

## Cross-reactive Carbohydrate Determinants - CCDs – To consider in IgE antibody testing?

### Summary

In most allergen sources, and especially from the plant kingdom, glycan-related IgE-reactivities have been demonstrated. Since glycoepitopes can share significant structural homologies beyond the limits of protein families they are prone to extensive cross-reactivity and they have, consequently, been called Cross-reactive Carbohydrate Determinants or CCDs.

Whether or not IgE antibodies against carbohydrate epitopes on glycoproteins have a clinical role is debated, but data supporting a clinical effect are emerging. As long as the demonstration of a clear *in vivo* effect remains to be confirmed, we must consider the sometimes confusing role of these epitopes in serum-based IgE antibody assays.

In certain subgroups of patients CCD-reactivity might, however, have clinical relevance and an awareness of a possible CCD response could therefore be of value when making diagnoses.

The first step to investigate potential CCD interference is to quantify CCD-specific IgE antibodies to see if they may contribute to the allergen-specific results. The risk for interference is greatest with food allergens of plant origin, latex, and *Hymenoptera* venoms.

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### Introduction

Glycan-related IgE-activity has been demonstrated in most allergen sources, especially in plants. Unlike classic peptide chain based epitopes, glycoepitopes can share significant structural homologies beyond the limits of protein families and are thus prone to extensive cross-reactivity.

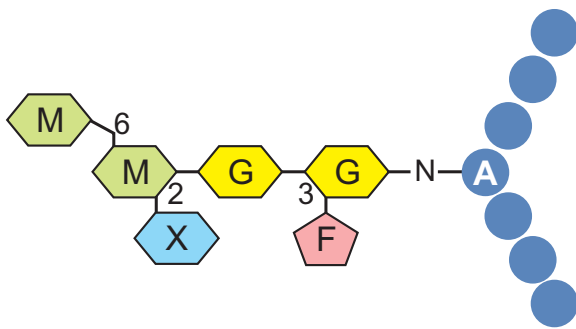
So far the opinion has prevailed that these epitopes have no clinical impact (1,2). In the light of recently obtained results, this opinion is now challenged (3). But as long as a clear clinical role of carbohydrate epitopes has not been confirmed, we must pay attention to Cross-reactive Carbohydrate Determinants, CCDs, and their potential IgE reactivity in serum-based assays.

The aim of this article is to give a brief factual background on glycoproteins and their cross-reactivity, some of the arguments for and against the clinical relevance of IgE antibodies against CCDs, and to describe the potential effects of such cross-reactivity on *in vitro* IgE-testing. Some facts are presented concerning when CCD interference could be expected and how such biological phenomenon can be measured.

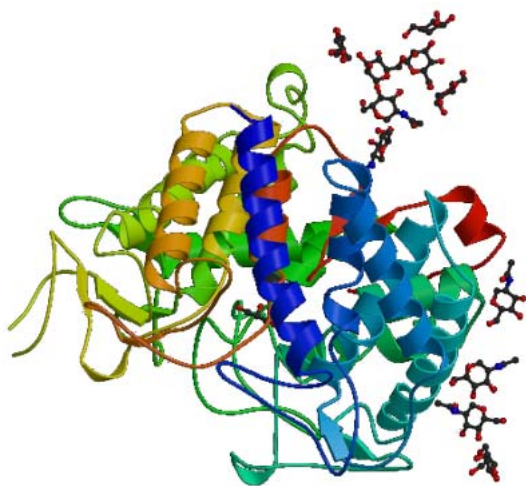
### Biochemical and immunogenic properties

Glycoproteins are proteins with attached carbohydrate chains. Glycosylation brings some important features to the protein, better hydrophilicity and stronger resistance to thermic shocks, among other things (4,5). Carbohydrate chains borne by membrane proteins also play a protective role and participate in the cell signature (e.g. blood groups).

The carbohydrate chains, or glycans, are attached to the peptide at specific sites. Depending on which kind of amino acid the glycan is attached to, a distinction is made between N-linked glycans and O-linked glycans. N-glycans are fixed to an asparagine, O-glycans to a serine, a threonine or an hydroxyproline. Glycans are functionally important, but often account only for a minor part of the protein weight. **Figure 1** shows an example of an N-glycan commonly occurring in plant proteins.



**Figure 1.** An example of N-glycan chain. The 6-sugar chain is linked to the peptide on an asparagine (A) and is composed of two N-acetyl-glucosamines (G), two mannose (M), one 1,3-fucose (F) and one 1,2-xylose (X). This "MUXF3" chain is often found in plant glycoproteins.



**Figure 2.** An example of glycoprotein. This mannosidase from *S. cerevisiae* presents three glycan chains at its outer surface. One of them has 7 sugars and could become an epitope. Protein Data Bank ID = 1DL2 (6).

Carbohydrate structures are often prominent and can therefore easily constitute an immunogenic site (T-cell epitope) and/or an IgE binding site (B-cell epitope) (see **figure 2**). The minimal length for a glycan to act as a B-cell epitope has not been established, but most carbohydrate epitopes characterized so far comprise at least four sugars.

One question that is still debated is if carbohydrate epitopes are restricted to the glycan chain, or if also adjacent amino acids can play a part in IgE binding (7). As some flexibility is possible at the paratope site of the IgE molecules, any given IgE molecule has an imperfect specificity towards a unique allergen epitope (8). In this respect, carbohydrate epitopes do not differ from peptide epitopes; they are able to bind to more than one IgE antibody (9), and these cross-reactive epitopes are called Cross-reactive Carbohydrate Determinants, CCDs.

### Glycan diversity and cross-reactivity

The structures of glycans are at least as diverse as peptide sequences. Each sugar offers several possible linkages with the following one. In fact, more than 1,000 different combinations can result from a chain consisting of only three sugars. Cells utilize this diversity to meet their metabolic and

defensive needs by means of finely modified glycoprotein structures.

Natural glycoproteins most often appear as a mix of variants. This gives rise to imprecise bands on immunoblots, especially when molecular weights exceed 30-40 kDa. Another diversity consists in the simultaneous presence of glycosylated and non-glycosylated isoforms, resulting in double bands.

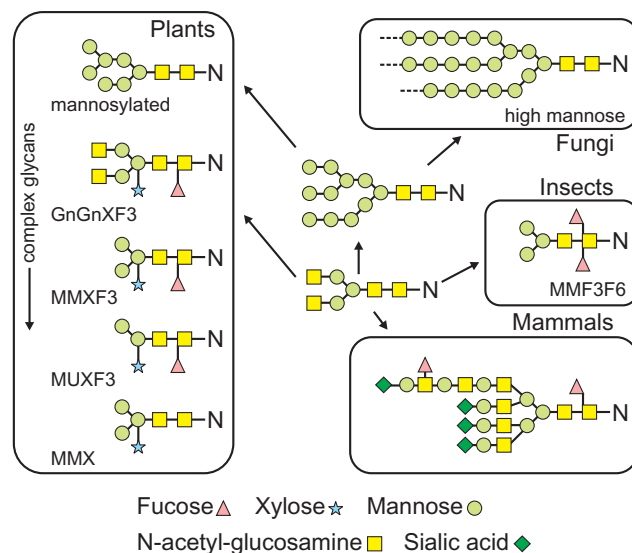
Contrary to what could be expected, this structural diversity does not prevent cross-reactivity between glycan chains. IgE reactivity is notably driven by the presence of certain sugars at specific positions in the chain. And certain basic sugar patterns are indeed found among IgE-reactive glycan chains, especially N-glycan ones (**figure 3**).

N-glycan synthesis starts with the addition of a basic 7-sugar chain, which is then elongated and/or trimmed. Xylose is a characteristic sugar of IgE-reactive plant N-glycans. A fucose is often found too, but a 1,3 spatial setting seems critical to its IgE-reactivity (10,11). Such 1,3-fucoses are a frequent finding in plant and some invertebrate N-glycan chains and this feature leads to potential cross-reactivity (e.g. between pollen and *Hymenoptera* allergens). Mammal N-glycans are 1,6-fucosylated and thus do not cross-react with 1,3-fucose N-glycans (see **figure 3**).

One could expect a similar tendency to cross-reactivity among O-glycan chains. But, except for some allergens such as mugwort pollen Art v 1 (12), this kind of glycans has been less well studied than N-glycans and the frequency of O-glycan-based CCD is largely unknown.

### The complexity of natural products

All natural products contain a large number of different proteins, some of these being glycoproteins. Among glycoproteins, some are known allergens, and among the epitopes of these allergens some consist of carbohydrate



**Figure 3.** Diversification of N-glycans among eukaryotes. The process starts with the addition of a 7-sugar chains at the N-glycosylation site (an asparagine). This oligosaccharide, common to all eukaryotes, is then modified by trimming and adding sugars. These modifications lead to a diversification of N-glycans along the phylogenetic branches of eukaryotes. A notation is used to describe the glycans, especially the presence and the position of mannose (M), xylose (X), fucose (F), and N-acetyl glucosamine + mannose (Gn).

chains. And among these glycoepitopes, certain are prone to cross-react with glycan chains present in other allergens, i.e. to behave as CCDs.

Any natural allergenic product can contain allergens and non-allergens, peptide- and/or glycoepitopes on each allergen molecule, and cross-reactive or not cross-reactive epitopes. The response observed during *in vitro* assays, as well as in clinical reactions, is thus quite complex.

Faced with the task of untangling this complexity, we can find some help in “glycomes”. A glycome corresponds to the percentage of all types of glycan chains present in the extract. For example, grass pollens are rich in MUXF3 glycans (see **figures 2 & 3**), and peanuts contain a noticeable proportion of high-mannose chains (13,14). This kind of information can give a useful clue in some situations of suspected cross-reactivity.

### Clinical relevance of glyco-epitopes

So far the view that these epitopes have no clinical impact has prevailed (1,2). Recently this opinion has been challenged, however (3,15-18). For example, a combined binding is conceivable, comprising a peptide epitope and a carbohydrate epitope of the same allergen molecule bound to two adjacent IgE on the mastocyte (10,19,20).

A work by van der Veen *et al* is classically cited as proof against any clinical impact of carbohydrate epitopes (21). These authors studied patients who had IgE antibodies to peanuts, but not clinically allergic to this food. They observed a histamine release after the contact of a peanut extract with the basophils of these patients, but only when very high concentrations of this extract were applied. This contrasted with the much lower concentrations needed with basophils of patients truly allergic to peanuts. It was concluded that the anti-glycan IgE detected by *in vitro* methods were clinically irrelevant.

This conclusion was supported by a study by Mari, where he tested the reactivity to bromelain in 4,535 subjects with a suspicion of respiratory allergy (2). He found 23% positive results *in vitro* but only 0.1% (four subjects) positive reactions on skin testing. These differences in test results were attributed to the monovalency of bromelain (only one glycan chain).

van der Veen *et al* used the same reasoning to explain their results (21); peanut proteins could polymerize and become “multivalent” at high concentration.

But these results can also be seen from a different point of view (10). van der Veen's patients (21) were primarily sensitized to grass pollens. Their anti-glycan IgE antibodies were thus more related to grass glycans than to peanut glycans. Perhaps only high concentrations of peanut could overcome this glycome inadequacy at the basophil level.

In the case of skin tests with bromelain (2), it is not surprising that so few positive results were observed in an unselected population. Degranulation requires the binding of at least two epitopes to two adjacent IgE antibody molecules (see **figure 4**). As bromelain has only one glycan chain, a positive skin test requires that the patient's IgE is directed to two peptide epitopes or to one glycan and one peptide epitope. In both cases only patients actually sensitized to bromelain could have anti-peptide antibodies and give a positive skin test, a situation only seen in rare cases of occupational allergies.

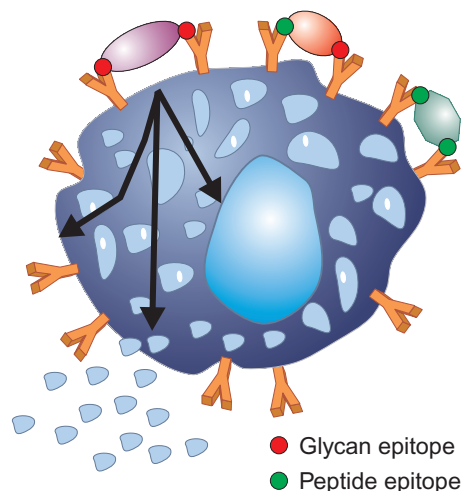
Ohta *et al* first published proofs for a clinical role of carbohydrate epitopes (22), observing positive skin tests with an oligosaccharide isolated from *Stylea plicata*, an oyster parasite responsible for occupational asthma in Japan. This work suffered from some methodological flaws, however (23).

More significant results have recently been obtained with plant products. For example, Vieths' team studied patients allergic to celery, tomato or zucchini. Basophils of these subjects degranulated in contact with bromelain glycans coupled to bovalbumin (17,24). Tomato Lyc e 2 lost its degranulating power if deglycosylated (15) and a positive oral challenge test was observed with celery and zucchini in some patients, although *in vitro* tests indicated that these patients were sensitized only to glycans in these foods (25,26).

Iacovacci *et al* have studied patients allergic to cypress and compared nCup a 1 with its nonglycosylated recombinant rCup a 1 (27). *In vitro*, all subjects positive to nCup a 1 were found negative to rCup a 1. Activated basophils of three patients responded similarly, degranulation being observed only with the natural glycosylated allergen.

Batanero *et al* have tested a glycopeptide obtained from the olive pollen major allergen, Ole e 1 (18). This glycopeptide was able to activate the basophils of five olive allergic patients. The same result was observed even with free glycans obtained from Ole e 1.

Data supporting a significant clinical effect of glyco-epitopes are thus emerging but are still preliminary. This contrasts with the high rate of CCD-mediated positive results observed with *in vitro* techniques such as ImmunoCAP™ or immunoblotting. One explanation could be methodological differences: in serum-based techniques the epitope/IgE ratio is typically high, which means that also low affinity bindings are revealed. In addition, IgE antibody molecules are free, an opportunity absent in cell-based systems (1,28).



**Figure 4.** Degranulation of mast cells require the binding of at least two epitopes to two adjacent IgE antibody molecules. This cross-linking may be achieved by two peptide epitopes, by one glycan and one peptide epitope, but also by two glycan epitopes (15).

## When could CCD interference be suspected?

- All plant products must be suspected to contain IgE-reactive glycoepitopes: pollens, foods, latex, etc. A cross-reactivity between these glycoepitopes is also possible, giving rise to plant CCD (p-CCD). The level and the frequency of p-CCD cross-reactivity is directly dependant on the degree of homology between the glycan chains present in the cross-reacting extracts. The main origin of IgE antibodies to p-CCDs is sensitization to pollens, especially in multiple pollen sensitized patients (2,11,29). Because pollen and food sensitization are often associated, it is difficult to distinguish the proper part of food glycoepitopes in the observed IgE-reactivity.
- Some allergens present in mammals are glycosylated: ovomucoid in egg, Fel d 1 in cat, etc. Their glycan chains seem unable to cross-react with p-CCD.
- Parvalbumins (found in fish) and tropomyosins (crustacea, mollusks, mites and many other invertebrates) have no significant carbohydrate-related IgE-reactivity.
- Some mollusk glycoproteins possess 1,3-fucosylated or xylosylated glycan chains (e.g. snail hemocyanin). They might cross-react with p-CCD, but such cross-reactivity still remains to be demonstrated.
- A significant carbohydrate-related IgE-reactivity seems unlikely with mites, at least cross-reactivity between mites and p-CCD.
- The relevance of glycan-induced IgE-reactivity has been proven for hymenoptera venom. The cross-reactivity between p-CCD and venom glycans needs further studies. Data are lacking about the insect-derived aeroallergens (e.g. cockroaches).
- Fungi contain high mannose glycan chains able to generate cross-reactions between different mould species. These mould-CCDs probably do not cross-react with p-CCD.

## How are *in vitro* test results affected?

In most cases CCDs are likely to have a marginal effect on the test results. However, in certain subgroups of patients CCD-reactivity may have clinical relevance (30) and an awareness of possible CCD response may therefore be of great value when making diagnoses.

### The test results could be affected in two ways:

- If the patient is not originally sensitized to the allergen tested, but the carbohydrate epitopes recognized by the patient's IgE are cross-reactive, the clinical relevance of the positive test result has to be considered. For example, in a pollen allergic patient not originally sensitized to latex, some glycan epitopes present in the latex extract can bind to anti-glycan IgE present in the patient's serum,

resulting in a positive latex test result (15,26,31). Thus, all *in vitro* tests must always be evaluated together with the clinical history in each individual patient.

- If the patient is in fact sensitized to the allergen tested, the presence of anti-glycan IgE in addition to anti-peptide IgE can result in an a higher quantitative result, indicating a more severe sensitization than is actually the case.

Serum-based assays, such as ImmunoCAP™ and other allergen-specific IgE tests, are at risk of carbohydrate interference. But this also applies to immunoblotting, as exemplified by the recent discovery of Hev b 13, a major latex allergen which for a long time has been mistaken for Hev b 7 (3).

## Testing for CCD-specific IgE-reactivity

A simple way to determine a possible IgE-reactivity to CCD in an *in vitro* IgE assay is to perform an ImmunoCAP™ CCD; MUXF3 test. The MUXF3 determinant is prepared from digested bromelain. Bromelain is a glycosylated allergen extracted from pineapple. This inhibitor is widely used for checking the cross-reactivity between a glycan and other glycoproteins, as its MUXF3 carbohydrate chain is also found in many other plant proteins and as true allergy to bromelain is very rare. If this test is positive, the patient's serum contains some anti-CCD IgE. A positive CCD; MUXF3 result means that the *in vitro* results for allergens containing these CCD structures partly or completely could be due to the glycoepitopes present in the allergen source tested and the clinical relevance has, as always, to be evaluated in relation to the symptoms of the patient.

A CCD; MUXF3 test could be useful when *in vitro* results do not match the clinical picture (symptoms, skin tests), especially in three types of situations:

- Sensitization to foods of plant origin, mainly vegetables and fruits, but also seeds such as peanuts;
- Sensitization to *Hevea* latex in a pollen allergic patient without occupational risk factors;
- In subjects tested positive for *Hymenoptera* venoms (32), or in subjects allergic to these venoms and tested positive for pollens (30).

## Conclusion

Carbohydrate epitopes and CCDs add another level to IgE cross-reactivity mechanisms, in addition to cross-reactivity arising from taxonomy closeness of plants and from proteins belonging to the same family.

Although generally of minor importance in everyday clinical practice, the *in vitro* reactivity of potential CCD-specific IgE antibodies must be considered in the diagnosis of certain patients.

A simple way to measure a possible IgE reactivity to CCD in an *in vitro* IgE assay is to perform an ImmunoCAP™ CCD; MUXF3 test.

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