

A new kind of carbohydrate array, its use for profiling antiglycan antibodies, and the discovery of a novel human cellulose-binding antibody

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Received on March 3, 2003; revised on May 8, 2003; accepted on May 30, 2003

In this study, we use a novel glycan array to analyze the glycan-binding antibody repertoire in a pool of affinity-purified IgG collected from a healthy human population. The glycan array used is based on mono- and oligosaccharides covalently linked to the surface via a long linker at their reducing ends. They are thus presented to the medium with a well-defined orientation and are accessible for specific binding by glycan-binding proteins, such as antibodies and lectins. A novel anticellulose antibody was detected that binds specifically to β 4-linked saccharides with a preference for gluco-pyranose over galactopyranose residues. We also found previously known antiglycan antibodies against mono- and oligosaccharides that are constituents of commonly occurring bacterial polysaccharides. We propose that this array can facilitate high-throughput screening of glycan-binding proteins and the search for biomarkers for personalized medicine.

Key words: antibodies/array/cellulose/saccharide/profile

Introduction

Glycans, due to the large number of saccharide building blocks and the variety of linkages between them, have an enormous potential to carry information—far exceeding that of nucleic acids or proteins (Laine, 1994; Sharon, 1975). These glycans are displayed on macromolecules and the surface of cells, where the information they encode is deciphered by glycan-binding proteins in numerous processes, such as the antigen recognition machinery, bacterial and viral adhesion to host cells and evasion from host immune system, and protein folding, stability, and trafficking (Crocker and Feizi, 1996; Feizi, 2000a,b; Helenius and Aebi, 2001; Karlsson, 1998).

The same attributes that confer the huge information-carrying capacity of the glycans make their synthesis difficult. Likewise, the analysis of protein–glycan interactions suffers from tedious methodology, such as erythrocyte agglutination, equilibrium dialysis, affinity chromatography, and so on. Thus, although much information has become available on the role of glycans in biological

systems, progress has been hampered due to difficulties in synthesizing and analyzing the function of these molecules. Much effort has been recently invested in the development of glycan arrays—analogue to the gene and protein array paradigm—and has yielded a number of methods for binding glycans to solid supports, all but one relying on non-covalent linkages (Bryan *et al.*, 2002; Fukui *et al.*, 2002; Houseman and Mrksich, 2002; Wang *et al.*, 2002; Willats *et al.*, 2002).

We report on a novel glycan array containing chemically defined, covalently bound mono- and oligosaccharides representing the carbohydrate moiety of naturally occurring glycoconjugates. In contrast to arrays based on non-covalently adsorbed saccharides, the carbohydrates in this array are coupled via a flexible linker to the array's surface. This technology enhances carbohydrate accessibility to protein binding while minimizing nonspecific binding. The carbohydrates are coupled to the solid phase, retaining their anomericity and presenting the nonreducing end of the carbohydrate to the solution, mimicking the orientation of glycans in natural glycoconjugates. The array exhibits robust reproducibility and facilitates rapid and convenient analysis of antibody samples on large numbers of glycans.

We identified antibodies in a human IgG pool against monosaccharides that are constituents of commonly occurring bacterial polysaccharides such as *N*-acetylglucosamine (GlcNAc) and rhamnose (Rha) and a novel antibody against β 1-4glucose oligomers. These antibodies were affinity-purified and further characterized using glycan arrays.

Results

The glycan array characteristics

The glycan array presents covalently attached mono- and oligo-saccharides (Figure 1A) in a microtiter plate format that allows for specific recognition and binding of glycan binding proteins. In the experiments described, up to 33 different glycans (in addition to glycerol and para-aminophenol, *p*AP) were immobilized as listed in Table I. Specific protein–glycan binding was demonstrated by application of lectins to the glycan array. For example, wheat germ agglutinin (WGA; Figure 1B), concanavalin A (ConA; Figure 1D), and *Bandeiraea simplicifolia* (*Griffonia simplicifolia*) lectin (BSI; Figure 1D) bound specifically only to their cognate ligands. Furthermore competition assays showed that the binding of ConA was specifically inhibited by mannose (Figure 1C). The specificity of lectin binding to their respective anomers is demonstrated clearly using the glycan array, as can be seen from the difference in signal intensity obtained from binding of ConA to α -Man versus β -Man

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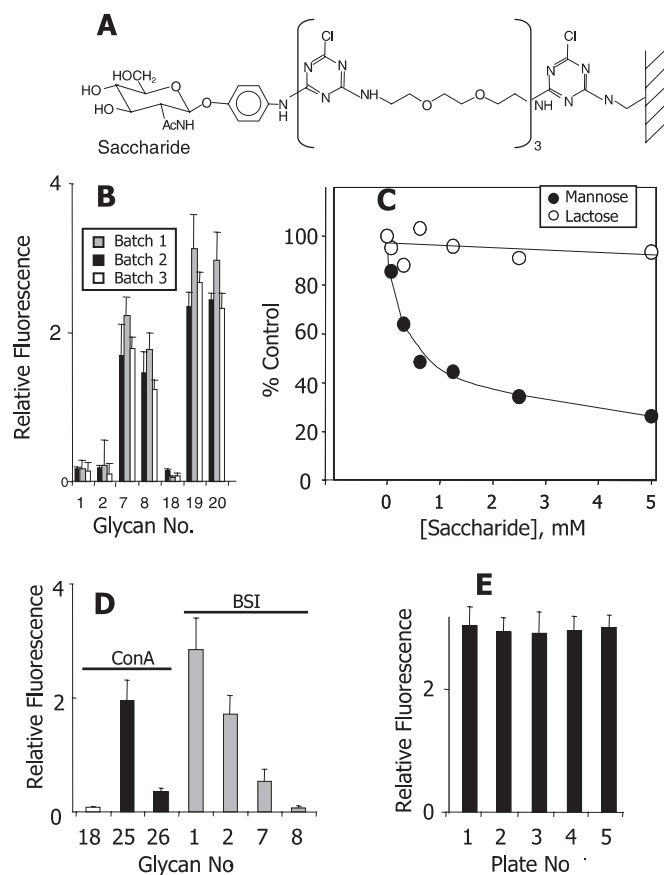


Fig. 1. The glycan array, chemical structure, specificity of lectin interaction, and reproducibility. (A) The pAP-saccharide is covalently linked at its reducing end to the solid surface via a linker. (B) Batch-to-batch reproducibility of binding of biotinylated WGA to the glycan array. Three separate batches of arrays were assayed simultaneously with biotinylated WGA. (C) Competition assay with ConA to bound mannose. Increasing concentrations of soluble mannose or lactose were incubated with biotinylated ConA (1.5 μ g/ml) for 1 h, and detected with streptavidin conjugated to europium. (D) Specificity of lectin binding to different anomers. ConA binding to negative control glycerol (18), α -Man (25), and β -Man (26). BSI binding to α -Gal (1), β -Gal (2), α -GalNAc (7), and β -GalNAc (8). (E) Plate-to-plate reproducibility of the glycan array. Five identical plates presenting β -GlcNAc were probed with biotinylated WGA.

and BSI binding to α -Gal versus β -Gal and α -GalNAc versus β -GalNAc (Figure 1D). Signal-to-noise ratios were around 10–20 for positive control wells.

Reproducibility

The median coefficient of variation of the quadruplicate samples was found to be between 5–20% (not shown). Using a well-characterized lectin-glycan pair, WGA and β -GlcNAc, the plate-to-plate coefficient of variation was determined to range between 2% and 10% (Figure 1E). Additionally, three different batches of glycan arrays were analyzed simultaneously to determine batch-to-batch variability. As can be seen in Figure 1B, excellent reproducibility was observed between batches; glycans containing β -GlcNAc gave rise to signals with a coefficient of variation of 16% between the batches.

Antiglycan antibodies in a human population

We analyzed the glycan binding profile of an IgG pool representing a healthy human population by applying it to 34 mono- and oligosaccharides as shown in Figure 2. Table I lists the oligosaccharides displayed in the array and notes the binding signal measured for the various antioligosaccharide antibodies. The strongest signals were recorded for antibodies against α -GlcNAc and α -L-Rha, whereas lower levels were observed against β 4-linked oligosaccharides of glucose, α -Gal and di-*N*-acetylchitobiose.

Specificity of the antiglycan antibodies

Binding of the IgG pool antibodies to immobilized α -L-Rha was inhibited by the corresponding para-nitrophenyl (pNP) α -L-Rha. In contrast, neither pNP-glycerol or other non-relevant pNP-glycosides had any effect on the binding (not shown). These results corroborate the specificity of the antiglycan antibodies as detected by the glycan array.

To further characterize the glycan-binding antibodies, affinity chromatography was performed on the IgG pool using Sepharose-bound α -GlcNAc, α -L-Rha, β -cellotriase, or β -di-*N*-acetylchitobiose. The affinity-purified antibodies against α -GlcNAc and α -L-Rha were highly specific to their respective antigens and bound significantly only to saccharides containing the monosaccharide against which they were purified (Figure 3A and 3B). The anticellotriase antibody, however, not only recognized the cello-oligosaccharides on the array but also bound significantly to lactose and β -di-*N*-acetylchitobiose (Figure 3C). Furthermore, the anticellotriase antibody was specifically inhibited by soluble cellotriase with an IC_{50} of 25 μ M (Figure 4A), and preincubation of the purified fraction with crystalline and amorphous cellulose removed most of the anticellotriase activity (Figure 4B). These results demonstrate that the anticellotriase antibody binds not only to β 4 glucose oligomers but also to cellulose. Antibodies purified on β -di-*N*-acetylchitobiose beads exhibited a different glycan binding profile than the anticellotriase antibody, binding primarily to β -GlcNAc containing glycans and to a smaller extent to β 1,4-linked glucose oligomers (Figure 3B and 3C).

Discussion

The field of glycobiology is following in the footsteps of genomics and proteomics in the development of tools for high-throughput detection and characterization of carbohydrate-binding proteins. Previously described arrays rely on adsorption of glycan or glycan conjugates to a nitrocellulose solid surface (Bryan *et al.*, 2002; Fukui *et al.*, 2002; Wang *et al.*, 2002; Willats *et al.*, 2002), whereas another covalently links activated sugars to self assembled monolayers (Houseman and Mrksich, 2002). In this article, we report on a novel glycan array and on findings regarding human antiglycan antibodies in the healthy population obtained by its use. This array possess a number of important features:

1. The glycan array utilizes simple and well-characterized chemistry to covalently link the glycans to the solid support.

Table I. Saccharides displayed on glycan array and level of anti-glycan antibody in healthy human population

Glycan no.	Glycan	Linear code ^a	Ab binding (RFU) ^b	Relative Ab level ^c
0	pNP-OH	—	NA	NA
1	Gal (α)	Aa	1,016,767	5
2	Gal (β)	Ab	596,747	9
3	Gal (β 1-3) GalNAc (α)	Ab3ANa	0	
4	Gal (β 1-3) GlcNAc (β)	Ab3GNb	0	
5	Gal (β 1-4) Glc (β)	Ab4Gb	325,733	
6	Gal (β 1-6) Gal (β)	Ab6Ab	395,470	10
7	GalNAc (α)	ANa	97,837	
8	GalNAc (β)	ANb	109,565	
9	Fuc (α)	Fa	46,639	
10	Fuc (β)	Fb	37,714	
11	Glc (α)	Ga	0	
12	Glc (α 1-4) Glc (α)	Ga4 Ga	0	
13	Glc (α 1-4) Glc (β)	Ga4Gb	58,722	
14	Glc (β)	Gb	201,748	
15	Glc (β 1-4) Glc (β)	Gb4Gb	634,489	7
16	Glc (β 1-4) Glc (β 1-4) Glc (β)	Gb4Gb4Gb	58,065	
17	Glc (β 1-4) Glc (β 1-4) Glc (β 1-4) Glc (β 1-4) Glc (β)	Gb4Gb4Gb4Gb4Gb	38,391	
18	Glycerol	Glycerol	0	
19	GlcNAc (α)	GNa	3,085,630	1
20	GlcNAc (β)	GNb	2,151,357	3
21	GlcNAc (β 1-3) GalNAc (α)	GNb3ANa	632,668	8
22	GlcNAc (β 1-4) GlcNAc (β)	GNb4GNb	871,049	6
23	L-Rha (α)	Ha	2,451,730	2
24	GalA (β)	Lb	362,266	
25	Man (α)	Ma	0	
26	Man (β)	Mb	0	
27	Neu5Ac (α)	NNa	0	
28	L-Araf (α)	Ra	0	
29	GlcA (β)	Ub	13,242	
30	X(α)	Xa	26,364	
31	X(α)	Xb	203,125	
32	Gal (β 1-3) [GlcNAc (β 1-6)] GalNAc (α)	Ab3(GNb6)ANa	81,154	
33	Gal (β 1-4) GlcNAc (α)	Ab4GNa	148,336	
34	Gal (α 1-3) Gal (β 1-4)GalNAc (β)	Aa3Ab4GNb	1,815,867	4

NA, not assessed.

^aAverage level of antibody (Ab) glycan binding in an IgG pool collected from a healthy human population measured in relative fluorescent units (RFU).

^bA syntax that describes the branched structure of glycans in a linear computer-friendly mathematical formula (Banin *et al.*, 2002).

^cFor the reader's convenience, the 10 most immunogenic glycans have been ranked, and 1 represents the highest level of antibody binding.

- The glycans are chemically defined and are bound to the linker by known anomeric bonds.
- A linker separates the glycan and the solid surface, conferring access to cells and glycan-binding proteins, such as antibodies, lectins, and glycosyltransferases.
- The saccharides are bound to the substrate via their reducing ends and are therefore presented to the medium with a well-defined orientation.
- The array allows for specific interaction between glycan-binding protein, such as lectins and antibodies, with their immobilized ligands.
- The array is produced in large batches with good well-to-well, plate-to-plate, and batch-to-batch reproducibility and gives excellent signal-to-noise ratios.
- The array format fits standard laboratory equipment, such as plate-washers and readers.

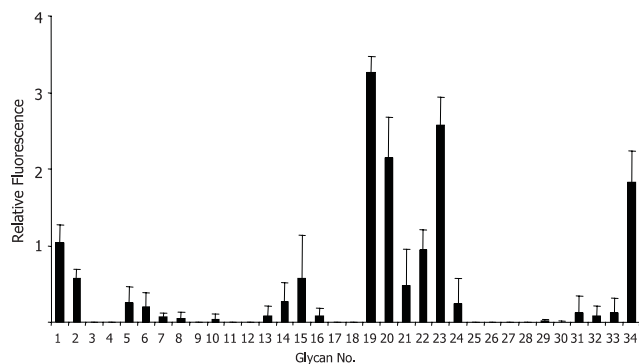


Fig. 2. Anticarbhydrate antibody profile in an IgG pool collected from a healthy human population. Bound antibodies were detected with biotinylated Protein A and europium-labeled streptavidin as described in *Materials and methods*. The graph shows average specific binding after subtraction of background (arbitrarily defined as the signal obtained from wells coated with glycerol) from four separate experiments. Error bars show standard deviation from average.

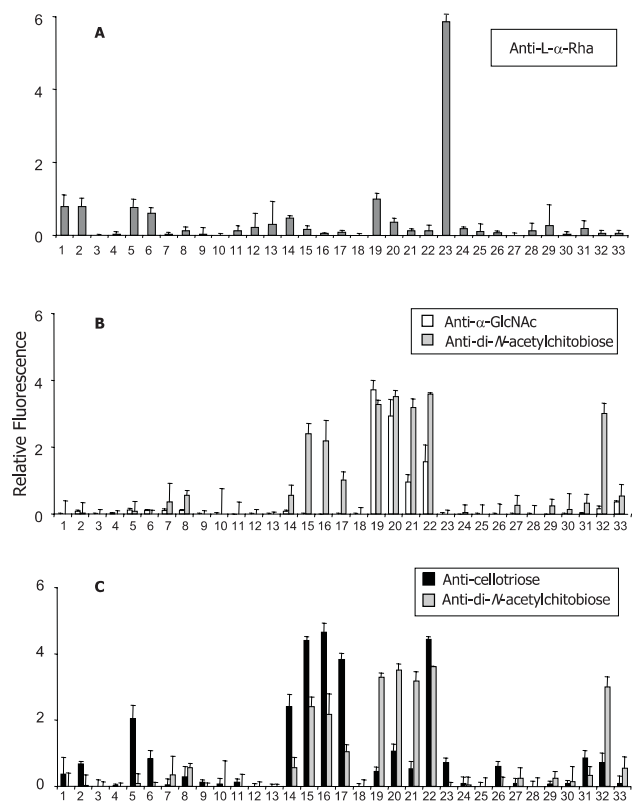


Fig. 3. Binding profile of affinity purified (A) anti- α -L-Rha (B) anti- α -GlcNAc and anti-di-N-acetylchitobiose and (C) anticellobiose and anti-di-N-acetylchitobiose antibodies to an array of 32 glycans and glycerol. Amount of antibody bound was measured using biotinylated goat anti-human IgG antibody.

8. The chemistry employed to link covalently glycans to the solid support is readily adapted to a variety of materials, such as plastic, glass, metal, and so on.
9. *p*NP-saccharides can be used as acceptors in enzymatic reactions to form complex carbohydrates either on the

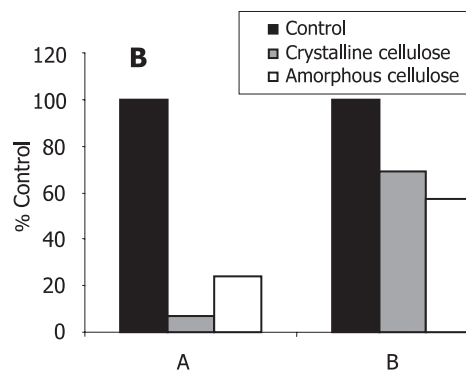
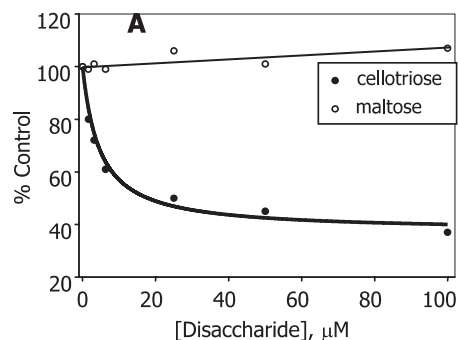


Fig. 4. Specificity of anticellobiose antibody. (A) Competitive inhibition of anticellobiose antibody binding. Inhibition of binding of affinity-purified anticellobiose antibody to *p*AP- β -cellobiose immobilized to the well surface as a function of cellotriose or lactose concentration. Amount of antibody bound was measured using biotinylated goat anti-human IgG antibody. (B) Binding of anticellobiose (A) and anti- α -L-Rha (B) antibodies to their cognate saccharide after incubation with crystalline or amorphous cellulose. Amount of antibody bound was measured using biotinylated goat anti-human IgG antibody.

glycan chip or in solution prior to chemical attachment to the chip surface, thus vastly extending the potential glycan diversity on the chip.

No other published glycan array exhibits all of these features. Notably most of the arrays are based on noncovalent adsorption of the sugar molecules to the solid support, a method that does not allow for control of the spatial orientation of the attached glycan and is limited to large saccharides. Thus the discovery and characterization of anticellobiose antibodies in human sera—the main finding of this report (Figures 2, 3C, and 4)—and characterization of binding specificity of lectins on the level of monosaccharides and anomericity of the glycosidic bonds, showed in Figure 1D, would not have been possible using glycan arrays based on adsorption. Furthermore, the Glycochip is the only glycan array that has been shown to retain good reproducibility between plates and between batches produced on industrial scale.

Commercially available glycoconjugates can be used to develop solid-phase protein–glycan interaction assays, but the glycoconjugates are not anomerically defined and must be attached to the solid phase by noncovalent adsorption, in

which there is no control over spatial direction. Furthermore, in contrast to glycoconjugates, the glycans of the GlycoChip are not coupled to the solid phase via a protein and are thus available for interaction without the context of protein antigens. The glycan array described is thus an important improvement to existing tools of glycan interactions and is well suited for screening of large numbers of samples, such as sera.

It has been shown that human sera from healthy individuals contain antiglycan antibodies directed against a variety of antigens, such as the blood groups ABO (Auf der Maur *et al.*, 1993; Rieben *et al.*, 1991), Gal α 1-3Gal (Galili *et al.*, 1999), N-glycolylneuraminic acid (Zhu and Hurst, 2002), and various bacterial glycans (Jann and Jann, 2001; Pazur, 1998). Relative to earlier studies, we expand the number of mono- and oligosaccharides used for the analysis of antiglycan antibodies and demonstrate the existence of a profile of antiglycan antibodies in a healthy population (Figure 2).

We chose to characterize antibodies against α -GlcNAc, α -L-Rha, and β 1,4-linked glucose oligomers by competitive inhibition studies and showed that they are specifically inhibited by their cognate ligands. In addition, microarray binding studies using affinity-purified anti- α -L-Rha and anti- α -GlcNAc antibodies showed high specificity among 35 immobilized glycans tested.

Unlike antibodies to α -L-Rha and α -GlcNAc, the presence of antibodies against β 1,4-linked glucose oligomers have not previously been described. The affinity-purified anticellotriose antibody was specifically inhibited by cellotriose and bound specifically to saccharides linked by β 4 bonds—preferring glucose over galactose residues (in Figure 3C, compare glycan no. 2 with glycan no. 15)—but not by α 4, α 3, β 3, or β 6 bonds. This suggests that the β 4-backbone is necessary but not sufficient to form the binding domain of the antibody. The anticellotriose antibody also bound to crystalline and amorphous cellulose raising the possibility that the original antigen against which the antibody was formed could have been cellulose.

The purified anti-di-*N*-acetylchitobiose antibody bound specifically to all GlcNAc containing saccharides but also to β -linked glucose oligosaccharides, which may suggest that the anti-di-*N*-acetylchitobiose antibody prefers the *N*-acetyl group but also recognizes the β 4-glucose backbone. This is in contrast to the anti- α -GlcNAc antibody, which binds solely to saccharides containing an *N*-acetyl moiety.

In summary, we used the glycan array to characterize the glycan-binding antibodies in a human IgG pool and revealed the existence of novel abundant antibodies. The demonstration that the technique used here is specific, sensitive, and reproducible enough to detect differences in levels of various antiglycan antibodies sets the stage for the quest for specific antiglycan antibody profiles to be used as biomarkers in personalized medicine.

Materials and methods

Glycan array

Antibodies were tested using GlycoChip (Glycominds, Lod, Israel). The glycans were covalently bound to the plastic

surface through a linker as described in Figure 1A (Dukler and Dotan, 2002). Briefly, an oligomer of 1,8-diamino-3,6-dioxaoctan (Sigma, St. Louis, MO) was synthesized and coupled to the solid support. Consequently, *p*NP-saccharide conjugates were reduced by sodium dithionite to *p*AP-saccharide derivatives and reacted with cyanochloride (Sigma)-activated linker.

A list describing the mono- and oligosaccharides present in the array is provided in Table I. The volume of all solutions added to the glycan array was 10 μ l/well.

Lectin binding assays

Biotinylated ConA (Sigma; 1.5 μ g/ml in 0.15 M Tris-HCl, pH 7.2, 0.085 M Mg₂SO₄, 0.05% Tween 20 [TBST], pH 7.2, +5% [v/v] glycerol), biotinylated WGA, and biotinylated BSI (Sigma; 15 μ g/ml in TBST, pH 7.2), were incubated on the glycan array 1 h at room temperature. Following washing with high-salt buffer (phosphate buffered saline with 0.1% Tween-20 [PBST], Sigma, supplemented with 2 M NaCl) binding was detected with europium-streptavidin as will be described.

Antiglycan antibody assay

The IgG pool (IgG affinity-purified from sera collected from some 10,000 healthy individuals) was kindly provided by Dr. Israel Nur, Omrix, Rehovot, Israel. The IgG pool was diluted 1:20 in TBST, dispensed into glycan array plates using a Tecan Genesis Workstation 200 automated handling system (Männedorf, Switzerland), and incubated for 60 min at 25°C.

The plates were then washed with 250 μ l/well of high-salt buffer in an automatic plate washer (Tecan, PowerWasher). At this point biotinylated Protein A (ICN Biomedicals) or biotinylated goat anti-human IgG (F_{cy}, biotin-SP-conj, Jackson, PA) was added. Both reagents were diluted in TBST and dispensed manually at 1 μ g/ml, and the plates were incubated for 60 min at 25°C. Following washing with high-salt buffer, binding was detected with europium-streptavidin.

Detection of glycan-binding proteins bound to the glycan array

The detection of binding of both antibodies and lectins are based on biotinylated probes detected with Europium conjugated Streptavidin (Perkin-Elmer, Boston, MA) and the enhancement of the signal by addition of Delfia Enhancer Solution (Perkin-Elmer). Thus biotinylated lectins and anti-antibodies were detected by addition of streptavidin-conjugated europium (0.1 μ g/ml) diluted in TBST to each well, followed by incubation for 30 min at 25°C in the dark and washing with high-salt buffer. Delfia enhancement solution was then added to the wells, and the plates were incubated for 30–45 min in the dark. The fluorescence of the wells was read with a Victor 1420 (Wallac, Finland) plate reader using time-resolved fluorescence settings of 340/612 nm (excitation/emission).

Inhibition of antibody binding to glycans

The IgG pool (1:40; a dilution giving approximately 75% of maximal binding) was preincubated with *p*NP- α -GlcNAc,

*p*NP- α -L-Rha, or *p*NP-glycerol at various concentrations for 1 h at 25°C. Affinity-purified anticellotriose antibody was incubated with cellotriose (Dextra, UK) or maltose (Sigma). Aliquots were dispensed into glycan array wells coated with the relevant saccharide and incubated at 25°C for 60 min. The bound antibodies were detected as described.

Affinity purification of antiglycan antibodies

Sepharose-*p*AP- α -GlcNAc, *p*AP- α -L-Rha, *p*AP- β -GlcNAc1-4 β -GlcNAc, and *p*AP-celotriose were prepared as described (Bloch and Burger, 1974). One milliliter of 10 \times TTBS (3 M NaCl, 0.2 M Tris, pH 7.8, 1% Tween 20) and 0.55 ml 4 M NaCl was added to 10 ml of the IgG pool. The resulting solution was incubated with 1 ml resin in a 15-ml tube for 16 h at 4°C with gentle agitation. The resin was packed in a column and washed extensively with 1 \times TTBS until the absorbance at 280 nm of the flow through was below 0.02 OD. The bound antibodies were eluted in 1-ml batches with 0.2 M glycine buffer at pH 2.8 into tubes containing 50 μ l 1 M Tris-HCl, pH 8.6. The pooled fractions were dialyzed overnight against phosphate buffered saline containing 0.02% sodium azide.

Acknowledgments

We thank Nathan Sharon for constructive guidance and Ronald L. Schnaar and Ajit Varki for helpful comments.

Abbreviations

BSI, *Bandeiraea simplicifolia* I; ConA, concanavalin A; *p*AP, para-aminophenyl; PBST, phosphate buffered saline with 0.1% Tween-20; *p*NP, para-nitrophenyl; Rha, rhamnose; TBST, Tris-buffered saline with 0.1% Tween-20; WGA, wheat germ agglutinin.

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