

Evidence of Hop Japanese pollinosis in Korea: IgE sensitization and identification of allergenic components

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Background: Hop Japanese (Hop J) pollens are abundant in the air of Korea during the autumn season. Their significance as a source of allergic sensitization is still unclear. **Objectives:** We sought to detect the sensitization rate to Hop J pollen as an inhalant allergen and to identify its allergenic components.

Method: We carried out skin prick tests with Hop J pollen extract in 1287 patients with respiratory allergy who visited our hospital over the course of 1 year. The serum-specific IgE antibody to Hop J pollen antigen was detected by ELISA, and its binding specificity was confirmed by the ELISA inhibition test. To confirm the respiratory sensitization, bronchoprovocation tests were performed in 16 asthmatic patients sensitive to this pollen. To characterize allergenic components, Hop J pollen extract was analyzed by means of sodium dodecylsulfate-polyacrylamide gel electrophoresis followed by IgE immunoblotting.

Results: A positive result on the skin prick test ($\geq 2+$ of the antigen to histamine ratio) was noted in 79 (6.1%) patients. The serum-specific IgE antibody was detected in 18 (41.9%) patients among 43 positive reactors tested. The ELISA inhibition test with the addition of Hop J pollen extract showed a dose-dependent response. Minimal inhibitions were noted with addition of ragweed and mugwort pollen extracts. Nine asthmatic patients showed significant bronchoconstriction after inhalation of the Hop J pollen extract (five early and four dual asthmatic responders), and all of them had high specific IgE binding. Immunoblot analysis revealed 12 IgE-binding components ranging from 13 to 89 kd. Three bands (13 kd, 74 kd, and 80 kd) were bound to the IgE among the sera tested from more than 50% of the patients.

Conclusion: We believe that the Hop J pollen should be considered as a relevant allergen during the autumn season and thus included in skin test batteries in Korea. Some patients diagnosed as having "intrinsic" asthma or rhinitis might be sensitized to this pollen and other previously unknown allergens. (*J Allergy Clin Immunol* 1997;100:475-9.)

Key words: Hop Japanese pollen, asthma, specific IgE, immunoblotting

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Supported by Hallym Academy of Sciences, Hallym University (1997). Received for publication Dec. 31, 1996; revised June 6, 1997; accepted for publication June 9, 1997.

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0091-6749/97 \$5.00 + 0 1/1/84001

Abbreviations used

A/H:	Antigen/histamine
Hop J:	Hop Japanese
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SPT:	Skin prick test
TBS:	Tris-buffered saline

Hop J (*Humulus Japonicus*), a weed of the Cannabaceae family and Urticales order, which grows 60 to 80 cm high (Fig. 1, *A* and *B*), is widespread in both rural and urban areas of Korea.¹ It is wind-pollinated, and the flowering period lasts from August to October in our region.² Kim et al.³ reported two cases of asthma showing positive responses to skin prick tests (SPTs) and bronchial challenges with this pollen. To the best of our knowledge, there have been very few reports dealing with Hop J pollinosis.

To evaluate the clinical significance of the Hop J pollen as a respiratory allergen in Korea, a prospective study was undertaken to assess the sensitization rate to this pollen in our area and to investigate its allergenic components.

METHODS

Patients

Patients were selected from the Allergy Clinic of Ajou University Hospital in Suwon, Korea (40 km south of Seoul). Between June 1995 and May 1996, 1287 patients admitted for treatment of asthma, rhinitis, or conjunctivitis were skin tested with Hop J pollen extract (Torii Co., Japan). Routine SPTs were performed on the backs of the patients with 50 common inhalant allergens and 20 food allergens used simultaneously. The results were read 15 minutes later. The size of the wheal produced by each antigen and histamine test was expressed as the maximum diameter and vertical length at the midportion of the maximal length. Skin reactivity was expressed as the ratio of wheal size of allergen to histamine according to the criteria of the Scandinavian Society of Allergy.⁴ If the antigen to histamine (A/H) ratio was from 0.1 to 1 and the erythema had a diameter of less than 21 mm, it was read as 1+. If the A/H ratio was from 0.1 to 1, but the erythema was greater than 21 mm, it was read as 2+. If the A/H ratio was from 1 to 2, it was read as 3+. If the A/H ratio was from 2 to 3, it was read as 4+. If the A/H ratio was greater than 3, it was read as 5+. A positive responder was

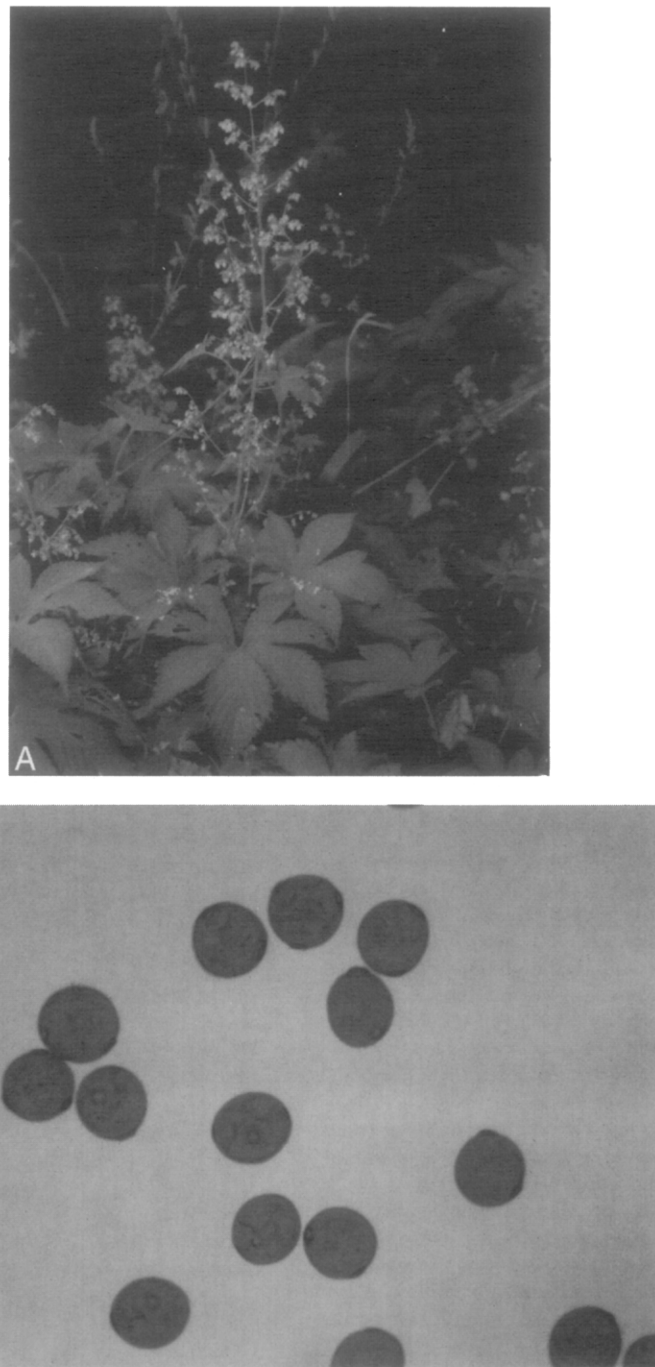


FIG. 1. Details of flowering Hop J weed (A) and its pollen (B, magnification $\times 400$). Pollen grains have smooth-surfaced walls and three germinal pores.

defined as a responder who demonstrated a response greater than 2+ on the SPT.

Preparation of Hop J pollen extract

The pollens were collected in the Suwon area in September of 1995 and 1996. Defatted pollen was extracted by magnetic stirring (24 hours at 4° C) in phosphate-buffered saline (PBS) pH 7.5, 1:5 wt/vol at 4° C for 1 hour followed by centrifugation

at 5000 rpm. The supernatant was used for the bronchoprovocation test at a concentration of 1:10 wt/vol. Some of the supernatant was dialyzed (the cutoff molecular weight was 6000 d) against 4 L of distilled water at 4° C for 48 hours and lyophilized at -70° C for the preparation of antigens used in the ELISA, the ELISA inhibition assay, and immunoblotting. Lyophilized Hop J pollen extract was determined to be 100% protein by the Bradford methods.⁵

TABLE 1. Clinical characteristics of Hop J-sensitive asthmatic patients

Patient	Sex	Age (yr)	Diagnosis	Skin reactivity	BPT response	Specific IgE (absorbance value)
				A/H ratio		
MY	F	26	BA	5	Early	0.22
YK	F	27	BA+AR	5	Early	1.88
KS*	F	35	BA	5	Early	1.98
LT	F	29	BA+AR+AC	5	Dual	0.88
CH	M	35	BA+AR+AC	5	Dual	1.95
JM	F	32	BA+AR	5	Early	1.82
KY*	F	35	BA+AR+AC	5	Dual	1.68
JK*	F	34	BA+AR+AC	4	Dual	1.60
KK*	M	49	BA+AC+BA	5	Early	0.25

Skin reactivity was presented as A/H ratio. Specific IgE level was determined by ELISA.

*Patients sensitized to only Hop J pollen.

BA, Bronchial asthma; AR, allergic rhinitis; AC, allergic conjunctivitis; BPT, bronchoprovocation test; ±, positive (absorbance ≥ cutoff value).

Bronchoprovocation test with Hop J pollen extract

Airway responsiveness to methacholine was tested according to the method described previously with some modifications.⁶ A specific bronchoprovocation test was performed according to the previous method in asthma studies.^{7,8} Normal saline solution was administered from a nebulizer 646 connected to a dosimeter (DeVilbiss Co.) operated at a pressure of 20 psi. The subject was asked to breathe the aerosol five times to vital capacity and was then observed for 7 hours. On a different day, Hop J pollen extracts were inhaled from 1:1000 wt/vol to 1:10 wt/vol. The pulmonary function tests, including functional vital capacity, FEV₁, and forced expiratory flow, mid-expiratory phase were measured with a spirometer (Multi SPLRO, USA) before and 10 minutes after each inhalation. Then, the FEV₁ and the forced expiratory flow, mid-expiratory phase were measured every 10 minutes during the first hour, and pulmonary function tests were performed every hour for 7 hours after the challenge. A positive reaction was defined when the FEV₁ decreased more than 20% from the baseline value.

ELISA for specific IgE antibodies to Hop J pollen extracts

The presence of specific IgE antibodies to Hop J extract was determined by ELISA. Microtiter plates (Dynatech) were first coated with 50 µl of Hop J (5 µg/well) extract and left at 4°C overnight. Each well was washed three times with 0.05% Tween PBS (PBS-Tween), and the remaining binding sites were blocked by incubation with 250 µl of 10% newborn calf serum for 1 hour at room temperature. The wells were then incubated for 2 hours at room temperature with 50 µl of either the patients' sera (undiluted) or control sera from 30 patients who showed negative SPT responses to common inhaled allergens and the Hop J pollen. After washing three times with PBS-Tween, 50 µl of 1:1000 vol/vol biotin-labeled goat anti-human IgE antibody (Sigma Co.) was added to the wells and incubated for 2 hours at room temperature. The wells were then washed three times with PBS-Tween and incubated with 1:1000 vol/vol streptavidin-peroxidase (Sigma Co.) for 30 minutes before another washing step, which was followed by incubation with 50 µl of 2,2'-azino-bis-3-ethylbenzthiazoline-sulfonic acid in a citrate phosphate buffer for 10 minutes at room temperature. The reaction was stopped by the addition of 50 µl of 2N sodium azide, and the absorbance was read at 410 nm by an automated

microplate reader. All assays were performed in triplicate. The cutoff value (0.06) of the positive IgE binding was determined from the mean + 3 standard deviations of absorbance value from the 30 control patients.

ELISA inhibition tests with mugwort, ragweed, and Hop J pollen extracts

Competitive ELISA inhibition was performed to determine the specificity of the IgE binding to the Hop J pollen antigens according to the previously described method.^{7,8} The sera of 10 patients who had high specific IgE binding were pooled. Of this pooled sera 50 µl was incubated with four concentrations (0.02 to 20 µg/ml protein concentration) of ragweed and mugwort extracts (Pharmacia, Uppsala, Sweden), as well as Hop J antigen, for 1 hour at room temperature. The serum pool was then incubated with the Hop J pollen-coated microtiter plate for 2 hours, and the serum steps of the experiments were followed as in the ELISA. After studying the control samples in which equal volumes of PBS, pH 7.5 were preincubated instead of inhibitors, the inhibition of the specific IgE binding was expressed as follows:

$$100 - \frac{\text{Absorbance of samples preincubated with antigens}}{\text{Absorbance of samples preincubated with PBS}} \times 100 (\%)$$

SDS-PAGE and immunoblot analysis

Hop J pollen extracts (10 mg/ml) were dissolved in a sample buffer (0.5 mol/L Tris-HCl, pH 6.8, 2.5 ml, glycerol 2.0 ml, 10% wt/vol SDS 4.0 ml, 0.1% bromophenol blue 0.5 ml, and distilled water 1.0 ml) and boiled. Ten microliters of standard markers (6 to 200 kd) (Novex, San Diego, Calif.) and antigen solutions were applied to a Novex precast Tris-glycine homogenous gel (14% acrylamide) to facilitate the separation of Hop J. Electrophoresis was performed with a Novex Mini-cell for 90 minutes at 125 constant voltage. The gel was fixed and stained with Coomassie Brilliant Blue (Novex, San Diego, Calif.). For immunoblotting the gel was soaked in a transfer buffer (12 mmol/L Tris, 96 mmol/L glycine in 20% methanol) for 10 minutes. Electroblothing was carried out for 90 minutes at 200 mA with a Novex western transfer apparatus. The blotted polyvinylidene difluoride membrane (Novex, San Diego, Calif.) was then treated with a 0.5% bovine serum albumin-Tris-buffered saline solution

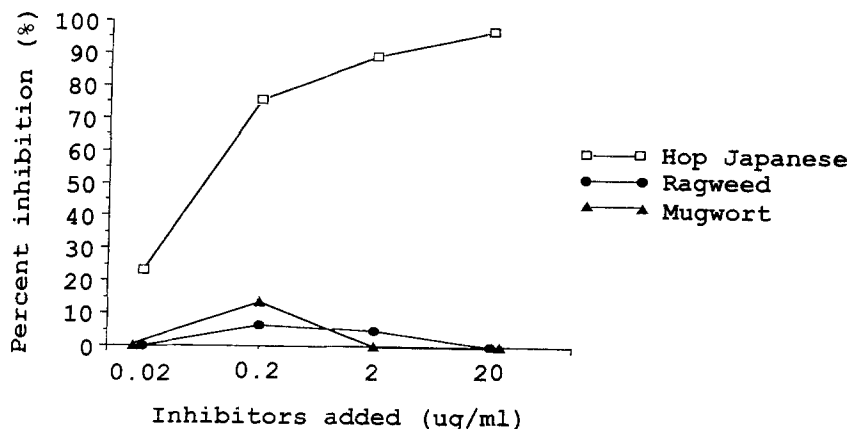


FIG. 2. Percent inhibition of Hop J ELISA with serial addition of Hop J, ragweed, and mugwort antigens.

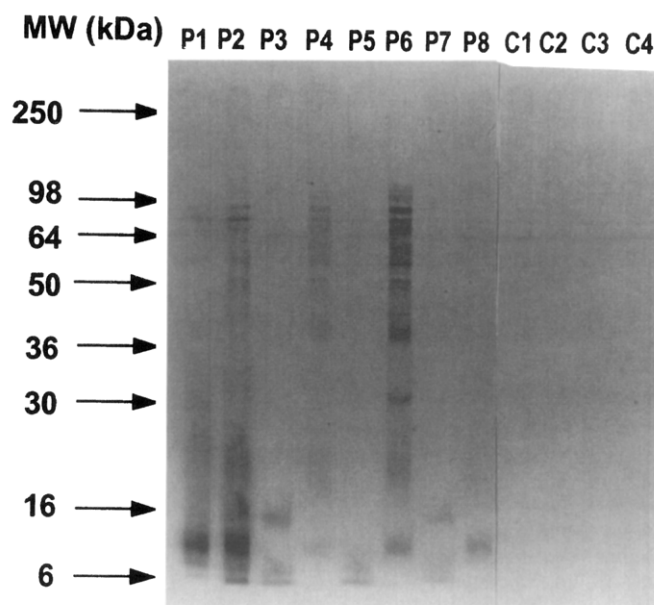


FIG. 3. Immunoblot analysis of Hop J pollen extract by using sera from four control samples (C1-C4) and eight patients who had high specific IgE binding (P1-P8). MW, molecular weight.

for 1 hour to block nonspecific protein binding. The patients' and control sera were diluted to 1:2 vol/vol with a Tris-buffered saline (TBS) (Tris 10 mmol/L, NaCl 150 mmol/L, 0.05% Tween-20). The membrane was then incubated with the patients' and control sera for 2 hours at room temperature in a special device (Multiscreen device; Bio-Rad, Hercules, Calif.). It was then washed with TBS with 0.1% Tween-20 (TBS-Tween) for 10 minutes and 0.5% NaCl in TBS-Tween for another 10 minutes. The biotin-conjugated anti-human IgE antibody (Vector) was diluted to 1:200 with TBS-Tween and incubated with the second antibody for 1 hour at room temperature and washed again as described above, and the membrane was incubated with an alkaline phosphatase-conjugated streptavidin (1:1000 vol/vol, Sigma Co.) solution for 1 hour at room temperature. Finally, after new washes, the substrate solution (NBT/BCIP kit, Sigma Co.) was applied to the membrane until positive bands appeared. After the reaction, the membrane was washed with distilled water and observed.

RESULTS

SPT

The results of skin prick testing throughout the year revealed that 79 (6.1%) of 1287 patients showed more than a 2+ A/H ratio to this pollen. Among them, 58 (73.4%) showed more than a 3+ A/H response. When we separated 404 patients with autumn pollinosis, 14.4% had greater than a 2+ A/H response to this pollen.

Clinical summary of patients with positive responses to challenge

Table I shows the clinical characteristics of nine asthmatic patients with a positive response to the bronchoprovocation test with Hop J pollen extract. Five patients had early asthmatic responses, four had dual asthmatic responses, and all had high specific IgE binding on the ELISA. Most subjects ranged in age from 20

to 40 years. Four (44%) were sensitized to only the Hop J pollen on the SPT and had an isolated positive asthmatic response to the Hop J bronchoprovocation test.

Specific IgE to Hop J pollen

When the ELISA was done in sera of 43 positive reactors as determined by SPTs, the prevalence of the specific IgE antibody was 33.3% in 2+ reactors, 27.3% in 3+ reactors, 33.3% in 4+ reactors, and 92.3% in 5+ reactors.

ELISA inhibition test

The dose-response pattern of the inhibitory effects with addition of the Hop J antigens was illustrated in the Hop J ELISA as shown in Fig. 2. No inhibition was noted with mugwort and ragweed pollen extracts.

SDS-PAGE and immunoblot analysis

To determine the protein components of the Hop J pollen, the extract was analyzed by 14% sodium dodecylsulfate-polyacrylamide gel electrophoresis, and resolved into bands ranging from 6 to 148 kd. Fig. 3 shows the binding of the specific IgE in the sera of eight asthmatic patients and in four control sera. There were 12 IgE-binding components with molecular weights ranging from 13 to 89 kd. Three bands (13, 74, and 80 kd) were bound to the IgE from more than 50% of the sera tested.

DISCUSSION

The pollination period of the Hop J plant in our geographic area is long-lasting (from August to October).² The Hop J pollen counts recorded in Seoul during the autumn season have been greater than those of mugwort and ragweed pollens.² Despite its high pollen count it has been underestimated as an allergenic source until now. To our knowledge one prospective study has been performed, resulting in failure to detect significant sensitization.⁹

This study was designed as a prospective trial to detect the sensitization rate to Hop J pollen. A positive SPT response was noted in 79 (6.1%) of the 1287 patients who visited throughout the year, and 14.4% of the patients who visited during the autumn season were sensitized to this pollen. Nine of 16 asthmatic patients tested showed a positive result on bronchial challenge test. Four of them were sensitized to only the Hop J pollen with only negative responses to common inhalant allergens on SPT. These patients had been diagnosed as having "intrinsic" asthma with influenza-like illness in the autumn season.

The prevalence of the specific IgE binding as determined by the ELISA increased according to skin reactivity. When the result of ELISA was compared with skin reactivity, the sensitivity was 54.5% of the reactors with scores of 2+ or greater and 60.7% of the reactors with

scores of 3+ or greater. The specificity was 100% of reactors with scores of 2+ or greater and 97% of reactors with scores of 3+ or greater. In addition, all of the nine asthmatic patients with positive bronchial challenges had high specific IgE binding. Finally, ELISA inhibition results indicate the specificity of the IgE binding in Hop J ELISA, and because cross-reactivity with mugwort and ragweed pollen extracts which pollinate during the same period could be excluded. These results suggested that the SPT and specific IgE determination by ELISA are valuable methods with which to identify patients allergic to Hop J.

The pattern of the IgE-binding components obtained after immunoblot analysis indicates the presence of 12 possible allergenic proteins reacting with the sera of the individuals with respiratory allergy symptoms during the pollination period. Three bands (13, 74, and 80 kd) bound to the IgE antibody from more than 50% of the patients' sera tested.

This study reveals Hop J pollen to be capable of inducing both sensitization and respiratory symptoms in this country. Thus this pollen should be taken into account as a relevant source of respiratory allergic disease in patients with seasonal aggravation in autumn because of its prevalence on the SPTs of 14.4% of patients tested during that season. Some patients diagnosed as having "intrinsic" asthma or rhinitis may, in fact, be sensitized to this allergen, unless it had been included in skin test batteries of common aeroallergens. These findings might encourage the research of hidden allergic sources in the etiology of rhinitis and asthma in this area.

We thank Ms. Jin-Yoo and Mrs. Seo-Young Ji for helping with technical assistance on the SPTs and the ELISA.

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