

Immunoglobulin G specifically binding plant *N*-glycans with high affinity could be generated in rabbits but not in mice

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Xylosylated and core α 1,3-fucosylated *N*-glycans from plants are immunogenic, and they play a still obscure role in allergy and in the field of plant-made protein pharmaceuticals. We immunized mice to generate monoclonal antibodies (mAbs) binding plant *N*-glycans specifically via the epitope containing either the xylose or the core α 1,3-fucose residue. Splenocytes expressing *N*-glycan-specific antibodies derived from C57BL/6 mice previously immunized with plant glycoproteins were preselected by cell sorting to generate hybridoma lines producing specific antibodies. However, we obtained only mAbs unable to distinguish fucosylated from xylosylated *N*-glycans and reactive even with the pentasaccharide core Man₃GlcNAc₂. In contrast, immunization of rabbits yielded polyclonal sera selectively reactive with either fucosylated or xylosylated *N*-glycans. Purification of these sera using glyco-modified neoglycoproteins coupled to a chromatography matrix provided polyclonal sera suitable for affinity determination. Surface plasmon resonance measurements using sensor chips with immobilized glyco-modified transferrins revealed dissociation constants of around 10⁻⁹ M. This unexpectedly high affinity of IgG antibodies toward carbohydrate epitopes has repercussions on our conception of the binding strength and significance of antiglycan IgE antibodies in allergy.

Key words: binding affinity/carbohydrate epitope/
glycoprotein/immunogenic *N*-glycan

Introduction

In complex-type *N*-glycans from plants, an α 1,3-fucose is attached to the proximal glucosamine residue and a β 1,2-xylose residue to the β -mannose. Being absent in mammals, these two residues are responsible for the fact that the glycan moiety of plant glycoproteins by itself constitutes an immunogenic determinant in mammals (Faye and Chrispeels,

1988; Lauriere *et al.*, 1988; Prenner *et al.*, 1992; Wilson *et al.*, 1998; van Ree *et al.*, 2000). Sera from around 20–30% of allergic patients contain IgE antibodies against plant *N*-glycans which cause an enormous *in vitro* cross-reactivity of such sera (Aalberse *et al.*, 1981; Tretter *et al.*, 1993; Wilson *et al.*, 1998; Bencurova *et al.*, 2004). The diagnostic and clinical consequences of the cross-reactivity of antiglycan IgE for allergic patients have been exposed recently (Foetisch and Vieths, 2001; van Ree, 2004; Kochuyt *et al.*, 2005; Malandain, 2005) and will be debated to some degree in the discussion. Among nonallergic blood donors, an even higher percentage was found to have IgG and IgM directed against xylosylated and α 1,3-fucosylated *N*-glycans (Bardor *et al.*, 2003).

Plants as production systems for recombinant biopharmaceuticals, such as antibodies, have received considerable attention during the last years (Raskin *et al.*, 2002; Fischer *et al.*, 2003; Yano *et al.*, 2004). However, glycoproteins will be furnished with complex-type *N*-glycans containing the said carbohydrate epitopes, unless special measures are undertaken to prevent a plant-like glycosylation (Koprivova *et al.*, 2004; Strasser *et al.*, 2004; Faye *et al.*, 2005). The presence of this immunogenic determinant on a glycoprotein therapeutic appears problematic if not intolerable because it will result in a reduced circulatory half-life and hence reduced efficacy of the drug. Antiglycan antibodies which were either preexisting or elicited by repeated administration of the drug may even lead to more severe adverse effects (Faye *et al.*, 2005). At the extreme, allergic individuals with IgE antibodies against plant-type *N*-glycans may experience anaphylactic reactions. It is at the moment difficult to quantitate these hazards arising from a plant-type glycosylation, as for example the biological significance of IgE to plant *N*-glycans is questionable (van der Veen *et al.*, 1997). Nevertheless, it is obvious that this issue deserves attention and considerable additional research before plant-made glycoprotein may become acceptable as topic pharmaceuticals for humans.

Following the discovery of the immunogenicity of plant *N*-glycans in rabbits and goats, it was soon discovered that the core α 1,3-fucose on one hand and the β 1,2-linked xylose on the other hand together with the lack of antennae (sugar chains on the nonreducing end) constituted the key features which made plant *N*-glycans immunogenic (Kaladas *et al.*, 1983; Lauriere *et al.*, 1988; McManus *et al.*, 1988; Kurosaka *et al.*, 1991; Prenner *et al.*, 1992). The importance of the fucose residue became evident from studies on bee venom-allergic patients (Weber *et al.*, 1987; Tretter *et al.*, 1993; Hemmer *et al.*, 2001). The separation of rabbit anti-plant *N*-glycan sera into a “fucose-” and a “xylose-specific” fraction using immobilized honeybee phospholipase A2 indicated that these oligosaccharides contained two independent immune

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determinants (Faye *et al.*, 1993), and this result was later corroborated by a study on commercial anti-horseradish peroxidase serum from rabbit with defined synthetic glycoantigens containing either xylose or fucose (Bencurova *et al.*, 2004). As expected, rabbit anti-bee venom serum exclusively bound to fucosylated glycans (Bencurova *et al.*, 2004). It is important to notice that the terms fucose-specific or xylose-specific are meant in the sense that these residues constitute an essential part of the epitope but together with other groups which, however, by themselves are not able to bind antibody (Kurosaka *et al.*, 1991).

Monoclonal antibodies (mAbs) of similar specificity have not been described so far. A mouse mAb raised against pollen extract from *Cupressus arizonica* was demonstrated to be directed against the plant *N*-glycan $\text{Man}_3\text{XylFucGlcNAc}_2$ (MMXF³) (for structures see Figure 1) (Iacovacci *et al.*, 2001); however, no detailed analysis about its sugar specificity had been performed.

A rat mAb (YZ1/2.23) raised against elderberry abscission tissues was also found to be specific for plant MMXF³ structure (McManus *et al.*, 1988), but further investigation showed that the paratope of YZ1/2.23 covered both the fucosyl and the xylosyl residues and that it bound to some degree even with the pentasaccharide core alone. As a consequence, YZ1/2.23 appears as an unsuitable tool for the detection of plant-specific glycosylation, and it certainly cannot be used to discriminate between fucosylated and xylosylated glycoproteins (Bencurova *et al.*, 2004). A likewise very broad reactivity was observed for an mAb obtained from *Schistosoma*-infected mice (van Remoortere *et al.*, 2003). Recently, C57BL/6 mice were reported to give a much stronger immune reaction toward plant glycoproteins than the more widely used BALB/c mice (Bardor *et al.*, 2003). This inability of BALB/c mice may also explain that the first and best-characterized monoclonal antiglycan antibody (YZ1/2.23) was from rat.

In none of the abovementioned studies, the affinity of binding between antibodies and glycoproteins has been determined. This is regrettable, as biological consequences of antibody reaction may depend on binding affinity at least as much as on antibody titer. Interactions between carbohydrates and proteins, for example lectins or antibodies, are usually considered to be much weaker (in the micromolar range) than protein–protein interactions (usually in the nanomolar range) (Wilson and Stanfield, 1995; Weis, 1997; Thomas *et al.*, 2002).

Binding affinities can be measured with various methods, the most attractive probably being surface plasmon resonance (SPR) (Haseley *et al.*, 1999). SPR and other techniques require the use of pure, well-defined carbohydrate structures. However, plant glycoproteins with uniform and defined glycosylation are hardly available, especially, if one aims at evaluating the relative contributions of fucose and xylose or other structural features to antibody binding. The previous success in glyco-modifying human transferrin with the help of recombinant xylosyl and fucosyltransferase opened the opportunity to study the binding kinetics of antiglycan antibodies to structurally defined glycoforms (Bencurova *et al.*, 2004).

In this article, we report on the attempt to produce mAbs against plant *N*-glycans in C57BL/6 mice and on the more successful attempt to produce structural specific polyclonal

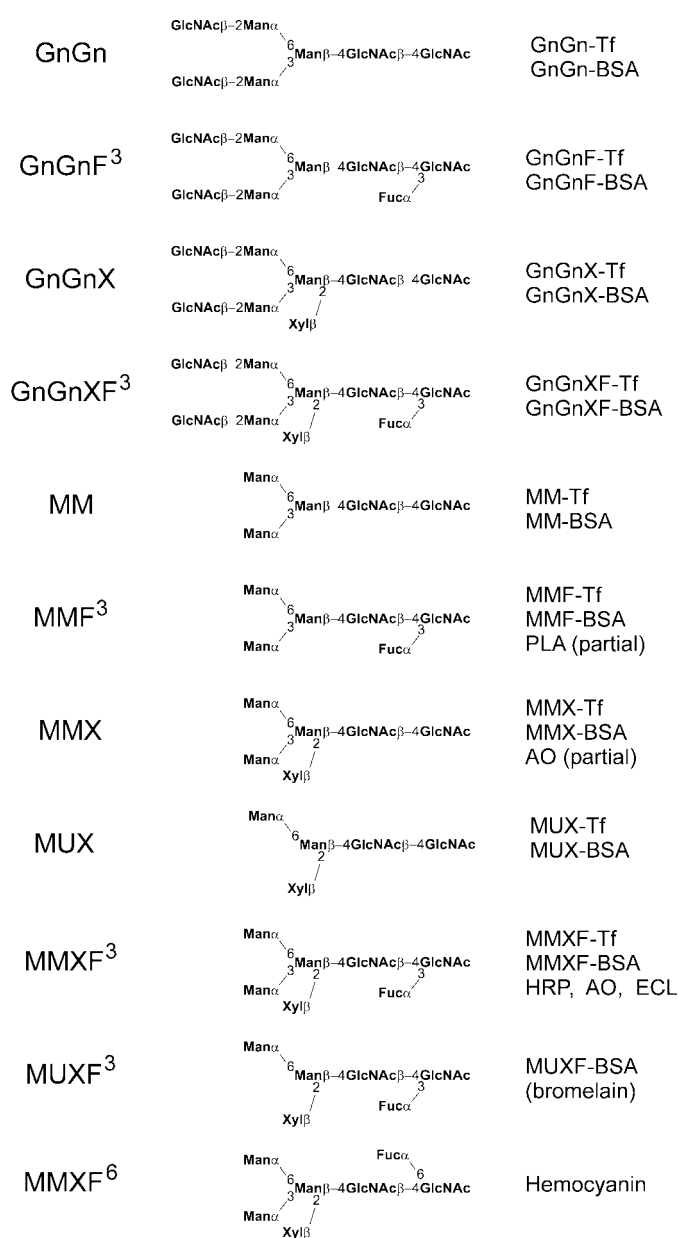


Fig. 1. Structures of oligosaccharides relevant to this study. *N*-glycans are shown with the abbreviations according to the proglycan system (<http://www.proglycan.com>) and their occurrence in semisynthetic and natural glycoproteins.

sera in rabbits. Last, but not least, the binding affinities of purified rabbit antibodies against MMX, MMF, and also MMXF glycans (see Figure 1 for structures) to their ligands were investigated by SPR technology, taking advantage of the availability of defined “plantified” glycoforms of human transferrin.

Results

Mouse mAbs

After repeated failure to generate hybridoma lines which produce mAbs against plant *N*-glycans (Tretter V and Altmann F,

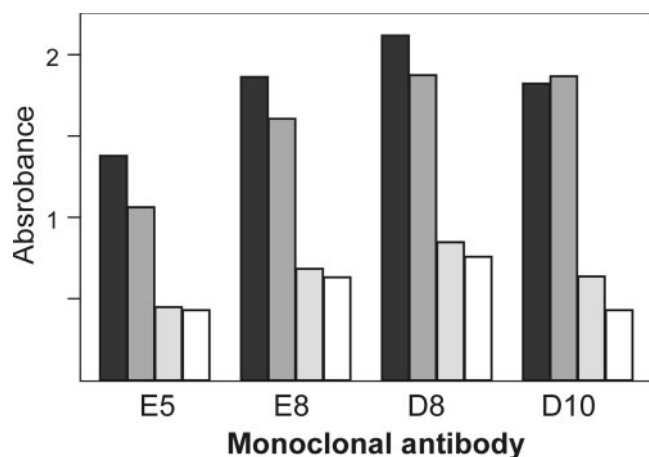


Fig. 2. ELISA of selected murine monoclonal antibodies (mAbs) with glycoforms of transferrin. The ELISA results of 4 out of 36 mAbs are shown here. Reactivity to MMF-Tf (dark gray), MMX-Tf (medium gray), MM-Tf (light gray), and unmodified transferrin (white bars) is shown. The concentration of IgG in each sample was very similar.

unpublished data), a recent report on the much higher immunogenicity of plant glycoproteins in C57BL/6 as compared with BALB/c mice (Bardor *et al.*, 2003) made us plan a new trial to obtain structure-specific antibodies against plant complex-type *N*-glycans. We wanted to specifically boost the glycan-directed immune response by challenging the mice at consecutive immunizations with different proteins bearing similar *N*-glycan structures. Even then, however, the immune response of the mice was far from overwhelming (data not shown). Nevertheless, spleen cells of a few mice were fused, and by applying a sophisticated sorting strategy (Böhm *et al.*, 2005), we got 164 clones, 36 of which secreted glycan-directed antibody as measured using MMX and MMF coupled to human transferrin (MMX- and MMF-Tf). However, a closer characterization of these mAbs revealed that the individual mAbs could bind both to MMX- and to MMF-Tf (Figure 2). Hence, they did not discriminate between fucosylated and xylosylated *N*-glycans. Moreover, the mAbs even exhibited high binding to MM-Tf and unmodified transferrin (Figure 2). Thus, despite great efforts and the use of C57BL/6 mice, we were not able to elicit a strong immune response against the plant *N*-glycans and to generate hybridoma lines producing structure-specific mAbs.

Rabbit polyclonal antisera

To overcome the problems encountered with mice, rabbits were chosen for the production of antiglycan sera. Specificity should be introduced by the immunization protocol using only MMF-Tf as antigen to obtain “fucose-specific” sera or MMX-Tf for “xylose-specific” sera. All rabbits showed immune response to the antigens, even though only one rabbit from each group of three developed a satisfyingly high titer, which may be attributed to the use of incomplete adjuvant. Notably, the same result was obtained for horseradish peroxidase (HRP), a usually very potent glyco-antigen as we judge from our long experience with commercial rabbit and goat anti-HRP serum (Tretter *et al.*,

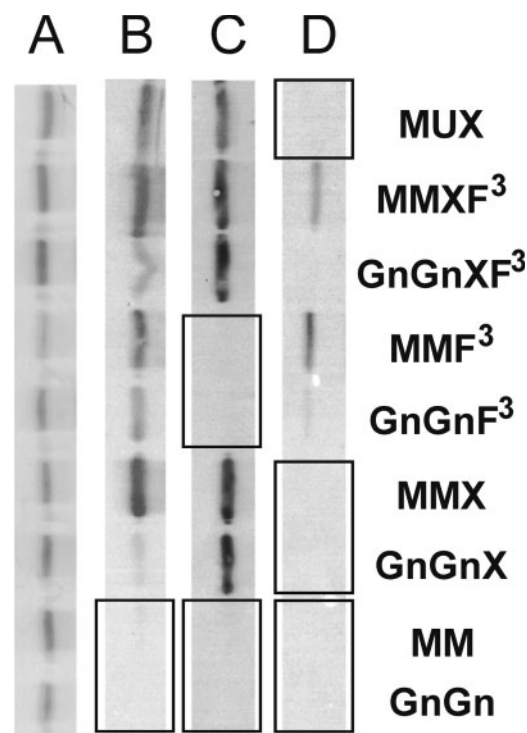


Fig. 3. Western blot with rabbit antiglycan antibodies. Equal amounts of various glycoforms of human transferrin were subjected to SDS-PAGE and blotted onto nitrocellulose. The Coomassie blue stain is shown in lane A. Lane B was incubated with anti-HRP serum, lane C with anti-MMX antibodies, and lane D with anti-MMF antibodies. The boxes indicate positions, which are critical for the structural specificity of the antibodies. With the possible exception of weak binding of anti-HRP to MM-Tf (not confirmed by ELISA), these spots remained absolutely unstained showing the strict need for xylose or fucose residues, respectively, for antibody binding. Terminal GlcNAc residues interfered with binding of anti-MMF antibodies and to some extent of anti-HRP serum. The *N*-glycan structures are depicted in Fig. 1.

1993; Wilson *et al.*, 1998) as well as for both transferrin glycoforms. Before immunization, no detectable binding activity to HRP, MMX- and MMF-Tf was found in control rabbits (not shown). Sera from rabbits which showed highest binding activity to xylose or fucose residues were used for a two-step affinity purification. To detect residual anti-transferrin antibodies in the purified Xyl- and Fuc-specific sera, they were tested by western blot with native and with glyco-modified transferrins (Figure 3). Obviously, the antibodies directed against the protein backbone of transferrin had been efficiently removed by affinity chromatography. After separation from (most of) the albumin, the three antiglycan antibodies were characterized with regard to their glycan binding using various transferrin glycoforms (Figure 1). In contrast to the mouse mAbs, the rabbit sera, even though polyclonal, exhibited high specificity and were able to discriminate between fucosylated and xylosylated glycoproteins. Remarkably, the anti-MMX serum bound xylosylated glycoforms irrespective of the presence or absence of non-reducing terminal GlcNAc residues. Anti-HRP also had this ability but to a lesser extent. In contrast, anti-MMF antibody only bound truncated fucosylated glycoforms (MMF, MUXF, and MMXF). The same results were obtained

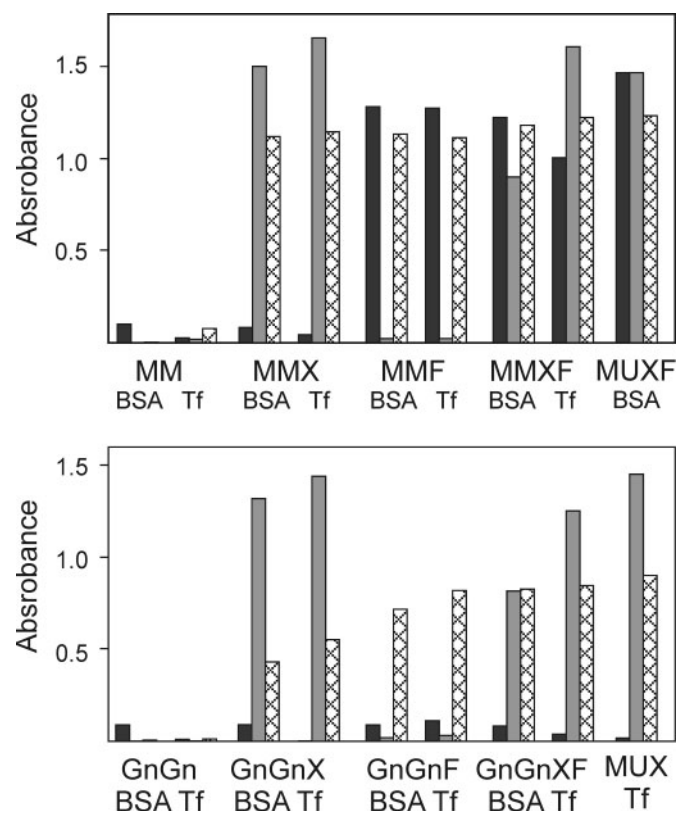


Fig. 4. Binding specificity of rabbit anti-HRP, anti-MMX, and anti-MMF antibodies. ELISA plate wells were coated with different BSA conjugates or glyco-modified transferrins. The *N*-glycan structures are depicted in Fig. 1. Black bars stand for anti-MMF, gray bars for anti-MMX, and the patterned bar for anti-HRP serum.

when the specificities of purified antiglycan antibodies were investigated by ELISA using glyco-modified transferrins as well as bovine serum albumin (BSA) conjugates (Figure 4) or glyco-modified human α 1-antitrypsins (data not shown). The binding to glyco-modified transferrins and to BSA conjugates with the same glycans was comparable, which argues against any essential influence of the protein backbone. Finally, the reaction of the purified antibodies with natural glycoproteins HRP (MMXF), ascorbate oxidase (MMXF and MMX; Altmann, 1998), hemocyanin from *Helix pomatia* (MMX and many undefined structures; van Kuik *et al.*, 1985), honeybee venom phospholipase A2 (PLA, an insect glycoprotein containing a core α (1,3)-fucose; Kubelka *et al.*, 1993), and lectin from *Erythrina corallodendron* (MMXF and other structures; Ashford *et al.*, 1991) was determined (Figure 5). Consistent with the structures of their carbohydrate chains, plant glycoproteins bound both the anti-MMF and the anti-MMX serum, whereas the insect and the snail glycoprotein selectively reacted with either anti-MMF or anti-MMX, respectively.

Goat anti-HRP serum

Commercial goat anti-HRP serum was subjected to ELISA with glyco-modified transferrins. This serum exhibited a similar specificity to that of rabbit anti-HRP, that is it bound strongly to all fucosylated and/or xylosylated transferrins

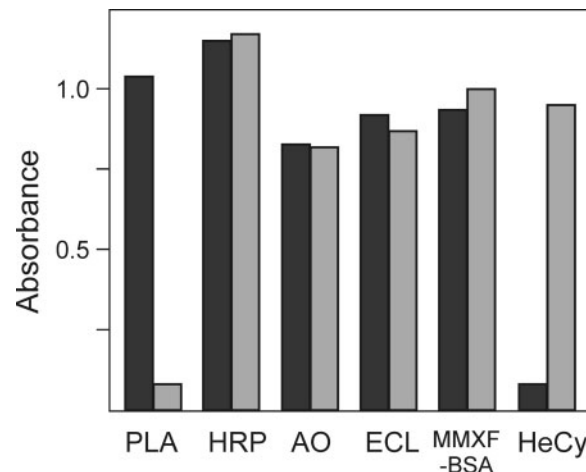


Fig. 5. Binding of anti-MMF and anti-MMX antibodies to natural glycoproteins. Antibody binding was observed for the same dilution of either anti-MMF Ab (black bars) or anti-MMX Ab (gray bars). The coated antigens were phospholipase A2 from honeybee venom (PLA), horseradish peroxidase (HRP), ascorbate oxidase from zucchini (AO), *Erythrina corallodendron* lectin (ECL), MMXF-BSA conjugate, and hemocyanin from *Helix pomatia* (HeCy). The reactivity of anti MMX with PLA corresponds to a blank value.

but almost not at all when these residues were absent (data not shown). The same results were obtained with BSA conjugates and glyco-modified human α 1-antitrypsins (data not shown).

Binding affinities of rabbit antibodies

The problems associated with the determination of binding affinities of polyclonal antibodies have recently been discussed, and a strategy to obtain representative average values has been devised (Bakker *et al.*, 1995; Sem *et al.*, 1999; Cachia *et al.*, 2004; Hantusch *et al.*, 2005). The situation in the present study is somewhat simpler as in the case of anti-MMF and anti-MMX as we are dealing with sera directed against a single epitope, whereas in natural allergens an unknown number of different epitopes is present.

The on- and off-rates of polyclonal xylose- and fucose-specific IgG and anti-HRP binding to MMX-, MMF-, and MMXF-Tf were determined in SPR experiments. In all instances, the reference cell of chip was coated with unmodified transferrin to subtract any trace unspecific binding to the transferrin backbone. Decreasing amounts of plant *N*-glycan-specific IgG and anti-HRP were injected in triplicates onto the sensor chip (Figure 6). Results of the association rate constant, k_a (1/Ms), dissociation rate constant, k_d (1/s), and the calculated equilibrium dissociation constant, K_D (M) are summarized in Table I. The values of k_a were determined from the association phase of the binding curve, whereas k_d were determined from the dissociation phase. The larger the association rate, the faster is the binding and in combination with a low dissociation rate, the higher is the affinity of an antibody. Notably, the binding of the xylose-specific antibody was faster to the MMXF surface than to the specific surface with MMX glycans (Table I). As the dissociation rates from the MMX and the MMXF surface were similar, this difference of the association rate

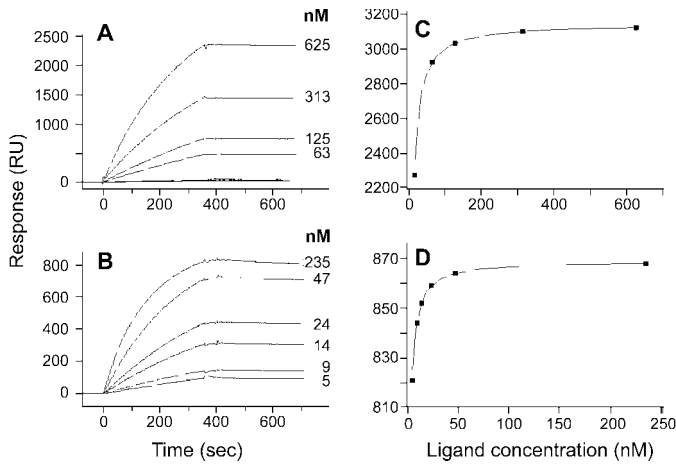


Fig. 6. Example of the SPR studies: Binding of rabbit anti-MMX to MMX-Tf. Overlay sensorgrams of injections with increasing concentrations of the “xylose-specific” antibody over the chip surface with immobilized MMX-Tf are shown in panel A, whereas panel B shows the result with immobilized MMXF-Tf. Panels C and D give the plots of steady-state binding values, R_{eq} , against xylose-specific antibody concentrations on MMX-Tf and MMXF-Tf surfaces. The control channel coated with unmodified transferrin did not show any anti-MMX binding.

caused the combination anti-MMX with MMXF-Tf to exhibit the highest binding affinities of 2.5×10^{-10} M (Table I). Possibly, the concomitant presence of xylose and fucose on the glycans favors a conformation that facilitates antibody binding to the epitope containing the rather flexible xylose residue (Lommerse *et al.*, 1995). The affinity of anti-HRP may be seen as sort of average value between those of anti-MMX and anti-MMF as anti-HRP actually consists of two antibody populations, one binding to xylosylated and one to fucosylated glycans.

Discussion

From this study as well as from previous experiences in our laboratory and from other groups (van Remoortere *et al.*, 2003), it appears that the immune system of mice and also of rats (Wilson *et al.*, 1998; Bencurova *et al.*, 2004) cannot respond to plant N-glycans with the production of structurally

specific antibodies as is seen with rabbits or goats. It also seems from this study and a previous one (Bardor *et al.*, 2003) that depending upon strain and maybe immunization regime, glycoproteins, which in other animals lead to a strong anti-carbohydrate immune response (Kaladas *et al.*, 1983; Faye and Chrispeels, 1988; Lauriere *et al.*, 1988; Prenner *et al.*, 1992; Faye *et al.*, 1993), do not necessarily lead to a nearly comparable antibody production in mice. In this light, the recent finding that a tobacco-produced mAb does not elicit an immune response in mice (Chargelegue *et al.*, 2000) cannot be taken as a last affirmation that plant-made glycoprotein pharmaceuticals may be regarded as immunologically safe for human therapy. In other words, mice must be rated an inappropriate animal model for the evaluation of the carbohydrate-related immunogenicity of plant-derived glycoproteins.

The immunological distinctiveness of mice unfortunately deprives us of the possibility of obtaining highly specific mAbs. In this study, we have therefore chosen affinity-purified polyclonal rabbit antibodies. Other options may be the use of phage display libraries, which, however, disrupts the pairing of heavy and light chain unless camelid antibodies are considered (Nguyen *et al.*, 2003). However, rabbits, when immunized with defined glyco-antigens such as our glyco-modified transferrins, respond in a very specific manner. The quality of the sera can be enhanced by removal of the inadvertently generated anti-protein antibodies by immobilized transferrin and/or by using immobilized glyco-antigen for affinity purification.

Another small but notable point can be made from the ELISA comparison of antibodies raised against HRP or modified transferrins and tested against a panel of glycoproteins. The protein backbone of the glyco-allergen on the ELISA does not affect the result. This argues very much against the sometimes heard notion that the sugars only modulate the binding of antibodies to nearby peptide epitopes.

The monovalent, albeit polyclonal, anti-MMF and anti-MMX antibodies were finally used to assess their binding affinities to glycoproteins. The motive for this effort lays in the enigmatic role of carbohydrate determinants in allergy. While on the one hand plant N-glycans have been shown to elicit effector release (Bublin *et al.*, 2003; Foetisch *et al.*, 2003; Westphal *et al.*, 2003; Wicklein *et al.*, 2004), they are considered as innocuous regarding their potency to evoke

Table I. Kinetics of antibody binding as measured by SPR

Glyco-modified transferrins	Antibody	k_a (1/Ms)	k_d (1/s)	K_D (M)
MMXF-Tf	Anti-HRP	$4.07 \pm 0.01 \times 10^4$	$1.48 \pm 0.02 \times 10^{-4}$	3.64×10^{-9}
	Anti-MMX	$8.58 \pm 0.03 \times 10^4$	$2.16 \pm 0.12 \times 10^{-5}$	2.52×10^{-10}
	Anti-MMF	$1.19 \pm 0.04 \times 10^5$	$1.77 \pm 0.02 \times 10^{-4}$	1.49×10^{-9}
MMX-Tf	Anti-HRP	$1.32 \pm 0.01 \times 10^4$	$1.00 \pm 0.05 \times 10^{-4}$	7.58×10^{-9}
	Anti-MMX	$6.00 \pm 0.01 \times 10^3$	$2.83 \pm 0.07 \times 10^{-5}$	4.72×10^{-9}
MMF-Tf	Anti-HRP	$5.49 \pm 0.02 \times 10^4$	$2.29 \pm 0.05 \times 10^{-4}$	4.17×10^{-9}
	Anti-MMF	$4.45 \pm 0.01 \times 10^4$	$8.26 \pm 0.03 \times 10^{-5}$	1.86×10^{-9}

Anti-MMX, xylose-specific antibody; anti-MMF, fucose-specific antibody. Each set of curves was simultaneously fitted using a 1:1 binding model to obtain the rate constants. k_a is the association rate, k_d the dissociation rate, and $K_D = k_d/k_a$ is the equilibrium dissociation constant. Experimental error was calculated from triplicate sensorgrams of the same antibody concentration.

clinical symptoms (van der Veen *et al.*, 1997). Does the carbohydrate nature of this determinant explain this observation? For immunologists, all epitopes are equal. But for allergologists, some epitopes—the peptide epitopes—appear to be more equal. A possible explanation may lay in a low binding affinity of antibodies to these epitopes as it was found for lectin–carbohydrate interactions, where the K_D often lies in the micromolar range (Haseley *et al.*, 1999). However, even for lectins, higher affinities have been reported for RCA₁₂₀ and DSA to terminal Gal residue in asialofetuin (K_D , 3.8×10^{-9} and 3.7×10^{-9} M, respectively) (Shinohara *et al.*, 1994). The affinities of antibodies against carbohydrate epitopes were often found to be quite high, for example in the case of a 3-deoxy-D-manno-octulosonic acid trisaccharide on *Chlamydia* outer membrane LPS, high-affinity mAbs could be obtained (K_D , $\sim 10^{-9}$ M) (Muller-Loennies *et al.*, 2000). Examples of lower affinity can be found as well, e.g. a K_D of 2.3×10^{-7} M for an mAb against O-polysaccharide from *Salmonella* serogroup B because of its rapid dissociation rates (MacKenzie *et al.*, 1996).

The antibodies investigated in this study exhibited K_D values of around 10^{-9} M and thereby almost reach the theoretical limit of antibody affinity ($\sim 10^{-10}$ M) (Foote and Eisen, 1995). Therefore, cross-reactive carbohydrate determinants (CCDs) can no longer be regarded as “less equal” in the sense of allowing only low-affinity binding. Even though the affinities of human IgE and IgG remain to be determined, we dare to speculate that low binding affinity of anti-CCD antibody drops out as an explanation for the usually observed inoffensiveness of CCDs as allergens. However, this should not remain a speculation for long as the current work forms the basis for the determination of binding affinities of anti-CCD antibodies in human sera.

Materials and Methods

Preparation of neoglycoproteins

BSA conjugates were prepared as previously described with some modifications (Wilson *et al.*, 1998). GnGn fibrin glycopeptides were first cross-linked to BSA to prepare GnGn-BSA. Then GnGn-BSA was modified by purified recombinant *Arabidopsis thaliana* core $\alpha 1,3$ -fucosyltransferase and rice $\beta 1,2$ -xylosyltransferase expressed in *Pichia pastoris* (Bencurova *et al.* 2003; Leonard *et al.*, 2004). The resultant GnGnX-, GnGnF-, and GnGnXF-BSA were treated with *N*-acetylglucosaminidase from *Streptococcus pneumoniae* (Sigma-Aldrich, St Louis, MO, USA) to obtain MMX-, MMF-, and MMXF-BSA. MUXF-BSA and MUX-BSA were generated by cross-linking bromelain glycopeptides or defucosylated glycopeptides to BSA (Wilson *et al.*, 1998). The BSA conjugates were subjected to amino sugar analysis (Altmann, 1992) and found to contain 2–3 mol glycopeptide per mol of BSA.

Glyco-modified human transferrins (MM-, MMX-, MUX-, MMF-, and MMXF-Tf) were prepared as previously described except using purified recombinant $\beta 1,2$ -xylosyltransferase from rice instead from *A. thaliana* (Bencurova *et al.* 2003; Leonard *et al.*, 2004). The purity and integrity of neoglycoproteins were verified by SDS-PAGE, and the structures of their glycan moieties were checked by matrix-assisted

laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (Kolarich and Altmann, 2000). It should be noted that the glycosyltransferases and glycosidases used were not of plant origin, and thus glyco-contamination from this source were excluded.

Generation and characterization of mouse mAbs

Nine eight-week-old C57BL/6 mice in three groups (Charles River Wiga, Sulzfeld, Germany) were randomly divided into three groups immunized with different combinations of natural or modified plant glycoproteins, mixed with incomplete Freund's adjuvant. The dosage per injection of the glycoproteins was as follows: 100 μ g of HRP (Sigma-Aldrich); 30 μ g of honeybee venom phospholipase (PLA, prepared as described; Tretter *et al.*, 1993); 50 μ g of zucchini ascorbate oxidase (AO, Sigma-Aldrich); 100 μ g of *Sophora japonica* lectin (SJA, Sigma-Aldrich); and 50 μ g of bromelain (BRO, prepared as described; Tretter *et al.*, 1993). The first three injections were given intraperitoneally on days 0, 21, 42, 63, and 84. The two final injections were given intravenously without adjuvant and were followed 4 days later by cell fusion.

The sequences of antigens were HRP-PLA-AO-HRP-SJL, HRP-BRO-AO-BRO-PLA, and HRP-AO-BRO-SJL-AO for the first, second, and third group of mice, respectively. The spleen cells from the three mice, which showed highest titers to glyco-modified transferrin (MMX- and MMF-Tf), were fused with an NS-0 myeloma cell line according to ClonaCell-HY for hybridoma production's protocol (Stem-cell Technologies, Vancouver, British Columbia, Canada).

After 10 days of hypoxanthine-aminopterin-thymidine (HAT) selection, cells were stained with the affinity matrix secretion assay (Böhm *et al.*, 2005) using a sheep-anti mouse IgG–biotin conjugate (Sigma-Aldrich) as capture antibody. Staining of specific hybridoma cells was achieved with the respective glyco-modified transferrin and an FITC-conjugated anti-transferrin (Capricorn Products, Portland, ME, USA). Cells secreting transferrin-specific antibodies were then sorted into 96-well plates at two to five cells per well. After approximately 2 weeks, samples of the culture supernatants of wells containing monoclonal colonies were taken and were screened by qualitative ELISA using MM-, MMX-, MMF-, MMXF-Tf, or NaNa-Tf (= unmodified transferrin) similarly as recently described (Bencurova *et al.*, 2004). However, 1:1000 diluted alkaline phosphatase-conjugated anti-mouse IgG γ -chain (Sigma-Aldrich) was used as the detection antibody. Bound antibody was detected by reaction with *p*-nitrophenylphosphate in 0.1 M diethanolamine at pH 9.8. The reaction was stopped after 20 min by addition of 5 M sodium hydroxide, and the absorbance at 405 nm was read.

Immunization of rabbits

Chinchilla Bastard rabbits were also divided into groups of three, but the animals in each group repeatedly received the same antigen, either HRP or MMX-Tf or MMF-Tf. All antigens were dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/mL. Intradermal injections consisting of 200 μ L antigen solution and the same volume of Freund's incomplete adjuvant were administered in 3-week

intervals. After the fourth immunization, the antisera were collected and kept at -20°C .

For ELISAs with rabbit sera, the plates were coated with plant glycoproteins or neoglycoprotein at a concentration of $5\text{ }\mu\text{g/mL}$ in 50 mM sodium carbonate, $\text{pH } 9.6$, for 60 min at 37°C . Bound antibody was detected by a $1:2000$ dilution of alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich) as described (Wilson *et al.*, 1998).

Affinity purification of xylose or fucose-specific IgG

From each group of three rabbits, the one serum which showed the highest antiglycan antibody titer was used for affinity purification. Affinity columns were prepared by coupling unmodified human transferrin, MMX-BSA or MMF-BSA conjugates to Affi-Gel 15 (BioRad, Richmond, CA) according to the manufacturer's instructions. The sera were diluted with 0.1 M Tris-HCl, $\text{pH } 7.2$, containing 0.1 M NaCl, 2.5 mM MgCl_2 , and 0.05% Tween 20 (TTBS) and applied at first to the transferrin column to eliminate anti-transferrin antibodies. The breakthrough was applied to either MMX-BSA- or MMF-BSA-coupled column depending on the antiserum. After extensive washing with TTBS, bound antibodies were eluted with 0.2 M glycine-HCl, $\text{pH } 2.5$, and neutralized immediately with 1 M Tris-HCl, $\text{pH } 7.5$. To remove rabbit serum albumin, effluents were loaded on a 1 mL bed of SP Sepharose Fast Flow (Amersham Biosciences AB, Uppsala, Sweden). Elution of IgG was performed with a gradient from 0.02 to 1.0 M NaCl in 50 mM MES, $\text{pH } 5.0$. Buffer exchange as well as concentration was performed immediately by filter devices with 10 kDa cut-off (Millipore, MA, USA).

The specificities of xylose or fucose-specific IgG were determined by ELISA and western blot. The amount of IgG was quantified by comparison of Coomassie-stained SDS-PAGE bands with a dilution series of pure human IgG. The xylose-specific IgG had an estimated concentration of 0.1 mg/mL , the fucose-specific pool of 0.2 mg/mL .

For western blot analysis, transferrin glycoforms and BSA conjugates ($0.5\text{--}1\text{ }\mu\text{g}$) were separated by SDS-PAGE, electroblotted to a nitrocellulose membrane and stained with the help of rabbit anti-HRP ($1:2000$) or purified rabbit xylose-specific or fucose-specific antibody ($1:500$) as described (Bencurova *et al.*, 2004).

SPR analysis

The binding kinetics of the purified antiglycan antibodies were determined by SPR using a Biacore X biosensor system (Biacore, Uppsala, Sweden). The running buffer used for all experiments was HBS-P buffer (Biacore), $\text{pH } 7.4$, containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 (Biacore). The immobilization to research grade CM5 sensor chips was carried out using a standard amine coupling procedure (Johnsson *et al.*, 1991). Transferrin glycoforms were applied in 10 mM sodium acetate buffer, $\text{pH } 4.0$. The two channels of each chip were set up as follows: MMX-, MMF-, and MMXF-Tf were immobilized on channel 1, whereas unmodified transferrin was bound to channel 2 of each chip as a control surface. Amounts of $3000 \pm 1000\text{ RU}$ of each glycoform were immobilized onto the chip surfaces. One RU corresponds to an

immobilized protein concentration of about 1 pg/mm^2 (Stenberg *et al.*, 1991).

All measurements were carried out in HBS-P buffer. Analyses were performed at 25°C and at a flow rate of $5\text{ }\mu\text{L/min}$. Between cycles, the chip surface was regenerated with two injections of $10\text{ }\mu\text{L}$ of 10 mM glycine-HCl, $\text{pH } 2.2$, or 0.5% SDS at a flow rate of $5\text{ }\mu\text{L/min}$ followed by washing the surface with HBS-P buffer until the signal reached baseline.

For the evaluation of association and dissociation rate constants, samples were diluted in HBS-P buffer and analyzed at various concentrations. Purified antibody at the specified concentration was pumped over the sensor chip surface for 6 min at a flow rate of $5\text{ }\mu\text{L/min}$ to observe the association phase, and dissociation phase was monitored after switching to protein-free buffer for another 6 min .

Sensorgrams were fitted to a single site-binding model ($1:1$ Langmuir binding), using the numerical integration functions of the BIAevaluation 3.1 software package. Association and dissociation rate constants (k_a and k_d) and the dissociation constant (K_D) were derived by both separate and simultaneous fitting of the association and dissociation phase of individual sensorgrams. Sensorgrams were also fitted globally using data collected at a series of concentrations. The steady-state binding values, R_{eq} , were then plotted as a function of the applied antibody concentrations and fitted to first-order kinetics, assuming a monovalent antibody-modified transferrin interaction (Karlsson *et al.*, 1991).

Supplementary data

Supplementary data are available at *Glycobiology* online (<http://glycob.oxfordjournals.org>).

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Abbreviations

BSA, bovine serum albumin; CCD, cross-reactive carbohydrate determinant; HAT, hypoxanthine-aminopterin-thymidine; HRP, horseradish peroxidase; SPR, surface plasmon resonance.

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Immunoglobulin G specifically binding plant N-glycans with high affinity could be generated in rabbits but not in mice

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Running title: Affinity of antibody binding to plant N-glycans

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Abstract

Xylosylated and core α 1,3-fucosylated N-glycans from plants are immunogenic and they play a still obscure role in allergy and in the field of plant-made protein pharmaceuticals. We immunized mice to generate monoclonal antibodies binding plant N-glycans specifically via the epitope containing either the xylose- or the core α 1,3-fucose-residue. Splenocytes expressing N-glycan-specific antibodies derived from C57BL/6 mice previously immunized with plant glycoproteins were pre-selected by cell sorting to generate hybridoma lines producing specific antibodies. However, we obtained only monoclonal antibodies unable to distinguish fucosylated from xylosylated N-glycans and reactive even with the pentasaccharide core $\text{Man}_3\text{GlcNAc}_2$. In contrast, immunization of rabbits yielded polyclonal sera selectively reactive with either fucosylated or xylosylated N-glycans. Purification of these sera using glyco-modified neo-glycoproteins coupled to a chromatography matrix provided polyclonal sera suitable for affinity determination. Surface plasmon resonance measurements using sensor chips with immobilized glyco-modified transferrins revealed dissociation constants of around 10^{-9} M. This unexpectedly high affinity of IgG antibodies towards carbohydrate epitopes has repercussions on our conception of the binding strength and significance of anti-glycan IgE antibodies in allergy.

Introduction

In complex-type N-glycans from plants, an α 1,3-fucose is attached to the proximal glucosamine residue and/ a β 1,2-xylose residue to the β -mannose. Being absent in mammals, these two residues are responsible for the fact that the glycan moiety of plant glycoproteins by itself constitutes an immunogenic determinant in mammals (Faye and Chrispeels, 1988, Lauriere *et al.*, 1988, Prenner *et al.*, 1992, van Ree *et al.*, 2000, Wilson *et al.*, 1998). Sera from around 20-30 % of allergic patients contain IgE antibodies against plant N-glycans which cause an enormous *in vitro* cross-reactivity of such sera (Aalberse *et al.* 1981, Bencurova *et al.* 2004, Tretter *et al.* 1993, Wilson *et al.* 1998). The diagnostic and clinical consequences of the cross-reactivity of anti-glycan IgE for allergic patients have been exposed recently (Foetisch and Vieths, 2001, Kochuyt *et al.*, 2005, Malandain, 2005, van Ree, 2004) and will be debated to some degree in the discussion. Among non-allergic blood donors, an even higher percentage was found to have IgG and IgM directed against xylosylated and α 1,3-fucosylated N-glycans (Bardor *et al.*, 2003).

Plants as production systems for recombinant biopharmaceuticals, such as antibodies, have received considerable attention during the last years (Fischer *et al.*, 2003, Raskin *et al.*, 2002, Yano *et al.*, 2004). However, glycoproteins will be furnished with complex type N-glycans containing the said carbohydrate epitopes, unless special measures are undertaken to prevent a plant-like glycosylation (Faye *et al.*, 2005, Koprivova *et al.*, 2004, Strasser *et al.*, 2004). The presence of this immunogenic determinant on a glycoprotein therapeutic appears problematic if not intolerable because it will result in a reduced circulatory half life and hence reduced efficacy of the drug. Anti-glycan antibodies which were either pre-existing or elicited by repeated administration of the drug may even lead to more severe adverse effects (Faye *et al.*, 2005). At the extreme, allergic individuals with IgE antibodies against plant-type N-glycans may experience anaphylactic reactions. It is at the moment difficult to quantitate these hazards

arising from a plant-type glycosylation, as *e.g.* the biological significance of IgE to plant N-glycans is questionable (van der Veen *et al.*, 1997). Nevertheless, it is obvious that this issue deserves attention and considerable additional research before plant-made glycoprotein may become acceptable as topic pharmaceuticals for humans.

Following the discovery of the immunogenicity of plant N-glycans in *e.g.* rabbits and goats, it was soon discovered that the core- α 1,3-fucose on one hand and the β 1,2-linked xylose on the other hand together with the lack of antennae (sugar chains on the non-reducing end) constituted the key features which made plant N-glycans immunogenic (Kaladas *et al.*, 1983, Kurosaka *et al.*, 1991, Lauriere *et al.*, 1988, McManus *et al.*, 1988, Prenner *et al.*, 1992). The importance of the fucose residue became evident from studies on bee venom allergic patients (Hemmer *et al.*, 2001, Tretter *et al.*, 1993, Weber *et al.*, 1987). The separation of rabbit anti-plant N-glycan sera into a “fucose-” and a “xylose-specific” fraction using immobilized honeybee phospholipase A2 indicated that these oligosaccharides contained two independent immune determinants (Faye *et al.*, 1993) and this result was later corroborated by a study on commercial anti-horseradish serum from rabbit with defined synthetic glyco-antigens containing either xylose or fucose (Bencurova *et al.*, 2004). As expected, rabbit anti-beevenom serum exclusively bound to fucosylated glycans (Bencurova *et al.*, 2004). It is important to notice that the terms “fucose-specific” or “xylose-specific” are meant in the sense that these residues constitute an essential part of the epitope but together with other groups which, however, by themselves are not able to bind antibody (Kurosaka *et al.*, 1991). Monoclonal antibodies (mAbs) of similar specificity have not been described so far. A mouse mAb raised against pollen extract from *Cupressus arizonica* was demonstrated to be directed against the plant N-glycan $\text{Man}_3\text{XylFucGlcNAc}_2$ (MMXF³) (for structures see Fig. 1.) (Iacovacci *et al.*, 2001), however, no detailed analysis about its sugar specificity had been performed.

Fig. 1

A rat mAb (YZ1/2.23) raised against elderberry abscission tissues was also found to be specific for plant MMXF³ structure (McManus *et al.*, 1988), but further investigation showed that the paratope of YZ1/2.23 covered both the fucosyl and the xylosyl residues and that it bound to some degree even with the pentasaccharide core alone. As a consequence, YZ1/2.23 appears as an unsuitable tool for the detection of plant-specific glycosylation and it certainly cannot be used to discriminate between fucosylated and xylosylated glycoproteins (Bencurova *et al.*, 2004). A likewise very broad reactivity was observed for a mAb obtained from *Schistosoma* infected mice (van Remoortere *et al.*, 2003). Recently, C57BL/6 mice were reported to give a much stronger immune reaction towards plant glycoproteins than the more widely used BALB/c mice (Bardor *et al.*, 2003). This inability of BALB/c mice may also explain that the first and best characterized monoclonal anti-glycan antibody (YZ1/2.23) was from rat.

In none of the above mentioned studies, the affinity of binding between antibodies and glycoproteins has been determined. This is regrettable, as biological consequences of antibody reaction may depend on binding affinity at least as much as on antibody titer. Interactions between carbohydrates and proteins, for example, lectins or antibodies, are usually considered to be much weaker (in the micromolar range) than protein-protein interactions (usually in the nanomolar range) (Thomas *et al.*, 2002, Weis, 1997, Wilson and Stanfield, 1995).

Binding affinities can be measured with various methods, the most attractive probably being surface plasmon resonance (SPR) (Haseley *et al.*, 1999). SPR and other techniques require the use of pure, well-defined carbohydrate structures. However, plant glycoproteins with uniform and defined glycosylation are hardly available, especially, if one aims at evaluating the relative contributions of fucose and xylose or other structural features to antibody binding. The previous success in glyco-modifying human transferrin with the help of recombinant xylosyl- and fucosyltransferase opened the opportunity to study the binding kinetics of anti-glycan antibodies to structurally defined glycoforms (Bencurova *et al.*, 2004).

In this article, we report on the attempt to produce monoclonal antibodies against plant N-glycans in C57BL/6 mice and on the more successful attempt to produce structural specific polyclonal sera in rabbits. Last, but not least, the binding affinities of purified rabbit antibodies against MMX-, MMF-, and also MMXF-glycans (see Fig. 1 for structures) to their ligands was investigated by surface plasmon resonance (SPR) technology taking advantage of the availability of defined “plantified” glycoforms of human transferrin.

Results

Mouse monoclonal antibodies

After repeated failure to generate hybridoma lines which produce mAbs against plant N-glycans (V. Tretter and F. Altmann, unpublished data), a recent report on the much higher immunogenicity of plant glycoproteins in C57BL/6 as compared to BALB/c mice (Bardor *et al.*, 2003), made us plan a new trial to obtain structure-specific antibodies against plant complex type N-glycans. We wanted to specifically boost the glycan-directed immune response by challenging the mice at consecutive immunizations with different proteins bearing similar N-glycan structures. Even then, however, the immune response of the mice was far from overwhelming (data not shown). Nevertheless, spleen cells of a few mice were fused and by applying a sophisticated sorting strategy (Böhm *et al.*, 2005) we got 164 clones, 36 of which secreted glycan-directed antibody as measured using MMX and MMF coupled to human transferrin (MMX- and MMF-Tf). However, a closer characterization of these mAbs revealed that the individual mAbs could bind both to MMX- and to MMF-Tf (Fig. 2). Hence, they did not discriminate between fucosylated and xylosylated N-glycans. Moreover, the mAbs even exhibited high binding to MM-Tf and unmodified transferrin (Fig. 2). Thus, despite great effort and the use of C57BL/6 mice, we were not able to elicit a strong immune

response against the plant N-glycans on hand, and to generate hybridoma lines producing structure specific mAbs.

Fig. 2

Rabbit polyclonal antisera

To overcome the problems encountered with mice, rabbits were chosen for the production of anti-glycan sera. Specificity should be introduced by the immunization protocol using only MMF-Tf as antigen to obtain “fucose-specific” sera or MMX-Tf for “xylose-specific” sera. All rabbits showed immune response to the antigens, even though only one rabbit from each group of three developed a satisfyingly high titer, which may be attributed to the use of incomplete adjuvant. Notably, the same result was obtained for horseradish peroxidase (HRP), a usually very potent glyco-antigen as we judge from our long experience with commercial rabbit and goat anti-HRP serum (Tretter *et al.*, 1993, Wilson *et al.*, 1998) as well as for both transferrin glycoforms. Before immunization, no detectable binding activity to HRP, MMX- and MMF-Tf was found in control rabbits (not shown). Sera from rabbits which showed highest binding activity to xylose or fucose residues were used for a two-step affinity purification. To detect residual anti-transferrin antibodies in the purified Xyl- and Fuc-specific sera, they were tested by Western blot with native and with glycomodified transferrins (Fig. 3). Obviously, the antibodies directed against the protein backbone of transferrin had been efficiently removed by affinity chromatography. After separation from (most of) the albumin, the three anti-glycan antibodies were characterized with regard to their glycan binding using various transferrin glycoforms (Fig. 1). In contrast to the mouse mAbs, the rabbit sera, even though polyclonal, exhibited high specificity and were able to discriminate between fucosylated and xylosylated glycoproteins. Remarkably, the anti-MMX serum bound xylosylated glycoforms irrespective of the presence or absence of non-reducing

terminal GlcNAc residues. Anti-HRP also had this ability but to a lesser extent. In contrast, anti-MMF antibody only bound truncated fucosylated glycoforms (MMF, MUXF, and MMXF). The same results were obtained when the specificities of purified anti-glycan antibodies were investigated by ELISA using glycomodified transferrins as well as BSA conjugates (Fig. 4) or glycomodified human α 1-antitrypsin (data not shown). The binding to glycomodified transferrins and to BSA conjugates with the same glycans was comparable, which argues against any essential influence of the protein backbone. Finally, the reaction of the purified antibodies with natural glycoproteins HRP (MMXF), ascorbate oxidase (MMXF and MMX; (Altmann, 1998)), hemocyanin from *Helix pomatia* (MMX and many undefined structure; (van Kuik *et al.*, 1985)), honeybee venom phospholipase A₂ (PLA₂, an insect glycoprotein containing a core α (1,3)-fucose; (Kubelka *et al.*, 1993) and lectin from *Erythrina corallodendron* (MMXF and other structure; (Ashford *et al.*, 1991) was determined (Fig. 5). Consistent with the structures of their carbohydrate chains, plant glycoproteins bound both the anti-MMF and the anti-MMX serum whereas the insect and the snail glycoprotein selectively reacted with either anti-MMF or anti-MMX, respectively.

Fig. 3

Fig. 4

Fig. 5

Goat anti-HRP serum

Commercial goat anti-horseradish peroxidase serum was subjected to ELISA with glycomodified transferrins. This serum exhibited a similar specificity like rabbit anti-HRP, *i.e.* it bound strongly to all fucosylated and/or xylosylated transferrins but almost not at all when these residues were absent (data not shown). The same results were obtained with BSA conjugates and glycomodified human α 1-antitrypsin (data not shown).

Binding affinities of rabbit antibodies

The problems associated with the determination of binding affinities of polyclonal antibodies have recently been discussed and a strategy to obtain representative average values has been devised (Bakker *et al.*, 1995, Cachia *et al.*, 2004, Hantusch *et al.*, 2005, Sem *et al.*, 1999). The situation in the present study is somewhat simpler as in the case of anti-MMF and anti-MMX as we are dealing with sera directed against a single epitope whereas in natural allergens an unknown number of different epitopes is present.

Fig. 6

The on- and off-rates of polyclonal xylose- and fucose-specific IgG and anti-HRP binding to MMX-, MMF- and MMXF-Tf were determined in surface plasmon resonance experiments. In all instances, the reference cell of chip was coated with unmodified transferrin in order to subtract any trace unspecific binding to the transferrin backbone. Decreasing amounts of plant N-glycan-specific IgG and anti-HRP were injected in triplicates onto the sensor chip (Fig. 6). Results of the association rate constant, k_a ($M^{-1}s^{-1}$), dissociation rate constant, k_d (s^{-1}), and the calculated equilibrium dissociation constant, K_D (M), are summarized in Table I. The association rate constants (k_a) were determined from the association phase of the binding curve, whereas the dissociation rate constants (k_d) were determined from the dissociation phase. The larger the association rate, the faster is the binding and in combination with a low dissociation rate, the higher is the affinity of an antibody. Notably, the binding of the xylose-specific antibody was faster to the MMXF surface than to the specific surface with MMX glycans (Table 1). As the dissociation rates from the MMX and the MMXF surface were similar, this difference of the association rate caused the combination anti-MMX with MMXF-Tf to exhibit the highest binding affinities of 2.5×10^{-10} M (Table 1). Possibly, the

concomitant presence of xylose and fucose on the glycans favors a conformation which facilitates antibody binding to the epitope containing the rather flexible xylose residue (Lommerse *et al.*, 1995). The affinity of anti-HRP may be seen as sort of average value between those of anti-MMX and anti-MMF as anti-HRP actually consists of two antibody populations, one binding to xylosylated, one to fucosylated glycans.

Discussion

From this study as well as from previous experiences in our laboratory and from other groups (van Remoortere *et al.*, 2003), it appears that the immune system of mice and also of rats (Bencurova *et al.*, 2004, Wilson *et al.*, 1998) cannot respond to plant N-glycans with the production of structurally specific antibodies as is seen with rabbits or goats. It also seems from this study and a previous one (Bardor *et al.*, 2003) that depending upon strain and maybe immunization regime, glycoproteins, which in other animals lead to a strong anti-carbohydrate immune response (Faye and Chrispeels, 1988, Faye *et al.*, 1993, Kaladas *et al.*, 1983, Lauriere *et al.*, 1988, Prenner *et al.*, 1992), do not necessarily lead to a nearly comparable antibody production in mice. In this light, the recent finding that a tobacco-produced mAb does not elicit an immune response [in mice \(Chargelegue *et al.*, 2000\)](#) cannot be taken as a last affirmation that plant-made glycoprotein pharmaceuticals may be regarded as immunologically safe for human therapy. In other words, mice must be rated an inappropriate animal model for the evaluation of the carbohydrate-related immunogenicity of plant-derived glycoproteins.

The immunological distinctiveness of mice unfortunately deprives us of the possibility of obtaining highly specific monoclonal antibodies. In this study, we have therefore chosen affinity purified polyclonal rabbit antibodies. Other options may be the use of phage display libraries, which, however, disrupts the pairing of heavy and light chain unless camelid antibodies are considered (Nguyen *et al.*, 2003). However, rabbits, when immunized with defined glyco-antigens such as our glycomodified transferrins, respond in a very specific manner. The quality of the sera can be enhanced by removal of the inadvertently generated anti-protein antibodies by *e.g.* immobilized transferrin and / or by using immobilized glyco-antigen for affinity purification.

Another small but notable point can be made from the ELISA comparison of antibodies raised against HRP or modified transferrins and tested against a panel of glycoproteins. The protein backbone of the glyco-allergen on the ELISA does not affect the result. This argues very much against the sometimes heard notion that the sugars only modulate the binding of antibodies to nearby peptide epitopes.

The monovalent, albeit polyclonal anti-MMF and anti-MMX antibodies were finally used to assess their binding affinities to glycoproteins. The motive for this effort lays in the enigmatic role of carbohydrate determinants in allergy. While on the one hand, plant N-glycans have been shown to elicit effector release (Bublin *et al.*, 2003, Foetisch *et al.*, 2003, Westphal *et al.*, 2003, Wicklein *et al.*, 2004), they are considered as innocuous regarding their potency to evoke clinical symptoms (van der Veen *et al.*, 1997). Does the carbohydrate nature of this determinant explain this observation? For immunologists, all epitopes are equal. But for allergologists, some epitopes - [the peptide epitopes](#) - appear to be more equal. A possible explanation may lay in a low binding affinity of antibodies to these epitopes as it was found for lectin-carbohydrate interactions, where the K_D often lies in the micromolar range (Haseley *et al.*, 1999). However, even for lectins, higher affinities have been reported, *e.g.* for RCA₁₂₀ and DSA to terminal Gal residue in asialofetuin (K_D , 3.8×10^{-9} and 3.7×10^{-9} M, respectively)

(Shinohara *et al.*, 1994). The affinities of antibodies against carbohydrate epitopes were often found to be quite high, *e.g.* in the case of a 3-deoxy-D-*manno*-octulosonic acid trisaccharide on *Chlamydia* outer membrane LPS, high-affinity mAbs could also be obtained (K_D , $\sim 10^{-9}$ M) (Muller-Loennies *et al.* 2000). Examples of lower affinity can be found as well, *e.g.* a K_D of 2.3×10^{-7} M for a mAb against O-polysaccharide from *Salmonella* serogroup B because of its rapid dissociation rates (MacKenzie *et al.*, 1996).

The antibodies investigated in this study exhibited K_D values of around 10^{-9} M and thereby almost reach the theoretical limit of antibody affinity ($\sim 10^{-10}$ M) (Foote and Eisen, 1995). Therefore, cross-reactive carbohydrate determinants (CCDs) can no longer be regarded as “less equal” **in the sense of allowing only low affinity binding**. Even though the affinities of human IgE and IgG remain to be determined, we dare to speculate that low binding affinity of anti-CCD antibody drops out as an explanation for the **usually observed inoffensiveness of CCDs** as allergens. However, this should not remain a speculation for long as the current work forms the basis for the determination of binding **affinities** of anti-CCD antibodies in human sera.

Materials and Methods

Preparation of neoglycoproteins

BSA conjugates were prepared as previously described with some modifications (Wilson *et al.*, 1998). GnGn fibrin glycopeptides were first cross-linked to BSA to prepare GnGn-BSA. Then GnGn-BSA was modified by purified recombinant *Arabidopsis thaliana* core α 1,3-fucosyltransferase and rice β 1,2-xylosyltransferase expressed in *Pichia pastoris* (Bencurova *et al.*, 2003, Leonard *et al.*, 2004). The resultant GnGnX-, GnGnF-, and GnGnXF-BSA were treated with *N*-acetylglucosaminidase from *Streptococcus pneumoniae* (Sigma-Aldrich, St. Louis, MO, USA) to obtain MMX-, MMF-, and MMXF-BSA. MUXF-BSA and MUX-BSA were generated by cross-linking bromelain glycopeptides or defucosylated glycopeptides to BSA (Wilson *et al.*, 1998). The BSA conjugates were subjected to amino sugar analysis (Altmann, 1992) and found to contain 2-3 mol glycopeptide per mol of BSA.

Glycomodified human transferrins (MM-, MMX-, MUX-, MMF- and MMXF-Tf) were prepared as previously described except using purified recombinant β 1,2-xylosyltransferase from rice instead from *A. thaliana* (Bencurova *et al.*, 2003, Leonard *et al.*, 2004). The purity and integrity of neoglycoproteins were verified by SDS-PAGE and the structures of their glycan moieties were checked by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (Kolarich and Altmann, 2000). It should be noted that the glycosyltransferases and glycosidases used were not of plant origin and thus glyco-contamination from this source were excluded.

Generation and characterization of mouse monoclonal antibodies

Nine eight-week old C57BL/6 mice in 3 groups (Charles River Wiga, Sulzfeld, Germany) were randomly divided in three groups immunized with different combinations of natural or modified plant glycoproteins, mixed with incomplete Freund's adjuvant. The dosage per injection of the glycoproteins was as follows: 100 µg of horseradish peroxidase (HRP, Sigma-Aldrich); 30 µg of honeybee venom phospholipase (PLA, prepared as described (Tretter *et al.*, 1993); 50 µg of zucchini ascorbate oxidase (AO, Sigma-Aldrich); 100 µg of *Sophora japonica* lectin (SJA, Sigma Aldrich); and 50 µg of bromelain (BRO, prepared as described (Tretter *et al.*, 1993)). The first three injections were given intraperitoneally on days 0, 21, 42, 63, and 84. The two final injections were given intravenously without adjuvant and were followed four days later by cell fusion.

The sequences of antigens were HRP-PLA-AO-HRP-SJL, HRP-BRO-AO-BRO-PLA, and HRP-AO-BRO-SJL-AO for the first, second and third group of mice, respectively. The spleen cells from the three mice, which showed highest titers to glycomodified transferrin (MMX- and MMF-Tf), were fused with a NS-0 myeloma cell line according to ClonaCell® -HY for hybridoma production's protocol (Stemcell Technologies, Vancouver, Canada).

After ten days of HAT selection, cells were stained with the affinity matrix secretion assay (Böhm *et al.*, 2005) using a sheep-anti mouse IgG-biotin conjugate (Sigma Aldrich) as capture antibody. Staining of specific hybridoma cells was achieved with the respective glycomodified transferrin and an anti-transferrin-FITC conjugate (Capricorn Products, Portland, Maine). Cells secreting transferrin-specific antibodies were then sorted into 96 well plates at 2 to 5 cells per well. After approximately 2 weeks, samples of the culture supernatants of wells containing monoclonal colonies were taken and were screened by qualitative ELISA using either MM-, MMX-, MMF-, MMXF-Tf or NaNa-Tf (= unmodified transferrin) similarly as recently described (Bencurova *et al.* 2004). However, 1 : 1000 diluted alkaline phosphatase conjugated anti-mouse IgG γ -chain (Sigma-Aldrich) was used as the

detection antibody. Bound antibody was detected by reaction with p-nitrophenylphosphate in 0.1 M diethanolamine at pH 9.8. The reaction was stopped after 20 min by addition of 5 M sodium hydroxide and the absorbance at 405 nm was read.

Immunization of rabbits

Chinchilla Bastard rabbits were also divided into groups of three but the animals in each group repeatedly received the same antigen, either HRP, MMX-Tf or MMF-Tf. All antigens were dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/mL.

Intradermal injections consisting of 200 μ L antigen solution and the same volume of Freund's incomplete adjuvant were administered in 3-week intervals. After the fourth immunization, the antisera were collected and kept at -20°C .

For ELISAs with rabbit sera, the plates were coated with plant glycoproteins or neoglycoprotein at a concentration of 5 $\mu\text{g/mL}$ in 50 mM sodium carbonate, pH 9.6 for 60 min at 37°C . Bound antibody was detected by a 1:2,000 dilution of alkaline phosphatase conjugated anti-rabbit IgG (Sigma-Aldrich) as described (Wilson *et al.*, 1998).

Affinity purification of xylose or fucose-specific IgG

From each group of three rabbits the one serum which showed the highest anti-glycan antibody titer was used for affinity purification. Affinity columns were prepared by coupling unmodified human transferrin, MMX-BSA or MMF-BSA conjugates to Affi-Gel 15 (BioRad, Richmond, CA) according to the manufacturer's instructions. The sera were diluted with 0.1 M Tris-HCl, pH 7.2 containing 0.1 M NaCl, 2.5 mM MgCl_2 , and 0.05 % Tween 20 (TTBS) and applied at first to the transferrin column in order to eliminate anti-transferrin antibodies. The breakthrough was applied to either MMX-BSA or MMF-BSA-coupled column depending on the antiserum. After extensive washing with TTBS, bound antibodies were eluted with 0.2 M glycine-HCl, pH 2.5 and neutralized immediately with 1 M Tris-HCl, pH

7.5. To remove rabbit serum albumin, effluents were loaded on a 1 mL bed of SP Sepharose Fast Flow (Amersham Biosciences AB, Uppsala, Sweden). Elution of IgG was performed with a gradient from 0.02 to 1.0 M NaCl in 50 mM MES, pH 5.0. Buffer exchange as well as concentration was performed immediately by filter devices with 10 kDa cut-off (Millipore, MA, USA).

The specificities of xylose or fucose-specific IgG were determined by ELISA and Western blot. The amount of IgG was quantified by comparison of Coomassie stained SDS-PAGE bands with a dilution series of pure human IgG. The xylose-specific IgG had an estimated concentration of 0.1 mg/mL, the fucose-specific pool of 0.2 mg/mL.

For Western blot analysis, transferrin glycoforms and BSA conjugates (0.5 - 1 µg) were separated by SDS-PAGE, electroblotted to a nitrocellulose membrane and stained with the help of the rabbit anti-HRP (1:2,000) or purified rabbit xylose-specific or fucose-specific antibody (1: 500) as described (Bencurova *et al.*, 2004).

Surface plasmon resonance (SPR) analysis

The binding kinetics of the purified anti-glycan antibodies were determined by SPR using a BIAcore X biosensor system (Biacore, Uppsala, Sweden). The running buffer used for all experiments was HBS-P buffer (Biacore), pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 (Biacore). The immobilization to research grade CM5 sensor chips was carried out using a standard amine coupling procedure (Johnsson *et al.*, 1991). Transferrin glycoforms were applied in 10 mM sodium acetate buffer, pH 4.0. The two channels of each chip were set up as follows: MMX-, MMF- and MMXF-Tf were immobilized on channel 1, whereas unmodified transferrin was bound to channel 2 of each chip as a control surface. Amounts of $3,000 \pm 1000$ RU of each glycoform were immobilized onto the chip surfaces. One RU corresponds to an immobilized protein concentration of about 1 pg/mm^2 (Stenberg *et al.*, 1991).

All measurements were carried out in HBS-P buffer. Analyses were performed at 25°C and at a flow rate of 5 μ L/min. Between cycles, the chip surface was regenerated with two injections of 10 μ L of 10 mM glycine-HCl, pH 2.2 or 0.5% SDS at a flow rate of 5 μ L/min followed by washing the surface with HBS-P buffer until the signal reached baseline.

For the evaluation of association and dissociation rate constants, samples were diluted in HBS-P buffer and analyzed at various concentrations. Purified antibody at the specified concentration was pumped over the sensor chip surface for 6 min at a flow rate of 5 μ L/min to observe the association phase, and dissociation phase was monitored after switching to protein free buffer for another 6 min.

Sensorgrams were fitted to a single site binding model (1:1 Langmuir binding), using the numerical integration functions of the BIAevaluation 3.1 software package. Association and dissociation rate constants (k_a and k_d) and the dissociation constant (K_D) were derived by both separate and simultaneous fitting of the association and dissociation phase of individual sensorgrams. Sensorgrams were also fitted globally using data collected at a series of concentrations. The steady-state binding values, R_{eq} , were then plotted as a function of the applied antibody concentrations and fitted to first-order kinetics, assuming a monovalent antibody-modified transferrin interaction (Karlsson et al. 1991).

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Abbreviations

BSA, bovine serum albumin;

CCD, cross-reactive carbohydrate determinant;

GnGn, GnGnX; MM, [MMX](#), [MMF](#), MMXF etc., N-glycan structures, depicted in Fig. 1;

HRP, horseradish peroxidase;

[MMX-Tf](#), [MMF-Tf](#) etc., artificial glycoforms of human serum (apo-)transferrin;

SPR, surface plasmon resonance.

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Figure legends

Fig. 1: Structures of oligosaccharides relevant to this study. N-glycans are shown with the abbreviations according to the proglycan system (www.proglycan.com) and their occurrence in semi-synthetic and natural glycoproteins.

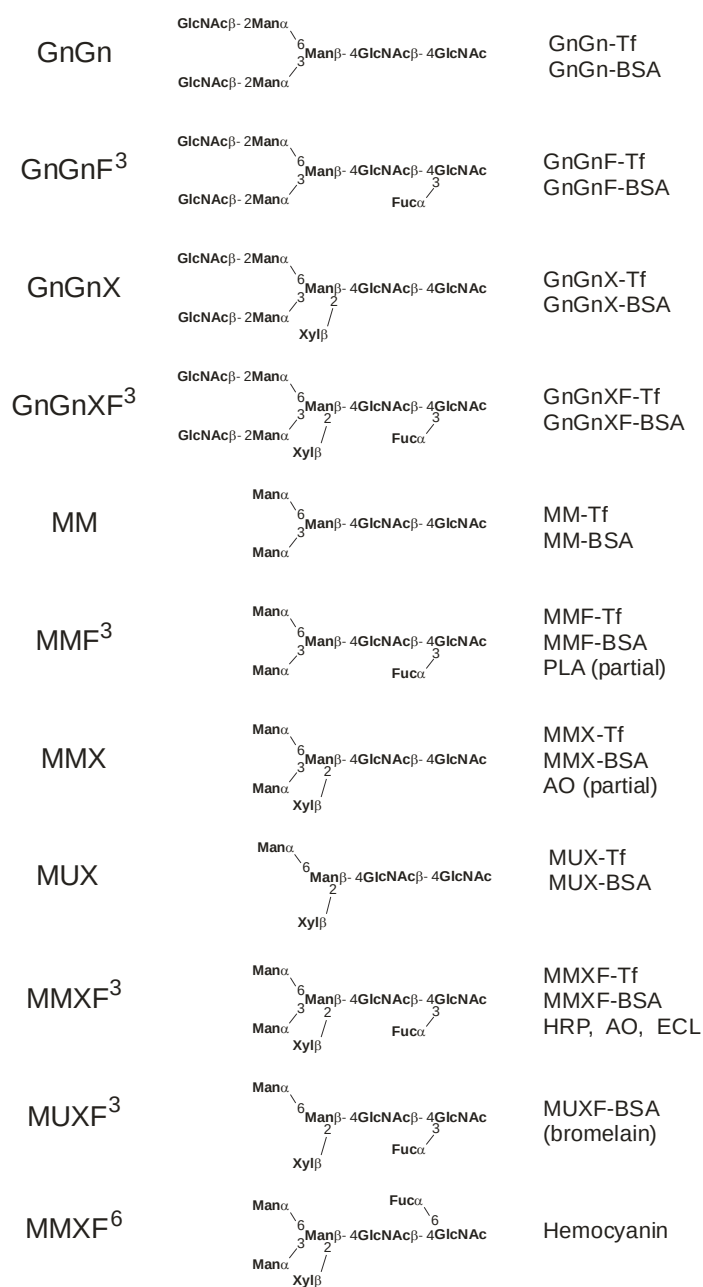


Fig. 2: ELISA of selected mAbs with glycoforms of transferrin. The ELISA results of four out of 36 mAbs are shown here. Reactivity to MMF-Tf (dark grey), MMX-Tf (medium grey), MM-Tf (light grey) and unmodified transferrin (white bars) is shown. The concentration of IgG in each sample was roughly equal.

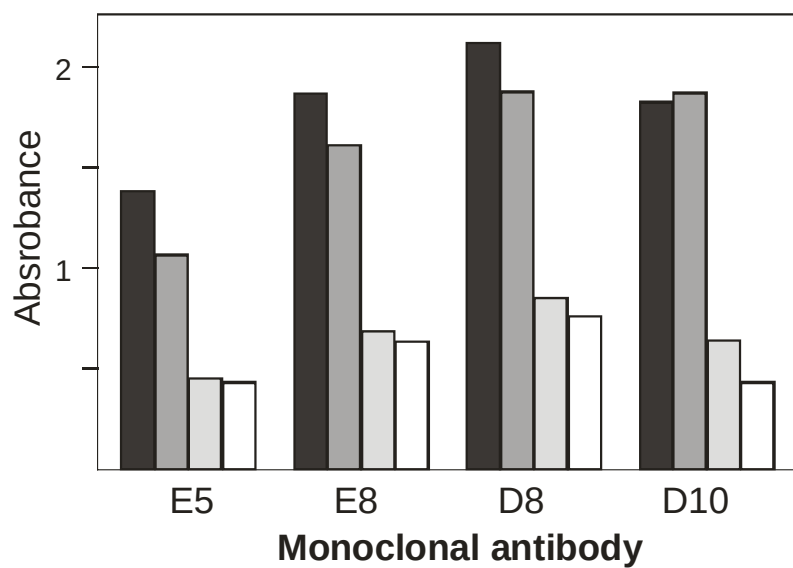


Fig. 3: Western blot with rabbit anti-glycan antibodies. Equal amounts of various glycoforms of human transferrin were subjected to SDS-PAGE and blotted to nitrocellulose. The Coomassie blue stain is shown in lane A. Lane B was incubated with anti-HRP serum, lane C with anti-MMX antibodies and lane D with anti-MMF antibodies. The boxes indicate positions, which are critical for the structural specificity of the antibodies. With the possible exception of weak binding of anti-HRP to MM-Tf (not confirmed by ELISA), these spots remained absolutely unstained showing the strict need for xylose or fucose residues, respectively, for antibody binding. Terminal GlcNAc residues interfered with binding of anti-MMF antibodies and to some extent of anti-HRP serum. The N-glycan structures are depicted in Fig. 1.

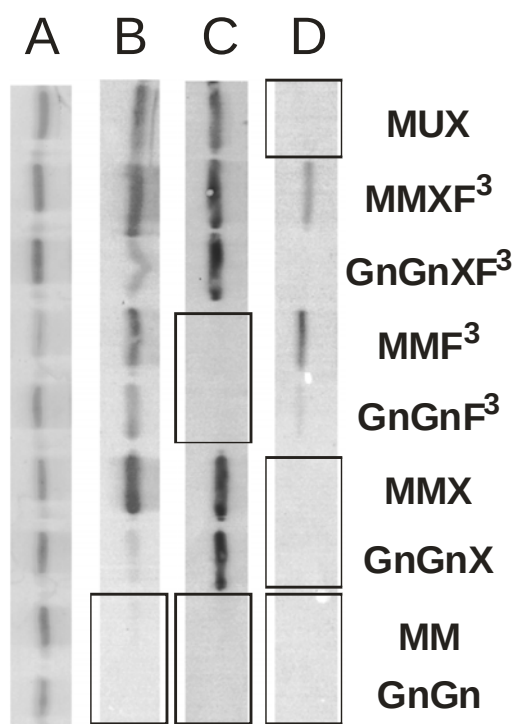


Fig. 4: ELISA of rabbit anti-HRP, anti-MMX and anti-MMF antibodies. ELISA plate wells were coated with different BSA conjugates or glycomodified transferrins. The N-glycan structures are depicted in Fig. 1. Black bars stand for anti-MMF, grey bars for anti-MMX and the patterned bar for anti-HRP serum. The upper panel proves the specificity of anti-MMF and anti-MMX sera. The lower panel shows that anti-MMX and anti-HRP can also react with glycans carrying GlcNAc residues at the non-reducing end.

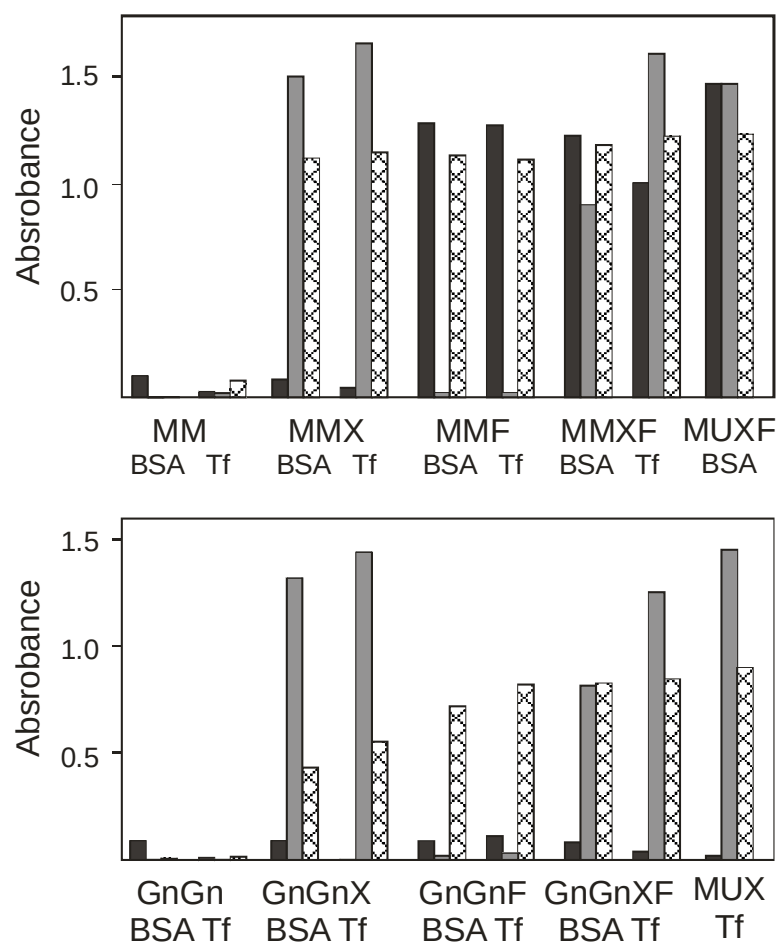


Fig. 5: Binding of anti-MMF and anti-MMX antibodies to natural glycoproteins. Antibody binding was observed for the same dilution of either anti-MMF Ab (black bars) or anti-MMX Ab (grey bars). The coated antigens were phospholipase A₂ from honeybee venom (PLA), horseradish peroxidase (HRP), ascorbate oxidase from zucchini (AO), *Erythrina corralodendron* lectin (ECL), MMXF-BSA conjugate, and hemocyanin from *Helix pomatia* (HeCy). The reactivity of anti MMX with PLA corresponds to a blank value.

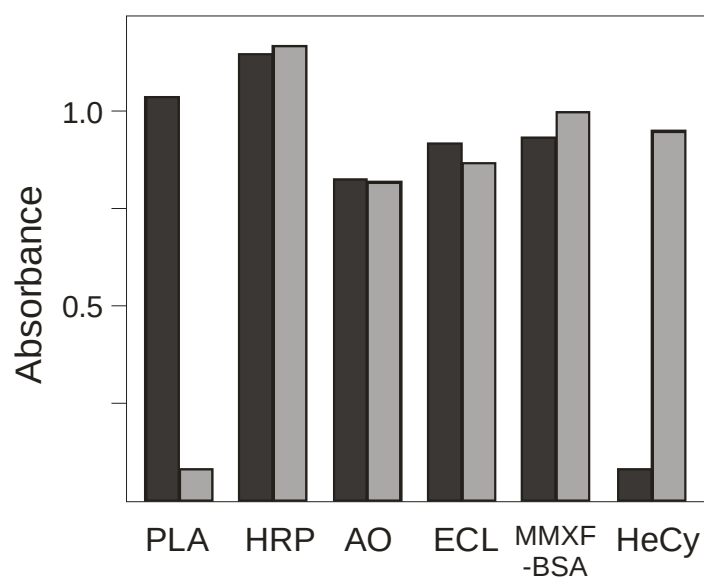


Fig. 6: Example of the SPR studies: Binding of rabbit anti-MMX to MMX-Tf. Overlay sensorgrams of injections of increasing concentrations of the “xylose-specific” antibody over the chip surface with immobilized MMX-Tf are shown in panel A, whereas panel B shows the result with immobilized MMXF-Tf. Panels C and D give the plots of steady-state binding values, R_{eq} , against xylose-specific antibody concentrations on MMX-Tf and MMXF-Tf surfaces. The control channel coated with unmodified transferrin did not show any anti-MMX binding.

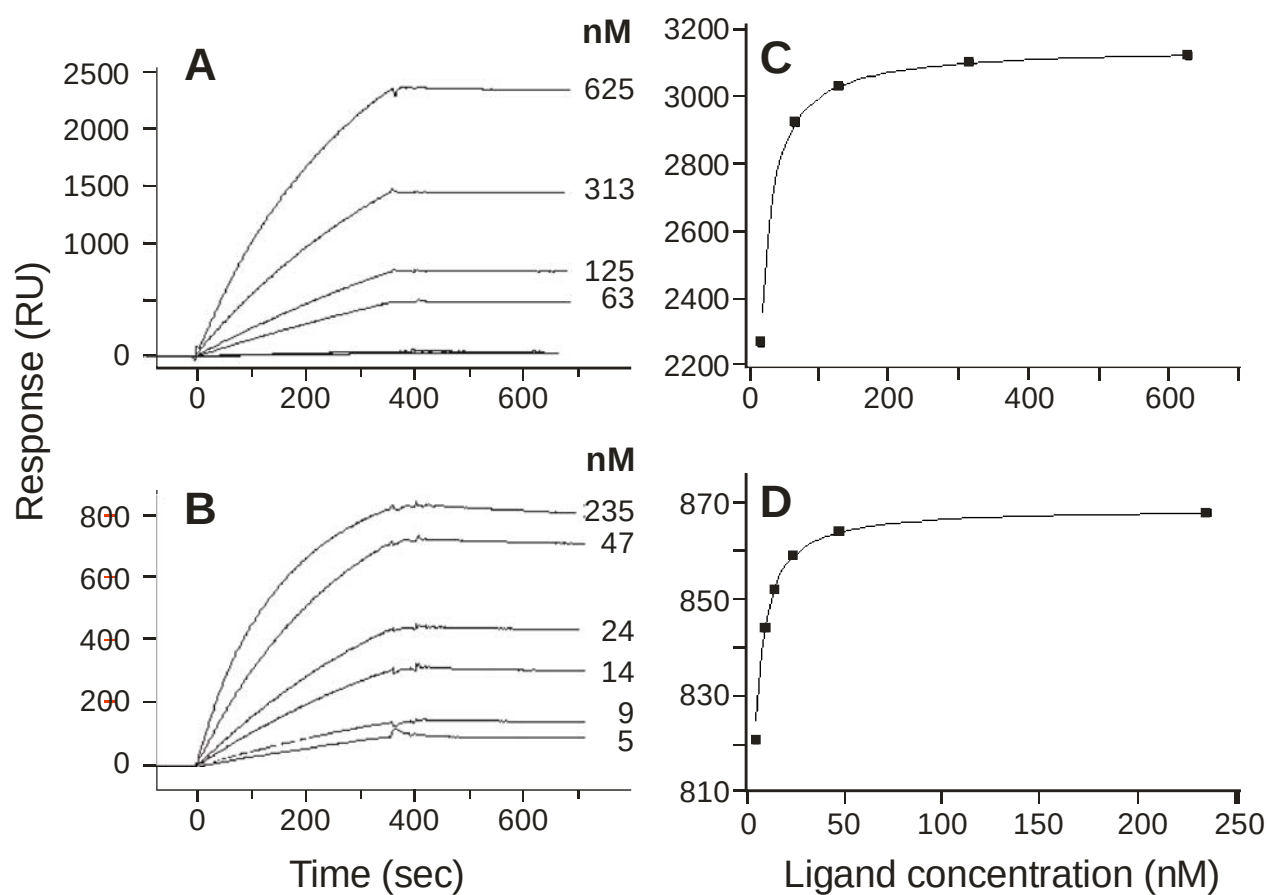


Table 1: Kinetics of antibody binding as measured by surface plasmon resonance (SPR)

Anti-MMX means xylose-specific antibody; Anti-MMF means fucose-specific antibody. Each set of curves was simultaneously fitted using a 1:1 binding model to obtain the rate constants. k_a is the association rate, k_d the dissociation rate and $K_D = k_d/k_a$ is the equilibrium dissociation constant. Experimental error was calculated from triplicate sensorgrams of the same antibody concentration.

Glycomodified transferrins	Antibody	k_a	k_d	K_D
		$M^{-1}s^{-1}$	s^{-1}	M
MMXF-Tf	Anti-HRP	$4.07 \pm 0.01 \times 10^4$	$1.48 \pm 0.02 \times 10^{-4}$	3.64×10^{-9}
	Anti-MMX	$8.58 \pm 0.03 \times 10^4$	$2.16 \pm 0.12 \times 10^{-5}$	2.52×10^{-10}
	Anti-MMF	$1.19 \pm 0.04 \times 10^5$	$1.77 \pm 0.02 \times 10^{-4}$	1.49×10^{-9}
MMX-Tf	Anti-HRP	$1.32 \pm 0.01 \times 10^4$	$1.00 \pm 0.05 \times 10^{-4}$	7.58×10^{-9}
	Anti-MMX	$6.00 \pm 0.01 \times 10^3$	$2.83 \pm 0.07 \times 10^{-5}$	4.72×10^{-9}
MMF-Tf	Anti-HRP	$5.49 \pm 0.02 \times 10^4$	$2.29 \pm 0.05 \times 10^{-4}$	4.17×10^{-9}
	Anti-MMF	$4.45 \pm 0.01 \times 10^4$	$8.26 \pm 0.03 \times 10^{-5}$	1.86×10^{-9}