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GLYCOARRAY SECTION

# Printed glycan array: antibodies as probed in undiluted serum and effects of dilution

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**Abstract** Using printed glycan array (PGA) we compared the results of antibody profiling in undiluted, moderately (1:15) and highly (1:100) diluted human blood serum. Undiluted serum is suitable for studying blood as a tissue in its native state, whereas to study the serum of newborns or small animals one usually has to dilute the starting material in order to have sufficient volume for PGA experimentation. The PGA used in this study allows for the use of whole serum without modifications to the protocol, and the background is surprisingly low. Antibodies profiles observed in undiluted serum versus 1:15 dilution were similar, with only a limited number of new signals identified in the undiluted serum. However, unexpected irregularities were found when IgG and IgM are measured separately, namely, at a 1:15 dilution more intensive IgG signals for many glycans are observed. We believe that in conditions of moderate dilution IgG and IgM antibodies can compete with each other for antigen and as a result, the higher affinity anti-glycan IgGs give rise to more intense signals. Therefore depending on the purpose, different dilutions of serum will be optimal: in competitive 1:15 conditions the observed IgG/IgM ratio corresponds to their titer, whereas at 1:100 dilution the measured ratio corresponds to real molar concentration of IgG and IgM.

**Keywords** Printed glycan array · Anti-glycan antibodies · Serum dilution

**Abbreviations**
PGA Printed glycan array
Abs Antibodies

## Introduction

The study of human blood serum using printed glycan arrays (PGA) [1] reveals a wide repertoire of natural anti-glycan antibodies (Abs) [2]. When aiming to study blood as a tissue it is probably more correct to probe it in its native undiluted state. On the other hand, when the amount of available sample to study is very low, *e.g.* serum of newborns or small animals it is necessary to dilute the starting material to a volume sufficient for analysis. In this study we have compared the results of PGA antibody profiling in: 1) undiluted, 2) moderately diluted (1:15) and 3) highly diluted (1:100) human blood sera.

## Methods

Two hundred seventy three glycans of 95–98% purity (Lectinity Holdings, Moscow, Russia) were printed at 50 uM concentrations, as 16 replicates each onto NHS-activated

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slides H (Schott AG, Mainz, Germany) and blocked as described previously [1]. The glycochips were incubated with the pool of sera from 15 blood group O donors (1:15 or 1:100 dilution in PBS/3%BSA/1%Tween-20, or without dilution in the presence of Tween-20) with gentle rocking for 1.5 h at 37°C. Serum antibodies bound to printed glycans were visualized with secondary goat antibodies against human IgG or IgM labeled with Alexa 647 (Invitrogen, USA). Fluorescence signal intensities were collected at 65% laser power and 53% PMT gain with 5 mm scanning resolution and quantified with ProScanArray v. 4.0 software (PerkinElmer, USA) using a "fixed circle" method with 70 mm diameter.

## Results and discussion

Surprisingly, very low background signal is observed for the undiluted samples – equal to that observed for the 15-fold dilution. In spite of its 15-fold higher concentration, only a limited number of new "responding" glycans appeared with the undiluted serum; Fig. 1(a, b) gives a general overview, whereas Fig. 1c gives specific examples. Antibodies (IgG, IgM or both) "appearing" in undiluted serum are presented in Table 1; most of the glycans were found to be sulfated or sialylated molecules.

Our experience of serum antibodies profiling with ELISA suggests that it is principally impossible to work with undiluted serum whereas the reasonable signal/background ratio is achieved at dilution 1:50 and higher. Presumably, the key role in the excellent properties of the PGA assay is played by the dense polyethylene glycol hydrogel acting as an interface between the glass surface and glycan residues. A consequence of very low background level is a possibility of reliable detection of low affinity and/or less presented Abs (see Fig. 1c). At the same time due to very wide dynamic range of the assay correct quantitation of highest rank antibodies is possible as well.

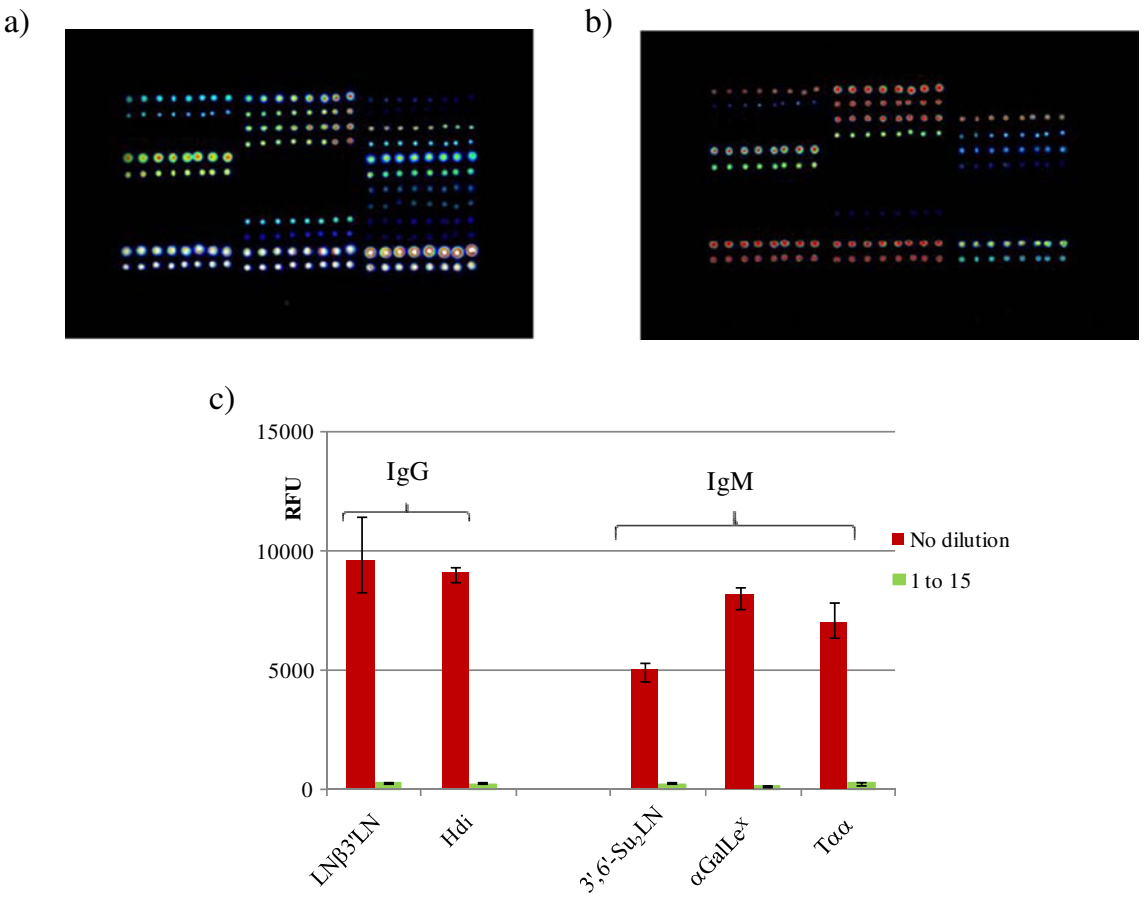


**Fig. 1** PGA analysis of serum IgG aliquots without dilution (**a**) and 1:15 dilution (**b**), all the other experimental conditions are the same; a part of the chip image in rainbow palette is shown. **c** Examples of glycans demonstrating binding only in undiluted sample, Tαα is Galα1-3GalNAcα

**Table 1** Glycans displaying human serum antibodies binding only in undiluted serum (in addition to five glycans displayed on Fig. 1c)

| glycan | abbreviation | IgG | IgM |
|---|---|---|---|
| 3-*O*-Su-Galβ | 3-*O*-Su-β-Gal | + | + |
| 3-*O*-Su-Galβ1-4GlcNAcβ | 3′-*O*-Su-LacNAc | + | + |
| 6-*O*-Su-Galβ1-4GlcNAcβ | 6′-*O*-Su-LacNAc | + | + |
| 3-*O*-Su-Galβ1-4(6-*O*-Su)GlcNAcβ | 3′,6-di-*O*-Su-LacNAc | + | + |
| 6-*O*-Su-Galβ1-4(6-*O*-Su)GlcNAcβ | 6,6′-di-*O*-Su-LacNAc | | + |
| Galβ1-4(6-*O*-Su)GlcNAcβ | 6-*O*-Su-LacNAc | | + |
| 3-*O*-Su-Galβ1-4 (Fucα1-3)GlcNAcβ | Su-Le$^x$ | + | |
| Neu5Acα2-3(6-*O*-Su)Galβ1-4(Fucα1-3)GlcNAcβ | 6$^{(Gal)}$-Su-SiaLe$^x$ | + | + |
| Neu5Gcα | Neu5Gc | + | |
| Neu5Acα2-6Galβ1-4Glcβ-Gly | 6′SL | | + |
| Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ | LNβ6′LN | | + |
| Galβ1-4GlcNAcβ | LacNAc | + | + |
| GlcNAcβ1-3Galβ1-4GlcNAcβ | GlcNAcβ3′LacNAc | + | + |
| (Glcα1-4/6)$_n$β-Gly | malto-/isomalto-OS | | + |
| Galβ1-4(Fucα1-3)GlcNAcβ | Le$^x$ | + | + |
| Fucα1-4(Fucα1-2Galβ1-3)GlcNAcβ | Le$^b$ | | + |

We have previously found [3] that the concentration of anti-glycan IgM in blood is several fold higher than that of IgG, and that IgM affinities are much lower. Thus it is reasonable to expect competition between IgM and IgG for glycans in PGAs [4].

Antibody patterns for three concentrations, undiluted serum, regular 1:15 dilution, and high dilution 1:100 were compared. With the exception of the degree of dilution all the other conditions (including the sample incubation time on chip) were the same. Comparison of data sets for these three concentrations shows that IgG binding to glycans decreases with dilution (Fig. 2a) whereas IgM signals at dilution 1:15 were unexpectedly low compared with 1:100 signals (Fig. 2b). More demonstrative were the IgG/IgM ratios: for ~20% of glycans at dilution 1:15 they were higher than for 1:100 dilution and undiluted sera (Fig. 3). The most representative in this unusual behavior were antibodies to blood group related antigens (left flank of Fig. 3), and antibodies to bacterial cell wall fragments (right flank).

We explain this phenomenon by the optimal conditions for competition between immunoglobulins G and M in case of dilution 1:15 – in favor of higher affinity IgG. We believe that optimal stoichiometry between immobilized glycan and antibody in solution [4] is important.

Indeed, the maximum value for glycan densely immobilized on surface as one spot is 30 fmol of glycan. The content of monospecific anti-glycan Ig (top or medium rank) per 1 mL of blood serum is about 1 μg [3]. A simple calculation shows that this is a considerable molar excess of antibodies related to immobilized glycan. In case of 1:100 dilution the situation is opposite: the amount of immobilized glycan is about one order of magnitude higher than the amount of cognate antibodies. This results in the absence of competition between IgG and IgM: all antibodies both IgG and IgM are captured by the excess of the antigen. The detected IgG/IgM ratios approximately correspond to that in blood – as in case of undiluted sample (Fig. 3). Only at moderate dilution (1:15), there are favorable conditions for competition resulting in preferable binding of higher affinity and smaller sized (and thus more mobile) IgG.

In four cases signals of IgM at dilution 1:100 were found to be much higher than at dilution 1:15 (Fig. 2b). From these four anti-GMDP-Lys and anti-6-Su-chitobiose can be regarded as adaptive rather than as natural antibodies as they are directed to common bacterial wall peptidoglycan, *i.e.* must be high affinity IgG antibodies; this is in accord with favored conditions for antibody competition at moderate serum dilution.

## Conclusions

The PGA version used here [1] allows for studying anti-glycan antibodies in whole serum without any additional reagents or modifications of the protocol. Antibodies profiles observed in 1:15 diluted vs. undiluted serum were similar. Dramatic differences in results were observed at different serum dilutions when IgG and IgM are measured separately. In conditions of

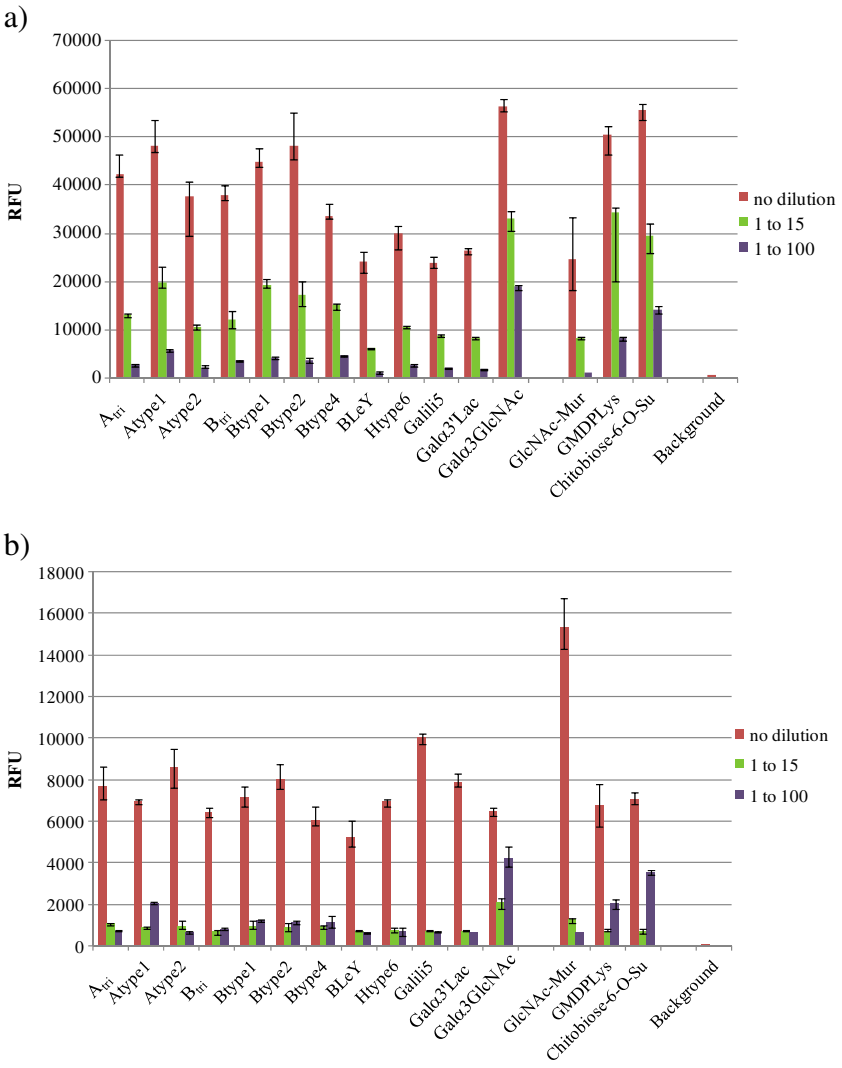


**Fig. 2** Binding of **a** IgG and **b** IgM with selected glycans at three serum dilutions. Galili5 is Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, Mur is muramyl, GMDP is glucosaminyl muramyldipeptide. Signals in glycan-free zones are shown as background

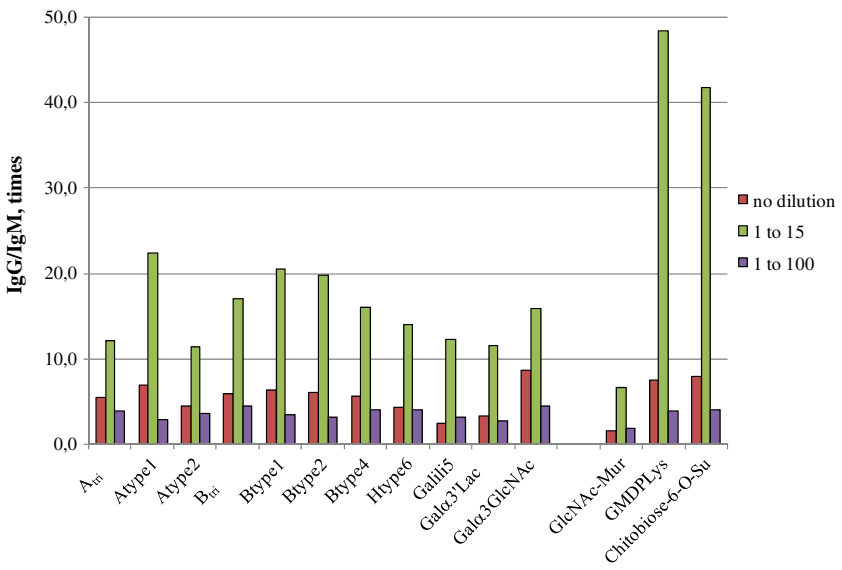


**Fig. 3** IgG/IgM signal ratio for the glycans shown on Fig. 2. The value is done in arbitrary units because affinities of secondary anti-IgM and anti-IgG are different

moderate dilution the IgG and IgM antibodies appear to compete for cognate antigen and due to this competition the observed IgG/IgM ratio corresponds to their titer (*i.e.* factor of concentration *x* affinity). At 1:100 dilution both IgG and IgM have a possibility to bind antigen, and thus the measured ratio corresponds to their real molar concentrations. We believe that for diagnostic purposes the measurement of the high affinity antibodies is most relevant, so competitive conditions are preferable.

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