Structural and functional analyses of antibodies specific for modified core N-glycans suggest a role in T_{H2} responses

Melanie Plum\textsuperscript{1,2} | Luna Tjerrild\textsuperscript{3} | Tim Raiber\textsuperscript{1,4} | Frank Bantleon\textsuperscript{1} | Sara Bantleon\textsuperscript{1,4} | Michaela Miehe\textsuperscript{1} | Frederic Jabs\textsuperscript{1,4} | Henning Seismann\textsuperscript{5} | Christian Möbs\textsuperscript{6} | Wolfgang Pfützner\textsuperscript{6} | Thilo Jakob\textsuperscript{7} | Gregers R. Andersen\textsuperscript{3} | Edzard Spillner\textsuperscript{1}

\textsuperscript{1}Immunological Biotechnology, Department of Biological and Chemical Engineering, Aarhus University, Aarhus, Denmark
\textsuperscript{2}Division of Clinical and Molecular Allergology, Research Center Borstel, Leibniz Lung Center, German Center for Lung Research (DZL), Borstel, Germany
\textsuperscript{3}Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark
\textsuperscript{4}Institute of Biochemistry and Molecular Biology, Department of Chemistry, University of Hamburg, Hamburg, Germany
\textsuperscript{5}Department of Oncology and Hematology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
\textsuperscript{6}Clinical & Experimental Allergology, Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany
\textsuperscript{7}Department of Dermatology and Allergy, University Medical Center Giessen, Justus Liebig University, Giessen, Germany

Correspondence
Edzard Spillner, Immunological Biotechnology, Department of Biological and Chemical Engineering, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark.
Email: e.spillner@bce.au.dk

Abstract

\textbf{Background:} Immune responses to N-glycan structures from allergens and parasites are often associated with pronounced, high affinity IgE reactivities. Cross-reactive carbohydrate determinants (CCDs) are constituted by modified N-glycan core structures and represent the most frequently recognized epitopes in allergic immune responses. Although recently accepted as potentially allergenic epitopes, the biological and clinical relevance as well as structural and functional characteristics of CCD-specific antibodies remain elusive.

\textbf{Methods:} In order to gain structural insights into the recognition of CCDs, two specific antibody fragments were isolated from a leporid immune repertoire library and converted into human/leporid IgE and IgG formats. The antibody formats were assessed by ELISA and surface plasmon resonance, structural and functional analyses were performed by X-ray crystallography, mediator release, and ELIFAB assays.

\textbf{Results:} The recombinant IgE exhibited highly specific interactions with different types of CCDs on numerous CCD-carrying glycoproteins. Crystal structures of two CCD-specific antibodies, one of which in complex with a CCD-derived disaccharide emphasize that mechanisms of core glycan epitope recognition are as specific as those governing protein epitope recognition. The rIgE triggered immediate cellular responses via FcεRI cross-linking and mediated facilitated antigen presentation by binding of IgE/antigen complexes to CD23, a process that also could be blocked by IgG of allergic patients.

\textbf{Conclusions:} Our study provides evidence for the relevance of N-glycan recognition in T_{H2} responses and corroborates that IgE and IgG antibodies to ubiquitous carbohydrate epitopes can be equivalent to those directed against proteinaceous epitopes with implications for diagnostic and immunotherapeutic concepts.

\textbf{KEYWORDS}
anaphylaxis, glycotopes, IgE and IgG, N-glycan, recognition

Abbreviations: CCD, cross-reactive carbohydrate determinant; Fab, fragment antigen binding; HRP, horseradish peroxidase; MUXF, the N-glycan from bromelain.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2022 The Authors. Allergy published by European Academy of Allergy and Clinical Immunology and John Wiley & Sons Ltd.
**GRAPHICAL ABSTRACT**

Monoclonal antibody fragments with specificity for CCD structure were generated. Derived CCD-specific IgE detect CCD-carrying allergens and are capable for mediating effector cell activation and facilitated allergen binding. A Fab in complex with an epitope surrogate provides direct insights into the binding mode of CCDs.

**Abbreviations:** CCD, cross-reactive carbohydrate determinant; Fab, fragment antigen binding; HRP, horseradish peroxidase; MUXF, the N-glycan from bromelain

1 | INTRODUCTION

Carbohydrate antigens of pathogens and environmental substances can provoke pronounced responses of the adaptive and innate immunity. Although carbohydrates mostly are considered to be T cell independent antigens and incompetent to raise high affinity responses, low affinities of carbohydrate-specific proteins are often compensated by multivalency to enable the physiologically intended effects. In contrast to their immunological significance, little is known about molecular aspects of carbohydrate recognition.

In pathological conditions associated with elevated levels of IgE such as type I allergy, pronounced IgE reactivities to carbohydrate structures can be found. IgE is an isotype that often exhibits outstanding affinities, which allow potent stimulation of immediate type immune responses by minute amounts of allergen.

The carbohydrate epitopes recognized by IgE, also called cross-reactive carbohydrate determinants (CCDs), have recently been included in the allergen database of the WHO/IUIS Allergen Nomenclature Sub-Committee as potentially allergenic epitopes. CCDs are established by specific glycosyltransferases of mostly non-mammalian species such as helminths, plants, and insects. The hallmark of classical CCDs is constituted by \( \alpha \)1,3-linked core fucose residues found on insect and plant glycans, and, additionally, spatially separated \( \beta \)1,2-linked xylose on plant- and helminth-derived CCDs (Figure 1A). These highly immunogenic glycotopes represent an universal principle for cross-reactivity of glycoproteins. Core-modified glycans were also shown to be part of the humoral response in helminth parasite infection and allergy and are potent inducers of Th2 immune responses. The exact role of the core modifications in allergy and parasite infection, however, still remains unclear.

Notably, anti-CCD antibodies are detected in up to 50% of the normal human population and IgE antibodies to CCDs are present in >30% of allergic patients. Hence, modified N-linked glycans represent the most frequently recognized IgE epitope. Although initial studies suggest high antibody affinities for core-modified carbohydrates, the clinical consequences of their binding to IgE antibodies however remain open. In contrast, IgE with specificity for the well-known \( \alpha \)-Gal carbohydrate epitope is the underlying principle of a novel type of anaphylaxis and high affinity of the IgE appears to be a crucial parameter. Apart from phenomenological knowledge, however, structural details on the interaction of IgE with carbohydrate antigens and the basis for in vivo activity remain scarce.

Generally, only a few structures of antibodies in complex with carbohydrate antigens-mostly LPS from different species-have been determined. Two structures of murine antibodies in complex with mammalian Lewis antigens are known. Analyses of human carbohydrate-specific antibodies are often hampered by low affinities, low serum concentrations, and broad unavailability. Molecular and structural data regarding carbohydrate-specific IgE antibodies are not available at all, since the very low number of IgE-producing cells renders hybridoma technology, single-cell technologies, and the establishment of IgE-derived immune repertoires highly difficult.

Antibodies against carbohydrate epitopes that are characterized by high immunogenicity and a defined architecture can also be obtained from animals and constitute a valuable tool if the recognized epitopes are identical to those of human antibodies. Although the immunogenicity of xenobiotic glycan core structures in rodents, for example, rabbits, is described, immunological analyses yielded contradictory findings on specificity and affinity so far.
Hence, the aim of our work was to obtain insights into the recognition of CCDs by specific antibodies using structural and functional analyses. Recombinant chimeric IgE and IgG formats and derived fragments were employed for characterization of the interaction, cellular activation tests, and molecular dissection of the glycotope by crystallographic analyses. This work contributes to the dissection of the antibody carbohydrate interaction and molecular aspects in carbohydrate-mediated anaphylaxis.

2 | MATERIAL AND METHODS

2.1 | Materials

Anti-V5 antibody was purchased from Life technologies. Polyclonal rabbit anti-horseradish (HRP) serum, anti-rabbit, and anti-mouse-IgG alkaline phosphatase (AP) conjugates, and HRP containing the MMXF (Manx1–6(Manx1–3)(Xylx1–2)Manx1–4GlcNAcβ1–4(Fucα1–3)GlcNAc) structure were obtained from Sigma-Aldrich. The anti-IgE antibody was purchased from BD Pharmingen. The MUXF-HSA conjugate (Manx1–6(Xylx1–2)Manj1–4GlcNAcβ1–4(Fucα1–3)GlcNAc-human serum albumin) was kindly provided by Siemens Healthcare Diagnostics.

2.2 | Structural analysis of the antibodies

The purification of the antibodies, crystallization and structure solution as well as the synthesis of the epitope disaccharide are described in the Appendix S1.

2.3 | Cellular mediator release assays

In vitro degranulation was analyzed as described previously. After sensitization of RBL-SX38 cells with IgE, CCD-carrying proteins were added and incubated for 60 min at 37°C. As reference, cross-linking was achieved by polyclonal goat anti-human IgE antibody (1 μg/ml, Sigma-Aldrich). β-Hexosaminidase release of viable versus lysed cells was assessed with p-nitrophenyl N-acetyl-glucosaminopyranoside (Sigma-Aldrich) as a substrate.
2.4 Analyses of CD23 binding using ELIFAB assay

In order to evaluate the capability of CCD-specific IgE for establishment of IgE/allergen complexes and their binding to CD23 the surface-based ELIFAB assay, a cell-free variant of the cellular FAB assay was performed. Moreover, the capacity to block IgE/allergen complex formation of insect venom-allergic patients’ sera containing HRP- and CCD-specific IgG antibodies was assessed. Therefore, 20 μl of rIgE (10 μg/ml) either mixed with 20 μl of individual patient serum or 20 μl of RPMI medium were preincubated with 5 μl HRP (1 μg/ml) at 37 °C for 1 h in order to allow for formation of IgE/allergen complexes. The rIgE was previously proven to exhibit an optimal antibody/allergen ratio for complex formation at a concentration of 1 μg/ml HRP. HRP was used as glycosylated allergen to allow defined assay conditions. After preincubation, IgE/allergen complexes were transferred to plates coated with soluble CD23 (R&D Systems) and incubated for 1 h at room temperature. After washing, IgE/allergen complexes bound to immobilized soluble CD23 (Sigma-Aldrich) were detected by adding biotin-conjugated anti-human IgE antibody (BD Biosciences), streptavidin-peroxidase and 3,3′,5,5′-tetramethylbenzidine (TMB; both Sigma-Aldrich). All samples were analyzed in duplicates.

Other methods including the generation, conversion, and production of the antibody formats as well as the analyses of immunoreactivity are described in the Appendix S1.

3 RESULTS

3.1 Generation and characterization of carbohydrate-specific IgE and IgG1 antibodies

After HRP immunization, leporid IgG variable region of the heavy and light chains (VH and VL) repertoires was amplified from spleen and bone marrow cDNA and combined to a scFv format using a cloning strategy allowing for an unbiased assembly of VH and VL. After iterative panning by phage display against HRP carrying the MMXF structure and a HSA conjugate carrying the MUXF structure found, for example, on bromelain, ELISA analyses demonstrated enrichment of the library and immunoreactivity of individual clones (Figure S1). The use of different targets for selection yielded two predominant antibody clones, for both of which additional sequences with single substitutions were found at lower frequency (Figure S2).

The predominant antibody fragments H1 and M5 then were converted into leporid/human IgG1 and IgE immunoglobulin formats comprising the scFv-based binding moieties and the IgE and IgG Fc constant domains and produced in mammalian cells. Notably, expression yields of the IgG and IgE format were found comparable. PAGE analyses verified the molecular masses suggesting proper folding and glycosylation, in particular for the highly glycosylated IgE as compared to the IgG (Figure 1B). The H1 IgG and IgE antibody exclusively detected the particular MMXF target structure of HRP and the M5 IgG and IgE antibody predominantly the MUXF structure as present on different glycoproteins in ELISA (Figure 1C–F). The ability to interact with recombinant soluble Ig Fc receptor proteins further suggested proper folding of the IgE as well as the IgG Fc domains (Figure 1C,E). These data underline that the recombinant antibodies represent functional equivalents of natural IgE and IgG antibodies and should exhibit comparable characteristics.

To further address specificity and sensitivity, the two rIgE were applied to diagnostically important allergens and extracts (Figure 2). In contrast to non-glycosylated and CCD-devoid proteins, allergens and extracts of known CCD reactivity exhibited pronounced immunoreactivity and also reflected the fine specificities of H1 and M5 IgE for the different CCD structures. Minor IgE reactivities were also demonstrated for parasitic Anisakis and Ascaris, but not Echinococcus species. As assessed by glycan arrays, the H1 and M5 IgE did not show immunoreactivity to 100 different carbohydrate epitopes (data not shown) including fucosylated and 1,3-linked oligosaccharides such as Lewis antigens and blood group antigens.

Against the tenet of lower affinities governing protein/carbohydrate interactions, robust interaction of the CCD-specific immunoglobulins was validated by SPR analyses yielding a K_{D} value of the H1 IgE of 1 × 10^{-7} M for HRP (Figure S3). The M5 IgE exhibited K_{D} values of 8 × 10^{-8} M and 8 × 10^{-9} M for HRP and MUXF conjugate, respectively. In an inverse setting mimicking receptor-bound antibodies on effector cell surfaces by immobilization of antibodies, a K_{D} value in a similar range was obtained for the H1 IgE and HRP (Figure S3). These values, however, have to be taken with care, as the bivalency of the IgE format and, in the inverse setting, the multiplicity of CCDs on HRP and the MUXF conjugate complicate the analyses.

3.2 Biological activity of CCD-specific antibodies

The potential of H1 and M5 IgE for activation and degranulation of effector cells by FcεRI cross-linking was assessed by determination of β-hexosaminidase release from RBL-SX38 cells (Figure 3). Since receptor cross-linking by monoclonal IgE demands the availability of more than one epitope per allergen we used the HRP and MUXF having multiple CCD epitopes. The two glycoproteins mediated clear-cut antigen-dependent activation as compared to anti-IgE-mediated antigen-independent activation (Figure 3A,B). Thereby, the two IgE exhibited clearly varying characteristics in line with their immunoreactivity to the different antigens. H1 IgE exclusively mediated degranulation by HRP. For M5 IgE, both antigens resulted in mediator release although to varying extend. Notably, even lowest concentrations of antigen (0.01 ng/ml MUXF conjugate) were highly efficient in mediating degranulation.

Binding, uptake, and presentation of allergens via CD23 in form of allergen/IgE complexes are considered key processes in allergic immune responses. Therefore, the M5 IgE was further evaluated for facilitated antigen binding (FAB) and inhibition by specific IgG using a surface-based variant of the FAB assay, the ELIFAB assay. Complexes of the M5 IgE and MUXF conjugate (Figure 4A) or the HRP (Figure 4B) bound efficiently to CD23, corroborating...
the capability of CCD-specific IgE to form immune complexes of higher-order and facilitate allergen binding via CD23. Addition of allergic patients’ sera with varying levels of CCD-specific IgG (Table S3) reduced the binding of the glycoprotein/IgE complexes to CD23, in contrast to sera without CCD-specific IgG (Figure 4A,B).

Notably, the mere CCD-specific IgG level did not correlate with the extent of reduction.

These data clearly suggest that CCD-specific IgE provide the entire intrinsic potential to interact with receptors, activate effector cells, and facilitate allergen presentation.
Structural insights into recognition of glycans by CCD-specific antibodies

In order to obtain molecular information on the recognition of N-glycan core structures, we aimed for structural analyses of the CCD-specific antibodies by crystallography. The clones H1 and M5 were produced as human IgG Fab antibodies in insect cells. PAGE and ELISA analyses demonstrated proper assembly of the two Fab fragments (Figure S4). The monovalent Fabs were subjected to SPR analysis, which revealed $K_D$ values of M5 Fab of $5.9 \times 10^{-7}$ M to HRP and $1.5 \times 10^{-7}$ M to MUXF. The $K_D$ value of $1 \times 10^{-6}$ M was obtained for the H1 Fab to HRP (Figure S3). These values reflect the relation of $K_D$ values observed for the bivalent rIgE.

The epitope surrogate disaccharide Fuc$\alpha_1$-3GlcNAc$\alpha_1$-OMe (Fuc-mNAG) (Figure S5, Tables S1 and S2) was used for co-crystallization with the Fab fragments, and we obtained diffraction data extending to at least 1.8 Å for both. Data collection and refinement statistics are summarized in Table 1.

Upon structure determination, it became clear that the M5 Fab had bound the disaccharide in the crystalline state. Difference $mF_o$ - $DF_c$ electron density calculated without the disaccharide included in the model (Figure S5A) unambiguously allowed us to place the fucose and the methylated N-acetylglucosamine in a manner compatible with their $\alpha_1,3$ glycosidic bond, the N-acetyl group on the glucosamine and both pyranose rings adopting a chair conformation. Due to the relative weak density $2mF_o$ - $DF_c$ obtained upon inclusion of

### Table 1 Statistics for data collection and refinement

|                      | H1 (S18K) | M5:Fuc-mGlcNAc (S18O) |
|----------------------|-----------|-----------------------|
| **Data collection**  |           |                       |
| Wavelength (Å)       | 0.8726    | 0.8726                |
| Space group          | P4,$2_1$  | P2,$2_1$              |
| a, b, c (Å)          | 62.647    | 63.447 63.587108.771 |
| $\alpha, \beta, \gamma$ (°) | 90 90 90  | 90 90 90           |
| Resolution range     | 48.33–1.77 (1.83–1.77) | 44.91–1.80 (1.86–1.80) |
| Total no of reflections | 457,333  | 273,313                |
| No of unique reflections | 45,033 (3813) | 41,512 (3833)         |
| Completeness (%)     | 99.21 (92.74) | 99.97 (100.00)        |
| Multiplicity         | 10        | 6                     |
| $(1/e(l))$           | 21.15 (1.71) | 9.05 (1.72)          |
| $R_{merge}$          | 0.077 (0.59) | 0.17 (0.982)         |
| Wilson B-factor (Å$^2$) | 19.66    | 16.14                |
| **Refinement**       |           |                       |
| $R_{work}$           | 0.1826 (0.2766) | 0.1643 (0.2685)     |
| $R_{free}$           | 0.2113 (0.3157) | 0.2047 (0.3456)     |
| No. of non-H atoms   | 3767      | 3899                  |
| Protein              | 3223      | 3250                  |
| Ligand               | –         | 26                    |
| Water                | 544       | 664                   |
| Protein residues     | 434       | 436                   |
| R.m.s. deviations    |           |                       |
| Bonds (Å)            | 0.003     | 0.007                 |
| Angles (°)           | 0.91      | 1.12                  |
| **Average B-factor (Å$^2$)** |         |                       |
| Protein              | 22.70     | 18.00                 |
| Ligand               | –         | 44.80                 |
| Solvent              | 34.70     | 31.40                 |
| **Ramachandran plot** |           |                       |
| Favored (%)          | 96        | 97.7                  |
| Additionally allowed (%) | 3.53  | 2.3                  |
| Outliers (%)         | 0         | 0                     |
| Clashscore           | 4.41      | 0.92                  |

**Note:** $R_{sym} = (\sum_i \sum_h |I(h)| - |\langle I(h) \rangle|/\sum_i \sum_h |I(h)|$ for the intensity of reflection $h$ measured $N$ times. Values in brackets are for outer resolution shell. $R$-factor = $(\sum_h ||F_o|| - k|F_c||/\sum_h |F_o||)$ where $F_o$ and $F_c$ are the observed and calculated structure factor, respectively, and $k$ is a scaling factor. $R_{free}$-factor is identical to the $R_{work}$ on a subset of test reflections not used in refinement. One crystal was used for each data set. Highest resolution shell is shown in parenthesis. Ramachandran plot analysis and clashscore were calculated with Molprobity. Both data sets were collected at the ESRF beamline 23-1.
the ligand at full occupancy (Figure 5B), we refined the occupancy of the disaccharide in phenix.refine, which turned out to be 70%. The reason as to why the H1 crystals did not contain the ligand remains unclear as crystal packing around the complementarity determining regions (CDR) appears to be compatible with a ligand of this size, but a subphysiological pH used for crystallization may explain the absence of the ligand.

In the M5 Fab complex, the disaccharide is recognized entirely by VH CDR and mainly through hydrogen bonds with donor-acceptor distances shorter than 3.3 Å, although both the fucose C6 methyl and mGlc acetyl group C8 methyl make van der Waals interactions shorter than 3.8 Å with tyrosines (Figure 5C). All three hydroxyl groups on the fucose are recognized by the Fab through hydrogen bonds with CDR1 or CDR2, while the pyranose O6 acts as an acceptor for a hydrogen from CDR1 Asn32 (Figure 5C). The N-acetylgalactosamine is recognized by a single hydrogen bond between the amino group and the main chain carbonyl of CDR3 Phe97. Notably, the observed binding mode is compatible with that an Asn-linked glycan could bind in the same manner. At the C1 atom, there is plenty of room for an Asn side chain to form the N-glycosidic bond and, at the O4 atom, there is also room for the glycosidic bond and the next N-acetylgalactosamine unit. In fact, the structure suggests that this could be recognized by, in particular, VL CDR1 and CDR3, while the protein Asn and perhaps the immediate surrounding residues potentially could interact with VL CDR2 and VH CDR1 and CDR3 (Figure 5D). In addition, a fucose bound to the first N-acetylgalactosamine with an α-1,6 glycosidic bond might be orientated towards VL CDR1 and CDR3. Hence, with a true Asn-linked glycan bound to the M5, it is likely that VL CDRs would contribute significantly to Fab-glycan interaction. Although we did not succeed in determining the structure of H1 with a glycan bound, the structure of the Fab alone reveals a cleft shaped by the CDRs of the VH and the VL, which similar to the M5 complex may contain the binding site (Figure 5D).

4 | DISCUSSION

The molecular recognition of carbohydrates by antibodies and the consequences of this interaction are important aspects of the human immune response but remain difficult to analyze due to the limited affinity of the majority of carbohydrate-specific antibodies. Hence, high affinity CCD-specific IgE represent an excellent basis for detailed molecular analyses. The high affinity response to HRP is predominantly directed to the CCD and anti-HRP serum is an important tool for analyses of these modifications. We therefore pursued the isolation of CCD-specific antibody fragments from leporid immune repertoire libraries. Subsequent conversion of the antibody fragments into IgG and IgE formats could then provide the Fc domains for receptor interaction, with the highly glycosylated IgE isotype as the more complex molecule to produce.

Notably, IgE is often considered a highly affinity matured isotype. Human polyclonal anti-CCD IgE is reported to have affinities of approx. 10^{10} M^{-1} (IgG 50-100-fold weaker). However, polyclonal anti-CCD IgG is also reported to have high affinities of approx. 10^{7} M^{-1}. These high affinities are in general agreement with the high affinities observed in this study. Hence, the H1 and M5 antibody bodies are prototypical for CCD-specific antibodies in mammals including humans.

For more than two decades, the relevance of IgE to classical CCDs provokes controversies and a variety of phenomenological studies has not shown coherent results. Recent analyses supported a weak activation of effector cells by anti-CCD IgE for insect venom and plant allergens. Clinical relevance however could not be shown conclusively. Recent studies on core glycans on allergens corroborated a role in strongly inducing Th_{2} immune responses in helminth parasite infection. Other studies suggested positive and negative impact on allergenic epitopes.

Independently, a clearly IgE-mediated anaphylaxis triggered by the well-known Gal-α1,3-Gal structure (α-Gal) has provided evidence for the detrimental allergenicity of glycans. α-Gal is also essential for red meat-induced delayed-type allergy and cross-reactivity to other mammalian allergens. Strong induction of α-Gal-specific IgE could be correlated with bites of distinct ticks. In a previous study, however, a recombinant IgE against α-Gal was found unable to mediate basophil degranulation. Apart from the antigen architecture, limited affinity and avidity might decrease effector cell sensitivity.

The CCD-specific IgEs generated in this study exhibited pronounced carbohydrate-dependent activation of effector cells in mediator release assays at very low concentration of antigen. This finding demonstrates that CCD-specific IgE is fully capable of exerting pronounced effects. Interestingly, the mediator release induced by the two antibodies is highly specific for particular carbohydrate structures as represented by HRP and MUXF conjugates. This variable activity suggests that also the general anti-CCD response relies on clonally independent antibodies specific to individual glycoforms of the allergens, a finding in line with varying results when using different glycoproteins for the determination of CCD-specific IgE levels. Competition by IgG has been hypothesized to act antagonistically to CCD-specific IgE, but broader studies on the presence of such IgG have not been performed.

Notably, efficient activation can also be induced by a combination of a high affinity IgE combined with a lower affinity IgE and even by lower affinity IgE only when using oligovalent antibodies. Hence, for diagnosis and intervention strategies, the entire IgE repertoire including carbohydrate-specific IgE has to be considered.

Beyond the effector mechanisms via FcRI, the ELIFAB analyses corroborate that CCD-specific IgE constitute a functional arm of the IgE repertoire. Binding of allergen/IgE complexes to CD23 is crucial for facilitated antigen presentation and transport across the epithelium, and amplifies the generation and epitope spread of specific IgE. Hence, the capability of CCD-specific IgE for complex binding clearly points to an uptake mechanism for glycosylated allergens. The observed inhibition of CD23 binding by patients’ serum IgG resembles the activity of blocking IgG induced by successful
allergen immunotherapy. However, similar to protein-specific IgG, the activity of carbohydrate-specific IgG correlates only in part with the serum level. The degree of blocking is probably linked not only to concentration, but also functional characteristics such as fine specificity, affinity maturation, and sterical factors such as the epitope density.

Until now, molecular information on the recognition of CCD epitopes by antibodies was unavailable. The use of purified or synthetic glycoproteins and carbohydrates however suggested CCD-specific antibody binding involving either the 1,3 fucose or the 1,2 xylose, with relevant contributions of a larger portion of the core glycan. Hence, the mode of interaction is clearly different from that of fucose-specific lectins such as *Aleuria aurantia* lectin, which does not differentiate fucose linkages and can be inhibited by a single fucose.

The abolishment of immunoreactivity with the glycan lacking the α-1,3 core fucose clearly suggests that specificity and affinity is mediated by the α-1,3 fucose residue. This finding is in accordance with the crystallographical analyses of the H1 and M5 Fabs. Using the disaccharide as epitope surrogate, we were able to describe the epitope for the M5 antibody. The fact that we were able to obtain crystals of the complex supports the idea that the α-1,3 core fucose and the adjacent N-acetylglucosamine residues represent the crucial part of the epitope of fucose-dependent anti-CCD antibodies in general. The manifold interactions of the fucose with several residues within the Fab heavy chain explain the loss of IgE reactivity upon absence of the α-1,3 core fucose and demonstrates how the fucose determines specificity as well as affinity. It is however of interest that the critical fucose is entirely bound by the heavy chain with providing sufficient space for further parts of the large glycan to be bound in a large cleft between VH and VL. The presence of clustered hydrophobic side chains, a key feature in carbohydrate recognition, can be found in both antibodies. The potential interaction with the VL however has to be taken with a grain of salt, since the VH/VL combination has been obtained by combinatorial approaches and might not fully correspond to the natural combination.

With the high prevalence of anti-CCD antibodies in allergic and healthy individuals, our findings clearly suggest that CCD-specific IgE can contribute to the complexity of the IgE repertoire and thereby the Th2 response in patients. Carbohydrate-specific IgG thereby could contribute to the outcome of disease and immunotherapy.

In summary, we have provided structural and functional evidence for the capability of carbohydrate-specific IgE antibodies for executing all biological effects so far only attributed to protein-specific IgE. The recognition of core glycans by IgE and other isoforms constitutes a general principle in the majority of allergies that needs to be reconsidered for linking diagnostic and therapeutic approaches. The obtained insights may contribute to dissecting the molecular aspects of the interaction of antibodies with carbohydrate antigens and understanding the complex network of allergic immune responses.
REFERENCES

1. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282(5396):2085-2088.

2. Avci FY, Li X, Tsuji M, Kasper DL. A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. Nat Med. 2011;17(12):1602-1609.

3. Platts-Mills TA, Jappe U, et al. Carbohydrate epitopes currently recognized as targets for IgE antibodies. Allergy. 2021;76:2383-2394.

4. Aalberse RC, Koshte V, Clemens JG. Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. J Allergy Clin Immunol. 1981;68(5):356-364.

5. Faveeuw C, Mallevaey T, Paschinger K, et al. Schistosome N-glycans containing core alpha 3-fucose and core beta 2-xylose epitopes are strong inducers of Th2 responses in mice. Eur J Immunol. 2003;33(5):1271-1281.

6. Shreffler WG, Castro RR, Kuczyk ZY, et al. The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. J Immunol. 2006;177(6):3677-3685.

7. Malandain H. IgE-reactive carbohydrate epitopes—classification, cross-reactivity, and clinical impact. Eur Ann Allergy Clin Immunol. 2005;37(4):122-128.

8. Bardor M, Faveeuw C, Fitchette AC, et al. Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha(1,3)-fucose and core xylose. Glycobiochemistry. 2003;13(6):427-434.

9. Holzweber F, Svehla E, Fellner W, et al. Inhibition of IgE binding to cross-reactive carbohydrate determinants enhances diagnostic selectivity. Allergy. 2013;68(10):1269-1277.

10. Jin C, Hantusch B, Hemmer W, Stadlmayr J, Altmann F. Affinity of IgE and IgG against cross-reactive carbohydrate determinants on plant and insect glycoproteins. J Allergy Clin Immunol. 2008;121(1):185-190.e2.

11. Chung CH, Mirakurh B, Chan E, et al. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha,1,3-galactose. N Engl J Med. 2008;358(11):1109-1117.

12. Gomery K, Müller-Loennies S, Brooks CL, et al. Antibody WN1 222-5 mimics Toll-like receptor 4 binding in the recognition of LPS. Proc Natl Acad Sci USA. 2012;109(51):20877-20882.

13. Czyler M, Rose DR, Bundle DR. Recognition of a cell-surface oligosaccharide of pathogenic Salmonella by an antibody Fab fragment. Science. 1991;253(5018):442-445.

14. Nguygen HP, Seto NOL, Mackenzie CR, et al. Germline antibody recognition of distinct carbohydrate epitopes. Nat Struct Biol. 2003;10(12):1019-1025.

15. Zdanov A, Li Y, Bundle DR, et al. Structure of a single-chain antibody variable domain (Fv) fragment complexed with a carbohydrate antigen at 1.7 Å resolution. Proc Natl Acad Sci USA. 1994;91(14):6423-6427.

16. Vyas NK, Vyas MN, Chervenak MC, et al. Molecular recognition of oligosaccharide epitopes by a monoclonal Fab specific for Shigella flexneri Y lipopolysaccharide: X-ray structures and thermodynamics. Biochemistry. 2002;41(46):13575-13586.

17. Villeneuve S, Souchon H, Riottot MM, et al. Crystal structure of an anti-carbohydrate antibody directed against Vibrio cholerae O1 in complex with antigen: molecular basis for serotype specificity. Proc Natl Acad Sci USA. 2000;97(15):8433-8438.

18. Jeffrey PD, Bajorath J, Chang CYY, et al. The x-ray structure of an anti-tumour antibody in complex with antigen. Nat Struct Biol. 1995;2(6):466-471.

19. van Roos AM, Pannu NS, de Vrij JP, et al. Structure of an anti-Lewis X Fab fragment in complex with its Lewis X antigen. Structure. 2004;12(7):1227-1236.

20. Calarrese DA, Scanlan CN, Zwick MB, et al. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science. 2003;300(5628):2065-2071.

21. Zürcher AW, Lang AB, Aebischer I, Miescher S, Stadler BM. IgE-producing hybridomas established after B-cell culture in the CD40 system. Immunol Lett. 1995;46(1-2):49-57.

22. Steinberger P, Kraft D, Valenta R. Construction of a combinatorial IgE library from an allergic patient. Isolation and characterization of human IgE Fabs with specificity for the major timothy grass pollen allergen, Phl p 5. J Biol Chem. 1996;271(18):10967-10972.

23. Laukkanen ML, Mäkinen-Kiljunen S, Isoherranen K, Haahtela T, Söderlund H, Takkinen K, Hevein-specific recombinant IgE antibodies from human single-chain antibody phage display libraries. J Immunol Methods. 2003;278(1-2):271-281.

24. Jin C, Bencúrová M, Borth N, et al. Immunoglobulin G specifically binding plant N-glycans with high affinity could be generated in rabbits but not in mice. Glycobiology. 2006;16(4):349-357.

25. Hecker J, Diethers A, Etzold S, et al. Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a. Mol Immunol. 2011;48(9-10):1236-1244.

26. Mobs C, Müller J, Rudzio A, et al. Decline of Ves v 5-specific blocking capacity in wasp venom-allergic patients after stopping allergen immunotherapy. Allergy. 2015;70(6):715-719.

27. Shamji MH, Francis JN, Würtzen PA, Lund K, Durham SR, Till SJ. Cell-free detection of allergen-IgE cross-linking with immobilized phase CD23: inhibition by blocking antibody responses after immunotherapy. J Allergy Clin Immunol. 2013;132(4):1003-1005 e1-e4.

28. Muller-Loennies S, Galliciotti G, Kollmann K, Glatzel M, Braulke T. A novel single-chain antibody fragment for detection of mannos-6-phosphate-containing proteins: application in mucolipidosis type II patients and mice. Am J Pathol. 2010;177(1):240-247.
29. Muller-Loennies S, CR MK, Patenaude SJ, et al. Characterization of high affinity monoclonal antibodies specific for chlamydial lipo-polysaccharide. Glycobiology. 2000;10(2):121-130.

30. Oberli MA, Tamborrini M, Tsai YH, et al. Molecular analysis of carbohydrate-antibody interactions: case study using a Bacillus anthracis tetrasaccharide. J Am Chem Soc. 2010;132(30):10239-10241.

31. Kurosaka A, Yano A, Itoh N, Kuroda Y, Nakagawa T, Kawai T. The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antisera. J Biol Chem. 1991;266(7):4168-4172.

32. Li Y, Cockburn W, Kilpatrick JB, Whitelam GC. High affinity ScFvs of horseradish peroxidase recognized by anti- horseradish peroxidase antiserum. Biochem Biophys Res Commun. 2000;268(2):398-404.

33. Ridder R, Schmitz R, Legay F, Gram H. Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast Pichia pastoris. Biotechnology (NY). 1995;13(3):255-260.

34. Boel E, Verlaan S, Poppelier MJJG, Westerdaal NAC, van Strijp JAG, Mertens M, Amler S, Moerschbacher BM, Brehler R. Cross-reactive carbohydrate determinants constructed from phage display library-derived single-chain Fv antibody fragments. J Immunol Methods. 2000;239(1-2):153-166.

35. Eberlein B, Krischan L, Darsow U, Ollert M, Ring J. Double positivity to bee and wasp venom: improved diagnostic procedure by recombinant allergen-based IgE testing and basophil activation test including data about cross-reactive carbohydrate determinants. J Allergy Clin Immunol. 2012;130(1):155-161.

36. Mittermann I. Glycosylation enhances allergenic activity of major bee venom allergen Api m 1 by adding IgE epitopes. Arch Allergy Immunol. 2009;123(5):1189-1191.

37. Tu Y, Perdue MH. CD23-mediated transport of IgE/immune complexes across human intestinal epithelium: role of p38 MAPK. Am J Physiol Gastrointest Liver Physiol. 2006;291(3):G532-G538.

38. Wilcock, L.K., J.N. Francis, and S.R. Durham, IgE-facilitated antigen presentation: role in allergy and the influence of allergen immunotherapy. Immunol Allergy Clin North Am. 2006. 26(2): 333–47, viii-ix.

39. Rauber MM, Wu HK, Adams B, et al. Birch pollen allergen-specific immunotherapy with glutaraldehyde-modified allergoid induces IL-10 secretion and protective antibody responses. Allergy. 2017;74(8):1575-1579.

40. Shamji MH, Ljørring C, Francis JN, et al. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. Allergy. 2012;67(2):217-226.

41. Collot M, Wilson IB, Blümel M, Hoffmann-Sommergruber K, Mallet JM. Synthesis of cross-reactive carbohydrate determinants fragments as tools for in vitro allergy diagnosis. Bioorg Med Chem. 2011;19(3):1306-1320.

42. Seissl H, Blank S, Braren I, et al. Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation. Mol Immunol. 2010;47(4):799-808.

43. Commins SP, James HR, Kelly LA, et al. The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose-alpha-1,3-galactose. J Allergy Clin Immunol. 2011;127(5):1286-1293.e6.

44. Plum M, Michel Y, Wallach K, et al. Close-up of the immunogenic alpha1,3-galactose epitope as defined by a monoclonal chimeric immunoglobulin E and human serum using saturation transfer difference (STD) NMR. J Biol Chem. 2011;286(50):43103-43111.

45. Christensen LH, Holm J, Lund G, Riise E, Lund K. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. J Allergy Clin Immunol. 2008;122(2):298-304.

46. Aalberse RC, Akkerdaas J, van Ree R. Cross-reactivity of IgE antibodies to allergens. Allergy. 2001;56(6):478-490.

47. Collins AM, Basil M, Nguyen K, Theilain D. Rat basophil leukaemia (RBL) cells sensitized with low affinity IgE respond to high valency antigen. Clin Exp Allergy. 1996;26(8):964-970.

48. Dembo M, Basil M, Nguyen K, Thelian D. Histamine release due to bivalent penicilloyl hapten: control by the basophil plasma membrane. J Immunol. 1978;121(1):354-358.

49. Willumsen N, Holm J, Christensen LH, Würtzen PA, Lund K. The complexity of allergic patients’ IgE repertoire correlates with serum concentration of allergen-specific IgE. Clin Exp Allergy. 2012;42(8):1227-1236.

50. Gould HJ, Sutton BJ. IgE in allergy and asthma today. Nat Rev Immunol. 2008;8(3):205-217.

51. Tu Y, Perdue MH. Cross-reactivity of IgE/immune complexes across human intestinal epithelium: role of p38 MAPK. Am J Physiol Gastrointest Liver Physiol. 2006;291(3):G532-G538.

52. Wilcock, L.K., J.N. Francis, and S.R. Durham, IgE-facilitated antigen presentation: role in allergy and the influence of allergen immunotherapy. Immunol Allergy Clin North Am. 2006. 26(2): 333–47, viii-ix.

53. Rauber MM, Wu HK, Adams B, et al. Birch pollen allergen-specific immunotherapy with glutaraldehyde-modified allergoid induces IL-10 secretion and protective antibody responses. Allergy. 2019;74(8):1575-1579.

54. Shamji MH, Ljørring C, Francis JN, et al. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. Allergy. 2012;67(2):217-226.

55. Collot M, Wilson IB, Blümel M, Hoffmann-Sommergruber K, Mallet JM. Synthesis of cross-reactive carbohydrate determinants fragments as tools for in vitro allergy diagnosis. Bioorg Med Chem. 2011;19(3):1306-1320.

56. Seissl H, Blank S, Braren I, et al. Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation. Mol Immunol. 2010;47(4):799-808.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Plum M, Tjerrild L, Raiber T, et al. Structural and functional analyses of antibodies specific for modified core N-glycans suggest a role in Tc1 responses. Allergy. 2023;78:121-130. doi: 10.1111/all.15417