

Specificity of IgG and IgE antibodies against plant and insect glycoprotein glycans determined with artificial glycoforms of human transferrin.

Monika Bencúrová, Wolfgang Hemmer², Margarete Focke-Tejkl², Iain B.H. Wilson, Friedrich Altmann¹

Glycobiology Division, Institute of Chemistry, University of Natural Resources and Applied Life Sciences (Universität für Bodenkultur), Vienna, Austria, and ²FAZ-Floridsdorf Allergy Center, Vienna, Austria

Key words:

anti-glycan antibody / core- α 1,3-fucose / insect glycoprotein / plant glycoprotein / cross-reactive carbohydrate determinant

¹Corresponding author: Friedrich Altmann, Institute of Chemistry, University of Natural Resources and Applied Life Sciences (Universität für Bodenkultur), Muthgasse 18, A-1190 Vienna, Austria

Tel.: +43-1-36006-6062

Fax: +43-1-36006-6059

Email: friedrich.altmann@boku.ac.at

Abstract:

Cross-reactive carbohydrate determinants of plants are essentially a mixture of N-glycans containing β 1,2-xylose and core α 1,3-fucose, the latter being also found in insect glycoproteins. In order to determine the relative contributions of these two sugar residues to antibody binding we have prepared an array of glycomodified forms of human apo-transferrin. Using core- α 1,3-fucosyltransferase (EC 2.4.1.214) and β 1,2-xylosyltransferase (EC 2.4.2.38) recombinantly expressed in *Pichia pastoris* and suitable glycosidases, glycoforms containing either only fucose (MMF), only xylose (MMX), both (MMXF) or none of them (MM) linked to the common pentasaccharide core were generated. Additional glycoforms were obtained by enzymatic removal of the α 1,3-linked mannosyl residue. These transferrin glycoforms served to define the binding specificity of antibodies in Western blot, ELISA and inhibition ELISA. Rabbit anti-horseradish peroxidase serum bound to both the fucosylated (MMF) and the xylosylated (MMX) glycoforms. Inhibition studies indicated two independent highly specific populations reacting with either of the two epitopes. In contrast, the monoclonal antibody YZ1/2.23 appears to recognize a larger structure including both the fucosyl and the xylosyl residue. The mannose-deficient glycoform was a poorer inhibitor for both antibodies. Terminal GlcNAc residues prevented antibody binding. Rabbit anti-bee venom serum reacted with fucosylated forms (MMF and MMXF) only. Experiments with sera from allergic patients suggest that glycomodified human transferrin, especially the MMXF glycoform, is a suitable reagent for the detection of antibodies against cross-reactive carbohydrate determinants. Within the panel studied, several sera contained high levels of fucose-reactive IgE but only a few sera showed any binding to MMX-transferrin.

Introduction

With regard to glycosylation, plants may appear as a fairly uninventive group since their glycoproteins carry the same limited set of structures almost regardless of the species (Lerouge *et al.*, 1998; Wilson *et al.*, 2001). However, just because of this and because some of the structural details are foreign to and hence immunogenic in mammals, plant N-glycans probably constitute the most frequently occurring set of epitopes to which we are exposed (Wilson *et al.*, 1998a). The vast potential for cross-reaction of antibodies with carbohydrate determinants is further extended by the occurrence of the same epitope structures on glycoproteins of insects, molluscs and parasitic worms (Wilson *et al.*, 1998a; Lommerse *et al.*, 1997; Altmann *et al.*, 1999; Wilson, 2002). In detail, plant N-glycans carry a fucose residue in α 1,3-linkage to the innermost GlcNAc (Ishihara *et al.*, 1979; Lerouge *et al.*, 1998) and this immunogenic feature also occurs on insect glycoproteins (Kubelka *et al.*, 1993; Altmann *et al.*, 1999) and even on N-glycans from *Schistosoma* and *Haemonchus* (Van Die *et al.*, 1999; Haslam *et al.*, 2001; Faveeuw *et al.*, 2003). In addition, a xylose residue, never seen in mammalian N-glycans, is present on plant N-glycans (Ishihara *et al.*, 1979; Lerouge *et al.*, 1998) but again, this feature is found in invertebrate animals such as snails (van Kuik *et al.*, 1985) and parasitic trematodes (Haslam *et al.*, 2001; Faveeuw *et al.*, 2003).

More than 20 years ago, the existence of a cross-reactive carbohydrate determinant (CCD) was proposed based on the promiscuous binding of allergic patients' sera to periodate sensitive, heat stable epitopes in a variety of allergens (Aalberse *et al.*, 1981). While these criteria might well have been misleading, the hypothesis was later confirmed by increasingly more sophisticated experiments. In 1988, a similarly cross-reactive rabbit antiserum was described (Faye and Chrispeels, 1988) and in 1991 anti-horseradish peroxidase serum was shown to bind to complex type plant N-glycans containing xylose and core α 1,3-fucose (Kurosaka *et al.*, 1991).

Not much later, Tretter *et al.* (1993) reported that, due to the presence of core α 1,3-fucose, plant N-glycans can bind IgE from many bee venom allergic patients. In this and in other studies bromelain glycopeptides were employed and the relative lability of the α 1,3-fucosyl linkage allowed the preparation of non-fucosylated glycopeptides, which could be tested in antibody-binding assays. As these defucosylated

glycopeptides - still containing xylose - were unable to bind IgE from bee venom allergic individuals we concluded that the core α 1,3-fucose is the key structural element for antibody binding. Identical results were obtained with patients sensitized against tomato, celery and other allergens (Petersen *et al.*, 1996, Fötisch *et al.*, 1999, Fötisch *et al.*, 2001, Westphal *et al.*, 2003). However, other important allergens such as Ara h 1, Ole e 1 or a vicillin-like 48 kDa protein from hazelnut, to which anti-CCD IgE binds, have been found to contain primarily structures with only xylose (Kolarich and Altmann, 2000, van Ree *et al.*, 2000; Müller *et al.*, 2000). Strong evidence in favor of the assumption that xylose also plays a role as (part of) a glyco-epitope came from a report on the fractionation of a polyclonal anti-horseradish peroxidase antiserum into a fucose specific and a xylose specific pool using immobilized honeybee phospholipase (Faye *et al.*, 1993). Unfortunately there was no well-defined glycoprotein containing xylose only that would have helped to assess the specificity of these fractions or the specificity of patients' sera IgE. Ascorbic acid oxidase from zucchini, occasionally used for that purpose (Batanero *et al.*, 1999), definitely also contains fucose (Altmann, 1998) whereas not necessarily all of the - partially very complex - structures occurring on hemocyanin from *Helix pomatia* have so far been elucidated (Lommerse *et al.*, 1997).

Thus, while it is clear that the term "cross-reacting carbohydrate determinants" essentially means complex type plant N-glycans (Foetisch and Vieths, 2001), the relative contributions of fucose and xylose or of other structural features to antibody binding are still unclear. Likewise, a sometimes postulated supportive role of the protein backbone for antibody binding has never been proven or disproven.

The biological significance of carbohydrate determinants in food, insect venom or pollen allergies, however, is to a much higher degree unclear and topic of a current debate (van der Veen *et al.*, 1997; Foetisch and Vieths, 2001; Foetisch *et al.*, 2003; Hemmer *et al.*, 2001). In many cases, anti-CCD IgE antibodies do not appear to trigger clinical symptoms (van Ree *et al.*, 1997; van Ree, 2002). This is contrasted by positive histamine release tests with some patients' sera (Foetisch *et al.*, 1999; Foetisch *et al.*, 2003; Westphal *et al.*, 2003; Bublin *et al.*, 2003) and by cases of patients with symptoms elicited by foods in the absence of detectable amounts of IgE against peptide epitopes (Foetisch *et al.*, 2003). The various aspects associated with carbohydrate determinants as allergens, *e.g.* the discrepancies between serum tests,

histamine release, skin tests and finally clinical symptoms have recently been reviewed and need not to be recapitulated here (van Ree, 2002). Clearly, CCDs play a role as a source of “false” positive serum test results in allergy diagnosis as the presence of anti-CCD IgE often has no clinical consequences (Mari *et al.*, 1999; Hemmer *et al.*, 2001; van Ree, 2002; Hemmer *et al.*, *submitted*). In keeping with earlier reports (Tretter *et al.*, 1993; Foetisch *et al.*, 1999), a recent survey indicated 23 % out of 1,831 patients have anti-CCD IgE (Mari, 2002). The criterion for CCD-reactivity was a positive ELISA and a negative skin prick test against bromelain – a plant glycoprotein with one complex type N-glycan lacking the α 1,3-mannose (Ishihara *et al.*, 1979). While this report impressively demonstrates the dimension of the problem, it also demonstrates the need for structurally defined and more easily applicable test antigens for the detection of anti-glycan IgE antibodies.

Here we report on the synthesis and characterisation of a glyco-modified human glycoprotein. Various glycoforms of human transferrin with or without xylose, fucose, terminal GlcNAc or 3-linked mannose have been prepared (Fig. 1) and their ability to bind anti-CCD IgG and anti-CCD IgE from patients' sera was tested.

RESULTS

Glyco-modification of human transferrin

Human transferrin (Tf) carries two N-glycans which are mainly disialylated and diantennary with only a small fraction being triantennary (Spik *et al.*, 1985). It therefore has a comparatively homogeneous glycosylation and it can be easily converted into a substrate for transferases requiring non-reducing terminal GlcNAc such as Fuc-T and Xyl-T by the use of sialidase and galactosidase. This GnGn-Tf was now subject to incubations with either Fuc-T or Xyl-T which were recombinantly expressed in *Pichia pastoris* as recently reported (Bencúrová *et al.*, 2003). While Xyl-T had been purified to homogeneity by Ni-chelate chromatography, Fuc-T was unstable under such conditions and hence untagged Fuc-T was enriched by dye-affinity chromatography.

Both Xyl-T and Fuc-T could be obtained in a form concentrated enough to allow an essentially complete conversion of several mg of GnGn-Tf to GnGnX-Tf or GnGnF-Tf, respectively (Figs. 1 and 2). Initially, we had problems to achieve complete fucosylation and speculations were made as to a restricted access of the transferase to the attachment on the native transferrin. However, the problem could be overcome by adding aliquots of the donor sugar GDP-fucose over the course of incubation and, thus, the incomplete conversion was due the instability of GDP-fucose rather than a decreasing activity of the enzyme. Although the *Pichia* strain used (GS115) secretes detectable amounts of protease (Brierly, 1998) the final products were essentially intact with only a small amount of a fragment band at 41.3 kDa (Fig. 4D).

To make sure that in following experiments any observed antibody binding could be exclusively attributed to the glycan moieties of Tf, the transferase incubation mixtures were done in duplicate, one with and one without the donor sugars UDP-xylose or GDP-fucose. However, as later described, there was absolutely no binding of anti-HRP and several patients' sera to these controls. Thus we believe that in future it will not be necessary to add Fuc-T or Xyl-T in the absence of the relevant nucleotide sugar in order to prepare MM-Tf from GnGn-Tf. An aliquot of the fucosylated GnGnF-Tf was subsequently xylosylated to yield GnGnXF-Tf.

Recent reports would suggest that the N-glycans recognised by anti-CCD did not contain terminal GlcNAc (Wilson *et al.*, 1998a; van Ree *et al.*, 2000; Foetisch and

Vieths, 2001). In addition, preliminary results of anti-HRP Western blots with fucosylated and xylosylated forms of GnGn-transferrin with or without prior hexosaminidase indicated that in fact the terminal GlcNAc residues had a weakening effect on antibody binding. Therefore, the primary transferase products were digested with hexosaminidase to finally yield the glyco-variants MM-, MMX-, MMF- and MMXF-Tf. The success of the various modification steps was finally verified by mass spectrometry of the glycopeptides around Asn 630 (Fig. 3). The glycopeptide 421-433 could not be observed in the MALDI spectrum. It should be noted that the glycosidases used (except the α -mannosidase) were not of plant origin, thus avoiding any “glyco-contamination” from this source.

Specificity of two rabbit antisera and a rat monoclonal antibody

With optimized amounts of fully converted transferrin and optimized dilutions of antiserum binding to GnGnF-Tf could not be observed (Fig. 4A). An inhibition experiment confirmed the attenuating effect of terminal GlcNAc residues (Table 1) and thus the further study was conducted with MMF-, MMX- and MMXF-Tf. We could not detect any cross-reactions of the sera with components from the expression system despite thorough controls, *e.g.* by making transferase incubations without or with the “wrong” nucleotide sugar.

A Western blot with MMX- and MMF-Tf revealed specific anti-HRP binding to both new glyco-epitopes (Fig. 4A). This is in keeping with a report on fractionation by immobilized bee venom phospholipase of anti-HRP serum into a fucose- and a xylose specific fraction (Faye *et al.*, 1993). Anti-bee venom serum showed binding to MMF-Tf as well as to MMXF-Tf, but not to MMX-Tf (Fig. 4B). This result reflects the absence of xylose in honeybee venom (Kubelka *et al.*, 1993; Kubelka *et al.*, 1995; Kolarich and Altmann 2000).

An ELISA titration of anti-HRP using MMX-, MMF-, and MMXF-transferrin as antigens revealed strikingly similar antibody titers against each of the three glycoforms. This could imply that this serum consists of one population of antibodies which although polyclonal all bind with similar affinity to each of the three glycoforms. However, subsequent inhibition studies revealed the opposite. Binding of

anti-HRP to MMX-Tf could not be inhibited by MMF-Tf and binding to MMF-Tf not by MMX-Tf (Table 1). In contrast binding of the rat monoclonal antibody YZ1/2.23 to MMX-Tf and MMF-Tf could be mutually cross-inhibited by MMX- and MMF-Tf (Table 1). Noteworthy, the absorbance measured for binding to MMX-Tf was only half that obtained with MMF-Tf. Apparently, YZ1/2.23 bears a paratope that covers both the fucosyl and the xylosyl residues, but with fucose contributing more to binding strength. Thus, YZ1/2.23 cannot be used to discriminate between fucosylated or xylosylated glycoproteins (Fig. 4C).

In previous work, defucosylation of bromelain glycopeptides led to a drastic decrease in their antibody binding capacity indicating negligible antibody binding by the glycopeptide carrying solely xylose (Tretter *et al.*, 1993; Petersen *et al.*, 1996; Wilson *et al.*, 1998a). The data presented here appear to contradict these earlier reports, as binding to MMX-Tf was displayed by both anti-HRP and YZ1/2.23 (Fig. 4 and Table 1). However, in the previous studies, defucosylated bromelain, which has a MUX structure, was used. Comparison of the inhibitory potency of MMX and MUX for anti-HRP binding to MMX-Tf revealed the significance of the α 1,3-mannosyl residue (Table 1 and also Fig. 4). In other words: while the MUXF structure of bromelain glycopeptides is suitable for measuring "anti-fucose" antibodies (Wilson *et al.*, 1998a) it does not appear to be useful for detecting "anti-xylose" antibodies.

Up to that point, only transferrin glycoforms had been used as coat antigens. A natural glycoprotein might contain additional structural features and thus we chose oil seed rape pollen extract as more natural example to explore the "natural history" of anti-CCD antibodies. Essentially complete inhibition of anti-HRP binding to oil seed rape pollen could be obtained by MMXF-Tf at a concentration ranging from 1 to 100 μ g / mL (table 1). HRP itself however inhibited more effectively on a weight per volume basis by a factor of about 30 to 60 (Table 1). Considering at least 7 N-glycans per molecule of HRP with a mass of 45 kDa for HRP and 1.6 fucosylated glycans per molecule of transferrin with a mass of 70 kDa, the difference shrinks, in molar terms, to between 4 and 9. This still significant difference in inhibitory potency could point to a contribution to antibody binding of the peptide regions in the neighbourhood of the N-glycan which of course differ between transferrin and HRP to which the antibody was actually raised. It could, however, simply reflect the difference in valency of these two glycoproteins which is known to be a critical factor for binding

strength of carbohydrate epitopes (Welply *et al.*, 1994; Yi *et al.*, 1998). Finally it should be mentioned that GnGnXF-Tf had only negligible inhibitory potency for anti-HRP (Table 1).

Detection and characterization of IgE against cross-reactive carbohydrate determinants (CCDs)

Finally, a small panel of allergic patients' sera was subject to measurement of specific IgE binding to the different transferrin glycoforms. All of the bee and wasp venom double positive patients' sera showed reaction with the fucosylated transferrins MMF and MMXF (the latter, however, could not be tested with all sera). Many sera of this group gave especially high readings in ELISA (Fig. 5A). Similarly, most of the rape pollen reactive sera also reacted with MMF and MMXF which corroborates the recent conclusion that oilseed rape pollen reactivity is in many cases due to anti-CCD IgE whereby rape pollen itself had not necessarily been the elicitor of this immune response (Hemmer *et al.*, 2001). None of the sera in these groups bound with MMX. In the case of the insect venom allergic patients this is not surprising. However, also these sera bound stronger to MMXF-transferrin which was, in addition to being fucosylated, also xylosylated (Fig. 5B).

None of the 20 sera from patients monosensitized to birch pollen reacted with any of the glycan-modified transferrins (data not shown), whereas four of the 24 sera from patients monosensitized to grass pollen reacted with MMF-Tf and/or MMXF-Tf. The highest prevalence of anti-CCD IgE was found in the sera from patients with multiple pollen allergy with at least 10 out of the 41 sera (24%) being positive (Fig. 5C).

To our surprise and disappointment, none of the sera recognized MMX to a significant degree with the exception of one heavily atopic patient whose serum reacted strongly even with MM-Tf. As this contrasts the results obtained for rabbit anti-HRP, we assume the lack of MMX-binding by human IgE to be the result of our serum selection rather than of a general "invisibility" of xylose for the human immune system. This is especially obvious for the insect venom group as insect glycoproteins do not contain xylose. Future studies with panels of patients allergic against primarily xylosylated allergens such as olive pollen, hazelnut or peanut may clarify this point.

Recently, human IgG levels against CCD structures have been measured using honeybee venom phospholipase and *Helix pomatia* hemocyanin as core α 1,3-fucosylated probe and as β 1,2-xylosylated standards, respectively (Bardor *et al.*, 2002). We compared the results obtained with the glycomodified transferrins with these naturally available probes. The results obtained with phospholipase and MMF- or MMXF-transferrin indeed were in agreement. The ELISA readings, however, were generally lower with phospholipase with the exception of a few sera where especially high values for phospholipase suggested the presence of anti-protein IgE (Fig. 6A). In other words, since the phospholipase polypeptide is the major allergen of bee venom, it cannot at all be regarded as a reliable probe for the measurement of anti-CCD antibodies. Hemocyanin, like MMX-Tf, was not bound significantly by any of the sera (data not shown).

DISCUSSION

Using the divalent human glycoprotein transferrin and recombinant glycosyltransferases, it was possible for the first time to generate and study plant glyco-epitopes containing either only fucose or only xylose rather than the usual mixture. In contrast to the use of naturally occurring glycoproteins such as phospholipase A₂ from honeybee venom (MMF and other structures), hemocyanin from *H. pomatia* (MMX and many undefined structures), ascorbic oxidase (erroneously supposed to contain only MMX; Altmann, 1998) or chemically defucosylated bromelain glycopeptides (Wilson *et al.*, 1998a) (MUX with possibly some residual MUXF), the approach presented in this paper allows the comparison of defined glycoforms all linked to the same carrier protein that had been subject to defined steps of modification. Hence, stringent controls can be introduced, *e.g.* by omission of the nucleotide sugar in the preparation scheme. While the influence of substitution of the terminal mannose residues by GlcNAc could be studied using GnGnF- and GnGnX-Tf, no natural glycoprotein with a complete substitution of the mannoses is known. Furthermore, degradation of MMX to MUX by mannosidase can be achieved on the whole protein.

As the glycans on human transferrin, especially in the GnGn, but also in the galactosylated form, are substrate for a variety of yet other glycosyltransferases, the same approach could obviously also be chosen for other glyco-determinants. In addition to antibody binding, the reactivity with animal lectins could be studied with such transferrin glycoforms using Western blot, ELISA or immunohistochemistry with transferrin specific antibodies. Indeed, a *Drosophila* lectin binding core α 1,3-fucose has been characterised using MMF-transferrin prepared in this laboratory (Bouyain *et al.*, 2002).

In this study the specificity of polyclonal rabbit sera, especially of an anti-HRP serum and of a rodent monoclonal antibody were studied. The latter, YZ1/2.23, exhibited a somewhat complicated epitope structure where both the fucose and the xylose residue play a role for antibody binding. In contrast, anti-HRP appears to consist of two distinct populations with paratopes either binding fucosylated or xylosylated glycans. Reflecting the absence of xylose in insect glycoproteins, the anti-honey bee serum only bound to core α 1,3-fucosylated transferrin.

The results obtained for GnGnF and GnGnXF show that the mere presence of core α 1,3-fucose is however not the only criterion for binding of these antibodies. The CCD-epitope can be hidden by additional modifications of the N-glycan as was already suggested as an explanation for the low anti-HRP binding of tree pollens which predominantly contain N-glycans with terminal GlcNAc residues (Wilson *et al.*, 1998a; Wilson *et al.*, 1998b; Wilson *et al.*, 2001).

Steric factors may also influence antibody binding. In Western blots, it appeared that the proteolytic fragment generated during enzymatic modifications of transferrin, binds slightly stronger than the full length transferrin (Fig. 4). A similar, albeit contrary, observation is made with honeybee phospholipase as compared to the much less abundant hyaluronidase (Hemmer *et al.*, 2003). Unfortunately, such effects of steric presentation on antibody binding are difficult to discern experimentally.

Another modulator of binding strength beyond glycan structure is the valency of the glyco-antigen. We suggest that this valency factor explains the difference in inhibitory potency of MMXF-Tf and HRP. Polyvalency is an important factor for the ability to trigger physiological reactions of effector cells in allergy. As transferrin is divalent it can be expected to be effective in biological test systems such as histamine release by granulocytes.

The analysis of the specificity of patients' sera revealed – once again – the importance of core α 1,3-linked fucose. This had to be expected in the bee and wasp venom reactive group but it was also observed for patients with oil seed rape and multiple pollen reactivity. Remarkably, however, MMXF-Tf consistently gave higher ELISA readings than MMF-Tf, even though no patient in our test groups exhibited significant binding with merely xylosylated glycans.

It should be emphasized that the bio-synthetic glyco-antigens used here are more reliable probes for anti-CCD IgE than *e.g.* honeybee phospholipase which also contains a number of highly important peptide epitopes. In the near future, larger numbers of patients' sera shall be analysed using the glycomodified transferrins with methods more suitable for IgE quantitation than ELISA. Apart from the maybe academic question whether there are xylose-reactive patients, a major issue will be to render allergy diagnosis more reliable by allowing discrimination between IgE binding to peptide or to carbohydrate epitopes. Although the overall prevalence of anti-CCD antibodies in our patients' sera was lower than that reported by others

(Mari, 2002), our data suggest that such antibodies may be commonly found in patients with multiple sensitization to many different allergens. However, there is no obvious correlation between anti-CCD titers as measured with, e.g., MMXF-Tf and total IgE levels (Fig. 6B) which contradicts the view that CCD-reactivity is merely a non-specific phenomenon observed in highly atopic patients.

Especially in the case of insect venom and food allergy, both including a certain risk for fatal or near-fatal reactions, it appears appropriate to develop tests which can result in reassuring patients where a positive laboratory result is merely caused by CCDs which supposedly have no clinical significance – at least in the absence of anti-peptide IgE against the respective allergen.

METHODS

Enzymes, antibodies and other materials

Recombinant forms of *Arabidopsis thaliana* β 1,2-xylosyltransferase and core α 1,3-fucosyltransferase were expressed in *Pichia pastoris* and purified as recently described (Bencúrová *et al.*, 2003). The enzyme unit is defined as transfer of 1 μ mol of xylose or fucose per min at 16 or 37°C, respectively, to a dabsylated or dansylated GnGn-tetrapeptide (Calbiochem) (Bencúrová *et al.*, 2003). β -Galactosidase from *Aspergillus oryzae* (Sigma-Aldrich) was purified before use (Zeleny *et al.*, 1997). Neuraminidase from *C. perfringens* and α -mannosidase from jack bean were also obtained from Sigma Aldrich. β -N-acetyl-glucosaminidase from *Streptococcus pneumoniae* (Calbiochem) was used instead of the enzyme from jack bean as the latter contained plant N-glycan structures interfering with the objectives of this work. Honeybee venom phospholipase was prepared as described (Kubelka *et al.*, 1993) and *Helix pomatia* hemocyanin was obtained from Serva (Heidelberg, Germany). GDP-fucose was purchased from Sigma-Aldrich and UDP-xylose was obtained from CarboSource Services at the University of Georgia.

The monoclonal antibody YZ1/2.23, raised against elderberry abscission tissue, was a gift of Dr. Daphne Osborne (Open University) and David Ashford (University of York). Polyclonal anti-horseradish peroxidase serum from rabbit, anti-bee venom serum from rabbit and the respective second antibodies have been described before (McManus *et al.*, 1988; Wilson *et al.*, 1998a).

Sera were collected from patients undergoing routine allergy testing for inhalant or insect venom allergy. Patients with inhalant allergy were skin prick tested with common inhalant allergens including pollens (hazel, alder, birch, grass, rye, ash, plantain, nettle, mugwort, ragweed, oilseed rape, plane tree), house dust mites, animal danders (cat, dog, horse, guinea pig), molds (*Cladosporium*, *Alternaria*, *Penicillium*), and rubber latex (all Soluprick, ALK, Denmark). Insect venom allergy was confirmed by positive radioallergosorbent test and subsequent skin testing.

Three groups of sera were used: (1) Sera from patients suffering from pollen allergy which according to radioallergosorbent and skin prick test were sensitized to a narrow

range of pollen allergens only, *i.e.* birch and other Fagales pollen only (n=20) and grass/rye pollen only (n=24); all patients from this group also had a positive radioallergosorbent test to the respective allergen; four of the 44 patients had a weakly positive skin test but negative serology to one of the tested indoor allergens; (2) sera from patients with multiple pollen sensitization, *i.e.* sensitization to at least four different pollen species (n=41); patients from this group had multiple positive radioallergosorbent test to pollen allergens although not all allergens reacting positively in the skin test have been tested by serology; 26 of the patients were also sensitized to one or more of the indoor allergens; (3) pre-selected sera which were assumed to contain anti-CCD IgE such as those reacting with HMW glycoallergens in oilseed rape pollen (Focke *et al.*, 1988) (n=9) or those exhibiting cross-reactivity with bee and wasp venom glycoallergens (Hemmer *et al.*, 2001; Hemmer *et al.*, *submitted*) (n=7).

Preparation of glyco-modified transferrins

10 mg of human apo-transferrin (Sigma-Aldrich) was treated for 16 h with 100 mU of neuraminidase in 0.5 mL of 50 mM sodium acetate buffer at pH 5.0 at 37 °C.

Subsequently, 4.2 U of β -galactosidase from *Aspergillus oryzae* (Zeleny *et al.*, 1997) was added and the sample was incubated overnight. The integrity of the resultant GnGn-transferrin was checked by SDS-PAGE and the N-glycans were analysed as described below.

For xylosylation, GnGn-transferrin (5 mg in a final volume of 1.6 mL) was incubated with 4 μ mol UDP-xylose and 13 mU of Xyl-T (in 25 mM 2(*N*-morpholino)ethanesulfonic acid, pH 7.0) at 16 °C for 48 h. For fucosylation, GnGn-transferrin (2 mg in a final volume of 0.8 mL containing 20 mM MnCl₂) was incubated with 2.8 μ mol of GDP-fucose (added in three aliquots over time) and 0.6 mU of Fuc-T (dissolved in 25 mM 2(*N*-morpholino)ethanesulfonic acid, pH 6.8) at 25°C for 48 h. A doubly modified glycoform, GnGnXF-transferrin, was obtained by fucosylation of GnGnX-transferrin.

In order to remove the terminal *N*-acetylglucosamine residues, solutions of GnGn-, GnGnX-, GnGnF-, and GnGnXF-transferrins were diluted threefold with 50 mM sodium citrate buffer of pH 5.0 and digested with β -*N*-acetyl-glucosaminidase (0.5 mU / mg of transferrin). For the preparation of glycoforms lacking the α 1,3-mannosyl residue, 0.45 mg of MM- and MMX-transferrin were digested for 24 h at 37°C with 20 mU of jack bean α -mannosidase in the above buffer containing 0.1 mM ZnCl₂.

Analytical methods

The structures of the N-glycans on the various transferrin glycoforms were verified in two ways. (1) In the first approach, 10 μ g of glycoproteins were digested for 4 h at 37°C with 0.5 μ g of pepsin (Sigma-Aldrich) in 30 μ L of 5 % formic acid. Then, this solvent was evaporated and the samples were deglycosylated with peptide:N-glycosidase A described (Kolarich and Altmann, 2000). Oligosaccharides were isolated and analysed by MALDI mass spectrometry on a linear time-of-flight instrument as described (Kolarich and Altmann, 2000). (2) Alternatively, glycoproteins were subjected to SDS-PAGE and bands were excised, S-alkylated and digested with described (Kolarich and Altmann, 2000; Katayama *et al.*, 2001). The (glyco-)peptides were analyzed by MALDI mass spectrometry on a Waters-Micromass Q-TOF GLOBAL system using α -cyano-4-hydroxycinnamic acid as the matrix.

Western blot

Transferrin glycoforms (0.1 μ g) were separated by SDS polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membrane. The membrane was blocked with 3 % non-fat milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween and subsequently incubated for 1 h with rabbit anti-horseradish peroxidase (anti-HRP) or anti-bee venom serum diluted 1: 2000. After washing in Tris-buffered saline, alkaline phosphatase conjugated anti-rabbit antibody at a dilution of 1: 2000 was added and bands were stained for 30 min with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

IgG ELISA

Transferrin glycoforms at a concentration of 5 μ g/mL or oilseed rape pollen extract (OSR) at a protein concentration of 1 μ g/mL was used for coating ELISA well plates

(Nunc maxisorp) for 1 h at 37 °C in 0.1 M sodium carbonate buffer, pH 9.6. After the washing and blocking steps, plates were incubated for 1 h at 37°C with either anti-HRP diluted 1:20,000 or YZ1/2.23 diluted 1: 40,000. The subsequent steps were performed as described previously (Wilson *et al.*, 2001).

For inhibition ELISA, sera were pre-incubated for 1 h at 37 °C with different glycoproteins at different concentrations.

IgE ELISA

Microtiter plates were coated with transferrin glycoforms overnight at 4 °C but otherwise as described above. The plates were washed twice with phosphate-buffered saline (PBS) containing 0.05 % Tween 20 and blocked with PBS containing 1 % bovine serum albumin for 2.5 h at room temperature. The plates were then incubated overnight at 4 °C with allergic patients' sera diluted 1:10 with PBS. After washing the plates five times with Tween-20 containing PBS, anti-human IgE-alkaline phosphatase conjugate (Pharmingen, San Diego) diluted 1: 2000 with PBS containing 0.05 % bovine serum albumin was added and the plates were incubated first for 1 h at 37 °C and then for 30 min at 4 °C. Plates were again washed five times as above and then stained with *p*-nitrophenyl phosphate dissolved to 1 mg/mL in 0.1 M diethanolamine of pH 9.7. The optical density at 405 nm was read after 2 h.

References

- Aalberse, R.C., Koshte, V. and Clemens, J.G. (1981) Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. *J Allergy Clin Immunol.*, **68**, 356-364.
- Altmann, F. (1998) Structures of the N-linked carbohydrate of ascorbic acid oxidase from zucchini. *Glycoconjugate J.*, **15**, 79-82.
- Altmann, F., Staudacher, E., Wilson, I.B.H. and März, L. (1999) Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconjugate J.*, **16**, 109-123.
- Bardor, M., Faveeuw, C., Fitchette, A.C., Gilbert, D., Galas, L., Trottein, F., Faye, L. and Lerouge, P. (2002) Immunoreactivity in mammals of two typical plant glyco-epitopes, core $\alpha(1,3)$ -fucose and core xylose. *Glycobiology*, **13**, 427-434.
- Batanero, E., Crespo, J.F., Monsalve, R.I., Martin-Esteban, M., Villalba, M. and Rodriguez, R. (1999) IgE-binding and histamine-release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen, Ole e 1. *J. Allergy Clin. Immunol.*, **103**, 147-153.
- Bencúrová, M., Rendić, D., Fabini, G., Kopecky, E.M., Altmann, F. and Wilson, I.B.H. (2003) Expression of eukaryotic glycosyltransferases in the yeast *Pichia pastoris*. *Biochimie*, **85**, 413-422.
- Bouyain, S., Silk, N.J., Fabini, G. and Drickamer, K. (2002) An endogenous *Drosophila* receptor for glycans bearing $\alpha 1,3$ -linked core fucose residues. *J. Biol. Chem.* **277**, 22566-22572.
- Brierley, R.A. (1998) Secretion of recombinant human insulin-like growth factor I (IGF-I). *Methods Mol. Biol.*, **103**, 149-177.
- Bublin, M., Radauer, C., Wilson, I.B.H., Kraft, D., Scheiner, O., Breiteneder, H. and Hoffmann-Sommergruber K. (2003) Cross-reactive N-glycans of Api g 5, a high molecular weight glycoprotein allergen from celery, are required for immunoglobulin E binding and activation of effector cells from allergic patients. *FASEB J.* **17**, 1697-1699
- Faveeuw, C., Mallevaey, T., Paschinger, K., Wilson, I.B.H., Fontaine, J., Mollicone, R., Oriol, R., Altmann, F., Lerouge, P., Capron, M. and Trottein, F. (2003) Schistosome N-glycans containing core $\alpha 3$ -fucose and core $\beta 2$ -xylose epitopes are strong inducers of Th2 responses in mice. *Eur. J. Immunol.*, **33**, 1271-1281.
- Faye, L., and Chrispeels, M.J. (1988) Common antigenic determinants in the glycoproteins of plants, molluscs and insects. *Glycoconjugate J.*, **5**, 245-256.
- Faye, L., Gomord, V., Fitchette-Laine, A.C. and Chrispeels, M.J. (1993) Affinity purification of antibodies specific for Asn-linked glycans containing $\alpha 1\rightarrow 3$ fucose or $\beta 1\rightarrow 2$ xylose. *Anal Biochem.*, **209**, 104-108.
- Focke, M., Hemmer, W., Hayek, B., Götz, M. and Jarisch, R. (1998) Identification of allergens in oilseed rape (*Brassica napus*) pollen. *Int. Arch. Allergy Immunol.*, **117**, 105-112.
- Foetisch, K., Altmann, F., Hausteiner, D. and Vieths, S. (1999) Involvement of carbohydrate epitopes in the IgE response of celery-allergic patients. *Int. Arch. Allergy Immunol.*, **120**, 30-42.
- Foetisch, K., Son, D.Y., Altmann, F., Aulepp, H., Conti, A., Hausteiner, D. and Vieths, S. (2001a) Tomato (*Lycopersicon esculentum*) allergens in pollen-allergic patients. *Eur. Food Res. Technol.*, **213**, 259-266.
- Foetisch, K. and Vieths, S. (2001b) N- and O-linked oligosaccharides of allergenic glycoproteins. *Glycoconjugate J.*, **18**, 373-390.

- Foetisch,K., Retzek,M., Westphal,S., Lauer,L., Altmann,F., Kolarich,K., Scheurer,S. and Vieths,S. (2003) Biological activity of IgE specific for cross-reactive carbohydrate determinants (CCD) *J. Allergy Clin. Immunol.*, **111**, 889-896.
- Haslam,S.M., Morris,H.R. and Dell A. (2001) Mass spectrometric strategies: providing structural clues for helminth glycoproteins. *Trends Parasitol.*, **17**, 213-235.
- Hemmer,W., Focke,M., Kolarich,D., Wilson,I.B.H., Altmann,F., Wöhrl,S., Götz,M. and Jarisch,R. (2001) Antibody binding to venom carbohydrates is a frequent cause for double-positivity of honeybee and yellow jacket venom in patients with stinging insect allergy. *J. Allergy Clin. Immunol.*, **108**, 1045-1052.
- Hemmer,W., Focke,M., Kolarich,D., Dalik,I., Götz,M. and Jarisch,M. (2003/2004) Identification by immunoblot of venom glycoproteins displaying IgE-binding N-glycans as cross-reactive allergens in honeybee and yellow jacket venom. *Clin. Exp. Allergy*, *submitted*
- Hsu,T.A., Takahashi,N., Tsukamoto,Y., Kato,K., Shimada,I., Masuda,K., Whiteley,E.M., Fan,J.Q., Lee,Y.C. and Betenbaugh,M.J. (1997) Differential N-glycan patterns of secreted and intracellular IgG produced in *Trichoplusia ni* cells. *J. Biol. Chem.*, **272**, 9062-9070.
- Ishihara,H., Takahashi,N., Oguri,S. and Tejima,S. (1979) Complete structure of the carbohydrate moiety of stem bromelain. An application of the almond glycopeptidase for structural studies of glycopeptides. *J. Biol. Chem.*, **254**, 10715-10719.
- Katayama,H., Nagasu,T. and Oda,Y. (2001) Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom.*, **15**, 1416-1421.
- Kolarich,D. and Altmann,F. (2000) N-glycan analysis by matrix assisted laser desorption/ionisation mass spectrometry of electrophoretically separated non-mammalian proteins. Application to peanut allergen Ara h 1 and olive pollen allergen Ole e 1. *Anal. Biochem.*, **285**, 64-75.
- Kubelka,V., Altmann,F., Staudacher,E., Tretter,V., März,L., Hård,K., Kamerling,J.P. and Vliegthart J.F.G. (1993) Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A₂. *Eur. J. Biochem.*, **213**, 1193-1204.
- Kubelka,V., Altmann,F. and März,L. (1995) The asparagine-linked carbohydrate of honeybee venom hyaluronidase. *Glyconjugate J.*, **12**, 77-83.
- Kurosaka,A., Yano,A., Itoh,N., Kuroda,Y., Nakagawa ,T. and Kawasaki,T. (1991) The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J. Biol. Chem.*, **266**, 4168-4172.
- Lerouge,P., Cabanes-Macheteau,M., Rayon,C., Fitchette-Lainé,A.C., Gomord,V. and Faye,L. (1998) N-glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol. Biol.*, **38**, 31-48.
- Lommerse,J.P., Thomas-Oates,J.E., Gielens,C., Preaux,G., Kamerling,J.P. and Vliegthart,J.F. (1997) Primary structure of 21 novel monoantennary and diantennary N-linked carbohydrate chains from α -hemocyanin of *Helix pomatia*. *Eur. J. Biochem.*, **249**, 195-222.
- Mari,A., Iacovacci,P., Afferni,C., Barletta,B., Tinghino,R., Di Felice,G. and Pini,C. (1999) Specific IgE to cross-reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases. *J. Allergy Clin. Immunol.*, **103**, 1005-1011.
- Mari,A. (2002) IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the in vivo and in vitro reactivity. *Int. Arch. Allergy Immunol.*, **129**, 286-295.

- McManus, M.T., McKeating, J., Secher, D.S., Osborne, D.J., Ashford, D., Dwek, R.A. and Rademacher, T.W. (1988) Identification of a monoclonal antibody to abscission tissue that recognises xylose/fucose-containing N-linked oligosaccharides from higher plants. *Planta*, **175**, 506-512.
- Müller, U., Lüttkopf, D., Hoffmann, A., Petersen, A., Becker, W.M., Schocker, F., Niggemann, B., Altmann, F., Kolarich, D. and Vieths, S. (2000) Allergens in native and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *Eur. Food Res. Technol.*, **212**, 2-12.
- Petersen, A., Vieths, S., Aulepp, H., Schlaak, M. and Becker, W.M. (1996) Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *J. Allergy Clin. Immunol.*, **98**, 805-815.
- Spik, G., Debruyne, V., Montreuil, J., van Halbeek, H. and Vliegthart J.F. (1985) Primary structure of two sialylated triantennary glycans from human serotransferrin. *FEBS Lett.*, **183**, 65-69.
- Tretter, V., Altmann, F., Kubelka, V., März, L. and Becker, W.M. (1993) Fucose linked α 1-3 to the core-region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. *Int. Arch. Allergy Immunol.*, **102**, 259-266.
- van der Veen, M.J., van Ree, R., Aalberse, R.C., Akkerdaas, J., Koppelman, S.J., Jansen, H.M. and van der Zee, J.S. (1997) Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J. Allergy Clin. Immunol.*, **100**, 327-334.
- van Die, I., Gomord, v., Kooyman, F.N.J., van den Berg, T.K., Cummings, R.D. and Vervelde, L. (1999) Core α 1 \rightarrow 3-fucose is a common modification of N-glycans in parasitic helminths and constitutes an important epitope for IgE from *Haemonchus contortus* infected sheep. *FEBS Lett.*, **463**, 189-193.
- van Kuik, J.A., van Halbeek, H., Kamerling, J.P. and Vliegthart, J.F. (1985) Primary structure of the low-molecular-weight carbohydrate chains of *Helix pomatia* α -hemocyanin. Xylose as a constituent of N-linked oligosaccharides in an animal glycoprotein. *J. Biol. Chem.*, **260**, 13984-13988.
- van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J.P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodriguez, R., Faye, L and Lerouge, P. (2000) β (1,2)-xylose and α (1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J. Biol. Chem.*, **275**, 11451-11458.
- van Ree, R. (2002) Carbohydrate epitopes and their relevance for the diagnosis and treatment of allergic diseases. *Int. Arch. Allergy Immunol.*, **129**, 189-197.
- Welpy, J.K., Abbas, S.Z., Scudder, P., Keene, J.L., Broschat, K., Casnocha, S., Gorka, C., Steininger, C., Howard, S.C. and Schmuke, J.J. (1994) Multivalent sialyl-LeX: potent inhibitors of E-selectin-mediated cell adhesion; reagent for staining activated endothelial cells. *Glycobiology*, **4**, 259-265.
- Westphal, S., Kolarich, D., Foetisch, K., Lauer, I., Altmann, F., Conti, A., Crespo, J.F., Miranda, E.E., Vieths, S. and Scheurer, S. (2003) Molecular Characterization and Allergenic Activity of Lyc e 2 (β -fructofuranosidase), a Glycosylated Allergen of Tomato. *Eur. J. Biochem.*, **270**, 1327-1337.
- Wilson, I.B.H., Harthill, J.E., Mullin, N., Ashford, D. and Altmann, F. (1998a) Core α 1,3-fucose is a key part of the epitope recognised by antibodies reacting against plant N-linked oligosaccharides. *Glycobiology*, **8**, 651-661.
- Wilson, I.B.H. and Altmann, F. (1998b) Structural analysis of N-glycans from allergenic grass, ragweed and tree pollens. Core α 1,3-fucose and xylose present in all pollens examined. *Glycoconjugate J.*, **15**, 1055-1070.
- Wilson, I.B.H., Zeleny, R., Kolarich, D., Staudacher, E., Stroop, C.J.M., Kamerling, J.P. and Altmann, F. (2001) Analysis of Asn-linked glycans from vegetable foodstuffs: Widespread occurrence of Lewis a, core α 1,3-linked fucose and xylose substitutions. *Glycobiology*, **11**, 261-274.

Wilson, I.B.H. (2002) Glycosylation of proteins in plants and invertebrates. *Curr. Opin. Struct. Biol.*, **12**, 569-577.

Yi, D., Lee, R.T., Longo, P., Boger, E.T., Lee, Y.C., Petri, W.A.Jr. and Schnaar, R.L. (1998) Substructural specificity and polyvalent carbohydrate recognition by the *Entamoeba histolytica* and rat hepatic N-acetylgalactosamine/galactose lectins. *Glycobiology*, **8**, 1037-1043.

Zeleny, R., Altmann, F. and Praznik W. (1997) A capillary electrophoretic study on the specificity of β -galactosidases from *Aspergillus oryzae*, *Escherichia coli*, *Streptococcus pneumoniae* and *Canavalia ensiformis* (jack bean). *Anal. Biochem.*, **246**, 96-101.

Acknowledgements

We thank Daniel Kolarich for acquiring the peptide spectra on the Q-TOF mass spectrometer which was funded by the Austrian Science Council. We acknowledge the help of Jin Chunsheng with immunoblots. This work was supported by a joint research program of the Austrian Science Fund (project S8803).

Abbreviations

CCD, cross-reactive carbohydrate determinant;

ELISA, enzyme linked immunosorbent assay;

Fuc-T, (core α 1,3-)fucosyltransferase;

GnGn, GnGnF, GnGnX, GnGnXF, N-glycans with terminal GlcNAc residues (see Fig. 1);

HRP, horseradish peroxidase;

MALDI-TOF MS, matrix assisted-laser desorption/ionization time-of-flight mass spectrometry;

MM, MMF, MMX, MMXF, MUX, N-glycans with terminal mannoses, see Fig. 1;

NaNa, disialylated, diantennary N-glycan;

OSR, oil seed rape pollen;

Tf, (human) transferrin;

Xyl-T, (β 1,2-)xylosyltransferase.

Fig. 1: Scheme of the glyco-modifications performed with human apo-transferrin. Open ellipses depict mannose residues, grey ellipses GlcNAc, white circles galactose, black pentangles sialic acid, black triangles fucose and black squares xylose. The structures of GnGnX and GnGnF are also shown in Fig. 2. NaNa-Tf denotes the untreated transferrin with disialylated N-glycans. Figures indicate the enzymes used: 1, neuraminidase; 2, β -galactosidase; 3, core- α 1,3-fucosyltransferase; 4, β -xylosyltransferase; 5, β -*N*-acetylglucosaminidase; 6, α -mannosidase.

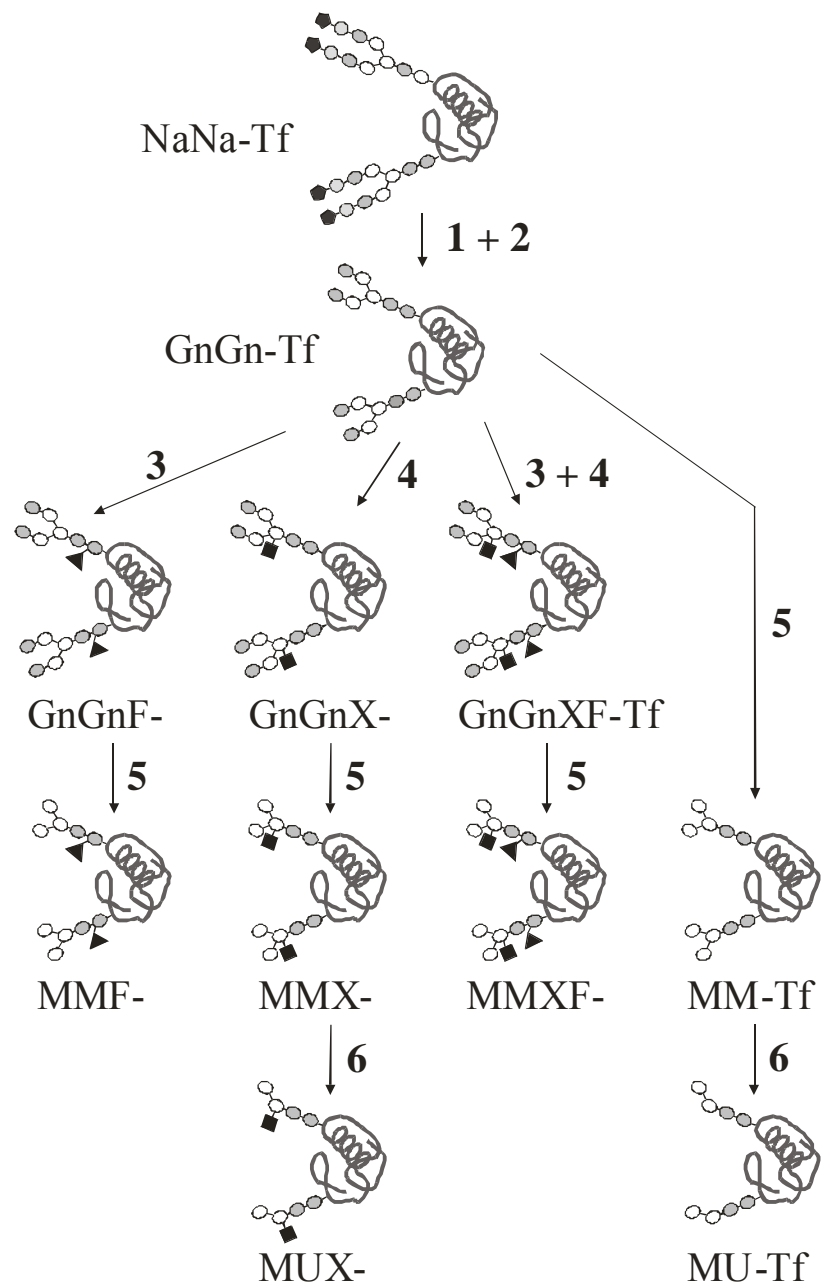


Fig. 2: Modification of the N-glycans of transferrin: The MALDI-TOF spectrum of oligosaccharides released after incubation of transferrin with xylosyltransferase (panel A) and fucosyltransferase (panel B) shows the (almost) complete conversion of the substrate glycan GnGn (1340 Da) to GnGnX (1472 Da) or GnGnF (1486 Da), respectively.

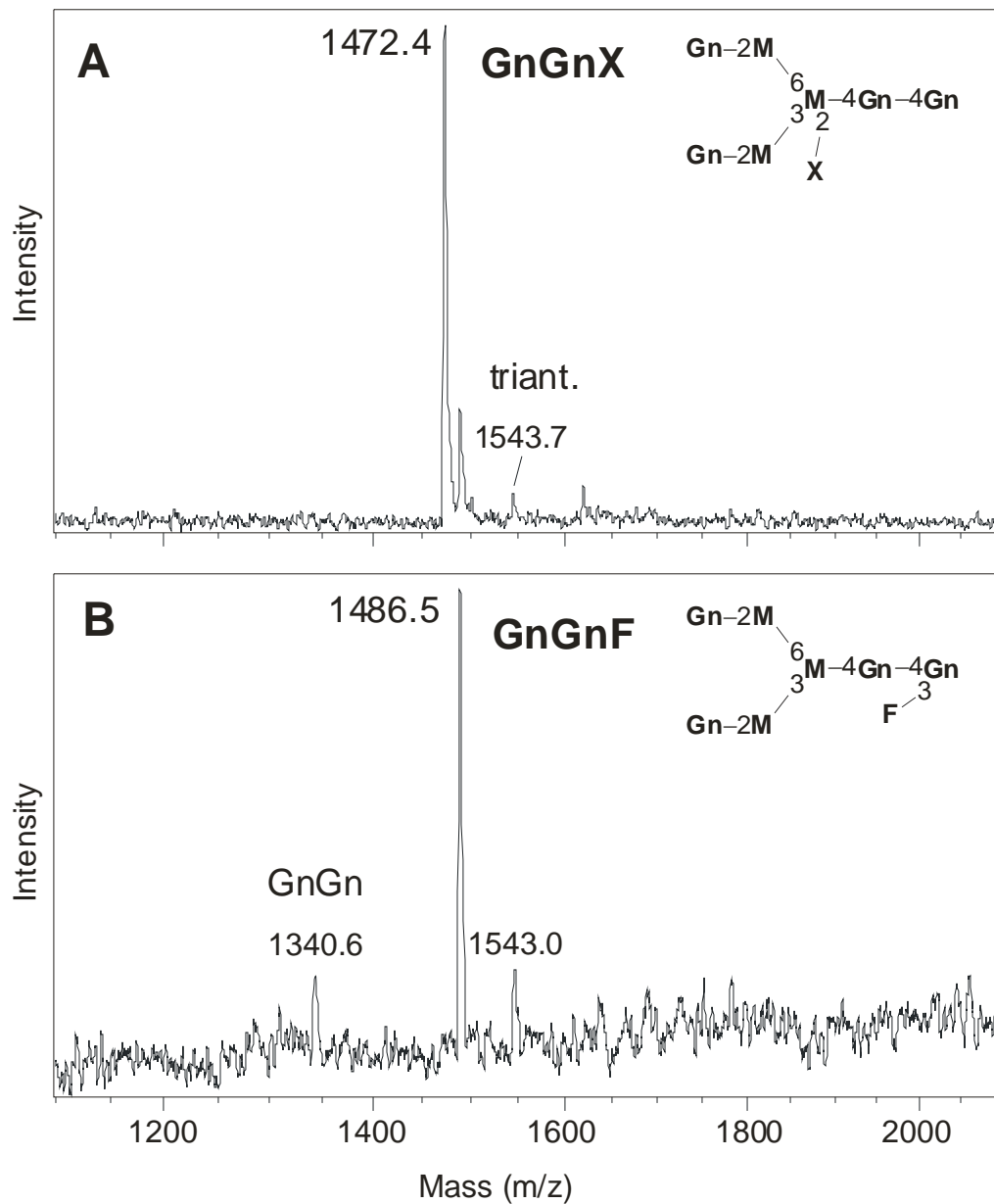


Fig. 3: MALDI-Q-TOF spectra of glycopeptides from MM-, MMX-, MMF-, and MMFX- transferrin reveal the almost quantitative conversion to the desired glycoforms. Major peaks correspond to peptide 622-642 with carboxamidomethylated cysteine (mass of $M+H^+ = 2515.125$). The mass differences indicate the composition of the attached oligosaccharide. The theoretical masses of the glycopeptides ($M+H^+$) of the peptides with MM-, MMX-, MMF-, and MMXF-glycans are 3407.44, 3539.49, 3553.50, and 3685.54, respectively.

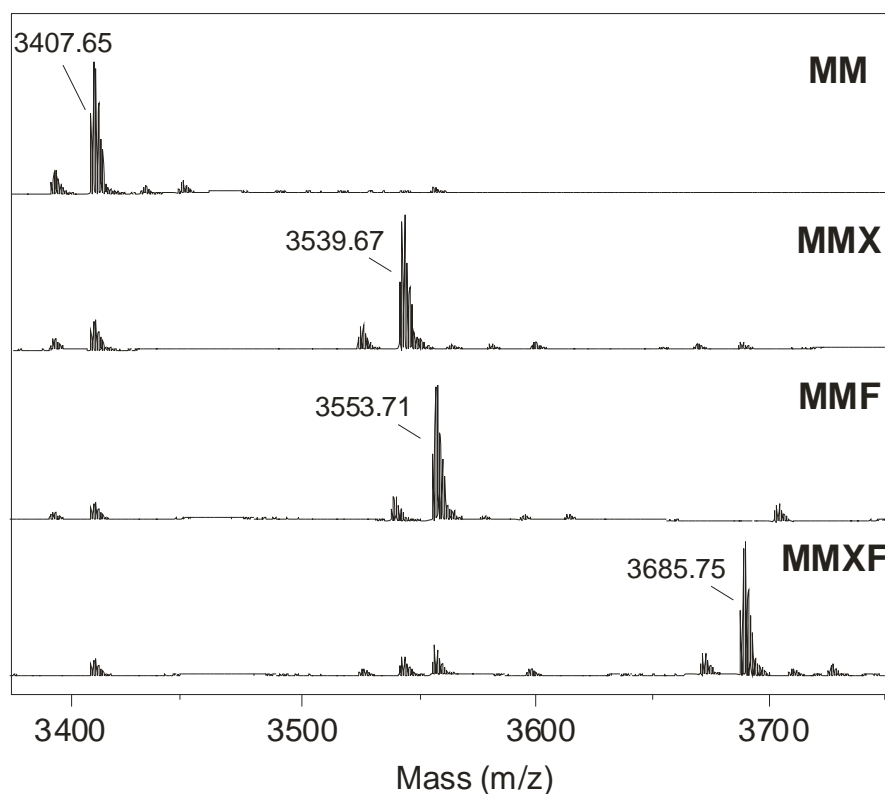


Fig. 4: Western Blot of glycomodified transferrins with anti rabbit anti-horseradish serum (panel A), anti-bee venom serum (panel B) and rat monoclonal YZ1/2.23 (panel C).. The silver stain of MMF-transferrin (panel D) reveals a major band at the expected size of ca. 70 kDa accompanied by a faint band at ca. 41 kDa. While both sera bind to MMF and MMFX, *i.e.* to the core α 1,3-fucosylated forms, only the anti-HRP serum recognizes the xylosylated forms MMX and MUX. Native sialylated transferrin (NaNa) as well as the GnGnX- and GnGnF glycoforms are not stained by either antiserum. The lanes of MU- and MUX-Tf exhibit a new band at ca. 60 kDa that originates from the mannosidase.

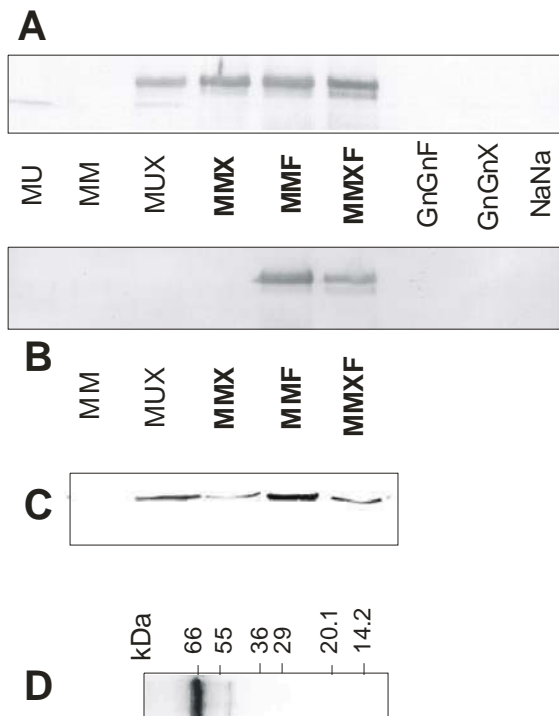


Fig. 5: Binding to MMXF-, MMF-, MMX-, and MM-transferrin of IgE from sera of honeybee/wasp venom double sensitized patients (panel A), of oilseed rape pollen reactive patients (panel B), and of patients with polyvalent reactivity (panel C). The great majority of these data were recorded in triplicate. The average standard deviation in absorbance units was 0,010. To mark those patients where experiments could only be performed with MMF-, MMX- and MM-Tf in panel A and B their acronyms have been put in a box. n.c. = normal healthy control.

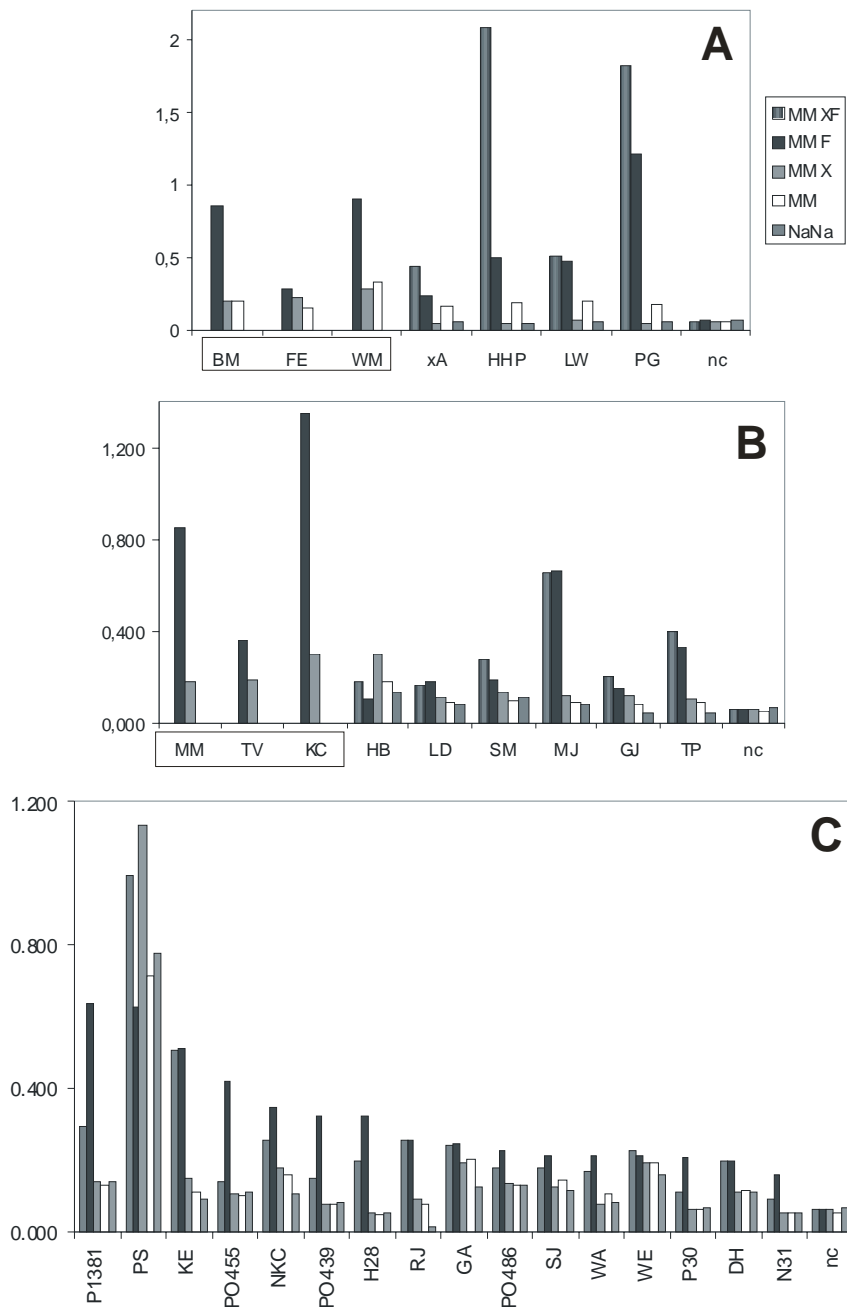


Fig. 6: Correlations for patients' IgE. The dots represent the absorbencies of patients from the oilseed rape pollen, the insect venom and the polyvalent pollen groups. Only sera showing binding to these antigens were considered. The line shows the correlation between data sets. Panel A depicts the correlation ($R = 0.41$) between results obtained with MMF-Tf and with bee venom phospholipase which likewise contains core α 1,3-linked fucose. Panel B shows the absence of a correlation between anti-CCD IgE as measured with MMXF-Tf.

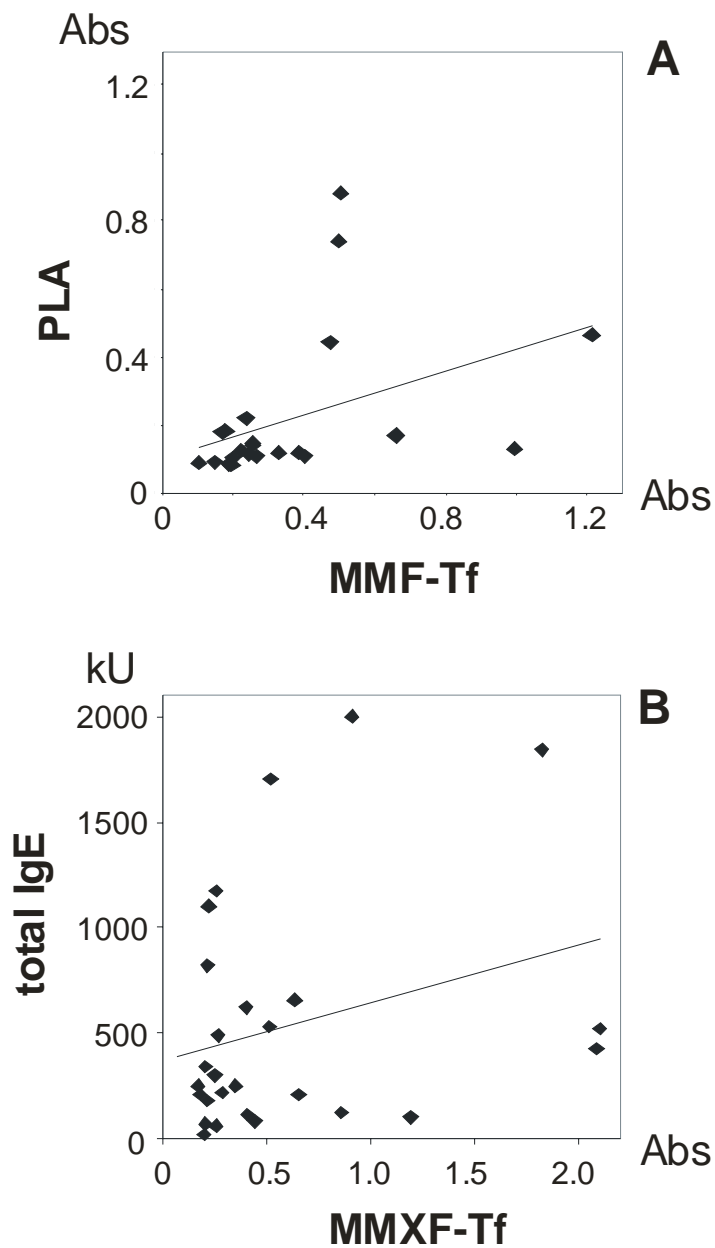


Table 1: Inhibition of antibody binding to glyco-antigens. Experiments were performed to give an absorbance in the range of 1.0 to 1.5 for the uninhibited serum with the exception of YZ1/2.23 reacting with MMX-transferrin where the absorbance was only ca. half of that obtained with MMF-Tf as coat. The standard error of the inhibition values was estimated to be 5 to 10 %. An inhibitor concentration of 1 $\mu\text{g}/\text{mL}$ translates to an N-glycan concentration of ca. 25 $\mu\text{mol}/\text{L}$.

Antibody	Coat antigen	Inhibitor concentration $\mu\text{g} / \text{mL}$	Inhibition (%) effected by:				
			MM-	MMX-	MMF-	MMXF-Tf	
rabbit anti-HRP	MMX-Tf	1	< 0	80.3	< 0	61.3	
		10	< 0	84.1	< 0	81.6	
		100	< 0	95.6	< 0	89.3	
rabbit anti-HRP	MMF-Tf	1	1.8	< 0	71.0	57.0	
		10	< 0	< 0	81.8	78.0	
		100	6.2	< 0	95.1	89.2	
YZ1/2.23	MMX-Tf	1	46.8	55.5	55.3	47.1	
		10	49.4	59.5	64.3	55.3	
		100	57.9	85.9	90.3	90.3	
YZ1/2.23	MMF-Tf	1	< 0	6.8	5.6	0.3	
		10	4.4	9.9	7.8	8.7	
		100	3.6	35.3	48.6	51.2	
rabbit anti-HRP	OSR pollen	0.1		MMXF-	MM-	GnGnXF-Tf	HRP
		1	76.3	< 0	10.3	88.3	
		10	86.5	< 0	14.4	91.4	
		100	94.8	< 0	25.0	93.6	
rabbit anti-HRP	MMX-Tf	200		MMX-	MUX-	MU-Tf	
	MMF-Tf	200		9.7	9.2	7.6	
YZ1/2.23	MMX-Tf	200		72.2	53.9	44.9	
YZ1/2.23	MMF-Tf	200		23.8	5.8	2.5	