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Review

Definition of immunogenic carbohydrate epitopes⋆

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Carbohydrates are known as sources of immunological cross-reactivity of allergenic significance. In celery and in cypress pollen, the major allergens Api g 5 and Cup a 1 are recognised by antisera raised against anti-horseradish peroxidase and by patients' IgE which apparently bind carbohydrate epitopes; mass spectrometric analysis of the tryptic peptides and of their N-glycans showed the presence of oligosaccharides carrying both xylose and core α1,3-fucose residues. Core α1,3-fucose residues are also a feature of invertebrates: genetic and biochemical studies on the fruitfly *Drosophila melanogaster*, the parasitic trematode *Schistosoma mansoni* and the nematode worm *Caenorhabditis elegans* indicate that these organisms possess core α1,3-fucosyltransferases. Various experiments have shown that fucosyltransferases from both fly and worm are responsible *in vivo* and *in vitro* for the synthesis of N-glycans which cross-react with anti-horseradish peroxidase; thus, we can consider these enzymes as useful tools in generating standard compounds for testing cross-reactive carbohydrate epitopes of allergenic interest.

Keywords: ***Caenorhabditis, Drosophila,*** plant, allergy, cross-reactivity

The role of carbohydrates in allergy has been much discussed in recent years and is still controversial. It is not disputed that patients with pollen, food and venom allergies often have IgE recognising N-glycan epitopes (Fötisch & Vieths, 2001); however, the clinical significance of these antibodies has been doubted (van Ree, 2002). Such lack of clarity only serves to show that suitable reagents are highly necessary in order to study the role of carbohydrates in allergy. Monovalent glycoconjugates, such as free oligosaccharides or glycoproteins such as pineapple bromelain which carry only a single glycan, may be suitable for testing binding of IgE, but are not suitable for studying histamine release, since they are not capable of cross-linking IgE receptors.

The IgE-binding carbohydrates are often N-glycans carrying core xylose and/or core α1,3-fucose; these are features absent in mammals (see Fig. 1) and are, therefore, immunogenic (Wilson, 2002). Core α1,3-fucose is also a feature of the N-glycans of many invertebrates, and antisera raised against N-glycosylated plant and invertebrate proteins, such as horseradish peroxidase, carrot fructosidase or bee venom, contain antibody species recognising these 'foreign' elements. Also there is immunological cross-reactivity between plant and insect glycoproteins; i.e., an antibody raised against a plant glycoprotein (e.g., horseradish peroxidase) can recognise insect glycoproteins (e.g., bee venom) and *vice versa* (Prenner *et al.*, 1992; Wilson *et al.*, 1998). Anti-horseradish peroxidase is probably the most widely used antiserum for detecting the presence of core xylose and/or core α1,3-fucose and for about twenty years it has been used to track neuronal pathways in invertebrates (Jan & Jan, 1982; Snow *et al.*, 1987; Siddiqui & Culotti, 1991; Haase *et al.*, 2001); however, only our recent studies have shown directly the role of core α1,3-fucose as the key cross-reactive determinant in the case of *Drosophila* (Fabini *et al.*, 2001).

It has also become clear that carbohydrates are a factor in parasitic diseases; nematode N-glycans and glycolipids sometimes carry phosphorylcholine, a component also present on the surfaces of some pathogenic bacteria, and appear to be immunomodulatory (Harnett & Harnett, 2001) — in this case, the phosphorylcholine may be acting as

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a "Tarnhelm", which, as in the Nibelungen legend, could confer invisibility on its bearer. Interestingly, too, insect glycolipids carry a related structure, phosphorylethanolamine (Seppo *et al.*, 2000), which is also present in some bacteria; however, although antibodies can be raised against this structure, no other immunological data is available. Furthermore, IgE recognising core α1,3-fucose has been detected in parasite-infected sheep (van Die *et al.*, 1999), whereas fucose-containing glycans may have a role in the TH2 response towards nematode proteins (Tawill *et al.*, 2004).

The biotechnological repercussions of the immunogenicity of these structures are many-fold. First, the use of insect cell or whole plant systems for the expression of therapeutic glycopharmaceuticals needs to be carefully considered, so that systems that either naturally do not express core xylose and/or core α1,3-fucose are chosen, or that the ability to express these epitopes is repressed. Secondly, the use of recombinant enzymes allows us for the first time to generate defined reagents with properties suitable for the adequate testing of antibody binding and immunological activity, as has been performed with recombinant *Arabidopsis* core xylosyl- and fucosyltransferases (Bencúrová *et al.*, 2004). Thirdly, using genetic approaches, we can now start to consider the role of core α1,3-fucose in invertebrate biology.

In our studies, we have examined core α1,3-fucosylation in plants and invertebrates using a variety of techniques: use of neoglycoconjugates, glycan analysis, expression of recombinant enzymes and DNA/RNA-based manipulation of fucosylation. These results pave the way for a better understanding of the biological and immunological significance of glycosylation.

ANTI-CARBOHYDRATE EPITOPES OF PLANT GLYCOPROTEINS

Celery is considered to be one of the major sources of food allergy and a number of celery proteins have been shown to bind IgE from patients' sera. Some of this binding can be inhibited by preoxidising the proteins with periodate, an indication that carbohydrate chains may be the epitopes for patients' IgE (Bublin *et al.*, 2003). To study this more specifically, patients' sera were preincubated with neoglycoconjugates: either bovine serum albumin carrying bromelain glycopeptides (called BSA-MUXF^3) or bovine serum albumin carrying the pentasaccharide core structure common to eukaryotic N-glycans (so-called BSA-MM). Indeed, the former, and not the latter, glycoconjugate prevented binding of IgE, in a manner similar to that mediated by intact native Api g 5 allergen. This suggested that Api g 5 may carry N-glycans with the MUXF^3 structure. Indeed, MALDI-TOF MS analysis verified that celery Api g 5 carries N-glycans with the probable compositions MUXF^3 and MMXF^3 (Bublin *et al.*, 2003). It was therefore concluded that the binding of IgE to Api g 5 is due to the presence of glycans carrying core xylose and α1,3-fucose.

In another study, the glycans of a cypress pollen allergen were investigated. Allergy to cypress pollen is common in Mediterranean countries and the binding of patients' IgE is often also sensitive to prior periodate oxidation (Afferni *et al.*, 1999). Purified Cup a 1 was examined by Western blotting, using anti-horseradish peroxidase as the primary antibody. The antibody was preincubated in the presence and absence of various concentrations of the aforementioned BSA-MUXF^3 as well as

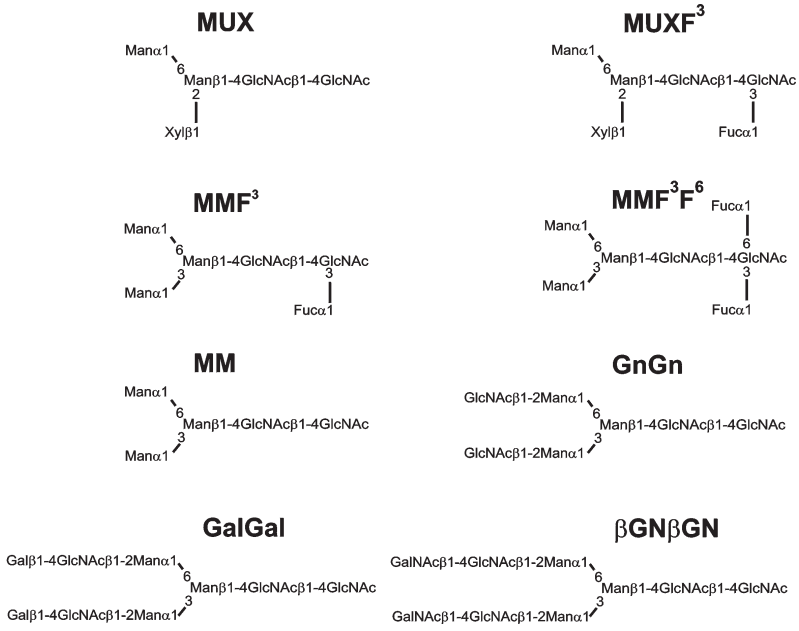

Figure 1. N-glycans referred to in this review.

a defucosylated variant BSA-MUX (Fig. 2). The results showed a strong inhibition, particularly with the BSA-MUXF^3 conjugate, suggesting that the allergen carries core α1,3-fucosylated N-glycans; this was compatible with a previously-published MALDI-TOF MS N-glycan analysis (Alisi *et al.*, 2001).

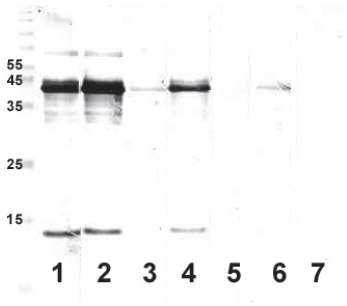

Figure 2. Binding of anti-horseradish peroxidase to cypress pollen Cup a 1 allergen.
Purified Cup a 1 (M_r 38000) was subjected to Western blotting with anti-horseradish peroxidase (1:50000) pre-incubated with either no inhibitor (1), 0.05 μM BSA-MUX (2), 0.05 μM BSA-MUXF^3 (3), 0.5 μM BSA-MUX (4), 0.5 μM BSA-MUXF^3 (5), 5 μM BSA-MUX (6) or 5 μM BSA-MUXF^3 (7). Alkaline-phosphatase conjugated anti-rabbit IgG was used for detection. Concentration-dependent inhibition was observed.

ANTI-CARBOHYDRATE EPITOPES OF INVERTEBRATE GLYCOPROTEINS

Using similar methods, N-glycan analysis and antibody inhibition assays, we began to examine the origin of anti-horseradish peroxidase cross-reactivity towards invertebrate glycoproteins. Glycan analyses of both *Caenorhabditis* and *Drosophila* indicated the presence of difucosylated N-glycans with probable MMF^{3}F^6-type structures (Fabini *et al.*, 2001; Haslam & Dell, 2003). Indeed, in the case of the *Drosophila* N-glycans, the presence of these glycans was also determined by RP-HPLC in conjunction with exoglycosidase digestion. Interestingly, the nematode has a far more complicated N-glycan spectrum than the fruitfly, even though one would expect a fly to be more complex than a small 'worm' which, however, has many more genes. Preincubation of anti-horseradish peroxidase with BSA-MUXF^3 inhibited binding to *Caenorhabditis* extracts in Western blots or to *Drosophila* embryonal neural tissue (Fabini *et al.*, 2001). BSA-MUX, however, was relatively ineffective as an inhibitor, a result compatible with the presence of core α1,3-fucose, and the absence of core xylose, in these species.

Our work with *Caenorhabditis* was expedited by the availability of fucosyltransferase mutants: we were able to find one mutant which did not display anti-horseradish peroxidase reactivity and which lacked certain fucosylated N-glycans (Paschinger *et al.*, 2004). This mutant (VC378) has a deletion in the *fut-1* (K08F8.3) gene. Therefore, we expressed the cDNA in both *Pichia pastoris* and in *Drosophila* Schneider S2 cells. In the case of the yeast expression system, the activity of the recombinant enzyme was tested with various N-glycan substrates (Fig. 3) and only activity towards MM was detected; with the Schneider cells system, it was shown that transfection of *fut-1* cDNA conferred ectopic expression of the anti-horseradish peroxidase epitope. The recombinant FUT-1 can also be used to re-create the anti-horseradish peroxidase epitope *in vitro* (Paschinger *et al.*, 2004). Similar studies with *Drosophila* suggested that FucTA (CG6869) gene was the fucosyltransferase responsible for the biosynthesis of this epitope in the fruitfly (Fabini *et al.*, 2001).

In contrast to the nematode and the fly, the trematode worm *Schistosoma mansoni* expresses glycoconjugates containing both core xylose and core α1,3-fucose (Khoo *et al.*, 1997). A previous study showed that we could detect both xylosyl- and fucosyltransferase activities that modify N-glycan substrates in schistosome egg extracts (Faveeuw *et al.*, 2003). Therefore, it was of no surprise to see that anti-horseradish peroxidase binding to glycoproteins in schistosome extracts was inhibited by both BSA-MUXF^3 and BSA-MUX (K. Paschinger, unpublished). However, the genes responsible for the presence of

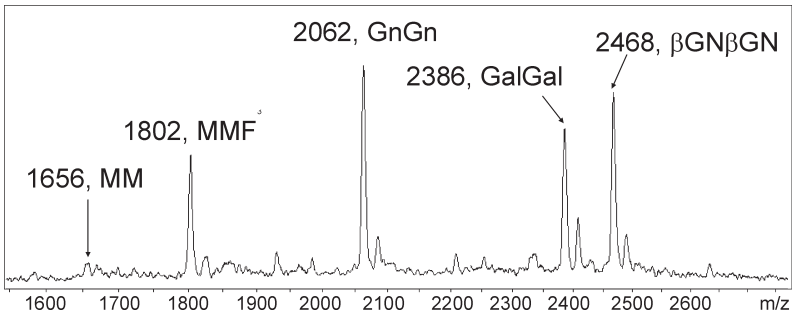

Figure 3. Assay of recombinant *Caenorhabditis* FUT-1 with N-glycans.
Recombinant yeast-expressed FUT-1 was incubated for 17 h at room temperature with a variety of dabsyl-glycopeptide substrates in the presence of GDP-fucose. The incubation was then analysed by MALDI-TOF MS. Only the MM substrate was converted to its fucosylated derivative. For an explanation of the N-glycan nomenclature, see Fig. 1.

these epitopes in this parasite are yet to be identified.

CONCLUSION

In our studies on plant and invertebrate glycosylation, we have dissected to some extent the origins of inter-species immunological cross-reactivity. A mixture of biochemical, analytical and genetic-based tools have been used and we are now at the threshold of being able to pursue the use of these in studies to uncover the wider biological and allergological significance of carbohydrate epitopes; results which will also have a bearing on the use of non-mammalian expression systems in biotechnology.

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