

Cha o 3, a cypress pollen allergen, does not activate basophils in Japanese cypress pollinosis



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Background: In Japan, pollinosis caused by the Japanese cypress (JCy) *Chamaecyparis obtusa* is among the very common seasonal allergies. In JCy pollinosis, Cha o 1 is the first major allergen, and Cha o 2 is the second major allergen. Recently, Cha o 3 was identified as a new JCy pollen allergen in JCy pollinosis. However, the relative contribution of Cha o 3 to JCy pollinosis compared with that of Cha o 1 and that of Cha o 2 has not been fully elucidated.

Objective: This study aimed to clarify the allergenicity of Cha o 3 compared with that of Cha o 1 and Cha o 2 in JCy pollinosis.

Methods: We recruited 27 patients with JCy pollinosis and performed the basophil activation test (BAT) with native (n) Cha o 1, Cha o 2, and Cha o 3 purified from JCy pollen. In addition, we performed JCy-specific IgE suppression test.

Results: In the BAT, 26 of 27 patients (96%) and 18 of 27 patients (67%) showed positive basophil activation in response to n Cha o 1 and n Cha o 2, respectively, as judged by CD203c expression. Little CD203c expression in response to n Cha o 3 was seen. The presence of n Cha o 3 marginally reduced the titer levels of JCy-specific IgE.

Conclusion: Cha o 3 showed little ability to activate basophils and suppress JCy-specific IgE titers compared with Cha o 1 or Cha o 2 in patients with JCy pollinosis. Thus, Cha o 3 may not be a major allergen in JCy pollinosis. (J Allergy Clin Immunol Global 2024;3:100198.)

Key Words: Japanese cypress pollinosis, Cha o 3, basophil activation test

Abbreviations used

BAT: Basophil activation test
Cha o: *Chamaecyparis obtusa*
Cry j: *Cryptomeria japonica*
JC: Japanese cedar
JCy: Japanese cypress

INTRODUCTION

Pollens from species of the Cupressaceae family are among the most important causes of respiratory allergies worldwide.¹ In North America, Mountain cedar (*Juniperus ashei*) is a major source of pollen of the genus *Cupressus* that causes allergic reactions,² whereas in the Mediterranean region, Arizona cypress (*Cupressus arizonica*) and Mediterranean cypress (*Cupressus sempervirens*) release pollens that cause allergic reactions.³

In Japan, pollinosis caused by the Japanese cypress (JCy), which belongs to the Cupressaceae family, is among the very common seasonal allergies.^{4,5} Around 15% of Japanese people experience symptoms of allergic rhinitis and conjunctivitis when JCy pollen is dispersed in Japan from March through early May. In JCy pollinosis, *Chamaecyparis obtusa* (Cha o) 1 is the first major allergen and Cha o 2 is the second major allergen.^{4,5} Cha o 1 is a glycoprotein comprising 374 amino acids; it is observed with molecular weights of 45 and 50 kDa by SDS-PAGE, which likely reflects glycosylation difference. Cha o 2 is expressed as a 514-amino acid proprotein, including a 50-mer signal peptide; mature Cha o 2 runs as an approximately 42- to 45-kDa band on SDS-PAGE. Cha o 1 and Cha o 2 are presumed to have pectate lyase activity or polygalacturonase activity based on their amino acid homology to pectate lyase allergens or polygalacturonases of other Cupressaceae pollens from around the world, respectively.

In 2016, Cha o 3, which has little homology to Cha o 1 and Cha o 2 in terms of amino acid sequences, was identified as a new JCy pollen allergen in patients with JCy pollinosis.⁶ Interestingly, Cha o 3 contains a cellulase domain and has been suggested to be the first plant cellulase with allergenic activity. In the first report on Cha o 3, 87.5% of patients with JCy pollinosis were positive for Cha o 3-specific IgE, and 3 of 3 patients with JCy pollinosis showed a level of basophil activation in response to Cha o 3 that was equal to or even higher than that in response to Cha o 1.⁶ However, there has not been enough evidence regarding the relative contribution of Cha o 3 to JCy pollinosis compared with that of Cha o 1 and Cha o 2.

This study aimed to clarify the allergenicity of Cha o 3 compared with that of Cha o 1 and Cha o 2 in JCy pollinosis. For this purpose, we recruited 27 patients with JCy pollinosis and performed a

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Received for publication July 30, 2023; revised September 24, 2023; accepted for publication October 2, 2023.

Available online December 1, 2023.

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2772-8293

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<https://doi.org/10.1016/j.jacig.2023.100198>

basophil activation test (BAT) with native (n) Cha o 1, n Cha o 2, and n Cha o 3 purified from JCy pollen. In addition, we performed a JCy-specific IgE suppression test using serum samples from the 27 patients. Details on our methods are available in this article's Online Repository at www.jaci-global.org.

RESULTS AND DISCUSSION

We first validated the identify of n Cha o 3 by liquid chromatography–tandem mass spectrometry (see Fig E1 in this article's Online Repository at www.jaci-global.org) and confirmed the specificity of n Cha o 1, n Cha o 2, and n Cha o 3 by Western blot analysis with anti-*Cryptomeria japonica* (Cry j) 1 (the first major allergen of Japanese cedar [JC] pollen, which shared allergenicity with Cha o 1),⁵ anti-Cry j 2 (the second major allergen of JC pollen, which shared allergenicity with Cha o 2),⁵ and anti-Cha o 3 antibody, respectively (see Fig E2 in this article's Online Repository at www.jaci-global.org). ImmunoCAP assay using n Cha o 1 and n Cha o 2 revealed that of the 27 patients, 26 (96%) had n Cha o 1–specific IgE and 24 (89%) had n Cha o 2–specific IgE (Table I). In addition, the serum titers of n Cha o 1– or n Cha o 2–specific IgE were correlated with the serum JCy-specific IgE titers, respectively (see Fig E3 in this article's Online Repository at www.jaci-global.org).

In the BAT, 26 of 27 patients (96%) showed positive basophil activation in response to 10 ng/mL of n Cha o 1 and 18 of 27 patients (67%) showed positive basophil activation in response to 10 ng/mL of n Cha o 2 (a $\geq 5\%$ increase of CD203c⁺ basophils compared with PBS following the stimulations was considered positive) (Fig 1). However, little or no detectable CD203c expression in response to n Cha o 3 was seen even after stimulation with a 10-fold higher concentration (100 ng/mL) than used for the allergens n Cha o 1 and n Cha o 2 (Fig 1). As a negative control, we confirmed that basophils derived from a normal subject did not respond to n Cha o 1, n Cha o 2, or n Cha o 3 (see Fig E4 in this article's Online Repository at www.jaci-global.org). Furthermore, the addition of n Cha o 3 did not affect n Cha o 1– or n Cha o 2–induced basophil activation in 3 patients with JCy pollinosis (see Fig E5 in this article's Online Repository at www.jaci-global.org), suggesting that n Cha o 3 did not contain any contaminants affecting basophil activation.

We also performed a JCy-specific IgE suppression test using the serum samples from the 27 patients (Fig 2). The level of suppression was assessed by JCy ImmunoCAP assay in the presence of competing allergen (JCy crude extract, n Cha o 1, n Cha o 2, and n Cha o 3) or PBS in the liquid phase. The suppression rates were calculated by the following formula: $1 - \text{each allergen-containing titer/PBS-containing titer}$. The titers according to the JCy ImmunoCAP assay were as follows: 13.7 ± 17.9 kU/L in the presence of PBS, 0.9 ± 1.1 kU/L in the presence of JCy crude extract (94% suppression), 5.7 ± 5.8 kU/L in the presence of n Cha o 1 (54% suppression), 7.6 ± 10.5 kU/L in the presence of Cha o 2 (46% suppression), and 12.3 ± 16.1 kU/L in the presence of Cha o 3 (10% suppression). The results were consistent with the BAT results.

The current findings of the BAT and the JCy-specific IgE suppression test are in striking contrast to the findings of previous reports.^{6–8} In their original study, Osada et al showed that 87.5% of patients with JCy pollinosis were positive for Cha o 3–specific IgE and 3 of 3 patients with JCy pollinosis

TABLE I. Patient profiles

Characteristic	Patients with JCy pollinosis (N=27)
Male, no. (%)	18 (67)
Age (y), mean $\pm$ SD	24.6 ± 5.1
Duration of JCy pollinosis (y), mean $\pm$ SD	13.3 ± 6.1
Specific IgE level (kU/L), JCy extract	
≥ 0.7 , no. (%) [*]	27 (100)
Mean $\pm$ SD	8.9 ± 11.6
Specific IgE level (kU/L), n Cha o 1	
≥ 0.7 , no. (%) [*]	26 (96)
Mean $\pm$ SD	16.0 ± 24.5
Specific IgE level (kU/L), n Cha o 2	
≥ 0.7 , no. (%) [*]	24 (89)
Mean $\pm$ SD	11.4 ± 15.9

^{*}Specific IgE value was measured by ImmunoCAP assay. JCy pollen sensitization was defined as each specific IgE level of 0.7 kU/L or higher.

showed positive basophil activation in response to Cha o 3 to the same or even higher extent than to Cha o 1.⁶ In their subsequent studies, 13 of 14 (92.9%) and 18 of 18 (100%) patients with JCy pollinosis were BAT-positive to Cha o 3.^{7,8} In addition, PBMCs from patients with JCy pollinosis produced significant IL-5 after Cha o 3 stimulation.⁸ Thus, on the basis of the findings of these studies, Cha o 3 appears to be a major allergen in JCy pollinosis.

The reason(s) for the difference between the previous studies and the current study remains unclear. We assume several possibilities, including patient cohort and purity of allergens. As for patient cohort, this study recruited patients in Yamanashi Prefecture, which is a highland prefecture to the west of Tokyo, whereas the previous study recruited patients who live in Saitama Prefecture, which is an inland prefecture located to the north of Tokyo.⁶ There are regional differences in the prevalence of sensitization to JCy in Japan, and Yamanashi Prefecture is the region with the highest prevalence of positivity for JCy.⁹ Therefore, the patient cohort might not be the primary factor responsible for the different outcomes between the 2 studies. As for the purity of allergens, we validated the identity of n Cha o 3 by liquid chromatography–tandem mass spectrometry as well as by using rabbit polyclonal antibody against Cha o 3 (see Figs E1 and E2). We also excluded contamination of n Cha o 3 by Western blot analysis showing that neither anti-Cry j 1 antibody nor anti-Cry j 2 antibody reacted to even a high dose (1000 ng/lane) of n Cha o 3 (see Fig E2) as well as by BAT analysis showing that basophils from a normal subject did not respond to n Cha o 3 (see Fig E4) and the addition of n Cha o 3 did not affect n Cha o 1– or n Cha o 2–induced basophil activation (see Fig E5). This issue should thus be clarified in future studies.

Recently, Yonekura et al reported that allergic symptoms caused by JCy pollen were suppressed by JC pollen sublingual immunotherapy tablets containing the major allergens Cry j 1 and Cry j 2, which have about 80% amino acid sequence homology to Cha o 1 and Cha o 2, respectively.¹⁰ The JC pollen sublingual immunotherapy tablets contain little Cry j 4 JC allergen having amino acid sequence homology to Cha o 3.⁴ The clinical findings may be consistent with the current findings that most of the patients with JCy pollinosis did not show responses to Cha o 3.

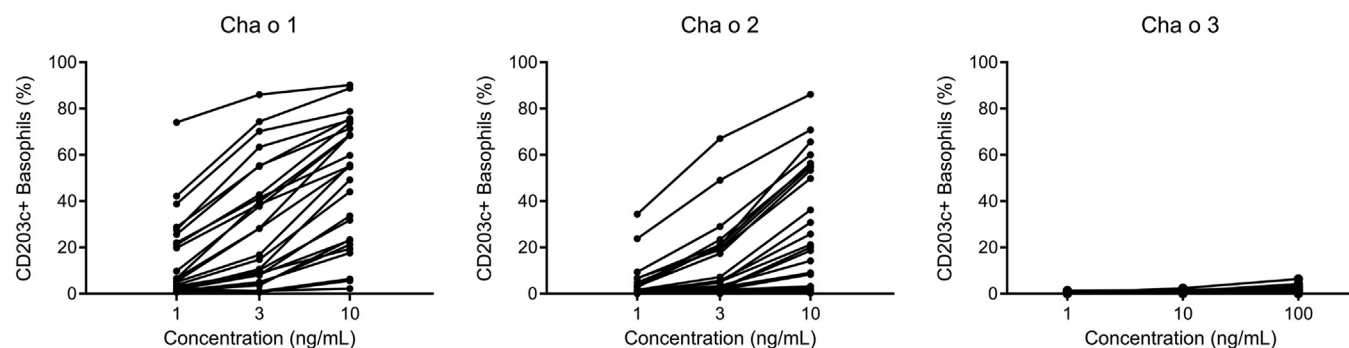


FIG 1. BATs with n Cha o 1, n Cha o 2, and n Cha o 3. The BAT was performed at concentrations of 1, 3, and 10 ng/mL for n Cha o 1 and n Cha o 2 and 1, 10, and 100 ng/mL for n Cha o 3.

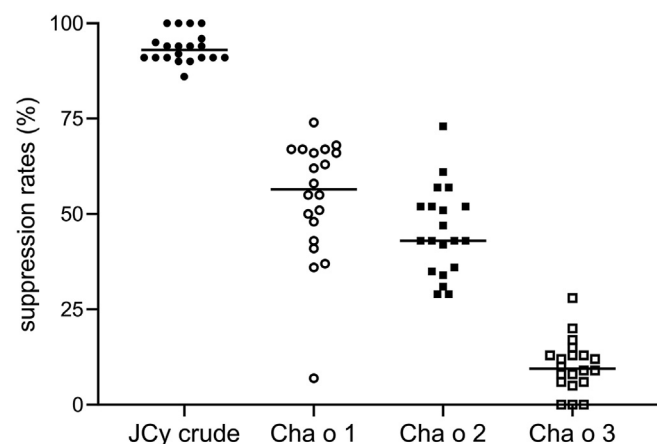


FIG 2. JCy-specific IgE inhibition test performed with n Cha o 1, n Cha o 2, and n Cha o 3. The inhibition assay was assessed by Japanese cypress ImmunoCAP assay in the presence of PBS, JCy extract, n Cha o 1, n Cha o 2, or n Cha o 3.

In summary, this study suggests that Cha o 3 may not play a major role in JCy pollinosis. Further extensive studies are thus required to establish Cha o 3 as an important allergen in JCy pollinosis and to advance our understanding of JCy pollinosis and its allergen immunotherapy.

DISCLOSURE STATEMENT

Supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan (grant-in-aid for scientific research 22K19427 [to A.N.]) and Torii Pharmaceutical Co, Ltd (to A.N.).

Disclosure of potential conflict of interest: A. Nakao received a research grant from Torii Pharmaceutical Co, Ltd. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Charpin D, Pichot C, Belmonte J, Sutra JP, Zidkova J, Chanez P, et al. Cypress pollinosis: from tree to clinic. *Clinic Rev Allergy Immunol* 2019;56:174-95.
2. Scala E, Alessandri C, Bernardi ML, Ferrara R, Palazzo P, Pomponi D, et al. Cross-sectional survey on immunoglobulin E reactivity in 23,077 subjects using an allergenic molecule-based microarray detection system. *Clin Exp Allergy* 2010;40:911-21.
3. Asam C, Hofer H, Wolf M, Aglas L, Wallner M. Tree pollen allergens-an update from a molecular perspective. *Allergy* 2015;70:1201-11.
4. Osada T, Okano M. Japanese cedar and cypress pollinosis updated: new allergens, cross reactivity, and treatment. *Allergol Int* 2021;70:281-90.
5. Ohashi-Doi K, Utsumi D, Mitobe Y, Fujinami K. Japanese cedar pollen allergens in Japan. *Curr Protein Pept Sci* 2022;23:837-50.
6. Osada T, Harada T, Asaka N, Haruma T, Kino K, Sasaki E, et al. Identification and gene cloning of a new major allergen Cha o 3 from *Chamaecyparis obtusa* (Japanese cypress) pollen. *J Allergy Clin Immunol* 2016;138:911-3.
7. Osada T, Tanaka Y, Yamada A, Sasaki E, Utsugi T. Identification of Cha o 3 homolog Cry j 4 from *Cryptomeria japonica* (Japanese cedar) pollen: limitation of the present Japanese cedar-specific ASIT. *Allergol Int* 2018;67:467-74.
8. Kikuoka H, Kouzaki H, Matsumoto K, Arai H, Yamamoto S, Tojima I, et al. Immunological effects of sublingual immunotherapy with Japanese cedar pollen extract in patients with combined Japanese cedar and Japanese cypress pollinosis. *Clin Immunol* 2020;210:108310.
9. Minami T, Fukutomi Y, Inada R, Tsuda M, Sekiya K, Miyazaki M, et al. Regional differences in the prevalence of sensitization to environmental allergens: analysis on IgE antibody testing conducted at major clinical testing laboratories throughout Japan from 2002 to 2011. *Allergol Int* 2019;68:440-9.
10. Yonekura S, Gotoh M, Okano M, Kurokawa T, Maekawa Y, Okubo K, et al. Japanese cedar pollen sublingual immunotherapy is effective in treating seasonal allergic rhinitis during the pollen dispersal period for Japanese cedar and Japanese cypress. *Allergol Int* 2022;71:140-3.

Online Repository (Methods)

Patients

We recruited twenty-seven Japanese cypress (JCy) pollinosis patients and one normal subject at the University of Yamanashi Hospital with written informed consent following the Helsinki Declaration of the World Medical Association. JCy pollinosis are defined by the presence of seasonal allergic symptoms and the serum JCy-specific IgE titer of ≥ 0.7 kU/L (ImmunoCAP, Phadia 200, Thermo Fischer Scientific). We regarded patients defined as Class 2-6 in the class system based on the ImmunoCAP (Class 0: from 0 to < 0.35 kU_A/L; Class 1: from 0.35 to < 0.7 kU_A/L; Class 2: from 0.70 to < 3.5 kU_A/L; Class 3: from 3.50 to < 17.5 kU_A/L; Class 4: from 17.5 to < 50 kU_A/L; Class 5: from 50 to < 100 kU_A/L; and Class 6: from ≥ 100 kU_A/L) as those who were sensitized to JCy pollen.^{E1} The patient profile is described in **Table 1**. None of the patients were treated with any medication at least 1 month before and during this study. The ethics committee of the University of Yamanashi Faculty of Medicine approved this study (No. 2504).

Preparation of Cha o allergens

Purification of Cha o 1 (native [n] Cha o 1)

Chamaecyparis obtusa pollen (Yamizo pollen study group, Ibaraki, Japan) was extracted at 1:20 (w/v) in 40 mM NH₄HCO₃, pH 7.8, for 3 hours at 4°C with gentle stirring. Supernatant was obtained by centrifugation (20,000xg, 30 min, 4°C). Proteins were precipitated in the presence of 80% (NH₄)₂SO₄. The precipitate was dissolved in small amount of 50 mM Tris-Cl (pH 7.8), dialyzed against 1.5 M (NH₄)₂SO₄, 50 mM Tris-Cl (pH 7.8) and applied onto a Butyl-S column (Cytiva, Marlborough, MA, US). Flow-through fraction was applied onto a HiTrap Phenyl HP column (Cytiva). Proteins were eluted from the column with a gradient of decreasing concentration of (NH₄)₂SO₄. Pooled fraction was dialysed against phosphate buffered saline (PBS). Concentrated protein (Vivaspin 15R (MWCO: 10,000HY, Sartorius) was further separated by a HiLoad 26/600 Superdex 75 pg gel filtration column (Cytiva). Fractions were examined by Western blotting with anti-Cry j 1 monoclonal antibody (Torii pharmaceutical) to choose fractions including Cha o 1 for further purification. Protein concentration was determined by the BCA method. Images were analyzed with ImageQuant TL (Cytiva) to estimate the purity of the protein.

Purification of Cha o 2 (n Cha o 2)

Cha o 2 was purified from Chamaecyparis obtusa pollen (Yamizo pollen study group) according to the previously described method with modification.^{E2-4} Pollen was extracted at 1:20 (w/v) in

40 mM NH_4HCO_3 , pH 7.8, for 3 hours at 4°C with gentle stirring. The precipitate obtained by centrifugation (20,000xg, 30 min) at 4°C was further extracted at 1:20 (w/v) in 40 mM NH_4HCO_3 , pH 7.8, 0.5 M NaCl for 24 h at 4°C with vigorous stirring. Supernatant was obtained by centrifugation (20,000xg, 30 min) at 4°C. Protein was precipitated by adding 80% $(\text{NH}_4)_2\text{SO}_4$ followed by centrifugation (20,000xg, 30 min) at 4°C. Dissolved precipitate was dialyzed against 2 M $(\text{NH}_4)_2\text{SO}_4$, 50 mM Tris-HCl, pH 7.8 and applied to a Hitrap Octyl FF (Cytiva) column equilibrated with 2 M $(\text{NH}_4)_2\text{SO}_4$, 50 mM Tris-HCl, pH 7.8. Protein was eluted from the column with a gradient of decreasing concentration of $(\text{NH}_4)_2\text{SO}_4$. Fractions were examined by Western blotting with anti-Cry j 2 monoclonal antibody (Torii pharmaceutical) to choose fractions including Cha o 2 for further purification. The fractions were pooled, dialyzed against 0.5 M NaCl, 30 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.8 and applied to a HiTrap IMAC HP column (Cytiva). Protein was eluted from the column with a gradient of decreasing pH by using 0.5 M NaCl, 30 mM Acetate/ $\text{Na}_3\text{Acetate}$ (pH 4.0). The fractions including Cha o 2 were pooled and dialyzed against 0.2 M NaCl, 30 mM Acetate/ $\text{Na}_3\text{Acetate}$, pH 4.0. The dialyzed sample was applied to a HiTrap SP HP (Cytiva). Protein was eluted from the column with a gradient of increasing concentration of NaCl.

Purification of Cha o 3 (n Cha o 3)

Chamaecyparis obtusa pollen (Yamizo pollen study group) was extracted at 1:20 (w/v) in 40 mM NH_4HCO_3 , pH 7.8, for 3 hours at 4°C with gentle stirring. Supernatant was obtained by centrifugation (20,000xg, 30 min, 4°C). Proteins were precipitated in the presence of 20% $(\text{NH}_4)_2\text{SO}_4$. The supernatant was applied onto a Phenyl HP column (Cytiva). Proteins were eluted from the column with a gradient of decreasing concentration of $(\text{NH}_4)_2\text{SO}_4$. Bands with apparent molecular weights of 50~75 Kd in SDS-PAGE were analyzed by nano-LC-MS/MS. Fractions including Cha o 3 were pooled, dialysed against 30 mM Acetate/ $\text{Na}_3\text{Acetate}$, pH 5.0 and applied to a HiTrap SP HP column (Cytiva). Protein was eluted from the column with a gradient of increasing concentration of NaCl. The fractions including Cha o 3 were selected by protein staining (Oriole, Bio-rad) of SDS-PAGE gel. Protein concentration was determined by the BCA method. Purified protein was further assessed by Western blot analysis with an anti-Cha o 3 rabbit antisera raised against Cha o 3 synthetic peptide (N-SVRSRWNYDASVKG-C) in our hands. We diluted the purified n Cha o 3 1000 times with PBS and used it for BAT.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses

Purified allergens were mixed with Sample buffer (TEFCO, Tokyo, Japan) and then denatured at 95°C for 5 minutes and separated by SDS-PAGE. Precision Plus ProteinTM unstained

Standards (Bio-Rad, CA) were used as molecular weight standards. Gel images stained with SyproRuby were captured by ImageQuant™ LAS 4000 (Cytiva, Uppsala, Sweden).

Allergens separated using SDS-PAGE were electrically transferred to Immobilon®-PSQ PVDF Membrane (Merck KGaA, Darmstadt, Germany). The membranes were blocked with BlockAce (KAC, Kyoto, Japan) for 1 hour. The membranes were subsequently incubated with a primary antibody or antiserum for overnight at 4°C. After washing 5 times with PBS-T (0.05% Tween20) buffer, Mouse IgG HRP-conjugated Antibody (R&D systems, MN) or Rabbit IgG HRP-conjugated Antibody (R&D systems) were used as a secondary antibody and incubated for 1 hour at room temperature. After washing 5 times with PBS-T, ECL Prime Western Blotting Reagent (Cytiva) was used as a chemiluminescent substrate. Chemiluminescence signals were captured using the ImageQuant™ LAS 4000. Images were analyzed with ImageQuant TL (Cytiva) to estimate the purity of the protein.

Basophil activation test

A commercial kit (Allergenicity kit; Beckman Coulter) was used to quantify basophil CD203c expression according to the manufacturer's instructions. Briefly, whole blood samples (100 µl) were incubated with PBS, anti-IgE antibody, Japanese cypress pollen crude extract (30 µg/ml), or various concentrations of purified n Cha o 1, n Cha o 2 (1, 3, 10 ng/ml) and n Cha o 3 (1, 10,

100 ng/ml) mixed with CRTH2-FITC/ CD203c-PE/ CD3-PC7 reagent for 15 minutes at 37°C.

Then, the percentage of CD203c^{high}/CRTH⁺/CD3⁻ cells was determined by BD AccuriTMC6

flow cytometry (Becton Dickinson). All blood samples were tested for CD203c expression

within 2 hours after collecting with EDTA as an anticoagulant. $\geq 5\%$ increase of CD203c⁺

basophils compared with PBS upon the stimulations was considered to be positive.

Measurement of allergen specific IgE titers

Plasma samples were analyzed for Japanese cypress Cha o 1 and Cha o 2 specific IgE titers by

ImmunoCAP (Phadia 200, Thermo Fischer Scientific) according to the manufacturer's

instructions. The inhibition assay was assessed by Japanese cypress ImmunoCAP in the

presence of competing allergen in the liquid phase. 50 μ L of plasma was mixed with 100 μ L of

PBS, JCy extract (10 μ g Protein/mL), purified n Cha o 1 (10 μ g/mL), purified n Cha o 2 (10

μ g/mL) or purified n Cha o 3 (10 μ g/mL) for 2 hours at 37°C prior to performing the JCy

ImmunoCAP assay.

Statistical analysis

For correlation coefficient determination, statistical analyses were performed using the Pearson

correlation coefficient. $p < 0.05$ was considered to be significant.

References

E1. Popescu FD, Vieru M. Precision medicine allergy immunoassay methods for assessing immunoglobulin E sensitization to aeroallergen molecules.

World J Methodol. 2018;8:17-36

E2. Kimura Y, Kuroki M, Maeda M, Okano M, Yokoyama M, Kino K. Glycoform analysis of Japanese cypress pollen allergen, Cha o 1: a comparison of the glycoforms of cedar and cypress pollen allergens. Biosci Biotechnol Biochem. 2008;72(2):485-91.

E3. Mori T, Yokoyama M, Komiyama N, Okano M, Kino K. Purification, identification, and cDNA cloning of Cha o 2, the second major allergen of Japanese cypress pollen. Biochem Biophys Res Commun 1999;263(1):166-71.

E4. Yasueda H, Saito A, Sakaguchi M, Ide T, Saito S, Taniguchi Y, et al. Identification and characterization of a group 2 conifer pollen allergen from *Chamaecyparis obtusa*, a homologue of Cry j 2 from *Cryptomeria japonica*. Clin Exp Allergy 2000;30(4):546-50.

Figure legends

Figure E1: The amino acid sequence of purified n Cha o 3 was verified by LC-MS/MS

The sequence of coverage of Cha o 3 was shown. 39% of the deduced amino acid sequence of Cha o 3 (C0HLA0) was identified by LC-MS/MS analysis of the purified protein (n Cha o 3) in this work. Matched peptides were shown as the bold red character.

Figure E2: SDS-PAGE and Western blotting of the purified n Cha o 1, 2 and 3.

A. SDS-PAGE analysis (left panel) and Western blot analysis (right panel) of purified Cha o 1 (n Cha o 1). As reported, Cha o 1 was migrated as doublet in SDS-PAGE gel (left panel).^{E2}

Purified Cha o 1 was detected with anti-Cry j 1 monoclonal antibody (mAb) in a dose-dependent manner (right panel). In contrast, Cha o 2 and 3 were not detected with anti-Cry j 1 mAb even when 1000 ng of the purified Cha o 2 and 3 were loaded (right panel).

B. SDS-PAGE analysis (left panel) and Western blot analysis (right panel) of purified Cha o 2 (n Cha o 2). Purified Cha o 2 was detected with anti-Cry j 2 mAb in a dose-dependent manner (right panel). In contrast, Cha o 1 and 3 were not detected with anti-Cry j 2 mAb even when 1000 ng of the purified Cha o 1 and 3 were loaded (right panel).

C. SDS-PAGE analysis (left panel) and Western blot analysis (right panel) of purified Cha o 3 (n Cha o 3). Single band with molecular weight in SDS-PAGE of about 65 KD Cha o 3 was

detected with anti-Cha o 3 peptide rabbit antiserum (right panel). The 45 or 50kDa band in the JCy extract lane of the SDS-PAGE (left panel) should be Cha o 1 protein, which were not detected in Western blot (right panel) by using the rabbit polyclonal serum against Cha o 3.

Figure E3: Correlation of n Cha o 1 and 2-specific IgE with JCy-specific IgE in 27 patients.

The serum titers of n Cha o 1 ($r^2=0.9622$, $p<0.0001$) and n Cha o 2 ($r^2=0.9439$, $p<0.0001$) - specific IgE show positive correlation to JCy crude extract-specific IgE, respectively (Pearson correlation coefficient).

Figure E4: Negative control for basophil activation test.

A representative basophil activation stimulated with PBS, anti-IgE antibody, Japanese cypress pollen (JCy) crude extract, or 10 ng/ml of n Cha o 1 or n Cha o 2 or n Cha o 3 from one subject who did not have JCy specific IgE.

Figure E5: n Cha o 3 did not affect n Cha o 1 and n Cha o 2-induced basophil activation in 3 patients with JCy pollinosis

Basophil activation test stimulated with 10 ng/ml of n Cha o 1 and n Cha 2 with or without Cha o 3 in 3 patients with JCy pollinosis (n=3).

References

- Charpin D. Pichot C. Belmonte J. Sutra J.P. Zidkova J. Chanez P. et al.
Cypress pollinosis: from tree to clinic.
Clin Rev Allerg Immunol. 2019; 56: 174–195
- Scala E. Alessandri C. Bernardi M.L. Ferrara R. Palazzo P. Pomponi D. et al.
Cross-sectional survey on immunoglobulin E reactivity in 23,077 subjects using an allergenic molecule-based microarray detection system.
Clin Exp Allergy. 2010; 40: 911–921
- Asam C. Hofer H. Wolf M. Aglas L. Wallner M.
Tree pollen allergens—an update from a molecular perspective.
Allergy. 2015; 70: 1201–1211
- Osada T. Okano M.
Japanese cedar and cypress pollinosis updated: new allergens, cross reactivity, and treatment.
Allergol Int. 2021; 70: 281–290
- Ohashi-Doi K. Utsumi D. Mitobe Y. Fujinami K.
Japanese cedar pollen allergens in Japan.
Curr Protein Pept Sci. 2022; 23: 837–850
- Osada T. Harada T. Asaka N. Haruma T. Kino K. Sasaki E. et al.
Identification and gene cloning of a new major allergen Cha o 3 from Chamaecyparis obtusa (Japanese cypress) pollen.
J Allergy Clin Immunol. 2016; 138: 911–913
- Osada T. Tanaka Y. Yamada A. Sasaki E. Utsugi T.

Identification of Cha o 3 homolog Cry j 4 from *Cryptomeria japonica* (Japanese cedar) pollen: limitation of the present Japanese cedar-specific ASIT.

Allergol Int. 2018; 67: 467–474

Kikuoka H. Kouzaki H. Matsumoto K. Arai H. Yamamoto S. Tojima I. et al.

Immunological effects of sublingual immunotherapy with Japanese cedar pollen extract in patients with combined Japanese cedar and Japanese cypress pollinosis.

Clin Immunol. 2020; 210: 108310

Minami T. Fukutomi Y. Inada R. Tsuda M. Sekiya K. Miyazaki M. et al.

Regional differences in the prevalence of sensitization to environmental allergens: analysis on IgE antibody testing conducted at major clinical testing laboratories throughout Japan from 2002 to 2011.

Allergol Int. 2019; 68: 440–449

Yonekura S. Gotoh M. Okano M. Kurokawa T. Maekawa Y. Okubo K. et al.

Japanese cedar pollen sublingual immunotherapy is effective in treating seasonal allergic rhinitis during the pollen dispersal period for Japanese cedar and Japanese cypress.

Allergol Int. 2022; 71: 140–143

Chao 3, a cypress pollen allergen, does not activate basophils in Japanese cypress pollinosis

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Volume 3 Issue 1 (February 2024)
DOI: 10.1016/j.jacig.2023.100198



Supplementary Figure E1

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101 TNATVAQTFA RLNLTEAASG IEHNNPELLD LGHVAAYHHV VAALSEAGVM
151 VILDNHVSKP KWCCAVIDDGN GFFGDRYFNP NTWVEGLGLM ATYFNNTFNV
201 VAMSLRNELR GNRSTPISWS RHMQWGAATV HKANPKVLVI LSGLQFDTDL
251 SFLPVLPVTL PFKEKIVYEG HWYSFGVPWR TGLPNDVCKN ETGRFKSNVG
301 FVTSSANATA APLFMSEFGI DQRYVNDNDN RYLNCILAYL AEEDLDWALW
351 TMGGSYYYRS DKQPVKDFEE TYGFFNHDWS RIRNPDFISR LKEIQQPIQD
401 PYLAPGPYYQ IIYHPASGLC VESGIGNTVH LGSCQSVRSR WNYDASVKGP
451 IGLMGSSSCI STQGNGLPAI MTENC SAPNN TLWSTVSSAQ LQLGTRVLGR
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551 LHQLQL

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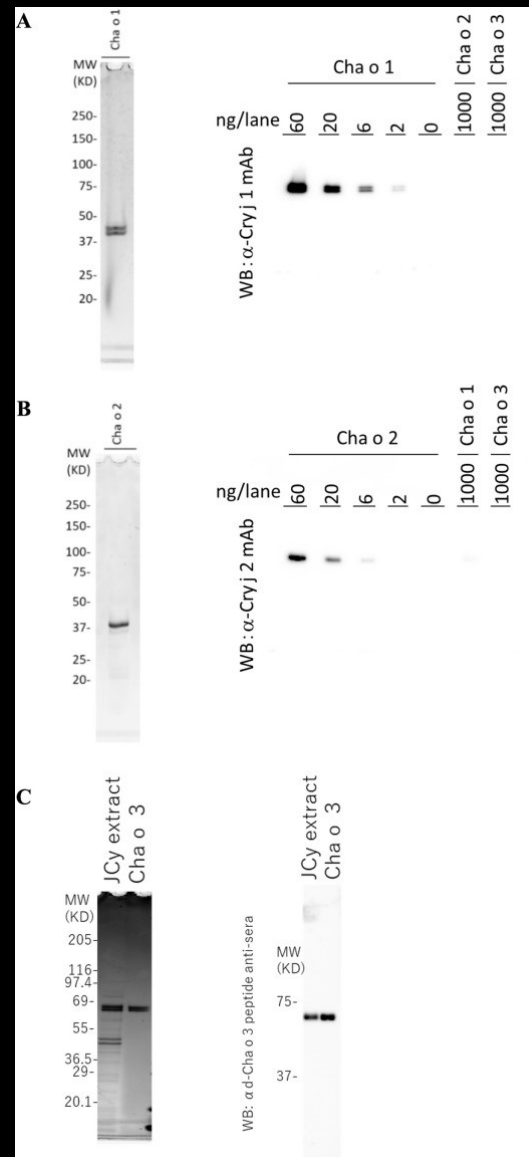
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Supplementary Figure E2



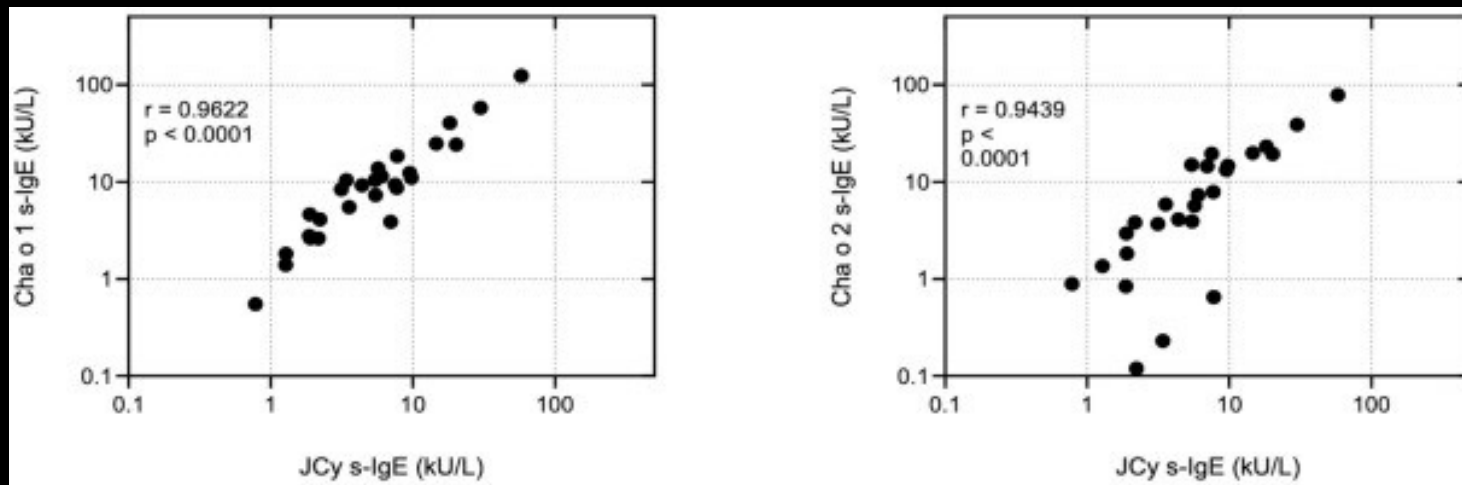
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Supplementary Figure E3



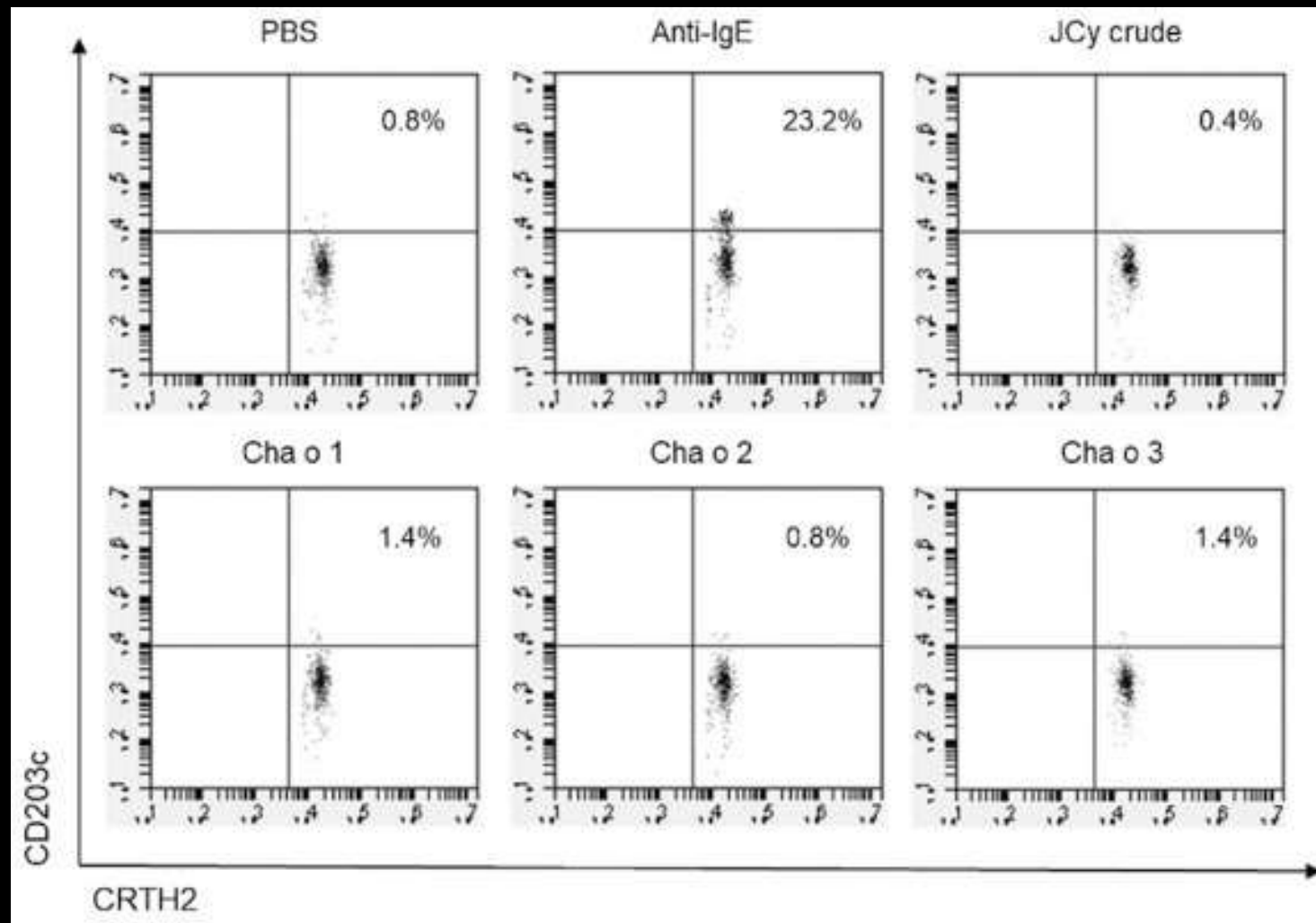
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Supplementary Figure E4



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Supplementary Figure E5

