

A new method of counting airborne Japanese cedar (*Cryptomeria japonica*) pollen allergens by immunoblotting

Takahashi Y, Nagoya T, Watanabe M, Inouye S, Sakaguchi M, Katagiri S. A new method of counting airborne Japanese cedar (*Cryptomeria japonica*) pollen allergens by immunoblotting. Allergy 1993; 48: 94–98. © Munksgaard 1993.

We have devised a new method of counting pollen allergen particles, modified from the fluorescent immunoblotting technique of Schumacher et al. Airborne Japanese cedar pollen allergens collected on Burkard's sampling tape were transferred onto a nitrocellulose membrane. The membrane was then treated with antiallergen mouse monoclonal antibody conjugated with alkaline phosphatase. Pollen allergens were detected as purple spots on the nitrocellulose membrane after phosphate substrate staining was performed. Pollen allergen particles were visible under a stereoscopic microscope or to the naked eye and could thus be counted easily. This new counting method takes less time than previous methods and requires no special skill.

Y. Takahashi¹, T. Nagoya²,
M. Watanabe², S. Inouye³,
M. Sakaguchi³, S. Katagiri¹

¹ Yamagata Prefectural Institute of Public Health, Yamagata, ² Kowa Research Institute, Kowa Co., Ltd, Tsukuba, and ³ Department of Microbiology, Institute of Public Health, Tokyo, Japan

Key words: airborne pollen; *Cry j* I; immunoblotting; pollen allergen.

Dr Yuichi Takahashi
Yamagata Prefectural Institute of Public Health
1–6–6, Tohkamachi, Yamagata City
Yamagata 990
Japan

Accepted for publication 19 August 1992

The conventional method of counting airborne pollen involves morphologic observation by light microscope after pollen staining (3). This method, however, requires skill and time, and, furthermore, when this method is used, it is rather difficult to discriminate between morphologically similar kinds of pollen that are dispersed during the same season.

The essential information for pollinosis patients is not the amount of airborne pollen, but the amount of allergens. Schumacher et al. (4) have reported a new method for counting pollen allergens, by which they counted Bermuda grass pollen grains after transferring the airborne pollen allergens onto a nitrocellulose membrane; the allergens were then stained by an indirect immunofluorescent technique, which used anti-Bermuda grass pollen rabbit serum. This method, however, is not particularly efficient, since it takes a long time to count the pollen grain spots, and, since this must be done under a fluorescence microscope in a dark room, it is very difficult.

In this study, we prepared enzyme-conjugated monoclonal antibodies against the major pollen allergen of Japanese cedar (*Cryptomeria japonica*), *Cry j* I; these antibodies were used to treat a nitrocellulose membrane onto which pollen allergens had been transferred. After staining the allergens, we counted the allergen particles visible to the naked eye or visible under a stereoscopic microscope.

Material and methods

Sampling airborne Japanese cedar pollen

Pollen sampling was performed with Burkard air samplers (Burkard Manufacturing Ltd, Rickmansworth, Hertfordshire, UK). The samplers were placed on the flat roof of a five-story building 20 m above ground level in the central part of Yamagata city during the Japanese cedar pollen season, from March to April, in 1991.

Production of monoclonal antibodies

Cry j I for immunization was purified from the pollen of *C. japonica* according to the method of Yasueda et al. (1).

Eight-week-old male BALB/C mice were given subcutaneous injections of 0.2 mg purified *Cry j* I plus Freund's complete adjuvant; they subsequently received subcutaneous booster injections of *Cry j* I (0.2 mg) plus Freund's incomplete adjuvant, twice, at 2-week intervals. Two weeks after the last injection, *Cry j* I was injected intravenously without Freund's adjuvant. Three days after this intravenous injection, spleen cells from these mice were fused with myeloma cells (P3-Ag8-γ), by means of an electrocell fusion apparatus (SSH-1, Shimadzu, Japan). The cloning of hybridoma cells was done by means

of "limiting dilution". Monoclonal antibodies were purified from the ascites of mice injected intraperitoneally with hybridoma cells. Purified antibodies were conjugated with alkaline phosphatase, as described by Harlow (2).

Immunoblotting

After sampling was completed, the Burkard tapes were cut into 24-h segments. These segments were placed in contact with nitrocellulose membranes (20 × 55 mm; Bio-Rad Laboratory, CA, USA), which were treated with 0.05 mol TRIS-0.04 mol glycine buffer (pH 7.6) containing 20% methanol for 1 h, to transfer the pollen allergens on the tape segment onto the membrane.

The membranes were removed and placed in 0.05 mol TRIS-buffered saline (TBS; pH 7.8 con-

taining 4% bovine serum albumin) for 1 h at 37°C. The membranes were then rinsed with TBS containing 0.25% Tween 20, and were treated with anti-*Cry j* I monoclonal antibody conjugated with alkaline phosphatase (1:500, diluted with TBS) at 25°C for 1 h (direct immunoblotting method). After the membranes were washed with a large volume of TBS containing 0.25% Tween 20, they were treated with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium), a phosphatase substrate kit (Kirkegaard & Perry Lab., Inc., Gaithersburg, MD, USA), after which the allergens were seen as purple spots.

To study cross-reactivity among different pollens, we sprinkled a number of different pollens onto a Burkard tape stained by the direct immunoblotting method outlined above. For indirect immunoblotting, anti-*Cry j* I mouse serum or normal mouse (spe-

Table 1. Cross-reactivity among 34 kinds of pollen and three anti-*Cry j* I antibodies (two monoclonal antibodies and one polyclonal antibody) determined by an immunoblotting technique

Family	Species	(Common name)	Antibody			Nonimmunized mouse serum
			Monoclonal		Polyclonal anti-Cry j I	
			KW-S91	KW-S10		
Pinaceae	<i>Picea pungens</i>	(Blue spruce)	+	-	+	-
Taxodiaceae	<i>Cryptomeria japonica</i>	(Japanese cedar)	++	++	++	-
	<i>Taxodium distichum</i>	(Bald cypress)	++	-	+	-
	<i>Sequoia</i> spp.	(Redwood)	++	-	+	-
	<i>Sequoia sempervirens</i>	(Sequoia)	++	-	+	-
Cupressaceae	<i>Metasequoia glyptostroboides</i>	(Metasequoia)	++	-	+	-
	<i>Chamaecyparis obtusa</i>	(False cypress)	++	-	+	-
	<i>Chamaecyparis pisifera</i>	(Sawara cypress)	++	-	+	-
	<i>Biota orientalis</i>	(Oriental arborvitae)	++	-	+	-
	<i>Thujopsis dolabrata</i>	(Hiba arborvitae)	++	-	+	-
	<i>Cupressus arizonica</i>	(Arizona cypress)	+	-	+	-
	<i>Cupressus macrocarpa</i>	(Monterey cypress)	+	-	+	-
	<i>Juniperus</i> spp.	(Cedar)	++	-	+	-
	<i>Juniperus rigida</i>	(Needle juniper)	+	-	+	-
	<i>Juniperus virginiana</i>	(Red cedar)	++	-	++	-
	<i>Juniperus sabinoides</i>	(Mountain cedar)	+	-	+	-
	<i>Juniperus occidentalis</i>	(Western juniper)	+	-	+	-
Cephalotaxaceae	<i>Cephalotaxus drupacea</i>	(Torreyia)	+	-	+	-
Taxaceae	<i>Taxus cuspidata</i>	(Yew)	±	-	±	-
Salicaceae	<i>Popula</i> spp.	(Poplar)	+	-	+	-
	<i>Salix</i> spp.	(Willow)	±	-	±	-
Juglandaceae	<i>Juglans regia</i>	(English walnut)	-	-	-	-
Betulaceae	<i>Corylus americana</i>	(Hazelnut)	-	-	-	-
	<i>Betula</i> spp.	(Birch)	±	-	-	-
	<i>Alnus</i> spp.	(Alder)	-	-	-	-
Fagaceae	<i>Fagus grandifolia</i>	(American beech)	-	-	-	-
	<i>Quercus</i> spp.	(Oak)	-	-	±	±
Moraceae	<i>Humulus japonicus</i>	(Japanese hops)	-	-	-	-
Rosaceae	<i>Prunus sargentii</i>	(Wild cherry)	-	-	+	+
Compositae	<i>Artemisia vulgaris</i>	(Mugwort)	±	-	+	+
	<i>Ambrosia artemisiifolia</i>	(Ragweed)	-	-	+	+
Typhaceae	<i>Typha orientalis</i>	(Bulrush)	-	-	-	-
Gramineae	<i>Dactylis glomerata</i>	(Orchard grass)	-	-	-	±
Cyperaceae	<i>Carex dispalata</i>	(Sedge)	-	-	-	-

cific pathogen-free) serum (1:1000) was used in the first reaction, and antimouse IgG conjugated with alkaline phosphatase (Tago, 9550), at 1:3000 dilution, was used in the second reaction.

Reference pollens

Regarding the reference pollens (Table 1), some were purchased from International Biological, Inc., Piedmont, OK, USA, some were donated by T. Oka, Tamano City Municipal Hospital, Okayama, Japan, and some we collected ourselves.

Results

Specificity of monoclonal antibodies

Ten kinds of anti-*Cry j* I monoclonal antibodies were obtained, and we examined the cross-reactivity of the monoclonal antibodies to 25 different kinds of pollen by an ELISA method. Five monoclonal antibodies reacted only with Japanese cedar pollen. KW-S10, one of 10 kinds of anti-*Cry j* I antibody, showed the strongest reactivity to Japanese cedar. Five other kinds of anti-*Cry j* I antibody broadly cross-reacted with pollens of the Taxodiaceae and Cupressaceae families. One of these antibodies, KW-S91, was most broadly cross-reactive with pollens of these families.

We then studied the specificity of the antibodies by an immunoblotting procedure. Thirty-four kinds of pollen reacted with two kinds of anti-*Cry j* I monoclonal antibodies, KW-S91 and KW-S10, and with anti-*Cry j* I mouse polyclonal antibody (Table 1). When the KW-S10 antibody was used, strong purple spots were formed only with Japanese cedar pollen. The KW-S91 antibody, on the other hand, formed purple spots with all the Pinophyta (Gymnospermae) pollens tested and with some kinds of Magnoliophyta (Angiospermae) pollens, such as poplar, willow (Salicaceae), and birch. Similar reactivity was observed with rabbit anti-*Cry j* I polyclonal antibody.

Detection of airborne pollen allergens on nitrocellulose membrane by immunoblotting

One example of the hourly variation in *Cry j* I spots stained with KW-S91 monoclonal antibody is shown in Fig. 1. With the naked eye, we clearly observed a large number of pollen grains that were sampled between 1200 and 1800 on 30 March 1991, and a smaller number that were sampled between 0500 and 0700 on the following day. Fig. 2 shows the daily counts of *Cry j* I spots stained with KW-S91 or KW-S10, and the morphologic Japanese cedar pollen counts. The number of KW-S91 spots was greater than the pollen count ($t = 3.18$; $P < 0.05$).

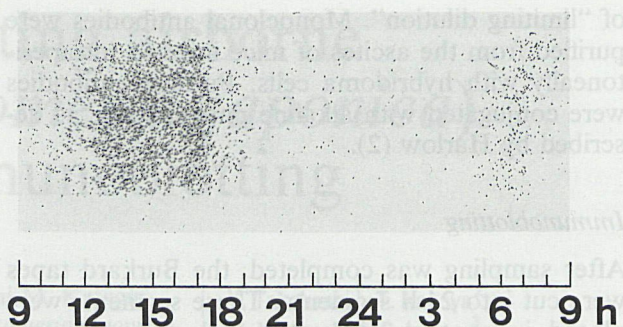


Fig. 1. *Cry j* I spots (KW-S91) obtained on 30 March 1991.

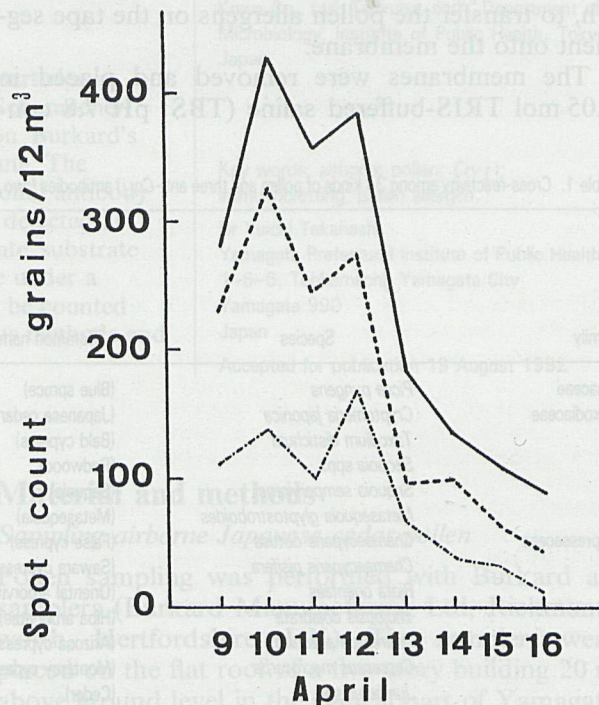


Fig. 2. Numbers of daily *Cry j* I spots (KW-S91 or KW-S10) and Japanese cedar pollen count. — *Cry j* I spot stained with KW-S91 monoclonal antibody; ---- *Cry j* I spot stained with KW-S10 monoclonal antibody; Japanese cedar pollen count. Spot count was done under light microscope. Classes I–III, but not Class IV, may be counted in this experimental setup. Classification of spots is shown in Fig. 3.

Microscopic examination of samples after immunoblotting

We then examined the stained spots in more detail under a light microscope. The size of the spots varied considerably, as shown in Fig. 3. We classified the spots into four classes, according to size. Class I spots were 60–90 μm in diameter and were visible to the naked eye. When the spot size was as large as Class I but had a weaker color, we classified it as Class II. Class III and Class IV spots were smaller than pollen grains, and were not visible to the naked

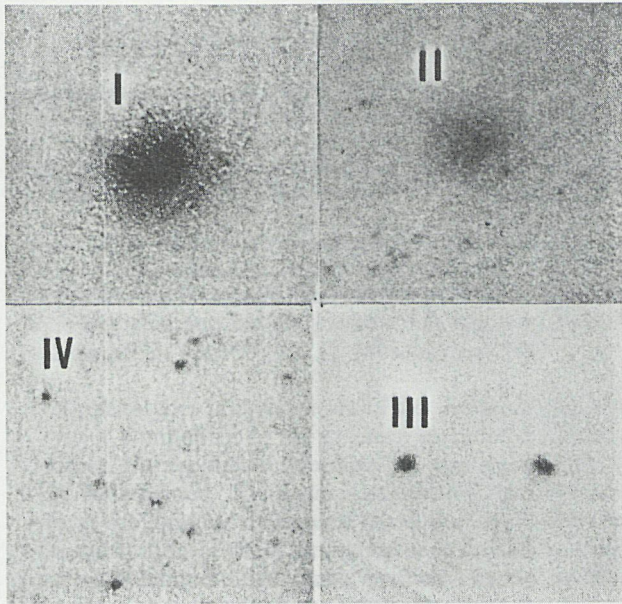


Fig. 3. Light microscopic view ($100\times$) of *Cryj* I spot (KW-S91). Spots were roughly classified into four classes, I, II, III, and IV, three of which were according to size (I, III, and IV). When spot size was as large as Class I but had a weaker color, it was designated Class II.

eye. Class IV spots were relatively uniform, at $2\text{--}4\text{ }\mu\text{m}$ in size. Class III and Class IV spots were observed predominantly during the latter half of the pollen season.

Discussion

Japanese cedar is the most widespread woody plant in mainland Japan, and Japanese cedar pollinosis is the most common pollinosis in Japan. We have established a method of separately counting the pollen allergen particles of Japanese cedar and false cypress, which are dispersed at the same time and are morphologically similar, by means of an immunoblotting technique which uses enzyme-conjugated monoclonal antibodies against *Cryj* I. This method requires less effort than the conventional morphologic technique. Our method involves the use of two kinds of monoclonal antibodies, i.e. KW-S10, which reacts only with Japanese cedar pollen, and KW-S91, which has a wide range of reactivity to conifer and some deciduous tree pollens. We consider that, with the KW-S91 antibody, it is possible to count most airborne Pinophyta pollen allergen particles other than those of Japanese cedar. It is possible that some of these counted pollen allergen particles were those of Magnoliophyta, which are dispersed during the same period as Cupressaceae family pollens; however, these pollens were rare in the atmosphere, and the numbers of grains derived from them can be regarded as negligible.

Weber & Nelson found no cross-reactivity between Pinophyta and Magnoliophyta pollens (6). Yoo reported (8) that no Cupressaceae antigen cross-reacted with rabbit antisera against redwood and Australian pine. However, *Cryj* I mouse antisera reacted widely to Pinophyta pollens other than Japanese cedar and to some Magnoliophyta pollens.

Some pollinosis patients suffering from allergies to Japanese cedar exhibit allergic symptoms when they inhale Cupressaceae family pollens. This phenomenon is explained by the cross-antigenicity that exists among Japanese cedar pollens and those of the Cupressaceae family (1). For this reason, it is desirable for the patient suffering from Japanese cedar pollinosis to have information on airborne allergens that includes information not only on Japanese cedar pollen but also on cross-reactive entities. For this purpose, the KW-S91 monoclonal antibody is useful for measuring airborne *Cryj* I-related allergen content.

The sizes of the immunoblot spots differ considerably, some of them being visible to the naked eye, while others are observable only with a microscope. It is conceivable that a spot could be 2–3 times the size of a pollen grain; that is, it could be $60\text{--}90\text{ }\mu\text{m}$, as a result of diffusion at the stage of allergen transfer. However, since we found that some spots were obviously smaller than pollen grains, they must have originated from entities other than intact pollen grains.

In a previous study, in which we used an Andersen sampler and a $5\text{-}\mu\text{m}$ pore diameter Nucleopore filter (5), we confirmed the presence of exine-free, micron-size *Cryj* I in airborne samples collected during the latter half of the Japanese cedar pollen season. We have now reconfirmed this finding by the immunoblotting technique described here. We found that the number of spots detected by this method was greater than the pollen count, as shown in Fig. 2; we believe this to be due to the counting of some spots which originated from entities other than intact pollen grains (5).

The time required for the immunochemical staining involved in this new method is about 3.5 h, and the counting time is one-third that required for the conventional morphologic method. We have also been using automatic counting methods with an image processor, a procedure on which we would like to report in the near future.

Acknowledgment

We are grateful to T. Oka of Tamano City Municipal Hospital, Okayama, Japan, for kindly supplying some of the pollen used in this study.

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