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# A plant-derived human monoclonal antibody induces an anti-carbohydrate immune response in rabbits

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**A common argument against using plants as a production system for therapeutic proteins is their inability to perform authentic *N*-glycosylation. A major concern is the presence of beta 1,2-xylose and core alpha 1,3-fucose residues on complex *N*-glycans as these nonmammalian *N*-glycan residues may provoke unwanted side effects in humans. In this study we have investigated the potential antigenicity of plant-type *N*-glycans attached to a human monoclonal antibody (2G12). Using glyco-engineered plant lines as expression hosts, four 2G12 glycoforms differing in the presence/absence of beta 1,2-xylose and core alpha 1,3-fucose were generated. Systemic immunization of rabbits with a xylose and fucose carrying 2G12 glycoform resulted in a humoral immune response to both *N*-glycan epitopes. Furthermore, IgE immunoblotting with sera derived from allergic patients revealed binding to plant-produced 2G12 carrying core alpha 1,3 fucosylated *N*-glycan structures. Our results provide evidence for the adverse potential of nonmammalian *N*-glycan modifications present on monoclonal antibodies produced in plants. This emphasizes the need for the use of glyco-engineered plants lacking any potentially antigenic *N*-glycan structures for the production of plant-derived recombinant proteins intended for parenteral human application.**

*Keywords:* anti-carbohydrate immune response/beta 1,2 xylose/core alpha 1,3 fucose/glyco-engineered plants/recombinant antibodies/*N*-glycosylation

## Introduction

In recent years plants have become an attractive alternative for the production of recombinant proteins (for recent reviews see Fischer et al. (2004); Ma et al. (2005)). Since many therapeutically relevant proteins need *N*-glycosylation to obtain full biological activity, plants are well suited to be used as an expression platform as these eukaryotes are able to synthesize *N*-glycans similar to mammalian cells. However, beside a common core structure present in mammals and plants, the structures of mature complex-type *N*-glycans differ to some extent: plant complex-type *N*-glycans are generally smaller; however, they contain β1,2-xylose and/or core α1,3-fucose residues. As these glycan-epitopes are absent in humans their potential antigenicity is a major concern in the context of a therapeutical use of plant-derived recombinant glycoproteins. Immunization of different laboratory animals with plant-derived glycoproteins elicits the production of xylose- and core α1,3-fucose-specific antibodies (Kurosaka et al. 1991; Prenner et al. 1992; Faye et al. 1993; Bardor et al. 2003; Jin et al. 2006). These results also indicate that the immune response to these carbohydrate epitopes varies between animal species, being apparently higher in rats and rabbits than in mice. BALB/c mice do not seem to develop any humoral response to these plant-specific *N*-glycan residues (Chargelegue et al. 2000; Bardor et al. 2003; Jin et al. 2006; Petruccelli et al. 2006). Furthermore, xylose and core α1,3 fucose epitopes are known to be important IgE binding carbohydrate determinants, so-called cross-reactive carbohydrate determinants (CCDs), of plant allergens (for a recent review see Altmann (2007)). IgE antibodies against these carbohydrate structures are induced upon exposure to pollen and result in extensive cross-reactivity to plant foods (van Ree 2002). Although the clinical relevance of these CCDs does not seem to be significant (van der Veen et al. 1997; Altmann 2007), their presence on therapeutic glycoproteins remains a pending issue. Another interesting observation is the presence of xylose and core α1,3-fucose specific antibodies in about 50% of human blood donors (Bardor et al. 2003). In other words, a systemically applied plant-made glycoprotein would immediately be bound by anti-carbohydrate antibodies in many individuals – irrespective of its own ability to elicit antibodies.

Monoclonal antibodies (mAbs) are amongst the most interesting candidate molecules for human therapy and the current expression capacities by far cannot meet the overall demand. A number of mAbs has been expressed in a variety of plant species (e.g., Stoger et al. 2002, 2004). Interestingly, plant-produced antibodies (pIgG) exhibit in vitro and more importantly in vivo activities comparable to their counterparts produced in animal cells (Ko et al. 2003; Ko and Koprowski 2005; Brodzik et al. 2006) not at least because of an appropriate *N*-glycosylation. It is well documented that the in vivo efficacy of IgGs largely depends on its glycosylation and varies between different glycoforms (Jefferis 2005). In this respect the natural lack of α1,6 fucose residues in plants seems to provide an advantage, since its elimination from CHO-produced IgGs caused a significant increase of effector activities such as antibody-dependent cellular cytotoxicity (ADCC) (Shields et al. 2002; Shinkawa et al.

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2003; Ferrara et al. 2006). Like other mammalian glycoproteins produced in plants (Dirnberger et al. 2001) pIgGs carry complex *N*-glycans containing xylose and core α1,3 fucose residues (e.g., Cabanes-Macheteau et al. 1999; Schähs et al. 2007). Since humans are constantly exposed to plant glycoproteins in the diet, plant-specific *N*-glycosylation of IgGs should be acceptable for a topical and oral application (Ma et al. 1998). However, as many therapeutic antibodies have to be repeatedly administered parenterally at high dosages the presence of nonhuman glycan epitopes may provoke unwanted side effects in humans. It has been proposed that due to the buried nature of *N*-glycans on IgG molecules (e.g., Burton and Dwek 2006) the immune system will not recognize the foreign carbohydrate epitopes. This hypothesis has been corroborated by studies from Chargelegue et al. (2000) where a plant-produced mouse antibody – when parenterally administered to BALB/c mice – did not induce any immune response to plant-specific *N*-glycans. However, recent studies indicate that mice and in particular BALB/c mice are not appropriate models to assess plant *N*-glycan immunogenicity (Jin et al. 2006; Petruccelli et al. 2006). No further studies investigated the potential antigenicity of the *N*-glycan moiety of parenterally administered plant-derived recombinant antibodies or other human glycoproteins in animals.

In the present work we have investigated the antigenicity of plant-specific *N*-glycans on a human monoclonal antibody against HIV in view of the ongoing controversial discussion about the significance of this issue for the use of plant-derived mAbs as therapeutics.

## Results

### *2G12 glycoforms*

The human monoclonal antibody 2G12, broadly neutralizing different HIV-1 strains (Trkola et al. 1995), was chosen since an efficient transient expression system allowed the production of amounts sufficient for the studies described here. In this context it has to be mentioned that the Fab region of 2G12 has a unique structure (Calarese et al. 2003). However, since 2G12 exhibits similar effector functions as other human mAbs (Trkola et al. 2005), significant structural changes to the Fc part, the site for *N*-glycosylation, are not expected. 2G12 was produced in different RNAi-derived glyco-engineered *Nicotiana benthamiana* lines (Strasser, Stadlmann, Schaehs, Stiegler, Quendler, Mach, Gloessl, Weterings, Altmann, Steinkellner, submitted). The following 2G12 glycoforms as determined by mass spectrometry (liquid-chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)) were obtained (see also Table I): (i) wt-2G12, main structure: complex-type *N*-glycans with xylose and core α1,3 fucose ($GnGnXF^3$); (ii) ΔXT-2G12 and (iii) ΔFT-2G12, main structures: complex *N*-glycans, mostly devoid of xylose ($GnGnF^3$) and fucose (GnGnX), respectively; and (iv) ΔXT/FT-2G12, main structure: complex-type *N*-glycans, lacking both xylose and fucose (GnGn). Furthermore, a CHO-produced 2G12 (CHO-2G12) carrying core α1,6-fucosylated complex *N*-glycans with either one or two terminal β1,4 galactose residues ($AGnF^6$ iso and $AAF^6$) was used as a control. A detailed description regarding expression and structural and functional integrity of these plant-derived 2G12 glycoforms is described in another study (Strasser, Stadlmann, Schaehs, Stiegler, Quendler, Mach, Gloessl, Weterings, Altmann, Steinkellner, submitted). We further confirmed the presence or absence of xylose and/or core α1,3 fucose on heavy chains ($C_H$) of 2G12 glycoforms by immunoblotting using rabbit antisera specific to either both (anti-HRP antiserum) or one of the two carbohydrate epitopes (Figure 1). As expected wt-2G12 exhibited a strong specific signal with all three antisera. ΔXT- and ΔFT-2G12 revealed a strong signal with anti-HRP and the respective specific antiserum, i.e., ΔXT-2G12 reacted with anti-fucose and ΔFT-2G12 with anti-xylose antiserum. A weak staining was obtained after long exposure reflecting the presence of smaller amounts of core α1,3 fucose and xylose residues in the respective RNAi lines (about 10% xylosylated structures and 20% fucosylated structures of 2G12 produced in ΔXT and ΔFT lines) which is in agreement with the mass spectrometric analyses (Table I). None of the sera reacted with ΔXT/FT-2G12 (and CHO-2G12) confirming the absence of xylose and core α1,3 fucose residues on either protein (data not shown).

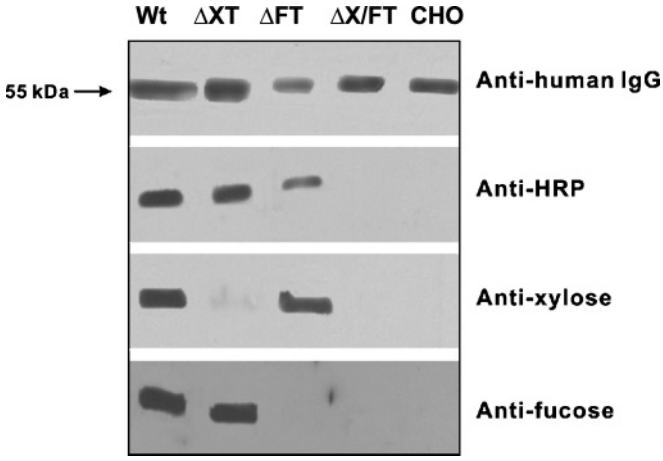


**Fig. 1.** Comparative Western blot analysis of 2G12 glycoforms ($C_H$) using different antisera. Purified 2G12 glycoforms were separated on reducing SDS–PAGE and blotted to nitrocellulose (50 ng per lane). Samples were probed with anti-IgG ($C_H$), -HRP, -xylose and -fucose antisera, respectively, to confirm the presence/absence plant-specific *N*-glycans.

**Table I.** *N*-Glycan composition of 2G12 expressed in *Nicotiana benthamiana* wt plants and glyco-engineered RNAi lines as determined by MS analysis. See http://www.proglycan.com for an explanation of Nglycan abbreviations

| | Relative abundance % | | | |
|---|---|---|---|---|
| *N*-glycan | wt | ΔXT | ΔFT | ΔXT/FT |
| $MMF^3$ | >1 | 2.5 | <1 | <1 |
| MMX | <1 | <1 | 1.7 | <1 |
| $MMXF^3$ | 3.4 | <1 | <1 | <1 |
| GnU | <1 | 2.2 | 1.1 | 7.8 |
| $GnUXF^3$ | 5.8 | <1 | 1.8 | <1 |
| GnM | <1 | 5.1 | 2.7 | 14.8 |
| $GnMF^3$ | <1 | 7.0 | <1 | <1 |
| GnMX | <1 | <1 | 9.3 | <1 |
| $GnMXF^3$ | 9.2 | 1.2 | 4.0 | <1 |
| GnGn | 1.9 | 22.8 | 6.4 | 72.6 |
| GnGnX | 5.5 | 1.4 | 46.3 | <1 |
| $GnGnF^3$ | 2.1 | 43.3 | 1.3 | <1 |
| $GnGnXF^3$ | 67.6 | 11.4 | 21.5 | <1 |
| Man8 | 4.0 | 3.1 | 4.0 | 3.1 |

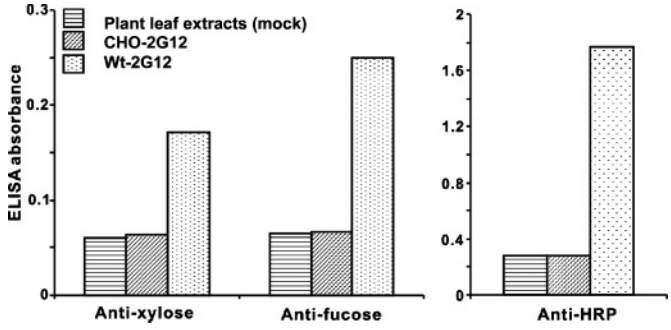


**Fig. 2.** ELISA of wt- and CHO-2G12 to estimate accessibility of xylose and α1,3 fucose on fully assembled 2G12. Wells were coated with antiserum against IgG $C_L$ and then incubated with total soluble proteins extracted from *N. benthamiana* wt expressing 2G12. Subsequently samples were incubated with anti HRP, -xylose and -fucose antiserum, respectively. As controls CHO-2G12 and soluble proteins from mock infected plants were used.

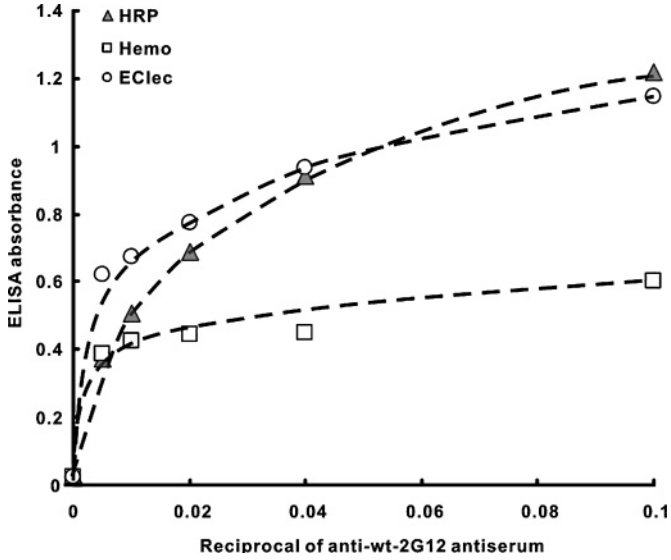


**Fig. 3.** Cross-reactivity of rabbit antiwt-2G12 antiserum. The binding with naturally occurring glycoproteins, with and without xylose and α1,3 fucose, was measured by ELISA. HRP: horseradish peroxidase ($MMXF^3$); Hemo: hemocyanin from *Helix pomatia* (MMX); EClec: *Erythrina corallodendron* lectin ($MMXF^3$).

*Accessibility of plant-type N-glycans on fully assembled 2G12*

The ability of xylose and core α1,3 fucose containing glycoproteins to induce a humoral response in various laboratory animals is well documented. However, proteins used in these studies (e.g., HRP or human transferrin) carry at least two complex *N*-glycan chains per protein backbone and expose them at the surface. In contrast to that IgGs possess only one glycan chain in the Fc Cγ2 domain, which is, due to the complex structure of IgGs, shielded by the protein backbone (Burton and Dwek 2006). Due to this unique feature of IgGs, it was questioned whether IgG *N*-glycans might be recognized by the mammalian immune system. Since rabbit anti-HRP, anti-xylose and anti-fucose-specific antisera clearly react with the heavy chain ($C_H$) of denatured plant-derived wt-2G12 in Western blot analyses (Figure 1), we wanted to determine whether this is also the case for fully assembled native molecules. Therefore a sandwich ELISA using nonpurified 2G12 transiently expressed in wild-type *N. benthamiana* leaves was carried out. To ensure that only fully assembled wt-2G12 is detected and to exclude possible IgG fragments present in plant cells, wells were coated with antiserum specific against IgG κ light chain ($C_L$). Wells were then incubated with protein extracts derived from 2G12 expressing wt-plants and subsequently probed with antisera against plant-specific *N*-glycans. The results revealed a specific binding of wt-2G12 to anti-HRP as well as to xylose and fucose specific antisera, although at different levels (Figure 2). Anti-HRP antiserum reacts significantly stronger than anti-xylose and anti-fucose antiserum. The estimated amount of wt-2G12 present in plant extracts was 20 ng/100 μL. No specific reaction was obtained when 30 ng/100 μL of CHO-2G12 was added to the control plant extract (Figure 2). These results clearly indicate that plant *N*-glycan epitopes are accessible for anti-carbohydrate antibodies on fully assembled wt-2G12 in vitro.

*Antigenicity of 2G12 with plant-type N-glycans*

To investigate the potential antigenicity of plant-derived 2G12 in vivo, rabbits were immunized with wt-2G12 and CHO-2G12 as a control. In a first set of experiments the obtained antiserum was tested for the presence of antibodies against the 2G12 protein backbone. ELISA quantification of anti human IgG1 in wt- and CHO-2G12 antisera revealed a titer of 1:2.000 for both antisera indicating a strong immune response to the heterologous protein.

Subsequently the cross-reactivity of the obtained rabbit antiserum with unrelated naturally occurring glycoproteins containing xylose and/or core α1,3 fucose residues was analyzed by ELISA (Figure 3). Anti wt-2G12 antiserum strongly cross-reacted with horseradish peroxidase (main *N*-glycan structure: $MMXF^3$), hemocyanin from *Helix pomatia* (contains MMX), and *Erythrina corallodendron* lectin ($MMXF^3$). Since all these glycoproteins do not have an apparent homology to 2G12 and the preimmunization serum did not reveal a significant signal (data not shown), the results indicate that wt-2G12 elicits the formation of cross-reactive anti-carbohydrate antibodies. ELISA quantification of anti-carbohydrate antibodies in wt-2G12 antiserum revealed a titer of 1:800. In contrast, no specific reaction was obtained with CHO-2G12 antiserum (data not shown).

To determine the specificity of wt-2G12 antiserum an immunoblot using glycoprotein variants carrying defined *N*-glycan structures was carried out. Several defined glycoforms of human transferrin (Tf) were prepared in vitro as described previously (Bencurova et al. 2004) and subjected to Western blot analyses: $GnGnXF^3$-Tf, $MMXF^3$-Tf, GnGnX-Tf, MMX-Tf, $GnGnF^3$-Tf, and $MMF^3$-Tf and MM-Tf (for *N*-glycan structures see Table II and http://www.proglycan.com). The experiments revealed that anti wt-2G12 antiserum bound to all Tf glycoforms carrying xylose and/or α1,3-fucose (Figure 4). Notably, both Tf glycoforms carrying only xylose or only fucose (GnGnX, $GnGnF^3$, MMX, and $MMF^3$) exhibited specific signals (Figure 4), indicating that both *N*-glycan epitopes are able to be individually recognized by anti-carbohydrate antibodies. In contrast, no significant reaction was obtained when the serum was blotted against Tf glycoforms that carry *N*-glycans without xylose and fucose (MM-Tf). No binding was obtained when glycan-modified Tf was incubated with anti-CHO-2G12 antiserum or with preimmunization sera

**Table II.** Schematic illustration of the predominant *N*-glycan structures of 2G12 expressed in *Nicotiana benthamiana* wt plants, glyco-engineered RNAi lines and CHO cells

| 2G12 | | Main *N*-glycan structure |
|---|---|---|
| Wt | $GnGnXF^3$ | GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc)-3(Fucα1-3)GlcNAcβ1-2Manα1-3(Xylβ1-2) |
| ΔXT | $GnGnF^3$ | GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc)-3(Fucα1-3)GlcNAcβ1-2Manα1-3 |
| ΔFT | GnGnX | GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc)-3GlcNAcβ1-2Manα1-3(Xylβ1-2) |
| ΔXT/FT | GnGn | GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc)-3GlcNAcβ1-2Manα1-3 |
| CHO | $AGnF^6$ | Galβ1-4GlcNAcβ1-2Manα1-6(Fucα1-6)-3(Manβ1-4GlcNAcβ1-4GlcNAc)-3GlcNAcβ1-2Manα1-3 |

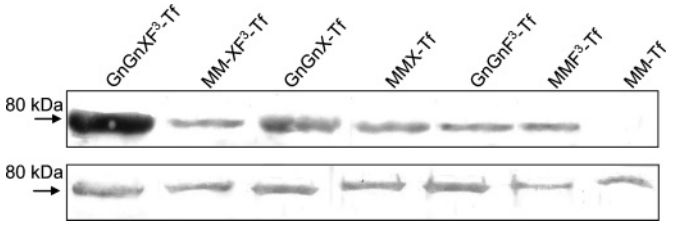


**Fig. 4.** (**A**) Specificity analysis of rabbit antiwt-2G12 by Western blot analysis: different human Tf glycoforms (500 ng) were separated on SDS–PAGE and blotted against rabbit anti-wt-2G12 antiserum. (**B**) Respective Tf glycoforms stained with Ponceau S as a control.

(data not shown), further confirming that the immune reactions are caused by plant-specific carbohydrates.

### *Human IgE immunoblots and cross-inhibition experiments with plant-derived 2G12*

The occurrence of cross-reactive human IgE recognizing carbohydrate epitopes is a well-known phenomenon (Altmann 2007). Several studies have shown that plant and insect glycoproteins bind to IgE from sera of allergic patients, and it has been suggested that core α1,3 fucose constitutes the major target for this binding (Bencurova et al. 2004; Jin et al. 2008). Here we describe IgE immunoblot analyses of different 2G12 glycoforms (wt-, ΔXT-, ΔFT-ΔXT/FT- and CHO-2G12) with serum pools derived from healthy and allergic blood donors suffering from pollen and insect venom allergy. No (or very faint) binding occurred when 2G12 glycoforms were incubated with sera from healthy blood donors (Figure 5, lanes 3, 6, 10, 12). However, when 2G12 glycoforms were incubated with the serum pool obtained from allergic patients, wt-, ΔXT-, and ΔFT-2G12 which contained either one or both of the carbohydrate epitopes displayed an IgE binding (Figure 5). However, no reaction was obtained when the serum was incubated with ΔXT/FT- (Figure 5) or CHO-2G12 (data not shown) glycoforms that do

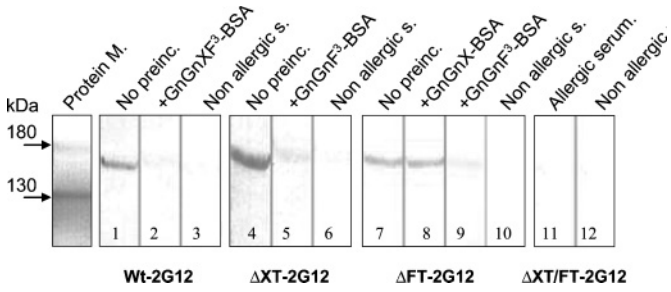


**Fig. 5.** Human IgE immunoblot using different 2G12 glycoforms. 2G12 glycoforms were separated on SDS–PAGE under nonreducing conditions and incubated with sera derived from allergic patients and healthy blood donors. Lanes 1, 4, 7, and 11: serum pool from allergic patients was directly blotted against the respective 2G12 glycoform (no preincubation); lanes 2, 5, 8, and 9: preincubation of the serum with different BSA glycoconjugates; lanes 3, 6, 10, and 12: respective 2G12 glycoforms were incubated with serum from healthy blood donors (nonallergic serum).

not carry plant-specific carbohydrates. To investigate whether the signal obtained with wt-, ΔXT-, and ΔFT-2G12 was provoked by plant-specific *N*-glycans, a cross-inhibition assay was carried out. Sera were preincubated with different neoglycoproteins carrying defined *N*-glycan structures (Jin et al. 2006). IgE binding to wt-2G12 (main *N*-glycan structure: $GnGnXF^3$) could be cross-inhibited with $GnGnXF^3$-BSA, while binding to ΔXT-2G12 ($GnGnF^3$) could be cross-inhibited with $GnGnF^3$-BSA (Figure 5), but not with GnGnX-BSA (data not shown). Interestingly, no inhibition of binding to ΔFT-2G12 (GnGnX) was obtained when the serum pool was preincubated with GnGnX-BSA, indicating that the IgE binding was not caused by xylose containing *N*-glycans. An explanation for the binding is the presence of residual amounts of core α1,3 fucose residues (20% of total *N*-glycan structures, corresponding to a reduction of 70% compared to wt-2G12) on ΔFT-2G12. In fact, a significant reduction of IgE binding was obtained when the allergic serum pool was preincubated with $MMF^3$-BSA, confirming that core α1,3 fucose but not xylose is actually involved in IgE binding to ΔFT-2G12 (Figure 5). No inhibition of IgE binding was obtained when the serum pool was preincubated with MM-BSA.

## Discussion

Here we show the antigenicity of nonmammalian *N*-glycan residues on a plant-derived monoclonal antibody. We demonstrate that both xylose and core α1,3 fucose residues present on wt-2G12 are able to elicit specific antibodies in rabbits after parenteral application. We further show that anti-CCD IgE present in sera from allergic patients do react with core α1,3 fucose on plant-derived 2G12. Among the hurdles to be taken before plants become a feasible alternative to mammalian cells for the production of therapeutic glycoproteins is the potential antigenicity of their nonmammalian *N*-glycan residues. Although various studies demonstrate the antigenicity of xylose and core α1,3 fucose in animal models and the presence of anti-CCD antibodies in humans, the significance of these *N*-glycan residues subsequent to parenteral application have not been evaluated in humans to date. A previous paper reported on undetectable "adverse reactions" in volunteers/individuals which had been systemically challenged with a plant-produced monoclonal

antibody (E. Grill, personal communication as cited in Ma et al. (2005)). However, as the details of this experiment have never been published, its significance cannot be judged. The present work shows that a plant-derived antibody evokes a xylose- and core α1,3-fucose-specific immune reaction in rabbits. However, it should be pointed out that the animals also developed a strong immune response to the protein backbone of the heterologous antigen, which could have assisted in the formation of the anti-carbohydrate antibodies. Thus, the antigenicity of the *N*-glycan moiety of plant-derived 2G12 might be different in humans.

However, nature on its own offers a free demonstration of the immunogenicity of plant and insect glycoproteins in humans. About 50% of healthy persons were found to have anti-CCD IgG and IgM (Bardor et al. 2003), an observation confirmed in our laboratory (unpublished results). It is currently unclear whether this percentage reflects the history of antigen challenge, e.g., insect stings, or the genetic background, but it certainly proves that humans can respond to CCDs with the formation of specific antibodies. Normally, in healthy persons, anti-CCD IgE are not detectable or very low; however, 15–30% of allergic patients contain anti-CCD IgE. Interestingly, sera of both healthy and allergic individuals contain anti-CCD IgG. Moreover, the latter antibodies have been shown to be of high affinity (Jin et al. 2008). Thus, already before the systemic application of a plant-derived glycoprotein may stimulate an immune response, in about every second individual there are anti-CCD antibodies present in the serum that may negatively interfere with applied therapeutics. Although the current opinion is that anti-CCD IgE are essentially incapable of eliciting allergic symptoms, nano- or microgram amounts of allergens on mucosal surfaces cannot be compared with milligram doses of a systemically applied therapeutic, as e.g., needed for therapeutic application of 2G12 (Trkola et al. 2005). In this regard it is noteworthy that even in the special situation of fully assembled wt-2G12 an anti-CCD reaction occurs. As many patients have been immunized to CCDs by unknown antigens, possibly insect stings (Altmann 2007), administered antibodies displaying CCD epitopes can interact with these pre-existing anti-CCD IgG and IgE. In the best case, this will cause reduction of efficacy of the antibody.

Can nonmammalian *N*-glycan residues be avoided on plant-made pharmaceuticals (PMPs)? Apart from targeting of PMPs to specific plant organelles (e.g., ER), major achievements in eliminating xylose and core α1,3 fucose have been made by the manipulation of the endogenous *N*-glycosylation pathway of the respective host plants. Beside the over-expression of the mammalian β1,4 galactosyltransferase which led to the generation of *N*-glycans with a significant reduction of xylose and core α1,3 fucose in tobacco (Bakker et al. 2006; Fujiyama et al. 2007), one of the most promising strategies is the specific elimination or downregulation of the enzymes, β1,2 xylosyltransferase (XylT) and core α1,3 fucosyltransferase (FucT), responsible for the attachment of the two epitopes to *N*-glycans. Recently, knock out and RNAi strategies were applied to eliminate or downregulate the expression of endogenous XylT and FucT genes (Koprivova et al. 2004; Strasser et al. 2004; Cox et al. 2006; Strasser, Stadlmann, Schaehs, Stiegler, Quendler, Mach, Gloessl, Weterings, Altmann, Steinkellner, submitted). The efficiency of these procedures enabled the production of monoclonal antibodies with a homogenous mammalian like the *N*-glycan structure lacking xylose and fucose residues (Cox et al. 2006; Schähs et al. 2007; Schuster et al. 2007; Strasser et al. in preparation). IgGs were efficiently expressed and secreted as functional molecules in these plants and, importantly, significantly enhanced ADCC activities of these IgGs were observed (Cox et al. 2006; Schuster et al. 2007). These findings indicate that the elimination of plant-specific *N*-glycan residues, most probably that of core α1,3 fucose, seem to have the same positive effect as the elimination of α1,6-fucose on CHO produced IgGs (Shields et al. 2002; Shinkawa et al. 2003; Ferrara et al. 2006). It remains to be shown, in how far the elimination of nonmammalian glyco-epitopes on PMPs not only prevents unwanted adverse reactions in humans but also contributes to the observed increased efficacy of plant-made antibodies in man.

To conclude, our results emphasize the adverse impact of xylose and core α1,3 fucose present on a plant-derived human monoclonal antibody for the mammalian immune system. Thus, we advocate for a careful consideration of nonmammalian glycosylation on plant-made pharmaceuticals intended for systemic human application. Importantly, these considerations do not hold for topically applied drugs, which may be well tolerated irrespective of the presence of CCDs.

## Material and methods

### *Comparative Western blotting*

Different 2G12 glycoforms (wt-, ΔXT-, ΔFT-, ΔXT/FT-, and CHO-2G12) were separated on 10% SDS–PAGE under reducing conditions (50 ng each). After transferring to nitrocellulose membrane and blocking, the membranes were incubated with different antisera with different specificities: (1) HRP-conjugated goat antihuman IgG (1:50,000; Promega, WI); (2) rabbit anti-HRP (1:15,000; Sigma-Aldrich, MO), (3) rabbit anti-xylose antiserum (1:500) (Jin et al. 2006); and (4) rabbit anti-fucose antiserum (1:500) (Jin et al. 2006). The bound rabbit IgG were detected by HRP-conjugated goat antirabbit IgG (1:100,000; Sigma-Aldrich). The peroxidase detection was carried out with Super Signal West Pico Chemiluminescent Substrate Kits (Pierce, IL).

### *Immunological detection of xylose and core α1,3 fucose on fully assembled wt-2G12*

In order to determine the accessibility of xylose and core α1,3 fucose on fully assembled wt-2G12 a sandwich ELISA was carried out. Leaves from *N. benthamiana* were agroinfiltrated with a 2G12 containing binary construct as described elsewhere (Schähs et al. 2007; Strasser et al. in preparation) to get transient expression. Three days after infiltration proteins were extracted in PBS, pH 7.4 (1 mg/10 μl) and added to antihuman κ chain antibody (1:10,000; Sigma-Aldrich)-coated plates. CHO-2G12 (30 ng 100 μL) and noninfiltrated plant extracts were used as a control. After blocking with PBS buffer containing 0.5% BSA, three rabbit polyclonal antisera against plant carbohydrates were applied: anti-HRP (1:2000; Sigma-Aldrich), xylose and fucose-specific antisera (Jin et al. 2006). For detection plates were incubated with alkaline phosphatase (AP)-conjugated goat antirabbit IgG ($C_H + C_L$) antibody (1:2000, Jackson ImmunoResearch, PA). Visualization was carried out with 50 μl of 0.1% p-nitrophenyl phosphate in 0.1 M diethanolamine, pH 9.8. Optical density at 405 nm was measured on a microplate reader.

*N-Glycan analysis of 2G12 by LC-ESI-MS*

In order to allow a highly reliable identification of glycan structures together with quick and sensitive quantitation, *N*-glycan analysis of 2G12 glycoforms was carried out as LC-ESI-MS of tryptic glycopeptides (De Jaeger et al. 2007; Schähs et al. 2007). Briefly, heavy chain of SDS–PAGE separated purified 2G12 was excised from the gel, *S*-alkylated and digested with trpysin. The tryptic peptides and glycopeptides were trapped on an Aquasil C18 pre-column (30 × 0.32 mm, Thermo Electron) using water as the solvent and then separated on a Biobasic C18 column (100 × 0.18 mm, Thermo Electron) with a gradient from 5 to 50% acetonitrile containing 0.1 formic acid. Positive ions of *m*/*z* from 200 to 2000 were monitored with a Q-TOF Ultima Global mass spectrometer (Waters). Identification and glycoform quantitation were performed on the summed and deconvoluted spectrum of the glycopeptide elution region.

*Immunization of rabbits*

In order to investigate the immunogenicity of wt-2G12, rabbits (young adult female white New Zealand rabbits 2.5–3 kg of body weight) were immunized s.c. and i.m. with 300 μg purified wt-2G12 per rabbit/per dose resuspended in incomplete Freund adjuvant, followed by two boosts with a 3-week interval. Please note that about 30% of wt-2G12 $C_H$ is not glycosylated (Strasser et al. in preparation). As a control 3 × 300 μg CHO-2G12 was injected as described above. A final bleed from each animal was obtained two weeks after the last boost. Preimmune serum was obtained from each rabbit before the initiation of the immunization. The serum was separated by centrifugation at 2000 × *g* at 4°C for 20 min and stored at −20°C until use.

*ELISA and Western blot using rabbit anti-wt 2G12 antiserum*

For ELISA, the plates were coated with 5 μg/mL of naturally occurring glycoproteins: horseradish peroxidase (HRP, $MMXF^3$) (Wuhrer et al. 2004), hemocyanin from *H. pomatia* (MMX and many undefined structures) (van Kuik et al. 1985), and *E. corallodendron* lectin ($MMXF^3$ and other structures) (Ashford et al. 1991) in 50 mM sodium carbonate, pH 9.6, for 60 min at 37°C. After incubation with a serial dilution of rabbit anti-wt-2G12 antiserum (from 1:10 to 1:100), the bound rabbit IgG was detected by AP-conjugated goat antirabbit IgG (Jackson ImmunoResearch) as described above.

For Western blot analysis, different human Tf glycoforms were prepared as previously described (Bencurova et al. 2004) and used for the detection of anti-carbohydrate antibodies in wt-2G12 antiserum. Different Tf glycoforms (500 ng) were subjected to SDS–PAGE (12.5%) under reducing conditions and subsequently transferred to a nitrocellulose membrane. Then the membrane was blocked with TTBS buffer (0.1 M Tris–HCl, pH 7.2, containing 0.1 M NaCl, 2.5 mM $MgCl_2$, and 0.05% Tween 20) containing 0.5% BSA and subsequently incubated with either rabbit anti-wt-2G12 or anti-CHO-2G12 antiserum in a dilution of 1:800. Detection was carried out using HRP-conjugated goat antirabbit IgG as described above.

*IgE immunoblotting and cross-inhibition experiments*

In order to investigate a possible cross-reactivity of allergic serum against plant-derived IgGs, a human IgE immunoblot was carried out. Purified 2G12 glycoforms (wt-, ΔXT-, ΔFT-, ΔXT/FT-, and CHO-2G12, 500 ng each) were subjected to SDS–PAGE (8%) under nonreducing conditions and transferred to a nitrocellulose membrane. After blocking strips were either incubated with serum pool from allergic or from healthy blood donors (1:10 dilution with TTBS buffer, 1 mL) overnight at 4°C. The allergic serum pool was collected from 19 allergic patients with allergy to either birch/grass pollen ($n = 12$) or insect venom ($n = 7$). The nonallergic serum pool was obtained from 10 healthy blood donors. To demonstrate the binding specificity, cross-inhibition with different BSA glyco-conjugates prepared as previously described (Wilson et al. 1998; Jin et al. 2006) was carried out. Allergic serum pool was preincubated with different BSA conjugates (20 μg/mL) bearing a defined *N*-glycan structure at 37°C for 1 h. The bound IgE was detected by AP-conjugated mouse antihuman IgE (mAb G7–26, BD Pharmingen, Heidelberg, Germany) and subsequent staining as described above.

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## Conflict of interest statement

None declared.

## Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; AKP, alkaline phosphatase; CCDs, cross-reactive carbohydrate determinants; HRP, horseradish peroxidase; LC-ESI-MS, liquid-chromatography-electrospray ionization-mass spectrometry; mAbs, monoclonal antibodies; PMPs, plant-made pharmaceuticals; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tf, transferrin.

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