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Impact of Humoral Immune Response on Distribution and Efficacy of Recombinant Adeno-Associated Virus-Derived Acid α-Glucosidase in a Model of Glycogen Storage Disease Type II

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ABSTRACT

Glycogen storage disease type II (GSDII) is a lysosomal storage disease caused by a deficiency in acid α-glucosidase (GAA), and leads to cardiorespiratory failure by the age of 2 years. In this study, we investigate the impact of anti-GAA antibody formation on cross-correction of the heart, diaphragm, and hind-limb muscles from liver-directed delivery of recombinant adeno-associated virus (rAAV5)- and rAAV8-GAA vectors. GAA$^{-/-}$ mice receiving 1×10^{12} vector genomes of rAAV5- or rAAV8-DHBV-hGAA were analyzed for anti-GAA antibody response, GAA levels, glycogen reduction, and contractile function. We demonstrate that restoration of GAA to the affected muscles is dependent on the presence or absence of the antibody response. Immune-tolerant mice had significantly increased enzyme levels in the heart and skeletal muscles, whereas immune-responsive mice had background levels of GAA in all tissues except the diaphragm. The increased levels of activity in immune-tolerant mice correlated with reduced glycogen in the heart and diaphragm and, overall, contractile function of the soleus muscle was significantly improved. These findings highlight the importance of the immune response to rAAV-encoded GAA in correcting GSDII and provide additional understanding of the approach to treatment of GSDII.

OVERVIEW SUMMARY

In this study, we investigate the potential to correct a mouse model of glycogen storage disease type II (GSDII) by intrahepatic delivery of recombinant adeno-associated virus 5 (rAAV5) and rAAV8 vectors expressing human acid α-glucosidase (hGAA) under the control of the duck hepatitis B viral promoter, for enhanced expression in liver. We specifically evaluated the level of humoral response to the rAAV-derived hGAA protein and the impact of this response on correction. With superphysiologic levels of liver GAA activity, we achieved significantly restored levels of GAA in the affected muscles of GSDII mice, without an immune response. These increased levels of GAA correlate with improved clearance of glycogen from the heart and diaphragm. Contractile function of the soleus was also significantly improved. Taken together, these results demonstrate the therapeutic potential of liver-directed rAAV-mediated therapy for GSDII and highlight the importance of understanding and controlling the anti-GAA immune response.

INTRODUCTION

GLYCOGEN STORAGE DISEASE TYPE II (GSDII) is an autosomal recessive lysosomal storage disorder caused by a complete or partial deficiency in the lysosomal hydrolase, acid α-glucosidase (GAA; EC 3.2.1.3). GAA deficiency leads to accumulation of lysosomal glycogen, in all tissues, with skeletal and cardiac muscle being the most severely affected (Hirschhorn and Reuser, 2000; Raben *et al*., 2002). The severity of GSDII is ultimately related to the degree of enzyme de-

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ficiency and represents a continuum of diseases. The most severe variant is the early-onset form, also known as Pompe disease. Pompe patients have complete or nearly complete deficiency of GAA and usually die within the first 2 years of life as a result of cardiorespiratory failure.

GAA is synthesized as a 110-kDa precursor protein, which is core glycosylated in the endoplasmic reticulum (ER) and acquires mannose 6-phosphate (M6P) residues in the *cis*-Golgi. Approximately 80–90% of the protein is trafficked to the lysosomes via the M6P receptor. The remaining 10–20% of GAA is secreted from the cell and can be internalized by other cells via M6P receptors on the cell membranes of adjacent and distant cells and subsequently trafficked to the lysosomes (Hoefsloot *et al*., 1990; Wisselaar *et al*., 1993). This secretion–reuptake mechanism provides the basis for both enzyme and gene replacement therapies in which systemic administration of recombinant enzyme or direct transduction of one cell population provides functional enzyme for another. On the basis of the relatively mild presentation of later onset patients with approximately 20% of normal GAA activity, it has generally been accepted that continuous restoration of this level of activity would ameliorate the more severe symptoms (Reuser *et al*., 1995).

Phase I/II clinical trials are currently underway to evaluate the efficacy of recombinant enzyme replacement therapy (ERT). However, the therapeutic efficacy and a clear understanding of the relationship between the immunologic background of patients and their potential to be successfully treated remain unknown. Recombinant AAV (rAAV) vectors have become a favorable gene delivery system for the treatment of a variety of diseases including cystic fibrosis (Flotte *et al*., 2003), hemophilia A and B (High, 2003; VandenDriessche *et al*., 2003), and at least five different storage disorders (Wei *et al*., 1994; Daly *et al*., 2001; Jung *et al*., 2001; Beaty *et al*., 2002; Fraites *et al*., 2002; Takahashi *et al*., 2002). Transgene expression from an rAAV vector has been observed out to 5 years posttreatment in a dog model of hemophilia B (Mount *et al*., 2002). Along with the ability of rAAV to persist in the host long-term, rAAV-mediated delivery of therapeutic transgenes is also considered to be safe, compared with other viral vector systems, because of the absence of viral open reading frames in the recombinant vector and the lack of association with any human disease (Berns and Linden, 1995).

Although there have been few reports of toxicity associated with the rAAV vector itself, there is the potential for an immune response to the encoded transgene product. Recombinant AAV-delivered transgenes capable of eliciting an immune response include pathogen-specific transgenes in the context of rAAV vectors designed as vaccines (Manning *et al*., 1997; Liu *et al*., 2000; Xin *et al*., 2001), naturally immunogenic transgenes including β-galactosidase (Lo *et al*., 1999; Zhang *et al*., 2000; Cordier *et al*., 2001) and ovalbumin (Brockstedt *et al*., 1999), and therapeutic transgenes such as factor IX for the treatment of hemophilia B (Fields *et al*., 2001; Herzog *et al*., 2002). The likelihood of an rAAV-delivered transgene eliciting an inhibitory immune response depends on several factors including the genetic background of the host, amount of protein secreted by transduced cells, presence or absence of residual mutant protein (Brooks, 1999), and route of vector administration (Brockstedt *et al*., 1999; Cordier *et al*., 2001; Mingozzi *et al*., 2003).

To date, little is known concerning the immune response to GAA after treatment of GSDII by ERT or rAAV-mediated protein production. Raben *et al*. reported formation of a humoral (Raben *et al*., 2003b) and cell-mediated response after ERT in GSDII mice ($GAA^{-/-}$), with a 100% fatality rate from anaphylaxis after the seventh injection of recombinant human GAA protein (Raben *et al*., 2003a). Others have shown that human GAA delivered by adenovirus (Ding *et al*., 2001, 2002) or AAV–adenovirus hybrid vectors (Sun *et al*., 2003) elicits an anti-GAA antibody response in $GAA^{-/-}$ mice. However, there have been no reported studies investigating the potential for and consequences of an immune response to rAAV-derived human GAA (hGAA). This information will be important as we approach potential clinical trials, as the most severely affected patients make little to no residual GAA that may confer immune tolerance.

Our current studies were designed to determine the potential for liver-directed, rAAV-mediated cross-correction of GSDII in both the presence and absence of an anti-GAA humoral response. We developed an rAAV-hGAA vector capable of high levels of hepatic expression by evaluating several liver-enhanced promoters. After packaging this vector into AAV5 or AAV8 capsids, we evaluated its therapeutic efficacy by intrahepatic delivery to $GAA^{-/-}$ mice with various degrees of anti-GAA immunity. We observed significantly greater levels of GAA activity in the heart, diaphragm, and hind-limb muscles in mice with no anti-GAA antibody response (immune-tolerant mice) compared with mice with an antibody response (immune-responsive mice). Immune-responsive mice had little to no increase in GAA activity levels in the heart and hind-limb muscles but had partially restored levels of GAA in the diaphragm. The increased activities in immune-tolerant mice resulted in complete glycogen clearance in the heart and partial clearance in the diaphragm. Significant improvement in contractile function was also observed, but was less dependent on terminal GAA levels.

MATERIALS AND METHODS

Plasmid construction

The 3.1-kb human GAA (hGAA) cDNA was constructed as described (Pauly *et al*., 1998) and subcloned into the p43.2 plasmid containing the two AAV serotype 2 inverted terminal repeats (ITRs). The pGHP3 plasmid containing 300 bp of the duck hepatitis B virus (DHBV) core promoter was constructed as described (Liu *et al*., 1991). The DHBV promoter, albumin promoter (gift of K. Zaret, Fox Chase Cancer Center, Philadelphia, PA), and human α_1-antitrypsin (hAAT) promoter (gift of K. Ponder, Washington University, St. Louis, MO) were subcloned into the pTR-hGAA vector by the following methods. 5′ *Bgl*II and 3′ *Hin*dIII sites were added to the promoter sequence by polymerase chain reaction (PCR) and the amplified products were initially subcloned into the PCR2.1 Topo cloning vector (Invitrogen, Carlsbad, CA). The *Bgl*II- and *Hin*dIII-flanked promoters were then subcloned into the p43.2-GAA plasmid to yield pTR-DHBV-hGAA, pTR-Alb-hGAA, and pTR-hAAT-hGAA.

Recombinant AAV vector packaging

The rAAV2 and rAAV2 *rep*/5 *cap* (rAAV5) vectors were packaged as previously described (Zolotukhin *et al*., 1999, 2002). Total particle titer was determined by dot-blot method as described (Zolotukhin *et al*., 1999). The rAAV2 *rep*/8 *cap* (rAAV8) vector was generated by triple transfection of 293 cells with the following plasmids: the hGAA expression cassette flanked by the AAV2 ITRs (shown in Fig. 1B); the pXX6 plasmid containing the adenoviral helper genes; and the *rep-cap* expression construct (p5E18-VD2/8; gift of J.M. Wilson, University of Pennsylvania, Philadelphia, PA), which contains the AAV8 *cap* and AAV2 *rep* genes. Vectors were purified on an iodixanol gradient and Q-Sepharose column and tittered by dot-blot method as previously described (Zolotukhin *et al*., 1999, 2002). Titers were reported as vector genomes (VG) per milliliter.

Animals and rAAV delivery methods

The $GAA^{-/-}$ mouse model used in these studies was generated by a targeted disruption of exon 6 and is maintained on a mixed C57BL/6 × 129X1/SvJ background, as described previously (Raben *et al*., 1998). Age- and sex-matched C57BL/6 × 129X1/SvJ mice were used as controls. All mice were housed in the University of Florida (Gainesville, FL) specific pathogen-free (SPF) animal facility and all animal procedures were done in accordance with the University of Florida's Institutional Animal Care and Use Committee-approved guidelines. Nine- to 10-week-old female $GAA^{-/-}$ mice were anesthetized with 2% isoflurane and restrained supine on a warmed operating surface. A midline incision was made and, using a 0.5-cm^3 insulin syringe, 200 μl of rAAV-hGAA diluted in lactated Ringer's saline was injected into the portal vein. Pressure was applied to the portal vein with a cotton applicator to stop bleeding. The incision was closed and analgesics and antibiotics were delivered subcutaneously.

Pretreatment of $GAA^{-/-}$ mice

Recombinant human GAA (25 μg) purified from the medium of transfected CHO cells was diluted in 50 μl of 0.9% saline and injected subcutaneously into the scruff of the neck of 15-

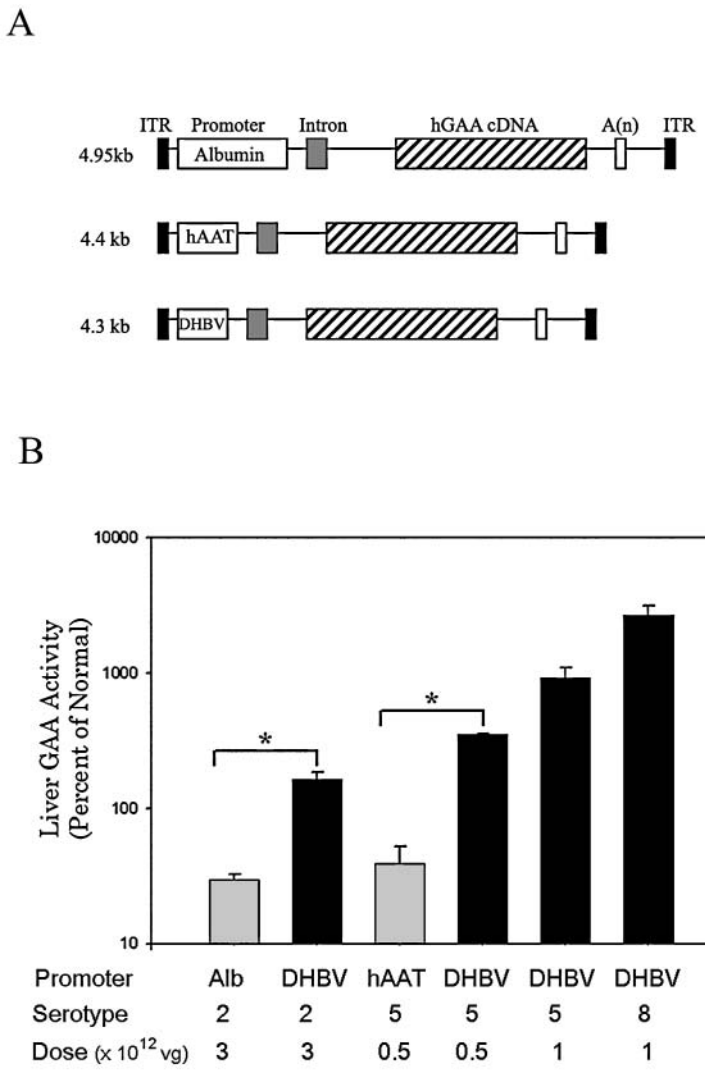

FIG. 1. *In vivo* evaluation of liver GAA expression levels from various promoters and rAAV serotypes. (**A**) Schematic of the rAAV-hGAA vector constructs. All constructs consist of the 145-bp AAV2 inverted terminal repeat (ITR) elements, 140-bp chimeric intron, 3.1-kb human GAA cDNA, poly(A) tail, and one of the three liver promoters: the 950-bp albumin promoter, the 400-bp human α_1-antitrypsin (hAAT) promoter, or the 300-bp duck hepatitis B virus (DHBV) core promoter. (**B**) Percentage of normal GAA activity levels in the liver after portal vein delivery of the vectors diagrammed in (**A**). An asterisk (*) indicates statistical significance ($p < 0.005$).

to 30-hr-old GAA$^{-/-}$ mice. Mice were immediately returned to the cage as a group.

Anti-GAA antibody ELISA

Serum samples were obtained weekly by tail vein bleeds of anesthetized animals.Microtiter plates were coated overnight at 4°C with 200 μl of human GAA (5.0 μg/ml) in 0.1 *M* $NaHCO_3$ (pH 8.2). Wells were washed three times with 300 μl of phosphate-buffered saline (PBS)–Tween 20 and blocked with 300 μl of 10% fetal bovine serum (FBS) for 2 hr at room temperature. Wells were washed again before adding samples. Serum samples were diluted from 1:100 to 1:10,000 in the blocking reagent and added to the wells in a total volume of 100 μl. Samples were incubated for 1 hr at room temperature. Washing was repeated and 100 μl of horseradish peroxidase (HRP)-linked anti-mouse IgG-antibody (from sheep; Amersham Biosciences, Piscataway, NJ) diluted 1:10,000 was added to the sample wells for 30 min at room temperature. After incubation, wells were washed again and 100 μl of tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO) was added to the wells for 1–3 min. The reaction was stopped with 100 μl of 1 *N* H_2SO_4 and absorbance was measured at 450 nm. Absorbance values were standardized to the dilution used. To control for assay variability, serial dilutions of rabbit anti-human GAA antibody were used to generate a standard curve. Absorbance values were converted to standardized units, using the equation for the standard curve. These values are reported as anti-GAA antibody titer fold over background with background equal to the standardized titer in serum from untreated GAA$^{-/-}$ mice.

Determination of anti-GAA isotypes

For the measurement of relative isotype levels of anti-GAA antibodies, the same enzyme-linked immunosorbent assay (ELISA) methods were performed with the following exceptions: after incubation of diluted serum on coated plates and washing, rabbit anti-mouse IgG1, IgG2a, or Ig2b (Zymed, South San Francisco, CA), diluted 1:10,000 in blocking reagent, was added to the wells for 30 min at room temperature. The remaining washing and detection steps were performed as described above. Absorbance values were standardized to the dilution and divided by background (measured in serum from untreated GAA$^{-/-}$ mice) to yield anti-GAA antibody titer (fold over background).

Tissue processing and enzymatic activity assay

Eight or 16 weeks postinjection, mice were killed and extracted tissues were immediately cut and frozen in liquid nitrogen. Tissues were homogenized with the FastPrep system (Qbiogene, Carlsbad, CA). Skeletal muscle tissue was first crushed to a powder with a hemostat in liquid nitrogen before homogenization. Homogenized lysates were subjected to three freeze–thaw cycles and centrifuged. Clarified lysate (20 μl) was used in the 4-methyl umbelliferyl β-D-glucuronide (4-MUG) cleavage assay for determination of GAA activity as described (Pauly *et al.*, 1998). Protein concentrations were determined with an *RC DC* protein assay kit (Bio-Rad, Hercules, CA) and standardized to serial dilutions of bovine serum albumin. GAA activity was calculated as nanomoles of 4-MU per hour per milligram of protein and represented as the percentage of GAA activity averaged from three age- and sex-matched C57B6/129 mice.

Immunoprecipitation and Western blot detection of serum GAA

Serum samples collected from tail bleeds of anesthetized mice were stored at −20°C. After thawing serum, 4 μg of purified goat anti-human GAA antibody was added to 100 μl of serum and 150 μl of Nonidet P-40 (NP-40) buffer (50 m*M* Tris-HCl [pH 8.0], 150 m*M* NaCl, and 1% NP-40) and incubated overnight at 4°C on a rotating platform. Protein G–agarose (Roche Diagnostics, Mannheim, Germany) was washed three times with NP-40 buffer and resuspended in an equal volume of buffer to generate 50% protein G. Fifty microliters of 50% protein G was added to the serum/anti-GAA antibody mixture and incubated for 4 hr at 4°C on an inverting platform. After centrifugation, the supernatant was removed and the pellet was washed with NP-40 buffer twice. The final pellets containing the protein G/goat anti-human GAA IgG/serum human GAA complexes were resuspended in an equal volume of 2× sodium dodecyl sulfate (SDS) loading buffer. Samples were heated to 100°C for 5 min and centrifuged. The total volume of supernatant was separated on an 8% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. After transfer, the membrane was blocked with 5% nonfat dry milk and washed with PBS–Tween 20. The membrane was probed with a 1:1000 dilution of rabbit anti-human GAA polyclonal serum for 1 hr at room temperature. Washing was repeated and the blot was probed with a 1:10,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences). After washing, hybridization was detected with the ECL Plus Western blotting detection system (Amersham Biosciences).

RNA isolation and analysis of human GAA transcript

RNA was isolated with an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions for RNA isolation for the respective tissue. The RNA was treated with DNase I (Ambion, Austin, TX) for 1 hr. First-strand cDNA synthesis was primed from 2.5 μg of DNase-treated RNA, using a first-strand cDNA synthesis kit (Amersham Biosciences) and 40 pmol of the reverse β-actin and hGAA primers described below. The cDNA product (1 μl) was used for PCR amplification of a 270-bp region of the human *GAA* cDNA, using the primer pair 5′-CCTTTCTACCTGGCGCTGGAGGAC-3′ and 5′-GGTGATAGCGGTGGAGGAGTA-3′. A separate PCR for amplification of a 300-bp region of the β-actin cDNA was performed under the same conditions, using the following primer pair: 5′-TCTAGGCACCAAGGTGTGAT-3′ and 5′-GTGGTACGACCAGAGGCATA-3′.

Histology

For liver histopathology, tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned (4 μm thick). Paraffin-embedded sections were stained with hematoxylin and counterstained with eosin and were examined and scored independently for histopathology by a veterinary pathologist at the University of Florida Pathology Core Laboratory. For histo-

logical assessment of glycogen, tissues were fixed overnight in 2% glutaraldehyde in PBS (TAAB Laboratories Equipment, Aldermaston, Berkshire, UK), embedded, sectioned (1 μm thick), and stained with periodic acid–Schiff (PAS) reagent (Richard-Allan Scientific, Kalamazoo, MI) according to the manufacturer's instructions. PAS-positive staining of glycogen on muscle cross-sections reveals distinct pink foci.

In vitro *force–frequency measurement*

In vitro contractile measurements were performed as described previously (Fraites *et al*., 2002). Briefly, the mouse was anesthetized with sodium pentobarbital. Soleus muscles were surgically excised and placed in a dissecting chamber with Krebs-Henseleit solution equilibrated with a 95% O_2/5% CO_2 gas mixture. The soleus muscle was suspended vertically between two lightweight Plexiglas clamps connected to force transducers (model FT03; Grass Instruments, West Warwick, RI) in a water-jacketed tissue bath containing Krebs-Henseleit solution equilibrated with a 95% O_2/5% CO_2 gas mixture (bath, 37°C, pH 7.4; osmolality, 290 mOsm). Transducer output was amplified and differentiated by operational amplifiers and underwent A/D conversion for analysis with a computer-based data acquisition system (Polyview; Grass Instruments). *In vitro* contractile measurements began with determination of the muscle's optimal length for isometric titanic tension development. The muscle was field-stimulated along its entire length with a stimulator (model S48; Grass Instruments) with platinum electrodes. Muscle length was progressively increased until maximal isometric twitch tension was obtained. Once the highest twitch force was achieved, all contractile properties were measured isometrically at optimal length. At the end of the study, the muscle strip length and weight were determined in order to calculate the normalized force generated.

Statistical analysis

An unpaired *t* test and unpaired *t* test with Welch's correction (InStat version 2.0; GraphPad Software, San Diego, CA) were used for analysis comparing the different groups, with statistical significance considered if $p < 0.05$.

RESULTS

Superphysiologic levels of hepatic hGAA expression achieved with the duck hepatitis B viral promoter

The limited packaging constraints of rAAV and the 3.1-kb size of the hGAA cDNA limit the size of a candidate promoter for these vectors to less than 1.0 kb. In addition, because of the inefficiency of human GAA secretion from transduced cells, the promoter must also have strong hepatocyte-enhanced expression capable of yielding 10- to 12-fold normal hepatic GAA levels (Raben *et al*., 2001). The 400-bp human α_1-antitrypsin (hAAT) promoter and the 950-bp murine albumin (Alb) promoter are two relatively small, commonly studied promoters for hepatocyte-enhanced expression of rAAV-delivered transgenes. The 300-bp duck hepatitis B virus (DHBV) core promoter has also been shown to have strong hepatic cell type specificity in tissue culture (Liu *et al*., 1991), yet has not been reported as a candidate promoter for gene delivery studies.

Two separate studies were performed to compare the DHBV promoter with the more commonly used albumin and hAAT promoters (Fig. 1A). In the first study, 3×10^{12} vector genomes (VG) of rAAV2-DHBV-hGAA or rAAV2-Alb-hGAA was delivered to the portal vein of 10-week-old $GAA^{-/-}$ mice (Fig. 1B). Hepatic hGAA expression from the DHBV promoter was 6-fold greater than that obtained with the albumin promoter (180 ± 21.7 versus 30 ± 3.4% of normal; $p = 0.003$). In a subsequent study comparing the DHBV promoter with the hAAT promoter cross-packaged in AAV5 capsids (rAAV5), hepatic GAA expression from the DHBV promoter was 8-fold greater after portal vein delivery of 5×10^{11} VG (324 ± 17.5 versus 40 ± 20% of normal; $p = 0.0015$). A 2-fold higher dose of the rAAV(5)-DHBV-hGAA vector yielded 3-fold higher hepatic-hGAA levels (926 ± 167% of normal; $p = 0.01$) (Fig. 1B), which is within the range Raben *et al*. proposed to be required for cross-correction of GSDII (Raben *et al*., 2001).

To determine whether liver GAA expression levels could be further optimized, we packaged the rAAV-DHBV-hGAA construct into AAV8 capsids and delivered 1×10^{12} VG to the portal vein of $GAA^{-/-}$ mice. Sixteen weeks postinjection, we observed 2676 ± 443% of normal GAA levels in the liver.

$GAA^{-/-}$ mice elicit a variable immune response to rAAV5- and rAAV8-derived human GAA

After analyzing GAA activity levels in distal tissues of naive $GAA^{-/-}$ mice receiving the rAAV2 or rAAV5 vectors described in Fig. 1, we observed the formation of anti-GAA antibodies and no increase in GAA levels in cardiac or skeletal muscles (not shown). Determining the reason for the lack of cross-correction was complicated, both because of the immune response and the relatively low liver GAA expression levels, which may not have been sufficient to achieve cross-correction even in the absence of an antibody response. In an effort to eliminate one of these variables, we attempted to induce human GAA-specific tolerance in $GAA^{-/-}$ mice by subcutaneously administering a low dose of inactive recombinant hGAA protein (rhGAA) to 1-day-old $GAA^{-/-}$ mice. In preliminary studies to test this method of tolerization, by subsequent challenge with rhGAA, we observed a 100-fold reduction in anti-GAA antibody titers to background levels in 100% of pretreated mice (not shown). Using this model, we next evaluated the ability of the rAAV5- and rAAV8-DHBV-hGAA vectors (1×10^{12} VG) to cross-correct affected heart, diaphragm, and hind-limb skeletal muscles after intrahepatic delivery.

In contrast to the success of neonatal pretreatment conferring tolerance to rhGAA protein challenge, rAAV-hGAA challenge in these mice resulted in tolerance in less than half of the treated mice. For these studies, immune tolerance was determined by the absence of an anti-GAA antibody response. Although this outcome was unexpected (and possibly due to structural, biochemical, or glycosylation differences between the directly infused rhGAA and the vector-produced and hepatic-secreted protein), it allowed us to analyze outcomes in both the presence and absence of an anti-GAA response.

In general, a higher percentage of mice elicited an immune response after rAAV5-hGAA treatment (40%) compared with

rAAV8-hGAA treatment (20%); however, this difference was not statistically significant ($p = 0.625$). Interestingly, of all the immune-responsive mice, anti-GAA antibody titers were both significantly higher and more rapidly detectable in rAAV8-hGAA-treated mice compared with rAAV5-treated mice (Fig. 2A). Specifically, animals that elicited an immune response to rAAV8-hGAA formed anti-GAA antibodies, beginning 2 weeks postinjection, that reached up to 100-fold background. Animals that elicited a response to rAAV5-hGAA formed antibodies, beginning 5 weeks postinjection, that peaked at only 10-fold over background.

The surprisingly high antibody titers in rAA8-treated mice led us to further evaluate the potential for a cell-mediated immune response to rAAV-derived hGAA in these mice. Hematoxylin and eosin (H&E)-stained liver sections were independently evaluated for inflammatory responses and revealed no signs of infiltration (data not shown). In addition, serum samples taken biweekly from treated mice were assayed for the presence of IgG1, IgG2b, and helper T cell type 1 (Th1)-indicating isotype IgG2a (Fields *et al*., 2000). We observed predominant levels of IgG1, followed by secondary levels of IgG2b and no detectable IgG2a (Fig. 2B) for the duration of the experiment, suggesting a Th2- and not a Th1-mediated response.

Anti-GAA antibodies inhibit cross-correction of distal tissues

To determine the effect of anti-GAA antibodies on the ability to cross-correct from liver-produced hGAA, we examined cardiac and skeletal muscles for restoration of GAA activity after rAAV5- or rAAV8-DHBV-hGAA delivery. A wide range of biochemical correction was observed in all examined tissues from both treatment groups. Overall, higher levels of correction were observed in all examined tissues of mice that did not elicit an immune response (Ab^-) compared with mice that did (Ab^+) (Fig. 3). All differences were statistically significant with the exception of the diaphragms of rAAV8-treated mice and quadriceps of rAAV5-treated mice. Cardiac correction in rAAV5- and rAAV8-treated mice was 12- and 15-fold greater, respectively, in Ab^- versus Ab^+ mice ($p < 0.001$), with Ab^+ mice having GAA activities below the proposed therapeutic threshold of $< 20\%$ of normal (Fig. 3A). GAA activity levels

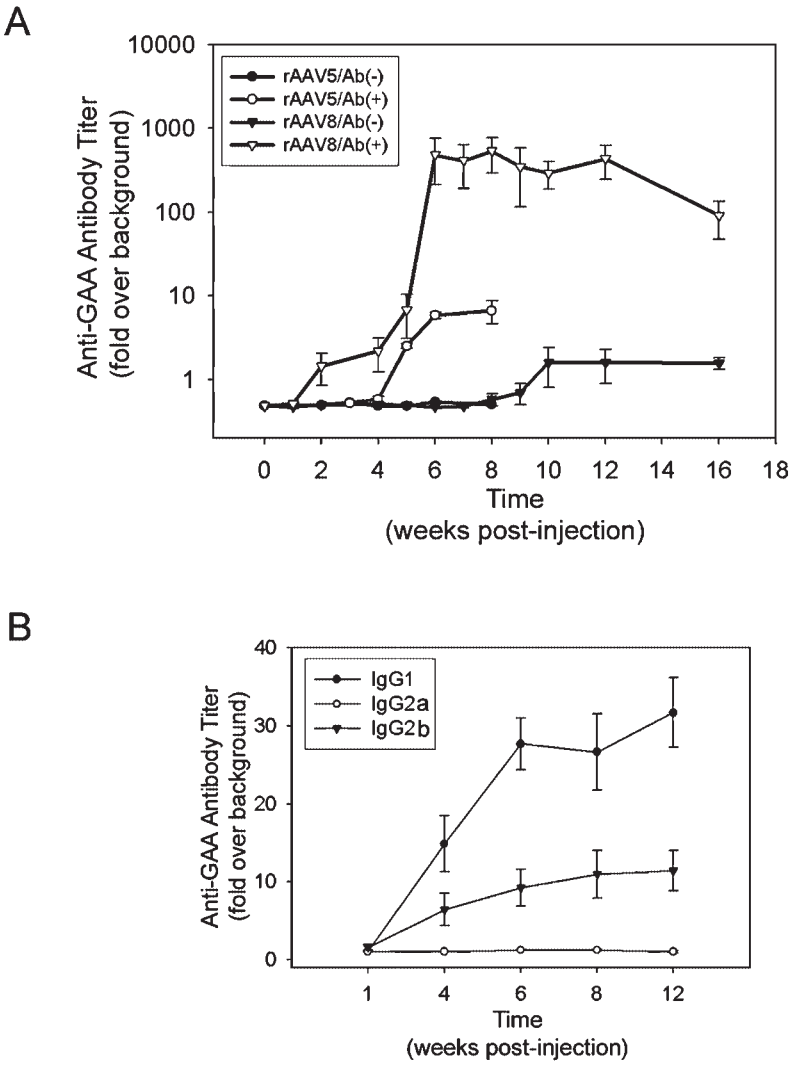

FIG. 2. Anti-GAA antibody response after intrahepatic delivery of 1×10^{12} VG of rAAV5- or rAAV8-DHBV-hGAA to $GAA^{-/-}$ mice. (**A**) Total IgG levels in rAAV5- and rAAV8-treated mice. Animals are divided into Ab^- and Ab^+ groups on the basis of the presence or absence of anti-GAA antibodies detectable by ELISA. For rAAV8-treated mice, $n = 4$ Ab^- mice and $n = 6$ Ab^+ mice. For rAAV5-treated mice, $n = 2$ Ab^- mice and $n = 8$ Ab^+ mice. (**B**) Levels of specific anti-GAA isotypes in rAAV8-treated mice as measured by ELISA. Error bars represent the standard error of the mean calculated for sample sizes ≤ 3.

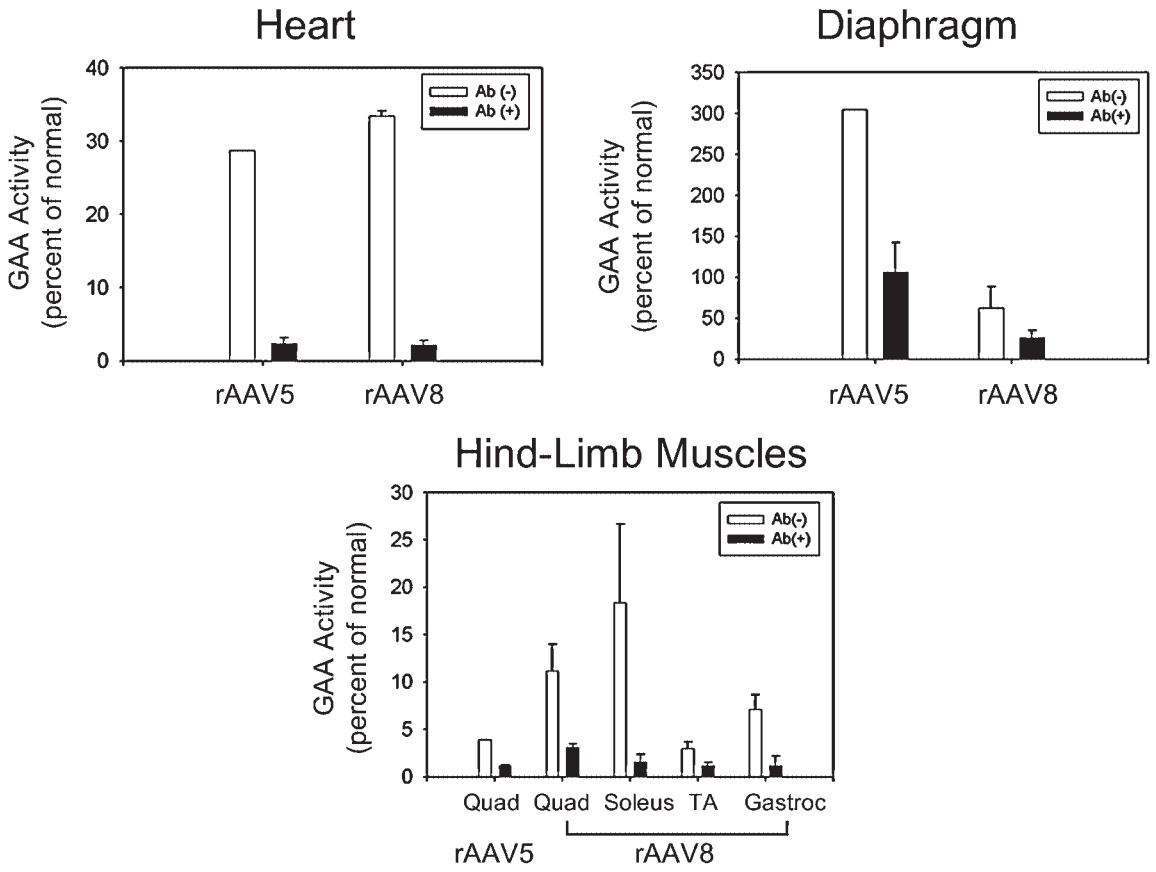

FIG. 3. Biochemical correction of affected tissues in $GAA^{-/-}$ mice after intrahepatic delivery of 1×10^{12} VG of rAAV5- or rAAV8-DHBV-hGAA. Animals are divided into Ab^- and Ab^+ groups on the basis of the presence or absence of anti-GAA antibodies. For rAAV8-treated mice, $n = 4$ Ab^- mice and $n = 6$ Ab^+ mice. For rAAV5-treated mice, $n = 2$ Ab^- mice and $n = 8$ Ab^+ mice. Differences between Ab^- and Ab^+ values are statistically significant ($p < 0.05$), with the exception of rAAV8 diaphragm and rAAV5 quadriceps. Error bars represent the standard error of the mean for sample sizes ≤ 3.

in the diaphragm were 3-fold greater in Ab^- versus Ab^+ mice after either rAAV5 or rAAV8 delivery (Fig. 3). However, even in the presence of an immune response, activity levels in the diaphragm were relatively high ($106 \pm 36.2\%$ for rAAV5 and $26 \pm 9.1\%$ for rAAV8).

The hind-limb skeletal muscles had lower levels of GAA activity than the heart and diaphragm, which is consistent with results reported for enzyme replacement therapy studies, and is likely due to lower mannose 6-phosphate receptor levels (Raben *et al*., 2003a). Among the rAAV5-treated mice, the quadriceps was the only muscle examined. Although quadriceps activity levels were 3-fold greater in Ab^- versus Ab^+ mice, this difference was not statistically significant, and even in Ab^- mice GAA levels were only 3% of normal.

After the rAAV5 studies, data were reported by Raben *et al*. (2003a) showing a relationship between fiber type and mannose 6-phosphate receptor density (and thus the ability to be cross-corrected). Therefore, we examined multiple muscle types in the subsequent rAAV8 studies; specifically the soleus, tibialis anterior (TA), gastrocnemius (gastroc), and quadriceps (quad). In all hind-limb muscles examined from this group, GAA activity levels in Ab^- mice were significantly higher than in Ab^+ mice ($p < 0.05$) (Fig. 3). The highest levels of activity were observed in the soleus of Ab^- mice, with values ranging from 14 to 26% of normal. The quadriceps and gastrocnemius had lower levels of activity restoration, with 11.2 ± 2.8 and $7.1 \pm 1.2\%$ normal, respectively (Fig. 3).

Interestingly, of the four leg muscles examined, the most easily corrected was the soleus, which has the highest proportion of type I fibers. The most difficult tissue to correct was the TA, which has the highest proportion of type II fibers. This relationship between fiber type and susceptibility to correction by exogenously supplied enzyme is consistent with findings of Raben *et al*. (2003a).

Presence of serum GAA correlates with immune response and cross-correction

To support the idea that GAA activity observed in the cardiac and skeletal muscle is derived from the circulating source of liver-produced GAA, serum from treated mice was analyzed for the presence or absence of GAA (Fig. 4). In rAAV8-treated mice that did elicit an immune response (Ab^+), serum GAA levels were undetectable, which is consistent with the absent to low levels of GAA activity in distal tissues. The presence of serum GAA and cardiac GAA in mouse 10 (marked as Ab^+)

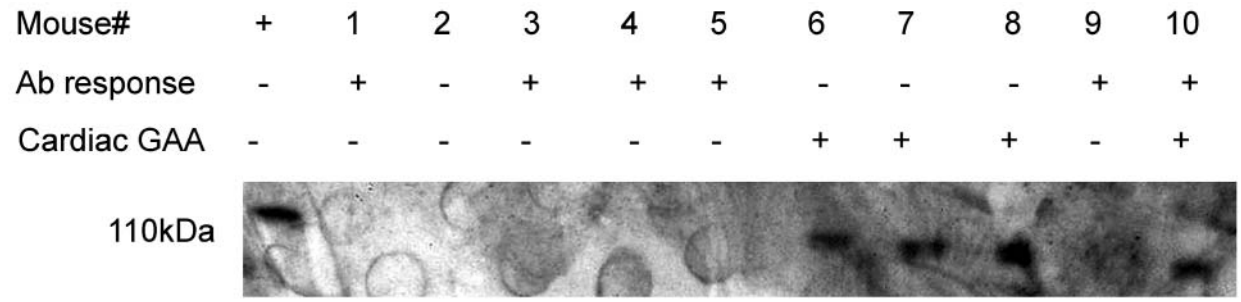

FIG. 4. Western blot detection of immunoprecipitated serum GAA in $GAA^{-/-}$ mice 16 weeks after intrahepatic delivery of 1×10^{12} VG of rAAV8-hGAA. The positive control sample (+) represents serum from a liver-overexpressing hGAA transgenic mouse (Raben *et al*., 2002). Ab response refers to the presence or absence of detectable anti-GAA antibodies by ELISA. Cardiac GAA refers to mice that have ≥20% of normal GAA activity in the heart.

was likely due to the delayed formation of antibodies in this mouse, 12 weeks postinjection. In addition, mouse 2 (which failed to have any cross-correction to the heart despite the absence of an immune response) had relatively low levels of liver GAA compared with the other Ab^- mice.

Serum collected from naive mice at earlier time points in the study were analyzed to determine whether GAA in the serum of Ab^+ mice was detectable before the inhibitory affects of antibody formation. No serum hGAA was detected at any time point in Ab^+ mice (not shown). This is not surprising, as antibodies were detected by 2 weeks postinjection, and liver expression levels between the onset of expression and this 2-week time point may not have been sufficient to produce detectable levels of serum GAA.

Human GAA was not detected in any serum samples in rAAV5-treated mice. This is also likely due to low to average

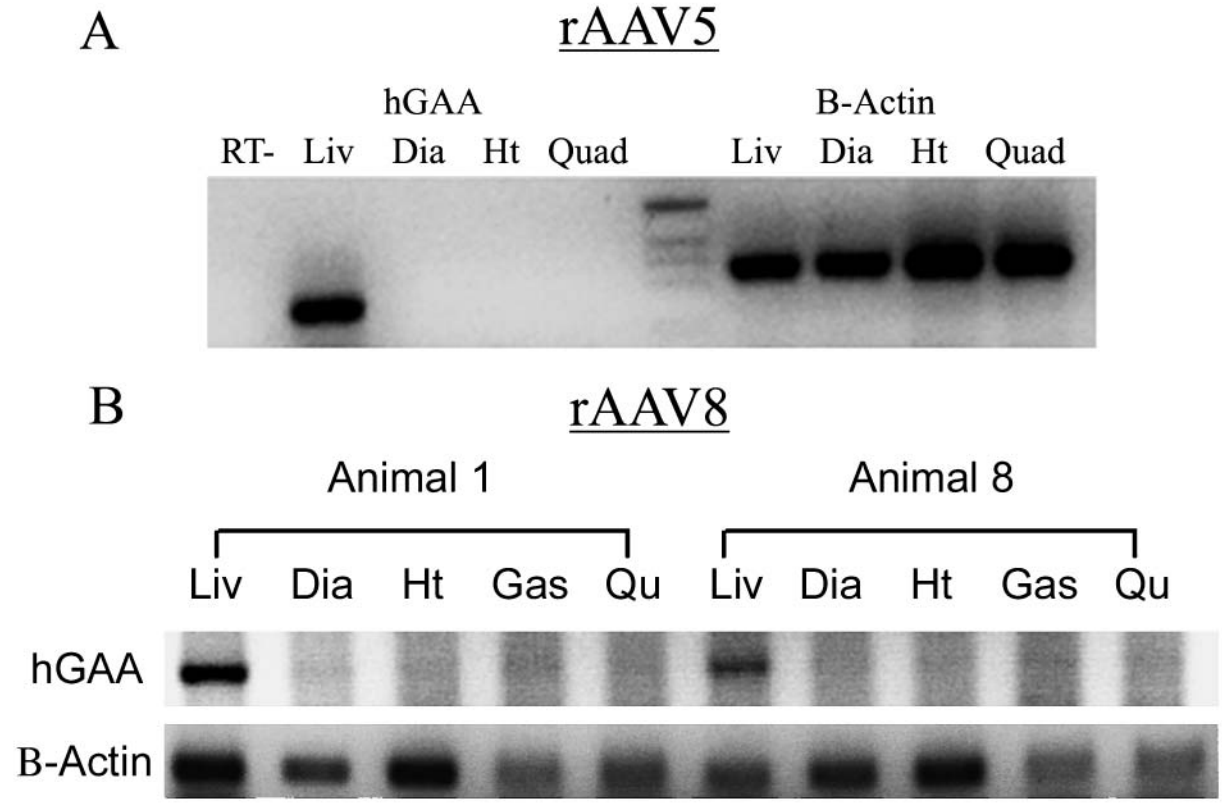

FIG. 5. RT-PCR of a 270-bp region of human GAA from rAAV5-hGAA-treated mice (**A**) and rAAV8-hGAA-treated mice (**B**). Results shown are representative of multiple animals from both Ab^- and Ab^+ groups. RT lanes represent RT-PCR of liver RNA in the absence of the reverse transcriptase enzyme. RT-PCR of mouse β-actin was performed as an internal control. Liv, liver; Dia, diaphragm; Ht, heart; Quad/Qu, quadriceps; Gas, gastrocnemius.

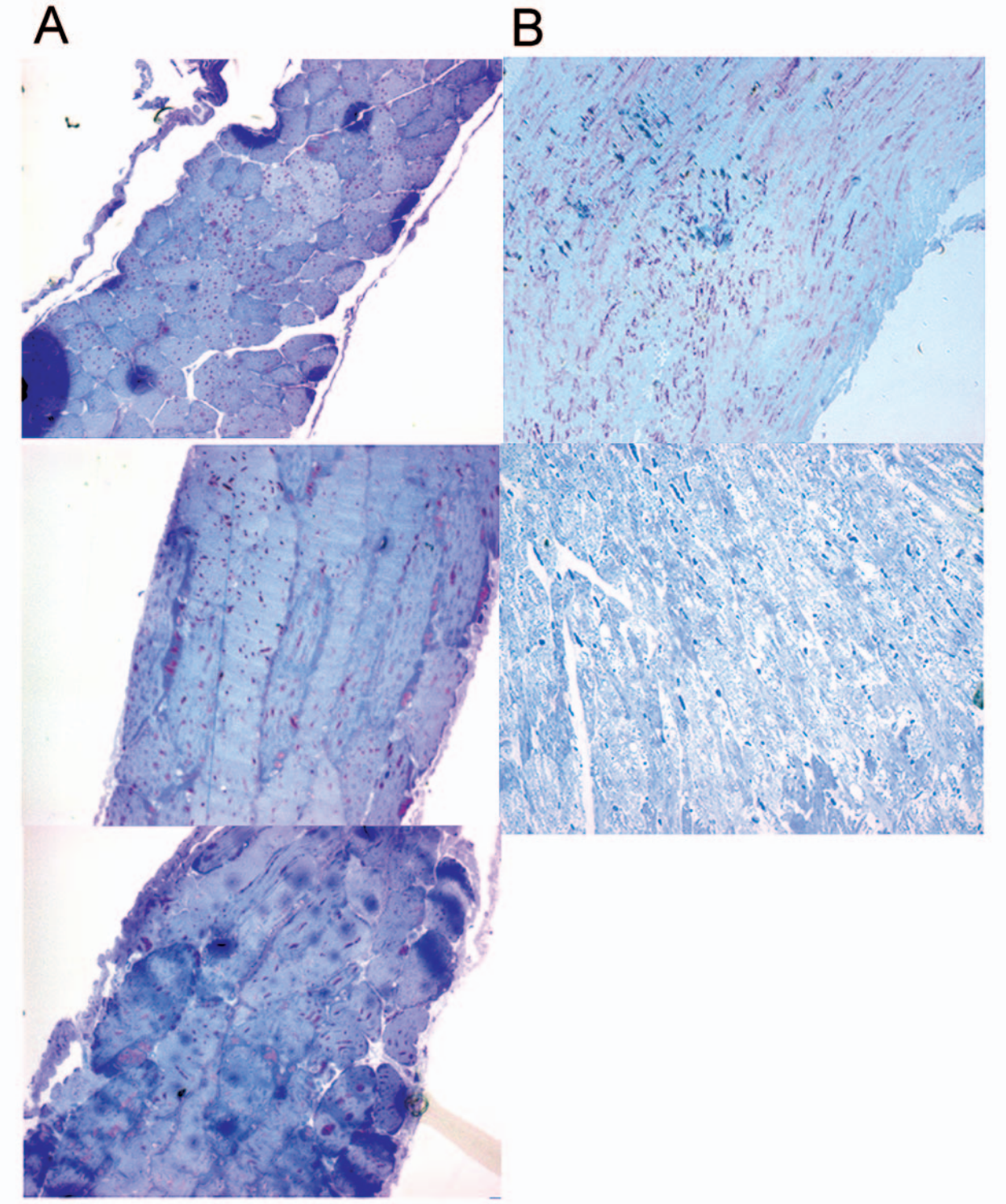

FIG. 6. Detection of glycogen by periodic acid–Schiff (PAS) staining of skeletal muscle (**A**) and cardiac muscle (**B**). (**A**) Diaphragm sections from an untreated age-matched $GAA^{-/-}$ mouse (*top*) and rAAV8-hGAA-treated $GAA^{-/-}$ mice with 40% of normal diaphragm GAA activity (*middle*) and 120% of normal diaphragm GAA activity (*bottom*). (**B**) Heart sections from an untreated, age-matched $GAA^{-/-}$ mouse (*top*) and an rAAV8-DHBV-hGAA treated $GAA^{-/-}$ mouse 16 weeks posttreatment with 33% of normal cardiac GAA activity (*bottom*). Original magnification: ×200 for diaphragm and ×400 for cardiac muscle. Pink staining represents glycogen.

levels of liver GAA expression in Ab^- mice from this group, which may not have been sufficient to yield detectable levels of serum GAA even in the absence of an immune response.

Human GAA protein detected in cardiac and skeletal muscles is hepatically derived

To confirm that the activity observed in cardiac and skeletal muscles is derived from liver-produced and -secreted hGAA, reverse transcription (RT)-PCR of hGAA was performed on liver, heart, diaphragm, and hind-limb skeletal muscles (gastrocnemius and quadriceps) (Fig. 5). In both rAAV5- and rAAV8-treated animals, hGAA transcript was detected in RNA isolated from the liver only. This is not surprising given the liver-specific properties of the DHBV promoter demonstrated *in vivo* by Liu *et al*. (1991).

Restored GAA activity results in partial to complete glycogen clearance in the heart and diaphragm

To determine the therapeutic effect of rAAV-delivered and liver-produced hGAA, heart and diaphragm sections (harvested 16 weeks after rAAV8-DHBV-hGAA delivery) were sectioned and stained for glycogen (Fig. 6). Complete glycogen clearance was observed in the heart with only 30–35% of normal GAA activity (Fig. 6B), whereas no glycogen clearance was observed in the Ab^+ mice, which expressed an average of 2% of normal GAA activity (data not shown). The more extreme variation in GAA activities in the diaphragm allowed us to evaluate the histologic correction with various degrees of activity. Immune-responsive mice with activity levels of 40–60% of normal had minimal glycogen clearance as shown by the representative sample. Mice with activity levels of 100–120% of normal had more obvious, yet not complete, clearance of glycogen in the diaphragm. This lower GAA activity requirement to clear accumulated glycogen in cardiac muscle, as compared with skeletal muscle, is consistent with studies done by Raben *et al*. (2002) in conditional, hGAA-expressing transgenic mice. Glycogen clearance was not detected in the hind-limb skeletal muscles examined (quadriceps, gastrocnemius, and tibialis anterior; data not shown). This was not surprising given the more stringent requirements for enzyme levels to clear skeletal muscle glycogen and the relatively low levels of enzyme activity observed in the hind-limb muscles. Understanding the specific level of GAA expression required to significantly reduce glycogen levels and the amount of glycogen clearance required to reverse the cardiac and respiratory pathology will depend on the duration of treatment and age at which treatment begins.

Partially restored GAA levels in soleus muscle improve contractile function

To determine whether rAAV8-mediated delivery of hGAA could lead to functional improvement, contractile function of the soleus muscle was measured 16 weeks after rAAV8-hGAA delivery. Fraites *et al*. (2002) were able to demonstrate a significant difference in soleus muscle function between normal and $GAA^{-/-}$ mice by this technique. Recombinant AAV8-treated $GAA^{-/-}$ mice showed significantly improved contractile function compared with untreated mice ($p < 0.05$) (Fig. 7). Surprisingly, there was a lack of correlation between force and measured GAA activity levels among treated mice, suggesting that unexpectedly low and/or transient levels of GAA (before antibody-mediated inhibition) are sufficient to improve soleus function, and that above this minimal threshold there is a loss of dose responsiveness.

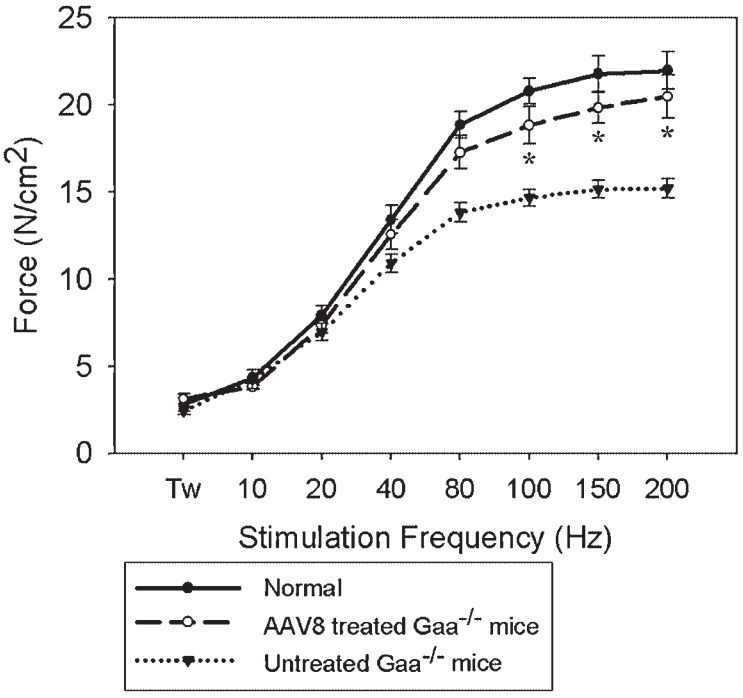

FIG. 7. Force–frequency relationship of intact soleus muscle 16 weeks after intrahepatic delivery of rAAV8-DHBV-hGAA. Four age-matched, C57BL/6/129 mice were used as normal controls. Four age-matched $GAA^{-/-}$ mice were used as untreated controls. An asterisk (*) represents statistical significance between treated and untreated mice ($p < 0.05$).

DISCUSSION

The goals of this study were to determine the optimal rAAV vectors for maximizing liver GAA expression levels and to evaluate the efficacy of these vectors in GSDII mice in both the presence and absence of a humoral immune response. We demonstrated superphysiologic levels of liver GAA activity from the DHBV promoter, using rAAV serotype 5, and were able to further increase these levels with rAAV serotype 8 to up to 47-fold over normal. However, mice from both rAAV5- and rAAV8-treated groups mounted a humoral response to the vector-derived protein and despite these extremely high levels of liver GAA expression, restoring GAA to the distal tissues was not possible in the presence of this immune response.

Overall, a smaller percentage of rAAV8-hGAA-treated mice elicited an immune response compared with mice treated with rAAV5-hGAA. However, the antibody titers observed in the rAAV8-treated mice were significantly higher. When the 10 individual mice from the rAAV8 group were analyzed, there was a clear relationship between liver expression levels and antibody response: mice with the lower levels of liver GAA expression had significantly higher anti-GAA antibody titers. Therefore, we speculate that tolerance induced by neonatal antigen exposure is maintained in the presence of higher levels of liver GAA expression (20- to 35-fold normal) whereas tolerance was not maintained in rAAV8-treated mice with lower levels of GAA expression (4- to 11-fold normal). In addition, the efficiency of antigen-presenting cell (APC) transduction by rAAV serotypes other than serotype 2 is not known. A potential increased advantage in APC transduction efficiency by rAAV8 vectors could explain the more robust immune response observed in immune-responsive mice from the rAAV8 group compared with the rAAV5 group. Our preliminary studies showed no evidence of a cell-mediated immune response to rAAV-derived hGAA; more in-depth immunologic assays would be required to answer these questions. Regardless of the reason for the enhanced immune response to rAAV8-derived hGAA, the impact of the lower antibody titers in rAAV5-treated mice and higher titer antibodies in rAAV8-treated mice was the same: significant inhibition of cross-correction.

To evaluate the efficacy of liver-directed, rAAV-mediated gene replacement, we examined the diaphragm, heart, and hindlimb muscles of treated mice and compared the activity levels of mice successfully tolerized to hGAA by neonatal pretreatment with those of mice that were not. Significantly higher levels of activity were observed in Ab^- mice than in Ab^+ mice. Even within these two groups, we observed various degrees of enzyme restoration among the different tissues. GAA activity levels were significantly increased in the hearts of Ab^- mice compared with Ab^+ mice from both rAAV5- and rAAV8-treated groups, with Ab^+ mice having only background levels of GAA activity. Restoring GAA activity levels to only 30% in the hearts of Ab^- mice led to complete glycogen clearance.

The highest levels of activity were observed in the diaphragm. This greater susceptibility to restoring GAA to the diaphragm than to other skeletal muscles or to the heart is possibly due to the high surface area and vascularity-to-mass ratio, allowing for both increased exposure to and maximal uptake of circulating GAA. Despite these high levels of diaphragm activity, glycogen clearance was only partially cleared (even at 100% of normal diaphragm GAA levels). The difficulty in achieving glycogen clearance in skeletal muscles (including the diaphragm) despite these high levels of GAA activity is consistent with studies by Raben *et al*. (2003a). The mechanism of increased resistance to glycogen clearance in skeletal muscle compared with cardiac muscle is unclear, but may be attributed to the structural and physiologic damage incurred on the cells before treatment. It is possible that this cellular damage may inhibit the processes necessary for functional GAA to efficiently clear glycogen, and extensive cellular remodeling may be required to restore normal functioning of the cell.

Although myopathies associated with cardiac and diaphragm muscles are the cause of death among GSDII patients, correction of the functional, antigravity muscles (including the quadriceps) would greatly improve the quality of life for these patients. Achieving significantly greater levels of activity in these muscles will likely require direct muscle transduction. However, our observation of improved contractile function in the soleus muscle with less than 20% of normal enzyme activity suggests that complete restoration of GAA is not necessary for functional improvement in some muscle groups.

These studies were not done to compare the rAAV5-hGAA vector with the rAAV8 vector (because of the difference in duration of treatments and the inability to accurately determine the infectious particle titers of these two vectors). However, it is interesting to note that the 2.7-fold increase in terminal liver GAA levels of rAAV8-treated mice compared with rAAV5-treated mice did not correlate with any significant increase in distal tissue activity. This suggests that there may be a maximal threshold for liver-derived and secreted GAA expression that was reached with the levels achieved with the rAAV5 vector, and that above this there is a loss of dose responsiveness. Alternatively, it is possible that the difference between the rAAV5 and rAAV8 liver expression levels observed in this study was insufficient to have an effect on cross-correction and that further optimizing expression levels will be necessary to improve cross-correction. In conclusion, we have demonstrated the significant impact of anti-GAA immunity on achieving liver-targeted cross-correction with rAAV vectors. Future studies will need to further analyze the reason for higher titer antibodies associated with rAAV8 delivery and determine whether the liver-directed, high-dose tolerance induction phenomenon observed in hemophilia (Mingozzi *et al*., 2003) and Fabry (Ziegler *et al*., 2004) disease models can be optimized for treatment of GSDII as well. Both of these studies demonstrated transgene product-specific tolerance by high-dose, liver-directed delivery of rAAV serotype 2 vectors. The success of this approach with other disease models will likely vary depending on the genetic (Mingozzi *et al*., 2003) and immunologic background of the mouse model along with the specific transgene and rAAV serotype used.

In addition to continuing to optimize liver-directed methods, we will also evaluate alternative delivery methods including peripheral intravenous delivery of muscle-specific or more ubiquitous promoters. If cross-correction is necessary to systemically treat GSDII, survival of the circulating GAA protein will be required. Developing immune-suppressive methods to avoid or inhibit the anti-GAA immune response (especially for patients with insufficient levels of GAA to confer tolerance) will be an important component for future clinical trials.

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