current studies. Transplantation 1992; 53: 957.
23. Lanir A, Jenkins RL, Caldwell C, Lee RGL, Khettry U, Clouse ME. Hepatic transplantation survival: correlation with adenine nucleotide level in donor liver. Hepatology 1988; 8: 471.
24. Gonzalez-Flecha B, Cutrin JC, Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. J Clin Invest 1993; 91: 456.
25. Ikeda T, Yanaga K, Kishikawa K, Kakizoe S, Shimada M, Sugimachi K. Ischemic injury in liver transplantation: differences in injury sites between warm and cold ischemia in rats. Hepatology 1992; 16: 454.
26. Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol 1991; 260: G355.

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REMOVAL OF ANTI-PORCINE NATURAL ANTIBODIES FROM HUMAN AND NONHUMAN PRIMATE PLASMA IN VITRO AND IN VIVO BY A Galα1–3Galβ1–4βGlc-X IMMUNOAFFINITY COLUMN

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Background. **Natural antibodies (NAbs) against a terminal α1–3 galactosyl (αGal) epitope have been identified as the major human anti-pig NAbs.**

Methods and Results. **We used two synthetic αGal trisaccharides—type 6 (αGal6) and type 2(αGal2)—linked to an inert matrix to remove NAbs from human plasma in vitro. Flow cytometry indicated that an average of 85% of the NAb binding activity was depleted by adsorption with αGal6. By measuring the binding of NAbs to pig peripheral blood mononuclear cells and bone marrow cells, we demonstrated that αGal6 was more effective than αGal2 in removing NAbs, and the combination of αGal6 + αGal2 did not further increase removal of NAbs. The specificity of the removal of NAbs (IgM and IgG) reactive with the αGal epitope by αGal6 matrix was shown by enzyme-linked immunosorbent assay. In vivo studies in nonhuman primates compared plasma perfusion through a αGal6 immunoaffinity column with hemoperfusion through a pig liver for changes in blood pressure, hematocrit, platelets, and NAb adsorption.**

Conclusions. **Both methods reduced the level of anti-pig IgM and IgG xenoreactive antibodies to nearly background, but column perfusion caused less hypotension and reduction in platelets than liver perfusion. Four pig kidneys transplanted into monkeys after column perfusion did not undergo hyperacute rejection, remaining functional for 2–10 days, with a mean functional period of 7 days, demonstrating that a pig kidney can support renal function in a primate.**

Preformed natural xenoreactive antibodies (NAbs*) of both IgM and IgG isotypes in human and most nonhuman primates (apes and Old World monkeys) represent a major barrier to discordant xenotransplantation by initiating hyperacute rejection within minutes to hours (*1*). The removal of NAbs by plasma exchange (*2*), organ perfusion (*3–5*), or immunoglobulin immunoadsorption (*6*) can prevent hyperacute rejection. Each of these antibody-depleting procedures has limitations. Plasma exchange nonspecifically removes all plasma proteins, which include immunoglobulins and coagulation proteins. Organ perfusion causes a significant loss of blood components and may lead to activation of the complement and coagulation cascades. Immunoadsorption technology leads to a significant reduction in the total IgM and/or IgG pool.

The predominant target epitope for human or nonhuman primate NAbs is the α1–3 galactosyl (αGal) epitope (*7–12*). Competitive blocking studies have shown that synthetic

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* Abbreviations: bkg, background MFI from negative controls; BSA, bovine serum albumin; BSA-αGal6, αGal6 conjugated to bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; αGal epitope, Galα1–3 galactosyl epitope; αGal6, Galα1–3Galβ1–4βGlc-X (trisaccharide type 6); αGal2, Galα1–3Galβ1–4βGlcNAc-X (trisaccharide type 2); IVC, inferior vena cava; MFI, mean fluorescence intensity (of antibody binding to pig cells by FACS); MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NAb, natural antibody; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline.

trisaccharides containing a terminal α1–3Gal residue can inhibit binding of NAbs to pig target cells (*7, 13*). In addition, natural or synthetic αGal can adsorb NAbs from plasma (*7, 13–15*).

Two synthetic αGal oligosaccharides are Galα1–3Galβ1–4βGlc-X (trisaccharide type 6; αGal6) and Galα1–3Galβ1–4βGlcNAc-X (trisaccharide type 2; αGal2). In the study reported here, we compare the in vitro adsorption properties of these two matrix-linked trisaccharides, which have the same linkage and density, in the removal of NAbs from the plasma of human and nonhuman primate donors. In addition, we demonstrate that immunoaffinity columns of αGal6 are able to remove NAbs from the blood of nonhuman primates and prevent the hyperacute rejection of transplanted pig kidneys.

MATERIALS AND METHODS

Animals. Ten cynomolgus monkeys, *Macaca fascicularis* (Charles River Laboratories, Wilmington, MA), weighing 5–8 kg, were used for perfusion studies and were recipients of porcine kidneys. Blood samples for in vitro studies were drawn from four male monkeys and from four female baboons (*Papio anubis*) (Biological Resources, Houston, TX) weighing 8–12 kg. Porcine donors for kidney transplantation and liver perfusion were selected from our herd of partially inbred miniature swine at 4–8 weeks of age. All surgical procedures and postoperative care of the animals were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Massachusetts General Hospital Subcommittee on Animal Research.

Peripheral blood cells and plasma. Heparinized peripheral blood was obtained from healthy donor pigs. Peripheral blood mononuclear cells (PBMCs) were harvested after separation over a Ficoll density gradient, and washed in Dulbecco's phosphate-buffered saline (PBS). Heparinized human blood was obtained from normal healthy donors after obtaining informed consent; plasma was isolated after centrifugation.

Adsorption material. The Biosynsorb αGal trisaccharide type 6 and type 2 matrix material and type 6 column were manufactured by the Alberta Research Council (Edmonton, Alberta, Canada). Trisaccharide was covalently linked to silica particles via a linker arm with a final biocompatible coating. Approximately 0.74 mg of trisaccharide was bound to each gram of silica beads. The density and linkage of the type 6 and type 2 matrices are the same. The αGal columns contained 50 g of αGal 6 matrix material. Type 6 trisaccharide was also conjugated to bovine serum albumin (BSA-αGal6) (Alberta Research Council) with approximately 17–20 trisaccharide moieties per molecule of BSA. This conjugate was used for competitive binding studies and as a capture reagent in enzyme-linked immunosorbent assay (ELISA).

In vitro adsorption. The in vitro adsorption assay was performed by mixing matrices with primate plasma at 4°C or room temperature for 2 hr with constant shaking. Various weight/volume ratios were evaluated, but 1 g of matrix per 1–3 ml of plasma was chosen as optimal. For comparison of αGal6 and αGal2 matrices for NAb depletion, increasing amounts of matrix were used to adsorb NAbs from 2 ml of plasma. For saturation studies, 1 g of αGal6 matrix was mixed with increasing volumes of human plasma. The resulting supernatants were checked for binding to pig PBMCs by a fluorescent-activated cell sorter (FACS). Undepleted plasma served as an internal positive control.

Isolation of human NAbs. After adsorption of plasma, as described above, matrix material obtained by centrifugation, was washed once with PBS, then once with saline. Preliminary testing showed that both low and high pH were able to elute the NAbs from the matrix. Because IgM was unstable at low pH, a high pH was used for elution. Adsorbed NAbs were eluted from the matrix by adding a volume of 1% NH_4OH (pH 11) equivalent to the original plasma volume, followed by immediate dialysis against PBS containing Ca^{++} and Mg^{++} at 4°C for 18 hr. The isolated NAb was therefore at a similar level to that in the original plasma sample.

NAb binding assays. Two assays were used to determine NAb levels: (a) FACS using pig PBMCs and (b) an ELISA. Serum (10 μl) was incubated with pig PBMCs (5×10^5cells) in PBS containing 1% BSA and 0.1% azide (FACS medium) for 1 hr at 4°C. Three different sources of NAb (at either 10 or 50 μl) were used, namely, plasma, adsorbed plasma, or plasma eluted from the matrix. The cells were then washed. For human and cynomolgus monkey NAb detection, 50 μl of fluorescein isothiocyanate-conjugated mouse anti-human IgM or IgG mAb (1:20 dilution) (Zymed Laboratories, South San Francisco, CA) were added for an additional 1-hr incubation at 4°C. For baboon and some cynomolgus monkey NAb detection, 50 μl of fluorescein isothiocyanate-conjugated goat anti-human IgM or IgG polyclonal antibody (Zymed) was used (1:20 dilution in FACS medium containing 10% normal goat serum). Cells were washed again and resuspended in FACS medium. Normal pig plasma was used as the negative control with the secondary antibodies. Pooled primate plasma adsorbed with pig PBMCs was also used as a negative control. Positive controls were the initial plasma sample and a plasma sample with previously documented high titers of NAb. Data were acquired and analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA). All the data were reported as mean fluorescence intensity (MFI). For competition studies, data were represented as % MFI (*MFI with competitor − background MFI from negative controls (bkg)/MFI without competitor − bkg*). For comparison of αGal6 and αGal2 for NAb depletion, data were presented as % adsorption (1 − (*post MFI − bkg*)/(*pre MFI − bkg*)).

ELISA for measurement of NAbs reactive with Galα1–3Gal consisted of loading a 0.016–2% concentration of human, cynomolgus monkey, or baboon serum on a Maxisorb plate (Nunc, Naperville, IL) coated with 5 μg/ml BSA-αGal6 (Alberta Research Council). Bound antibodies were detected using polyclonal donkey anti-human IgG (Accurate Chemical & Scientific, Westbury, NY) or rabbit anti-human IgM (Dako, Copenhagen, Denmark) conjugated to horseradish peroxidase. Color development was achieved by using o-phenylenediamine dihydrochloride (Sigma Chemical, St. Louis, MO) as a substrate at 0.9 mg/ml in PBS with urea hydrogen peroxidase (Sigma). Absorbance at 490 nm was determined by a THERMOmax plate reader (Molecular Devices, Menlo Park, CA).

NAb cytotoxicity. Complement-mediated NAb cytotoxicity after incubation of live cells with NAbs and complement was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (tetrazolium salt) colorimetric assay (Promega, Madison, WI). Briefly, PK15 cells were plated at 5×10^4/well into a tissue culture 96-well plate 1–3 days before the experiment. When the cells were in log phase growth and just before use, they were washed with PBS containing Ca^{++} and Mg^{++} and incubated with an increasing concentration of serum or PBS (control) for 1 h at 4°C. Plates were washed again with PBS and incubated with rabbit complement (Pel-Freez, Brown Deer, WI) for 1 hr at 37°C. Substrate was then added according to kit instructions. MTS is taken up by live cells and reduced to formazan. Absorbance of formazan at 490 was measured by THERMOmax plate reader (Molecular Devices).

Surgical procedures. Surgical procedures were carried out under inhalation anesthesia. Blood pressure was continuously monitored through an arterial line. Each animal had a central venous silastic catheter placed into the external or internal jugular vein by direct cutdown and tunneled to exit on the dorsolateral thorax. These catheters were used for measurement of central venous pressure, administration of medications, and blood sampling.

For in vivo immunoaffinity column or liver perfusion, a medial laparotomy was performed. The infrarenal aorta and vena cava were dissected, and the vessels retracted by suture loops. Silastic tubing was inserted into the lumen of the aorta and inferior vena cava (IVC), respectively, and connected to the column or liver (afferent

limb into the portal vein; efferent limb into the suprahepatic vena cava). The column or liver was placed into a bowl containing warm saline to prevent temperature loss resulting from the extracorporeal blood flow. Blood samples for analysis of NAbs and other parameters were collected during the perfusion and postoperative recovery period.

After 60 min of perfusion, the tubing was removed from the aorta and IVC, and the nascent ostia in these vessels were used for anastomosis of the pig kidney. The donor renal artery and vein were anastomosed end-to-side to the recipient aorta and IVC, respectively. The donor ureter was anastomosed to the anterior surface of the recipient's bladder. The operation was completed after splenectomy and ligation of the monkey ureters to prevent urine production by the native kidneys.

Hematologic studies. Complete blood cell counts were performed on blood samples taken before, during and after column or liver perfusion and kidney transplantation using a hematology cell counter.

RESULTS

Comparison of efficiency of αGal6 and αGal2 trisaccharides for removal of NAbs. Initially, the capacity of both type 6 and type 2 matrices to remove NAbs reactive with the Galα1–3Gal epitope was tested. This was achieved by at first eluting antibodies bound to the matrices after adsorption of human serum. The reactivity of the eluted antibodies in the presence or absence of αGal6 conjugated to BSA (BSA-αGal6) was determined by flow cytometry. The results presented in Figure 1 indicate that anti-αGal IgM eluted from either matrix bound pig cells. A similar result was obtained with anti-αGal IgG (data not shown). Binding of the antibodies eluted from αGal6, αGal2, or both matrices was inhibited equally by the addition of BSA-αGal6. Because the only epitope shared by the matrices and BSA-αGal6 is αGal6, this demonstrates that the matrix-bound antibodies were specifically reactive with the αGal6 epitope.

The ability of the two different trisaccharide matrices to adsorb NAbs from pooled human plasma was compared in an in vitro adsorption experiment. The pig cell reactivity of serum pre and postadsorption on the trisaccharide matrices was measured by flow cytometry. Figure 2 shows the percent adsorption versus amount of matrix used. The αGal6 matrix is more efficient than the αGal2 matrix in removing NAbs. Whereas it took 0.2 g of the αGal6 matrix to cause greater than 80% depletion, 16-fold more of the αGal2 matrix (3.2 g) were needed to achieve equivalent adsorption. Moreover, the results depicted in Table 1 demonstrate that even at the matrix dose designed to achieve maximum NAb depletion, αGal2 was less effective than αGal6 in removing NAbs (both IgG and IgM). To determine whether a combination of αGal6 + αGal2 could be more effective than αGal6 alone, 1 g of an equal mixture of the matrix materials was used to adsorb NAbs from the plasma. This mixture did not remove more NAbs than αGal6 alone (Table 1). Similar results were obtained when pig bone marrow cells (rather than PBMCs) were used as target cells (Table 1).

When individual human plasma were tested, there was considerable variation in the capacity of the matrices to adsorb NAbs (Table 2). With only one exception, αGal6 matrix was more effective than αGal2 in removing NAbs, and in that one individual the efficiency of the matrices was equivalent (64% vs. 66% IgG). Immunoadsorption with αGal6 resulted in a mean of 92% and 83% reduction in IgM and IgG, respectively, whereas adsorption with αGal2 resulted in 64% and 65% reduction in both immunoglobulins.

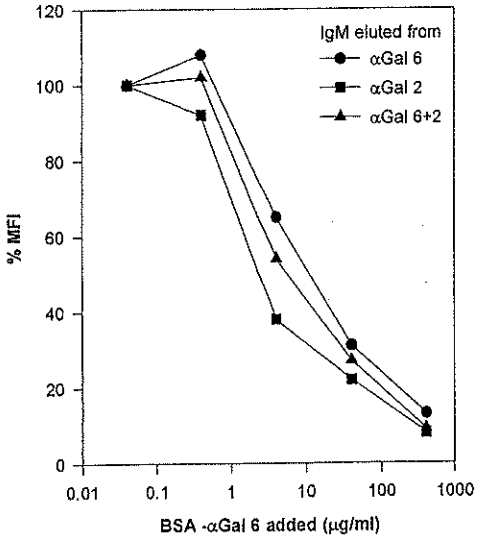

FIGURE 1. IgM eluted from αGal6, 2, and both matrices recognize the same αGal specificity. Binding of eluted antibodies was inhibited equally by BSA-αGal6. Concentration of BSA-αGal6 and % MFI are shown.

Specificity of the αGal6 matrix. After establishing that the αGal6 matrix was more effective than αGal2 in removing NAbs, the remaining in vitro and in vivo studies were conducted using this matrix. Additional evidence of the specificity of the αGal6 matrix consisted of the demonstration by ELISA that the reactivity of human serum to BSA-αGal6 was completely removed after adsorption with the matrix (Table 3). Subsequent adsorption with pig cells did not remove additional reactivity, indicating that the αGal6 matrix had removed all NAbs from the serum.

Adsorption of serum with αGal6 caused minimal removal of other immunoglobulins. Only 5–10% of total IgG or IgM

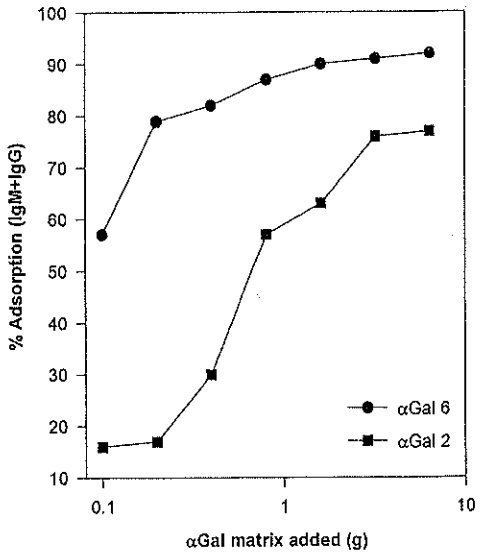

FIGURE 2. αGal6 matrix is more effective than αGal2 matrix for NAb depletion. Increasing amounts of matrix (grams) were used to adsorb NAb from 2 ml of plasma. Percent adsorption by matrices is shown.

TABLE 1. In vitro efficiency of αGal6, αGal2, and αGal6+2 matrix material for NAb (IgM) depletion from pooled human plasma[a]

	Pig target cells			
	PBMCs		Bone marrow cells	
	MFI	Depletion (%)	MFI	Depletion (%)
Unmodified plasma	211		579	
Post-αGal6	15	95	102	86
Post-αGal2	39	83	205	67
Post-αGal6+2	15	95	104	86
(Background)	(5)		(24)	

[a] NAb binding to pig PBMCs and bone marrow cells are shown as MFI. NAb depletion is presented as % depletion (postadsorption MFI − bkg)/(pre-MFI − bkg), where bkg is MFI from negative controls.

TABLE 2. In vitro efficiency of αGal6 and αGal2 matrix material for NAb (IgM and IgG) depletion from individual human plasma[a]

Plasma (blood type)	Depletion postadsorption (%)			
	IgM		IgG	
	αGal6	αGal2	αGal6	αGal2
1 (A)	96	93	92	84
2 (A)	89	46	98	82
3 (B)	95	31	78	44
4 (B)	98	60	90	70
5 (O)	98	88	93	80
6 (O)	84	73	64	66
7 (O)	85	74	78	56
8 (O)	92	76	80	60
Mean	92	64	83	65

[a] PBMCs were used as target cells. Percent depletion was calculated as in Table 1.

were removed by matrix adsorption (unpublished results). Furthermore, when pig allostimulated sera were adsorbed on the matrix, binding to allogeneic pig PBMCs was not reduced (data not shown).

Effect of NAb depletion on serum cytotoxicity. These same samples (as in Table 3) were incubated with pig cells in a cell viability assay (MTS). The results (Fig. 3) show that, after adsorption of serum with αGal6 matrix, the cytotoxicity of NAb is inhibited dramatically. Only 20–30% cytotoxicity can be detected at the highest serum concentration (50%). Additional incubation with pig cells completely removed the cytotoxicity of the serum, indicating the presence of some anti-pig antibodies not reactive with αGal determinants.

Capacity of αGal6 for NAbs. Because the αGal6 matrix was to be used in vivo, the quantity of matrix material required to remove NAbs was assessed. Incremental increases of pooled human plasma were added to 1 g of matrix material and incubated at room temperature, after which an aliquot of the adsorbed plasma was removed for analysis. In Figure 4, the binding activities (reported as MFI) for both IgM and IgG are plotted against the volume of plasma added. When the plasma volumes exceeded 25 ml, the MFI increased, indicating that, at this point, the αGal6 matrix was becoming saturated with NAbs. From this study, we estimated that each preclinical-sized column (50 g of matrix material) could maximally adsorb NAbs from 1250 ml of plasma. As the monkeys to be used in our studies had an estimated blood volume of 400–600 ml and the baboons had an estimated blood volume of 600–900 ml, we estimated that one column (50 g) was sufficient to remove the NAbs from one animal.

To confirm this in vitro prediction, we subsequently took an aliquot of matrix from a 50-g column after in vivo monkey NAb depletion, and assessed the capacity of the matrix to remove additional NAbs from human plasma in vitro. The matrix used in vivo was still able to remove NAbs from human plasma, although it was not as efficient as unused matrix (data not shown). This result indicated that the matrix epitopes were not fully saturated because the matrix retained the capacity to adsorb additional NAbs.

Depletion of NAbs from nonhuman primate plasma in vitro. The initial in vitro studies to characterize the binding properties of αGal6 were performed using human plasma. Before evaluating the column in vivo in nonhuman primates, we documented that the matrix could effectively remove NAbs from their plasma. In vitro depletion of NAbs by αGal6 from nonhuman primate plasma demonstrated that αGal6 was effective in removing both IgM and IgG NAbs, and was equally effective in cynomolgus monkeys and baboons (data not shown).

TABLE 3. Specificity of NAb adsorption from serum by αGal6 matrix[a]

Serum	Absorbance (490 nm)	
	IgG	IgM
Pre-αGal6 adsorption	0.489	0.259
Post-αGal6 adsorption	0.015	0.002
Post-pig cell adsorption	0.019	0.011

[a] Aliquots from serum before and after adsorption with αGal6 matrix and after subsequent adsorption with pig cells were tested by ELISA. The results were corrected for background binding.

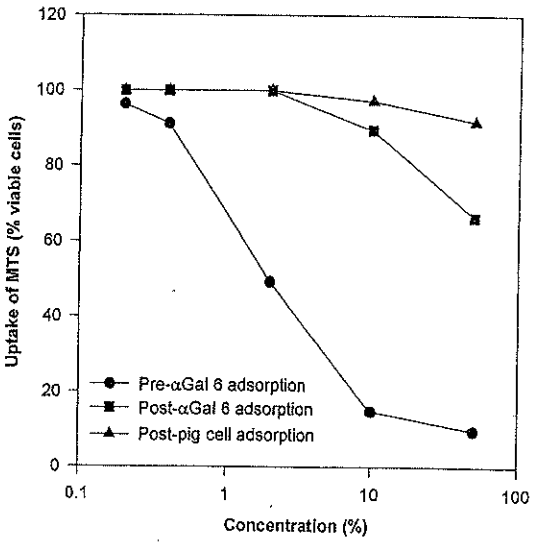

FIGURE 3. αGal6 matrix adsorption reduces cytotoxicity mediated by NAb and complement. Adsorption of sera over αGal6 matrix dramatically reduced serum cytotoxicity to pig cells. Cytotoxicity was reduced further by additional adsorption with pig cells. Serum concentration and % uptake of MTS (% viable cells) are shown.

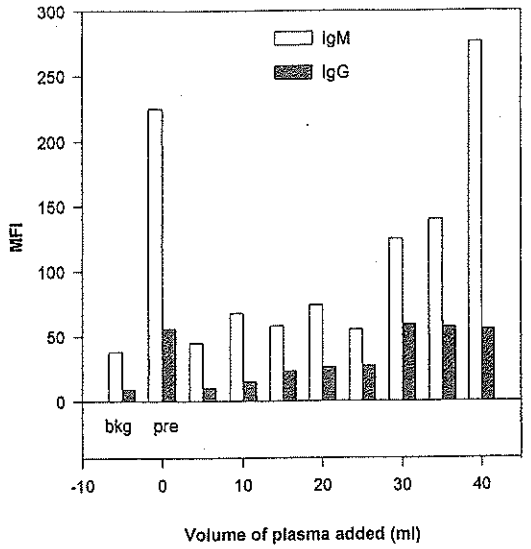

FIGURE 4. Matrix saturation study. The volume of plasma that can saturate 1 g of αGal6 matrix material and still achieve maximal NAb depletion was determined. Volume of plasma added to 1 g of the matrix vs. MFI is indicated. Plasma before depletion (pre) and background binding (bkg) are also shown as controls.

Clinical course during extracorporeal immunoadsorption of NAbs in monkeys. In vivo perfusion of blood through an αGal6 column was compared with perfusion of blood through a porcine liver (Figs. 5-7). The effect of column and liver perfusion on systemic blood pressure is compared in Figure 5. A dramatic fall in blood pressure was seen within minutes of initiating liver perfusion. In contrast, there was only a moderate decrease in blood pressure during column perfusion. Both perfusion techniques caused a significant decrease in hematocrit (Fig. 6) and platelet count (Fig. 7). A combination of hemodilution and nonspecific removal of red blood cells caused the hematocrit to be reduced by 30% within 30 min,

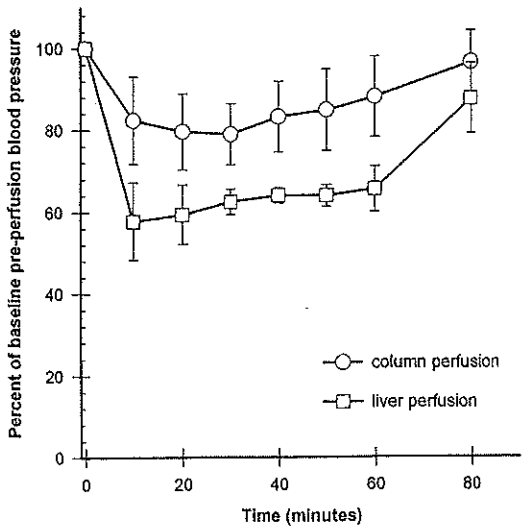

FIGURE 5. Comparison of effect of αGal6 immunoaffinity column (n=4) vs. liver perfusion (n=3) on systolic blood pressure (monitored continuously through an arterial catheter and illustrated as % baseline pressure±SD) during immunoadsorption in cynomolgus monkeys.

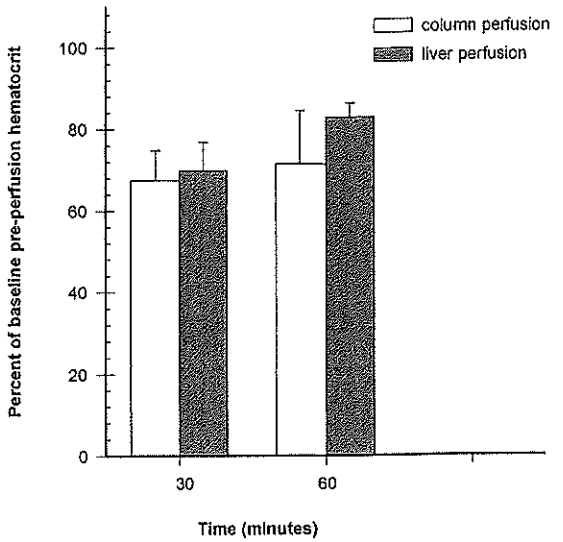

FIGURE 6. Comparison of effect of αGal6 immunoaffinity column (n=4) vs. liver perfusion (n=3) on hematocrit (presented as % baseline hematocrit±SD) during immunoadsorption in cynomolgus monkeys.

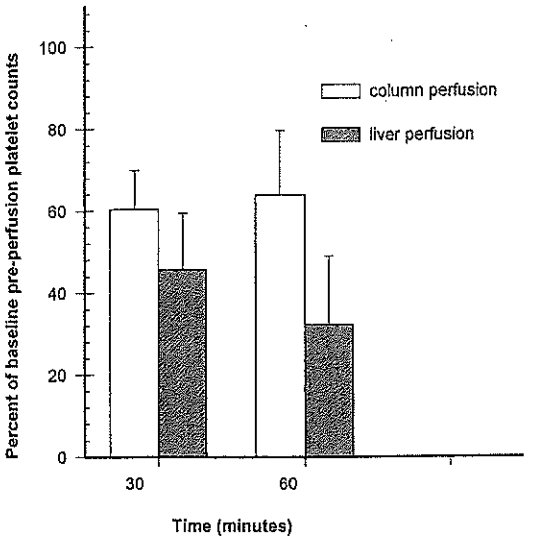

FIGURE 7. Comparison of effect of αGal6 immunoaffinity column (n=4) vs. liver perfusion (n=3) on platelet count (presented as % baseline counts±SD) during immunoadsorption in cynomolgus monkeys.

though there was some recovery by the end of the perfusion. Similarly, the platelet count was significantly reduced by 40–50% within 30 min. In monkeys undergoing liver perfusion, platelet loss continued throughout the procedure.

Both forms of hemoperfusion also resulted in transient reductions in total immunoglobin, platelets, complement, total protein, albumin, and fibrinogen; increases in prothrombin and partial thromboplastin times were also documented (data not shown). All of these temporary changes were associated with hemodilution, and were largely corrected within 24 hr as the hematocrit returned toward the prehemoperfusion level. Some nonspecific adsorption, however, could not be excluded.

In vivo removal of NAbs from nonhuman primates. To assess the efficiency of the immunoadsorption techniques to deplete NAbs from nonhuman primates, we performed sequential binding analyses using flow cytometry during and after immunoadsorption (Table 4). Plasma drawn before and after column perfusion (at 60 min) was compared for NAb level. Good NAb depletion was demonstrated in both monkeys (n=4) and baboons (n=4). To confirm the complete removal of NAb, postperfusion samples were further adsorbed in vitro by αGal6 matrix. No additional reduction in binding to pig cells was observed.

Immunoadsorption by column perfusion was compared with liver perfusion. The typical courses for the elimination of IgM and IgG NAbs by column and liver perfusion are shown in Figures 8 and 9. The majority of the NAb was eliminated within 30 min with either technique.

Clinical course and changes in NAb level after pig kidney transplantation in monkeys. Hyperacute rejection was not seen after the successful transplantation of a pig kidney into a monkey that had undergone prior plasma immunoadsorption through an αGal6 column (n=4) or liver (n=6). The kidney xenografts functioned for 2–10 days (mean, 7 days) after column perfusion, and for 4–12 days (mean, 7 days) after liver perfusion. NAbs (both IgM and IgG) remained at background levels after transplantation (presumably because returning NAb was adsorbed onto the transplanted organ), and did not recover for 5–8 days. All kidneys made urine for at least 48 hr, and there were no clinical signs of rejection during this period, demonstrating that a pig kidney can support renal function in a primate. However, all kidneys ultimately rejected when circulating NAb returned. As an example, Figure 10 illustrates the NAb profile after column adsorption and a pig kidney transplant with immunosuppression in a cynomolgus monkey. After column perfusion on day 0, both IgM and IgG were reduced, and remained so after pig kidney transplantation. NAb remained low from days 1 to 7, presumably due to adsorption onto the pig kidney. After the rejected kidney was excised on day 7, an immediate

TABLE 4. In vivo depletion of NAb (IgM and IgG) in nonhuman primates by extracorporeal immunoadsorption using an αGal6 column[a]

Primate	Plasma sample	MFI (% depletion)	
		IgM	IgG
Monkey			
1	Pre-IA	85	30
	Post-IA	10 (96%)	12 (100%)
	Bkg	7	14
2	Pre-IA	262	194
	Post-IA	32 (98%)	22 (100%)
	Bkg	26	37
3	Pre-IA	160	103
	Post-IA	18 (100%)	16 (100%)
	Bkg	19	36
4	Pre-IA	427	284
	Post-IA	65 (87%)	22 (97%)
	Bkg	12	14
Baboon			
1	Pre-IA	825	194
	Post-IA	199 (81%)	64 (96%)
	Bkg	51	58
2	Pre-IA	255	91
	Post-IA	80 (82%)	25 (84%)
	Bkg	41	12
3	Pre-IA	441	60
	Post-IA	80 (91%)	28 (97%)
	Bkg	44	27
4	Pre-IA	117	63
	Post-IA	48 (80%)	23 (85%)
	Bkg	31	16

[a] NAb levels from pre- and post-immunoadsorption (IA) plasma are shown as MFI (and % depletion), measured by binding to pig PBMCs. Postadsorption plasma were tested after 60 min of column perfusion.

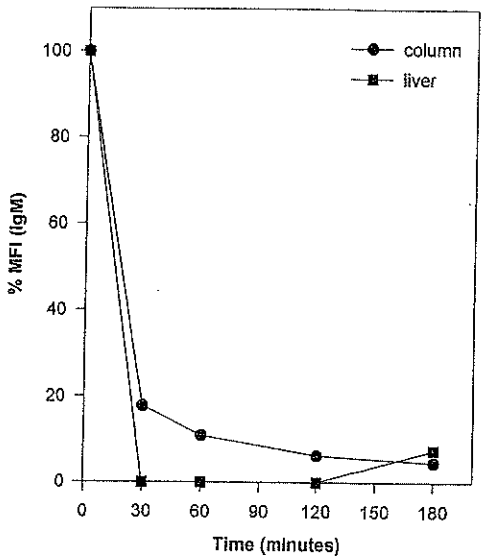

FIGURE 8. Effect of αGal6 immunoaffinity column vs. liver perfusion on the level of IgM (measured by FACS) in monkey sera. Serum samples were taken before (pre), during, and after a 1-h period of extracorporeal perfusion. Percent changes in MFI in relation to the preperfusion value are shown (representative of four studies.).

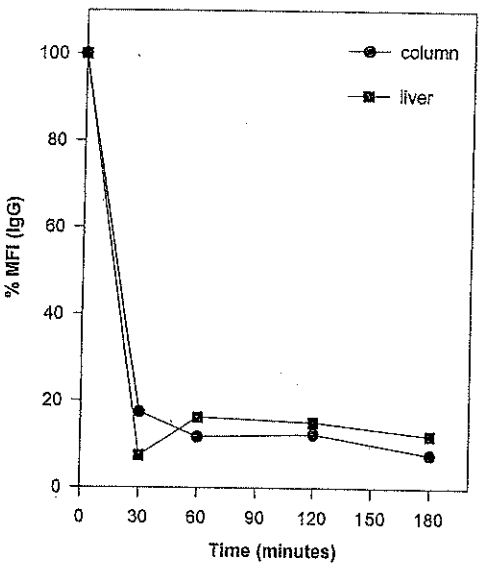

FIGURE 9. Effect of αGal6 immunoaffinity column vs. liver perfusion on the level of IgG (measured by FACS) in monkey sera. Serum samples were taken before (pre), during, and after a 1-h period of extracorporeal perfusion. Percent changes in MFI in relation to the preperfusion value are shown (representative of four studies.).

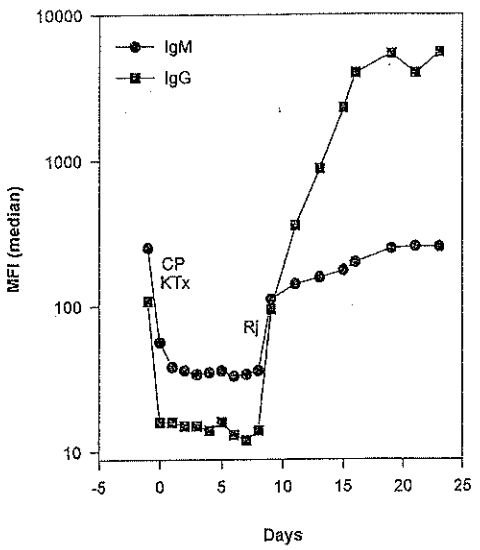

FIGURE 10. Changes in anti-pig IgM and IgG (measured by FACS) in a cynomolgus monkey undergoing column perfusion (CP) and pig kidney transplantation (KTx) (both on day 0). The pig kidney was rejected and excised on day 7 (Rj).

increase in NAb was documented. Details of some of these transplant experiments using liver hemoperfusion have been reported by Sablinski et al. (*16*). Further experience using column perfusion in baboons will be reported by Kozlowski et al.[4] As reductions in hematocrit, platelets, and complement are only transient after either liver or column perfusion (see above), it is not thought that these factors contributed significantly to prolonged pig xenograft survival.

DISCUSSION

In this article, we demonstrate in vitro immunoadsorption of NAbs using synthetic specific carbohydrate epitopes (αGal6 and αGal2). We further demonstrate that the in vivo extracorporeal perfusion of nonhuman primate plasma through an αGal6 immunoadsorbent column can reduce the circulating NAbs to a nearly undetectable level and prevent hyperacute rejection of a subsequently transplanted pig kidney.

Anti-αGal antibodies were originally described by Galili et al. (*17*). Attention to their important role in xenotransplantation followed the work of Good et al. in 1991 (*7–10*). This group also suggested the use of immunoaffinity columns of an αGal oligosaccharide bound to a matrix for the removal of anti-pig antibodies to prevent the hyperacute rejection of a transplanted pig organ. Some heterogeneity in anti-αGal antibody was observed (*7, 18*), and has also been documented by others (*19, 20*). Subsequent in vitro studies confirmed the feasibility of immunoadsorption as a clinical approach to prevent hyperacute rejection (*13*).

The first in vivo study was by Ye et al. (*21*), who, using a melibiose (αGal1–6Glc) column, were able to remove anti-αGal antibodies and, after the intravenous infusion of melibiose or arabinogalactan, demonstrated delay in rejection of a pig heart transplanted into a baboon. Subsequent studies using αGal oligosaccharide columns (*22, 23*) have supported the potential of this form of therapy, and also documented prolonged pig heart graft survival in one baboon. To our knowledge, no other in vivo studies utilizing specific αGal immunoadsorption columns have been reported.

The present study demonstrates that the αGal6 trisaccharide is a more efficient adsorbent of anti-pig antibodies than the αGal2 trisaccharide. As the target epitope on pig vascular endothelium for human anti-pig antibodies has been demonstrated to be identical to the αGal2 structure, the greater efficacy of αGal6 adsorption over αGal2 was an unexpected result. It is nevertheless fortuitous as the αGal6 trisaccharide is significantly easier to synthesize than the αGal2 structure. Neethling et al. (*13*) found that αGal2 to be an efficient inhibitor of NAb, but did not test αGal6, and it should be emphasized that both oligosaccharides are efficient when compared with non-Galα1–3Gal structures. The efficiency of the αGal6 trisaccharide in adsorbing anti-pig antibodies (both IgM and IgG) is high, with significant reductions in both antibody level and cytotoxicity of the adsorbed plasma.

Galili et al. (*24, 25*) drew attention to the presence of anti-αGal antibody in apes and Old World monkeys as well as in humans, and evidence that synthetic αGal trisaccharides were as effective against baboon anti-pig antibodies as against human anti-pig antibodies was provided by Good et al. (*7, 18*). We demonstrate here that αGal trisaccharides are equally effective in relation to cynomolgus monkey anti-pig antibody both in vitro and, importantly, in vivo.

Immunoadsorption of nonhuman primate plasma by an αGal immunoaffinity column not only depletes the animal of anti-αGal antibody but, importantly, prevents the hyperacute rejection of a transplanted pig organ. The consistent survival in excess of 48 hr of the pig kidneys reported here is, with the exception of a single experiment reported previously (*23*), the first definite in vivo evidence that removal of anti-αGal antibody alone (by specific synthetic oligosaccharides in the absence of removal of any other antibodies or complement) is sufficient to prevent the hyperacute rejection of a transplanted pig organ. Furthermore, these experiments are an additional demonstration that an unmodified pig kidney can support renal function in a primate (*2*). These important observations open up the possibility of using such columns in clinical organ xenotransplantation in association with other forms of therapy.

Anti-pig antibody removal by hemoperfusion through a pig organ has been described by several previous groups (*3–5*). This laboratory's experience with in vivo hemoperfusion through a pig liver, reported by Sablinski et al. (*16*), demonstrated efficiency in the removal of anti-pig antibodies. We here demonstrate that the in vivo efficiency of a synthetic αGal trisaccharide column is comparable to that of a pig liver, and is associated with fewer disadvantages, including hypotension and thrombocytopenia.

We conclude that the immunoadsorption system utilized in the present study (i) efficiently removes human and nonhuman primate anti-pig antibodies in vitro and, in nonhuman primates, in vivo, (ii) prevents the hyperacute rejection of transplanted pig kidneys in cynomolgus monkeys, (iii) is associated with little morbidity, and (iv) has advantages over immunoadsorption by hemoperfusion of a pig liver or other

[4] Kozlowski T, Ierino FL, Lambrigts D, Foley A, Andrews D, Awwad M, Monroy R, Cosimi AB, Cooper DKC, Sachs DH. Depletion of anti-Galα1-3Gal antibody in baboons by specific immunoaffinity columns. Manuscript submitted for publication.

organ. Despite other procedures and therapy that result in immune suppression of the recipient monkey, the subsequent return of anti-pig antibodies leads to delayed xenograft rejection. Attention must now be focused on this problem if xenotransplantation is to become a clinical reality.

REFERENCES

1. Platt JL, Bach FH. Mechanism of tissue injury and hyperacute xenograft rejection. In: Cooper DKC, Kemp E, Reemtsma K, et al., eds. Xenotransplantation. Heidelberg: Springer, 1991: 69.
2. Alexandre GPJ, Gianello P, Latinne D, et al. Plasmapheresis and splenectomy in experimental renal xenotransplantation. In: Hardy MA, ed. Xenograft 25. New York: Elsevier, 1989: 259.
3. Moberg AW, Shons AR, Gewurz H, Mozes M, Najarian JS. Prolongation of renal xenograft by the simultaneous sequestration of preformed antibody, inhibition of complement, coagulation, and antibody synthesis. Transplant Proc 1971; 3: 538.
4. Shons AR, Bier M, Jetzer T, Najarian JS. Techniques of in vivo plasma modification for the treatment of hyperacute rejection. Surgery 1973; 73: 28.
5. Cooper DKC, Human PA, Lexer G, et al. Effects of cyclosporine and antibody adsorption on pig cardiac xenograft survival in the baboon. J Heart Transplant 1988; 7: 238.
6. Leventhal JR, John R, Fryer JP, et al. Removal of baboon and human antiporcine IgG and IgM natural antibodies by immunoadsorption: results of in vitro and in vivo studies. Transplantation 1995; 59: 294.
7. Good AH, Cooper DKC, Malcolm AJ, et al. Identification of carbohydrate structures which bind human anti-porcine antibodies: implications for discordant xenografting in man. Transplant Proc 1992; 24: 559.
8. Cooper DKC. Depletion of natural antibodies in nonhuman primates: a step towards successful discordant xenografting in man. Clin Transplant 1992; 6: 178.
9. Cooper DKC, Good AH, Koren E, et al. Identification of αgalactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. Transplant Immunol 1993; 1: 198.
10. Oriol R, Ye Y, Koren E, Cooper DKC. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433.
11. Sandrin MS, Vaughan HA, Dabkowski PL, McKenzie IFC. Anti-pig IgM antibodies in human serum react predominantly with Galα(1–3)Gal epitopes. Proc Natl Acad Sci USA 1993; 90: 11391.
12. Galili U. Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunol Today 1993; 14: 480.
13. Neethling FA, Koren E, Ye Y, et al. Protection of pig kidney (PK15) cells from the cytotoxic effect of anti-pig antibodies by αgalactosyl oligosaccharides. Transplantation 1994; 57: 959.
14. Rieben R, Von Allmen E, Korchagina EY, et al. Detection, immunoadsorption, and inhibition of cytotoxic activity of anti-αGal antibodies using newly developed substances with synthetic Galα1–3Gal disaccharide epitopes. Xenotransplantation 1995; 2: 98.
15. Li SF, Neethling FA, Taniguchi S, et al. Glycans derived from porcine stomach mucin are effective inhibitors of natural anti-α-galactosyl antibodies in vitro and after intravenous infusion in baboons. Transplantation 1996; 62: 1324.
16. Sablinski T, Latinne D, Gianello P, et al. Xenotransplantation of pig kidneys to nonhuman primates. I. Development of the model. Xenotransplantation 1995; 2: 264.
17. Galili U, Rachmilewitz EA, Peleg A, Flechner I. A unique natural human IgG antibody with anti-α-galactosyl specificity. J Exp Med 1984; 260: 1519.
18. Cooper DKC, Koren E, Oriol R. Oligosaccharides and discordant xenotransplantation. Immunol Rev 1994; 141: 31.
19. Cairns T, Lee J, Goldberg L, Hacking A, Weymouth-Wilson A, Taube O. Polymorphism within the human IgG anti-pig repertoire [Abstract]. Br Transplant Soc 1995.
20. Parker W, Lateef J, Everett ML, Platt JL. Specificity of xenoreactive anti-Galα1–3Gal IgM for α-galactosyl ligands. Glycobiology 1996; 6: 499.
21. Ye Y, Neethling FA, Niekrasz M, et al. Evidence that intravenously administered αgalactosyl carbohydrates reduce baboon serum cytotoxicity to pig kidney cells (PK15) and transplanted pig hearts. Transplantation 1994; 58: 330.
22. Taniguchi S, Neethling FA, Korchagina EY, et al. In vivo immunoadsorption of anti-pig antibodies in baboons using a specific αGal1–3Gal column. Transplantation 1996; 62: 1379.
23. Cooper DKC, Cairns TJ, Taube DH. Extracorporeal immunoadsorption of anti-pig antibody in baboons using αGal oligosaccharide immunoaffinity columns. Xenotransplantation 1996; 4: 27.
24. Galili U, Clark MR, Shohet SB, Buehler J, Macher BA. Evolutionary relationship between the natural anti-Gal antibody and the Galα1–3Gal epitope in primates. Proc Natl Acad Sci USA 1987; 84: 1369.
25. Galili U, Shohet SB, Kobrin E, Stults CLM, Macher BA. Man, apes and Old World monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J Biol Chem 1988; 263: 17755.

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