Antigen Binding of an Ovomucoid-specific Antibody is Affected by a Carbohydrate Chain Located on the Light Chain Variable Region

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We cloned the variable regions of heavy and light chain genes of an anti-ovomucoid monoclonal antibody (MAb-OM21) produced by the mouse hybridoma cell line OM21. DNA sequence analysis showed that the light chain of the MAb-OM21 has only one potential N-glycosylation consensus sequence in the complementarity determining region 2 of the light chain. To find whether carbohydrate chains are located on the light chain, we assayed for the size of the light chain, after treatment with N-glycosidase, by western blotting, and also detection of the carbohydrate chains on the light chain was done using the lectin blot assay. A N-linked carbohydrate chain has been shown to bind to the light chain. To clarify the role of this carbohydrate chain in the light chain, we produced carbohydrate variant antibodies by N-deglycosylation using glycosidase or by expressing the antibody from different host cells. The N-deglycosylated variant antibody has greater antigen binding, and the antibody produced from the different host cells showed a reduced antigen binding activity and acquired the ability to react to ovalbumin. These results suggest that antigen binding of the ovomucoid specific antibody MAb-OM21 can be affected by the carbohydrate chain on the light chain variable region.

Key words: antigen binding; glycosylation; light chain variable region; human IgG; ovomucoid

Food allergies are inflammatory reactions that are induced by various inflammatory mediators, such as histamine and metabolites of arachidonic acid, which are released from basophils and mast cells when a specific allergen associates with an allergen-specific IgE that has bound to a high affinity IgE receptor on the cell surface.1,2) Bovine milk, egg, and soybean are three major food sources which have a history of causing allergic reactions. Of the three, allergy to eggs occurs at an especially high frequency. Ovomucoid, a glycoprotein in egg white, is one of the major egg allergens, and antibodies specific to ovomucoid are detected frequently in the sera from patients suffering from egg allergy. Many monoclonal antibodies to ovomucoid have so far been reported and analysis of the ovomucoid epitope has been done, but there is little information pertaining to the structures of the antibodies available. Therefore, to gain further insights into food allergies, it is important to identify the structure of antigen binding site of food allergen-reactive antibodies.

The immunoglobulin molecules are glycoproteins of which the carbohydrates are usually located in the constant regions of their heavy chains. These carbohydrates have been suggested to be significantly important for effector functions, but not for antigen recognition.3,4) It is generally known that amino acid sequence variations in the variable regions of heavy and light chains (VH and VL) contribute to diverse antigen recognition of immunoglobulin molecules. However, the role of carbohydrates in antigen recognition has not yet been established.5) The carbohydrate chains have so far not been found in the constant regions of the immunoglobulin light chains, but carbohydrate chains attached to the variable regions have been found. Recently, we reported that a carbohydrate chain is located on the light chain of an anti-adenocarcinoma human antibody, and that this glycosylation on the light chain variable region could influence antigen binding of the antibody.5-9)

Here, we have found such an antibody among the ovomucoid-specific antibodies. Antigen binding assays showed that the carbohydrate chain linked to the light chain affects not only the binding strength to antigen but also the specificity. To our knowledge, this is the first paper to show that the antigen binding of a food allergen-specific antibody can be affected by a carbohydrate located on the light chain.

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Role of Carbohydrates on the VL in Antigen Binding

Materials and Methods

Reagent. Horse radish peroxidase conjugated (HRP)-lectins, lentil lectin (LCA), erythroagglutinating phytohemagglutinin (E-PHA), concanavalin A (Con A), Ricinus communis agglutinin I (RCA-1), wheat germ agglutinin (WGA), and peanut agglutinin (PNA) were all purchased from Seikagaku Kogyo, Japan. HRP-conjugated goat anti-human IgG antibody and HRP-conjugated goat anti-mouse κ chain antibody were obtained from Biosource (Camarillo, CA, USA) or Southern Biotechnology Associates Inc (Birmingham, USA), respectively. Ovomucoid, ovalbumin, hypoxanthine, and 2,2'-azino-di-3-ethylbenz-thiazoline sulfonic acid (ABTS) was purchased from Wako (Osaka, Japan).

Cells and cell culture. The hybridoma cell line OM21, which secretes the mouse MAb-OM21 (IgG1, κ) directed against ovomucoid, was generated by fusing a B lymphocyte from an ovomucoid-immunized mouse with the mouse myeloma cell line P3-X63-Ag8-U1. Chinese hamster ovary (CHO) cells were cultured in ERDF (Kyokuto Pharmaceutical, Tokyo, Japan) medium with 5% fetal bovine serum (Intergen, Purchase, NY), 100 μg/ml hypoxanthine, and 16 μM thymidine. COS-7 and OM21 cells were cultured in ERDF medium containing 5% fetal bovine serum. All cell lines were cultured under humidified 5% CO₂/95% air atmosphere at 37°C.

Cloning and sequencing of the MAb-OM21 heavy and light chain variable region genes. Total RNA was isolated from OM21 cells using Trizol reagent (GIBCO/BRL, MD, USA) according to the manufacturer's instructions. The cDNA was synthesized from 10 μg of the total RNA using 20 units of MMLV-reverse transcriptase (Amersham, UK) and 0.5 μg of (dT20) primer in the reaction volume of 20 μl. The resultant cDNA samples were amplified by 30 cycles of PCR in the presence of specific sense and antisense primers for variable region of the heavy chain (VH) or full length κ light chain genes (1 μM each), 200 μM dNTP, 0.05 U/μl Taq DNA polymerase (Perkin Elmer, USA), and PCR buffer (1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 0.08% Nonidet P40). Samples were denatured at 95°C for 9 min followed by amplification at 95°C for 30 sec, 55°C for 1 min and 72°C for 2 min for 30 cycles, followed by a final 10 min extension step at 72°C. Sequences of PCR primers were as follows: for the VH gene: sense primer P-MHV2 5'-ATG GGA TGG AGC T(A or G) ATC AT(C or G) (C or T)TC TT-3', and antisense primer P-MHVC2b 5'-CAG TGG ATA GAC TGA TGG GGG-3'; for the full length κ light chain gene: sense primer P-MKV5 5'-ATG GAT TT (A or T) CAG GTG CAG ATT (A or T) TC AGC TTC-3', and antisense primer P-MOKLR 5'-TTG GCT CTA ACA CTC ATT CC-3'. Purified PCR products were cloned into the pGEM-T sequence vector (Promega, USA) using the TA cloning method. The cloned genes were sequenced by the dideoxynucleotide chain termination method using the ABI Prism Dye Terminator Cycle Sequencing FS Ready Reaction Kit (ABI, USA) according to the manufacturer's protocol. The ABI DNA sequencer model 310 was used for cycle sequencing.

Construction of the chimeric heavy chain gene. The human IgG constant region fragment was obtained from the human IgG-producing hybridoma cell line BD9D12 (data not shown). The cloned VH region of MAb-OM21 and the human IgG constant region genes were amplified by PCR using overlapping oligonucleotides to generate a chimeric heavy chain gene (1st PCR). Overlapping oligonucleotides were designed to construct two DNA fragments that have overlapping ends. Sequences of overlapping oligonucleotides were as follows: for the VH region gene: antisense primer P-HUOM721GR 5'-GAT GGG CCC TTG GTG CTA GCT GAG GAG ACT GTG AGA GTG G-3'; for the human IgG constant region gene: sense primer P-HUOM721GF 5'-CCA CTC TCA CAG TCT CCT CAG CTA GCA CCA AGG GCC CAT C-3'. The VH fragment with the overlapping end was obtained using P-MHV2 and P-HUOM721GR, and the human IgG constant fragment with the overlapping end was obtained using P-HUOM721GF and P-HC4GR (5'-ACT-AGC-GGC-GCG-AGT-CAT-TTA-CCC-GGA-G-3'). Ten times diluted VH and constant fragments were mixed to hybridize the overlapping ends to each other. The fused DNA fragments were amplified by Taq DNA polymerase (Takara, Osaka, Japan) to produce a chimeric heavy chain gene using P-MHV2 and P-HC4GR (2nd PCR). PCR condition were as follows: for the 1st PCR, samples were denatured at 94°C for 2 min followed by amplification at 95°C for 30 sec, 60°C for 1 min and 72°C for 25 cycles, followed by a final 10 min extension step at 72°C; for the 2nd PCR, samples were denatured at 94°C for 2 min followed by amplification at 96°C for 20 sec, 68°C for 2 min for 25 cycles, followed by a final 10 min extension step at 72°C.

Expression of light and chimeric heavy chain genes. The light chain or the chimeric heavy chain genes were cloned into the expression vector pRc/CMV (Invitrogen, CA, USA). pRc/CMV contains the neomycin resistance gene as a selectable marker in mammalian cells, and the cytomegalovirus (CMV) promoter placed immediately upstream of the cloned gene to drive expression. The expression plasmids for the light chain and chimeric heavy chain genes were co-transfected into COS-7 or CHO cells using the electroporation method. CHO cells were
cultured for 2 days after the electroporation, and were cultured in the medium with 1 mg/ml of G418 (Gibco/BRL, MD, USA) added to select for transformants. COS-7 cells were cultured for 3 days after transfection and the culture supernatant was then used for further experiments.

Antigen binding assay. The concentrations of antibodies were measured by the ELISA method using anti-human IgG antibody. Reactivities of the antibodies to ovomucoid or ovalbumin were also assessed by ELISA. Ovomucoid or ovalbumin was dissolved in 50 mM sodium bicarbonate, pH 9.6, at a concentration of 10 μg/ml, and used to coat 96-well immunoplates (Nunc, Roskilde, Denmark). After the wells were washed with phosphate buffered saline containing 0.05% Tween 20 (TPBS), the wells were blocked with Block Ace (blocking reagent, Dainippon Pharmaceuticals, Japan) for 1 h at 37°C. Antibodies were added and incubated for 1 h at 37°C, then the plates were washed with TPBS. Bound antibodies were detected by HRP-anti-human IgG antibody. The enzyme color reaction was done by addition of a substrate solution containing 0.33 mg/ml 2,2'-azino-di-3-ethylbenz-thiazoline sulfonic acid (ABTS), 0.03% H2O2 in 0.1 M citrate buffer, pH 4.0. The absorbance of the color was measured at 415 nm.

Electrophoresis and western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the antibody samples was done under reducing conditions. The antibodies were then electro-transferred onto a nitrocellulose membrane. The blotted nitrocellulose membrane was blocked with Block Ace overnight at 4°C. After being washed with TPBS, the heavy and light chains were detected with HRP-anti-human IgG or HRP-anti-mouse κ antibodies. The antibodies were stained with an ECL kit (Amersham, UK).

Deglycosylation. N-deglycosylation of the OM21 κ light chain was done by treatment with the enzyme peptide N-glycosidase F (PNGase F) from Flavobacterium meningosepticum (Boehringer Mannheim, Germany) for 24 h at 37°C in 80 mM sodium phosphate buffer, pH 7.5.

Lectin blot assay of the carbohydrate chains on the light chain. Antibodies were electrophoresed on SDS-PAGE, and were electro-transferred onto a nitrocellulose membrane at 100 V for 1 h in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. The blotted membrane was cut into several strips. The strips were incubated in TPBS at 37°C for 1 h. After incubation, each strip was incubated with a 1:200 dilution of HRP-lectins or HRP-anti-mouse κ light chain antibodies at 4°C overnight. The color was developed by 4-chloro-1-naphthol.

Results

DNA sequence of the MAb-OM21VH and VL regions

The genes for the light chain and the variable region of heavy chain of MAB-OM21 were obtained by PCR using the appropriate primers. The cDNA sequences encoding the variable regions of the heavy and light chains were analyzed. The DNA and the deduced amino acid sequences of the two variable regions are shown in Fig. 1. Sequence analysis showed that the VH and VL of MAB-OM21 belong to subgroup IIB and κ subgroup VI, respectively, as described by Kabat et al.10 The light chain has only one N-glycosylation consensus sequence (N-X-S/T) in the complementarity determining region 2 (CDR2).
at asparagine-52. No N-glycosylation consensus sequence was found in the \( V_h \) region. These results suggested the possibility that at least one carbohydrate chain is located on the light chain variable region.

Cloning and expression of the MAb-OM21 heavy and light chain genes

It is known that IgE plays a central role in the allergic reaction, and also IgG plays an important role in inhibiting the release of various inflammatory mediators.\(^{11-17}\) Here we constructed a human IgG type chimeric heavy chain gene for the use of the MAb-OM21 in various applications. The constant region of the original heavy chain was replaced by the human IgG constant region, and the genes of this chimeric heavy chain and the light chain were co-expressed in COS-7 cells to confirm whether the cloned genes were able to produce a functional antibody reactive to ovomucoid. Generally, it has been thought that allergen specificity, which is essential to display the functions of antibodies, is one of the main properties for deciding how the antibody is used. For example, in an IgE-dependent allergic reaction, non specific IgE antibodies can increase the ability of mast cells and basophils to degranulate, but can't induce degranulation.\(^{18,19}\) Non specific IgG has been shown to be unrelated with the induction or inhibition of degranulation.\(^{12,13}\) Therefore, the reactivity of secreted immunoglobulin from COS-7 cells transfected with the engineered genes to ovomucoid was assessed by ELISA. As shown in Fig. 2, the secreted recombinant antibody could bind to ovomucoid, while conventional human IgG could not, suggesting that the cloned light chain gene and the chimeric heavy chain gene were able to produce a functional recombinant MAb-OM21 antibody.

Detection of carbohydrate chains on the MAb-OM21 light chain

Since the light chain of MAb-OM21 has only one N-glycosylation consensus sequence in the CDR2, we examined whether or not carbohydrate chains are located on the light chain. If N-linked carbohydrate chains are located on the light chain, a smaller size light chain should be observed after treatment with PNGase \( F \), which can remove the N-linked carbohydrate chains. The light chain of the antibody expressed in COS-7 cells was treated with PNGase \( F \), followed by immunoblot analysis. A smaller size light chain was detected, suggesting that N-linked carbohydrate chains are located on the light chain (Fig. 3). We further attempted to detect the carbohydrate chains directly by using various lectins that recognize specific carbohydrate structure. As shown in Fig. 4, the light chain was reactive to all lectins examined (Con A, LCA, E-PHA, RCA120, WGA, and PNA). These results demonstrated that a N-linked carbohydrate chain is located on the light chain.

Fig. 2. Antigen Binding Assay of the Recombinant MAb-OM21 Produced from COS-7 Cells Transfected with the Cloned Heavy and Light Chain Genes.

Reactivity of the recombinant antibody to ovomucoid was assessed by ELISA. Antibody produced from the COS-7 cells (circle) and conventional human IgG (triangle) were tested against ovomucoid, followed by incubation with HRP-anti-human IgG antibody. Enzyme activity of HRP bound to the plate was assayed by the addition of ABTS, and the color reaction was measured at 415 nm.

Fig. 3. Western Blot Analysis of the \( \kappa \) Light Chain Treated with PNGase \( F \).

N-deglycosylation of the recombinant MAb-OM21 produced from COS-7 cells was done by incubation with PNGase \( F \) for 24 h at 37°C in 0.08 M sodium phosphate buffer, pH 7.5. The light chains derived from the antibodies treated with (lane 2) or without (lane 1) PNGase \( F \) were detected with HRP-anti-mouse \( \kappa \) chain antibody.

Fig. 4. Detection of MAb-OM21 Light Chains Using Various Lectins.

The recombinant MAb-OM21 produced from COS-7 cells or conventional mouse IgG was electrophoresed and then transferred to nitrocellulose membrane. The membranes were cut into several strips. Each strip was stained with anti-\( \kappa \) light chain antibody (a and h), Con A (b), LCA (c), E-PHA (d), RCA120 (e), WGA (f), or PNA (g).
Role of the carbohydrate chain on the light chain of MAb-OM21

The variable regions of antibodies are the central regions for antigen recognition, in particular, the CDR of the antigen binding site is where antigens directly come in contact with antibodies. We postulated that the carbohydrate chain on the light chain affects the antigen binding strength of MAb-OM21. To clarify the effects of the carbohydrate chain on the light chain in relation to antigen binding activity, we examined the reactivity of the COS-7 cells-derived antibody treated with PNGase F to ovomucoid. The reactivity of the deglycosylated antibody increased about four times as compared to the intact antibody (Fig. 5). This result indicates that the carbohydrate chain on the light chain of MAb-OM21 affects the reactivity of the antibody to ovomucoid. It has been shown that glycosylation of glycoproteins in mammalian cells is dependent on the host cells. Then, we produced the recombinant MAb-OM21 in CHO cells, and lectin blot analysis was done to examine whether N-linked carbohydrate chain is located on light chain of the CHO cells-derived antibody. As shown in Fig. 6A, the light chain was reactive to all lectins examined (Con A, LCA, E-PHA, RCA120, WGA, and PNA). This result demonstrated that a N-linked carbohydrate chain is located on the light chain. We also examined the size of the light chains of recombinant MAb-OM21 obtained from COS-7 and CHO cells. The size of the two light chains differed, suggesting that structures of the carbohydrate chains on the light chains obtained from COS-7 and CHO are different (Fig. 6B). To clarify the relationship between antigen binding activity and the difference of structures of the carbohydrate chain on the light chain, we compared the antigen binding activity of the two recombinant antibodies (Fig. 7A). The reactivity of the antibody produced from CHO cells was significantly reduced as compared to the antibody produced from COS-7 cells. Moreover, the antibody generated from CHO cells, but not COS-7 cells, was found to be cross-reactive to ovalbumin (Fig. 7B). These results indicate that the difference of structures of the carbohydrate on the light chain of MAb-OM21 affects not only the binding strength to antigen but also the specificity of the antibody.

Discussion

We have characterized a monoclonal antibody specific to ovomucoid, which is one of the major food allergens. Sequence and western blotting analysis demonstrate that the light chain of the anti-ovomucoid antibody does have a N-linked carbohydrate chain in the CDR2 site. Generally, it is known that carbohydrate chains linked to proteins is related to cell differentiation, growth, adhesion, and metastasis, and play an important role in cancer, virus infection, and inflammation reactions through the interaction with carbohydrate recognition molecules. Immunoglobulin is a glycoprotein, and the
Fig. 7. Antigen Binding Assay of the Recombinant MAb-OM21 Obtained from COS-7 and CHO Cells.

Reactivities of the antibodies obtained from COS-7 and CHO cells to ovomucoid (A) and ovalbumin (B) were assessed by ELISA. Antibodies obtained from COS-7 (closed circle) or CHO cells (open circle) and conventional human IgG (open triangle) tested against ovomucoid or ovalbumin, followed by incubation with HRP-anti-human IgG antibody was incubated. Enzyme activity of HRP bound to the plate was assayed by the addition of ABTS, and the color reaction was measured at 415 nm.

carbohydrate chains have been shown to be located only on the heavy chain constant region. However, especially, variable regions of the heavy and light chains have been shown to be modified with glycosylation only when the variable regions have a consensus sequence for N-glycosylation. Carbohydrate chains in constant regions are thought to be important for the effector functions of the antibody such as complement activation and antibody dependent cell mediated cytotoxicity.\(^3\)\(^4\) However, there have been a few reports showing the relationship between carbohydrate chains located in the variable region and antigen binding. It has been reported that the presence of a N-linked carbohydrate at asparagine-58 in the heavy chain variable region of an antibody specific for dextran increased the affinity for dextran 15-fold.\(^2\) We have recently reported that a N-linked carbohydrate chain is located on the light chain variable region of a lung adenocarcinoma specific human monoclonal antibody, and changes in the light chain glycosylation affect the antigen binding of that antibody.\(^6\)\(^9\) To investigate the relationship between carbohydrates on the light chain and antigen binding of MAb-OM21, we obtained carbohydrate variants of MAb-OM21 using two different methods. One is N-deglycosylation of the secreted antibody, another is producing the recombinant antibody from a different host cell. In a previous study, we showed that the variants, which trim the carbohydrate chain by glycosidases to different lengths, result in altered reactivity or acquisition of the ability to bind other antigens.\(^3\) Investigation of N-deglycosylation of MAb-OM21 by N-glycosidase showed that the reactivity of the N-deglycosylated antibody to ovomucoid increased as compared to the intact antibody. The increase of reactivity of the COS-7 cells-derived antibody by N-glycosidase treatment may be due to the removal of the carbohydrate chains, which have an inhibitory role for the ovomucoid binding of the antibody, from various glycoforms of the light chain.

It has been shown that the glycosylation of glycoproteins in mammalian cells is dependent on the host cells; this is ascribed to differences in glycosyltransferases or carbohydrate-modifying enzymes, and mutation of such enzymes were found in lectin-resistant variants.\(^6\)\(^9\)\(^27\)\(^29\) To examine the role of carbohydrate chains in the light chain variable region on the antigen recognition by changing the structure of carbohydrate, we previously produced an antibody from a glycosylation mutant cell line which is resistant to cytotoxic lectin Con A. This examination showed that the Con A-resistant variants produced variant antibodies consisting of an altered light chain which differs in size from the original antibody, and that these variant antibodies significantly altered the original binding activity or totally lost the antigen binding activity, indicating that varying the host cells can lead to structural changes of carbohydrate chains.\(^6\) Therefore, we produced the recombinant MAb-OM21 in both COS-7 and CHO cells. The difference in host cells lead to changes of the size of light chains. Considering such difference in the size and the results from lectin blot analysis of both light chains, it is speculated that difference in the size is due to the difference in the length of polyglactosamine in the carbohydrate chains on both COS-7 and CHO-derived light chains. The specificity of the recombinant antibody produced from COS-7 cells was conserved. On the other hand, the antibody generated from CHO cells showed diminished ability to bind to ovomucoid but acquired the ability to bind with ovalbumin. These results suggest that the difference in structures of carbohydrates in the light chain variable
region can influence the reactivity of food allergen-specific antibodies to their allergens. It has also been thought that the heavy chain is critical for the diversity of antigen recognition rather than the light chain since the amino acid sequence of the heavy chain variable regions is more varied compared to the light chains. However our findings show that the light chain can also influence antigen recognition through the carbohydrate chains.

It has been reported that abnormal glycosylation on the constant regions of IgG contributes to rheumatic diseases. The results shown here suggest a possibility that modulation of antigen recognition of food allergen-reactive antibodies through carbohydrates may also contribute to immunological disorders such as food allergy.

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