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17. For the calculation of apparent association constants, we used the integration of peaks of the guest inside and outside of the capsule that were obtained by ^{1}H NMR. There is an estimated 10% error in these measurements. The equilibrium can be described as follows:

$$H_{aggregate} + G \overset{K_a(app)}{\rightleftharpoons} H_4G$$

The following assumptions were made: (i) the amount of tetramer (unfilled or filled with solvent) present before addition of the guest is negligible; (ii) after addition of the guest, all of the host material not assembled into the capsule (including solid material) is in the aggregate state; and (iii) the association of the guest with itself is negligible.

$$K_a(app) = \frac{[H_4G]}{[H_{aggregate}][G]} = \frac{xV}{(h - x)(g - x)}$$

$$\Delta G° = -RT \ln K_a(app)$$

$$x = g(I_{gi}/I_{gt})$$

$$I_{gt} = I_{go} + I_{gi}$$

In these equations, $H_{aggregate}$ is the host as a low-ordered aggregate, G is the guest, H_4G is the tetramer complex, x is the amount of guest (in millimoles) in the complex H_4G, V is the total volume (in milliliters), h is the initial theoretical amount of tetramer (in millimoles), g is the amount of guest (in millimoles) added to the solution, $\Delta G°$ is the free energy of formation, R is the ideal gas constant, T is temperature (kelvin), I_{gt} is the sum of all the integrals corresponding to the guest, I_{go} is the integral for the signal of the guest outside of the tetramer complex, and I_{gi} is the integral for the signal of the guest inside of the tetramer complex. To obtain K_a(app) for guest **10**, we added 0.5 equivalents of the guest with respect to the monomer **1b** in CD_2Cl_2, sonicated the heterogeneous solution for 30 min, and measured the ^{1}H NMR spectrum after 30 hours.
18. The competition experiments were performed by the addition of different amounts of one guest (G′) to compound **1b** with another guest (G) present. The ratio of the two complexes H_4G and H_4G' was obtained by integration of the N-H signals of the two species. The equilibrium may be described as follows:

$$H_4G + G' \overset{K}{\rightleftharpoons} H_4G' + G$$

$$K = \frac{[H_4G'][G]}{[H_4G][G']} = \frac{K_a'(app)}{K_a(app)}$$

19. These compounds were prepared by monoalkylation of the corresponding urea and cyclic sulfamide with 1-bromodecane. The presence of the long alkyl chain is necessary for good solubility in noncompetitive solvents.

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Inhibition of Xenoreactive Natural Antibody Production by Retroviral Gene Therapy

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The major barrier to transplantation across discordant species, such as from pig to human, is rejection mediated by xenoreactive natural antibodies (XNA) that bind the carbohydrate epitope Galα1-3Galβ1-4GlcNAc-R (αGal) on donor tissues. This epitope is synthesized by the enzyme glucosyltransferase uridine 5′-diphosphate galactose:β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminide α(1-3)galactosyltransferase (E.C. 2.4.1.151), or simply αGT. When a functional αGT gene was introduced by retroviral gene transfer into bone marrow cells, αGal XNA production in a murine model ceased. Thus, genetic engineering of bone marrow may overcome humoral rejection of discordant xenografts and may be useful for inducing B cell tolerance.

A shortage of human organs has stimulated a great deal of research into the possibility of using nonhuman donors for transplantation. Pigs are the most likely species to serve as donors for clinical xenotransplantation because of their similarity to humans (*1, 2*). However, humoral rejection mediated by αGal XNA remains a major barrier to successful xenotransplantation (*3, 4*). Although several approaches have been developed to prevent hyperacute rejection mediated by αGal XNA, delayed rejection caused by these antibodies remains a formidable challenge (*5–8*). For xenotransplantation to be successful, methods must be developed to overcome organ damage mediated by αGal XNA.

Induction of mixed chimerism by bone marrow transplantation can be used to tolerize B cells producing αGal XNA (*9*), but this is not a practical solution (*10*). We hypothesized that production of αGal XNA may be inhibited by introducing a functional αGT gene by retroviral gene transfer into autologous bone marrow cells. αGal epitopes would then be expressed on the surface of bone marrow–derived cells or on secreted proteins that could potentially tolerize B cells making αGal XNA. Thus, establishing molecular rather than cellular chimerism could circumvent difficulties associated with engraftment of pig bone marrow in primates for the purpose of inducing tolerance.

To construct a retroviral vector (*11*) carrying the gene encoding porcine αGT, we isolated from the plasmid pSal3GT1 an 1145–base pair (bp) Eco RI–Cac 8I DNA fragment containing the coding region of pig αGT (*12*) and cloned it together with the murine phosphoglycerate kinase promoter into the N2A retroviral vector (*13*) to construct LGTRV (Fig. 1A). The LGTRV construct was transfected into the amphotropic retroviral packaging cell line AM12 (*14*) to derive the virus producer cell line LGTA7. The viral titer of supernatants harvested from the LGTA7 line was approximately 5×10^4 to 1×10^5 colony-forming units per milliliter. To test whether LGTA7-derived virus could transfer functional αGT expression, we transduced Vero cells with LGTA7 or a control virus containing only the neomycin resistance (neo^r) gene, and surface expression of αGal epitopes was analyzed by staining with the isolectin BSI-B_4 (IB4 lectin) isolated from *Bandeiraea simplicifolia*, which specifically binds αGal (*15*). G418-resistant Vero cell clones infected with LGTA7 expressed αGal epitopes on the cell surface at levels detectable by staining with IB4 lectin (Fig. 1B). Surface expression of αGal epitopes was stable and could be detected on the surface of transduced cells for at least 4 months in culture. In contrast, no surface expression of αGal epitopes was detected on Vero cells transduced with control virus. Treatment of LGTA7-transduced Vero cell clones

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with α-galactosidase specifically reduced αGal expression detectable by staining with IB4 lectin (Fig. 1B), but not major histocompatibility complex class I expression on the same cells, as detected by staining with the monoclonal antibody W6/32 (*16*). Thus, LGTA7-derived retroviruses transferred stable expression of porcine αGT, which catalyzed the addition of αGal epitopes to existing carbohydrate side chains.

To examine whether genetic engineering of bone marrow could be used to inhibit production of αGal XNA, we made use of αGT knockout mice (*17*) generated by gene targeting in embryonic stem cells (*18, 19*). Mice homozygous for a targeted disruption in the αGT gene (GT^0 mice) do not express the αGal epitope and produce αGal-reactive serum antibodies (*18, 20*). GT^0 mice were treated in vivo with 5-fluorouracil (150 mg/kg of body weight). At 7 days, bone marrow cells were harvested and transduced with LGTA7 or GN24 (neo^r-only) control virus (*21*). Transduced bone marrow cells were then recovered, and 10^6 LGTA7- or GN24-transduced bone marrow cells were used to reconstitute lethally irradiated (10.25 Gy) syngeneic GT^0 mice. To examine transduction efficiency, we reconstituted additional groups of GT^0 mice with limiting numbers of transduced bone marrow cells, and 12 days after reconstitution, individual spleen colonies were examined for integrated viral DNA by semi-nested polymerase chain reaction (PCR) using primers specific for the LGTA7 virus (*22*). Approximately 78% (7/9) of splenic colonies harvested from mice reconstituted with LGTA7-transduced bone marrow cells contained LGTA7 DNA, indicating that bone marrow–derived cells were carrying the transduced pig αGT gene (Fig. 2A).

At 7 weeks after reconstitution, mice were bled weekly and sera were analyzed for the presence of αGal XNA by binding to enzyme-linked immunosorbent assay (ELISA) plates coated with αGal oligosaccharides conjugated to bovine serum albumin (BSA) (*9*). Seven weeks after transplantation, αGal XNAs were detected in the sera of control mice reconstituted with neo^r-transduced bone marrow, but not in the sera of mice reconstituted with LGTA7-transduced bone marrow (Fig. 2B) (*23*). At week 15 after transplantation, the level of αGal XNA in mice reconstituted with neo^r-transduced bone marrow reached stable levels similar to that observed in control GT^0 mice, while the level of αGal XNA in mice reconstituted with LGTA7-transduced bone marrow remained undetectable (Fig. 2C). The failure of mice receiving LGTA7-transduced bone marrow to produce αGal-reactive antibodies was not due to a failure of B cell reconstitution, because by 4 weeks after bone marrow transplantation, the number of peripheral blood B cells was identical in both groups, on the basis of cell surface staining with monoclonal antibody to CD19 and flow cytometry (*24*).

We further examined the effect of gene therapy on αGal XNA production, at between 9 and 10 weeks after bone marrow transplantation, by analyzing, by flow cytometry, serum antibodies from untreated GT^0 and $GT^{+/+}$ controls, as well as GT^0 mice reconstituted with LGTA7- or neo^r-transduced syngeneic bone marrow, for their ability to bind to pig blood cells (*25*). Control GT^0, but not $GT^{+/+}$ mice contained in their sera antibodies that bind pig cells (Fig. 2D). Essentially, all detectable pig cell–binding antibodies in the serum of GT^0 mice were αGal-reactive. The binding of serum antibodies from GT^0 mice reconstituted with neo^r-transduced bone marrow to pig cells was indistinguishable from that observed with control untreated GT^0 mice (Fig. 2D). In contrast, we were unable to detect binding of serum antibodies from mice reconstituted with LGTA7-transduced bone marrow to pig cells (Fig. 2D).

We examined whether αGal-reactive antibodies, below the detection limit of our ELISA or pig cell binding assays, were present in mice reconstituted with LGTA7-transduced bone marrow by use of a fluorescence-based complement-mediated cytotoxicity assay (*26*). Sera from control GT^0 (Fig. 3A) and GT^0 mice reconstituted with neo^r-transduced bone marrow (Fig. 3B) contained antibodies that mediated killing of the porcine kidney cell line PK-15 in the presence of complement, whereas no significant killing of PK-15 cells was detected with sera from control $GT^{+/+}$ or GT^0 mice reconstituted with LGTA7-transduced bone marrow (Fig. 3, C and D, respectively). After

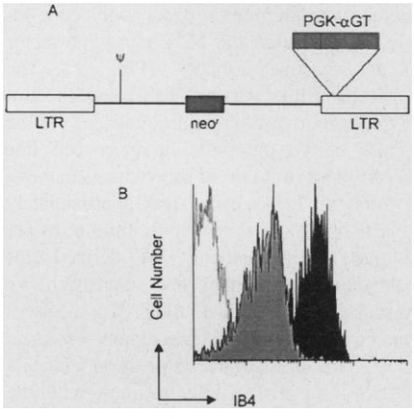

Fig. 1. (**A**) The LGTRV retroviral construct. Drawing not to scale. (**B**) Analysis of αGal expression on transduced Vero cell clones by staining with FITC-labeled IB4 lectin. Shown is staining of G418-resistant selected Vero cell clones. Staining of Vero cell clones transduced with neo^r control virus (NB5) is shown in white. Staining of Vero cell clones transduced with LGTA7 is shown in black. Shown in gray is staining of LGTA7-transduced cells following treatment with α-galactosidase. Enzymatic removal of αGal epitopes was not complete because the reactions were performed at suboptimal pH to preserve cell viability.

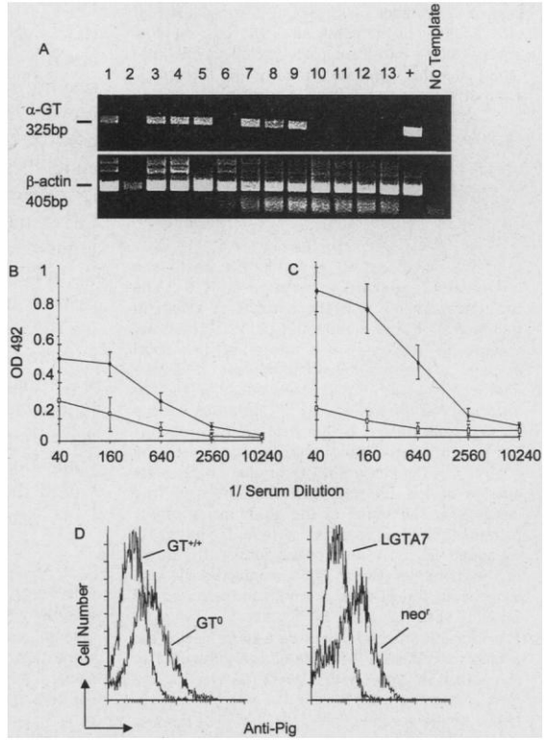

Fig. 2. (**A**) Analysis of day 12 splenic colonies for the presence of LGTA7-derived αGT or the endogenous β-actin gene by PCR. Lanes 1 through 9, samples from two individual mice reconstituted with LGTA7-transduced bone marrow. Lanes 10 through 13, samples from two individual mice reconstituted with GN24 (neo^r)–transduced bone marrow. DNA from a LGTA7-transduced Vero cell clone was used as a positive control (+). Results obtained using primers specific for LGTA7-encoded αGT (upper panel) and β-actin (lower panel) are shown. Inhibition of αGal XNA production in mice reconstituted with LGTA7-transduced bone marrow (**B** through **D**). Analysis of αGal-reactive IgM antibodies at (B) 7 and (C) 15 weeks after reconstitution. Solid diamonds (top trace), sera from mice reconstituted with neo^r-transduced bone marrow (n = 12); open squares (bottom trace), sera from mice reconstituted with LGTA7-transduced bone marrow (n = 11). Shown are the mean and standard deviation of combined results from two independent experiments. OD 492, optical density at 492-nm wavelength. (**D**) Staining of pig blood cells with sera from control GT^0 or $GT^{+/+}$ mice, and mice reconstituted with neo^r- or LGTA7-transduced bone marrow. Serum samples from reconstituted mice were analyzed at week 9 following reconstitution. Shown are samples taken from individual representative mice.

incubation of PK-15 cells with sera from control GT^0 mice, 70.00 ± 22.63% of the cells were killed, whereas only 3.36 ± 1.29% were killed after incubation with sera from $GT^{+/+}$ mice. Sera from mice reconstituted with neo^r-transduced bone marrow killed 77.92 ± 19.28% of PK-15 cells. In contrast, sera from mice reconstituted with LGTA7-transduced bone marrow killed 2.58 ± 1.31% of the PK-15 cells. The difference in percent killing observed using sera from mice reconstituted with neo^r- or LGTA7-transduced bone marrow was significant ($P < 0.0001$). Thus, αGal XNA production is inhibited in GT^0 mice reconstituted with LGTA7-transduced bone marrow.

To date, approaches to overcome antibody-mediated xenograft rejection have included modifying the host by either depleting or neutralizing preexisting serum αGal XNA (*7*) or by transgenic modification of donor pigs (*27*), but neither approach allows permanent graft survival (*28*). Thus, although host conditioning regimens and organs from transgenic pigs will likely play a role in clinical xenotransplantation, production of αGal XNA by the host must be overcome to achieve discordant xenotransplantation. We have focused on developing a genetic engineering approach to prevent production of host αGal XNA. XNAs to αGal were undetected in mice reconstituted with LGTA7-transduced bone marrow. The level of αGal-reactive XNA remained undetectable in mice up to 51 weeks after transplantation (*29*). Antibodies reactive with αGal are believed to be produced in response to normal bacterial flora present in the host (*30*). Thus, even with constant antigenic stimulation, the effect of gene therapy on αGal XNA is maintained. The use of genetically modified autologous bone marrow to establish molecular chimerism eliminates the potential for graft-versus-host disease that is associated with transplantation tolerance established by induction of cellular chimerism, and may permit the use of less toxic host-conditioning regimens. Similar approaches may also be applicable to induction of tolerance in other disorders, including autoimmune diseases.

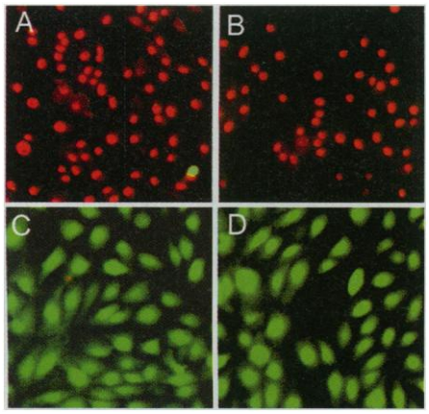

Fig. 3. Analysis of cytolytic αGal XNA production in gene therapy mice. Shown are results obtained using complement-mediated fluorescence-based cytotoxicity. Live cells fluoresce green while nuclei from dead cells fluoresce red. Killing of PK-15 cells using sera from (**A**) control GT^0 mice, (**B**) GT^0 mice reconstituted with neo^r-transduced bone marrow, (**C**) control $GT^{+/+}$ mice, and (**D**) GT^0 mice reconstituted with LGTA7-transduced bone marrow. Representative results are shown (200× magnification).

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23. Full B cell reconstitution was not observed until 4 weeks after transplantation. Production of αGal reactive XNA in controls receiving neo^r-transduced bone marrow was first observed at 5 weeks after transplantation. The level of αGal XNA reactivity in mice reconstituted with LGTA7-transduced bone marrow was indistinguishable from that observed by ELISA using lactosamine-conjugated BSA-coated plates. Lactosamine-BSA is identical to αGal-BSA except the terminal αGal carbohydrate residue is absent.
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25. Serum antibody binding to pig cells was analyzed by indirect immunofluorescence. We added 10 μl of undiluted mouse serum to 5×10^5 pig peripheral blood cells resuspended in 90 μl of Hanks' balanced salt solution (HBSS) and incubated them for 30 min at 4°C. Binding of serum antibodies was revealed by staining with fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse immunoglobulin M. To analyze cell surface expression of αGal epitopes, we stained 5×10^5 cells with FITC-labeled IB4 lectin (Sigma, St. Louis, MO) for 30 min at 4°C. For α-galactosidase assays, after staining with IB4 lectin, 2×10^5 cells were washed in HBSS and resuspended in 100 μl galactosidase buffer [0.1 M citric acid, 0.2 M Na_2HPO_4, 3% glycerol (pH 6.0)] containing 0.5 unit of α-galactosidase (Sigma) or buffer alone as a control for 1 hour at 4°C. The cells were washed in HBSS and analyzed by flow cytometry. Stained cells were analyzed on a Becton Dickinson FACscan. All staining data was analyzed using WinList software (Verity Software House, Topsham, ME). All serum samples used in binding assays were obtained from sex- and age-matched mice.
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