High expression reduces an antibody response after neonatal gene therapy with B domain-deleted human factor VIII in mice

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Summary. Background: Gene therapy could prevent bleeding in patients with hemophilia A, but might induce antibodies that block factor VIII (FVIII) function. Objectives: To test the efficacy of gene therapy in the newborn period for preventing a response to human FVIII (hFVIII) because of immaturity of the immune system. Methods: Varying doses of a retroviral vector (RV) expressing a B domain-deleted hFVIII cDNA were injected i.v. into newborn hemophilia A C57BL/6 or normal C3H mice. Mice were evaluated for hFVIII expression, hemostasis, and development of anti-hFVIII antibodies with inhibitory activity. Results and conclusions: Injection of a high RV dose [10^10 transducing units (TU) kg^-1] into newborn hemophilia A or C3H mice resulted in 61% and 13% of normal hFVIII antigen in plasma, respectively; most mice did not produce anti-hFVIII antibodies, and hemophilia A mice did not bleed. Furthermore, most mice with > 20 ng mL^-1 of hFVIII in plasma (10% normal, 1 x 10^{-10} m) were tolerant to hFVIII, as an antibody response was markedly reduced after challenge with hFVIII with or without adjuvant. However, most RV-treated animals with lower antigen levels developed antibodies before or after challenge. Thus, initiation of a gene therapy trial with low RV doses might increase inhibitor formation. Furthermore, frequent hFVIII infusions in newborns with hemophilia A might reduce inhibitor formation. Finally, difficulties in achieving tolerance after gene therapy for hemophilia A as compared to hemophilia B may relate to lower expression of FVIII than FIX, as high antigen levels are most effective at inducing tolerance.

Keywords: factor VIII, hemophilia A, inhibitor, neonatal, retroviral vector.

Introduction
Hemophilia A is due to factor VIII deficiency and occurs in one in 5000 males [1]. Patients with <1% of normal activity have frequent and severe spontaneous bleeding, those with 1–5% have less frequent spontaneous bleeding, and those with >5% have relatively mild bleeding. Hemophilia A is treated with human FVIII (hFVIII) protein, which is expensive and inconvenient to administer, and can induce inhibitory antibodies that block FVIII function in 25% of patients [2]. The most successful long-term therapy for inhibitors is immune tolerance induction, which involves frequent infusions of high FVIII doses, which can induce apoptosis of lymphocytes in vivo and in vitro in mice [3].

Gene therapy could permanently cure hemophilia A by achieving stable FVIII expression from liver, endothelial cells or hematopoietic cells from gamma retroviral vectors (RVs), or from lentiviral, adenoviral, adeno-associated virus (AAV) or plasmid vectors [4–6]. However, inhibitors often develop in both mice and dogs. We previously demonstrated that neonatal injection of an RV-expressing human FIX (hFIX) induced tolerance in mice, dogs and cats if sufficient expression was achieved [7,8], although high hFIX levels can also induce tolerance after gene therapy in adults [4–6,9].

Although neonatal gene therapy induced tolerance to hFIX, it was unclear whether this approach would be effective for the highly immunogenic hFVIII. Both protein and gene therapy have been given to newborn mice in an attempt to establish tolerance to hFVIII and/or prevent bleeding manifestations, with variable results. A single dose of hFVIII protein given within 24–30 h after birth resulted in tolerance after adult protein injections in 89% [10] and 93% [11] of mice or after adult injection of a transposon expressing hFVIII in 82% of mice [12]. However, when hFVIII protein was injected at 3 days after birth, only 46% of mice were later tolerant [11]. Furthermore, administration of a transposon expressing hFVIII from endothelial cells within 24 h after birth failed to induce tolerance after subsequent challenge as adults with an additional transposon [13]. Finally, i.v. injection of an RV at 2–3 days after birth resulted in antibodies in 46% of mice [14].
Thus, neonatal protein or gene transfer is not uniformly effective at inducing tolerance to hFVIII in mice. The goal of this study was to further evaluate the effect of hFVIII levels upon antibody formation after neonatal gene transfer.

**Materials and methods**

Reagents were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified. Albumin-free ReFacto [15] with specific activity of 10 438 U mg$^{-1}$ was provided by D. Roth of Wyeth Pharmaceuticals.

**hAAT-BDD-hFVIII WPRE**

The 4.5-kb B domain-deleted (BDD) cDNA [16] was cloned into the NotI site of hAAT-WPRE-767 (hAAT, human $\alpha_1$-antitrypsin promoter; WPRE, Woodchuck hepatitis virus post-transcriptional regulatory element) [17] to generate hAAT-BDD-hFVIII-WPRE. An amphotropic GP$^+\text{AM12-based}$ packaging cell line was used to prepare replication-incompetent RVs as reported previously [17].

**Animal care**

Hemophilia A mice with an exon 16 insertion on a C57BL/6 background [18] and normal C3H mice from Jackson Laboratory (Bar Harbor, ME, USA) were injected via the temporal vein with 100 $\mu$L of RV at 2 or 3 days after birth. Adult animals were injected i.p. with 25 or 250 U kg$^{-1}$ of ReFacto in 200 $\mu$L phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4). For some mice, ReFacto was mixed with 200 $\mu$L of the adjuvant RIBI MPL+TDM emulsion (Corixa, Hamilton, MT, USA) in PBS. For adult transduction, mice were treated with 25 mg kg$^{-1}$ of hepatocyte growth factor (HGF) and 10$^{10}$ transducing units (TU) kg$^{-1}$ of RV [19]. Tail-clip was performed as previously described [19], and the percentage of mice that were still bleeding after 10 min or rebled within 6 h was determined. Blood from the retro-orbital sinus was anticoagulated with a 1/10 volume of 3.2% sodium citrate. Hemophilia A mice that bled excessively into the peritoneum after i.p. injections were killed.

**hFVIII antigen**

The first and second antibodies were a mouse monoclonal antibody (mAb) 10104 directed against the hFVIII light chain (QED Bioscience, Inc; San Diego, CA, USA) and a horseradish peroxidase (HRP)-conjugated mouse mAb ESH8 directed against the C2 domain of the light chain (American Diagnostica, Inc., Stamford, CT, USA), respectively. Samples were diluted in blocking buffer with 10 U mL$^{-1}$ of heparin (Baxter Healthcare, Deerfield, IL, USA) and assayed as previously described [7]. Standards were dilutions of normal human plasma (NHP; George King, Overland Park, KS, USA) with 200 ng mL$^{-1}$ of hFVIII. The sensitivities in hemophilia A and normal C3H mice were 2 and 5 ng mL$^{-1}$, respectively.

**hFVIII activity**

The Coatest FVIII activity assay was performed at room temperature using a kit from DiaPharma (West Chester, OH, USA) as previously described [20], except that kinetic readings were used here. Standards contained dilutions of ReFacto in hemophilia A mouse plasma.

**Anti-hFVIII antibody**

Enzyme-linked immunosorbet assay (ELISA) wells were coated with 5 $\mu$g mL$^{-1}$ of hFVIII, and assays were performed as previously described [7], using HRP-conjugated goat antimouse total IgG, or antimouse IgG1, IgG2A or IgG2B (Roche Molecular Biochemicals, Indianapolis, IN, USA). For standards, total mouse IgG or purified mouse IgG subclasses were used. The sensitivity was 0.02 mg mL$^{-1}$ of anti-hFVIII antibody.

**Inhibitor**

Ten microliters of mouse plasma was heat-inactivated at 56 °C for 1 h and mixed with 10 $\mu$L of NHP for 2 h at 37 °C; 50 $\mu$L of hFVIII-deficient human plasma and 30 $\mu$L of PBS were added, and activated partial thromboplastin time was performed as previously described to determine the Bethesda units (BU) per milliliter [8].

**Statistics**

Statistical comparisons were performed with SIGMASTAT software.

**Results**

**RV vector**

The RV designated hAAT-BDD-hFVIII-WPRE is shown in Fig. 1. NIH3T3 cells that were transduced at a multiplicity of...
infection of 1 transducing particle per cell secreted 0.41 ± 0.01 [standard deviation (SD)] units (U) per milliliter of hFVIII activity per 24 h per 10^6 cells, where 1 U is the amount of activity found in 1 mL of NHP.

**Expression in hemophilia A mice**

Hemophilia A mice were injected at 2–3 days after birth with high (10^10 TU kg^{-1}), medium (10^9 TU kg^{-1}) or low (10^8 TU kg^{-1}) dose of hAAT-BDD-hFVIII-WPRE. No further treatment was given until 3–4 months, when challenge was initiated with the recombinant BDD-hFVIII, called ReFacto, with or without adjuvant. hFVIII antigen and anti-hFVIII total IgG antibody levels are shown for individual mice in Fig. 2, and as average values in Fig. 3. At 2–3 months, mice that received a high RV dose had 123 ± 76 ng mL^{-1} (61% normal) of hFVIII in plasma (Figs 2A,H and 3A) and 1.9 ± 1.2 U mL^{-1} of FVIII activity (190% normal; Fig. 3B). The discrepancy in the ratio to normal may reflect the use of human plasma for the ELISA standard and ReFacto for the activity standard, as ReFacto has ~2-fold higher specific activity in a Coatest assay than does full-length hFVIII [21]. Human plasma was used for the antigen assay, as this study was initiated before ReFacto was available. Untreated hemophilia A mice had <1 ng mL^{-1} of hFVIII antigen (Fig. 3A) and <0.01 U mL^{-1} of FVIII activity (Fig. 3B). Medium-dose RV mice achieved 12 ± 16 ng mL^{-1} of hFVIII (6% normal; Figs 2B,I and 3A) and 0.23 ± 0.26 U mL^{-1} of Coatest activity (Fig. 3B) in plasma at 2 months, whereas low-dose RV mice achieved 9 ± 9 ng mL^{-1} of hFVIII (4% normal; Figs 2C,J and 3A) but did not have detectable hFVIII activity (Fig. 3B).

**Fig. 2.** Antigen and antibody responses in individual hemophilia A mice after neonatal gene therapy. Newborn hemophilia A mice were injected i.v. with a high (10^{10} TU kg^{-1}), medium (10^9 TU kg^{-1}), or low (10^8 TU kg^{-1}) dose of hAAT-BDD-hFVIII-WPRE (hAAT, human α1-antitrypsin promoter; BDD, B domain-deleted; WPRE, Woodchuck hepatitis virus post-transcriptional regulatory element). Some control hemophilia A mice did not receive gene therapy (D,K). Each line represents values for an individual mouse. Starting at 3–4 months after birth, mice in (A)–(G) received i.p. injection of ReFacto (BDD-hFVIII) without adjuvant at the times indicated by the thin vertical lines at the top. Mice in (H)–(N) received ReFacto with the adjuvant RIBI, as indicated by the thick vertical lines. For all panels, short and long vertical lines indicate doses of 25 and 250 U kg^{-1}, respectively. (A)–(C) and (H)–(J) hFVIII antigen. Plasma hFVIII antigen levels were determined by immunoassay. (D)–(G) and (K)–(N) Anti-hFVIII antibody levels. Anti-hFVIII total IgG antibody levels were determined by immunoassay.

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Bleeding in hemophilia A mice

The percentages of mice with prolonged bleeding after tail-clip at 2–3 months of age were 0%, 14% and 20% for the high-dose, medium- and low-dose RV groups, respectively (Fig. 3C). These values were statistically lower than the 100% frequency in untreated hemophilia A mice. The frequencies of severe bleeding after i.p. injection during ≥8 months of observation were 0%, 42% and 58% for the high-dose, medium- and low-dose RV groups, respectively, and the frequency was 59% in untreated hemophilia A mice (Fig. 3D). Thus, high-dose RV eliminated bleeding, but some bleeding still occurred with the lower doses.

Challenge with hFVIII (ReFacto)

Mice were challenged with ReFacto, starting at 3–4 months of age, to determine whether they were truly tolerant. Some received multiple hFVIII injections without adjuvant, which resembles the fashion in which human patients are treated. Others received the adjuvant RIBI in addition to hFVIII, which is a more stringent test of tolerance. Animals were injected i.p. for convenience, which differs from the i.v. route used in patients. The initial plan was to give 25 U kg\(^{-1}\) per dose. However, antibodies did not develop in control hemophilia A mice that did not receive gene therapy after six doses of ReFacto given once a week (Fig. 2D) or two doses of ReFacto/RIBI separated by 3 weeks (Fig. 2K). The dose was therefore increased to 250 U kg\(^{-1}\), and 10 more doses of ReFacto alone or two more doses of ReFacto/RIBI were given. This dose resulted in 17.5 ± 7 ng mL\(^{-1}\) of hFVIII in plasma at 2 h after i.p. injection (data not shown).

Antibodies in hemophilia A mice

Most hemophilia A mice that did not receive gene therapy produced high levels of anti-hFVIII antibodies after challenge with ReFacto only (Fig. 2D) or ReFacto/RIBI (Fig. 2K), with average anti-hFVIII total IgG of 0.78 ± 0.06 and 0.62 ± 0.77 mg mL\(^{-1}\) (Fig. 3E) and inhibitor titers of 350 ± 357 and 319 ± 59 BU mL\(^{-1}\) (Fig. 3F), respectively, at the last time of collection. This was primarily a Th2 response, as 83% and 75%, respectively, of the IgG was IgG1 (Fig. 3G). Mice that did not receive gene therapy or protein challenge did not develop anti-hFVIII antibodies or inhibitors (not shown).

No hemophilia A mice that received neonatal injection of 10\(^{10}\) TU kg\(^{-1}\) of RV at birth produced anti-hFVIII antibodies after gene therapy alone (Fig. 2E.L). Furthermore, only one of nine produced anti-hFVIII antibodies in response to ReFacto alone (Fig. 2E), the average total anti-hFVIII IgG antibody
(Fig. 3E) and inhibitor titers (Fig. 3F) were very low at the end of the challenge period (P < 0.001 vs. No RV-ReFacto), and IgG subclass assays were negative. Although four of six mice that received high-dose RV developed antibodies in response to ReFacto/RIBI (Fig. 2L) that were temporally associated with a fall in hFVIII antigen levels (Fig 2H), the total IgG level of 0.20 ± 0.19 mg mL \(^{-1}\) (Fig. 3E) and the inhibitor titers of only 14.2 ± 11 BU mL \(^{-1}\) (Fig. 3F) were lower than in hemophilia A mice that received ReFacto/RIBI without gene therapy (P = 0.045 and P < 0.001, respectively). This antibody response was primarily Th2, as 74% was IgG1 (Fig. 3G). None of the hemophilia A mice that received 10⁹ TU kg \(^{-1}\) of RV developed antibodies in response to the gene therapy (Fig. 2F,M). None of seven of these mice developed high levels (>0.05 mg mL \(^{-1}\)) of anti-hFVIII antibodies in response to ReFacto only (Fig. 2F), and the total IgG level of 0.01 ± 0.01 mg mL \(^{-1}\) (Fig. 3E) and the inhibitor titer of only 3 ± 2 BU mL \(^{-1}\) (Fig. 3F) were significantly less than in mice that received ReFacto without gene therapy (P < 0.001). However, six of seven medium-dose RV mice developed high levels of anti-hFVIII antibodies in response to ReFacto/RIBI (Fig. 2M). Furthermore, their total anti-hFVIII IgG of 2.2 ± 1.7 mg mL \(^{-1}\) (85% IgG1; Fig. 3E,G) and inhibitor titers of 250 ± 100 BU mL \(^{-1}\) (Fig. 3F) were very high and were not significantly different from those in No RV-ReFacto/RIBI controls.

None of the hemophilia A mice that received 10⁹ TU kg \(^{-1}\) of RV developed antibodies after gene therapy alone (Fig. 2G,N). Only one of three developed antibodies after ReFacto administration (Fig. 2G), whereas two of two developed antibodies after ReFacto/RIBI administration (Fig. 2N). However, the small number of animals evaluated, because of the high bleeding rate, complicated statistical comparisons.

Expression in C3H mice

As C3H mice often produce more antibodies to foreign proteins than do C57BL/6 mice [22], the effect of neonatal gene therapy was also tested in hemostatically normal C3H mice, as hemophilia A C3H mice were not available in this laboratory. Mice received varying doses of hAAT-BDD-hFVIII-WPRE at 2–3 days after birth. The initiation of challenge with ReFacto with or without the adjuvant RIBI at 7 months was later than for hemophilia A mice; this was due to a delay in obtaining ReFacto and the initiation of the C3H study first. C3H mice that received the high dose (10¹⁰ TU kg \(^{-1}\)) achieved 26 ± 8 ng mL \(^{-1}\) of hFVIII antigen in plasma (13.1% of normal) at 1.5 months (Fig. 4A–C), and this was stable for at least 7 months. Values in individual mice fluctuated substantially, which was probably due to difficulties in consistently anticoagulating the blood during collection. The lower expression in C3H than in the C57BL/6 mice is similar to the result observed with an hFIX-expressing vector [7]. hFVIII antigen levels were not above background in C3H mice that received the medium (10⁹ TU kg \(^{-1}\)) or low (10⁸ TU kg \(^{-1}\)) dose of RV. FVIII activity could not be evaluated, because of the background FVIII activity in normal mice.

Antibodies in C3H mice

All C3H mice that did not receive gene transfer developed high levels of antibodies in response to ReFacto alone (Fig. 4D) or ReFacto with RIBI (Fig. 4H), with total IgG of 1.5 ± 1.7 and 4.4 ± 3.4 mg mL \(^{-1}\), respectively (Fig. 4L), and inhibitor titers of 191 ± 11 and 185 ± 21 BU mL \(^{-1}\) (Fig. 4M). This was primarily a Th2 response, as 81% and 77%, respectively, of the total antibody was IgG1 (Fig. 4N). None of the mice that received 10¹⁰ TU kg \(^{-1}\) of RV developed high levels (>0.05 mg mL \(^{-1}\)) of anti-hFVIII antibodies or inhibitors either before or after challenge with ReFacto alone or ReFacto/RIBI (Fig. 4E,IL,M). In contrast, 70% of mice that received the medium RV dose developed anti-hFVIII antibodies after gene transfer alone (Fig. 4F,J). Although challenge with ReFacto alone did not substantially affect the antibody levels (Fig. 4F), challenge with ReFacto/RIBI resulted in very high antibody and inhibitor levels. Although both medium-dose RV groups developed a Th2 response, the Th1 response was also substantial, as 52% and 66% of the total IgG was of the IgG2A subclass for those that were challenged with ReFacto alone and ReFacto/RIBI, respectively.

Few mice (20%) that received neonatal low-dose RV produced anti-hFVIII antibodies in response to the gene therapy alone, suggesting that the expression level was insufficient to induce an immune response. However, challenge with ReFacto alone (Fig. 4G) or ReFacto/RIBI (Fig. 4K) resulted in very high levels of anti-hFVIII antibodies (Fig. 4L) and inhibitors (Fig. 4M). Both groups developed both Th2 and Th1 responses, as 62% and 41% of the total antibody was IgG1 (Fig. 4N), whereas 35% and 58% of the antibody was IgG2A (Fig. 4O) for the ReFacto and ReFacto/RIBI groups, respectively.

Response in adult C3H mice

To compare responses in newborns with those in adults, adult C3H mice were injected i.v. with 10¹⁰ TU kg \(^{-1}\) of hAAT-BDD-hFVIII-WPRE, as detailed in Fig. 5. Some mice received HGF to induce hepatocyte replication and augment hepatocyte transduction. Mice that did not or did receive HGF prior to RV had a peak hFVIII antigen level of 6 ± 2 or 21 ± 6 ng mL \(^{-1}\), respectively, at 1 week after transduction (Fig. 5A,B), which became undetectable at 2 months or earlier. At 1 month after transduction, both groups developed high anti-hFVIII total IgG levels of 7.3 ± 5.3 and 3.4 ± 0.1 mg mL \(^{-1}\), respectively, (Fig. 5C,D), and high levels of inhibitors of 284 ± 41 and 216 ± 56 BU mL \(^{-1}\), respectively (Fig. 5E,F). We conclude that the magnitude of the immune response to hFVIII in C3H mice that receive medium- or low-dose neonatal gene therapy followed by protein challenge with adjuvant is similar to what occurs after adult gene therapy.
Discussion

High expression reduces antibody formation after neonatal gene therapy

In this study, high-dose ($10^{10}$ TU kg$^{-1}$) neonatal gene therapy administered to hemophilia A C57BL/6 mice resulted in 61% of normal hFVIII antigen, eliminated bleeding, and failed to induce antibodies. Furthermore, most mice failed to produce antibodies after multiple doses of ReFacto, and produced relatively low amounts of antibody after administration of ReFacto/RIBI. However, mice that received the medium RV dose ($10^9$ TU kg$^{-1}$) developed very high anti-hFVIII antibody and inhibitor levels after ReFacto/RIBI challenge, although...
antibody levels were low after challenge with ReFacto without adjuvant. High-dose neonatal gene transfer to C3H mice resulted in 13% of normal hFVIII antigen, failed to induce anti-hFVIII antibodies, and resulted in tolerance to the administration of ReFacto with or without adjuvant. However, C3H mice that received medium-dose RV produced anti-hFVIII antibodies in response to the gene therapy, suggesting that the neonatal immune system can respond to hFVIII in mice. C3H mice with high expression appeared to be more tolerant to administration of protein with adjuvant than were hemophilia A C57BL/6 mice with high expression, which may reflect more time for clonal deletion because of the later age of challenge in C3H mice, or intrinsic differences between the strains. A Th2 response is predominant in patients with inhibitors [23], and was also the predominant response in mice that developed antibodies after challenge in adulthood. However, both Th1 and Th2 responses developed in C3H mice that developed antibodies early after neonatal gene therapy, suggesting that neonatal mice may be more efficient at producing a Th1 response to hFVIII than are older mice.

In general, mice that achieved 20 ng mL\(^{-1}\) of hFVIII (10% of normal, 1 × 10\(^{-10}\) M) were tolerant, although there were some exceptions to this rule. The finding that high expression of hFVIII was more effective at avoiding antibody formation and/or reducing the immune response to protein challenge than was low expression is similar to that in our previous study with hFIX in mice [7,8]. However, the RV dose needed in this study was higher than was required in the previous hFIX experiment. This difference may relate to the relative expression levels of the two proteins: 10\(^{10}\) TU kg\(^{-1}\) of hAAT-BDD-hFVIII-WPRE resulted in 121 ng mL\(^{-1}\) of hFVIII (0.5 nm) for hemophilia A C57BL/6 mice, whereas the same dose of hAAT-hFVIII-WPRE resulted in 8200 ng mL\(^{-1}\) (130 nm) of hFIX for hemophilia B mice on a mixed C57BL/6-129S background. These results are consistent with the finding that 0.1–1 nm of a protein in blood is required in transgenic mice to induce tolerance to proteins [24]. The antibody development observed in some hemophilia A mice after neonatal RV-mediated gene therapy in a previous study [14] could have been due to an inadequate injection resulting in a relatively low level of expression.

**Implications for treating newborns with hFVIII**

There has been concern that initiation of protein replacement therapy within a year after birth increases the risk of inhibitor formation as compared with initiation at older ages [25,26], although other studies have suggested that the specific hFVIII mutation and/or initiation with on-demand therapy rather than prophylaxis may be critical [27–30]. Our study demonstrates that achieving constant high levels of hFVIII in blood in newborn mice reduces the chance of inhibitor formation, suggesting that initiation of hFVIII prophylaxis in newborns might reduce inhibitor formation. However, it is unclear whether the human immune system would behave in the same fashion, and performing a trial to address this issue is complicated by problems with i.v. access in newborns.

**Implications for gene therapy in patients**

It is unlikely that gene therapy trials will be initiated in the near future with an integrating vector for hemophilia A, because of concerns about insertional mutagenesis. However, if a safe vector can be identified, this study demonstrates that the newborn immune system is more likely to respond to hFVIII after neonatal gene therapy if expression is relatively low, and suggests that standard dose escalation might increase the chance of inhibitor formation. It remains possible that transient immunosuppression at the time of gene transfer, as was carried out successfully in adult mice [31], might be necessary, as tolerance to hFVIII was not consistently obtained with neonatal gene or protein therapy [10–14]. Studies in large animal models are in progress, and may help to address this issue.

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Disclosure of Conflict of Interests

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