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Xenotransplantation: *in vitro* analysis of synthetic α-galactosyl inhibitors of human anti-Galα1→3Gal IgM and IgG antibodies

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Pig-to-human xenotransplantation might be an option to overcome the increasing shortage of human donor organs. However, naturally occurring antibodies in human blood against the Galα1→3Gal antigen on pig endothelial cells lead to hyperacute or, if prevented, acute or delayed vascular rejection of the pig graft. The purpose of this study was therefore to evaluate synthetic oligosaccharides with terminal Galα1→3Gal to inhibit antigen-binding and cytotoxicity of anti-αGal antibodies against pig cells. Different oligosaccharides were synthesized chemically and by a combined chemico-enzymatic approach. These included monomeric di–, tri–, and pentasaccharides, a polyacrylamide-conjugate (PAA-Bdi), as well as di–, tetra–, and octamers of Galα1→3Gal. All were tested for inhibitory activity by anti-αGal ELISA and complement-dependent cytotoxicity tests. PAA-Bdi was the best inhibitor of binding as well as cytotoxicity of anti-αGal antibodies. Monomeric oligosaccharides efficiently prevented binding of anti-αGal IgG, but less well that of anti-αGal IgM, with tri- and pentasaccharides showing a better efficacy than the disaccharide. The two trisaccharides Galα1→3Galβ1→4GlcNAc and Galα1→3Galβ1→3GlcNAc were equally effective. Oligomers of Galα1→3Gal were more effective than monomers in blocking the binding of anti-αGal IgG. However, they could not block IgM binding, nor could they match the efficacy of PAA-Bdi. We conclude that oligosaccharides with terminal Galα1→3Gal, most effectively as PAA-conjugates, can prevent binding and cytotoxicity of human anti-αGal *in vitro*. The PAA-Bdi conjugate might be most suited for use as a Sepharose-bound immunoabsorption material.

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Introduction

During the last decade, the increasing shortage of human donor organs for transplantation has boosted the interest in xenotransplantation, i.e., the transplantation of animal organs into humans (Auchincloss and Sachs 1998). Because of some similarities in terms of anatomy and physiology with humans, their high replication rate and easy breeding, but also because of ethical and virological considerations, pigs are seen today as the most promising organ donor candidates (Ye *et al.*, 1994; Hammer *et al.*, 1998). However, pigs—and with them all mammals except humans, apes, and Old World monkeys—carry the terminal disaccharide Galα1→3Gal on the surface of their endothelial cells (Oriol *et al.*, 1999). All humans and primates have naturally occurring antibodies against this epitope in their bloodstream (Galili *et al.*, 1984). These carbohydrate-specific, xenoreactive antibodies (anti-αGal), both of the IgM and IgG isotype, have been identified to be an important immunological barrier in pig-to-human xenotransplantation (Good *et al.*, 1992; Oriol *et al.*, 1993). Together with incompatibilities of the complement system (Cooper *et al.*, 1988; Dalmasso *et al.*, 1991; Platt *et al.*, 1991; Yang *et al.*, 1992), binding of anti-αGal to the endothelial cells of a pig organ leads to hyperacute rejection of the latter (Cooper *et al.*, 1994; Sandrin and McKenzie 1994; Collins *et al.*, 1995).

It was recently demonstrated that organs derived from pigs transgenic for human complement regulatory proteins, such as decay accelerating factor (DAF), CD59, or membrane cofactor protein (MCP), are protected against hyperacute rejection when transplanted into baboons (McCurry *et al.*, 1995; White and Yannoutsos 1996). However, as the terminal Galα1→3Gal epitope is still present on the endothelium of such organs, anti-αGal antibodies are binding nonetheless, leading to either hyperacute, acute, or delayed xenograft rejections, which may resemble the rejections seen in ABO-incompatible transplantations (Kobayashi *et al.*, 1997; Palmetshofer et al., 1998a,b). Two principally different strategies to prevent anti-αGal binding to the endothelial cells have therefore been followed by various research groups. The first is the genetic manipulation of donor pigs in order to block expression of the Galα1→3Gal epitope. Mouse experiments showed that both the transgenic expression of human α1→2–fucosyltransferase (H-transferase) and the inactivation of the α1→3–galactosyltransferase gene by homologous recombination successfully prevented the appearance of the Galα1→3Gal epitope (Sandrin *et al.*, 1995; Chen *et al.*, 1996; Sharma *et al.*, 1996; Osman *et al.*, 1997; McKenzie *et al.*, 1998). The second possibility is the use of synthetic antigens either as immunoabsorption substances to remove anti-αGal from the recipient's circulation or as soluble substances to inhibit the binding of anti-αGal to the α–galactosyl epitope on the cell surface. The use of immunoabsorption substances was shown to be successful in the clinical setting of ABO-incompatible transplantation (Bensinger *et al.*, 1981; Aeschbacher *et al.*, 1987; Mendez *et al.*, 1992) and also in experimental xenotransplantation models (Rieben *et al.*, 1995; Taniguchi *et al.*, 1996; Kozlowski *et al.*, 1998; Xu *et al.*, 1998). Infusion of soluble oligosaccharides to block anti-αGal binding was shown to effectively delay rejection in ABO-incompatible transplantation (Cooper *et al.*, 1993) and in anti-αGal dependent xenotransplantation models as well (Simon *et al.*, 1998).

Human anti-αGal that bind to pig endothelial cells or the pig kidney cell line PK15, which abundantly expresses the α-galactosyl epitope, were shown to preferentially react with more complex structures than the Galα1→3Gal disaccharide (Galili and Matta 1996; Neethling *et al.*, 1996). In fact, as compared with the Galα1→3Gal disaccharide the trisaccharide Galα1→3Galβ1→4GlcNAc was shown to have an up to 10-fold higher inhibitory effect on anti-αGal mediated cytotoxicity against PK15 cells *in vitro* (Neethling *et al.*, 1996). The experiments presented here were therefore designed to extend the search for optimal synthetic oligosaccharide inhibitors of human anti-αGal, including oligo- and polymeric variants thereof. As the difficulties and also the costs for direct chemical synthesis of oligosaccharides increase considerably with increasing chain length, a combined chemico-enzymatic strategy for oligosaccharide production was developed and used for the production of one of the substances analyzed in this report.

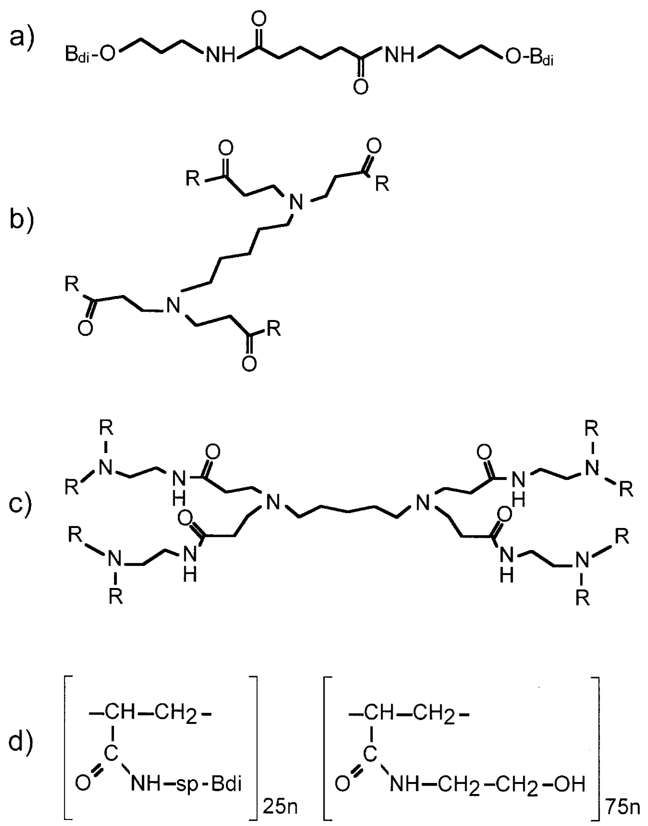

Fig. 1. Structure of neoglycoconjugates: (**a**) Bdi-dimer, MW 1161; (**b**) Bdi-tetramer, R = $NHCH_2CH_2NH(O)C(CH_2)_4C(O)NH(CH_2)_3OBdi$, MW 1607; (**c**) Bdi-octamer R = $CH_2CH_2C(O)NHCH_2CH_2NH(O)C(CH_2)_4C(O)NH(CH_2)_3OBdi$, MW 5555; (**d**) polyacrylamide-Bdi conjugate of 25% molar substitution rate. The spacer (sp) is $CH_2CH_2CH_2O$, a C_2-tail (CH_2CH_2OH) is linked to the CONH-sites which are not occupied by the αGal disaccharide. The relative molecular weight of the PAA-Bdi conjugate is ~30 kDa, i.e., about 40 Bdi-haptens are present per conjugate molecule.

Results

Combined chemico-enzymatic synthesis of Galα1→3Galβ1→3GlcNAc.

A chemically synthesized Galβ1→3GlcNAc disaccharide was incubated with α1→3-galactosyltransferase and UDP-Gal to produce the Galα1→3Galβ1→3GlcNAc trisaccharide as described in *Materials and methods*. The product eluted as a single peak from gel filtration on Bio-Gel P-4 and from HPLC on a Lichrosorb-NH_2 column. Based on HPLC and phenol-sulfuric acid assay yields ranged from 60–90%. For product characterization, part of the material was analyzed by 400 MHz ^{1}H-NMR spectroscopy. Assignments were made by comparison with spectral data of known compounds. The $J_{1,2}$ of 3.9 Hz for H-1 of the terminal Gal residue in the trisaccharide product, together with the characteristic position of its resonance (δ 5.138 p.p.m.), is diagnostic of an α–anomeric linkage. Furthermore, the change in the chemical shift of the H–1 signals of Galβ3 (Δδ H-1(α) + 0.062, and Δδ H-1(β) + 0.067 p.p.m.), together with the effect on the chemical shift of its H–4 (Δδ H-4(α) + 0.095, and Δδ H-4(β) + 0.116 p.p.m.), are characteristic of a Gal1→<u>3</u> substitution of the Galβ3. Similar changes were observed in the H-1 and H-4 signals of the β4-linked galactose in Galβ1→4GlcNAc upon conversion into Galα1→3Galβ1→4GlcNAc (Joziasse *et al.*, 1990). Changes in the chemical shifts of the GlcNAc H-1(α) and N-acetyl proton signals were too small (<0.005 p.p.m.) to be significant. Taken together, the NMR data confirm that the substrate Galβ1→3GlcNAc was α1→3–galactosylated at the terminal β3-linked Gal residue.

Immunoabsorption of human serum on PAA-Bdi Sepharose: effects on titers of anti-αGal as detected by ELISA and PK15-cytotoxicity test

Human serum was absorbed over a column of PAA-Bdi Sepharose, and the reduction of both anti-αGal and anti-A trisaccharide antibody titers monitored isotype-specifically by ELISA (Figure 2) and by PK15 cytotoxicity test (Figure 3). PAA-Bdi Sepharose absorbed 96% (IgG) to 99% (IgM) of the anti-αGal antibody as assessed by ELISA with PAA-Bdi (structure: Figure 1d) as coating antigen, whereas the same column absorbed only 23% of anti-A IgG and 20% of anti-A IgM (assay with PAA-Atri as coating antigen).

The cytotoxicity of human serum against pig cells before and after immunoabsorption was measured in the presence of 10% rabbit serum as additional complement source. After immunoabsorption, anti-PK15 cytotoxicity was 11 ± 5% of the level of

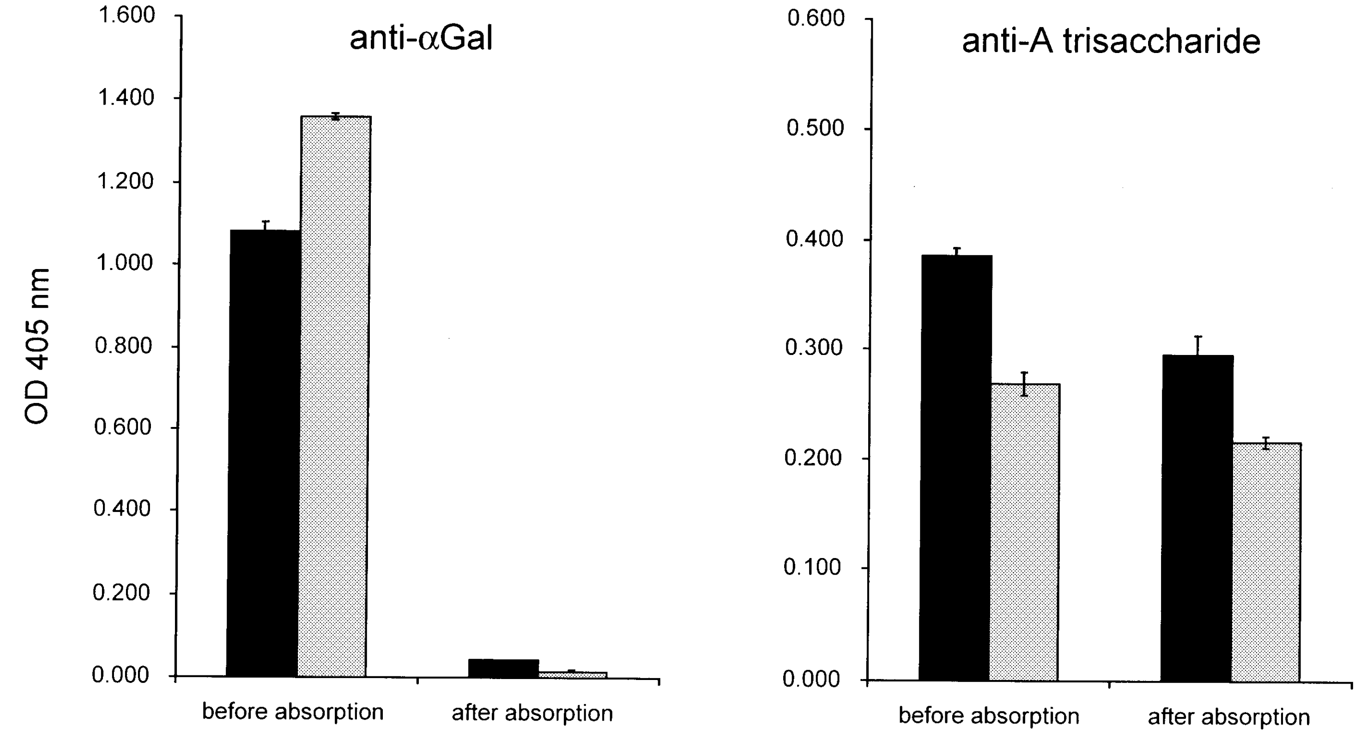

Fig. 2. Immunoabsorption of anti-αGal on PAA-Bdi Sepharose (Xenotran): efficacy and specificity as detected by ELISA. Human anti-αGal and anti-Atri antibodies were measured isotype-specifically by ELISA before and after immunoabsorption. Averages of duplicate optical density values at 405 nm of the respective IgM- (black columns) and IgG-isotypes (gray columns) are represented with indication of the standard deviations.

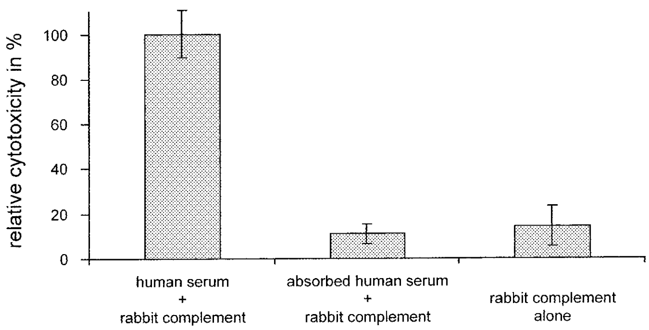

Fig. 3. Immunoabsorption of anti-αGal on PAA-Bdi Sepharose (Xenotran): influence on PK15-cytotoxicity. The cytotoxic activity of human serum against the PK15 cell line was measured before and after immunoabsorption. Rabbit serum (10%) was added as complement source. The columns represent relative cytotoxicities of the native serum (set to 100%), the absorbed serum, and the 10% rabbit serum in cell culture medium. Average values of 5-fold testing are given with indication of the standard deviations.

the untreated serum, which was not significantly different from the 14 ± 9% background cytotoxicity induced by the complement source alone (Figure 3).

Isotype-specific differences in inhibition of anti-αGal binding by oligosaccharides and glycoconjugates with terminal Galα1→3Gal

The inhibitory effects of different oligosaccharides and glycoconjugates on human anti-αGal binding to PAA-Bdi coated microtiter plates was analyzed isotype-specifically by ELISA. Inhibition experiments were performed with the disaccharide Galα1→3Gal, the trisaccharides Galα1→3Galβ1→**4**GlcNAc and Galα1→3Galβ1→**3**GlcNAc, and the pentasaccharide Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc. As shown in Figure 4, binding of anti-αGal IgG (Figure 4, lower panel) was inhibited more easily than binding of the respective IgM isotype (Figure 4, upper panel). For both isotypes, the pentasaccharide antigen was the most effective inhibitor, followed by the tri– and disaccharides. The two different trisaccharide isomers with β1→4 and β1→3 linkages to GlcNAc were equally active as inhibitors of both anti-αGal IgM and –IgG binding (Figure 4, data shown only for the β1→4 linked trisaccharide).

Oligomeric glycoconjugates (di–, tetra–, and octamers, see Figure 1a–c for structures) and PAA-Bdi were also tested as inhibitors in the ELISA. Like the monomeric disaccharide, the tested oligomeric conjugates thereof were unable to inhibit anti-αGal IgM binding at concentrations up to 10 μM. In contrast, PAA-Bdi showed a pronounced inhibitory effect at the same molar concentrations of Galα1→3Gal residues, with an IC50 of about 1 μM (Figure 5, upper panel). The capacity of both the tetra– and octameric compounds to block anti-αGal IgG binding was enhanced as compared to the monomer (Figure 4, lower panel), revealing IC50 values of ~8 μM (Figure 5, lower panel). However, also for inhibition of IgG binding the PAA-Bdi conjugate, with an IC50 of ~1 μM, was the most active substance.

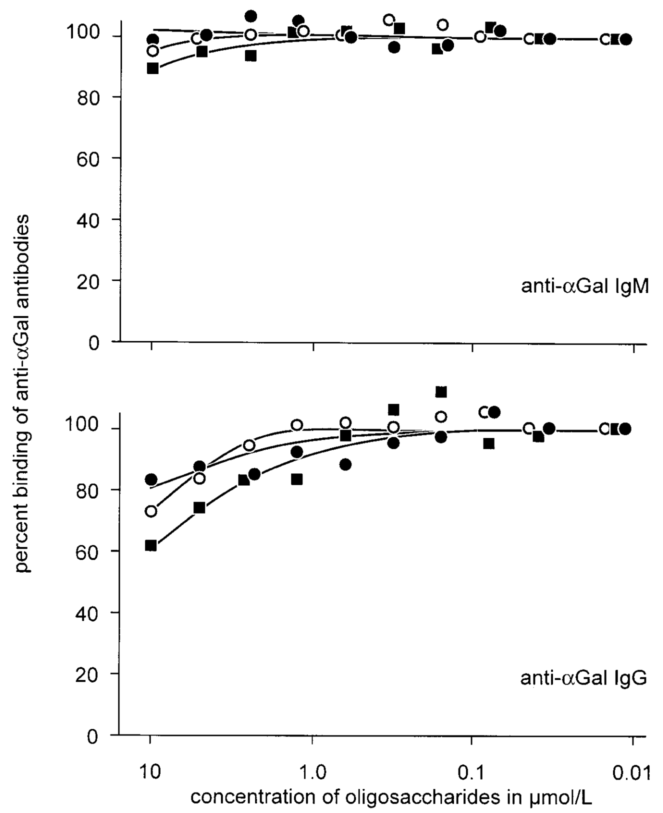

Fig. 4. Inhibition of anti-αGal binding by monomeric oligosaccharides in ELISA. Oligosaccharides were added to 1:400 diluted human serum, the mixtures incubated on PAA-Bdi coated ELISA plates, and the binding of IgM (upper panel) and IgG (lower panel) visualized with monoclonal antibodies. The oligosaccharides used were Galα1→3Gal (solid circles), Galα1→3Galβ1→4GlcNAc (open circles), and Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc (solid squares). Representative data are shown of three experiments with similar results.

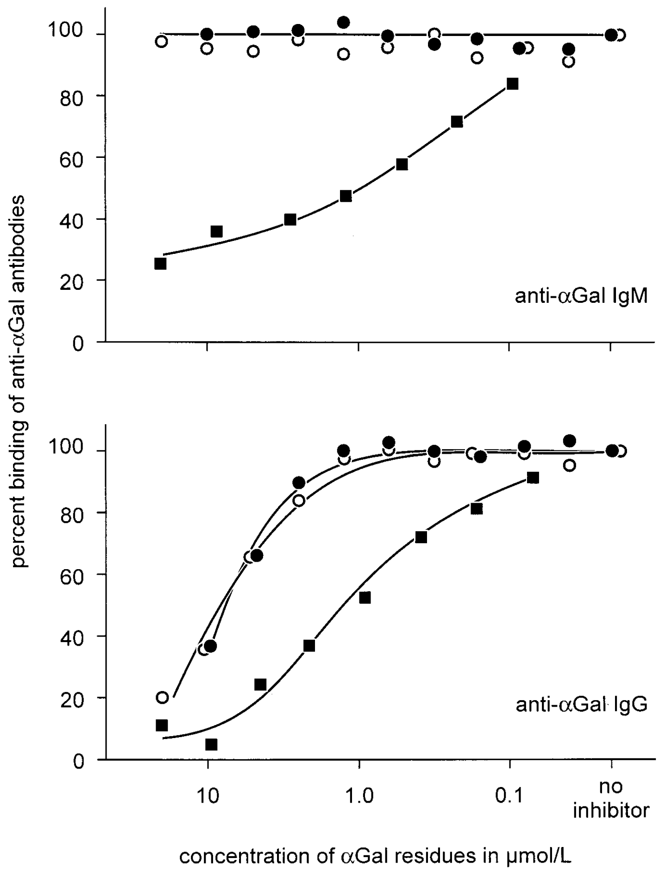

Fig. 5. Inhibition of anti-αGal binding by oligo– and polymeric glycoconjugates of Galα1→3Gal. Tetrameric (solid circles) and octameric (open circles) conjugates as well as PAA-Bdi (solid squares) were used to block human anti-αGal binding in an ELISA system. Binding of anti-αGal IgM is shown in the upper panel (no inhibition with tetra- and octamers), and binding of IgG in the lower panel. The units on the x-axis refer to molar concentrations of Galα1→3Gal residues. Representative data of three experiments.

Inhibition of anti-αGal mediated cytotoxicity

Glycoconjugates and oligosaccharides with terminal Galα1→3Gal were serially diluted in human serum and their inhibitory effect on cytotoxicity against the pig kidney cell line PK15 was measured. Figure 6 shows the inhibition curves for the disaccharide and the two different trisaccharide isomers Galaα1→3Galβ1→4GlcNAc and Galα1→3Galβ1→3GlcNAc. Similar to the inhibition of anti-αGal IgM in the ELISA, a better blocking of cytotoxicity was seen for the trisaccharides than for the disaccharide. The inhibitory effects of the two isomeric trisaccharides were indistinguishable also in this assay, with IC50 values of ~200 µM.

The inhibitory capacity per mol αGal of the oligomeric substances was similar to the Galα1→3Gal monomer (IC50 ~500 µM, Figure 7, lower panel). In contrast, the PAA-conjugated Galα1→3Gal disaccharide (PAA-Bdi) showed an enhanced inhibitory effect on a per mol αGal basis as compared with the monomeric substance (IC50 ~500 µM vs. ~0.8 µM; Figure 7, upper panel). As a control of the specificity of PAA-Bdi for blocking anti-αGal antibodies a PAA-conjugate of the blood group H disaccharide Fucα1→2Gal, PAA-Hdi, was included in some of the experiments. This substance did not inhibit the cytotoxicity of human serum against PK15 cells (Figure 7, upper panel).

Discussion

Unlike chemical synthesis, the small-scale application of glycosyltransferases allows for a quick, regio-selective, and stereospecific synthesis of micromolar to millimolar quantities of glycoconjugates; quantities are limited by the availability of the various enzymes only. In this report we have taken advantage of the known relaxed acceptor preference of some glycosyltransferases (Hokke *et al.*, 1993; de Vries *et al.*, 1997), and applied α1→3-galactosyltransferase in the synthesis of the non-physiological trisaccharide Galα1→3Galβ1→3GlcNAc. Although the enzyme prefers type II precursor chains (Galβ1→4GlcNAc) over type I chains (Galβ1→3GlcNAc), as seen from a 10-fold difference in K_m for these substrates (Blanken and Van den Eijnden 1985), reasonable yields of

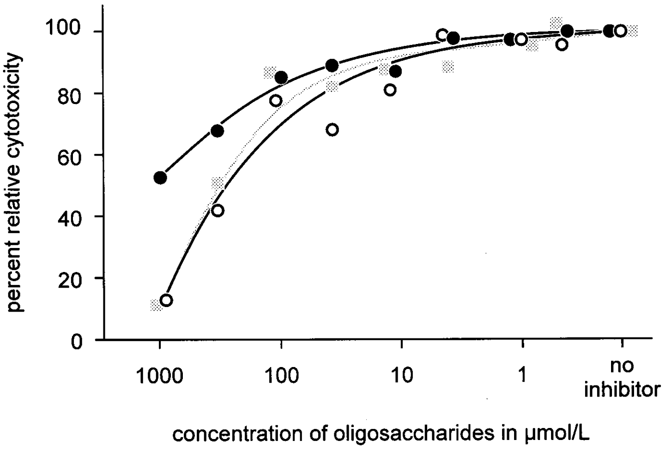

Fig. 6. Inhibition of anti-αGal mediated cytotoxicity of human serum against PK15 cells. The disaccharide Galα1→3Gal (solid circles) as well as the trisaccharides Galα1→3Galβ1→**4**GlcNAc (open circles) and Galα1→3Galβ1→**3**GlcNAc (shaded squares) were added to undiluted human serum, 10% rabbit serum added as a complement source, and the cytotoxicity measured in a fluorescence-based assay. Representative data of 5 assays are shown.

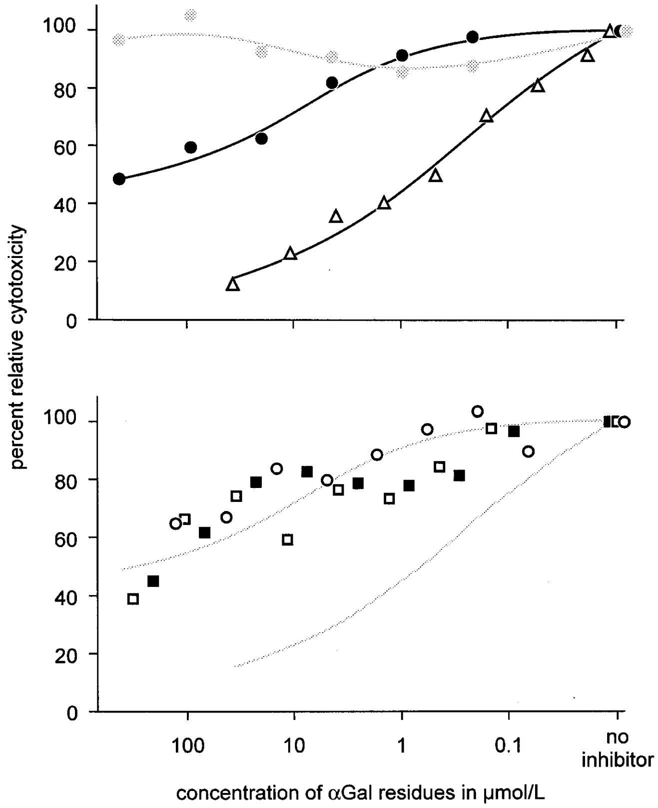

Fig. 7. Inhibitory effects of αGal oligo- and polymers on PK15 cytotoxicity of human serum. Inhibition curves for the Galα1→3Gal disaccharide (solid circles) and its PAA-conjugate, PAA-Bdi (open triangles), as well as PAA-Hdi (as a negative control, shaded circles) are shown on the upper panel. Cytotoxicity-inhibition by dimers (open circles), tetramers (solid squares), and octamers (open circles) of Galα1→3Gal is represented on the lower panel, with superposition of the inhibition curves for the monomeric disaccharide and PAA-Bdi (gray lines). Representative data of three experiments with similar results.

products can be obtained by combining relatively high amounts of enzyme with increased concentrations of acceptor substrate.

The chemico-enzymatically produced Galα1→3Galβ1→3GlcNAc was compared with its physiological β1→4 linked counterpart, Galα1→3Galβ1→4GlcNAc, as well as the disaccharide Galα1→3Gal and the pentasaccharide Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc for inhibitory activity on anti-αGal binding in ELISA and PK15 cytotoxicity test. Our results confirm previous observations (Neethling *et al.*, 1996; Parker *et al.*, 1996; Simon *et al.*, 1998) that the tri– and pentasaccharide structures are better inhibitors of human anti-αGal binding than the disaccharide. In fact, the pentasaccharide, which was originally identified as the main αGal-bearing oligosaccharide species in pig kidney endothelium glycolipids by the group of Samuelsson (Holgersson *et al.*, 1992; Samuelsson *et al.*, 1994), had the highest inhibitory capacity of all tested monomeric oligosaccharides. Interestingly, the β1→3 and β1→4 linked trisaccharides showed no difference in inhibitory strength, neither in the ELISA system nor in the PK15 cytotoxicity test. Because the β1→3 linked trisaccharide is easier and therefore cheaper to synthesize by organic synthesis, this substance might be a valuable alternative for future (pre–)clinical xenotransplantation experiments which will require relatively large amounts of oligosaccharides as anti-αGal blockers.

An important part of this study was devoted to a comparison of the isotype-specific inhibitory effects of mono–, oligo–, and polymeric variants of oligosaccharides. In general, monomeric antigens, including the tri– and pentasaccharide, were poor inhibitors of anti-αGal IgM binding, with only zero (disaccharide) to 10% (pentasaccharide) inhibition at the highest tested concentration. Whereas the di–, tetra–, and octameric constructs were better inhibitors of anti-αGal IgG than the monomeric disaccharide this was not the case for inhibition of anti-αGal IgM, nor the PK15-specific cytotoxicity. However, an approximately 1000-fold enhancement of inhibitory activity, based on calculation per αGal residue, was achieved by using the flexible, hydrophilic PAA-Bdi polymer, which contains ~40 Galα1→3Gal epitopes per molecule. The anti-αGal inhibition by PAA-Bdi was specific as assessed by a PAA-Hdi, which did not inhibit PK15 cytotoxicity up to a concentration of 1000 µM, and by the use of Sepharose-bound PAA-Bdi, which efficiently absorbed anti-αGal from human serum, but not antibodies against the blood group A trisaccharide.

This study was not designed to allow an exact calculation of the number of anti-αGal IgM or –IgG molecules that can be bound by the tested substances, nor of their affinities for human anti-αGal antibodies. However, it can be assumed that the oligomeric constructs of Galα1→3Gal used in this study—while being good inhibitors of anti-αGal IgG—were not able to efficiently block enough of the 10 binding sites of an anti-αGal IgM molecule to prevent its binding to either PAA-Bdi coated ELISA plates or PK15 cells. In contrast to the relatively compact and rigid oligomers, the PAA-conjugate seems to be large and/or flexible enough to be a highly efficient inhibitor of anti-αGal IgM as well as PK15 cytotoxicity. Larger oligomeric structures, up to 64-mer dendrimers of Galα1→3Gal, are currently under investigation and preliminary experiments showed an enhancement of anti-αGal IgM binding properties with increasing size; these results will be published elsewhere.

The use of PAA-Bdi as a soluble substance for infusion into a patient might be problematic because of the possibility of immune complex formation and will therefore need careful evaluation. However, the excellent anti-αGal IgM- and IgG-binding properties of PAA-Bdi were also retained when it was used coupled to Sepharose as an immunoabsorption material, whereas direct coupling of Galα1→3Gal to Sepharose via a C3- or C9 spacer produced less efficient immunoabsorption substances (results not shown). In fact, Sepharose-based immunoabsorption substances have a long tradition in clinical application and Sepharose coated with a PAA-conjugate of the blood group B trisaccharide was recently used at our clinic to treat a patient of blood group O who accidentally received a blood group B heart transplant. The treatment proved to be safe and efficient and the patient is still alive and well more than 2 years after transplantation (Mohacsi *et al.*, 1998). Similarly, PAA-Bdi Sepharose might be used in the future to remove anti-αGal antibodies prior to pig-to-human xenotransplantation. In view of the results presented here it is likely that the ligand (Bdi) can be further optimized: tri– or pentasaccharides, or a mixture thereof as proposed by others (McKane *et al.*, 1998), conjugated to PAA might be superior to PAA-Bdi. In addition, organic chemical synthesis of the ligand may be facilitated by use of the Galα1→3Galβ1→3GlcNAc isomer. In conclusion, immunoabsorption on PAA-Bdi Sepharose derivatives for now seems to be the most promising approach for medical application of oligosaccharides with terminal Galα1→3Gal in xenotransplantation in the near future.

Materials and methods

Oligosaccharides, glycoconjugates and immunoabsorption material

The linear B-disaccharide Galα1→3Gal (Bdi), the trisaccharide Galα1→3Galβ1→4GlcNAc, and the pentasaccharide Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc were obtained from Dextra Laboratories Ltd., Reading, UK. Conjugates of Bdi and poly-N-hydroxyethylacrylamide (PAA-Bdi) as well as PAA-Bdi covalently linked to Sepharose 6FF (PAA-Bdi Sepharose, Xenotran) were produced by the laboratory of carbohydrate chemistry in Moscow (Bovin *et al.*, 1993) and obtained through Syntesome GmbH, Munich, Germany. PAA-conjugates of the blood group A trisaccharide GalNAcα1→3(Fucα1→2)Gal (PAA-Atri) as well as the H disaccharide Fucα1→2Gal (PAA-Hdi) were used for control experiments (both from Bovin/Syntesome). Dimeric Bdi, namely $B_{di}O(CH_2)_3NH$-$CO(CH_2)_4CONH(CH_2)_3OB_{di}$,, was synthesized by reaction of $B_{di}O(CH_2)_3NH_2$ with bis-nitrophenyl ester of adipinic acid. Synthesis of di–, tetra–, and octameric B_{di} (see structures in Figure 1a) was described earlier (Tsvetkov *et al.*, 1999).

Chemical reagents, human sera, and antibodies

Recombinant bovine UDP-Gal:Galβ1→4GlcNAc α1→3-galactosyltransferase was produced in an insect cell culture system as described earlier (Joziasse *et al.*, 1989, 1990), and purified by affinity chromatography on UDP-Sepharose. Human sera were obtained from healthy volunteer donors and selected for high titers of anti-αGal and a pronounced cytotoxic activity against PK15 cells. Monoclonal antibodies against human IgM or –IgG (clones HB57 and HB43) were obtained from the American Type Culture Collection (ATCC), Manassas, VA. Biotinylated goat anti-mouse IgG_1 antibody was from Southern Biotechnology Associates, Birmingham, AL, and streptavidin conjugated to alkaline phosphatase from Amersham Life Science (Amersham Pharmacia Biotech, Bucks, UK). All other chemicals were from Merck (E. Merck, Darmstadt, Germany) or Sigma (Sigma Chemical Co., St. Louis, MO).

Synthesis of Galα1→3Galβ1→3GlcNAc

The trisaccharide Galα1→3Galβ1→3GlcNAc was produced by chemico-enzymatic synthesis. The disaccharide Galβ1→3GlcNAc was chemically synthesized by Bovin et al. (Bovin and Khorlin, 1984), and α1→3-galactosylated by use of recombinant bovine α1→3-galactosyltransferase in a final reaction volume of 2.50 ml containing: 5.0 μmol Galβ1→3GlcNAc, 0.25 mmol Tris-maleic acid buffer pH 6.8, 10 μmol ATP, 50 μmol γ–galactonolacton, 100 μg bovine serum albumin, 20 μmol $MnCl_2$, 7.5 μmol UDP–Gal, and 150 mU bovine α1→3-galactosyltransferase. After incubation at 37°C for 16 h the reaction was stopped on ice. The mixture was chromatographed on a column (bed volume 3 ml) of Dowex 1-X8 (Cl^-), the flow-through collected and lyophilized. The dry residue was dissolved in 1.0 ml 50 mM ammonium acetate buffer at pH 5.2, and purified by gel filtration on a calibrated column of Bio-Gel P-4 (100–200 mesh, 1.6 × 200 cm), equilibrated and run in 50 mM ammonium acetate buffer pH 5.2. Fractions of 3.6 ml were collected, and the elution position of the product was determined based on orcinol assay. Fractions containing hexose, eluting in the trisaccharide region, were collected and lyophilized.

The total amount of product was assayed by the phenol-sulfuric acid hexose assay, and on the basis of the detector response upon HPLC analysis (Lichrosorb-NH_2 column, elution with acetonitrile/buffer 80/20 at a flow rate of 0.2 ml/min; detection based on UV absorption at 195 nm). Part of the product was analyzed by 400 MHz ^{1}H-NMR spectroscopy as described earlier (Joziasse *et al.*, 1990).

Immunoabsorption of human serum on PAA-Bdi Sepharose

Polypropylene chromatography columns (Poly-Prep, Bio-Rad Laboratories, Hercules, CA) were packed with 2 ml of PAA-Bdi Sepharose and rinsed with PBS. Nine milliliters of human serum were absorbed over the column and the amount of anti-αGal as well as anti-blood group A trisaccharide antibodies (as a control) measured by ELISA before and after absorption. For the ELISAs, microtiter plates were coated with PAA-Bdi and –Atri, respectively, and the sera were diluted 1:40 in PBS-BSA-Tween. Detection of bound antibodies was performed analogously to the oligosaccharide-inhibition test described below. Serum samples before and after absorption were also analyzed in the cytotoxicity test with PK15 cells.

Inhibition of anti-αGal antibody binding by different oligosaccharides and glycoconjugates: isotype-specific detection by ELISA

The degree of inhibition of human anti-αGal binding by the different oligosaccharide inhibitors was analyzed isotype-specifically by ELISA (Rieben *et al.*, 1995). Human serum was diluted 1:400 in phosphate-buffered saline pH 7.4 (PBS) containing 1% bovine serum albumin (BSA) and 5% Tween 20 (PBS-BSA-Tween). A serial dilution of the oligosaccharide or glycoconjugate to be tested was added to the serum and the mixture incubated for 120 min at 37°C or overnight at 4°C. Polystyrene microtiter plates (NUNC MaxiSorp, NUNC A/S, Roskilde, Denmark) were coated overnight at 4°C with 5 µg/ml of PAA-Bdi in 0.1 M carbonate buffer pH 9.6 and then washed with PBS containing 0.02% Tween 20. The serum-oligosaccharide mixture was added to the coated wells and incubated for 90 min at 37°C. After washing, the bound human antibodies were revealed isotype-specifically with monoclonal antibodies against IgM (HB 57) or IgG (HB 43), followed by biotinylated goat anti-mouse IgG_1, streptavidin–alkaline phosphatase conjugate, and 4-nitrophenyl phosphate substrate. The development of yellow color was measured with a microplate reader at 405 nm and the data analyzed in Microsoft Excel; values for 50% inhibition (IC50) were calculated by log-logit curve fitting.

Culture of PK15 cells

The PK15 cell line (order no. CCL 33) was obtained from ATCC. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Inc., Rockville, MD) with addition of 10% fetal bovine serum (FCS, Life Technologies) and 200 IU/ml of penicillin/streptomycin (Pen/Strep, Life Technologies); $DMEM^{++}$. Cells were grown in 75 cm^2 polystyrene culture flasks (Becton Dickinson Franklin Lakes, NJ) until they were used in the cytotoxicity assay (see below).

Inhibition of anti-αGal antibody-mediated cytotoxicity by different oligosaccharides and glycoconjugates: quantitation by nonradioactive cytotoxicity assay with PK15 cells

The cytotoxicity test was performed analogously to the one described by Neethling and Cooper (Neethling *et al.*, 1999). PK15 cells were seeded at ~150,000/ml in 10 µl $DMEM^{++}$ into 60 well Terasaki plates (Robbins Scientific, Sunnyvale, CA) and incubated for 24–48 h. The oligosaccharide or glycoconjugate to be tested was serially diluted in human serum and the mixture incubated over night at 4°C. Immediately before use in the assay 10% rabbit serum (Sigma) was added as additional complement source and the mixture then incubated for 10 min in the Terasaki plates with the PK15 cells. The plates were washed and the amount of cytotoxicity was revealed with a two-color fluorescent live/dead stain (calcein AM/ethidium homodimer 1, Molecular Probes Europe BV, Leiden, The Netherlands).

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Abbreviations

Anti-αGal, antibodies recognizing epitopes with terminal Galα1→3Gal; Atri, blood group A-trisaccharide GalNAcα1→3(Fucα1→2)Gal; Bdi, Galα1→3Gal; BSA, bovine serum albumin; $DMEM^{++}$, Dulbecco's modified eagle medium with 10% FCS and Pen/Strep; ELISA, enzyme-linked immunosorbent assay; FCS, fetal bovine serum; Hdi, blood group H disaccharide Fucα1→2Gal; IC50, concentration resulting in a 50% inhibition of binding (of human anti-αGal); PAA, poly-N-hydroxyethylacrylamide; PAA-Atri, conjugate of Atri to PAA; PAA-Bdi, conjugate of Bdi to PAA; PBS, phosphate-buffered saline; Pen/Strep, penicillin/streptomycin.

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