

Mucopolysaccharidosis I Cats Mount a Cytotoxic T Lymphocyte Response after Neonatal Gene Therapy That Can Be Blocked with CTLA4-Ig

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Although gene therapy has reduced manifestations of genetic diseases, immune responses can abrogate the effect. One approach to inducing tolerance is to perform gene transfer in newborns when the immune system is immature. We demonstrate here that the dose of retroviral vector (RV) is important in mice, as mucopolysaccharidosis I (MPS I) mice that received neonatal intravenous gene therapy with a high dose of a canine α -L-iduronidase (cIDUA)-expressing RV had stable expression, while those that received a low dose did not. It was unclear, however, if neonatal transfer with any dose could induce tolerance in large animals. Therefore, newborn MPS I cats were injected intravenously with the RV expressing cIDUA. Although this resulted in high serum IDUA activity due to secretion by transduced cells, expression fell due to a CTL response. Cats that transiently received the immunosuppressive agent CTLA4-Ig did not develop a CTL response. In contrast, MPS I dogs, which can respond immunologically to canine IDUA, had stable serum IDUA activity after neonatal gene therapy. We conclude that cats, but not dogs, mount a potent CTL response to canine IDUA after neonatal gene therapy, which can be prevented with transient CTLA4-Ig.

Key Words: gene therapy, lysosomal storage disease, retroviral vector, mucopolysaccharidosis, glycosaminoglycan, cytotoxic T lymphocytes, blocking immune response, neonatal, liver

INTRODUCTION

Mucopolysaccharidosis I (MPS I) is caused by deficient activity of α -L-iduronidase (IDUA; EC 3.2.1.76) [1], which results in the inability to degrade the glycosaminoglycans (GAGs) heparan and dermatan sulfates [1]. The incidence of MPS I is 1:100,000 live births [2]. Patients with severe disease (Hurler syndrome; OMIM 607014) have cardiac lesions, skeletal deformities, hearing and visual abnormalities, and mental retardation.

Treatments that are currently used for humans with MPS include hematopoietic stem cell transplantation [3] and enzyme replacement therapy (ERT) [4]. Gene therapy has also reduced clinical manifestations in animal models of MPS [5,6]. One approach involved intravenous (iv) injection of a viral vector expressing the appropriate enzyme, which resulted in efficient transduction of hepatocytes and variable transduction of other cell types. Transduced cells secreted mannose 6-phosphate (M6P)-

modified enzyme into blood, which was taken up by cells in other parts of the body via the M6P receptor [4]. The advantages of neonatal gene therapy include: (1) treatment is initiated early; (2) liver cells are replicating, which is important for vectors that transduce only dividing cells; and (3) the immune system is less mature in newborns than in adults. Indeed, neonatal administration of an AAV vector, lentiretroviral (lentiviral) vector, or γ retroviral vector (RV) resulted in long-term expression and reduced manifestations of disease in MPS I mice [7–9] and MPS VII mice (reviewed in [5,6]). In contrast, iv injection of lentiviral vector or γ RV into adult MPS I mice resulted in unstable expression [9,10] or expression that was low at a late time of evaluation [8]. This suggests that the immaturity of the newborn immune system was critical for achieving stable expression in mice after neonatal transduction. However, the dose of RV needed for tolerance induction was unclear.

Therefore, studies were performed in MPS I mice to define the effects of RV dose upon neonatal tolerance induction. These mice have an insertion in exon 6 of the 14-exon gene [11] and have mounted cytotoxic T lymphocyte (CTL) responses after gene therapy to adults [9,10].

Although it is encouraging that neonatal gene therapy reduced immune responses in mice, the mouse immune system is less mature at birth than that of other species such as human [12]. We previously reported that neonatal delivery of a canine β -glucuronidase (GUSB)-expressing RV to dogs with MPS VII resulted in transduction and expression in liver, spleen, and blood cells for up to 5 years and improved many manifestations [13,14]. However, these dogs have a missense mutation [15] and might be tolerized to most epitopes of GUSB. Testing immune responses in large animals with a greater chance of an immune response will be important prior to using gene therapy in humans with null mutations. In this study, immune responses after neonatal RV-mediated gene therapy with the canine *IDUA* cDNA were evaluated in cats and dogs with MPS I. MPS I cats have a 3-bp deletion in the feline *IDUA* gene, which results in loss of the aspartate analogous to D349 of human *IDUA* [16,17]. Since the canine and feline proteins have ~130 differences in 653 total amino acids, the canine protein could induce an immune response in MPS I cats. MPS I cats have developed antibody responses after ERT with human *IDUA* [18]. The MPS I dogs have very low *IDUA* activity due to a 5' splice site mutation in the first intron

and lack full-length mRNA [19–21]. MPS I dogs developed antibody responses after ERT with human or canine *IDUA* [22] and probably developed antibody and CTL responses after *ex vivo* transduction of hematopoietic stem cells (HSCs) with a canine *IDUA*-expressing RV in adults [23]. Thus, MPS I cats and dogs should be able to respond immunologically to canine *IDUA* and should be stringent models in which to assess the ability of neonatal gene therapy to induce tolerance in large animals.

RESULTS

Effects of RV Dose on Tolerance Induction in Mice

The amphotropic RV designed hAAT-cIDUA-WPRE contains the human α_1 -antitrypsin promoter, the canine *IDUA* cDNA, and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), as described previously [9]. We have reported that 100% ($N = 23$) of MPS I mice that received 10^9 transducing units (TU)/kg hAAT-cIDUA-WPRE had stable expression [9], but expression was stable in only 76% of mice ($N = 17$) that received 10^8 TU/kg. Mice that lost expression had low RV DNA copies and *IDUA* activity in liver and did not develop antibodies. These data are consistent with the hypothesis that a CTL response occurred in mice that lost expression and that lower doses of RV were less effective at inducing tolerance than higher doses.

To test this hypothesis, we gave additional MPS I mice neonatal gene therapy with varying doses of RV and

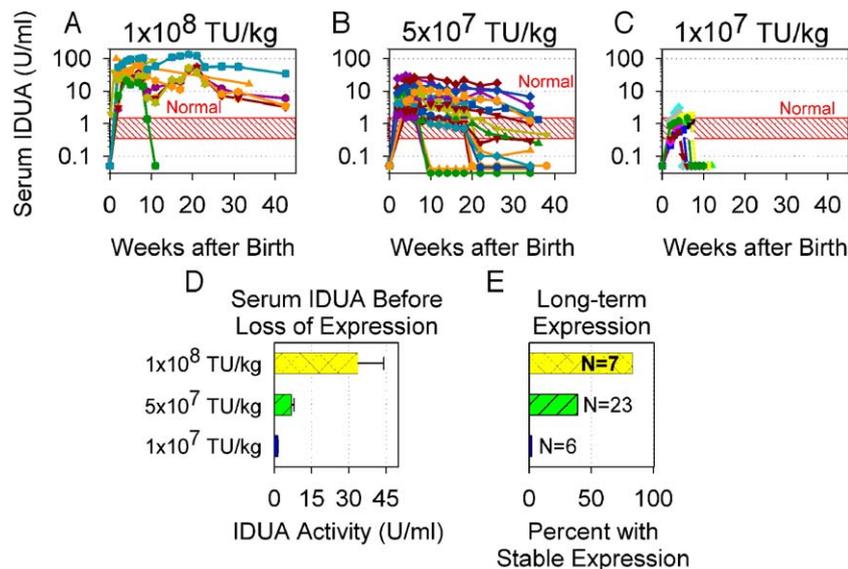


FIG. 1. Effects of RV dose on stability of expression in mice after neonatal gene therapy. MPS I mice were injected iv at 2 to 3 days after birth with different doses of hAAT-cIDUA-WPRE. (A–C) Serum IDUA activity. The serum IDUA activities at the indicated weeks after transduction with 1×10^8 (A), 5×10^7 (B), or 1×10^7 TU/kg (C) are shown. Each line represents an individual mouse. Homozygous normal mice have 0.9 ± 0.6 U/ml (average ± 2 standard deviations; $N = 5$) serum IDUA activity, as indicated by the striped box. Although untreated MPS I mice do not have detectable IDUA activity, samples without activity are shown as 0.05 U/ml on this semilog scale. (D) Average peak IDUA activity. The average serum IDUA activity \pm SEM prior to any loss of activity was determined for each group. (E) Percentage of mice with stable expression. The percentage of mice from each group that had stable expression over time is shown.

assessed the stability of serum IDUA activity, as shown in Figs. 1A, 1B, and 1C. Fig. 1D shows the average serum IDUA activity prior to loss of expression, and Fig. 1E shows the percentage of mice with stable expression. For mice that received 10^8 TU/kg of hAAT-cIDUA-WPRE, the average serum IDUA activity was 33.7 ± 10.4 (SEM) units (U)/ml, and 83% exhibited stable expression for up to 8 months ($N = 7$). For mice that received 5×10^7 TU/kg RV, the average serum IDUA activity was 7.0 ± 1.0 U/ml, and 39% had stable expression ($N = 23$). For mice that received 1×10^7 TU/kg, the average serum IDUA activity was 1.5 ± 0.2 U/ml, and all lost expression by 5 to 8 weeks ($N = 6$). These data are consistent with the hypothesis that higher doses of RV are more effective than lower doses at inducing tolerance to canine IDUA in mice.

Serum IDUA Activity Falls over Time in MPS I Cats After Neonatal Gene Therapy

Although the above data demonstrate that neonatal gene therapy can induce tolerance in mice if expression is sufficiently high, it was unclear if this would be effective in higher animals with more mature systems at birth. Therefore, we injected hAAT-cIDUA-WPRE iv into newborn MPS I cats. The group that received $1.3 \pm 0.3 \times 10^9$ TU/kg had high serum IDUA activity at 1 month (Fig. 2A), which averaged 286 ± 416 (SD) U/ml and ranged from 12 to 907 U/ml. These averages were higher than the serum IDUA activity in homozygous normal [26.8 ± 3.2 U/ml (SEM); $N = 7$] or homozygous-deficient MPS I (0.7 ± 0.2 U/ml; $N = 5$) cats. The cats (cat 7028 and cat 7034) that received

10^{10} TU/kg achieved IDUA activity at 1 month that was even higher at 2355 ± 2763 (SD), with values that ranged from 401 to 4308 U/ml. However, serum IDUA activity fell in all RV-treated cats to very low levels (3.7 ± 1.5 U/ml) at 1 to 3 months. Although RV-treated cats did not develop anti-canine IDUA antibodies (data not shown), they developed a CTL response directed against cells expressing canine IDUA, as will be shown below.

Human CTLA4-Ig is an immunosuppressive agent that can block immune responses in feline lymphocytes [24,25]. In an attempt to prolong expression, we gave two MPS I cats human CTLA4-Ig in addition to neonatal transfer of 10^{10} TU/kg of hAAT-cIDUA-WPRE. As diagrammed in Fig. 2B by the green bars, they received four doses of human CTLA4-Ig over 2 weeks starting on the day of gene transfer. Cat 6986 and cat 7031 achieved IDUA activity of 73 and 301 U/ml, respectively, in serum at 1 month after transduction, which is an average of 187 ± 161 U/ml (SD), and has been stable for 10 months. In contrast, two littermates of cat 7031 (cat 7028 and cat 7034; see Fig. 2A) that received the same batch and dose of RV without immunomodulation lost expression by 2 to 3 months. This suggests that short-term administration of CTLA4-Ig at the time of neonatal gene therapy can result in long-standing tolerance to canine IDUA.

Cytotoxic T Lymphocyte Assays

We performed CTL assays to test the hypothesis that the loss of serum IDUA activity in RV-treated MPS I cats was

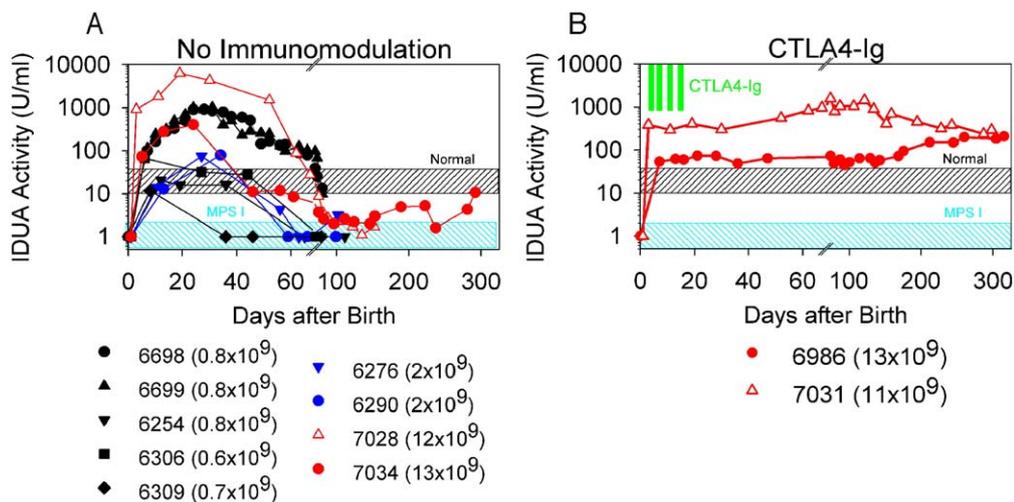


FIG. 2. Serum IDUA activity in MPS I cats after neonatal transduction. MPS I cats were injected iv with a low dose (doses of 0.6 to 0.8×10^9 TU/kg are shown as closed black symbols, doses of 2×10^9 TU/kg as blue symbols) or a high dose (12×10^9 to 13×10^9 TU/kg; shown as red symbols) of hAAT-cIDUA-WPRE at 2 to 5 days after birth. Serum IDUA activity was determined at the indicated days after birth. The average serum IDUA activity ± 2 standard deviations in homozygous normal cats (Normal; 26.4 ± 15.4 U/ml; $N = 7$; black-hatched box) and in homozygous-deficient MPS I cats (MPS I; 0.7 ± 0.9 U/ml; $N = 5$; blue-hatched box) is shown. (A) RV without immunomodulation. Serum IDUA activity in cats that received neonatal RV without immunosuppression. (B) RV/CTLA4-Ig. Serum IDUA activity in cats that received neonatal RV and immunosuppression with human CTLA4-Ig. The green bars at the top indicate the days after birth of CTLA4-Ig administration. Cat 6986 received 25 mg/kg CTLA4-Ig for each dose. Cat 7031 received 25 mg/kg for the first two doses, and lower doses for doses 3 and 4 as detailed under Materials and Methods.

due to a CTL response. Lymphocytes from naive cats that did not receive gene therapy had no specific killing of autologous canine IDUA-expressing fibroblasts, as shown in Figs. 3A and 3B and as summarized at an effector:target ratio (E:T) of 20:1 in Table 1. We collected lymphocytes at 3 months of age from the two cats that received high-dose neonatal RV without immunomodulation (cat 7028 and cat 7034), at which time serum IDUA activity had fallen to

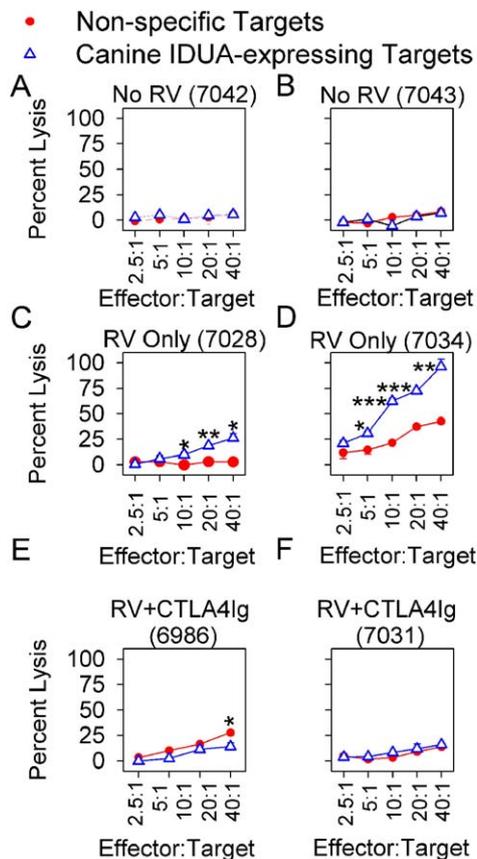


FIG. 3. CTL assays in cats. Lymphocytes from blood were stimulated *in vitro* for 6 days with autologous canine IDUA-expressing fibroblasts. Stimulated lymphocytes were then incubated at the indicated effector (lymphocyte) to target (fibroblast) (E:T) ratio with ^{51}Cr -labeled autologous fibroblasts that did not express canine IDUA (nonspecific targets) or with ^{51}Cr -labeled autologous fibroblasts that did express canine IDUA (canine IDUA-expressing targets). The percentage lysis was plotted as the average of triplicate samples \pm SEM. Statistical comparisons were performed between values for nonspecific targets and canine IDUA-expressing targets at each E:T ratio using the Student *t* test; **P* = 0.005 to 0.05, ***P* = 0.0005 to 0.005, and ****P* < 0.0005. (A and B) CTL response in naive cats. Lymphocytes were obtained at 5 months of age from normal cats that did not receive gene transfer. There were no significant differences in the lysis of nonspecific targets and canine IDUA-expressing targets at any E:T ratio. (C and D) CTL response after RV treatment without immunomodulation. Lymphocytes were obtained at 3 months of age from cat 7028 and cat 7034, which received neonatal gene therapy without immunosuppression. (E and F) CTL response after RV/CTLA4-Ig treatment. Lymphocytes were obtained at 5 and 3 months of age, respectively, from cat 6986 and cat 7031, which received neonatal gene therapy and were immunosuppressed with CTLA4-Ig, as detailed in Fig. 2B.

very low levels (Fig. 2A). Lymphocytes from cat 7028 lysed $19 \pm 2\%$ of canine IDUA-expressing autologous fibroblasts at an E:T ratio of 20:1, which was sixfold the lysis of the nonspecific autologous targets of $3 \pm 1\%$ (*P* = 0.003 for specific vs. nonspecific lysis), as summarized in Table 1. There was also specific lysis at E:T ratios of 10:1 and 40:1, as shown in Fig. 3C. Similarly, lymphocytes from cat 7034 lysed $73 \pm 2\%$ of specific targets at an E:T ratio of 20:1, which was higher than the value of $37 \pm 2\%$ for nonspecific targets (*P* < 0.0001 for specific vs. nonspecific lysis), as shown in Table 1. There was also specific lysis of targets at an E:T ratio of 5:1, 10:1, and 40:1, as shown in Fig. 3D. Lymphocytes isolated from these RV-treated cats at 5 months of age were also able to lyse specifically canine IDUA-expressing autologous fibroblasts, as summarized in Table 1. The variation in the percentage lysis of specific targets between the two assays was likely due to technical issues. These data demonstrate that canine IDUA-specific CTLs were present in cats that received neonatal gene therapy without immunomodulation.

We also performed CTL assays with lymphocytes from the RV/CTLA4-Ig-treated cats that exhibited the stable expression shown in Fig. 2B. For cat 6986 (Fig. 3E and Table 1), the killing of autologous canine IDUA-expressing fibroblasts by lymphocytes isolated at 3.5 months at an E:T ratio of 20:1 was actually less at $14 \pm 1\%$ than the killing of nonspecific targets ($18 \pm 1\%$; *P* = 0.022), although there were no differences between the groups at other E:T ratios. Similarly, the lysis of specific targets by lymphocytes isolated at 5 months of age from cat 6986 at an E:T ratio of 20:1 was less than the lysis of nonspecific targets (Table 1), although this difference was not significant. Again, there were no differences between the two groups at other E:T ratios (data not shown). For the other RV/CTLA4-Ig-treated cat (cat 7031), there were no significant differences at either time of evaluation between the lysis of specific and nonspecific targets at an effector:target ratio of 20:1 (Table 1) or other ratios (Fig. 3F and data not shown). These data suggest that neither of the RV/CTLA4-Ig-treated cats developed a CTL response. The apparent reduction in lysis of specific targets by cat 6986 at 3.5 months is intriguing and could be consistent with the presence of suppressor T cells. However, studies will need to be performed in additional cats to see if this occurs in other animals.

Liver IDUA Activity

The liver is an important site of expression in mice and dogs that receive neonatal gene therapy with an RV [9,13]. Since a CTL response should eradicate expressing cells, we analyzed livers obtained from biopsies of hAAT-cIDUA-WPRE-transduced cats for IDUA activity, as shown in Fig. 4A. The RV/CTLA4-Ig-treated cats had 107.0 ± 16.5 (SD) U/mg, which was 5-fold the value of 21.5 ± 2.1 U/mg found in homozygous normal cats and 191-fold the value of 0.6 ± 0.1 U/mg found in untreated

TABLE 1: Summary of CTL responses in cats

| Treatment | Cat | Age (months) | E:T ratio | Specific lysis | Nonspecific lysis | P value |
|-----------------|------|--------------|-----------|----------------|-------------------|---------|
| Naive | 7042 | 5 | 20:1 | 4 ± 1 | 3 ± 6 | 0.738 |
| | 7043 | 5 | 20:1 | 3 ± 0.2 | 5 ± 2 | 0.514 |
| RV only | 7028 | 3 | 20:1 | 19 ± 2 | 3 ± 1 | 0.003 |
| | | 5 | 20:1 | 45 ± 5 | 1 ± 1 | 0.011 |
| | 7034 | 3 | 20:1 | 73 ± 2 | 37 ± 2 | <0.001 |
| | | 5 | 20:1 | 18 ± 0.3 | 11 ± 0.2 | <0.001 |
| RV and CTLA4-Ig | 6986 | 3.5 | 20:1 | 14 ± 1 | 18 ± 1 | 0.022 |
| | | 5 | 20:1 | 14 ± 4 | 28 ± 3 | 0.101 |
| | 7031 | 3 | 20:1 | 12 ± 5 | 9 ± 1 | 0.575 |
| | | 5 | 20:1 | 7 ± 1 | 11 ± 2 | 0.089 |

Cats were normal cats that did not receive gene therapy (naive), MPS I cats that received neonatal RV without immunosuppression as described in Fig. 2A (RV only), or MPS I cats that received neonatal RV with CTLA4-Ig as described in Fig. 2B (RV and CTLA4-Ig). PBMC were obtained at the indicated months after birth, and the lysis of autologous specific targets that expressed canine IDUA or nonspecific autologous targets that did not express IDUA was determined at an E:T ratio of 20:1, which was a ratio that generally gave a robust response when one occurred. All assays were performed in triplicate, and averages ± SEM are shown. The P value for comparison of the lysis of specific and nonspecific targets at an E:T ratio of 20:1 is shown.

MPS I cats. In contrast, RV-treated cats that did not receive CTLA4-Ig had very low liver IDUA activity at 1 ± 0.5 U/mg.

Liver β -hexosaminidase Activity and GAG Levels

We also evaluated livers for biochemical evidence of correction of lysosomal storage. MPS I results in an elevation in the activity of other lysosomal enzymes and in sulfated GAGs, and normalization of these elevations by effective treatment correlates with reduction in lysosomal storage. As shown in Figs. 4B and 4C, untreated MPS I cats had 3- and 32-fold higher levels of total β -hexosaminidase (β -hex) activity and GAG in the liver than normal cats ($P < 0.01$ with ANOVA for MPS I vs. normal). β -Hex activity and GAG levels were normalized in RV/CTLA4-Ig-treated cats ($P < 0.01$ for RV/CTLA4-Ig vs. MPS I), but were not significantly reduced in RV-treated cats that did not receive CTLA4-Ig.

Vector DNA and RNA Levels in Liver

We also evaluated livers of MPS I cats that received neonatal gene therapy for RV DNA and RNA sequences, as a CTL response would be expected to result in the loss of vector copies. Fig. 4D shows that RV/CTLA4-Ig-treated cats had 6.9 ± 3.1 copies/100 cells of RV DNA. Somewhat surprisingly, cats that received high-dose RV without immunosuppression had substantial amounts of RV DNA in the liver at 0.66 ± 0.04 copies/100 cells (9% of the value in RV/CTLA4-Ig-treated cats) despite the fact that their expression had fallen to very low levels. However, RV RNA levels were very low, as RV-treated cats that did not receive immunosuppression had $0.035 \pm 0.005\%$ as many copies of RV RNA as the RV/CTLA4-Ig-treated cats after normalization to the β -actin signal, as shown in Fig. 4E. Thus, the amount of RNA per copy of DNA was 300-fold higher for RV/CTLA4-Ig-treated cats than for cats that received RV

without immunosuppression. This suggests that cells with high expression of canine IDUA were selectively destroyed by a CTL response in cats that did not receive immunosuppression, while cells with silent or near-silent integrations survived.

Mps I Dogs Have Stable Expression of IDUA After Neonatal Gene Therapy

We also evaluated the stability of expression of canine IDUA in MPS I dogs after neonatal gene therapy. We injected MPS I dogs iv with 3 to 10×10^9 TU/kg hAAT-cIDUA-WPRE at 2 or 3 days after birth. All six dogs exhibited stable serum IDUA activity for the duration of evaluation (3 to 16 months) at levels that varied from 25 to 1300 U/ml, as shown in Fig. 5. We conclude that dogs do not appear to develop a CTL response to canine IDUA after neonatal iv injection of an RV.

DISCUSSION

Induction of Tolerance in Mice Is Potentiated by High Expression

Identification of the dose of RV needed to induce tolerance will be important for designing gene therapy experiments. The data presented here suggest that high doses of RV are better at inducing tolerance than low doses in MPS I mice. Our data are consistent with the observation that high doses of allogeneic cells or infectious virus in the neonatal period are more effective at inducing tolerance in mice than lower doses of the same reagent [26–30]. In this study, we did not formally demonstrate that a CTL response to canine IDUA-expressing cells occurred in MPS I mice that lost expression after neonatal transduction. However, in our previous study, animals that lost expression after neonatal transduction had very low IDUA activity and RV DNA copies in liver and did not develop anti-canine

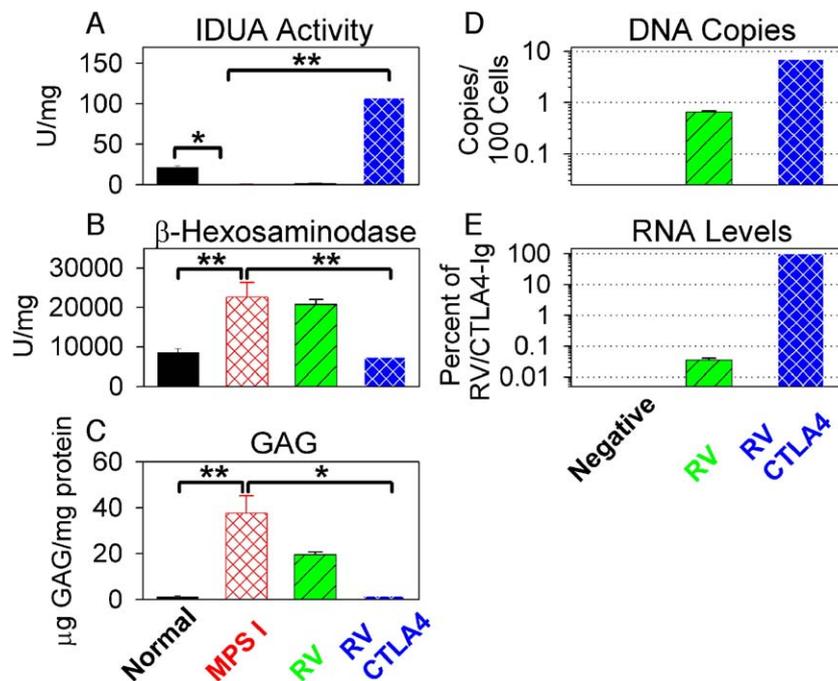


FIG. 4. Evaluation of livers from transduced cats. MPS I cats were transduced with high-dose RV without immunosuppression (RV; cat 7028 and cat 7034; see Fig. 2A) or with high-dose RV with CTLA4-Ig immunosuppression (RV CTLA4; cat 6986 and cat 7031; see Fig. 2B). Livers were obtained at 4 to 6 months after transduction by biopsy. Livers were also obtained from homozygous normal (Normal; $N = 4$) and untreated MPS I (MPS I; $N = 4$) cats. (A) IDUA activity. Samples from each cat were assayed in duplicate, and the average values for all cats of each group were averaged to give the IDUA activity in $U/mg \pm SD$. Statistical comparisons were performed with ANOVA with Tukey post hoc analysis. Values in untreated MPS I cats were compared with values in the other groups. $*P = 0.01$ to 0.05 and $**P < 0.01$, and the brackets indicate the two groups that were being compared. (B) β -Hexosaminidase. The liver β -hex activity in U/mg was determined and statistical analyses performed as in (A). (C) GAG levels. The liver GAG levels in μg GAG/mg protein were determined and statistical comparisons performed as in (A). (D) Liver RV DNA copy number. DNA was isolated from liver, and real-time PCR was performed in duplicate to give the average value for each cat. Average values for all cats in each group were averaged to give the RV DNA copy number $\pm SD$. Negative indicates nontransduced cats (one normal and one MPS I cat), which did not have a detectable signal. (E) Liver RV RNA levels. RNA was isolated from liver, and real-time RT-PCR was performed. The cycle number to reach the threshold (C_T) for the WPRE and the C_T for the β -actin RNA was used to estimate that the amount of RV RNA was $\sim 12.5\%$ the level of β -actin mRNA for RV/CTLA4-Ig-treated cats. No signal was obtained for RNA from RV/CTLA4-Ig-treated cats without RT treatment (not shown) or for RT-treated RNA from normal or MPS I cats that did not receive gene transfer (Negative). The values in the RV-treated cats that did not receive immunosuppression are shown as the average relative to that found in RV/CTLA4-Ig-treated cats with stable expression $\pm SD$.

IDUA antibodies [9]. Therefore, it is likely that a CTL response resulted in the loss of expression.

Mps I Cats but Not Dogs Develop a CTL Response to Canine Idua after Neonatal Gene Therapy

We demonstrate here that MPS I cats that received neonatal gene therapy with 5×10^8 to 1×10^{10} TU/kg of a canine IDUA-expressing RV achieved high IDUA activity in serum at 1 month after transduction. However, serum IDUA activity consistently fell to very low levels by 2 to 3 months ($N = 9$). This fall was not due to an antibody response, as serum was negative for anti-canine IDUA IgG antibodies (data not shown). Evidence that this decline was due to a CTL response includes: (1) lymphocytes from peripheral blood of transduced cats that lost expression could specifically lyse autologous fibroblasts expressing canine IDUA and (2) IDUA activity and RV RNA sequences were very low at 4 to 6 months after

transduction in livers of transduced cats that lost expression. The presence of substantial copies of DNA at late times in livers of cats that lost expression is likely due to silent or near-silent integrations. These data suggest that the cat has a relatively mature immune system at birth and may be a valuable model for testing gene therapy approaches in newborns. Although little is known about the immune system of the cat, cats are slightly closer phylogenetically to humans than dogs are to humans and are substantially closer to humans than rodents are to humans [31].

In contrast to the results in cats, neonatal iv injection of RV resulted in stable serum IDUA activity in MPS I dogs. Our results in MPS I dogs differ from the unsuccessful *ex vivo* attempts at HSC-directed gene therapy with a canine IDUA-expressing RV in adult MPS I dogs [23]. In this previous study, a CTL response was likely, as there was no expression in transduced blood cells, and

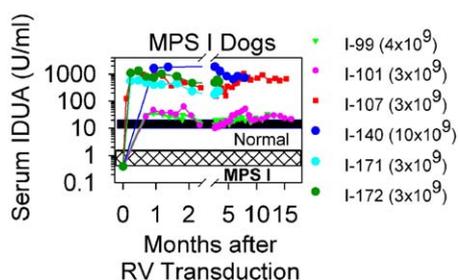


FIG. 5. Serum IDUA activity in RV-treated MPS I dogs. MPS I dogs were injected iv with 3 to 10×10^9 TU/kg hAAT-cIDUA-WPRE. Serum IDUA activity in U/ml was determined at the indicated months after transduction. The average values ± 2 standard deviations in homozygous normal dogs (17 ± 6.4 U/ml) and homozygous MPS I dogs (1.2 ± 0.8 U/ml) are shown.

lymphocytes proliferated *in vitro* in response to canine IDUA-expressing cells. In addition, transduced dogs developed anti-canine IDUA antibodies. Therefore, we believe that neonatal gene therapy was successful in MPS I dogs in our study due to the immaturity of the canine immune system at birth rather than the presence of tolerance in dogs to canine IDUA. This is the first report of stable expression of IDUA in the canine MPS I model. Midgestation *in utero* injection of canine IDUA-transduced allogeneic HSC [32] or an RV encoding the human IDUA cDNA [33] failed to result in expression in adults, which was attributed to low transduction efficiency and/or low expression.

Since CTL responses can occur in mice that receive neonatal gene therapy but have low levels of expression, it is possible that RV-treated MPS I cats developed a CTL response because expression was low. However, the average peak serum IDUA activity in the RV-transduced cats was 286 ± 416 (SD) U/ml for the low-dose (10^9 TU/kg) RV group and 2355 ± 2763 (SD) U/ml for the high-dose (10^{10} TU/kg) RV group. Both of these values are considerably higher than the level of 33.7 ± 10.4 U/ml found in the mice that received 10^8 TU/kg of RV, of which 83% had stable expression. Similarly, this level of expression in RV-treated cats is similar to the expression of 580 ± 216 U/ml observed in MPS I dogs that received neonatal gene therapy and maintained stable expression. Therefore, we doubt that MPS I cats developed a CTL response because expression was too low, although it is possible that the threshold for inducing tolerance varies among species.

CTLA4-IG Prolongs Expression After Neonatal Gene Therapy in MPS I Cats

It is intriguing and exciting that short-term (2 weeks) administration of CTLA4-Ig prevented a CTL response to canine IDUA-expressing cells after neonatal gene therapy and allowed expression to be stable for over 10 months. CTLA4-Ig is a fusion protein between CTLA4 and IgG, which can reduce immune responses by binding to CD80

or CD86 (CD80/86) and preventing them from interacting with CD28 [34]. CD80/86 are induced in B cells, dendritic cells, and macrophages during inflammatory responses, while CD28 is constitutively present on T cells. It is likely that the immune system of newborn cats is still relatively immature and that blockade of CD80/86:CD28 costimulation at this age may synergize with deficiencies in immune responses in newborns. Although little is known about the cat immune system at birth, newborn humans and/or mice have reduced secretion of cytokines by lymphocytes [35–37], have fewer dendritic cells [29], have reduced responses of dendritic cell precursors to cytokines [38], and have low levels of another costimulatory molecule, CD40 ligand [39,40]. In contrast to the deficiency of other molecules important for an immune response, neonatal human T cells have normal or increased levels of CD80/86 and CD28 [41]. As some have reported that neonatal tolerance involves clonal deletion [42,43], which can occur when cells encounter antigen in the absence of co-stimulation [44], it is possible that CTLA4-Ig induces tolerance in this model by clonal deletion. Future studies will attempt to define the mechanism of induction of tolerance in cats by evaluating lymphocyte responses *in vitro* and will evaluate if the RV/CTLA4-Ig-treated cats are improved clinically. Additional studies will also test if administration of gene therapy with CTLA4-Ig will result in long-term expression in older cats. Finally, we plan to evaluate the ability of transient cyclosporin and azathioprine to induce tolerance, as this prevented antibody formation in response to human IDUA during ERT in adult MPS I dogs [45].

Development of infectious diseases could be an adverse effect of an immunosuppressive agent such as CTLA4-Ig. Both CTLA4-Ig-treated cats were vaccinated with a killed parvovirus vaccine at 6, 9, and 12 weeks after birth. When these cats were rechallenged at 8 to 9 months of age, there was a 16-fold increase in their anti-parvovirus titer, which indicates an effective immune response. In addition, these cats did not develop overt infections at any time. These data suggest that the transient administration of CTLA4-Ig did not result in prolonged generalized immunosuppression.

Implications for Patients With MPS I

A high percentage of patients with severe MPS I (Hurler syndrome) have null mutations such as W402X and Q70X [46] and will have a high risk of developing immune responses after gene therapy. The studies presented here suggest that neonatal tolerance is not effective in all species, as cats developed a potent CTL response to canine IDUA after neonatal gene therapy. The results in cats may be more predictive of what will occur in humans, who have the ability to mount immune responses at birth, albeit inefficiently. Therefore, this model may prove to be very valuable for testing

approaches to block immune responses in this setting. It is very encouraging that short-term administration of CTLA4-Ig was effective at preventing an immune response in cats. Future studies will evaluate the immune response to other proteins in cats and will test if neonatal gene therapy with or without CTLA4-Ig can induce tolerance in primates.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated. The amphotropic RV hAAT-cIDUA-WPRE was prepared as described previously [9]. Human CTLA4-Ig was generously provided by Bristol-Myers Squibb (Princeton, NJ, USA).

MPS I mice. MPS I mice [11] in a C57BL/6 background were injected iv with varying doses of hAAT-cIDUA-WPRE at 2–3 days after birth as described previously [9].

Transduction of cats. MPS I kittens [16] were identified by IDUA assay of serum and were injected iv at 2 to 5 days after birth with one to four doses of ~2 ml of RV per dose. RV/CTLA4-Ig cats received human CTLA4-Ig iv in addition to RV. Cat 6986 received 25 mg/kg just prior to injection of RV on day 0 and received the same dose on days 5, 7, and 11 after transduction. Cat 7031 received 25 mg/kg on days 0 and 5, 6.4 mg/kg on day 10, and 10.2 mg/kg on day 14 after transduction. Lower doses were used due to insufficient amounts of human CTLA4-Ig.

CTL assay. SV40 large T antigen immortalization of feline fibroblasts and CTL assays were performed as described [47]. Skin biopsies were minced and incubated for 30 min at 37°C with Hanks' balanced salt solution containing 1 mg/ml collagenase, 0.25% trypsin, and 1 mM EDTA, and cells were cultured in RPMI 1640 with 18% fetal bovine serum, L-glutamine, and penicillin-streptomycin. Primary fibroblasts were transfected with pBABE-neo-T [48], which contains the SV40 large T antigen and the neomycin resistance gene, using the CalPhos Mammalian Transfection Kit from BD Biosciences (Palo Alto, CA, USA), and cells were selected with 200 µg/ml G418. Cells were transduced with hAAT-cIDUA-WPRE with 8 µg/ml Polybrene and a clone with high IDUA activity was identified after limiting dilution and was used for *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) and as the specific target cells for the CTL assay. Nontransduced autologous fibroblasts were used as nonspecific targets.

PBMCs were separated by gradient centrifugation using Histopaque with a density of 1.066 g/ml. For *in vitro* stimulation, 5×10^6 PBMCs were cultured in 24-well plates for 6 days with 2×10^5 canine IDUA-expressing autologous fibroblasts that were irradiated with 2000 rad and were used as effector cells in the CTL assay. Canine IDUA-expressing or nonexpressing autologous immortalized fibroblasts were plated at 5×10^3 cells/well and labeled overnight with 0.375 µCi per well of sodium [⁵¹Cr]chromate (Perkin-Elmer, Billerica, MA, USA) at 37°C in a 96-well round bottom plate and washed three times. The appropriate number of effector cells was added to the ⁵¹Cr-labeled target cells in a final volume of 200 µl to give E:T ratios of 2.5:1 to 40:1 in triplicate. After 6 h, the radioactivity in 100 µl of supernatant was determined with a gamma counter. The percentage specific lysis = [(mean experimental cpm – mean spontaneous cpm)/(mean maximum cpm – mean spontaneous cpm)] × 100. Maximum and spontaneous release were determined by incubating the labeled targets with 2% Triton X-100 and with medium alone, respectively.

IDUA activity, β-hexosaminidase activity, and GAG levels. Five microliters or less of serum was tested for IDUA activity using 4-methylumbelliferyl-α-L-iduronide (Calbiochem, San Diego, CA, USA) as the substrate [9]. One unit of enzyme releases 1 nmol of 4-methylumbelliferone per hour. Livers were homogenized in lysis buffer as described [9] and used for IDUA assay, for β-hex assay with 4-methylumbelliferyl-2-acetamido-2-deoxy-β-glucopyranoside as the substrate [9], and for GAG

assays using a sulfated glycosaminoglycan kit from Blyscan (Newtownabbey, Northern Ireland) [14]. The protein concentration was determined with the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time PCR. DNA was obtained from liver samples, and high-molecular-weight fragments were selected with a QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA) as described previously [14]. Real-time PCR for the WPRE sequence in RV DNA was performed as described previously [49] using a TaqMan PCR core reagent kit (Applied Biosystems, Rockville, MD, USA). For normalization to cat β-actin sequences, a forward primer (5'-GACCCTCAAGTACCCCATCGAAC-3'), a reverse primer (5'-GCGTCGAGCAACATCTTCCA-3'), and a TaqMan probe (5'-TTGtACCAACTGGGACGACATGGAGAAG-3') were used. The TaqMan probe was derived from the mouse β-actin sequence and had 1-nt difference (indicated as a lowercase letter in bold, which is C in the cat) between the mouse and the cat sequence. RNA levels were evaluated using reverse transcription with a Superscript III kit (Invitrogen, Carlsbad, CA, USA) and the reverse WPRE and reverse cat β-actin primers, followed by real-time PCR using a SYBR green PCR core reagent kit (Applied Biosystems).

MPS I dogs. MPS I dogs [19] were identified at birth by IDUA assay of serum. MPS I dogs were injected iv with one dose of 5 ml of hAAT-cIDUA-WPRE at 2 or 3 days after birth.

Statistics. Statistical comparisons were performed using Sigma Stat (Sigma Chemical). ANOVA with Tukey post hoc analysis was used to compare values for three or more groups. For comparisons of two groups, the two-tailed Student *t* test was used.

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