



Research article

Incidence in plasma of low level antibodies against three xenotransplantation and immunotherapeutic glycan antigens

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Abstract: Antibodies against xeno-glycan antigens terminating with the saccharides Gal α , GalNAc α and Rha α are ubiquitous in human blood. Although originating as barriers to infection some of these naturally occurring complement-activating antibodies also contribute to disease processes, hinder xenotransplantation and have potential medical roles in immuno-oncotherapy. Because concentration of antibody is important in determining biological activity, there is a need to understand population variation in naturally occurring antibody levels, and to be able to rapidly and accurately determine levels in individuals. Xeno-glycan antigens in the form of function-spacer-lipid constructs were used to modify human red cells (kodecytes) to have on their surface micromolar equivalents of the xeno-glycan antigens Gal α 1-3Gal β 1-4GlcNAc, GalNAc α 1-3Gal β 1-4GlcNAc and Rha α . The methodology used was based on a previously validated kodecyte method used for quantifying IgM and IgG ABO human blood group antibodies in undiluted plasma. We tested plasma samples from 100 healthy individuals against these three different xeno-glycan kodecytes with each at three different loading concentrations of antigen to determine relative levels of these antibodies in human plasma. Sixty-one samples were also independently tested by enzyme immunoassay to correlate levels of anti-Gal α . Results demonstrate independence between antibody specificities and substantial variation between individuals in levels of these antibodies, with >92% of the population having medium or high levels of at least one specificity. However, of particular importance was that 5–8% of the population had low levels of both IgM and IgG to at least one specificity and these individuals would probably have a poor immediate response when challenged by the corresponding antigen.

Keywords: xeno-glycan antigens; function-spacer-lipid constructs; kodecytes

1. Introduction

There are a large range of natural antibodies present in blood, most of which are ubiquitous in humans [1–5]. Many of these antibodies are directed against non-human carbohydrate (xeno-glycan) antigens [1–5] present on microbes [2,5,6], pollen [7], parasites [8] and insects [9], which are probably the source of their initial stimulation and continued maintenance [2,6]. Together this wide-ranging repertoire of xeno-glycan antibody specificities [1,4] contributes to a continuous and effective barrier against attack from pathogens, particularly those of microbial origin [2,5]. Although many individual glycan antibody specificities are against unknown targets [5], a few are of well recognized specificity; such as those directed against the ABO blood group system antigens [10] and the xeno-glycan antigen Gal α 1-3Gal β 1-R [11]. With the exception of ABO most anti-glycan antibodies are not routinely screened. However, with some xeno-glycan antibody specificities now recognized for their role in wound healing [12,13], vaccine augmentation [14,15], oncology diagnostics [16,17], immuno-oncotherapy [15,18,19], as biomarkers of autoimmune and inflammatory disease [20,21] and barriers to xeno-transplantation [15,22], there is a need to easily and accurately quantify an individual's specific anti-glycan profile. There are several reports measuring xeno-glycan antibodies in the general population [1,4,23], primarily using solid phase arrays which require specialized laboratory equipment and procedures [1,4,16–18,20,23–26]. The ability to use function-spacer-lipid constructs to modify cell membranes to create cells (kodecytes) with precise and equimolar levels of chemically defined glycans [27–29], and then use them to quantify antibody levels in undiluted plasma on routine serological platforms is an alternative and validated approach [28]. We made three different xeno-antigen kodecytes with human red blood cells (RBC) and quantified xeno-glycan antibodies against trisaccharides Gal α 1-3Gal β 1-4GlcNAc and GalNAc α 1-3Gal β 1-4GlcNAc, and monosaccharide L-rhamnose (Rha), present in human plasma. We also compared the kodecyte method with enzyme immunoassay (EIA)-determined levels of antibody to Gal α 1-3Gal β 1-4GlcNAc. The primary aim of this study was to evaluate if human RBCs modified with xeno-glycan antigens are able to quantify relative levels of antibody in undiluted plasma using standardized routine blood typing equipment, as can be performed with ABO antibodies [28].

2. Materials and methods

2.1. Xeno-glycan kodecytes

Terminology used to describe and synthesize Kode Technology constructs and kodecytes is as reported elsewhere [27,30]. Function-spacer-lipid (FSL) constructs FSL-Gal α 1-3Gal β 1-4GlcNAc, FSL-GalNAc α 1-3Gal β 1-4GlcNAc, and FSL-Rha α were supplied by Kode Biotech Materials, Auckland New Zealand (see Figure 1 for a description and MW of these constructs). The purity of initial chemically synthesized glycans and the final FSL constructs were confirmed by 700 MHz NMR and HPLC as > 95% pure. Dilutions (40, 10 and 0.5 μ mol/L) of FSL constructs were made to prepare kodecytes from fresh blood group O RBC (with the resultant kodecytes referred as 40-, 10- and 0.5-kodecytes) as previously described [27,30]. The quality of the insertion of the FSL constructs

and their quantitative dilutions in kodeocytes was unable to be validated due a lack of standardized antibody reagents, but serological results were consistent with expected quantitative insertion [28]. Kodeocytes in preservative solution CellStab (BioRad, Cressier, Switzerland) are stable and were stored for up to 6 weeks at 4 °C [28].

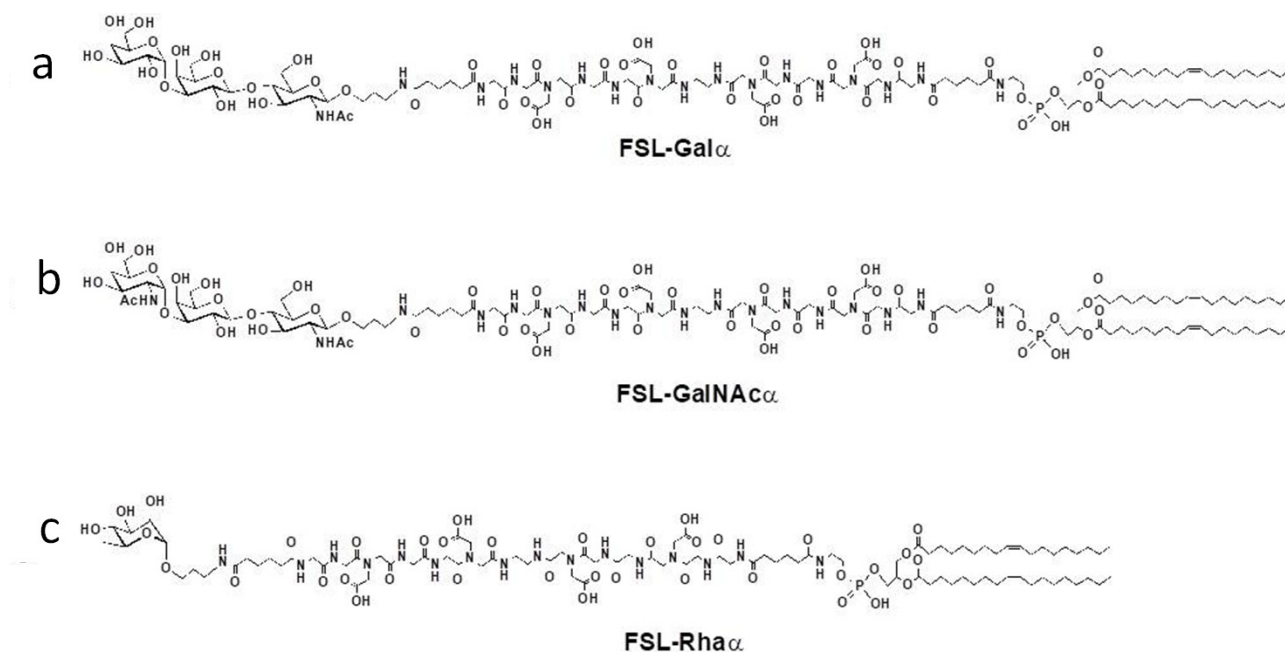


Figure 1. Gal α 1-3Gal β 1-4R, GalNAc α 1-3Gal β 1-4R and Rha α function-spacer-lipid (FSL) constructs. These FSL constructs were used in equimolar concentrations to prepare kodeocytes with a group O RBC. All FSL constructs have the same carboxymethylglycine spacer and a 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) lipid tail. Shown are the constructs without sodium, MW given are for the FSL constructs as supplied with sodium salts. a: FSL-Gal α , FSL-Gal α 1-3Gal β 1-R, MW 2654. b: FSL-GalNAc α , FSL-GalNAc α 1-3Gal β 1-R, MW 2695. c: FSL-Rha α , FSL-L-Rha α -R, MW 2272.

2.2. Samples

Random plasma samples from 100 group O blood donors were provided by the New Zealand Blood Service. Ethnicity and age of donors was not available as samples were provided unlinked under ethical approval (AUTEC 15/366), although the samples would be expected to be predominantly of European ancestry within the age range of 16–65 years. RBC from a single blood group O donor were used to make all kodeocyte preparations.

2.3. Antibody testing—kodeocytes

Antibody testing was performed by manual serology in test tubes by standard and validated blood bank procedures [31] as previously described [28]. All 100 samples were unreactive with the unmodified cell used to prepare the kodeocytes, indicating reactivity when observed was due only to the kodeocyte xeno-glycan antigen. The procedure involved adding 50 μ L of a 4% suspension of each

kodeocyte (equivalent to 2 μ L of packed cells) to 100 μ L aliquots of each test plasma. Reactants were incubated at 37 °C for 1 h then briefly centrifuged (ImmuFuge, American Dade, Miami-Dade, Florida). Saline (IgM) activity was determined as positive or negative by direct observation of agglutination with a 10 \times magnification eyepiece [31]. According to accepted practice direct saline agglutination reactions were considered as being predominantly due to IgM content, with the caveat that IgG could potentially contribute to direct agglutination [32]. The indirect anti-IgG assay (IAA) for the detection of IgG was only performed when the direct saline IgM reaction was negative; as existing positive IgM reactions mask the underlying IgG reactions. Negative saline reactions were washed four times in phosphate buffered saline (isotonic) followed by addition of 50 μ L of anti-IgG (bioCSL, Melbourne, Australia) and centrifugation. IgG activity was determined as positive or negative by observation of agglutination with a 10 \times magnification eyepiece.

Antibody levels were reported as high (+++), medium (++) or low (+) depending on their reaction patterns against three different kodeocyte preparations (based on established and validated criteria using kodeocyte reactivity profiles) [28]. The low antigen level 0.5 μ mol/L kodeocytes were chosen to react only with high levels of antibody, while the medium antigen level 10 μ mol/L antigen level kodeocytes reacted with a broad range of medium to medium-high levels of antibody. The high antigen level 40 μ mol/L kodeocytes were chosen to react with low levels of antibody. If a plasma reacted with all kodeocytes (0.5, 10 and 40 μ mol/L) the antibody level was deemed high (+++); if it reacted with both the 10 and 40 μ mol/L but not the 0.5 μ mol/L kodeocytes it was deemed medium (++) and if it only reacted with the highest strength 40-kodeocytes then it was deemed low (+). If the sample did not react with any kodeocyte the antibody level was reported as undetectable.

2.4. Antibody testing—EIA

Solid phase EIA using immobilized polyacrylamide Gal α 1-3Gal β 1-4GlcNAc (070-PA; GlycoNZ, Auckland) was performed as previously described [33] to quantify IgG and IgM anti-Gal α levels in 61 of 100 samples, where sufficient sample was available.

3. Results

3.1. Saline reactivity—IgM activity—kodeocytes

The overall distribution of high, medium, low and undetectable saline (IgM) reactions for the 100 samples is shown in Table 1. The Rha α -kodeocytes showed the highest level of reactivity with 12 samples having high levels of antibody. When combined with the 74 medium level results a total of 86% of samples showed medium or greater levels of anti-Rha α (Table 1). The GalNAc α -kodeocytes revealed high level antibody with 5 samples. When combined with the 84 samples giving medium reactions a total of 89% of reactions were medium or greater. Similarly Gal α -kodeocytes had 4 high level antibody results and when combined with medium results 79% of samples had medium or greater levels of antibody (Table 1). The single sample to be saline non-reactive with any 40-kodeocyte was directed against Gal α kodeocytes (Table 1). Analysis of specificity combinations revealed that 54 samples had medium levels of antibody with all three kodeocytes (Table 2) and 97 had at least one medium reaction against any one of the three different specificities (Tables 2 and 3).

Table 1. Differentiation of 100 plasma samples into high, medium and low antibody levels by saline (IgM) technique.

Antibody level	Kodecyte reactivity	Kodecyte defined antibody levels		
	FSL $\mu\text{mol/L}$	Gal α	GalNAc α	Rha α
High (+++)	0.5 + 10 + 40	4	5	12
Medium (++)	10 + 40	75	84	74
Low (+)	40	20	11	14
Undetectable (0)	-	1	0	0

Table 2. Number and kodecyte profiles of samples without a low level antibody.

n	Saline reactive kodecytes—IgM		
	Gal α	GalNAc α	Rha α
54	++	++	++
1	+++	++	++
1	+++	+++	++
3	++	+++	++
1	++	+++	+++
1	+++	++	+++
10	++	++	+++

++: Medium antibody levels; +++: High antibody level.

Table 3. Number and kodecyte profiles of samples with one or more low level antibody.

n	Saline reactive kodecytes—IgM		
	Gal α	GalNAc α	Rha α
3	+	+	+
1	0	++	+
4	+	+	++
4	+	++	+
9	+	++	++
2	++	+	+
2	++	+	++
3	++	++	+
1	+++	++	+

0: Undetectable antibody; +: Low antibody levels; ++: Medium antibody levels; +++: High antibody levels.

No sample had high levels of IgM antibody against all three kodecyte specificities. Three samples had a high level of IgM antibody against two specificities, while 18 samples had a high level of IgM antibody against one specificity (Tables 2 and 3).

There was a poor association of high level antibody against one specificity with low level antibody against another specificity, with only one sample having both a high and low result; in this case high activity against Gal α -kodecytes and low activity against Rha α -kodecytes (Table 3).

Of the 100 plasma samples 29 had at least one low level reaction against one or more of the three different kodecytes tested and three samples had low levels of IgM antibody against all three

kodecyte panels (Table 3). The lowest overall reactivity was seen with the Gal α -kodecytes against 21 samples (Tables 1 and 3) of which one sample was non-reactive indicating very low or no IgM anti-Gal α 1-3Gal β 1-4GlcNAc (Table 3). Further IAA analysis of this sample indicated the presence of low levels of IgG anti-Gal α 1-3Gal β 1-4GlcNAc. This sample was also poorly reactive against Rha α -kodecytes, although it had medium reactivity against GalNAc α -kodecytes. The Rha α -kodecytes revealed low level antibody with 14 plasma samples while the GalNAc α -kodecytes revealed low antibody with 11 plasma samples (Table 1).

3.2. Indirect anti-IgG assay (IAA)—kodecytes

The IAA, which detects the presence of IgG antibody in the absence of pre-existing direct agglutination, was conducted on samples which were saline non-reactive and was able to establish IgG bioactivity when it exceeded IgM reactivity. All samples with a medium or low antibody level result were tested against 0.5-kodecytes and those with a low antibody level result were also tested against 10-kodecytes (Table 1). In the samples with low IgM saline activity about half of them reacted with the 10-kodecytes (13/20 Gal α -kodecytes; 6/11 GalNAc α -kodecytes; 7/14 Rha α -kodecytes) indicating more IgG than IgM, but none reacted with any 0.5-kodecyte, indicating none had high levels of IgG (Table 4). In the samples with medium IgM saline activity very few had more IgG than IgM reactivity as seen with reactivity against 0.5-kodecytes (9/75 Gal α -kodecytes; 1/84 GalNAc α -kodecytes; 1/74 Rha α -kodecytes) (Table 4).

Table 4. Indirect anti-IgG assay (IAA) of samples which were classified low and medium IgM antibody positive kodecyte reactivity*.

IgM	Saline low samples			Saline medium samples	
	n	10-kodecytes	0.5-kodecytes	n	0.5-kodecytes
Gal α	20	13	0	75	9
GalNAc α	11	6	0	84	1
Rha α	14	7	0	74	1

*: Samples are only tested for IAA IgG reactions when the saline direct IgM agglutination reaction is negative.

Overall observed reactivity with 10 and/or 0.5-kodecytes by any method (Table 5) demonstrating medium or high level IgM and/or IgG reactivity was seen with 92% of Gal α -kodecytes, 95% of GalNAc α -kodecytes and 93% of Rha α -kodecytes. Similarly reactivity with 0.5-kodecytes by any method (Table 5), demonstrating only high level IgM and/or IgG reactivity was seen with 13% of Gal α -kodecytes, 6% of GalNAc α -kodecytes and 13% of Rha α -kodecytes.

Table 5. Differentiation of 100 plasma samples into high, medium and low antibody levels by either saline or IAA results (IgM+IgG).

Antibody level*	Kodecyte reactivity	Kodecyte defined antibody levels		
	FSL $\mu\text{mol/L}$	Gal α	GalNAc α	Rha α
High	0.5 + 10 + 40	13	6	13
Medium	10 + 40	79	89	80
Low	40	8	5	7

*: Highest category by either technique.

3.3. Enzyme immunoassay (EIA) versus kodecyte assay

Scatter plots of the levels of anti-Gal α defined by the kodecyte assay versus EIA optical densities (OD) for the same samples were determined (Figure 2). Comparison of IgM levels with the kodecytes against EIA IgM OD's found 3 of the 61 samples were outliers (Figure 2a). These 3 samples gave high IgM OD's but were defined as having low IgM levels with kodecytes. Repeat testing of both assays confirmed these discordant results. Total antibody results (IgG/IgM) were also compared. However, due to dissimilarities in assay design only the highest EIA OD value observed for either IgG or IgM was compared against the combined IgG+IgM kodecyte result, which does not measure IgG without IgM. Two of the three IgM outliers (Figure 2a) were still outliers with high EIA OD's and a low antibody kodecyte grade. There was a new outlier which changed from a medium level for IgM to a high level for IgM+IgG with the kodecytes, but had low EIA OD's for both IgM (OD 1.3) and IgG (OD 1.2) (Figure 2b). However, if the EIA OD's for both the IgG and IgM reactions were cumulative, then this sample would no longer be an outlier.

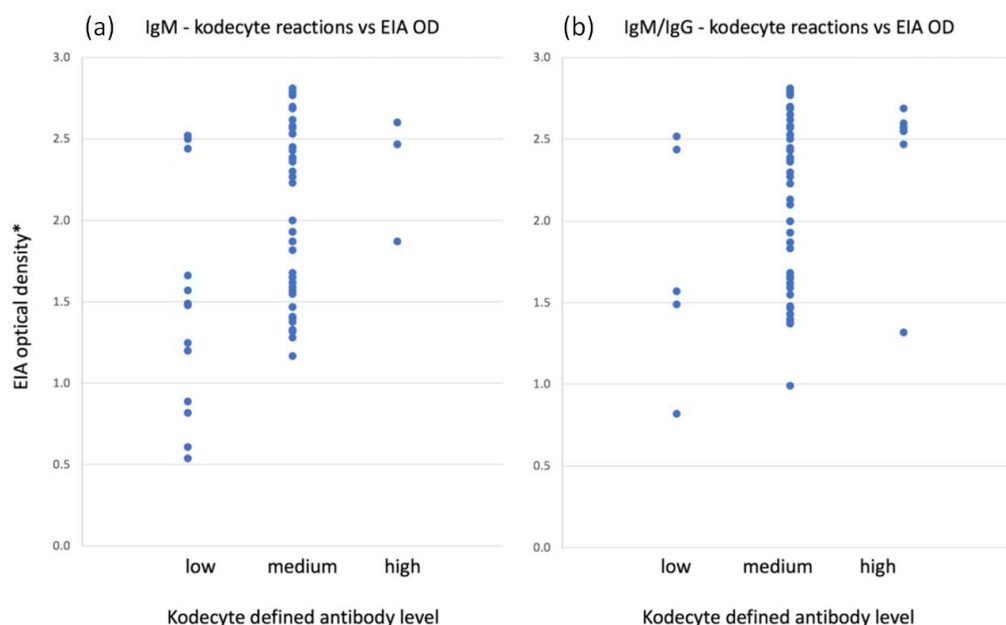


Figure 2. Scatter plot correlations between EIA optical densities and kodecyte defined anti-Gal α levels for the same samples. (a) IgM anti-Gal α , (b) IgM/IgG anti-Gal α where the EIA OD value is the highest OD observed for either IgG or IgM.

4. Discussion

The levels of xeno-glycan antibodies in human plasma determine if an immediate in vivo immunological reaction will occur in an individual, whether that be in a natural setting such as prevention of infection [2,5], or a medical intervention such as xeno-transplantation where one is trying to avoid the consequences of antigen-antibody interaction [15,22], or where one is trying to exploit these consequences, as in immunotherapy [15,18,19]. The xeno-antigens Gal α 1-3Gal β 1-R and L-rhamnose-containing bacterial polysaccharides are immunologically important xeno-antigens, both in a variety of disease processes and medical interventions [12,14,15,18–21] as well as being targets for oncological immunotherapy [15,18,19]. The xeno-antigen GalNAc α 1-3Gal β 1-4GlcNAc has not been previously studied at a population level (although studies have been performed using related GalNAc α compounds and disaccharides) [4,34] and it was included in this study because of its potential as an immunotherapeutic target. Although only Gal α 1-3Gal β 1-R is considered important to xenotransplantation two other xeno-antigens (L-Rha and GalNAc α 1-3Gal β 1-4GlcNAc) were included in this study, primarily to expand knowledge on other xeno-glycan antibodies which are expected to be present in a high proportion of individuals.

The three xeno-glycan antigens in this study, as well as all other xeno-glycan antigens are by their nature not naturally present in humans so screening for antibodies against them cannot be performed with natural human cells. Previously solid phase antigens bound to surfaces such as micro-chips have been used [1,4,16–18,20,23–26]. However with the recent observations that ABO antigen bearing kodeocytes can be used to accurately determine levels of antibodies in undiluted plasma by simply controlling the level of FSL-antigens on cells [28] we used this technology to examine levels of three xeno-glycan antibodies. Recognising the somewhat arbitrary classification of high, medium, and low antibody levels in both assays, comparison of the kodeocyte assay with the generally accepted EIA assay for anti-Gal α showed reasonable correlation (Figure 2). The use of FSL constructs for controllable and quantitative attachment of small molecules onto the outside of cells, creating so-called kodeocytes is well established and validated [27–29]. Standardised kodeocytes have previously been used in multiple antibody validation studies including comparisons with EIA [35,36], flow cytometry [37,38] and serologic agglutination [28,35–38] including use as routine quality quantitative control reagents for the ABO reagents [38]. These studies support the use of FSL-glycan antigens to quantify antibody present in samples by simply varying the quantity of FSL attached to cell membranes as kodeocytes. The potential methodological impact of different IgM:IgG ratios in samples was previously observed with kodeocytes [28], but as the kodeocyte method involves the use of undiluted plasma against intact cells it is potentially more representative of clinical significance than observations with methods (including EIA) that use diluted plasma [28].

With FSL technology the xeno-glycan antigens Gal α 1-3Gal β 1-4GlcNAc, GalNAc α 1-3Gal β 1-4GlcNAc and L-rhamnose monosaccharide were prepared and attached to human group O RBCs creating xeno-glycan Gal α -kodeocytes, GalNAc α -kodeocytes and Rha α -kodeocytes. These xeno-glycan kodeocytes, with the exception of the xeno-glycan antigen they carry, are still human group O RBC compatible with human plasma and can therefore be used in routine serological antibody detection systems [28]. Although the xeno-glycan antigens attached to RBCs by the FSL are not natural, FSLs have similarities with glycolipids which can naturally label cells with ABO & Lewis glycans [39] and FSL modified cells behave like mimics of natural cells [28,37] and solid phase assay targets [36]. Most importantly; because quantitative equivalent

kodeocyte variants can be made from the same cell [28,37], direct comparisons of different antigens at controlled levels can be made and variation associated with plasma dilution avoided [40,41].

In this paper, human group O RBC kodeocytes bearing three selected xeno-glycan antigens were created in molar equivalents over three concentrations. The kodeocyte preparations were selected for their capability to differentiate high, medium and low levels of antibody in plasma. These antibody levels were chosen as no known xeno-glycan antibody standards exist and are based on equivalent serological reactions observed for the ABO blood group system and validated in the ABO kodeocyte antibody quantitation assay [28]. Serological dogma holds true that if an antibody is unable to agglutinate a cell with a strong antigen expression (either directly or indirectly) then the level of antibody is low [10]. The primary consideration of the kodeocyte assay was to identify those samples with low or undetectable levels of antibody, as it is expected these individuals will respond poorly when facing an immediate challenge from the corresponding glycan antigen [42,43], which is important in determining the efficacy of medical interventions and immunotherapy. In contrast individuals with medium or high levels of antibody are expected to respond immediately to an antigenic challenge [10,42,43].

Plasma from 100 random blood group O donors was tested for the levels of antibodies against the xeno-glycan antigens Gal α 1-3Gal β 1-4GlcNAc, GalNAc α 1-3Gal β 1-4GlcNAc and L-rhamnose monosaccharide. Only group O samples were chosen to ensure that the results were not confounded by ABO phenotype variations and because they would be expected to have a larger repertoire of antibodies against A & B related antigens [34], and these related specificities might cross-react with the non-fucosylated xeno-glycan antigens GalNAc α 1-3Gal β 1-R, and Gal α 1-3Gal β 1-R [34,44]. Therefore the antibody level results reported here with group O samples against Gal α and GalNAc α kodeocytes are potentially higher than those that may be observed in group A, B and AB individuals [44], but this has yet to be established.

Antibodies against the xeno-glycan antigens Gal α 1-3Gal β 1-R and L-rhamnose-containing polysaccharides are well reported using solid phase assays [1,4,18,23,45,46]. These assays show that antibodies against these xeno-glycans (or closely related compounds) are both of high frequency in a population [1,4,18,23,45,46] and of a relatively high level [1,4,18,23]. Similarly we found that the majority of individuals have medium or high levels of antibodies to all three of the xeno-glycan antigens we studied. Where possible (when reactions were negative in the saline kodeocyte assay), we also reported the samples' IgG level, as Galili et al [47] reported that IgG is the dominant class (98%) of antibody against Gal α 1-3Gal β 1-R and that this antibody may constitute as much as 1% of total IgG [15,26,48] (although this has been challenged by others) [45,46].

With kodeocytes, we found that only about 65% of samples had more IgG than IgM bioactivity for Gal α 1-3Gal β 1-4GlcNAc and similarly only 50% of samples had more IgG than IgM directed against GalNAc α 1-3Gal β 1-4GlcNAc and Rha α . Only a small number of samples had high levels of IgG antibody (Table 4). Based on these observations we do not find IgG antibodies to be as dominant as previously reported [47], and instead observe a substantial contribution of IgM to the total amount of bioactivity observed for the xeno-glycan antibodies studied.

In agreement with other studies [4,23] we also find xeno-glycan antibodies are ubiquitous, but despite this we find that 5–8% of individuals (Table 5) have relatively low levels of specific antibody (both IgG and IgM), and are therefore less likely to respond immediately to antigenic challenge [42,43]. These low level results were independent of each other, that is no individual was low (for combined IgG and IgM) for all three specificities, and therefore low results observed are not

a consequence of generic low antibody levels or a poor response to xeno-glycans. As individual donor information was not available, it was not possible to identify whether low levels were associated with age or ethnicity. Low xeno-glycan antibody levels are potentially very clinically significant, especially where the xeno-glycan antigen is a target for medical intervention, (e.g. Gal α 1-3Gal β 1-R in oncotherapy [15,19] and xeno-transplantation [15,22]), and highlights the need for good diagnostic assays for xeno-glycan antibodies. The potential reason for the differences in our study compared to others [4,23] is likely to relate to the different testing methods to measure interaction of antibody and antigen. We used a cellular assay to measure antigen-antibody interaction, whereas others [4,23] used solid phase assays. Whilst the solid phase assay may be more sensitive than the agglutination assay [49], it does not necessarily present the antigen in the same way as does a cell membrane, which we postulate may be a better predictor of in vivo significance [19,50].

Although in this study we use only three different xeno-glycan antigens on the kodeocytes, the flexibility of this technology allows for the potential attachment of any carbohydrate xeno-glycan antigen. Furthermore with the ability to make FSL peptide antigens [51,52], this work could also be extended beyond glycans.

5. Conclusions

By using human RBCs modified to quantitative and qualitative variants bearing xeno-glycan antigens we were able to quantify relative levels of antibody in undiluted plasma using standardized routine blood typing equipment. Importantly this research identified that although xeno-glycan antibodies are ubiquitous in plasma, a small but significant proportion of the population would respond poorly when challenged by the corresponding xeno-glycan antigen, and this may have important clinical consequences.

Conflict of interests

All authors declare no conflicts of interest in this paper.

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