Therefore, Dac g 5 was considered to be one of the suitably defined antigens for our purpose.

As for antibody, several categories of antibodies are available for the airborne antigen measurements, e.g., antibodies created from crude antigen extracts, antibodies created from defined antigens, monoclonal antibodies, and polyclonal antibodies. There are no commercial products for antibodies against grass pollen antigens in Japan. First of all, we must prepare antibodies against grass pollen antigens. Monoclonal antibodies are easy to manage concerning quality control as indicated by the example that the MONALISA project has selected them. However, we prepared polyclonal antibodies because we want to use antibodies that have wide specificities related to local grass pollinosis, and it can be made easily. There is, certainly, a problem with quality control among preparations. However, we do not worry about this matter concerning the standpoint of pollen allergen information, because outstanding sensitivity of the ESR method made it possible to dilute the HRP-conjugated antibody to 10,000-fold. So, we can use the same preparation for many years. Addition to this, we think suitable antibody will differ from place to place as discussed in the next paragraph.

Some patients show symptoms before the flowering of *D. glomerata*. The amounts of airborne allergens causing grass pollinosis can be roughly estimated to quantify the airborne Dac g levels, because the antibody used in the present study reacts not only to Dac g pollen antigens, but also to grass pollen antigens cross-reactive with Dac g from other plants of the Poöideae subfamily. Thus, the airborne grass pollen antigens observed before *D. glomerata* flowering season that bloom until late May (Takahashi et al. 1993) may not have been Dac g itself, but may have been other species having cross-reactive antigens with Dac g antigen. Two early blooming species of grasses in our region are *Alopecurus aequalis* and *Poa annua*. However, early blooming species are not

limited to the above-mentioned species; for example, *Anthoxanthum odoratum* blooms prior to the major grass pollen season in some areas in Japan (Sudo et al. 2005). It would be desirable to investigate antibodies with a broad range of specificity against major allergens related to local grass pollinosis. Moreover, it would be helpful to examine antibodies in immunization with a mixture of grass pollen antigens from different species. Antibodies suitable for such studies will be those against the most important species in a particular region, and we think the suitable antibody will differ from place to place. Further research is still needed to clarify the differences among applied antibodies and to identify the appropriate antibodies for the measurement.

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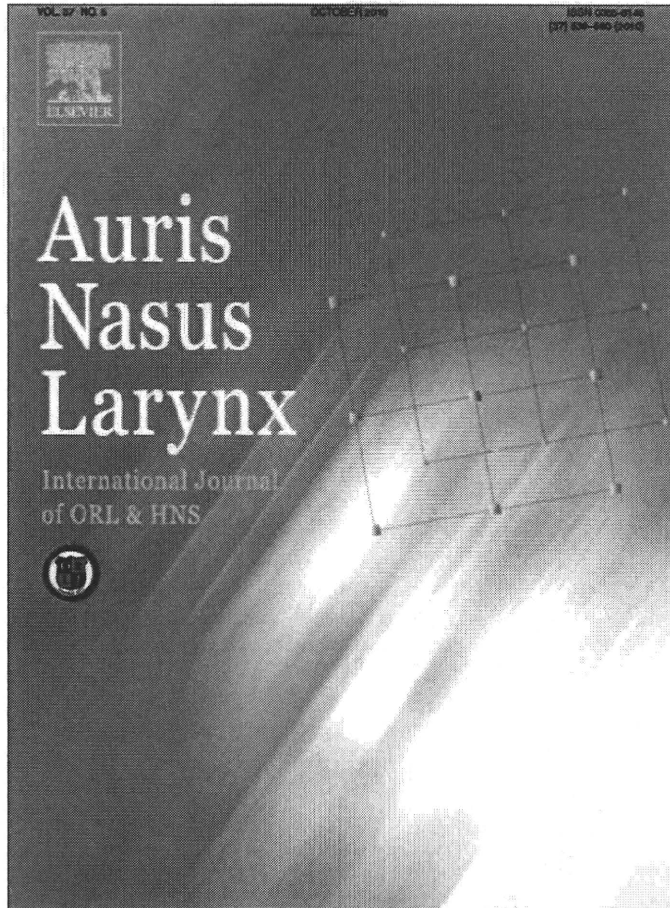


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ORIGINAL ARTICLE

# Analysis of the Comorbidity of Bronchial Asthma and Allergic Rhinitis by Questionnaire in 10,009 Patients

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## ABSTRACT

**Background:** Bronchial asthma (BA) and allergic rhinitis (AR) are thought to share a common pathogenesis. However, reports concerning the comorbidity of the two diseases in a large-scaled population are rare in Japan. In the present study, we performed an analysis on the two diseases using questionnaires that addressed the diagnosis, symptoms and period of occurrence in more than 10,000 patients with BA or AR.

**Methods:** Patients with BA (adult: $n = 2,781$, childhood: $n = 3,283$) and AR ($n = 3,945$) were enrolled in the present study during the 3 months from August 1, 2006 to October 31, 2006.

**Results:** Sixty one percent of the patients with adult BA showed symptoms of AR. Among them, 68% of the patients were diagnosed with AR. Among the patients with childhood BA, 68% showed AR symptoms and 60% were diagnosed with AR. On the other hand, 49% of AR patients showed BA symptoms and 35% of them were diagnosed with BA. The symptoms of both BA and AR in the BA and AR patients were frequent in two seasons, March and April, and September and October. In addition, BA and AR symptoms often co-occurred in the patients with BA and AR.

**Conclusions:** Comorbidity of BA and AR was high in both populations of BA and AR. The symptoms of both BA and AR co-occurred on both a daily and seasonal basis. These results suggested that BA and AR share a common immuno-pathogenesis in the airway and need to be treated as a single airway disease.

## KEY WORDS

allergen, allergic rhinitis, asthma, exacerbation, pollen

## *INTRODUCTION*

The high comorbidity of bronchial asthma (BA) and allergic rhinitis (AR) has been reported.[1-5] Since Th2 lymphocytes, mast cells and eosinophils are known to infiltrate the mucosal layer of the upper and lower air-

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**Table 1** The ratio of the subjects with AR symptoms in BA patients and BA symptoms in AR patients

| | Number of cases | With syms | Without complications | Not answered |
|---|---|---|---|---|
| Adult asthma | 2,781 | 1,693 (60.8%)* | 1,044 (37.5%) | 44 (1.6%) |
| Childhood asthma | 3,283 | 2,238 (68.2%)** | 1,035 (31.5%) | 10 (0.3%) |
| Allergic rhinitis | 3,945 | 1,935 (49.0%) | 2,010 (51.0%) | — |

The following questions to the patients with adult BA, childhood BA and AR;

1) Question to BA patients: Have you had an experience in which symptoms such as sneezing, runny nose and stuffy nose developed repeatedly when you did not have a cold?

2) Question to AR: Have you had an experience in which asthma-like symptoms such as a wheezing sound, cough, sputum, and exercise-induced breathing difficulty developed repeatedly when you did not have a cold?

#; The complications mean AR symptoms in BA patients and BA symptoms in AR patients.

*Adult asthma *vs* Allergic rhinitis, $p < 0.001$, **Childhood asthma *vs* Allergic rhinitis, $p < 0.001$ by $\chi2$ analysis.

ways of these two diseases, they have been thought to share a common pathogenesis.[6,7] Inhalant allergens common to BA and AR have been also evaluated.[5]

Recently, BA and AR have come to be considered as "one airway disease" and therapeutic strategies have been considered consistent with this concept.[8]

To date, epidemiological studies on the comorbidity of BA and AR have been reported globally. Greisner, *et al.* reported that among college students in the US, 85.7% of patients with BA had a history of AR. On the other hand, the frequency of asthma was 16.2% among individuals with rhinitis in a European population.[5] There have been few reports on the comorbidity of BA and AR in a large Japanese population. The present study examined more than 10,000 patients, including patients with adult BA, child BA and AR, in the same period in the Tohoku district of Japan.

## METHODS

*Subjects*: The subjects enrolled in the present study were patients who visited private medical offices, public hospitals and university hospitals during the 3 month period from August 1, 2006 to October 31, 2006, in the Tohoku district of Japan. The patients with BA ($n$ = 2,781) were diagnosed by internal medicine physicians according to ATS guidelines. The patients with childhood BA ($n$ = 3,283) who were less than the age of 16 years were diagnosed by pediatricians according to the Japanese Pediatric Guideline for the Treatment and Management of Asthma 2005.[9] The patients with AR ($n$ = 3,945) who included both children and adults were diagnosed by otolaryngologists according to Practical Guideline for the Management of Allergic Rhinitis in Japan.[10]

*Questionnaire*: The patients were requested to answer a questionnaire based on the following questions: for patients with adult BA and child BA, patients were asked "Have you had an experience in which symptoms such as sneezing, runny nose and stuffy nose developed repeatedly when you did not have a cold?"; "Have you been diagnosed with perennial allergic rhinitis or seasonal allergic rhinitis?"; "Do you have symptoms such as sneezing, runny nose and stuffy nose when asthma is aggravated?"; "In which months do you have symptoms such as sneezing, runny nose and stuffy nose?"; and "In which months do you have aggravated symptoms of asthma?". In these questionnaires, the patients could answer "all year" when they had the symptoms perennially. For patients with AR, patients were asked: "Have you had an experience in which asthma-like symptoms such as a wheezing sound, cough, sputum, and exercise-induced breathing difficulty developed repeatedly when you did not have a cold?"; "Have you been diagnosed with asthma?"; "Do you have asthma-like symptoms when allergic rhinitis is aggravated?"; "In which months do you have symptoms such as sneezing, runny nose and stuffy nose?"; and "In which months do you develop asthma-like symptoms?". In the patients with childhood BA, the mothers or adult attendants answered the questions if the patients seemed unable to understand the questionnaire.

*Statistics*: Data in the present study were analyzed by McNemar Analysis and $\chi2$ analysis.

## RESULTS

### COMORBIDITY OF BA AND AR

Among the patients with adult BA ($n$ = 2,781), 60.8% answered that they had had symptoms of AR (Table 1). Among the adult BA patients with AR symptoms ($n$ = 1,693), 68.2% were diagnosed with AR (Table 2).

Among patients with childhood BA ($n$ = 3,283), 68.2% answered that they had had symptoms of AR (Table 1). Among the childhood BA patients with the AR symptoms ($n$ = 1,335), 59.7% were diagnosed with AR (Table 2).

On the other hand, among patients with AR (n = 3,945), 49% answered that they had ever had symptoms of BA (Table 1). Among AR patients with BA symptoms ($n$ = 1,935), 34.8% had been diagnosed with BA (Table 2).

The ratios of subjects with AR symptoms among both adult and childhood BA patients were signifi-

**Table 2** The ratio of the subjects diagnosed as AR in BA patients with AR symptoms and BA in AR patients with BA symptoms

| | Number of cases | Diagnosed | Not diagnosed | Not answered |
|---|---|---|---|---|
| Adult asthma | 1,693 | 1,155 (68.2%)* | 509 (30.1%) | 29 (1.7%) |
| Childhood asthma | 2,238 | 1,335 (59.7%)** | 873 (39.0%) | 30 (1.3%) |
| Allergic rhinitis | 1,935 | 674 (34.8%) | 1,219 (63.0%) | 42 (2.2%) |

The following questions to the patients with adult BA, childhood BA and AR;

1) Question to asthma patients: Have you been diagnosed with perennial allergic rhinitis or seasonal allergic rhinitis?

2) Question to patients with allergic rhinitis: Have you been diagnosed with asthma?

*Adult asthma *vs* Allergic rhinitis, $p<0.001$, **Childhood asthma *vs* Allergic rhinitis, $p<0.001$ by $\chi2$ analysis.

**Table 3** The ratio of the subjects who aggravated both AR and BA symptoms in BA patients with AR symptoms and AR patients with BA symptoms

| | Number of cases | Aggravated | Not aggravated | Not answered |
|---|---|---|---|---|
| Adult asthma | 1,693 | 886 (52.3%)* | 769 (45.4%) | 38 (2.2%) |
| Childhood asthma | 2,238 | 1,391 (62.2%)** | 810 (36.2%) | 37 (1.7%) |
| Allergic rhinitis | 1,935 | 1,449 (74.9%) | 402 (20.8%) | 84 (4.3%) |

The following questions to the patients with adult BA, childhood BA and AR;

1) Question to asthma patients: Do you have symptoms such as sneezing, runny nose and stuffy nose when asthma is aggravated?

2) Question to patients with allergic rhinitis: Do you have asthma-like symptoms when allergic rhinitis is aggravated?

*Adult asthma *vs* Allergic rhinitis, $p < 0.001$, **Childhood asthma *vs* Allergic rhinitis, $p < 0.001$ by $\chi2$ analysis.

cantly higher than that of subjects with BA symptoms among AR patients. In addition, the ratios of subjects diagnosed with AR among both adult and childhood BA patients were significantly higher than that of subjects diagnosed with BA among AR patients. In the current study, the complications of AR in adult and child BA patients, and BA in AR patients were diagnosed according to the questionnaire.

## CO-OCCURRENCE OF THE SYMPTOMS OF BA AND AR

Among patients with adult BA ($n$ = 1,693), 52.3% showed AR symptoms when their BA symptoms were aggravated. Sixty two percent of the patients with childhood BA ($n$ = 2,238) also showed AR symptoms when their BA symptoms were aggravated.

On the other hand, among patients with AR ($n$ = 1,935), 74.9% showed BA symptoms when their AR symptoms were aggravated.

The ratios of subjects with both aggravated AR and BA symptoms among both adult and childhood BA patients were significantly lower than that of those among AR patients (Table 3).

## FREQUENCY OF SYMPTOMS OF BA AND AR

Among patients with adult BA, symptoms of BA occurred frequently in spring (March and April) and autumn (September and October). These two peaks in the frequency of symptoms were statistically significant compared with the month with the lowest frequency. Among those patients, the symptoms of AR occurred frequently in two seasons such as March and April, and September and October, the same as with the BA symptoms (Fig. 1). The two peaks of AR symptoms among the adult BA patients were also significant. The ratio of the adult BA patients with perennial symptoms of BA was 13.6% and 33.7% of these showed perennial symptoms of AR.

Among the patients with childhood BA ($n$ = 2,238), the symptoms of BA also occurred frequently in spring and autumn, similar to those of adult BA (Fig. 2). Among these, the symptoms of AR occurred frequently in two seasons such as March and April, and September and October, similar to that seen in the adult BA patients (Fig. 2). The two peaks of AR symptoms in the childhood BA patients were also seen in two seasons such as March and April, and September and October. Among the child BA patients, 5.9% showed perennial symptoms of BA and 30.2% of these showed perennial symptoms of AR.

On the other hand, in the AR patients, AR symptoms occurred frequently also in spring and autumn, similar to those of adult BA and childhood BA patients. In the same periods, the BA symptoms in the AR patients also occurred frequently, and the two peaks of frequency were significantly high (Fig. 3). Thirty five percent of the AR patients showed perennial symptoms of AR and 23.7% of these showed perennial symptoms of BA.

## *DISCUSSION*

The present study confirmed the high comorbidity of

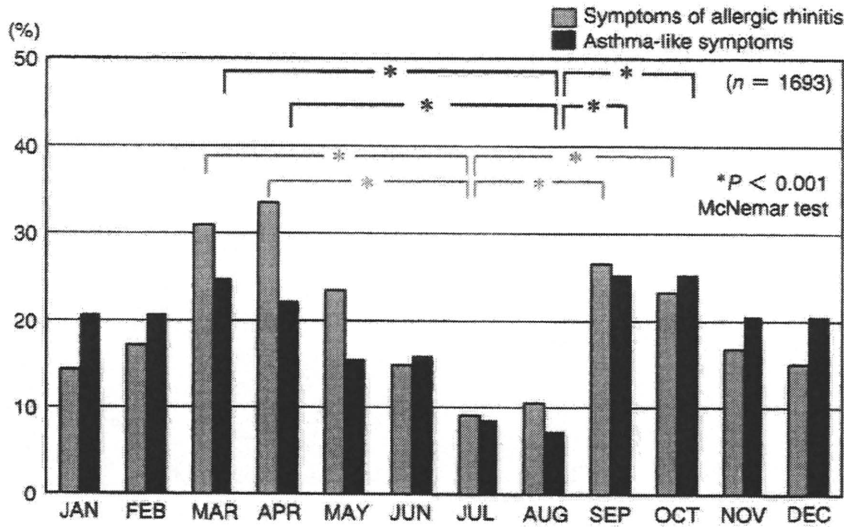


**Fig. 1** Frequency of BA and AR symptoms in adult BA patients. The following questions were given to the adult BA patients who repeatedly developed symptoms of sneezing, runny nose or stuffy nose without having a cold: 1) In which months do you have symptoms such as sneezing, runny nose and stuffy nose?; 2) Do you have aggravated symptoms of asthma in specific months?

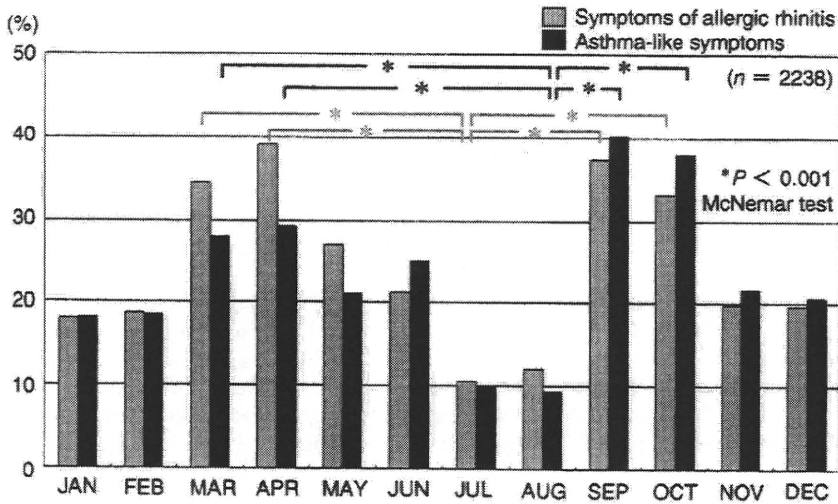


**Fig. 2** Frequency of BA and AR symptoms in childhood BA patients. The following questions were given to the childhood BA patients who repeatedly developed symptoms of sneezing, runny nose or stuffy nose without having a cold: 1) In which months do you have symptoms such as sneezing, runny nose and stuffy nose?; 2) Do you have aggravated symptoms of asthma in specific months?

BA and AR. The symptoms of BA and AR frequently occurred in the same periods such as spring and autumn. The co-occurrence of the symptoms of the two diseases was demonstrated. These results tend to confirm that AR and BA share common pathogenesis in the upper and lower airway.

Based on the AR symptoms, the ratio of comorbidity of AR was suggested to be 60.8% among the patients with adult BA and 68.2% among those with childhood BA. Greisner *et al.* reported that 85.7% of patients with BA had a history of AR in the US.[1] Soler *et al.* reported that 63.4% of the patients with asthma

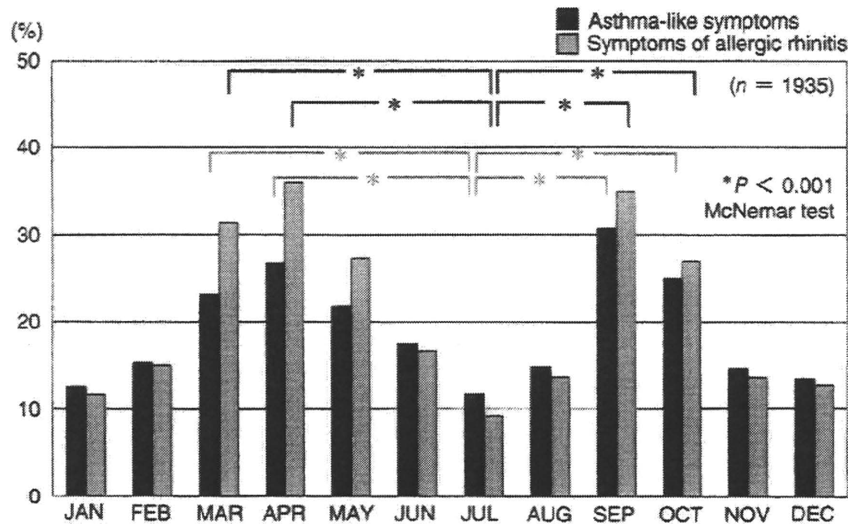


**Fig. 3** Frequency of BA and AR symptoms in AR patients. The following questions were given to patients with AR who repeatedly developed asthma-like symptoms without having a cold: 1) Do you have aggravated symptoms of allergic rhinitis in specific months?; 2) In which months do you develop asthma-like symptoms?

(*n* = 546) had seasonal AR and 77.3% of these had perennial AR.[11] Linneberg *et al.* reported that 89–100% of patients with allergic BA (*n* = 734) had allergic rhinitis in Denmark.[5] These reported ratios of comorbidity of AR in subjects with BA were higher than those in our study. In the study by Linneberg *et al.*, the ratio of comorbidity of AR was based on pollen-sensitized allergic asthma. In the present study, the adult BA population included both atopic and non-atopic BA. The comorbidity with AR is thought to be less frequent among non-atopic BA subjects compared to those with atopic BA.[4] The ratio of comorbidity of AR among BA subjects may, therefore, depend on the ratio of atopic BA patients in the population. Masuda *et al.* reported that 77.7% of 130 children with asthma (ages 2 through 10) had co-existing AR based on objective findings in a Japanese population.[12] Our data showed a slightly lower ratio of comorbidity of AR (68.2%) in patients with childhood BA. In this case, the difference in the ratio of comorbidity may be caused by the age of the subjects and by the way of diagnosing AR. In the present study, the ratio of comorbidity of BA in AR patients (49.0%) was lower that of AR in BA patients (60.8% in adult BA patients, 68.2% in childhood BA patients). However, we have no data concerning the allergic disposition of the BA patients in the current study. Mullarkey *et al.* reported that 58.8% of patients with AR had histories or findings consistent with asthma.[2] Globally, the population size of AR seemed larger than that of BA. In addition, AR from cedar pollen occupies a dominant position in Japan.[13-15] However, Japanese cedar pollen is not thought to be closely associated with BA compared to other allergens, such as orchard grass, ragweed, or mite.[16] This may account for the fact that the ratio of the comorbidity of BA in AR patients appeared to be lower than that of AR in BA patients. However, we have no data to specify the AR patients with Japanese cedar pollen in the current study. This study was performed based on a questionnaire in a large population. Therefore, the diagnosis of AR in adult and child BA patients, and BA in AR patients may have some limitations. However, we believe that the obtained results including comorbidity of BA and AR have some meaning.

The present study demonstrated that there were two seasonal peaks of frequency of both AR and BA symptoms, in spring and autumn among both the adult and the childhood BA patients. In addition, these two seasonal peaks in the frequency of both AR and BA symptoms in AR patients were also evaluated. We have no clear evidence to answer to the question of why the AR and BA symptoms co-occurred in the same two seasons in the adult BA, childhood BA and AR patients. However, we can speculate that possible causes include seasonal pollen, change of temperature, change of weather, viral infection etc. Among them, seasonal pollen are important allergens that induce AR and BA symptoms in the spring and autumn. Japanese cedar pollen is known to be a major allergen that induces AR symptoms in the spring all over Japan. While Japanese cedar pollen is not closely associated with BA,[16] other seasonal pollen such as ragweed, mugwort, orchard grass, birch etc. are thought

to be common seasonal allergens associated with AR and BA. In this context, the pollen allergens common to AR and BA might play a role in inducing both AR and BA symptoms in spring and autumn.

The present study also revealed that AR and BA symptoms co-occurred both seasonably and perennially among 52.3% of adult BA, 62.0% of childhood BA and 74.9% of AR patients by asking in the questionnaire whether AR symptoms were perennial or seasonal. The common triggers including allergens as described above were the probable causes. Beside these, AR exacerbation has been thought to provoke airway inflammation in the lower respiratory tract or to induce an increase in airway hyperresponsiveness.[17-19] Our results revealed that the ratio of AR patients with BA symptoms when patients experienced aggravated AR was significantly higher than those of adult and childhood BA patients with AR symptoms when they experienced aggravated BA. These findings indicate that more AR patients showed BA symptoms with AR exacerbation as compared with the BA patients with AR symptoms with BA exacerbation. These results support the idea that allergic inflammation in the upper airway influences airway hyperresponsiveness in BA.[18,19] In addition, this result seems to indicate that the upper airway symptoms tended to induce the lower airway symptoms more than the lower airway symptoms influenced the upper airway symptoms.

Our results also revealed that BA aggravation induced AR symptoms and, *vice versa*, AR aggravation induced BA symptoms in BA and AR patients. It is hypothesized that the inflammation in the upper airway in AR and that in the lower airway in BA influence each other *via* the systemic circulation and nervous system.[20-22] This mechanism may contribute, at least in part, to the co-occurrence of AR and BA symptoms both seasonally and perennially among the patients with adult BA, child BA and AR.

In conclusion, the high comorbidity of BA and AR was confirmed in a large Japanese population. The co-occurrence of the symptoms of the two diseases suggests that AR and BA share a common pathogenesis and should be treated as a single airway disease.

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アレルギー 58(12),
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原　著

# 山形市におけるアレルギー性鼻炎患者の花粉抗原陽性率の検討

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【背景・目的】花粉症の原因抗原としてはスギ・イネ科が有名であるが，その他の花粉症も注目されている．今回我々は当科を受診したアレルギー性鼻炎患者の花粉抗原陽性率について調査・検討したので報告する．
【対象・方法】対象はアレルギー性鼻炎症例の男性90例，女性61例．ハウスダスト・ダニ・スギ・カモガヤ・チモシー・ブタクサ・ヨモギ・カナムグラ・アルテルナリア・カンジタ・アスペルギルスに加え，シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギのエキスを用いてスクラッチテストを施行した．
【結果】スギ花粉陽性率（45%）よりもカモガヤ花粉陽性率（51%）のほうが高く，シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉はそれぞれ13%，8%，9%，11%，10%とスギ・イネ科花粉に比べると低いが真菌より高い陽性率であった．
【考察】イネ科花粉や，イネ科花粉と飛散時期の重なるシラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉は陽性率が高く重要な抗原と考えられた．重複陽性率も高い傾向があり，抗原間の交差反応性の検索も必要と考えられた．

Key words: allergic rhinitis —— overlapping positive ratio —— positive ratio of pollen antigen —— scratch test

## 1. 緒　言

アレルギー性鼻炎は患者数が多く，その症状も強くQOLも低下することなどにより近年大きな社会的注目を集めている．原因抗原の全国平均陽性率はハウスダスト・ダニが53.0%，スギ42.0%，イネ科16.4%と報告され，主要な抗原であると考えられる[1)]．

しかし，スギ，イネ科以外に花粉症の原因となる花粉には多くのものがあり，季節や地域によっては様々な花粉が飛散している．本邦でI型アレルギー疾患の抗原として，花粉抗原が約60種，職業性抗原が約80種，ハウスダスト・ダニ・真菌抗原・ペット抗原など合わせて20種以上が報告されている[1)]．

地域に根ざしたアレルギー性鼻炎の診療を考えるとき，その地域特性を知っておく必要がある．山形県は首都圏と異なりスギ花粉症患者よりもイネ科花粉症患者のほうが多い傾向があり[2)]，その他様々な花粉が季節ごとに飛散している．山形県では山形県衛生研究所のホームページにて各種花粉飛散予想，各種花粉飛散開花情報，スギ花粉の

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利益相反（conflict of interest）に関する開示：著者全員は本論文の研究内容について他者との利害関係を有しません．
鈴木祐輔：山形大学医学部情報構造統御学講座耳鼻咽喉・頭頸部外科学分野〔〒990-9585　山形県山形市飯田西2-2-2〕
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総飛散数・飛散開始日及び花粉シーズン中の毎日の飛散数が公開されている[3]．この中ではスギ・ヒノキ・カモガヤ・ブタクサ・ヨモギ・カナムグラなどの一般的な花粉抗原の飛散時期・飛散状況の他にもシラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギなどの花粉抗原の飛散時期・飛散状況も公開されている．過去に山形市におけるスギ・キク科やイネ科花粉抗原陽性率に関する報告はなされている[2]がシラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉の実際の陽性率に関する検討はなされていなかった．これらの花粉の飛散時期は春～夏，特に5～6月にあたり，山形市においてはスギ花粉よりも患者数の多いイネ科花粉の飛散時期と重なる．イネ科花粉飛散の時期にアレルギー性鼻炎症状が悪化する患者の中で，イネ科花粉には陽性とはならずにこれらの花粉抗原に陽性反応を示す者やイネ科花粉とともに重複して陽性を示し症状を悪化させている者も多数存在しうる可能性も考えられ臨床の場において注意すべき花粉である．

今回，我々は主要花粉抗原陽性率のほかに，シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉の陽性率と重複陽性率について調査，検討したので報告する．

## 2. 対象と方法

2003年5月から2006年2月までに山形大学附属病院耳鼻咽喉科アレルギー外来と協力いただいた山形市内開業医を受診した男性90例，女性61例を対象に行った．年齢は2歳～83歳，平均年齢は30.3歳であった．陽性の判定方法はスクラッチテストを用いて行った．消毒した前腕屈側皮膚に皮内注射針を用いて傷をつけ，診断用エキスを1滴滴下し，15分後に膨疹と発赤の大きさを測定した．判定は奥田の基準に準じ，紅斑21mm以上，膨疹9mm以上を陽性と判定した．

ハウスダスト・ダニ・アルテルナリア・アスペルギルスおよびスギ・ヒノキ・カモガヤ・チモシー・ヨモギ・カナムグラ・ブタクサ各花粉のスクラッチエキス（トリイ薬品製）を使用した．シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ各花粉のスクラッチエキスは安枝の方法に準じて作製した．シラカンバ（*Betula platyphylla* var. japonica）とヤナギ（*Salix* spp.）花粉はInternational Biological INC（USA）社製，ヒメスイバ（*Rumex acetosella*）花粉はAllergon社（Sweden）社製，コナラ（*Quercus serrata*）とオニグルミ（*Juglans mandshurica*）花粉は開花期に雄花序を水栽培して集めた花粉を使用した．花粉の純度はカルベル液で染色し顕微鏡下で確認したところほぼ100％の純度であることが確かめられた．それぞれ花粉重量の20倍量の0.125M重炭酸アンモニウム液を加えて，4℃で48時間静置し時々用手攪拌しながら抽出した．抽出液は蒸留水に対して2日間透析後0.45μのミリポアフィルターを通して除菌した．除菌後，試料を滅菌したセルローズチューブ（Visking社）に入れ，50％グリセリン-5%NaCl溶液を外液として，4℃に1週間放置し，溶媒交換を行った．溶媒交換後内液の容量が減少するので，最終的に50％グリセリン-5%NaCl溶液で20倍液に調製し，診断用花粉エキスとした．

対照液はアレルゲンスクラッチエキス「トリイ」対照液を使用した．

各抗原の陽性率を性別・年齢・検査施行時期別に比較し，性別はMann-Whitney's U検定，年齢・検査施行時期はKruskal-Wallis検定にて比較した．またシラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉陽性者の重複陽性率についてもMann-Whitney's U検定にて比較した．

## 3. 結　果

ハウスダスト，ダニの陽性率はそれぞれ79％，57％と高値を示した．スギ花粉は45％，イネ科花粉はカモガヤ51％，チモシー36％と以前の報告と同様に[2]スギよりもイネ科のほうが陽性率が高い傾向が認められた．ブタクサ30％，ヨモギ19％，カナムグラ37％，アルテルナリア7％，カンジタ5％；アスペルギルス5％であった．シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギはそれぞれ13％，8％，9％，11％，10％であり，真菌アレルギーより高い陽性率であった．シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ少なくともどれか一つに反応する抗原陽性率は26％も見られた（Fig. 1）．

男性90例，女性61例各々の抗原陽性率の検討

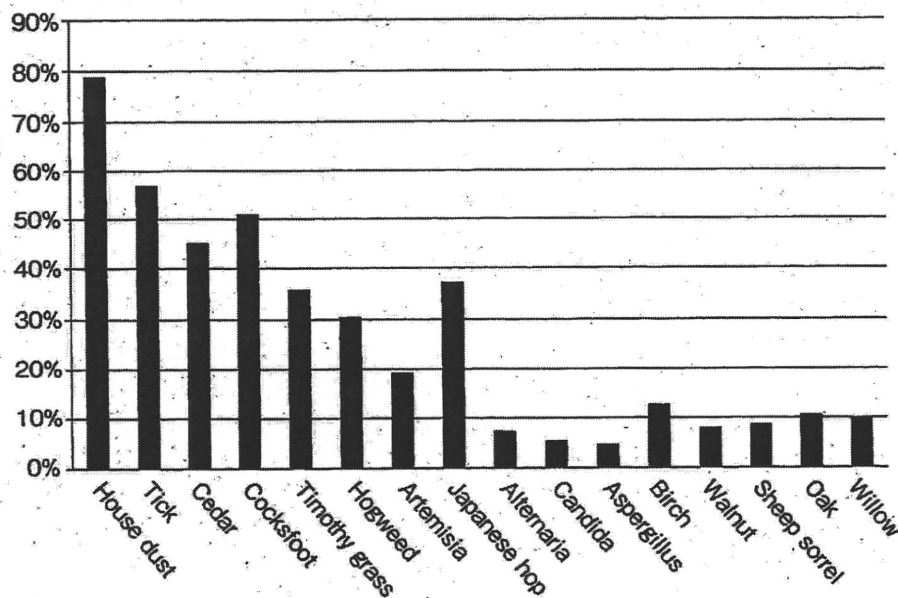


Fig. 1. Positive ratio of pollen antigen in our study (n=151).
This figure shows that Positive ratio of Cocksfoot (51%) was higher than that of Cedar (45%). Each positive ratio of Birch, Walnut, Sheep sorrel, Oak and Willow was 13%, 8%, 9%, 11% ,10%, respectively. These were lower than that of Cocksfoot or Cedar, but higher than these of Fungi: Alternaria, Candida and Aspergillus.

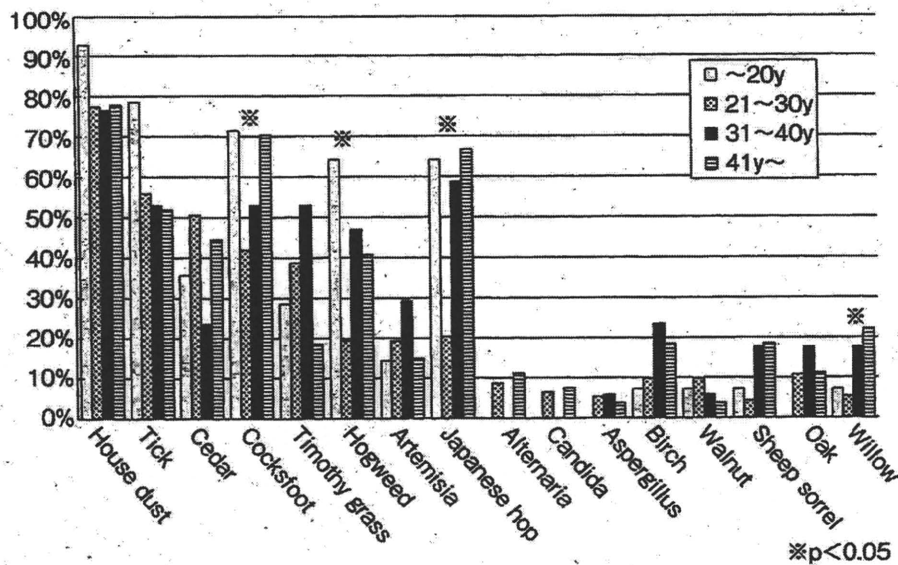


Fig. 2. Positive ratio of pollen antigen by age bracket.

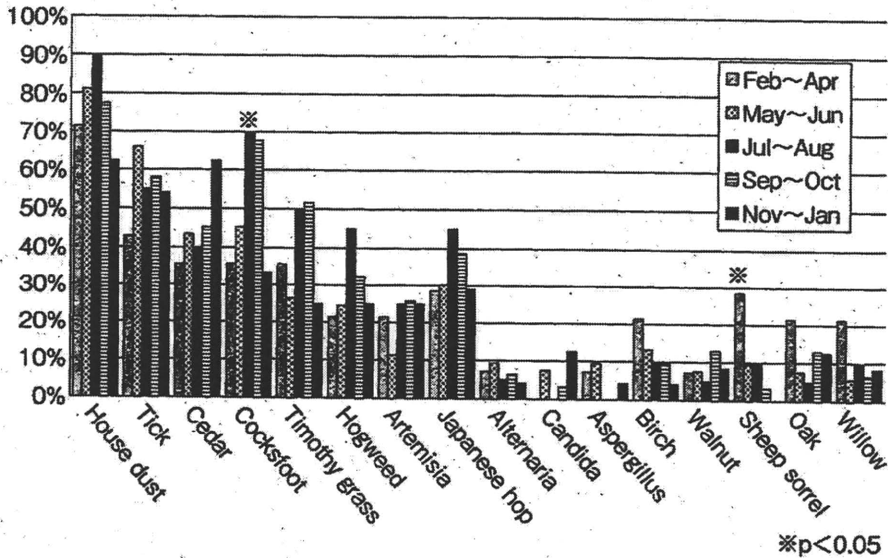


**Fig. 3.** Positive ratio of pollen antigen by consultation timing.

もおこなったが，性別によっては特に差は認められなかった．

年齢別でも検討をおこなった．〜20歳が14例，21〜30歳が93例，31〜40歳が17例，41歳〜が27例であった．カモガヤ，ブタクサ，カナムグラ，ヤナギ陽性率にて年齢間に有意差を認め（p<0.05）ヤナギについては31歳以上で陽性率が高い傾向が認められた（Fig. 2）．

スクラッチテスト施行時期別でも検討を加えた．主にスギ花粉飛散時期である2〜4月，イネ科花粉・シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉飛散時期である5〜6月，7〜8月，キク科花粉飛散時期の9〜10月，11〜1月に分けて検討した．2〜4月は14例，5〜6月は53例，7〜8月は20例，9〜10月は31例，11〜1月は24例であった（施行時期不明者9例を除く）．カモガヤ，ヒメスイバ陽性率にて施行時期間に有意差を認め（p<0.05），ヒメスイバについては2〜4月に検査を施行した群で陽性率が高い傾向が認められた（Fig. 3）．

各々の重複陽性率についても調査を行った．ハウスダスト陽性者，ダニ陽性者における重複陽性率はFig. 1に示す山形市における花粉抗原陽性率とほぼ同様の傾向を示した．

スギ花粉陽性者ではカモガヤ・チモシー花粉への重複陽性率が有意に高く（p<0.05），シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉との重複陽性率はそれぞれ10〜21%であった．

イネ科花粉陽性者における重複陽性率はカモガヤ陽性者ではスギ・チモシー・ブタクサ・カナムグラにおいて有意差を認め（p<0.05），シラカンバ・ヒメスイバが19，14%と高い傾向があった．チモシー花粉陽性者ではスギ・カモガヤ・ヨモギ・クルミ花粉への重複陽性率が有意に高く（p<0.05），シラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギ花粉との重複陽性率も15〜22%と高い傾向を認めた（data not shown）．

イネ科花粉陰性者における各種花粉抗原陽性率についても調査した．特筆して高い陽性率は認められず，今回新たに調査したシラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギについても2〜9%とFig. 1で示した山形市における花粉抗原陽性率と比較しても同様かやや低い陽性率であった（data not shown）．

シラカンバ陽性者における重複陽性率はスギ・カモガヤ・チモシー・ブタクサ・クルミ・ヒメスイバ・コナラ・ヤナギで有意差を認めた（p<0.05）（Fig. 4）．

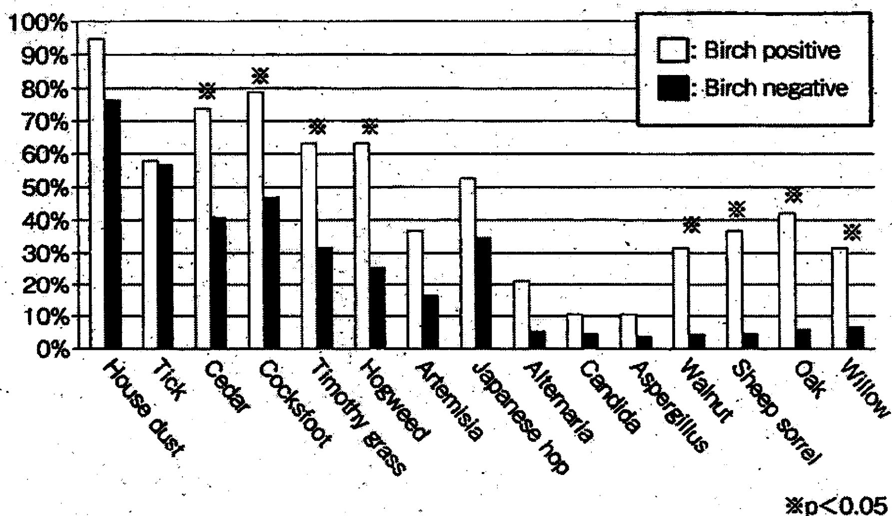


Fig. 4. Overlapping positive ratio with Birch.

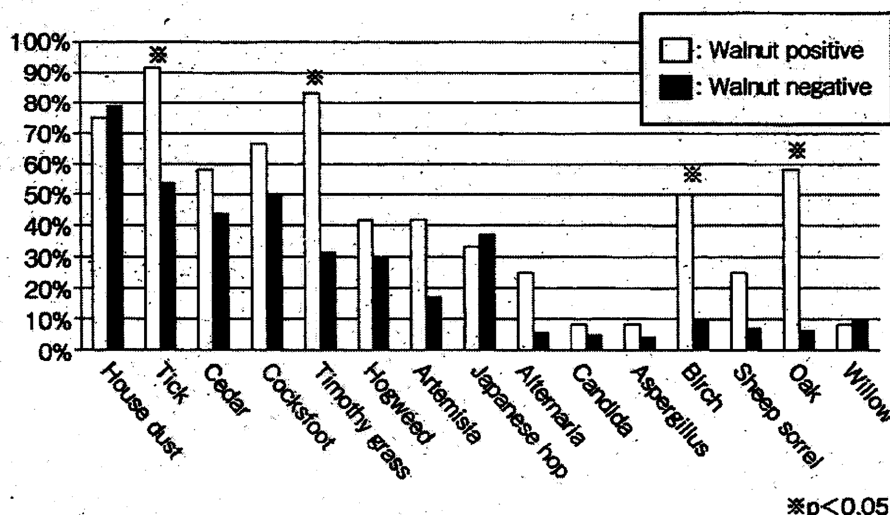


Fig. 5. Overlapping positive ratio with Walnut.

クルミ陽性者における重複陽性率はダニ・チモシー・シラカンバ・コナラで有意差を認めた ($p<0.05$) (Fig. 5).

ヒメスイバ陽性者における重複陽性率はスギ・カモガヤ・チモシー・ブタクサ・ヨモギ・カナムグラ・シラカンバ・コナラ・ヤナギで有意差を認めた ($p<0.05$) (Fig. 6).

コナラ陽性者における重複陽性率はチモシー・シラカンバ・クルミ・ヒメスイバで有意差を認めた ($p<0.05$) (Fig. 7).

ヤナギ陽性者における重複陽性率はチモシー・カナムグラ・シラカンバ・ヒメスイバで有意差を認めた ($p<0.05$) (Fig. 8).

## 4. 考 察

花粉抗原については全国的にスギが最も重要な花粉抗原であり，次いでイネ科のカモガヤ・チモシー，さらにキク科のブタクサ・ヨモギであると

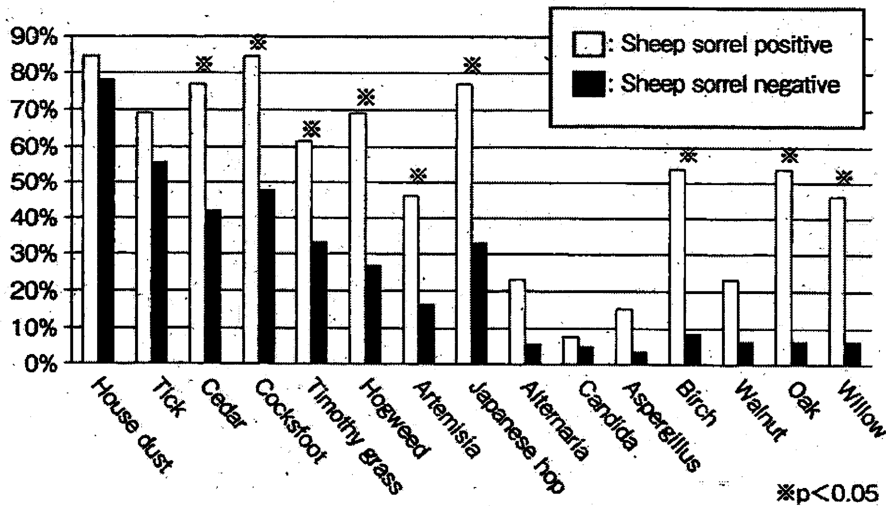


Fig. 6. Overlapping positive ratio with Sheep sorrel.

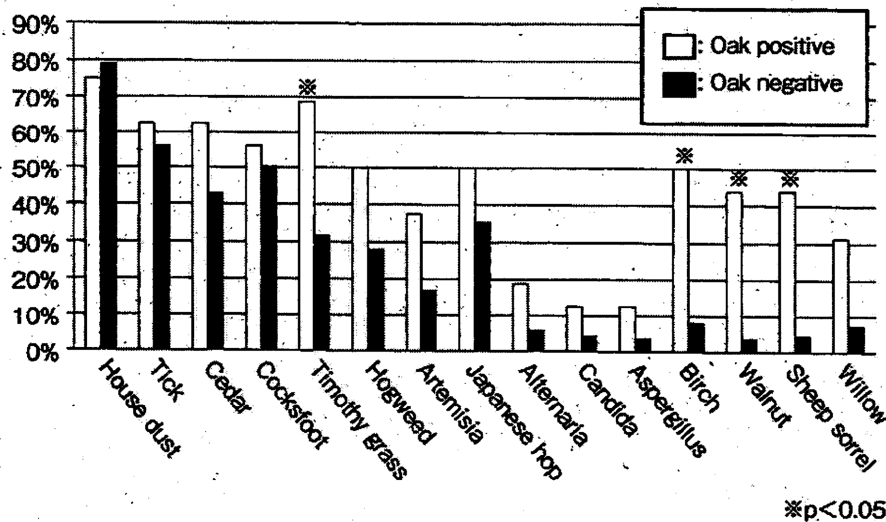


Fig. 7. Overlapping positive ratio with Oak.

する報告が多い[4,5]．今回，花粉アレルギーの抗原陽性率をスクラッチテストを用いて検討した結果，以前の報告と同様にスギ花粉よりもイネ科のカモガヤ花粉の陽性率が高いという傾向が認められ，イネ科花粉も重要な抗原であると考えられた[2]．また，チモシー，ブタクサ，ヨモギ，カナムグラの陽性率も相当数認め，以前の報告と同様の傾向を認めた．今回新たに調査項目に加えたシラカンバ・クルミ・ヒメスイバ・コナラ・ヤナギの陽性率は従来ほとんど検討されておらず，当科を受診された限られた症例数ではあるが，その陽性率はスギ，イネ科，キク科花粉と比較すると低いものの真菌よりも高く，注目すべき抗原と考えられた．

シラカンバ花粉症は1972年[6]我妻らによって本邦ではじめて報告された．北海道におけるシラ