

Immunoreactivity in mammals of two typical plant glyco-epitopes, core $\alpha(1,3)$ -fucose and core xylose

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The presence of nonmammalian core $\alpha(1,3)$ -fucose and core xylose glyco-epitopes on glycans *N*-linked to therapeutic glycoproteins produced in plants has raised the question of their immunogenicity in human therapy. We address this question by studying the distribution of these *N*-glycans in pea, rice, and maize (which are the crops intended for the production of therapeutic proteins) and by reinvestigating their immunogenicity in rodents. We found that immunization with a model glycoprotein, horseradish peroxidase, elicits in C57BL/6 mice and rats the production of antibodies (Abs) specific for core $\alpha(1,3)$ -fucose and core xylose epitopes. Furthermore, we demonstrated that about 50% of nonallergic blood donors contains in their sera Abs specific for core xylose, whereas 25% have Abs against core $\alpha(1,3)$ -fucose. These Abs probably result from sensitization to environmental antigens. Although the immunological significance of these data is too speculative at the moment, the presence of such Abs might introduce some limitations to the use of plant-derived biopharmaceutical glycoproteins, such as an accelerated clearance during human therapy.

Key words: immunogenicity/*N*-glycans/recombinant proteins/transgenic plants

Introduction

Plants are potential cost-efficient and contamination-safe factories for the production of recombinant biopharmaceutical proteins. Plants are able to produce proteins with complex *N*-linked glycans having a core bearing two GlcNAc residues as observed in mammals. However, this core is substituted in plant glycoproteins by a $\beta(1,2)$ -linked xylose residue (core xylose), Lewis^a epitopes, and an $\alpha(1,3)$ -linked

fucose (core $\alpha[1,3]$ -fucose), instead of an $\alpha(1,6)$ -linked core fucose as in mammals (Lerouge *et al.*, 1998). Consequently, expression of mammalian glycoproteins in plants results in the production of glycoproteins bearing glycans that reflect the differences in the *N*-glycan processing between plants and mammals, as recently demonstrated for monoclonal antibodies (Abs) produced in tobacco plants (plantibodies) (Cabanes-Macheteau *et al.*, 1999; Bakker *et al.*, 2001). *In vivo* experiments using BALB/c mouse have shown that plantibodies produced in tobacco do not elicit immunological response against their *N*-linked glycans of plant origin (Chargelegue *et al.*, 2000). Moreover, a plantibody produced in plants was found to be highly efficient *in vivo* for prevention of vaginal herpes simplex virus 2 infection in C57BL/6 mouse, without eliciting an adverse immunological response (Zeitlin *et al.*, 1998). Altogether, these preliminary data obtained in mouse suggest that plantibodies may be suitable for immunotherapy in humans.

On the other hand, core xylose and core $\alpha(1,3)$ -fucose epitopes are known to be important IgE binding carbohydrate determinants of plant allergens (Aalberse *et al.*, 1981; Faye and Chrispeels, 1988; van Ree and Aalberse, 1995; Garcia-Casado *et al.*, 1996; Wilson and Altmann, 1998; van Ree *et al.*, 2000). Furthermore, we and others have reported that immunization of goats (Kurosaka *et al.*, 1991) or rabbits (Faye *et al.*, 1993) with plant glycoproteins elicits the production of core xylose- and core $\alpha(1,3)$ -fucose-specific Abs. A rat monoclonal Ab (YZ1/2.23) raised against elderberry abscission tissues was found to be specific for core $\alpha(1,3)$ -fucose (McManus *et al.*, 1988).

Altogether, the immunogenicity of plant *N*-glycans remains a major pending issue. In the context of human therapy using therapeutic proteins produced in plants, elicitation of immune responses in humans by specific plant glyco-antigens could be a major concern if people have prolonged exposure to large quantities of plant-derived glycoproteins, as may be required for certain *in vivo* treatments. In this article, we show that crops, such as pea, rice, and maize, that are intended for the production of therapeutic proteins, introduce plant-specific core xylose and core $\alpha(1,3)$ -fucose on their natural or recombinant glycoproteins. Data on the immunogenicity of these plant glyco-epitopes in immunized rodents as well as in nonallergic humans are also reported.

Results

Core xylose and core $\alpha(1,3)$ -fucose N-glycan epitopes are widely distributed in pea, rice, and maize

Crop plants such as pea, rice, and maize are intended for the production of biopharmaceutical glycoproteins. Therefore,

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structural studies were performed to investigate the *N*-glycosylation of glycoproteins that accumulate in the seeds of these plants as well as the *N*-glycosylation of recombinant egg-white avidin produced in transgenic maize (Hood *et al.*, 1997). Analysis of the *N*-glycosylation was first carried out by western blotting using Abs specific for plant *N*-glycan epitopes, that is, core xylose, core $\alpha(1,3)$ -fucose (Faye *et al.*, 1993), and Lewis^a (Fitchette *et al.*, 1999) epitopes. Proteins from pea, maize, and rice seeds, as well as from recombinant avidin, were immunodetected by these glycan-specific Abs, which indicates that they carry mature *N*-glycans (data not shown).

To further investigate their glycosylation, *N*-glycans were released from natural glycoproteins or from recombinant avidin by PNGase A treatment and analysed by mass spectrometry (MS). The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS of the pools of *N*-glycans isolated from pea, rice, and maize seed glycoproteins (Figure 1A, B, and C, respectively) shows a mixture of ions that were assigned to $(M + Na)^+$ adducts of high-mannose-type *N*-glycans ranging from Man-5 to Man-9 (indicated by a star) as well as $\alpha(1,3)$ -fucose- and $\beta(1,2)$ -xylose-containing *N*-glycans from the truncated paucimannosidic structure **a** to the complex *N*-glycan **h** harbouring two Lewis^a epitopes (Table I). Assignments were done on the basis of the molecular masses of *N*-glycans by homology with previous data on plant *N*-glycosylation (Lerouge *et al.*, 1998) and considering that glycoproteins from these plants were immunodetected with antibodies specific for core xylose, core $\alpha(1,3)$ -fucose, and Lewis^a epitopes. These assignments were confirmed by enzyme sequencing using β -*N*-acetylglucosaminidase and α -mannosidase combined to MALDI-TOF MS analysis of the resulting digests, as reported in Bakker *et al.* (2001).

Chicken avidin is a 15.5-kDa polypeptide having one *N*-glycosylation site exhibiting an extensive glycan microheterogeneity (Bruch and White, 1982). We have investigated the detailed *N*-glycosylation of the chicken avidin produced in transgenic maize (Hood *et al.*, 1997). The MALDI-TOF MS (Figure 1D) of the pool of *N*-glycans indicates that the chicken avidin produced in maize also exhibits on its unique *N*-glycosylation site a wide variety of *N*-glycans. Major ions were assigned to *N*-glycans **b** to **h** (Table I), and their structures were confirmed by enzyme-sequencing (Bakker *et al.*, 2001). To confirm the structure of major *N*-linked oligosaccharides of the recombinant maize avidin, the pool of *N*-glycans was then reduced and permethylated. $(M + Na)^+$ ion species of the permethylated oligosaccharides **c**, **d**, and **f** were fragmented by collision-induced dissociation (CID). Fragment ions at *m/z* 490 and 284 were detected in the resulting MALDI-post source delay (PSD) mass spectra (data not shown), which confirms that the fucose residue is linked to O-3 of the reducing GlcNAc residue of the *N*-linked glycans, according to Melo *et al.* (1997). In addition, a daughter ion at *m/z* 660 was specifically observed in the spectra, resulting from the fragmentation of the permethylated oligosaccharide **g**. This ion arises from the fragmentation of the terminal Lewis epitope present on these molecules (Melo *et al.*, 1997). Altogether, these analyses demonstrate that pea, rice, and maize glycoproteins, as well as the recombinant egg-white avidin produced in transgenic maize, harbor

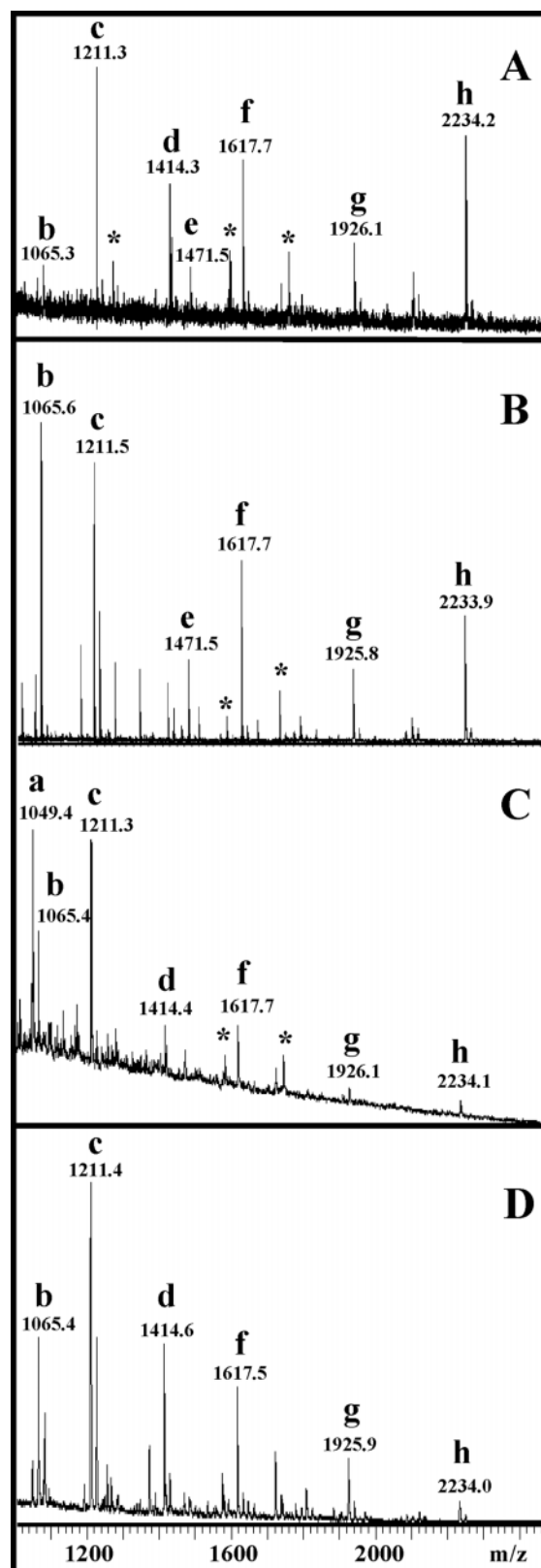
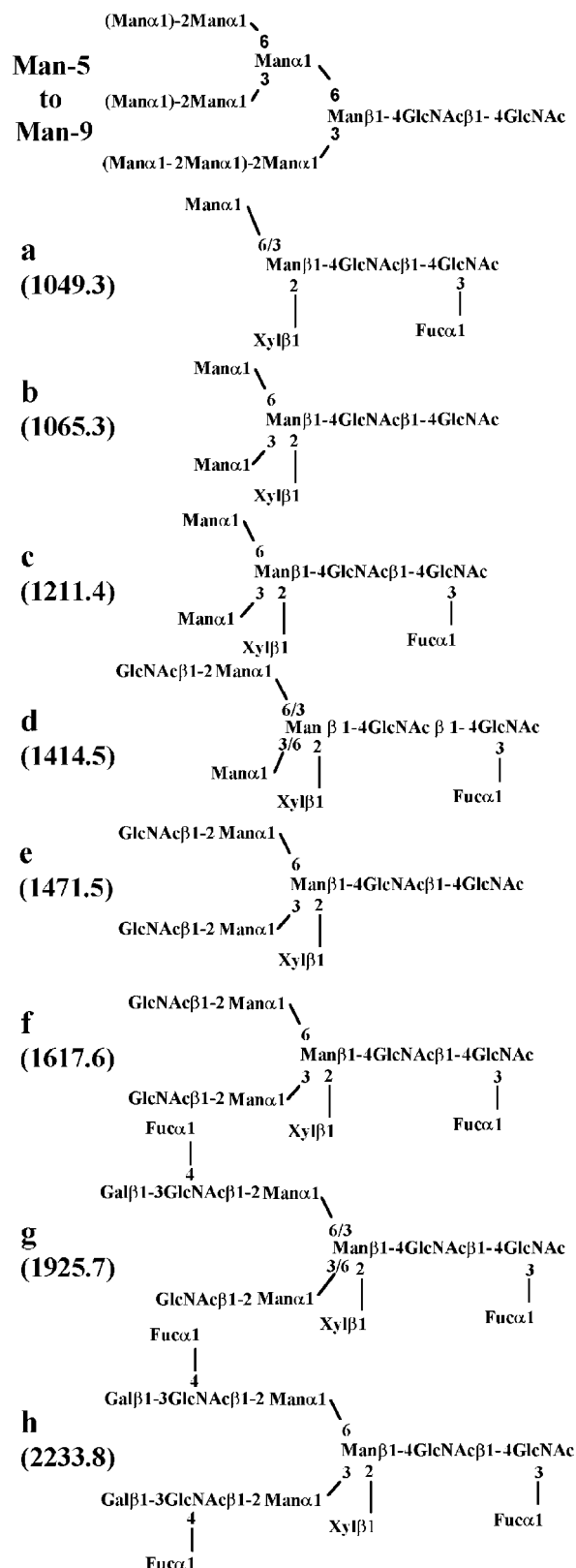


Fig. 1. MALDI-TOF mass spectra of *N*-linked glycans isolated from glycoproteins of pea (**A**), maize (**B**), and rice (**C**) seeds, and from recombinant avidin (**D**) produced in maize seeds. Nonassigned ions correspond to $(M + K)^+$ adducts or to minor complex *N*-glycans and high-mannose-type *N*-glycans ranging from Man-5 to Man-9. See Table I for structures. *High-mannose-type *N*-glycans.

Table I. Structure and theoretical (M + Na)⁺ value of paucimannosidic, complex, and high-mannose-type *N*-glycans isolated from pea, maize, and rice natural glycoproteins, and from recombinant chicken avidin produced in maize



core xylose and/or core α -(1,3)-fucose epitopes on their *N*-linked glycans, as well as terminal Lewis^a.

Immunogenicity of core xylose and core α -(1,3)-fucose epitopes in rodents

We investigated the immunogenicity of core xylose and core α -(1,3)-fucose epitopes of plant *N*-glycans in two different mouse strains and in rats. To this end, BALB/c and C57BL/6 mice were immunized with horseradish peroxidase (HRP), a plant glycoprotein harboring six identical *N*-glycans represented in Figure 2A (referred to as **c** in Table I) (Kurosaka *et al.*, 1991). The production of anti-glycan

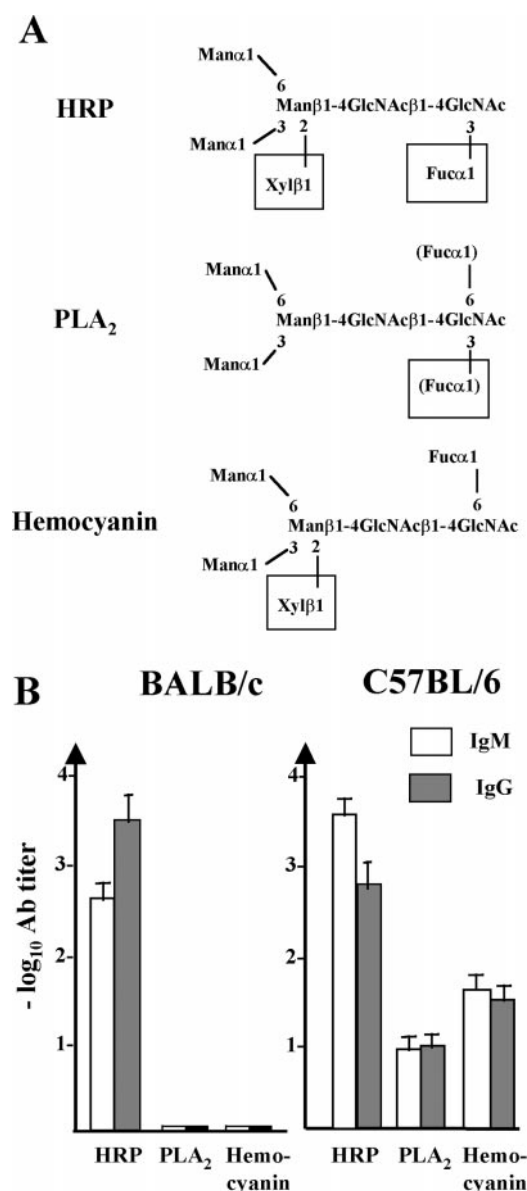


Fig. 2. (A) Structures of glycans *N*-linked to HRP, honeybee venom PLA₂, and snail hemocyanin. (B) Ab titers of sera from BALB/c and C57BL/6 mice immunized with HRP. Humoral responses to core α -(1,3)-fucose (PLA₂) and core xylose (hemocyanin) determined by ELISA experiment using HRP-conjugated anti-mouse IgM (1/10,000) and IgG (1/10,000) as a secondary Abs.

Abs in their sera was then determined by enzyme-linked immunosorbent assay (ELISA) using two model glycoproteins having a unique common glycan epitope with HRP: honeybee venom phospholipase A₂ (PLA₂), an insect glycoprotein containing a core $\alpha(1,3)$ -fucose (Kubelka *et al.*, 1993), and *Helix pomatia* hemocyanin, a snail N-linked glycoprotein containing a core xylose (van Kuik *et al.*, 1985) (Figure 2A). Because no glycan-specific responses were obtained in the experiments reported by Chargelegue *et al.* (2000) using 30 μ g of HRP, BALB/c mice were immunized with a higher amount of HRP (100 μ g) using complete Freund's adjuvant to maximize the immune response. Results are expressed as $\log_{10}$ Ab titers after deducing the values obtained for the pre-immune sera.

Figure 2B shows that immunized BALB/c and C57BL/6 mice developed a strong humoral response (IgG and IgM) against HRP, whereas IgM/IgG responses against core xylose and core $\alpha(1,3)$ -fucose, determined by measuring the cross-reactivities of sera with hemocyanin and PLA₂, respectively, were detected in C57BL/6 mice but not in BALB/c mice. No detection of these reporter glycoproteins was observed with the pre-immune C57BL/6 mouse sera, which indicates that production of Abs specific for plant N-glycans was induced by immunization with HRP. Altogether, these results indicate that immunized BALB/c mice developed a humoral response against the HRP protein backbone but not against the plant N-glycans, whereas C57BL/6 immunized mice produced IgM and IgG Abs against the protein as well as a weak response against the core xylose and core $\alpha(1,3)$ -fucose epitopes.

We then studied the immunogenicity of HRP in rats. In addition to the humoral response directed against the HRP protein backbone, high titers of IgM and of IgG (isotypes IgG₁ and IgG_{2a}) against core xylose and core $\alpha(1,3)$ -fucose were measured by ELISA using the approach developed for mouse serum analysis (Figure 3A). A comparison of the Ab reactivity with the chicken or the recombinant maize-derived avidin further demonstrates that part of the humoral response is raised against plant N-glycans introduced onto the recombinant protein.

To confirm these data, sera from HRP-immunized rodents were analyzed by western blotting. In addition to strong immunodetection of HRP (not included in Figure 3B), the sera exhibit different reactions against the model glycoproteins. As depicted in Figure 3B, maize-derived avidin, PLA₂, and hemocyanin are immunodetected using sera from HRP-injected rats. As control, the degradation of oligosaccharides by periodate treatment abrogated the binding to model glycoproteins of antibodies from HRP-immunized rat (Figure 3B), thus confirming that these Abs are glycan-specific. In contrast, we could not detect any reactivity using sera from immunized BALB/c and C57BL/6 mice, confirming the absence or weak humoral response in mouse (data not shown). In contrast to rats, the weak glycan-specific antibody titers obtained in C57BL/6 mice did not allow detection of model glycoproteins by western blot analysis.

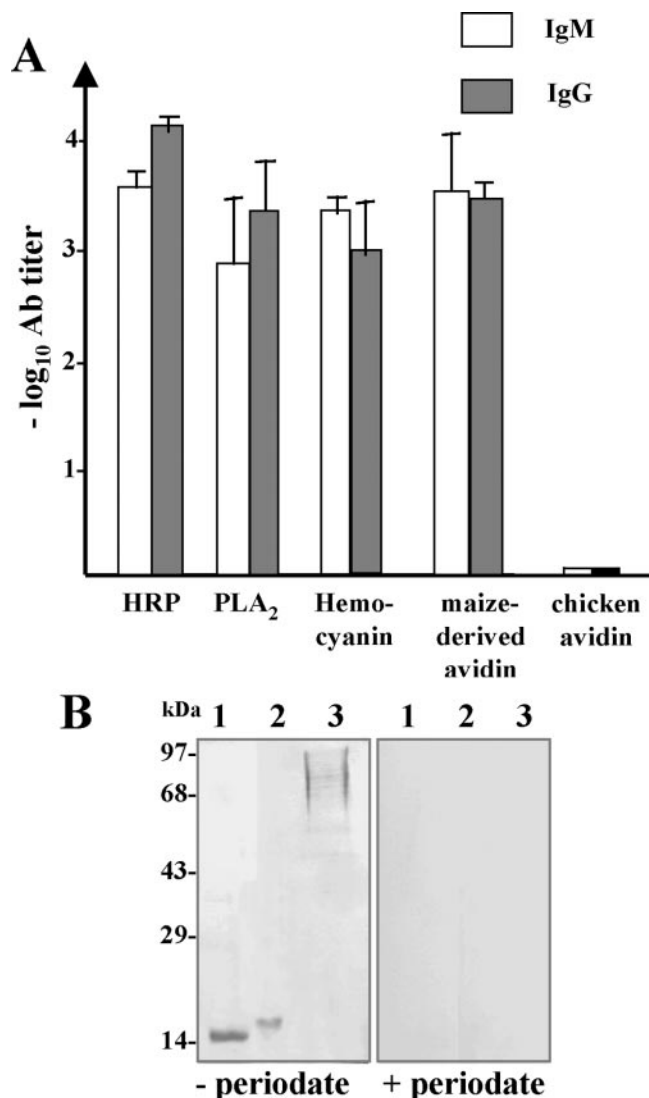


Fig. 3. (A) Ab titers of sera from rats immunized with HRP. Humoral responses to core $\alpha(1,3)$ -fucose (PLA₂) and core xylose (hemocyanin) determined by ELISA experiment using HRP-conjugated anti-rat IgM (1/1500) and IgG (1/1500) as a secondary Abs. (B) Western blot analysis of sera (1/500) of rats immunized with HRP before and after periodate treatment of the model glycoproteins. Lane 1: maize-derived avidin (16.8 kDa). Lane 2: honeybee venom PLA₂ (18 kDa). Lane 3: snail hemocyanin: broad band between 80 and 120 kDa.

A proportion of human nonallergic blood donors have Abs directed against the core xylose or core $\alpha(1,3)$ -fucose epitopes

Because humans are exposed daily to plant glycoprotein antigens in edible plant material or to other environmental glycoprotein antigens, we investigated the presence in humans of Abs raised against the core $\alpha(1,3)$ -fucose and/or the core xylose epitopes. Sera from 53 nonallergic human blood donors were analyzed by ELISA using PLA₂ and snail hemocyanin as probes. Sera were considered positive when their log of IgM or IgG₁ titers were superior to 2. Surprisingly, up to 27 sera were found to react against the core xylose or core $\alpha(1,3)$ -fucose epitopes (Figure 4A

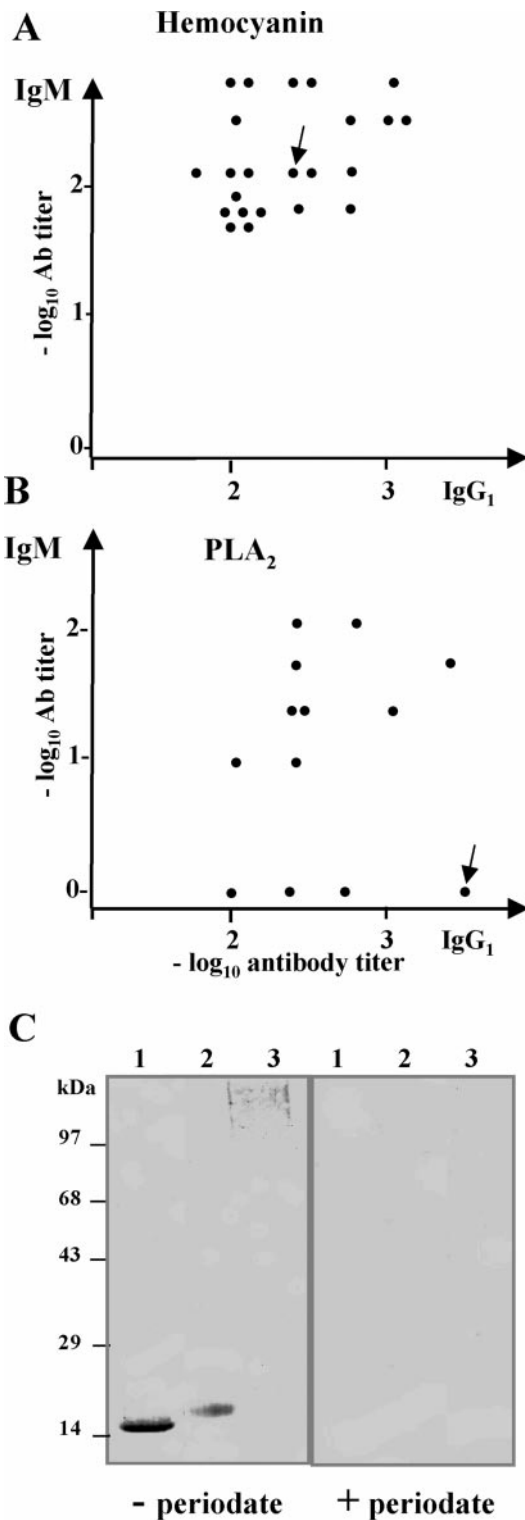


Fig. 4. Ab titers of nonallergic human blood donors exhibiting positive IgM and/or IgG₁ immune responses determined by ELISA using biotinylated anti-human IgM (1/10,000) and IgG₁ (1/10,000). (A) Immune responses to core xylose (hemocyanin). (B) Immune responses to core $\alpha(1,3)$ -fucose (PLA₂). Ab titers indicated by an arrow correspond to serum 52. (C) Western blot analysis before and after periodate treatment of the model glycoproteins of serum 52 using biotinylated anti-human IgG₁ as a secondary Ab. Lane 1: maize-derived avidin (16.8 kDa). Lane 2: honeybee venom PLA₂ (18 kDa). Lane 3: snail hemocyanin: broad band between 80 and 120 kDa.

and B). Among these sera, 23 were positive for hemocyanin and 13 for PLA₂, 9 of them being both PLA₂- and hemocyanin-positive. Isotype analysis indicated that Abs are almost exclusively IgM and IgG₁. However, although IgG₄ and IgE were undetectable, IgG₂ or IgG₃ were weakly detected in four sera (not shown).

As illustrated in Figure 4C, western blot analysis of a human serum (no. 52), exhibiting both PLA₂ and hemocyanin reactions, also results in the specific recognition of the core xylose and core $\alpha(1,3)$ -fucose epitopes that are found on these model glycoproteins, as well as on maize-derived avidin (Figure 4C). Alteration of oligosaccharides by periodate treatment abrogated Ab binding, confirming that these human IgG₁ are glycan-specific. In conclusion, half of the tested population of nonallergic human has in their sera low titers of IgG specific for core xylose and/or core $\alpha(1,3)$ -fucose epitopes that are present on glycans N-linked to plant glycoproteins.

Discussion

Crop plants, such as pea, rice, or maize, are intended to be used for the production in seeds of recombinant glycoproteins devoted to therapeutic applications in humans (Hood *et al.*, 1997; Stoger *et al.*, 2000). In contrast to plants intended for the production of therapeutic proteins in leaves such as tobacco, only limited data were available concerning the N-glycosylation in these plants (Hayashi *et al.*, 1990; Wilson *et al.*, 2001). Consequently, we analyzed the structure of their N-glycans and found that these oligosaccharides carry core xylose and core $\alpha(1,3)$ -fucose residues on their N-glycans. Together with previous studies on plant N-glycosylation (Lerouge *et al.*, 1998; Wilson and Altmann, 1998; Wilson *et al.*, 2001), this confirms that core xylosylation and core $\alpha(1,3)$ -fucosylation are ubiquitous N-glycan modifications in plants. These glyco-epitopes are absent in humans, so their potential immunogenicity is a major concern in the context of therapies with plant-derived recombinant glycoproteins. For this reason, the immunogenicity of plant N-glycans was reinvestigated in rodents. Consistent with a previous study (Chargelegue *et al.*, 2000), we showed that BALB/c mice do not develop any humoral response to plant N-glycans even using a high concentration of HRP and complete Freund's adjuvants to maximize the immune response. In contrast, core xylose and core $\alpha(1,3)$ -fucose induce a weak but significant IgM/IgG production in C57BL/6 mice. These glyco-epitopes are also immunogenic in rats as previously observed for goats (Kurosaka *et al.*, 1991) or rabbits (Faye *et al.*, 1993).

The presence of nonmammalian core $\alpha(1,3)$ -fucose and core xylose epitopes onto glycans N-linked to therapeutic glycoproteins produced in plants, in addition to the observed immunogenicity of these glyco-epitopes in some laboratory animals, raised the question of their immunogenicity in the context of a human therapy using plant-derived biopharmaceuticals. This might be investigated by the analysis of human sera following administration of a human protein bearing plant N-glycans. Indeed, the stimulation of Ab production is a concern only if the protein carrier itself turns out to be immunogenic. Immunization of rodents as described herein is far from the context of

human therapy with plant-derived proteins because a plant glycoprotein, rather than a self-antigen carrying plant *N*-glycans, is administrated. Furthermore, to stimulate anti-glycan responses, the immunization of rodents was carried out with complete Freund's adjuvants, which is a usual protocol to generate immune responses against almost any target molecules. Such adjuvants are unlikely to be used in clinical applications during human therapy.

Because humans are exposed daily to plant glycoprotein antigens through food and inhalation, the presence in humans of Abs raised against plant glyco-epitopes was questionable. As a consequence, to address this question, sera from a healthy human population were analyzed. Surprisingly, 27 out of 53 sera were found to contain IgG₁ raised against plant glyco-epitopes. About 50% of sera were positive for core xylose and 25% for core $\alpha(1,3)$ -fucose. The presence of such Abs could be related to an immune response to the core $\alpha(1,3)$ -fucose and/or the core xylose ubiquitously present on plant *N*-glycans. In the same way, other environmental antigens, such as *N*-glycoproteins from insects (Kubelka *et al.*, 1993) or parasites (Khoo *et al.*, 1997; van Die *et al.*, 1999), may also contribute to Ab production against these glyco-epitopes.

The observed human serum titers as determined by ELISA (1:100 to 1:1000) are weak but significant. Whatever the origin of glycoproteins responsible for the human immunostimulation, this demonstrates that core $\alpha(1,3)$ -fucose and/or the core xylose, existing on plant or other glycoprotein antigens, are able to elicit immune responses in humans.

The fact that humans have Abs against plant *N*-glycan epitopes does not necessarily mean that there will be adverse effects when administrating plant-derived therapeutic glycoproteins. The presence of low-titer antiplant glycan Abs might be a neutralizing effect. Although the immunological significance of anti-core $\alpha(1,3)$ -fucose and anti-core xylose Abs is too speculative at the moment, the presence of such Abs may well induce an accelerated clearance of recombinant plant glycoproteins from plasma, resulting in a therapeutic failure. In addition to this accelerated clearance, clinical effects resulting from the administration of plant-derived therapeutic glycoproteins in allergic patients are also questionable. Indeed, because these plant *N*-glycan epitopes appear to trigger IgE response in allergic patients (Wilson and Altmann, 1998; van Ree *et al.*, 2000) and in parasite-infected mammals (van Die *et al.*, 1999), the presence of such glyco-epitopes on plant-derived therapeutic glycoproteins could induce clinical troubles in allergic populations. As a consequence, for a more detailed evaluation of safety concerns related to the use of plant-derived therapeutic proteins, further experiments have to be carried out in an appropriate model animal as well as in human by administering a therapeutic glycoprotein produced in a plant and analyzing immune responses to the plant glyco-epitopes in allergic and nonallergic populations.

Materials and methods

Materials

Hemocyanin from *H. pomatia* was from Serva (Heidelberg, Germany). Natural chicken avidin and chicken avidin

produced in maize, HRP, and honeybee venom PLA₂ were from Sigma (St Quentin-Fallavier, France). BALB/c and C57BL/6 mice and Wistar rats were from Iffa-Credo (L'Arbresle, France). Sera from human blood donors were obtained at the Centre de Médecine Préventive (Institut Pasteur de Lille).

N-linked glycan analysis

Protein extraction from meal of pea, rice, or maize seeds and isolation of *N*-glycans were carried out according to Fitchette *et al.* (1999). Enzyme sequencing of plant *N*-glycans was carried out as reported by Bakker *et al.* (2001). For permethylation, the pool of *N*-glycans was reduced with NaBH₄ and permethylated according to Ciucanu and Kerek (1984).

MS analysis

MALDI-TOF mass spectra of *N*-glycans were acquired on a Micromass (Manchester, UK) ToF Spec E MALDI-TOF mass spectrometer equipped with a nitrogen laser. Mass spectra were performed in the reflector mode using 2,5-dihydroxybenzoic acid as matrix. Assignments to both (M+Na)⁺ and (M+K)⁺ adducts were confirmed by running an additional spectrum with CsCl. In these conditions both (M+Na)⁺ and (M+K)⁺ adducts were converted into a single (M+Cs)⁺ ion.

CID and MALDI-PSD experiments, selection of the precursor ion was carried out using a Bradbury-Nielsen ion gate with a $m/\delta m = 100$ resolution. Extraction with a delay time of 600 ns was used. CID was carried out by collision with helium at a pressure of 10^{-6} mbar in the collision cell. To record the full PSD spectra, reflector voltage was decreased into successive 25% steps leading to 10 spectral segments using 30 laser shots per segment. PSD was calibrated using ACTH (fragment 18–39).

Immunization of mice and rats

Four inbred 6–7-week-old female BALB/c (H-2^d) or C57BL/6 (H-2^b) mice were immunized by subcutaneous injection of, respectively, 100 μ g and 30 μ g of HRP in complete Freund's adjuvant, followed by boosts with the same dose of immunogen in incomplete Freund's adjuvant 2, 4, and 6 weeks after the first immunization. Sera were collected 1 week after the last injection. Immunization of four 8-week-old Wistar rats was carried out according to the same protocol and using 100 μ g HRP.

Western blot analysis of model glycoproteins with different sera

The glycoproteins were separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and blocked overnight at room temperature with 3% gelatin dissolved in Tris buffered saline buffer. The membranes were incubated with the sera of mice (1/500), rats (1/1000), and humans (1/500) for 2 h as previously described (Faye *et al.*, 1993). The membranes were incubated with either the anti-mouse, anti-rat, anti-human IgG Abs (1/1000) conjugated with HRP or biotinylated anti-human IgG₁ Abs. For detection of biotinylated anti-human IgG₁ Abs, the membranes were further

incubated with streptavidin conjugated with HRP. For detection of HRP activity, the membranes were revealed with 4-chloro 1-naphtol (Faye *et al.*, 1993). Pre-immune sera were used as negative controls. Alteration of the glycan moiety of the model glycoproteins was carried out by oxidation using sodium periodate (Fitchette-Lainé *et al.*, 1998).

ELISA

Ninety-six-well plates were coated overnight at 4°C with either 1 µg HRP or recombinant avidin, 3 µg PLA₂, or 10 µg of hemocyanin in 50 mM sodium carbonate buffer, pH 9.6. The microplates were then washed with phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBS/Tween) and blocked with PBS plus 1% bovine serum albumin for 1 h at 37°C. The plates were then incubated with serial dilutions of mice, rat, and human sera in PBS/Tween/bovine serum albumin for 2 h at 37°C. The microplates were washed with the same buffer and incubated for 2 h at 37°C with appropriate secondary Abs. Dilutions used for the secondary antibodies are: HRP-conjugated anti-mouse IgM and IgG Abs, 1/10,000; HRP-conjugated anti-rat IgM and IgG Abs, 1/1500; and biotinylated anti-human IgM and IgG₁ Abs, 1/10,000.

For biotinylated conjugates, a third incubation was carried out with streptavidin conjugated to HRP (1/1500) for 1 h at 37°C. HRP activity was detected by addition of 100 µl/well of a substrate solution (15 mg of o-phenylenediamine dihydrochloride tablets in 15 ml 0.05 M citrate buffer, pH 5.6, supplemented with 15 µl H₂O₂). After 30 min at room temperature in the dark, the enzymatic reaction was stopped with 50 µl 2 M H₂SO₄, and OD was determined at 492 nm. The same plates were prepared and incubated with pre-immune sera, and the OD obtained for pre-immune sera were deduced from values obtained for immunized animals for the determination of the titers. Ab titer was determined as the highest dilution that gives an absorbance value twofold superior compared to that of background.

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Abbreviations

Ab, antibody; CID, collision-induced dissociation; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish

peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PBS, phosphate buffered saline; PSD, post source decay; PLA₂, phospholipase A₂.

References

- Aalberse, R.C., Koshte, V., and Clemens, J.G. (1981) Cross-reactions between vegetable foods, pollen and bee venom due to IgE antibodies to a ubiquitous carbohydrate determinant. *Int. Arch. Allergy Appl. Immunol.*, **66**, 259–260.
- Bakker, H., Bardor, M., Molhoff, J., Gomord, V., Elbers, I., Stevens, L., Jordi, W., Lommen, A., Faye, L., Lerouge, P., and Bosch, D. (2001) Galactose-extended glycans of antibodies produced by transgenic plants. *Proc. Natl Acad. Sci. USA*, **98**, 2899–2904.
- Bruch, R.C. and White, H.B. (1982) Composition and structural heterogeneity of avidin glycopeptides. *Biochemistry*, **21**, 5334–5341.
- Cabanes-Macheteau, M., Fitchette-Lainé, A.-C., Loutelier-Bourhis, C., Lange, C., Vine, N., Ma, J., Lerouge, P., and Faye, L. (1999) *N*-glycosylation of a mouse IgG expressed in transgenic tobacco plants. *Glycobiology*, **9**, 365–372.
- Chargelegue, D., Vine, N.D., van Dolleweerd, C.J., Drake, P.M.W., and Ma, J. (2000) A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. *Transgenic Res.*, **9**, 187–194.
- Ciucanu, I. and Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.*, **131**, 209–217.
- Faye, L. and Chrispeels, M.J. (1988) Common antigenic determinants in the glycoproteins of plants, molluscs and insects. *Glycoconj. J.*, **5**, 245–256.
- Faye, L., Gomord, V., Fitchette-Lainé, 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.
- Fitchette, A.-C. Cabanes-Macheteau, M., Marvin, L., Martin, B., Siatat-Jeunemaitre, B., Gomord, V., Crooks, K., Lerouge, P., Faye, L., and Hawes, C. (1999) Biosynthesis and immunolocalization of Lewis A-containing *N*-glycans in the plant cell. *Plant Physiol.*, **121**, 333–343.
- Fitchette-Lainé, A.-C., Denmat, L.A., Lerouge, P., and Faye, L. (1998) Analysis of *N*- and *O*-glycosylation of plant proteins. In Cunningham, C. and Porter, A.J.R. (eds.), *Recombinant proteins from plants*. Methods in Biotechnology vol 3. Humana Press, Totowa, NJ, pp. 271–289.
- Garcia-Casado, G., Sanchez-Monge, R., Chrispeels, M.J., Armentia, A., Salcedo, G., and Gomez, L. (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology*, **6**, 471–477.
- Hayashi, M., Tsuru, A., Mitsui, T., Takahashi, N., Hanzawa, H., Arata, Y., and Akazawa, T. (1990) Structure and biosynthesis of the xylose-containing carbohydrate moiety of rice α -amylase. *Eur. J. Biochem.*, **191**, 287–295.
- Hood, E.E., Witcher, D.R., Maddock, S., Meyer, T., Baszczynski, C., Bailey, M., Flynn, P., Register, J., Marshall, L., Bond, D., and others. (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Mol. Breeding*, **3**, 291–306.
- Khoo, K.H., Chatterjee, D., Caufield, J.P., Morris, H.R., and Dell, A. (1997) A structural mapping of the glycans from the egg glycoproteins of *Schistosoma mansoni* and *Schistosoma japonicum*: identification of novel core structures and terminal sequences. *Glycobiology*, **7**, 663–667.
- Kubelka, V., Altmann, F., Staudacher, E., Tretter, V., März, L., Hard, K., Kamerling, J.P., and Vliegenhart, J.F.G. (1993) Primary structures of the *N*-linked carbohydrate chains from honeybee venom phospholipase A₂. *Eur. J. Biochem.*, **213**, 1193–1204.
- 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: recent development and future trends. *Plant Mol. Biol.*, **38**, 31–48.
- 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.
- Melo, N.S., Nimtz, M., Conradt, H.S., Fevereiro, P.S., and Costa, J. (1997) Identification of the human Lewis a carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium myrtillus* L.). *FEBS Lett.*, **415**, 186–191.
- Stoger, E., Vaquero, C., Torres, E., Sack, M., Nicholson, L., Drossard, J., Williams, S., Keen, D., Perrin, Y., Christou, P., and Fischer R. (2000) Cereal crops as viable production and storage systems for pharmaceutical antibodies. *Plant Mol. Biol.*, **42**, 583–590.
- van Die, I., Gomord, V., Kooyman, F.N.J., van den Berg, T.K., Cummings, R.D., and Vervelde, L. (1999) Core α 1–3 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., and Vliegenhart, J.F.G. (1985) Primary structure of the low-molecular-weight carbohydrate chains of *Helix pomatia* α -hemocyanin. *J. Biol. Chem.*, **260**, 13984–13988.
- van Ree, R. and Aalberse, R.C. (1995) Demonstration of carbohydrate-specific immunoglobulin G₄ antibodies in sera of patients receiving grass pollen immunotherapy. *Int. Arch. Allergy Immunol.*, **106**, 146–148.
- van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J.P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodriguez, R., and others. (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.
- Wilson, I.B.H. and Altmann, F. (1998) Structural analysis of *N*-glycans from allergenic grass, ragweed and tree pollens: core α 1,3-linked fucose and xylose present in all pollens examined. *Glycoconj. 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.
- Zeitlin, L., Olmsted, S.S., Moench, T.C., Co, M.S., Martinell, B.J., Paradkar, V.M., Russell, D.R., Queen, C., Cone, R.A., and Whaley, K. (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat. Biotechnol.*, **16**, 1361–1364.