Gene Transfer as an Approach to Treating Hemophilia

Katherine A. High

Abstract—Hemophilia is an X-linked bleeding diathesis caused by a deficiency of either factor VIII or factor IX. Present treatment for hemophilia involves intravenous infusion of either recombinant or plasma-derived clotting factor concentrates. Problems with this treatment method, including the expense, need for intravenous access, and risks of blood-borne disease transmission, have fueled an interest in developing a gene-transfer approach to treatment. On the basis of experience with protein concentrate therapy, it seems likely that even modest elevations in circulating levels of factor VIII or factor IX can prevent most of the mortality and much of the morbidity associated with the disease. Hemophilia has a number of advantages as a model system for working out strategies for gene transfer as an approach to the treatment of genetic diseases; these include wide latitude in choice of target tissue, a wide therapeutic window for levels of circulating factor, ease of determining therapeutic endpoints, and existence of excellent animal models of the disease. Preclinical studies over the last decade have recently culminated in the initiation of clinical trials of gene transfer for hemophilia A and B. Three trials, each using different vectors and target tissues, are presently underway, and two additional trials are in late planning stages. This report reviews the preclinical data underlying these strategies and the design of the ongoing and proposed clinical trials. (Circ Res. 2001;88:137-144.)

Key Words: hemophilia A ■ hemophilia B ■ gene therapy ■ factor VIII ■ factor IX

Hemophilia is an X-linked bleeding diathesis resulting from a deficiency of blood coagulation factor VIII (F.VIII) (hemophilia A) or factor IX (F.IX) (hemophilia B). Clinically, the disease is characterized by frequent spontaneous bleeding episodes, mostly into joints or soft tissues. Bleeding can also occur into other critical closed spaces, such as the intracranial space or the retroperitoneal space, where it can be rapidly fatal. Hemophilia A occurs in \( \approx 1 \) in 5000 male births; hemophilia B is less common, occurring in \( \approx 1 \) in 30 000 births. Still, hemophilia is one of the most common genetic disorders, and prevalence of the disease is the same in all populations studied. Hemophilia is classified as mild, moderate, or severe on the basis of circulating levels of clotting factor; severe disease is defined as \(< 1\%\) of normal levels, moderate as \(1\% \) to \(5\%\), and mild as \(> 5\%\). Life expectancy for individuals with hemophilia increased dramatically with the introduction of clotting factor concentrates in the 1960s, but contamination of these with hepatitis viruses and later with human immunodeficiency virus (HIV) has had devastating effects for the hemophilia population. Thus, in the 1950s, the leading cause of death in hemophilia was fatal bleeding episodes, whereas today the two leading causes of death are HIV-related disease and end-stage liver disease. Other disadvantages of the present protein-based therapy include the expense of the product, which can reach $50 000 to $100 000 per year for an individual with severe disease, and the inconvenience of managing a chronic disease with a medication that must be infused intravenously. These considerations have fueled an interest in developing a gene-based approach to treating hemophilia.
Experience with prophylactic regimens of protein concentrates over the last 30 years has established that continuous maintenance of circulating levels of clotting factor $>1\%$ is adequate to prevent most of the mortality and much of the morbidity associated with the disease. These data provide a strong rationale for the potential for success of a gene-based approach. Compared with other genetic diseases, hemophilia has several characteristics that are likely to facilitate the development of a gene-transfer approach to treatment. Biologically active clotting factors can be synthesized in many different cell types, so that there is latitude in choice of target cells. The therapeutic window is wide, because factor levels as low as $1.5\%$ of normal are likely to improve the clinical symptoms of the disease, and levels of $100\%$ are still within normal limits. (However, recent studies suggest that levels in excess of $100\%$ may predispose to thrombosis and should probably be avoided.) There are large and small animal models of the disease (genetically engineered mice and naturally occurring dog models), and the murine and canine F.VIII and F.IX genes have been cloned and are available, allowing detailed feasibility studies before moving to clinical trials. Finally, determination of therapeutic efficacy is straightforward in the case of hemophilia, because circulating levels of clotting factor are easy to measure and correlate well with clinical manifestations of the disease.

**Considerations for Clinical Trials**

**Clinical Endpoints**

Determination of clinical efficacy has been a source of difficulty for some diseases treated through a gene-therapy approach but is unlikely to present a problem in the case of hemophilia. A wealth of clinical and laboratory data document that plasma F.VIII and F.IX activity levels correlate well with clinical severity of disease. As noted above, the Swedish prophylaxis studies, using as a goal maintenance of trough factor levels of $>1\%$, have documented prevention of joint disease and life-threatening bleeds at this level. Thus, levels of $1\%$ to $5\%$ are likely to result in considerable improvement of disease phenotype, and levels of $5\%$ to $20\%$ are likely to free patients from any substantial need for clotting factor concentrates except in the setting of surgery or trauma. It is unclear at this time whether it is prudent to aim for normal levels without incorporating a control element that would allow for downregulation of expression in the event of an overshoot. The use of secondary endpoints, such as total factor usage and number of bleeding episodes, would also be of interest, but such data are unlikely to be considered convincing in the absence of measurable activity levels in the circulation. An exception to this would be strategies that are targeted to specific cell types (platelets or synovial cells) that are abundant at bleeding sites but are unlikely to yield detectable circulating levels of F.VIII or F.IX.

**Inhibitory Antibodies**

An important issue facing all gene therapy trials for hemophilia is the risk of forming inhibitory antibodies to the transgene product. Presently, formation of neutralizing antibodies, or inhibitors, is the most common complication of protein-based therapy, occurring in $\approx20\%$ of patients with hemophilia A and $\approx3\%$ of those with hemophilia B. Despite years of study, it is still not possible to predict with certainty which patients will develop inhibitory antibodies in the setting of protein-infusion therapy, but certain risk factors have been identified, including the nature of the underlying mutation in the clotting factor gene, inherited characteristics of the individual’s immune response, and the circumstances surrounding exposure to the clotting factor protein (ie, presence of tissue injury or inflammation).

It is likely that there are differences in antigen presentation of clotting factor epitopes in gene-based versus protein-based treatment. When clotting factor is infused intravenously, antigen presentation occurs primarily in the setting of major histocompatibility complex class II determinants, which display peptides derived from proteins taken up from the environment. In the setting of gene therapy, though, antigen presentation may also occur through major histocompatibility complex class I, which presents peptides derived from proteins synthesized within the cell that displays them. Thus, gene therapy, which results for the first time in endogenous synthesis of the wild-type protein in a recipient, may be characterized by a different immune response to the transgene product compared with responses seen in protein infusion therapy.

Factors that are likely to influence inhibitor formation in the setting of gene therapy include the vector itself, target tissue used, dose of vector, and inclusion of tissue-specific promoter elements. Vectors that elicit a strong immune response to the viral proteins (eg, adenoviral vectors) are more likely to elicit an immune response to the transgene product as well. Target tissues rich in professional antigen-presenting cells might also predispose to inhibitor formation. Although a clear understanding of the immunologic mechanisms underlying antigen presentation and immune response in the setting of gene therapy is not yet available (the same may be said of the present protein-based method of treatment), a few comments can be made. First, it is clear that each therapeutic system, consisting of a specific transgene, vector, and target tissue, constitutes a different problem. It will in general not be accurate to make statements about any one of these without defining the other parameters as well. Second, the immune response will be influenced by characteristics of the subject, including the underlying mutation, which will determine the level of tolerance to the transgene product, and inherited characteristics of the immune response, which will determine details of antigen processing and presentation in the recipient. These facts underscore the critical importance in cases of genetic diseases like hemophilia of carrying out preliminary experiments in animal models of the disease, where tolerance to the transgene product may be lacking or altered, as is the case for humans with the disease. A more comprehensive discussion of immune response to the transgene product in the setting of gene therapy for hemophilia is available.

**Influence of Comorbid Conditions: Hepatitis and HIV**

Among adults with severe hemophilia, the prevalence of HIV infection is high ($\approx82\%$ for patients with hemophilia A and $48\%$ for those with hemophilia B), as is the prevalence of infection with hepatitis B (90%) and hepatitis C (80%). These prevalent comorbid conditions are likely to have
implications for gene therapy for hemophilia. For example, the presently recommended antiretroviral therapy (highly active antiretroviral therapy) is likely to block transduction with retroviral and lentiviral vectors, but stopping the medication is generally contraindicated, because it may facilitate emergence of resistant strains (although this is a rapidly evolving area, and brief interruption of therapy in individuals who are fully suppressed [<50 copies virus/mL] may be acceptable). Similarly, the effect of coexisting hepatitis on liver-directed gene therapy is unknown. It is possible that the presence of ongoing inflammation in the liver may predispose to inhibitor formation; alternatively, patients with hepatitis C may be less likely to mount an immune response. Efforts to assess this question may be facilitated by the recent development of animal models of hepatitis infection.17

Risks Associated With Integration
For many of the viral and nonviral treatment strategies under consideration, successful gene transfer will result in integration of the donated transgene at random sites within the recipient genome. The long-term consequences of such events are unknown, but the concern exists that integration into a crucial gene sequence, eg, a tumor-suppressor gene, could result in inactivation of the critical gene and an increased likelihood of malignant transformation. Data specifically addressing this point continue to accrue as the number of long-term survivors of therapy with integrating vectors increases. It should be recognized, however, that extended periods of follow-up will be required to determine whether any substantial risk attaches to the theoretical concerns regarding insertional mutagenesis. A conservative approach, until more data are available, would be to limit initial trials with integrating vectors to older subjects who have relatively fewer years of potential life remaining. Such an approach would allow accrual of additional information on effects of integrating vectors while protecting the population that would be most affected by late-appearing complications.

Risk of Inadvertent Germline Transmission
A potential consequence of gene therapy is the introduction of foreign DNA into the gonads of recipients and, therefore, potentially into the germ cells of these individuals.18,19 This could result in the transmission of the donated gene sequences to subsequent generations. If this resulted in permanent correction of the genetic defect, it could hardly be viewed as a deleterious side effect, but because most vectors integrate randomly, the concern exists that the donated sequences may result in harm to the offspring if, for example, the site of integration disrupts a critical gene sequence in the developing embryo19 or if expression of the donated gene sequence somehow disrupts the normal program of development. Thus, a part of the safety assessment of every gene-therapy strategy is a determination of the likelihood that donated gene sequences will be transmitted to future generations. In general, these risks are lower for ex vivo strategies than for in vivo gene transfer; there has been no evidence of germline transmission of vector sequences in the ongoing hemophilia trials, two of which use in vivo gene delivery. It will be important to continue to collect data in this area.

Clinical Gene Transfer for Hemophilia
On the basis of considerations discussed above, it is likely that in the case of hemophilia, more than one successful gene-based approach to treatment can be developed. In a sense, this can be viewed as analogous to the situation with antibiotics: multiple drugs are available, and physicians choose the most suitable one on the basis of the organism to be treated and the side effects of the drug. In the case of hemophilia, the disease state to be treated is the same, but patients differ considerably in comorbid conditions that may influence the choice of treatment. Thus some patients are on antiretroviral drugs and cannot be treated with retroviral or lentiviral regimens, whereas those with liver disease from hepatitis infection may not be good candidates for liver-directed treatment approaches. At this point, the hemophilia population is probably best served by the continued simultaneous development of multiple approaches; one of the major challenges of the next few years of clinical research in this area will be to define which subgroups of patients can be most safely and effectively treated with which approaches. If some approaches are much safer and more effective than others, then these will become the benchmark standards against which new strategies will need to be assessed.

Gene transfer strategies are characterized by 3 essential elements: the gene delivery vehicle, or vector; the gene to be transferred, sometimes referred to as the transgene; and a specific target cell, which may determine the route of administration of vector. Gene transfer strategies are often referred to by a shorthand that lists these 3 key elements, eg, adeno-associated viral (AAV)-F.IX-liver. Another important characteristic of gene transfer is whether it is given ex vivo (outside the body), as is generally done when hematopoietic stem cells are the target, or in vivo, ie, vector is injected directly into the recipient. Both ex vivo and in vivo approaches are currently under investigation as methods of treating hemophilia.

In the rest of this review, discussion of therapeutic strategies will be divided into 3 parts. The first section will review preclinical data and early clinical results for approaches that are already in clinical trials. The second section will review other promising strategies that are presently under review by the regulatory agencies and intended for initiation in 2001. The third section will review strategies that are at earlier stages and not yet validated in large animal models or contemplation for clinical trials but are attractive according to early preclinical studies.

Ongoing Clinical Trials
Retroviral-Mediated Approaches
Regarding clinical trials in the field of gene therapy, by far the largest volume of experience has been with retroviral vectors. The extant peer-reviewed and published preclinical data using retroviral vectors to treat hemophilia are best summarized in 2 studies, one from 199321 and the other from 1999.22 In the first, Kay et al21 prepared a retroviral vector expressing canine F.IX and infused it into the portal vein of hemophilic dogs that had undergone partial hepatectomy to induce replication (required for retroviral transduction) in the
remaining hepatocytes. They were able to demonstrate long-term expression of canine F.IX (>2 years) but at levels that were far too low to be therapeutic in humans (2 to 4 ng/mL, <0.1% normal human plasma levels). Because even these low levels required antecedent partial hepatectomy, this strategy was not acceptable as a treatment approach in humans. VandenDriessche et al found one way around this obstacle by using newborn mice, in which the rate of hepatocyte proliferation is very high. They demonstrated that intravenous injection of a retroviral vector expressing human F.VIII into newborn mice with hemophilia A was associated with high-level expression of F.VIII in mice that did not develop inhibitory antibodies (detected in 7 of 13 mice). Other investigators have explored the use of growth factors to enhance retroviral transduction of the liver in animal models. Experimental evidence indicates that some baseline level of retroviral transduction can occur in adult animals even in the absence of a stimulus to hepatocyte replication, but it is not entirely clear which cells are transduced in this setting. A presently ongoing phase I trial sponsored by the Chiron Corporation is designed to assess the safety of intravenous infusion of a retroviral vector expressing human F.VIII into adult subjects with severe hemophilia A. The trial has an open-label dose-escalation design. As of June 2000, 10 subjects had been enrolled, with a projected total enrollment of ~20 subjects. No serious adverse events have been reported, and enrollment is continuing.

Plasmid-Based Approach
A second approach that is currently being evaluated for treatment of hemophilia A is ex vivo introduction of a plasmid expressing B-domain–deleted (BDD) F.VIII into autologous fibroblasts, which are then reimplanted on the omentum. In this strategy, a skin biopsy from the patient serves as a source of autologous fibroblasts, which are then transfected by electroporation with a plasmid expressing BDD F.VIII and a selectable marker. After transfection, F.VIII-expressing cells are selected, expanded, and additionally characterized. These maneuvers require ~7 weeks; when adequate numbers of cells are available (on the order of 10^9 to 10^10), the cells are reimplanted on the omentum in a laparoscopic procedure. This trial is sponsored by Transkaryotic Therapy, Inc, and is being conducted at the Beth Israel Deaconess Medical Center in Boston. As of the spring of 2000, 6 subjects with severe hemophilia A had been enrolled in the trial, and no major safety problems had been encountered. A preliminary report at the 42nd annual meeting of the American Society of Hematology noted F.VIII levels of 1% to 2% in 3 of 6 subjects studied, with expression lasting for months. There are no published reports of preclinical studies of F.VIII expression with this strategy, and thus it is difficult to predict the likelihood of success. Some scientific aspects of the present protocol are unclear. The cells that are eventually implanted have undergone many divisions, on the order of 30 to 50. It is unclear whether this fact has safety implications. Because experiments have not been carried out in immunocompetent hemophilic animal models, it is not clear what the risk of inhibitor formation will be in null patients who undergo treatment. On the other hand, this approach has several attractive features. Gene transfer occurs ex vivo, so that risk of germline transmission of vector sequences is virtually nil. Second, because a single integrant is selected for expansion, risks of insertional mutagenesis are minimized. The results of the present clinical trial should help to clarify the safety and feasibility of the approach.

AAV Vector–Expressing F.IX Delivered to Skeletal Muscle
A third trial, this one for hemophilia B, makes use of an AAV vector. AAV vectors in present use are engineered from a parvovirus, AAV serotype 2, with a small (4.7 kb) single-stranded DNA genome. Many individuals are infected with the wild-type virus as children, but infection is not associated with any known illness. The virus is naturally replication-defective, and the engineered vector is completely devoid of viral coding sequences. Preclinical studies by several groups have shown that AAV vectors can direct sustained expression of a transgene introduced into skeletal muscle, liver, or central nervous system. In the case of F.IX, preclinical studies in support of this approach are published and demonstrate that doses of ~10^12 vector genomes (vg)/kg introduced into skeletal muscle in the hindlimbs of mice resulted in F.IX levels of 250 to 350 ng/mL (5% to 7% normal circulating levels), whereas similar doses in hemophilic dogs (~8.5×10^12 vg/kg) resulted in levels of 70 to 80 ng/mL (~1.5% normal levels). On the basis of these efficacy studies and additional safety studies in mice, rabbits, rats, and hemophilic dogs, a phase I study was initiated using an AAV vector expressing human F.IX under the control of the cytomegalovirus promoter. The study has an open-label dose-escalation design, with 3 subjects in each of 3 dose cohorts. Initial subjects were injected with a dose of 2×10^11 vg/kg, with a planned dose escalation of 1 log between the low- and mid-dose groups. However, because one of the subjects in the low-dose group consistently showed levels of >1% beginning ~10 weeks after treatment (consistent with the known time course of AAV expression), the pace of dose escalation was slowed to one-half log between cohorts. Muscle biopsies obtained 2 months after injection have shown evidence of gene transfer by polymerase chain reaction and Southern blot analysis and evidence of expression of the donated gene by immunohistochemical staining. Treatment of subjects in the high-dose group (2×10^12 vg/kg) has recently begun. As of the date of this review, there have been no serious adverse events associated with vector administration, including no evidence of inhibitory antibody formation or germline transmission of vector sequences. It is expected that dose escalation will continue until all subjects within a cohort achieve F.IX levels >1% or until toxicity is encountered. A recent development of interest is the report that higher levels of transgene expression can be obtained using a different serotype of AAV. Xiao et al have reported a 3- to 10-fold increase, whereas Chao et al have reported a 1000-fold increase in circulating levels of a secreted transgene product.

Trials in Planning Stages
AAV Vectors Introduced Into Liver
In January 1999, Snyder et al reported sustained expression of F.IX levels of 1% to 2% in hemophilic dogs after injection...
of AAV into the portal vein. A report by Wang et al in February 2000 documented levels of 4% using an AAV vector with a liver-specific promoter, and higher levels have since been achieved in dogs through additional engineering of the construct at doses that are lower (in vg/kg) than those presently being used in the high-dose subjects in the muscle trial. Such trials are now under review by regulatory agencies and will likely be structured as open-label dose-escalation studies, with vector to be infused into the hepatic artery under radiographic visualization. AAV vectors have not previously been infused into the liver in human subjects. Safety studies by several groups have demonstrated an absence of liver-related toxicity after infusion of AAV-F.IX into dogs and nonhuman primates. In addition, there has been no evidence of vector-related toxicity in human subjects injected with AAV-F.IX at intramuscular sites. These preclinical data seem quite promising, but it must be noted that the effects of coexisting hepatitis (>80% of individuals with severe hemophilia are hepatitis C–positive) are difficult to predict and cannot easily be modeled in other animals. The effect of preexisting antibodies to AAV serotype 2 (neutralizing antibodies present in 32% of normal adults) also cannot be predicted on the basis of animal studies, although one would expect that these may reduce the efficiency of gene transfer, because the antigenic determinants on capsid proteins are identical in the vector and the wild-type virus.

Efforts are also underway to extend the use of a liver-directed AAV approach to F.VIII, but the size of the transgene presents a problem in this case, because AAV vectors cannot accommodate inserts above ≈5 kb and the B domain–deleted F.VIII cDNA (without promoter, intron, or viral-inverted terminal repeats) is 4.4 kb. Because of these size constraints, several novel strategies have been devised to allow expression of F.VIII from an AAV vector. In the first of these, described by Burton et al, two vectors are constructed, one expressing the heavy chain (A1 and A2 domains) and the other the light chain (A3, C1, and C2) of F.VIII. After introduction of vectors into the portal circulation of mice, bioactively active F.VIII is produced in the circulation at supraphysiologic levels (200 to 400 ng/mL), presumably from hepatocytes that are cotransduced with both vectors. Chao et al have approached the problem differently by constructing a single vector with a small promoter. Using a minigene consisting of BDD F.VIII driven by the thymidine kinase promoter linked to a hepatitis B enhancer, this group showed F.VIII levels of 55 ng/mL in nonobese diabetic/severe combined immunodeficiency mice after portal-vein injection of vector at a dose of 6×10^12 viral particles/kg. A third strategy proposed by Duan et al takes advantage of the molecular configuration of recombinant AAV within a transduced cell. Because the vector genome is present within transduced cells as head-to-tail concatamers, two vectors can be constructed: one containing regulatory elements and a splice donor, and the other containing a splice acceptor, the transgene of interest, and a polyadenylation signal. Whether this strategy will be successful for AAV-mediated expression of F.VIII remains to be seen.

Both the liver-directed and muscle-directed approaches have shown efficacy in the canine model, and it is not yet clear which will be more useful clinically. There is a clear dose-advantage in favor of liver on the order of 1 to 2 logs with presently available constructs; this is likely accounted for primarily by the more efficient transit of F.IX into the circulation from the hepatocyte compared with transit from skeletal muscle fibers. In addition, it is likely that all posttranslational modifications will be executed accurately and efficiently in hepatocytes, whereas some modifications affecting F.IX recovery are not efficiently performed in skeletal muscle. On the other hand, introduction of vector into skeletal muscle can be done by simple intramuscular injections, whereas introduction of vector into the liver will require an invasive procedure in which a catheter is introduced into the hepatic artery. An additional uncertainty regarding the liver-directed approach is the effect of underlying hepatitis on vector transduction and vice versa (vide supra). Among adults with severe hemophilia, >90% have been exposed to hepatitis B and >80% to hepatitis C. The effect of ongoing inflammation and altered cytokine profiles within the target tissue may increase the likelihood of inhibitor formation in the setting of gene transfer. Finally, if sites of injection into skeletal muscle are judiciously selected, one could conceivably reverse the procedure by resecting the injected sites if some unanticipated adverse event were to occur; on the other hand, introduction of vector into the liver is an irreversible event. Given these considerations, it is appropriate that skeletal muscle was selected as the initial site for parenteral injection of AAV vector, but liver may eventually be the better target, especially for individuals who are not infected with hepatitis. At this point, the best course of action is to continue efforts to develop clinical approaches using both of these target tissues. It may be that both will be useful but will have different indications, for example, depending on presence of hepatitis or other factors.

Adenoviral Vectors

Adenoviral vectors have several attractive features as gene delivery vehicles, including ease of preparation and efficient transduction of liver after introduction of vector into the peripheral circulation. These characteristics were exploited by Kay et al to obtain high-level expression of canine F.IX in hemophilic dogs as an early proof of principle for this approach. However, expression was short-lived, and work by many other groups has now established that the immune response to the vector, characterized by a cytotoxic T-lymphocyte response against cells harboring the vector, will make it problematic to obtain long-term expression using early-generation adenoviral vectors. However, several important insights about adenoviral vectors have been gained through the work of Connelly and colleagues, who have explored the use of earlier generation adenoviral vectors as an approach to treating hemophilia A. Using an adenoviral vector expressing B domain–deleted F.VIII, these workers were able to demonstrate phenotypic correction of the bleeding diathesis in mice with hemophilia A. Levels of expression were initially >2000 mU/mL and, as expected, declined gradually over 9 months to ≈100 mU/mL; lower doses of
vector resulted in longer-term expression, presumably attributable to a less vigorous immune response. Attempts to extend this approach to the canine model have been hampered by hepatotoxicity and inhibitor formation, the latter perhaps linked to the former (vide supra).51 Studies by several groups has been directed to the task of deleting all of the adenoviral backbone genes with the goal of diminishing toxicity and prolonging expression.52–58 Balagüé et al59 have described the use of a fully deleted adenoviral vector to correct the phenotype of mice with hemophilia A. Using a construct containing the full-length human F.VIII cDNA under the control of a 12.5-kb albumin promoter, this group has demonstrated long-term expression of therapeutic levels of F.VIII (100 to 800 ng/mL) in 3 of 16 hemophilic mice. The remaining 13 mice developed antibodies to human F.VIII within 3 to 8 weeks of vector administration, so that F.VIII levels could no longer be measured. Administration of the vector was not associated with any evidence of hepatotoxicity in the mice, a finding that has been noted by others working with fully deleted adenoviral vectors.60

A finding documented with earlier-generation adenoviral vectors is a cytokine release syndrome, which occurs within hours of systemic administration of vector. In studies using an E1,E4-deleted vector, Wilson61 documented release of very high levels of interleukin-6 in mice, nonhuman primates, and humans. The underlying cause of the dose-dependent cytokine release is unclear; similar findings have been documented in mice and nonhuman primates after infusion of ultraviolet-irradiated adenoviral vector. Ongoing studies will determine whether cytokine release is seen with fully deleted adenoviral vectors.

Two other issues that will need to be addressed before gutted adenoviral vectors are used in the setting of hemophilia are the degree of contamination of clinical grade preparations by helper virus and the existence of a threshold effect, which has been described by Bristol et al62 for both earlier-generation adenoviral vectors and gutted vectors. The threshold effect refers to a phenomenon where the dose response is linear down to a certain dose, below which there is no expression.63 The existence of this phenomenon has implications for dosing in a clinical trial.

A phase I study of a fully deleted adenoviral vector for hemophilia A is now in late planning stages. The trial is sponsored by the Genstar Corporation and is structured as an open-label dose-escalation study, with 3 subjects in each of 3 dose cohorts. Vector is to be infused intravenously at doses ranging from 4.3×10^10 vector particles (vp)/kg to 4.3×10^13 vp/kg.

**Other Strategies in Early Preclinical Stages**

**Lentiviral Vectors**

Lentiviral vectors,64 a newer gene delivery vehicle based on HIV, have also been shown to transduce liver, muscle, and hematopoietic cells and thus could potentially be used for gene therapy for hemophilia. Work published 3 years ago by Kafri et al65 demonstrated stable expression (22 weeks) of a humanized GFP after direct intraparenchymal injection into liver of a lentiviral vector. The authors showed that the percent of cells transduced at the site of injection was high (~90%), comprising up to 3% to 4% of hepatocytes, and that the vector could be successfully re injected. Park et al67 recently reported that antecedent partial hepatectomy increases lentiviral transduction efficiency by ~30-fold in mouse hepatocytes after infusion of vector into the portal vein; in addition, in colabeling experiments with bromodeoxyuridine and a lentiviral vector expressing lacZ, it was shown that ~90% of cells expressing lacZ were colabeled with bromodeoxyuridine. These findings raise the possibility that DNA synthesis may be required for efficient lentiviral transduction in vivo in mice. The demonstration that dose-dependent increases in serum transaminases occurred with lentiviral vector infusion suggests a mechanism (liver injury) for increased cell cycling. Park et al67 have carried out studies using a lentiviral vector expressing F.IX under the control of an EF1α promoter to direct sustained expression of the transgene after introduction of vector into the portal veins of C57Bl/6 mice. Consistent with their earlier studies, preparative partial hepatectomy resulted in a 4- to 6-fold increase in levels of expression compared with nonhepatectomized mice. Similar experiments attempted with a F.VIII transgene resulted initially in levels of expression of ~36 ng/mL (18% of normal plasma levels), but F.VIII levels became undetectable by week 8 because of the development of anti-F.VIII antibodies. There are as yet no published data using lentiviral vectors in the canine model of hemophilia, although Naldini68 has reported such experiments in preliminary form. Again, levels of expression could not be determined, because the treated dog developed inhibitory antibodies to the canine transgene product (vide infra).

Lentiviral vectors have not yet been used in human trials. Work by Dull et al69 has been directed at minimizing the possibility of generating replication-competent recombinants through the use of aconditional packaging system that uses only a fractional set of HIV genes. The recent development of a sensitive assay for replication-competent recombinants (which can detect as little as 1 fg of p24) should also be useful for eventual clinical testing of these vectors.

**Oral Administration of Plasmid DNA Encoding F.IX**

Okoli et al70 have presented a preliminary report in which F.IX plasmid DNA contained within a chitosan-DNA nanosphere is embedded within gelatin cubes and fed to mice at a dose of 25 μg plasmid in a single treatment. Treated mice showed levels of 45 ng/mL (~1% normal plasma levels), although levels gradually declined to undetectable over a 14-day period. The effects of repeated oral administration are under study. Although many questions remain (eg, site of synthesis, biological activity of the protein, and ability to achieve adequate levels in a larger animal), this study raises the exciting possibility of a noninvasive method for achieving gene transfer.

**Summary**

After years of preclinical studies, clinical trials of gene therapy for hemophilia are now underway. Although none of the trials have yet been completed, early results are encouraging in that safety has been demonstrated and evidence of
gene transfer and expression have been reported. These results are important in that they confirm findings in animal models and thus suggest that data derived from animal studies will serve as a reliable guide to results in humans. Continued engineering of vectors and minigene cassettes is presently resulting in substantial increases in clotting factor levels in hemophilic animal models. Present experience would suggest that these preclinical findings can be translated successfully into clinical results. Despite these encouraging early results, it should be kept in mind that at this time there are no licensed gene therapy products, and that even for well-established classes of pharmaceuticals such as small molecules and proteins, the time from initial clinical trials to licensing of a product may be anywhere from 2 to 10 years. Nevertheless, these trials have ushered in a new era of treatment for hemophilia that holds the promise not only of improved treatment for bleeding disorders but for a variety of other genetic diseases as well.

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