COMMUNICATION

High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems

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Progress toward understanding the biological roles of carbohydrates has been remarkably slow, and efforts to exploit this class of biopolymers as diagnostic and therapeutic targets have proven extremely challenging. Both basic and clinical research rely heavily on identifying and monitoring expression levels of carbohydrates. Over the last 30 years, the majority of expression information has been derived from antibody- and lectin-binding studies. Using a carbohydrate microarray containing 80 different glycans and glycoproteins, the specificities of 27 antiglycan antibodies were evaluated, including antibodies to histo-blood group A, B, and H antigens (81FR2.2, CLCP-19B, B389, 92FR-A2, B480, B460, B376, and B393), Lewis antigens (7LE, 15C02, 28, ZC-18C, 121SLE, CA199.02, PR.5C5, 2-25LE, BR55, T174, T218, F3, A70-C/C8, FR4A5, and K21), and other tumor-associated antigens (B389, 1A4, B1.1, and 5B5). In total, evaluation of over 2000 individual carbohydrate−protein interactions was carried out. More than half of the antibodies considered to be specific for their designated antigen were found to cross-react with other glycans. The cross-reactive glycans could be mistaken for the designated antigen in biopsy samples or other biological samples, leading to inaccurate conclusions.

Key words: antibodies/blood group antigens/glycan array/ Lewis antigens/tumor-associated carbohydrate antigens

Introduction

Carbohydrates play important roles in a wide range of biological processes. In addition, cells undergo dramatic changes in carbohydrate expression during the onset and progression of diseases such as cancer and rheumatoid arthritis. For example, altered expression of ABH and Lewis histo-blood group antigens, glycosphingolipids, and mucin-related antigens (e.g. TF, Tn, STn) in cancer tissue is well known (Dube and Bertozzi 2005; Fuster and Esko 2005). Over the last 30 years, there have been numerous studies aimed at understanding the biological roles of carbohydrates and determining how changes in carbohydrate expression contribute to diseases. In some cases, carbohydrate antigens have been found to play key roles in processes such as cell−cell adhesion, inflammation, and metastasis. In most cases, however, the biological effects and the relationships between structure and function are not well understood. Carbohydrate antigens have also become important molecular targets for the development of diagnostic and therapeutic agents (Dube and Bertozzi 2005; Fuster and Esko 2005). For example, a number of carbohydrate-based cancer vaccines (Keding and Danishefsky 2004; Slovin et al. 2005), anticarbohydrate antibodies (Pai et al. 1996; Posey et al. 2002), and lectins (Valdimarsson 2003; Schoffski et al. 2005) are currently in clinical trials. Although there is tremendous potential, development of effective diagnostic and therapeutic agents has proven to be very challenging. With carbohydrate-based cancer vaccines, for example, one frequently observes good clinical responses in a subset of patients but a modest overall response for the entire patient group. When monitoring carbohydrate expression for diagnostic−prognostic purposes, different studies frequently report conflicting results. As a result, only a small number of carbohydrate antigens are used clinically as biomarkers.

One of the most fundamental requirements for both basic and translational research is determining when and where a carbohydrate is expressed. To understand the biological roles of a particular carbohydrate or to evaluate a carbohydrate antigen as a disease biomarker, one must first locate the antigen. Reliable information is also critical for clinical trials. For example, the successful application of carbohydrate-based cancer vaccines requires identification of patients with antigen-positive tumors. Unfortunately, carbohydrate expression levels are extremely difficult to measure using direct detection methods, to infer from gene expression levels, or to gauge from protein expression patterns. As a result, expression levels have primarily been monitored indirectly by probing the binding of anticarbohydrate antibodies and/or lectins using techniques such as immunohistochemistry and western blotting. The quality of the information obtained from antibody and lectin-binding studies depends largely on the specificity of these proteins. Consequently, specificity has been studied at length over the years. Most lectins are known to have broad specificity. As a result, they have primarily been used to monitor general changes of carbohydrate expression and to track families of

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antigens (e.g. various structures containing a terminal fucose). In contrast, anticarbohydrate antibodies typically have much better specificity, and many are reported to be completely specific for their designated antigen. Therefore, antibody binding has been used extensively to monitor expression of individual carbohydrate antigens (e.g. Lewis Y).

Although antibody and lectin specificity has been studied frequently (for some examples, see Oriol et al. 1990; Mollicone et al. 1996; Pochechueva et al. 2002), only a tiny fraction of all the natural carbohydrate structures found in nature have been evaluated as potential ligands for any given protein. The two primary obstacles have been a lack of access to structurally defined glycans for analysis and a lack of high-throughput methods to evaluate binding. Advances in the fields of combinatorial carbohydrate synthesis and automated carbohydrate synthesis are rapidly expanding the number of glycans available. However, traditional methods for analyzing carbohydrate–protein interactions, such as X-ray crystallography, NMR, isothermal calorimetry, ELISA, and mono- or oligosaccharide inhibition studies can be slow, require large amounts of material, and are not well suited to analysis of thousands of potential carbohydrate–protein interactions.

Carbohydrate microarrays are a new technology being developed by a number of groups for high-throughput evaluation of carbohydrate–macromolecule interactions (Paulson et al. 2006). Carbohydrate microarrays contain many different glycans immobilized on a solid support in a miniaturized format. One can simultaneously evaluate binding of proteins, viruses, or cells to every component on the array under identical conditions. In addition, the microarray format has the capacity to accommodate thousands of unique glycans, although consuming only tiny amounts of precious materials. Our group has developed a carbohydrate microarray and highly sensitive assay (Manimala et al. 2005, 2006). The array contains 80 different components including 61 structurally defined synthetic carbohydrates [in the form of bovine serum albumin (BSA)/human serum albumin (HSA) conjugates], 15 natural glycoproteins, and 4 controls (see Table I for a detailed list). The glycans are printed on glass microscope slides using a robotic microarrayer. To maximize throughput, a 16-well slide format is utilized, with an entire array printed in each well. Therefore, 16 independent array experiments can be conveniently carried out on each slide.

In this paper, carbohydrate microarray profiling of 27 carbohydrate-binding antibodies is described. In addition to providing key information for scientists using these antibodies or using the information derived from the antibodies, the results have broad implications for the field.

Results

The 27 antibodies chosen for this study are listed in Table II. All the antibodies are known to bind antigens with altered expression in tumors. These included 8 antibodies to ABH histo-blood group antigens, 15 antibodies to Lewis antigens, and 4 antibodies to Tn, TF, and Galα1-4Galβ1-4Glc-HSA (Gb3) (Table II). The panel consisted of 21 IgM antibodies and 6 IgG antibodies, reflecting the higher proportion of IgM antibodies available. All the antibodies are reported to be specific for their listed antigen by the companies that sell

them. However, four of these have previously been reported to have some cross-reactivity.

Antibodies were evaluated using an ELISA format adapted for the carbohydrate microarray (Manimala et al. 2005, 2006). Briefly, mouse monoclonal antibodies were incubated in wells at eight or more different concentrations. Next, wells were incubated with goat antimouse horseradish peroxidase (HRP) conjugate, followed by incubation with a Cy3-labeled tyramide substrate. Fluorescence intensities for each spot were measured using a DNA microarray scanner, and the antibody titers were determined for each component on the array. For this study, the titer was set as the largest dilution that produced a signal 5 times higher than the background. This cutoff was typically around 1000 and was chosen to avoid possible concerns related to low signal-to-noise ratios. Throughout the assay, the slides are washed extensively. Therefore, any signal observed on a slide arises from a fairly strong binding event. In total, more than 2000 potential protein–carbohydrate interactions were evaluated in the study.

Overall, the antibodies were far more selective than most lectins. However, over half displayed inappropriate binding relative to the listed specificity (Table II). Fourteen of the antibodies had measurable cross-reactivity with at least one other glycan on the array; 10 of these were regarded as having substantial cross-reactivity (i.e. ≤20-fold selectivity for the designated antigen; antibodies T174, T218, F3, A70-C/C8, 92FR-A2, B386, FR4A5, B480, 1A4, and K21). Two antibodies did not bind any glycan on the array (antibodies B460 and 5B5). Finally, one antibody recognized only a specific form of its designated antigen.

Of the eight antibodies to blood group A, B, and H antigens, only 81FR2.2 displayed the expected specificity on the array. B480, also known as 3-3A, cross-reacted with blood group B, whereas CLCP-19B cross-reacted weakly with blood group H1. 92FR-A2 was found to bind both blood group H2 and Fucα1-2Galβ1-4[Fucα1-3)GlcNac-HSA (Le^y). These results are in agreement with a previous publication (Mollicone et al. 1996) but not with information provided by the company supplying the antibody. B389, also known as A70-A/A9, was found to bind Le^y. This antibody is listed as binding blood group H2, Le^y, and Fucα1-2Galβ1-3[Fucα1-4)GlcNacβ1-3Galβ1-4Glcβ-BSA (Le^b) by the manufacturer. In contrast, a recent report showed strong binding to Le^y, 30-fold weaker binding to Le^b, and no binding to blood group H2 (Christensen et al. 2007). Antibodies B376 (also known as A63-D/B12) and B393 (also known as A46-B/B10) from Biomeda (Foster City, CA) did not show any binding on the array. Two different batches of antibodies yielded identical results. However, the same antibodies obtained from Glycotope (Berlin, Germany) showed substantial binding on the array. As an interesting note, Biomeda lists these antigens as specific for blood group H2, whereas Glycotope lists them as cross-reacting with Le^y. On our array, they were found to bind both blood group H2 and Le^y. Antibody B460 did not show any binding on our array even after concentrating the antibodies. The lack of binding is not due to the absence of the glycans on the array since results from this study and previous studies verify that every glycan on the array is present and accessible for binding (Manimala et al. 2006). The lack of binding could be due to a production/manufacturing and/or shipping problem of the

Table I. Components of the microarray and abbreviations

Cy5-BSA	Glcα1-6Glcβ-BSA (isomalt)	Galβ1-3GlcNAcβ1-3Galβ-BSA (LNT)
Bovine serum albumin (BSA)	Manα1-6[Manα1-3]Man-BSA (ManT)	Sia2-3Galβ1-3GlcNAcβ1-3Galβ1-BSA (LSTa)
Human serum albumin (HSA)	Galα1-4Galβ-BSA	Galβ1-3(Sia2-6)GlcNAcβ1-3Galβ-BSA (LSTb)
Glc-β-BSA	Sialyl2-3Galβ1-4GlcNAc-BSA (3′SLacNAc)	Sia2-6Galβ1-3GlcNAcβ1-3Galβ-BSA (LSTc)
GalNAc-α-BSA	GalNAα1-3(Fucα1-3)(Fucα1-2)Galβ-BSA (BG-A)	Galβ1-3GalNAcβ1-4Galβ-BSA (GA1)
Glcα-BSA	Galα 1-3(Fucα1-2)Galβ-BSA (BG-B)	Keyhole limpet hemocyanin (KLH)
Man-α-BSA	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-HSA (BG-H1)	oxKLH
GlcNAc-β-BSA	Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ-BSA (Le^a)	Bovine submaxillary mucin (BSM)
GalNAc-β-BSA		Asialo-BSM (aBSM)
Fuc-β-BSA	Fucα1-2Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ-BSA (Le^b)	Deacetylated-BSM (deAcBSM)
Fuc-α-BSA		Ovine submaxillary mucin (OSM)
Rha-α-BSA	Fucα1-2Galβ1-4[Fucα1-3)GlcNAc-HSA (Le^y)	asialo-OSM (aOSM)
Rha-β-BSA	Galβ1-4[Fucα1-3)GlcNAc-HSA (Le^x)	Glycophorin (Gn)
Gal-α-BSA	SLe^x-BSA	asialo-glycophorin (aGn)
Gal-β-BSA	Galα1-4Galβ1-4Glc-HSA (Gb3)	Prostate-specific antigen (PSA)
GalNAcα1-Thr-Gly-BSA (Tn)	Xylβ1-4Xylβ1-4Xylβ1-4Xylβ1-BSA (Xyl4)	Heat shock protein 90 (hsp90)
GalNAcα1-6Galβ-BSA	Araα1-5Araα1-5Araα1-5Araα1-BSA (Ara5)	Thyroglobulin (Tgl)
GalNAcα1-3Galβ-BSA (A$_{di}$)	Xylα1-6Glcβ1-4(Xylα1-6)Glcβ1-4(Xylα1-6)Glcβ1-BSA (X3Glc3)	Alpha fetoprotein (AFP)
GlcNacα1-4Galβ-BSA		Carcinoembryonic antigen (CEA)
Galα1-3Gal-BSA (Bdi)	3′Sialyllactose-HSA (GM3)	Fatty acid-binding protein (FABP)
Galβ1-4GlcNAc-BSA (LacNAc)	6′Sialyllactose-HSA (6′SLac)	Fucα 1-2Galβ1-4GlcNAcβ-HSA (BG-H2)
Galβ1-3GlcNAc-HSA (TF$_{di}$)	Sia-LeA-HSA (SLe^a)	AcTn-Tn-Tn-Gly-Hex-BSA (Tn3, clustered Tn)
Galβ1-4Glcβ-BSA (Lac)	Di-LeX-BSA	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-BSA (Chito3)
Galβ1-3GlcNAcβ-BSA (Le^c)	Manα1Manβ1-4Manβ1-4Manβ1-BSA (Manβ4)	Galα 1-3Galβ1-4Galα-BSA (Gal3)
Galβ1-6Man-α-BSA		Manα1-6(Manα1-3)Manβ1-4GlcNAc-BSA (Man3)
Manα1-6Man-α-BSA	Manβ1-4(Galα1-6)Manβ1-4(Galα1-6)Manβ-BSA (G2M4)	AcSerSerGly-BSA (SSS)
GalNAcβ1-4Galβ-BSA (GA2$_{di}$)		AcSerTnSerGly-BSA (STnS)
Glcβ1-4Glcβ-BSA (cellobiose)	Glcα1-6Glcα1-4Glcα1-4Glcβ-BSA	Cy3-BSA
Glcα1-4Glcβ-BSA (maltose)		

supplier. Alternatively, the antibody may only recognize the antigen in a specific context. Carbohydrate antigens can be presented on N-linked oligosaccharides, O-linked oligosaccharides, or glycolipids. Differences in presentation can lead to differences in accessibility, spacing of epitopes, and orientation of epitopes. In this case, there are at least some contexts that are not recognized by these antibodies. As a result, they can produce false-negative results (i.e. failure to detect expression of the antigen).

Of the 15 Lewis antigen-binding antibodies, 6 (7LE, 121SLE, CA199.02, 15C02, 28, and ZC-18C) displayed excellent specificity within the context of the array. Of the Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ-BSA (Le^a)-binding antibodies, T174 cross-reacted with sialyl Le^a, whereas PR.5C5 cross-reacted weakly with Galα1-4Gal and rhamnose. Of the Le^b-binding antibodies, 2-25LE displayed weak cross-reactivity with Le^a, an observation that has also been seen previously (Good et al. 1992; Amano et al. 1997), whereas T218 cross-reacted significantly with both Le^y and blood group H1. One of the Galβ1-4[Fucα1-3)GlcNAc-HSA (Le^x) antibodies, FR4A5, cross-reacted with Glcα1-6Glcα1-4Glc, Tn, blood group H1, and maltose. All three Le^y-binding antibodies showed at least some cross-reactivity to other antigens. F3 bound well to blood group H2 and Le^x. A70-C/C8 was found to cross-react substantially with blood group B. Previous studies on the specificity of this antibody had found it to be specific for Le^y (Cao et al. 2001; Pochechueva et al. 2002). BR55 displayed the best specificity but still showed weak cross-reactivity to Le^x and DiLe^x, a feature that is not uncommon for Le^x-binding antibodies. K21, a Galβ1-3GlcNAcβ-BSA (Le^c)-binding antibody, bound well to Glcα1-6Glcα1-4Glc, maltose, and rhamnose but did not bind the Le^c disaccharide on the array.

Of the four other tumor antigen-binding antibodies, none displayed binding consistent with the listed specificity. Antibody 1A4 bound well to Gb3 but also cross-reacted with blood group B. 5B5, a different Gb3-binding antibody, did not bind any glycan on the array. Lack of binding to Gb3 has been observed previously (Dorken et al. 1989) and appears to be assay dependent and/or context dependent. Antibody B1.1 is listed as a general Tn binder but was only found to bind the clustered form of the antigen, sequences containing two or more GalNAcα1-Ser/Thr (Tn) residues linked consecutively on a peptide chain. Although the antibody displayed good selectivity and could be very useful, previous conclusions drawn from binding data may be inaccurate as a result of the difference between the listed specificity and the actual specificity. B386, also known as A68-B/A11, was found to bind blood group B and blood group H1, but no binding to the TF disaccharide or asialo-glycophorin (a protein known to display the TF antigen) was observed. Previous studies have also seen cross-reactivity to blood group B as well as Galβ1-3Gal (Pochechueva et al. 2002).

Several factors confirmed that the observed specificity problems were due to the antibodies and not artifacts arising from the microarray. First, the binding profiles were reproducible and observable over a range of concentrations for each antibody. Second, binding preferences were verified by ELISA with individual carbohydrates to ensure that the observed binding was not a result of a misprinted slide or an artifact (see Supplementary data). Third, results for different antibodies to the same antigen were compared to ensure that unanticipated signals were not due to contamination (Figure 1). For example, BR55, F3, and A70-C/C8 all bind well to Le^y on the microarray. However, F3 cross-reacted with BG-H2, and A70-C/C8 cross-reacted with BG-B. If

Table II. Antibody-binding results from the carbohydrate microarray

Clone	Isotype	References	Listed antigen[a]	Titer[b] for listed antigen	Cross-reactive carbohydrates and their titers[b]	Glycoproteins bound and their titers[b,c]
81FR2.2	IgM	Unpublished	BG-A	1:20 000	None observed	BSM = 1:4000; aBSM = 1:2000; deAcBSM = 1:2000
B480	IgM	Bara et al. (1986)	BG-A	1:200 000	BG-B = 1:10 000	BSM = 1:20 000; aBSM = 1:10 000; deAcBSM = 1:10 000
CLCP-19B	IgM	Unpublished	BG-B	1:2000	BG-H1 = 1:50	None observed
B460	IgM	Vanak et al. (1989)	BG-B	No binding	None observed	None observed
B389	IgG1	Christensen (2005)	BG-H2, Le^y, Le^b	Le^y = 1:10 000; No binding to BG-H2 or Le^b	None observed	None observed
92FR-A2	IgM	Biomeda, unpublished	BG-H2	1:320 000	Le^y = 1:320 000	None observed
B376 (A63-D/B12)	IgM	Christensen (2005)	BG-H2[d]	No binding	None observed	None observed
A63-D/B12	IgM	Christensen (2005)	Le^y, BG-H2[d]	Le^y = 1:32 000; BG-H2 = 1:32 000	None observed	None observed
B393 (A46-B/B10)	IgM	Karsten et al. (1988)	BG-H2[d]	No binding	None observed	None observed
A46-B/B10	IgM	Karsten et al. (1988)	Le^y, BG-H2[d]	Le^y = 1:32 000; BG-H2 = 1:32 000	None observed	None observed
7LE	IgG1	Daher et al. (1987)	Le^a	1:40 000	None observed	None observed
PR.5C5	IgG1	Richman and Bodmer (1987)	Le^a	1:100	Galα1-4Gal = 1:1; Rhaα = 1:1	OxKLH = 1:1; KLH = 1:1
T174	IgG1	Sakamoto et al. (1986)	Le^a	1:1000	SLe^a = 1:1000	None observed
121SLE	IgM	Herrero-Zabaleta et al. (1987)	SLe^a	1:160 000	None observed	None observed
CA199.02	IgM	Unpublished	SLe^a	1:4000	None observed	None observed
2-25LE	IgG1	Bara et al. (1986)	Le^b	1:100 000	Le^a = 1:1000	None observed
T218	IgM	Sakamoto et al. (1986)	Le^b	1:30 000	Le^y = 1:20 000; BG-H1 = 1:10 000	None observed
K21	IgM	Rettig et al. (1985)	Le^c	No binding	Glcα1-6Glcα1-4 = 1:50; maltose = 1:40; Rhaα = 1:5	None observed
15C02	IgM	Unpublished	Le^x	Le^x = 1:2000; DiLe^x = 1:8000	None observed	None observed
28	IgM	Hogg et al. (1984)	Le^x	Le^x = 1:4000; DiLe^x = 1:20 000	None observed	None observed
ZC-18C	IgM	Zola et al. (1981)	Le^x	Le^x = 1:500; DiLe^x = 1:4000	None observed	None observed
FR4A5	IgM	Unpublished	Le^x	1:10	Glcα1-6Glcα1-4 = 1:100; Tn = 1:50; BG-H1 = 1:50; maltose = 1:50	None observed
BR55	IgG2a	Unpublished	Le^y	1:128 000	Le^x = 1:4000; DiLe^x = 1:1000	None observed

Continued

Table II. *Continued*

Clone	Isotype	References	Listed antigen[a]	Titer[b] for listed antigen	Cross-reactive carbohydrates and their titers[b]	Glycoproteins bound and their titers[b,c]
F3	IgM	Lloyd et al. (1983)	Le^y	1:6000	BG-H2 = 1:800; Le^x = 1:200	None observed
A70-C/C8	IgM	Christensen (2005)	Le^y	1:4000	BG-B = 1:2000	None observed
B1.1	IgM	Biomeda, unpublished	Tn	Tn3 = 1:2000; No binding to STnS, Tn	None observed	aOSM = 1:4000; aBSM = 1:2000
B386	IgM	Karsten et al. (1995)	TF	No binding	BG-B = 1:20 000; BG-H1 = 1:400	aGn = No binding
1A4	IgM	Unpublished	Gb3	1:180	BG-B = 1:180	OxKLH = 1:180
5B5	IgM	Fyfe et al. (1987)	Gb3	No binding	None observed	None observed

[a]For abbreviations, see Table I and Supplementary data.
[b]The titer is the largest dilution that produced a signal 5 times higher than the background.
[c]More detailed information on the carbohydrate compositions of the glycoproteins can be found in the Supplementary data. Briefly, bovine submaxillary mucin (BSM) contains a high content of STn antigen, STF, and sialyl-GlcNAcβ1-3GalNAc; 22% of the sialic acids are acetylated at position 7, 8, or 9; KLH contains a complex variety of glycans including mannose, fucose, and galactose terminal structures; asialo-ovine submaxillary mucin (aOSM) and asialo-bovine submaxillary mucin (aBSM) contain a high content of Tn antigen; asialo-glycophorin (aGn) contains a high content of TF antigen.
[d]Biomeda states that the antibody is specific for blood group H2, whereas Glycotope states that the antibody binds both blood group H2 and Le^y.

BG-H2 or BG-B was contaminated with Le^y, all three antibodies would give a positive signal for the contaminated component. As a second example, T174 gives a positive signal with both Le^a and SLe^a. In contrast, 7LE only binds Le^a, and 121SLE only binds SLe^a, demonstrating that neither of these components is contaminated with the other carbohydrate antigen. Representative microarray images for all the antibodies can be found in the Supplementary data.

Fig. 1. Comparisons of microarray data for different antibodies. Representative microarray data for (**A**) three Le^y-binding antibodies: BR55 (best selectivity), F3 (cross-reacts with BG-H2), and A70-C/C8 (cross-reacts with BG-B) and for (**B**) Le^a- and SLe^a-binding antibodies: 7LE (selective for Le^a), T174 (Le^a-binding antibody that cross-reacts with SLe^a), and 121SLE (selective for SLe^a). Each array component is printed in duplicate. Binding is detected using an ELISA-based assay with Cy3 tyramide as the substrate (see *Materials and methods* section). Green spots indicate fluorescent values ranging from 1000 to 65 000. White spots indicate a saturated signal (>65 000). Arrows and labels designate the locations for the Cy3–BSA (green) and Cy5–BSA (red) controls, as well as other relevant components.

Discussion

Antibody binding has been used extensively to monitor the expression of carbohydrate antigens, modulate their biological activity, and target specific glycans for clinical applications. Conclusions drawn from these studies are frequently based on the perception that the antibodies are highly specific for their listed antigen. Using a carbohydrate microarray, we show that many of the antibodies currently in use do not have the expected specificity. More than half the antibodies cross-react with other carbohydrate antigens. With these antibodies, the cross-reacting carbohydrate(s) could easily be mistaken for the listed antigen in a biopsy sample, leading to a false-positive or an overestimation of antigen expression levels. Several other antibodies did not bind their listed antigen at all and, therefore, could produce false-negative results. Even the antibodies that were found to be specific within the context of the array may cross-react with glycans that are not present on the array.

Both false-positive and false-negative results can have a major effect on basic and clinical research. For example, information regarding expression of carbohydrate tumor antigens in different tissues has been used to select patient groups for clinical trials. If that information is incorrect, a suboptimal group of patients may be chosen. When studying the biological roles of carbohydrates, properties or functions attributed to a particular carbohydrate antigen may actually be due to a cross-reactive epitope. Given the importance of this information, one must be extremely cautious drawing conclusions from antibody-binding data and interpreting previous conclusions reported in the literature. To avoid false-positive and false-negative results, verification of conclusions with one or more independent analytical methods would be best. However, analysis of carbohydrate expression with two or more different antibodies that bind the same glycan could also yield more reliable information. The data reported in this paper should be a useful resource for selecting appropriate
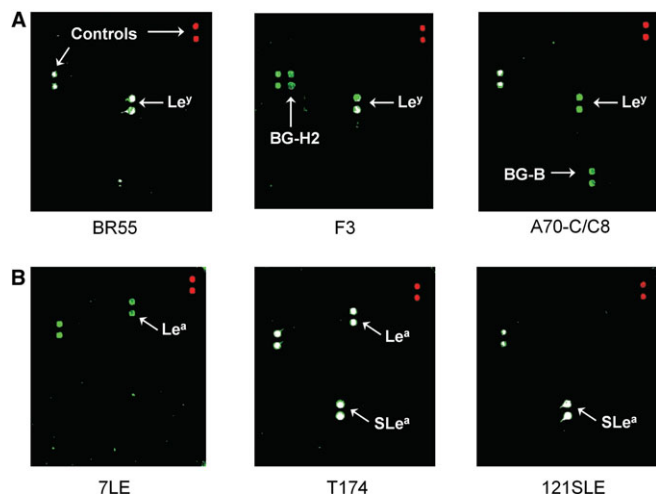
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antibodies for studies and for interpreting experimental results arising from those antibodies. Efforts to re-evaluate previous conclusions regarding carbohydrate expression and to develop more effective and reliable methods for monitoring carbohydrate expression should be a high priority for the field.

Finally, the results presented in this paper illustrate the power of carbohydrate microarray technology. Thousands of potential carbohydrate−protein interactions were rapidly analyzed using only nanograms of each neoglycoconjugate. As access to glycans increases into the thousands, the carbohydrate microarray format can easily accommodate the added diversity and provide the throughput necessary to evaluate large numbers of potential interactions. Moreover, the carbohydrate microarray is a powerful tool for screening new antibodies and lectins to identify proteins with improved specificity.

Materials and methods

Materials

81FR2.2 was purchased from DakoCytomation (Carpinteria, CA). CLCP-19B, F3, T218, and T174 were purchased from EMD Biosciences, Inc. (San Diego, CA). B369, B460, B386, B389, B480, B1.1, B393, and B376 were purchased from Biomeda. PR5C5, 28, and ZC-18C were purchased from Chemicon International, Inc. (Temecula, CA). 7LE, 2-25LE, 15C02, 121SLE, and CA199.02 were purchased from Lab Vision Corporation (Fremont, CA). K21 was purchased from Genetex, Inc. (San Antonio, TX) and 5B5 was purchased from BD Biosciences (San Jose, CA). BR55 was purchased from Glycotech (Gaithersburg, MD). A63-D/B12 and A46-B/B10 were purchased from Glycotope. Goat anti-mouse immunoglobulin (Ig)−HRP (was purchased from SouthernBiotech (Birmingham, AL). The epoxide-derivatized Nunc ArrayCote 16-well microarray slides were purchased from Nalge Nunc International (Rochester, NY), and the arrays were printed by KamTek Inc. (Gaithersburg, MD).

Antibody-binding assay and analysis

The assay is based on previously reported protocols (Manimala et al. 2005, 2006). Briefly, the printed array slides (see Supplementary data for fabrication of the arrays) were incubated with blocking buffer (3% BSA in PBS, 200 µL/well) for 2 h. Serial dilutions of antibodies (50 µL/well, diluted in PBS with 0.3% BSA) were incubated for 2 h in individual wells. Slides were washed 3 times with PBS, incubated for 1 h with goat antimouse Ig (H + L)−HRP (diluted 1:500 in PBS containing 3% BSA), washed 7 times with PBS, and then incubated with 0.1% cyanine 3-tyramide-labeling reagent in PBS containing 1% BSA, 0.01% H_2O_2, 0.005% Tween 20, and 0.01% polyvinylpyrrolidone for 10 min. Wells were washed with PBS, and the detachable well casings were removed. The slides were incubated in PBS for 5 min and then dried by centrifugation at 900g for 5 min. The slides were scanned on a GenePix 4000B scanner (Molecular Devices Corporation, Union City, CA). The fluorescence was quantified by using GenePix Pro 6.0 software with a GenePix Array List file. The mean values minus the background (typically around 200) for each of the two spots for a particular sample were averaged. Spot-to-spot variability is typically less than 20%. All antibodies were assayed on at

least two different slides and data were fully consistent from slide to slide.

Supplementary data

Supplementary data are available at Glycobiology online (http://glycob.oxfordjournals.org/).

Conflict of interest statement

None declared.

Abbreviations

aBSM, asialo-bovine submaxillary mucin; aGn, asialo-glycophorin; aOSM, asialo-ovine submaxillary mucin; BSA, bovine serum albumin; BSM, bovine submaxillary mucin; deAcBSM, deacetylated-BSM; Gb3, Galα1-4Galβ1-4Glc-HSA; HRP, horseradish peroxidase; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; Le[a], Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ-BSA; Le[b], Fucα1-2Galβ1-3[Fucα1-4)GlcNacβ1-3Galβ1-4Glcβ-BSA; Le[c], Galβ1-3GlcNAcβ-BSA; Le[x], Galβ1-4[Fucα1-3)GlcNAc-HSA; Le[y], Fucα1-2Galβ1-4[Fucα1-3)GlcNac-HSA

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