

Coping with cross-reactive carbohydrate determinants in allergy diagnosis

FRIEDRICH ALTMANN

Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria

Abstract

A relevant proportion of allergy diagnosis is accomplished by *in vitro* determination of specific immunoglobulin E (sIgE) to extracts from suspected allergens. Such extracts inevitably contain glycoproteins, which may react with patients' IgE. In the case of plant and insect allergens, the relevant epitope structure is an α -1,3-fucose on the Asn-linked sugar residue of so-called N-glycans. Due to their wide distribution, N-glycans carrying this epitope are known as "cross-reactive carbohydrate determinant(s)" (CCD[s]). About 15 years of awareness allow the conclusion that anti-CCD IgE does not cause noticeable clinical symptoms. In consequence, diagnostic results arising from CCD reactivity must be rated as false positives. With up to 30% of CCD reactive patients, this can be regarded as a serious problem.

Another cross-reactive carbohydrate determinant became notorious as a potential cause of anaphylactic reactions to a recombinant glycoprotein drug carrying α -1,3-galactose. This galactose-containing determinant (GalCD, galactose containing cross-reactive carbohydrate determinant) was supposed as a trigger for delayed allergic reactions to red meat

in several cases. Thus, α -1,3-galactose may have clinical relevance in certain cases – possibly as a result of tick bites. Often, however, GalCDs probably cause false-positive results with milk and meat extracts. No clear evidence for the role of other non-human carbohydrate structures such as N-glycolylneuraminic acid as CCD has been presented so far.

Remedies for sIgE based *in vitro* diagnosis come in the form of non-glycosylated recombinant allergen components or of specific CCD inhibitors. The high potential of recombinant allergens is optimally realized in the context of component resolved diagnosis using allergen arrays with more than 100 components, whereas CCD inhibitors increase the specificity of conventional extract-based diagnosis. Reagents for the detection and inhibition of CCDs from plants and insects have been developed, whereas tools for GalCDs of milk and meat lag behind.

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Introduction

The obvious purpose of any diagnostic measure is to obtain an accurate impression of the patient's condition. In case of allergy diagnosis this entails the identification of the true culprit allergen while avoiding to indict harmless allergens. Among the various "mimickers of allergy" [1], protein-linked carbohydrates are a prominent and well defined cause of false-positive reactions. The following chapters will deal with plant/insect fucose-containing CCDs, how they became accepted as being clinically irrelevant, and how CCD-based false-positive

results can be avoided. Finally, the possibly more serious role of α -1,3-galactose containing GalCDs will be discussed.

History and structures of plant/insect CCDs

More than 30 years ago, Aalberse and co-workers incubated patients' sera with an unusual array of allergens and supposed allergens (e. g., potato and buckwheat). They observed an almost ubiquitous cross-reactivity of some sera [2]. More precisely, these sera reacted with extracts from pollens, vegetable foods, and – noteworthy – also hymenoptera venoms. The

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reactivity of the allergen extracts could be greatly reduced by periodate treatment, which destroys (mainly) terminal sugars in complex carbohydrates. While not absolutely specific, periodate sensitivity is a good indication for the carbohydrate nature of an epitope.

Meanwhile, several reports on cross-reactive IgG antibodies appeared. The most relevant was on an antiserum against horseradish peroxidase [3]. Astonishingly, this serum stained the neuronal chord in *Drosophila* embryos. The authors defined the epitope in horseradish as an Asn-linked oligosaccharide (N-glycan) with xylose and a fucose linked α -1,3 to the innermost GlcNAc residue (now called MMXF) (Fig. 1). Both elements are foreign to mammals, which explains how this N-glycan can be an epitope. A similar structure had been described earlier for pineapple bromelain [4], which is among the few plant glycoproteins available in decent purity and quantity.

The elucidation of the N-glycans of honeybee venom phospholipase revealed core α -1,3-fucose as the structural basis for the cross-reactivity between insect and plant allergens [5]. A parallel study exploited the comparable sensitivity of the fucose linkage to acid degradation to substantiate the role of this fucose residue. A quarter of 122 insect venom positive patients reacted with glycopeptides isolated from protease-degraded bromelain as shown by the ability of these glycopeptides to inhibit the binding of IgE to honeybee venom phospholipase [6]. This ability was largely abolished when the glycopeptides were treated with mild acid just to that point when the fucose residues were essentially removed. Apparently, a highly cross-reactive “allergenic” structure was detected. In the following years, a number of trials to proof its biological significance were undertaken.

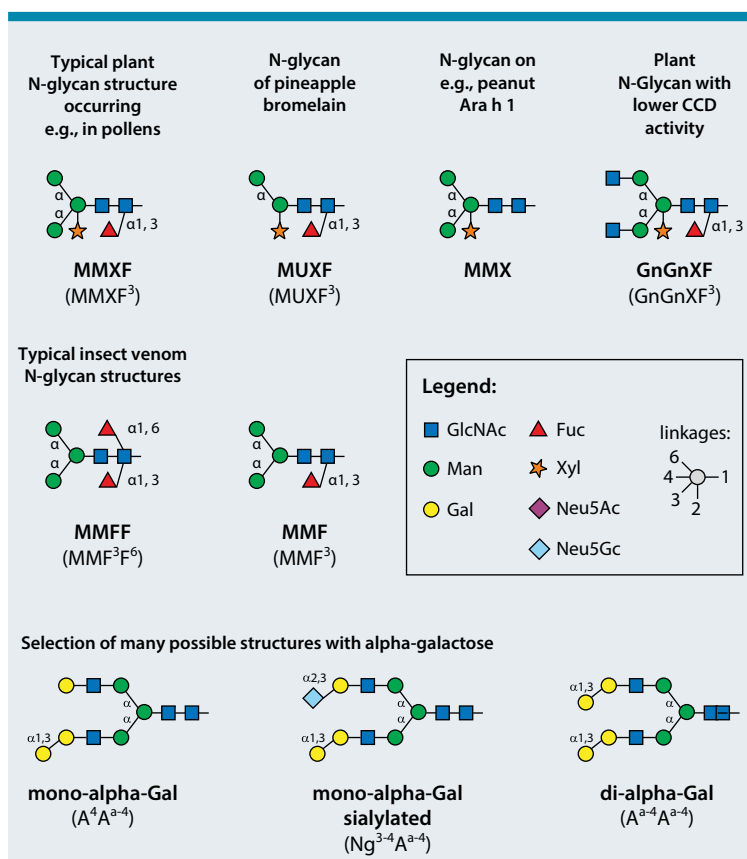


Fig. 1: Selection of relevant cross-reactive carbohydrate determinants. The glycans are composed of galactose (Gal, A), mannose (Man, M), N-acetylglucosamine (GlcNAc, Gn), fucose, (Fuc, F), xylose (Xyl, X), and/or N-glycolylneuraminic acid (Neu5Gc, Ng). Linkages are to be understood as β , except where indicated as α . The abbreviations of structures are according to the proglycan system. Briefly, all terminal sugars are listed starting with the one in the upper left corner and then moving counter-clockwise. In some cases, the linkage of the terminal sugar is unambiguous (e.g., with Man, Xyl, GlcNAc residues), in others the options are specified by superscripts. Further information can be found at www.proglycan.com.

Abbreviations

BAT	Basophil activation test
CCD	Cross-reactive carbohydrate determinant
Fuc (F)	Fucose
Gal (G)	Galactose
GalCD	Galactose containing cross-reactive carbohydrate determinant
GlcNAc (Gn)	N-acetylglucosamine
Man (M)	Mannose
Neu5Gc (Ng)	N-glycolylneuraminic acid
NRL	Natural rubber latex
sIgE	Specific immunoglobulin E
Xyl (X)	Xylose

High specificity and affinity of anti-CCD antibodies

Chemical deglycosylation is a destructive process of limited specificity. Thus, modifications other than the one intended may occur. More evidence for the role of individual sugar moieties could be provided by generating the structure rather than destroying it. This was achieved using recombinant, pure xylosyl- and α -1,3-fucosyltransferase and N-glycan acceptors from mammals, which are certainly free of the sugar residues under investigation. Soluble forms of these two glycosyltransferases were expressed in *Pichia pastoris* and used to modify human transferrin. Before, transferrin was trimmed with sialidase and galactosidase to get the so-called GnGn glycan structure [7]. Thus, glyco-variants of transferrin were generated specifically containing a

structural feature that was certainly not present on the native transferrin.

These neo-glycoproteins were then used to detect CCD-specific IgG and IgE in patients' sera. Among the patients with multiple grass sensitization, 24% were positive for α -1,3-fucosylated transferrin with or without xylose (MMF³ and MMXF³ glycan moiety), whereas none of these sera clearly reacted with the xylosylated transferrin [7, 8]. Remarkably, rabbit IgG can contain an antibody fraction specific for xylosylated only (MMX) glycans [7, 9].

As a final characterization of human IgE against CCDs, affinity-purified anti-CCD IgE and anti-CCD IgG were analyzed for their binding affinity to MMF³ and MMXF³ glycans [8]. In great contrast to other carbohydrate proteins such as lectins with their notoriously low binding affinities, the anti-CCD antibodies exhibited very high affinity. In the case of IgE, the dissociation constants were comparable to protein allergens. In the case of IgG, the affinity was clearly higher [8].

Biological significance of plant/insect CCDs

The process leading to the clinical symptoms of allergy can be re-enacted by test monitoring the degranulation of basophilic granulocytes either by measuring released histamine [10], interleukin 4 [11], or vesicle specific protein markers [12]. Such histamine release tests were performed with tomato β -fructofuranosidase or albumin coupled CCD-glycopeptides [10, 11] and pointed at biological activity very similar to that of undoubted allergens such as Bet v 1. The obvious conclusion was that core α -1,3-fucosylated N-glycans are relevant allergenic epitopes causing clinical symptoms.

Even earlier, van der Veen and co-workers performed histamine release with peanut extracts and sera from peanut allergic patients [13]. With sera that were peanut reactive solely on the basis of CCDs, degranulation only occurred at concentrations much higher than needed for protein based reaction with peanut extract. Due to this result, they concluded that CCDs lack biological significance [13].

However, one may challenge this test system for two reasons: First, the major peanut allergen Ara h 1 is mono-glycosylated and thus cannot perform the cross-linking of Fc ϵ receptors required for triggering degranulation. Second, peanut glycoproteins contain almost no fucose [14]. Anti-Xyl IgE never found above baseline – even in peanut allergic patients [Eiwegger T, Altmann F, Vienna, Austria; unpublished results]. Though the science of this work was built on rather uncertain grounds, the conclusion has prevailed. No clear evidence for CCDs provoking allergic reactions has ever been brought forward. As a possible exception the somewhat special

case of a tomato allergic patient could be mentioned for whom a xylosylated N-glycan appeared to be part of a peptide epitope [15]. A large study by Adriano Mari, who tested 1,831 subjects of which 23% reacted towards plant protein N-glycans, did not provide evidence for relevant CCD-based reactions [16]. Of the CCD-positive individuals, 21% showed but very weak skin reactions when tested with horseradish peroxidase, but not with phospholipase A² or ascorbate oxidase [16]. Given the up to eight N-glycosylation sites on peroxidase as opposed to one and two on PLA and ascorbate oxidase, this result can be taken as a corroboration of the concept that it requires multivalent binding of an allergen to trigger mast cell degranulation. Moreover, these results confirm the assumption that CCDs do not cause notable clinical symptoms.

Ebo and co-workers came with a different approach to the same conclusion [1]. Much later, the biological significance of anti-CCD IgE was examined by a commercial basophil activation test (BAT), in which CCDs could in fact lead to basophil activation as indicative of biological efficacy [12]. As the patients had been carefully diagnosed, this positive diagnosis against natural rubber latex (NRL) and apple could clearly be rated as false-positives.

Still unclear is the reason for this essentially complete inability of eliciting manifest allergic symptoms. Jin and co-workers speculated on an inhibitory role of IgG, as the anti-CCD IgE exhibited a higher affinity than IgG against well-known protein allergens [8]. It may well be envisaged that the inevitably frequent uptake of vegetable food (including cereals!) could desensitize against plant N-glycans by a mechanism akin sublingual immune therapy.

It is unknown why certain people develop anti-CCD IgE. A link to hymenoptera stings is often discussed [17], whereas the increased risk for anti-CCD IgE development among heavy drinkers supposedly does not explain the whole phenomenon [18]. An interesting link to the physiological function of IgE may be posed by a study, which found increased anti-CCD IgE as a result of parasite infection [19].

False positive diagnosis due to CCDs and possible solutions

The percentages of allergic individuals sensitized to plant/insect CCDs may be estimated from two large studies, where sera were collected rather unbiased and in which anti-CCD IgE was found in 23% [16] and 22% [20] of patient cohorts numbering well over 1,000 individuals. In the young adult cohort, the prevalence reached 30% [20]. Based on an admittedly only semi-quantitative strip test, most of the reactions were RAST class 2 or higher [20]. For-

tunately for the patients, their anti-CCD IgE does not bring them any inconvenience.

Already in 1998, Aalberse stated “These results support the concept that the accuracy of serological allergy tests will improve if CCD-related reactivity can be avoided” [21]. However it required the fundamental study of Mari [16] to anchor the clinical insignificance of CCDs in the consciousness of the experts. Mari’s conclusions were supported by a study on “mimickers of allergy”, which concluded “Sensitization to profilin and/or bromelain-type CCD, caused by pollen (timothy grass, mugwort) or hymenoptera venom allergens, can elicit false-positive IgE antibodies against natural rubber latex and apple” [1].

A particular problem is the assignment of the culprit insect in the case of apparent double sensitization against bee and wasp venom, which often is solely based on CCD cross-reactivity and only rarely a true double sensitization [22]. Nine years later, the idea of the innocence of CCDs consolidated further and Hemmer wrote “For the time being, we appreciate judging these antibodies as clinically insignificant as a useful hypothesis, ...” [23]. A consistent diagnosis of vespid venom allergy should eliminate the CCD problem, e. g., “by CCD-blocking” [24].

Taken together, CCDs found in virtually all plant allergen extracts and in all hymenoptera venoms are a frequent cause of false-positive diagnostic results in extract-based sIgE determinations.

Four possible remedies for the CCD problem:

1. In vivo testing such as skin-prick test or placebo controlled food challenges.
2. Ex vivo testing such as the basophil activation test, which, however, is not entirely unaffected by CCDs [1].
3. In vitro testing with recombinant allergens. This is increasingly done with the label “component resolved diagnosis” either in single allergen tests or on strips or biochip arrays, the most prominent of which currently is the ImmunoCAP ISAC system (Phadia, Uppsala, Sweden) comprising 112 components from 51 allergen sources. Component resolved allergy diagnosis very often gives a better guidance with regard to the steps to be taken as a consequence of a positive reaction, e. g., because it helps to unveil the primary sensitizing agent [25, 26, 27]. Recombinant allergens are typically produced in *Escherichia coli* and thus are devoid of any glycosylation. Therefore, there is no CCD problem anymore – in theory? However, the ISAC array contains a number of allergen components purified from natural extracts. Indeed, a recent study revealed nPhl p 4, nCyn d 1, nJug r 2, nCry j 1, nCup a 1, nOle e 1 and nPla a 1 (the “n” indicating natural

as opposed to recombinant array component) as being recognized by anti-CCD IgE, whereas nGly m 5, nGly m 6, nArt v 1, nAmb a 1, nJug r 1 and nJug r 3 were not [20]. So, even the ImmunoCAP ISAC is prone to deliver false positive results based on CCD-reactive IgE. But for most components, the sentence “... yields the impression of a discrepancy as positive findings in extract-based diagnosis were not verified by the ISAC results.” [28] will hold true.

Single allergen ImmunoCAP assays will show the same problem with the above mentioned natural components. Moreover, with CCD-positive sera, ImmunoCAP tends to yield +/- threshold readings for recombinant proteins despite their production in bacteria. The astonishing reason is that the cellulose sponges do not only consist of cellulose but also of some cotton glycoproteins carrying CCD epitopes [29]. The supplier may meanwhile have overcome this drawback.

4. In vitro testing with competitive inhibition with a specific CCD-inhibitor (**Fig. 2**). Any plant glycoprotein carrying an MMXF or MUXF structure could be used as inhibitor. The glycoprotein should contain several CCD structures to exploit the multivalency effect and it should be very pure to avoid an adverse influence by impurities that may have homologies to the allergens to be investigated. Similarly, the glycoprotein itself must not contain (cross-)reactive protein epitopes. This requirement can be met by proteolytic degradation of the glycoprotein, purification, and finally coupling of the glycopeptide to an immunologically inert carrier.

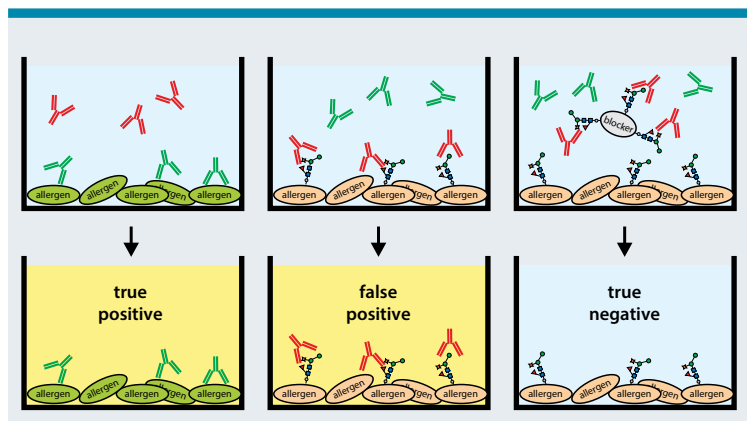


Fig. 2: Principle and effect of competitive inhibition of anti-CCD IgE. The left cup shows a reaction between a protein allergen and its specific IgE. The middle cup was coated with a glycoprotein that cross-reacts with anti-CCD IgE. Both samples give positive results. In the right cup, a polyvalent inhibitor (e. g., the semisynthetic glycoprotein used by Holzweber and coworkers [20]) was added resulting in competitive inhibition with coat allergen for the binding of CCD-specific IgE and thus in a – justifiably – negative result.

An example for the use of natural glycoproteins is the CCD inhibitor cocktail offered by Mediwiss Analytic GmbH (Moers, Germany). It consists of a cocktail of three glycoproteins (bromelain, horseradish peroxidase, and ascorbate oxidase) and is mixed with twice the volume of serum to give a total inhibitor protein concentration of about 300 µg/mL. Concerns about the rather high amount of plant protein and about the use of an active protease may be expressed.

An example for a protease treated inhibitor is the “proglycan” CCD-blocker (www.proglycan.com) consisting of bromelain glycopeptide coupled to human serum albumin [20]. This semi-synthetic inhibitor is added to serum in a volume ratio of 1 : 50 to arrive at a final concentration of 20 µg/mL. Extensive tests were performed with allergen strips (Fig. 3), the ImmunoCAP single allergen system, the ImmunoCAP ISAC (both Phadia, Uppsala, Sweden), and the Immulite 2000 (Siemens Healthcare, Erlangen, Germany) [20]. Generally, CCD inhibition led to a vast reduction of the number of positive, recte false-positive results in all test systems, even in the ISAC system for the reasons detailed above. Unsatisfactory results where the reading remained above or only slightly below the threshold of 0.35 U/mL were notably obtained for honeybee venom and the venom component Api m 1 (Tab. 1).

The positive reaction of Api m 1 in the single allergen test remained an enigma as the same component did not show any IgE binding in ISAC, which is much more in line with recombinant Api m 1 not being glycosylated. Could the cotton glycoprotein problem be responsible for such a high reading? More interesting is the case of the two insect venom extracts (aka complete allergens), where the non-inhibited result did not allow to decide on the culprit insect. The CCD-inhibited readings still saw honeybee venom as positive, but the difference to wasp venom now was huge (Tab. 1). The remaining 1.1 U/mL could arise from incomplete inhibition owed to the fact that bee venom has MMF and MMFF structures rather than MUXF (Fig. 1) as found on the semi-synthetic CCD-blocker [20]. Given the homology between bee and wasp venom proteins, the 1.1 U/mL might also result from cross-reactivity of a part of the anti-wasp venom IgE population. If this diagnosis was performed to decide on which venom to use for specific immune therapy, the result could hardly be clearer.

This review is not the place to discuss economic aspects or the difficulties of interpreting array data. It shall nevertheless be pointed out that combining extract-based sIgE determinations with CCD-inhibitions has the potential to resolve ambiguities arising from polysensitization.

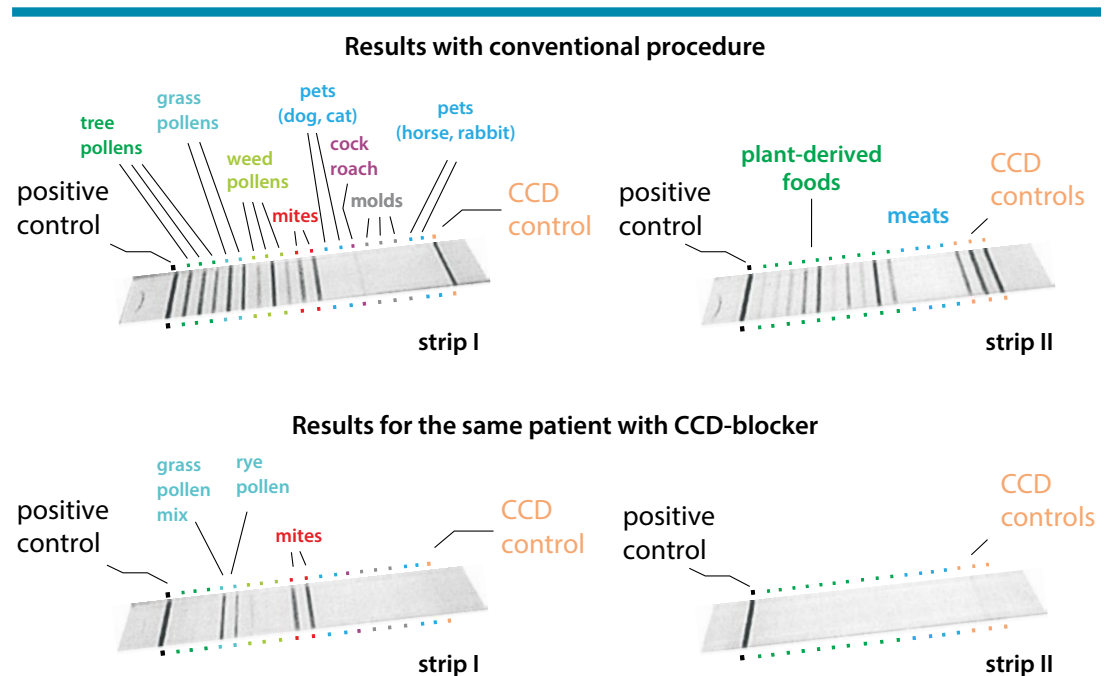


Fig. 3: Example of the effect of CCD inhibition. Serum of a 46-year-old male from Carinthia, Austria, was tested with custom made multi-allergen strips (Mediwiss Analytics, Moers, Germany) containing one or three CCD reporter bands. Experimental details can be found in [20]. In the presence of inhibitor only allergens with anamnestic substantiation appeared as positive.

Alpha-1,3-galactose – a mammalian CCD

A decade ago, two at first independent observations led to the discovery of yet another cross-reactive carbohydrate structure: allergic reactions towards a glycoprotein drug and delayed anaphylaxis after meat consumption. Severe, even lethal anaphylactic reactions were caused by the then novel anti-cancer antibody Cetuximab [30]. It was concluded that these reactions were based on pre-existing IgE against the alpha-galactose epitope (Gal- α -1,3-Gal- β -1,4-GlcNAc) [30]. While antibodies against this epitope prevail in the population [31], heavy adverse effect against Cetuximab were only seen in certain areas of USA's Midwest (later also Kenya) and the current guess for this phenomenon is sensitization by tick bites [32].

At the same place and time episodes of meat allergy were observed, which appeared strange as the clinical symptoms developed only several hours after the carnal meal and as they sprang up without warning and re-appeared unpredictably [33]. With the raised awareness due to the Cetuximab case, IgE to alpha-Gal was identified as the likely culprit epitope of meat allergy [32]. Pork kidney turned out as a particularly potent elicitor of anaphylactic reactions [34]. There appears to be a correlation between meat allergy and alpha-Gal IgE that allows to regard alpha-Gal as a carbohydrate epitope with clinical relevance [34, 35]. It should nevertheless be added that alpha-Gal IgE can also be found in healthy individuals [Soukop K, Altmann F, Hemmer W, Vienna, Austria; unpublished results]. While most people exhibit IgG against the alpha-Gal epitope, it may require some special trigger to obtain sensitization and IgE production [36]. This trigger could come from ticks, an idea that is further substantiated by patients' history and tick habitat range in eastern Austria [Swoboda I, Vienna, Austria; personal communication].

It must be stated that the macro-structure of an alpha-Gal IgE epitope (e. g., the effect of the number of alpha-Gal residues per glycan or its occurrence on O-glycans and glycolipids in addition to N-glycans) has not yet been defined [32]. Skin tests with an alpha-Gal containing non-allergenic protein have not yet been performed. The use of Cetuximab and bovine thyroglobin appears as a rather awkward approximation to a specific and defined tool for anti alpha-Gal-IgE determination or inhibition as both are a mixture of various structures and both contain N-glycolylneuraminic acid, another potential IgE epitope. Neo-glycoproteins with various trisaccharides containing α -1,3-galactose (Dextra Laboratories, Reading, United Kingdom) may be useful but have not been tested,

Tab. 1: Serum of a 46-year-old male analyzed by various methods with and without application of a CCD inhibitor (Extracted from [20]).*

CAP with extracts	CAP n U/mL	CAP i U/mL	Evaluation
Bee venom	28.5	1.1	drastic reduction, still pos.
Yellow jacket venom	28.5	18.3	clearly positive
CAP with components			
rApi m1	1.63	0.31	conflict with ISAC
rVes v1	11.8	9.7	clearly positive
rVes v5	48.7	44.6	clearly positive
ISAC (selected results)			
rApi m1	0	0	clearly negative
rPol d5	1.0	1.5	clearly positive
rVes v5	6.2	8.9	clearly positive

*Sera were tested by either the conventional ImmunoCAP test (Phadia, Uppsala, Sweden) or the multi-component array ImmunoCAP ISAC (Phadia, Uppsala, Sweden) either in the normal format (CAP n, ISAC n) or with CCD-inhibitor (CAP i, ISAC i).

certainly not in comparison to N-glycans with one or two α -1,3-galactose residues (**Fig. 1**).

Other potential CCDs

A variety of other non-human carbohydrate determinants could emerge as cross-reactive carbohydrate determinants. But so far, neither N-glycolylneuraminic acid (as found on Cetuximab [37], nor the Lewis a epitope found on plant glycoproteins, nor arabinogalactans on allergens [38] have as yet been described as IgE epitopes, even less as cross-reactive carbohydrate determinants. β -Arabinoses on weed pollen allergens can bind IgE but do not seem to be widely occurring [38].

Conclusion

Specificity of diagnostic tests is a big issue. Thus, it is surprising that in the case of CCDs the maximum achievement so far are products for the detection of anti-CCD IgE. Obviously, there is little pressure to react. Indeed, in the case of single allergen testing both physician and patient are contented if the four or five allergens tested provide positive results. The physician was – apparently – right and the patient is impressed by her/his uncanny instinct. Should the “allergen” later on never cause any discomfort it is taken by the

patient as a confirmation of the treatment's quality. With multi-allergen tests, however, the need for a rational management of the CCD issue arose. Hopefully, this review facilitates dealing with CCD-based false polysensitization.

Dr. Friedrich Altmann

Department of Chemistry
University of Natural Resources and Life Sciences, Vienna
Muthgasse 18
1190 Vienna
Austria
E-Mail: friedrich.altmann@boku.ac.at

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Conflict of interest

The author declares that there are no conflicts of interest.

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