

Roles of carbohydrates on Cry j 1, the major allergen of Japanese cedar pollen, in specific T-cell responses

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Background: Carbohydrates expressed on allergens are known to be important for allergenicity. However, little is known about whether the carbohydrates drive the T_H2 response.

Objective: We sought to determine a role for carbohydrates expressed on Cry j 1, which is the major allergen of *Cryptomeria japonica* pollen and causes the most prevalent pollinosis in Japan, in *in vitro* cellular responses.

Methods: Carbohydrates on Cry j 1 were destroyed by periodate-oxidation under mild conditions. Proliferative responses and cytokine productions against native, periodate-treated, and mock-treated Cry j 1 were compared in peripheral blood mononuclear cells, Cry j 1-specific T-cell lines, and clones from patients with Japanese cedar pollinosis.

Results: We found that peripheral blood mononuclear cells from patients with Japanese cedar pollinosis displayed a significant decrease in proliferation and IL-5 production in response to periodate-treated Cry j 1 in comparison with native and mock-treated Cry j 1. Decreased proliferative responses against periodate-treated Cry j 1 were also seen in polyclonal T-cell lines, and the responses showed a heterogeneity. In addition, Cry j 1-specific CD4+ T-cell clones also displayed a significant decrease in proliferation and IL-4 and IL-5 production—but not IFN- γ production—in comparison with the control antigens. However, most of the clones showed decreased but positive proliferative responses against periodate-treated Cry j 1. Blockade of the mannose receptor had no effect on cellular responses.

Conclusion: The results suggest that carbohydrates on Cry j 1 play a major role in promoting Cry j 1-specific T_H2 response *in vitro*, though they are not major targets as T-cell epitopes. (J Allergy Clin Immunol 2001;108:101-8.)

Key words: Allergen, carbohydrate, Cry j 1, T cell, clones, T_H2, mannose receptor

Carbohydrates expressed on allergens are known to participate in both initiation and exacerbation of allergic

Abbreviations used

APC:	Antigen-presenting cell
CM:	Culture medium
PBMC:	Peripheral blood mononuclear cell
PBMCx:	Irradiated autologous PBMC
TCC:	T-cell clone
TCL:	T-cell line
TCR:	T-cell receptor

responses. Several carbohydrates on allergens are recognized by specific IgE and occasionally function as cross-reactive carbohydrate determinants.¹⁻⁴ Carbohydrates on allergens can elicit specific T-cell responses. For example, carbohydrates on phospholipase A2 from honeybee venom⁵ and pollen extract from *Parietaria judaica*⁶ were recognized by CD4+ and CD8+ T cells, respectively. In addition, we have recently demonstrated that several carbohydrates on allergens were essential for the initiation of T_H2 response *in vivo*.^{7,8} Furthermore, a recent study revealed that alveolar macrophages phagocytosed allergen-containing pollen starch granules via C-type lectins.⁹

Japanese cedar pollinosis is the most prevalent allergic disease in Japan. A recent epidemiologic study demonstrated that approximately 20% of people living in urban areas have the disease.¹⁰ Cry j 1 is one of the major allergens in Japanese cedar pollen, and up to 95% of patients have Cry j 1-specific IgE in serum.^{11,12} Cry j 1 is known as a glycoprotein with a 6% (wt/wt) carbohydrate content.¹¹ Five possible N-linked glycosylation sites have been found in the primary amino acid sequence of this allergen.¹³ Furthermore, an N-linked oligosaccharide structure of Cry j 1 has been proposed.¹⁴ Inhibition assay revealed that carbohydrates on Cry j 1 did not act as epitopes for specific IgE in humans.¹⁴ However, little is known about whether the carbohydrate moiety expressed on Cry j 1 is involved in Cry j 1-specific cellular immunity.

In the present study, we investigated the role of carbohydrates on Cry j 1 in specific T-cell recognition and activation by comparing the cellular responses caused by native and carbohydrate-destroyed Cry j 1 stimulation. We believe that the findings presented here might provide new insights not only into the pathogenesis of Japanese cedar pollinosis but also into the differences in cellular responses against pollens.⁶

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Supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (12770969).

Received for publication January 14, 2001; revised March 13, 2001; accepted for publication March 14, 2001.

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0091-6749/2001 \$35.00 + 0 1/83/115757

doi:10.1067/mai.2001.115757

METHODS

Subjects

A total of 16 Japanese patients (7 male and 9 female; age range, 19 to 66 years; mean age, 37.1 years) with Japanese cedar pollinosis were examined. Informed consent was obtained from each subject. The patients showed elevations of serum IgE specific for Japanese cedar pollen in CAP-RAST (Pharmacia, Uppsala, Sweden) ranging from 1.08 to 46.20 UA/mL (mean, 17.68 UA/mL). None of the patients used immunosuppressive drugs or underwent immunotherapy during the study. Proliferative responses of peripheral blood mononuclear cells (PBMCs) against Cry j 1 antigens were determined in all of the subjects. Moreover, the number of samples for further investigation, such as cytokine production assay, was based on the amount and frequency of individual blood donation.

Preparation of Cry j 1 and periodate-oxidation

Cry j 1 was purified from crude extracts of *Cryptomeria japonica* pollen in a well-established procedure.¹¹ Periodate-oxidation was performed under mild conditions as described previously,⁸ and the resulting Cry j 1 was then dialyzed against PBS for 3 days at 4°C. Mock-treated Cry j 1 was produced by following the identical periodate-oxidation protocol but with the omission of sodium metaperiodate (Sigma Chemical, St Louis, Mo). Mock-treated Cry j 1 was used as a buffer control of the periodate-oxidized Cry j 1. Protein concentrations of these antigens were determined by means of bicinchoninic acid assay (Pierce, Rockford, Ill) according to the manufacturer's instructions.

Binding activity of mAbs against peptides representing major T-cell epitopes on Cry j 1 to Cry j 1 antigens

To determine whether the periodate treatment can alter the antigenicity of peptide moieties, we used 2 mouse mAbs, K8.238 (IgG1/κ) and G1.635 (IgG1/κ), which recognize major human T-cell epitopes p211-225 (KSMKVTVAFNQFGPN) and p81-95 (KMP-MYIAGYKTFDGR) on Cry j 1, respectively,¹⁵ for binding ELISA. Each well of a 96-well ELISA plate (Corning Laboratories, Corning, NY) was coated with 2 μg/mL of native, periodate-treated, and mock-treated Cry j 1 in 0.05 mol/L carbonate buffer, pH 9.6 (Sigma), and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBS/Tween 20, Sigma) and blocked with PBS containing 10% FCS (Gibco BRL, Grand Island, NY) for 2 hours at 37°C. K8.238 and G1.635 were then plated in triplicate in 10-fold serial dilution, beginning at 1:100, and incubated for 2 hours at 37°C. Bound antibodies were detected by incubation with goat antimouse IgG1 diluted to 1/4000 (Boehringer-Mannheim, Indianapolis, Ind). After a further incubation period at 37°C for 1 hour, tetramethylbenzidine substrate solution (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, Md) was added to the plates. The reaction was stopped by 5% phosphoric acid after approximately 10 minutes, and the optical density at 450 nm was read on a microplate reader (Bio-Rad Laboratories, Hercules, Calif).

Peripheral blood mononuclear cell proliferation and cytokine production assay

PBMCs were separated by Ficoll-Paque (Pharmacia-Upjohn, Uppsala, Sweden) density gradient centrifugation from heparinized blood of the 16 patients during the pollen season (March through April) in 1999. The culture medium (CM) used throughout the study was RPMI1640 (Sigma) supplemented with 10% human AB serum (ICN Biomedicals, Aurora, Ohio), 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 mmol/L l-glutamin (Gibco BRL). All cultures were performed in triplicate. Antigen-driven proliferation and

cytokine production of PBMCs were tested in vitro, as described previously.¹⁶ Results are shown as stimulation index (SI) values, the SI being calculated as the ratio of the mean counts per minute (cpm) obtained in the presence and the absence of antigens. Culture supernatants were collected after 72 hours' incubation with or without antigens and stored at -80°C until the assay for cytokine production.

Characterization of Cry j 1-specific T-cell lines and T-cell clones

Short-term, cultured, Cry j 1-specific T-cell lines (TCLs) were generated from PBMCs of 7 patients during the pollen season in 1999. First, 2×10^6 PBMCs were cultured with 10 μg/mL native Cry j 1 in 1.5 mL CM in 24 well plates (Corning) for 7 days. T-cell blasts were then washed twice with RPMI1640 and resuspended with CM containing 20 U/mL human recombinant IL-2 (Boehringer-Mannheim GmbH, Mannheim, Germany) (IL-2 CM) for an additional 7 days. One half of the IL-2 CM was replaced 3 days after the resuspension. Then 2×10^5 blastoid T cells were restimulated with 2 μg/mL native Cry j 1 and 1×10^6 30-Gy-irradiated autologous PBMCs (PBMCx) in 1.5 mL of IL-2 CM. Cells were cultured for an additional 2 weeks, and one half of the IL-2 CM was replaced every 3 days. The established TCLs were then used for proliferation and cytokine production assays. In addition, T-cell clones (TCCs) were generated from 3 patients by micromanipulation methods, as described previously.¹⁷

Antigen-driven proliferation and cytokine production of Cry j 1-specific TCLs and TCCs were tested in vitro, as described previously.⁷ To determine the restriction molecule, 2×10^4 T-cell blasts mixed with 1×10^5 autologous PBMCx and 10 μg/mL native Cry j 1 in 0.2 mL of CM were cultured with anti-DR (L243), anti-DQ (Leu-10), or anti-DP (B7/21) mAb (Beckton-Dickinson, Mountain View, Calif) concentrated at 0.6 μg/mL for 72 hours. Proliferative responses were determined as described above.

The phenotypes of TCLs and TCCs were determined by means of flow cytometry through use of a FACSCalibur with CellQuest software (Beckton, Dickinson) after staining with FITC-conjugated anti-CD4 and phycoerythrin-conjugated anti-CD8 mAb (Dako Japan, Kyoto, Japan).

Cytokine determination

Levels of IL-4, IL-5, and IFN-γ in culture supernatants were measured by means of Opt EIA sets (PharMingen, San Diego, Calif) according to the manufacturer's instructions. The detection limits in these assays were 3 pg/mL for IL-4, 10 pg/mL for IL-5, and 20 pg/mL for IFN-γ.

Statistical analysis

Data were analyzed through use of the Bartlett test followed by the Wilcoxon signed-rank test. *P* values less than .05 were considered statistically significant. Each value was given as the mean ± SEM.

RESULTS

Treatment with sodium metaperiodate does not affect the antigenicity of peptides representing major human T-cell epitopes

The 2 mouse mAbs, K8.238 and G1.635, could bind to both periodate-treated Cry j 1 and mock-treated Cry j 1 at the same magnitude (Fig 1). These results indicate that the antigenicities of peptide moieties on periodate-treated and mock-treated Cry j 1 were similar. In addition, the binding of G1.635 to native Cry j 1 was strong in comparison with the binding to mock-treated Cry j 1 (Fig 1, B), suggesting

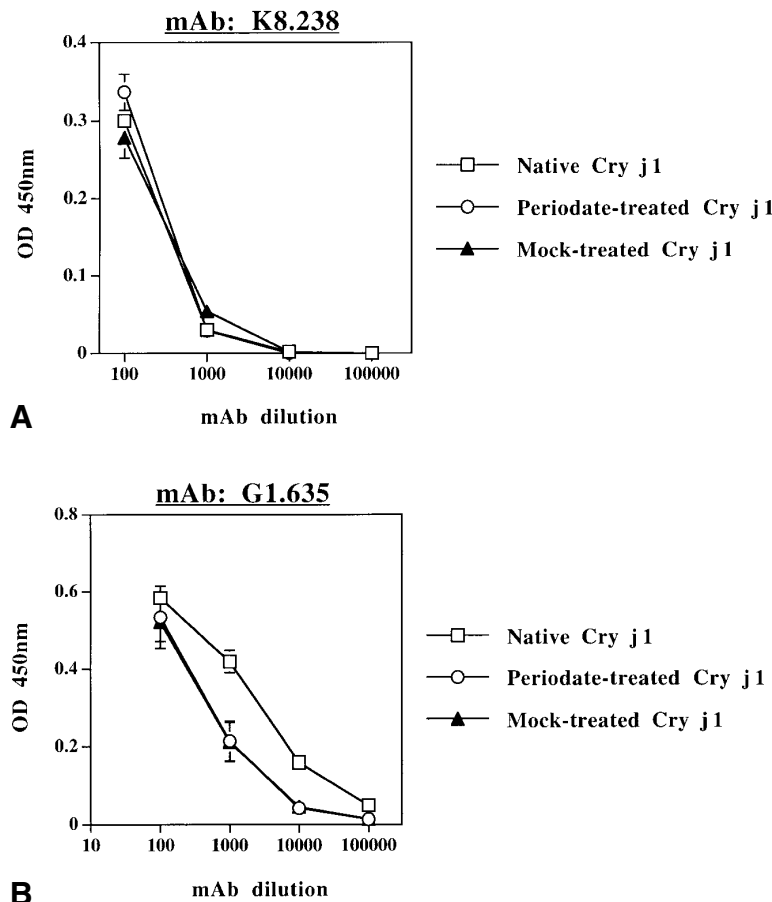


FIG 1. Effect of periodate treatment on antigenicity of peptides representing major human T-cell epitopes. Binding of 2 mAbs K8.238 (A) and G1.635 (B), which recognize peptides representing human T-cell epitopes,¹⁵ to native (open square), periodate-treated (open circle), and mock-treated (closed triangle) Cry j 1 was determined by ELISA, as described in Methods section. Data are representative for 2 separate experiments.

that 1 of 2 peptides representing major T-cell epitopes reduced the antigenicity by the dialysis with acetate buffer.

Responses of PBMC against carbohydrates on Cry j 1

In our preliminary study, PBMCs from 4 allergic patients with Japanese cedar pollinosis showed proliferative responses to native Cry j 1 in a dose-dependent manner, and all samples showed positive responses to the Ag at 10 μ g of protein per milliliter (data not shown). We therefore used this protein concentration for further analysis.

During the 1999 pollen season (March through April), PBMCs of 16 allergic patients were collected and stimulated in vitro with native, periodate-treated, or mock-treated Cry j 1. All patients except 2 showed positive responses to native Cry j 1 (mean SI, 8.54 ± 2.29). Mock-treated Cry j 1 induced amounts of the responses (mean SI, 7.35 ± 2.02) similar to those induced in response to native Cry j 1 ($P = .756$ by Wilcoxon signed-rank test). However, the responses against periodate-treated Cry j 1

were significantly decreased (mean SI, 3.83 ± 1.15) in comparison with native ($P = .001$) and mock-treated ($P = .002$) Cry j 1 (Fig 2).

Next, we investigated in vitro IL-4, IL-5, and IFN- γ production of PBMCs against Cry j 1 stimulation. Although PBMCs from 3 and 5 of 11 patients produced detectable levels of IL-4 and IFN- γ , respectively, in response to native Cry j 1, mean levels of these cytokines were not significant in comparison with nonstimulation (IL-4, $P > .999$; IFN- γ , $P = .722$). On the contrary, a significant production of IL-5 was induced in in vitro culture with Cry j 1 antigens. The mean concentration of IL-5 after 72 hours without antigen stimulation was 17.56 ± 7.20 pg/mL, whereas the concentrations in response to native, periodate-treated, and mock-treated Cry j 1 were 224.84 ± 49.85 pg/mL ($P = .003$), 184.03 ± 58.90 pg/mL ($P = .006$), and 251.82 ± 73.19 pg/mL ($P = .003$), respectively. Interestingly, the production in response to periodate-treated Cry j 1 was significantly decreased in comparison with the production in response to native Cry j 1 ($P = .051$) and mock-treated Cry j 1 ($P = .003$; Fig 3).

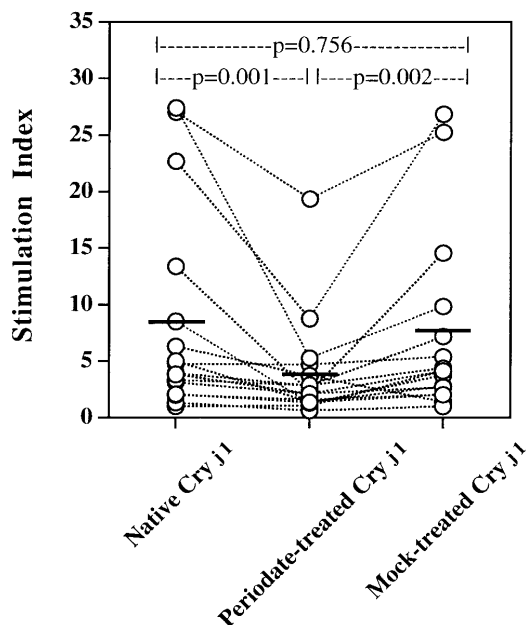


FIG 2. Proliferative responses of PBMCs against stimulation with Cry j 1 antigens. PBMCs from 16 allergic patients were cultured with 10 μ g of protein per milliliter of native, periodate-treated, or mock-treated Cry j 1 for 7 days. For the final 16 hours, ^{3} H-thymidine was added to each well. After harvest of the cells, incorporation of ^{3} H-thymidine was measured. Results are shown as SI values. Background cpm ranged from 237 to 2230 (mean 795 ± 152). *P* value was obtained through use of Wilcoxon signed-rank test.

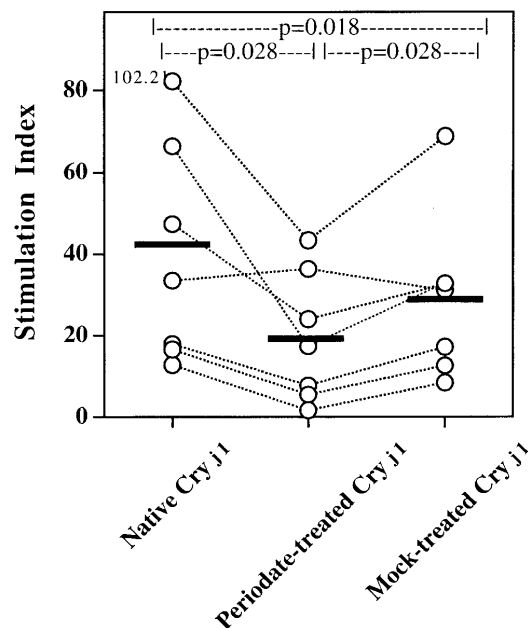


FIG 4. Proliferative responses of TCLs against stimulation with Cry j 1 antigens. Seven short-term TCLs from different allergic patients were cultured with 10 μ g of protein per milliliter of native, periodate-treated, or mock-treated Cry j 1 for 3 days. ^{3} H-thymidine was added, and incorporation of ^{3} H-thymidine was measured as described above. Results are shown as SI values. Background cpm ranged from 55 to 806 (mean 356 ± 271). *P* value was obtained through use of Wilcoxon signed-rank test.

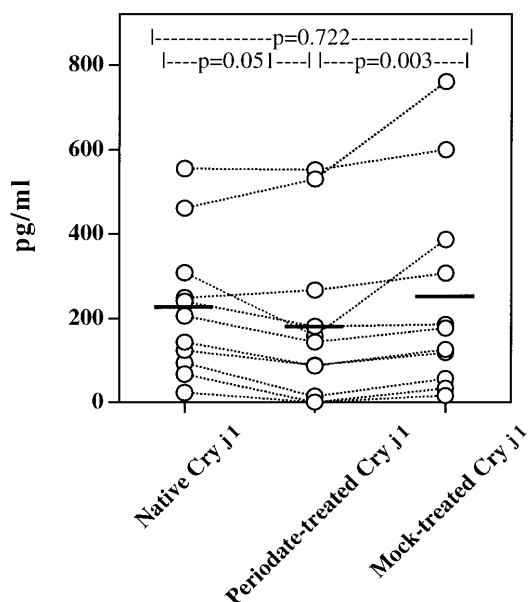


FIG 3. IL-5 production of PBMCs against stimulation with Cry j 1 antigens. PBMCs from 11 allergic patients were cultured with 10 μ g of protein per milliliter of native, periodate-treated, or mock-treated Cry j 1 for 3 days. After incubation, supernatants were collected and IL-5 concentration was determined by means of ELISA. *P* value was obtained through use of Wilcoxon signed-rank test.

Response of Cry j 1-specific T-cell lines to carbohydrates on Cry j 1

We generated 7 Cry j 1-specific TCLs from 7 different individuals. The CD4/CD8 ratios for these TCLs ranged from 1.70 to 110.28 (mean, 23.36 ± 15.16). All TCLs except 1 showed a decreased proliferative response to periodate-treated Cry j 1 in comparison with native or mock-treated Cry j 1 (Fig 4). The mean SI values for proliferative responses of TCLs against 10 μ g of protein per milliliter of native, periodate-treated, and mock-treated Cry j 1 were 42.39 ± 12.34 , 19.45 ± 6.04 , and 29.09 ± 7.64 , respectively. These results showed that periodate-treated Cry j 1 was poorly immunogenic for the induction of proliferative responses of specific TCLs in comparison with native (*P* = .028) and mock-treated (*P* = .028) Cry j 1.

Recognition of carbohydrates on Cry j 1 by T-cell clones

The results described above enabled us to determine whether carbohydrates on Cry j 1 could be epitopes for specific T cells; thus we generated a panel of Cry j 1-specific TCCs from 3 patients. We established 7, 3, and 9 TCCs from patients IM, MO, and NK, respectively; cloning efficiency was 17.5%, 10.0%, and 15.0% for patients IM, MO, and NK, respectively. All of the TCCs generated were CD4+, CD8-, and T-cell receptor (TCR) $\alpha\beta$ + (data not shown). As a whole, the mean SI values for the proliferative responses of 19 TCCs to native, periodate-

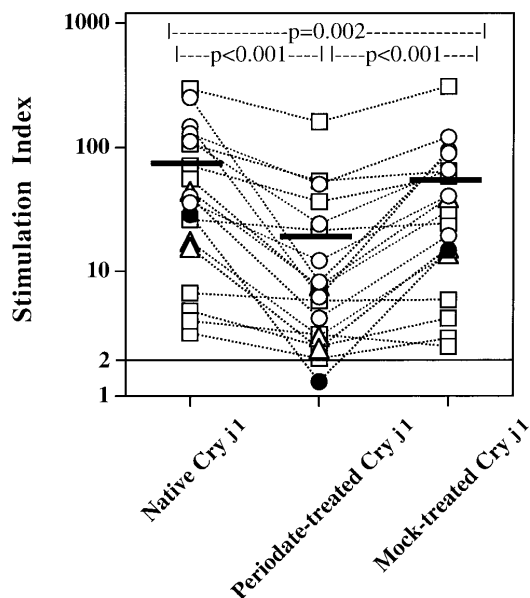


FIG 5. Proliferative responses of 19 Cry j 1-specific TCCs against stimulation with Cry j 1 antigens. Results are shown as SI values. Background cpm ranged from 38 to 2479 (mean 557 ± 156). Clones from patients IM, MO, and NK are indicated with circles, triangles, and squares, respectively; among these, IM1.3 is indicated with closed circles.

treated, and mock-treated Cry j 1 were 73.05 ± 19.17 , 21.69 ± 8.54 , and 53.33 ± 16.20 , respectively (Fig 5). These results indicated a significant decrease of proliferation in response to periodate-treated Cry j 1 in comparison with native ($P < .001$) and mock-treated ($P < .001$) Cry j 1.

Seven TCCs from patient IM and 8 TCCs from patient NK were investigated for IL-4, IL-5, and IFN- γ production after in vitro stimulation with native, periodate-treated, and mock-treated Cry j 1. All TCCs produced detectable levels of IL-4 and IL-5 in response to native Cry j 1, whereas only 1 TCC produced a marginal amount of IFN- γ (26.3 pg/mL), suggesting that most TCCs were T_H2 subsets. As in the results seen in the proliferative responses, production of both IL-4 and IL-5 by TCCs was significantly decreased in response to periodate-treated Cry j 1 in comparison with native and mock-treated Cry j 1 (Fig 6). The mean concentrations of IL-4 after stimulation with native, periodate-treated, and mock-treated Cry j 1 were 43.47 ± 10.36 , 9.61 ± 2.79 , and 27.51 ± 8.06 pg/mL, respectively. In addition, the mean concentrations of IL-5 after stimulation with native, periodate-treated, and mock-treated Cry j 1 were 800.96 ± 229.76 , 140.42 ± 66.68 , and 581.92 ± 187.71 pg/mL, respectively.

Although TCCs showed a decreased proliferative response to periodate-treated Cry j 1, 18 of 19 TCCs showed significant proliferative responses, as indicated by SI values greater than 2; this suggests that carbohydrates on Cry j 1 were not the major T-cell epitopes. However, we did establish 1 TCC, designated IM1.3, that showed carbohydrate-dependent recognition of Cry j 1 (Fig 5). IM1.3 did not recognize periodate-treated Cry j 1 even at the concentration of 100 μ g of protein per mil-

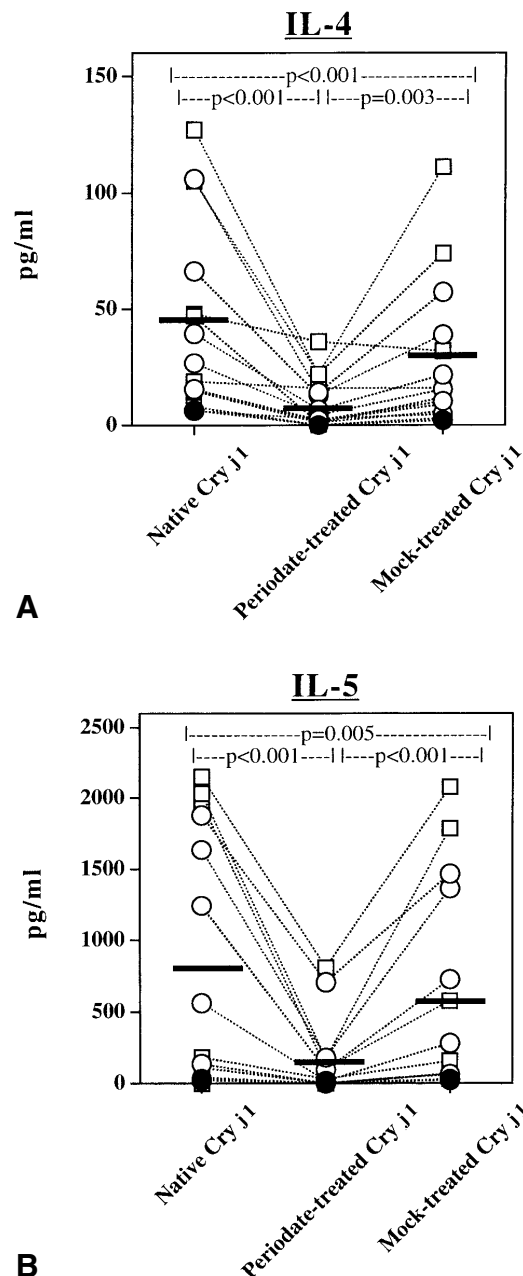


FIG 6. IL-4 (A) and IL-5 (B) production of Cry j 1-specific TCCs against stimulation with Cry j 1 antigens. Seven clones from patient IM and 8 clones from patient NK were cultured with 10 μ g of protein per milliliter of native, periodate-treated, or mock-treated Cry j 1 for 3 days. After incubation, supernatants were collected and cytokines were determined by means of ELISA. *P* value was obtained through use of Wilcoxon signed-rank test. Clones from patients IM and NK are indicated with circles and squares, respectively; among these, IM1.3 is indicated with closed circles.

liliter (SI = 1.26). This clone produced IL-4 and IL-5 but not IFN- γ , although the amounts were relatively smaller than those produced by other TCCs that could recognize periodate-treated Cry j 1 (Fig 6). In addition, inhibition assay revealed that IM1.3 was restricted by HLA-DR, which is similar to other TCCs (data not shown).

Involvement of mannose receptor in specific T-cell responses

Finally, we sought to determine whether the carbohydrate moiety of Cry j 1 might be involved in an effective antigen presentation via a carbohydrate-specific receptor. The mannose receptor (MR) is one of the most well-characterized lectinlike receptors that are found mainly on macrophages.¹⁸ We therefore investigated whether the blockade of MR could inhibit the in vitro antigen-specific activation of Cry j 1-specific T cells. Proliferative responses of specific T cells against native Cry j 1 stimulation by the addition of anti-MR mAbs were similar to those induced by the addition of control mouse IgG1. For example, the SI values of TCL IM2 on 10 µg/mL native Cry j 1 stimulation in the presence of 50 µg/mL anti-MR mAb and of 50 µg/mL mouse IgG1 were 57.57 and 61.70, respectively. These results suggest that the involvement of MR was negligible in Cry j 1-specific T-cell responses.

DISCUSSION

The present study demonstrates that carbohydrates on Cry j 1 selectively promote Ag-specific T_H2 responses in Cry j 1-specific TCLs and TCCs as well as the PBMCs of patients with Japanese cedar pollinosis. Surprisingly, few reports have demonstrated roles of carbohydrates expressed on allergens in specific cellular responses using PBMCs, although several reports have demonstrated such roles using allergen-specific TCCs.^{5,6} PBMCs are considered to reflect the physiological in vivo situation better than cultured T cells, though the magnitude of the cellular responses is smaller.¹⁵⁻¹⁷ PBMCs from patients with Japanese cedar pollinosis displayed a significant decrease in proliferation and IL-5 production in response to periodate-treated Cry j 1 in comparison with native and mock-treated Cry j 1, suggesting that carbohydrates on Cry j 1 are involved in antigen-specific T_H2 response in PBMCs. The level of IL-5 produced by CD4+ T cells in primary culture is known to correlate with allergic disease severity.¹⁹ Thus carbohydrates on Cry j 1 might participate in the allergic inflammation and subsequent illness severity in Japanese cedar pollinosis.

A study using short-term, cultured, Cry j 1-specific polyclonal TCLs confirmed the results seen in PBMCs. Of note, 6 TCLs showed a decreased proliferative response against periodate-treated Cry j 1 in comparison with native and mock-treated Cry j 1, whereas 1 TCL displayed similar levels of responses among the 3 Cry j 1 antigens. These results indicate a heterogeneity of cellular responses against carbohydrates on Cry j 1. The heterogeneity of polyclonal T-cell responses to allergens was well known, and a difference in peptide motifs has often been the focus of attention.^{15,20} In fact, Dormann et al²⁰ demonstrated that 1 of 14 TCLs specific for birch pollen extracts reacted with natural Bet v1 but not with recombinant Bet v1. Our current data might provide an additional suggestion that not only peptide but also car-

bohydrate moiety might be responsible for the heterogeneity of T-cell responses.

Flowcytometric analysis revealed that Cry j 1-specific TCLs were a mixture of CD4+/CD8- and CD4-/CD8+ TCR αβ+ T cells. This result is not consistent with findings described elsewhere.^{15,21} The difference might be due either to the time required to generate TCLs or to culture conditions such as antigen concentration and the ratio of T cells/antigen-presenting cells (APCs), inasmuch as these factors are known to be involved in T-cell activation and differentiation.^{22,23} Furthermore, the existence of CD8+ T cells in short-term TCLs encouraged us to investigate whether carbohydrates on Cry j 1 are recognized by CD4+ or CD8+ T cells at clonal level, because Corinti et al⁶ demonstrated that human CD8+ αβ T cells recognize carbohydrates on allergenic extract from *P. judaica* pollen.

We generated 19 Cry j 1-specific TCCs from 3 patients. However, we could not establish any CD8+ clones. All of the TCCs were CD4+, CD8-, and TCR αβ+. Indeed, other reports have demonstrated that all of the established TCCs specific for Cry j 1 showed the same phenotype, and no Cry j 1-specific CD8+ TCC has been established at present.^{24,25} Unlike CD8+ TCCs specific for the extracts from *P. judaica* pollen, survival and proliferation of CD8+ T cells specific for Cry j 1 might need the help of specific CD4+ T cells.

As a whole, 19 Cry j 1-specific TCCs displayed a significant decrease of proliferation in response to periodate-treated Cry j 1 in comparison with native and mock-treated Cry j 1. In addition, periodate-treated Cry j 1 induced a significant decrease in IL-4 and IL-5 production—but not IFN-γ production—in these TCCs. These data confirm that carbohydrates on Cry j 1 are selectively promoting in vitro antigen-specific T_H2 response at the clonal level. Several reports have demonstrated that allergen-specific TCCs show less response to recombinant allergen than to native allergen.^{26,27} An absence or alteration of acetylation and glycosylation has often been seen in recombinant antigens because of posttranslational modification.²⁸ In fact, deglycosylation of the 45/47-kDa Ag complex (Apa) of *Mycobacterium tuberculosis* and recombinant Apa are reported to induce a marked decrease in CD4+ T cell proliferation and delayed-type hypersensitivity response in comparison with native Apa.^{28,29} Thus our present data might provide a key to why allergen-specific T cells show a lesser response to recombinant allergen.

There are several explanations of how carbohydrates on Cry j 1 might promote in vitro Cry j 1-specific T_H2 responses. The most likely explanation is that carbohydrates on Cry j 1 are T-cell epitopes. There are several reports demonstrating that allergen-specific T cells recognize carbohydrates on allergens.^{5,6} In our case, however, most (18 of 19) TCCs showed a positive proliferative response as indicated by SI; this compared with > 2 against periodate-treated Cry j 1 stimulation, suggesting that carbohydrates on Cry j 1 are not major T-cell epitopes.

A second possible explanation is that carbohydrates on Cry j 1 might bind to carbohydrate-specific receptors on

APCs, which might lead to efficient antigen presentation.^{18,30-32} We thus focused on the involvement of MR in Cry j 1-specific cellular responses, inasmuch as MR can bind to mannose, glucose, fucose, and N-acetylglucosamine and the latter 2 residues are located at the branched end of the carbohydrates on Cry j 1.^{14,18} Our results show that a blockade of MR induced few changes in in vitro Cry j 1-specific T-cell responses, suggesting that the involvement of MR was negligible. However, these results do not exclude the possibility that other carbohydrate-specific receptors expressed on APCs interact with carbohydrates on Cry j 1.¹⁸

Another possible explanation is that carbohydrates on Cry j 1 can influence the recognition of peptides by T cells by altering the structural conformation of the peptides. However, this possibility appears to be trivial in our case, because the mouse mAbs K8.238 and G1.635, which can recognize major human T-cell epitopes p211-225 and p81-95 on Cry j 1,¹⁵ respectively, can bind to both periodate-treated and mock-treated Cry j 1 at the same magnitude (Fig 1). These results also suggest that periodate treatment did not affect the structure or antigenicity of major T-cell epitopes on Cry j 1 in comparison with mock treatment.

Although the population seems to be minor, we established 1 CD4+, CD8-, and TCR $\alpha\beta$ + TCC, which displayed a carbohydrate-dependent recognition to Cry j 1. This clone showed HLA-DR restriction and produced T_H2 type cytokines. Dudler et al⁵ identified several CD4+ TCCs that are specific for phospholipase A2 from honeybee venom and recognize the allergen in a carbohydrate-dependent, HLA-DR-restricted manner. Corinti et al⁶ demonstrated that CD8+ TCR $\alpha\beta$ + TCCs specific for the extracts of *P. judaica* pollen recognize carbohydrates on the antigen and that the recognition was major histocompatibility complex-independent. Our result was consistent with the former and suggests that carbohydrates on Cry j 1 have a potential for T-cell epitopes and there is a heterogeneity of T-cell recognition against the carbohydrate moiety of pollen antigens.

In summary, we have provided in vitro evidence that carbohydrate residues on Cry j 1 play a major role in antigen-specific T_H2 response. Moreover, the carbohydrates appear not to have been major T-cell epitopes. These results have implications for future investigations on the induction, exacerbation, and pathophysiology of allergic rhinitis and asthma as well as of allergy caused by infectious diseases.

We thank Dr Yoshio Hiraki for his invaluable direction and assistance with the irradiation of PBMCs, Dr Eiichi Nakayama for critical review and comments, Natsuyo Komura and Mayumi Ishii for technical assistance, and Yuko Okano for help during preparation of the manuscript.

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