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GLYCOARRAY SECTION

Profiling of serum antibodies with printed glycan array: room for data misinterpretation

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Abstract Using an example of Galβ1-3GlcNAc (Le^C) related glycans, we here demonstrate a risk of data misinterpretation when polyclonal antibodies are probed for their glycan-binding specificities with help of a printed glycan array (PGA). Affinity isolation of antibodies from human serum using Le^C-Sepharose or 3′-O-SuLe^C-Sepharose in conditions of excess of the adsorbents generated identical material regardless of the affinity ligand, with the antibodies equally capable of binding to Le^C and to 3′-O-SuLe^C disaccharides, as well as to 3′-O-SiaLe^C trisaccharide. More detailed profiling has shown that the isolated antibodies bind to the inner part of Galβ1-3GlcNAc disaccharide. We therefore conclude that serum does not contain different subsets of antibodies specific either to Le^C or to 3′-O-SuLe^C, despite their visibly different binding signals to these glycans on PGA.

Keywords PGA · ELISA · Glycans · Human antibodies · Le^C

Abbreviations

Abs	Antibodies
ELISA	Enzyme-linked immunosorbent assay
GBP	Glycan binding protein
PGA	Printed glycan array
PAA	Polyacrylamide
Glyc	Glycan

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Introduction

Probing of individual glycan-binding proteins such as lectins and certain monoclonal antibodies for their glycan-binding specificity using printed glycan array, results in clear and rather unambiguous profile of the given protein-glycan binding specificity. However, when serum is probed for the presence of anti-glycan auto-antibodies, the interpretation of the profile of bound autoantibodies is not so univocal due to the enormous complexity of the serum composition. For instance, when we observe an antibody-binding signal at Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc (Le^BLac) spot on the PGA, does it mean that the serum actually contains antibodies against Le^BLac? Actually, Abs binding only to an inner Galβ1-4Glc (Lac) fragment could generate a binding signal as "true" antibodies recognizing the entire Le^BLac hexasaccharide [1]. In order to avoid such "false-positive" interpretation, the following methodology can be applied: affinity isolation of serum Abs binding to the glycan (such as Le^BLac in [2, 3]) in conditions of exhausting adsorption by an excess of the adsorbent, followed by elution of affinity-bound Abs, and finally ELISA- or PGA-based analysis of the eluted material [2, 3]. Obviously, this analytical approach cannot be easily applied to all species of anti-glycan antibodies such as at least 100 found in healthy humans [1]. However, such approach is necessary in cases of natural Abs of expected specific biological activities. Here, we discuss potential pitfalls in interpretation of antibody-binding signals to arrayed glycans, using

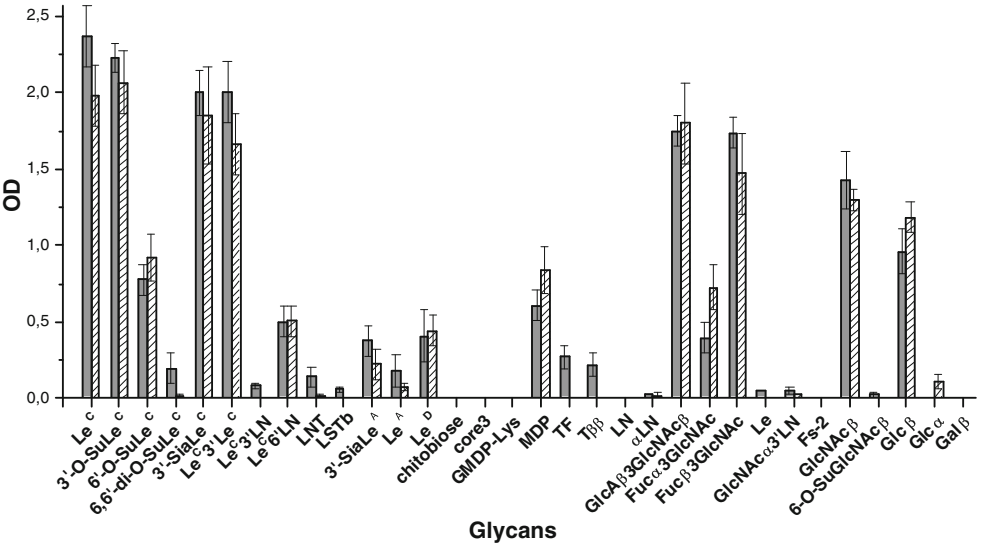

Fig. 1 Comparison of Abs isolated from pools of healthy donor's sera using adsorbent Le^C-Sepharose (*gray bars*) and adsorbent 3′-O-$SuLe^C$-Sepharose (*striped bars*), ELISA. Loading concentration of Ig was 1 μg/ml. Glycans (structures see in Table 1) were used as polyacrylamide (PAA) glycoconjugates. The background (binding to glycan-free PAA) is subtracted. *Error bars* are standard deviations of replicates

as an example the results of our study of specificity of human Abs, which show one of the highest binding signals to 3′-O-$SuLe^C$ spot on PGA.

Materials and methods

Sera and fresh red blood cells from healthy adult donors with blood groups 0, A, B and AB, were obtained from Sklifosofsky Research Institute of Emergency Care (Moscow, Russia). Goat anti-human Ig (IgM+IgG+IgA) antibodies conjugated with horseradish peroxidase were purchased from Southern Biotechnology Associates, Inc. (USA). Goat anti-human Ig (IgM+IgG+IgA) antibodies conjugated with biotin were from Pierce (USA); streptavidin-Alexa555 was from Invitrogen (USA). Tween-20 and bovine serum albumin were from Sigma (USA). All other chemicals were of analytical grade and were purchased from Fluka (Switzerland) or Merck (Germany). MaxiSorp 96-well microtiter immunoplates were from Nunc (Denmark). Glycan-polyacrylamide conjugates Glyc-PAA [4] (~30 kDa, 20 mol% of glycan), and affinity adsorbents [4] were obtained from Lectinity (Moscow, Russia). Milk oligosaccharides were obtained from Glycoseparations (Moscow, Russia). Purity of glycans used in inhibition assay, as well as glycans used for conjugation was 95–97% according to NMR and HPLC data. ELISA was performed as described [1]. All the tests were performed at least in duplicate; the differences did not exceed 5%. Printed glycan array was fabricated at Cellexicon, Inc. (CA, USA) or at the University of Copenhagen (Denmark) as described [1, 5]. Amersham (UK) spectrophotometer Ultrospec 3100 pro was used.

Purification of human anti-glycan antibodies Pooled serum (25 ml) was incubated for 30 min at 56°C, and centrifuged at 4°C (13,000 g; J2-21 centrifuge, Beckman (USA)). The supernatant was diluted 1:1 with phosphate buffered saline (PBS, 0.15 M, pH 7.4) containing 0.02% NaN_3 and applied on the column with Glyc-PAA-Sepharose 6FF (2 ml), equilibrated with PBS/0.02% NaN_3, at 20–22°C at flow rate 0.3 ml/min. The column was extensively washed with PBS/0.02% NaN_3, then PBS/0.02% NaN_3/0.5% Tween-20, until no absorbance at 280 nm of the effluent was detected, and finally with PBS/0.02% NaN_3 in 10–15 vol of column. Antibodies were eluted [1] with Tris-OH (0.2 M, pH 10.4) containing 0.02% NaN_3, flow rate of eluting buffer 60 to 90 μl/min. Fractions were neutralized immediately using Gly-HCl buffer (2 M, pH 2.4). Column with carbohydrate-free PAA-Sepharose 6FF alone was used for removal of nonspecific antibodies. The antibody concentration was measured at 280 nm. Antibodies were stored at 4°C for 1 week or kept frozen at −20°C.

Results and discussion

Glycans of Le^C group, *i.e.* the disaccharide Galβ1-3GlcNAc, its sialylated Neu5Acα2-3Galβ1-3GlcNAc (3′-$SiaLe^C$), and particularly its sulfated 3-O-Su-Galβ1-3GlcNAc (3′-O-$SuLe^C$) derivatives demonstrate very high signals[1] of serum antibodies binding to them on PGA; this is true also for intravenous immunoglobulins, which contain predominantly immunoglobulins G [IVIG; 6]. These antibodies were found in each of more than 200 tested donors, indicating their physiological significance. Statistical analysis revealed high correlation of antibody-binding signals between all three mentioned glycans in all individuals within a large group of over 100 donors [not shown].

[1] High binding signal could be caused by either elevated content or high affinity of the Abs, or both.

Table 1 Glycans used for epitope profiling of affinity isolated antibodies

Glycan	Used abbreviation
Galβ1-3GlcNAcβ-	Le^C
3′-O-Su-**Galβ1-3GlcNAcβ-**	3′-O-$SuLe^C$
6-O-Su-**Galβ1-3GlcNAcβ-**	6′-O-$SuLe^C$
6-O-Su-**Galβ1-3**(6-O-Su)**GlcNAcβ-**	6,6′-di-O-$SuLe^C$
Neu5Acα2-3**Galβ1-3GlcNAcβ-**	3′-$SiaLe^C$
Galβ1-3GlcNAcβ1-3**Galβ1-3GlcNAcβ-**	$Le^C3'Le^C$
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-	$Le^C3'LN$
Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-	$Le^C6'LN$
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-NHGly-	LNT
Neu5Acα2-6 (**Galβ1-3)GlcNAcβ**1-3Galβ1-4Glcβ-NHGly-	LSTb
Neu5Acα2-3**Galβ1-3**(Fucα1-4)**GlcNAcβ-**	3′-$SiaLe^A$
Fucα1-4(**Galβ1-3)GlcNAcβ-**	Le^A
Fucα1-2**Galβ1-3GlcNAcβ-**	Le^D
GlcNAcβ1-4GlcNAcβ-	chitobiose
GlcNAcβ1-3GalNAcα-	core 3
GlcNAcβ1-4Mur-L-Ala-D-i-Gln-Lys	GMDP-Lys
(Mur-L-Ala-D-I-Gln)β-O$(CH_2)_4$NH-	MDP
Galβ1-3GalNAcα	TF
Galβ1-3GalNAcβ-	$T_{ββ}$
Galβ1-4GlcNAcβ-	LN
Galα1-4GlcNAcβ-	αLN
GlcAβ1-3GlcNAcβ-	GlcAβ1-3GlcNAc
Fucα1-3GlcNAcβ-	Fucα1−3GlcNAc
Fucβ1-3GlcNAcβ-	Fucβ1−3GlcNAc
Fucα1-4GlcNAcβ-	Le
GlcNAcα1-3Galβ1-4GlcNAcβ-	GlcNAcα1-3′LN
GalNAcα1-3GalNAcβ-	Fs-2
GlcNAcβ-	GlcNAcβ
6-O-SuGlcNAcβ-	6-O-SuGlcNAcβ
Glcβ-	Glcβ
Glcα-	Glcα
Galβ-	Galβ
GlcNAcβ-O-Ser	GlcNAc-Ser[a]
Galβ1-4GlcNAcβ1-6(**Galβ1-3GlcNAc**β1-3)Galβ1-4Glc	LNH[a]
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6 (**Galβ1-3GlcNAc**β1-3)Galβ1-4Glc	iLNO[a]
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6 (**Galβ1-3GlcNAc**β1-3)Galβ1-4Glc	MF(1–3)iLNO[a]

Le^C disaccharide fragment is represented in bold

[a] In inhibitory ELISA only, as free glycans

Affinity chromatography on adsorbents Le^C or 3′-O-$SuLe^C$ was performed using several samples of pooled sera differing by the number of individual donors in a given sample; the same experiments were carried out using sera of individual donors. In all cases the specificity profiles of isolated antibodies were practically identical. Moreover, as it can be seen from ELISA (Fig. 1[2]), Abs isolated on the adsorbent 3′-O-$SuLe^C$ were similar to those isolated on the adsorbent Le^C. Fine binding specificities of the obtained antibodies are discussed below in terms of contributions of various sites within the given glycan molecules to their interaction with antibodies.

GlcNAcβ site Hydroxyl groups at C6 and C4 are essential for antibody binding; their replacement completely abolishes the interaction with antibodies. A structurally-important consequence of these hydroxyl groups is the lack of antibody recognition of trisaccharide Le^A and its more complex derivatives. Hydroxyl at C4 must be in equatorial position—since $T_{ββ}$ disaccharide displays practically no antibody interaction; the effect of acetamide group at C2 is negligible as binding to glucose does not differ significantly from binding to *N*-acetylgalactosamine. Hydroxyl at C3 can be substituted by a monosaccharide residue; according to inactivity of α-anomers axial H-C1 site is important for binding.

Galβ site The input of this site is not as essential as that of GlcNAc—as demonstrated by the lack of binding to monosaccharide Gal; however—the binding signal improves in the presence of the second (Galβ) monosaccharide residue. Substitution of Gal residue at O3 does not abolish binding; substitution by O6 produces varied effect: on one hand the substitution $–CH_2OH \rightarrow –COOH$ is allowed but on the other hand sulfate in this position decreases the binding considerably; substitution by O2 leads to nearly complete loss of binding (see Le^D), *i.e.* equatorial OH (or axial hydrogen) at C2 is essential for interaction.

Aglycon site The most intriguing aspect of epitope specificity of the antibodies studied here is that the motif Galβ1-3GlcNAc can be recognized by them only at the reducing end of a more complex glycan. This is proven by complete

Table 2 Glycans used for epitope profiling of affinity isolated antibodies in inhibitory assay

Glycan	IC_{50}, mM
Galβ1-3GlcNAcβ-$OCH_2CH_2CH_2NH_2$	0.12
Galβ1-3GlcNAcβ-$OCH_2CH_2NH_2$	0.23
GlcNAcβ-$OCH_2CH_2CH_2NH_2$	0.72
GlcNAcβ-$OCH_2CH_2NH_2$	0.91
GlcNAcβ-O-Ser	2.20
Galβ1-3GlcNAcα-$OCH_2C_6H_5$	NI
Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3) Galβ1-4Glc	NI
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6 (Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	NI
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3) GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	NI

[2] PGA results (not shown) are analogous for the same glycans

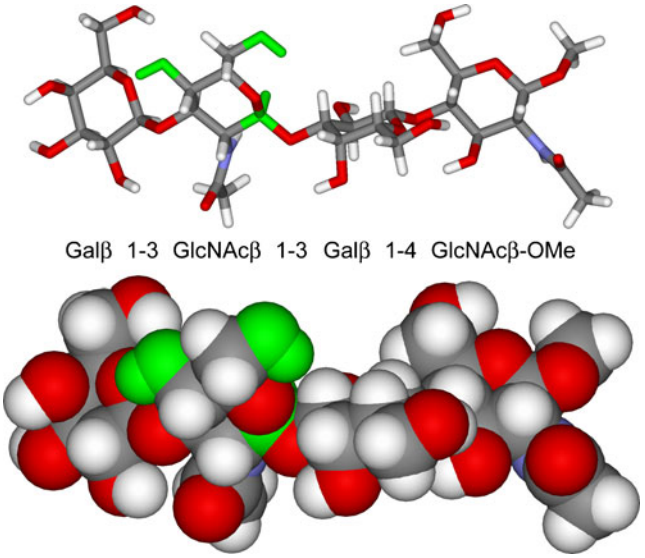

Fig. 2 Tetrasaccharide Le^C-3′LN (as a methyl glycoside), H-C1 site and hydroxyl groups at C4 and C6 of GlcNAc (in composition of Le^C) are highlighted in *green*. Structure sized to the van der Waals radii of atoms (bottom) shows that *N*-acetyllactosamine fragment causes considerable hindrance for Le^C to Abs binding; indeed, H-C1 site of *N*-acetylglucosamine (in composition of Le^C) is crypted entirely, and hydroxyl group at C6 is shielded. The tetrasaccharide conformation was calculated and downloaded using Glycam Biomolecule Builder (http://glycam.ccrc.uga.edu/, Complex Carbohydrate Research Center, research group of Dr. Robert J. Woods). The hindrance is more obvious when a computer viewer is applied (the structure can be downloaded from http://www.carbohydrate.ru/LeC-LN.zip (in msv and c3d formats))

lack of interaction with tetrasaccharides of common structure Le^C-Lac(NAc) and more complicated milk oligosaccharides (Table 1) in inhibitory ELISA (Table 2). Moreover, only weak interaction with complex-type glycoprotein N-chains where Le^C is present as mannose-containing fragment Galβ1-3GlcNAcβ1-2Man has been demonstrated in [6]. Oxygen atom of aglycon is obviously important because GlcNAcβ-NH- loses the activity completely, whereas GlcNAcβ-O- preserves it. Possibly the following methylene unit affects the binding because tetrasaccharide Le^{C}β1-6′LN is the only one in Le^C-Lac(NAc) series displaying any affinity to antibodies. Analysis of tetrasaccharide Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβOMe (Le^C-3′LacNAc, Fig. 2) conformation demonstrates that the LacNAc fragment masks the site of GlcNAc moiety recognized by antibodies. In summary, based on the epitope specificity mapping, the following three conclusions can be made in context of the question addressed in Introduction.

Two subpopulations or double specificity? As mentioned above, the isolated antibodies can bind both the disaccharide motif and its fragment GlcNAc; this can be explained in two ways: first, by the presence of two populations of antibodies, one of which possesses specificity to GlcNAc; second, by broad specificity of single Abs species inseparable into two sub-fractions. From the point of view of the posed problem it is important to select between these two variants, so we have carried out an additional experiment where primary isolated material was subjected to a second chromatography procedure on GlcNAc adsorbent. The result (Fig. 3) favors the first version, *i.e.* primary antibodies really contain a fraction capable of recognizing monosaccharide fragment GlcNAc in composition of the Le^C disaccharide. The presence of these Abs does not impact the interpretation of PGA data of serum antibody-binding, because a major fraction of antibodies recognizes the entire disaccharide.

Are there specific antibodies to a sulfated form, 3′-O-SuLe^C? In this context we call *specific* only the Abs to the sulfated variant presuming that they recognize sulfate. In reality, serum does not contain such antibodies, with this conclusion derived from the fact of complete identity of profiles for the Abs isolated using 3′-O-SuLe^C and non-substituted disaccharide Galβ1-3GlcNAc adsorbents (Fig. 1). The same is true for anti-SiaLe^C, since we did not find in

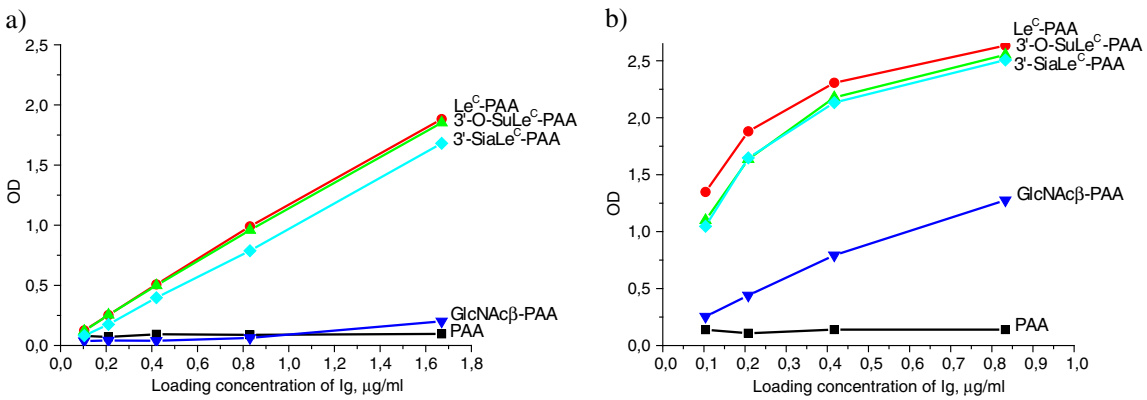

Fig. 3 Isolation of monospecific antibodies, ELISA data. Abs isolated on Le^C-Sepharose were loaded on GlcNAc-Sepharose. **a** Flow-through (second column) monospecific Abs, no GlcNAc-binding fraction; **b** GlcNAc-Sepharose-bound antibodies (eluted material) capable of binding both GlcNAcβ and Le^c disaccharide

human serum antibodies discriminating between sialylated and asialo forms of Le^C.

Are there antibodies to Le^C? Finally, we make a paradoxical conclusion about lack of true antibodies to Le^C *i.e.* immunoglobulins specific to the naturally occurring Le^C epitope. Indeed, it is known that disaccharide fragment Le^C can be present only in composition of longer glycans (glycoproteins, glycolipids, milk oligosaccharides). No variants with disaccharide Galβ1-3GlcNAc attached directly to a protein or lipid have been found in humans. At the same time we have observed no binding of the isolated Abs to tetrasaccharides of type Galβ1-3GlcNAcβ1-XGalβ1-4Glc(NAc) and longer glycans (Table 2), what suggests that there are no interactions of antibodies with natural carbohydrate chains of glycoproteins or glycolipids in undistorted conformation. Thus, Abs isolated on the column with adsorbent Le^C, can be considered as antibodies binding to Le^C in experimental conditions, while their real, natural entire epitope remains unknown. We also believe that there is more room for misinterpretation of antibody binding signals when profiling sera using PGA, for instance, mode of spots integration, pooling of sera samples, influence of other (varying) blood components, spacer arm in glycan composition, *etc*. We will discuss these factors in the following communications.

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