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Fucose α 1,3-Linked to the Core Region of Glycoprotein N-Glycans Creates an Important Epitope for IgE from Honeybee Venom Allergic Individuals

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Key Words

Bee venom
Glycoprotein
N-linked oligosaccharides

Abstract

The reactivity of sera from honeybee venom allergic patients with the N-glycan of phospholipase A₂ was investigated using neoglycoproteins with an enzyme-linked immunosorbent assay. Of 122 sera with appreciable levels of IgE antibodies directed against bee venom as measured by radioallergosorbent test 34 sera exhibited significant amounts of glycan-reactive IgE. These sera cross-reacted with the N-glycan from the plant glycoprotein bromelain. The interaction of IgE with the N-glycan from phospholipase could be inhibited with glycopeptides from bromelain which shares the α 1,3-fucosylation of the asparagine-bound N-acetylglucosamine with bee venom phospholipase. Since defucosylated bromelain glycopeptides or glycopeptides containing a Man:GlcNAc₂ oligosaccharide were not recognized by most of these sera, we conclude that α 1,3-fucosylation of the innermost N-acetylglucosamine residue of N-glycoproteins forms an IgE-reactive determinant. This structural element is frequent in glycoproteins from plants, and it occurs also in insects. It is suspected to be one of the major causes of the broad allergenic cross-reactivity among various allergens from insects and plants.

Introduction

Despite the finding of cross-reactions between vegetable foods, pollen, and insect venom due to IgE against a ubiquitous cross-reactive carbohydrate determinant [1, 2], the involvement of the carbohydrate moiety of glycoproteins in allergic reactions has received minimal attention. Sea squirt O-glycans as well as N-glycans from a honeybee venom glycoprotein have been reported to bind IgE from allergic individuals [3, 4].

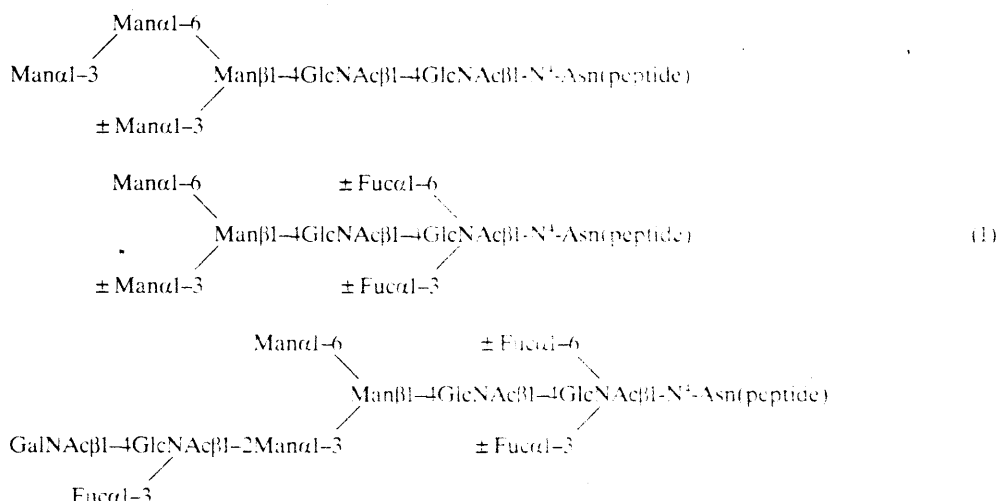
Honeybee venom allergy is primarily an IgE-mediated type of hypersensitivity. Due to possible anaphylactic reactions to honeybee venom, bee stings are a lethal threat to allergic persons [e.g., 5, 6]. Among the various components of bee venom, phospholipase A₂ (PLA) is the major allergen [7], although other components such as, e.g., hyaluronidase may likewise play an important role [8, 9]. PLA accounts for 12% of the dry weight of bee venom and is thus the major protein component [10]. It is a glycoprotein composed of 134 amino acids and a single carbohydrate

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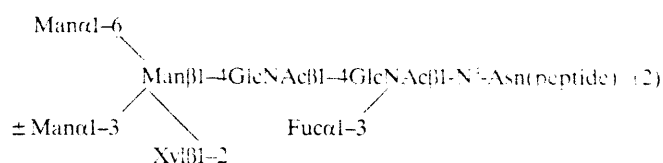
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side chain linked to asparagine 13 [11]. About 12% of PLA occur as a non-glycosylated isoform [4, 12]. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the glycosylated fraction is separated into two

bands: PLA-18, the main glycoform, and PLA-20, containing N-acetylgalactosamine [12]. The structures of the heterogeneous N-glycan of the glycosylated fraction of PLA have now been elucidated [13] (formula 1: Fuc = fucose).



The occurrence of Fuc $\alpha 1,3$ -linked to the innermost GlcNAc is a remarkable analogy to the N-glycans of plants which often additionally display a xylose linked to the β -mannosyl residue. Typical examples of this structural type are the N-glycans of pineapple stem bromelain (two mannoses) and of horseradish peroxidase (three mannoses) [14, 15] (formula 2).



Such N-glycans are immunogenic, leading to highly cross-reactive antisera [16, 17]. The participation of the Fuc $\alpha 1,3$ and Man $\alpha 1,6$ residues in the formation of the immunogenic determinant has been revealed in the case of a rabbit antiserum [15, 18], although other structural features, e.g., the xylose residue, may likewise constitute the heart of an epitope region [17, 19]. Immunization with PLA has been shown to elicit an immune response which was partially directed against the carbohydrate moiety of PLA [20]. Again, this serum cross-reacted with a number of plant glycoproteins. In analogy to antisera raised against plant glycoproteins, the reaction of anti-PLA serum with the N-glycan was shown to involve the Fuc $\alpha 1-3$ GlcNAc-Asn group [20].

Weber et al. [4] recognized that the sera of some bee venom allergic individuals contained IgE antibodies directed against the carbohydrate portion of PLA. At that time no reliable information on the structures of these N-glycans was available. Since the structures of the PLA N-glycan now have been elucidated [13], we reinvestigated these early findings and tried to identify the structural feature(s) responsible for the IgE-carbohydrate interaction. In this report we have investigated the interaction of IgE antibodies of 122 individual sera from bee venom allergic patients with the carbohydrate moieties of honeybee PLA and of plant glycoproteins using, among others, neoglycoproteins as coat antigens with an enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Allergic Patient's Sera and Other Materials

One set of sera (A and B sera) from 51 bee venom allergic patients was a kind gift of Drs. H. Ebner and D. Kraft (Ambulatorium für Allergie und klinische Immunologie, Vienna, Austria). 20 sera (C sera) were donated by Dr. R. Einarsson (Pharmacia, Uppsala, Sweden). Another 51 sera (D sera) were kindly provided by Dr. J. Ring (Dermatologie, Division of the Universitätskrankenhaus Hamburg-Eppendorf, FRG), and one serum (B1) was obtained from a bee venom allergic collaborator of the Universität für Bodenkultur. The sera of group A had radioallergen sorbent test (RAST) scores classes against bee venom of at least 3, the other sera of 2 or higher. As far as the RAST class is given, it was determined with the CAP system (Pharmacia).

Free and alkaline phosphatase conjugated mouse antihuman IgE monoclonal antibody (Pharmingen) and goat antimouse IgG (H+L) coupled to alkaline phosphatase were obtained from Dianova (Hamburg), and a human IgE standard was delivered from Behring (Marburg, FRG).

Lyophilized bee venom was purchased from Nectarcop (Sofia, Bulgaria). Pineapple stem bromelain, horseradish peroxidase, pronase E from *Streptomyces griseus*, β -N-acetylhexosaminidase from jack beans, and rabbit antihorseradish serum were from Sigma. β -Galactosidase from *Aspergillus oryzae* was purified as described [13]. Glycoamidase A from almonds was purchased from Seikagaku Kogyo (Tokyo, Japan).

Preparation of Antigens, Neoglycoproteins, and Inhibitors

PLA was purified from honeybee venom by gel filtration and ion exchange [12]. The unglycosylated variant, termed PLA-16 referring to the molecular mass [12], or PLA-0 according to its affinity towards concanavalin A [4], was prepared by lectin affinity chromatography [4]. Its purity was ascertained by SDS-PAGE.

Pineapple stem bromelain and horseradish peroxidase were purified as described [21]. Glycopeptides were prepared from unfractionated PLA, from bromelain, and from bovine fibrin by two cycles of digestion with pronase followed by gel filtration over Sephadex G-25 using 1% (v/v) acetic acid as the eluent. Further purification was achieved by anion-exchange chromatography on Sephadex DEAE-A25 using 0.01–0.8 M sodium borate (linear gradient, pH 8.8). The main products were Asn(CHO)-Lys₁₆ in the case of PLA and Asn₁₆-Asn(CHO)-Glu-Ser-Ser₁₆ of bromelain as deduced from amino acid composition (data not shown).

Bromelain glycopeptides were defucosylated with trifluoroacetic acid overnight at room temperature and subsequently subjected to gel filtration [15, 20]. As shown by monosaccharide analysis using GLC-MS [21], the resulting glycopeptide bromelain-Fuc contained less than 1% of the fucose originally present.

Fibrin glycopeptides were desalted with sulfuric acid (50 mM, 80°C, 30 min) and desalted by gel filtration. Exhaustive digestion with β -galactosidase and β -N-acetylhexosaminidase yielded a glycopeptide with the core pentasaccharide Man₅GlcNAc₂ peptide [13].

Glycopeptides were coupled to bovine serum albumin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in phosphate-buffered saline using a 25-fold molar excess of glycopeptide [15]. Thereby, 3–4 mol oligosaccharide was attached per mole of protein as determined by gas chromatography-mass spectrometry and high-performance liquid chromatography analysis of aminosugars [22].

Free oligosaccharides were obtained from bromelain glycopeptides by digestion with glycoamidase A followed by passage over Dowex 50W-X2 in 0.5% acetic acid. Part of this preparation was reduced to oligosaccharitol with sodium borohydride. Another part was reductively aminated with 2-aminopyridine [13, 23]. These samples were purified by gel filtration.

ELISA and Immunoblot

Generally, an amount of 0.2 ml of the respective solution was incubated at room temperature if not otherwise stated. Antigens (0.01 mg/ml) were coated on 96-well microtiter plates (NUNC) at 37°C for 2 h using 0.1 M sodium carbonate buffer (pH 9.6). The wells were saturated with bovine serum albumin. Patient sera were diluted appropriately (5–50-fold) with Tris MS-buffered saline (TBS; 0.1 M Tris, HCl, 0.1 M NaCl, 2.5 mM MgCl₂, pH 7.4) containing 0.5% (w/v) bovine serum albumin and were allowed to react overnight. The wells

were then incubated with the alkaline phosphatase conjugated anti-human IgE antibody for 3 h. Sodium-4-nitrophenylphosphate (1 mg/ml) in 0.1 M Tris/HCl, 0.1 M NaCl, 2.5 M MgCl₂, pH 9.6) was added, and the reaction was stopped after 1 h by addition of 50 μ l of 5.0 M NaOH. All measurements are referenced to an IgE standard which was subjected to ELISA using antihuman IgE as the coat. Since no corrections for differences in antigen density and binding affinities were made, the results, though expressed in nanograms per milliliter, must be regarded as apparent concentrations.

ELISA inhibition assays were performed accordingly, except that the sera were preincubated with inhibitor for 3 h at room temperature. For inhibition assays, the sera were diluted to give 80% of maximum binding of IgE to the coated antigen. The inhibitor concentrations were determined by high performance liquid chromatography of N-acetylglucosamine [22].

For immunoprints, the isoforms of PLA were separated by SDS-PAGE using a 14% gel [12], and the protein bands were transferred to nitrocellulose. Blots were incubated overnight with diluted sera (1:20 in TBS containing 0.1% Tween 20). After washing, the strips were incubated with mouse anti-IgE (1:1,000 in TBS-Tween) followed by goat antimouse IgG conjugated to alkaline phosphatase (1:5,000 in TBS-Tween). Bound IgE was now visualized using the NBT/BCIP dyeing solution [24]. A reference strip was stained with India ink.

Results

Selection of Sera for Further Experiments

122 sera of individuals classified as bee venom allergic were tested for their reactivity against unfractionated honeybee venom PLA using an ELISA test system. 47 of these sera exhibited an apparent content of more than 20 ng/ml of IgE against PLA (table 1). Sera containing appreciable levels of IgE antibodies directed against the carbohydrate portion of PLA were not found in this group. Therefore, the specificity of these 47 sera was further investigated. The other sera may have contained no or very low levels of PLA-specific IgE. There is some risk that conformational epitopes of PLA may be disrupted by the adsorption of the antigen to the surface of the microtiter plate. IgE antibodies directed against such epitopes would not be recognized in the applied test system. Additionally, the IgE antibodies from the 75 sera which did not significantly react with PLA in ELISA may have been directed against bee venom components other than PLA.

IgE Binding to the Carbohydrate Portion of PLA and of Plant Glycoproteins

The unglycosylated fraction PLA-16 and PLA neoglycoprotein (PLA glycopeptides coupled to bovine serum albumin) were used as coat antigens in IgE ELISA to estimate the amounts of IgE directed towards the polypeptide and the carbohydrate moiety, respectively (table 1). The 47 sera thus fell into three groups exhibiting measurable

Table 1. Allergen-specific IgE content of sera from 47 honeybee venom allergic individuals

Serum code	RAST class against bee venom	Coat antigen					
		PLA	PLA-16	PLA glycopeptide	bromelain glycopeptide	bromelain -Fuc	fibrin (core)
A1	≥ 3	20	17.5	2.5	2.5	-	n.d.
A2	≥ 3	20	20	-	-	-	n.d.
A4	≥ 3	50	40	-	-	-	n.d.
A9	≥ 3	25	25	8	9	-	n.d.
A10	≥ 3	50	-	2	50	-	n.d.
A11	≥ 3	12.5	2	6	6	-	n.d.
A13	≥ 3	10	2	8	15	-	n.d.
A14	≥ 3	20	15	2.5	5	-	n.d.
A15	≥ 3	12.5	10	2.5	7.5	-	n.d.
A20	≥ 3	40	40	-	-	-	n.d.
A21	≥ 3	5	2.5	2	2.5	-	n.d.
A23	≥ 3	30	20	2	15	-	n.d.
A24	≥ 3	2.5	2.5	2.5	10	-	n.d.
A26	≥ 3	5	2.5	-	-	-	n.d.
A28	≥ 3	28	16	-	-	-	n.d.
A32	≥ 3	10	10	-	-	-	n.d.
A38	≥ 3	10	8	2.5	5	-	n.d.
B1	3	25	20	2	2	-	n.d.
B3	3	10	2	3.5	2.5	-	n.d.
B7	3	5	-	2.5	5	-	n.d.
B9	3	3.5	-	5	5.5	-	n.d.
B12	3	3.5	2	5	5	-	n.d.
B15	4	70	-	9	60	-	n.d.
B16	3	30	12.5	2	10	5	n.d.
B17	3	5	3.5	2	2	-	n.d.
C4	4	35	5	-	-	-	-
C5	4	2.5	-	2.5	7.5	-	-
C6	6	225	135	180	180	30	170
C7	3	10	-	2.5	65	10	10
C8	4	35	5	-	-	-	-
C10	4	2.5	-	2.5	7.5	-	-
C12	3	30	-	35	85	-	-
C13	n.d.	40	37.5	-	2.5	-	-
C14	4	32	-	30	56	-	-
C17	n.d.	30	20	-	-	-	-
C18	6	2,800	2,400	2.5	-	-	-
C19	n.d.	25	15	-	-	-	-
C20	4	10	-	7.5	7.5	-	-
D4	2	7.5	-	3	10	-	-
D16	6	30	-	20	30	-	-
D23	3	45	45	-	-	-	-
D24	3	-	-	5	22.5	-	-
D26	n.d.	15	12.5	6	5	n.d.	n.d.
D31	3	12.5	-	2.5	20	-	-
D32	6	60	-	80	50	-	-
D44	3	10	10	-	-	-	-
D48	3	7.5	-	3	15	-	-

Values represent the apparent content of antigen-specific IgE (ng/ml).

From 122 sera investigated only those are listed which reacted with PLA in the applied ELISA test system. Due to the special constraints of ELISA measurements, the values given have to be interpreted as apparent concentrations. Natural proteins were coated in case of unfractionated PLA and nonglycosylated isotorm PLA-16. Neoglycoproteins were used as coat antigens in case of PLA glycopeptide, bromelain glycopeptide, bromelain Fuc (glycopeptide), and fibrin (glycopeptide). Fibrin contained the Man-GlcNAc core pentasaccharide. - = ELISA readings below the level of significance; n.d. = not determined.

binding: (1) to the polypeptide only (13 sera), (2) to both peptide and carbohydrate (19 sera), and (3) to the PLA glycopeptide only (15 sera). The carbohydrate specificity of these IgEs is also evident on immunoblots (fig. 1), since the unglycosylated fraction PLA-16 and the glycosylated main fraction PLA-18 (and PLA-20) separate on SDS-PAGE, although they have identical polypeptide chains [12].

Initial experiments revealed a cross-reactivity of the antivenom sera with bromelain and horseradish peroxidase which both share the α 1,3-fucosylation of their N-linked oligosaccharides with PLA. In order to avoid any polypeptide-based cross-reaction, bromelain glycopeptides coupled to bovine serum albumin were used as coat antigen for IgE ELISA. 33 of the 34 sera which reacted with PLA glycopeptides also bound to the bromelain neoglycoprotein, and in many cases the binding was even stronger (table 1). One factor contributing to this difference may be the heterogeneity of the PLA glycopeptides, of which only 20% are α 1,3-fucosylated at the innermost GlcNAc [13]. This fucosyl residue is primarily responsible for the antibody binding, as is impressively demonstrated by the fact that defucosylated bromelain glycopeptides were no longer recognized by 30 (serum D26 was already exhausted) of the 34 patient sera which bound to PLA and/or bromelain glycopeptides (table 1). The same picture emerged when $\text{Man}_3\text{GlcNAc}_2$ peptide, derived from a mammalian glycoprotein, was offered as the hapten (table 1). Apart from serum B16 which has not been available for this experiment, the sera which recognized defucosylated bromelain also bound to the core pentasaccharide. The three sera appear to contain antibodies with a specificity differing from that of the other 30 sera.

Inhibition of the Carbohydrate-Based IgE Binding

Since the IgE antibodies of the patient sera have to be considered polyclonal, the reactivity to PLA and to bromelain glycopeptides is not necessarily caused by the same subset of antibodies. Therefore, the binding of IgE to PLA neoglycoprotein was measured in the presence of bromelain glycopeptide. Glycopeptides were chosen as the inhibitory substance despite the particular advantages of oligosaccharides or pyridylaminated oligosaccharides with regard to their purification and homogeneity [15].

The reason for this decision shall be briefly substantiated: Preceding experiments with a commercial anti-horseradish peroxidase serum revealed large differences in the potency of various glycan derivatives to inhibit the reaction with PLA. In detail, 50% inhibition was achieved with 0.3 μM bromelain glycopeptide, whereas bromelain

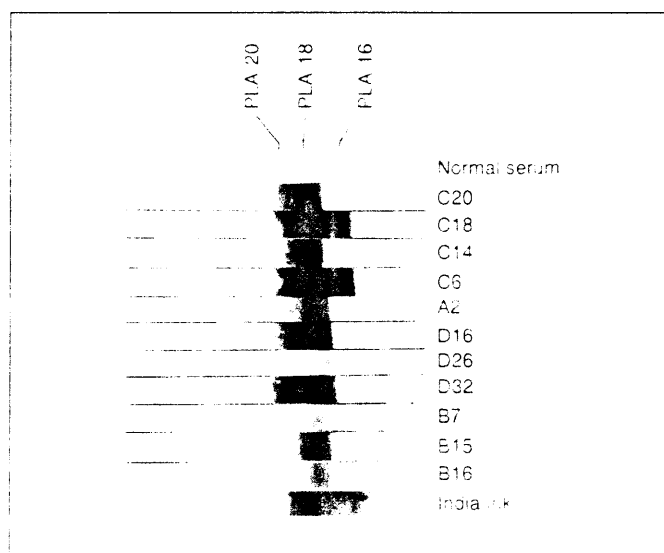
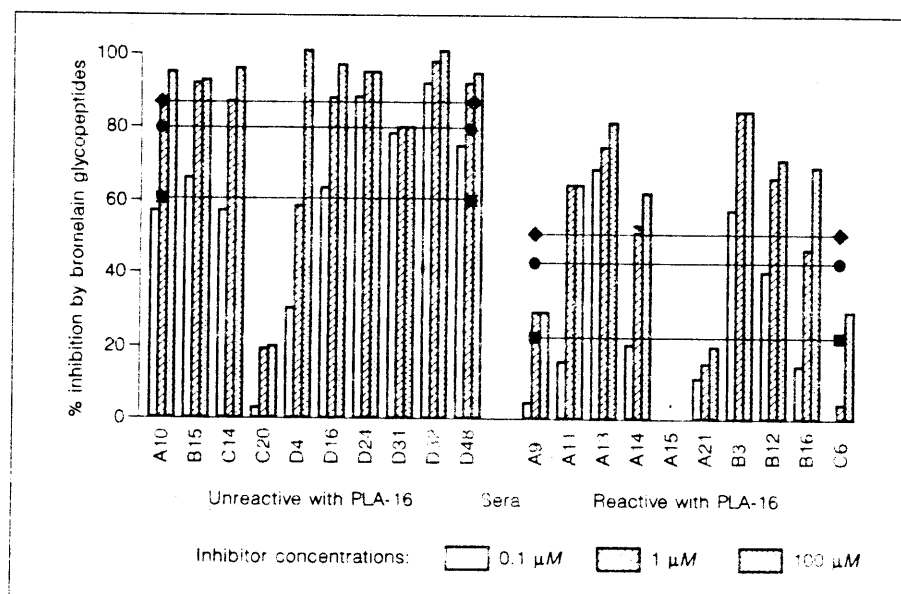


Fig. 1. IgE immunoblot of patient sera versus PLA fractions. PLA was separated by SDS-PAGE, and blots were incubated with diluted patient sera. Bound IgE was visualized by mouse antihuman IgE followed by goat antimouse IgG enzyme conjugate. The arrows indicate the positions of the three glycoforms of PLA, i.e., the unglycosylated variant PLA-16, the glycosylated main fraction PLA-18, and the second glycoform PLA-20 [12]. Both glycoforms exhibit the same pattern of fucosylation, but the glycan of PLA-20 contains a β -GalNAc-(Fuc-)GlcNAc antenna [13].

oligosaccharide, reduced bromelain oligosaccharide, and pyridylaminated oligosaccharide had to be applied at concentrations of 3, 10, and 160 μM , respectively. Defucosylated bromelain glycopeptide was the worst inhibitor. It had to be used at a concentration of 300 μM which may well reflect the small residual content of fucose. It is assumed that other antibodies reacting with α 1,3-fucosylated N-glycans will likewise be sensitive to structural changes of the innermost GlcNAc residue.

Twenty patient sera with high antigen-specific IgE titers were selected for inhibition experiments. PLA neoglycoprotein was used as the coat, and sera were preincubated with varying concentrations of bromelain glycopeptides. The binding of IgE from most of the sera could be inhibited to a remarkably high percentage even with moderate concentrations of heterologous hapten (fig. 2). It should be added that, for unknown reasons, complete inhibition could not be achieved in every case even with 100 μM PLA glycopeptides, though this homologous hapten yielded generally somewhat higher inhibition rates (data not shown). The essence of these results is that, on the whole, the same antibodies appear to be responsible for binding to PLA as well as to bromelain glycan.

Fig. 2. Inhibition of IgE binding to PLA glycopeptides. Glycopeptides from honeybee PLA coupled to bovine serum albumin were used as coat antigen. Antigen was incubated with properly diluted patient IgE in the presence of three different concentrations of bromelain glycopeptides as is indicated at the bottom of the figure. In this presentation, sera are arranged according to their reactivity with the unglycosylated fraction PLA-16, in other words, according to the absence or presence of IgE antibodies directed against the polypeptide moiety of PLA as measured by ELISA. Horizontal bars mark the mean values of inhibition achieved with each inhibitor concentration within the two groups of sera. Despite the large variation of inhibition achieved with individual sera, the mean values of the two groups differ statistically significant in all three concentration series ($\alpha = 0.005$).



Discussion

Glycoprotein N- and O-glycans are, by the nature of their biosynthesis, variations of a common theme, and, therefore, they represent a source of immunological cross-reactivity between evolutionary related as well as unrelated species. N-glycans from plant glycoproteins have repeatedly been shown to be immunogenic in mammals [15–19, 25]. This is explained by different structural motifs, since, e.g., xylosylation or α 1,3-fucosylation of the asparagine-bound N-acetylglucosamine has not yet been found in higher animals. N-glycans of glycoproteins from honeybee venom and most probably also from other insect materials [26] exhibit the α 1,6-fucosylation typical of mammalian cells and the α 1,3-fucosylation [13] hitherto believed to occur in plants only.

In this report we have shown that the carbohydrate chain of PLA constitutes an important target for IgE from honeybee venom allergic patients. 34 out of 122 sera reacted with the asparagine-linked glycans from both PLA and plant protease bromelain. Only three of these sera reacted at least weakly with defucosylated bromelain glycopeptides, indicating a different specificity of these sera. The other sera exhibited no measurable reaction, neither with defucosylated bromelain nor with the pentasaccharide core $\text{Man}_3\text{GlcNAc}_2$ peptide. This demonstrates that the α 1,3-linked fucosyl residue is the gist for the immunological cross-reactivity between insect and plant glycoproteins.

However, it should be emphasized that while the α 1,3-fucosyl residue is the big difference between mammalian and insect or plant glycoprotein glycans, it may by itself not be sufficient to elicit an immune response or to confer antibody binding. Other parts of the molecule may contribute to the binding affinity of antibodies, as it may be the case with the α 1,6-mannosyl residue [15]. Additionally, antibodies may be directed against other parts of the molecule, as is indicated by the reaction of at least two sera with the naked pentasaccharide core.

Irrespective of such considerations, the present study demonstrates α 1,3-fucosylation of the innermost GlcNAc residue to be a structural detail capable of creating an IgE epitope. The 'cross-reactive carbohydrate determinant' proposed in earlier studies [1, 2] can now be associated with the action of one particular glycosyl transferase activity, i.e., (GDP-fucose:GlcNAc-N-Asn)- α 1,3-fucosyltransferase [27]. Due to its now perceptible wide distribution in nature, it must be considered an important source of cross-reactivity in a wide variety of allergens. Such cross-reactivities actually have been impressively demonstrated [1, 2, 16, 28]. Like most other in vitro test systems, ELISA measures monovalent reactions. Therefore, they cannot reflect the clinical significance of the carbohydrate-based IgE reactivity. This will have to be investigated by other – e.g., cellular – test systems. However, one thing can be deduced from the present results with considerable certainty: if a serum containing antibodies against fucosylated N-glycans is subjected to diagnostic tests such as

RAST, CAP, or ELISA, a positive result will be obtained whenever the allergen contains oligosaccharides with α 1,3-fucose linked to the Asn-bound GlcNAc residue. The occurrence of this immunogenic and obviously also allergenic determinant in plant and insect glycoproteins is well established and most probably it is not restricted to these organisms. From a positive in vitro reaction a conclusion on the allergenic stimulus can, therefore, not be drawn unless it is established that the reaction involves peptide determinants which in general are more species specific. The inhibition experiment (fig. 2) actually tempts us to speculate that for some of the patients bee venom may not have been the stimulus. The reasoning for this view takes into consideration that bromelain and PLA glycans are structurally similar but not identical. An antibody population raised against honeybee N-glycans may contain immunoglobulin species with low affinity to the plant N-glycan or an antibody subpopulation reacting with a different determinant such as the GalNAc antenna of PLA [20].

The reaction of this serum with PLA neoglycoprotein can consequently not be fully inhibited. In contrast, if the reaction with PLA neoglycoprotein is only a cross-reaction of antibodies originally elicited by a plant antigen, then it should be easily inhibited by the bromelain glycan. Such sera would of course not react with PLA polypeptide.

The molecular identification of a major immunogenic determinant may help to find rational solutions to the issue of cross-reactivity caused by this carbohydrate structure. Finally, the capacity of insect cells to furnish recombinant glycoproteins with a potentially immunogenic and/or allergenic determinant must not be ignored.

Acknowledgments

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