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## THE HEMOPHILIAS — FROM ROYAL GENES TO GENE THERAPY

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OF the various types of hemophilia, the most common of these lifelong bleeding disorders are due to an inherited deficiency of factor VIII or factor IX (Table 1). The genes for these blood coagulation factors lie on the X chromosome, and when mutated, they cause the X-linked recessive traits hemophilia A and B. Since these disorders are X-linked, they usually occur in males. Usually, the affected boy has inherited the mutant gene ( $X_H$ ) from his carrier mother ( $X_H/X$ ), but about 30 percent of cases arise from a spontaneous mutation, and there is no family history of hemophilia.

The incidence of hemophilia A is 1 in 5000 male live births, and that of hemophilia B is 1 in 30,000.<sup>1</sup> By contrast, a deficiency or dysfunction of the adhesive glycoprotein von Willebrand factor causes the most frequent bleeding disorder, von Willebrand's disease, which may affect 1 in 1000 or even more.<sup>1</sup>

Hemophilia is well known for its effect on the royal houses of Europe. Queen Victoria, a clinically normal carrier, had one son, Leopold, who had hemophilia and two daughters, Alice and Beatrice, who were carriers and who, in turn, transmitted the disease to the Russian, Prussian, and Spanish royal families. Since the two X-linked hemophilias are clinically indistinguishable and none of the descendants of Queen Victoria who were known to be affected are alive (the last one, Waldemar, died in 1945), we may never know which type of hemophilia they had. Victoria's great-great-granddaughter Olympia, from the Spanish branch, had a son, Paul Alexander, who died in childhood of a "blood" disorder, and she may therefore be the last surviving carrier.

Hemophilias occur in mild, moderate, and severe forms (corresponding to plasma coagulation factor levels of 6 to 30 percent, 2 to 5 percent, and 1 percent

**TABLE 1.** GENERAL FEATURES OF INHERITED DEFICIENCIES OF COAGULATION FACTOR ASSOCIATED WITH BLEEDING DISORDERS.

DEFICIENT COAGULATION FACTOR	INCIDENCE IN GENERAL POPULATION	CHROMOSOME INVOLVED	MODE OF INHERITANCE
Fibrinogen	1:1 million	4	Autosomal recessive
Prothrombin	1:2 million	11	Autosomal recessive
Factor V	1:1 million	1	Autosomal recessive
Factor VII	1:500,000	13	Autosomal recessive
Factor VIII	1:10,000	X	X-linked recessive
Factor IX	1:60,000	X	X-linked recessive
Factor X	1:1 million	13	Autosomal recessive
Factor XI	1:1 million	4	Autosomal recessive
Factor XIII	1:1 million	6 (subunit A) 1 (subunit B)	Autosomal recessive

or less of normal, respectively). Although patients with mild hemophilia usually bleed excessively only after trauma or surgery, those with severe hemophilia have an annual average of 20 to 30 episodes of spontaneous or excessive bleeding after minor trauma, particularly into joints and muscles. In some such patients the episodes are more frequent. These symptoms differ substantially from those of bleeding disorders due to platelet defects or von Willebrand's disease, in which mucosal bleeding predominates.

The modern management of hemophilia began in the 1970s, with the availability of plasma concentrates of coagulation factors. The widespread adoption of home-administered replacement therapy led to the early control of hemorrhages and thereby reduced or prevented the musculoskeletal damage typical in patients with inadequately treated disease. Hemophilia care became one of the most gratifying examples of the successful secondary prevention of a chronic disease. However, concentrates manufactured from pooled plasma obtained from thousands of donors were invariably contaminated with hepatitis B or C virus, and they caused post-transfusion hepatitis in practically all patients with hemophilia who received them. Chronic hepatitis was common but was thought to be relatively mild and nonprogressive, so that the benefits of concentrates seemed to outweigh the risks. This optimistic perception changed dramatically in the early 1980s, when 60 to 70 percent of patients with severe hemophilia in Western Europe and the United States became infected with human immunodeficiency virus (HIV), which had contaminated plasma concentrates.

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For the past 15 years safer plasma concentrates of coagulation factors have been produced, and genetically engineered recombinant factors are now available. Moreover, molecular techniques can now be used to identify the genetic lesions that cause hemophilia and thus facilitate prevention of the disease through the identification of carriers and antenatal diagnosis. New treatments have substantially improved the prognosis of patients in whom alloantibodies against factor VIII or factor IX (inhibitors) develop as a result of treatment with these factors.

Hemophilia A was reviewed in the *Journal* in 1994.<sup>2</sup> Since then there have been important advances in its treatment. Experience with recombinant antihemophilic factors has increased, and plasma-derived factors have become safer. In the past two years studies of the use of somatic gene therapy have been initiated, and the preliminary results are promising. But new issues of concern have emerged, particularly regarding the long-term consequences of the infectious complications of replacement therapy. This article will review recent progress in the diagnosis and treatment of hemophilia; more general information is available elsewhere.<sup>2</sup>

#### EFFECTS OF DNA AND BIOCHEMICAL TECHNIQUES

Cloning of the genes for factor VIII and factor IX made it possible to search for mutations in these genes in patients with hemophilia.<sup>3-5</sup> Progress was slow at first,<sup>6</sup> but the situation changed with the introduction of the polymerase chain reaction.<sup>7</sup> Given the ability to amplify and sequence each exon, numerous mutations in the genes for factor VIII, factor IX, and other coagulation factors were identified. It soon became apparent that about 40 to 50 percent of the mutations causing severe hemophilia A had been undetected as a result of an inversion of DNA sequences within intron 22 that disrupted the factor VIII gene.<sup>8</sup> Specific mutations are now used for the direct identification of genes for purposes of antenatal diagnosis and carrier analysis.

An analysis of mutations can also be used to predict to some extent the likelihood of the development of antibodies that inactivate factor VIII or factor IX. Among a large cohort of patients with hemophilia A, one fourth of whom had inhibitors, those carrying missense mutations and small deletions had a low incidence of inhibitors (4 percent and 7 percent, respectively), whereas those with the inversion involving intron 22, large deletions, and nonsense mutations that led to stop codons had a much higher incidence (34 percent, 36 percent, and 38 percent, respectively).<sup>9</sup> Nonsense mutations cause truncation of factor VIII or prevent its production, whereas with missense mutations or small deletions, some factor VIII is produced. The presence of even minute amounts of the factor lessens the likelihood that exogenous factor VIII

will be recognized as a foreign antigen. In patients with hemophilia B, the presence of inhibitors is almost always associated with large deletions and nonsense mutations.

Studies of the structure and function of factor VIII continue to yield new insights (Fig. 1 and 2). Some parts of the factor VIII molecule (the A2 domain, the C2 domain, and to a lesser degree, the A3 domain) are more immunogenic than others (the A1 and B domains).<sup>12</sup> Many of these immunogenic regions are located at the end of the C2 and A3 domains. These findings have implications for preventing the development of inhibitors through the use of recombinant factor VIII that has been modified to be less immunogenic.<sup>13</sup>

In plasma samples from some patients with hemophilia, the factor VIII level differs according to the type of assay: it is higher when measured by the one-stage assay than by the two-stage assay.<sup>14</sup> The discrepancy can be so great as to cause misdiagnosis, since the one-stage assay can give a normal value.<sup>15-17</sup> The mutations in patients with such results have been identified,<sup>15-17</sup> and all were found to lie at or very close to the interface of the A domains of the gene for factor VIII. Biochemical studies revealed the mechanism of the discrepancy in the assays. The activated mutant factor VIII is intrinsically less stable than normal activated factor VIII.<sup>18</sup> In the two-stage assay, this instability is made evident by the prolonged incubation used to maximize the formation of activated factor X, whereas the one-stage assay measures the generation of thrombin without preincubation.

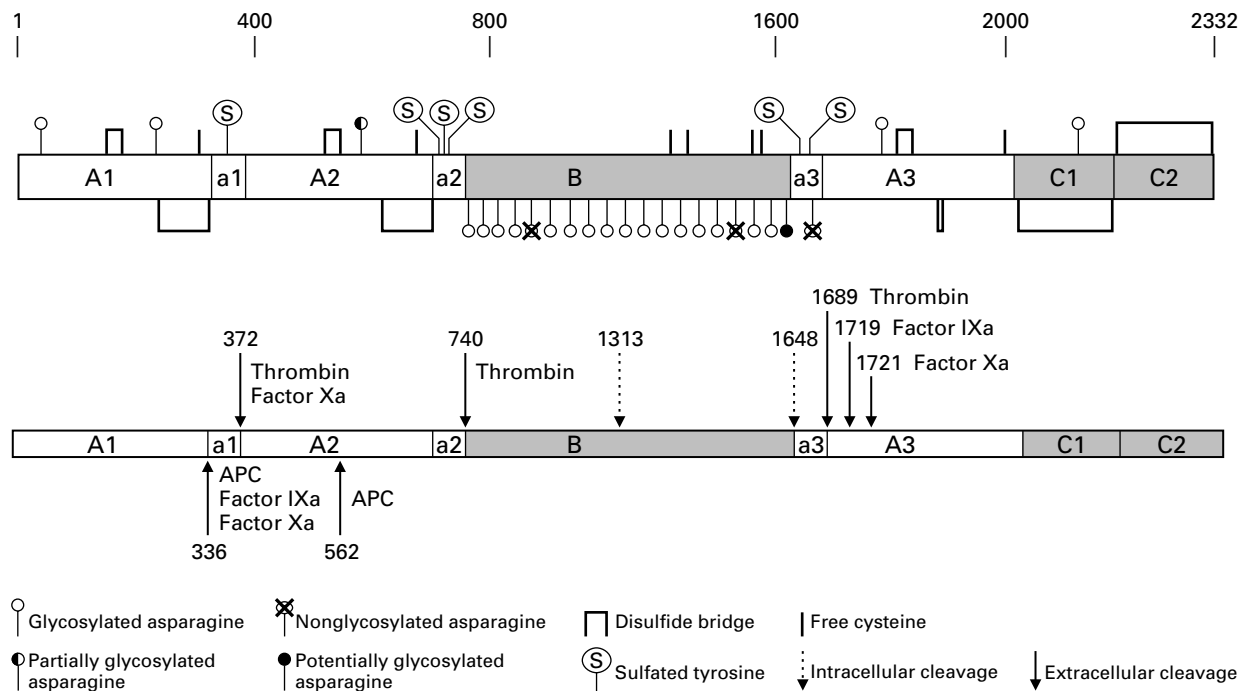
Increasingly detailed knowledge of the structure and function of factor VIII and of the tenase complex of activated factor VIII and activated factor IX (Fig. 2) permits deeper insights into the molecular abnormalities that cause hemophilia and has led to the production of a second-generation recombinant clotting factor that has no B domain. Eventually, it may be feasible to use a highly modified and structurally simpler protein with a longer plasma half-life to treat hemophilia.

#### THE CHOICE OF REPLACEMENT THERAPY

Whether to choose plasma-derived or recombinant factor VIII or factor IX is a dilemma for clinicians involved in the care of patients with hemophilia. Safety, cost, and availability are the determining factors. Because the limited availability of recombinant factors precludes their exclusive use, plasma-derived factors, which are much safer than in the past, are still commonly used.

#### Plasma-Derived Factors

The safety of plasma-derived factors depends on the viral load in the plasma concentrate and the degree of inactivation of these viruses. The procedures currently used to inactivate viruses include heating of concentrates at high temperatures (80°C or more), heating



**Figure 1.** The Structure of Human Factor VIII.

The upper panel shows the main post-translational modifications of the primary protein sequence. The sulfated tyrosine residues shown in short acidic domains (a1, a2, and a3) are essential for efficient binding of activating and inactivating proteases and for binding to von Willebrand factor. The B domain is heavily glycosylated, and the reason for this is unknown. The lower panel indicates the sites of proteolytic cleavage that occur intracellularly before secretion or extracellularly on activation by thrombin, activated factor IX (IXa), or activated factor X (Xa) or inactivation by activated protein C (APC). Adapted from Lenting et al.<sup>10</sup> with the permission of the publisher.

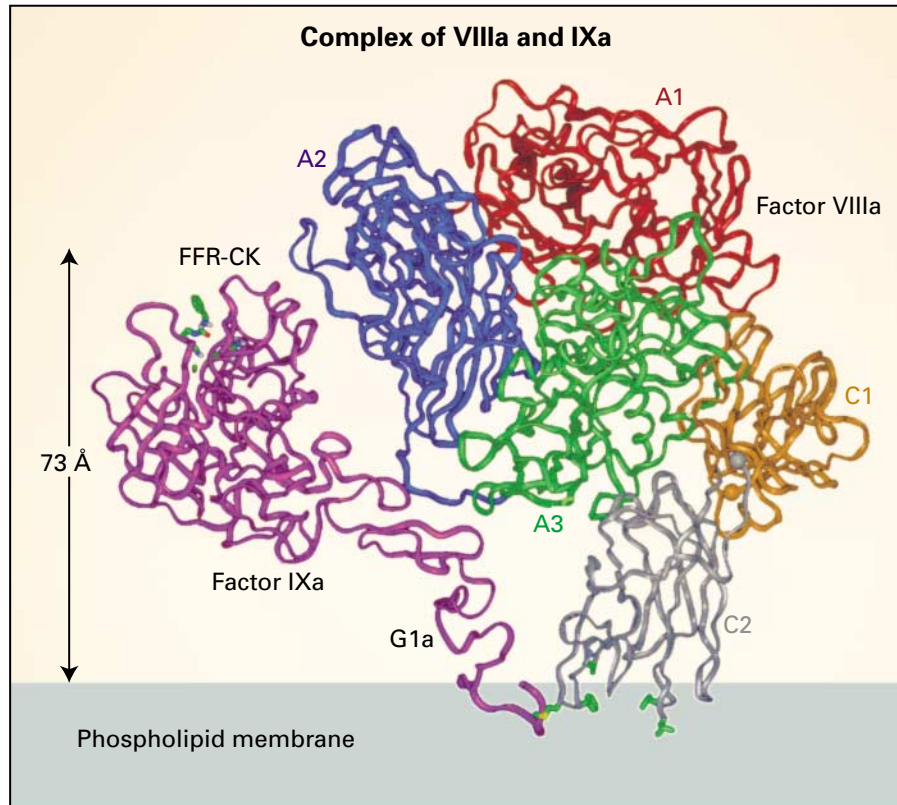
concentrates at 60°C in solution or with vapor, and adding a mixture of an organic solvent and a detergent to the concentrates.<sup>19,20</sup> The solvent–detergent mixture is widely used because it is highly efficacious in inactivating hepatitis B and C virus and HIV, but it does not inactivate viruses without a lipid envelope. This technical fault has led to outbreaks of hepatitis A in patients with hemophilia.<sup>21</sup> As a result, concentrate manufacturers now use at least two virus-inactivation procedures. To assess the viral load, pooled plasma or single units of plasma are screened with the use of assays involving the amplification of nucleic acids. This procedure has become obligatory in the United States and Europe.

These measures do not prevent the transmission of the highly thermoresistant parvovirus B19 by plasma concentrates.<sup>22</sup> Even though parvovirus B19 infection is normally of little consequence in patients with hemophilia, a few clinically significant complications have been reported.<sup>23,24</sup> In addition, the finding of parvovirus B19 infection is a signal that other bloodborne viruses may still be transmitted. Another perceived threat is the outbreak of new-variant Creutzfeldt–Jakob disease in the United Kingdom, which has raised the fear that prion proteins might be contained

in and transmitted by the human albumin used in the manufacture and formulation of some recombinant factors.<sup>25,26</sup> Several studies conducted in patients with hemophilia who have received multiple transfusions have conclusively shown that sporadic Creutzfeldt–Jakob disease has not been transmitted through transfusion blood or its derivatives.<sup>27–30</sup> However, these data are not completely reassuring, because new-variant Creutzfeldt–Jakob disease is caused by a different strain of prion, with a different incubation period. The number of blood donors who might carry such prions may be much higher than the number of donors who carry the prions that cause the sporadic form of the disease.

**Recombinant Products**

Two preparations of full-length recombinant factor VIII were licensed in the early 1990s. Clinical studies have demonstrated their excellent efficacy and the high correlation between the dose given and the level of factor VIII reached in plasma.<sup>31–34</sup> No antibodies were formed against the animal proteins used in producing the recombinant factor, nor was transmission of bloodborne or animal viruses demonstrated. In patients with hemophilia who have previously received



**Figure 2.** The Complex of Activated Factor VIII (VIIIa) and Activated Factor IX (IXa) Bound to the Phospholipid Membrane.

The points of contact between activated factor IX and activated factor VIII are derived from biochemical data, and the overall orientation is derived from two-dimensional structural analysis.<sup>11</sup> G1a denotes the  $\gamma$ -carboxyglutamic acid residue containing the domain of factor IX that binds to negatively charged phospholipids in cell membranes. Phe-Phe-Arg chloromethylketone (FFR-CK), an inhibitor necessary for crystallization, is covalently bound in the active site of activated factor IX. The active site is held at a specific distance from the membrane in order to optimize the activation of substrate factor X. Hydrophobic residues on the C2 domain of factor VIII are shown in green and are important for the binding of factor VIII to phospholipid membranes. Model courtesy of G. Kemball-Cook.

plasma-derived products, recombinant factor VIII infrequently triggers the formation of new inhibitors.<sup>31,34</sup> By contrast, in 25 to 30 percent of previously untreated patients inhibitors developed within the first 10 to 20 infusions.<sup>32,33</sup> However, in one third to one half of these patients, the inhibitors quickly disappeared spontaneously or remained at low titers; ultimately, the incidence of persistent inhibitors at high titers was no more than the expected 10 to 15 percent.<sup>35,36</sup> The current view is that recombinant factors are no more immunogenic than plasma-derived factors.<sup>37,38</sup>

Recombinant factor IX, licensed for the treatment of patients with hemophilia B,<sup>39</sup> is unique because no human or animal protein is used in its preparation or formulation. This feature should eliminate the risk of transmission of infectious agents of human or animal origin. Moreover, the high purity of the product should reduce the risk of thrombotic complications

that occur with other products used to treat hemophilia B (such as prothrombin-complex concentrates and plasma-derived factor IX concentrates). Studies of the pharmacokinetics and efficacy of recombinant factor IX in patients who had previously received plasma-derived products reported satisfactory results,<sup>39</sup> even though plasma levels after infusion are lower than those found after the infusion of plasma-derived factor IX, probably because of minor alterations in the structure of the recombinant protein.<sup>39</sup> There is no evidence that inhibitors of factor IX develop with high frequency in patients treated with recombinant factor IX.<sup>39</sup>

Recently, a new recombinant factor VIII, formulated in sucrose instead of human albumin, has been licensed in the United States and Europe.<sup>40</sup> Another new product lacks the large B domain of the full-length protein but retains coagulant activity.<sup>41,42</sup> Factor VIII that lacks the B domain is secreted more efficiently from cultured cells than is full-length factor

VIII and is less sensitive to proteolytic degradation.<sup>43,44</sup> No human albumin is added in the final formulation of this product. The manufacturing process includes a virus-inactivation step. Clinical trials of a preparation of recombinant factor VIII manufactured and formulated without human or animal protein are in progress.

#### Selecting the Appropriate Product

The choice of the product for replacement therapy must take into account three facts: plasma-derived factors are becoming ever safer, recombinant factors cost two to three times as much as plasma-derived factors, and the limited capacity to produce recombinant factors often causes periods of shortage. Because recombinant factors are perceived as safer than plasma-derived factors, nearly all patients with hemophilia in some countries receive recombinant products. In the United States, approximately 60 percent of patients with severe hemophilia use recombinant products. Italy and the United Kingdom give priority for the use of recombinant factors to patients with newly diagnosed, previously untreated hemophilia and to patients in whom bloodborne infections have not developed despite previous exposure to plasma-derived factors.

#### TREATMENT OF PATIENTS WHO HAVE INHIBITORS

Until the 1980s, the risk of death from uncontrollable bleeding was high in patients with hemophilia who had inhibitors, particularly in the event of emergency surgery or when limb-threatening hemarthroses and muscle hematomas could not be treated optimally. Treatments that bypass factor VIII or factor IX have lowered the risk. The principle underlying these treatments is that the defect in intrinsic coagulation is bypassed by the use of activated forms of factors VII, IX and X. These activated factors are contained in the prothrombin-complex concentrates used in the routine treatment of factor IX deficiency. They are also manufactured in products containing factor VIII inhibitor-bypassing activity.<sup>45-47</sup> Randomized, placebo-controlled trials have shown that both types of products control 50 to 60 percent of episodes of spontaneous bleeding in patients with inhibitors.<sup>45-47</sup> This rate of success is lower than the rate of 85 to 90 percent obtained with one or two doses of factor VIII or factor IX in patients with hemophilia who do not have inhibitors.<sup>31-35</sup>

A new bypassing product, recombinant activated factor VII, has been licensed. Activated factor VII is thought to ensure hemostasis by binding, directly or in complex with tissue factor, to negatively charged phospholipids exposed on the surface of activated platelets.<sup>48</sup> Alternatively, its effect may be due to an increase in the ratio of activated factor VII to factor VII.<sup>49</sup> The product stops spontaneous hemorrhages and prevents excessive bleeding during complex surgical procedures in 70 to 75 percent of patients with

inhibitors.<sup>50,51</sup> Home therapy with activated factor VII makes early intervention possible in patients with inhibitors.<sup>52</sup>

Recombinant activated factor VII costs approximately \$1 per microgram. At the recommended dosages of 90  $\mu\text{g}$  per kilogram of body weight every two to three hours, the cost of treating a single episode of bleeding can easily exceed \$50,000. Few clinical centers can afford to use this product exclusively in patients with inhibitors. We recommend it as a first-choice treatment for patients who must undergo major surgery and for those with life-threatening hemorrhages. For hemarthroses, plasma-derived bypassing products are preferred.

#### SECONDARY DISEASES IN PATIENTS WITH HEMOPHILIA

Before the advent of virus-inactivation procedures, most patients with hemophilia who were treated with plasma factors became chronically infected with the hepatitis B virus, hepatitis C virus, and HIV. These viruses can have long-term consequences other than cirrhosis or acquired immunodeficiency; the most prominent of these is the development of tumors. A cohort study of HIV-infected patients with hemophilia found that the frequency of Kaposi's sarcoma was 200 times that in the general population<sup>53</sup> and the frequency of non-Hodgkin's lymphoma was 29 times that in the general population.<sup>53</sup>

Cirrhosis, which develops in 10 to 20 percent of patients with hemophilia who are chronically infected with hepatitis B virus or hepatitis C virus, increases the risk of hepatocellular carcinoma. In patients with hemophilia this risk is 30 times that in the general population.<sup>54</sup>

The introduction of highly active antiretroviral therapy has sharply reduced mortality and morbidity in HIV-infected patients with hemophilia.<sup>55,56</sup> However, combination therapies that include protease inhibitors appear to increase the susceptibility of these patients to spontaneous bleeding, particularly at unusual sites, such as finger and wrist joints.<sup>57,58</sup> The possibility that antiretroviral therapy might increase the risk of premature cardiovascular disease in patients with hemophilia<sup>59</sup> is also a cause for concern.

A complication of replacement therapy has been reported in 5 percent of patients with severe hemophilia B. Soon after an infusion of factor IX, life-threatening symptoms of anaphylaxis (bronchospasm, angioedema, and hypotension) develop.<sup>60</sup> The reaction is elicited by treatment with highly purified recombinant or purified plasma-derived factor, and also by less pure prothrombin-complex concentrates.

The use of replacement therapy with preparations containing factor IX is dangerous and usually not feasible in patients with a history of anaphylactic reactions or inhibitors, and in some cases repeated infusions have led to the development of the nephrotic syndrome.<sup>60</sup>

Recombinant activated factor VII containing no factor IX is often the only therapeutic option in these patients.

### GENE THERAPY

Of all the genetic diseases, the hemophilias probably represent the combination of features that makes a favorable response to gene-replacement therapy most likely. The clinical manifestations of these conditions are entirely attributable to the lack of a single gene product, which circulates in minute amounts in plasma. Tightly regulated control of gene expression is not essential; a small increase in the plasma level of the protein markedly ameliorates the symptoms in severe cases. Animal models are available. Many different types of cell are capable of making coagulation factors, and the site of synthesis is not critical to function.

Three trials of gene therapy in patients with hemophilia A or B are now under way. One study in patients with hemophilia B is evaluating the safety of intramuscular injections of an adenovirus vector.<sup>61</sup> At the lowest dose of the adenovirus vector containing the gene for factor VIII, plasma levels of factor IX ranged from 0.5 to 1.6 percent of normal and less use of concentrates was evident.<sup>61</sup> The second study involves patients with severe hemophilia A and entails the intravenous injection of a vector based on a murine leukemia retrovirus containing complementary DNA (cDNA) for factor VIII that lacks the B domain. The results of this study have not been released. In the third study dermal fibroblasts from patients with severe hemophilia A were grown in culture, transfected with cDNA for factor VIII that lacked the B domain, and then laparoscopically reimplanted into peritoneal fat.<sup>62</sup> Six patients with severe hemophilia A (all of whom had chronic hepatitis C and four of whom had HIV infection) have been treated so far, and four of them had measurable plasma levels of factor VIII and a decreased frequency of bleeding or transfusion requirements. However, these favorable effects were transient and lasted no longer than 10 months.

Although the preliminary data are encouraging, several questions remain. The plasma levels of factor VIII or IX reached so far with the use of gene therapy (1 to 2 percent of normal, but often less) are insufficient to free patients from the need for exogenous coagulation factors. Levels of at least 5 percent of normal are required to prevent episodes of spontaneous bleeding and to guarantee that supplementary factors are needed only in cases of trauma or surgery. The risk of developing inhibitors is still of concern, particularly in previously untreated patients. HIV-infected patients who are receiving highly active antiretroviral therapy may have a poor response to retrovirus-based approaches; those with chronic hepatitis may have a poor response to the insertion of viral vectors into the liver.

### CONCLUSIONS

In the past three decades, hemophilia has moved from the status of a neglected and often fatal hereditary disorder to that of a defined group of disorders with a molecular basis for which safe and effective treatment is available. Hemophilia is likely to be the first common severe genetic condition to be cured by gene therapy. Apart from the long-term consequences of viral infections transmitted by infected blood products, there seem to be only two remaining problems.

First, there is the difficult problem presented by patients with high titers of antibodies against factor VIII or factor IX. Perhaps methods of inducing early immune tolerance (some of which have already been tested in animal models<sup>63</sup>) or the use of recombinant factors that lack immunogenic regions of factor VIII or factor IX<sup>13</sup> will prevent inhibitors from developing in patients with newly diagnosed hemophilia.

Second, it remains a challenge to society that four fifths of all patients with hemophilia, mainly those in developing countries, receive no treatment.<sup>64</sup> Large-scale production and purification of factor VIII and factor IX from the milk of transgenic farmyard animals could provide a less expensive source of replacement therapy for developing countries. Affordable gene therapy will be the ultimate solution for all patients with hemophilia. We can confidently predict that the new millennium will see an end to this ancient scourge.

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