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## Anti-carbohydrate antibodies of normal sera: Findings, surprises and challenges

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### ABSTRACT

We have used microchip format glycan array to characterize the individual carbohydrate recognition patterns by antibodies (Ab) in sera of 106 healthy donors. The glycan library included blood group antigens and other most frequent terminal oligosaccharides and their cores of mammalian N- and O-linked glycoproteins and glycolipids, tumor-associated carbohydrate antigens, and common components of bacterial/pathogenic polysaccharides and lipopolysaccharides, totally 205 glycans. The serum Ab interacted with at least 50 normal human glyco-motifs. Apart from expected blood group-, xeno- (heterophil) and infection-related binding activities, we observed a number of new and unexpected features. The surprising, relatively high antibody binding was found to the blood group P<sub>1</sub> and P<sup>k</sup> trisaccharides and H(type 2) trisaccharide. Novel and very high binding activities have been observed towards Galβ1-3GlcNAc (Le<sup>C</sup>) related glycans, especially 3'-O-Su-Le<sup>C</sup>, and towards 4'-O-sulfated lactosamine. Relatively high and uniform Ab binding to GalNAcα1-3Gal disaccharide demonstrated absence of correlation with fucosylated blood group A GalNAcα1-3(Fucα1-2)Gal antigen—similarly to well known relationship between Galα1-3Gal and true, fucosylated blood group B Galα1-3(Fucα1-2)Gal antigen. The binding intensity to Galα1-3Galβ1-4GlcNAc xenoantigen was shown to be rather modest. Absence or very low Ab binding was found against oligosialic acid, sialooligosaccharides except SiaT<sub>n</sub>, type 2 backbone glycans such as Le<sup>y</sup>, and biantennary N-chain as well as its truncated forms, i.e. without terminal Sia, SiaGal, and SiaGalGlcNAc motifs. We have also found that Ab are capable of recognizing the short inner core typical for glycolipids (–Galβ1-4Glc) and glycoproteins (–GalNAcα) as a fragment of bigger glycans.

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### 1. Introduction

A success of the immune system depends largely on the presence of a competent and well-equipped repertoire of carbohydrate-binding antibodies that are the first line of defense against pathogens, and remove dysfunctional or malignant cells and particles [Cheng, 1998; Vollmers and Brandlein, 2007]. These immunoglobulins belong to IgG, IgM, IgA, and IgD subtypes and are called natural antibodies (NAb), since they are present in sera of all individuals in the absence of deliberate immunization [Shoenfeld,

1989], and are thus a part of the innate immune system [George and Shoenfeld, 1996]. NAb recognizing carbohydrates including blood group antigens are not found during first weeks of life, and a hypothesis referred as “bacterial paradigm” has been proposed by G.F. Springer who postulated that anti-carbohydrate NAb appear in response to stimulation of the immune system by bacterial O-antigens and LPS of gastrointestinal bacteria [Springer and Horton, 1969; Springer, 1984].

Although it has been shown that rather high levels of natural IgG antibodies remain practically unchanged during lifetime, and their repertoire is conservative [Lacroix-Desmazes et al., 1995], the factors involved in the regulation of composition of circulating NAb are less known. It is now well established that most NAb are encoded by their genes in germline configuration by B cells, which have not been subjected to somatic hypermutation and affinity maturation [Coutinho et al., 1995]. Many NAb are directed to carbohydrate antigens found in normal human tissues, and can be

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called auto-antibodies [Shoenfeld, 1989]. The best known and studied NAb are directed to blood group antigens A and B [Milland and Sandrin, 2006], and to antigens known also as xeno-(heterophil), such as “Galili epitope” Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc [Cooper, 1998; McKane et al., 1998], Forssman (Fs) glycolipid antigen GalNAc $\alpha$ 1-3GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc [Kano and Milgrom, 1977; Young et al., 1979], and Hanganutziu–Deicher (H–D) antigen Neu5Gc $\alpha$ 2-3Gal $\beta$ 1-4Glc [Ezzelarab et al., 2005]. Anti-carbohydrate NAb appear in number of pathologies, the group of particular attention includes antibodies to tumor-associated carbohydrate antigens such as Gal $\beta$ 1-3GalNAc $\alpha$  (TF, Thomsen–Friedenreich antigen), GalNAc $\alpha$ 1-OSer/Thr (T<sub>n</sub>) [Springer, 1984], and several related ones [Lloyd, 1991]. Antibodies to ganglioside GT3 (Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc) were found in patients with type 1 diabetes mellitus [Gaillard et al., 1989]; circulating islet cells Ab recognize pancreatic glycolipids including sulfatide [Cabrera-Rode et al., 1995]. Number of neuropathies is associated with characteristic pattern of antibodies to gangliosides, sulfated glycolipids and in some cases neutral glycosphingolipids such as Fs antigen [Yuki, 1999]. Antibodies against Glc $\alpha$ 1-4Glc $\alpha$  (glycogen fragment) were found to be specific for multiple sclerosis patients, enabling differentiation between patients with different neurological diseases [Dotan et al., 2006]. Patients with peptic ulcers have anti-Le<sup>x</sup> and anti-Le<sup>y</sup> antibodies [Kamiya et al., 1999]. Antibodies against poly-lactosamines (blood group Ii system) [Feizi and Childs, 1978] and in some cases against H-antigen (fucosylated I or i) [Nydegger et al., 1994] are known as pathological factor of cold agglutination disease, whereas anti-globoside Ab were found in paroxysmal cold hemoglobinuria; several cases of other autoimmune disorders are reviewed in [Feizi, 1981]. Antibodies to limited repertoire of glycolipids were revealed in patients with hemolytic anemia [Yasuda et al., 1982], anti-GlcNAc $\beta$  antibodies were found to be indicative for rheumatoid arthritis [Parekh et al., 1985], Ab directed against oligomannose sequences are markers of Crohn’s disease [Standaert–Vitse et al., 2006], and antibodies elicited by SARS vaccine were found to have an undesired autoimmune reactivity to a glycan fragment common in humans [Wang and Lu, 2004].

Although the above findings point into the significance of the repertoire and properties of NAb, only limited data on anti-carbohydrate antibodies in humans have been available, largely due to the laborious nature of ELISA in large-cohort studies. Development of multi-hundred glycan arrays printed on chip platform [Alvarez and Blixt, 2006; Blixt et al., 2004; Duk et al., 2003; Fukui et al., 2002] offers finally a possibility of systematic, large-population based research. The present study, in which profiles of carbohydrate-binding Ab in a cohort of 106 healthy donors were evaluated using glycochip, confirms a number of previous findings on anti-carbohydrate antibodies, and it also reveals unexpected facts and opens questions pointing into further investigations.

## 2. Materials and methods

### 2.1. Study subjects

Sera of 106 healthy female donors without known malignancies or malignancy history were included in the study. The study subjects were enrolled between 2000 and 2007 at the U.T., M.D. Anderson Cancer Center in accordance with the approved IRB protocol, after obtaining patients’ informed consent. Age range was 50–76, median age was 59. This population included 97 white females (91.5%), 5 African American females (4.7%), 3 Asian females (2.8%) and 1 Hispanic female (0.9%).

Glycochips were printed and developed in Cellexicon, Inc., La Jolla, CA. The 205 glycan structures of 95–98% purity (Lectinity Holdings, Moscow, Russia) were printed as previously described [Blixt et al., 2004], at 50  $\mu$ M concentration, at eight replicates each.

PGA chips have been produced in a process that has been standardized and optimized for clinical applications. The quality control covered intra-chip quality analysis of replicates, and inter-chip analysis of PGA reproducibility. The former included computation of coefficients of variation and the overall concordance correlation coefficients [Barnhart et al., 2002] in order to monitor and control the PGA measurement precision, while the latter included computation of the Lin’s concordance correlation coefficients [Lin, 1989] for PGA printed on different batches of slides and incubated with the same sera in different development schedules. The quality analysis has also extended to analysis of variances resulting in the interclass correlation coefficients (ICC) which provides the final test whether the platform and the particular cohort qualifies for further statistical analysis. The glycochips were incubated with human serum (1:15 dilution in PBS/3%BSA/1% Tween-20) with gentle rocking for 2 h at 37 °C. Serum Ab bound to printed glycans were visualized simultaneously with the “combo” biotinylated secondary Ab against human IgG, IgM and IgA (Pierce, Rockford, IL) followed by streptavidin–Alexa<sup>555</sup> (Invitrogen/Molecular Probes, Carlsbad, CA). Fluorescence signal intensities corresponding to Ab bound to printed glycans were collected at 95% laser power, and quantified with BioDiscovery/ImaGene software (El Segundo, CA). The range of total values of the relative fluorescence signal intensities of bound antibodies was 0–210  $\times 10^5$  RFU. In order to analyze the statistical correlation between the raw signal intensities the Pearson cross-correlation coefficient was computed for all glycans pairs. This has resulted in a 205  $\times$  205 cross-correlation matrix, which has further revealed several groups of glycans that are statistically correlated at various levels, ranging from moderate to high correlation ( $\rho = 0.3$ –0.999). The significance of the cross-correlation coefficients was sufficiently high for all correlated groups considered ( $p < 10^{-10}$ ) due to relatively large number of samples.

## 3. Results

The library of glycans arrayed on glycochip used in this study is composed of 205 structures, and includes the most frequent terminal oligosaccharides, as well as backbones and cores of mammalian N- and O-linked glycoproteins and glycolipids. About 20 of these oligosaccharides (OS) are well known to be tumor-associated [Hakomori, 1984], i.e. they are synthesized by cancer cells considerably more frequently than by the corresponding normal cells. Numerous structures including oligomers of GlcNAc $\beta$ 1-4 (chitobiose and higher chito-OS), GMDP-Lys, GlcNAc-Mur and other common components of bacterial polysaccharides and lipopolysaccharides (LPS) are also present. Majority of the arrayed structures are listed in Table 1. About 30 other synthetic glycans hereinafter called ‘monsters’ (for compactness, we excluded from Table 1 most cases when the same glycan is synthesized with two different spacers, and most of ‘monsters’) have also been included in the glycan library: these OS differ from natural mammalian glycans either by unnatural glycosidic bond position, e.g. Neu5Ac $\alpha$ 2-3GalNAc $\alpha$  instead of natural Neu5Ac $\alpha$ 2-6GalNAc $\alpha$  (SiaT<sub>n</sub>) or opposite anomeric configuration of glycosidic bond, e.g. Neu5Ac $\beta$ 2- instead of natural Neu5Ac $\alpha$ 2-. Aminoglucitol, an opened, reduced form of D-glucose which is not present as a structural component of glycosylation, was used here as a negative and background binding control.

To obtain information about person-to-person variability within profiles of anti-glycan auto-antibodies, each of 106 serum samples has been tested individually. Combined fluorescence corresponding to IgA, IgM and IgG binding to every individual glycan was measured, without antibody isotyping. The serum samples were not grouped according to ABH status, race or age of donors. Selected antibody binding data obtained here using PGA qualitatively coin-

cided with data of common ELISA [Obukhova et al., 2007] (data not shown).

Fig. 1 demonstrates an integral presentation of antibody binding to all arrayed structures where glycans are arranged on X-axis according to ascending order of means and corresponding standard deviations of fluorescence intensity of antibody binding for all 106 donors. The second important set of parameters which describes properties of immunoprofiles of carbohydrate-binding antibodies are median and median absolute deviation, MAD, shown in Table 1. Low median indicates that most of donors show low intensities of antibodies bound to the give glycan, while large median suggests that there is a significant number of donors with large antibody binding intensities to a given glycan.

To simplify general classification of antibody binding levels, such as non-binding to minimal, low or high binding, it was necessary to define the background level as well as fluorescence threshold levels for the given groups of glycans. In the absence of a “natural” grouping as shown by RFU of means in Fig. 1, we have selected the RFU value of  $\sim 6 \times 10^5$  as the cutoff level that separates glycans with low binding intensities that are less than  $\sim 5\%$  of maximal observed RFU, from the group of high-binding glycans. It can be seen that the cutoff assigns about a half of 205 glycans to the group of non-binding to weakly binding glycans. As further shown in Fig. 1, logarithmic version of the means suggests the same threshold. The cutoff is also marked in Table 1 as a thick line between structures 80 and 81, and it is supported by the fact that H(type 1) and H(type

**Table 1**

List of glycans, their median binding to antibodies in sera of 106 healthy donors, corresponding median absolute deviation (MAD) values, and number of donors exceeding cutoff.

|    | Structure of glycan with spacer group                                                                                                       | Common name                             | Median as<br>RFU $\times 10^{-5}$ ,<br>(MAD $\times 10^{-5}$ ) | <sup>a</sup> Number of<br>donors<br>showing<br>RFU > $6 \times 10^5$ |
|----|---------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------------|
| 1  | Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc $\beta$ -sp <sup>b</sup>                                                                           | 6'SLN                                   | 0.7(0.8)                                                       | 3                                                                    |
| 2  | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3<br>GalNAc $\alpha$ -sp<br>Neu5Ac $\alpha$ 2-6                                                            | Sia <sub>2</sub> -3',6-TF               | 0.7(0.4)                                                       | 0                                                                    |
| 3  | Gal $\beta$ 1-4GlcNAc $\beta$ 1-3<br>Gal $\beta$ 1-4GlcNAc $\beta$ -sp<br>Gal $\beta$ 1-4GlcNAc $\beta$ 1-6                                 | I                                       | 0.7(0.7)                                                       | 5                                                                    |
| 4  | (Neu5Ac $\alpha$ 2-8) <sub>3</sub> -sp                                                                                                      | (Sia) <sub>3</sub>                      | 0.8(0.5)                                                       | 0                                                                    |
| 5  | Neu5Gc $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                                                                        | 6'SLN(Gc)                               | 0.8(0.6)                                                       | 1                                                                    |
| 6  | Fuc $\alpha$ 1-3<br>GlcNAc $\beta$ -sp<br>Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4                                                                | SiaLe <sup>x</sup>                      | 0.8(0.9)                                                       | 3                                                                    |
| 7  | Man $\alpha$ 1-3<br>Man $\alpha$ 1-6(Man $\alpha$ 1-3)Man $\beta$ -sp1<br>Man $\alpha$ 1-6                                                  | Man5                                    | 0.8(1.5)                                                       | 4                                                                    |
| 8  | Man $\alpha$ 1-3<br>Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ -sp1<br>Man $\alpha$ 1-6                                                | 5-OS                                    | 0.8(1.1)                                                       | 1                                                                    |
| 9  | Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-sp                                                                                                     | (Sia) <sub>2</sub>                      | 0.9(0.6)                                                       | 3                                                                    |
| 10 | Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ -sp                                                                                         | Le <sup>x</sup>                         | 0.9(1.5)                                                       | 3                                                                    |
| 11 | 3-O-Su-Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ -sp                                                                                  | Su-Le <sup>x</sup>                      | 0.9(1.2)                                                       | 4                                                                    |
| 12 | Fuc $\alpha$ 1-2Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                                                                           | H (type 2)                              | 0.9(2.3)                                                       | 11                                                                   |
| 13 | Fuc $\alpha$ 1-3<br>6-O-Su-GlcNAc $\beta$ -sp<br>Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4                                                         | 6-Su-SiaLe <sup>x</sup>                 | 0.9(1.3)                                                       | 5                                                                    |
| 14 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ -sp1                                                                     | SiaLe <sup>a</sup>                      | 0.9(1.7)                                                       | 18                                                                   |
| 15 | GlcNAc $\beta$ 1-6<br>Gal $\beta$ 1-4GlcNAc $\beta$ -sp<br>Gal $\beta$ 1-4GlcNAc $\beta$ 1-3                                                |                                         | 0.9(1.0)                                                       | 1                                                                    |
| 16 | (Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man $\alpha$ 1) <sub>2</sub> -3,6-Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ -sp1 | 11-OS                                   | 0.9(1.2)                                                       | 13                                                                   |
| 17 | (Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man $\alpha$ 1) <sub>2</sub> -3,6-Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ -sp1                    | 9-OS                                    | 1.0(1.3)                                                       | 8                                                                    |
| 18 | (GlcNAc $\beta$ 1-2Man $\alpha$ 1) <sub>2</sub> -3,6-Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ -sp1                                   | 7-OS                                    | 1.0(0.8)                                                       | 2                                                                    |
| 19 | Fuc $\alpha$ 1-2Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ -sp                                                                         | Le <sup>y</sup>                         | 1.0(0.8)                                                       | 1                                                                    |
| 20 | Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ -sp                                                                                         | Le <sup>a</sup>                         | 1.1(4.3)                                                       | 18                                                                   |
| 21 | Neu5Ac $\alpha$ -sp                                                                                                                         |                                         | 1.1(1.6)                                                       | 9                                                                    |
| 22 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3-(6-O-Su)GalNAc $\alpha$ -sp                                                                              | 6-Su-3'SiaTF                            | 1.1(6.2)                                                       | 16                                                                   |
| 23 | (Gal $\beta$ 1-4GlcNAc $\beta$ 1-3) <sub>3</sub> $\beta$ -sp                                                                                | LN <sub>3</sub> , (LacNAc) <sub>3</sub> | 1.1(10.6)                                                      | 15                                                                   |
| 24 | Man $\alpha$ 1-6Man $\beta$ -sp1                                                                                                            |                                         | 1.2(2.2)                                                       | 12                                                                   |
| 25 | Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4-(6-O-Su)GlcNAc $\beta$ -sp                                                                               |                                         | 1.2(6.2)                                                       | 21                                                                   |
| 26 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4-(6-O-Su)GlcNAc $\beta$ -sp                                                                               |                                         | 1.2(6.4)                                                       | 20                                                                   |
| 27 | Fuc $\alpha$ 1-3<br>GlcNAc $\beta$ -sp<br>Neu5Ac $\alpha$ 2-3(6-O-Su)Gal $\beta$ 1-4                                                        | 6'-Su-SiaLe <sup>x</sup>                | 1.2(6.2)                                                       | 16                                                                   |
| 28 | Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ -OCH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> -p-sp1                                                   |                                         | 1.3(2.6)                                                       | 4                                                                    |
| 29 | 3-O-Su-Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ -sp                                                                                  | Su-Le <sup>a</sup>                      | 1.3(4.2)                                                       | 15                                                                   |
| 30 | Glc $\alpha$ -sp                                                                                                                            |                                         | 1.4(1.8)                                                       | 6                                                                    |
| 31 | 3-O-Su-Gal $\beta$ -sp                                                                                                                      |                                         | 1.4(1.9)                                                       | 6                                                                    |
| 32 | GlcN(Ge) $\beta$ -sp1                                                                                                                       |                                         | 1.4(1.4)                                                       | 5                                                                    |
| 33 | Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                                                                                           | LacNAc                                  | 1.4(5.1)                                                       | 19                                                                   |
| 34 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                                                                        | 3'SLN                                   | 1.4(4.8)                                                       | 14                                                                   |

Table 1 (Continued)

|    |                                                                                               |                             |            |    |
|----|-----------------------------------------------------------------------------------------------|-----------------------------|------------|----|
| 35 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc $\alpha$ -sp                                         | 3'-Sia-TF                   | 1.4(8.3)   | 28 |
| 36 | GlcNAc $\beta$ 1-3<br>Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                       |                             | 1.4(3.5)   | 12 |
| 37 | Neu5Ac $\alpha$ 2-3Gal $\beta$ -sp                                                            | GM4                         | 1.5(3.7)   | 13 |
| 38 | Man $\alpha$ 1-3(Man $\alpha$ 1-6)Man $\beta$ -sp1                                            | Man3                        | 1.5(2.0)   | 7  |
| 39 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc $\beta$ -sp                                             | 3'SL                        | 1.5(3.2)   | 14 |
| 40 | Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc $\beta$ -sp                            | LN <sub>2</sub> , LN3'LN, i | 1.5(6.0)   | 16 |
| 41 | 3-O-Su-Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc-sp                              |                             | 1.6(16.1)  | 20 |
| 42 | Man $\alpha$ -sp                                                                              |                             | 1.7(3.1)   | 17 |
| 43 | 3-O-Su-Gal $\beta$ 1-4Glc $\beta$ -sp                                                         | SM3                         | 1.7(4.3)   | 20 |
| 44 | Neu5Gc $\alpha$ -sp                                                                           |                             | 1.9(2.1)   | 10 |
| 45 | (Glc $\alpha$ 1-4) $\beta$ -sp1                                                               | maltotriose                 | 1.9(4.5)   | 20 |
| 46 | GlcNAc $\beta$ 1-3(GlcNAc $\beta$ 1-6)Gal $\beta$ 1-4GlcNAc $\beta$ -sp                       | Tk                          | 1.9(3.7)   | 18 |
| 47 | 3-O-Su-Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                                      |                             | 2.0(7.0)   | 22 |
| 48 | GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                           |                             | 2.0(7.8)   | 22 |
| 49 | (Glc $\alpha$ 1-6) $\beta$ -sp1                                                               | isomaltotetraose            | 2.0(5.6)   | 25 |
| 50 | GlcA $\beta$ -sp                                                                              | glucuronic acid             | 2.1(3.3)   | 17 |
| 51 | Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4Glc $\beta$ -sp1                                            | 6'SL                        | 2.1(8.5)   | 36 |
| 52 | (Neu5Ac $\alpha$ 2-8) $\beta$ -sp                                                             | (Sia) $\beta$               | 2.1(10.8)  | 31 |
| 53 | Fuc $\alpha$ 1-2Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ -sp                           | Le <sup>b</sup>             | 2.1(8.1)   | 32 |
| 54 | Fuc $\alpha$ 1-3<br>GlcNAc $\beta$ 1-3Gal $\beta$ -sp<br>Neu5Ac $\alpha$ 2-3 Gal $\beta$ 1-4  | SiaLe <sup>x</sup> -penta   | 2.2(9.7)   | 26 |
| 55 | 6-H <sub>2</sub> PO <sub>3</sub> Man $\alpha$ -sp                                             | Man6P                       | 2.6(4.2)   | 22 |
| 56 | 3-O-Su-Gal $\beta$ 1-4(6-O-Su)GlcNAc $\beta$ -sp                                              |                             | 2.9(8.8)   | 34 |
| 57 | Gal $\beta$ 1-4GlcNAc $\beta$ 1-6Gal $\beta$ 1-4GlcNAc $\beta$ -sp                            | LN6'LN                      | 2.9(6.4)   | 35 |
| 58 | 3,6-O-Su <sub>2</sub> -Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                      |                             | 3.0(7.4)   | 34 |
| 59 | Glc $\alpha$ 1-6Glc $\alpha$ 1-6Glc $\beta$ -sp1                                              | isomaltotriose              | 3.2(7.2)   | 42 |
| 60 | Gal $\beta$ 1-4GlcNAc $\beta$ 1-6(Gal $\beta$ 1-3)GalNAc $\alpha$ -sp                         | 6-LN-TF                     | 3.2(12.5)  | 38 |
| 61 | Neu5Ac $\alpha$ -OCH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> -p-sp1                        |                             | 3.4(11.8)  | 40 |
| 62 | 6-O-Su-Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                                      |                             | 3.5(5.2)   | 29 |
| 63 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ -sp                        | SiaLe <sup>a</sup>          | 3.7(11.5)  | 37 |
| 64 | GlcA $\alpha$ -sp                                                                             | $\alpha$ -glucuronic acid   | 3.8(6.5)   | 43 |
| 65 | Gal $\beta$ 1-4(6-O-Su)GlcNAc $\beta$ -sp                                                     |                             | 3.8(8.8)   | 43 |
| 66 | Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc $\beta$ -sp1                         | GD3                         | 3.9(14.9)  | 45 |
| 67 | Fuc $\alpha$ -sp                                                                              |                             | 4.1(8.5)   | 40 |
| 68 | 3-O-Su-Gal $\beta$ 1-3GalNAc $\alpha$ -sp                                                     | 3'-O-Su-TF                  | 4.1(8.9)   | 46 |
| 69 | 6-O-Su-Gal $\beta$ 1-4(6-O-Su)GlcNAc $\beta$ -sp                                              |                             | 4.5(10.3)  | 46 |
| 70 | 3,6-O-Su <sub>2</sub> -Gal $\beta$ 1-4(6-O-Su)GlcNAc $\beta$ -sp                              |                             | 4.6(11.9)  | 49 |
| 71 | 3-O-Su-Gal $\beta$ 1-4(6-O-Su)Glc $\beta$ -sp                                                 |                             | 4.8(8.9)   | 50 |
| 72 | 6-O-Su-Gal $\beta$ 1-4(6-O-Su)Glc $\beta$ -sp                                                 |                             | 4.9(7.3)   | 46 |
| 73 | Neu5Ac $\alpha$ 2-6(Gal $\beta$ 1-3)GalNAc $\alpha$ -sp                                       | 6-SiaTF                     | 5.2(12.7)  | 52 |
| 74 | (Glc $\alpha$ 1-4) $\beta$ -sp1                                                               | maltotetraose               | 5.2(7.9)   | 48 |
| 75 | Neu5Ac $\alpha$ 2-6(Gal $\beta$ 1-3)GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc $\beta$ -sp1         | LSTb                        | 5.2(11.9)  | 43 |
| 76 | Gal $\beta$ 1-4GlcNAc $\beta$ 1-3<br>GalNAc $\alpha$ -sp<br>Gal $\beta$ 1-4GlcNAc $\beta$ 1-6 |                             | 5.3(13.8)  | 50 |
| 77 | Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc $\beta$ -sp                                             | Le <sup>d</sup> (H type 1)  | 5.5(14.0)  | 53 |
| 78 | Fuc $\alpha$ 1-2Gal $\beta$ 1-3GalNAc $\alpha$ -sp                                            | H (type 3)                  | 5.5(11.2)  | 51 |
| 79 | 6-H <sub>2</sub> PO <sub>3</sub> Glc $\beta$ -sp1                                             | Glc6P                       | 5.6(6.2)   | 50 |
| 80 | 6-O-Su-Gal $\beta$ 1-4Glc $\beta$ -sp                                                         |                             | 5.7(6.7)   | 47 |
| 81 | Gal $\alpha$ 1-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ -sp                           | $\alpha$ GalLe <sup>x</sup> | 6.7(10.2)  | 57 |
| 82 | GlcNAc $\beta$ 1-3Gal $\beta$ 1-3GalNAc $\alpha$ -sp                                          |                             | 6.8(7.8)   | 61 |
| 83 | GalNAc $\alpha$ 1-3(Fuc $\alpha$ 1-2)Gal $\beta$ 1-4GlcNAc $\beta$ -sp                        | A (type 2)                  | 7.6(20.5)  | 55 |
| 84 | GalNAc $\alpha$ 1-O-Ser                                                                       | T <sub>n</sub>              | 7.7(7.1)   | 60 |
| 85 | GlcNAc $\beta$ 1-6Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                           |                             | 8.2(9.2)   | 61 |
| 86 | GlcNAc $\beta$ 1-6(Gal $\beta$ 1-3)GalNAc $\alpha$ -sp                                        | core 2                      | 9.0(12.0)  | 63 |
| 87 | GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc $\beta$ -sp                                              |                             | 9.4(9.2)   | 66 |
| 88 | Gal $\beta$ 1-4GlcNAc $\beta$ 1-3GalNAc $\alpha$ -sp                                          |                             | 9.5(11.0)  | 67 |
| 89 | GlcA $\beta$ 1-3Gal $\beta$ -sp                                                               |                             | 9.7(7.1)   | 67 |
| 90 | Neu5Ac $\alpha$ 2-8Neu5Ac $\beta$ -OCH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> -p-sp1      |                             | 10.4(15.5) | 62 |
| 91 | Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc $\beta$ -sp1                                            | 3'SL                        | 10.7(20.4) | 64 |
| 92 | Fuc $\alpha$ 1-2Gal $\beta$ -sp                                                               | H <sub>di</sub>             | 10.9(12.0) | 71 |
| 93 | 3,4-O-Su <sub>2</sub> -Gal $\beta$ 1-4GlcNAc $\beta$ -sp                                      |                             | 11.1(16.0) | 65 |
| 94 | Neu5Ac $\alpha$ 2-6Gal $\beta$ -sp                                                            |                             | 11.7(11.5) | 70 |
| 95 | Gal $\alpha$ 1-3Gal $\beta$ -sp                                                               | B <sub>di</sub>             | 11.9(6.8)  | 76 |
| 96 | Gal $\beta$ 1-4Glc $\beta$ -sp                                                                | Lac                         | 12.2(6.7)  | 79 |

Table 1 (Continued).

|     |                                                        |                         |             |     |
|-----|--------------------------------------------------------|-------------------------|-------------|-----|
| 97  | GalNAcβ1-3Galβ-sp                                      |                         | 12.3(9.8)   | 70  |
| 98  | Galα-sp                                                |                         | 12.8(6.8)   | 82  |
| 99  | GlcNAcβ1-3Manβ-sp1                                     |                         | 12.8(8.3)   | 66  |
| 100 | Galβ1-3GalNAcα-sp                                      | TF                      | 13.5(10.9)  | 74  |
| 101 | Galα1-3GalNAcα-sp                                      | T <sub>αα</sub>         | 14.8(10.2)  | 75  |
| 102 | Neu5Gcα2-6GalNAcα-sp                                   | Neu5Gc-T <sub>n</sub>   | 16.7(10.9)  | 73  |
| 103 | Galα1-4Galβ1-4GlcNAcβ-sp                               | P <sub>1</sub>          | 17.0(9.8)   | 81  |
| 104 | GalNAcα1-3(Fucα1-2)Galβ-sp                             | A <sub>tri</sub>        | 18.4(17.7)  | 72  |
| 105 | Glcβ-sp                                                |                         | 18.9(7.7)   | 87  |
| 106 | Glcβ1-4Glcβ-sp1                                        | cellobiose              | 19.3(8.0)   | 94  |
| 107 | GlcAβ1-6Galβ-sp                                        |                         | 19.7(6.6)   | 93  |
| 108 | GalNAcβ-sp                                             |                         | 19.9(9.6)   | 96  |
| 109 | GalNAcβ1-4GlcNAcβ-sp                                   | LacdiNAc                | 20.1(9.7)   | 86  |
| 110 | Glcα1-4Glcβ-sp1                                        | maltose                 | 20.9(10.4)  | 79  |
| 111 | Galα1-3GalNAcβ-sp                                      | T <sub>αβ</sub>         | 21.2(11.4)  | 79  |
| 112 | Neu5Acα2-6GalNAcα-sp                                   | SiaT <sub>n</sub>       | 21.6(13.6)  | 79  |
| 113 | Galβ1-4(6-O-Su)Glcβ-sp                                 |                         | 21.6(5.6)   | 99  |
| 114 | Galβ1-3Galβ-sp                                         |                         | 22.0(6.9)   | 95  |
| 115 | GalNAcα-sp                                             | T <sub>n</sub>          | 22.4(10.5)  | 92  |
| 116 | Galα1-6Glcβ-sp                                         |                         | 22.4(4.0)   | 106 |
| 117 | Manβ1-4GlcNAcβ-sp1                                     |                         | 22.4(6.3)   | 98  |
| 118 | Glcβ1-6Glcβ-sp1                                        | gentiobiose             | 22.7(6.3)   | 102 |
| 119 | GlcNAcβ1-4GlcNAcβ-Asn                                  | chitobiose-Asn          | 23.1(8.8)   | 93  |
| 120 | Galα1-3Galβ1-4GlcNAcβ-sp                               | “Galili”                | 23.1(11.2)  | 90  |
| 121 | Fucα1-2Galβ1-4Glcβ-sp1                                 | H (type 6)              | 23.1(11.2)  | 93  |
| 122 | Fucα1-4<br>GlcNAcβ1-3Galβ1-4Glcβ-sp1<br>Fucα1-2Galβ1-3 | Le <sup>b</sup> (hexa)  | 23.7(18.0)  | 87  |
| 123 | Galβ1-2Galβ-sp                                         |                         | 24.5(8.9)   | 102 |
| 124 | Galα1-4Galβ1-4Glcβ-sp                                  | P <sup>k</sup>          | 24.8(4.6)   | 105 |
| 125 | Galβ-sp                                                |                         | 25.1(7.1)   | 101 |
| 126 | Galβ1-3Galβ1-4GlcNAcβ-sp1                              |                         | 25.6(7.1)   | 98  |
| 127 | Galα1-2Galβ-sp                                         |                         | 27.0(5.8)   | 105 |
| 128 | 6-O-Su-GlcNAcβ-sp                                      |                         | 27.6(16.9)  | 93  |
| 129 | GlcNAcβ1-4GlcNAcβ-sp1                                  | chitobiose              | 28.4(12.2)  | 102 |
| 130 | Galβ1-4GlcNAcβ1-6GalNAcα-sp                            |                         | 28.5(13.1)  | 98  |
| 131 | GlcAβ1-3GlcNAcβ-sp                                     |                         | 28.6(6.7)   | 104 |
| 132 | Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp1                       | LNnT                    | 29.5(16.4)  | 94  |
| 133 | Fucα1-3<br>GlcNAcβ1-3Galβ1-4Glcβ-sp1<br>Fucα1-2Galβ1-4 | Le <sup>v</sup> (hexa)  | 29.9(16.4)  | 94  |
| 134 | Galβ1-3GalNAcβ-sp                                      | T <sub>ββ</sub>         | 31.1(7.2)   | 105 |
| 135 | GlcNAcβ1-3GalNAcα-sp                                   | core 3                  | 31.2(8.3)   | 103 |
| 136 | Galα1-3(Fucα1-2)Galβ-sp                                | B <sub>tri</sub>        | 31.3(12.2)  | 88  |
| 137 | GalNAcβ1-4Galβ1-4Glcβ-sp                               | GA2, GgOse <sub>3</sub> | 31.7(9.0)   | 101 |
| 138 | GlcNAcβ1-6GalNAcα-sp                                   | core 6                  | 32.0(10.6)  | 104 |
| 139 | Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp1                       | LNT                     | 32.1(16.4)  | 99  |
| 140 | GalNAcα1-3(Fucα1-2)Galβ-sp2                            | A <sub>tri</sub>        | 33.0(29.3)  | 79  |
| 141 | GlcNAcβ1-4-(Mur)-3-O-GlcNAcβ-sp1                       | GlcNAc-Mur              | 33.3(16.5)  | 100 |
| 142 | GlcNAcβ-sp                                             |                         | 35.8(13.8)  | 102 |
| 143 | Galβ1-3GlcNAcβ-sp                                      | Le <sup>c</sup>         | 35.8(12.9)  | 100 |
| 144 | GalNAcα1-3Galβ-sp                                      | A <sub>di</sub>         | 36.3(10.2)  | 105 |
| 145 | GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα-sp                       | core 4                  | 36.9(10.6)  | 101 |
| 146 | Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ-sp                      | B (type 2)              | 39.7(15.0)  | 91  |
| 147 | Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp                        | asialo-GM1              | 41.2(8.4)   | 106 |
| 148 | Galα1-4GlcNAcβ-sp                                      | “αLacNAc”               | 43.0(9.7)   | 106 |
| 149 | Fucα1-4GlcNAcβ-sp                                      |                         | 43.0(13.0)  | 106 |
| 150 | Fucα1-3GlcNAcβ-sp                                      |                         | 44.0(12.8)  | 106 |
| 151 | Rhaα-sp                                                |                         | 44.6(17.4)  | 106 |
| 152 | Galα1-3(Fucα1-2)Galβ-sp2                               | B <sub>tri</sub>        | 44.7(18.5)  | 92  |
| 153 | GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-sp1                        | chitotriose             | 48.7(27.7)  | 102 |
| 154 | 4-O-Su-Galβ1-4GlcNAcβ-sp                               |                         | 51.5(24.0)  | 97  |
| 155 | GalNAcα1-3GalNAcβ-sp                                   | Fs-2                    | 53.9(20.6)  | 106 |
| 156 | (GlcNAcβ1-4) <sub>6</sub> β-sp1                        | chitohexaose            | 62.1(27.7)  | 102 |
| 157 | Neu5Acα2-3Galβ1-3GlcNAcβ-sp                            | SiaLe <sup>c</sup>      | 75.0(30.3)  | 105 |
| 158 | (GlcNAcβ1-4) <sub>5</sub> β-sp1                        | chitopentaose           | 83.6(31.5)  | 104 |
| 159 | GlcNAcβ1-4Mur-L-Ala-D-i-Gln-Lys                        | GMDP-Lys                | 117.0(47.2) | 106 |
| 160 | 3-O-Su-Galβ1-3GlcNAcβ-sp                               | Su-Le <sup>c</sup>      | 134.2(63.9) | 106 |

<sup>a</sup>RFU cutoff value of  $6 \times 10^5$  has been selected according to Fig. 1.<sup>b</sup>sp,  $-(\text{CH}_2)_3\text{NH}_2$  or  $-(\text{CH}_2)_2\text{NH}_2$ ; sp1,  $-\text{NHCOCH}_2\text{NH}_2$ ; sp2,  $-(\text{CH}_2)_3\text{NHCO}(\text{CH}_2)_5\text{NH}_2$ ; Gc, glycolyl; GlcA, glucuronic acid; Mur, muramoyl; OS, oligosaccharide; P, phosphate; Ser, serine; Sia, Neu5Ac; Su, sulfate.

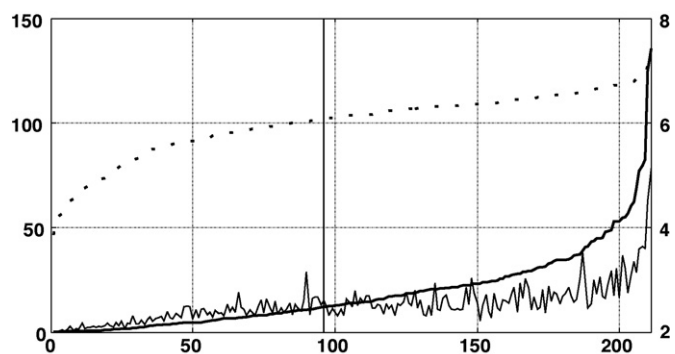


**Fig. 1.** Left y-axis: mean values (bold solid line) and corresponding standard deviations (thin solid line) of total intensities for all 205 glycans,  $\text{RFU} \times 10^5$ . Right y-axis: the  $\log_{10}$  of the mean values (bold dotted line). X-axis represents glycans arranged in ascending order of their mean values. The logarithmic version of the means assumes an S-shape revealing an inflection after  $\sim 100$  glycans, marked here by a vertical cut-off line. The means and the standard deviations are calculated for 106 subjects, for eight replicates of each glycan independently.

3), to which humans are unable to generate Abs according to general considerations, are below the cutoff level, whereas the blood group antigen A(type 2), antibodies to which must be present in significant amounts in sera of donors with blood groups O and B is also located near the cutoff though at the opposite side. Thus, we can conclude that significant and not random response to at least 100 glycans has been observed in serum of healthy donors. Since about a half of these glycans are typical for human cells, we can further conclude that at least 50 species of carbohydrate-binding auto-antibodies are present in the serum.

We identified only a limited number of normal human glycans to which minimal or very sporadic antibody binding was observed in this study population, such as to: (1) 6'SLN and bi-antennary sialylated 11-OS; (2) Sia<sub>2</sub> and Sia<sub>3</sub>; (3) type 2 backbone oligosaccharides such as Le<sup>y</sup>, Le<sup>x</sup>, SiaLe<sup>x</sup> and Su-Le<sup>x</sup>; (4) mannose and manno-oligosaccharides; and (5) oligolactosamine derivatives, for example Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc. We will refer to these glycans as 'low binders'. Majority of other glycans showed quite a variety of binding patterns that could be exemplified as shown in Fig. 2. The binding to a 'negative control' aminoglucitol is indeed very low and uniform, not exceeding level of  $0.5 \times 10^5$  RFU, and represents the pattern 'minimal to none'. High and variable binding is shown in Fig. 2B, whereas Fig. 2C demonstrates pattern observed for blood group glycans, shown here on an example of blood group A trisaccharide. Fig. 2D shows uniform distribution of relative medium binding across the entire donor population on an example of binding to a P<sup>k</sup> trisaccharide. Finally, Fig. 2E illustrates a very characteristic 'bi-polar' binding pattern, where  $\sim 10\%$  subjects show very high antibody binding levels as compared to the levels found in the other donors; this pattern is represented here by the blood group i tetrasaccharide.

Overall comparative analysis of carbohydrate-binding antibody immunoprofiles in a relatively large group of 106 individuals demonstrates existence of certain regularities, such as uniform binding of antibodies to a given glycan in all examined sera, e.g. Fig. 2D, and on the other hand—allows identification of the individual features within personal immunoprofiles, as shown in Fig. 2E. The former brings several new, sometimes surprising, findings as discussed below. The latter provides a clue to potential pathologies such as those that might result from the pathogen exposure of an individual donor. In a future, immunoprofiling of carbohydrate-binding antibodies might allow prediction of potential transfusion problems, vaccination limitations, appearance of malignancy or other pathological conditions.

### 3.1. Effects of glycan size and charge, and nature of spacer-arm

We have evaluated a potential impact of glycan size, charge, and a nature of spacer arm on antibody binding. It is considered that typical size of glycotope is tetra- to pentasaccharide [Newman and Kabat, 1985]. A simple calculation demonstrates that average mean of binding for 16 monosaccharides on our PGA practically coincides with average mean for 29 trisaccharides, i.e. saccharide size by itself is not critical.

NAb are characterized by the presence of many arginine and lysine residues in their hypervariable regions [Avrameas, 1991] and many of polyreactive NAb in fact tend to bind negatively charged antigens [Lekakh et al., 2001]. In our PGA several glycans possess two or three negative charges, therefore we have investigated the possibility of number of charge moieties as non-specific factor and found that glycan a number of charges – similarly to above mentioned glycan size – is statistically insignificant.

Spacer-arm is also a potentially critical matter. We have indeed observed that the long aliphatic spacer increases Ab binding considerably as exemplified by high binding to A and B trisaccharides with elongated spacer  $-\text{O}(\text{CH}_2)_3\text{NHCO}(\text{CH}_2)_5\text{NH}_2$  as compared to  $-\text{O}(\text{CH}_2)_3\text{NH}_2$  (Fig. 3). At the same time the binding patterns for glycan derivatized with aminoethyl or aminopropyl spacer were practically identical.

### 3.2. ABH blood group antigens and related structures

Antibody binding to blood group A and B related antigens are presented in Fig. 3, where MAD is given for each glycan besides the mean of binding. Trisaccharides GalNAcα1-3(Fucα1-2)Gal and Galα1-3(Fucα1-2)Gal are known to be minimal determinants of blood group antigens A and B respectively, whereas tetrasaccharides GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAc and Galα1-3(Fucα1-2)Galβ1-4GlcNAc are major A or B erythrocyte antigens. Natural antibodies to the type 2 tetrasaccharides are responsible for agglutination and for mismatch transfusion/transplantation complications. In addition to these structures, corresponding fucose-free disaccharides GalNAcα1-3Gal (A<sub>di</sub>) and Galα1-3Gal (B<sub>di</sub>), the last one known as the most important xenotransplantation antigen [Cooper, 1998], were also included in the PGA. The data presented in Fig. 3 provide an evidence that blood group A glycans, both tri- and tetrasaccharides, bind donor's Ab weaker than the corresponding blood group B glycans, whereas variability between donors, as indicated by MAD, is considerably higher in case of anti-A, in particular for tetrasaccharide.

In the latter case of A(type 2), MAD is unique between all 205 glycans with the value threefold higher than median, whereas typical MAD is close or considerably less than median for 'high binders'. According to vast literature human blood group A antigens display far greater heterogeneity as compared to B antigens; it is likely that observed here diversity of NAb reflects the diversity of cognate antigens. In contrast to antibody binding patterns to A and B tri- and tetrasaccharides best represented in Fig. 2C, binding of antibodies to A and B disaccharides are best reflected by pattern in Fig. 2D. Because anti-disaccharide Ab exist in all tested sera, their presence is unrelated to the ABH blood group system.

Level of Ab to A disaccharide-related Fs disaccharide, GalNAcα1-3GalNAcβ, is one of the highest in our PGA; the antibody binding signals to these two A and Fs disaccharides are statistically correlated ( $r > 0.69$ ,  $\text{CI}_{95\%} = [0.58, 0.78]$ ). High and uniform donors' response to Fs disaccharide, as well as wide occurrence of this antigen in animals suggests potential contribution of anti-Fs in antibody-mediated xenograft rejection.

It is well documented that repertoire of antibodies to αGal-containing oligosaccharides is quite broad in humans [Obukhova et al., 2007; Wieslander et al., 1990]. Glycoarray study has confirmed

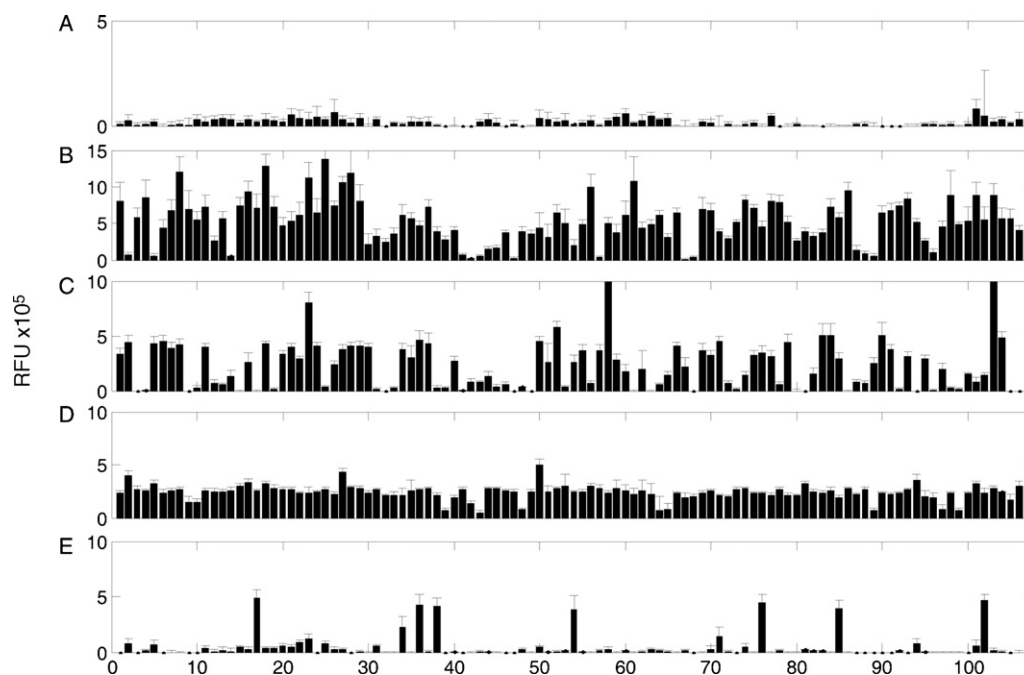


**Fig. 2.** Five representative patterns of serum antibodies binding. A: binding minimal to none: aminoglucitol,  $\text{HOCH}_2(\text{HOCH})_4\text{CH}_2\text{NH}_2$ ; B: high and variable binding to 4'-Su-Gal $\beta$ 1-4GlcNAc; C: blood group binding pattern, shown here for A trisaccharide, GalNAc $\alpha$ 1-3(Fuc $\alpha$ 1-2)Gal; D: relatively uniform antibody binding to P<sup>k</sup> trisaccharide, Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc; E: "bi-polar" binding pattern, represented by the blood group i tetrasaccharide, Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc. Whiskers represent standard deviations of eight replicates.

these data: we observe the antibody binding to  $\alpha$ Gal monosaccharide as well as to all  $\alpha$ Gal-containing oligosaccharides as presented in entry 1 of Table 2, where Table 2 is a condensed form of Table 1, for the sake of convenience of the following discussions. Notably, the rank of classical 'anti-Galili' antibodies on our PGA is modest as compared to all the other anti- $\alpha$ Gal Ab with an exception of  $\alpha$ GalLe<sup>x</sup>. The leader between  $\alpha$ Gal-containing glycans is disaccharide Gal $\alpha$ 1-4GlcNAc (entry 1), in agreement with ELISA results [Obukhova et al., 2007]. This structure is alien for mammals, but it is known to be a pendant chain of PS from several *Bacillus* bacteria, for example, widely spread *Lactobacillus* [Yamamoto et al., 1994], with very close structure Gal $\alpha$ 1-3GlcNAc, found in *Bacillus anthracis* [Choudhury et al., 2006].

The next section is related to the discussion of blood group H antigens. Trisaccharide H(type 2), Fuc $\alpha$ 1-2Gal $\beta$ 1-4GlcNAc, is

widely present on erythrocytes and endothelium irrespectively of ABH blood group, and thus should not be antigenic (except extremely rare homozygous *h/h*, so called Bombay phenotype). Surprisingly, four of 106 sera contained corresponding auto-Ab—three individuals with moderate and one with rather high level, in spite of "taboo" for anti-H antibodies. Investigation of an entire immunoprofile of this donor revealed also very high levels of Ab against various lactosamines what is also quite unusual (see Section 3.4). Presence of high levels of these Ab suggests recent infection-related disease of this 'healthy donor', since several viral and mycoplasma infections are known to cause immune response to oligolactosamines including fucosylated ones, i.e. H antigen [Feizi and Childs, 1978; Nydegger et al., 1994]. Thus, these could be anti-oligolactosamine Ab with contaminant anti-H activity.

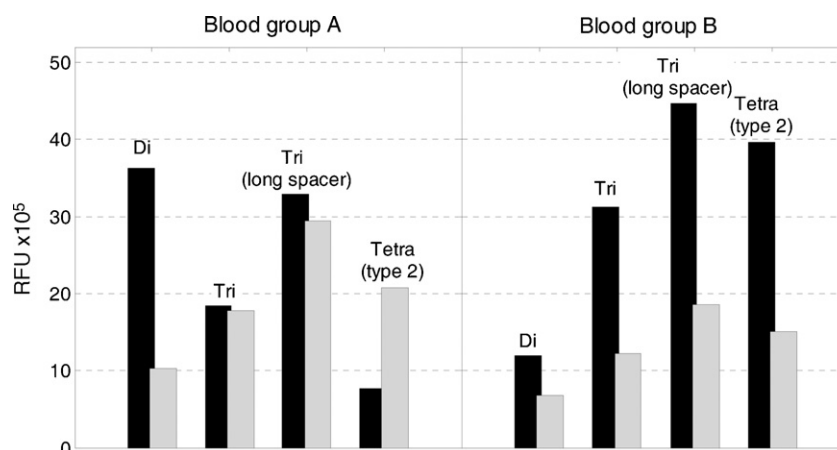


**Fig. 3.** Medians (black bars) and corresponding MADs (grey bars) for binding of 106 sera to blood group A and B di-, tri- and tetrasaccharides. Trisaccharides were presented in the array in normal and elongated (more hydrophobic) forms.

**Table 2**  
'Silent' (left) and structurally related high-binding glycans (right).

| Glycans, demonstrating absence, very low, or sporadic binding |                                                                                                                                                                                                                                                                                                              | Related glycans with high or very high (in bold) binding                                                                                                                                                                                                                                                    |
|---------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1                                                             | Fuc $\alpha$ 1-3<br>Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc ( $\alpha$ GalLe <sup>s</sup> )                                                                                                                                                                                                                    | $\alpha$ Gal, T <sub><math>\alpha\beta</math></sub> , T <sub><math>\alpha\alpha</math></sub> , Gal $\alpha$ 1-(2,3,4 or 6)Gal, <b>Gal<math>\alpha</math>1-4GlcNAc</b> ,<br>Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc, <b>Gal<math>\alpha</math>1-4GlcNAc<math>\beta</math>1-3Gal<math>\beta</math>1-4GlcNAc</b> |
| 2                                                             | Fuc $\alpha$ 1-2Gal $\beta$ 1-4GlcNAc (H type 2)                                                                                                                                                                                                                                                             | Fuc $\alpha$ 1-2Gal (H <sub>di</sub> )                                                                                                                                                                                                                                                                      |
| 3                                                             | Fuc $\alpha$ 1-3<br>Gal $\beta$ 1-4GlcNAc (Le <sup>s</sup> )<br>Fuc $\alpha$ 1-2Gal $\beta$ 1-4<br>Fuc $\alpha$ 1-3GlcNAc (Le <sup>v</sup> )<br>3-O-Su-Gal $\beta$ 1-4<br>Fuc $\alpha$ 1-3GlcNAc (Su-Le <sup>s</sup> )<br>Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4<br>Fuc $\alpha$ 1-3GlcNAc (SiaLe <sup>s</sup> ) | <b>Fuc<math>\alpha</math>1-3GlcNAc</b>                                                                                                                                                                                                                                                                      |
| 4                                                             | Gal $\beta$ 1-4GlcNAc $\beta$ 1-3<br>Gal $\beta$ 1-4GlcNAc $\beta$ 1-6<br>Gal $\beta$ 1-4GlcNAc (I antigen)<br>Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc (i antigen, LN <sub>2</sub> )                                                                                                          | Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc (LNnT)                                                                                                                                                                                                                                                  |
| 5                                                             | Fuc $\alpha$ 1-4<br>3-O-Su-Gal $\beta$ 1-3GlcNAc (Su-Le <sup>s</sup> )<br>3-O-Su-Gal $\beta$ 1-4GlcNAc<br>3-O-Su-Gal $\beta$ 1-3GalNAc $\alpha$                                                                                                                                                              | <b>3-O-Su-Gal<math>\beta</math>1-3GlcNAc (SuLe<sup>c</sup>)</b>                                                                                                                                                                                                                                             |
| 6                                                             | Fuc $\alpha$ 1-4<br>Gal $\beta$ 1-3GlcNAc (Le <sup>s</sup> )                                                                                                                                                                                                                                                 | Gal $\beta$ 1-3GlcNAc (Le <sup>c</sup> )<br>Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc<br><b>3-O-Su-Gal<math>\beta</math>1-3GlcNAc (Su-Le<sup>c</sup>)</b><br><b>Neu5Ac<math>\alpha</math>2-3Gal<math>\beta</math>1-3GlcNAc (SiaLe<sup>c</sup>)</b>                                             |
| 7                                                             | 3-O-Su-Gal $\beta$ 1-4GlcNAc<br>6'-O-Su-Gal $\beta$ 1-4GlcNAc<br>6-O-Su-Gal $\beta$ 1-4GlcNAc                                                                                                                                                                                                                | <b>4'-O-Su-Gal<math>\beta</math>1-4GlcNAc</b>                                                                                                                                                                                                                                                               |
| 8                                                             | GlcA                                                                                                                                                                                                                                                                                                         | GlcA $\beta$ 1-3Gal, GlcA $\beta$ 1-6Gal, GlcA $\beta$ 1-3GlcNAc                                                                                                                                                                                                                                            |
| 9                                                             | Neu5Ac $\alpha$ , Neu5Ac $\alpha$ -OS                                                                                                                                                                                                                                                                        | Neu5Ac $\beta$ -anomers, SiaT <sub>n</sub>                                                                                                                                                                                                                                                                  |
| 10                                                            | Neu5Gc $\alpha$ , Neu5Gc $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc                                                                                                                                                                                                                                                   | Neu5Gc-T <sub>n</sub>                                                                                                                                                                                                                                                                                       |
| 11                                                            | GlcNAc $\beta$ 1-4Man $\alpha$ 1-6<br>GlcNAc $\beta$ 1-4Man $\alpha$ 1-3<br>Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc (7-OS)                                                                                                                                                                                   | GlcNAc, <b>chito-OS</b> , <b>GMDP-Lys</b>                                                                                                                                                                                                                                                                   |
| 12                                                            | GlcNAc $\beta$ 1-6<br>GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc (Tk)                                                                                                                                                                                                                                           | GlcNAc $\beta$ 1-4Man, GlcNAc $\beta$ 1-6Gal $\beta$ 1-4GlcNAc<br>GlcNAc $\beta$ 1-6<br>Gal $\beta$ 1-3GalNAc $\alpha$ (core 2)<br>GlcNAc $\beta$ 1-3GalNAc $\alpha$ (core 3)<br>GlcNAc $\beta$ 1-6<br>GlcNAc $\beta$ 1-3GalNAc $\alpha$ (core 4)<br>GlcNAc $\beta$ 1-6GalNAc $\alpha$ (core 6)             |
| 13                                                            | Man <sub>s</sub> , Man $\alpha$ 1-6Man, $\alpha$ Man, M6P<br>Man $\alpha$ 1-6<br>Man $\alpha$ 1-3Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc (5-OS)                                                                                                                                                              | Man $\beta$ 1-4GlcNAc                                                                                                                                                                                                                                                                                       |
| 14                                                            | [Glc $\alpha$ 1-4] <sub>n</sub> , n>2; [Glc $\alpha$ 1-6Glc] <sub>n</sub>                                                                                                                                                                                                                                    | Glc $\beta$ 1-6Glc, Glc $\beta$ 1-4Glc                                                                                                                                                                                                                                                                      |
| 15                                                            | Fuc $\alpha$ 1-4<br>Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc (Le <sup>b</sup> )<br>Fuc $\alpha$ 1-3<br>Fuc $\alpha$ 1-2Gal $\beta$ 1-4GlcNAc (Le <sup>v</sup> )                                                                                                                                                 | Fuc $\alpha$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc (Le <sup>b</sup> -Lac)<br>Fuc $\alpha$ 1-2Gal $\beta$ 1-3<br>Fuc $\alpha$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc (Le <sup>v</sup> -Lac)                                                                                                               |

### 3.3. Lewis and related antigens

Lewis blood group system is represented by two antigens with type 1 backbone structure, namely Fuc $\alpha$ 1-4(Gal $\beta$ 1-3)GlcNAc (Le<sup>a</sup>) and Fuc $\alpha$ 1-4(Fuc $\alpha$ 1-2Gal $\beta$ 1-3)GlcNAc (Le<sup>b</sup>). Their biosynthetic precursor Gal $\beta$ 1-3GlcNAc, known as Le<sup>c</sup>, is not significant for blood typing [Henry et al., 1995]. We observe very low median binding both for Le<sup>a</sup> trisaccharide and Le<sup>b</sup> tetrasaccharide, in fact much less than the cutoff value. However, several individuals display moderate response—that follows expected distribution for blood group antibodies. These results are consistent with numerous literature data pointing to low Ab levels against Le<sup>a</sup> and Le<sup>b</sup>.

Type 2 backbone glycans, Le<sup>x</sup> and Le<sup>y</sup> (Table 2, entry 3), positional isomers of respectively Le<sup>a</sup> and Le<sup>b</sup>, could only formally be related to Lewis antigen system because they are not important for Lewis serology, and in addition are synthesized by different set of glycosyltransferases. We observe no or very rare Ab to these antigens. Lack of auto-antibodies to them as well as to other fucosylated glycans with type 2 backbone such as SiaLe<sup>x</sup> and Su-Le<sup>x</sup> is in full concordance with their high occurrence as terminations in numerous human glycoconjugates, and with crucial role of SiaLe<sup>x</sup> in leukocyte recruiting. At the same time, their constituent, disaccharide Fuc $\alpha$ 1-3GlcNAc (entry 3, Table 2), is one of the highest-ranked glycans on our PGA (#150, see Table 1), whereas Ab to monosaccharide L-fucose are below cutoff. The absence of Ab to Le<sup>x</sup> trisaccharide and high response to partial disaccharide can be explained by conformational compactness of Le<sup>x</sup>. Indeed, fucose residue neighbors bulky Gal substituent here, which constrains the approach of anti-FucGlcNAc antibodies to the trisaccharide. Similarly, Ab binding to disaccharide Fuc $\alpha$ 1-4GlcNAc is exceptionally high, while minimal to no binding is observed to conformationally constrained oligosaccharides Le<sup>a</sup> and Le<sup>b</sup> which are the normal glyco-components in most individuals.

### 3.4. Blood group li and N-acetyl lactosamine derivatives

Blood group system li consists of two antigens. Linear glycan, Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc (i), in adults is substituted partially or completely by branched version, Gal $\beta$ 1-4GlcNAc $\beta$ 1-3(Gal $\beta$ 1-4GlcNAc $\beta$ 1-6)Gal $\beta$ 1-4GlcNAc (I) (Table 2, entry 4). Frequency of the ii phenotype is very low, <0.0001, however, frequency of individuals with anti-I Ab (i.e. auto-antibodies) is much higher, supposedly as a result of immune response to structurally related viral glycans and bacterial LPS [Feizi and Childs, 1978]. In a good agreement with these facts, we observe sharp bi-polar distribution of Ab binding to all of the oligolactosamines present on the array, i.e. we see very limited numbers of individuals with high Ab levels, as illustrated by Fig. 2E. Generally, N-acetyl lactosamine (LN), its oligomers as well as 3'- and 6'-sialylated derivatives proved to be in the group of 'silent' glycans, in accordance to their high occurrence on the surface of all normal cells. At the same time, Ab against many other Gal-terminated OS, such as Gal $\beta$ 1-2Gal, Gal $\beta$ 1-3Gal, Gal $\beta$ 1-3Gal $\beta$ 1-4GlcNAc, Gal $\beta$ 1-3GlcNAc (Le<sup>c</sup>), Gal $\beta$ 1-3GalNAc—as common fragment of TF, aGM1 and T $\beta$  $\beta$ , and even to monosaccharide  $\beta$ Gal have been revealed in normal sera. Notably, a 'monster' combination of two LN residues, when they are connected with an  $\alpha$ -bond, displays antigenic properties in contrast to normal  $\beta$ -connected variant, i-antigen; another close analog of i-antigen without one inner acetamido group, Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc (LNNT), displayed significant binding in PGA.

### 3.5. Real epitope specificity of antibodies to complex glycans

The dramatic difference of Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc vs. Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc takes more detailed consider-

ation, because analogously, replacement –GlcNAc  $\rightarrow$  –Glc leads to antigenicity in case of several other pairs, in particular H(type 2) vs. H(type 6), GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc vs. GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc, and Gal $\beta$ 1-4GlcNAc vs. Gal $\beta$ 1-4Glc. Moreover, addition of lactose motif to tetrasaccharides Le<sup>b</sup> or Le<sup>y</sup> at the reducing end, giving rise to Le<sup>b</sup>Lac or Le<sup>y</sup>Lac hexasaccharides (Table 2, entry 15), converts these structures to antigenic. We suppose that antibodies recognizing inner lactose-containing glycotopes are responsible for interaction with all these glycans. The lactose motif –Gal $\beta$ 1-4Glc is obviously non-alien for humans, as it is almost obligatory in composition of glycosphingolipids, so antibodies should not recognize it in composition of normal cells. We explain this non-recognition by masking of –Gal $\beta$ 1-4Glc fragment by a bulky ceramide residue in composition of glycosphingolipids, more exactly—by the obstructive neighboring membrane components. However, less constrained forms, as presented on PGA, are now 'visible' to these Ab.

The above data raise a general question concerning correct interpretation of direct binding of polyclonal Ab to complex oligosaccharides. For example, when we observe signal at Le<sup>b</sup>Lac spot on the PGA, does it mean that the serum actually contains anti-Le<sup>b</sup>Lac? Indeed, Ab to an inner Lac fragment could cause the same signal as true antibodies against entire Le<sup>b</sup>Lac hexasaccharide. In order to avoid such 'false-positive' interpretation, we synthesized Le<sup>b</sup>Lac-Sepharose affinity resin and isolated Ab from pooled serum in conditions of exhausting adsorption provided by an excess of the resin, followed by elution of affinity-bound antibodies. ELISA of eluted material with adsorbed Le<sup>b</sup>Lac hexasaccharide, Le<sup>b</sup> tetrasaccharide, Lac and several related glycans demonstrated that Ab to hexasaccharide do exist. Similar results showing a presence of Ab binding to entire complex oligosaccharides were obtained when this methodology [Korchagina et al., 2005; Lemieux et al., 1981; Obukhova et al., 2007] was applied to numerous except one tested glycans (Obukhova et al., in preparation). Thus, returning to the initial question about the correctness of interpretation of serum antibodies binding data, we can give rather positive answer.

### 3.6. Blood group P<sub>1</sub> and P<sup>k</sup>

Blood group P consists of three antigens, Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc (P<sub>1</sub>), Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc (P<sup>k</sup>, or Gb3), and GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc (P, or Gb4). Approximately 75% of normal individuals belong to blood group P<sub>1</sub> phenotype, they possess all three antigens of this blood group system and therefore should not have any antibodies against blood group P glycans. According to literature, the hemagglutination strength and the Ab titration scores are low [Bailly and Bouhours, 1995]. Two of three antigens, namely P<sub>1</sub> and P<sup>k</sup>, were included in our array. Unexpectedly, binding to both of these trisaccharides was rather high and unusually uniform for anti-blood group antibodies, with median 17.0 (MAD = 9.8) and 24.8 (MAD = 4.6), correspondingly, i.e. we observe anti-P<sup>k</sup> and anti-P<sub>1</sub> in practically all individuals as demonstrated in Fig. 2D. Thus, in contrast to discussed above PGA data on blood group systems ABH, Lewis and li, our results on P blood group antibodies are discordant with literature. To verify the epitope specificity, we have isolated Ab using P<sup>k</sup> affinity column and tested purified material. Firstly, the results demonstrate high specificity of Ab to cognate antigen, namely, Ab bind to Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc, whereas they bind neither to Gal $\alpha$ —nor to –Gal $\beta$ 1-4Glc glycotopes. Secondly, the antibodies proved to be not anti-NOR [Duk et al., 2003]. We suppose, that revealed antibodies are capable of recognizing oligosaccharide antigen in 'free' form but binding is constrained when the same saccharide in the glycolipid form, is located in plasma membrane,—a situation close to mentioned above H<sub>di</sub>/H<sub>tri</sub> and LN<sub>2</sub>/LNNT pair (entry 4 in Table 2). So, in order to explain tolerance of the Ab to self-

antigens more detail investigation is required. Noteworthy, P<sup>k</sup>/CD77 antigen in composition of erythrocytes is difficult for detection with antibodies [Furukawa et al., 2002].

### 3.7. Negatively charged glycans

Three types of negatively charged carbohydrates are present in our PGA: sulfated, sialylated and glucuronic acid-containing glycans. Overall below cutoff and sporadic binding to sulfated glycans was observed, with two exceptions. One of them, disaccharide Su-Le<sup>C</sup>, i.e. 3-O-Su-Galβ1-3GlcNAc, is 'all-round champion' between all the 205 glycans of the PGA. Interestingly, there is only a minimal binding to its fucosylated derivative Su-Le<sup>a</sup>, as well as to closely related disaccharides 3-O-Su-Galβ1-4GlcNAc and 3-O-Su-Galβ1-3GalNAcα (Table 2, entry 5). There are no structural data concerning occurrence of fucose-free Su-Le<sup>C</sup> fragment in composition of glycoconjugates, though it is expected to be a biosynthetic precursor of the well-known Su-Le<sup>a</sup> motif [Galustian et al., 1999] as it was revealed histochemically with the help of monoclonal Ab [Veerman et al., 1997]. Since type 1 core glycans such as Galβ1-3GlcNAc are generally less frequent in humans and in addition Su-Le<sup>a</sup> and SiaLe<sup>a</sup> are well-known tumor-associated antigens, the existence of auto-antibodies to type 1 structures is not surprising. What is truly amazing—is the level of antibody binding to both Le<sup>C</sup> and to Su-Le<sup>C</sup> (entry 6).

Besides Su-Le<sup>C</sup>, another sulfated glycan 4-O-Su-Galβ1-4GlcNAc occupies one of the highest ranks (entry 7). Specificity of the Ab seems to be high, as deduced from the minimal binding to isomeric sulfated glycans. Whether 4-O-Su-Galβ1-4GlcNAc occurs in human cells remains unclear, though related structure 4-O-Su-GalNAcβ1-4GlcNAc is known to be a termination of lutropin glycans that mediate the glycoprotein clearance by hepatic reticuloendothelial cell receptor [Fiete et al., 1991], and found as capping motif in several other glycoproteins [Fiete et al., 2007].

Next group of charged glycans includes molecules containing glucuronic acid, GlcA (entry 8). The presence of relatively high levels of antibodies to a disaccharide GlcAβ1-3Gal is expected and it could be well explained in the context of Springer's paradigm of intestinal bacteria, since glucuronic acid is an abundant component of bacterial polysaccharides [<http://www.glyco.ac.ru/bcsdb/start.shtml>]. On the other hand, this motif in composition of HNK-1 antigen 3-O-Su-GlcAβ1-3Galβ1-4GlcNAc is unlikely to be targeted by anti-GlcAβ1-3Gal antibodies, since it is masked with a sulfate group. The observed high level of antibodies against related GlcAβ1-3GlcNAc motif is unexpected, because this disaccharide is a repeating unit of highly abundant hyaluronic acid occurring in numerous serum and basement membrane glycosaminoglycans [An et al., 2006]. The observed lack of Ab to monosaccharide GlcA is also easy to explain: simple glycosides of glucuronic acid represent a wide group of metabolites, so there should be a tolerance towards the GlcA β-connected to simple aglycons.

Third and a wide-spread group of charged glycans consists of numerous sialo-molecules. Ab to most of α-sialosides (entry 9) have not been revealed, which is expected since Neu5Ac is presented only as α-anomer in nature. An exception is observed in case of a high Ab binding to a tumor-associated Neu5Acα2-6GalNAcα (SiaT<sub>n</sub>) antigen, and it can be explained by the drastic structural differences from all the other sialo-glycans, such as the presence of αGalNAc unit instead of regular βGal, and, additionally, proximity to peptide backbone.

On the other hand, high levels of Ab have been detected to all sialosides in β-configuration (described above as 'monsters'). This finding can also be explained by Springer's paradigm since Gram-negative bacteria contain LPS with βKDN- and/or βKDO which are very close analogs of Neu5Ac that are found in β-connected form [<http://www.glyco.ac.ru/bcsdb/start.shtml>].

Unexpectedly, we found minimal to none Ab to monosaccharide Neu5Gc and trisaccharide Neu5Gcα2-6Galβ1-4GlcNAc (entry 10), in spite of this form of neuraminic acid being alien to humans [Varki, 2001]. There are many reports on the presence of antibodies to Neu5Gc when in HD-antigen, with 2-3 bound glycolylneuraminic acid, Neu5Gcα2-3Galβ1-4Glc or Neu5Gcα2-3Galβ1-4GlcNAc [Merrick et al., 1978; Padler-Karavani et al., 2008] in humans. These 2–3 bound glycans are absent in our array, but both of them are presented in the array of Consortium for Functional Glycomics (CFG) [[www.functionalglycomics.org](http://www.functionalglycomics.org)], and CFG data confirm low levels of Ab to all Neu5Gc glycans except for SiaLe<sup>C</sup> analog, Neu5Gcα2-3Galβ1-3GlcNAc. Finally, only Ab to Neu5Gcα2-6GalNAcα (Neu5Gc-T<sub>n</sub>) were detected on our array at the rather high levels, which were close, for example, to the levels of anti-A<sub>tri</sub> and anti-P<sup>k</sup>, however, binding to Neu5Gc-T<sub>n</sub> proved to correlate statistically with binding to Neu5Ac-T<sub>n</sub>, thus, these Ab are likely to actually be anti-Neu5Ac-T<sub>n</sub>.

### 3.8. GlcNAc-terminated carbohydrate chains

Glycans with terminal βGlcNAc residue are widely represented in our array. The molecules with the highest Ab-binding rank (Table 2, entry 11), are chito-oligosaccharides, a fragment of bacterial peptidoglycan GMDP-Lys, and its peptide free form. High level of Ab has also been observed to monosaccharide βGlcNAc. Certainly, Ab to βGlcNAc are not auto-antibodies since βGlcNAc termination is not typical for human glycans, most of which are capped with Gal or more complex moiety. Nevertheless, terminal GlcNAcβ occurs in mammalian glycans, such as: (1) bisecting N-chains, (2) *agalacto* forms of biantennary IgG glycans, typical for pathology-associated IgG, (3) minor N-chains of several glycoproteins [Yoon et al., 2007], (4) GlcNAc-terminated O-chains that are represented by incompletely glycosylated cores 2, 3, 4 and 6, and finally, as (5) GlcNAcβ1-OSer in composition of numerous intracellular glycoproteins. In our array Ab of normal donors bind to GlcNAc but not to the *agalacto* heptasaccharide fragment of biantennary N-chain with core chitobiose and the two terminal GlcNAc residues (entry 11, Table 2).

Pathogenic 'anti-GlcNAc' Ab have been observed in rheumatoid arthritis [Parekh et al., 1985] and other autoimmune diseases, however, a fine specificity of these Ab remains unclear. The level of anti-GlcNAc is also increased after streptococcus (group A) infection [Briko et al., 1997]. Streptococcus core polysaccharide chain bears pendant GlcNAc substituents, and this bacterium is an immunogen causing formation of anti-GlcNAc antibodies in humans. GlcNAcβ polymers are also known as typical components of bacterial biofilms.

Apart from above mentioned Ab to β1-4 connected oligomers of GlcNAc and bacterial peptidoglycan fragment, our array reveals binding to several auto-antigens (Table 2, entry 12), particularly, GlcNAcβ1-3Man, as well as cores 2, 3, 4 and 6 of O-chains. Notable is a high median binding to core 4 trisaccharide in contrast to minimal binding to Tk tetrasaccharide, despite a shared trisaccharide motif (see structure in Table 2, entry 12).

### 3.9. Other glycans

Antibodies to manno-OS, at least to those included in the array, have not been observed (entry 13), in agreement with occurrence of high concentration of mannose-rich N-chains in composition of immunoglobulins, see below. Single exception is a disaccharide Manβ1-4GlcNAc with a high binding rank, but mannose residue is here connected in a β-form, in contrast to all other glycans in our array. Close structural similarity to GlcNAcβ1-4GlcNAc and statistical correlation ( $r = 0.45$ ,  $CI_{95\%} = [0.28, 0.59]$ ) suggest that antibodies binding to Manβ1-4GlcNAc might recognize an inner part of this disaccharide rather than its terminal part.

Glc $\alpha$ 1-6Glc and Glc $\alpha$ 1-6Hex are common outer core motifs for bacterial lipid A [Knirel, 1990]. Nevertheless, we observe only low levels of Ab to oligosaccharides [Glc $\alpha$ 1-4] $_n$  ( $n=3$  and more) and [Glc $\alpha$ 1-6Glc] $_n$  (entry 14, Table 2) in PGA, perhaps because they are regular fragments of mammalian polysaccharide glycogen. At the same time, there are antibodies binding to  $\beta$ -glucose-terminated oligosaccharides, as expected from the wide occurrence of corresponding gluco-polysaccharides of bacteria and yeast [<http://www.glyco.ac.ru/bcsdb/start.shtml>] and in an agreement with the literature [Schwarz et al., 2003].

#### 4. Discussion

In this study, we have used a standardized glycan array including 205 well defined, high-purity carbohydrate structures to characterize the individual carbohydrate recognition patterns by antibodies in sera of 106 healthy donors. The glycan library included blood group antigens and other most frequent terminal oligosaccharides and their cores of mammalian N- and O-linked glycoproteins and glycolipids, tumor-associated carbohydrate antigens, and common components of bacterial/pathogenic polysaccharides and lipopolysaccharides. As demonstrated by the PGA, sera of healthy individuals interact with at least 50 normal human glyco-motifs. Apart from well-known blood group-, xeno- (heterophile) and infection-related binding activities we observe a number of new and unexpected features. These “surprises” include (1) relatively high levels of antibodies binding to the blood group P $_1$  and P $^k$  trisaccharides, (2) antibodies to the blood group H glycans and (3) the presence of antibodies recognizing a short inner core of glycolipid or glycoprotein as a fragment of the larger glycan structure. As to certain expected high binding intensities, Gal $\alpha$ 1-3Gal xenoantibodies occupy rather modest position, conceding, for instance, to anti-Fs and anti-GalNAc $\alpha$ 1-3Gal antibodies by factor of 4 and 3, respectively; similarly, blood group B and especially A antibodies also achieve unexpectedly low rank. Completely novel activities are found towards Le $^c$  related glycans, especially 3-O-Su-Gal $\beta$ 1-3GlcNAc, and also 4'-O-sulfated lactosamine. High and uniform levels of Ab binding to GalNAc $\alpha$ 1-3Gal disaccharide demonstrate absence of correlation with fucosylated blood group A antigens—similarly to earlier reported lack of relationship between Gal $\alpha$ 1-3Gal and true blood group B antigens. Since the current study group of 106 donors is considered to be representative of “normal, healthy” general population, reported above features of their immunoprofiles may be considered as relating to the innate, NAb-type rather than to antibodies generated as the result of the cognate, or acquired, immunity. Yet, when comparing individual immunoprofiles of 106 healthy donors, we observe both striking individual features as well as certain regularities. This is a direct demonstration of advantage offered by “immunoprofiling” using PGA that allows identification of potentially biologically and clinically significant individual features that are otherwise undetectable in antibody samples obtained through affinity-purification from pooled sera. While the individual features reflect better specificities and the dynamics of the personal immuno-status, the regularities represent largely antibodies that were inspired by the similar immunogens. Noteworthy, minor and sporadic if any, binding of antibodies has been observed to oligosialic motif, and type 2 backbone glycans such as Le $^y$  and SiaLe $^a$ , many of which are known to be tumor-associated antigens.

Antibodies to biantennary N-chain, (Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man $\alpha$ ) $_2$ -3,6-Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc, as well as to its truncated asialo- and agalacto-versions have not been observed at all. Complex-type glycans, as well as manno-OS (also sporadic and low-binding), are typical for human IgG that represent about one third of all serum proteins [Arnold et al., 2007], i.e. concentration of biantennary complex-type and mannose-rich

glycans in serum is extremely high. If NAb to biantennary or mannose chains do exist, their content is many orders of magnitude less than content of the glycans, and thus their binding to cognate glycans on a chip should be ultimately inhibited. In other words, there is no need for a hypothesis suggesting a selective B-cell deletion [Dighiero and Rose, 1999] in order to explain the visible lack of antibodies to various complex-type and mannose-rich N-glycans, since these Ab can exist in the same medium with cognate cell/tissue antigens without any risk of autoimmune reaction while buffered by glycans of circulating IgG and other major glycoproteins. Proposed buffering mechanism looks very plausible: diverse enterobacterial polysaccharides induce wide repertoire-anti-carbohydrate response so necessary for efficiency of innate immunity, whereas specific ‘gap’ in this comprehensive pattern is created by glycans of immunoglobulins. Glycan repertoire of IgG, IgM, IgD and secretory IgA are different, with more than a hundred of well-characterized N- and O-chains found in these Ig types [Arnold et al., 2007]. Lack of visible binding to Le $^x$  and other type 2 structures (see Section 3) could also be explained by their presence as a component of IgA glycosylation. Thus, selective masking of undesirable auto-antibodies is a one more general function of Ig carbohydrate chains, in addition to their numerous other functions (see [Arnold et al., 2007] for the review). Low (~5%) sialylation and high level of agalacto-glycans distincts a repertoire of IgG complex N-chains from most of other glycoproteins. Possibly, these unusual glycoforms have been destined for selective buffering of self-reactive ‘anti-galactose’ and ‘anti-N-acetylglucosamine’ auto-antibodies.

Several proposed stimuli causing formation of glycan auto-antibodies were mentioned above, namely: Gal $\alpha$ 1-4GlcNAc (*Bacillus*), Ii (mycoplasma, viruses), GlcA (numerous bacteria), Neu5Ac $\beta$  (bacterial KDN/KDO), GlcNAc $\beta$  (O-polysaccharide of *Streptococci*, peptidoglycan of Gram-positive bacteria, biofilms), and Glc $\beta$  (numerous bacteria and yeast). Here, we discuss agents rising other, highly binding auto-antibodies revealed by PGA. The genesis of anti-Fuc $\alpha$ 1-3/4GlcNAc Ab can be explained in several ways. Firstly, humans acquire anti-Fuc $\alpha$ 1-3GlcNAc in response to an exposure to *Helicobacter pylori*, LPS of which is known to have Le $^x$  or Le $^y$  termini. However, several *H. pylori* strains possess Fuc $\alpha$ 1-3GlcNAc variant, without galactose substituent in position 4 of GlcNAc [<http://www.glyco.ac.ru/bcsdb/start.shtml>]. Secondly, Fuc $\alpha$ 1-3GlcNAc motif, so called horseradish peroxidase (HRP)-epitope, is typical for various parasites as a core of their glycoprotein N-chains, whereas humans possess exclusively Fuc $\alpha$ 1-6GlcNAc fragment instead [Paschinger et al., 2009]. Thirdly, arabinopyranoside-terminated PS, widely spread in bacteria, could induce Ab to fuco-OS motifs since D-arabinose is a stereochemical analog of L-fucose by all chiral atoms except C5. Binding of Ab against 4-O-Su-Gal $\beta$ 1-4GlcNAc is also in the top rank, as mentioned above. Importance of these antibodies as an element of innate immunity is expected to be significant since their enormously high level (alternatively, quantity is modest but affinity is outstanding, which cannot be determined at present, since we have only ‘integral’ data of the solid phase assay). Sulfate group in composition of 4-O-Su-Gal $\beta$ 1-4GlcNAc is key point: LacNAc without sulfate or with sulfate in different position found to be low binders. Bacterial stimuli capable of generating antibodies against 4-O-Su-Gal $\beta$ 1-4GlcNAc remain unknown; only one bacterial PS with 4-O-Su-Gal $\beta$  fragment and 17 structures (mostly, *Salmonella pneumoniae*) with -P-4-Gal $\alpha$  (a possible mimetic) are described in bacterial polysaccharide databank [<http://www.glyco.ac.ru/bcsdb/start.shtml>]. Perhaps, we should search for a causative antigen in other, not bacterial, field. Ab of the highest rank observed here are directed against Su-Le $^c$  and SiaLe $^c$ , i.e. sulfated and sialylated forms of Le $^c$  disaccharide substituted at position 3'. The level of antibodies (RFU value in the PGA) to unsubstituted Le $^c$  is also high but substantially lower as compared

to the charged versions. The impact of sulfate moiety itself on the binding affinity is not so large, as determined by epitope profiling of Ab isolated with Le<sup>C</sup>-Sephacrose resin from the pooled 20 sera of healthy individuals. The Ab recognized equally Le<sup>C</sup>, SiaLe<sup>C</sup> and Su-Le<sup>C</sup>, and less but at the comparable level—also a monosaccharide GlcNAc $\beta$ . So, the determinant part of this glycotope is situated in a core fragment of the antigen.

Analysis of data presented in Table 1 allows identification of several other auto-antibodies directed to a core part of oligosaccharide chain. We have already discussed above antibodies capable of recognizing –Lac fragment, or, rather more sophisticated –Lac containing glycotope in composition of an X-X-X-Lac oligosaccharide (entry 15, Table 2). Similarly, inspection of Ab binding to glycans with –GalNAc $\alpha$  at the reducing end revealed numerous examples when this fragment functions as antigenic determinant of a large oligosaccharide, in spite of ‘obvious’ masking, as discussed for core 4/Tk (entry 12, Table 2). According to our estimation, serum antibodies do bind to core fragments of up to 1/3 of ‘high binding’ glycans in the PGA, therefore this phenomenon seems to be general, and in contradiction to a conventional paradigm stating that terminal fragment of glycan possesses antigenic properties. If so, what is the target of anti-core antibodies? Intriguingly, most of tumor-associated carbohydrate antigens are glycolipids with core –Lac fragment or glycoprotein O-chains with core –GalNAc $\alpha$  fragment [Hakomori, 1984]. Therefore, we hypothesize a surveillance role of anti-core NAb, for identification of: (1) tumor-specific membrane-bound architectures where normal glycolipids in combination with adjacent normal molecules form abnormal glycotopes, and (2) aberrant conformational glycotopes formed by –GalNAc $\alpha$  in combination with adjacent peptide chain fragments. Overview of wide repertoire of PGA-detected antibodies in light of known chemical structures of bacterial polysaccharides [<http://www.glyco.ac.ru/bcsdb/start.shtml>] suggests immediately that hypothesis about enterobacteria being a driving force for anti-carbohydrate NAb [Springer and Horton, 1969] is grossly incomplete, and that it should be complemented at least by a hypothesis proposing a contribution of anti-glycan NAb in surveillance over sporadically appearing transformed cells – recognizing and eliminating cells with abnormal cell surface glycosylation [Shoenfeld, 1989]. Such combined hypotheses would then create a foundation of a search for the origin of identified in this work antibodies against glycan core motifs, certain sulfated oligosaccharides, as well as to other numerous glycans present on the array and not known previously as biologically active structures.

When our manuscript was completed, a publication reporting Ivlg tested with similar glycoarray appeared [von Gunten et al., 2009]. Many activities were found similar, although only binding of IgG was investigated. This observation confirms the general finding of our study demonstrating that there is a magnitude of antibodies capable of interacting with unexpectedly large variety of glycotopes.

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## Glossary

- NA*: natural antibodies.  
*CI*: confidence interval.  
*PGA*: printed glycan array.  
*OS*: oligosaccharide.  
*MAD*: median absolute deviation.  
*PS*: polysaccharide.  
*Sia*: sialic acid.  
*Su*: O-sulfate residue.  
*P*: O-phosphate residue.  
*RFU*: relative fluorescence units.  
 see also abbreviations in tables, and glycan structures in Table 1.