JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 6851−6857 **6851**

Reduced T Cell Response to β-Lactoglobulin by Conjugation with Acidic Oligosaccharides

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We have previously reported that the conjugation of β-lactoglobulin (β-LG) with alginic acid oligosaccharide (ALGO) and phosphoryl oligosaccharides reduced the immunogenicity of β-LG. In addition, those conjugates showed higher thermal stability and improved emulsifying properties than those of native β-LG. We examine in this study the effect of conjugation on the T cell response. Our results demonstrate that the T cell response was reduced when mice were immunized with the conjugates. The findings obtained from an experiment using overlapping synthetic peptides show that novel epitopes were not generated by conjugation. One of the mechanisms for the reduced T cell response to the conjugates was found to be the reduced susceptibility of the conjugates to processing enzymes for antigen presentation. We further clarify that the β-LG−ALGO conjugate modulated the immune response to Th1 dominance. We consider that this property of the β-LG−ALGO conjugate would be effective for preventing food allergy as well as by its reduced immunogenicity. Our observations indicate that the method used in this study could be applied to various protein allergens to achieve reduced allergenicity with multiple improvements in their properties.

KEYWORDS: β-Lactoglobulin; acidic oligosaccharides; allergenicity; protein conjugation; susceptibility to enzymes

INTRODUCTION

β-Lactoglobulin (β-LG) is a major whey protein that is widely used as a food additive because it has such useful functions as emulsifying, foaming, and gelling properties (*1*, *2*). However, β-LG is a potent allergen of milk allergy (*3*). Food allergy, a major allergic disease for children, is elicited through the type I allergic reaction. Reduction of the allergenicity of allergenic proteins has been attempted by several methods involving enzymatic digestion (*4*), denaturation (*5*, *6*), genetic modification (*9*), and chemical modification of the proteins (*8*, *9*). Enzymatic digestion and denaturation are useful for destroying those structures in an allergen that are recognized by the immune system, because they can be easily achieved for any type of protein. However, the methods also have such defects as generating peptides that taste bitter and the loss of other valuable functional properties for food applications. Genetic modification of allergenic proteins can be expected to effectively achieve reduced allergenicity without any major loss of valuable functions. However, considerable cost and time are involved, because the modification must be individually performed on each protein.

The conjugation of proteins with carbohydrates has been well-investigated in previous studies (*8*, *9*). We have also attempted to make novel protein−saccharide conjugates and have demonstrated multiple functional improvements in the conjugates (*10*). β-LG conjugated with carboxymethyl dextran (CMD) showed a higher thermal stability and improved emulsifying properties than those of native β-LG (*11*, *12*). In addition, the immunogenicity of the conjugate was significantly lower than that of native β-LG (*13*). We have also clarified that conjugation with CMD of a higher molecular weight was more effective for reducing the immunogenicity of β-LG (*14*, *15*). However, we used water soluble carbodiimide for conjugating β-LG with CMD in those studies. Although conjugation was easy to achieve, however, the safety of the conjugates produced with water soluble carbodiimide has not yet been established for application to food materials.

We therefore next prepared conjugates of β-LG that were covalently bound with two types of acidic oligosaccharides, alginic acid oligosaccharide (ALGO) and phosphoryl oligosaccharide (POs), by the Maillard reaction (*16*, *17*). ALGO is the lyase-lysate of alginic acid obtained from seaweed or algae, and many physiological functions of ALGO have been reported (*18*−*20*). POs were obtained from potato starch by enzymatic digestion (*21*) and have also been reported to have some physiological functions (*22*). We found that these conjugates showed improved properties as good as those of β-LG−CMD conjugates. Furthermore, the immunogenicity of the conjugates was lower than that of native β-LG, as we had expected. These

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10.1021/jf050772k CCC: $30.25 © 2005 American Chemical Society
Published on Web 08/02/2005

observations demonstrated that we could successfully obtain two edible neoglycoconjugates of β-LG, which had improved properties with respect to their allergenicity and function for food applications.

The mechanism for the reduced immunogenicity of β-LG by conjugating with ALGO or POs is considered to involve the shielding effect on the epitopes on β-LG by the bound oligosaccharides. We have previously reported that the conjugation of β-LG with high molecular weight CMD was more effective than with low molecular weight CMD (*14*), indicating that covering the epitopes by the CMD chain might be critical for reducing the immunogenicity. However, the affinity of the conjugates for some monoclonal antibodies indicated that recognition of the β-LG−ALGO and β-LG−POs conjugates by antibodies was not altered at several sites, suggesting that a mechanism other than shielding of the epitopes might also be involved in the reduced immunogenicity of the conjugates.

We further examine in the present study the mechanism for the reduced immunogenicity of edible conjugates of β-LG with acidic oligosaccharides, focusing on the T cell response. Our results demonstrate that the conjugation of β-LG with acidic oligosaccharides successfully reduced the immunogenicity with respect to both the T cell response and the antibody production. One of the mechanisms for the suppression of T cell response seemed to involve the low susceptibility of the conjugates to those enzymes that are necessary for processing in the antigen-presenting cells (APC). It is expected that the conjugation of protein allergens will be useful for preparing novel food materials with improved functions.

MATERIALS AND METHODS

Mice. Female BALB/c and C57BL/6 mice (Clea Japan, Tokyo, Japan) at 6 weeks of age were used. These mice were maintained and used in accordance with the guidelines for the care and use of experimental animals of Tokyo University of Agriculture and Technology.

Preparation of β-LG. Crude bovine β-LG (genotype AA) was prepared from fresh milk of a Holstein cow according to the method of Armstrong et al. (*23*). This crude β-LG was purified in a DEAE-Sepharose Fast Flow column (2.5 cm i.d. × 50 cm; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) by the method previously described (*11*). The purity of β-LG was confirmed by polyacrylamide gel electrophoresis (PAGE) performed by the method of Davis (*24*).

Acidic Oligosaccharides. ALGO ($\overline{DP} = 4$) was supplied by Meiji Seika Co. Ltd. (Tokyo, Japan). POs ($\overline{DP} = 4$) were prepared from potato starch as previously described (*25*).

Preparation and Purification of the β-LG−Acidic Oligosaccharide Conjugates. The β-LG−acidic oligosaccharide conjugate was prepared by the Maillard reaction according to the method previously described (*16*). In brief, ALGO (1 g) and β-LG (1 g) were dissolved in 600 mL of distilled water, and the solution was lyophilized. The mixture was incubated at 50 °C in a relative humidity of 79% for 24 h. After dialyzing against distilled water and lyophilizing, a crude β-LG−ALGO conjugate was obtained.

The β-LG−POs conjugate was prepared by adding POs (1.5 g), β-LG (1.5 g), and 25 mL of penicillin−streptomycin (5000 units/mL; Life Technologies, Carlsbad, CA) to 600 mL of distilled water and then lyophilizing. The mixture was incubated at 50 °C in a relative humidity of 79% for 480 h. After dialyzing against distilled water and lyophilizing, a crude β-LG−POs conjugate was obtained.

Free oligosaccharides were removed by salting out. Each crude sample was dissolved in distilled water, and ammonium sulfate was added to a final concentration of 5 M. The precipitate was recovered by centrifuging (20000 rpm for 30 min) at 20 °C. The purified conjugate was obtained after dialyzing against distilled water and lyophilizing.

The composition of the conjugates determined according to the Bradford (*26*) and phenol−sulfuric acid methods (*27*) indicated respective molar ratios of β-LG to ALGO and POs in the conjugates of 1:6 and 1:8, respectively.

Immunization. We used two strains of mice, BALB/c and C57BL/6, for this study. Our previous study had indicated that the immunogenicity of the conjugates was lower than that of native β-LG in these strains. The contribution of the shielding effect of epitopes or another mechanism for the reduced immunogenicity of the conjugates could be separately evaluated by using these mice, because each strain has completely distinct T cell epitopes against β-LG. We therefore considered that it would be beneficial to use these different strains of mice in this study. The mice were subcutaneously immunized in the hind footpads and base of the tail with β-LG or a conjugate (100 μg as protein) emulsified in Freund's complete adjuvant (H37Ra; Difco Laboratories, Detroit, MI). After 7 days, the inguinal and popliteal lymph nodes were removed.

In the experiment for antibody production, the mice were intraperitoneally immunized with β-LG or a conjugate (100 μg as protein) emulsified in Freund's complete adjuvant (Difco Laboratories). These mice were boosted with the same antigen (100 μg as protein) emulsified in Freund's incomplete adjuvant (Difco Laboratories) 14 days after the first immunization. The mice were then bled 7 days after the second immunization. The blood samples were centrifuged, and the sera were collected and stored at −30 °C until needed.

T Cell Proliferation Assay and T Cell Epitope Scanning. A T cell proliferation assay and T cell epitope scanning were performed in 96 well culture plates with 200 μL/well of an RPMI 1640 medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 0.03% glutamine, 0.2% $NaHCO_3$, 2-mercaptoethanol (2-ME; 50 μM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 1% autologous normal mouse serum. T cell epitope scanning involved synthesizing a series of overlapping 15-mer peptides and moving one amino acid residue at a time in accordance with the amino acid sequence of β-LG, with a five-in-one B cell and T cell epitope scanning kit (Chiron Mimotopes, Clayton, Victoria, Australia) as previously described (*13*). The concentration of each synthesized peptide was approximately 1 nmol/μL from the results of an amino acid analysis. The lymph node cells were suspended at 5×10^5 cells/well in the culture plates and then stimulated with protein (β-LG or a conjugate) at various concentrations or by a synthesized peptide solution (5 μL). Cultures were set up in triplicate for stimulation with the proteins, while one well for each peptide was tested for the peptide series. After the cells were cultured in a humidified atmosphere of 5% CO_2 in air for 3 days at 37 °C, the T cell proliferation was measured with a BrdU proliferation kit (Roche Molecular Biochemicals, Basel, Switzerland). In short, the inguinal and popliteal lymph node cells from β-LG-immunized mice in the culture plates were pulsed with a 100 μM BrdU solution (20 μL/well) for 2 h. The culture plates were centrifuged at 1250 rpm for 10 min at 4 °C, and the supernatant was removed, before the plates were dried for 1 h at 60 °C. A FixDenat solution (200 μL) was added to each well, and the plates were incubated for 1 h at 25 °C. The FixDenat solution was removed, and a peroxidase-labeled anti-BrdU mAb solution was added, before the plates were incubated for 2 h at 25 °C. The peroxidase-labeled anti-BrdU mAb solution was then removed, and each well was washed three times with PBS (200 μL each). A tetramethylbenzidine solution (100 μL/well) was added, and the plates were incubated for 5−10 min. After 1 M H_2SO_4 (25 μL/well) was added to stop the enzymatic reaction, the absorbance at 450 nm was measured.

The following criteria were used for the peptides adopted as positive in determining the T cell epitopes: (i) those that showed a response greater than the mean value plus three times the standard deviation of the absorbance to the peptide (PLAQGGGGGGGGGG) in the absence of the β-LG sequence (*28*), (ii) those that showed a positive response to at least two consecutive overlapping peptides, and (iii) those that showed reproducibility in two individual experiments (*28*). The common amino acid sequences among the peptides that fulfilled these criteria were identified as the epitopes according to the method of Gammon et al. (*29*).

Serum Antibody Detection. β-LG specific antibody titers in the sera were measured by enzyme-linked immunosorbent assay (ELISA).

Maxisorp immunoplates were coated with a 0.01% β-LG solution. After washing and blocking, the sample sera and standards were added to the plates. The bound antibody was detected with the biotin-labeled rabbit anti-mouse IgG1 or anti-mouse IgG2a antibody (Zymed, San Francisco, CA), before incubating with alkaline phosphatase−streptavidine (Zymed). A substrate (*p*-nitrophenyl-phosphate; Wako Pure Chemical Industries, Tokyo, Japan) was added, and the absorbance was determined at 405 nm.

Digestion of the β-LG-Acidic Oligosaccharide Conjugates with Cathepsin B and Cathepsin D. β-LG or each conjugate [0.1% (w/v) as the protein concentration] was dissolved in a 0.2 M citric acid/Na_2HPO_4 buffer (pH 5.0) containing ethylenediaminetetraacetic acid (1 mM) and 2% (v/v) 2-ME, and the solution was incubated at 37 °C for 12 h. Cathepsin B (EC 3.4.22.1) or cathepsin D (EC 3.4.23.5) from bovine spleen (Sigma Chemical Co., St. Louis, MO) was added to the solution (enzyme:substrate = 1:10), and the mixture was incubated at 37 °C for various times. Digestion was stopped by adding the loading buffer for sodium dodecyl sulfate (SDS) PAGE and by heating at 100 °C for 5 min. The digested sample was applied to SDS-PAGE (*30*), and the gel was stained with Coomassie Brilliant Blue R-250. After destaining, the digestibility of each sample was evaluated by densitometry.

RESULTS

Reduced T Cell Response by Conjugating β-LG with Acidic Oligosaccharides without the Emergence of Any Novel Immunogenicity. We had previously prepared two conjugates, β-LG−ALGO and β-LG−POs, to reduce the immunogenicity of β-LG. The immunogenicity of these conjugates was investigated by immunizing mice with them and was found to be reduced for the induction of antibody production in the previous study (*17*). One of the mechanisms for the reduced immunogenicity of the conjugates seemed to involve shielding of the epitopes resulting from the conjugation. We anticipated that the induction of regulatory T cells and the reduced susceptibility of the conjugates to endosomal/lysosomal enzymes in the APCs would be important. We therefore examined the T cell response induced by these conjugates in this study. After BALB/c and C57BL/6 mice had been immunized with β-LG or a conjugate, the lymph node cells from the mice were stimulated with β-LG or the conjugate. T cells from BALB/c mice that had been immunized with the β-LG−POs conjugate showed a lower response than those from β-LG-immunized (control) mice (**Figure 1a**). On the other hand, the T cell response of the mice immunized with the β-LG−ALGO conjugate was at a similar level to that of the control mice (**Figure 1a**). The T cell response of mice immunized with a conjugate against each conjugate was lower than that against native β-LG (**Figure 1a**). T cells from C57BL/6 mice that had been immunized with a conjugate showed a lower response than those from the control mice (**Figure 1b**). The T cell response to the respective conjugate was lower in the mice immunized with the β-LG−POs conjugate than in those to native β-LG, while being at a similar level in the mice immunized with the β-LG−ALGO conjugate (**Figure 1b**). These results demonstrate that the T cell response induced by these conjugates was lower without generating any marked degree of novel immunogenicity.

T Cell Epitope Profiles of the β-LG-Acidic Oligosaccharide Conjugates. To investigate the mechanism for the reduced T cell response induced by the β-LG−acidic oligosaccharide conjugates, we next examined in detail the T cell epitope profiles of these conjugates. After the lymph node cells from BALB/c and C57BL/6 mice immunized with β-LG or a conjugate had been stimulated by the overlapping peptides synthesized on the basis of the sequence of β-LG, the T cell proliferation in response to the peptides was measured by BrdU ELISA. **Figure 2** shows the T cell epitope profiles of β-LG and the β-LG−acidic oligosaccharide conjugates. The horizontal axis indicates the N-terminal amino acid residue number of each 15-mer peptide corresponding to the position in the β-LG sequence, and the vertical axis indicates the T cell proliferation in response to each peptide. The T cell epitopes identified according to the method of Gammon et al. are summarized in **Figure 3**, in which the horizontal axis indicates the sequence number in β-LG and the line thickness indicates the intensity of the response to each epitope. The T cells from BALB/c mice immunized with β-LG showed a proliferative response to peptides 4−7, 24−29, 62−66, 68−69, and 73−76 (**Figure 2a**). Therefore, the T cell epitopes of β-LG that had been recognized in BALB/c mice were determined to be ^{7}Met-^{18}Thr, ^{29}Ile-^{38}Pro, ^{66}Cys-^{76}Thr, ^{69}Lys-^{82}Phe, and ^{76}Thr-^{87}Leu (**Figure 3**). In the same way, the T cell epitopes of the conjugates recognized in BALB/c mice were determined to be ^{6}Thr-^{19}Trp, ^{40}Arg-^{53}Asp, ^{64}Asp-^{74}Glu, ^{70}Lys-^{80}Ala, ^{77}Lys-^{89}Glu, and ^{139}Ala-^{150}Ser for β-LG−ALGO and ^{6}Thr-^{19}Trp, ^{70}Lys-^{75}Lys, ^{75}Lys-^{88}Asn, ^{103}Leu-^{116}Ser, and ^{139}Ala-^{150}Ser for β-LG−POs (**Figure 3**). No difference in the epitope distribution was apparent for T cells from the mice immunized with either of the conjugates, but the proliferative response to each peptide was lower throughout the entire amino acid sequence than that of the control mice (**Figure 2a−c**).

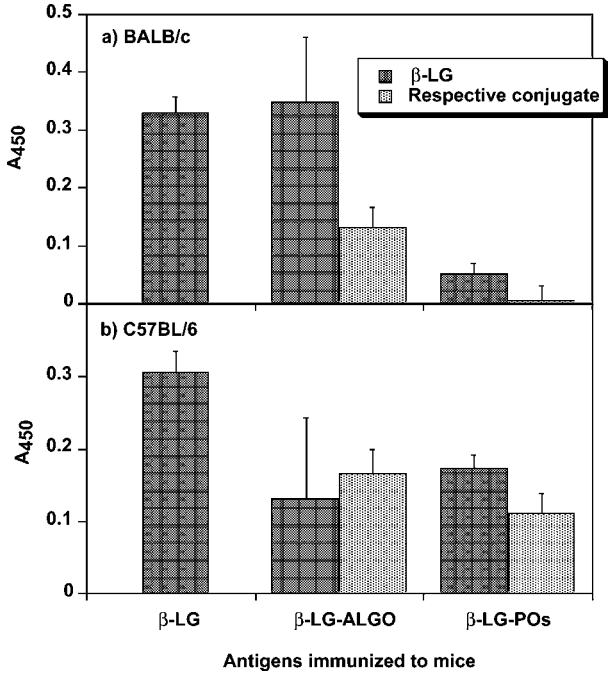

Figure 1. Proliferative response to the antigen of lymph node cells obtained from mice immunized with native β-LG or a conjugate. BALB/c (**a**) and C57BL/6 (**b**) mice were immunized with β-LG or a conjugate. Lymph node cells of these mice were obtained and cultured in the presence of β-LG or the respective conjugate at 0.1 μM for BALB/c and 0.3 μM for C57BL/6. The proliferative response of these cells was measured by BrdU ELISA. The result is representative of two independent experiments.

The results obtained with C57BL/6 mice are shown in **Figure 2d−f**. The T cell epitopes of β-LG were determined to be ^{122}Leu-^{132}Ala (**Figure 3**). The T cell epitopes of each conjugate recognized in C57BL/6 mice were determined to be ^{120}Gln-^{132}Ala for β-LG−ALGO and ^{13}Gln-^{26}Ala and ^{122}Leu-^{131}Glu for β-LG−POs (**Figure 3**). The epitope distribution of both these conjugates was similar to that of β-LG, whereas the proliferative response to each epitope was lower throughout the entire sequence than that of the control mice.

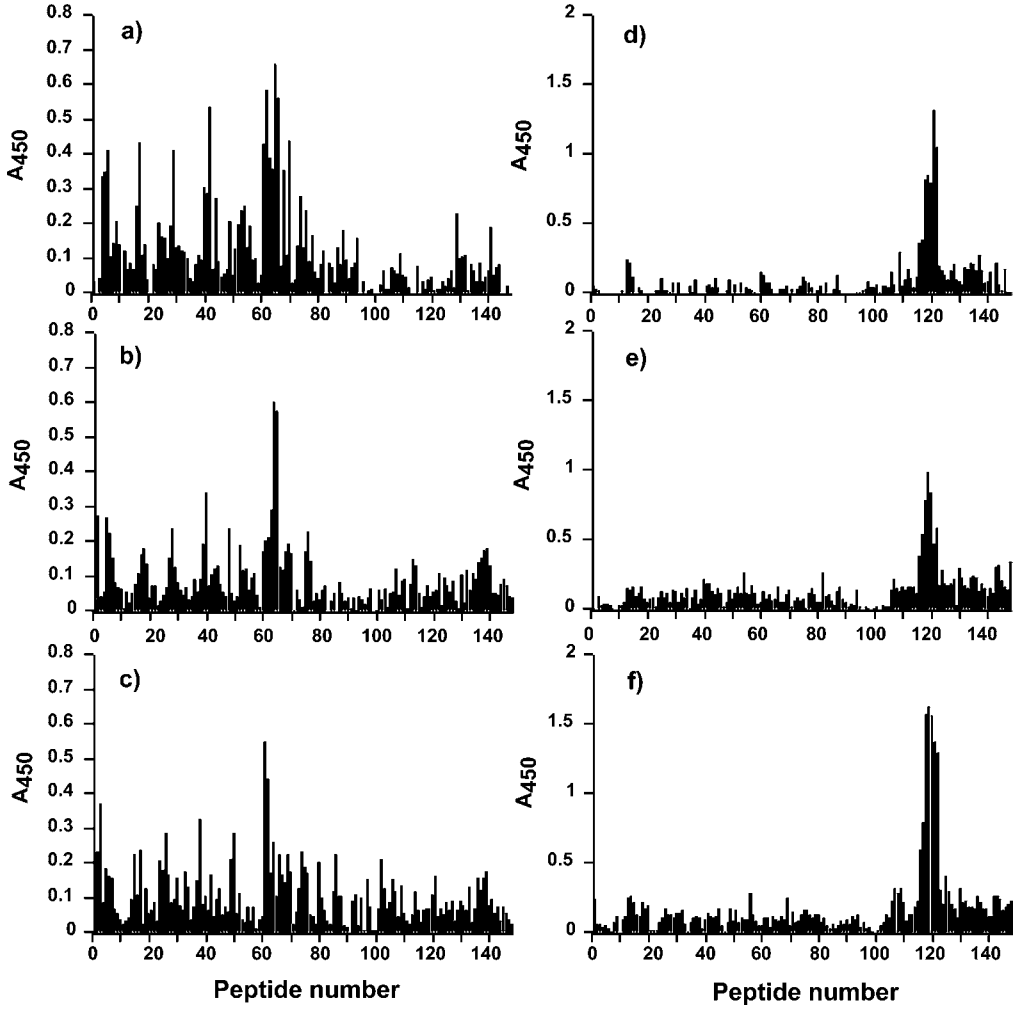

Figure 2. Proliferative response to the synthetic peptides of the lymph node cells obtained from mice immunized with native β-LG or a conjugate. BALB/c (**a**–**c**) and C57BL/6 (**d**–**f**) mice were immunized with β-LG (**a** and **d**), β-LG-ALGO (**b** and **e**), or β-LG-POs (**c** and **f**). Lymph node cells of these mice were obtained and cultured in the presence of overlapping 15-mer peptides covering the amino acid sequence of β-LG. The proliferative response of these cells was measured by BrdU ELISA. The result is representative of two independent experiments.

Susceptibility of the β-LG-Acidic Oligosaccharide Conjugates to Cathepsin B and Cathepsin D. We have previously partially identified the binding sites of carbohydrates in the β-LG−ALGO conjugate to be ^{60}Lys, ^{77}Lys, ^{100}Lys, ^{138}Lys, and ^{141}Lys (*17*). However, we could not observe the preferential reduction of T cell response to the epitopes close to the carbohydrate-binding sites in mice immunized with the β-LG−ALGO conjugate. It was considered that blocking the binding of antigenic peptides to the MHC molecule or T cell receptor by conjugation with an oligosaccharide was not the major reason for the reduction in T cell response induced by the conjugates. We therefore next examined the susceptibility of the conjugates to cathepsin B and cathepsin D, which are both regarded as important processing enzymes for antigen presentation (*31*, *32*). We found that about 60 and 80% of β-LG was, respectively, digested with cathepsin B and cathepsin D after 4 h (**Figure 4a**,**b**). On the other hand, no more than 50% of the conjugate was digested (**Figure 4a**,**b**), and the susceptibility to cathepsin D was significantly reduced by conjugation with the acidic oligosaccharides (**Figure 4b**).

In Vitro Antigen Presentation of the β-LG-Acidic Oligosaccharide Conjugates to β-LG-specific T Cells. Our results suggested that the reduced susceptibility of the conjugates to processing enzymes might have resulted from the reduced T cell response induced by immunization with the conjugates. We

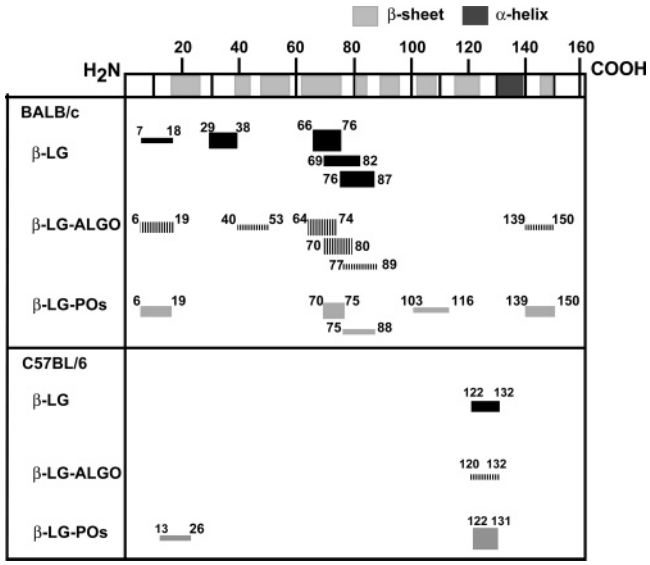

Figure 3. T cell epitope profiles of β-LG and the conjugates. The common regions of overlapping peptides, which induced significant proliferation in lymph node cells, are identified as epitopes according to the method of Gammon et al. The thickness of the lines indicates the intensity of response to each epitope.

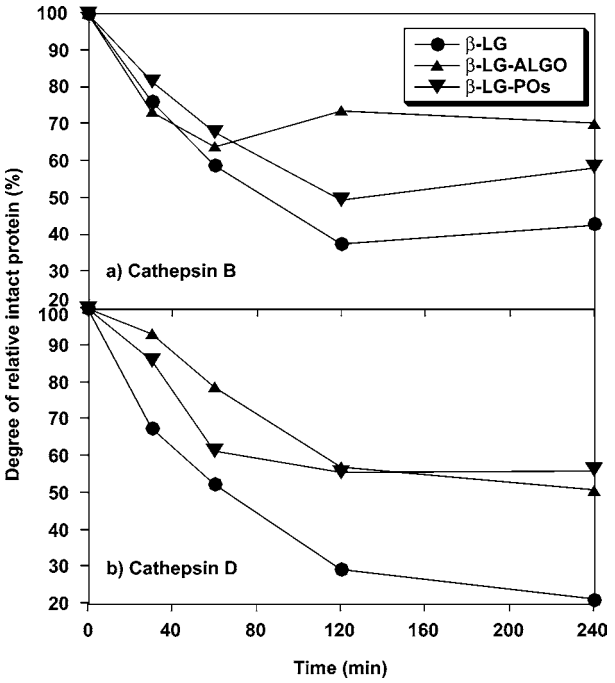

Figure 4. Susceptibility of β-LG and the conjugates to cathepsin B and cathepsin D. β-LG, β-LG-ALGO, and β-LG-POs were digested with cathepsin B or cathepsin D for various times in the presence of 2-ME. The digested samples were applied to SDS–PAGE, and the digestibility of each sample was evaluated by densitometry. The intensity of the band of each sample at 0 min is taken as 100%, and that of the samples at subsequent times is relative to the value at 0 min. All results are expressed relative to the intact protein (%).

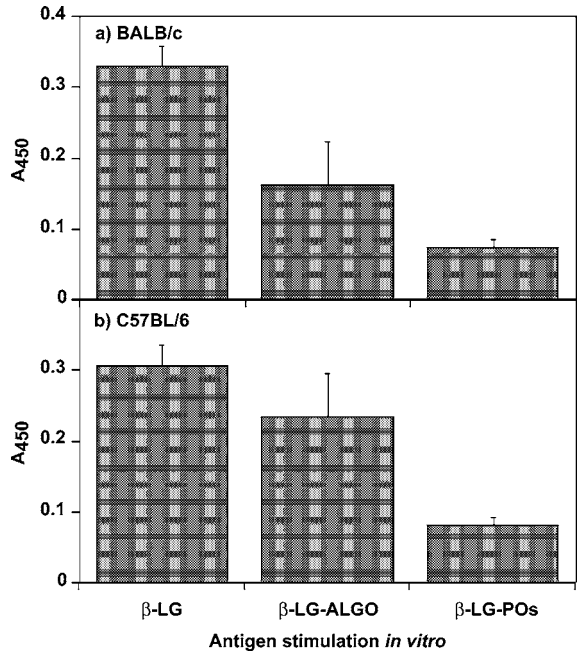

Figure 5. Proliferative response to the conjugates of lymph node cells obtained from mice immunized with native β-LG. BALB/c (**a**) and C57BL/6 (**b**) mice were immunized with β-LG. Lymph node cells of these mice were obtained and cultured in the presence of β-LG or a conjugate at 0.1 μM for BALB/c and 0.3 μM for C57BL/6. The proliferative response of these cells was measured by BrdU ELISA. The result is representative of two independent experiments.

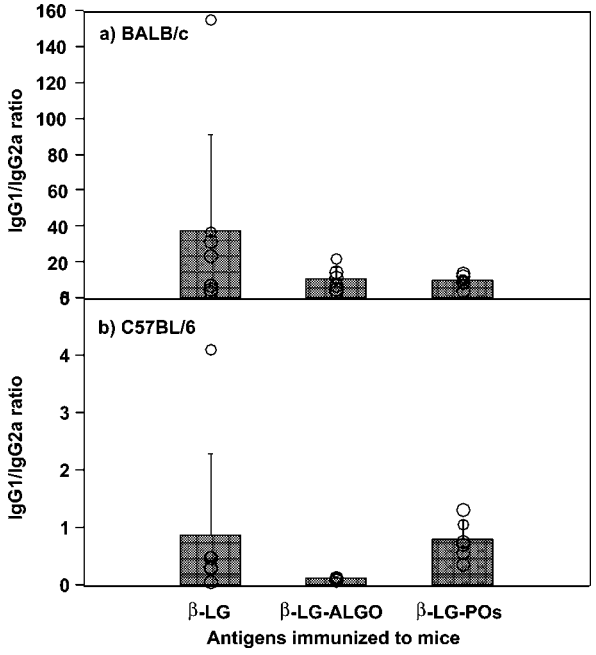

Figure 6. Antibody production in mice immunized with native β-LG or a conjugate. BALB/c (**a**) and C57BL/6 (**b**) mice were immunized with β-LG or a conjugate. The mice were bled after the second immunization with the respective antigen. The levels of β-LG specific IgG1 and IgG2a in the sera were measured by ELISA. The ratio of IgG1 and IgG2a is shown. The result is representative of two independent experiments.

therefore next investigated the in vitro T cell proliferation induced by the antigen presentation of the conjugates. After the two strains of mice had been immunized with β-LG, the lymph node cells were stimulated with β-LG or a conjugate. The efficiency of antigen presentation was evaluated as the in vitro T cell proliferation by BrdU ELISA. In both strains of mice, the proliferative response of β-LG specific T cells to the conjugates was lower than that to native β-LG (**Figure 5a**,**b**). The proliferation induced by the β-LG−POs conjugate was particularly reduced in both strains. These results demonstrate that the lower efficiency of the conjugates for antigen presentation, which was due to their reduced susceptibility to processing enzymes, should be a critical mechanism for the reduced immunogenicity of the conjugates.

Th1 Dominant Response Induced by the β-LG-Acidic Oligosaccharide Conjugates. We have previously reported that ALGO modulated the balance of Th1 and Th2 to Th1 dominance (*20*). We therefore tested the cytokine production of T cells obtained from mice that had been immunized with β-LG or a conjugate. However, there was no detectable amount of IL-4, which is the major cytokine produced by Th2, from T cells of the mice that had been immunized with either β-LG or a conjugate (data not shown). We next investigated the antibody production in the sera from mice that had been immunized with β-LG or a conjugate. The production of the IgG1 antibody is known to be enhanced by IL-4, whereas the IgG2a antibody is induced by IFN-γ, which is a cytokine produced by Th1. Our results show that the ratio of IgG1 and IgG2a was lower in the sera from mice that had been immunized with the β-LG−ALGO conjugate than from β-LG-immunized mice of both strains (**Figure 6a**,**b**). This result indicates that the β-LG−ALGO conjugate induced a Th1 dominant response. With respect to the β-LG−POs conjugate, although the ratio of IgG1 and IgG2a induced by the β-LG−POs conjugate tended to be lower in the BALB/c mice (**Figure 6a**), there was no reduction in the ratio in the C57BL/6 mice (**Figure 6b**).

DISCUSSION

The aim of this study was to investigate the mechanism for the reduced immunogenicity of β-LG by conjugating with acidic oligosaccharides. Our results demonstrate that the conjugation of β-LG with ALGO and POs altered the susceptibility of β-LG to the processing enzymes, cathepsin B and cathepsin D. The response of T cells induced by these conjugates was significantly suppressed for that reason. We conclude that this is at least partially responsible for the reduced immunogenicity of β-LG by conjugating with acidic oligosaccharides.

It has been indicated in some reports that the conjugation of protein antigens with other substances would generate novel immunogenicity independent of that of the native proteins (*33*). However, we did not find the generation of any novel epitopes by using overlapping peptides. Furthermore, the response of T cells from mice that had been immunized with each conjugate was lower when the T cells were stimulated with the conjugate than they were when stimulated with native β-LG. These results indicate that the reduced immunogenicity of β-LG was not actually due to any change in the immunogenicity. Similar results were also apparent from our study on the B cell response induced by these conjugates (*17*).

T cells cannot directly recognize an antigen but can when it is presented on APC bound with the MHC molecule (*31*). APC processes the antigen after its intake by the endosome and subsequent presentation (*31*, *34*). Therefore, the susceptibility of the antigen to endosomal proteases would be very important for determining the immunogenicity of the antigen. We found that the susceptibility of the conjugates of β-LG and acidic oligosaccharides to cathepsin B and cathepsin D had been reduced. These enzymes are reported to be involved in the processing of antigens in APC. Cathepsin D is an endoprotease and may reveal and release antigens, whereas cathepsin B is an exopeptidase and may trim epitopes to their final size (*31*, *32*). This evidence suggests that the reduction of T cell response to our conjugates would mainly have been due to inhibition of the generated antigenic peptides in APC and not to interference with the binding of the antigenic peptides to the MHC molecule or of the peptide−MHC complex to TCR by oligosaccharides conjugated with the peptides. Indeed, the binding sites of ALGO in the conjugate have been partially determined to be ^{60}Lys, ^{77}Lys, ^{100}Lys, ^{138}Lys, and ^{141}Lys (*17*), and we found that the T cell response to the epitopes adjacent to the binding sites of ALGO was not preferentially inhibited. Another possibility can also be considered as the mechanism for the decreased antigen presentation of the conjugates: APC usually takes up antigens by phagocytosis, so the saccharides conjugated to proteins might interrupt that process, thus leading to reduced immunogenicity.

We have previously reported that ALGO induced IL-12 production and modulated the immune response to Th1 dominance (*20*). The type I allergy, including almost all food allergies, is mediated by an allergen specific IgE antibody, and the induction of the IgE antibody is dependent on Th2 immune response (*35*). Therefore, modulation of the balance of the Th1 and Th2 response to Th1 dominance would be effective for preventing allergic diseases. This study has shown that the β-LG−ALGO conjugate preferentially induced Th1 dominant antibody production as compared with native β-LG. It is expected that the β-LG−ALGO conjugate will be able to suppress allergic diseases by both its reduced immunogenicity and its improved activity for enhancing the Th1 response. We have reported in the previous study that the effect of the β-LG−POs conjugate on the reduction of antibody production was stronger than that of the β-LG−ALGO conjugate (*17*). We also obtained similar results for the T cell response, although the difference was not significant. We consider that this might have been due to the difference in the number of binding oligosaccharides of 1:6 for the β-LG−ALGO conjugate and 1:8 for the β-LG−POs conjugate. However, it cannot be discounted that another mechanism could have been involved, because there was hardly any difference in the susceptibility of these conjugates to cathepsin B and D.

ACKNOWLEDGMENT

We are indebted to Dr. Masao Hirayama of Meiji Seika Co. Ltd. (Tokyo, Japan) for presenting the ALGO sample and to Drs. Takashi Kuriki and Kenji To-o of Ezaki Glico Company Ltd. (Osaka, Japan) for presenting the POs sample.

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Received for review April 6, 2005. Revised manuscript received June 11, 2005. Accepted June 17, 2005. This work was supported in part by grants from JSPS. KAKENHI (14560092) and Asahi Breweries Foundation.

JF050772K