

The Role of Protein Glycosylation in Allergy

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Key Words

Allergens, clinically irrelevant · Carbohydrate epitopes · Cross-reactive carbohydrate determinant · Glycoprotein · N-glycosylation · O-/N-linked glycans

Abstract

The asparagine-linked carbohydrate moieties of plant and insect glycoproteins are the most abundant environmental immune determinants. They are the structural basis of what is known as cross-reactive carbohydrate determinants (CCDs). Despite some structural variation, the two main motifs are the xylose and the core-3-linked fucose, which form the essential part of two independent epitopes. Plants contain both epitopes, insect glycoproteins only fucose. These epitopes and other fucosylated determinants are also found in helminth parasites where they exert remarkable immunomodulatory effects. About 20% or more of allergic patients generate specific anti-glycan IgE, which is often accompanied by IgG. Even though antibody-binding glycoproteins are widespread in pollens, foods and insect venoms, CCDs do not appear to cause clinical symptoms in most, if not all patients. When IgE binding is solely due to CCDs, a glycoprotein allergen thus can be rated as clinical irrelevant allergen. Low binding affinity between IgE and plant N-glycans now drops out as a plausible explanation for the benign nature of CCDs. This rather may result from blocking antibodies induced by an incidental 'immune therapy' ('glyco-specific immune therapy') exerted by everyday contact with plant ma-

terials, e.g. fruits or vegetables. The need to detect and suppress anti-CCD IgE without interference from peptide epitopes can be best met by artificial glycoprotein allergens. Hydroxyproline-linked arabinose (single β -arabinofuranosyl residues) has been identified as a new IgE-binding carbohydrate epitope in the major mugwort allergen. However, currently the occurrence of this O-glycan determinant appears to be rather restricted. Copyright © 2007 S. Karger AG, Basel

Introduction

The word carbohydrate is a potent stop signal for the 'readosome' of a typical allergist. This is unfortunate because carbohydrate determinants are almost certainly the most frequently encountered individual epitope structures for IgE. They have therefore been baptized cross-reactive carbohydrate determinants (CCDs) [1]. Fortunately for patients who have developed IgE against CCDs, the clinical effect of these antibodies appears to be negligible in most cases [2–5]. At first instance, the carbohydrate nature of CCDs appears to hold a simple explanation for this phenomenon, but for the impartial biological chemist, carbohydrates are not by nature molecules of minor significance. This review deals with (i) the history and current status of the knowledge on the structure(s) of CCDs in plants, insects and also parasites; (ii) the specificity of animal and human anti-CCD anti-

bodies; (iii) the current view of the clinical significance of CCDs and anti-CCD IgE and possible reasons for the innocuous nature of carbohydrate epitopes; (iv) strategies for the improvement of in vitro measurement of IgE by recognition and inhibition of anti-CCD IgE, and (v) the new but small area of O-glycan epitopes on plant allergens.

The review is confined to protein-linked glycans of known structure. The possibly large and interesting, but to date rudimentary, area of fungal glyco-allergens has been touched elsewhere [6] and will not be presented here.

The Malaise of Allergy Diagnosis by in vitro Measurement of IgE

It is common ground that type I hypersensitivities (in simple terms: most phenomena usually understood as allergy) are caused by immunoglobulins of class E [7]. Depending on the eliciting agent, IgE from different patients binds to very different antigens, then called allergens. Remarkably, and not untypical for the current status of this science, the term allergen equally means a single type of molecule containing the IgE-binding determinants as well as the natural package of various molecules and structures, e.g. venoms, pollens or hair, which may then contain several allergens in the molecular sense. On scientific grounds, to support a patient's anamnesis, type I allergies are diagnosed by skin prick testing (SPT) or by in vitro determination of allergen-specific IgE, as far as methods are concerned. The in vitro method has the advantages of being less time consuming (at least for the patient), less painful and of allowing a larger number of allergens to be screened. In theory, reaction of a patient's IgE with a certain allergen extract points to sensitization towards this allergen. The concentration of IgE indicates the severity of allergic symptoms to be expected on contact with the allergen. This prognostic value is of importance especially for allergies having life-threatening potential, e.g. insect venom or peanut allergy. Patients with a high radioallergosorbent test (RAST) class towards bee or wasp venom are advised to always carry with them an emergency set to rescue them in the event of an insect sting.

Unfortunately, the correlation between specific IgE (sIgE) levels and the severity of real clinical symptoms is remarkably loose. On the one side, in vitro tests may fail to detect sIgE and thereby overlook sensitization towards the allergen. This has been observed in the diagnosis of

food allergies, e.g. against apple and carrot [8, 9], and the reasons may be quite trivial ones such as low stability of the allergen or varying concentrations in the raw material used for extraction. Standardization of allergen extracts for prick testing and serum IgE determination is therefore a challenging and serious task [10]. On the other side, the observation has been made that the mere binding of IgE to components of an allergen extract does not always correlate with the clinical diagnosis obtained by SPT or even by more physiological tests such as a double-blind placebo-controlled food challenge [2, 3, 11, 12]. In this review, we will show that >20% of allergic patients have IgE that binds to carbohydrate compounds, which are essentially unable to elicit clinical symptoms. These supposedly clinically irrelevant carbohydrate determinants compromise the significance of serum IgE determinations. In vitro reactivity with protein panallergens such as profilin likewise cannot always be taken as an unambiguous indication of the clinical role of carbohydrate determinants [4, 13–15].

A Short History of CCDs

It all began in the 1970s when a Japanese group elucidated the strange structure of a protease from pineapple [16]. Later, it was confirmed that this bromelain (Brl) contained an oligosaccharide with two structural features, which had not been found in mammalian glycoproteins, i.e. core α 1,3-fucose and xylose (fig. 1) [17]. Of note, bromelain is a special case as glycans with three mannose residues are more usually found in plant glycoproteins, e.g. on horseradish peroxidase (HRP; fig. 1) [18–21].

The link between plant glyco-biology and allergy was made in 1981 by some short but pioneering work from Aalberse et al. [1]. They showed that IgE from patients' sera cross-reacted with extracts from various allergenic foods as well as with insect venoms, but treating the extracts with periodate abolished the reaction. Conclusions from periodate oxidation experiments must be drawn with caution as this harsh treatment can also destroy peptide epitopes [22]. However, their finding was corroborated by later reports. The first one showed N-glycans were involved in the IgE binding to honeybee venom phospholipase A₂ (PLA₂; Api m 1) [23]. Aalberse et al. [1] had already perceived a connection between reactivity to plant CCDs and a history of insect stings. The structural basis for the cross-reaction of IgE with insect and plant glycoproteins only became evident once the structural particularities of insect N-glycans, i.e. the presence of a

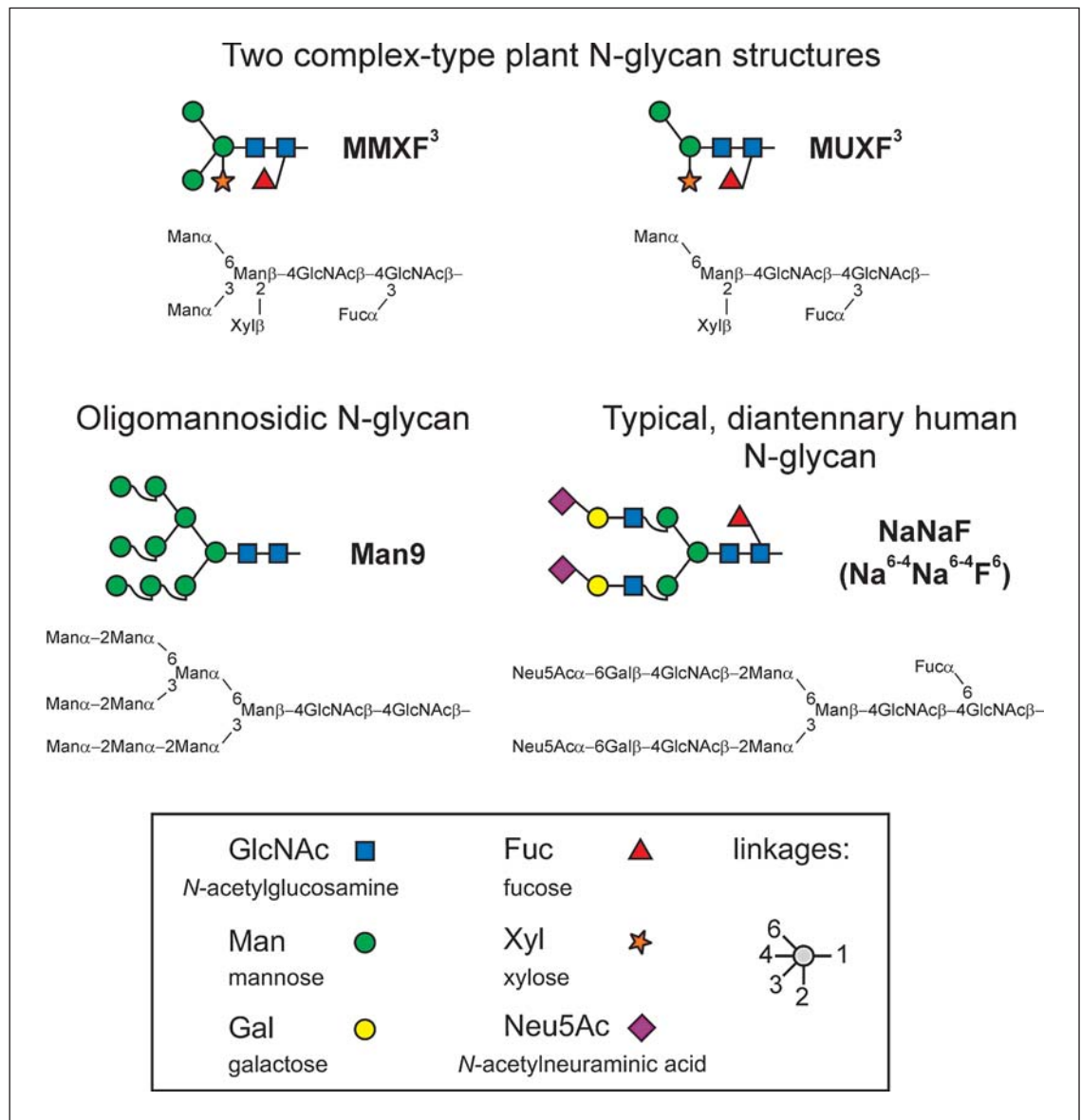


Fig. 1. Comparison of plant and mammalian N-glycans. The oligosaccharide structures are shown in the symbolic depiction suggested by the Consortium of Functional Glycomics (www.functionalglycomics.org) with the additional information about linkage positions. The structure codes comply with the proglycan system (www.proglycan.com). The upper two structures MMXF³ and MUXF³ represent typical complex-type plant N-glycans, e.g. HRP or bromelain, respectively. The Man₉ structure is one example of the series of oligomannose-type glycans Man₅ to Man₉ (Man₅GlcNAc₂ to Man₉GlcNAc₂), which occur in all eukaryotic organisms. The glycan NaNaF constitutes a typical mammalian complex-type N-glycan with sialic acids. Note the different position of the fucose residues.

core α 1,3-fucose just like in plants [24], had been revealed. In sera from patients allergic to bee venom, glycopeptides (GPs) made from pineapple stem Brl were shown to inhibit IgE from binding to Api m 1 [24]. As the peptide moieties of such GPs comprise only two to four

amino acids at maximum, they hardly constitute epitopes on their own. The conclusiveness of this approach appears much stronger than that of destructive methods such as periodate oxidation or chemical deglycosylation. In the succeeding years, an ever growing number of pa-

pers reported that anti-CCD IgE was involved in in vitro reactivity of patients' sera to a wide variety of allergens.

Asn-Linked Oligosaccharides in Plants

Different taxonomic groups are distinguished by different structural features of their Asn-linked glycans (N-glycans) as well as their ability to generate certain types of O-glycans, which will be dealt with in a separate chapter. The biosynthesis of N-glycans is textbook knowledge allowing us to start with the assertion that the first steps of protein N-glycosylation are essentially conserved in all eukaryotic organisms [25, 26]. The first large phylogenetic group to leave the common trail are fungi, which lack the enzymes that lead to complex-type glycans, e.g. GlcNAc transferase I – at least, this holds true for yeasts [27]. In plants and animals, the Man5 structure is processed by GlcNAc transferase I and subsequently Golgi α -mannosidase (often referred to as α -mannosidase II) and GlcNAc transferase II. But already at this stage the 'higher animals', i.e. the deuterostomia, begin to deviate from the taxonomic protostomia (e.g. insects), acoelomata (e.g. *Caenorhabditis elegans* and many parasitic worms) and noteworthy plants [28].

The term plant must be restricted again, as except from the moss *Physcomitrella patens* [29], all other plants whose N-glycan structures have been investigated belong to the *Tracheophyta* (vascular plant). However, within these taxonomic limitations, we can claim that all plant species have the same spectrum of N-glycan structures, certainly the angio- and gymnosperms [20], but also ferns and horsetails [Altmann, unpubl. results]. The most important differences from human N-glycans are: (1) the core α 1,3-fucose, i.e. the fucosylation of the innermost GlcNAc at position 3; (2) the xylosylation of the β -mannosyl residue, and (3) the 'premature' termination of the antennae with mannose, GlcNAc or galactose residues instead of sialic acids (fig. 2). A further feature of certain plant glycoproteins is the occurrence of Lewis a determinants, which are branched, terminal trisaccharides made of galactose, fucose and N-acetylglucosamine (fig. 2). Lewis structures are also found in mammals, albeit usually together with sialic acid.

Plants have not been very inventive with their glycan structures. Essentially every plant and plant material contains the entire spectrum of N-glycans but with large quantitative differences [20, 30]. The very large Lewis a type structures are rarely found on soluble allergens and are currently thought to play only a minor, if any, role in

IgE binding even though many plant foods (apple, banana, celery, onion, orange, pear, and strawberry) contain large amounts of these near-mammalian N-glycans [20]. The cedar pollen allergens Cry j 1 and Jun a 1 are the only allergens known to contain Lewis a antennae [31, 32]. Complex-type glycans without terminal galactose/fucose comprise the most abundant species in most (food) plants. For isolated allergen proteins, the prototypical N-glycan structure is the so-called MMXF³ (fig. 1). This structure occurs so frequently on plant proteins that trying to list all known carriers would be futile. Examples among non-allergens are HRP [18] and many lectins [33, 34], and among allergens, Phl p 1, Phl p 13 [35], Lyc e 2 [36] Hev b 4 [37], Lol p 11 [38], and Api g 5 [39]. Tree pollens [30] and tree pollen allergens [39] tend to bear terminal non-reducing GlcNAc residues, a feature which may reduce IgE binding (see paragraph on specificity of IgG). Olive pollen Ole e 1, hazelnut Cor a 11 and peanut Ara h 1 differ by essentially lacking the core fucosyl residue [38, 40, 41]. In contrast, Cyn d 24 and other glycoproteins from Bermuda grass pollen carry non-xylosylated glycans, mainly MMF³ (fig. 2) [42, 43].

Important to understand is that the nature of the protein to which such sugar chains are attached is irrelevant, comparable to a traffic light for which it hardly matters where and how exactly it is mounted. Having said this, cases where the protein moiety may affect presentation of carbohydrate determinants can admittedly be found. An example is phospholipase, the major protein of honeybee venom (see also next paragraph). In immunoblots with anti-CCD sera, the much less abundant hyaluronidase stains stronger [44]. The glycan profiles of these allergens hardly pose an explanation for this difference [45]. To stay with the above picture, the traffic light could be shielded by the branch of a tree.

Asn-Linked Oligosaccharides (N-Glycans) of Insects

Core α 1,3-fucose is regularly found in insect glycoproteins, e.g. in honeybee venom PLA₂ [46, 47] and hyaluronidase [40, 45], and in yellow jacket venom hyaluronidases [48]. Most often it is accompanied by a second, α 1,6-linked fucose (fig. 2). Apart from insect venoms, this cross-reactive structure is found in neuronal cells of insects [18, 28]. Xylosylation has never been found in insect glycoproteins. Proteins from house dust mites, despite their phylogenetic relationship, are probably not core 3-fucosylated as they fail to bind rabbit anti-CCD antibodies [19].

Asn-Linked Oligosaccharides of Snails and Parasitic Worms

Immunogenic glycans with both xylose and core α 1,3-fucose have been found in parasitic worms (helminths) such as *Schistosoma mansoni* and *S. japonica* or *Haemonchus contortus* (fig. 2) [49–53]. Given the considerable problems associated with the analysis of glycoproteins from such sources, a large number of unknown cases can be expected. Core α 1,3-fucosylated glycans have not been found in cercaria of *S. mansoni* [49], which may point to a stage- and/or tissue-specific expression of this determinant. Snail glycoproteins have been shown to contain xylose residue [54–56]. Although the relevant fucosyltransferase could be detected in the snail *Lymnea stagnalis* [57], only traces of core α 1,3-fucosylated glycans could hitherto be found in snails [55].

It is crucial to appreciate that parasitic helminths, e.g. schistosomes, express additional glyco-antigens on the non-reducing side of N-glycans as well as O-glycans [50, 58–63]. Apart from the well-known Lewis x structure, several determinants have been found with GalNAc-containing LacdiNAc antennae instead of the usual Gal-containing LacNAc antennae [reviewed in ref. 58, 59]. LacdiNAc structures may be fucosylated or difucosylated and they may form repeats. Even substitution of fucosyl residue by fucose has been found [63, 64]. Remarkably, some of these glyco-determinants also appear in mollusks, which are phylogenetically quite distant to helminths [60, 65]. These – for mammals – highly unusual structures elicit generation of antibodies of all kinds of classes [58, 66] and they induce granuloma formation [60]. Most importantly, they skew the host immune system towards a Th2 response [66–70]. At the same time, the course of the Th2 response is attenuated by down-regulatory cytokines (e.g. IL-10) [58, 59, 66, 71].

Sheep infected with parasites generate mainly IgE against glyco-determinants, some of which are also found in plant and insect glycoproteins [52]. Nevertheless, the immune response to such parasites does not resemble allergic hypersensitivity. On the contrary, there is evidence that a history of infection by parasitic worms strongly reduces the allergy risk by restraining the Th2 responses against allergens [59, 72]. Parasite glycoconjugates have not yet been directly associated with allergy. However, one could meditate if the immunomodulatory nature of parasite glycans on the one hand and the apparently benign conduct of glyco-allergens on the other are purely coincidental phenomena.

Specificity and Affinity of IgG Raised against Plant Glycoproteins

Despite the long history of carbohydrates as immunogens – just think of blood group antigens or bacterial polysaccharides (used for example in the *Haemophilus influenzae* type b vaccine) – allergologists originally met the idea of anti-glycan antibodies of the IgE class with considerable skepticism if not sheer rejection. Differences in serum reactivity between glycosylated and non-glycosylated allergens were discounted as changes in protein structure induced by the glycans or just as another kind of non-specific binding. Therefore, the thorough characterization of the epitope structure recognized by rabbit, goat or rat antibodies was an important prerequisite for the appreciation of plant N-glycans as specific antibody determinants on their own, both for IgG and IgE.

Concomitant with the first observations on the immunogenicity of plant N-glycans, conclusions about the chemical nature of the carbohydrate epitope were drawn based on the structural difference between plant and human glycans. At first, the xylose residues were implicated as the essential element [73, 74]. Detailed work on the HRP epitope corroborated this finding and added the core α 1,3-linked fucose as a second antibody-binding element [18]. With the discovery of core α 1,3 fucose in insect glycoproteins [45, 46], it became apparent that this widely distributed residue is involved in cross-reactions between plant and insect glycoproteins. Using different forms of Brl-GPs and oligosaccharides, it could be shown that only reducing glycans or GPs – but not reductively aminated or reduced oligosaccharides – inhibit rabbit anti-HRP from binding to various glycoproteins including honeybee venom PLA (Api m 1) [24]. Besides, 0.3 μ M GP was required to achieve 50% inhibition of antibody binding [24]. At about that time, rabbit serum raised against carrot β -fructosidase or HRP was successfully fractionated into a 'fucose-specific' and a 'xylose-specific' fraction by affinity chromatography on immobilized PLA [75]. This work re-introduced xylose as a CCD. Interestingly, later work on the cross-reaction of anti-HRP and of a rat monoclonal antibody did not indicate any substantial contribution of xylose [19, 76]. The conclusions on the structure of the CCD were drawn from inhibition experiments with Brl-GP or defucosylated Brl-GP. A reason for the inconsistent findings about the role of xylose may be that Brl contains MUXF³ (or simpler: MUXF), a truncated version lacking one mannose residue, instead of the more common MMXF³ (or MMXF) structure (fig. 1). In fact, MUXF was recently found to be

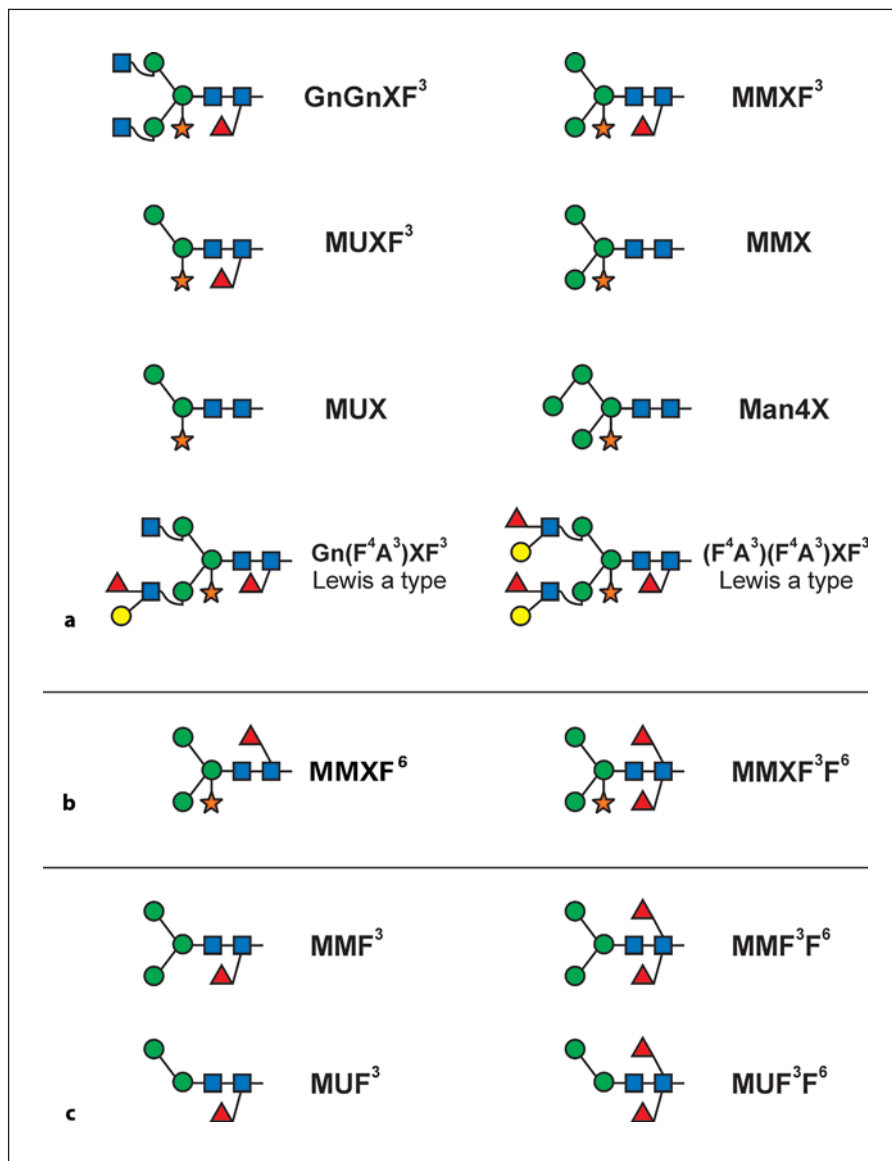


Fig. 2. Glycans of plants, insects and parasites. **a** Some complex-type N-glycans as found in plants. The lack of terminal GlcNAc or Lewis a determinants of either of the two antennae generates additional combinations. The non-xylosylated MMF³ glycan found in Bermuda grass [42] is shown in **c**. **b** Examples of N-glycans found in snails and parasitic helminths. **c** Typical core 3-fucosylated N-glycans from insect venoms.

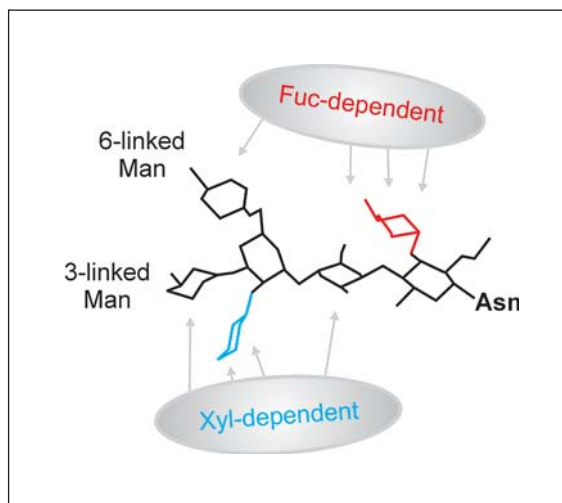


Fig. 3. Model of a CCD. The cartoon depicting the 3D structure of the most abundant CCD N-glycan MMXF³ was emulated from Bouwstra et al. [79]. It shows that the core α 1,3-fucose (shown in red) and the 6-linked mannose lie on the same side of the glycan whereas the xylose (in blue) is located at the opposite side. Thus xylose and fucose form independent epitopes. The cartoon shall, however, not be understood the way that the two types of antibody can bind simultaneously to the same molecule.

a poorer ligand for Xyl-dependent antibodies than MMXF [77]. In this work, recombinant xylosyl-transferase and core α 3-fucosyl-transferase were used to 'plantify' human apotransferrin that had been treated with glycosidases to carry GnGn oligosaccharides, which serve as substrate for the glycosyltransferases [77]. No hint of a comparable influence of the presence or absence of the α 3-linked mannose on the binding of fucose-dependent antibodies has been found. The different dependence of the xylose and the fucose epitopes on α 3-mannose may be explicable by the different orientation of xylose and α 3-fucose as found in NMR-based modeling studies of the N-glycan of Brl [78, 79].

Another important finding was that efficient binding of anti-HRP, for example, to re-modelled transferrin was only obtained after removal of the GlcNAc-residues at the non-reducing termini, i.e. after the conversion of GnGnF and GnGnX to MMF and MMX, respectively [77]. This means, that terminal substituents shield the Fuc epitopes and therefore larger plant N-glycans may not function as CCD despite their content of Xyl and Fuc. Exceptions to this rule may however be found as immunization with MMX glycoprotein, which also contained some GnGnX, elicited antibodies able to bind to GnGnX [80].

Taken together, recent work produced conclusive evidence that plant N-glycans harbor two essentially independent epitopes, one containing core α 1,3-fucose and the other one the β 1,2-linked xylose (fig. 3) [75, 77, 80]. Since in both cases other parts of the oligosaccharide confer individually weak, but in concert important, contributions to the binding of antibodies [18, 77], such anti-CCD immunoglobulins should not be termed Fuc or Xyl specific but rather Fuc or Xyl dependant. Also, there may be differences in binding strength of similar N-glycans to the same serum as well as small differences in the substrate specificity of antibody populations. However, apart from the above example with MMX, MUX and GnGnX (with or without Fuc), no such differences have been demonstrated.

Species-Related Aspects of the Immune Response

Some of the above-mentioned studies were probably done on the implicit understanding that what is found for rabbit antibodies would also hold true for human IgG and, hopefully, even IgE. There is, however, no guarantee of such a similarity. For example the murine immune system behaves very different from that of rabbits [77, 81]. Although a monoclonal antibody against *Cupressus arizonica* glycoproteins could be generated from Balb/c mice [82], this strain is usually found not to react measurably

against the glycans when immunized with plant glycoproteins [81]. Raising anti-CCD antibodies and generating hybridoma cell lines that produce monoclonal antibodies is apparently easier in C57BL/6 mice and rats than elsewhere [19, 81–85]. However, as far as analyzed in detail, these murine antibodies cannot discriminate different truncated complex-type ('paucimannosidic') N-glycans such as MM, MMF³ or MMX [77, 80, 85]. Human IgE, however, has proved able to discriminate MMF³ from MM or MMX [77]. A similar picture can be seen in a study with non-allergic people, where 25% exhibited IgG binding to honeybee PLA (core α 3-fucosylated) whereas 50% reacted with *Helix pomatia* hemocyanin, which is known to contain xylose but also a panel of other immunogenic carbohydrate determinants [65, 81, 86].

Anti-CCD IgE in Allergic Patients

With growing awareness among allergologists of the existence of IgE-binding carbohydrates, the number of papers implicating glycans as part of an allergen is steadily increasing. Such IgE binding has been observed with insect venoms, grass and tree pollens and foods [2, 11, 35, 36, 38, 39, 44, 76, 87–104]. Possibly the first work measuring anti-CCD IgE in individual sera was on a panel of patients allergic to bee venom [24]. From 122 sera, 28% bound to GPs from bee venom PLA and from Brl, which were coupled to bovine serum albumin (BSA; fig. 4). Only two sera also reacted with a pentasaccharide core devoid of xylose and fucose [24]. The anti-CCD IgE from many of the bee-venom-allergic patients could be inhibited by GPs prepared from pineapple Brl, but not if the GPs had been defucosylated [24]. A similar percentage of CCD reactivity was found in a large study on patients allergic to insect venom [5]. Here, CCD-positive patients were detected on the basis of a positive CAP test with Brl and negative SPT with different pollens. Thus, as admitted by the authors, the 16% of CCD-positive patients did not include all those who had anti-glycan IgE and at the same time a SPT reaction with pollens – regardless of which allergen molecule had caused the reaction [5]. In studies of carrot and celery allergens, 45–55% of the sera were CCD positive when tested with Brl-GPs [11, 105]. Using proteinase-K-digested grass pollen extract as the CCD source, van der Veen et al. [2] showed that one third of 32 patients allergic to grass pollen exhibited anti-CCD IgE, which was responsible for reactivity with peanut in vitro.

The major work on CCD reactivity is that of Mari [12], where a large cohort of allergic patients who showed reactivity to Brl in vitro was challenged with Brl by SPT. In a CAP assay, 23% of the sera from 1,831 patients showed IgE to Brl. The prevalence of CCD reactivity somehow correlated with the number of pollens with which the patients gave a positive SPT. However, almost none of the patients had a positive SPT against Brl (see also next paragraph).

The specificity of human IgE may at first be considered similar to that of rabbit IgG. This can be deduced from studies where Brl-GPs and their defucosylated form were used to inhibit IgE binding to plant glycoproteins [24, 77] and from a recent study with biosynthetic glycoallergens [77]. Interestingly, only Fuc-dependant IgE could be detected [77], which may just reflect a coincidental bias in serum selection or the postulated role of insect stings as elicitors of anti-CCD IgE [2, 5].

In conclusion, many allergic patients develop specific IgE directed against plant/insect protein-linked glycans. This anti-CCD IgE will lead to 'false-positive' results in in vitro testing whenever natural allergen extracts are used [3, 5, 6, 72, 77].

On the Clinical (In)Significance of anti-CCD IgE

Any patient with anti-CCD IgE would be a very poor creature if glycan epitopes were to trigger severe clinical symptoms like notoriously strong allergens, e.g. Ara h 1 and 2, Bet v 1 or Api m 1, do. Obviously, and fortunately, this is not the case. Several recent studies make a clear point about the clinical insignificance of carbohydrate determinants [2, 4, 5, 12, 38, 106, 107]. In these studies, the discrepancies between RAST/CAP and SPT results or other in vivo tests formed the basis of the conclusion. Using proteinase-K-digested grass pollen as the CCD source, van der Veen et al. [2] observed that about one third of patients allergic to grass pollen had a positive RAST with peanut extract but a negative SPT with the same extract or with isolated Ara h 1 or 'Ara h 2'. [From the sequence given, it is rather Ara h 6 (Q9SQG5) than what is now termed Ara h 2 (Q8GV20). For both allergens, the glycoprotein nature is not evident.] The in vitro reactivity was shown to be due to CCD epitopes. As Ara h 1 is monovalent, the soundness of this rationale is arguable. However, a later study by Mari [12] who selected over 1,000 sera with positive CAP against Brl corroborated the observation. The patients from whom the sera had been taken were challenged with three monovalent allergens, i.e. Brl,

ascorbate oxidase (P37064) and honeybee phospholipase and the multivalent glycoprotein HRP. In accordance with the theory that cross-linking of IgE receptors on mast cells triggers an allergic response, only HRP elicited a positive SPT in at least 21% of the patients. The response, which may or may not have actually been caused by the glycans, hardly ever exceeded SPT class II, which is below the threshold above which people visit their doctor [12]. Not to be overlooked, almost 80% of the patients did not even react with the polyvalent HRP. As a side note, a few patients did react with phospholipase but probably because of a protein-directed sensitization. Thus, as long as HRP is not convicted of being a particularly poor elicitor for whichever reasons, these results score a clear point against a clinical significance of N-glycans.

Kochuyt et al. [5], who concentrated on insect-sting-allergic patients, found that allergic patients who were CCD positive to insect stings did not react towards grass pollens in a nasal provocation test. Similar results were found for the glycoproteins orange germin-like protein (Cit s 1) and latex Hev b 2 [95, 96]. Also, the carbohydrate of vicilin (mainly MMX) was found not to contribute to eliciting symptoms [41]. Because of this clinical insignificance, CCD epitopes and in many cases also profilin can be seen as 'mimickers of allergy' [4].

Degranulation by Glyco-Allergens

These observations contrast with several reports on histamine release by glycoproteins and anti-CCD IgE. One of the first of these papers compared the histamine release capabilities of Brl-GPs coupled to BSA (Brl-BSA; fig. 4), defucosylated Brl-BSA, rBet v 1, and celery extract were compared [101]. The concentration of Brl-BSA required to release histamine was about 10 times higher than that of Bet v 1, whereas the celery extract concentration required was up to 1,000 times higher. In other words, natural extracts may be a rather poor source of polyvalent CCD allergens and their comparison with pure protein allergens may be misleading. In a study on natural and recombinant Cup a 1 in several patients, the concentration dependence curves were very similar [92]. Studies with tomato allergens, HRP and Brl-BSA conjugate also favor the idea of a similar potency of CCD [36, 108]. Other studies have not emphasized the quantitative aspect so much [35, 39, 89]. In the case of the olive allergen work, it should be added that deglycosylation by PNGase F cannot be expected to be successful because of the enzyme's restricted substrated specificity and hence the interpretation of the results is difficult [89, 109]. For patients allergic to peanut, rather high threshold concen-

Fig. 4. Preparation of CCD reagents. Starting point are glycoproteins containing different N-glycans. **a** The work flow for bovine fibrin, which contains diantennary N-glycans as shown in figure 1 from which GPs with GnGn and MM structures can be obtained. **b** Brl with its MUXF N-glycan. **c** A glycoprotein with a MMXF structure. Digestion with an unspecific protease (usually from *Streptomyces griseus*) yields GPs with just a few amino acids as depicted in the middle line. These can be chemically coupled to a carrier protein or a solid support, which is symbolized by an oval area. The dotted line from the fibrin GnGn-GP to the MMXF-GP indicates an alternative route, where recombinant glycosyltransferases are used to 'plantify' the mammalian substrate by the addition of xylose and fucose residues to yield GnGnXF GPs in the first place. Removal of GlcNAc residues by *N*-acetylglucosaminidase finally leads to MMXF structures.

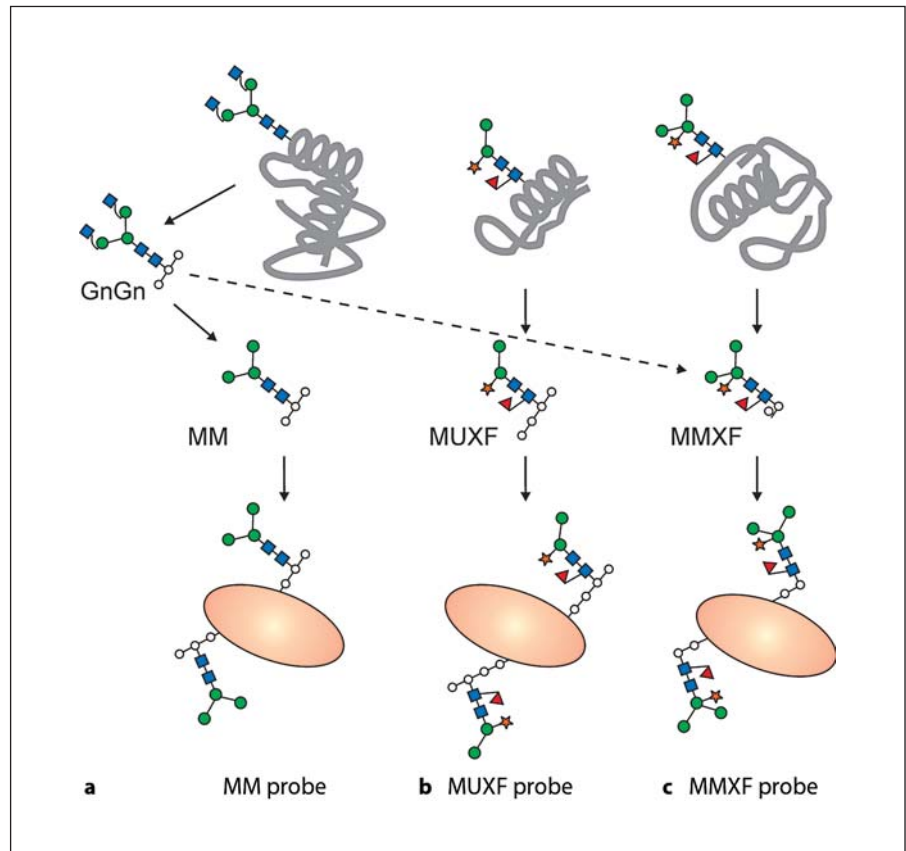
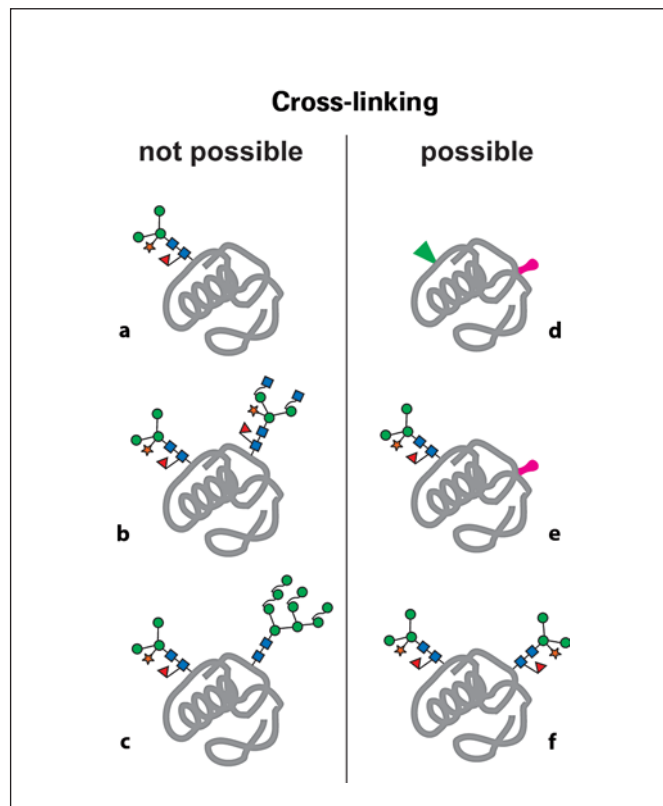


Fig. 5. Effective valency of glyco-allergens. Mediator release from mast cells or basophils requires IgE molecules to be cross-linked. Therefore, allergens have to be at least divalent. The left cartoons show models of glycoproteins, which can be considered ineffective because they carry only one glycan (**a**), or even two glycans but one of them cannot bind IgE because the CCD epitopes are masked by terminal GlcNAcs (blue squares; **b**) or it is of the oligomannose type (**c**). The cartoons on the right side depict cases where cross-linking could happen (provided spatial requirements are fulfilled) because of two peptide epitopes (**d**), a peptide and a CCD epitope (**e**) or two glycans with CCDs (**f**). This latter case represents a glycoprotein, which is unrelated to the sensitizing agent and where IgE binding occurs solely on the basis of CCDs.



trations were found for peanut extract, Ara h 1 (monovalent) and 'Ara h 2' (or rather Ara h 6) [2]. On the basis of these high threshold concentrations, van Ree [110] proposed low binding affinity of anti-CCD IgEs as an explanation for their weak clinical relevance. Similarly, rice-produced human lactoferrin was able to effect basophil histamine release only at very high concentrations, but it was anyway ineffective in SPT up to 0.5 mg/ml [presentation by Mari et al. at the XXVth Annual Congress of the European Academy of Allergology and Clinical Immunology (held on June 10–14, 2006, in Vienna)]. Lactoferrin can carry two N-glycans. The major structure in the rice-produced protein was MMXF, however, in mixture with other glycans devoid of fucose. Therefore, the question remains whether a large fraction actually had been at least divalent in terms of core fucose.

In summary, despite conflicting observations about the required dosage for histamine release or disputes about proper test systems, there are strong in vitro indications that glycoproteins can trigger IgE-mediated degranulation of granulocytes and/or mast cells. At the same time, it appears that the in vitro effects are mild. Although the carbohydrate nature of CCDs may have sometimes been taken as an answer to this discrepancy, it does not hold an explanation. Besides, such an attitude affronts the enthusiastic glyco-biologist. Carbohydrates stood at the cradle of immunology and they continue to be useful as bacterial antigens, e.g. for *Haemophilus influenzae* vaccination. How then can the benign nature of plant/insect N-glycans as allergenic determinants be explained?

Reasons for the Clinical Insignificance of CCDs

A first explanation comes from the requirement of cross-linking of IgE receptors (FcεR) on effector cells, which is only met by allergens that are at least divalent. For glycoproteins, this can mean that the allergen harbors peptide epitopes in addition to a CCD (fig. 5). This could be the case if the glycoprotein actually were the sensitizing agent. The sugars are just one additional epitope and their efficacy does not need separate consideration. The other possibility is that the patient's serum solely recognizes glycan determinants on the allergen. Then it would not be sufficient that the glycoprotein carries two, three or more glycan chains, which also must exhibit structures that facilitate antibody binding. Given the structural variety on plant glycoproteins, this may not be so easily fulfilled, and therefore many plant glycoproteins may have little potential to trigger degranulation even though they bind IgE in in vitro tests for which monovalent binding is sufficient. Besides, many of the well-

known glyco-allergens are monovalent, e.g. Api m 1 (PLA), Ara h 1, or Phl p 1.

A second, more general hypothesis is based on the relatively high (glyco)protein concentrations required for histamine release, which van Ree [110] suggested was due to an assumed low binding affinity of IgE to glycan epitopes. This hypothesis may have been fuelled by earlier findings on the affinity of animal lectins and their carbohydrate ligands [111]. It does, however, not apply to antibody glycan interactions. In particular, the affinity of rabbit anti-CCD antibodies has recently been determined to be around 10^{-9} M [80]. Human anti-CCD IgE likewise appears to have a rather high binding affinity [Jin et al., unpubl. results]. Thus, low binding affinity drops out as an explanation.

A third hypothesis could be formulated if patients' sera were found to contain considerable amounts of blocking antibodies (presumably IgG₄) of sufficient affinity. Preliminary results from our laboratory favor this idea. Interception of IgE binding by blocking antibodies is one important mechanism contributing to the efficacy of allergy vaccination (specific immunotherapy = SIT) [112–114]. CCD-specific IgG₄ antibodies have indeed been found in patients who have received grass pollen immunotherapy [115]. However, the more than 1,000 people examined by Mari [12] and patients in similar studies mentioned above did not receive such treatment. However, these people just like everybody else will have had contact with plant material probably every day of their life. So, many people may undergo an incidental or natural 'immune therapy' ('glyco-SIT') with CCDs before, while or after they are sensitized to glyco-allergens. The situation might resemble that recently described for occupational exposure to animal plasma proteins where some sensitized patients had developed IgG₄ levels to apparently protect them from adverse effects [116] and it reminds of the old observation that beekeepers develop high levels of IgG, which protect them from allergic reactions against bee venom. A recent work estimated 25% of normal healthy people have Fuc-dependent IgG (IgG₁ and IgM) [81]. Phospholipase (Api m 1) was used as a Fuc-containing test antigen which, however, led to false-positive results due to peptide determinants as well as underestimation because Api m 1 is a relatively poor ligand of anti-CCD IgE [44]. Even more sera reacted with *H. pomatia* hemocyanin, which carries Xyl-containing glycans [56], but binding to other epitopes of a peptide or carbohydrate nature cannot be strictly excluded.

The blocking antibody concept does not contravene positive histamine release as in this semi-physiological

test, IgG would largely be removed from the assay system.

Regrettably, little is known about the patients' usual age at IgG induction, about the subclass distribution (although a bias towards IgG4 may be assumed), about the relation between IgE and IgG levels and about the relative binding affinities of these antibodies in humans. Can we safely assume that a sufficient level of blocking antibodies is generated in each patient to prevent relevant allergic reactions? Put another way, can we find patients who do respond to CCDs and can conditions be defined where CCDs play a clinical role? Some CCD-reactive, celery-allergic patients were found to develop true allergic symptoms on contact with carrot despite the absence of measurable anti-protein IgE [105]. In some patients, moderate SPT reactions with rape pollen extract likewise could not be explained by epitopes other than CCDs [117]. However, the overwhelming majority of patients with anti-CCD IgE appear to be protected against severe symptoms. It is highly unlikely that physicians would fail to notice if up to 20% of their patients had allergic episodes after they had ingested virtually every kind of vegetable food. A glycoprotein which binds IgE by CCDs only must therefore be rated as just a clinically irrelevant allergen.

A fascinating side aspect of the glyco-SIT hypothesis is the speculation that the innocuous nature of highly conserved protein panallergens such as profilin could derive from an analogous mechanism. Remember that panallergens from different sources exhibit increasingly different structures with growing phylogenetic distance whereas CCD structures do not vary in this way.

A fourth line of reasoning could be developed from the observation that fucosylated glycoconjugates of parasites interact with components of the innate immune system to facilitate the induction of a Th2 response, which, however, is quantitatively restricted (see paragraph on parasite N-glycans). Although a connection of parasite immune response and allergic response to CCDs is entirely speculative, it might pay to keep an eye on the adjacent borders of these subjects.

Discrimination between Anti-Peptide and Anti-CCD IgE

Specificity is a core quality of any diagnostic test. Regrettably, because of interference from cross-reactive carbohydrate determinants, serum IgE diagnosis is afflicted by an awkwardly high degree of unspecificity [5, 12, 38, 88, 91]. This has to be taken into account whenever extracts

of natural allergens from plants and insects are used as antigens as in current *in vitro* allergy diagnosis. While this may pose a case for recombinant allergens [118, 119], more instantaneous solutions are required to improve diagnoses. A good start might be to detect false-positive serum diagnosis of insect venom allergies in patients who react with insect venoms solely because of anti-CCD IgE.

To our mind, a proper strategy would detect whether a particular serum contains anti-CCD IgE and, if so, specifically prevent this IgE fraction from binding to the allergen. These two steps can either be performed consecutively or simultaneously, which means that all sera would be treated in the same way at the outset. For detection, a plant glycoprotein or a 'plantified' mammalian protein (to be explained later) could be used and introduced as an additional 'allergen' in a serum diagnosis work flow. We believe it is high time to solve the CCD problem in serum IgE diagnosis. Considering the high cost of specific IgE determinations, a tiny extra expenditure must not be allowed to hinder securing more specific and reliable results. Moreover, current developments towards miniaturization of serum diagnosis by microarray methodology should meet any objections based on cost [120, 121]. Recognizing a serum as containing anti-CCD IgE is only the first step. The compulsive consequence is to perform sIgE determination in a way that eliminates the reactivity of anti-CCD IgE. This is probably best achieved by adding an inhibitor (other options are discussed below). It must be emphasized that while the presence of anti-CCD IgE will lead to many positives only because of sugar-IgE interaction, it does not at all exclude the presence of anti-peptide antibodies capable of triggering adverse reactions against a few allergens. CCD inhibition will allow this anti-protein IgE to be detected in spite of the presence of cross-reactive anti-CCD IgE. The suppression of signal from clinically irrelevant IgE should increase the reliability of serum IgE diagnosis by eliminating most if not all false-positive results.

The CCD inhibitor as well as the CCD pseudo-allergen should have no protein-based IgE reactivity, it should contain the relevant glycan structures, and, to provide high inhibitory potency, it should be a large and polyvalent molecule. Free GPs for example have a poorer inhibitory potency than the same GP coupled to BSA with moderate density has (<2 mol/mol) [Dalik and Altmann, unpubl. results]. The first demand is fulfilled by protease digestion of the CCD source, which could be any relatively pure and well-characterized plant glycoprotein, e.g. HRP or pineapple stem Brl [19]. Important results have

recently been obtained with proteinase-K-digested grass pollen extract [77, 110]. However, this extract is a rather expensive and heterogeneous source where the absence of residual peptide-based IgE binding has to be ensured. Brl is an inexpensive source but lacks the 3-arm mannose [16, 19, 24]. As this residue may both have beneficial [77, 110] and inhibitory effects [77, 110], especially for Xyl-dependant antibodies, a mixture of MUXF-GP from Brl and MMXF-GPs from another plant glycoprotein might best fulfill the requirements. Digestion with protease (e.g. from *Streptomyces griseus*) followed by purification of the resulting GPs ensures absence of peptide determinants (fig. 5) [19, 24, 39, 44, 76]. Coupling of such GPs to an inert carrier increases the inhibitory potency per mole of glycan chain about 100-fold [Dalik and Altmann, unpubl. results]. In addition, these conjugates can be used for ELISA and, due to their polyvalency, for histamine release assays [35]. Preparing control conjugates from mammalian glycoprotein (bovine fibrin) that lack the plant-specific sugars is also possible [24, 77]. Alternatives could be plant glycoproteins, e.g. Brl or HRP (multivalent with mainly MMXF glycans), but negative controls, i.e. the protein without plant-specific sugar residues, are not available for them. Therefore, protein-based reactivity cannot be recognized. Besides, Brl may be an odd choice because firstly it is a protease, secondly it just has one glycan, which thirdly has the rare MUXF structure.

Instead of making use of ready-made CCDs of plant glycoproteins, a mammalian glycoprotein can be modified with glycosidases and recombinant glycosyltransferases [77, 80]. This approach avoids artificial linkers and allows reliable controls to be prepared and, for scientific purposes, distinction between Fuc-dependant and Xyl-dependant antibodies.

In the following, alternative strategies for interfering with IgE binding to CCDs and their inherent drawbacks are presented.

Other Approaches for Anti-CCD IgE Detection

Carbohydrate determinants can be destroyed by either periodate or glycosidase treatment [6]. Periodate is a powerful reagent, which abolishes glycan-based IgE reactivity. However, its specificity is occasionally questioned. Indeed, we have recently observed that Art v 1 loses its protein-based, IgE-binding capacity upon mild periodate treatment [22]. Periodate treatment strongly reduced IgE binding by Mal d 1 even though Mal d 2 is not glycosylated [129]. The use of glycosidases is less hampered by unspecificity – although proteases can be an issue – than by the sad fact that peptide N-glycosidases (PNGases) A

or F are inefficient, but for different reasons [109]. Treatment with exo-glycosidases, especially α -mannosidase, might attenuate the glycan epitopes [18, 19]. However, both terminal α -mannosidases must be removed quantitatively, which can only be achieved by rather high enzyme doses [45]. An only temporary solution could be the use of proteinase K for example to destroy any protein-bound reactivity. Persisting IgE binding indicates carbohydrate epitopes [6]. However, converting a large and polyvalent allergen into small monovalent fragments may in itself interfere with the test system in various ways leading to lower IgE binding.

Discrimination of Glycan and Peptide Epitopes of Allergens

One important route to understanding allergy is the molecular characterization of individual allergens, which in its ultimate format includes the definition of all relevant epitopes. Although this an ambitious and laborious task, it is pretty simple to assess the relative importance of IgE binding to peptide versus carbohydrate epitopes using the above-described CCD allergens as inhibitor. It is suspected that many high-molecular-weight proteins currently listed as minor allergens merely fell victim to cross-reactive antibodies.

O-Glycans in Plants

Much has now been said about N-glycans but these are not the only carbohydrates on allergens with the ability to bind IgE even though in our view they will remain the most prominent class of carbohydrate determinants in allergy. Recently, Art v 1, the major allergen of mugwort (*Artemisia vulgaris*), has been shown to be O-glycosylated in two ways [22]. The prominent glycan, a hydroxyproline-linked arabinogalactan, did not bind serum IgE from allergic patients but the single β -arabinosyl residues linked to the many, often adjacent, hydroxyproline residues of the non-globular domain of Art v 1 did. This new type of carbohydrate determinant explained the different performance of recombinant and natural Art v 1 in RASTs [122]. The somewhat lower in vivo reactivity of recombinant as compared to purified Art v 1 may indicate a contribution of the β -arabinoses to the development of clinical symptoms [123]. The O-glycans of potato lectin have been implicated in IgE binding some time ago [1, 2]. A cross-reaction of sera binding to Art v 1 with potato lectin was, however, not observed [22].

O-glyco-allergens from other weeds are currently unknown. Related plant species might be assumed to equip their proteins with similar determinants. However, what can be guessed from recent work with rabbit anti-Art v 1 serum and various allergen extracts is that the volume of arabinose-based cross-reactivity is not at all comparable with that caused by N-glycans [Léonard, pers. commun.].

Glycans and T Cells/Jokes Played by Lectins/Mammalian CCD

The immune response to polysaccharides is T-cell independent and therefore rather weak. In contrast, Asn-linked oligosaccharides of honeybee PLA have been found to contribute to the proliferation of some human T-cell clones, which did not respond to unglycosylated PLA [124]. The involvement of the glycan moiety in T-cell responses is well established in cancer immune therapy [125]. Here it should be mentioned that oligosaccharides of Cry j 1 were found to have an inhibitory potential for Cry-j-1-specific T cells [103].

A totally different effect of protein glycosylation was found when calf intestine alkaline phosphatase conjugates were used to identify cat allergens. A strong staining of cat IgA and IgM was revealed to be caused by IgE-independent cross-linking with the phosphatase via patients' anti-glycan IgM [126]. A similar case of reverse glycan binding occurred in immunoblots of banana and pea extracts, where lectins led to direct binding of IgG₄ or IgE antibodies, respectively [2, 127].

Finally, a hint at the existence of a yet undiscovered third CCD in mammalian tissues was presented by Wong et al. [128].

Concluding Remarks

Our knowledge of CCDs in the form of Asn-linked glycans from plants and insects precludes us from ignoring this large group of IgE determinants for any longer. It is high time to implement anti-CCD IgE detection in allergy diagnosis to prevent misdiagnosis. Patients allergic to grass pollen may all too often have been scared by being erroneously categorized as insect venom allergic. To avoid the however apparently small risk of errors in the other direction, the possibility of clinically relevant reactions based on CCDs alone must be investigated by careful evaluation of the IgE specificity data from large numbers of patients. The glyco-SIT hypothesis does not exclude the possibility of severe symptoms due to CCDs. It would, however, suggest that the allergic status of a patient persists only for a short period and that the severity of symptoms decreases over time. Then glycoproteins are clinically irrelevant allergens for this patient.

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