Immunology of Neonatal Gene Transfer

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Abstract: Gene therapy could result in the permanent correction or amelioration of the clinical manifestations of many genetic diseases. However, immune responses to the therapeutic protein pose a significant hurdle for successful gene therapy. Problematic immune responses can include the development of a cytotoxic T lymphocyte (CTL) response that results in the destruction of genetically-modified cells and/or the formation of antibodies directed against the therapeutic protein. One approach to avoid an immune response is to perform gene therapy in newborns, which takes advantage of the fact that the immune system is relatively immature at birth. This approach has been highly effective in mice, and has resulted in stable expression without antibody formation for proteins that are highly immunogenic after transfer to adults. High levels of expression after neonatal gene therapy were more effective at inducing tolerance than low levels of expression in mice, which suggests that high antigen levels are more efficient at inducing tolerance. A criticism of this approach is that the murine immune system is less mature at birth than the immune systems of larger animals. Indeed, neonatal gene therapy to cats with mucopolysaccharidosis I resulted in a CTL response that destroyed expressing cells. Nevertheless, the immune system was still relatively immature, as transient administration of a single immunosuppressive agent at the time of neonatal gene therapy resulted in stable expression. Neonatal administration can reduce, but not eliminate, immune responses after gene therapy.

Keywords: Tolerance, hemophilia, mucopolysaccharidosis, lysosomal storage disease, and T regulatory cells.

INTRODUCTION

Gene therapy has corrected the clinical manifestations of several genetic diseases in animal models and in some human patients [Lillicrap *et al.,* 2006; Herzog *et al.,* 2006; Ponder, 2006b; Ponder and Haskins, 2007; Pierce *et al.,* 2007]. However, the immune system can recognize the therapeutic protein as foreign, which can result in the development of a cytotoxic T lymphocyte (CTL) response that can destroy genetically-modified cells, or the production of antibodies that block the function of blood proteins [Dobrzynski and Herzog, 2005]. CTL responses require that antigen-presenting cells (APC) present peptides derived from the protein on MHC class I molecules, which results in stimulation of CD8+ cells that can destroy target cells in a MHC class I-restricted fashion. These peptides are usually derived from proteins that are expressed in APCs, although peptides obtained from proteins that are taken up from the extracellular space can also be presented. CTL responses are generally induced by a so-called Th_1 response, which involves production of interleukin 2 (IL-2) and interferon gamma (IFN-γ) as well as other cytokines. Antibody responses require that cells present peptides from the protein on MHC class II molecules. Some APCs such as B cells are quite efficient at taking up antigen from the extracellular space and presenting peptides on class II molecules [Harvey *et al.,* 2007], which explains why antibodies can be efficiently induced after administration of protein. A Th_2 response results in production of IgG_1 antibodies and involves cytokines such as IL-4 and IL-10, while a Th_1 responses involves IL-2 and IFNγ and results in production of IgG_{2a}, IgG_{2b}, and IgG_3.

A variety of factors influence the probability and type of an immune response. Animals or patients with missense mutations are much less likely to develop antibodies to coagulation factors than are those with nonsense mutations or deletions [Fakharzadeh and Kazazian, 2000; Sabatino *et al.,* 2004; Zhang *et al.* 2007], which likely reflects the fewer number of epitopes with the potential to interact with the immune system.

The specific gene can also influence the type of immune response, as peptides derived from the protein may have a variable ability to interact with MHC molecules, and the final destination of the protein (cytosol, an intracellular compartment such as the lysosome, or extracellular secretion) may also be important. For example, CTL responses are most problematic after gene therapy for the lysosomal storage disease mucopolysaccharidosis I (MPS I) [Ponder and Haskins, 2007], while antibody responses appear to predominate after gene therapy for blood protein deficiencies such as hemophilia [Lillicrap *et al.,* 2006; Herzog *et al.,* 2006; Ponder, 2006b; Ponder and Haskins, 2007; Pierce *et al.,* 2007]. It is possible that a CTL responses is more profound for a lysosomal enzyme that traffics through endosomal pathways [Moron *et al.,* 2003; Gromme *et al.,* 2002] than for a protein that is secreted efficiently, although alternative explanations could explain the difference in these models. The target organ is a factor, with gene therapy to the liver being less likely to induce an immune response than transfer to other sites such as muscle [Nathwani *et al.,* 2001]. Finally, the age of gene transfer can be important, as the im-

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mune systems of a variety of mammalian species are relatively immature at birth.

This review will focus on using neonatal gene therapy to induce tolerance to a therapeutic gene. It will discuss the results obtained with two representative genes, α-L-iduronidase (IDUA) and Factor VIII (FVIII). IDUA is a lysosomal enzyme, and the results of gene therapy with a vector expressing this gene serve as a model for CTL responses. FVIII is a blood protein that contributes to coagulation of blood, and the results of gene therapy with a vector expressing this gene serve as a model for antibody responses.

NEWBORN ANIMALS HAVE REDUCED IMMUNE RESPONSES

Newborn mammals have an immature immune system at birth, although the degree of maturity differs among species, and in general, the human immune system is more mature than that of mice at birth [Landers *et al.,* 2005; West *et al.*, 2002; Schelonka and Infante 1998; Adkins *et al.,* 2004A]. Neonatal tolerance will be divided into three sections: 1) CTL responses; 2) antibody responses to T cell-dependent antigens; and 3) antibody responses to T cell-independent antigens.

CTL RESPONSES IN NEWBORNS

Most studies that have evaluated neonatal tolerance have involved transplantation of cells from a donor strain of mice into a host strain with a different MHC class I or class II molecule, followed by transplantation of an organ from the donor strain into the host during adulthood. Indeed, it was first reported in 1953 that transplantation between mouse strains at the time of birth could induce tolerance (Billingham *et al.,*1953]. This approach has generally allowed the graft to survive in mice if sufficient numbers of cells are transplanted at birth. Interestingly, however, transplantation of fewer cells did not prevent subsequent graft rejection [Adkins *et al.,* 2004b; Ridge *et al.,* 1996; Marshall-Clarke *et al.*, 2000], suggesting that a relatively high dose of antigen is needed to establish tolerance. Similarly, a high dose of an infectious retrovirus at birth failed to induce a CTL response in mice, while a low virus dose induced a CTL response that resulted in clearance of the virus [Sarzotti *et al.,*1996].

CTL tolerance has not been as effective in newborn humans as in mice. During the 2nd trimester, human immune cells acquire the ability to mount a mixed lymphocyte-reaction, which is a potent response to HLA-mismatched cells, and newborn humans can reject organ and tissue allografts [West, 2002]. Nevertheless, the chance of rejection of organ transplants is reduced in patients that receive organs before 6 months of age. Thus, the ability to produce a CTL response at birth varies among species, but is at least partially impaired in humans as well as mice.

ANTIBODY RESPONSES TO T CELL-DEPENDENT ANTIGENS IN NEWBORNS

Newborn mice have a reduced ability to produce antibodies to T cell-dependent antigens. FVIII, which will be a focus of this review, is one such T cell-dependent antigen (Wu *et al.,* 2001; Reding *et al.,* 2002; Qian *et al.,*1999). Indeed, although human FVIII is highly immunogenic in adult mice after intravenous (IV) injection, administration of hFVIII protein within 24 to 30 hours after birth resulted in tolerance after subsequent protein injections to adults in ~90% of mice [Pittman *et al.,* 1993; Madoiwa *et al.,* 2004]. Although it has been reported that neonatal tolerance involves deviation to a Th_2 rather than a Th_1 response [Adkins *et al.,* 1993; Abramowicz *et al.*, 1993; Singh *et al.,* 1996; Forsthuber *et al.,* 1996), this would be expected to induce Th_2-type antibodies, which has not been seen in several studies.

The human newborn immune system is also immature in its ability to respond to T cell-dependent antigens, as several vaccines fail to induce significant antibody responses to T-dependent antigens in >90% of human neonates (Siegrist, 2001). Although hepatitis B vaccine is often mentioned as an exception to this general observation, the percentage of seroconversion after the first vaccine dose given at birth is low (Belloni, *et al.,* 1993). Thus, antibody responses are deficient to T cell-dependent antigens, albeit not absent, in newborn humans.

ANTIBODY RESPONSES TO T CELL-INDEPENDENT ANTIGENS IN NEWBORNS

The immune system of humans is unable to produce responses to T cell-independent antigens at birth, which include carbohydrate antigens such as those found on blood groups. Because of this deficiency, heart transplants were accepted in ABO-incompatible newborn humans without formation of antibodies against the blood group carbohydrate moieties [Fan *et al.,* 2004]. Thus, responses to T cell-independent antigens are absent at birth.

BASIC MECHANISMS OF TOLERANCE

Immunological tolerance occurs when an animal does not mount a CTL or antibody response to an antigen [Borde *et al.,* 2006]. Central tolerance is due to the death of developing lymphocytes that encounter self-antigens in the thymus and/or the development of T_{reg} cells in the thymus. Peripheral tolerance can be due to clonal deletion, suppression, anergy, or ignorance. Clonal deletion can occur when antigen stimulation occurs in the absence of co-stimulation and/or cytokines, and results in apoptosis of the cell [Zhang *et al.,* 2004a]. Suppression is due to a subset of cells known as regulatory T (T_{reg}) cells that inhibit the activation of T cells. A population of T_{reg} cells that co-express CD4 and CD25 play a key role in the maintenance of immunologic tolerance by suppressing T cell responses in an IL-10-dependent fashion [Roncarolo *et al.,* 2006; Miyara and Sakaguchi, 2007; Aluvihare and Betz, 2006]. Indeed, T_{reg} cells play an important role in establishing tolerance after gene therapy to adult mice (Cao *et al.,* 2007). Anergy is present when a potentially reactive cell fails to respond to antigen. Ignorance can occur because the self-antigen is anatomically sequestered from immunocompetent lymphocytes, the antigen is expressed at levels that are insufficient to induce an immune response, or the receptor has low avidity for the antigen.

MECHANISM OF REDUCED IMMUNE RESPONSES IN NEWBORNS

A variety of factors appear to contribute to the reduced immune responses found in newborns. Cytokines are proteins that can induce innate and adaptive immune responses. Expression or secretion of many cytokines, including IL-2, IFN-γ, and IL-4, are reduced in lymphocytes from newborn patients or animals [Adkins *et al.*, 1993; von Freeden *et al.*, 1991; Splawski and Lipsky, 1991; Splawski *et al.*, 1998; Marodi, 2006; Ma *et al.*, 2007].

Co-stimulatory molecules are proteins that potentiate immune responses [Snanoudj *et al.*, 2006]. Two of the most important co-stimulatory pathways involve the interaction between CD80 or CD86 on APCs with CD28 on lymphocytes, and the interaction of CD40 on APCs with CD40 ligand (CD154) on lymphocytes. Newborn mice have a marked reduction in the levels of RNA for CD28 and CD40 ligand [Ma *et al.*, 2007], and should therefore be deficient in both of these pathways. In contrast, although newborn humans have low levels of CD40 ligand [Splawski *et al.*, 1996; Elliott *et al.*, 2000], they have normal levels of CD80, CD86, and CD28 [Elliott *et al.*, 1999]. Thus, the greater ability of newborn humans than newborn mice to mount immune responses at birth may relate to the fact that one co-stimulatory pathway is already mature in humans.

Antigen presenting cells (APC) need to present peptides derived from a protein on their MHC molecules in order for an immune response to occur. Dendritic cells are from the macrophage lineage, and are the most efficient type of APC. Newborn mice and/or humans have a reduced number of dendritic cells [Ridge *et al.*, 1996)], a deficiency in the ability of dendritic cell precursors to respond to cytokines [Pihlgren *et al.*, 2003], an impaired response of macrophages to IFN-γ [Marodi *et al.*, 2001], and an impaired response of macrophages to lipopolysaccharide and other ligands for toll-like receptors [Marodi, 2006]. In addition, neonatal B cells can inhibit dendritic cell function [Walker *et al.*, 2007].

Although there are deficiencies in other cell types at birth, CD4(+) and CD8(+) T_{reg} cells are present in newborns at normal levels [Kim *et al.*, 2007; Reibke *et al.*, 2006]. In addition, CD4(+) lymphocytes from newborn mice have reduced levels of proteins that induce T cell survival, OX40 ligand (OX40L) and CD30 ligand (CD30L) [Kim *et al.*, 2005]. Thus, reduced immune responses in newborns appear to be due to reduced production of cytokines, reduced levels of co-stimulatory molecules, and reduced maturity and responsiveness of APC in a setting where T_{reg} cells are still present and active.

REDUCED IMMUNOLOGICAL RESPONSE AFTER NEONATAL GENE THERAPY FOR MPS I

The results of gene therapy in animals with MPS I will serve as an example of a model where CTL responses appear to predominate in adults. IDUA is a lysosomal enzyme that traffics to endosomal compartments and then to the lysosome of the cell via the mannose 6-phosphate receptor. As shown in (Fig. **1A**), a retroviral vector expressing canine IDUA was injected IV into adult MPS I mice [Liu *et al.*, 2005; Ma *et al.*, 2007]. Serum IDUA activity was used to follow the stability of expression, as some of the enzyme produced by transduced hepatocytes or other cells is secreted into blood. Adult gene therapy resulted in high serum IDUA activity at 1 week after gene transfer, but expression fell to very low levels by one month. The loss of expression in this and a similar study [Di Domenico *et al.*, 2005] was associated with a very low level of retroviral vector DNA and/or RNA in the liver, and with a cytotoxic T lymphocyte response (B. Wang, X. Ma, K. Ponder, unpublished results). In contrast, anti-canine IDUA antibodies were not detected.

To attempt to prevent an immune response, the retroviral vector expressing canine IDUA was injected IV into mice at 2 to 3 days after birth. Administration of a relatively high dose [10^9 transducing units (TU)/kg] of retroviral vector resulted in high serum IDUA activity (Fig. **1B**), which has been stable for 8 months or longer in all mice that have been evaluated to date [Liu *et al.*, 2005; X. Ma and KP Ponder, unpublished data]. Thus, neonatal gene therapy was remarkably effective at inducing tolerance if a high dose of vector was used. Neonatal gene therapy was also successful with lentiviral [Kobayashi *et al.*, 2005] and AAV [Hartung *et al.*, 2004] vectors.

To evaluate the effect of retroviral vector dose on the efficiency of inducing tolerance, mice were injected with varying doses of the retroviral vector expressing canine IDUA, and the percentage of mice with stable expression was determined. As shown in (Fig. **1C**), lower doses of the retroviral vector were less effective at inducing tolerance, and none of the mice that received a very low dose (10^7 TU/kg) of vector achieved stable expression [Liu *et al.*, 2005; Ponder *et al.*, 2006a]. These data are consistent with data from the transplantation [Adkins *et al.*, 2004b; Ridge *et al.*, 1996; Marshall-Clarke *et al.*, 2000] and viral infection [Sarzotti *et al.*,1996] literature, where high levels of cells or virus were needed to establish immunological tolerance. These data have implications for how to initiate a gene therapy trial, as low doses of a vector may actually increase the chance of an immune response and reduce the probability of success.

CTL RESPONSE TO IDUA IN LARGE ANIMALS

Large animals often mount a more-potent immunological response to foreign proteins than mice, and may have a more mature immune system at birth. Neonatal administration of a retroviral vector expressing canine IDUA to MPS I cats resulted in high serum IDUA activity at 1 to 2 weeks [Ponder *et al.*, 2006a]. However, all cats lost expression at 1 to 2 months, which was associated with a loss of DNA and RNA in the liver, and with a CTL response directed against canine IDUA. In contrast, none of the cats produced antibodies to canine IDUA. The cat immune system was still relatively immature, however, as this CTL response was prevented by transient (2 week) administration of CTLA4-Ig, which blocks co-stimulation between CD80/CD86 and CD28 [Alegre and Fallarino, 2006]. The ability of a single immunosuppressive agent to prevent a CTL response differs from the result after gene therapy to adult MPS I mice, where CTLA4-Ig needed to be given indefinitely long-term, or to be given with a second immunosuppressive agent [Ma *et al.*, 2007]. Immune responses were not observed after neonatal

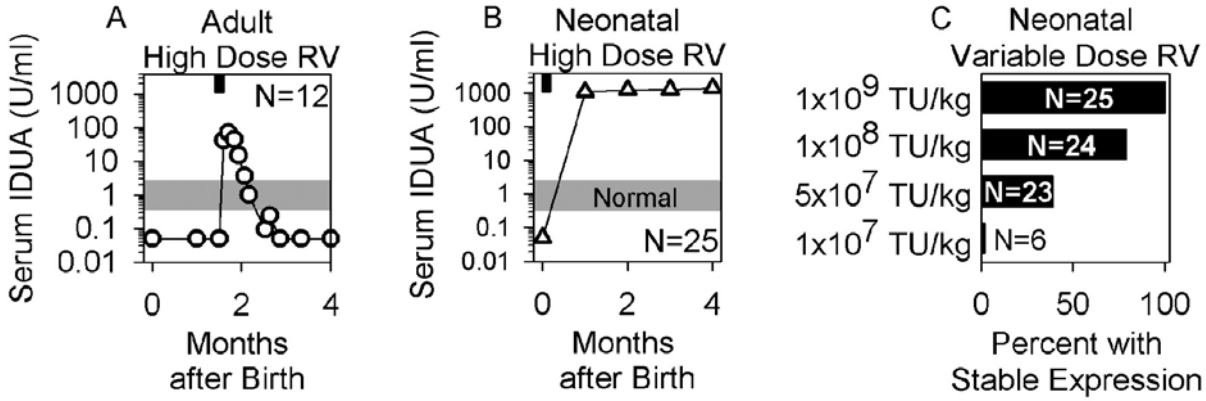

Fig. (1). Effect of adult or neonatal gene therapy with a retroviral vector expressing canine IDUA in MPS I mice. MPS I mice on a C57BL/6 background received adult or neonatal gene therapy. Mice were evaluated for serum IDUA activity, as some of the enzyme produced by liver or other organs can be secreted into blood. One unit (U) can convert 1 nmole of substrate into product in 1 hour. The IDUA activity in untreated MPS I mice was very low at <0.05 U/ml. Normal IDUA activity in serum is 1 U/ml, as indicated in panels A and B by the shaded region. The bar at the top of panels A and B indicates the age of retroviral vector administration. **A. Adult gene therapy.** Six week-old MPS I mice were injected with hepatocyte growth factor to induce hepatocyte replication, followed by administration of $2x10^9$ TU/kg of the retroviral vector hAAT-cIDUA-WPRE as detailed previously [Liu *et al.,* 2006]. The average serum IDUA ± the standarad deviation (SD) is plotted *vs.* the months of age after birth for 12 mice. The loss of expression was due to a CTL response. **B. Neonatal gene therapy.** Newborn MPS I mice were injected with 10^9 TU/kg of hAAT-cIDUA-WPRE at 2 to 3 days after birth as detailed previously [Liu *et al.,* 2006]. The average serum IDUA ± SD is shown for 25 mice. **C. Effect of retroviral vector dose on the stability of expression after neonatal gene therapy.** Newborn MPS I mice were injected with the indicated dose of hAAT-cIDUA-WPRE at 2 to 3 days after birth, as described previously [Liu *et al.,* 2006; Ponder *et al.,* 2006a]. All mice had detectable IDUA activity at 0.5 months after birth. The percentage of mice with stable IDUA activity in serum at 3 months after birth is shown for the indicated number of mice in each group.

administration of a retroviral vector expressing canine IDUA to MPS I dogs, although it is unclear if this was due to immaturity of the immune system or to tolerance to canine IDUA epitopes [Traas *et al.,* 2007]. Thus, the neonatal cat immune system is somewhat more mature than the mouse immune system at birth, and may serve as a very important model for predicting responses in humans. The ability to prevent a CTL response in cats with transient administration of a single agent at birth is encouraging that such an approach might also be effective in humans.

IMMUNOLOGICAL RESPONSES AFTER GENE THERAPY FOR HEMOPHILIA A

The results of gene therapy in animals with hemophilia will serve as an example of a model where antibody responses appear to predominate. This review will focus on results obtained with FVIII in animals with hemophilia A, as FVIII appears to be more immunogenic in adults than the Factor IX (FIX) protein that is deficient in hemophilia B. Gene therapy to adult mice or dogs with hemophilia A with a variety of vectors that express FVIII has often evoked a very potent antibody response [Ponder, 2006b; Lillicrap *et al.,* 2006; Herzog *et al.,* 2006; Pierce *et al.,* 2007]. These antibodies can inhibit the coagulation function of FVIII, in which case they are referred to as inhibitors. Development of inhibitors can make bleeding episodes very difficult and expensive to treat [Key, 2004].

In one study [Xu *et al.,* 2007a], IV injection of a retroviral vector to adult mice resulted in transient expression of human FVIII (Fig. **2A**), high levels of anti-human FVIII antibodies (Fig. **2B**), and high levels of inhibitors (not shown). Similarly, an antibody response to FVIII was observed after adult gene therapy to some or all strains of mice tested with plasmid, adenoviral, and AAV vectors in some other studies [Rawle *et al.,* 2004; Kang *et al.,* 2005; Jiang *et al.,* 2006; Miao *et al.,* 2006].

Although gene transfer of a vector expressing human FVIII resulted in a potent immune response in most adults, transfer of a high dose (10^{10} TU/kg) of a retroviral vector to newborns resulted in stable expression in all mice without antibody formation (Xu *et al.,* 2007a), as shown in (Fig. **2C** and **2D**). As was the case for neonatal gene therapy for MPS I, the dose of retroviral vector was extremely important. Mice that received a lower dose (10^9 TU/kg) of retroviral vector failed to have detectable expression of human FVIII (Fig. **2E**), and most produced high levels of antibodies (Fig. **2F**) with inhibitory activity (not shown). Indeed, the effect of expression levels may explain the results of the first neonatal gene therapy study for hemophiliia A, where half the mice had stable expression without inhibitors after neonatal administration of a retroviral vector, while the rest developed inhibitors [VandenDriessche *et al.,* 1999]. Another study successfully used neonatal protein injection to induce tolerance for transposon-based gene therapy performed in adults [Ohlfest *et al.,* 2005]. In contrast, administration of a transposon expressing hFVIII from endothelial cells within 24 hours after birth failed to induce tolerance when an additional dose of transposon was given to adults [Liu *et al.,* 2006]. This failure may relate to the relatively low level of expression achieved. In summary, neonatal gene therapy can induce tolerance to human FVIII in mice, but this requires that high expression be achieved.

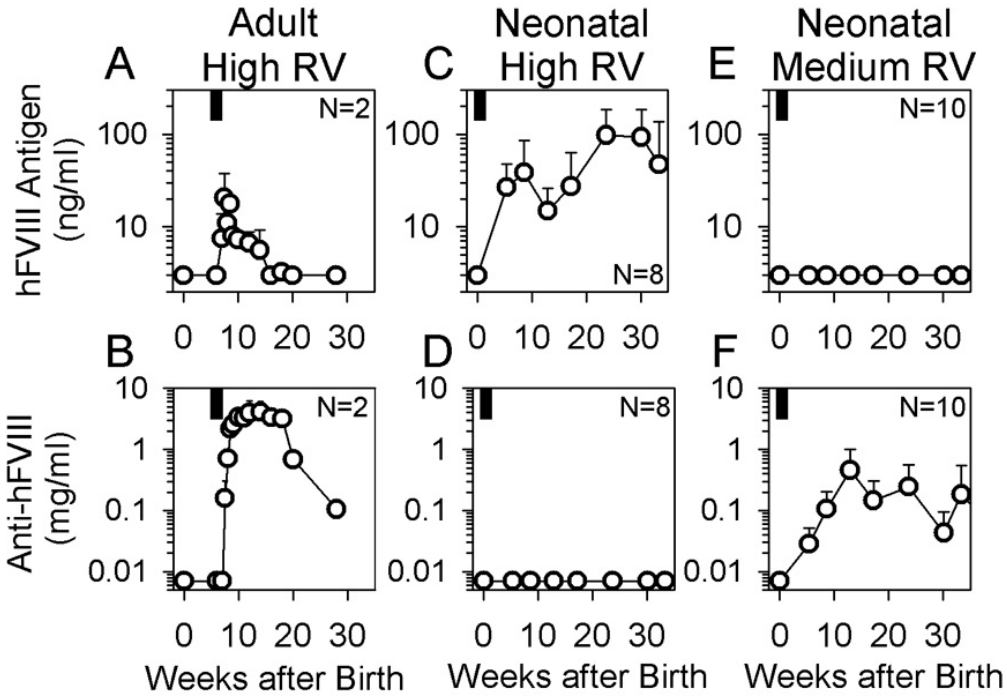

Fig. (2). Effect of adult or neonatal gene therapy with a retroviral vector expressing human FVIII in mice. Normal C3H mice received adult or neonatal gene therapy. Mice were evaluated for plasma FVIII antigen levels (panel A, C, and E) and for total anti-human FVIII IgG antibodies (panels B, D, and F) at the indicated age after birth. The black bar at the top of the panels indicates the time of administration of retroviral vector (RV). Normal levels of human FVIII in human plasma are 200 ng/ml. **A and B. Adult gene therapy.** Six week-old normal C3H mice were injected with hepatocyte growth factor to induce hepatocyte replication, followed by administration of a high dose of the retroviral vector (High RV;10^{10} TU/kg) hAAT-hFVIII-WPRE as detailed previously [Xu *et al.,* 2007a]. **C and D. Neonatal gene transfer with a high dose of retroviral vector.** Newborn normal C3H mice were injected with a high dose (High RV; 10^{10} TU/kg) of hAAT-hFVIII-WPRE at 2 to 3 days after birth as detailed previously [Xu *et al.,* 2007a]. **E and F. Neonatal gene transfer with a medium dose of retroviral vector.** Newborn normal C3H mice were injected with a medium dose (Medium RV; 10^{9} TU/kg) of hAAT-hFVIII-WPRE at 2 to 3 days after birth as described previously [Xu *et al.,* 2007a]. Although human FVIII antigen levels were not detectable at any time, the average anti-human FVIII antibody IgG level was high.

EFFICACY OF NEONATAL TOLERANCE TO HUMAN FVIII IN HUMANS

Although no gene therapy studies have been performed in newborn patients with hemophilia A, the response to protein administration might provide clues as to whether or not early administration of a gene therapy vector might induce tolerance in humans. Somewhat paradoxically, it was reported that inhibitor formation was more frequent in patients that initiated IV injections of human FVIII before 6 months of age than for those that started therapy after 6 months [Lorenzo *et al.,* 2001; van der Bom *et al.,* 2003], which would appear to argue against using early administration of protein therapy to prevent inhibitors. However, other studies suggest that the specific hFVIII mutation and/or initiation with episodic therapy in response to bleeds rather than frequent prophylaxis may be critical [Rivard *et al.,* 2005; Chalmers *et al.,* 2007; Santagostino *et al.,* 2005; Kulkarni *et al.,* 2006]. A trial in which protein therapy is initiated with high and frequent administration of human FVIII would be difficult due to problems with IV access in newborn patients. Thus, it is unclear if neonatal gene therapy will prevent an antibody response to human FVIII in humans, although data in mice suggest that achieving a high level of expression should be necessary, and there is substantial concern that neonatal gene therapy would not consistently induce tolerance. It may therefore be necessary to give one or more immunosuppressive agents transiently at the time of gene transfer, as was done for adult hemophilia A mice [Miao *et al.,* 2006, Miao, 2007].

IMMUNOLOGICAL RESPONSES AFTER GENE THERAPY FOR HEMOPHILIA B

There has been a substantial amount of work in which immune responses to FIX were evaluated after neonatal or adult gene therapy [Ponder, 2006b; Lillicrap *et al.,* 2006; Herzog *et al.,* 2006; Pierce *et al.,* 2007]. In general, it has been easier to achieve tolerance to human FIX than to human FVIII after fetal or neonatal gene therapy in mice, and high expression was more effective than low expression for tolerance induction [Zhang *et al.,* 2004b; Themis *et al.,* 2005; Waddington *et al.,* 2004; Xu *et al.,* 2007b; Sabatino *et al.,* 2007]. This approach was also usually effective at inducing tolerance in large animals, as all dogs that received varying doses of RV achieved stable expression without inhibitors, while 3 of 4 newborn cats that received a high dose of neonatal gene therapy achieved stable expression [Zhang *et al.,* 2004b; Xu *et al.,* 2007b]. Expression was unstable in one cat after neonatal gene therapy, which was likely due to a CTL response against transduced cells and was not associated with high levels of antibodies to human FIX.

In one study [Xu *et al.*, 2007b], clonal deletion of reactive cells appeared to be the main mechanism of tolerance after neonatal gene therapy with a retroviral vector, as adoptive transfer from tolerant mice did not confer tolerance. Furthermore, lymphocytes from tolerant mice did not secrete IL-10, which is usually produced by T_{reg} cells. This is consistent with a study that evaluated the mechanism of tolerance induction after neonatal protein administration, where clonal deletion was believed to be the major mechanism [Gammon *et al.*, 1986]. However, these studies cannot rule out the possibility that T_{reg} cells contribute to the establishment of tolerance. In contrast, both clonal deletion and T_{reg} cells contributed to tolerance development after gene therapy to adults [Mingozzi *et al.*, 2003; Cao *et al.*, 2007].

One possible explanation for why it is easier to achieve tolerance to FIX than to FVIII is that the molar level of FIX that was achieved (100 nM, which is the normal level of human FIX) was higher than for FVIII (0.5 nM, which is 50% of the normal level of human FVIII), and that it is the molar level of a protein in blood that is critical for establishing tolerance. This hypothesis is consistent with the fact that achieving 0.1 to 1 nM of a protein in blood is required in transgenic mice to induce tolerance to proteins [Cabaniols *et al.*, 1994].

IMPLICATIONS FOR GENE THERAPY IN PATIENTS

Gene therapy has the potential to prevent or reduce the clinical manifestations of genetic diseases. Neonatal gene therapy might be necessary for otherwise-lethal diseases, and would reduce the period of clinical manifestations for non-lethal diseases. In addition, performing gene therapy in newborns could reduce the chance of developing an immune response, which is a very important issue for patients. Indeed, neonatal gene therapy has reduced immune responses in mice, although achieving high levels of expression appears to be very important for establishing tolerance. Although neonatal gene therapy has not been consistently effective in large animals including cats, the immune system of large animals still appears to be relatively immature, as transient administration of a single agent was effective at blocking immune responses in newborn cats. Since it is unclear as to whether or not immune responses in cats and dogs will be predictive of results in humans, it will be important to test the efficacy of neonatal gene therapy in non-human primates prior to proceeding with trials in newborn humans that are at high risk of developing an immune response.

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ABBREVIATIONS

CTL = Cytotoxic T lymphocyte

APC = Antigen presenting cells

IDUA = α-L-iduronidase

FVIII = Factor VIII

MPS = Mucopolysaccharidosis

T_{reg} cells = T regulatory cells

TU = Transducing units

U = Units

SD = Standard deviation

IV = Intravenous

FIX = Factor IX

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