

IgE immune response to *Ginkgo biloba* pollen

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Background: The ginkgo (*Ginkgo biloba* L.) continues to be planted as a shade tree in preference to other species in Seoul, Korea. The proportion of ginkgo to total shade trees was 43.2% in 1998, but the allergenic characteristics of ginkgo pollen has not been elucidated.

Objectives: This study was undertaken to obtain information regarding the skin reactivity rate to ginkgo pollen in a population of Korean subjects with respiratory allergy. Possible ginkgo pollen allergens and the cross-reactivity of ginkgo pollen with other prevalent pollens were also examined.

Methods: Four hundred and forty-seven patients with asthma and/or allergic rhinitis were skin prick tested with extract of ginkgo pollen (1:20 wt/vol). Of these patients, positive skin responders (A/H ratio $\geq 2+$) were selected for ELISA and immunoblot experiments.

Results: A total of 21 patients (4.7%) showed skin reactivity (A/H ratio $\geq 2+$) to ginkgo pollen in the skin prick test. They were also cosensitized to many other tree, grass, and weed pollens. Sixteen (76%) of the 21 positive skin responders showed specific IgE responses to ginkgo pollen in ELISA. In inhibitory ELISA, IgE binding to ginkgo pollen was inhibited by more than 80% by oak, ryegrass, mugwort, and ragweed; and 34% by hop Japanese; and 10% by rBet v 2 at 10 $\mu\text{g/mL}$. In immunoblot, 10 out of 21 sera (48%) reacted to the 15-kD protein of ginkgo pollen, 9 (43%) to 33–35 kD, and 8 (38%) to 36–38 kD. In inhibitory immunoblot, IgE binding to ginkgo pollen proteins was almost completely inhibited by oak, ryegrass, mugwort and ragweed, but only partially by hop Japanese and rBet v 2.

Conclusion: The skin reactivity rate to ginkgo pollen is approximately 4.7% in a population of Korean subjects with respiratory allergy. Since ginkgo pollen has a high cross-reactivity with other prevalent pollens, it could cause clinical symptoms during its pollen season by cross-reacting with the IgE produced in response to other pollens in patients sensitized to multiple pollens.

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INTRODUCTION

The maidenhair or ginkgo tree (*Ginkgo biloba* L.) is the world's oldest living tree and the sole survivor of the family Ginkgoaceae, order Ginkgoales.¹ The ginkgo was almost destroyed during the Ice Age but survived in China where it was cultivated as a sacred tree. It became known to Western science in the 18th century when the first specimens were planted in Europe. There are separate male and female trees. Ginkgo trees grow well in most temperate regions except for the cold

north.² The ginkgo is among the most resistant of trees to insects, disease, city smoke, and industrial fumes and is frequently planted as a shade tree.¹ The fruit of the ginkgo has been known from ancient times to irritate the skin.³ Contact dermatitis has been reported after ingestion or physical contact with the fruit.^{4–6} The shell and kernel are also known to produce positive patch test reactions.⁷ Extracts from the leaves of the ginkgo have been used therapeutically for centuries because they have an antioxidant effect, an antagonistic effect on platelet activating factor, and a beneficial effect on neurotransmitter disturbances.⁸

The ginkgo pollen, however, has not been a subject of significant scientific interest, and only one report has been

published concerning the allergenicity of ginkgo pollen.⁹ The ginkgo is common in urban and rural areas throughout Korea. It sheds its windborne pollen during late April in Korea. The ginkgo pollen is rice bran shaped with a central longitudinal groove and on average is 36 μm long and 16 μm wide (Fig 1 a and b). The proportion of the ginkgo to total shade trees in Seoul has steadily increased from 13.4% to 43.2% during the past 15 years (26,186 ginkgoes in a total of 195,198 shade trees in 1983, and 111,025 ginkgoes in a total of 257,139 shade trees in 1998). Our interest in ginkgo stems from the facts that its pollen is readily airborne and being dioecious it can be treated as a suspect allergen. This study was undertaken to obtain information regarding the skin reactivity rate to ginkgo pollen in patients with asthma and/or allergic rhinitis and the cross-reactivity of ginkgo pollen with other prevalent tree, grass, and weed pollens.

MATERIALS AND METHODS

Subjects

Of the patients who were skin prick tested at our Allergy Clinic sequentially between May 1997 and July 1998, 447 patients with asthma and/or allergic rhinitis were recruited into this study, mean age 38.3 ± 15.7 years, and age range 10 to 87 years. Their serum samples were collected with informed consent and were stored at -20°C . Of these, sera from positive skin responders to the extract of ginkgo pollen were selected for ELISA and immunoblot experiments. Five sera were collected from non-atopic subjects who were recruited as control subjects for the experiments. The non-atopic subjects were defined as negative responders to a series of 50 common inhalant allergens (Bayer Co, USA) by the skin prick test. Skin reactivity was expressed as the ratio of the wheal sizes of allergen and hista-

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mine according to the criteria of the Scandinavian Society of Allergy.¹⁰ If the antigen to histamine (A/H) ratio lay between 0.1 and 1 and the erythema had a diameter of less than 21 mm, it was interpreted as 1+. If the A/H ratio was in the range of 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 4+, it was read as 5+. A positive responder was defined as one who exhibited a response greater than 2+ in the skin prick test.

Preparation of Pollen Extracts

The ginkgo pollen was collected at our campus in late April. Dry pollen (1 gram) was defatted with ethylether and mixed with 1:20 wt/vol carbonate buffer (0.125 mol/L NH_4HCO_3 , 0.015 mol/L NaN_3 , pH 7.5) at 4°C for 24 hours. The extract was centrifuged at 4000 rpm for 20 minutes and its supernatant was re-centrifuged at 15,000 rpm for 30 minutes. The extract was then dialyzed against distilled water with semipermeable membrane of pore size 3.5 kD (Spectrum, Houston, TX, USA) at 4°C for 48 hours, which was followed by centrifugation at 15,000 rpm for 30 minutes. The supernatant so obtained was lyophilized and used as a source of crude protein.

Oak and hop Japanese pollens were also collected at our campus when in season; in the case of ryegrass, mugwort and ragweed, commercial pollens were used (Allergon, A Pharmacia and Upjohn, Sweden). The extracts of these five pollens were also prepared as described above. Recombinant birch profilin (rBet v 2) was donated by Valenta R.

ELISA for Specific IgE/Inhibitory ELISA

ELISA was performed as described by Crowther.¹¹ The extract of ginkgo pollen was coated overnight at 4°C onto a 96-well polyvinyl microtiter plate (Costar, Cambridge, MA, USA) by the addition of 50 μL of 0.1 mol/L carbonate buffer (pH 9.6) containing 10

$\mu\text{g/mL}$ of the crude protein. After blocking with 1% bovine serum albumin, the antigen-coated immunoplate was incubated for 1 hour at room temperature with 50 μL of undiluted sera from those patients that showed positive skin reactions to the ginkgo pollen extract. After washing with phosphate buffered saline containing 0.1%

Tween-20 (PBST), the immunoplate was incubated with 1:500 biotinylated polyclonal anti-human IgE (Vector, Burlingame, CA, USA) for 1 hour followed by incubation with 1:500 streptavidin-peroxidase (Sigma, St. Louis, MO, USA) for 30 minutes at room temperature. After washing with PBST, 100 μL of ABTS solution [25



Figure 1. *Ginkgo biloba* L. (Upper panel) flower in bud and (Lower panel) ginkgo pollen (by scanning electron microscope, $\times 700$, approximately 36 μm long and 16 μm wide).

For inhibitory ELISA, a pool of sera from 21 skin responders were pre-incubated with oak, ryegrass, mugwort, ragweed, hop Japanese pollens, and recombinant birch profilin overnight at 4°C at several concentrations (10^{-2} , 10^{-1} , 1, 10 $\mu\text{g/mL}$, respectively), before being added to the antigen-coated immunoplate. The remainder of the procedure was performed as described above.

Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed as described by Laemmli.¹² Briefly, 10 μ g of crude protein per lane, or 100 to 150 μ g per gel, were mixed with sample buffer containing 2% sodium dodecylsulfate, 14.4 mmol/L 2-mercaptoethanol, 60 mmol/L Tris-HCl, 25% glycerol, and 0.1% bromphenol blue; boiled for 5 minutes; and electrophoresed (Small mighty, Hoeffer, San Francisco, CA, USA) in 13.5% polyacrylamide separation gel with 4% stacking gel. The separated proteins were stained with Coomassie brilliant blue or transferred (Transpor, Hoeffer, San Francisco, CA, USA) to nitrocellulose membrane (pore size 0.45 μ m, Amersham, Buckinghamshire, UK). Blotted membrane strips were stained using Ponceau's solution or blocked with 5% defatted milk in PBST for 1 hour. Sera diluted 1:4 in PBST were incubated overnight at room temperature with the blotted membrane strips and washed 3 times with PBST. Bound IgE was detected after incubating for 1 hour with goat polyclonal anti-human IgE conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA) diluted 1:2000 in PBST followed by 0.66% 4-nitroblue

For inhibitory immunoblot, a pool of sera from 21 skin responders were pre-incubated with oak, ryegrass, mugwort, ragweed, hop Japanese pollens, and rBet v 2 overnight at 4°C at several concentrations (10^{-1} , 1, 10 $\mu\text{g/mL}$, respectively). The concentrations were then incubated with blotted membrane strips. The remaining steps were performed as described previously.

Of the 447 patients with asthma and/or allergic rhinitis that were skin prick tested with the extract (1:20 wt/vol) of ginkgo pollen and 50 common inhalant allergens (Bayer Co, USA), a total of 21 patients (4.7%) showed skin reactivity to ginkgo pollen. These patients also showed cosensitization to many other tree, grass, and weed pollens including oak (86%), ryegrass (62%), mugwort (62%), ragweed (67%), and hop Japanese (38%).

ELISA assays were performed upon the extract of ginkgo pollen (Fig 2). Sixteen (76%) of 21 skin responders showed ELISA values that exceeded the cut-off level for a positive response (absorbency 0.01) and exhibited no

In inhibitory ELISA, the IgE binding to ginkgo pollen was inhibited dose dependently by the ginkgo pollen itself and other pollens such as oak, ryegrass, mugwort, and ragweed (Fig 3). At 10 $\mu\text{g/mL}$, oak inhibited IgE binding to ginkgo pollen by 86%, ryegrass by 88%, mugwort by 86%, and ragweed by 83%. In addition to these pollens, hop Japanese and recombinant birch profilin (rBet v 2) inhibited the IgE binding to ginkgo pollen by 34% and 10%, respectively, at 10 $\mu\text{g/mL}$.

To identify the allergenic components of ginkgo pollen, IgE immunoblot was performed using the sera of the 21 skin responders (Fig 4). Of these 21 sera, 10 (48%) reacted to the 15-kD protein of ginkgo pollen, 9 (43%) to 33-35 kD, and 8 (38%) to 36-38 kD, respectively. The range of IgE binding to the ginkgo pollen proteins was from 14 to 94 kD, and approximately 15 protein bands exhibited binding to IgE.

Cross-reactive allergens between ginkgo pollen and other prevalent pollens were investigated by inhibitory immunoblot (Fig 5). The extracts of oak, ryegrass, mugwort, and ragweed pollens almost completely inhibited IgE binding to the ginkgo pollen proteins at 10 μ g/mL. On the other hand, hop Japanese pollen extract partially

Figure 2. Ginkgo pollen specific IgE by ELISA according to the results of the skin prick test. No correlation existed between the level of specific IgE for ginkgo pollen and skin reactivity.

inhibited IgE binding to the ginkgo pollen proteins even at 10 $\mu\text{g/mL}$. Recombinant birch profilin (rBet v 2) slightly inhibited IgE binding to the 15-kD protein of ginkgo pollen irrespective of concentration levels.

DISCUSSION

While the leaves and fruit of the ginkgo have been the object of study, ginkgo pollen has not been investigated previously. Because of the widespread practice of planting the ginkgo as a shade tree, ginkgo pollen has significant potential to act on the respiratory tract as an inhalant allergen. To date only one report has been published about the allergenicity of this pollen,⁹ and so far there is no literature on the cross-reactivity of ginkgo pollen with other pollens. Long et al⁹ purified a crude extract of ginkgo pollen by gel filtration with Sephadex G25, then G75 and observed two elution peaks. On SDS-PAGE, the molecular weights of the first peak and the valley were 30 to 42kD and 13 to 18kD, respectively, and the molecular weight of the second peak was less than 13kD. Using several fractions from gel filtration, the skin prick test was performed on 40 asthma and/or allergic rhinitis patients. These patients had the greatest skin reaction to the first peak, whereas the second peak produced only a slight reaction. This result was further corroborated by inhibitory ELISA experiment.

In our study, the skin reactivity rate to ginkgo pollen for Korean respiratory allergic patients was approximately 4.7%, and those patients who were sensitized to ginkgo pollen were also displayed cosensitization to many other tree, grass, and weed pollens. According to our previously published findings on sensitization rates to prevalent pollens by skin prick test among Korean respiratory allergic patients,¹³ 14.2% (45/317) of patients showed positive responses to sagebrush (*Artemisia vulgaris*), 10.4% to ragweed (*Ambrosia elatior*), 5.0% to grass (*Zoysia japonica*), 4.1% to oak (*Quercus alba*), 3.8% to alder (*Alnus rhombifolia*), 1.9% to poplar (*Populus tri-*

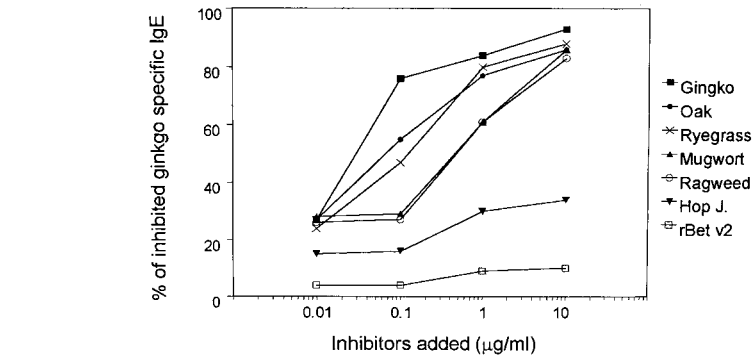


Figure 3. Inhibitory ELISA. Oak inhibited IgE binding to the ginkgo pollen proteins by 86%, ryegrass by 88%, mugwort by 86%, ragweed by 83%, hop Japanese by 34%, and rBet v 2 by 10% at 10 $\mu\text{g/mL}$.

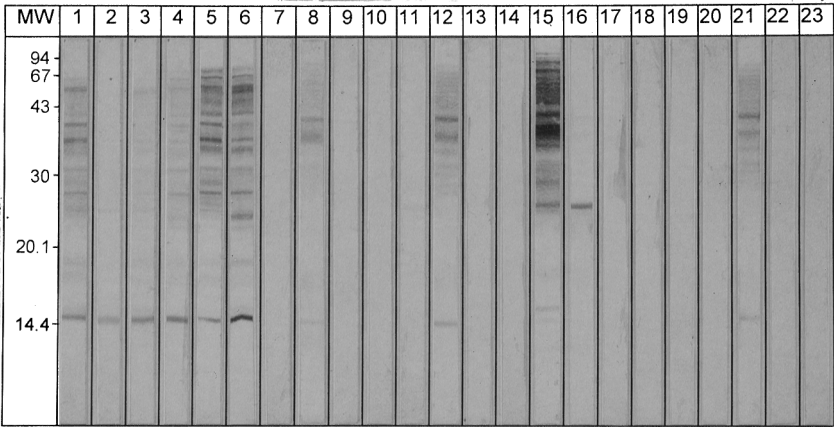


Figure 4. IgE immunoblot. Lanes 1 through 21: sera from positive skin responders to the extract of ginkgo pollen, lane 22: a pool of five sera from non-atopic control subjects, and lane 23; buffer control.

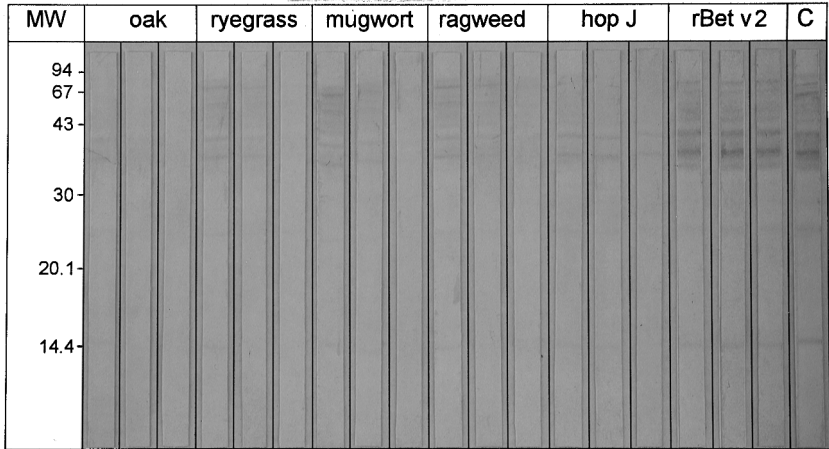


Figure 5. Inhibitory immunoblot. A pool of 21 sera from positive skin responders to the extract of ginkgo pollen was pre-incubated with oak, ryegrass, mugwort, ragweed, hop Japanese pollens, and recombinant birch profilin (rBet v 2) at several concentrations (in the order 0.1, 1, and 10 $\mu\text{g/mL}$, C: buffer control).

chocarpa), and 0% to pine (a mixture of *Pinus contorta*, *Pinus ponderosa*, and *Pinus strobus*). At that time (1987), the shade trees in Seoul were composed of *Platanus* (49.3%), ginkgo (25.6%), and weeping willow (7.9%) in a total of 235,391 shade trees. Since that time, the ginkgo has steadily been planted as a shade tree in preference to *Platanus*, and now represents 43.2% of the shade trees in Seoul (111,025 ginkgoes in a total of 257,139 shade trees in 1998, data presented by the Landscape Division, Urban Planning Bureau of the Seoul Metropolitan Government). If Korean patients with respiratory allergy are further exposed to ginkgo pollen, it is expected that the skin reactivity rate will increase further and it is believed that a follow-up study focusing on this increased reactivity will provide more evidence of its allergenicity.

The peak daily ginkgo pollen count has been reported by the Korean Pediatric Society¹⁴ to be 9 grains/m³/day and the mean total annual count of 122 grains/m³ was reported during April and May. These levels are low as compared with alder (49 grains/m³/day, 900 grains/m³), birch (39 grains/m³/day, 1036 grains/m³), juniper (71 grains/m³/day, 802 grains/m³), poplar (14 grains/m³/day, 569 grains/m³), and hazelnut (27 grains/m³/day, 390 grains/m³) during March, and oak (114 grains/m³/day, 1285 grains/m³), maple (52 grains/m³/day, 1014 grains/m³), elm (18 grains/m³/day, 466 grains/m³), willow (15 grains/m³/day, 304 grains/m³), and sycamore (12 grains/m³/day, 228 grains/m³) during April and May in Seoul, Korea.

Specific IgE antibodies to ginkgo pollen were present in 76% of positive skin responders. In an immunoblot experiment, it was difficult to discriminate between the possible ginkgo pollen allergens among the multiple IgE binding components and further confirmatory data are needed. In inhibitory ELISA and inhibitory immunoblot

experiments, ginkgo pollen highly cross-reacted with other prevalent pollens such as oak, ryegrass, mugwort, and ragweed, but which components are responsible for cross-reactivity is not certain. Profilin is a well-known allergenic component that induces cross-reactivity,¹⁵⁻¹⁷ but in our inhibitory immunoblot experiment, recombinant birch profilin could not totally inhibit the IgE binding to the 15-kD protein of ginkgo pollen. Based upon this finding, we are not sure whether the 15-kD protein of ginkgo pollen is a profilin.

In conclusion, on the basis of its rather low skin reactivity (4.7%), ginkgo pollen does not seem to act as a sole potent allergen. Because ginkgo pollen has high cross-reactivity with other prevalent pollens, it could cause clinical symptoms during its pollen season by cross-reacting with the IgE produced in response to other pollens in sensitized patients to multiple pollens.

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