

Crossreactive Carbohydrate Determinants

R. C. Aalberse* and R. van Ree

*Central Laboratory of the Netherlands Red Cross Blood Transfusion Service
and Laboratory for Experimental and Clinical Immunology, University
of Amsterdam, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands*

Introduction

Multireactivity of IgE has been an issue in IgE serology almost from the earliest days of the RAST. Three explanations have been offered, each of which has proven to be true in some cases:

1. Nonspecific/nonimmune binding of IgE to the allergosorbent; this phenomenon is particularly relevant when the total IgE of the serum is high (as is often, but not always, the case).
2. Independent sensitization of the patient to many different allergens; this will lead to an (often substantial) increase in total IgE and may therefore often be found in combination with the aforementioned nonimmune binding.
3. Crossreactivity of the IgE antibody. Some cases of crossreactivity are predictable, because the antigens are closely related. An example is the crossreactivity between cat serum albumin and dog serum albumin. Other cases of crossreactivity were unexpected; e.g., the crossreaction between profilin in pollen and in vegetable foods (1–6) or, even less predictable, the crossreaction between components in grass pollen extract and honeybee venom (7–9). Such reactions suggest that IgE in these cases lacked the specificity normally associated with antibody–antigen reactions. Extensive investigations of this type of crossreactivity indicated that it was not the IgE that lacked specificity, but the antigenic determinant. This ubiquitous antigenic determinant was found to consist of sugars rather than amino acids.

*Author to whom all correspondence and reprint requests should be addressed.
E-mail: aalberse@clb.nl

Lectins in Relation to Multispecific IgE Binding

Before turning to the main topic of this paper, (i.e., immunologically specific crossreactivity owing to evolutionary conserved sugar structures), it may be useful to discuss the conceptual and experimental distinction between IgE that is binding as a glycoprotein to a lectin, (10), and as an antibody to a protein-bound glycan.

This is important to discuss, because many vegetable allergenic source materials contain lectins, some of which bind well to the sugars that occur on IgE. By way of example, we will discuss the similarities and differences between IgE binding to: the *Pisum sativum* lectin (10) in green peas (this interaction does not depend on the immunological specificity of the IgE, but on its sugars); and the IgE binding to the lectin in potato (this interaction is immunologically specific: it is caused by IgE antibodies to the glycan found on the potato lectin [7]).

The lectin in green peas has a binding affinity for mannose-containing glycans. It binds to crosslinked dextrans such as Sephadex G75, from which it can be eluted using a low-mol-wt sugar, for which typically α -methyl-mannoside is used. When this lectin is coupled to produce an allergosorbent and used in a RAST-type protocol, it will bind IgE. Similarly, the lectin in potato, which has an affinity for chitin-like sugars (11–13), can be purified using chitin powder (e.g., from crab shell as the adsorbent), from which it can be eluted using chito-tetraose, or, more conveniently, an acid digest of chitin. When this lectin is coupled to produce an allergosorbent, it will also bind IgE.

Superficially, these RAST-type IgE-binding reactions are similar, particularly because both types of reaction can be inhibited by a soluble glycan. However, a closer look indicates marked differences:

1. Preincubation of the soluble inhibiting glycan with the solid-phase-coupled lectin, followed by a washing step, will still block the pea lectin. However, this will give no inhibition of the binding of IgE to potato lectin. This indicates that the inhibitor in the latter case reacts with IgE, rather than with the lectin. This is confirmed by the observation that the chitin-derived substrate for the potato lectin binding site does not interfere with IgE binding.
2. Relation with total serum IgE: A close correlation is found for IgE binding to the pea lectin, whereas the majority of the sera with a high total IgE do not bind to the potato lectin.
3. Specificity of lectin-binding IgE, as detected by affinity purification on lectin agarose: Pea lectin will bind IgE to cat or nonglycosylated invertebrate allergens, as well as IgE to glycosylated vegetable or invertebrate allergens, whereas potato lectin will bind only IgE to glycosylated vegetable and invertebrate macromolecules.

4. Heat and protease lability of the lectin: IgE binding to pea lectin is destroyed by these treatments, whereas IgE binding to potato lectin is not affected.
5. Susceptibility to procedures to destroy sugars on the lectin (such as periodate; *see below*): IgE binding to pea lectin is almost unaffected, whereas IgE binding to potato lectin is lost.

It proved difficult to produce deglycosylated IgE. However, the prediction is that such material would have lost its ability to bind to pea lectin but would still bind to potato lectin. IgE that binds to potato lectin also binds to many other, often well-characterized, nonmammalian glycoproteins, such as the enzymes bromelain and horseradish peroxidase—proteins without lectin activity.

The conclusion is that there is a similarity with lectin–glycoprotein interactions. The IgE that concerns us in this paper, however, is an antibody with a specificity for sugars, which functions as a lectin-like protein, rather than as the substrate for a lectin.

Types of Antigenic Determinants

The interaction between antigens and antibodies depends on close contact between surface structures of the antigen and the antibody. The contact area of the antigen is called the antigenic determinant or epitope. It is convenient to subdivide epitopes into peptidic and nonpeptidic epitopes.

Peptidic Epitopes

Peptidic epitopes are formed by amino acids in a folded protein. Such epitopes used to be subdivided into linear/sequential epitopes and conformational epitopes. Immunoblots prepared from denaturing gels were often assumed to show only epitopes formed by sequential amino acids. Similarly, reactivity of an antibody with a short (6–20 amino acids) synthetic peptide were interpreted to indicate that this antibody exclusively recognized the corresponding linear sequence in the antigen. However, the crystallographic elucidation of the structure of several antigen–antibody complexes clearly showed that the distinction between linear or sequential epitopes and conformational epitopes is of limited relevance: all peptidic epitopes studied so far show some contributions of amino acids that are distant in the linear sequence. Strictly sequential epitopes are likely to be the exception. The reactivity of an antibody to a conformational epitope on a denaturing immunoblot indicates that the polypeptide chains are extensively refolding during the immunoblot procedure. Short peptides will usually mimic only a small part of the epitope, and the reaction of the antibody with such a peptide has an affinity that is several orders of magnitude lower than that to the complete protein.

Nonpeptidic Epitopes

An important class of nonpeptidic epitopes are haptens—small structures (usually less than 5 kDa) that will not induce antibodies unless coupled to an immunogenic carrier structure. Antihapten antibodies will react with the isolated hapten and also to the hapten coupled to virtually any carrier structure—provided, of course, that the hapten is accessible rather than buried in the interior of the carrier.

Hybrid Epitopes

In addition, examples of hybrid epitopes are known. They are defined by antibodies that react preferentially to a hapten coupled to a particular (site on the) carrier. They can also be defined by antibodies that react to a hapten-modified carrier but not to the free carrier, nor to the hapten coupled to a different carrier.

Crossreactive Carbohydrate Determinants

In this section, we will focus on a particular group of nonpeptidic epitopes called crossreactive carbohydrate determinants (CCDs): glycans (i.e., sugar chains) that are found on a wide variety of non-mammalian glycoproteins. As with other haptens, pure glycans are usually poor immunogens, but the same glycan may elicit antiglycan antibodies, when coupled to a protein backbone. The glycan-part of mammalian glycoproteins is usually not immunogenic, because most glycans are common to all mammals; their immune systems develop tolerance to their own structures. However, glycans from nonmammalian sources (e.g., vegetable glycoproteins) are distinct from mammalian glycans, and strong antiglycan antibody responses are often found (7,8,13,14). Antibodies to vegetable glycans are often, but not always, crossreactive (i.e., these antibodies do not clearly distinguish different vegetable glycans). Consequently, these antiglycan antibodies often react with very different glycoproteins from nonrelated plants. Crossreactivity may even be found with invertebrate glycoproteins or glycans (7,15,16). The structure of some strongly immunogenic cross-reactive nonmammalian N-linked glycans has been elucidated (Fig. 1); for selected antibodies, differences in immune reactivity have been found to depend on a fucose and a xylose (13–15,17–23). In addition to the relatively well-characterized asparagine-linked mannose-containing glycans, there is a group of O-linked arabinose-containing glycans. These are often found in plant proteins rich in 4-hydroxyproline. The aforementioned potato lectin is a particularly good source for this glycan structure. When we first encountered the carbohydrate-related crossreactivity of IgE, we found strong reactivity with the potato; since

	Asp - GlcNAc - GlcNAc - Man - R (3/6) (2) Fuc Xyl		
	Fuc 1,6	Fuc 1,3	Xyl 1,2
Horseradish Peroxidase:	-	+	+
Ascorbate Oxidase:	-	-	+
Haemocyanine:	+	-	+
PLA2:	+	+	-

Fig. 1. Proposed structures for N-linked nonmammalian glycoproteins. PLA2, phospholipase A2 from honey bee; Asp, asparagine (linkage with the protein backbone); GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose; Xyl, xylose; fucose linkage can either be a 1,6-linkage or a 1,3-linkage; xylose (if present) is 1,2-linked.

this reactivity was often, but not always, recovered in the potato lectin fraction, we at that time proposed that these alkali-resistant O-linked arabinose-containing structures were good candidates for observed crossreactivity (7). However, the N-linked mannose/xylose/fucose glycans have also been identified as strongly immunogenic and cross-reactive structures. This crossreactivity was found not only for IgE antibodies, but also for hyperimmune rabbit antibodies (13,14,18). A full classification of all glycan-type crossreactive structures is likely to be very complicated, because of the variability of the sugar structures within a single glycoprotein, as well as the different crossreactivity patterns of individual antibodies. For the current discussion, it is relevant that CCDs are found in virtually all allergenic materials from vegetable sources, including food (7,24–28) and pollen extracts (7,29–31), as well as with purified pollen allergens from grass (32,33), olive (30), and Parthenium (31). Unexpectedly, insect venom is a frequent cause of the induction of IgE antibodies to CCD (7,24). However, the most frequently observed serological reactions related to CCD concern food allergens and the relation with food allergy.

Food Allergens

As discussed elsewhere (34), the terminology food allergen is ambiguous, as it has at least three interpretations:

1. A food substance that reacts with IgE antibodies.
2. A food substance that induces allergic reactions.
3. A food substance that induces allergic sensitization.

Some food substances do all three of these things and are called complete allergens. However, some will do interpretation 1, but not 2 or 3 (nonelicitors); whereas others will do 1 and 2, but not 3 (nonsensitizing elicitors).

Two Types of Incomplete Food Allergens

Nonsensitizing Elicitor

A food substance that induces allergic reactions, or nonsensitizing elicitor (e.g., the apple allergen that crossreacts with the major birch pollen allergen Bet v 1 [35–37]) is usually not responsible for the induction of IgE antibodies. The lymphoid cells of the immune system never see the apple allergen; the IgE antibodies are induced by a crossreacting allergen. In the example mentioned, birch pollen grains in the nose release Bet v 1 molecules, which results in an immune response, including IgE antibodies, to many epitopes on this molecule. Most of these antibodies are specific for the birch protein, but some are crossreactive, e.g., with the homologous protein from apple. These IgE antibodies circulate through the body and will fix to mast cells in the oral area. Upon subsequent oral contact with apple proteins, the local mast cells will react. This will induce perioral symptoms.

Another well-known vegetable protein in this category is profilin (1–6), for which pollen from grasses, trees, and weeds are relatively common sensitizers, and raw vegetables are good elicitors (e.g., carrots).

Nonelicitors

A nonelicitor (4,7–9,25,38–43) (usually, but not necessarily, nonsensitizing) reacts with IgE on the mast cell, but this interaction does not trigger the mast cell to degranulate. The reasons for this lack of mast cell stimulation are unclear, but it might possibly be related to monovalency of the allergen. One of the dogmas of allergy is that an allergen has to bind via at least two epitopes in order to crosslink two IgE antibodies on the mast cell surface. A monovalent allergen will bind only a single IgE antibody; therefore, this interaction will not result in IgE receptor crosslinking or mast cell stimulation.

Relevance of Anti-CCD Antibodies for Allergy

Three aspects make the CCD-structure require special attention:

1. The carbohydrate structure is more stable than most peptidic structures, both in relation to heat as well as to gastrointestinal enzymes. In stan-

dard laboratory tests (e.g., immunoblotting) the CCD structure stands out prominently and may mask clinically more relevant structures.

2. As mentioned earlier, anti-CCD antibodies tend to be highly crossreactive; consequently, a serum that, owing to whatever type of immunizing contact, has IgE antibodies to CCD will be positive in IgE antibody assays for a very wide range of vegetable sources.
3. CCD-containing foods are usually well-tolerated by patients with IgE antibodies to CCD.

These three points indicate the confusion that CCD-reactive antibodies may cause in relation to allergy diagnosis.

How to Deal with CCD Reactivity in Specific Allergy Serology

To avoid the above-mentioned diagnostic confusion, it is desirable to have two artificial types of reagents:

1. CCDs without a relevant, potentially IgE-binding protein backbone; and
2. Protein backbones without CCDs.

Using exhaustive protease digestion, CCD, with only a few amino acids attached, may be obtained with less than 0.1% of the peptidic immune reactivity; but this procedure also destroys part of the CCD immune reactivity, possibly by traces of glycosidase activity in the protease used (proteinase K). The second type of reagent can be obtained using recombinant proteins expressed in a microbial or mammalian host (*see below*). Alternatively, the CCD structure may in principle be removed and/or degraded by enzymatic or chemical means. However, none of the commercially available glycosidases we tested so far is very efficient in cleaving off CCD from native glycoprotein. Presumably, this is caused by shielding by the peptidic part. Periodate (7) is usually efficient in destroying CCD immune reactivity, but some loss of peptidic immune reactivity may occasionally be found.

CCD in Relation to Recombinant Allergens

The glycosylation pattern of a recombinant protein is determined largely by the host used for its expression. This means that these IgE-binding epitopes will not be found when vegetable glycoproteins are expressed in microbial expression systems, but the reverse. Expression of microbial or mammalian glycoproteins in plant or invertebrate expression systems will induce CCD-type epitopes on these proteins.

If the lack of symptom elicitation via CCD-anti-CCD can be substantiated, the aforementioned differences in glycosylation pattern will have to be interpreted in a way very different from the more familiar

situation, in which IgE binding is associated with clinical allergy. The use of recombinant plant glycoproteins lacking the CCD-structure owing to their expression in a microbial host may be a distinct advantage from the diagnostic point of view, because the test system becomes more specific and more relevant. In many, but not all, cases, glycosylation does not have a major effect on the antigenic structure of the protein, but the effect of glycosylation on the overall structure of a protein needs attention for each allergen.

Prevalence of IgE to CCDs

As with all such studies, prevalence numbers depend on the population sample and on the detection sensitivity. To our knowledge, large random population surveys with explicit tests for CCD reactivity have not been published. However, population surveys for serological reactivity to foods, insect venom, or latex give some indications, particularly if these results can be combined with tests for reactivity to pollen. Such studies indicate that the prevalence of all three types of positive serology (foods, venom, and latex) is substantially higher (range 1–10%) than expected on the basis of the prevalence of objective symptomatology. Moreover, an association with IgE to pollen is evident.

We have some preliminary information on the prevalence of IgE to CCD in a grass pollen-positive population ($n = 214$). Of this sample 21% had IgE to profilin and/or CCD (17% to profilin: 14% to CCD). In another study, we selected sera for IgE antibodies to pollen and/or vegetable foods ($n = 520$); 23% reacted in the RAST to birch-derived CCD, 10% to mugwort-derived CCD, and 14% with grass-derived CCD.

Future Directions

The chemical and serological studies have clarified much of the structural basis of CCD-type crossreactivities, but many details are still unknown. A major issue is the heterogeneity of the crossreactivity patterns of the glycans, which markedly depends on the antibody used. Some antibodies, particularly polyclonal antisera, have a broad cross-reactivity; whereas others, particularly some monoclonal antibodies, have a much more restricted specificity profile. When we tested a panel of 19 sera that were RAST-positive both to birch and to apple, we found 7 sera virtually negative for celery and carrot (sera A-G in Fig. 1). Of the 12 celery/carrot positive sera, 4 were positive for bee venom (sera P-S in Fig. 2). When we performed a statistical clustering solely on the basis of the direct RAST data without any inhibition analysis, we obtained the pattern shown in Figure 3. This pattern fits quite well with what we now know about the major crossreactive structures of Bet v 1, Bet v 2/profilin, and CCD. However, it is also evident that the individual pat-

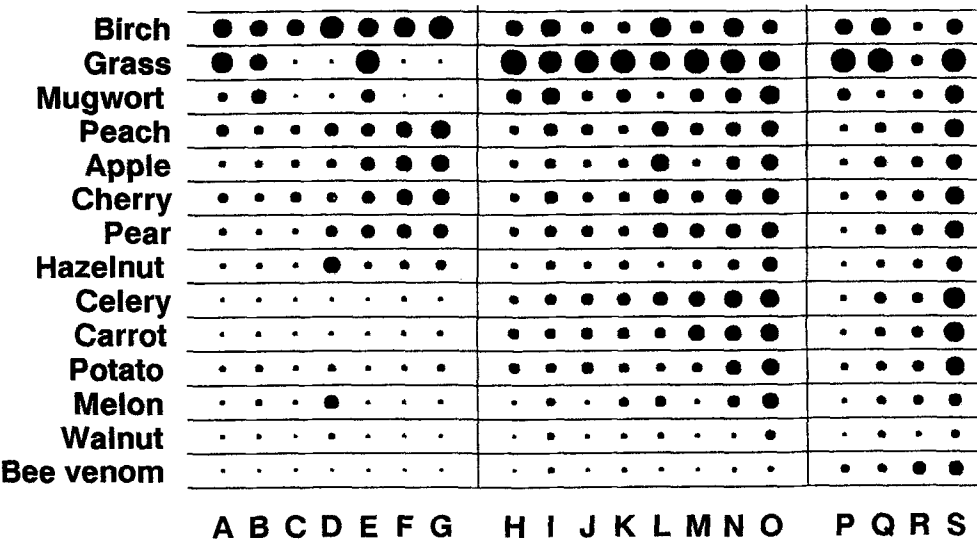


Fig. 2. RAST reactivity of 19 sera (coded A–S), selected on the basis of a positive RAST for both birch and apple. In order to present the results in a clear way, the RAST results have been translated into dot diameters. Serum A–G, negative for celery/carrot; serum P–S, positive for honeybee venom.

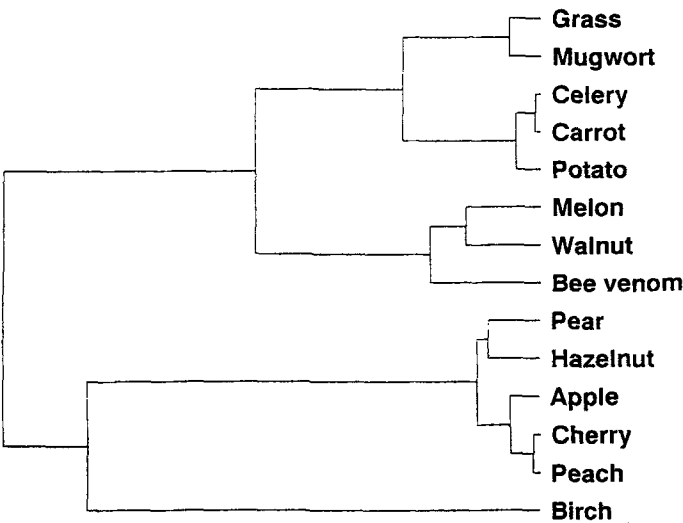


Fig. 3. Statistical clustering of allergen extracts based on the RAST results shown in Fig. 1. The upper five represent the profilin-positive, low-Bet v 1 set; the next three the low-profilin, low-Bet v 1 set (but, as all the other extracts, CCD-positive); the lower six the Bet v 1-positive, profilin-positive set.

terns are complex. This is further illustrated by the data presented in Fig. 4, where we show RAST data for 16 sera with a positive buckwheat and RAST. Some of these sera reacted very well with buckwheat honey.

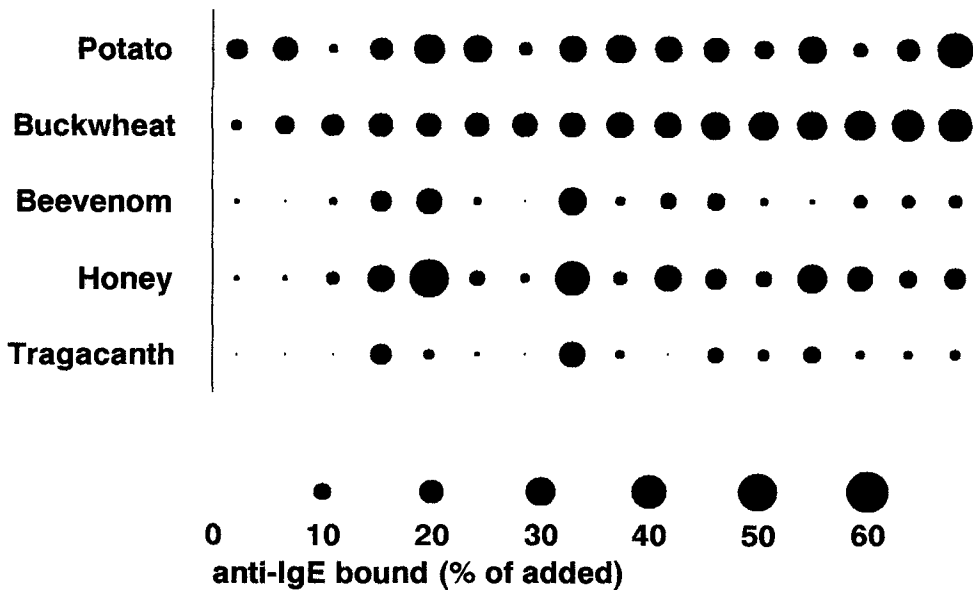


Fig. 4. Sixteen sera with a positive buckwheat/potato RAST tested for reactivity to buckwheat honey, honeybee venom, and tragacanth gum. The batch of honey used had little reactivity with two sera that were strongly reactive with pollen profilin (the two sera to the left).

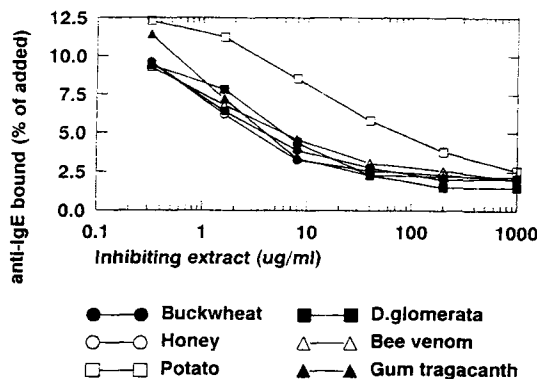


Fig. 5. Inhibition of the buckwheat RAST by the inhibitors shown.

Honey may often be contaminated with pollen, but this batch proved to contain very little profilin. The honey RAST correlated not only with the RAST for bee venom, but also with a RAST for tragacanth gum, a polysaccharide with very little protein. Figure 5 shows by RAST-inhibition that these extracts actually crossreact. In Fig. 6, it is shown that this crossreactivity is found not only with IgE antibodies, but also with hyperimmune rabbit antibodies. These natural gums are very complicated glycan structures, but not all anti-CCD antibodies react with this gum.

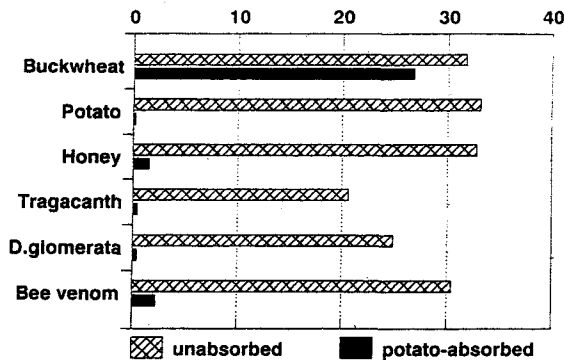


Fig. 6. A rabbit antiserum obtained after immunization with buckwheat extract was tested before and after absorption with Sepharose-coupled potato extract. The antibody activity (50- μ L serum 1:1000) was tested using the indicated six CCD-sources coupled to Sepharose in combination with iodinated antirabbit IgG.

It would be very helpful to have well-characterized glycans rather than the mixed structures that are currently mostly used. The immunological relation with other nonmammalian glycan structures, including O-linked sugars and other structures on the ubiquitous proline-rich glycoproteins, needs to be elucidated. Most importantly, the lack of cell-triggering activity of IgE-CCD interactions needs to be more fully understood. A better insight into these matters will undoubtedly contribute substantially to the reliability of IgE serology.

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