

Carbohydrate Epitopes and Their Relevance for the Diagnosis and Treatment of Allergic Diseases

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Key Words

IgE · Carbohydrate epitopes · Recombinant allergens · Diagnostic tests · Immunotherapy

Abstract

Allergenicity of plant and invertebrate N-glycans has been shown to be caused by the presence of two typical nonmammalian substitutions: an $\alpha(1,3)$ -fucose linked to the proximal N-acetylglucosamine and a $\beta(1,2)$ -xylose linked to the core mannose. IgE antibodies against these carbohydrate structures are induced upon exposure to pollen or after insect stings, and result in extensive cross-reactivity to plant and invertebrate foods. These cross-reactive IgE antibodies have been shown to possess variable degrees of biological activity, but have never been convincingly shown to induce clinical food allergy. The most likely explanation for this lack of clinical relevance has to be sought in a combination of epitope valency and antibody affinity. In diagnostic tests, these antibodies are at the basis of many false-positive test results for food allergy. Recombinant technologies offer the possibility to produce allergens that do not carry IgE-binding glycans. Whether their absence or presence is of importance for the application of recombinant allergens in immunotherapy is still largely unknown.

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Introduction

The existence of IgE antibodies against carbohydrate determinants was first reported about two decades ago [1]. Binding of IgE antibodies was shown to be lost upon periodate oxidation but to persist unchanged upon heating of allergen extracts. These are two typical characteristics of carbohydrate structures. For quite some time, many allergists remained skeptical about the concept of IgE antibodies against carbohydrate determinants. It was suggested that the interaction between IgE and allergen extract was not based on a paratope-epitope, but on a carbohydrate (on IgE molecule) lectin (in the allergen extract) interaction [2]. Others thought that it was impossible to have carbohydrate-specific IgE antibodies because carbohydrate determinants were supposed to be T cell independent. Several groups have confirmed the existence of IgE antibodies against N-glycans on glycoproteins from plants and invertebrate animals since they were first reported [3–27]. Immunologically, antibody responses to these structures can best be compared to the classical concept of haptens. This review gives an overview of the current knowledge on IgE-binding carbohydrate epitopes and deals with the implications for the diagnosis of allergy and biotechnological approaches for allergy treatment.

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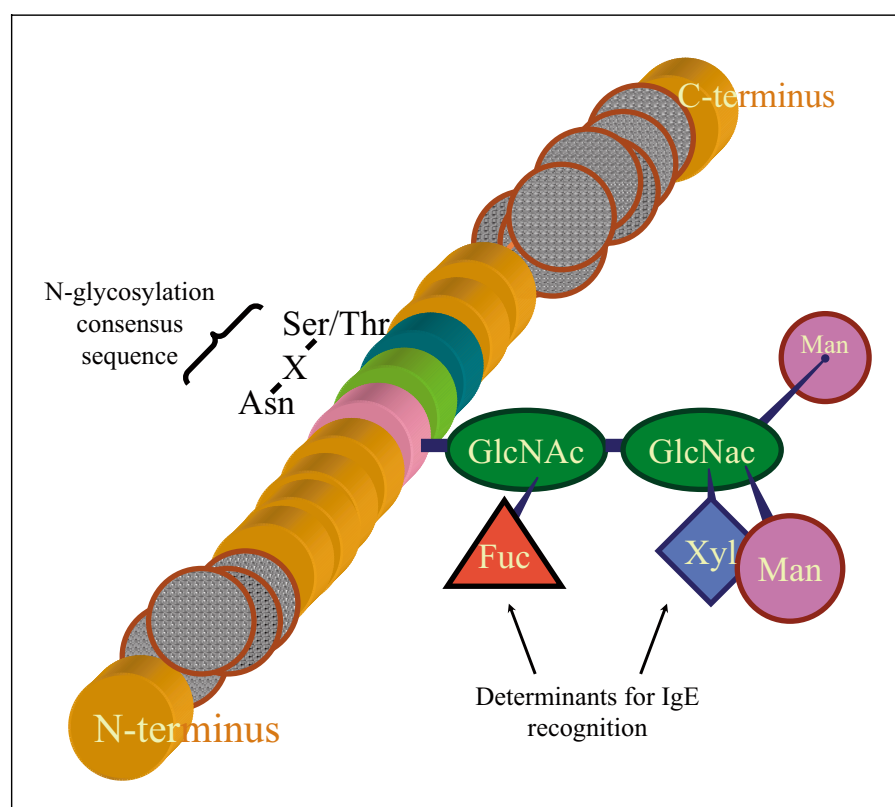
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Fig. 1. Typical IgE-binding N-glycan. N-glycan with typical features of plants and invertebrate animals: an $\alpha(1,3)$ -fucose linked to the proximal N-acetyl glucosamine and a $\beta(1,2)$ -xylose linked to the core mannose. PLA₂ from bee venom misses the xylose, bromelain does not carry the $\alpha(1,3)$ -mannose neighboring the $\beta(1,2)$ -xylose. This mannose has been suspected of blocking recognition of N-glycans on e.g. horseradish peroxidase by xylose-specific IgE [45].



Structural Characteristics of IgE-Binding Glycans

Glycoproteins can carry two basic types of glycans, i.e. N-linked and O-linked glycans [28–30]. N-glycans are always linked to the protein backbone through the free amino group of asparagine (Asn). Not every Asn can be glycosylated though. Only if the second amino acid located C-terminally after the Asn is a serine (Ser) or threonine (Thr), can glycosylation occur, on the condition this Ser/Thr is not preceded by a proline (Pro). This Asn-X-Ser/Thr combination is referred to as the N-glycosylation consensus sequence. The presence of such a sequence is still no guarantee for glycosylation [31]. The pathway a protein under construction takes in the endoplasmic reticulum and the Golgi determines whether (and how) a protein is substituted with carbohydrates. O-glycans can be linked to serine, threonine or hydroxyproline [28, 29, 32]. Though some reports claim that consensus sequences exist also for O-glycosylation, they are certainly less defined than is the case for N-glycans [33–36].

Until now, the study of IgE antibodies with carbohydrate specificity has focused almost exclusively on N-

linked glycans. The first allergenic glycoprotein studied in some more detail for its allergenic glycan was the phospholipase PLA₂ from bee venom [3, 7, 37]. The core of its carbohydrate determinant, like all known N-glycans, consists of two N-acetyl-glucosamines and a mannose (fig. 1). At this mannose, the structure branches by substitution with two additional mannose residues. Each branch can further be substituted with several different combinations of monosaccharides [38]. An $\alpha(1,3)$ -fucose is linked to the proximal N-acetyl-glucosamine. This linkage is not found in mammals, making it a highly immunogenic structure [39]. This feature is not limited to bee venom PLA₂, but is also found on glycoproteins from other insects and invertebrates, plants and parasites [40–44]. A second non-mammalian substitution, a $\beta(1,2)$ -xylose linked to the core mannose, is also widespread among glycoproteins from these sources, but is absent from PLA₂ [41]. It is now well established that fucose and xylose are crucial elements for IgE antibody recognition of plant and invertebrate animal N-glycans (fig. 1) [45]. Some glycoproteins carry both substitutions, others just either one of them [45–51]. Differences in carbohydrate-mediated IgE recognition of plant extracts and plant glycoproteins can at

least partly be explained by this variability [45]. In some cases, recognition of the $\beta(1,2)$ -xylose by xylose-specific IgE antibodies can be hampered by the presence of an $\alpha(1,3)$ -mannose (branch) linked to the core mannose [45]. Finally, it is not unlikely that variations in the peptidic environment surrounding the carbohydrate epitope also influence IgE recognition [52]. In conclusion, the IgE-reactive N-glycosidic epitope is not a single uniform entity.

Carbohydrate Epitopes, Sensitization and Cross-Reactivity

The widespread presence of $\alpha(1,3)$ -fucose and $\beta(1,2)$ -xylose on N-glycans of plants and invertebrates is the basis for the high degree of cross-reactivity that has been reported for carbohydrate-specific IgE antibodies [1, 9, 15, 24, 45, 53–63]. Carbohydrate-reactive IgE is predominantly found in serum from patients allergic to pollen or insect venom [1, 56, 60]. These IgE antibodies recognize a broad spectrum of plant foods, but also foods of invertebrate origin like mollusks, shrimps and snails. It is now well accepted that pollen exposure and to a lesser extent insect stings are the primary inducers of anti-carbohydrate IgE antibodies, leading to cross-reactive recognition of similar glycans in foods [54]. This mechanism of ‘cross-sensitization’ is certainly characteristic but not unique to sugar epitopes, as is most clearly exemplified by the non-glycosylated allergens from birch pollen, Bet v 1 (belonging to the pathogenesis-related (PR) proteins referred to as PR10) and Bet v 2 (profilin) [64]. Both are also instrumental in extensive cross-reactivity to their homologues in plant foods [53, 65]. Carbohydrate-specific IgE antibodies are rarely observed in sera of patients with isolated food allergies, i.e. in sera of patients without pollinosis or insect venom allergy. The same is true for IgE antibodies against PR10 proteins and profilins [64]. Sensitization to foods via the gastrointestinal route is thought to be the cause of non-pollen-related food allergies and to be dependent on protease resistance of food allergens [66]. Both B- and T-cell epitopes should reach the immune system in a physically linked fashion. PR10 allergens like Mal d 1 from apple and Pru p 1 from peach are not capable of reaching the intestinal immune system in an immunogenic fashion, because they are extremely sensitive to pepsin digestion [67, 68]. No pepsin digestion studies for profilin have been reported so far. N-glycans are not only linked to labile food proteins but also to real pepsin-resistant food allergens, like e.g. Ara h 1 [45, 66]. It is therefore not really understood why the glycan moiety of stable glycoproteins, in

contrast to their peptide backbone, apparently does not manage to trigger the intestinal immune system.

Clinical Relevance of IgE Antibodies against N-Glycans

There has been a long-standing debate about the clinical relevance of anti-carbohydrate IgE antibodies [69]. For this discussion, it is important to make a clear distinction between clinical relevance (role in causing symptoms) and biological activity (role in triggering mediator release from effector cells). The capacity of carbohydrate IgE to induce mediator release does not automatically translate into clinical allergy. Until this moment, no convincing evidence has been reported to support clinical relevance of anti-carbohydrate IgE. As mentioned, these IgE antibodies are usually part of a polyclonal IgE response to pollen together with a broad variety of anti-protein IgE antibodies. Patients with anti-pollen IgE antibodies that exclusively react with carbohydrate epitopes are extremely rare. It is impossible to determine whether IgE antibodies against pollen N-glycans are involved in the induction of symptoms of pollinosis when they are mixed with anti-peptide antibodies. The resulting cross-reactivity to plant foods is more informative. Van der Veen et al. [59] reported a group of patients ($n = 32$) with grass pollen rhinoconjunctivitis and carbohydrate-mediated cross-reactivity to peanut. None of these patients had symptoms of peanut allergy, supporting the view that anti-carbohydrate IgE lacks clinical relevance. The absence of clinical food allergy was explained by poor biological activity of IgE antibodies against carbohydrates, both in skin tests and in *in vitro* basophil histamine release tests. Only at unusually high peanut allergen concentrations were these antibodies able to trigger mediator release.

A limited number of reports shows that cross-reactive IgE antibodies against plant food N-glycans have normal biological activity in basophil histamine release assays. Fötisch et al. [17] performed such experiments using a neo-glycopeptide as a trigger. Their reagent was composed of BSA molecules conjugated with IgE-binding N-glycans derived from pineapple stem bromelain [46, 47]. Using basophils from a patient having clinical celery allergy as well as anti-carbohydrate IgE, they convincingly demonstrated that, though moderate, histamine release was induced by these neo-glycopeptides. Unfortunately, this observation was only made in a single patient. It was not established whether these glycan-specific IgE antibodies were instrumental in the patient’s clinical symptoms.

Nevertheless, the study implies that anti-glycan IgE might have clinical relevance in individual cases.

Batanero et al. [16] studied the biological activity of anti-glycan IgE by a different approach. They purified the complex N-glycan from the major olive pollen allergen Ole e 1. It was demonstrated that the free glycans could potentially liberate histamine from basophils of patients with anti-carbohydrate IgE. This suggests that a very small (1.3–1.6 kD) structure accommodated the binding of at least two IgE molecules, the prerequisite for cross-linking of high-affinity IgE receptors on basophils [70]. Although sterically perhaps possible, the efficiency by which these tiny molecules induced histamine release is quite surprising. An alternative mechanism different from classical high-affinity IgE-receptor cross-linking should perhaps not be ruled out completely. Even if these experiments indeed prove that IgE antibodies against plant N-glycans have 'normal' biological activity, they still do not prove clinical relevance. Unfortunately, the authors did not describe the clinical background of the patients used for their experiments in detail. They suffered from olive pollen allergy, but there was no mention of any food allergies. If clear biological activity translated into clinical allergy, these patients would be expected to have food allergy to a broad spectrum of plant foods. Since this was not reported, they probably were not food allergic.

In summary, the overall impression still is that IgE antibodies against carbohydrate determinants are of poor biological activity for the majority of patients. Clinical relevance in individual cases cannot be ruled out but still awaits experimental proof. What is the explanation for the poor biological activity of carbohydrate-specific IgE antibodies? Efficient cross-linking of FcεRI is dependent on many different factors [70]. At least two receptors should be cross-linked, but simultaneous IgE binding to 3 or 4 epitopes on an allergen increases the efficacy of mediator release (epitope valency). From this perspective it is not surprising that monoglycosylated bromelain did not give positive skin tests in patients with IgE antibodies recognizing the glycan moiety of this glycoprotein [15]. Then, the affinity of the interaction between allergen and IgE also plays a role [70, 71]. High-affinity interactions seem to be needed for the triggering of effector cells. The observation that only high concentrations of allergen give positive skin tests or induce in vitro histamine release from basophils is in line with low-affinity IgE antibodies not becoming biologically active unless high allergen concentrations are administered [59]. The lack of clinical relevance of anti-carbohydrate IgE could be explained by a combination of limited epitope valency and low affinity [69].

Finally, other factors extrinsic to the specific interaction between allergen and IgE antibodies could be involved. It has been reported that high total IgE levels become negatively correlated with allergen-induced mediator release, simply when receptors are saturated with irrelevant IgE [72]. Anti-carbohydrate IgE certainly is not only found in sera with high total IgE, making this a less plausible explanation of their poor biological activity. Also specific IgG4 antibodies have been shown to inhibit allergen-induced histamine release (blocking antibodies) [73]. Specific IgG4 antibodies against plant N-glycans have been demonstrated in sera of patients allergic to grass pollen, but only after grass pollen immunotherapy [74]. It is unlikely that natural exposure to pollen results in glycan-specific blocking antibody titers sufficiently high to explain the lack of biological activity of anti-carbohydrate IgE.

Diagnostic Implications of IgE Antibodies against Carbohydrate Determinants

In vitro food allergy diagnostic tests frequently have a false-positive outcome. This has especially been reported for plant foods like cereals and legumes. Anti-carbohydrate IgE antibodies can partly explain this poor performance. Obviously, IgE antibodies without clinical relevance would preferably go undetected in diagnostic tests. A method to avoid detection of anti-carbohydrate IgE has recently been proposed [68]. Addition of protease-digested pollen extract as soluble inhibitor during the incubation of allergen source and serum sample in diagnostic procedures can efficiently inhibit IgE binding to carbohydrates of the allergen source. Routine application of such an approach, however, still awaits firm proof that glycan-specific IgE is indeed of negligible clinical relevance. Even then, it will never completely solve the problem of false-positive test results. Cross-reactive IgE antibodies against profilins have recently been reported to be of clinical relevance in a minority of the cases only [75]. Clinically irrelevant IgE antibodies against profilins and carbohydrates frequently go hand in hand. Even cross-reactive IgE antibodies against PR10 proteins are not always translating into clinical fruit allergy [75, 76]. To improve the clinical relevance of in vitro food allergy diagnostics, test formats are needed that measure biological activity. Of course such assays have been available for some decades now, i.e. basophil histamine release tests [77, 78]. More recently, possibilities for monitoring the activation of basophils have expanded beyond the measurement of histamine

and other mediators and cytokines. FACS analysis of activation markers like CD63 offers an elegant alternative [79], which was recently shown to allow distinction between clinically relevant and irrelevant (anti-carbohydrate) anti-latex IgE antibodies [80]. Cellular tests certainly offer possibilities to improve the clinical relevance of food allergy diagnostics. The disadvantage, however, is that cellular tests are far from ideal for high-throughput routine laboratory testing. Serum-based immunoassays that can estimate biological activity of IgE antibodies are needed to fulfill the requirements of routine testing with increased clinical relevance.

Recombinant Allergens for Application in Diagnostics and Immunotherapy

Recombinant molecules of most major inhalant allergens, and an increasing number of food allergens, have been produced (for an updated list, see website of IUIS Allergen Nomenclature Subcommittee: www.allergen.org). These proteins are proposed to replace current allergen extracts in diagnostic tests and in products for allergen-specific immunotherapy [81–85]. Glycosylation of recombinant proteins is dependent on the expression system used [86–95]. The first recombinant allergens were produced in *Escherichia coli*, resulting in nonglycosylated molecules [96–102]. This prokaryotic expression system is still applied for the production of many different recombinant allergens [103–108]. In more recent years, several allergens were produced in eukaryotic expression systems like the yeast *Pichia pastoris* [109–127], insect cells [128–136], and tobacco plants [137, 138]. These systems facilitate post-translational modifications that do not (easily) occur in *E. coli*, like disulfide-bridge formation, hydroxylation of prolines, glycosylation. In the frame of this review, we will focus on the implications of the use of different expression systems for the glycosylation of allergens.

As mentioned, *E. coli* does not facilitate glycosylation. Apart from the fact that this can hamper correct folding of a recombinant protein, it certainly does not deliver allergens carrying IgE-binding N-glycans. Provided that correct folding is achieved, *E. coli*-derived recombinant allergens are interesting candidates to prevent detection of clinically irrelevant carbohydrate-specific IgE antibodies in diagnostic tests. If correct folding is not achieved, eukaryotic expression systems usually offer the solution. As mentioned above, several allergens have now been produced in the yeast *P. pastoris* [109–127]. In general, yeast

is known for hyperglycosylation with large high-mannose glycans [90, 94]. The system does not substitute recombinant proteins with fucose/xylose-containing complex N-glycans. In this respect, *P. pastoris* offers a good eukaryotic alternative to *E. coli* for application in diagnostic tests. On the other hand, *P. pastoris* glycans can occasionally be recognized by IgE antibodies in sera of patients with yeast- or mold-related allergies [manuscript in preparation]. This introduces a new risk of false-positive diagnostic tests, when nonmold/nonyeast glycoproteins are expressed in *P. pastoris*.

Expression in tobacco is the appropriate system to use if typical IgE-binding complex glycans are to be present on the allergen. Close mimics of pollen or food allergens with IgE binding complex N-glycans can only be produced in plant (or insect) expression systems [88, 93]. For reasons similar to those described for yeast, expression of non-plant glycoproteins like e.g. Fel d 1 or Der p 1 in tobacco will potentially introduce IgE-binding glycans to these allergens resulting in false-positive diagnostic tests. In summary, to achieve the optimal diagnostic performance, the right expression system needs to be evaluated for each allergen.

Immunotherapy with recombinant allergens is on the horizon now [84, 85]. The importance of correct glycosylation for immunotherapy is largely a black box. Does successful immunotherapy require correctly folded allergens? If so, correct glycosylation might be necessary. Immunotherapy has been shown to induce glycan-specific IgG4 antibodies [74]. Do they play a role in the beneficial effect of immunotherapy? The mechanism of allergen-specific immunotherapy is slowly being unraveled. A shift from Th2 to Th1 was originally proposed as the key event in immunotherapy [139–141]. More recently, the induction of regulatory T cells (Tr) has been proposed to be instrumental in the beneficial effect of immunotherapy [142, 143]. In addition, the role of dendritic cells in driving the development of T cells to Th1, Th2 or Tr has gained a lot of attention [144–152]. These antigen-presenting cells carry Toll-like receptors that seem to have specialized in telling the immune system the origin of an intruder [153, 154]. Some Toll-like receptors recognize carbohydrate-containing structures like glycolipids [155–157]. Whether such mechanisms play a role in the mechanism of allergen-specific immunotherapy is still unknown, but it is attractive to speculate that plant-derived carbohydrate determinants interact with dendritic cells, that in turn polarize T cell responses to the desired Th1 or Tr profile. From this perspective, correct glycosylation could turn out to be important for successful immunotherapy.

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