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Microarrays with Varying Carbohydrate Density Reveal Distinct Subpopulations of Serum Antibodies

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Abstract

Antigen arrays have become important tools for profiling complex mixtures of proteins such as serum antibodies. These arrays can be used to better understand immune responses, discover new biomarkers, and guide the development of vaccines. Nevertheless, they are not perfect and improved array designs would enhance the information derived from this technology. In this study, we describe and evaluate a strategy for varying antigen density on an array and then use the array to study binding of lectins, monoclonal antibodies, and serum antibodies. To vary density, neoglycoproteins containing differing amounts of carbohydrate were synthesized and used to make a carbohydrate microarray with variations in both structure and density. We demonstrate that this method provides variations in density on the array surface within a range that is relevant for biological recognition events. The array was used to evaluate density dependent binding properties of three lectins (*Vicia villosa* lectin B₄, *Helix pomatia* agglutinin, and soybean agglutinin) and three monoclonal antibodies (HBTn-1, B1.1, and Bric111) that bind the tumor-associated Tn antigen. In addition, serum antibodies were profiled from 30 healthy donors. The results show that variations in antigen density are required to detect the full spectrum of antibodies that bind a particular antigen and can be used to reveal differences in antibody populations between individuals that are not detectable using a single antigen density.

Keywords

microarray; carbohydrate; antibodies; multivalency; antigen density; glycan array; lectin; Tn

Introduction

Antigen arrays contain many different molecules, such as proteins, peptides, and carbohydrates, immobilized on a solid support in a spatially-defined arrangement.^{1, 2} The technology allows one to examine binding to numerous potential antigens in parallel while using only tiny amounts of precious materials. Antigen arrays have become powerful tools for profiling the repertoire of antibodies in serum and this technology has proven useful for basic research, antigen discovery, and vaccine development.^{1, 2} While antigen arrays are becoming a mainstay of basic and clinical research, improved array design and enhanced capabilities are still needed. First, detection is limited to the antigens found on the array. Second, antibody binding can be dependent on how the antigen is displayed. Therefore, detection is further limited to those antigens with appropriate presentation. Third, each antigen on the array may be recognized by an ensemble of molecularly distinct antibodies. Each of the antibodies within this collection can have different biological activities, protective effects, and/or biomarker

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properties; however, competition between these antibodies can make it difficult to distinguish these populations. For instance, changes in levels of one subpopulation of antibodies may be offset by opposing changes in another population or obscured by high concentrations of a competing antibody. To overcome these limitations, it is desirable to increase antigen content, optimize presentation on the array, and develop methods to distinguish different antibodies that bind the same antigen

Antibodies have multiple binding sites and are capable of forming multivalent interactions when two or more antigens are present. Formation of a multivalent complex can have a substantial effect on the overall affinity (avidity) of the interaction as well as the selectivity of the recognition event.³ The formation of a multivalent complex requires optimal spacing and orientation, thus making antigen density a critical factor. Antigen density can have a significant effect on recognition, especially for certain classes of antigens such as carbohydrates.⁴ Monovalent binding events between carbohydrates and proteins are typically weak with dissociation constant (K_d) values in the high micromolar to millimolar range. To compensate, most carbohydrate binding proteins either possess multiple binding sites or oligomerize to form assemblies containing multiple binding sites. Simultaneous binding of two or more carbohydrates on a cell surface or glycoprotein results in formation of a high avidity multivalent complex. The importance of density has been recognized and studied at length in a variety of multivalent systems.^{3–6} These studies have primarily focused on the relationship between density and avidity, but density can also have a significant effect on selectivity. For example, Kiessling^{7–9} and others^{10–12} have observed enhanced selectivity for multivalent interactions relative to monovalent binding. In other work, Kahne *et al.* have observed a switch in selectivity with changing density.^{13–15} Although these systems have focused on lectin-carbohydrate interactions, similar effects for antibodies are possible and might permit one to distinguish different antibodies that bind the same antigen but have differing density requirements. In addition, optimal density may be required to detect certain antibodies.

In this study, we describe a method for varying both structure and density on a carbohydrate antigen microarray and then use the array to evaluate density-dependent binding effects for tumor-binding lectins, monoclonal antibodies, and serum antibodies. We show that variations in antigen density are required to detect the full spectrum of antibodies that bind a particular antigen and can be used to reveal differences in antibody populations between individuals that are not detectable using a single antigen density.

Materials and Methods

Unless otherwise stated, reagents were obtained from commercial suppliers and used without purification. All aqueous solutions were prepared from distilled deionized water filtered with a Milli-Q purification system and sterile filtered through a 0.2 μ m filter. BSA (bovine serum albumin) and HSA (human serum albumin, cat# A8763) were purchased from Sigma (St. Louis, MO). Biotinylated soybean agglutinin (SBA) and *Vicia villosa* lectin (VVL-B₄) were purchased from Vector Laboratories (Burlingame, CA). *Helix pomatia* agglutinin (HPA) was purchased from Sigma (St. Louis, MO). Monoclonal antibody B1.1 was purchased from Biomedica (Foster City, CA). Monoclonal antibody Bric111 was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY). HBTn-1 was obtained from Dako Cytomation (Carpenteria, CA). Streptavidin-Cy3 was purchased from Zymed Laboratories of Invitrogen Corporation (Carlsbad, CA). Cy3-labeled AffiniPure goat anti-mouse IgM and goat anti-mouse IgG; Cy3-conjugated goat anti-human IgA + IgG + IgM (H+L); Cy3-conjugated goat anti-human IgG, Fc γ Fragment Specific; and Cy3-conjugated goat anti-human IgM, Fc γ Fragment Specific were purchased from Jackson ImmunoResearch (West Grove, PA). Human serum samples were purchased from Valley Biomedical Products and Services (Winchester, VA) and were accompanied by a certification that all samples were tested by an

FDA-approved test and found to be negative for HBsAG, HIV 1/2, HIV-1 AG, or HIV-1 NAT, HCV, and Syphilis. Aliquots of samples were made and stored at -20°C .

Carbohydrate Microarray Fabrication

Epoxide-derivatized ArrayIt® SuperEpoxy 2 Protein microarray slides were purchased from TeleChem International, Inc. (Sunnyvale, CA) and the arrays were printed with SMP3 Stealth Micro Spotting Pins from TeleChem International, Inc. using a Biorobotics MicroGrid II 600/610, Genomic Solutions (Ann Arbor, MI) robotic microarrayer at the Laboratory of Molecular Technology, SAIC-Frederick (Frederick, MD).

45 glycoconjugates and 4 controls were distributed into 384-well plates at 4 wells per sample and 20 μL per well. Each component was prepared at 125 $\mu\text{g/mL}$ in print buffer (1X phosphate-buffered saline (PBS), 2.5% glycerol, 0.006% Triton-X 100) onto glass slides. 4 micro-spotting pins were used for the print, with each pin printing 4 complete arrays per slide. The pins were blotted 4 times before printing. The humidity level in the arraying chamber was maintained at about 50–60% during printing. Each of the 49 components was printed in duplicate in a 20×5 grid of 110 μm diameter spots. 16 complete arrays were printed on each slide. Printed slides were stored at -20°C until use.

Determination of Apparent Kd on Carbohydrate Microarray

The binding experiment was carried out in triplicate. Slides were assembled on 16-well slide holders and blocked with 3% BSA/PBS overnight at 4°C , then washed $6 \times 200 \mu\text{L}$ PBST0.05 (PBS with 0.05% Tween 20). A dilution series of biotinylated lectin solutions (HPA, SBA, and VVL- B_4) was prepared in 0.3% BSA, 0.01 mM Mn^{2+} , 1 mM Ca^{2+} , 1X PBS. HPA was prepared at 4.8 pM to 1.3 μM in 4-fold dilutions, SBA was prepared at 15.9 pM to 4.23 μM in 4-fold dilutions, and VVL- B_4 was prepared at 72.7 pM to 1.4 μM in 3-fold dilutions. Lectin solutions were added to arrays, covered tightly with a seal strip, and allowed to incubate for 2 h at room temperature. After washing unbound lectin with $3 \times 200 \mu\text{L}$ PBST0.05, detection of bound lectin was carried out by incubating with Cy3-streptavidin in 3% BSA/PBS (5 $\mu\text{g/mL}$) for 2 h at room temperature. Slides were then washed $7 \times 200 \mu\text{L}$ PBST0.05, removed from holders, immersed in wash buffer for 5 min then centrifuged at $453 \times g$ for 5 min. For determination of apparent binding constant for B1.1 and Bric111, the slide was blocked by 3% BSA/PBS (200 μL per well) for two hours at room temperature. The array was then incubated at room temperature for two hours with a total of eight four-fold diluted concentrations starting from 40 $\mu\text{g/mL}$ (for B1.1), 49 $\mu\text{g/mL}$ (HBTn-1), or 200 $\mu\text{g/mL}$ (for Bric111) (100 μL per well). The slide was then washed and incubated with secondary antibody Cy3-labeled AffiniPure goat anti-mouse IgM (for B1.1, HBTn-1) or IgG (for Bric111) for two hours at room temperature, washed, and dried as described above.

Slides were scanned with a Genepix 4000B microarray scanner at 10 μm resolution (Molecular Devices Corporation, Union City, CA) at PMT voltage settings where no saturated pixels were obtained. Image analysis was carried out with Genepix Pro 6.0 analysis software (Molecular Devices Corporation, Union City, CA). Spots were defined as circular features with maximum diameter of 100 μm . Local background subtraction was performed and features were allowed to be resized to 70 μm as needed. The background-corrected median feature intensity, *F532median-B532*, was used for initial data processing, which was performed with Microsoft Excel. The mean for replicate spots for each array component was calculated and used for calculation of apparent Kd.

Apparent dissociation constants were determined using the scientific graphing and analysis software Origin 7.5 (OriginLab, Northampton, MA). For each glycoconjugate, the mean

fluorescence intensity was plotted as a function of protein concentration on a logarithmic scale. Nonlinear curve-fitting to the equation

$$F_{\text{obs}} = \frac{F_{\text{max}}}{\frac{K_D}{[L]} + 1}$$

was performed, where F_{obs} is the mean intensity of the replicate spots for protein binding at a particular concentration, F_{max} is the maximum fluorescence intensity, K_D is the apparent dissociation constant for interaction between protein and immobilized glycoconjugate, and $[L]$ is the concentration of protein. For each lectin, the reported values are the average of apparent K_D s derived from 3 replicate experiments and the error is the standard deviation of the 3 measurements.

Human Serum Profiling on Carbohydrate Microarray

Slides were fitted with 16-well slide holders (Grace Bio-Labs) and blocked with 3% BSA/PBS overnight at 4°C, then washed with PBST0.05. Serum samples were diluted 1:50 in 3% BSA/PBST0.05, added to arrays, and allowed to incubate with gentle shaking for 4 h at 37°C. Each sample was analyzed on duplicate slides within the same experiment. A reference sample (an unrelated healthy human serum sample) was also analyzed in one well of each slide. After washing unbound protein with PBST0.05, detection of bound Ig was carried out by incubating with Cy3-conjugated goat anti-human IgA + IgG + IgM (H+L) in 3% HSA/1% BSA/PBS (2 µg/mL) at 37°C. For IgG and IgM detection, slides were incubated with Cy3-conjugated goat anti-human IgG and Cy3-conjugated goat anti-human IgM, respectively, in 3% HSA/1% BSA/PBS (2 µg/mL) at 37°C. After 2 h, slides were washed with PBST0.05, removed from holders, immersed in wash buffer for 5 min before being centrifuged at $453 \times g$ for 5 min.

Image Processing and Data Analysis

Slides were imaged and analyzed as described above with the following modifications. Intensities above 50,000 were corrected using the algorithm of Lyng et al.¹⁶ Briefly, each slide was scanned twice. The first scan was obtained at a PMT voltage setting such that Cy3-BSA spot intensities were about 20,000 (I_{scan1}). The second scan was obtained with a PMT voltage setting such that there were no saturated pixels on the slide (I_{scan2}). For each slide separately, spots having intensities between 20,000 and 30,000 in scan 1 were identified and the ratio $I_{\text{scan1}}/I_{\text{scan2}}$ for each spot was calculated. The average of these ratios afforded the correction factor, F_{cor} .

$$F_{\text{cor}} = \frac{1}{m} \sum_{i=1}^m \frac{I_{\text{scan1}}}{I_{\text{scan2}}}$$

where m is the number of spots having intensities in scan 1 between 20,000 and 30,000. Corrected intensities were calculated for spots having intensities above 50,000 in scan 1 via the formula

$$\text{Corrected intensity} = I_{\text{scan2}} * F_{\text{cor}}$$

For each component in each well, the average of duplicate spots was calculated to obtain a value for the slide. Since each sample was run on two slides, the final signal for a sample is the average of two slides (4 spots). The mean of all the signals (excluding controls) for the reference sample on each slide was scaled to a common value of 10,000, then all other samples on the slide were normalized to the reference via the scaling factor.

Results and Discussion

Rationale and approach for varying density

Carbohydrate microarrays contain many different glycans immobilized on a surface in a spatially-defined arrangement.^{17–22} The miniaturized format permits high-throughput evaluation of carbohydrate-macromolecule interactions while using only minuscule amounts of materials. In addition to varying structure, arrays provide an advantageous platform for varying density. To be successful, the approach used should 1) produce variations in density on the surface, preferably in a controllable way, and 2) provide variations in density on an appropriate scale for biological recognition events. Ideally, the approach used to vary density should also allow one to readily translate array results to other assays, experiments, and objectives such as the development of multivalent inhibitors or probes. Addressing these issues is not trivial since a number of factors, such as the slide surface and printing conditions, can affect the density and uniformity of each spot. First, the maximum capacity of the surface will limit the maximum density achievable. Second, when printing material such that the surface is not “saturated”, there is a potential to obtain density gradients over the area of a spot. For example, as a liquid droplet evaporates, it can produce higher densities at the center of the spot relative to the perimeter regions. Under other conditions, one can obtain higher densities around the perimeter, sometimes referred to as “ring-like spots”.²³ Care must be taken to minimize these effects. Third, with certain surface attachment methods, the carbohydrates can move and adopt a range of densities within a spot. While this imparts flexibility to accommodate lectins with varying architectures, the density is not well defined making it difficult to transfer information to other scaffolds.

Several strategies have been examined for varying carbohydrate density on an array, but an optimal method has not yet emerged.^{9, 24–29} The most common approach for generating carbohydrate microarrays involves attaching different monovalent carbohydrates to a surface via a linker. One simple strategy for modulating density is to vary the print concentration of the monovalent carbohydrates. Several groups have examined this approach, but it is not yet clear that this method meets the appropriate criteria. First, certain experimental observations suggest that this approach is either not producing variations in density at the molecular level or is not producing biologically relevant variations. For example, Wong *et al.* printed a mannose derivative at concentrations ranging from 0.6 μM to 100 μM and then evaluated binding to Concanavalin A (ConA).²⁷ Based on calculations and experimental evidence, the authors concluded that at print concentrations of 10 μM or lower, the mannose residues are too far apart to form a multivalent complex with ConA. While one would expect a large increase in K_d when going from a multivalent interaction to a monovalent interaction, they observe only about a 3-fold change in both K_d and maximum signal. The marginal change observed and the relatively low K_d values (nanomolar) suggest that they are still obtaining a multivalent interaction on the array surface, thus indicating that printing different concentrations of carbohydrates does not necessarily result in changes in density in a range that allows one to measure differences in affinity. Second, the approach of varying the print concentration of monovalent carbohydrates onto a surface is not well-suited for translating array results to other experiments or using array results to obtain inhibitors or probes. With this method, the array surface acts as a multivalent scaffold. As a result, one must identify a multivalent scaffold with similar spacing and presentation of carbohydrates as the array surface.

An alternative approach, presented here, involves printing multivalent carbohydrates onto the array surface (Figure 1). To construct our arrays, we attach carbohydrates to a carrier protein such as bovine serum albumin (BSA) and then print these neoglycoconjugates, along with natural glycoproteins, on the slide surface (Figure 1).^{30–35} In this method, the carrier protein serves both as a linker for immobilization and as a multivalent scaffold. Although there are a variety of methods to attach molecules to BSA, we have focused on attaching oligosaccharides

to free amine groups via a) reductive amination of oligosaccharide lactols, or b) synthesis of oligosaccharides with a free carboxylic acid at the end of a linker and coupling via activation with EDC/NHS. BSA contains 60 amines (59 lysines and the N-terminus), 54 of which are expected to be solvent exposed based on the crystal structure of the homologous human serum albumin (HSA).^{36, 37} The amines on HSA (and based on homology, BSA) are fairly evenly distributed on the protein surface, with an average distance from one amine group to the nearest neighboring amine group of $11 \pm 3 \text{ \AA}$.^{36, 37} To vary density, one can modulate the number of carbohydrates that are attached to BSA. It should be noted that the conjugation is not done in a site specific manner and the resulting neoglycoconjugates have a mixture of attachment sites. In addition, the linkers on the carbohydrates and the side chains of the lysines are flexible. This provides some extension from the protein surface as well as some modest mobility to the carbohydrates. For these reasons, the neoglycoconjugates provide variations in average density rather than a precise, defined arrangement of oligosaccharides. Nevertheless, the ease of synthesis, variety of methods for attachment of oligosaccharides, and variety of methods to attach neoglycoproteins to surfaces make them an attractive platform for production of arrays. Importantly, neoglycoconjugates can often be used directly in other assays (ELISA/ELLA, Western blots), as immunogens, and as soluble inhibitors,^{38, 39} thereby facilitating the translation of array information and discoveries into useful multivalent reagents without having to identify a multivalent scaffold that mimics the spacing, density, and presentation on the array surface.

Preparation and evaluation of the carbohydrate arrays

For this study, we focused on recognition of the Tn antigen, a tumor-associated antigen composed of a GalNAc residue alpha linked to either threonine or serine of a polypeptide chain (Figure 2).⁴⁰ The Tn antigen has been studied extensively as a vaccine target,^{41, 42} and Tn-based vaccines have progressed into clinical trials for the treatment of breast and prostate cancer.^{43–45} Furthermore, serum antibody levels to the Tn antigen vary with the onset and progression of cancer.^{46–49} Tn-binding lectins and antibodies have been used at length to measure Tn expression, identify patients with Tn positive tumors for clinical trials, and target Tn positive cells. Even with the recognized potential of this antigen, progress in the development of Tn-based vaccines, detection of Tn binding antibodies, and analysis of Tn expression has been hampered, in part, by the fact that the antigen can be present in various forms. In addition to serine/threonine variants, the Tn antigen may be found as a single unit or as multiple Tn residues linked consecutively, referred to as a clustered Tn antigen (Figure 2). Antigen density is thought to play a key role for recognition of the Tn antigen, but it has been difficult to determine the optimal density and structure for vaccines or for probes used to detect serum antibodies because the density of the Tn antigen on tumor cells is not known.

To begin to address these issues, we constructed a microarray containing variations in both structure and density comprising a variety of Tn and other GalNAc terminal carbohydrate antigens as well as some galactose and fucose terminal structures for comparison. For this array, 11 glycans (Figure 2) were coupled to BSA at a variety of different densities to produce a set of 45 conjugates (see Supporting Information). Several forms of the Tn antigen were included along with a series of structurally-related and unrelated carbohydrates. Conjugation ratios were determined using MALDI-TOF MS and the average ratios are listed in Table 1 and Table S1 (in supplementary materials). The conjugates were then printed on an epoxide coated glass slide to produce an array of conjugates with varying densities. To obtain a uniform surface coating, we use a print solution of 125 $\mu\text{g/mL}$ which provides an excess of protein relative to the surface capacity, and we add 0.006% Triton-X 100 to facilitate even surface immobilization of proteins as recommended by Zhu et al.²³ Using these conditions and this method of varying density, we were able to obtain consistent signal intensities over the area of the spots for both low and high density conjugates when evaluating lectin and antibody binding. In addition, the

spots did not decrease in size at low density or low signal intensity (for an example, see Figure 3). The conjugation ratio did not appear to significantly affect the amount of protein immobilized on the surface, presumably due to the use of excess conjugate in the printing procedure (see Supporting Information). Although the exact spacing between carbohydrates on the surface is not known, a rough estimate can be calculated based on the surface area of BSA, approximately 30,000 Å². If the sugars are attached evenly on the surface, a conjugate with 4 sugars per BSA should have an approximate spacing of 85 Å between ligands while a conjugate with 20 sugars per BSA should have an approximate spacing of 40 Å.

As an initial test of the array format, we evaluated binding to a plant lectin, *Vicia villosa* B₄ (VVL-B₄). This lectin has been used for many years to detect expression of the Tn antigen in tumors samples and the specificity has been examined previously.^{50–56} Binding for VVL-B₄ has been shown to be highly density dependent and would thus provide a test case to determine whether we were successfully obtaining variations in density and if those variations were in a range where the binding properties of a protein could be affected. Our objective was to determine the effects of density; however, changes in density also result in differences in the total amount of sugar on each spot. The measured signal at any single lectin concentration can vary as a function of the amount of sugar. Since affinity constants are not dependent on the amount or concentration of carbohydrate, these values can provide a comparison of the effects of density. Therefore, it was necessary to evaluate binding at a series of lectin concentrations and measure apparent K_ds following the method of MacBeath (Table 1).⁵⁷ VVL-B₄ showed large density-dependent binding preferences for a number of carbohydrates. For example, the apparent K_d for the lowest density S-Tn(Ser)-S conjugate was greater than 1430 nM while the apparent K_d for the highest density form of S-Tn(Ser)-S was 26 nM. The observed variation in K_d implies that we were able to vary the density of the carbohydrate ligands within a biologically relevant range such that one could detect density-dependent binding of the lectins. Interestingly, density effects were dependent on the structure of the carbohydrate. For example, a much smaller density preference was observed for the closely related glycopeptide, S-Tn(Thr)-S.

Next, we examined density-dependent binding properties for two other plant lectins, *Helix pomatia* agglutinin (HPA) and soybean agglutinin (SBA). These lectins have also been used extensively to monitor carbohydrate expression on tumors (for some examples, see refs.^{58–61}) and HPA is known to block metastasis of breast cancer cells.^{62, 63} The specificities of these lectins have been examined previously,^{30, 31, 50, 53–55, 58, 64} but little is known about the effects of ligand density on selectivity. Apparent K_ds for these lectins are listed in Table 1. In contrast to VVL-B₄, HPA bound many of the low density conjugates with high avidity. For HPA, the apparent K_ds for the low and high density forms of Adi, Forssman, clustered Tn, S-Tn(Ser)-S, and S-Tn(Thr)-S were all in the range of 3–31 nM. Nevertheless, some larger density effects were observed for HPA binding to GA2di and GalNAc-α. Of the three lectins investigated, SBA showed the largest density-dependent effects. The apparent K_ds for SBA for the highest density Forssman antigen and Adi were each 280-fold lower than the low density forms of the same carbohydrates (>4200 vs 15 nM). Again, observed variations in K_d imply that we were able to vary the density of the carbohydrate ligands within a biologically relevant range.

Taken together, two general trends were observed for the lectins. First, the selectivity of a lectin for a particular carbohydrate over other carbohydrates generally deteriorated at high density. VVL-B₄ bound many different GalNAc containing sugars at high density but bound selectively to Tn containing conjugates at low density. In spite of this loss of selectivity among preferred ligands at high density, all the lectins retained specificity for carbohydrates containing a GalNAc residue at the non-reducing terminus over those lacking a terminal GalNAc such as Fuc-β, LNT, and GA1. Second, the selectivity of a conjugate for an individual lectin over other

lectins typically deteriorated at high density. For example, only HPA bound low density S-Tn (Ser)-S whereas all 3 lectins bound the high density variant. These trends were only detectable by evaluating many combinations of structure and density for multiple lectins, thus highlighting one advantage of the microarray format.

Density-dependent properties of monoclonal antibodies

We next evaluated the density-dependent binding properties of a set of monoclonal antibodies to the Tn antigen. Like the lectins, these reagents are used to measure Tn expression and choose appropriate patients for clinical trials with Tn-based vaccines, but little is known about the effects of density on recognition. We chose three monoclonal antibodies to the tumor-associated Tn antigen, B1.1, HBTn-1 and Bric111.^{65, 66} All three antibodies bind best to the clustered form of the Tn antigen (structures containing 2 or more Tn residues linked consecutively on a polypeptide chain; see Figure 2), so our evaluation of density dependence focused on recognition of this antigen.³² B1.1 exhibited a strong preference for the high-density form of the clustered Tn antigen with only minimal binding to the lowest density variant. The Kds for 6/BSA, 16/BSA, and 27 per BSA were 5.4, 3.7, and 3.0 nM respectively. The Kd for the lowest density conjugate, 3/BSA, could not be accurately determined since we were unable to reach a high enough concentration of antibody to saturate the signal; however, it was at least 10 fold higher. In contrast, antibody Bric111 bound low and high density forms of the clustered Tn antigen almost equally (Kds ranged from 113–198 nM). HBTn-1 had an intermediate dependence on antigen density (Kds ranged from 2.0 nM for high density to 6.7 nM for the lowest density). These results show that antibodies to the same carbohydrate antigen can recognize density in significantly different ways.

It is important to note that the density-dependent binding properties observed for the lectins and monoclonal antibodies should be taken into account when using these reagents. Our studies show that some antibodies and lectins, such as SBA, B1.1, and VVL-B₄, do not bind low density forms of the Tn antigen well. Therefore, an absence of binding observed with these proteins does not necessarily demonstrate an absence of the antigen, since low density forms of the antigen may be present. However, little is known about the density of the Tn antigen on tumor cells and proteins. The information on density preferences provided in this study should facilitate the use of these antibodies and lectins to study Tn density in biological systems, by, for example, comparing binding of lectins/antibodies that bind both low and high density Tn with lectins/antibodies that only bind high density Tn in an immunohistochemical experiment.

Density-dependent binding properties of serum antibodies

To evaluate the effects of density on serum antibody recognition of GalNAc-terminated carbohydrate antigens, we measured binding properties of serum antibodies from 30 healthy donors. Serum was diluted to a concentration of 1:50 and incubated on the array. Antibody binding was measured by incubating with Cy3-labeled goat antihuman Ig followed by fluorescence detection with a microarray scanner. Good signals were obtained for many of the antigens on the array for all individuals, and the profiles are shown in Figure 4 (see also Supporting information).

Variations in antigen density revealed significant differences in serum antibodies across the 30 subjects in our study. To visualize differences between individuals, the ratio of the signals for a high density conjugate to the signals for a lower density conjugate was graphed (see Figure 5). Serum antibodies from some individuals bound both high and low density forms of a given antigen while antibodies from other individuals only bound the high density form. For example, antibodies from subject 6 bound both high and low density forms of S-Tn(Thr)-S nearly equally, resulting in a ratio of 1.4. In contrast, antibodies in subject 29 bound much better to S-Tn(Thr)-S – 24 than to S-Tn(Thr)-S –04, resulting in a ratio of about 200 (Figure 5a).

Similarly, subjects 6 and 22 had comparable signals to both densities of Tn3, while subject 23 had almost 100-fold higher signal for Tn3 – 27 over Tn3 – 03 (Figure 5b). Large variations were also observed for Fuc- β (Figure 5c) indicating that density-dependent differences between individuals were not restricted to the Tn antigen.

It is important to note that binding preferences could not be readily extrapolated from evaluating binding at only one density. For example, subjects 16 and 29 had similar signals to a high density form of S-Tn(Ser)-S but a 6-fold difference in binding to a low density form of the antigen (Figure 6a). Similarly, subjects 1 and 2 had nearly equal signals to a high density form of clustered Tn but almost 10-fold difference in binding to a low density form of Tn3 (Figure 6b). This trend also holds true in the opposite direction. Subjects 8 and 12 had similar levels of antibody binding to low density S-Tn(Ser)-S but a 4-fold difference in signal denoting binding to high density S-Tn(Ser)-S (Figure 6c). For Adi, subjects 5 and 10 had almost equal signal to the low density conjugate but a 3-fold difference in signal at high density (Figure 6d). These differences between individuals could only have been detected using multiple densities of each antigen.

The results above show that modulation of antigen density can be used to reveal differences in antibody populations between individuals. The ability of an individual's serum antibodies to recognize both high and low density forms of an antigen could be due to several factors. One possibility is that they have a single population of antibodies that bind independently of density. A second possibility is that they have some antibodies that bind high density and others that bind low density; the combination in serum produces a signal to both densities. If this were the case, assays that use only a single antigen density might not detect the full spectrum of antibodies to that antigen. IgG and IgM antibodies have different architectures and valencies. We therefore hypothesized that the immune system might use different antibody isotypes to target different densities of the same antigen. To test this possibility, we evaluated IgG and IgM binding separately for a subset of 6 individuals (see Figure 7 and Supporting Information).

There were certain cases where IgG and IgM antibodies showed distinct density preferences within an individual. For example, the signals to the low density clustered Tn conjugate for subjects 6 and 22 were primarily due to IgG binding while the signals for the corresponding higher density conjugates were primarily the result of IgM binding (see Figure 8). Thus, at least in some cases, there are distinct subpopulations of antibodies that bind different densities of antigen. Again, it was necessary to use multiple densities to detect the full spectrum of antibodies.

Conclusions

In this study, we show that modulation of antigen density can be used to distinguish between different antibodies and lectins that bind the same antigen. In addition, we show that arrays with varying antigen density can be used to reveal distinct subpopulations of serum antibodies. The strategy used for varying antigen density should be useful for other objectives as well. First, information on the specificities of carbohydrate-binding lectins and antibodies is vital for identifying a protein's natural ligand(s), designing probes/inhibitors, and interpreting biological results and biochemical assays. Both structure and density contribute significantly to affinity and selectivity; however, the relationships between carbohydrate structure, density, and recognition are highly complex, unpredictable, and must be determined empirically. The array format described in this study provides a rapid and convenient tool to examine the many permutations while using only minimal amounts of each carbohydrate. It is especially well-suited for identification of probes/inhibitors since neoglycoproteins can be used as multivalent reagents and immunogens without having to identify a multivalent scaffold that mimics the spacing and presentation of ligands on the array surface. Second, there is substantial interest

in identifying new carbohydrate-binding proteins from the proteome. Since binding is highly dependent on both structure and density and the appropriate combination cannot be predicted, arrays with a diverse collection of carbohydrates and densities are more likely to possess a suitable combination to bind an unknown lectin. Therefore, inclusion of antigen density as an element of diversity on antigen arrays should be useful for functional proteomics as well as serum antibody profiling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Casiano CA, Mediavilla-Varela M, Tan EM. Tumor-associated antigen arrays for the serological diagnosis of cancer. *Mol. Cell. Proteomics* 2006;5(10):1745–1759. [PubMed: 16733262]
2. Robinson WH. Antigen arrays for antibody profiling. *Curr. Opin. Chem. Biol* 2006;10(1):67–72. [PubMed: 16406767]
3. Mammen M, Choi SK, Whitesides GM. Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Ed. Engl* 1998;37(20):2755.
4. Lundquist JJ, Toone EJ. The cluster glycoside effect. *Chem. Rev* 2002;102(2):555. [PubMed: 11841254]
5. Brewer CF, Miceli MC, Baum LG. Clusters, bundles, arrays and lattices: Novel mechanisms for lectin-saccharide-mediated cellular interactions. *Curr. Opin. Struct. Biol* 2002;12(5):616–623. [PubMed: 12464313]
6. Lee RT, Lee YC. Affinity enhancement by multivalent lectin-Carbohydrate interaction. *Glycoconj. J* 2000;17(7–9):543–551. [PubMed: 11421347]
7. Mortel KH, Weatherman RV, Kiessling LL. Recognition Specificity of Neoglycopolymers Prepared by Ring-Opening Metathesis Polymerization. *J. Am. Chem. Soc* 1996;118:2297.
8. Gestwicki JE, Cairo CW, Strong LE, Oetjen KA, Kiessling LL. Influencing Receptor-Ligand Binding Mechanisms with Multivalent Ligand Architecture. *J. Am. Chem. Soc* 2002;124:14922–14933. [PubMed: 12475334]
9. Smith EA, Thomas WD, Kiessling LL, Corn RM. Surface Plasmon Resonance Imaging Studies of Protein-Carbohydrate Interactions. *J. Am. Chem. Soc* 2003;125(20):6140–6148. [PubMed: 12785845]
10. Wolfenden ML, Cloninger MJ. Mannose/glucose-functionalized dendrimers to investigate the predictable tunability of multivalent interactions. *J. Am. Chem. Soc* 2005;127(35):12168. [PubMed: 16131163]
11. Roseman DS, Baenziger JU. The Mannose/N-Acetylglactosamine-4-SO₄ Receptor Displays Greater Specificity for Multivalent than Monovalent Ligands. *J. Biol. Chem* 2001;276(20):17052. [PubMed: 11279168]
12. Adler P, Wood SJ, Lee YC, Lee RT, Petri WA, Schnaar RL. High Affinity Binding of the *Entamoeba histolytica* Lectin to Polyvalent N-Acetylglactosaminides. *J. Biol. Chem* 1995;270(10):5164–5171. [PubMed: 7890626]
13. Horan N, Yan L, Isobe H, Whitesides GM, Kahne D. Nonstatistical binding of a protein to clustered carbohydrates. *Proc. Natl. Acad. Sci. USA* 1999;96(21):11782. [PubMed: 10518527]
14. Liang R, Loebach J, Horan N, Ge M, Thompson C, Yan L, Kahne D. Polyvalent binding to carbohydrates immobilized on an insoluble resin. *Proc. Natl. Acad. Sci., USA* 1997;94(20):10554–10559. [PubMed: 9380673]

15. Liang R, Yan L, Loebach J, Ge M, Uozumi Y, Sekanina K, Horan N, Gildersleeve J, Thompson C, Smith A, Biswas K, Still WC, Kahne D. Parallel synthesis and screening of a solid phase carbohydrate library. *Science* 1996;274(5292):1520–1522. [PubMed: 8929411]
16. Lyng H, Badiie A, Svendsrud DH, Hovig E, Myklebost O, Stokke T. Profound influence of microarray scanner characteristics on gene expression ratios: Analysis and procedure for correction. *BMC Genomics* 2004;5. [PubMed: 14720308]
17. Liang P-H, Wu C-Y, Greenberg WA, Wong C-H. Glycan arrays: biological and medical applications. *Curr. Opin. Chem. Biol* 2008;12(1):86. [PubMed: 18258211]
18. Paulson JC, Blixt O, Collins BE. Sweet spots in functional glycomics. *Nat. Chem. Biol* 2006;2(5):238–248. [PubMed: 16619023]
19. Park S, Lee M-R, Shin I. Carbohydrate microarrays as powerful tools in studies of carbohydrate-mediated biological processes. *Chem. Comm* 2008;(37):4389–4399. [PubMed: 18802572]
20. Laurent N, Voglmeir J, Flitsch SL. Glycoarrays-tools for determining protein-carbohydrate interactions and glycoenzyme specificity. *Chem. Comm* 2008;(37):4400–4412. [PubMed: 18802573]
21. Horlacher T, Seeberger PH. Carbohydrate arrays as tools for research and diagnostics. *Chem. Soc. Rev* 2008;37(7):1414. [PubMed: 18568167]
22. Oyelaran O, Gildersleeve JC. Application of carbohydrate array technology to antigen discovery and vaccine development. *Expert Rev. Vaccines* 2007;6(6):957–969. [PubMed: 18377358]
23. Deng Y, Zhu XY, Kienlen T, Guo A. Transport at the air/water interface is the reason for rings in protein microarrays. *J. Am. Chem. Soc* 2006;128(9):2768–2769. [PubMed: 16506733]
24. Houseman B, Mrksich M. Carbohydrate Arrays for the Evaluation of Protein Binding and Enzymatic Modification. *Chem. Biol* 2002;9:443–454. [PubMed: 11983333]
25. Ngundi MM, Taitt CR, McMurphy SA, Kahne D, Ligler FS. Detection of bacterial toxins with monosaccharide arrays. *Biosens. Bioelectron* 2006;21(7):1195–1201. [PubMed: 15946840]
26. Chevotot Y, Bouillon C, Vidal S, Morvan F, Meyer A, Cloarec JP, Jochum A, Praly JP, Vasseur JJ, Souteyrand E. DNA-based carbohydrate biochips: A platform for surface glycoengineering. *Angew. Chem. Int. Ed* 2007;46(14):2398–2402.
27. Liang PH, Wang SK, Wong CH. Quantitative Analysis of Carbohydrate-Protein Interactions Using Glycan Microarrays: Determination of Surface and Solution Dissociation Constants. *J. Am. Chem. Soc* 2007;129(36):11177–11184. [PubMed: 17705486]
28. Mercey E, Sadir R, Maillart E, Roget A, Baleux F, Lortat-Jacob H, Livache T. Polypyrrole oligosaccharide array and surface plasmon resonance imaging for the measurement of glycosaminoglycan binding interactions. *Anal. Chem* 2008;80(9):3476. [PubMed: 18348577]
29. Song X, Xia B, Lasanajak Y, Smith DF, Cummings RD. Quantifiable fluorescent glycan microarrays. *Glycoconj. J* 2008;25(1):15. [PubMed: 17763939]
30. Manimala J, Li Z, Jain A, VedBrat S, Gildersleeve JC. Carbohydrate array analysis of anti-Tn antibodies and lectins reveals unexpected specificities: Implications for diagnostic and vaccine development. *ChemBioChem* 2005;6(12):2229–2241. [PubMed: 16252298]
31. Manimala JC, Roach TA, Li Z, Gildersleeve JC. High-throughput carbohydrate microarray analysis of 24 lectins. *Angew. Chem. Int. Ed. Engl* 2006;45:3607–3610. [PubMed: 16639753]
32. Manimala JC, Roach TA, Li Z, Gildersleeve JC. High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems. *Glycobiology* 2007;17(8):17C–23C.
33. Hsu KL, Gildersleeve JC, Mahal LK. A simple strategy for the creation of a recombinant lectin microarray. *Mol. Biosyst* 2008;4(6):654–662. [PubMed: 18493664]
34. Gildersleeve JC, Oyelaran O, Simpson JT, Allred B. Improved procedure for Direct Coupling of Carbohydrates to Proteins via Reductive Amination. *Bioconjugate Chem* 2008;19(7):1485–1490.
35. Li Q, Anver M, Butcher D, Gildersleeve J. Resolving Conflicting Data on Expression of the Tn Antigen and Implications for Clinical Trials with Cancer Vaccines. *Mol. Cancer. Res* 2009;8(4)in press
36. He XM, Carter DC. Atomic structure and chemistry of human serum albumin. *Nature* 1992;358(6383):209–215. [PubMed: 1630489]

37. Sugio S, Kashima A, Mochizuki S, Noda M, Kobayashi K. Crystal structure of human serum albumin at 2.5 Å... resolution. *Protein Engineering* 1999;12(6):439–446. [PubMed: 10388840]
38. Stowell CP, Lee VC. Neoglycoproteins: the preparation and application of synthetic glycoproteins. *Adv. Carbohydr. Chem. Biochem* 1980;37:225–281. [PubMed: 6996450]
39. Roy R. Syntheses and some applications of chemically defined multivalent glycoconjugates. *Curr. Opin. Struct. Biol* 1996;6(5):692–702. [PubMed: 8913693]
40. Dausset J, Moullec J, Bernard J. Acquired Hemolytic Anemia with Polyagglutinability of Red Blood Cells Due to a New Factor Present in Normal Human Serum (Anti-Tn). *Blood* 1959;14(10):1079–1093. [PubMed: 13814231]
41. Desai P. Immunoreactive T and Tn antigens in malignancy: Role in carcinoma diagnosis, prognosis, and immunotherapy. *Transfusion Med. Rev* 2000;14(4):312–325.
42. Springer GF. Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. *J. Mol. Med* 1997;75:594–602. [PubMed: 9297627]
43. Slovin SF, Ragupathi G, Fernandez C, Diani M, Jefferson MP, Wilton A, Kelly WK, Morris M, Solit D, Clausen H, Livingston P, Scher HI. A polyvalent vaccine for high-risk prostate patients: "Are more antigens better?". *Cancer Immunol. Immunother* 2007;56(12):1921–1930. [PubMed: 17619878]
44. Slovin SF, Ragupathi G, Musselli C, Olkiewicz K, Verbel D, Kuduk SD, Schwarz JB, Sames D, Danishefsky SJ, Livingston PO, Scher HI. Fully synthetic carbohydrate-based vaccines in biochemically relapsed prostate cancer: Clinical trial results with alpha-N-acetylgalactosamine-O-Serine/Threonine conjugate vaccine. *J. Clin. Oncol* 2003;21(23):4292–4298. [PubMed: 14645418]
45. Springer GF, Desai PR, Spencer BD, Tegtmeier H, Carlstedt SC, Scanlan EF. T/Tn antigen vaccine is effective and safe in preventing recurrence of advanced breast carcinoma. *Cancer Detect. Prev* 1995;19(4):374–380. [PubMed: 7553680]
46. Desai PR, Ujjainwala LH, Carlstedt SC, Springer GF. Antibodies to pancarcinoma Tn epitope: Their altered levels in breast carcinoma patients. *Onkologie* 1997;20(6):472.
47. Von Mensdorff-Pouilly S, Petrakou E, Kenemans P, Van Uffelen K, Verstraeten AA, Snijdwint FGM, Van Kamp GJ, Schol DJ, Reis CA, Price MR, Livingston PO, Hilgers J. Reactivity of natural and induced human antibodies to MUC1 mucin with MUC1 peptides and N-acetylgalactosamine (GalNAc) peptides. *Int. J. Cancer* 2000;86(5):702. [PubMed: 10797294]
48. Smorodin EP, Kurtenkov OA, Sergeyev BL, Lilleorg AL, Chuzmarov VI. Antibodies to tumor-associated carbohydrate epitopes in sera of cancer patients and blood donors. *Exp. Oncology* 2001;23(2):109.
49. Chen Y, Jain RK, Chanrasekaran EV, Matta KL. Use of sialylated or sulfated derivatives and acrylamide copolymers of Gal β 1,3GalNAc β - and GalNAc β - to determine the specificities of blood group T- and Tn-specific lectins and the copolymers to measure anti-T and anti-Tn antibody levels in cancer patients. *Glycoconj. J* 1995;12(1):55. [PubMed: 7795413]
50. Tollefsen S, Kornfeld R. The B4 Lectin from *Vicia Villosa* Seeds Interacts With N-AcetylGalactosamine Residues α -Linked to Serine or Threonine Residues in Cell Surface Glycoproteins. *J. Biol. Chem* 1983;258:5172–5176. [PubMed: 6403544]
51. Puri KD, Gopalakrishnan B, Surolia A. Carbohydrate Binding Specificity of the Tn-Antigen Binding Lectin from *Vicia Villosa* Seeds (VVLB4). *FEBS Lett* 1992;312:208–212. [PubMed: 1426254]
52. Medeiros A, Bianchi S, Calvete JJ, Balter H, Bay S, Robles A, Cantacuzăne D, Nimtz M, Alzari PM, Osinaga E. Biochemical and functional characterization of the Tn-specific lectin from *Salvia sclarea* seeds. *Eur. J. Biochem* 2000;267(5):1434–1440. [PubMed: 10691981]
53. Osinaga E, Bay S, Tello D, Babino A, Pritsch O, Assemet K, Cantacuzene D, Nakada H, Alzari P. Analysis of the Fine Specificity of Tn-binding Proteins Using Synthetic Glycopeptide Epitopes and a Biosensor Based on Surface Plasmon Resonance Spectroscopy. *FEBS Lett* 2000;469:24–28. [PubMed: 10708749]
54. Babino A, Tello D, Rojas A, Bay S, Osinaga E, Alzari PM. The crystal structure of a plant lectin in complex with the Tn antigen. *FEBS Lett* 2003;536(1–3):106–110. [PubMed: 12586347]
55. Wu AM. Polyvalency of Tn (GalNAc α -Ser/Thr) Glycotope as a Critical Factor for *Vicia Villosa* B4 and Glycoprotein Interactions. *FEBS Lett* 2004;562:51–58. [PubMed: 15044001]

56. Kato K, Takeuchi H, Ohki T, Waki M, Usami K, Hassan H, Clausen H, Irimura T. A lectin recognizes differential arrangements of O-glycans on mucin repeats. *Biochem. Biophys. Res. Comm* 2008;371(4):698–701. [PubMed: 18455506]
57. Gordus A, MacBeath G. Circumventing the problems caused by protein diversity in microarrays: Implications for protein interaction networks. *J. Am. Chem. Soc* 2006;128(42):13668–13669. [PubMed: 17044677]
58. Lescar J, Sanchez JF, Audfray A, Coll JL, Breton C, Mitchell EP, Imberty A. Structural basis for recognition of breast and colon cancer epitopes Tn antigen and Forssman disaccharide by Helix pomatia lectin. *Glycobiology* 2007;17(10):1077. [PubMed: 17652409]
59. Reese MR, Chow DA. Tumor progression in vivo: Increased soybean agglutinin lectin binding, N-acetylgalactosamine-specific lectin expression, and liver metastasis potential. *Cancer Res* 1992;52(19):5235–5243. [PubMed: 1394127]
60. Konska G, Guillot J, de Latour M, Fonck Y. Expression of Tn antigen and N-acetylglucosamine residues in malignant and benign human breast tumors detected by lectins and monoclonal antibody 83D4. *Int. J. Oncol* 1998;12(2):361–367. [PubMed: 9458363]
61. Konska G, Guerry M, Caldefie-Chez F, De Latour M, Guillot J. Study of the expression of Tn antigen in different types of human breast cancer cells using VVA-B4 lectin. *Oncology reports* 2006;15(2):305. [PubMed: 16391846]
62. Valentiner U, Hall DMS, Brooks SA, Schumacher U. HPA binding and metastasis formation of human breast cancer cell lines transplanted into severe combined immunodeficient (scid) mice. *Cancer Lett* 2005;219(2):233–242. [PubMed: 15723724]
63. Brooks SA, Leatham AJC. Expression of N-acetyl galactosaminylated and sialylated glycans by metastases arising from primary breast cancer. *Invas. Metast* 1999;18(3):115–121.
64. Olsen LR, Dessen A, Gupta D, Sabesan S, Sacchettini JC, Brewer CF. X-ray crystallographic studies of unique cross-linked lattices between four isomeric biantennary oligosaccharides and soybean agglutinin. *Biochemistry* 1997;36(49):15073. [PubMed: 9398234]
65. The production of B1.1 and HBTn1 have not been published.
66. King M, Parsons S, Wu A, Jones N. Immunochemical studies on the differential binding properties of two monoclonal antibodies reacting with Tn red cells. *Transfusion* 1991;31(2):142–149. [PubMed: 1847560]

Figure 1.

Neoglycoproteins with variations in carbohydrate structure and density are printed on an epoxide-coated glass microscope slide. Binding of lectins (or antibodies) to each of the neoglycoproteins is evaluated in parallel to determine the effects of structure and density on recognition.

Figure 2.
Carbohydrates chosen for the study.

Figure 3.

Comparison of spot intensities and sizes for low (3/BSA) and high (27/BSA) density Tn3. The array was incubated with human serum at a dilution of 1:50 followed by Cy3-labeled goat anti-human Ig, then imaged using a GenePix scanner. A full array image can be found in the supporting information.

Figure 4.

Profiles for 30 healthy individuals. Glycoconjugates are indicated in the rows and subjects are indicated in the columns. The number after the array component name refers to the average number of haptens per molecule of BSA (e.g. “Adi – 04” refers to the conjugate having an average of 4 Adi molecules per BSA). Fluorescence intensities are background-corrected and transformed to log base 2 to compress the scale. Each rectangle represents the mean of two measurements (4 spots). The color of the rectangle corresponds to values on the color scale to the right of the heat map.

Figure 5.

Binding properties of serum antibodies from 30 normal subjects; bars represent the ratio of the signal for a high density conjugate to the signal for a lower density conjugate for each individual. Graphs (a), (b), and (c), density-dependent binding preferences for S-Tn(Thr)-S, Tn3, and Fuc- β , respectively. Error bars represent the standard error of the ratio of the signal for high density conjugate to the signal for the lower density conjugate.

Figure 6. Binding properties of serum antibodies to antigens of different densities; bars represent background subtracted fluorescence values. Panels (a) S-Tn(Ser)-S and (b) Tn3, different individuals can have similar signals to high density conjugates but different levels to the corresponding low density conjugate. Panels (c) S-Tn(Ser)-S and (d) Adi, different individuals can also have similar signals to low density conjugates but different levels to the corresponding high density conjugate. Error bars represent standard deviation of 4 spots.

Figure 7.

IgG and IgM profiles for 6 healthy individuals. Glycoconjugates are indicated in the rows and subjects are indicated in the columns. The number after the array component name refers to the average number of haptens per molecule of BSA (e.g. "Adi - 04" refers to the conjugate having an average of 4 Adi molecules per BSA). Fluorescence intensities are background-corrected and transformed to log base 2. Each rectangle represents the mean of two measurements (four spots). The color of the rectangle corresponds to values on the color scale to the right of the heat map.

Figure 8. Comparison of IgG and IgM signals. Signals to clustered Tn (Tn3) at different conjugation ratios for subject 6 (a) and 22 (b). Error bars represent standard deviation of 4 spots.

Table 1Summary of Apparent Kds (mean $\pm$ SD, nM) for the Lectins

Carbohydrate	#BSA	VVL-B4 ^a	HPA ^a	SBA ^a
Adi	4	>1430	~20	>4200
	8	>1430	7 $\pm$ 1	128 $\pm$ 13
	17	207 $\pm$ 29	5 $\pm$ 0.4	35 $\pm$ 5
	33	42 $\pm$ 7	3 $\pm$ 0.3	15 $\pm$ 12
GalNAc α 1-6Gal	4	>1430	~50	>4200
	15	56 $\pm$ 5	4 $\pm$ 0.5	43 $\pm$ 7
	21	42 $\pm$ 4	4 $\pm$ 0.4	34 $\pm$ 4
Forssman Di	4	>1430	6 $\pm$ 0.9	>4200
	8	>1430	4 $\pm$ 0.4	171 $\pm$ 32
	15	>1430	4 $\pm$ 0.3	41 $\pm$ 8
	21	>1430	5 $\pm$ 0.3	25 $\pm$ 6
	31	>1430	5 $\pm$ 0.4	15 $\pm$ 4
GA2di	4	>1430	>1270	>4200
	7	>1430	~500	>4200
	16	149 $\pm$ 20	~318	451 $\pm$ 11
	22	90 $\pm$ 18	105 $\pm$ 12	257 $\pm$ 114
	37	41 $\pm$ 5	63 $\pm$ 8	93 $\pm$ 11
GalNAc- α	4	>1430	>1270	>4200
	9	>1430	132 $\pm$ 12	194 $\pm$ 24
	13	129 $\pm$ 11	42 $\pm$ 8	74 $\pm$ 10
	22	62 $\pm$ 6	9 $\pm$ 2	38 $\pm$ 4
	37	35 $\pm$ 4	5 $\pm$ 0.5	19 $\pm$ 2
Clustered Tn	3	110 $\pm$ 13	8 $\pm$ 2	~200
	6	57 $\pm$ 6	4 $\pm$ 0.4	60 $\pm$ 8
	16	26 $\pm$ 3	4 $\pm$ 0.4	30 $\pm$ 5
	27	16 $\pm$ 1	4 $\pm$ 0.4	18 $\pm$ 2
S-Tn(Ser)-S	4	>1430	20 $\pm$ 4	>4200
	7	149 $\pm$ 19	8 $\pm$ 1	~200
	11	93 $\pm$ 10	6 $\pm$ 0.5	99 $\pm$ 16
	22	37 $\pm$ 4	5 $\pm$ 0.4	32 $\pm$ 3
	33	26 $\pm$ 3	4 $\pm$ 0.3	22 $\pm$ 4
S-Tn(Thr)-S	4	129 $\pm$ 13	31 $\pm$ 8	>4200
	7	66 $\pm$ 7	8 $\pm$ 2	257 $\pm$ 19
	13	38 $\pm$ 4	6 $\pm$ 0.7	95 $\pm$ 7
	24	19 $\pm$ 2	5 $\pm$ 0.5	51 $\pm$ 6

^aNo binding was observed to LNT, GA1, or Fuc- β .