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ORIGINAL ARTICLE

Carbohydrate residues downstream of the terminal Galα(1,3)Gal epitope modulate the specificity of xenoreactive antibodies

Julie Milland[1], Elizabeth Yuriev[2], Pei-Xiang Xing[3], Ian FC McKenzie[1], Paul A Ramsland[3] and Mauro S Sandrin[1]

Carbohydrates are involved in many immunological responses including the rejection of incompatible blood, tissues and organs. Carbohydrate antigens with Galα(1,3)Gal epitopes are recognized by natural antibodies in humans and pose a major barrier for pig-to-human xenotransplantation. Genetically modified pigs have been established that have no functional α1,3-galactosyltransferase (α1,3GT), which transfers αGal to *N*-acetyllactosamine (LacNAc) type oligosaccharides. However, a low level of Galα(1,3)Gal is still expressed in α1,3GT knockout animals in the form of a lipid, isoglobotrihexosylceramide (iGb3), which is produced by iGb3 synthase on lactose (Lac) type core structures. Here, we define the reactivity of a series of monoclonal antibodies (mAb) generated in α1,3GT−/− mice immunized with rabbit red blood cells (RbRBC), as a rich source of lipid-linked antigens. Interestingly, one mAb (15.101) binds weakly to synthetic and cell surface-expressed Galα(1,3)Gal on LacNAc, but strongly to versions of the antigen on Lac cores, including iGb3. Three-dimensional models suggest that the terminal α-linked Gal binds tightly into the antibody-binding cavity. Furthermore, antibody interactions were predicted with the second and third monosaccharide units. Collectively, our findings suggest that although the terminal carbohydrate residues confer most of the binding affinity, the fine specificity is determined by subsequent residues in the oligosaccharide.
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INTRODUCTION

Carbohydrates are important to many of the immunological processes in medicine. For example, natural preformed and induced antibodies can recognize carbohydrate antigens, such as ABO blood group antigens [1] and the xenograft antigen, Galα(1,3)Gal,[2–7] which causes hyperacute rejection and later rejection events. Therapeutically, there is also the possibility of the treatment of cancer using antibodies that bind tumour-associated carbohydrate antigens.[8–10] In both the transplantation/transfusion and cancer immunotherapy settings, it is crucial to develop a detailed molecular understanding of the interactions between carbohydrates and antibodies.

Our group has been examining a model system for antibody interactions with carbohydrates based on the major xenograft antigen, Galα(1,3)Gal. Formation of the Galα(1,3)Gal epitope mostly occurs by the transfer of a galactose in an α(1,3)-glycosidic linkage to an *N*-acetyllactosamine (LacNAc or Type-II precursor) acceptor molecule presented on protein and lipid. This reaction is catalysed by α1,3-galactosyltransferase (α1,3GT) [11] that is encoded by the *GGTA1* gene,[12] which is widely expressed and functional in most species, except humans and Old World monkeys.[13] Consequently, humans have natural preformed antibodies that recognize the Galα(1,3)Gal epitope, and this specificity constitutes around 1% of circulating antibodies in healthy individuals.[4,14] As expected, α1,3GT−/− knockout mice [15–17] and pigs [18–20] also produce natural and induced Abs to the Galα(1,3)Gal epitope. However, while Galα(1,3)Gal on LacNAc was considered the major xenoantigen, more recently Galα(1,3)Gal was characterized on lactose (Lac or Type-V precursor) containing lipid isoglobotrihexosylceramide (iGb3) and synthesized by iGb3 synthase (iGb3S).[21] The iGb3 glycolipid may be important to rejection processes in pig-to-human xenotransplantation [5] and of great significance to NK cell biology where it may be recognized by NKT cells.[22–25]

The difference between the two naturally occurring forms of Galα(1,3)Gal provides a useful system for studying the fine specificity of carbohydrate interactions with antibodies. One form of Galα(1,3)-Gal is attached to a LacNAc core structure (Galβ(1,4)GlcNAc) and the

[1]Department of Surgery (Austin Health), University of Melbourne, Heidelberg, Victoria, Australia; [2]Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University, Parkville, Victoria, Australia and [3]Structural Immunology and Cancer Immunotherapy Laboratories, Macfarlane Burnet Institute for Medical Research and Public Health (Austin Health), Heidelberg, Victoria, Australia
Correspondence: Professor MS Sandrin, Department of Surgery, Austin Health, University of Melbourne, Kronheimer Building, PO Box 5555, Heidelberg, Victoria 3084, Australia.
E-mail: m.sandrin@unimelb.edu.au
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other is Galα(1,3)Gal attached to a Lac core (Galβ(1,4)Glc). Most preformed antibodies recognize the LacNAc form of Galα(1,3)Gal rather than the Lac form (as found in iGb3) even though the terminal disaccharide moiety is identical. Other evidence shows that human serum contains antibodies that can bind Galα(1,3)Gal found on iGb3 lipid[21] suggesting that carbohydrate determinants downstream of the two terminal Gal residues (that is, either GlcNAc or Glc) may affect binding of antibodies. The work presented here investigates the possible effect of carbohydrate residues downstream of the Galα(1,3)-Gal antigen on the fine specificity of anti-carbohydrate antibodies.

The α1,3GT−/− mice are a valuable resource as a small animal model with relevance to pig-to-primate transplantation and have included studies of Galα(1,3)Gal-dependent hyperacute rejection of xenografts,[26,27] delayed xenograft rejection,[17,28] islet transplantation,[29–31] role of NK cells in xenotransplantation,[32] antibody production,[33] T- and B-cell tolerance induction [34–37] or to rapidly evaluate the efficacy of combining different transgenic strategies to produce the optimal donor animal.[38,39]

Here we describe the binding properties of a panel of Galα(1,3)Gal binding monoclonal antibodies (mAb) that were derived from α1,3GT−/− mice immunized with rabbit red blood cells (RbRBC). Like humans and Old World primates, α1,3GT−/− mice also have natural anti-Gal antibodies, however at lower levels. The α1,3GT−/− mice were immunized to increase the chances of producing anti-Gal antibody secreting hybridomas. Interestingly, one mAb was found to bind the Lac form of Galα(1,3)Gal (synthesized by iGb3S) with substantially greater selectivity when compared to the LacNAc form of Galα(1,3)Gal (synthesized by α1,3GT). Using molecular modelling, we show that these antibodies are likely to recognize xenograft antigens predominantly by end-on insertion and tight binding of the terminal α-linked Gal residue. Furthermore, we demonstrate that the antibody-binding sites can easily accommodate the second and third carbohydrate residues of the oligosaccharide chain, and discriminate between Glc and GlcNAc in the third position. Thus, the probable involvement of carbohydrate residues other than the dominant antigenic epitope may modulate the fine specificity of antibodies towards their carbohydrate antigens.

RESULTS

Production of mAbs

Supernatants of hybridomas were screened using Galα(1,3)Gal-BSA and BSA-coated plates by enzyme-linked immunosorbent assay (ELISA). Those reacting with Galα(1,3)Gal-BSA, but not with BSA, were cloned and retested by ELISA. Positive wells were expanded, cloned by limiting dilution, and the clones were retested. Eight mAbs were generated from independent primary hybridomas and isotypes examined (data not shown): 8.17 (IgM), 22.121 (IgG_3), 24.7 (IgG_1), 15.101 (IgG_1), 12.15 (IgM), 6.13 (IgM), 16.34 (IgM) and 25.20 (IgG_3).

Antibodies bind terminal Galα(1,3)Gal

Initially, the mAbs were tested for specificity to the Galα(1,3)Gal epitope by ELISA using synthetic glycoconjugates consisting of a carbohydrate covalently attached to BSA (Figure 1 and Table 1). Typical results are shown for four of the mAbs (8.17, 15.101, 16.34 and 25.20), together with a negative control mAb, FE14.1, which binds haptoglobin[40] (Figure 1). Data for all eight mAbs are summarized in Table 1. Not surprisingly, all the mAbs reacted with the Galα(1,3)Gal-BSA, which was used in screening protocols to select the hybridomas. In addition, the antibodies also bound Galα(1,3)Galβ(1,4)GlcNAc-BSA, Galα(1,3)Galβ(1,4)Glc-BSA and Galα(1,3)Galβ(1,4)GlcNAcβ(1,3)-Galβ(1,4)Glc-BSA (except 8.17), but did not interact with Galβ(1,4)Glc-BSA (Lac-BSA), Galβ(1,4)GlcNAc-BSA (LacNAc-BSA), Man-BSA and unconjugated BSA. Thus, all mAbs displayed a requirement for a terminal Galα(1,3)Gal to be present in the oligosaccharide ligand. If terminal Galα(1,3)Gal was present, the mAbs could react with various short and long oligosaccharide chains. This finding suggested that, in general, carbohydrate residues downstream of the dominant Galα(1,3)Gal epitope have minimal impact on binding. However, while 15.101 bound the disaccharide Galα(1,3)Gal conjugated to BSA with similar strength to 8.17 and 16.34 (Figure 1a), it bound weaker than the other antibodies to the trisaccharide Galα(1,3)Galβ(1,4)GlcNAc conjugated to BSA (Figure 1b). Since differences in binding of Galα(1,3)Gal antigens are important in the xenotransplantation setting, the molecular basis for carbohydrate recognition by the series of mAbs was investigated further.

Requirement for the terminal α-linked Gal for binding to the antibodies

To confirm and quantify the binding of the antibodies to Galα(1,3)-Gal, we examined the ability of different carbohydrates to inhibit the binding of the mAbs to Galα(1,3)Gal-BSA. The full inhibition curve for one mAb (8.17) is shown as an example in Figure 2. The 8.17 mAb was inhibited, in a dose-dependent manner, by D-Gal (mixed α/β-anomers), methyl α-D-galactopyranoside (Me-αGal), Galα(1,3)Gal and Galα(1,6)Glc (melibiose), but not by any of the other mono- or disaccharides tested (Figure 2). The ELISA results for four mAbs (8.17, 15.101, 16.34 and 25.20) were used to calculate the relative affinity for the carbohydrates. The antibodies were used at two times the concentration required to give 50% of the maximum OD_{405} reading. The concentration of carbohydrate, which inhibited the binding of antibody by 50% (I_{50}), was calculated as a measurement of relative affinity (Table 2). Using this approach, the affinity for the free Galα(1,3)Gal disaccharide was comparable for all antibodies (I_{50} ranging from 0.08 to 0.23 mM). The saccharide inhibition studies confirmed that a terminal Gal in the α-configuration is an absolute requirement for recognition by the mAbs. However, binding does not appear to be as tight to the second carbohydrate residue since Galα(1,6)Glc (melibiose), with Glc in an α(1,6)-glycosidic linkage at the second position, inhibited the binding of the mAbs. Further differences in mAb recognition of carbohydrates are suggested by considering the relative inhibition (that is, I_{50} (carbohydrate)/I_{50} (Galα(1,3)Gal)). Binding of the terminal α-linked Gal residue fell into two broad categories where interactions with this residue (1) accounted for most of the affinity (15.101 and 16.34) or (2) only partially accounted for the affinity (8.17 and 25.20). Experiments with melibiose suggested that the capacity for the mAb binding sites to accommodate variations to the carbohydrate at the second position (for example, Glc) was 15.101 > 8.17 > 16.34 > 25.20. It was therefore considered worthwhile to investigate the effects of the carbohydrate at the third position from the non-reducing end on the binding of the mAbs. As mAb 15.101 had shown variation in binding compared with the other mAbs, it was used in further comparative studies.

Effects of the third carbohydrate residue in the chain on binding to the 15.101 mAb

The ELISA data showed that the 15.101 mAb bound a trisaccharide of Galα(1,3)Galβ(1,4)GlcNAc-BSA with lower affinity, when compared to the other Galα(1,3)Gal-specific mAbs (Figure 1). In the context of xenotransplantation, this trisaccharide represents Galα(1,3)Gal on LacNAc (that is, synthesized by α1,3GT) as opposed to Galα(1,3)Gal on Lac (that is, synthesized by iGb3S). In contrast to α1,3GT that can utilize LacNAc on either glycoproteins or glycolipids as substrate,

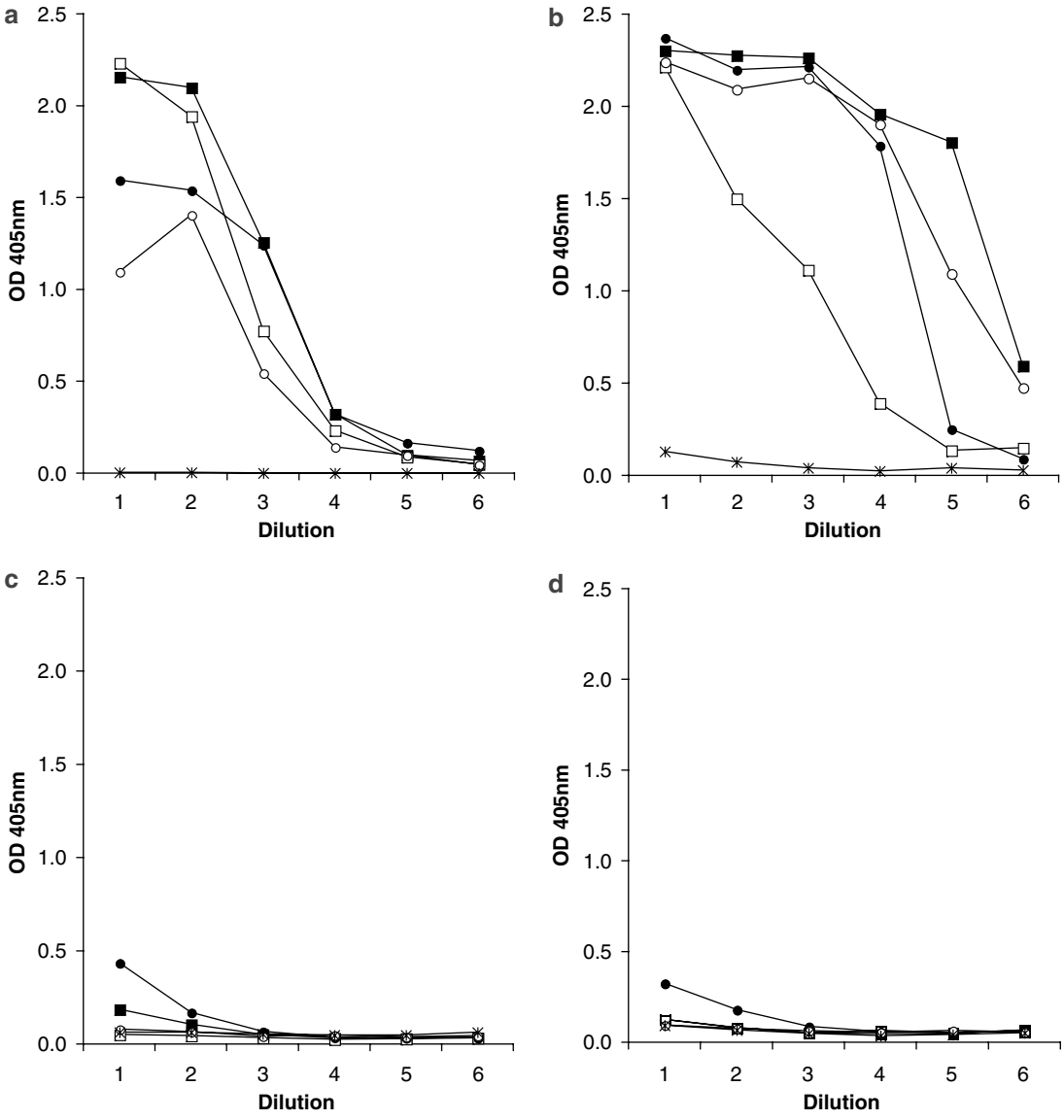

Figure 1 Titration of mAbs on synthetic glycoconjugates-BSA. The monoclonal antibodies were serially diluted (threefold starting with neat supernatant) and analysed for binding to various glycoconjugates by ELISA. Panels show: (**a**) Galα(1,3)Gal-BSA, (**b**) Galα(1,3)Galβ(1,4)GlcNAc-BSA, (**c**) LacNAc-BSA and (**d**) BSA. The antibodies are graphed as follows: 8.17 (■), 15.101 (□), 25.20 (○), 16.34 (●) and FE14.1 (*).

Table 1 Binding of monoclonal antibodies to various glycoconjugates measured by ELISA[a]

	mAb							
Glycoconjugate	*8.17*	*22.121*	*24.7*	*15.101*	*25.20*	*16.34*	*12.15*	*6.13*
Galα(1,3)Gal-BSA	+	+	+	+	+	+	+	+
Galα(1,3)Galβ(1,4)GlcNAc-BSA	+	+	+	+	+	+	+	+
Galα(1,3)Galβ(1,4)Glc-BSA	+	+	+	+	+	+	+	+
Penta-Gal-BSA[b]	–	+	+	+	+	+	+	+
Galβ(1,4)GlcNAc-BSA	–	–	–	–	–	–	–	–
Galβ(1,4)Glc-BSA	–	–	–	–	–	–	–	–
Man-BSA	–	–	–	–	–	–	–	–
BSA	–	–	–	–	–	–	–	–

[a]Binding of mAb: +, positive reactivity; –, no reactivity.
[b]Penta-Gal-BSA, Galα(1,3)Galβ(1,4)GlcNAcβ(1,3)Galβ(1,4)Glc-BSA.

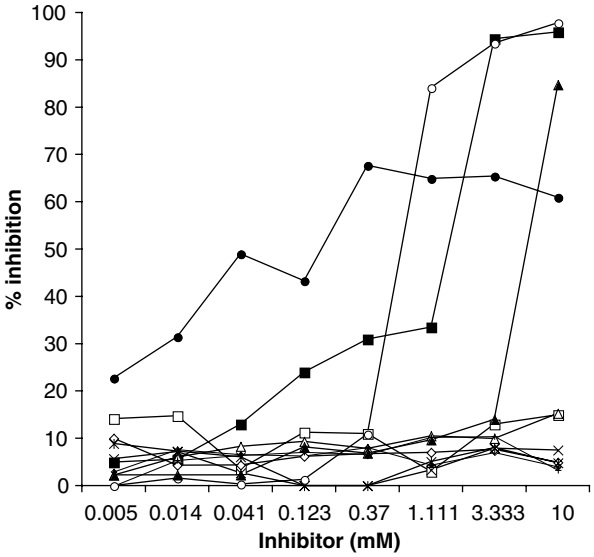

Figure 2 Inhibition of mAb 8.17 binding by oligosaccharides. Varying concentrations of inhibitors (shown in mM on the horizontal axis) were examined by ELISA for inhibition of mAb 8.17. Inhibitors used: Me-αGal (■), Me-βGal (□), Me-αGlc (♦), Me-βGlc (◇), Me-αMan (△), Me-βMan (×), LacNAc (*),Galα(1,3)Gal (●), melibiose (○), lactose (+) and Gal (▲).

Table 2 Relative affinities of carbohydrates for monoclonal antibodies

	I_{50} (mM)[a]			
Inhibitor	*8.17*	*15.101*	*16.34*	*25.20*
D-Gal	6	1.2	1.2	10
D-Glc	—[b]	—	—	—
D-Man	—	—	—	—
L-Fuc	—	—	—	—
Me-αGal	1.3	0.2	0.3	2.5
Me-βGal	—	—	—	—
Me-αGlc	—	—	—	—
Me-βGlc	—	—	—	—
Me-αMan	—	—	—	—
Me-βMan	—	—	—	—
Galα(1,3)Gal	0.14	0.08	0.10	0.23
Galα(1,6)Glc	0.60	0.11	0.80	3.8
Galβ(1,4)GlcNAc	—	—	—	—
Galβ(1,4)Glc	—	—	—	—
Galα(1,4)Fru	—	—	—	—

[a]I_{50} is the concentration of carbohydrate required to give 50% inhibition of antibody binding.
[b]—, no inhibition observed at 20 mM.

iGb3S can only use the glycolipid lactosylceramide as an acceptor. Furthermore, while α1,3GT can utilize Lac as a substrate, it cannot utilize lactosylceramide. Thus, we tested the possibility that the 15.101 mAb could interact selectively with the iGb3 glycolipid antigen. Reactivity was examined with a series of di- and trisaccharides conjugated to BSA for 15.101 (Figure 3a) and 8.17 (Figure 3b). For 15.101, the titres on the Galα(1,3)Gal-BSA and Galα(1,3)Galβ(1,4) Glc-BSA (iGb3 oligosaccharide) were similar, whereas 1/8th of this titre was observed on Galα(1,3)Galβ(1,4)GlcNAc-BSA (α1,3GT oligosaccharide). In contrast, similar titres of 8.17 were observed for all

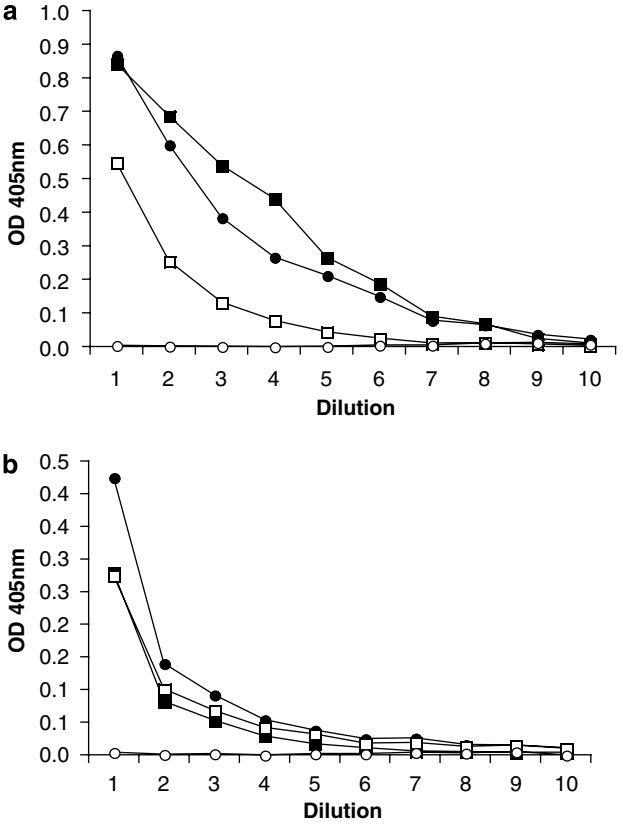

Figure 3 Titration of mAbs on synthetic Galα(1,3)Gal containing glycoconjugates-BSA. The monoclonal antibodies 15.101 (**a**) and 8.17 (**b**) were serially diluted (twofold starting with neat supernatant) and analysed for binding to various αGal-containing glycoconjugates by ELISA. Glycoconjugates used were: Galα(1,3)Gal-BSA (■), Galα(1,3)Galβ(1,4) GlcNAc-BSA (□), Galα(1,3)Galβ(1,4)Glc-BSA (●) and BSA (○).

carbohydrate–BSA conjugates. As the disaccharide Galα(1,3)Gal is common to both xenograft antigens, this suggests that the third sugar moiety modulates the enhanced binding of iGb3 oligosaccharides by the 15.101 mAb, that is 15.101 has a greater selectivity for Glc rather than GlcNAc. In addition, the difference can be viewed as a reduced permissiveness of the binding site for accepting an *N*-acetyl group on the third carbohydrate unit in the oligosaccharide chain (GlcNAc vs Glc).

Differences of carbohydrate antigen reactivity of the antibodies *in vivo*

To assess the mAbs binding to cell-surface glycoproteins and glycolipids displaying Galα(1,3)Gal antigens, the transferases responsible for Galα(1,3)Gal synthesis on both proteins and glycolipids (α1,3GT) or glycolipids only (iGb3S) were expressed in the human 293 cell line, which lacks Galα(1,3)Gal antigens. Cell-surface staining for Gb3 (Galα(1,4)Galβ(1,4)Glcβ(1,1)Cer), that is produced by Gb3 synthase (Gb3S), was used as a control. The α1,3GT-293 and iGb3-293 cells showed cell-surface expression of the Galα(1,3)Gal determinant, as demonstrated by their reactivity with the IB4 lectin (Figure 4a), with the positive cell population having a median fluorescence unit (mfu) of 346 and 145, respectively. In contrast, non-transfected 293 (Figure 4a) or Gb3-293 (not shown) cells did not react with the lectin. The α1,3GT-293 and iGb3-293 cells reacted equivalently with

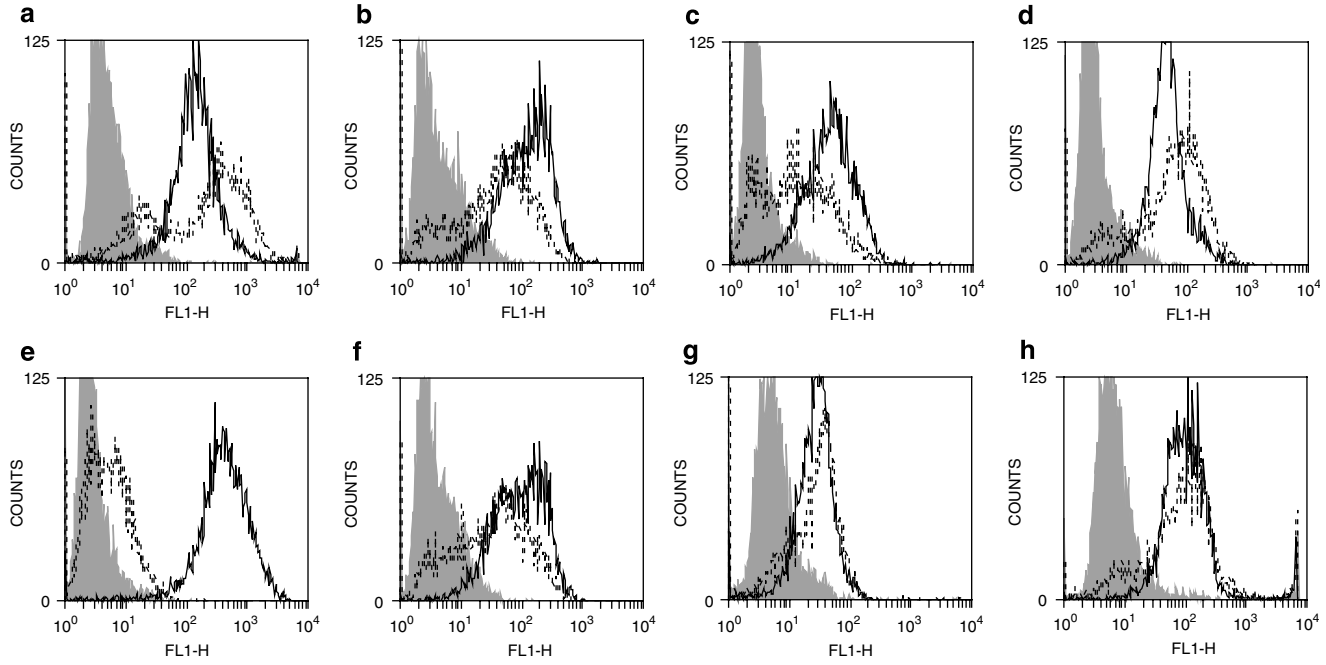

Figure 4 Cell-surface staining of carbohydrate antigens. Human 293 cells (grey filled) and those stably expressing either α1,3GT (broken line) or iGb3 synthase (solid line) were stained for cell-surface expression with the anti-Galα(1,3)Gal reagents or mAbs. Panels show binding with: (**a**) IB4 conjugated to FITC, (**b**) 22.121 mAb, (**c**) 8.17 mAb, (**d**) 24.7 mAb, (**e**) 15.101 mAb, (**f**) 25.2 mAb, (**g**) anti-Galα(1,3)Gal chicken IgY and (**h**) anti-Galα(1,3)Gal human IgG. Primary antibodies were detected with secondary sheep anti-mouse-FITC or anti-Ig-FITC specific for chicken and human, respectively. The cells were analysed by flow cytometry.

both a chicken anti-Galα(1,3)Gal IgY (Figure 4g, 35 and 29 mfu, respectively) and purified human anti-Galα(1,3)Gal IgG (Figure 4h, 113 and 98 mfu, respectively). The 22.121 mAb reacted strongly with the α1,3GT-293 and iGb3-293 cells (Figure 4b, 55 and 141 mfu, respectively), as did mAbs 24.7 (Figure 4d, 76 and 46 mfu, respectively) and 25.2 (Figure 4f, 48 and 105 mfu, respectively). The mAb 8.17 (Figure 4c) reacted strongly with iGb3-293 cells (48 mfu), but on α1,3GT-293 cells showed a broader distribution (18.1 mfu). In contrast to these results, mAb 15.101 (Figure 4e) reacted strongly with iGb3S-293 cells (445 mfu), but only very weakly with α1,3GT-293 cells (8.5 mfu). Thus, we can conclude that all the mAbs can bind to Galα(1,3)Gal antigens produced by α1,3GT (protein and lipid) and iGb3S (lipid) on the cell surface. However, of all the antibodies studied, 15.101 binds cell surface-expressed Galα(1,3)Gal produced by α1,3GT only weakly, whereas it binds intensely to Galα(1,3)Gal produced by iGb3S (that is, iGb3).

Structural basis for antibody recognition of terminal Galα(1,3)Gal residues on oligosaccharides

To investigate the structural basis of Galα(1,3)Gal antigen recognition, we have generated homology models of the Fv portions of 8.17 and 15.101, which were based on their previously reported translated amino-acid sequences.[41] These antibodies differ in amino-acid sequence at only three positions, namely Ser/Arg (8.17/15.101) at H56 in HCDR2, Gly/Ser at H101 in HCDR3 and Lys/Arg at L40 in LFR2. Consequently, the homology models are almost identical in overall three-dimensional structure. Similar to other carbohydrate-specific antibodies,[42–47] the binding sites of anti-Galα(1,3)Gal antibodies contain a large and relatively shallow pocket or cavity, which is formed by the complementarity determining regions (CDRs). With respect to the locations of the amino acids that differ between 8.17 and 15.101, only Gly/Ser at H101 falls within the binding cavity, while Ser/Arg at H56 is at the periphery of the binding site and Lys/Arg at L40 is distant from the binding site near the bottom of the VL–VH interface.

Automated docking studies were performed and the highest-scoring docked solutions for carbohydrates Galα(1,3)Gal, Galα(1,3)-Galβ(1,4)Glc and Galα(1,3)Galβ(1,4)GlcNAc have been analysed with respect to their binding modes. As hypothesized,[48] the carbohydrates were found to bind via an end-on insertion mechanism, with the terminal α-linked Gal monosaccharide penetrating deepest into the binding cavity (Figure 5). It should be noted that the antibody-binding site also participated in interactions with the second and third carbohydrate moieties of the di- and trisaccharides, respectively. As we proposed earlier,[48] the following residues were found to be important players in carbohydrate recognition: aromatic residues occupying six of the nine structurally conserved locations in the antibody-binding pocket and Pro and Ser residues located near the floor of the binding pocket. Aromatic residues (in particular, Trp L92, Tyr H32, Trp H33, and Tyr H102) were found to display a multitude of stabilizing van der Waals (vdW) interactions directed to the hydrophobic faces of the carbohydrate residues and a range of specific hydrogen-bonding interactions instrumental in selecting particular binding modes.

A detailed comparative analysis of hydrogen-bonding interactions exhibited by antibodies 8.17 and 15.101 in their docked complexes with Galα(1,3)Galβ(1,4)Glc and Galα(1,3)Galβ(1,4)GlcNAc was performed. Specifically, the number of hydrogen-bonding interactions, displayed by each amino acid to each carbohydrate residue in the top 100 docked poses, was tallied as per the procedure described previously.[49] These contacts, displayed by both antibodies in their docked complexes are summarized in Table 3. It is clear that 8.17 utilizes different hydrogen-bonding arrangements to bind to Galα(1,3)-Galβ(1,4)GlcNAc, as compared to Galα(1,3)Galβ(1,4)Glc (Table 3 and Figures 6a and b) However, the obvious differences, such as the

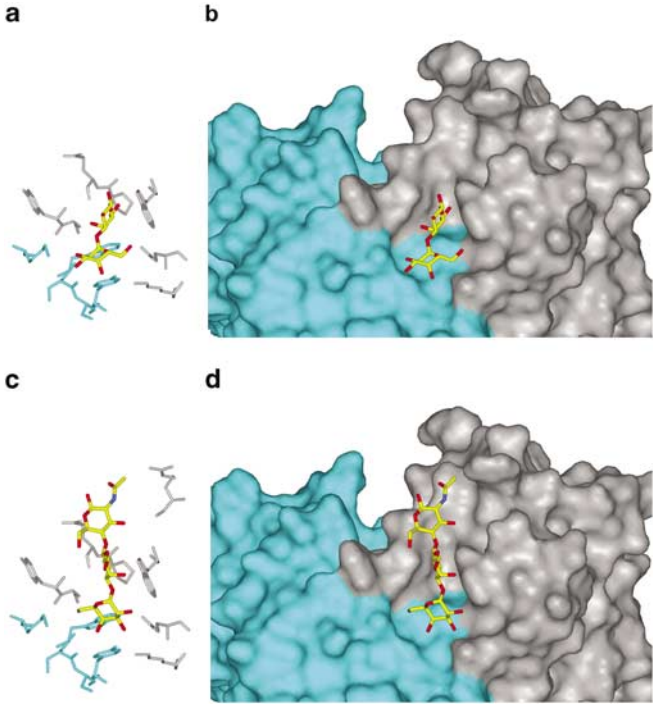

Figure 5 Models of the 8.17 Fv with bound carbohydrates. Docked complexes of Galα(1,3)Gal (**a**–**b**) and Galα(1,3)Galβ(1,4)GlcNAc (**c**–**d**). Panels a and c show details of the predicted interactions between antibody-binding site residues (VL, cyan; VH, grey) and carbohydrate ligands (coloured by atom type: carbon, yellow; oxygen, red; nitrogen, blue; hydrogens omitted for clarity). Panels b and d show solvent-accessible surface views of the 8.17 Fv region.

decreased role of Trp L92, Arg H31 and Gln H53, are compensated by increased hydrogen bonding to Ser L94 and Tyr H102. In the case of 15.101, the overriding feature is the decreased binding of all critical residues to Galα(1,3)Galβ(1,4)GlcNAc, as compared to Galα(1,3)-Galβ(1,4)Glc, the most pronounced being Trp L92 and Tyr L97 (Table 3 and Figures 6c and d). The slight increase in the binding of Arg H31 and Glu H50 is too minor to compensate for this dramatic decrease.

Hydrogen-bonding patterns displayed by the 8.17 and 15.101 mAbs with only the Glc and GlcNAc residues further illustrate the carbohydrate selectivity of the 15.101 mAb (Table 4). In both antibodies, some amino acids were predicted to perform similar roles in recognition: Trp L92 and Glu H100 are critical for recognizing Glc, whereas Ser L93 is important for recognizing GlcNAc. Conversely, there is a range of binding site amino acids (Lys L33, Ser L94, Trp H33 and Tyr H102), which form hydrogen bonds with GlcNAc in 8.17, but not in 15.101. On the other hand, there is only one amino acid (Lys H59), which forms hydrogen bonds with GlcNAc in 15.101, but not in 8.17. These observations suggest that both antibodies have comparable structural capacities for recognizing Glc, but 15.101 has a reduced structural capacity for recognizing GlcNAc. Binding features of amino acids Glu H100 and Gly/Ser H101 give further insight into this reduced capacity. While Gly H101 (8.17) does not hydrogen bond to any part of the trisaccharide ligand, Ser H101 is the major contributor to Glc recognition in 15.101 (39 contacts), with significantly less preference for GlcNAc (11 contacts). Glu H100, which is a foremost recognition element for Glc in both Abs, also contributes extensively to recognition of GlcNAc in 15.101. Thus, Glu H100 and Lys H59 may provide compensatory mechanisms for 15.101 binding to GlcNAc, but these are clearly not sufficient to offset the loss of binding by Lys L33, Ser L94, Trp H33 and Tyr H102.

Table 3 Number of hydrogen bonding contacts to the trisaccharide ligand

Protein residue	*8.17*		*15.101*	
	GalGalGlc[a]	*GalGalGlcNAc*[b]	*GalGalGlc*[a]	*GalGalGlcNAc*[b]
Lys L33	51	28	34	34
Trp L92	65	4	64	7
Ser L93	48	67	72	55
Ser L94	0	28	0	0
Tyr L97	21	39	75	43
Arg H31	30	3	2	16
Tyr H32	0	0	0	2
Trp H33	37	31	52	8
Glu H50	16	40	0	9
Asn H52	5	0	0	0
Gln H53	35	1	2	0
Lys H59	9	37	41	22
Pro H99	1	1	2	0
Glu H100	61	37	61	56
Gly/Ser H101	0	0	41	34
Tyr H102	20	70	35	35
Gly H103	15	1	0	0

[a]GalGalGlc, Galα(1,3)Galβ(1,4)Glc.
[b]GalGalGlcNAc, Galα(1,3)Galβ(1,4)GlcNAc.

DISCUSSION

Humans have natural preformed antibodies to the carbohydrate Galα(1,3)Gal, mainly IgM and to a lesser extent IgG. [4,14] It was believed that these antibodies were uniformly specific for the terminal Galα(1,3)Gal structure, but McKane *et al.*[50] suggested that binding of these antibodies is influenced by the particular composition and the steric configuration of oligosaccharides, including those with a Glc or a GlcNAc in the third position. Similarly, using inhibition of whole human serum, Neethling *et al.*,[51] demonstrated increased inhibition by extending the carbohydrate length. This and more recent data, showing binding of human anti-Galα(1,3)Gal antibodies to iGb3 lipid [21] or to tissues in α1,3GT−/− pigs,[52] suggest that the ability of the third monosaccharide unit to alter the fine specificity of antibodies to iGb3 may have implications for xenotransplantation.

In this paper, we have examined the specificity of a series of carbohydrate-binding mAbs, derived from α1,3GT−/− mice immunized with RbRBC. The mAbs were selected for binding to the minimal Galα(1,3)Gal epitope, which is present on either a LacNAc or Lac core structure that differ by the presence or absence of an *N*-acetyl group on the third sugar (Glc vs GlcNAc) from the non-reducing terminal end. The IB4 lectin displayed preferential binding to Galα(1,3)Gal on LacNAc and, as previously shown, this binding is different from the broader specificity for Galα(1,3)Gal antigens inherent in the natural antibody repertoire.[53] The chicken and human anti-Galα(1,3)Gal antibodies and all the remaining mAbs tested bound similarly to Galα(1,3)Gal on both LacNAc and Lac. In contrast, the 15.101 mAb bound preferentially to Galα(1,3)Gal on Lac. The fine specificity of the 15.101 mAb appears to be due to the ability of its binding site to differentiate between Glc and GlcNAc in the third sugar unit in the oligosaccharide chain.

The 15.101 mAb is the first reported to bind selectively to Galα(1,3)Gal on Lac and the iGb3 lipid. The Gal-13 mAb binds

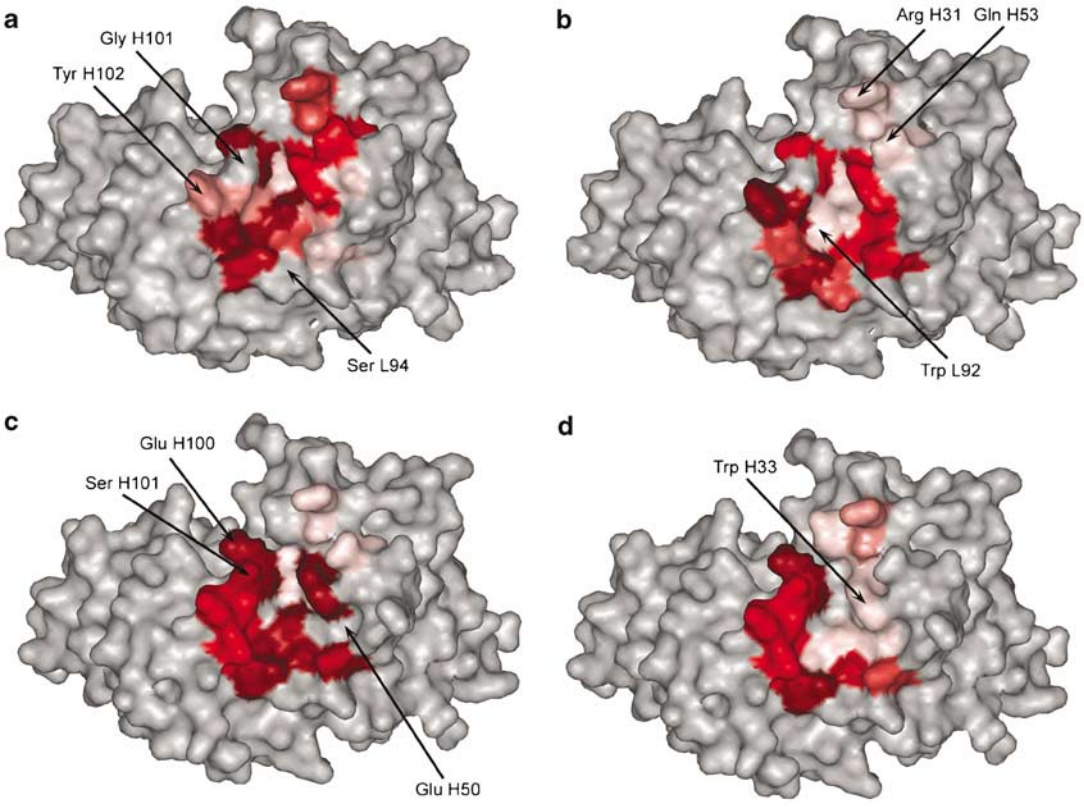

Figure 6 Surface views of 8.17 and 15.101 binding sites mapped with residues involved in hydrogen bonding to carbohydrate ligands. Hydrogen bonding was tallied for the top 100 docked poses for: (**a**) 8.17 mAb with Galα(1,3)Galβ(1,4)Glc, (**b**) 8.17 mAb with Galα(1,3)Galβ(1,4)GlcNAc, (**c**) 15.101 mAb with Galα(1,3)Galβ(1,4)Glc and (**d**) 15.101 mAb with Galα(1,3)Galβ(1,4)GlcNAc. Depth of colour (white-to-red) indicates number of hydrogen bonds between an amino acid and carbohydrate ligand.

Table 4 Number of hydrogen bonding contacts to the third residue

Protein residue	*8.17*		*15.101*	
	Glc	*GlcNAc*	*Glc*	*GlcNAc*
Lys L33	12	27	2	1
Trp L92	34	2	23	5
Ser L93	5	27	1	22
Ser L94	0	28	0	0
Tyr L97	12	0	1	0
Arg H31	0	2	0	11
Tyr H32	0	0	0	2
Trp H33	5	30	0	1
Glu H50	0	1	0	0
Gln H53	5	0	0	0
Lys H59	0	7	0	22
Pro H99	1	1	0	0
Glu H100	46	1	59	31
Gly/Ser H101	0	0	39	11
Tyr H102	0	29	0	0
Gly H103	0	0	0	0

lipid terminating in Galα(1,3)Gal in the form of ceramide pentahexoside (CPH), which contains the LacNAc core, but does not bind iGb3.[54] Most other mAbs also bind Galα(1,3)Gal on LacNAc core structures. It is probably the combination of the immunogen used (RbRBC) and the screening method that has enabled us to isolate 15.101, an mAb with unique specificity for iGb3. Others have also used RbRBC to immunize mice, however the hybridomas were screened using CPH [54] or trisaccharide Galα(1,3)Galβ(1,4)GlcNAc-BSA[55] and the resulting mAbs recognized CPH and Galα(1,3)Gal on LacNAc, respectively. Cell-membrane fractions purified on an IB4 affinity column [56] or transplantation of α1,3GT+/+ mouse or rat hearts into α1,3GT−/− mice [57,58] have also been employed as immunization methodologies and were followed by screening on Galα(1,3)Galβ(1,4)GlcNAc-BSA. The mAbs generated by these approaches appear to only recognize Galα(1,3)Gal on LacNAc core structures. In our approach, RbRBC, that are rich in lipid-linked Galα(1,3)Gal, were used to immunize α1,3GT−/− mice. Hybridoma supernatants were screened with the Galα(1,3)Gal disaccharide, thus giving the greatest opportunity to find an mAb that recognizes iGb3. The use of the disaccharide potentially enabled the 15.101 mAb to preferentially bind to a different structure, namely Glc rather than GlcNAc, at position three of the carbohydrate chain. Differences in fine specificity of anti-Galα(1,3)Gal antibodies to different carbohydrate structures is not restricted to mAbs, but has similarly been reported for polyclonal human anti-Galα(1,3)Gal antibodies.[59] Human anti-Galα(1,3)Gal IgM antibodies present in normal serum can be divided into two groups based on the ability to bind/not bind to Galα(1,6)Glc and related structures.[59]

In this paper, we have carried out a detailed examination of intermolecular interactions exhibited by the 15.101 mAb in its docked complexes with Galα(1,3)Galβ(1,4)Glc and Galα(1,3)Galβ(1,4)GlcNAc and compared them to the interactions displayed by the 8.17 mAb. This comparative analysis showed that these two antibodies

utilize different hydrogen-bonding arrangements to bind to the two carbohydrates. It also revealed that, whereas in 8.17 the overall number of contacts made was similar, for the 15.101 mAb the overriding feature was the decreased binding of all critical residues to Galα(1,3)-Galβ(1,4)GlcNAc, as compared to Galα(1,3)Galβ(1,4)Glc. Furthermore, the focus on position three of the carbohydrate chain illustrated that the mechanisms employed in the 15.101 mAb to bind to GlcNAc, as compared to Glc, were not compensated to the same extent as they were in the 8.17 mAb.

There is precedence for carbohydrate-binding antibodies or lectins to differentially bind to similar oligosaccharides, differing by only an *N*-acetyl group. For instance, the isolectins I–A and I–B of *Griffonia (Bandeiraea) simplicifolia* (IA4 and IB4) have 89% identity and have specificity for terminal αGalNAc (A blood group and Forssman antigens) and terminal αGal (B blood group and the Galα(1,3)Gal xenoantigen), respectively.[60] The IB4 lectin recognizes only terminal Galα(1,3)Gal, whereas the IA4 lectin predominantly recognizes GalNAcα(1,3)Gal yet can also bind Galα(1,3)Gal. Using X-ray crystallography, homology modelling and docking techniques, this fine specificity was shown to be due to a single amino-acid difference at residue 106 in the binding site.[60]

The α1,3GT−/− mice have lower levels of natural anti-Galα(1,3)-Gal antibodies compared to humans and Old World primates. Furthermore, α1,3GT−/− mice which express iGb3 do have an increase in the titre of anti-Galα(1,3)Gal antibodies after immunization. Therefore, in a similar fashion it is not unexpected that α1,3GT−/− pigs can make anti-Galα(1,3)Gal antibodies. The lack of data showing increased primate anti-Galα(1,3)Gal after sensitization to α1,3GT−/− pig organs is more difficult to reconcile. Review of the literature shows that the definition of non-Galα(1,3)Gal antigens is by FACS analysis of α1,3GT−/− pig cells using sera at one dilution.[61–66] The α1,3GT−/− pigs were defined as being Galα(1,3)Gal-negative based on IB4 or mAb M86 staining. We have previously shown that IB4 is not very effective at detecting iGb3 [67] and that the level of iGb3 on α1,3GT−/− mouse cells is at least 1/20th the level of α1,3GT-synthesized Galα(1,3)Gal.[5] In the α1,3GT−/− pig-to-primate transplant studies, conducted in immunosuppressed recipients, 1/2 baboons had an increase in anti-Galα(1,3)Gal antibodies post-transplant [62] and absorption of post-transplant sera with type 6 trisaccharide (Galα(1,3)Galβ(1,4)Glc-) showed some decrease in binding.[63] Furthermore, there is a trend to decrease ADCC of α1,3GT−/− pig cells by absorption of anti-Gal from the sera,[66] and the authors comment that this may be due to iGb3 on the α1,3GT−/− pig cells. Thus, the issue of iGb3 expression on α1,3GT−/− pig cells remains unresolved, particularly as these cells are used to define antibodies directed to non-Galα(1,3)Gal epitopes. Therefore, the 15.101 mAb is a unique and promising reagent due to its preferential binding of the iGb3 glycolipid. Given the potential importance of iGb3 to xenotransplantation [21,52,66] and NKT cell biology,[25] 15.101 mAb would be a more suitable reagent than the IB4 lectin and other mAbs that often preferentially bind Galα(1,3)Gal on LacNAc core structures. In the era of α1,3GT knockout pigs, the specificity of antibodies to identify additional carbohydrate antigens, including iGb3, may be of importance to the future success of clinical xenotransplantation.

METHODS

Animals

The α1,3GT−/− mice were obtained from Dr John Lowe [15] and were maintained at the Biological Research Facilities, Austin Health. All procedures were approved by the Austin Health's Animal Ethics Committee.

Carbohydrates and cDNA clones

The following glycoconjugates were used: Galα(1,3)Gal-BSA (di-Gal-BSA), Galα(1,3)Galβ(1,4)GlcNAc-BSA (tri-Gal-BSA), Galα(1,3)Galβ(1,4)Glc-NAcβ(1,3)Galβ(1,4)Glc-BSA (penta-Gal-BSA), Galβ(1,4)GlcNAc-BSA (Lac-NAc-BSA), Galβ(1,4)Glc-BSA (Lac-BSA), Gal-BSA, Man-BSA (Dextra Laboratories, Reading, UK), all other carbohydrates were obtained from Sigma Chemicals, St Louis, MO, USA. The following cDNA encoding pig α1,3GT,[68] rat iGb3S,[69] rat Gb3 synthase (Gb3S) [67] were ligated the pcDNA1 expression vector (Invitrogen, Mount Waverly, VIC, Australia) and used for transfection studies.

Immunization and fusion procedures

The α1,3GT−/− mice were immunized three times with 200 µl of packed RbRBC by intraperitoneal (i.p.) injections at 2-week intervals. Antibody responses were monitored by ELISA (see below). Mice with anti-Galα(1,3)Gal antibody titres greater than 1:20 000 were used for fusions. Three days after an intravenous injection of RbRBC, spleen cells were isolated and fused with NS1 cells. The resulting hybridoma supernatants were analysed for anti-Galα(1,3)-Gal-reactive antibody by ELISA. Only those hybridomas that exhibited reactivity to Galα(1,3)Gal-BSA and not to BSA were cloned by limiting dilution and characterized further.

Enzyme-linked immunosorbent assay

An ELISA was used to detect antibody binding. Glycoconjugate–BSA or BSA was coated onto 96-well polyvinyl chloride plates (Costar, Cambridge, MA, USA) at 20 µg ml^{-1} in 0.05 M carbonate buffer, pH 9.6, for 2 h at 37 °C. To block the non-specific binding sites on the plates, the plates were incubated with 2% (w/v) human serum albumin (Sigma) in phosphate-buffered saline for 1 h at room temperature. The anti-Galα(1,3)Gal antibodies to be tested were added to the wells either as mouse serum or as culture media supernatant, containing the secreted mAbs. The amount of antibody bound was quantitated by the addition of sheep anti-mouse immunoglobulin, conjugated to horseradish peroxidase (HRP) (Amersham, Piscataway, NJ, USA) and the HRP substrate [0.03% 2,2-azino-di-3-ethylbenzthiazoline sulphonate, 0.02% $H2O_2$] and the optical density was measured using an ELISA reader (Biotek EL312e, Bio-Tek Instruments, Winooski, VT, USA) at 405 nm. An anti-haptoglobin IgG1, FE14.1,[40] was used to determine the level of non-specific interaction between the mouse antibodies and the antigen.

An ELISA was also used to determine the antibody isotype of each mAb. Hybridoma supernatant culture media containing the secreted mAbs were added to Galα(1,3)Gal-BSA-coated plates and incubated as described above. The mAbs were detected by using isotype-specific biotin-conjugated rabbit anti-mouse IgG_1, IgG_2a, IgG_2b, IgG_3, IgA and IgM antibodies (Pharmingen, San Diego, CA, USA) at 1/1000 dilution. Detection of the bound isotype antibodies was detected by streptavidin-labelled HRP (Pharmingen) and measured as described above.

Carbohydrate inhibition assays

To test the specificity of the reactions and better define the epitopes of the mAbs, inhibition assays were performed. Briefly, 25 µl of antibody at one dilution less than that of the 50% titre was incubated at 4 °C for 16 h with 25 µl of the inhibitors (Table 2), serially diluted from 20 to 0.005 mM, and the mixture was then used in the ELISA. The percentage inhibition for a given inhibitor concentration was calculated as: ((1-OD mAb bound in the presence of inhibitor)/(total OD bound in the absence of inhibitor)×100). The relative affinity of the antibodies for the carbohydrates was calculated as the molar concentration of carbohydrate giving 50% inhibition (I_{50}) of the maximal binding of antibody.

Cell culture, transfection and serology

Human embryonic kidney cells (293) were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum and transfected with the cDNA clones described above using LipofectAMINE Plus (Life Technologies, Gaithesburg, MD, USA) as recommended by the manufacturer. Stable expressing 293 clones were produced by selection in puromycin to give the following clones: α1,3GT-293, iGb3S-293 and Gb3S-293. Cell-surface expression of carbohydrate epitopes was analysed by flow cytometry using a FACScalibur (Becton

Dickinson, Franklin Lakes, NJ, USA). Cells were stained with either FITC-labelled IB_4 lectin (Sigma), the mAbs. All mAbs were detected with FITC-labelled sheep anti-mouse IgG (Dako, Glostrup, Denmark). Cells were also stained with purified human anti-Galα(1,3)Gal IgG [70] and detected with FITC-labelled sheep anti-human IgG (Dako) or anti-Galα(1,3)Gal IgY detected with FITC-labelled anti-chicken Ig (Dako).

Molecular modelling

Comparative models of the Fv molecules of 8.17 and 15.101 mAbs were prepared using the Homology module of the Insight II program, version 98.0 (Accelrys, San Diego, CA, USA) following previously developed procedures.[71] An anti-galactan mAb (J539) shared high amino-acid identities (>85%) with the VL and VH domains of 8.17 and 15.101. Since the three-dimensional structure of the J539 Fab was determined by X-ray diffraction analysis at 2.6 Å (PDB code 2FBJ),[42] we used the atomic coordinates of its Fv portion as a template for two prototypic anti-Galα(1,3)Gal mAb. For predicting conformations of CDRs, templates sharing the lengths and canonical structures were used. Conformations of the 9-residue HCDR3 regions of 8.17 and 15.101 were predicted using well-resolved templates with similar loop and base structures.[72] The Fv coordinates were subjected to energy minimization, to remove any steric clashes introduced into the initial template-based models, using the CNS program package, version 0.9.[73]

Automated docking procedures were similar to those we have previously employed to predict antibody interactions with peptides.[49,74,75] Carbohydrate structures Galα(1,3)Gal, Galα(1,3)Galβ(1,4)Glc and Galα(1,3)Galβ(1,4)GlcNAc were generated using SYBYL (Tripos, St Louis, MO, USA), version 6.9.1. Automated docking was implemented with DOCK, version 4.0 (DOCK4),[76] the intermolecular interactions between carbohydrates and mAbs in the docked complexes were examined with the program LIGPLOT,[77] using default parameters. Automated docking was performed with the following DOCK4 routines: (1) *anchor first* approach for conformational sampling; (2) *uniform sampling* for orientational search and (3) electrostatic and vdW interactions within the AMBER force field for scoring and ranking. Solvation effects were implicitly included into the force-field energy function through the use of a distance-dependent dielectric coefficient. Structural validation of carbohydrate docking, including detailed description of the procedure and different approaches to treatment of the conformational flexibility, is to be published elsewhere (Yuriev *et al*., manuscript submitted to *Molecular Simulation*). Coordinates for the docked complexes are available from the authors upon request.

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