

Roles of major oligosaccharides on Cry j 1 in human immunoglobulin E and T cell responses

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Summary

Background We have demonstrated that carbohydrates in Cry j 1, the major allergen of *Cryptomeria japonica* pollen, play a major role in promoting Cry j 1-specific Th2 response. However, little is known as to whether the carbohydrates directly participate in allergic responses.

Objective We sought to determine whether Cry j 1-related oligosaccharides function as IgE and/or T cell epitopes. In addition, the regulatory effect of Cry j 1-related oligosaccharide on Cry j 1-specific T cell responses was investigated.

Methods Two monovalent oligosaccharides largely found on Cry j 1, Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc (M3FX), and GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc (GN2M3FX) were prepared. Man α 1-2Man α 1-6(Man α 1-2Man α 1-3)Man α 1-6(Man α 1-2Man α 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc (M9A) was used as control. Competitive inhibition ELISA for Cry j 1-specific IgE was performed using these oligosaccharides as inhibitors. In addition, T cell lines specific for Cry j 1 or purified protein derivative of *Mycobacterium tuberculosis* (PPD) were established, and cellular responses against these oligosaccharides were investigated in the presence or absence of the respective antigens.

Results Overall, neither M3FX nor GN2M3FX displayed inhibitory effect on the binding between IgE and Cry j 1. In addition, M3FX did not by itself stimulate Cry j 1 or PPD-specific T cells. However, M3FX significantly inhibited Cry j 1-induced proliferation and IL-4 production in Cry j 1-specific T cells. Such an inhibitory effect was not seen in PPD-specific T cell responses.

Conclusion These results suggest that Cry j 1-related oligosaccharides are not major epitopes for IgE or T cells. However, these oligosaccharides have a novel potential to inhibit Cry j 1-specific T cell responses selectively.

Keywords IgE, oligosaccharide, pollen, T cell, Th2

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Introduction

Carbohydrates expressed on allergens are known to participate in both initiation and exacerbation of allergic responses. Several carbohydrates on allergens/antigens such as *Aspergillus fumigatus* (Af) and *Schistosoma mansoni* egg antigen participate in *in vivo* induction of Th2 response [1–3]. For example, we have recently demonstrated that deglycosylation of Af antigen by periodate treatment significantly abrogates its ability to induce *in vivo* Af-specific IgE production and nasal eosinophilia in a murine model of allergic rhinitis [3]. On the contrary, carbohydrates on phospholipase A2, a major allergen of honeybee venom, are not involved in the induction of Th2 responses *in vivo* [4].

Several reports demonstrated that carbohydrates on allergens such as phospholipase A2 from bee venom, sea squirt allergen, Lol p 11 from rye grass, Bermuda grass pollen BG60 and Ole e 1 from olive pollen have potential as IgE epitopes [5–10]. In addition, carbohydrate structures such as β [1, 2]-xylose and α [1, 3]-fucose are shared by a number of unrelated glycoallergens, and can act as cross-reactive carbohydrate determinants (CCD) which lead to the multiple sensitization detected *in vitro* [11, 12]. On the other hand, it remains controversial whether the carbohydrates show biological activity [9, 10, 13–15].

Further, carbohydrates on allergens can elicit specific T cell responses. For example, carbohydrates on phospholipase A2 and the pollen extract from *Parietaria judaica*, respectively, were recognized by CD4⁺ and CD8⁺ T cells from allergic patients [16, 17]. Also, several T cell clones specific to Bet v 1 from birch pollen and Phl p 5 from *Phleum pratense* do not recognize recombinant isoallergens that do not contain carbohydrate structures unlike the natural isoforms [18,19]. However, little is known whether the carbohydrates on

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allergen directly act as T cell epitopes or whether they contribute to the immunogenicity of glycosylated allergens.

Japanese cedar pollinosis is the most prevalent allergic disease in Japan. A recent epidemiological study demonstrated that approximately 20% of people living in urban areas suffer from the disease [20]. Cry j 1 is one of the major allergens on the pollen, and up to 95% of the patients have Cry j 1-specific IgE in serum [21, 22]. Cry j 1 is known as a glycoprotein with a 6% (W/W) carbohydrate content [21]. Five possible *N*-linked glycosylation sites have been found in the primary amino acid sequence of this allergen although it is not known whether all these sites are glycosylated [23]. Furthermore, *N*-linked oligosaccharide structures of Cry j 1 have been proposed (Fig. 1; [24]). Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc (M3FX), and GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc (GN2M3FX) but not Man α 1-2Man α 1-6(Man α 1-2Man α 1-3)Man α 1-6(Man α 1-2Man α 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc (M9A) were largely found on the allergen. Inhibition assay revealed that carbohydrates on Cry j 1 contribute little or only conformationally to the reactivity of specific IgE in humans [24]. On the other hand, Taniai et al. [25] reported that glycosylation of NGNATPQLTKNAGVLTCSLSKR sequence on Cry j 1 might be required for IgE binding to the peptide moiety. In addition, we have demonstrated that carbohydrates on Cry j 1 play a major role in promoting Cry j 1-specific Th2 response *in vitro* [26]. However, in such investigations, structurally related glycopeptide and/or periodate-treated glycoproteins were used, and it cannot be determined whether the carbohydrates directly function as IgE and/or T cell epitopes or whether they affect the allergenicity of peptide portion.

In the present study, we investigated the direct roles of major oligosaccharides on Cry j 1 in specific IgE and T cell responses by using monovalent oligosaccharides. We believe that findings presented here may provide new insights into not only the pathogenesis of pollinosis, but also the development of novel tools for pollen-specific immunotherapy since M3FX seems to be one of the common oligosaccharides shared by unrelated pollen allergens [11, 12].

Methods

Antigens and monovalent oligosaccharides

Cry j 1 was purified from crude extracts of *Cryptomeria japonica* pollen (JCP) in a well-established procedure [21]. Protein concentration of Cry j 1 was determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Purified protein derivative of *Mycobacterium tubecurosis* (PPD) was purchased from Nihon BCG Seizo Co. (Tokyo, Japan). Cry-consensus, a recombinant polypeptide containing major T cell epitopes of p106–120 and p211–225 on Cry j 1, was constructed as described previously [27]. Monovalent GN2M3FX (GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA (pyridylaamino)), M3FX (Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA), and M9A (Man α 1-2Man α 1-6(Man α 1-2Man α 1-3)Man β 1-6(Man α 1-2Man α 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA) were prepared and purified from some plant

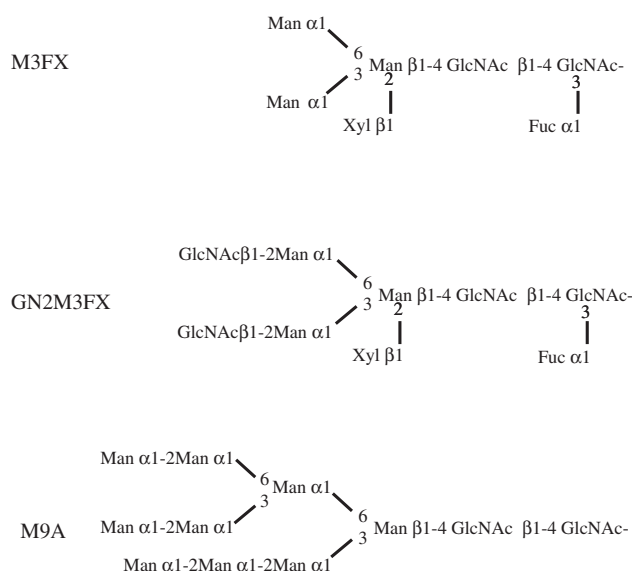


Fig. 1. Structures of oligosaccharides.

glycoproteins (*Ginkgo biloba* pollen glycoprotein [28], *Ricinus communis* agglutinin (RCA 120) [29], and royal jelly glycoproteins [30]) (Fig. 1). In brief, *N*-glycans were released by hydrazinolysis (100 °C, 12 h, in anhydrous hydrazine) from each plant glycoproteins (100–300 mg). After *N*-acetylation of the hydrazinolysate with saturated ammonium bicarbonate (20 mL) and acetic anhydride (0.8 mL), the acetylated hydrazinolysate was desalted by Dowex 50 × 2 resin. Pyridylamination (PA) of the sugar chains was done by the method of Kondo et al. [31]. Separation of PA-sugar chains was done by HPLC on a Jasco 880-PU HPLC apparatus with a Jasco (Tokyo, Japan) 821-FP Intelligent Spectrofluorometer, using a Cosmosil 5C18-AR column (1.0 × 25-cm, Nacalai Tesque Inc., Kyoto, Japan) or a Shodex Asahipak NH2P-50 column (10 × 25 cm, Showa Denko Co., Tokyo, Japan). On the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 20% in 60 min at a flow rate 1.5 mL/min. In the case of size-fractionation HPLC using the Asahipak NH2P-50 column, the PA-oligosaccharide was eluted by increasing the water content in the water–acetonitrile mixture from 30% to 50% linearly in 60 min at a flow rate of 1.5 mL/min.

Serum samples

Forty-five patients with Japanese cedar pollinosis (mean age, 33.60 ± 19.61 years; range, 7–81 years; 22 males and 23 females) were enrolled in this study. Sensitization to JCP was confirmed by the presence of specific-IgE antibodies determined by CAP [32] (Pharmacia, Uppsala, Sweden; ranging from 1.27 to 146.34 (mean 28.47 ± 31.36) UA/mL). Of these, 12 patients were sensitized to JCP alone or both JCP and Hinoki cypress (*Chamaecyparis obtuse*) whereas the remaining 33 patients were sensitized not only JCP but also to other unrelated pollens such as orchard grass, short ragweed, mugwort, and Japanese alder as determined by CAP. None of the patients were sensitized to mites, molds, insects, animal

danders, or foods. In addition, these patients underwent skin scratch test to JCP extract (Torii Co., Tokyo, Japan). A positive result was defined as a skin reaction with a erythema of greater than or equal to 10 mm in combination with a negative response to control fluid (Torii). Based on this evaluation, eight out of 45 patients showed negative results in skin scratch test to Japanese cedar pollen. None of the patients had received immunotherapy or used immunosuppressive drugs, including topical steroids, prior to the enrollment. The present study was approved by the Human Research Committee of Okayama University Medical School.

Cry j 1-specific IgE ELISA and inhibition

The Cry j 1-specific IgE was measured by captured ELISA according to the methods as previously reported [33]. In brief, ELISA plates (Corning Inc., Corning, NY, USA) were coated overnight at 4 °C with 100 µL of 5 µg/mL rat anti-human IgE mAb (clone LO-HE-17; Biosource, Camarillo, CA, USA) in 0.05 M carbonate buffer pH 9.6. Then plates were blocked for 2 h at 37 °C with 200 µL of PBS containing 10% FCS. After blocking, serum samples serially diluted with PBS containing 10% FCS were added to the plates, and the plates were incubated for 2 h at 37 °C. Thereafter, plates were incubated with 6 nM biotinylated Cry j 1 (Hayashibara Biochemical Laboratories Inc., Okayama, Japan) for 2 h at 37 °C, followed by 1/1000 extravidin–peroxidase conjugate (Sigma Chemical Co., St Louis, MO, USA) for 1 h at 37 °C. After the final washing, tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) was added. Finally, 5% phosphoric acid (Wako Pure Chemical Industries Ltd, Osaka, Japan) was added to stop the reaction. The absorbance at 450 nm was measured using an automatic microplate reader (Bio-Rad, Hercules, CA, USA). Between each steps, plates were washed with PBS containing 0.05% Tween 20 four times, except the final wash, which was repeated six times.

Competitive inhibition ELISA for Cry j 1-specific IgE was performed as follows. ELISA plates were coated with rat anti-human IgE mAb as described above. After the blocking, serum samples were added in triplicate wells, then the plates were incubated for 2 h at 37 °C. In a separate plate, biotinylated Cry j 1 (final 6 nM) was mixed with serial concentrations (final 0, 30, 300 and 3000 nM) of M3FX, GN2M3FX or M9A as control. Then it was added to the IgE-bound plate for 2 h at 37 °C, followed by extravidin–peroxidase conjugate for 1 h at 37 °C. Finally, plates were developed with TMB substrate and stopped with 5% phosphoric acid. Plates were read in a microplate reader at 450 nm. Results were presented as percentage binding calculated as follows: (OD 450 nm with M3FX or GN2M3FX/OD 450 nm with M9A) × 100.

Antigen-specific T cell lines

Short-term cultured Cry j 1-specific T cell lines (TCLs) were generated from peripheral blood mononuclear cells (PBMCs) of 7 patients with Japanese cedar pollinosis (four males and three females: age 21–43, mean 31.29 ± 8.48 years old) as

described previously [26]. PPD-specific TCLs were also generated from these patients in the same manner.

Cellular responses to Cry j 1-related oligosaccharide

M3FX-driven proliferation and cytokine production of Cry j 1-specific TCL was tested *in vitro* as described previously [34]. Briefly, in flat-bottomed microtitre plates (Corning), 2×10^4 T cell blasts mixed with 1×10^5 autologous PBMCs in the presence or absence of either 0.28 µM of Cry j 1 or 10 µg/mL of Cry-consensus were cultured in 0.2 mL CM with serial concentrations (final 0, 0.5, 5.0 and 50 µM) of M3FX or M9A as negative control. In addition, these cells were cultured with 0.28 µM of Cry j 1 alone. Culture supernatants were collected after 65 h incubation, and stored at –80 °C until the assay for cytokine production. For the last 7 h of the 72 h culture, 1 µCi ^3H -thymidine (ICN Radiochemicals, Irvine, CA, USA) was added to each well. Uptake of ^3H -thymidine by responding cells was counted by liquid scintillation spectrometry (Aloka Co., Tokyo, Japan). The data recorded was the mean count per minutes (c.p.m.) of triplicate cultures.

In separate experiments, 2×10^4 T cell blasts specific for PPD co-cultured with 1×10^5 autologous PBMCs were cultured in 0.2 mL CM with 2 µg/mL PPD in the presence or absence of serial concentrations (final 0, 0.5, 5.0 and 50 µM) of M3FX or M9A. Proliferative responses and cytokine production by PPD-specific TCLs were determined as described above.

Cytokine determination

Levels of IL-4 and IFN-γ in culture supernatants were measured by means of Opt EIA sets (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions [26]. The detection limit in these assays was 3 pg/mL for IL-4 and 20 pg/mL for IFN-γ.

Statistical analysis

Data were analysed using the Bartlett test followed by Wilcoxon's signed-rank test and Fisher's exact probability test. $P < 0.05$ was considered statistically significant. Values were given as mean ± standard deviation (SD).

Results

Inhibition of binding between IgE and Cry j 1 by pollen-related oligosaccharides

In captured ELISA, pooled serum from patients with pollinosis reacts with biotinylated Cry j 1 when the serum was diluted at 1:32, and the signal was lost when it was heated at 56 °C for 1 h (data not shown). On the other hand, that from healthy subjects did not react with biotinylated Cry j 1, so that individual sera at 1:32 dilution were examined for further experiments. Cry j 1-specific IgE titre is significantly and positively correlated with JCP-specific IgE titre determined by CAP (Fig. 2a, $r = 0.615$, $P < 0.0001$), and 40 out of 45 samples showed a positive IgE signal against Cry j 1 twice

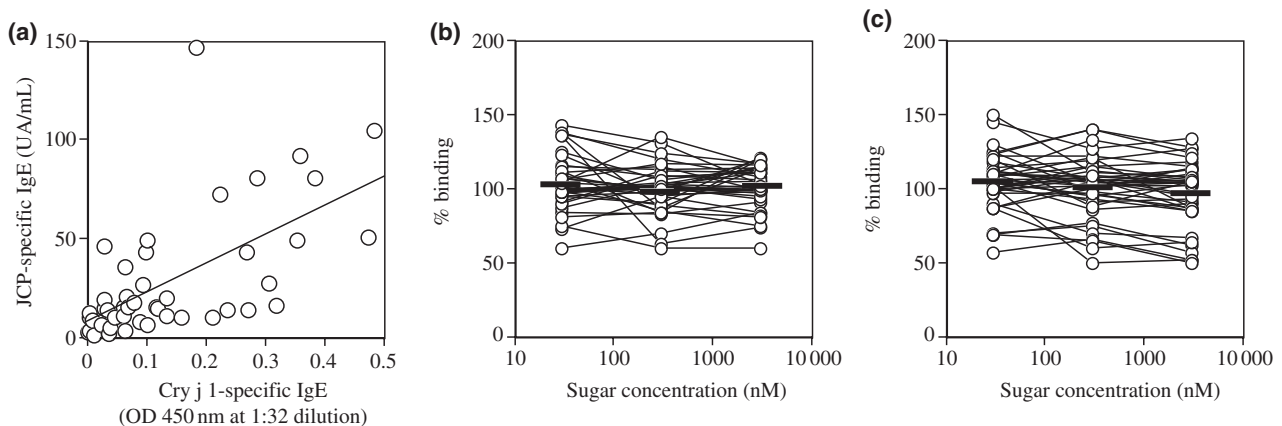


Fig. 2. (a) Correlation of IgE-binding to crude extract of Japanese cedar pollen (JCP) against IgE-binding to Cry j 1. Titres for JP-specific IgE were expressed as UA/mL determined by CAP [31]. Titres for Cry j 1-specific IgE were presented as the absorbance at 450 nm from triplicate wells of 1 : 32 serum dilution. (b, c) Competitive inhibition of the binding between Cry j 1 and IgE by monovalent oligosaccharides. Biotinylated Cry j 1 (6 nM) was mixed with M3FX (b), GN2M3FX (c), or M9A at 30, 300 and 3000 nM, then they were added to the IgE-bound plate. Results are presented as percentage binding. *P*-value was obtained through use of the Wilcoxon signed-rank test.

that from the background. Thus we examined inhibition ELISA with these 40 samples.

We used M3FX, GN2M3FX and M9A as an inhibitor at the concentration of 30, 300 and 3000 nM which yields the ratio between carbohydrates on biotinylated Cry j 1 and free carbohydrates of about 1 : 1, 1 : 10 and 1 : 100, respectively, since five possible *N*-linked glycosylation sites are found in the primary sequence of Cry j 1 molecule [23]. Overall, no significant inhibition was observed in the presence of M3FX and GN2M3FX, the Cry j 1-related oligosaccharides, as compared with the response in the presence of M9A, the Cry j 1-unrelated oligosaccharide (Figs 2b and c). The mean absorbances at 450 nm in the presence of 30, 300 and 3000 nM of M9A were 0.191 ± 0.290 , 0.194 ± 0.286 and 0.189 ± 0.294 , respectively. These results suggest that neither M3FX nor GN2M3FX were the major IgE epitopes in these patients with Japanese cedar pollinosis.

Tentatively defining a positive inhibition as that in which the binding between IgE and Cry j 1 was inhibited more than 20% in the presence of M3FX or GN2M3FX as compared with M9A, most sera (31/40:77.5%) showed no inhibition. However, 2 (5.0%), 4 (10.0%) and 3 (7.5%) samples showed a positive inhibition in the presence of M3FX alone, GN2M3FX alone, and both M3FX and GN2M3FX, respectively. These results suggest that pollen-related oligosaccharides may function as a minor IgE epitope in nine out of 40 subjects (22.5%).

Clinical characterization of patients sensitized to Cry j 1-related oligosaccharides

In 40 patients showing sensitization to Cry j 1, 12 patients were sensitized to Japanese cedar alone or both Japanese cedar and Hinoki cypress, whereas the remaining 28 patients were sensitized to other unrelated pollens such as orchard grass, short ragweed, mugwort, and Japanese alder. Eight out of nine patients (88.9%) who were sensitized to M3FX and/or GN2M3FX showed multiple sensitization to unrelated pollens whereas 20 out of 31 patients (64.5%) who were not sensitized to the oligosaccharides showed the multiple

sensitization. However, there was no significant difference regarding multiple sensitization to unrelated pollens between patients with or without sensitization to Cry j 1-related oligosaccharides ($P = 0.1617$). In addition, seven out of 40 patients showed the negative results in skin scratch test to Japanese cedar pollen. There was also no significant difference regarding reactivity in skin scratch test against Japanese cedar pollen between patients with or without sensitization to Cry j 1-related oligosaccharides ($P = 0.8246$).

T cell responses against Cry j 1-related oligosaccharide

In order to determine whether Cry j 1-specific T cells recognize Cry j 1-related oligosaccharide as a T cell epitope, we established a panel of Cry j 1-specific T cell lines from seven patients with Japanese cedar pollinosis. All of the Cry j 1-specific T cell lines responded to native Cry j 1 at the concentration of $0.28 \mu\text{M}$ (mean: $32\,592 \pm 32\,527$ c.p.m., $P = 0.0180$), and produced a higher amount of IL-4 (mean: 563 ± 466 pg/mL, $P = 0.0180$) and a lesser amount of IFN- γ (mean: 93 ± 161 pg/mL, $P = 0.0431$). However, these T cells responded to neither M3FX nor M9A at the concentration of 0.5, 5.0 or 50 mM (Fig. 3a). Two out of seven patients were sensitized to GN2M3FX and/or M3FX, and we found no significant differences in the cellular responses to M3FX whether the patients were sensitized or not to the oligosaccharides. In addition, production of neither IL-4 or IFN- γ was induced in response to these oligosaccharides (Figs 3b and c). PPD-specific TCLs also did not respond either to M3FX or to M9A (data not shown).

Selective inhibition of Cry j 1-specific T cell responses by Cry j 1-related oligosaccharide

Finally, we decided to determine whether Cry j 1-related oligosaccharide affect specific T cell responses. Thus serial concentrations of M3FX or M9A were added in the interaction between specific T cells and Cry j 1. Presence of M9A did not affect the Cry j 1-specific cellular responses (Figs 4a–c).

However, presence of M3FX significantly inhibited specific T cell responses in a dose-dependent manner (Fig. 4d). In addition, M3FX at 50 μM inhibited IL-4 production in Cry j 1-specific T cell (Fig. 4e). On the other hand, the presence of M3FX did not play any role in IFN- γ production (Fig. 4f). On the contrary, addition of either M3FX or M9A did not affect Cry-consensus-specific T cell proliferation or cytokine production (Fig. 5). Addition of M3FX had no effect on either PPD-specific T cell proliferation or cytokine production (data not shown).

Discussion

Several reports demonstrated that carbohydrates on allergens contribute to the binding capacity of IgE to the allergen [5–12, 14, 15]. For example, 14 out of 17 Cup a 1-positive sera abolished the IgE binding to the periodate-treated allergen [10]. On the contrary, other reports showed that the carbohydrates on allergens such as Pla 1 1 from *Plantago lanceolata* pollen do not constitute a relevant allergenic epitope [4, 13, 35]. However, it remains unclear whether the carbohydrates expressed on allergen directly act as IgE epitopes or indirectly contribute the epitope activity of a

peptide moiety by affecting the conformational structure. Allergens expressed in prokaryotic or eukaryotic vectors may be potential tools for investigating the role of carbohydrates on allergen in the immune responses. In addition, the effect of glycosylation of the individual glycosylation sites can be studied using engineered mutants expressed in prokaryotic and eukaryotic systems. However, these investigations may reflect indirect roles of carbohydrates, and cannot exclude the possibility that alteration of conformational structure of protein moieties such as refolding may affect the responses. For example, Akdis et al. [36] demonstrated that non-refolded recombinant allergen alters both IgE binding and proliferation/cytokine production by T cells. And Taniai et al. [25] reported that some conformational structure might be involved in the effective binding between IgE and Cry j 1. In this manuscript, we focused on the direct roles of carbohydrates on Cry j 1 in specific IgE and T cell responses using monovalent oligosaccharides. The result presented here that GN2M3FX and M3FX, the major and core oligosaccharide expressed on Cry j 1, respectively, did not display the significant inhibition of the binding between Cry j 1 and human IgE (Fig. 3) is consistent with the report by Ogawa et al. [24], and gives direct evidence that these two oligosaccharides are not the major IgE epitopes in patients

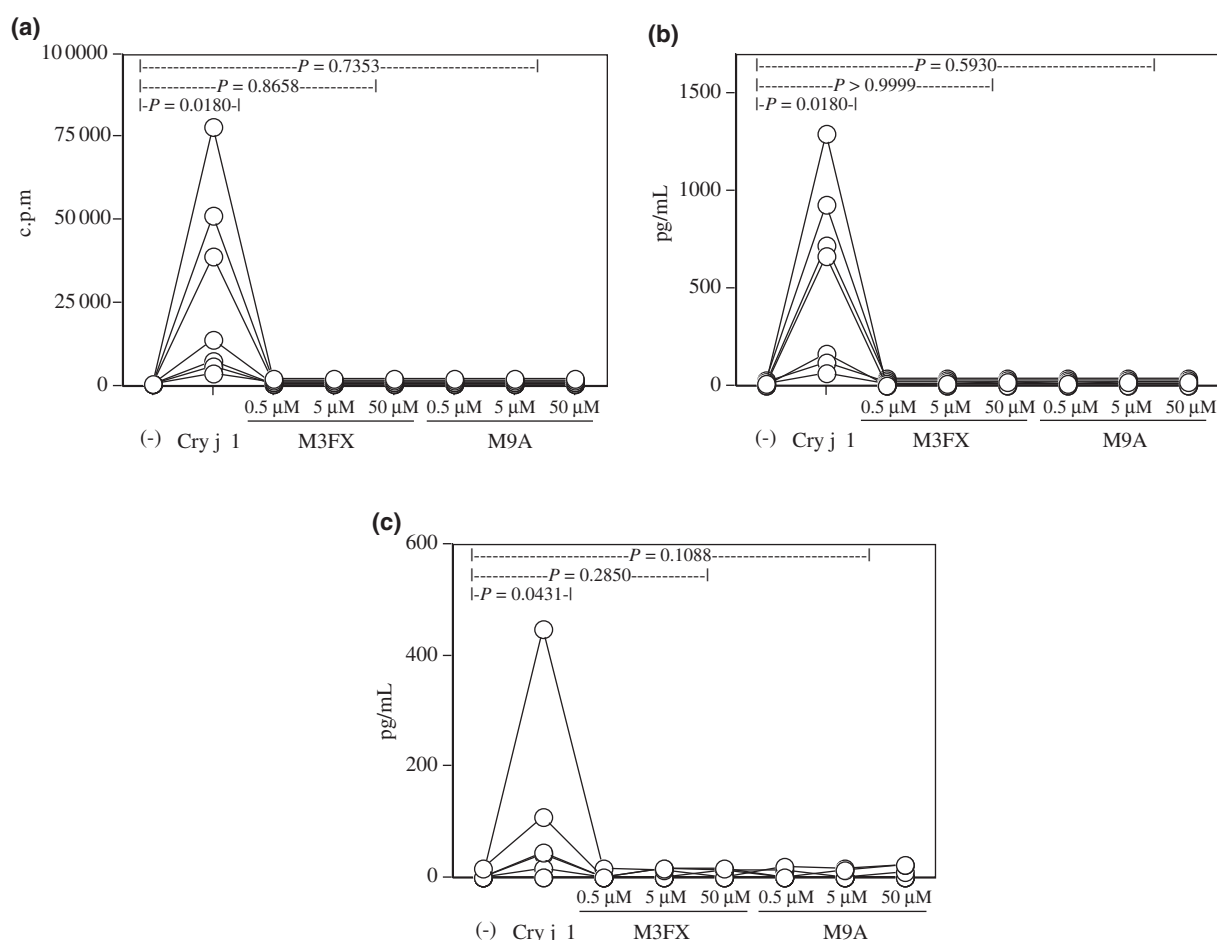


Fig. 3. Proliferation (a) IL-4 production (b) and IFN- γ production (c) of Cry j 1-specific T cells against stimulation with monovalent oligosaccharides. Seven individual T cells were cultured with M3FX or M9A at serial concentrations for 72 h. As a positive control, these cells were cultured with 0.28 μM of Cry j 1. P -value was obtained through use of Wilcoxon signed-rank test.

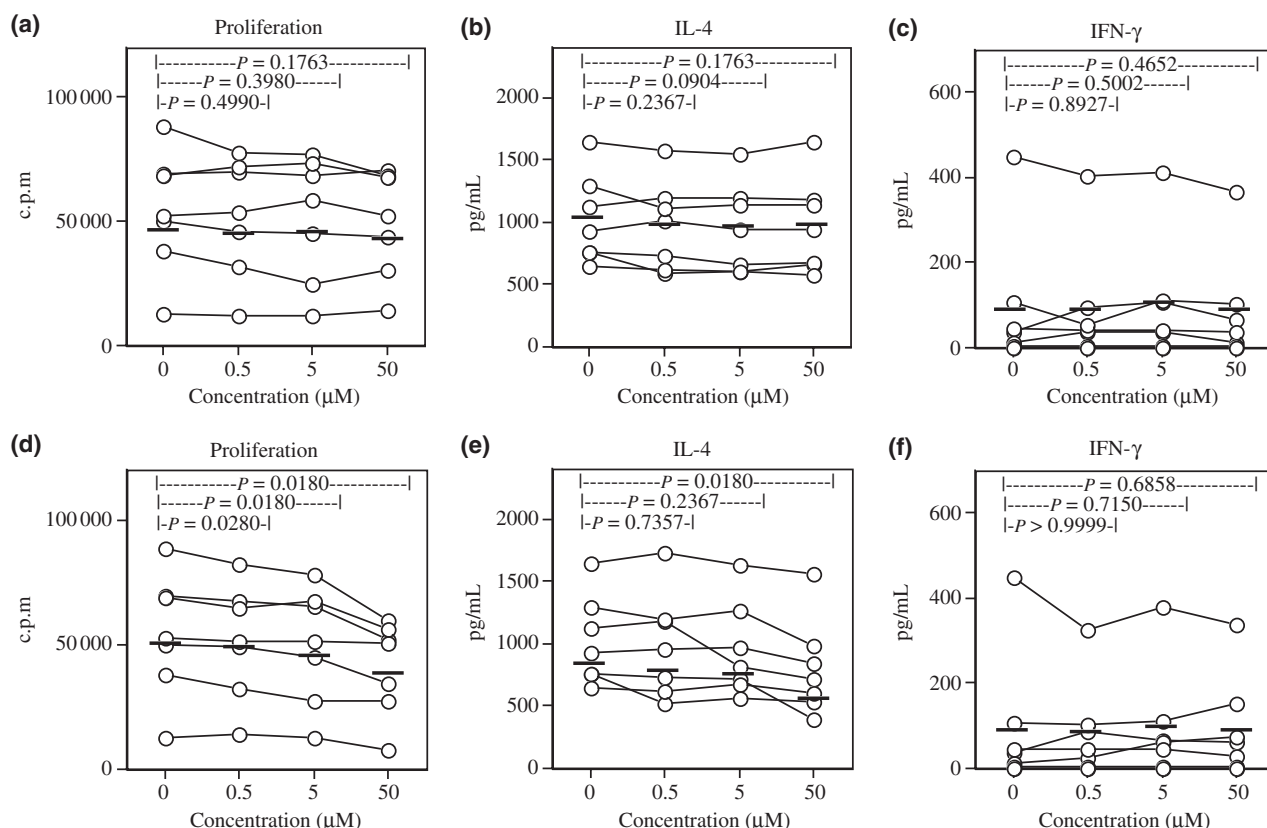


Fig. 4. Inhibition of Cry j 1-specific T cell responses by monovalent oligosaccharide. Seven individual TCLs were cultured with $0.28 \mu\text{M}$ of Cry j 1 in the presence of M9A (a–c) or M3FX (d–f) at serial concentrations for 72 h. Proliferation (a, d), IL-4 production (b, e) and IFN- γ production (c, f) of Cry j 1-specific T cell responses were determined as described in Methods. *P*-value was obtained through use of the Wilcoxon signed-rank test.

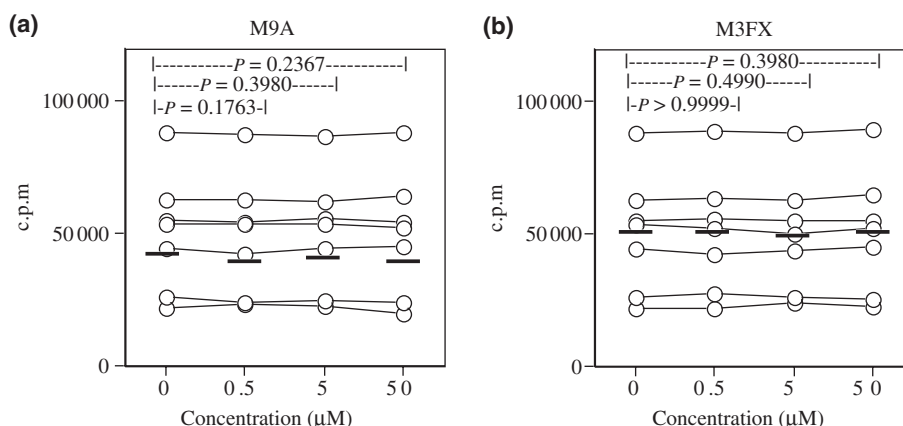


Fig. 5. Effect of momovalent oligosaccharide on peptide-driven T cell responses. Seven individual TCLs were cultured with $10 \mu\text{g/mL}$ of Cry-consensus in the presence of M9A (a) or M3FX (b) at serial concentrations for 72 h. Proliferation of peptide-specific T cell responses was determined as described in Methods. *P*-value was obtained through use of Wilcoxon signed-rank test.

with Japanese cedar pollinosis. In these experiments, we used M9A as a negative control of inhibition since high concentration of oligosaccharides may affect the inhibition via changing the culture condition such as osmolarity, and M9A is suitable as a negative control since Cry j 1 does not contain this oligosaccharide [24].

However, these oligosaccharides may function as minor IgE epitopes since a partial inhibition of the binding between IgE

and Cry j 1 was observed in the presence of GN2M3FX and/or M3FX in 22.5% of the patients sensitized to Cry j 1 (Fig. 4). van Ree et al. [7] demonstrated that sera of 38.8% of patients allergic to Lol p 11 contained anti-carbohydrate IgE. And more recently, Calabozo et al. [34] reported that sugar residues of Pla 11 participate in the binding of IgE epitopes that are minor allergic determinants, contributing in a low proportion to the total IgE-binding capacity of the allergen.

On the other hand, Batarero et al. [9] reported that 65% and 100% of patients sensitized to olive pollen and Ole e 1, respectively, recognized carbohydrate determinant on Ole e 1. In terms of the prevalence of anti-carbohydrate IgE, our results were similar to the first two reports in this paragraph. This may be due to the composition, valency and/or structural homology of carbohydrates among the allergens. Both Cry j 1 and Lol p 11 contain M3FX, which has fucose α 1-3 linked to the innermost GlucNAc, but Ole e 1 is not known to contain such oligosaccharide or fucose [13, 24].

Carbohydrates on allergens are known to act as CCD, which lead to the broad cross-reactivity of IgE in food pollen and invertebrate animal allergens [11, 12, 14, 15]. Iacovacci et al. [37] established mAb 5E6, which recognized M3FX on Cup a 1 from *Cupressus arizonica* pollen. This mAb also reacted with not only pollens from Cupressaceae family, but also taxonomically unrelated pollens such as olive and grass pollen. We divided 40 patients sensitized to Cry j 1 into two groups. The first group enrolled patients who were sensitized to *C. japonica* alone or to both *C. japonica* and *Chamaecyparis obtusa*. *C. japonica* and *C. obtusa* are taxonomically related tree pollens, and we have previously reported that 76.4% of patients with spring pollinosis were sensitized to both pollens [38]. The second group enrolled patients who were sensitized not only to *C. japonica*, but also to other unrelated pollens such as orchard grass, short ragweed, mugwort, and Japanese alder. There was no significant difference regarding multiple sensitization to unrelated pollens between patients with or without sensitization to pollen-related carbohydrates. This result suggests that M3FX and/or GN2M3FX is not acting significantly as CCD in Japanese cedar pollinosis.

The role of carbohydrates on allergens in the biological implications of IgE-mediated reactions remains controversial [9, 10, 13–15]. Natural and recombinant PLA2 show similar histamine-releasing capability [13]. And recently, Mari et al. [15] reported that positive ELISA results of antigens eliciting negative skin test responses could be ascribed to IgE for carbohydrate epitopes. On the other hand, blood cells from patients with olive pollinosis gave specific histamine release after stimulation of isolated carbohydrate from Ole e 1. And there was no observed histamine release by basophils from Cupressaceae-allergic subjects whose reactivity was essentially a reactivity against carbohydrate moiety after stimulation with recombinant Cup a 1 [10]. Further, more recently, Foetisch et al. [39] reported that some patients with tomato allergy have biologically relevant CCD-specific IgE. In order to determine whether the recognition of oligosaccharides on Cry j 1 are involved in the biologic activity, we divided the patients according to their reactivity in skin scratch test to *C. japonica* pollen extract. No significant correlation was observed between the reactivity in skin scratch test against Japanese cedar pollen and sensitization to M3FX and/or GN2M3FX, suggesting that these oligosaccharides are not important in these biological activities. Further analysis such as histamine release assay using these oligosaccharides and selection of subjects with positive IgE in CAP and negative symptoms against *C. japonica* may be required for precise investigation.

In the present study, we stimulated Cry j 1-specific T cells with M3FX, the core oligosaccharide on Cry j 1 containing α 1-3 fucose and β 1-2 xylose (Fig. 3). We have previously shown

that 18 out of 19 T cell clones specific for Cry j 1 showed significant proliferative responses as indicated by values of stimulation index greater than 2, suggesting that carbohydrates on Cry j 1 are not the major T cell epitopes [26]. The result presented here that Cry j 1-specific T cell lines did not display proliferation or cytokine production in response to M3FX is consistent with our former report as well as the report by Dudler et al. [16] that carbohydrate-dependent T cell clones acting against PLA2 did not respond to asparagine-linked oligosaccharide alone. Although the carbohydrate structures of Cry j 1 and PLA2 are not similar, e.g. with respect to the presence or absence of β 1-2 xylose, these results suggest that the oligosaccharides on allergens appear not to be the major T cell epitopes [23, 24, 40]. In fact, we have previously found six major T cell epitopes (p16–30, p81–95, p106–120, p111–125, p211–225, and p301–315) in Cry j 1, and no glycosylation sites exist adjacent to these T cell epitopes [27].

On the other hand, addition of M3FX inhibited the Cry j 1-specific T cell responses of proliferation and IL-4 production (Fig. 5). Our study has for the first time demonstrated that monovalent oligosaccharide can regulate allergen-specific T cell responses. It is known that monovalent oligosaccharides can inhibit cellular responses against glycoproteins. For example, Velupillai and Harn [41] reported that, in spleen cells from mice infected with *Schistosoma mansoni*, proliferation in response to human serum albumin conjugated with lacto-*N*-fucopentaose III (LNFIII) and lacto-*N*-neotetraose (LNnT) could be inhibited by the addition of monovalent LNFIII and LNnT, respectively, in a sugar-specific manner. In addition, M3FX did not inhibit proliferation and cytokine production in PPD-specific TCLs. PPD may contain several immunogenic glycoproteins such as Apa [42]. Although the composition and structure of carbohydrates of Cry j 1 and PPD may differ, these results suggest that M3FX selectively regulates cellular responses against glycoproteins containing this oligosaccharide [23, 24, 43]. Result that addition of M3FX did not affect Cry-consensus-specific T cell responses (Fig. 5) may support this since Cry-consensus is a prokaryotically expressed polypeptide and did not contain carbohydrates. This in turn suggests the explanation that carbohydrate-specific ligand on antigen presenting cells and/or T cells can affect Cry j 1-specific T cell responses [44]. However, we have previously reported that a blockade of mannose receptor (MR), one of the most characteristic carbohydrate-specific receptors, induced few changes in Cry j 1-specific T cell responses, suggesting that the involvement of MR was negligible [26]. Identification of the receptor specific for M3FX must be made in further investigation. It then may be found that this orphan receptor is not simply involved in antigen uptake but also controls Th differentiation, since the addition of M3FX affects the IL-4 but not IFN- γ production by Cry j 1-stimulated TCLs.

In summary, we have provided *in vitro* evidence that pollen-related oligosaccharides from Cry j 1 are not the major IgE or T cell epitopes. However, monovalent core oligosaccharide containing α 1-3 fucose and β 1-2 xylose selectively inhibits Cry j 1-specific T cell responses. These observations may provide a basis for future therapeutic approaches in the management of pollinosis by using monovalent oligosaccharides since these oligosaccharides do not induce IgE reaction in the majority of patients.

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References

- Okano M, Statoskar AR, Nishizaki K, Abe M, Harn DA. Induction of Th2 responses and IgE is largely due to carbohydrates functioning as adjuvants on *Schistosoma mansoni* egg antigen. *J Immunol* 1999; 163:6712–7.
- Okano M, Satoskar AR, Nishizaki K, Harn DA. Lacto-*N*-fucopentaose III found on *Schistosoma mansoni* egg antigen functions as adjuvant for proteins by inducing Th2-type responses. *J Immunol* 2001; 167:442–50.
- Yamashita Y, Okano M, Yoshino T et al. Carbohydrate expressed on *Aspergillus fumigatus* induce *in vivo* allergic Th2-type response. *Clin Exp Allergy* 2002; 32:776–82.
- Okano M, Nishizaki K, Satoskar AR, Yoshino T, Masuda Y, Harn DA. Involvement of carbohydrate on phospholipase A2, a bee venom, in *in vivo* antigen-specific IgE synthesis in mice. *Allergy* 1999; 54:811–8.
- Weber A, Schroder H, Thalberg K, Marz L. Specific interaction of IgE antibodies with a carbohydrate epitope of honeybee phospholipase A2. *Allergy* 1987; 42:464–70.
- Ohta N, Matsuura F, Kobayashi Y, Shigeta S, Ono K, Oka S. Further characterization of allergenically active oligosaccharitols isolated from a sea squirt H-antigen. *Arch Biochem Biophys* 1991; 290:474–83.
- van Ree R, Hoffman DR, van Dijk W et al. Lol p XI, a new major grass pollen allergen, is a member of a family of soybean trypsin inhibitor-related proteins. *J Allergy Clin Immunol* 1995; 95:970–8.
- Su S, Shu P, Lau G, Yang S, Huang S, Lee Y. Immunologic and physicochemical studies of Bermuda grass pollen antigen BG60. *J Allergy Clin Immunol* 1996; 98:486–94.
- Batanero E, Crespo JF, Monsalve RI, Martin-Esteban M, Villalba M, Rodriguez R. IgE-binding and histamine release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen. *J Allergy Clin Immunol* 1999; 103:147–53.
- Iacovacci P, Afferni C, Butteroni C et al. Comparison between the native glycosylated and recombinant Cup a 1 allergen: role of carbohydrates in the histamine release from basophils. *Clin Exp Allergy* 2002; 32:1620–7.
- Batanero E, Vilalba M, Monsalve RI, Rodriguez R. Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen. *J Allergy Clin Immunol* 1996; 97:1264–71.
- van Ree R, Cabanes-Macheteau M, Akkerdaas J et al. Beta (1,2)-xylose and alpha (1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J Biol Chem* 2000; 275:11451–8.
- Forster E, Dudler T, Gmachl M, Aberer W, Urbanek R, Suter M. Natural and enzymatically active or inactive bee venom phospholipase A2 has the same potency to release histamine from basophils in patients with hymenoptera allergy. *J Allergy Clin Immunol* 1995; 95:1229–35.
- van der Veen MJ, van Ree R, Aalberse RC et al. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J Allergy Clin Immunol* 1997; 100:327–34.
- Mari A, Iacovacci P, Afferni C et al. Specific IgE to cross-reactive carbohydrate determinants strongly affect the *in vitro* diagnosis of allergic diseases. *J Allergy Clin Immunol* 1999; 103:1005–11.
- Dudler T, Altmann F, Carballido JM, Blaser K. Carbohydrate-dependent, HLA class II-restricted human T cell response to the bee venom allergen phospholipase A2 in allergic patients. *Eur J Immunol* 1995; 25:538–42.
- Corinti S, Palma RD, Fontana A, Gagliardi C, Pini C, Sallusto F. Major histocompatibility complex-independent recognition of a distinctive pollen antigen, most likely a carbohydrate, by human CD8+ a/b T cells. *J Exp Med* 1997; 186:899–908.
- Schenk S, Hoffmann-Sommergruber K, Breiteneder H et al. Four recombinant isoforms of Cor a 1, the major allergen of hazel pollen, show different reactivities with allergen-specific T-lymphocyte clones. *Eur J Biochem* 1994; 224:717–22.
- Wurtzen PA, Wissenbach M, Ipsen H, Bufer A, Arned J, van Neerven RJJ. Highly heterogeneous Phl p 5-specific T cells from patients with allergic rhinitis differentially recognize recombinant Phl p 5 isoallergens. *J Allergy Clin Immunol* 1999; 104:115–22.
- Nishihata S, Inouye S, Saiga T et al. Prevalence rate of allergy to Japanese cedar pollen in Tokyo – from field investigation in 1996 by Tokyo Japanese Cedar Pollen Allergy Measurements and Review Committee. *Arerugi* 1999; 48:597–604.
- Yasueda H, Yui Y, Shimizu T, Shida T. Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. *J Allergy Clin Immunol* 1983; 71:77–86.
- Hashimoto M, Nigi H, Sakaguchi M et al. Sensitivity to two major allergens (Cry j I and Cry j II) in patients with Japanese cedar (*Cryptomeria japonica*) pollinosis. *Clin Exp Allergy* 1995; 25:848–52.
- Sone T, Komiyama N, Shimizu K, Kusakabe T, Morikubo K, Kino K. Cloning and sequencing of cDNA coding for Cry j I, a major allergen of Japanese cedar pollen. *Biochem Biophys Res Commun* 1994; 199:619–25.
- Ogawa H, Hijikata A, Amano M et al. Structures and contribution to the antigenicity of oligosaccharides of Japanese cedar (*Cryptomeria japonica*) pollen allergen Cry j I: relationship between the structures and antigenic epitopes of plant N-linked complex-type glycans. *Glycoconj J* 1996; 13:555–66.
- Taniai M, Kayano T, Takakura R et al. Epitopes on Cry j I and Cry j II for the human IgE antibodies cross-reactive between *Cupressus sempervirens* and *Cryptomeria japonica* pollen. *Mol Immunol* 1993; 30:183–9.
- Okano M, Kino K, Takishita T et al. Roles of carbohydrates on Cry j I, the major allergen of Japanese cedar pollen, in specific T cell responses. *J Allergy Clin Immunol* 2001; 108:101–8.
- Sone T, Morikubo K, Miyahara M et al. T cell epitopes in Japanese cedar (*Cryptomeria japonica*) pollen antigens: Choice of major T cell epitopes in Cry j 1 and Cry j 2 toward design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis. *J Immunol* 1998; 161:448–57.
- Kimura Y, Suzuki M, Kimura M. N-linked oligosaccharides of glycoproteins from *Ginkgo biloba* pollen, an allergenic pollen. *Biosci Biotechnol Biochem* 2001; 65:2001–6.
- Kimura Y, Hase S, Kobayashi Y, Kyogoku Y, Ikenaka T, Funatsu G. Structures of sugar chains of *Ricinus communis* agglutinin. *Biochim Biophys Acta* 1988; 966:248–56.
- Kimura Y, Ohno A, Takagi S. Structural analysis of N-glycans of storage glycoproteins in soybean (*Glycine max.*) seed. *Biosci Biotechnol Biochem* 1997; 61:1866–71.
- Kondo A, Suzuki J, Kuraya N, Hase S, Kato I, Ikenaka T. Improved method for fluorescence labeling of sugar chains with sialic acid residues. *Agric Biol Chem* 1990; 54:2169–70.

- 32 Pastorello EA, Incorvaia C, Pravettoni V et al. A multicentric study on sensitivity and specificity of a new *in vitro* test for measurement of IgE antibodies. *Ann Allergy* 1991; 67:365–70.
- 33 Okano M, Nishizaki K, Abe M et al. Strain-dependent induction of allergic rhinitis without adjuvant in mice. *Allergy* 1999; 54:593–601.
- 34 Okano M, Nagano T, Ono T, Masuda Y, Ohta N. Clonal analysis of human T-cell responses to fractionated house dust mite antigens (*Dermatophagoides pteronyssinus*). *Int Arch Allergy Immunol* 1993; 101:82–8.
- 35 Calabozo B, Barber D, Polo F. Studies on the carbohydrate moiety of Pla 1.1 allergen. Identification of a major *N*-glycan and significance for the immunoglobulin E-binding activity. *Clin Exp Allergy* 2002; 32:1628–34.
- 36 Akdis CA, Blesken T, Wymann D, Akdis M, Blaser K. Differential regulation of human T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants. *Eur J Immunol* 1998; 28:914–25.
- 37 Iacovacci P, Pini C, Afferni C et al. A monoclonal antibody specific for a carbohydrate epitope recognizes an IgE-binding determinant shared by taxonomically unrelated allergic pollens. *Clin Exp Allergy* 2001; 31:458–65.
- 38 Okano M, Nishioka K, Nagano T, Ohta N, Masuda Y. Clinical characterization of allergic patients sensitized to *Camaecyparis obtusa* – using AlaSTAT system. *Alerugi* 1994; 43:1179–84.
- 39 Foetisch K, Westphal S, Lauer I et al. Biological activity of IgE specific for cross-reactive carbohydrate determinants. *J Allergy Clin Immunol* 2003; 111:889–96.
- 40 Kubelka V, Altmann F, Staudacher E, Tretter V, Marz L, Hard K et al. Primary structures of the *N*-linked carbohydrate chains from honeybee venom phospholipase A2. *Eur J Biochem* 1993; 213:1193–204.
- 41 Velupillai P, Harn DA. Oligosaccharide-specific induction of interleukin 10 production by B220⁺ cells from schistosome-infected mice: a mechanism for regulation of CD4⁺ T-cell subsets. *Proc Natl Acad Sci USA* 1994; 91:18–22.
- 42 Romani F, Horn C, Pescher P et al. Deglycosylation of the 45/47-kilodalton antigen complex of *Mycobacterium tuberculosis* decreases its capacity to elicit *in vivo* or *in vitro* cellular immune responses. *Infect Immun* 1999; 67:5567–72.
- 43 Dobos KM, Khoo KH, Swiderek KM, Brennan PJ, Belisle JT. Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. *J Bacteriol* 1996; 178:2498–506.
- 44 Stahl PD. The mannose receptor and other macrophage lectins. *Curr Opin Immunol* 1992; 4:49–52.