

# Removal of anti-Gal $\alpha$ 1,3Gal xenoantibodies with an injectable polymer

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Preformed and elicited Ab's against the Gal $\alpha$ 1,3Gal terminating carbohydrate chains ( $\alpha$ Gal Ab's) are the primary cause of hyperacute and acute vascular xenograft rejection in pig-to-primate transplantation.  $\alpha$ Gal Ab's are produced by long-lived Ab-producing cells that are not susceptible to pharmacological immunosuppression. We reasoned that antigen-specific elimination of  $\alpha$ Gal Ab's might be achieved in vivo by systemic administration of nonimmunogenic polyvalent  $\alpha$ Gal structures with high avidity for  $\alpha$ Gal Ab's. We devised GAS914, a soluble trisaccharide-polylysine conjugate of approximately 500 kDa that effectively competes for  $\alpha$ Gal binding by  $\alpha$ Gal IgM (IC<sub>50</sub>, 43 nM) and IgG (IC<sub>50</sub>, 28 nM) in vitro. Injections of GAS914 in cynomolgus monkeys, at the dose of 1 mg/kg, resulted in the immediate decrease of more than 90% of circulating  $\alpha$ Gal Ab's and serum anti-pig cytotoxicity. In baboons, repeated injections of GAS914 effectively reduced both circulating  $\alpha$ Gal Ab's and cytotoxicity over several months. Studies with [<sup>14</sup>C]GAS914 in rhesus monkeys and Gal<sup>-/-</sup> mice indicate that GAS914 binds to circulating  $\alpha$ Gal Ab's and that the complex is quickly metabolized by the liver and excreted by the kidney. Remarkably, posttreatment  $\alpha$ Gal Ab titers never exceeded pretreatment levels and no sensitization to either  $\alpha$ Gal or the polylysine backbone has been observed. Furthermore there was no apparent acute or chronic toxicity associated with GAS914 treatment in primates. We conclude that GAS914 may be used therapeutically for the specific removal of  $\alpha$ Gal Ab's.

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## Introduction

The most immediate hurdle in pig-to-primate transplantation is the presence of naturally occurring Ab's against the Linear B trisaccharide (Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc) that is expressed on pig but not on the tissues of man and Old World monkeys (ref. 1, for review see ref. 2).

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**Nonstandard abbreviations used:** Gal $\alpha$ 1,3Gal terminating carbohydrate chains ( $\alpha$ Gal);  $\alpha$ 1,3-galactosyltransferase-deficient (Gal<sup>-/-</sup>); hemolytic anti-pig Ab (HAPAb); alanine aminotransferase (ALT); aspartate aminotransferase (AST).

Gal $\alpha$ 1,3Gal terminating carbohydrate chains ( $\alpha$ Gal) Ab's destroy pig organs via activation of complement, macrophage, and NK cell recruitment and endothelial cell activation (2, 3). When transplanted into primates, transgenic pig organs expressing human complement inhibitors resist hyperacute rejection (4, 5) but succumb to acute vascular rejection principally caused by induced  $\alpha$ Gal Ab's (6). It is now accepted that anti-complement strategies are not sufficient to protect the xenograft from acute vascular rejection and that removal of the  $\alpha$ Gal barrier will be necessary to achieve extended xenograft survival. Pending the generation of  $\alpha$ Gal-deficient pigs (7), elimination of  $\alpha$ Gal Ab's is a promising method for overcoming this barrier.

In  $\alpha$ 1,3-galactosyltransferase-deficient (Gal<sup>-/-</sup>) mice,  $\alpha$ Gal Ab's are produced by a B-1b-like splenic cell population and not by conventional B2-derived plasma cells (8). The analogous population in human and nonhuman primates is not responsive to conventional immunosuppression, cyclophosphamide, or treatment with B cell-depleting Ab's (9, 10), and their phenotype remains elusive. In the absence of effective pharmacological control of Ab synthesis by these cells, extracorporeal immunoabsorption has been examined as a means of  $\alpha$ Gal Ab depletion (11).

Extracorporeal immunoabsorption reduces circulating  $\alpha$ Gal Ab's before transplant, but the procedure is associated with significant morbidity in humans and mortality in nonhuman primates (6). A soluble inhibitor that can bind and clear  $\alpha$ Gal Ab's systemically might compete better with the xenograft for binding of Ab's and prove more effective in protecting it from humoral damage. Such an inhibitor should bind to  $\alpha$ Gal Ab's at least as tightly as xenogeneic tissue antigens; it should be nonimmunogenic; and the resulting immune complexes should be rapidly cleared from the circulation to avoid immune-complex deposition disease.

$\alpha$ Gal Ab's have high avidity for multivalent antigens, glycoproteins, and glycolipids on cell surface membranes, but low affinity for single oligosaccharides (12). Inhibition with monovalent compounds is therefore inefficient. This is particularly relevant for  $\alpha$ Gal IgMs that have only millimolar affinity for monomeric  $\alpha$ Gal oligosaccharides, but account for most of the anti-pig cytotoxicity in primate serum. We have prepared a series of linear polymers designed to provide flexible backbones for the multivalent presentation of Linear B monomers and maximize avidity to  $\alpha$ Gal Ab (IgG and IgM). Avidity, measured on a per monomer basis, was increased by the length of the backbone, and was also influenced by the loading of the carbohydrate monomer and choice of spacer (13). Maximal increase in avidity relative to the monomer was achieved with GAS914, a linear polylysine backbone with an average length of 1,000 lysines and with 25% of side chains conjugated to Linear B trisaccharide. In vitro, GAS914 is a potent inhibitor of the binding of both  $\alpha$ Gal IgG and IgM as well as the complement-dependent hemolysis of pig erythrocytes by human serum (Figure 1).

In the present study, we show that GAS914 is also highly efficacious in vivo in eliminating circulating  $\alpha$ Gal Ab's in primates without side effects and with minimal complement activation and no immune response against either the carbohydrate or the backbone. This mode of antigen-specific treatment promises to protect pig organs transplanted into primates.

## Methods

**Sera.** A pool of human AB serum from several individuals with high  $\alpha$ Gal IgM titers was used as an internal standard in assays of primate and *Gal*<sup>-/-</sup> mouse IgM and hemolytic anti-pig Ab (HAPAb) titers. Another pool with high  $\alpha$ Gal IgG titers was used as standard for IgG determinations. All titers reported here are relative to these standards. A third batch of pooled human AB serum (Sigma-Aldrich, St. Louis, Missouri, USA) was used for all in vitro inhibition assays with GAS914.

**$\alpha$ Gal ELISA.** Flat bottomed, 96-well PolySorb plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 50  $\mu$ l  $\alpha$ Gal-HSA (NGP3334; Dextra Laboratories, Reading, United Kingdom) at

5  $\mu$ g/ml in PBS and blocked with 200  $\mu$ l 0.5% Tween 20 in PBS at room temperature for 2 hours. After washing, 50  $\mu$ l of serum serial dilutions were made directly on the plate in PBS containing 0.2% Tween 20 (starting at 1:15 in twofold steps). After 1 hour, the plate was washed and incubated for 1 hour with either anti-human IgG or anti-human IgM peroxidase conjugates (Sigma-Aldrich) at 1:200 dilution in PBS with 0.1% Tween 20. After washing, the plates were developed for 5 minutes with *ortho*-phenylenediamine (Sigma-Aldrich) and absorbance at 420 nm was measured. Duplicate plates were run in blank wells (blocked wells with no  $\alpha$ Gal-HSA coating) and the background signal thus obtained was subtracted from the values obtained from the positive wells. Absorbance was plotted against log serum dilution for both experimental and standard human sera, and the titer of Ab in the experimental serum (relative to the standard serum) was estimated by the parallel lines method (standard titer, 1.0).

**HAPAb assay.** Ice-cold complement fixation diluent (Oxoid Ltd., Basingstoke, United Kingdom) was used for all steps. Pig blood (2.5 ml collected in standard heparin tubes) was diluted in 50 ml buffer and centrifuged for 6 minutes at 2,000 g. The erythrocyte pellet was washed twice and suspended in 60 ml buffer. All sera to be tested were heat inactivated for 15 minutes at 56°C. In a 96-well round-bottomed plate, 50  $\mu$ l of serum was serially diluted across a row starting at well 1 with a dilution of 1:2.5 and proceeding in twofold steps to well 10. Well 11 received 100  $\mu$ l water, and well 12 received 50  $\mu$ l complement fixation diluent. All wells received 50  $\mu$ l of washed erythrocyte suspension. Baby rabbit complement (Serotec Ltd., Kidlington, United Kingdom) was diluted 1:10 in buffer and 50  $\mu$ l was added to wells 1–10 and well 12. The plate was incubated at 37°C for 1 hour with shaking and centrifuged for 10 minutes at 2,000 g. Supernatants were transferred into new 96-well flat-bottomed plates and absorbance at 420 nm was measured. Well 11 represented the maximum value possible (100% lysis), and well 12 represented background lysis (complement alone). All sera were assayed in duplicate. Average values for each well minus the average value for well 12 were plotted, and the area under the curve was calculated as the cytotoxicity value. Values reported are relative to human standard serum assayed at the same time (human standard set at 1,000).

**Equivalent weight calculation.** Values for inhibition in the  $\alpha$ Gal Ab ELISA or hemolytic assay are based on the equivalent weight per Linear B trisaccharide, rather than on the average molecular weight of the polymer. This equivalent weight is a function of the fraction ( $x$ ) of glycosylated monomer (determined by NMR) and is independent of the degree of polymerization ( $n$ ). Equivalent weight is calculated by the formula:  $[(\text{mol wt}_{\text{glycosylated monomer}})x + (\text{mol wt}_{\text{thioglycerol monomer}})(1 - x)]/x$ . For  $x = 0.25$ , the equivalent weight is calculated as:

$[(872)0.25 + (276)0.75]/0.25 = 1,700$ . A polymer of average degree of polymerization ( $n$ ) = 1,000 and  $x = 0.25$  would have an average  $nx$  of 250 carbohydrate antigens per polymer molecule. The average molecular weight for  $x = 0.25$  is calculated as  $[(872)0.25 + (276)0.75]n$ , e.g. 425,000 when  $n = 1000$ . This method allows direct comparison of potencies between different size and loading of polymers.

**Administration of GAS914 to nonhuman primates.** Primate housing and all experiments were performed in accordance with national guidelines for experimentation on nonhuman primates. Cynomolgus monkeys (*Macaca fascicularis*), 3–5 kg, were obtained from Siconbrec Ltd. (Makati City, Philippines). Baboons (*Papio anubis*), 8–12 kg, were purchased from Consort Bioservices Ltd. (Steyning, United Kingdom). For injections and blood sampling, cynomolgus monkeys were restrained in a primate chair and baboons were put in a squeeze-back cage. GAS914 in saline (0.5 mg/ml) was administered to conscious animals at the indicated dose by slow injection (5 ml/min) via the cephalic or saphenous vein. Peripheral blood samples were taken at another site before and after GAS914 treatment for analysis of serum Ab's and biochemistry and C3a in plasma. Samples were stored at  $-70^{\circ}\text{C}$ .  $\alpha\text{Gal}$  Ab and HAPAb analyses were performed at the same time for all samples from a given animal. Serum biochemical parameters were determined immediately using a Synchron CX system (Beckman Coulter Inc., Miami, Florida, USA) and included total protein, albumin, creatinine, urea, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), leucine aminopeptidase,  $\gamma$ -glutamyl transferase, bilirubin, cholesterol, sodium, potassium, calcium, magnesium, chloride, and phosphate. C3a assays were performed using a commercial kit (Quidel Corp., San Diego, California, USA). In all terminal experiments, a complete necropsy was performed. Kidney, liver, and spleen tissues from each animal were investigated microscopically.

**Administration of  $[^{14}\text{C}]\text{GAS914}$  to mice and rats.** Labeled GAS914 was prepared as described previously, using chloroacetaldehyde labeled with  $^{14}\text{C}$  on the first carbon for the synthesis of perchloroacetylated polylysine intermediate. Male mice and rats were dosed intravenously with 1 mg/kg  $[^{14}\text{C}]\text{GAS914}$ . The animals were sacrificed ( $n = 3$  per timepoint) at 0.086, 0.25, 0.5, 1, 2, 4, 8, 24, and 48 hours, and blood and selected tissues were collected at various times up to 96 hours after administration. Whole-body frozen sections were obtained at various times after injection. Urine and feces were quantitatively collected daily over 4 days.

$\text{Gal}^{-/-}$  mice (14) were obtained from J. Lowe (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Michigan, USA) and bred under specific pathogen-free conditions. Eight-week-old mice received  $1 \times 10^7$  washed rabbit erythrocyte membranes intraperitoneally once

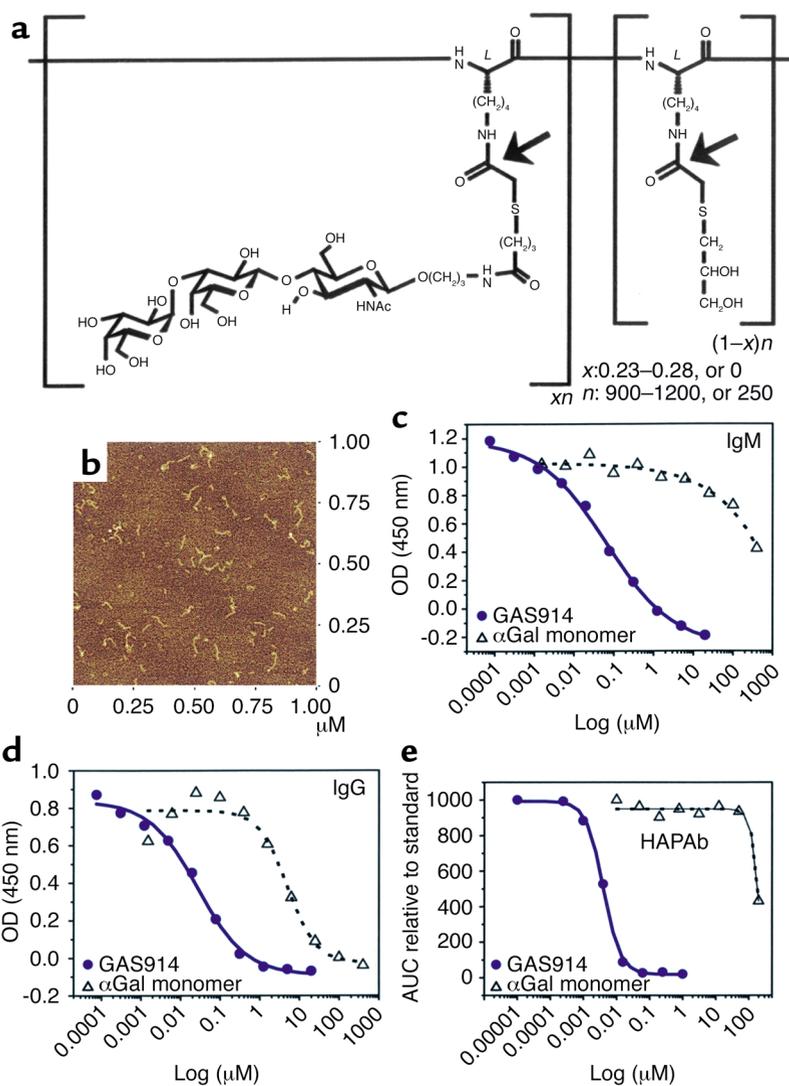
a week for 3 weeks (15). A week after the last injection, animals were bled and  $\alpha\text{Gal}$  Ab titers were determined. Animals with high  $\alpha\text{Gal}$  Ab titers were grouped ( $n = 3$ ), injected with 1 mg/kg intravenous  $[^{14}\text{C}]\text{GAS914}$ , and analyzed as indicated above for wild-type mice and rats.

**Administration of  $[^{14}\text{C}]\text{GAS914}$  to rhesus monkeys.** Three rhesus monkeys received a single 1 mg/kg intravenous injection of  $[^{14}\text{C}]\text{GAS914}$  in saline. Blood was sampled at 0.086, 0.25, 0.5, 0.75, 1, 2, 4, 8, 24, 48, and 72 hours. Urine and feces were quantitatively collected. The animals were sacrificed at 72 hours after dosing and perfused with cold saline before tissue collection. Total radioactivity was measured in all samples collected. Tissue samples were also frozen at  $-70^{\circ}\text{C}$  for immunohistochemistry.

**Immunohistochemistry.** Monoclonal anti-polylysine Ab's were generated in female BALB/c mice by conventional techniques. Thioglycerol-blocked polylysine (compare Figure 1a, where  $x = 0$ , with 3% of residues coupled to thiopropionic acid, and 97% of residues thioglycerol capped), amidically linked to ovalbumin, was used as the immunogen. The selected Ab's were shown to bind specifically on the polylysine backbone of GAS914 independently of the presence of carbohydrate chains. Immunohistochemistry was carried out on paraffin sections from tissues of selected animals with monoclonal anti-GAS914 Ab and either T cell (CD3; DAKO Corp., Carpinteria, California, USA) (dilution 1:400) or B cell Ab's (CD79a; DAKO Corp.) (dilution 1:20) in an immunostainer (Ventana Medical Systems, Tucson, Arizona, USA). Prior to the immunohistochemical procedure, sections were placed in a 0.02 M boric acid solution (pH 7.0) and heated for 2 minutes in a pressure cooker followed by a 20-minute cooling period under pressure. Sections were incubated with normal goat serum (DAKO Corp.) and immunostained first with B cell or T cell Ab's and biotinylated Ig/avidin-horseradish peroxidase (Ventana Medical Systems), and then with anti-GAS914 Ab (dilution 1:250) and the Alkaline Phosphatase Fast Red detection kit (Ventana Medical Systems).

## Results

**In vitro inhibition of  $\alpha\text{Gal}$  Ab's and HAPAb.** We have previously shown that multivalent presentation of carbohydrates on polylysine backbones potentiates the affinity of carbohydrate receptors (16). By optimizing the degree of polymerization (average number of lysines per polymer) and the carbohydrate density, we obtained GAS914. This compound is a flexible linear polymer and shows a dramatic fold increase of  $1 \times 10^3$  to  $1 \times 10^4$  in avidity with both the IgG and IgM classes of  $\alpha\text{Gal}$  Ab's compared with the monomer. This increased affinity is also reflected in a potent inhibition of HAPAb (Figure 1, c and e). The values reported in Figure 1 are on a per carbohydrate basis. That is, the concentrations are based on the



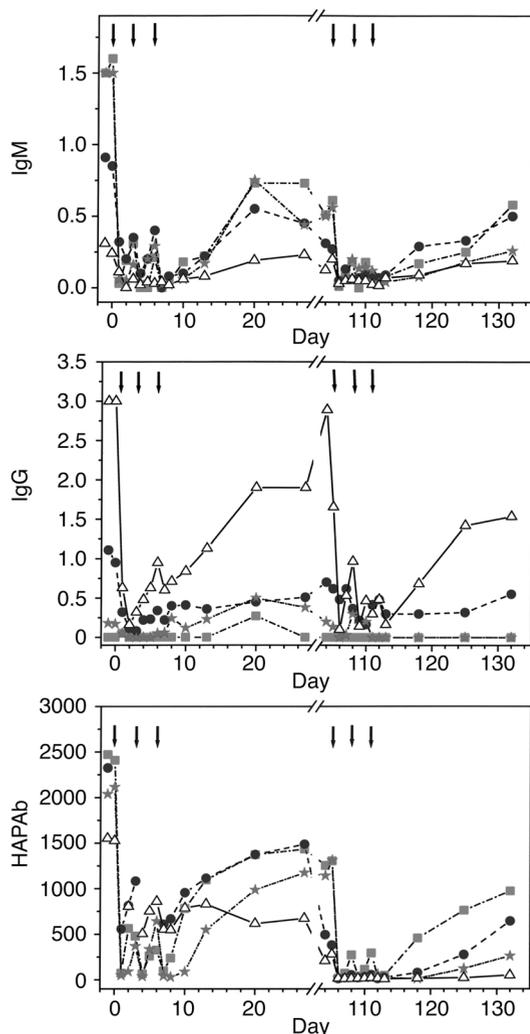
**Figure 1**  
Structure of GAS 914 and inhibition of  $\alpha$ Gal IgG, IgM, and HAPAb. (a) Chemical structure of random copolymer with:  $n$ , average degree of polymerization;  $x$ , fraction of glycosylated monomer;  $1 - x$ , fraction of thiolglycerol-capped monomer. Arrows indicate the positions labeled with  $^{14}\text{C}$  for pharmacokinetic studies. (b) Atomic force microscopy of GAS914 shows a chain-like molecule with an average chain diameter of  $4.0 \pm 0.2$  nm and average chain length of  $79 \pm 24$  nm. (c) Inhibition curves of  $\alpha$ Gal IgG ( $\text{IC}_{50}$  monomer =  $54 \mu\text{M}$ ,  $\text{IC}_{50}$  GAS914 =  $0.028 \mu\text{M}$ ). (d) Inhibition curves of  $\alpha$ Gal IgM ( $\text{IC}_{50}$  monomer =  $332 \mu\text{M}$ ,  $\text{IC}_{50}$  GAS914 =  $0.043 \mu\text{M}$ ). (e) Inhibition curves of HAPAb ( $\text{IC}_{50}$  monomer =  $400 \mu\text{M}$ ,  $\text{IC}_{50}$  GAS914 =  $0.004 \mu\text{M}$ ). Concentrations shown are on a per oligosaccharide basis as indicated in Methods. Curves are representative of more than seven experiments performed on different days.

equivalent weight of Linear B trisaccharide and not on the molecular weight of the polymer and thus directly relate to the avidity between Ab's and the carbohydrate hapten. Polylysine backbone with no carbohydrate or with lactosamine attached inhibits neither  $\alpha$ Gal Ab's nor anti-pig cytotoxicity up to concentrations of 1 mM (data not shown). To our knowledge, GAS914 is the most potent inhibitor of  $\alpha$ Gal Ab's reported to date. Previously published polyacrylamide polymers conjugated with  $\alpha$ Gal moieties have reported in vitro affinity to  $\alpha$ Gal IgM that is similar to GAS914, but have lower affinity to  $\alpha$ Gal IgG, and no in vivo data have been reported for this class of compounds (17). However, recently reported polyethylene glycol  $\alpha$ Gal conjugates lead to good in vivo reduction of  $\alpha$ Gal IgM Ab's at 50 mg/kg (18).

**Removal of  $\alpha$ Gal Ab's in vivo.** Although GAS914 is an excellent in vitro inhibitor of  $\alpha$ Gal Ab's, it was unclear whether it could be used safely in vivo due to potential risk factors of immunogenicity and immune complex formation. Indeed  $\alpha$ Gal-HSA was found to be a potent immunogen in cynomolgus monkeys (R.G. Warner, unpublished data). To avoid such problems,

we initially injected smaller polylysine derivatives (average degree of polymerization = 250; see Figure 1a) into cynomolgus monkeys. Although the smaller polylysine conjugates are good in vitro inhibitors of  $\alpha$ Gal Ab's, they are ineffective in vivo, resulting in the incomplete removal of circulating  $\alpha$ Gal Ab's and HAPAb (data not shown).

In subsequent experiments, intravenous injections of 1 mg/kg GAS914 were administered to a group of four cynomolgus monkeys on days 0, 3, and 6. Ab and biochemical analyses were performed until day 28. GAS914 injections resulted in an immediate disappearance of circulating  $\alpha$ Gal Ab's and HAPAb; these began to return slowly starting 12 hours after injection and reached approximately 30% of pretreatment values by day 3 after injection (Figure 2). Repeated injections with GAS914 further lowered the circulating  $\alpha$ Gal titers with equal efficiency. Circulating titers slowly rose after the third injection but by day 28 were still below pretreatment levels (Figure 2). A similar response was observed when the same group of animals was injected intravenously with 1 mg/kg on days 105, 108, and 111 after the first



**Figure 2**

Cynomolgus monkeys ( $n = 4$ ) received intravenous injections of 1 mg/kg GAS914 on days 0, 3, 6, 105, 108, and 111 (indicated by arrows). The animals were bled at the indicated times and serum was assayed for anti- $\alpha$ Gal Ab's and anti-pig hemolytic activity. IgM, IgG, and HAPAb titers shown are relative to human standard. Symbols represent titers of the same animal in all three panels.

was observed throughout the observation period, indicating that no anti-backbone Ab's were elicited by GAS914 injections.

In a control study, two cynomolgus monkeys received 1 mg/kg intravenously of a thioglycerol-capped polylysine (Figure 1a,  $x = 0$ ) on days 0, 3, and 6 and were followed for 28 days. No change in the circulating levels of  $\alpha$ Gal Ab's or HAPAb was observed over the treatment period, indicating that the polylysine backbone itself does not cause Ab depletion. Similar to the GAS914 study, no Ab's against the backbone were detected by ELISA during the control study.

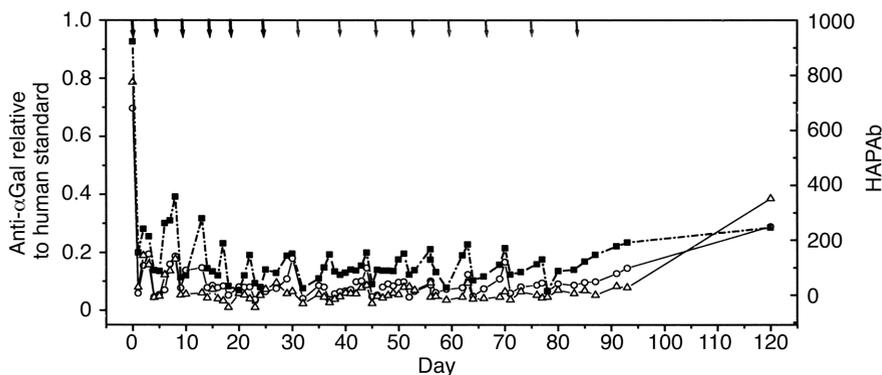
No clinically adverse effects were observed during treatment with GAS914 and during the subsequent observation period. Serum urea, creatinine, and blood phosphate remained normal throughout the observation period. Similarly, serum ALT and AST also remained within normal ranges, with the exception of a single animal that had mildly elevated baseline levels of ALT (50 mU/ml) and displayed consistently elevated ALT values (80 mU/ml) throughout treatment. All other biochemical markers were normal in this animal. Since clinical signs of immune complex-mediated glomerulonephritis might include proteinuria, urine was collected daily on days -1 to 28 and 105-130. Urinary volume and protein levels were always within normal range for all animals. Histological examination of kidney, spleen, and liver sections collected at necropsy (day 138) showed no significant pathological findings.

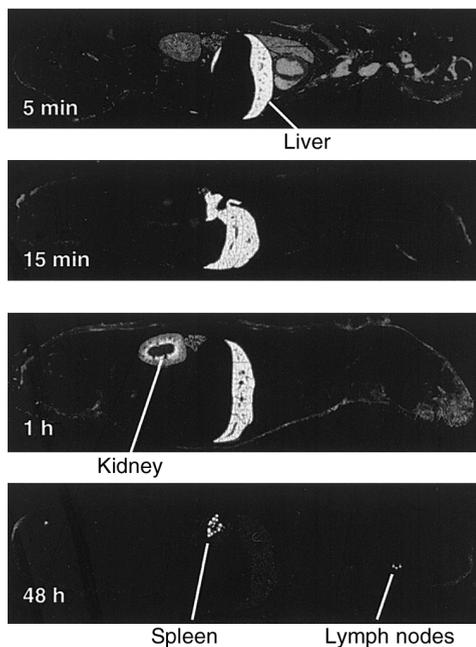
Levels of the anaphylatoxin C3a-desArg were followed during the second set of GAS914 injections (days 105-132) as a measure of systemic complement activation. In two of the four animals (the two with the highest levels of  $\alpha$ Gal IgM), small increases in C3a levels appeared immediately after the first GAS914 injection, but the levels returned to baseline within 12-24 hours.

GAS914 injection to test for sensitization (Figure 2). Surprisingly, GAS914 injections did not elicit an immune response against  $\alpha$ Gal. In fact, both  $\alpha$ Gal and HAPAb titers showed a sustained decrease throughout and remained below pretreatment levels at the end of the observation period. Sera from this study were analyzed on ELISA plates coated with control thioglycerol-capped polylysine (Figure 1a,  $x = 0$ ). No change in the signal against control polylysine

**Figure 3**

Baboons ( $n = 3$ ) received intravenous injections of GAS914 at the times indicated by arrows. Heavy arrows indicate injection of 5 mg/kg and light arrows indicate injection of 1 mg/kg. Anti- $\alpha$ Gal IgM (circles), IgG (triangles), and HAPAb (squares) titers relative to human standard are shown (see Methods). Data from a single baboon are shown; the other two animals had a similar response.





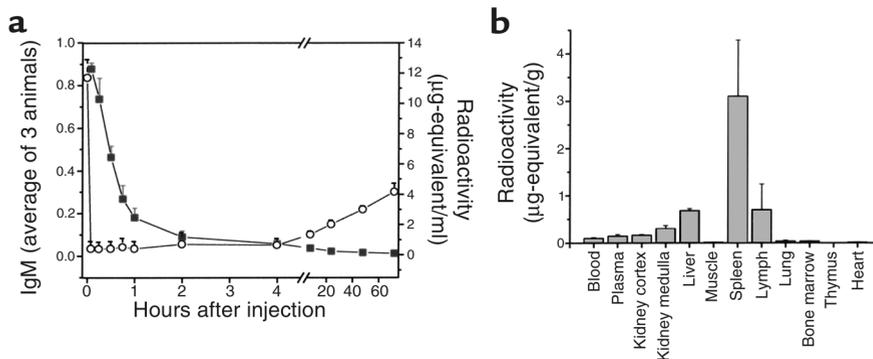
**Figure 4**  
Whole-body autoradioluminographs of *Gal*<sup>-/-</sup> mice immunized with rabbit erythrocytes and injected with 1 mg/kg intravenous [<sup>14</sup>C]GAS914. The bright areas indicate GAS914 or its metabolites. Times indicated are after injection.

Subsequent injections were associated with much smaller increases in C3a levels. C3a production was also measured as a percentage of total C3 (measured after *ex vivo* complement activation with cobra venom factor). It was calculated that the highest activation levels represented less than 0.5% activation of the total C3 available.

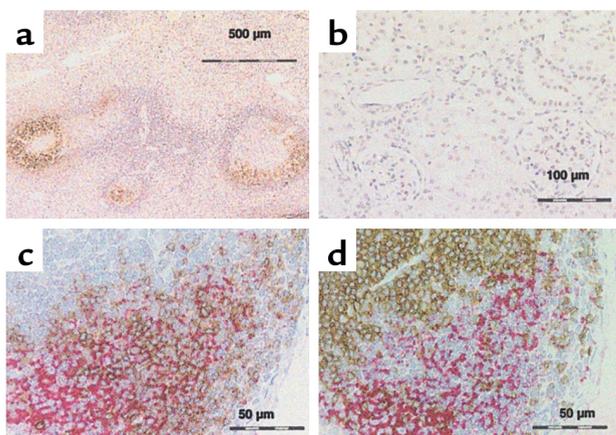
*Long-term αGal Ab suppression in baboons using GAS914.*  
In order to achieve extended xenograft survival, it is anticipated that effective long-term treatment will be required after transplantation until either the αGal response of the recipient is neutralized or the transplanted organ accommodates to its new environment (6). To determine the ability of GAS914 to control αGal Ab levels chronically, we treated a group of three baboons with repeated GAS914 injections over 3 months. Animals received a single intravenous injection of GAS914 (5 mg/kg during the initial 3 weeks and 1 mg/kg thereafter), when HAPAb titers reached 15% of the human standard level. Circulating αGal Ab and

HAPAb titers for a single representative animal over the complete observation period are shown in Figure 3. Injections of GAS914 in baboons showed an efficacy similar to that obtained in cynomolgus monkeys, leading to rapid elimination of circulating αGal Ab's followed by a slow return. Initially, injections every 2–3 days were required to keep circulating HAPAb within the target level. After the initial few weeks of continuous treatment, less frequent injections (once a week) sufficed to maintain both αGal Ab and HAPAb levels below target levels. One month after treatment was discontinued, a reduction of αGal Ab's and HAPAb persisted, with levels below 40% of the human standard (Figure 3).

*In vivo half-life of GAS914.* For pharmacokinetic studies, GAS914 was labeled with <sup>14</sup>C (see Figure 1a) to allow detection of the backbone even if the carbohydrate is metabolized. The half-life of [<sup>14</sup>C]GAS914 was determined after a single intravenous injection in normal mice and rats (species with no circulating αGal Ab's). In both species, radioactivity was eliminated in a biphasic manner. In the first phase, more than 95% of radioactivity was eliminated with extremely rapid kinetics from systemic circulation (half life of first elimination phase = 3.7 ± 0.2 min). From 30 minutes onward, a much slower multiexponential elimination was evident (half life of terminal elimination phase = 30 ± 7 h). Whole-body autoradioluminographs of rats at different times showed that radioactivity was quickly taken up by the liver and then excreted primarily through the kidneys. Analysis of blood samples at various times by PAGE suggests that GAS914 is metabolized by the liver and recycled into the circulation as two fragments of lower molecular weight. The GAS914 fragments are then taken up by the kidney where they are further metabolized and excreted in urine.



**Figure 5**  
Three rhesus monkeys received a single intravenous injection of 1 mg/kg [<sup>14</sup>C]GAS914. Blood was collected at the indicated times. (a) Radioactivity (squares) in whole blood disappeared rapidly from circulation in a biphasic manner. The proportion of radioactivity in plasma versus whole blood was constant (90%), suggesting that GAS914 is not significantly taken up by blood cells (data not shown). Almost no anti-αGal IgM was detected in serum by ELISA after the first bleeding at 5 minutes after injection. There was a slow return of anti-αGal IgM (circles) starting approximately 12 hours after injection. (b) Tissue concentration of total radioactivity 72 hours after administration of 1 mg/kg [<sup>14</sup>C]GAS914.



**Figure 6**  
Cynomolgus monkeys received daily intravenous GAS914 for 3 days (a and b) or 4 weeks (c and d) and were sacrificed 72 hours after the last injection. (a) Spleen of animal receiving 0.1 mg/kg GAS914 and (b) kidney of animal receiving 1 mg/kg GAS914. Both sections were stained with anti-GAS914 Ab (brown). Note the presence of GAS914 staining in the spleen and absence in the kidney. (c and d) Bronchial lymph node of animal receiving 1 mg/kg stained with anti-GAS914 Ab (red) and (c) anti-B cell Ab (brown) or (d) anti-T cell Ab (brown).

In view of the short circulating half-life of GAS914 in rodents with no  $\alpha$ Gal Ab's, it was important to determine the pharmacokinetics of this compound in the presence of circulating  $\alpha$ Gal Ab's. Immunized *Gal*<sup>-/-</sup> mice with titers of circulating  $\alpha$ Gal Ab (both IgM and IgG) comparable to those of primates were injected intravenously with 1 mg/kg [<sup>14</sup>C]GAS914. The same short circulating half-life and essentially identical excretion kinetics were observed in these animals as in normal mice, suggesting that circulating  $\alpha$ Gal Ab's do not substantially alter GAS914 metabolism. Whole-body autoradioluminograms (Figure 4) show that, as in the wild-type mice and rats, the liver is the primary target organ and the kidney is the secondary target organ. In strong contrast to the complete clearance of radioactivity from all tissues of normal mice and rats, significant levels of GAS914-associated radioactivity accumulated in the spleen and lymph nodes of immunized *Gal*<sup>-/-</sup> mice and were also evident in the whole-body autoradioluminograms (Figure 4). Immunohistochemical analysis of tissues from the *Gal*<sup>-/-</sup> mice showed that GAS914 is associated with B cells in the spleens of these animals.

[<sup>14</sup>C]GAS914 injected intravenously in rhesus monkeys was effective in clearing  $\alpha$ Gal Ab's and had a circulating half-life of 32 ± 12 minutes (Figure 5a), which is comparable to that observed in rodents. Circulating  $\alpha$ Gal IgM was undetectable by ELISA at 5 minutes after [<sup>14</sup>C]GAS914 injection (Figure 5a). Distribution and excretion of radioactivity in rhesus monkeys was the same as observed in *Gal*<sup>-/-</sup> mice. At 72 hours after injection, radioactivity was essentially cleared from all tissues, with the exception of the spleen and lymph nodes, where small amounts of the

labeled compound were still detected (Figure 5b). Immunohistochemistry of tissues from animals receiving GAS914 injections using an Ab recognizing the polylysine backbone confirmed the presence of GAS914 in spleen and lymph nodes but its absence from all other tissues examined, such as kidney, liver, lung, heart, gut, and skin. Double labeling with anti-GAS914 Ab and anti-T cell or anti-B cell Ab's revealed that GAS914 immunoreactivity is associated with the B cell area in lymph nodes (Figure 6).

## Discussion

The high levels of  $\alpha$ Gal Ab's in primates (19) are a formidable barrier for pig organ transplantation into these species. Our results demonstrate that GAS914 can lead to rapid and long-lasting antigen-specific removal of  $\alpha$ Gal Ab's from circulation without activation of B cells. The GAS914 polymer has a rapid uptake and metabolism accompanied by very low levels of complement activation. The physical and pharmacokinetic properties of the GAS914 polymer are likely to be important contributors to the in vivo efficacy, low toxicity, and lack of immune response to this compound. Lack of complement activation may be influenced by the shape, structural features such as lack of lipophilicity and charge, and size of the polylysine derivative, or may result from the short circulating half-life. Lack of immunogenicity may be the result of low complement activation, short half-life, or unproductive engagement with B cell receptors.

In  $\alpha$ Gal knockout mice, production of elicited  $\alpha$ Gal Ab's is T cell dependent (20). In this system, xenopeptides are highly efficient in eliciting T cell help, whereas allopeptides bearing  $\alpha$ Gal residues are ineffective (21). GAS914 may act as an allopeptide that fails to elicit T cell help. In the primate setting, elicited  $\alpha$ Gal Ab's are also T cell dependent, as evidenced by the efficacy of anti-CD154 treatment to inhibit elicited Ab's (22). However, production of  $\alpha$ Gal Ab's from existing plasma cells is not inhibited by anti-CD154 treatment and is very rapid after immunoabsorption. GAS914 has a dual function since it not only fails to elicit new  $\alpha$ Gal Ab's but also substantially limits new production of circulating  $\alpha$ Gal Ab's.

Continuous infusions of oligosaccharide monomers at very high doses have been used to inhibit circulating  $\alpha$ Gal Ab's in pig-to-primate xenotransplantation, but have provided only incomplete protection against vascular rejection (23, 24). In contrast to this approach, small dosages of GAS914 efficiently eliminate  $\alpha$ Gal Ab's from circulation. An important advantage of GAS914 is that it is a well-defined polymer that can be synthesized in large amounts with restricted physicochemical variability, thereby offering a relevant therapeutic option for xenotransplantation.

In considering the development of antigen-specific inhibitors of xenoreactive Ab's, the carbohydrate specificity is crucial. The great majority of naturally

occurring primate anti-pig Ab's bind to carbohydrate structures terminating with  $\alpha$ Gal (25, 26), but have variable specificities for di-, tri- or pentasaccharides and also for type II (Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc) versus type VI (Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc) core structures (27). We synthesized GAS914 with the Linear B type II trisaccharide, and therefore it binds and eliminates  $\alpha$ Gal Ab's with affinity for this carbohydrate. In numerous in vitro studies with human, cynomolgus monkey, and baboon sera, we find that GAS914 consistently eliminates more than 80% of natural anti-pig Ab's. In humans, natural Ab's against porcine antigens are mainly against  $\alpha$ Gal, and there is no evidence to suggest that new anti-carbohydrate specificities emerge in the immediate posttransplant period (28). We therefore expect that in animals where GAS914 achieves good pretransplant removal of circulating  $\alpha$ Gal Ab's, it will continue to control these Ab's effectively after transplant. This was recently demonstrated in a life-supporting pig kidney to baboon model (29) and in several ongoing solid organ transplantation models (R. Mañez et al., R. Morris et al., unpublished results). Even with GAS914 treatment, however, exposure to pig organs leads to the development of elicited Ab's against non- $\alpha$ Gal antigens. This has been well described in several nonhuman primate models.

Naive primates have small amounts of  $\alpha$ Gal Ab's that are not cross-reactive with GAS914 and may expand after transplant. Such Ab's may be removed with polylysine conjugates analogous to GAS914 but carrying different  $\alpha$ Gal structures. To this end, we have conjugated Linear B type VI to the polylysine backbone of GAS914 and have shown that this reagent is a similarly potent in vitro inhibitor that preferentially inhibits type VI-specific Ab's. Ongoing studies with type II (GAS914) and type VI GAS914-like polymer conjugates in primates indicate a more complete removal of  $\alpha$ Gal Ab's when the two injectable polymers are combined. The effect of this combination in pig-to-primate solid organ and bone marrow transplantation is under investigation.

Antigen arrays have been shown to eliminate Ab's and lead to antigen-specific B cell unresponsiveness (30). There is evidence that they function by engaging the B cell receptor in a manner that generates apoptotic instead of activation signals (31–33). It has been suggested that the size and hapten valence of antigen arrays is important for eliciting an inhibitory instead of an activating response (30).

The GAS914 polymer was optimized for its ability to bind  $\alpha$ Gal Ab's and not for its effect on B cells. We show here accumulation of GAS914 in the lymphoid organs of treated primates and *Gal*<sup>-/-</sup> mice (Figure 4, Figure 5, and Figure 6), and suppression of circulating  $\alpha$ Gal Ab's for periods far in excess of the period of time GAS914 is measurable in the blood. Simple removal of Ab's is probably not the only effect of GAS914, since removal of  $\alpha$ Gal Ab's by immunoadsorption leads to a quick rebound of circulating Ab's

as homeostatic mechanisms produce more Ab. We speculate that the GAS914 accumulated in the lymphoid organs is important for the long-term lowering of circulating  $\alpha$ Gal Ab's. GAS914 may associate with B cells and temporarily inhibit  $\alpha$ Gal Ab production. Alternatively, GAS914 may absorb newly formed  $\alpha$ Gal Ab's as they are produced in the lymphoid organs and prevent them from reaching the circulation. In agreement with the former hypothesis, GAS914 inhibits formation of plaques on pig erythrocyte-coated plates by splenocytes from immunized *Gal*<sup>-/-</sup> mice, even when the splenocytes are washed after incubation with GAS914 so that no free GAS914 is present in the solution (A. Katopodis, unpublished results). In the mouse, the  $\alpha$ Gal IgM is produced by splenic B cells with  $\alpha$ Gal Ab receptors (8) to which GAS914 may bind in a manner that suppresses Ab production. However, the deposition of GAS914 in the lymphoid organs of *Gal*<sup>-/-</sup> mice and primates is consistent with immune complexes in the network of follicular dendritic cells in secondary follicles and not direct association with B cells as such. Interestingly, immune complexes in secondary follicles are believed to be required for effective B cell stimulation and immunoglobulin class switch, but GAS914 is clearly nonimmunogenic. Additional polylysine conjugates of varying length and ligand density are currently being tested for their ability to elicit B cell hyporesponsiveness in *Gal*<sup>-/-</sup> mice.

Many autoimmune diseases are the result of the generation of pathogenic Ab's against specific antigens. Elimination of auto-Ab's has been shown to be associated with disease remission (34). The polylysine backbone of GAS914 offers an excellent scaffold for antigen-specific therapies in Ab-mediated diseases where the immunogenic epitope is defined.

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