

## Commentary

# Gene therapy for the hemophilias

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The hemophilias are a group of severe bleeding disorders resulting from the lack of functional blood coagulation factor VIII (hemophilia A) or factor IX (hemophilia B). Factor IX, when activated, catalyzes the conversion of factor X to its activated form, and activated factor VIII serves as a cofactor in this reaction. Clinically the disorders are indistinguishable, and they were not distinguished in the laboratory until the 1950s, when two research teams independently demonstrated that plasma from one group of patients could correct the clotting defect of the other group (1, 2). Clinically the disease is characterized by frequent spontaneous bleeds into joints and soft tissues, with the potential to result in a chronic and debilitating arthropathy. More serious complications, and death, can result from bleeding into other critical closed spaces, such as the intracranial or the retroperitoneal space. The disease is classified as severe, moderate, or mild, based on circulating-factor levels, with severe defined as <1% of normal activity, moderate as 1–5%, and mild as 5–30%.

Current treatment for hemophilia is based on intravenous infusion of clotting-factor concentrates, either prophylactically or at the time of a bleed. Problems with these protein concentrates, including the expense, the inconvenience, and, in the case of plasma-derived concentrates, the risk of transmission of viral blood-borne diseases such as hepatitis and HIV, have fueled interest in gene-based approaches to therapy. Indeed, hemophilia has a number of features that make it attractive as a model for gene therapy. The therapeutic window is wide, because the natural history of the disease, as well as a generation of experience with clotting-factor concentrates (3), makes it clear that even modest elevation of factor levels, e.g., to 2%, will result in substantial improvement in clinical phenotype, and elevations to as high as 150% are still within normal limits and thus are unlikely to be harmful. Second, although clotting factors are normally synthesized in the liver, it is clear on the basis of studies from a number of laboratories that biologically active clotting factors can be synthesized in a wide variety of tissues, including muscle cells, fibroblasts, and endothelial cells (4–6). Finally, there are excellent animal models of hemophilia, including both genetically engineered mice (7–10) and naturally occurring dog models (11–13), and the species-specific transgenes have been cloned and are available, facilitating feasibility studies before moving to clinical trials.

In this issue of the *Proceedings*, VandenDriessche *et al.* (14) present the first full-length report of sustained expression of therapeutic levels of factor VIII in a hemophilic animal model achieved through the use of a retroviral vector. The report is timely because a clinical trial that uses a similar strategy is now under way. The study reported in the *Proceedings* used a Moloney retroviral vector expressing the B-domain deleted factor VIII cDNA from the promoter contained within the viral long-terminal repeat. Because retroviruses integrate, targeting a long-lived cell or a progenitor offers the chance for life-long therapy with a single administration. In contrast to most previous *in vivo* retroviral studies, the advantage of this study was the absence of complex and sophisticated delivery routes or pretreatment of the animals with agents or surgical procedures designed to promote

hepatocyte proliferation, which are undesirable strategies for treatment. Thus, for example, in an earlier preclinical study in which a retroviral vector was introduced into liver, hemophilia B dogs underwent a partial hepatectomy before retroviral vector administration to achieve a partial but persistent correction (15).

Gene therapy for factor VIII deficiency has been relatively more difficult than for factor IX deficiency because of the large size of the factor VIII coding region. Early efforts were hampered by the inability to obtain a high-titer retrovirus expressing factor VIII; at the time this was presumed to be caused by sequences within factor VIII that caused RNA instability (16, 17). This problem was subsequently overcome by the inclusion of the endogenous viral envelope splice site (18), which resulted in titers in a more conventional range ( $1 \times 10^6$ /ml). Although improved, this was still not practical for *in vivo* gene delivery. To increase the titer of the virus, physical concentration of the vector was performed by using the vesicular stomatitis virus (VSV)-G envelope to pseudotype the virus (19). This allows the virus to be concentrated by ultracentrifugation with high rates of vector recovery. In addition, this envelope, unlike many others, will not undergo complement inactivation after intravenous infusion. Furthermore, the VSV-G envelope does not require a specific receptor for cellular entry. Although the broad cell-type specificity is useful, the absence of tissue specificity has negative implications (see below). An additional disadvantage is that the vesicular stomatitis virus-G envelope is fusogenic with membranes and can be toxic at high concentrations (20, 21).

The study of VandenDriessche *et al.* (14) used a high-titer preparation ( $\approx 1 \times 10^8$  colony-forming units) of the concentrated vector that was administered by a simple systemic intravenous administration into 2- to 3-day-old mice with hemophilia A. The results showed that in some animals, normal or supranormal concentrations of plasma factor VIII were achieved with resulting phenotypic correction of the bleeding diathesis.

As noted earlier, a limiting factor in the use of Moloney retroviral vectors *in vivo* is that they require the target cells to be cycling or undergoing cell division at the time of retroviral delivery to achieve transduction (22). The authors here circumvented this problem by using neonatal animals (23). During neonatal life, the hepatocyte, the major target cell, undergoes rapid proliferation; however, in mature animals at any specific time, the number of hepatocytes in cycle is estimated to be 1/10,000 to 1/20,000 (24). In the current study, it was estimated by PCR analysis that 10–60% of liver cells were transduced by the vector, and the reverse transcription-PCR (measurement of mRNA) results suggest that most of the factor VIII production is derived from the liver. These data can be confirmed in future studies by Southern and Northern blot analysis. Nevertheless, on the basis of current knowledge, similar therapy in mature animals would not likely reach a therapeutic level of gene transfer into the liver without the addition of growth factors (25, 26) or other stimuli to induce hepatocellular proliferation. Alternative retro-

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viral vectors include the lentiviral class, which at least in some cell types does not require cellular division for transduction (27).

A major issue facing all gene therapy trials for hemophilia is the risk of forming inhibitory antibodies to the transgene product. Currently, formation of neutralizing antibodies is the most common complication of protein-based therapy, occurring in 20% of patients with factor VIII deficiency and  $\approx 3\%$  of patients with factor IX deficiency. These antibodies, referred to clinically as inhibitors, complicate treatment of acute bleeding episodes, because they neutralize the activity of the infused clotting factor, making it difficult to establish effective hemostasis. Despite years of study, it is not yet possible to predict which patients will develop inhibitory antibodies, but certain risk factors have been identified. These include the nature of the underlying mutation, inherited characteristics of the individual's immune response, and circumstances surrounding exposure to the clotting-factor protein. The role of the underlying mutation first became evident in studies of patients with factor IX deficiency (28, 29); sequencing of the entire Swedish hemophilia B population showed that, although the *a priori* risk of inhibitor formation was  $\approx 3\%$ , the risk for patients with missense mutations approached zero, whereas that for patients with extensive loss of coding information (gene deletions, early stop codons) was  $\approx 20\%$ . This observation helps explain the discrepancy between the incidence of inhibitors in hemophilia A and hemophilia B; large gene deletions are a relatively uncommon mutation in hemophilia B, but a gene inversion accounts for 40% of severe hemophilia A (30). However, family studies indicate that the underlying mutation is not the only risk factor, because, within a kindred, it is not uncommon to observe that one member of the family develops an inhibitor, while other members, presumably with the same underlying mutation, do not (reviewed in refs. 31 and 32). This observation points up the role of genetically determined characteristics of the immune response in inhibitor formation. Some authors have suggested that extensive tissue injury or inflammation at the time of factor administration may also modulate the immune response (32).

The influence of these factors on inhibitor formation may relate to current concepts of tolerance and antigen presentation (33, 34). Induction of inhibitors, i.e., antibodies to clotting factors, is promoted by T helper cells. Normally, self-reactive T cells (such as those against clotting factors) are deleted or anergized during T cell development. Individuals with hemophilia, however, may not express the sequences (i.e., the epitopes) that are recognized by T cells specific to the wild-type protein. Thus, these T cells mature in hemophilic patients and, on encounter with the antigen during therapy with clotting factor, promote the induction of a neutralizing antibody response. Activation of T cells requires, in addition to the specific antigen, an activation signal for antigen presenting cells, which, in their resting stage, are unsuited to optimally activate a T cell response. Such activation signals (also referred to as danger signals) can be provided by tissue injury or by inflammatory reactions after common viral or bacterial infections.

An additional layer of complexity characterizes antigen presentation in the setting of gene therapy, where the protein is now synthesized endogenously; for protein-based therapy, antigen presentation occurs primarily in the setting of MHC Class II, which displays peptides derived from proteins taken up from the environment. In the setting of gene therapy, though, antigen presentation also occurs through MHC Class I, which presents peptides derived from proteins synthesized within the cell that displays them. Current management of inhibitors requires the daily infusion of high doses of the offending clotting factor, which results in 70–90% of patients in the disappearance of the inhibitor (35). This has led some to argue that gene therapy could actually be used to treat patients with inhibitors, because it provides a steady source of factor (36). Whether a gene therapy approach, resulting in a steady stream of antigen, will be more effective than repeated bolus injections of the protein (the current method of

treating inhibitors) remains to be seen. However, in support of the gene therapy approach are experimental data in which an inhibitor to factor IX in a hemophilic dog treated with an adeno-associated virus-factor IX vector disappeared with no specific treatment (37). Other factors that are likely to influence inhibitor formation in the setting of gene therapy include the choice of vector, the target tissue used, dose of vector, and inclusion of tissue-specific promoter elements. Vectors that elicit a strong immune response to the viral proteins (e.g., adenoviral vectors) may be more likely to elicit an immune response to the transgene product as well. Target tissues rich in antigen presenting cells might also predispose to inhibitor formation. Some evidence suggests that inclusion in the vector of tissue-specific elements that restrict expression to the target tissue alone (and thus do not allow expression in antigen presenting cells) may reduce formation of inhibitory antibodies (38). Finally, it is possible that transient immunomodulation at the time of vector administration may block formation of inhibitory antibodies (ref. 39).<sup>§</sup> These are active areas of investigation, and it will be critical for the success of gene therapy for the hemophilias to understand more clearly the immunologic mechanisms underlying antigen presentation and the immune response in the setting of gene therapy.

The studies of VandenDriessche *et al.* (14) raise some intriguing questions about antigen presentation in the setting of gene therapy. The concentrated vector preps contain not only vector but also factor VIII protein. In the initial experiments, 7/13 mice developed inhibitory antibodies to the human (h)factor VIII transgene product, but when transduction was prevented by repressing vesicular stomatitis virus (VSV)-G expression in the vector producer cells and blocking vector infectivity with VSV-G-specific monoclonal antibodies, none of the mice developed antibodies to hfactor VIII. At first glance, this may seem to suggest that it is the endogenous synthesis that results in inhibitor formation, but several confounding variables complicate interpretation of this experiment. Normally it is necessary to use a species-specific transgene to avoid an immune response (these experiments used a human transgene in a mouse), but there are strain-specific exceptions to this rule, and C57BL/6 mice do not generally mount an immune response to human secreted proteins (40, 41). Presumably the presence of antibodies to factor VIII in the initial experiment reflects the fact that the experimental hemophilia A knockout mice had been crossed into C57BL/6 mice for only five generations. In addition, generation of an antibody response to infused clotting-factor protein generally requires multiple exposures, and these mice received only one exposure. Nonetheless, this is an interesting result that can profitably be pursued in this experimental system, especially with the use of additional strains of mice and a species-specific transgene.

The most important conclusion of the study of VandenDriessche *et al.* (14) is that it does appear possible, by using high-titer retroviral preparations, to achieve therapeutic levels of factor VIII in neonatal mice. Whether these findings can be extended to mature animals is currently being investigated, both by this group in animal models and by others in a clinical trial in which a highly concentrated factor VIII-expressing Moloney virus pseudotyped with an amphotropic envelope is infused into patients with severe hemophilia A over 3 days.<sup>¶</sup>

After years of disappointments and setbacks in the field of gene therapy, the stage appears set for success, and many observers agree that the first convincing demonstration of efficacy for gene therapy will likely be for hemophilia. Three clinical trials are currently underway; in addition to the retroviral trial mentioned

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<sup>¶</sup>Jolly, D., Second Annual Meeting of the American Society of Gene Therapy, June 9–13, 1999, Washington, DC, p. 52.

above, another trial for hemophilia A involves the implantation of genetically modified autologous fibroblasts expressing B domain-deleted factor VIII, and a third involves intramuscular injection of a recombinant adeno-associated vector (rAAV) expressing factor IX. Additional trials in which an AAV vector is infused into the hepatic circulation for liver-directed gene transfer are in the planning stages and are based on strong preclinical data in hemophilia B mice and dogs (42–44). At this time, it is still not clear whether a high-titer functional factor VIII cDNA will fit within the limited confines of a rAAV vector. Liver-directed gene therapy with rAAV vectors has not yet taken place, and indeed issues of safety have been raised in the current adult hemophilia population, most of whom are infected with hepatitis viruses. These patients have ongoing inflammation and immunological factors that may predispose to inhibitor formation. Currently it would appear that the most prudent approach is to foster the development of all of these strategies. The hemophilia patient population is a heterogeneous one; many patients are infected with hepatitis and may not be candidates for a liver-directed approach and others, infected with HIV, are on antiretroviral medications and may not be candidates for retroviral or lentiviral-based strategies. The simultaneous development of different strategies is likely to offer the best solution for those suffering from the disease.

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