

IGE-REACTIVE CARBOHYDRATE EPITOPES - CLASSIFICATION, CROSS-REACTIVITY, AND CLINICAL IMPACT (2ND PART)

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HAVE GLYCO-EPITOPES ANY CLINICAL IMPACT?

Are carbohydrate epitopes, and thus CCD, able to lead to a degranulation? Up to now, the thesis that these epitopes have no clinical impact has prevailed (4, 31). But, considering recently obtained experimental results, this opinion has been challenged (101). For example, a combined binding is conceivable, comprising a peptide epitope and a carbohydrate epitope of the same allergen molecule bound to 2 adjacent IgE on the mastocyte (5, 6, 160).

Arguments against a significant clinical role

A work by Van der Veen *et al.* is classically cited as a proof against any clinical impact of carbohydrate epitopes (94). These authors studied patients who were RAST-positive to peanuts but clinically not 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 (or of individual allergens like Ara h 1 or Ara h 2) 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.

Mari tested the reactivity to bromelain in 4535 subjects with a suspicion of respiratory allergy (31). He found 23% positive results *in vitro* but only 0.1% (4

subjects) positive reactions on skin testing. So, nearly all positive CAP results were without clinical significance.

A subset of the same cohort (1076 patients) was also skin tested with other glycoproteins (31): none patient was positive for ascorbate oxidase, 6 were positive for honeybee PLA2, and 90 for horseradish peroxidase. These differences in skin test results were assigned to the respective valencies of the proteins (31): horseradish peroxidase is a "multivalent" glycoprotein (7 glycan chains), whereas bromelain is "monovalent" (only 1 glycan chain).

Van der Veen *et al.*, used the same reasoning to explain their results (94): peanut proteins could polymerize and become "multivalent" at high concentrations. Tenants for a negligible clinical impact of CCD also argue that the few positive results obtained during *ex vivo* experiments could be related to the multivalency of the glycoproteins used (4): for example, celery Api g 5 bears at least 3 glycan chains (59) and tomato Lyc e 2 has 4 glycan chains (84).

But the results obtained by van der Veen (94) and by Mari (31) could be seen from a different point of view (6):
➤ in the case of peanuts (94), the patients were primarily sensitized to grass pollens: their anti-glycan IgE were thus more related to grass glycans than to peanut glycans (mostly of M0XF3 type vs high-mannose type, respectively). And perhaps only high concentrations of peanuts could overcome this glycome inadequacy at the basophil level;

➤ in the case of skin tests with bromelain (31), it is not surprising that nearly no positive result was observed in an unselected population. A degranulation requires the binding of 2 epitopes to 2 adjacent IgE. As long as bromelain bears only one glycan chain,

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a positive skin test requires that patient's IgE are directed to 2 peptide epitopes or to one glycan and one peptide epitopes. In both cases only patients actually sensitized to bromelain could have anti-peptide antibodies and would give a positive skin test, a situation only seen in rare cases of occupational allergies.

Similar remarks could be made about the results Mari obtained with other glycoproteins (31): ascorbate oxidase is not known as an allergen; PLA2 positive rate (0.6%) could match the actual prevalence of sensitization to honeybee in unselected populations; and horseradish peroxidase figures could be related to the sensitization to food peroxidases such as those found in legumes or flours (161).

Facts favouring a clinical role of glyco-epitopes

Ohta *et al.* first published a proof for a clinical role of carbohydrate epitopes (162): these authors observed positive skin tests with an oligosaccharide isolated from *Styela plicata*, an oyster parasite responsible for occupational asthma in Japan. But this work suffered from methodological flaws (119). More significant results have been recently obtained with plant products:

➤ Vieths's team studied patients allergic to celery, tomato or zucchini. Basophils of these subjects degranulated in contact with bromelain glycans coupled to bovalbumin (84, 163). Tomato Lyc e 2 lost its degranulating power if deglycosylated (34). And a positive oral challenge test (DBPCFC) was observed with celery and zucchini (3 and 1 subjects, respectively), although these patients were sensitized *in vitro* only to glycans in these foods (164, 165).

➤ Iacovacci *et al.* studied patients allergic to cypress and compared nCup a 1 with its non-glycosylated recombinant counterpart rCup a 1 (53). *In vitro*, all subjects positive to nCup a 1 were found negative to rCup a 1. Passively activated basophils of 3 patients responded similarly, a degranulation being observed only with the natural glycosylated allergen.

➤ Batanero *et al.* tested a glycopeptide obtained from the olive pollen major allergen, Ole e 1 (166). This glycopeptide was able to activate the basophils of 5 olive allergic patients. And the same result was even observed with free glycans obtained from Ole e 1.

➤ A histamine release was demonstrated with Api g 5 in a patient allergic to celery (59). After deglycosylation of Api g 5 this effect was lost. But these results were disputed on the grounds that the deglycosylating agent used could have affected the protein spatial conformation (6).

➤ Although Hev b 13 is a major allergen in hevea latex, its IgE-reactivity was shown restricted to glyco-

epitopes (101). A similar finding has been reported for Hev b 4 (167). How many glycan chains are actually present on Hev b 13 is unknown, but this allergen could be "multivalent" because it has 3 potential glycosylation sites.

➤ Timothy grass Phl p 13 allergen was able to elicit a mediator release from the basophils of a patient whose IgE-reactivity was restricted to glycans (45). Because Phl p 1 gave no response in the same patient, the authors stated that multivalency is mandatory for any glycan-based cell reactivity (4 glycans are present on Phl p 13, compared to only one chain on Phl p 1) (45).

➤ Finally, an *in vivo* glycan-related activity was recently suggested in mugwort pollen allergic patients (168): responses to skin and to nasal provocation tests were higher with natural Art v 1 allergen than with its non-glycosylated recombinant counterpart.

Data supporting a significant clinical effect of glyco-epitopes are thus on the rise but still preliminary. This contrasts with the high rate of CCD-mediated positive results observed with *in vitro* techniques such as RAST or immunoblotting. An explanation could rest on methodological differences: in serum-based techniques the epitope/IgE ratio is typically high so that even low affinity bindings are revealed; in addition IgE molecules are free, an opportunity absent in cell-based systems (4, 169).

A low IgE-reactivity does not infer that carbohydrate epitopes are poorly immunogenic, however: it seems that glycan chains have an important role as an adjuvant during the sensitization process (170-172).

PRACTICAL CONSEQUENCES FOR ALLERGY DIAGNOSIS

As long as the demonstration of a clear *in vivo* effect of carbohydrate epitopes is still to be confirmed, one should consider these epitopes as potential interferants in serum-based assays. *In vitro* results could be affected in two different ways:

➤ the patient is sensitized to the extract allergen tested: anti-glycan IgE are measured in addition to anti-peptide IgE: the quantitative result is exaggerated. For example, the patient is mono-sensitized to peanuts and her/his peanuts RAST result is the addition of some kU/l related to peptide epitopes and of some kU/l related to glycan epitopes;

➤ the patient is not sensitized to the extract/allergen tested but the carbohydrate epitopes recognized by the patient's IgE are cross-reactive: they behave as CCD. For example, in a pollinic patient not sensitized to latex some glycan epitopes present in the latex

extract can bind to anti-glycan IgE present in the patient's serum. The RAST result is "falsely" positive.

Serum-based assays, such as RAST and other "specific" IgE tests, are typically at risk of a carbohydrate interference. But this also applies to immunoblot as exemplified by the recent discovery of Hev b 13, a major latex allergen which has been mistaken for Hev b 7 for a long time (101).

Cell-based tests (eg. basophil activation, histamine release, or flow cytometry) are much less prone to this carbohydrate/CCD interference (173).

One must stress some points about the interference in "specific" IgE assays:

- the diversity and the concentration of the epitopes contained in a commercial "specific" IgE assay differ from a manufacturer to another one (origin of the extract, binding on a solid phase, etc.). The impact of glyco-epitopes can thus greatly vary between commercial reagents, some of them being less affected (174);
- these differences can also extend to uncharacterized proteins present in the extract (and in the reagent) along with known allergens. These seemingly harmless proteins can cross-react with anti-glycan IgE as easily as well-known glycosylated allergens present in the extract (figure 5). An exaggerated response or a positive "specific" IgE test can thus result from a wide array of natural products.

Such matrix-induced problems have been poorly studied. The rare published data dealt with a different situation, ie. the lectin effect of certain extracts (eg. legumes) which cause a non-specific IgE (IgE molecules are themselves glycoproteins) (175-177);

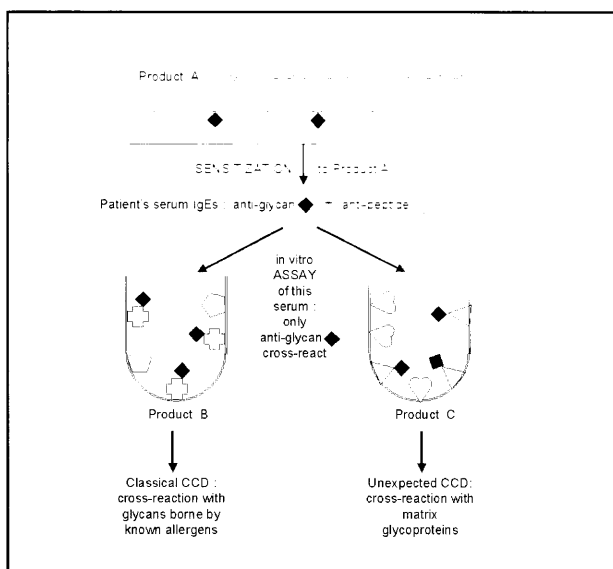


Figure 5: CCD reactivity is not restricted to allergens

- the risk of a CCD interference is higher when several products are mixed in the same reagent, especially when these products are of too heterogeneous origins: for example, beef, shrimp, kiwifruit and sesame in the same reagent.

In the example shown, sensitization to the product A gave rise to IgE directed to glycans in A (black diamonds). These IgE could cross-react with the CCD of already known allergens in the product B. But glycans present on non-allergenic proteins could also cross-react, generating a positive response in the case of product C.

Finally, with the growing use of recombinant allergens for diagnostic purposes, careful attention should be paid to checking the equivalence between recombinant allergens and their natural counterparts: for example, *Pichia pastoris*-based expression systems are able to add IgE-reactive mannosylated chains onto naturally non-glycosylated such as grass Dac g 5 (178) and mite Der p 1 (179) allergens. A similar phenomenon was observed with recombinant fire ant Sol i 3 allergen expressed in a baculovirus/insect cell system (180).

When could a CCD interference be suspected?

- **With plant-derived products:** All plant products must be suspected to contain IgE-reactive glyco-epitopes: pollens, foods, latex, etc.. A cross-reactivity between these glyco-epitopes 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 cause of p-CCD is pollinosis, especially in multiple pollen sensitized patients (22, 31, 181). Because pollen and food sensitizations are often associated, it is difficult to distinguish the proper part of food glyco-epitopes into the observed IgE-reactivity. In some cases, however, one can find help in paying attention to glycomes: for example, legume seeds contain highly mannosylated glycomes (182) which are only slightly prone to cross-react with grass glycan chains (22). The same could apply to non-fucosylated vicilins such as peanut Ara h 1 or hazelnut Cor a 11 (39, 183).

- **With other products:**

- some allergens present in mammals are glycosylated: ovomucoid in egg, Fel d 1 in cat hair, etc... Their glycan chains seem unable to cross-react with p-CCD;
- parvalbumins (found in fish) and tropomyosins (crustacea, mollusks, mites, and many other inverte-

brates) have no significant carbohydrate-related IgE-reactivity;

- some mollusk glycoproteins possess 1,3-fucosylated or xylosylated glycan chains (eg. snail hemocyanin). They might cross-react with p-CCD. But such a cross-reactivity is still to be demonstrated;
- a significant carbohydrate-related IgE-reactivity seems unlikely with mites, at least any cross-reactivity between mites and p-CCD;
- the relevance of glycan-induced IgE-reactivity is proved for hymenoptera venom. The cross-reactivity between p-CCD and venom glycans needs further studies. Data are lacking about the insect-derived aeroallergens (eg. cockroaches);
- fungi contain high mannose glycan chains able to generate cross-reactions between different mould species. These mould-CCD probably do not cross-react with p-CCD.

What is the quantitative effect of CCD on *in vitro* results?

This question has not been properly studied up to now. Few data are available:

- bromelain CAP results were found < 3.5 kU/l in pollen allergic subjects (n=14) (35);
- 75-90% patients positive for CCD in birch, mugwort or ryegrass pollen extracts had anti-CCD IgE concentrations < 1 kU/l (39);
- but 42% CCD RAST were > 1 kU/l in pollen allergic patients with an apple CAP result > 2 kU/l (184).
- very recently, Kochuyt *et al.* reported convincing data in 14 patients allergic to hymenoptera venoms but with false positive *in vitro* tests for pollens (185): CAP bromelain results ranged from 0.49 kU/l to 10.8 kU/l. A good parallelism was found between CAP bromelain and CAP pollen results in these patients.

In addition, some *in vitro* results could be turned positive only by the presence of CCD reactivity (34, 78, 165).

Are there simple methods to point out a CCD interference?

As long as commercial reagents for "specific" IgE assays are interfered by glyco-epitopes, the simplest way to point out such an interference consists in performing a bromelain RAST. If this test is positive the patient's serum contains some anti-CCD IgE (or the patient is actually sensitized to bromelain, an extremely rare event). A positive bromelain result means that certain other *in vitro* results could be exaggerated, or even positivized, by the glyco-epitopes present in the extract tested.

One must keep in mind that bromelain is an imperfect CCD model. This glycoprotein bears a glycan chain closer to grass pollen allergens than to tree pollen allergens (M0XF3 vs. GnGnXF3 or MMX3, respectively) (16, 186-188). The type of pollinosis has thus its importance (39) and it could help to take into account the glycomes of the natural products.

A bromelain RAST could be useful when *in vitro* results do not match the clinical picture (symptoms, skin tests), especially when numerous RAST are found positive. Checking the possible presence of anti-CCD IgE is advisable with three kinds of natural products:

- foods of plant origin: this mainly applies to vegetables and fruits but could be also useful to seeds such as peanuts;
- sensitization to hevea latex in a pollen allergic patient without occupational risk factors;
- in subjects both positive for honeybee and for wasp venoms (141).

CONCLUSION AND FUTURE PROSPECTS

Carbohydrate epitopes and CCD add a third level to IgE cross-reactivity mechanisms. A cross-reaction can arise:

- from taxonomy closeness (eg. inside a botanical family);
- from proteins belonging to the same family (eg. birch Bet v 1 and apple Mal d 1 are PR-10 proteins);
- but also from carbohydrate epitopes, ie. with neither taxonomical nor molecular relatedness.

While peptide epitopes have a clear impact on *in vivo* IgE-reactivity, glyco-epitopes still wait for confirmation of a clinical role. Their *in vitro* reactivity is thus to be considered as a potential complication in allergy diagnosis. This is especially troublesome when a positive result is only due to the presence of CCD, ie. to epitopes unrelated to the actual patient's sensitization.

For the same reasons, the problems induced by glyco-epitopes could complicate the pre-marketing assessment of genetically modified organisms (GMO). One step of the WHO/FAO protocol about GMO requires to check the absence of any *in vitro* reactivity of patients sera with the protein to be transferred to the GMO (189): Must CCD-positive sera been avoided or pre-treated, as suggested in the protocol? Opinions are divided about this point (190-192).

As long as the question of the clinical impact of glyco-epitopes is unanswered, efforts should be urgently made for correcting or measuring the interference of

these epitopes in commercially available RAST-type assays. To this end, some attempts have been recently made (22).

Other progress would be desirable, such as better delineating the importance of CCD among animal products, or improving our knowledge about O-glycan IgE-reactivity. For example, the unexpected IgE-reactivity of beta-arabinofuranoses on mugwort Art v 1 allergen (193) opens new avenues for studying O-glycan cross-reactivity. It could be also of interest to study the part played by anti-glycan IgG (25), especially in patients treated with immunotherapy, and to solve the question of whether adjacent peptide- and carbohydrate-epitopes are able to co-operate at the cell level (45, 169, 187).

One can expect significant advances in our understanding of CCD-related phenomena from the fast progress made in the domain of glycan chain mapping. Just as genome mapping efforts have benefitted to phylogenetics and to allergen classification, the accumulation of glycomes analyzed for glycobiology research aims could give better clues to correctly interpret *in vitro* results.

In addition, unraveling glycans and glycosylation could speed up works such as the synthesis of glycan-humanized proteins or the production of non-glycated allergens suitable for diagnostic or therapeutic purposes (29, 194, 195).

Finally, glyco-epitopes open up other exciting areas of research:

- because carbohydrate epitopes can cross-react outside the boundaries of protein families, they could behave as "panepitopes" and, as such, could be a powerful means of expanding the sensitization spectrum in an individual (196);
- are some subjects genetically predisposed to develop an IgE response towards CCD (197) Such "CCD phenotype" could represent a risk to become polysensitized (31), a risk which would be important to screen as early as possible in infancy.

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