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# The reducing end of αGal oligosaccharides contributes to their efficiency in blocking natural antibodies of human and baboon sera

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**Abstract** Synthetic galactosyl oligosaccharides were tested for their ability to inhibit the cytotoxic reaction of human and baboon natural antibodies on PK15 cells in culture. Methyl-α-Gal gave weak inhibition, Galα1-3Gal substantially inhibited the reaction (400 μM), and Galα1-3Galβ1-4GlcNAc was ten times more efficient (30 μM). The modification from α to β anomeric configuration of the nonreducing end resulted in a complete loss of activity, while substitutions at the reducing end induced only a partial loss of activity. These observations suggest that natural anti-αGal antibodies recognize the epitope from its nonreducing end, but that substitutions at the reducing terminus can modify the antibody-binding capacity. Modified tri- and tetrasaccharides are better inhibitors than the disaccharide but not as good as Galα1-3Galβ1-4GlcNAc. The reducing terminus therefore contributes some energy to the reaction, indicating that certain oligosaccharides will be of more potential clinical use than others.

**Key words** Xenotransplantation, natural antibodies, oligosaccharides · Oligosaccharides, xenotransplantation · Natural antibodies, xenotransplantation · Baboon, xenotransplantation

## Introduction

New World monkeys and lower mammals express the Galα1-3Gal epitope on vascular endothelium [5, 14, 18]. Old World monkeys and humans have nonfunctional α1,3-galactosyltransferase genes [7, 9, 12], do not express the Galα1-3Gal epitope in tissues, and have developed natural antibodies that react with this antigen [6]. These antibodies can be responsible for the hyperacute rejection of pig vascularized organs transplanted into higher primates and for the cytotoxic reactions obtained on pig cells incubated in the presence of human or baboon normal serum and complement [1, 3, 4, 6, 11, 13, 15, 19, 20].

The main αGal glycolipid extracted from pig vascular endothelium has been shown to be the neutral pentaglycosylceramide Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer [17], and a similar oligosaccharide structure may be present on pig cell membrane glycoproteins. However, the components of this pentasaccharide that contribute most significantly to the binding of natural anti-αGal antibodies to the tissue target epitopes have not yet been defined.

The recent chemical synthesis (Chembiomed, Dextra Laboratories, and Syntesome) [10] and enzymatic synthesis [8] of some di-, tri-, and tetrasaccharides with terminal nonreducing αGal structures have allowed us to investigate various αGal oligosaccharides for their efficiency as blockers of the "in vitro" cytotoxic reaction of natural anti-αGal antibodies on the pig kidney cell line (PK15) that expresses the αGal epitope.

## Materials and methods

### Live/dead cytotoxicity test

PK15 cells were seeded in Terasaki microcytotoxicity plates at 750 cells per well and grown for 24 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum, penicillin/streptomycin 10000 U/ml, and glutamine 200 mM. After washing, 10 µl of human, baboon, or owl monkey serum in serial dilutions was added and the mixture was incubated for 60 min at 37 °C, washed again, and incubated for 30 min at room temperature with a mixture of 10 µl of live/dead fluorescent reagents, calcein AM 1 µM, and ethidium homodimer 2 µM (Molecular Probes, Eugene Ore., USA). Live (green cytoplasm) and dead (red nuclei) cells were counted in an inverted fluorescence microscope as previously described [11, 13].

### Human, baboon, and owl monkey serum

Fresh sera were used as the source of natural anti-αGal antibodies and normal rabbit serum as the source of complement when needed.

### Inhibition of cytotoxicity

Serial dilutions of the oligosaccharides, starting at a concentration of 10 mg/ml, were added to baboon or human serum aliquots, incubated for 10 min, and then added to cultures of PK15 cells in the microcytotoxicity trays. After incubation, the cells were stained with the live/dead reagents as in the direct cytotoxicity test. The concentration of oligosaccharide needed to obtain 50 % inhibition of the cytotoxic reaction was calculated from the regression lines obtained from the points comprised between 20 % and 80 % killing of cells in each test.

### Oligosaccharides

Monosaccharides were obtained from Sigma Chemicals (St. Louis, Mo., USA). Four synthetic oligosaccharides with αGal on the reducing end – G203 (Galα1-3Gal), G334 (Galα1-3Galβ1-4Gal), G443 (Galα1-3Galβ1-4Galα1-3Gal), and GN334 (Galα1-3Galβ1-4GlcNAc) – were obtained from Dextra Laboratories (Reading, UK). Other related oligosaccharides were obtained from Syntesome (Munich, Germany) and the solid immunoabsorbent Galα1-3Galβ1-4GlcNAcβ-Synsorb was obtained from Chembiomed (Alberta Research Council, Edmonton, Canada).

## Results

### Cytotoxic reaction

The PK15 cell line expressed large amounts of αGal epitopes, which were stained with the labeled lectin I-B4 of *Griffonia simplicifolia* (GSIB4, Vector Laboratories, Burlingame, Calif., USA) or with labeled human or baboon anti-αGal antibodies, affinity-purified on the Galα1-3Galβ1-4GlcNAc-Synsorb immunoabsorbent (Chembiomed, Alberta Research Council, Edmonton, Canada).

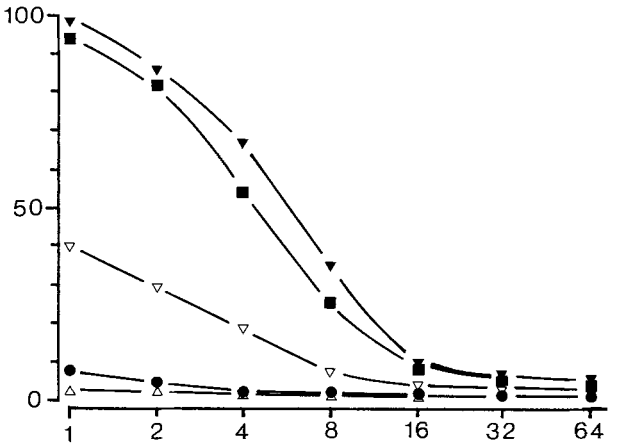


**Fig. 1** Cytotoxicity of unmodified fresh serum *(solid symbols)* from human (▼), baboon (■), and owl monkey (●) on PK15 pig cells in culture. The *unshaded symbols* indicate the cytotoxic reaction of human serum after one absorption (▽) and three absorption (△) on the Galα1-3Galβ1-4GlcNAcβ-Synsorb. Percent dead cells is represented on the ordinate and the reverse of the serum dilution on the abscisa

Incubation of PK15 cells in the presence of fresh human or baboon serum resulted in almost 100 % lysis of cells (Fig. 1). This reaction is complement-mediated since inactivation of complement by heating at 56 °C for 30 min or by the addition of EDTA abolished the reaction. The full cytotoxic reaction was restored by the addition of fresh rabbit serum as a source of complement. Incubation of cultured PK15 cells under similar conditions with owl monkey serum (*Aotus trivirgatus*, a New World monkey) did not give a significant cytotoxic reaction (Fig. 1).

Two-thirds of natural anti-αGal antibodies were eliminated from normal human serum after a single absorption on a column of Galα1-3Galβ1-4GlcNAc-Synsorb (3 ml of serum per gram of immunoabsorbent) and more than 95 % of the cytotoxic reaction was abolished after three consecutive absorptions of the same human serum on the regenerated immunoabsorbent (Fig. 1). The addition of rabbit serum as an extra source of complement did not increase the cytotoxic capacity of this absorbed human serum fraction (not shown). The affinity-purified anti-αGal antibodies were eluted from the Galα1-3Galβ1-4GlcNAc-Synsorb with $NH_4OH$ 1 % (pH 11). After dialysis against culture medium (DMEM), these affinity purified antibodies were able to kill cultured PK15 cells in the presence of normal rabbit serum as a source of complement (3 µl/well; Fig. 2).

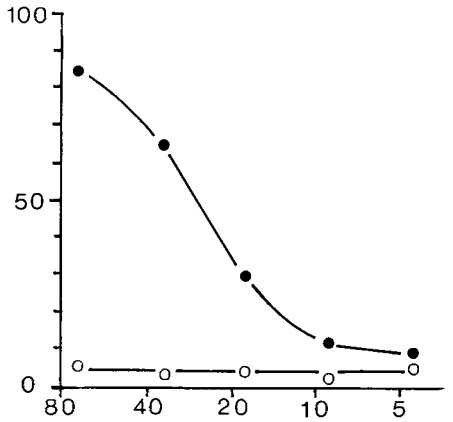


**Fig. 2** Cytotoxicity on PK15 cells of human anti-αGal antibodies (affinity-purified on Galα1-3Galβ1-4GlcNAc-Synsorb; ●) after addition of normal rabbit serum as a source of complement. Neither the affinity-purified human antibodies (○) nor rabbit serum alone (not shown) had any cytotoxic effect. Percent dead cells on the ordinate and concentration of affinity-purified antibody (μg/ml) on the abscisa

**Table 1** μM concentration of each oligosaccharide needed to obtain 50 % inhibition of cytotoxicity of unmodified human or baboon serum on PK15 cells

| Inhibitor oligosaccharide | Serum | |
|---|---|---|
| | Human | Baboon |
| **Fucα1-2Galβ1-R** | > 10000 | > 10000 |
| **Galβ1-R** | > 10000 | > 10000 |
| Galα1-**2Galβ1-R′** | 7000 | > 10000 |
| Galα1-3Gal | 386 ± 149[a] | 301 ± 44[e] |
| Galα1-3Galβ1-4**Gal** | 163 ± 73[b] | 141 ± 60[f] |
| Galα1-3Galβ1-4**Galα1-3Gal** | 54 ± 31[c] | 119 ± 30[g] |
| Galα1-3Galβ1-4GlcNAc | 27 ± 11[d] | 31 ± 4[h] |

Bold type indicates structural differences of the oligosaccharide with the major pig vascular endothelium glycolipid Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer [16]. R represents 1–3 or 1–4 linkages to Gal or to GlcNAc; R′ is $-O(CH_2)_3NHCOCF_3$. The results of the strong inhibitors (a–h) are expressed as mean ± SD ($n = 3$). Statistical significance: a vs c ($t = 3.0$); a vs d ($t = 3.9$); b vs d ($t = 3.1$); e vs h ($t = 9.7$); f vs h ($t = 3.1$); g vs h ($t = 5.4$), all with $P < 0.02$. The other comparisons did not reach the $P = 0.05$ level of significance, but both human and baboon serum inhibition tests follow a similar trend

### Inhibition of the cytotoxic reaction with synthetic oligosaccharides

None of the monosaccharides tested (methyl-β-galactose, galactose, glucose, fucose, mannose, *N*-acetylgalactosamine or *N*-acetylglucosamine) significantly inhibited the cytotoxicity of human or baboon serum, with the exception of methyl-α-galactose, which gave a weak and partial inhibition at very high concentrations ( > 30 mM).

The Galα1-3Gal structure was the best disaccharide for blocking the cytotoxic reaction of human or baboon natural anti-αGal antibodies. Fifty percent inhibition of the reaction was reached with concentrations of the order of 300–400 μM (Table 1). Other disaccharides with the non-reducing terminal galactose in β anomeric conformation (Galβ1-3GlcNAc and Galβ1-4 GlcNAc) or trisaccharides such as H type 1 (Fucα1-2Galβ1-3GlcNAc), H type 2 (Fucα1-2Galβ1-4GlcNAc), $Le^x$ (Galβ1-4(Fucα1-3)GlcNAc), and $Le^a$ (Galβ1-3(Fucα1-4)GlcNAc) did not significantly inhibit the reaction. The disaccharide Galα1-2Gal did reach 50 % inhibition of the cytotoxic reaction but only with human sera and at a high oligosaccharide concentration (7 mM). This kind of weak crossreactivity has also been reported for melibiose and stachyose, which are saccharides with a Galα1-6Glc and Galα1-6Gal nonreducing terminus, respectively [13, 19].

The trisaccharide Galα1-3Galβ1-4GlcNAc, which is identical to the terminal structure of the major pig vascular endothelium glycolipid, inhibited 50 % of the cytotoxic reaction at a concentration of about 30 μM for both human and baboon serum and, therefore, proved to be about ten times more efficient than the disaccharide (Table 1). Replacement of the GlcNAc of this trisaccharide by galactose diminished the inhibition efficacy of the trisaccharide fivefold (Table 1). A tetrasaccharide with a repetitive disaccharide Galα1-3Gal motif resulted in a two- to fourfold loss of inhibiting activity as compared to the trisaccharide Galα1-3Galβ1-4GlcNAc (Table 1).

## Discussion

Among the monosaccharides, only the methyl-α-Gal showed some weak inhibitory activity. Any modification of the nonreducing terminal structure of the disaccharide resulted in dramatic loss of the cytotoxic inhibitory capacity, confirming that the nonreducing end of the pig oligosaccharide is the main epitope for natural anti-αGal antibodies.

The synthetic disaccharide Galα1-3Gal has been shown to be efficient in removing and blocking anti-αGal antibodies from sera [16]. However, the synthetic trisaccharide Galα1-3Galβ1-4GlcNAc was ten times more efficient as an inhibitor, indicating that the reducing end of the trisaccharide does also contribute to the binding of natural antibodies. We conclude that the binding must be more permissive on this side of the oligosaccharide because compounds in which the GlcNAc residue is replaced by Gal or by Galα1-3Gal are better inhibitors than the disaccharide, although not as good as the Galα1-3Galβ1-4GlcNAc trisaccharide (Table 1).

The blocking activity is lost by a change in the anomeric configuration of the terminal nonreducing galac-

tose from α to β. The linkage of the second galactose in position 3 is also important since a decrease in biological activity has also been found with the second galactose linked in position 2 or 6. In contrast, after the addition of an alternative monosaccharide or disaccharide on the reducing terminus of the disaccharide, there is still high biological activity of the resulting tri or tetrasaccharides, indicating that the presence of bulky structures at the reducing end does not hinder recognition of the antibody but, in fact, slightly improves the binding of antibodies when compared to the disaccharide (Table 1). This observation will be important for the design of the best inhibitor if an attempt is to be made to block the humoral hyperacute vascular rejection of xenotransplants by the intravenous infusion of soluble oligosaccharides "in vivo" [2, 21].

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