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ARTICLE

Effects of Various Saccharides on the Masking of Epitope Sites and Uptake in the Gut of Cedar Allergen Cry j 1−Saccharide Conjugates by a Naturally Occurring Maillard Reaction

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A major allergen of Japanese cedar, Cry j 1, was conjugated with galactomannan (M_w of 15 kDa), dextran (M_w of 12 kDa), xyloglucan (M_w of 1.4 kDa), and various monosaccharides through the Maillard reaction by dry-heating in 65% relative humidity. The Cry j 1−galactomannan conjugate completely masked the epitopes of the allergen in Cry j 1. The Cry j 1−dextran conjugate also masked the epitopes of Cry j 1. The small size of oligosaccharide (xyloglucan) and various monosaccharides cannot mask the epitopes of allergen Cry j 1. This suggests that the higher molecular size of attached saccharides is important to mask sterically the epitope sites. The Cry j 1−galactomannan and Cry j 1−mannose conjugates were effectively trafficked in the gut and co-localized with immune cells, such as dendritic cells in the gut, suggesting that Cry j 1−saccharide conjugates are phagocytosed via the mannose receptor in immune cells. These results suggest that the Cry j 1−galactomannan conjugate is suitable for masking the epitope sites of Cry j 1 and trafficking to immune cells in gut lumen.

KEYWORDS: Japanese cedar pollen allergen Cry j 1; allergen Cry j 1−saccharide conjugate; Maillard reaction; masking of epitope; immune cell; dendritic cell; mannose receptor; galactomannan

INTRODUCTION

Pollen from Japanese cedar (*Cryptomeria japonica*) is a potent seasonal aeroallergen in Japan. About 25−30% of Japanese suffer from the pollinosis in early spring. It has been reported that a major allergen of the Japanese cedar pollen is Cry j 1, designated according to the World Health Organization (WHO) allergen nomenclature (*1*). Although many people suffer from pollinosis, the effective therapy to reduce or cure the symptoms has not been established. Most patients were treated with antihistamine and steroid drugs to suppress allergenic symptoms; however, it is desirable to develop a fundamental therapy without any side effects. As a fundamental therapy, the sublingual therapy has recently been attempted in Europe by the recommendation of the WHO. This therapy is expected to induce the tolerance to pollen allergen by the adsorption of allergen proteins through sublingual immune cells. Similarly, oral immunotherapy is a promising method as one of the therapies for modulating immune response (*2*−*4*). Oral tolerance induces a state of active inhibition of immune responses to an antigen by prior exposure to the antigen through oral and intestinal routes. The intestinal tract is known as the largest immunological organ in the body. Around 30−40% of immune cells of the whole body are localized in the intestinal track, because many heterogeneous proteins are normally treated in the gut. Once antigen proteins contact the intestinal surface, it is sampled by different immune cells. Antigens might be taken up by microfold cells (M cells) overlying Peyer's patches, dendritic cells, and epithelial cells. Different cells in the immune system of gut lumen participate in oral tolerance induction to activate regulatory T cells, resulting in the suppression of an immune response (*3*). However, the sublingual and oral immunotherapies have a risk to cause anaphylaxis when the allergens are administrated in a native form. Therefore, it is desirable to develop a new method to suppress anaphylaxis when allergens are administrated in the gut.

We have reported that the IgE epitopes of food allergen proteins, such as soy allergen (*5*), egg white lysozyme (*6*), and the major allergen of Japanese cedar pollen (*7*), can be masked by the attachment of polysaccharides using the naturally occurring Maillard reaction in the dry-heating state between the ε-amino group in the protein and the reducing-end carbonyl group in the polysaccharide. The binding of sera IgE of the patient to these allergens were completely inhibited by conjugation with galactomannan (GM). These inhibitions are mainly due to the masking of IgE epitopes by conjugated polysaccharide (*7*). In addition, we observed that the intraperitoneal primed mice with these allergen−polysaccharide conjugates reduced the production of IgE against lysozyme and soy proteins (*5*, *6*). Therefore, the oral administration of allergen protein Cry j 1−galactomannan conjugate may be used as a new immunotherapy agent without any anaphylaxis. To further expand the use of allergen−saccharide conjugates as an immunotherapy drug of various allergies, the optimal saccharide and its size should be investigated.

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This paper describes how the long size of saccharides is effective at masking the epitopes of Cry j 1 and what kind of saccharides are effective to uptake the saccharide-attached antigen in gut immune cells.

MATERIALS AND METHODS

Mice. Female, 6-week-old BALB/c mice were purchased from Chiyoda Kaihatsu Co., Ltd. (Tokyo, Japan) and housed under conventional conditions. Experiments were performed according to the Guidelines of Laboratory Animals at Yamaguchi University.

Saccharides Used for the Conjugation with Cry j 1. Mannose, galactose, and glucose were obtained from Wako Co., Osaka, Japan. Galactomannan (M_w of 15 kDa) was obtained by dialyzing a mannase hydrolyzate of guar gum from Taiyo Chemical Co., Japan. Dextran (M_w of 12 kDa) was obtained from Sigma Chemical Co. Xyloglucan (M_w of 1.4 kDa) was obtained from Dainihon Medical Co., Japan.

Isolation of Major Allergen Cry j 1 from Japanese Cedar Pollen. Cry j 1 was purified from Japanese cedar pollen according to the procedure described previously (*1*). Crude extracts of cedar pollen allergen were extracted from 100 g of cedar pollen in 2 L of 0.1 M phosphate buffer at pH 8.0 containing 0.14 M NaCl and 0.01 M KCl. The mixtures of cedar pollen and extract solution were stirred gently for 1 h. The pollen extract solution was separated by centrifugation (15000*g* for 30 min). The supernatant was further filtered to remove contaminant pollen. Then, solid ammonium sulfate was added to the extracts with stirring to attain 80% saturation (561 g/L). After stirring overnight at 4 °C, the precipitate was collected by centrifugation (18500*g* for 40 min). The precipitate was dissolved in distilled water and then dialyzed against distilled water. Further purification was performed as described in a previous paper (*7*). The dialyzate was further purified by CM-Toyopearl after removal of impure proteins by DEAE-Toyopearl and stored as crude extracts of Cry j 1.

Human Sera of Cedar Pollinosis Patients. The anti-sera from Japanese cedar pollinosis patients were provided by Dr. M. Muto, Medical School of Yamaguchi University, Yamaguchi, Japan.

Preparation of Cry j 1−Saccharide Conjugates. The attachment of mono-, oligo-, and polysaccharides to pollen allergen Cry j 1 was carried out using the Maillard reaction in the dry state according to the method by Usui et al. (*7*). The pollen allergen−saccharide powder mixtures in the saturated saccharide were dissolved in distilled water at 0.1% (w/v) and freeze-dried. The Cry j 1−dextran powder mixtures in the molar ratio of 1:20 were dissolved at 0.1% (w/v) and freeze-dried. The mixtures were dry-heated at 60 °C under 65% relative humidity (RH) for 2 weeks in a similar condition as the Cry j 1−galactomannan mixture. The Cry j 1−xyloglucan and Cry j 1−glucose, Cry j 1−mannose, and Cry j 1−galactose powder mixtures in the molar ratio of 1:20 were dissolved at 0.1% (w/v) and freeze-dried. Because the smaller molecular weight of saccharides easily reacts with allergen protein Cry j 1, the reaction was carried out in an optimal condition as follows. The Cry j 1−xyloglucan mixture was dry-heated at 40 °C for 14 days under 65% RH, and Cry j 1−monosaccharide mixtures were at 50 °C for 1 day under 65% RH. The Cry j 1−saccharide conjugates are finally purified by gel filtration on a Sephadex G-100 column (2.6 × 100 cm, Pharmacia Uppsala, Sweden).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was carried out using the method by Laemmli (*8*) with a 12.5% acrylamide separating gel and a 5% acrylamide stacking gel containing 0.1% SDS. The attachment of saccharides to Cry j 1 was followed by SDS−PAGE. Allergen protein Cry j 1−carbohydrates were dissolved in distilled water, and then a 0.1% sample (10 μL) was prepared in Tris-glycine buffer at pH 8.8 containing 1% SDS. These solutions were treated in the presence of 2-mercaptoethanol and carried out SDS−PAGE. Electrophoresis was performed at a current of 10 mA for 0.5 h on stacking gel and 20 mA for 1 h on separating gel in electrophoretic Tris-glycine buffer (pH 8.8) containing 0.1% SDS. After electrophoresis, the gel sheets were stained with 0.2% Coomassie Brilliant Blue R250 and destained with 10% acetic acid containing 20% methanol.

Measurement of Free Amino Groups. Changes in free amino groups of Cry j 1 and Cry j 1−saccharide conjugates were determined by the spectrophotometric assay (A_{344}) using 2,4,6-trinitrobenzenesulfonic acid sodium salt dihydrate (TNBS) according to the method by Haynes et al. (*9*). A total of 500 μL of 4% $NaHCO_3$ at pH 8.5 and 500 μL of 0.1% TNBS were added to 500 μL of protein solution (1 mg/mL) in water. The solution was incubated at 37 °C for 2 h under shade. Then, 500 μL of 10% SDS was added, followed by 250 μL of 1 N HCl. The absorbance of the solution was read at 344 nm. The number of attached saccharides was determined by the difference in free amino groups between native and saccharide-modified Cry j 1.

Measurement of IgE Binding of Cry j 1−Saccharides. The binding of patient IgE antibody with Cry j 1−saccharide conjugates were measured by dot blotting. Dot blotting was performed using the biotin−avidin method. Cry j 1−saccharide mixtures and conjugates were diluted to 0.000 25%, and then 10 μL of these samples were dotted onto the PVDF membrane, which was washed with methanol. Detection of IgE binding to Cry j 1 was carried out using biotin−avidin interactions. After the membrane was dried, it was incubated with blocking solution [0.5% bovine serum albumin (BSA) in PBS-Tween] for 1 h. Then, the membrane was washed 3 times with PBS-Tween to remove the excess BSA. The membrane was incubated overnight with patient sera diluted to 1:20 in PBS-Tween. After the membrane was washed with PBS-Tween 4 times, a $^1/_{20\,000}$ dilution of biotinylated anti-human IgE antibody was added to the membrane and incubated for 1 h. After the membrane was washed with PBS-Tween 20 4 times, bound IgE antibodies were detected using a $^1/_{10\,000}$ dilution of horseradish peroxidase (HRP)−streptavidin and electrochemiluminescence (ECL)−western blotting.

Histology and Microscopy of Gut Lumen. Cry j 1 and Cry j 1−saccharide conjugates were biotinylated with Biotin Labeling Kit (Roche, Indianapolis, IN) to visualize their uptake in gut lumen. A total of 200 μL of 0.01 M phosphate buffer at pH 7.4 containing 20 μg of each biotinylated sample was orally administrated into mice, and phosphate buffer alone was administrated as a control experiment. After 3 h, the small intestine was cut and frozen samples of intestine were sliced into 10 μm sections fixed in cold acetone and then rehydrated in 0.01 M phosphate buffer at pH 7.4. Each section was incubated with 2% BSA in 0.01 M phosphate buffer containing 0.05% Tween 20 for 1 h at room temperature. After washing with 0.01 M phosphate buffer containing Tween 20, sections were incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Southern Biotech, Birmingham, AL), R-Phycoerythrin (RPE)-conjugated hamster anti-mouse CD11c (Serotec, Ltd., Oxford, U.K.), and 4′,6-diamidino-2-phenylindole (Wako, Osaka, Japan) at room temperature for 1 h in the shade. The washed and stained sections were mounted in Aqueous Gel Mount (Biomeda Corp., Foster City, CA) and observed with a fluorescent microscope (E600, Nikon, Tokyo, Japan) with a magnification of 200× object lens (Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

The conjugation of saccharides with Cry j 1 was carried out using controlled dry-heating, which was carried out in a naturally occurring Maillard reaction between ε-amino groups of allergen protein and the carbonyl group of saccharides at various temperatures and a controlled humidity of 65% without the use of any chemicals (*7*). We have reported that the allergen protein−polysaccharide conjugates are effective at reducing the allergenicity (*5*−*7*). Because four epitope sites of Cry j 1 are located on the molecular surface, the binding of saccharides can mask the epitope sites sterically. However, the optimal saccharide and molecular size to mask the epitope sites are still uncertain. Thus, the effect of the molecular size of attached saccharides on the masking of the epitope of cedar allergen Cry j 1 was investigated using various kinds and sizes of saccharides.

Table 1 shows the binding number of various saccharides with cedar allergen Cry j 1 in controlled conditions. The number of attached saccharides was calculated from the decrease in N-terminal and ε-amino groups (18 residues) of Cry j 1. As shown in **Table 1**, monosaccharides were easily reacted with Cry j 1 within 1 day of incubation at 50 °C and resulted in the binding of four to five molar monosaccharides. Further incubation brought about the insoluble materials to be impossible for further analysis. Oligosaccharide xyloglucan also easily reacted with Cry j 1.

Therefore, the reaction was carried out in a lower temperature at 40 °C for 2 weeks to obtain an optimum conjugation. Polysaccharides, such as dextran and galactomannan, reacted at 60 °C for 2 weeks, as reported in a previous paper (*7*), to obtain conjugates with Cry j 1. The properties of Cry j 1−saccharide conjugates thus obtained were investigated as follows.

Figure 1a shows SDS−PAGE patterns of monosaccharides (mannose, galactose, and glucose)−Cry j 1 conjugates in an optimized dry-heating condition. The soluble conjugates were obtained after 1 day of incubation at 50 °C. A small amount of higher molecular bands of SDS−PAGE patterns were observed in **Figure 1a**. **Figure 1b** shows SDS−PAGE patterns of oligosaccharide (xyloglucan)−Cry j 1 conjugates during 2 weeks of incubation at an optimized temperature of 40 °C. Although the reaction was performed at a lower temperature, the formation of a slightly higher molecular size of conjugates was shown during the reaction time. **Figure 1c** shows SDS−PAGE patterns of dextran−Cry j 1 and galactomannan conjugates during 2 weeks of incubation at 60 °C, suggesting the formation of a higher molecular conjugate between Cry j 1 and dextran in a manner similar to galactomannan−Cry j 1 conjugate reported in a previous paper (*7*).

Figure 2 shows the dot blot patterns of the Cry j 1 conjugates with various mono-, oligo-, and polysaccharides. As shown in **Figure 2**, monosaccharide−Cry j 1 conjugates and xyloglucan−Cry j 1 conjugate could not reduce the antigenicity (IgE binding capacity). On the other hand, polysaccharide-attached Cry j 1 greatly reduced the allergenicity. The reduction of allergenicity in galactomannan−Cry j 1 conjugate was much higher than that of dextran−Cry j 1 conjugates. The difference in the reduction of allergenicity between galactomannan and dextran may be due to their branched structure and the attached number of saccharides. The number of branched saccharides in galactomannan is much higher than that of dextran. The difference of the branched structure may affect the masking of allergenic epitopes.

Thus, the antigenicity of cedar major allergen Cry j 1 was greatly diminished by the attachment of galactomannan with Cry j 1 through the aminocarbonyl reaction between ε-amino groups in Cry j 1 and the reducing end of the carbonyl group in polysaccharides. The five attached polysaccharides to the ε-amino groups of the molecular surface in the allergen protein can sterically mask four epitopes of Cry j 1: amino acids 71−83, 218−230, 231−237, and 296−308 (*10*, *11*). It is likely that the attached five polysaccharide chains are effective at masking the molecular surface of epitopes in Cry j 1.

The galactomannan−Cry j 1 conjugate can be used as an oral tolerance agent without anaphylaxis because of the remarkable diminishment of antigenicity. Thus, the galactomannan−Cry j 1 conjugate can be carried to gut lumen, where immune cells, such as dendritic and macrophage cells, widely distribute. The fate of the saccharide−Cry j 1 conjugates in gut lumen was investigated using a fluoro-immune-staining method, as described below.

Table 1. Binding Number of Various Saccharides with Cedar Allergen Cry j 1 in Controlled Dry Heating at 65% RH

Cry j 1−carbohydrate conjugate	reaction conditions[a] temperature (°C)	time (days)	number of attached saccharides/mol
Cry j 1−mannose	50	1	4.6
Cry j 1−galactose	50	1	4.3
Cry j 1−glucose	50	1	4.3
Cry j 1−xyloglucan	40	14	6.2
Cry j 1−dextran	60	14	4.3
Cry j 1−galactomannan	60	14	5.0

[a] Cry j 1−saccharide conjugates are constructed in mild heat conditions to obtain soluble conjugates, because mono- and oligosaccharide conjugates with Cry j 1 easily form insoluble coagulates.

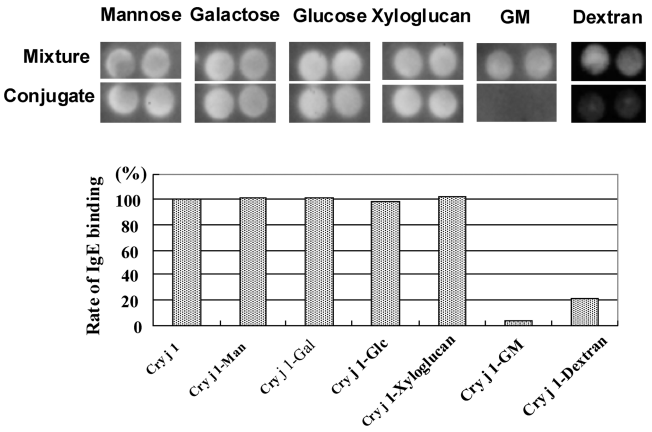

Figure 2. Reduction of antigenicity of Cry j 1 by various saccharide−Cry j 1 conjugates estimated from dot blot analysis. (Top) Dot blots of saccharide−Cry j 1 conjugates. (Bottom) Density of dot blots.

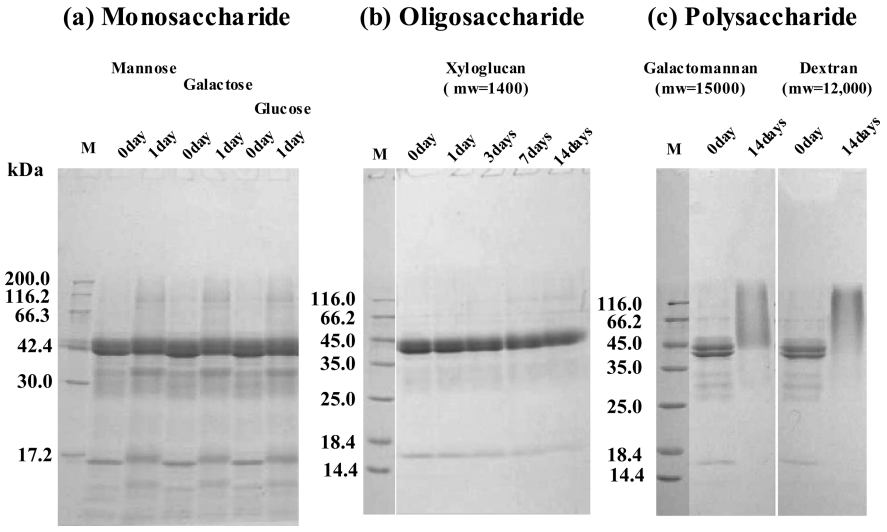

Figure 1. SDS−PAGE patterns of Cry j 1−saccharide conjugates formed at (a) 50 °C, (b) 40 °C, and (c) 60 °C for given days.

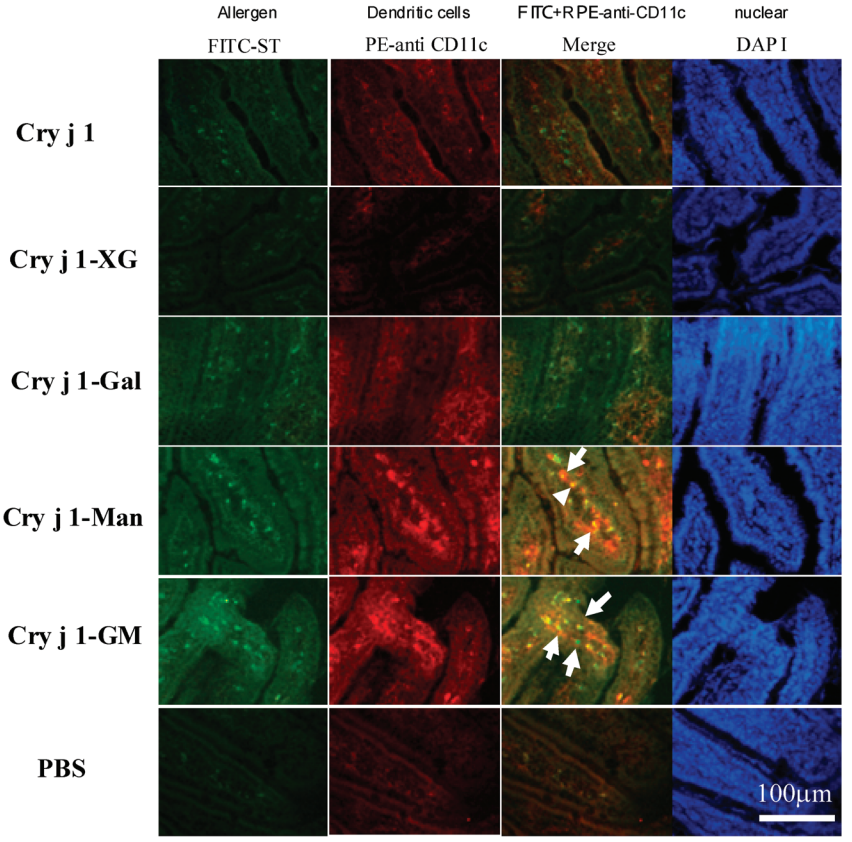

Figure 3. Co-localization of biotinylated Cry j 1−saccharide conjugates with dendritic cells in the intestine. White arrows indicate the co-localized sites of biotinylated allergen with dendritic cells. Sections (10 micrometer) were used in the analysis with a magnification of 200×.

As shown in **Figure 3**, the co-localization of glactomannan−Cry j 1 and mannose−Cry j 1 conjugates (green) with dendritic cells (red) was observed in the site-localized analysis. The histological analysis suggests that the galactomannan−Cry j 1 and mannose−Cry j 1 conjugates were effectively co-localized to phagocytose by dendritic cells. On the other hand, the co-localization of Cry j 1 with immune cells was not observed in the case of the oral administration of non-glycosylated Cry j 1, xyloglucan−Cry j 1, and galactose−Cry j 1 conjugates.

Thus, acceleration of trafficking and uptake of antigen Cry j 1 in immune cells (dendritic cells) of gut lumen was observed in mannose−Cry j 1 and galactomannan−Cry j 1 conjugates. It has been reported that immune cells in intestine lumen as well dendritic cells or macrophages have a mannose receptor (*12*, *13*). These results and reports suggest that attachments of mannose or galactomannan to Cry j 1 increased the co-localization of antigen with immune cells, such as dendritic cells, which have a mannose receptor. Therefore, it is expected that Cry j 1−mannnose and Cry j 1−galactomannan conjugates are easily caught in antigen-presenting cells, such as dendritic cells, by mediation of a mannose receptor, leading to sequent antigen presenting to regulatory T cells.

To elucidate the possibility, it should be investigated whether regulatory T cells are induced by oral administration of galactomannan−Cry j 1 to mice. Thus, galactomannan binding to Cry j 1 is effective at reducing the risk of anaphylaxis and accelerating the uptake of antigen into gut immune cells. This result suggests that galactomannan−Cry j 1 conjugate can be used as a safe oral tolerance-inducing agent.

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