



IgG2 dominance and carbohydrate recognition specificity of C3H/He mouse antibodies directed to cross-reactive carbohydrate determinants (CCDs) bearing β -(1,2)-xylose and α -(1,3)-fucose

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ABSTRACT

Few common carbohydrate epitopes consisting of terminal β -(1,2)-xylose and/or α -(1,3)-fucose residues are shared by a variety of glycoproteins from plants, insects and parasitic worms, termed cross-reactive carbohydrate determinant (CCD), and frequently recognized by IgE antibodies of patients with food and/or respiratory allergy, though clinical relevancy of such CCD-specific IgE is still controversial. Attention has also been focused on CCDs from the undesired post-translational modification of recombinant therapeutic proteins produced by transgenic plants and insects. In the present study, to clarify immunogenic potentials of CCD-bearing glycoproteins, the antibody response to a model plant glycoprotein, horseradish peroxidase (HRP) was investigated in a mouse model. C3H/He mice were immunized with HRP plus Al(OH)₃ or Freund's adjuvant, and IgG and IgE responses to CCDs in addition to HRP were analyzed by ELISA using some distinct glycoproteins with known N-glycan structures. IgE response to HRP was induced remarkably, whereas that to CCD was weaker and delayed. Moreover, apparent ratio of the CCD-specific antibodies to HRP-specific ones tended to be higher in IgG2a and IgG2b isotypes than IgG1, IgG3 and IgE. In contrast to rabbit antibodies, the CCD-specific antibodies from the mice gave poor reactivity with bromelain and honeybee phospholipase A2, suggesting the critical role of both β -(1,2)-xylose and α -(1,3)-mannose in the CCD-recognition by the mouse antibodies. Moreover, the mouse antibodies showed weaker cross-reactivity to pollen- and insect-derived glycoproteins than the rabbit ones. Thus, in this mouse model, not only IgE but also IgG2 antibody responses to CCDs were induced by immunizing with a CCD-bearing glycoprotein, suggesting that CCDs affected not only Th2-type but also Th1-type antibody response at least in C3H/He mice.

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1. Introduction

Some plant N-glycans have unique structures that distinct from those found in their mammalian counterpart and are characterized by the absence of sialic acid and the presence of β -(1,2)-xylose and α -(1,3)-fucose residues [1]. Same or similar glycan structures are present also on glycoproteins of insects, molluscs, and parasitic worms [2–4]. The N-glycans containing such β -(1,2)-xylose and α -(1,3)-fucose residues are known to be recognized by mammalian immune system [5]. It has become evident that few common epi-

topes consisting of β -(1,2)-xylose and α -(1,3)-fucose residues are shared by a variety of glycoproteins recognized by IgE antibodies of patients with food and/or respiratory allergy [6]. These common glycan epitopes on distinct proteins are termed cross-reactive carbohydrate determinants (CCDs) of allergens [5,7], though definition of CCDs is ambiguous and a variety of carbohydrate epitope structures are speculated. The clinical relevancy of CCDs is still controversial in IgE-mediated allergies, while attention has again been focused on the CCDs as undesired post-translational modification products of recombinant therapeutic proteins from transgenic plants and insects.

Production and characterization of antibodies specific for CCDs have been investigated in some animal models [8,9]. Immunizing rabbits or goats with a typical CCDs-bearing plant glycoprotein,

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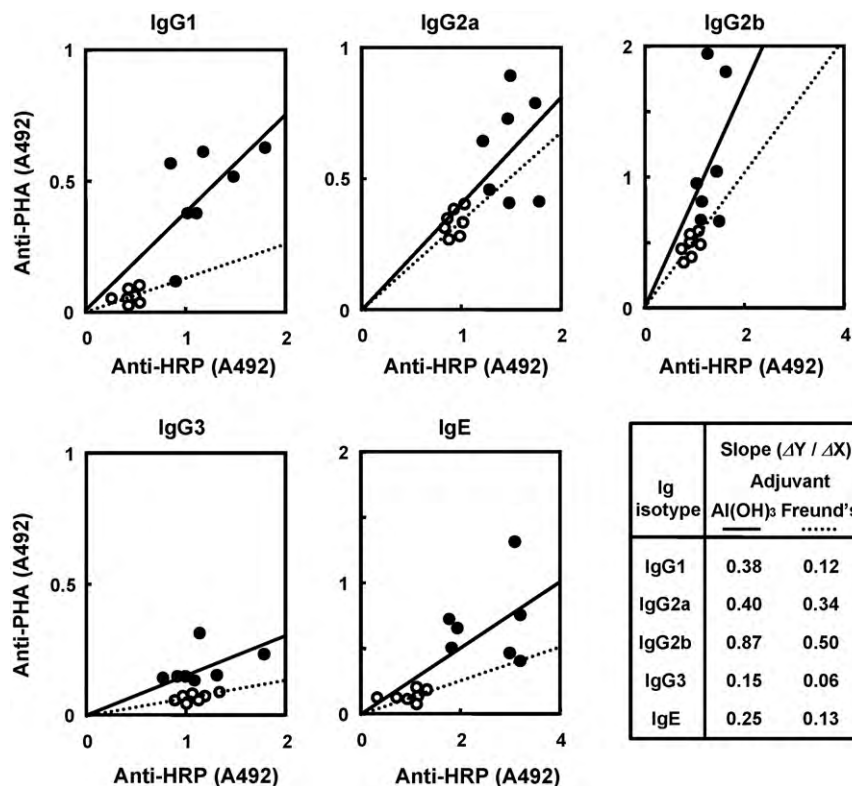


Fig. 2. The anti-PHA/anti-HRP plot for IgG and IgE antibodies from the C3H/He mice immunized with HRP. The serum antibody titer to PHA (Y axis) and HRP (X axis) of each blood sample collected from seven C3H/He mice in each of the Al(OH)₃ (closed circles) and Freund's adjuvant (open circles) experimental groups was estimated by ELISA as described in Section 2. Regression lines which cross intersection of the axis were obtained by the method of least-squares. The slope ($\Delta Y / \Delta X$) of each line is listed in the bottom-right panel.

rabbit IgG antibodies specific for each subclass (Nordic immunological Laboratories) were used as the secondary antibodies, and HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) as the tertiary antibody. When HRP-derived glycopeptides described below were used as coated antigens, Reacti-Bind™, maleic anhydride-activated polystyrene 96-well plates (PIERCE, Rockford, USA), were used according to manufacturer's instruction. In the present study, the antibodies cross-reactive to PHA among the mouse and rabbit anti-HRP antibodies were regarded as the CCD-specific antibodies, because both of HRP and PHA share the common carbohydrate epitope consisting of β -(1,2)-xylose and α -(1,3)-fucose residues in their *N*-linked sugar chains (Fig. 1), and because the cross-reactivity was lost by deglycosylation of the carbohydrate moiety from PHA [11]. No reactivity of PHA with mouse immunoglobulin sugar chains was confirmed by using heat inactivated PHA without the lectin activity. To inactivate enzymatic activity of HRP used as a coated antigen, the wells coated with HRP were treated with 3% H₂O₂ containing 1% NaN₃ for 30 min at 25 °C.

2.4. Preparation of glycopeptides from HRP

HRP (10 mg in 3 ml PBS) was incubated with 1 mg of thermolysin (SIGMA) for 48 h at 70 °C, and then subjected to gel filtration chromatography on a BioGel P-4 (Bio-Rad, Richmond, USA) column (1.3 cm $\times$ 65 cm) equilibrated with distilled water. Carbohydrate-containing fractions as determined by the phenol-sulfuric acid method [19] were pooled and freeze-dried. The dried sample was dissolved in 1 ml PBS, further digested with 1 mg pronase (SIGMA) at 37 °C for 48 h to make residual peptide smaller, and subjected again to the gel filtration chromatography as above. The

carbohydrate-containing fractions were pooled and kept frozen until use.

3. Results

3.1. IgG2 dominance in the CCD-specific antibody response of C3H/He mice

In a preliminary experiment using three strains of mice (BALB/c, C3H/He and B10.A), C3H/He was found to be a high responder strain to CCDs. Therefore, two groups of C3H/He mice (9 mice each) were immunized, respectively with HRP plus Al(OH)₃ and Freund's adjuvant, and the serum antibody responses were analyzed to HRP and CCDs. The CCD-specific antibodies became to be produced several weeks behind the anti-HRP antibodies, and increased considerably 26 weeks after the priming (data not shown).

On the day 35 and 11 weeks after the initial immunizations with HRP plus Al(OH)₃ and Freund's adjuvant, respectively, serum IgG and/or IgE responses to CCDs were detected for all mice. Two mice among nine immunized with HRP plus Al(OH)₃ produced IgG and IgE antibodies with extraordinarily high reactivity to many distinct glycoprotein antigens tested and, therefore, these two mice were eliminated from the analyses. As shown in Fig. 2, the immunization with Al(OH)₃ induced much stronger response to HRP of Th2 antibodies, IgG1 and IgE, as compared to the immunization with Freund's adjuvant. To analyze dominance of the CCD-specific response among immunoglobulin classes, each ELISA value of PHA-specific antibody was plotted against that of HRP-reactive one (Fig. 2). The ratio of anti-PHA ELISA values to the anti-HRP as demonstrated with the slope of each regression line was low in the IgG1, IgG3 and IgE isotypes and high in the IgG2a and IgG2b.

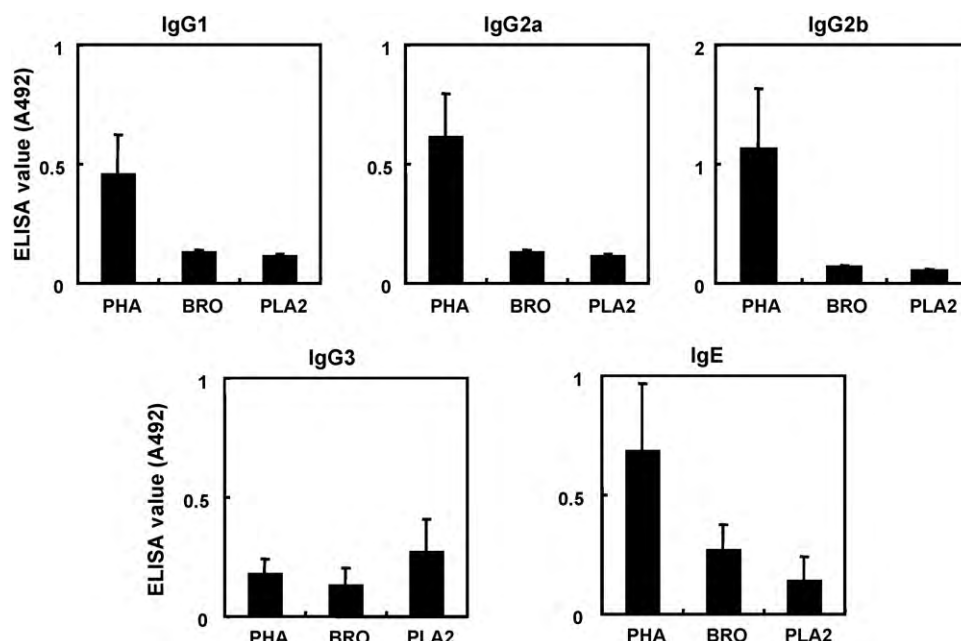


Fig. 3. Cross-reactivity to the three model glycoproteins of the antibodies from the C3H/He mice immunized with HRP plus Al(OH)₃. Reactivity of IgG1, IgG2a, IgG2b, IgG3 and IgE antibodies to the three glycoproteins, phytohemagglutinin (PHA) with MMXF, bromelain (BRO) with MUXF, and phospholipase A2 (PLA2) with MMUF, was measured for the antisera from the mice. The ELISA values are represented as mean $\pm$ SD of the seven mice.

Such tendency was remarkable in the mice immunized with HRP plus Freund's adjuvant.

3.2. Glycan-binding specificity of the mouse anti-CCD antibodies

The glycan-binding specificity of antibodies of the C3H/He mice immunized with HRP plus Al(OH)₃ was analyzed using the three glycoproteins with structurally defined *N*-glycans, PHA (MMXF), AsOX (MMXF), BRO (MUXF) and PLA2 (MMUF) (see Fig. 1). All of IgG1, IgG2a, IgG2b and IgE antibodies from the mice reacted considerably with PHA with the glycan containing both of the β -(1,2)-xylose and α -(1,3)-fucose, whereas these antibodies poorly reacted with BRO and PLA2 with glycans lacking α -(1,3)-mannose and β -(1,2)-xylose, respectively (Fig. 3).

To determine whether such specificity of the mouse antibodies depends on adjuvant used for immunization, the reactivity to PHA, AsOX, BRO, PLA2 and the HRP-derived glycopeptides (HRP-gp), was analyzed for serum IgG antibodies from the mice immunized with HRP plus Freund's adjuvant and Al(OH)₃, respectively. A commercially available rabbit anti-HRP IgG was also analyzed for comparison. HRP-gp was prepared from HRP by protease digestion followed by the size-exclusion chromatography (data not shown). The carbohydrate-containing fractions following after the void-volume UV absorbance peak were pooled and used as HRP-gp for the following experiments. Molecular masses of the glycopeptides preparation were estimated to be between 800 and 4000 based on the packed gel property. As shown in Fig. 4A, the mouse IgG from the Freund's adjuvant group reacted to BRO (MUXF) and PLA2 (MMUF) significantly weaker than PHA (MMXF), AsOX (MMXF) or HRP-derived glycopeptides (HRP-gp). This binding specificity resembled that of IgG from those of the Al(OH)₃ group. As expected based on some previous studies [8,20], rabbit IgG contained antibodies well reactive to all of the four glycoproteins as well as the HRP-derived glycopeptides. To confirm difference in the binding specificity between the mouse and rabbit antibodies, the IgG binding to PHA, AsOX, BRO and PLA2 was analyzed at several dilutions of each antiserum (Fig. 4B). The mouse IgG showed

higher reactivity to PHA and AsOX than BRO and PLA2 at any dilutions. By contrast, the rabbit IgG reacted almost equally to the four glycoproteins even under the lower antibody-concentration conditions, where the coated antigen was excess for and not saturated with the specific antibodies. The ELISA using varied concentrations (0–2.5 μ g/ml) of glycoprotein antigens for the plate coating produced the same results, that is, the mouse antibodies poorly reacted with BRO and PLA2 (Fig. 4C). The characteristic specificity in CCD-binding of the mouse antibodies prompted us to analyze cross-reactivity of the antibodies to some other glycoproteins from pollen allergen extracts with more complicated and undefined glycan chains. As shown in Fig. 5A, the mouse IgG antibodies from both of the Al(OH)₃ and Freund's adjuvant groups showed higher reactivity to the cypress allergen extract and lower reactivity to the ragweed one. In contrast, the rabbit IgG reacted with the ragweed more strongly than the cypress.

Additionally, cross-reactivity of the CCD-specific antibodies was analyzed also to several recombinant mammalian glycoproteins expressed by insect High-Five cells as well as silkworm (Fig. 5B), which were expected to produce insect-type *N*-glycans bearing α -(1,3)-fucose [21]. As for the recombinant proteins from the High-Five cells, the mouse IgG antibodies from either the Al(OH)₃ or the Freund's adjuvant group reacted with rGM-CSF and rIL-2A more strongly than rIFN- γ . In contrast, the rabbit IgG reacted almost equally with all the three recombinant proteins. All of the mouse and rabbit antibodies showed the lower binding activity to rIL-2B from silk worm as compared with rIL-2A from High-Five cells.

4. Discussion

Immunization of mice with soluble protein antigens plus Al(OH)₃ generally induces Th2-type immune response, resulting in elevation of serum level of specific IgG1 and IgE antibodies. In fact, in the present study, IgG1 and IgE responses to HRP and CCDs (PHA) were observed considerably. At the same time, however, IgG2a and IgG2b antibodies also were induced remarkably (Fig. 2). In the ELISA-based assay, restrict comparison in the response intensity could not be done among immunoglobulin isotypes, because of

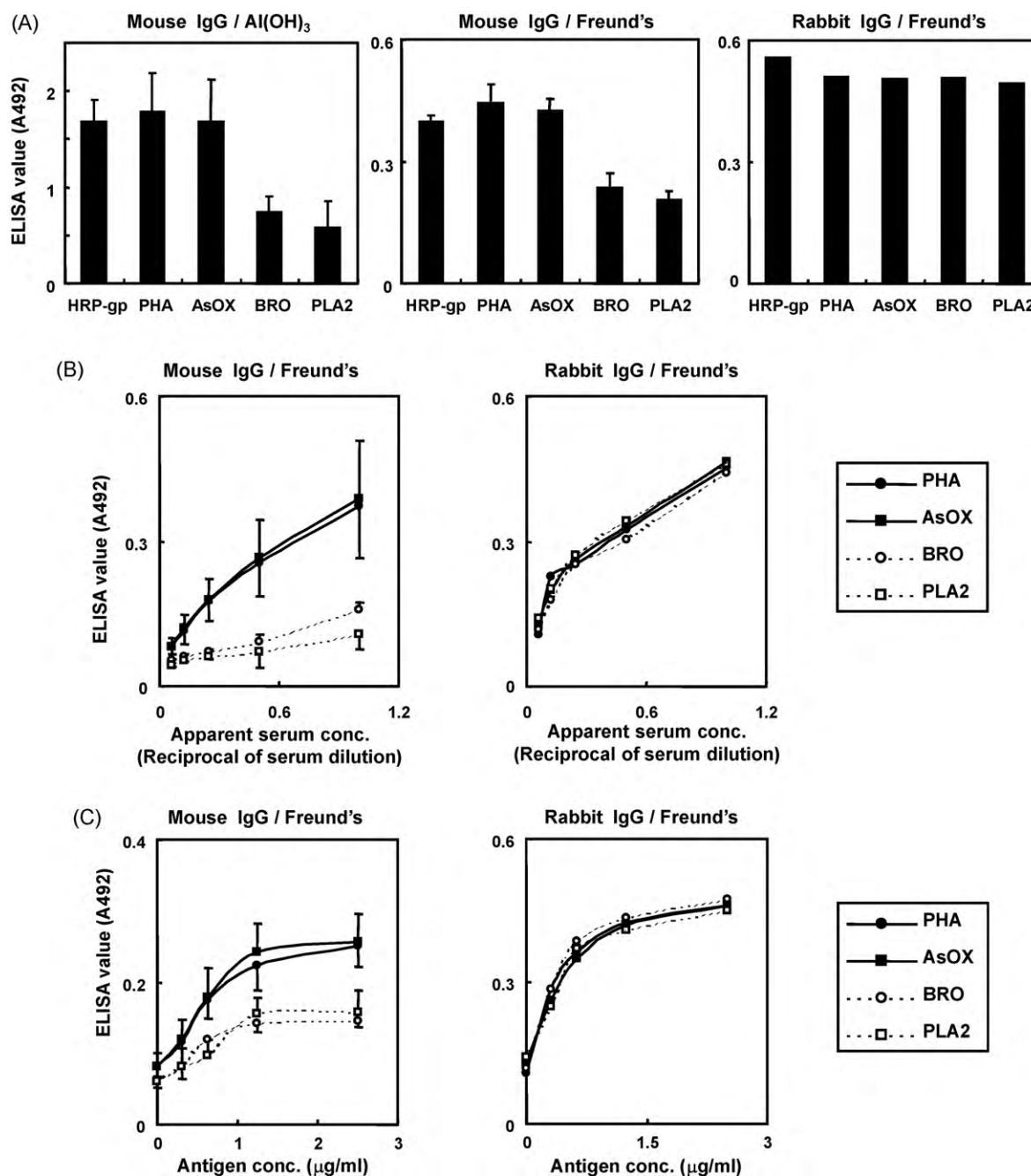


Fig. 4. Cross-reactivity to HRP-derived glycopeptides (HRP-gp) and the model glycoproteins of the IgG antibodies from the C3H/He mice and a rabbit immunized with HRP. (A) Reactivity of IgG antibodies to HRP-gp and the four glycoproteins (PHA, AsOX, BRO and PLA2) was measured for the antisera from the mice immunized with HRP plus Al(OH)₃ or Freund's adjuvant. The ELISA values are represented as mean $\pm$ SD of the seven mice. The reactivity of IgG from a rabbit immunized with HRP plus Freund's adjuvant was also analyzed for comparison. (B) The reactivity to PHA, AsOX, BRO and PLA2 of mouse and rabbit antibodies was analyzed using serially diluted antiserum. Each antiserum was 1:50 diluted (mouse) or 1:1000 diluted (rabbit), and then further diluted serially by twofold. The X-axis scale is represented as the reciprocal of the antiserum serial dilution. (C) The reactivity to PHA, AsOX, BRO and PLA2 of mouse and rabbit antibodies was analyzed at varied concentrations (0–2.5 μ g/ml) of the glycoproteins for coating.

the relative values obtained using each enzyme-labeled secondary antibody with different affinity and enzyme-activity. Nevertheless, it would be worth noting that the ratio of CCD-specific antibody, as represented with PHA-specific one, to HRP-specific one was higher in IgG2a and IgG2b than the Th-2 type antibodies, IgG1 and IgE (Fig. 2). These results suggested that the glycan chains of HRP stimulate mouse immune cells and enhance IgG2 response, at least to the CCD portion of HRP. Molecular and cellular mechanisms remain uncertain to explain the isotype-dependent differential response of antibodies to a specific epitope (CCD) of a single

molecule. The Th1-dominant anti-CCD antibody response, as compared with the anti-HRP Th2-response, may indicate involvement of Th1 cytokines produced by T cells activated with CCDs. A question remains whether such Th1 cytokines activate CCD-specific B cell clones more strongly or preferentially among B cell repertoire, which recognize HRP. One possible hypothesis is that binding of multiple CCDs on a HRP molecule to the receptors of a CCD-specific B cell clones may stimulate the B cells and affect immunoglobulin class switch in the stimulated B cells. In the case of humans, many patients with allergic symptoms frequently have serum IgE anti-

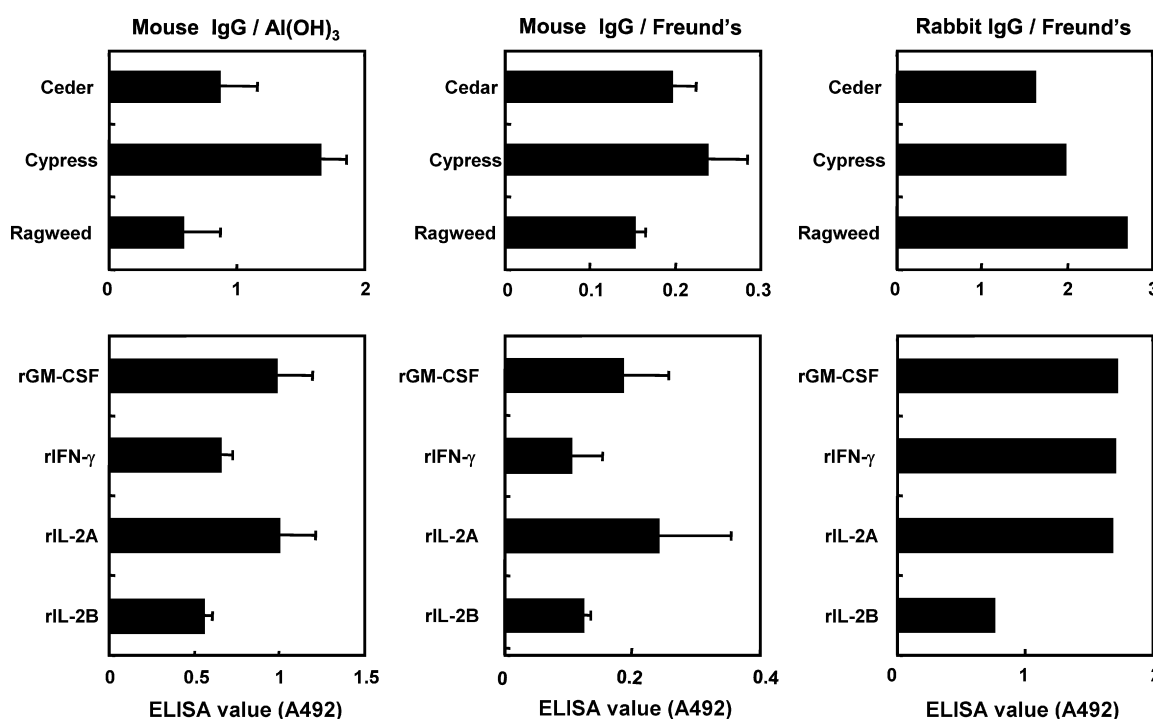


Fig. 5. Cross-reactivity to pollen-allergen extracts and insect-derived recombinant glycoproteins of the IgG antibodies from the C3H/He mice and a rabbit immunized with HRP. Reactivity of IgG antibodies to the pollen allergens (cedar, cypress, and ragweed) and the insect-derived recombinant glycoproteins (rGM-CSF, rIFN- γ , rIL-2A and rIL-2B) was measured for the antisera from the mice immunized with HRP plus Al(OH)₃ or Freund's adjuvant. The ELISA values are represented as mean $\pm$ SD of the seven mice. The reactivity of IgG from a rabbit immunized with HRP plus Freund's adjuvant was also analyzed for comparison.

bodies to CCDs [6], but the other isotypes of antibodies to CCDs have not been well investigated except for IgG4 [22]. It seems difficult to estimate human antibodies to each of the CCD and protein portions of a glycoprotein antigen, because human anti-CCD antibodies are considered to be mixture of those against various glycoprotein antigens with CCDs.

Recently, recombinant therapeutic proteins have become to be expressed widely in transgenic plants and insects [23]. However, immunogenic potential of such plant- or insect-derived CCD-bearing glycoproteins are still poorly understood, despite of its clinical defect from viewpoints of allergic response to and blood clearance of the therapeutic glycoproteins. The IgG2-dominant antibody response to CCDs of HRP observed in the present study may suggest that *N*-glycosylation done in plant and insect expression systems affect immunoglobulin isotype dominance in the CCD-specific antibody response at least to non-self-recombinant proteins. It would be of interest to investigate the immunogenic potential of CCD-bearing self-proteins produced in plant and insect expression systems. The animal model of C3H/He mice may be useful for the evaluation of such recombinant therapeutic proteins.

In our recent study, the mouse anti-HRP antisera containing CCD-specific IgE did not always induce degranulation of sensitized rat basophilic RBL-2H3 cells after challenging with a glycoprotein, PHA, bearing CCDs (MMXF) [11]. The reason why the combination of CCDs and their specific IgE did not induce degranulation of the RBL-2H3 cells is uncertain at this moment. The high-affinity IgE receptor, Fc ϵ RI, of RBL-2H3 cells and rat peritoneal mast cells are known to bind with not only IgE but also cross-linked IgG2a [24]. Moreover, mouse and human mast cells express low-affinity receptors for mouse IgG [25,26]. More detailed studies using not only antiserum but also purified IgE and IgG2 antibodies would be required to elucidate physiological relevance of the CCD-specific IgG2 antibodies produced by C3H/He mice immunized with HRP.

CCD-specific antibodies are well-known to exist in antisera produced by immunizing rabbits and goats with HRP, and a recent

study showed that rabbit anti-HRP sera contained IgG antibodies with high affinity ($K_d = 10^{-9}$ M) to either β -(1,3)-xylosylated or α -(1,3)-fucosylated *N*-glycans [27]. Commercially available goat anti-HRP antibodies were reported to exhibit similar specificity to that of rabbit anti-HRP [27]. In consistent with these previous results, the rabbit anti-HRP antibody showed high reactivity to most of plant- and insect-derived glycoproteins, including BRO (MUXF) and PLA2 (MMUF) (Figs. 2–4). By contrast to such rabbits and goats antibodies, the anti-CCD antibody from the C3H/He mice exhibited a characteristic carbohydrate-binding specificity, i.e., weak or no cross-reactivity to BRO (MUXF), PLA2 (MMUF), and pollen and insect glycoproteins (Figs. 2–4), suggesting that the mouse antibodies do not strongly bind with a β -(1,2)-xylose or an α -(1,3)-fucose residue alone. Such characteristic binding to CCDs was observed for antibodies from mice immunized with either Al(OH)₃ or Freund' adjuvant. Therefore, CCDs-recognition specificity might be ascribed to difference in rather animal species than the adjuvant used, though mouse strain dependency could not be ruled out. Utilization of the C3H/He mouse anti-CCD antibodies in combination with rabbit ones might be effective to estimate whether CCDs consist of all or each one of α -(1,3)-mannose, β -(1,2)-xylose and α -(1,3)-fucose residues.

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