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Gene Therapy: A Status Report

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Many people continue to look to gene therapy as a potential cure for a diverse array of conditions. Initially, gene therapy was considered appropriate for only single gene disorders. Currently, strategies are being developed for many different conditions. Although an inability to demonstrate clinical efficacy has dimmed the shining promises from gene therapy's early years, the obstacles faced by this exciting field are being defined and studied. It is just a matter of time before effective gene therapy is possible. Research is now focusing on ways to conquer the most challenging of these hurdles, and it appears that successful gene therapy will become a reality.

If you stop to think about it, organ transplantation was a form of gene therapy. Bone marrow transplants for individuals with hereditary immunodeficiencies and liver transplantation for familial hyperlipidemia or urea cycle defects are both performed to supply a missing gene function or product. One current definition of gene therapy was established at the Food and Drug Administration (FDA)-National Institutes of Health (NIH) gene therapy forum in 1996.[1] This states that gene therapy is "a medical intervention based on modification of the genetic material of living cells." Gene therapy generally requires the following steps. First, it is necessary to define what product you want the patient's own cells to produce. This might be a protein the patient cannot produce enough of like factor IX in hemophilia, or it might be something extra that will help fight the disease directly (like an anti-inflammatory agent) or indirectly (like a protein that helps a therapeutic agent get into cells). The next step is to produce the nucleic acid sequence that codes for the product you want the patient to produce. Next, and most difficult, is getting that nucleic acid sequence into the patient's cells where it can function. A "vector" is used to modify the patient's cells. This may be done ex vivo for sub-

EDUCATIONAL OBJECTIVES

1. To determine what conditions are currently being targeted for gene therapy.
2. To understand how "therapeutic" genes are being delivered.
3. To appreciate the past problems encountered and what obstacles need to be overcome before gene therapy can be used routinely in clinical practice.

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sequent administration or altered in vivo by gene therapy products given directly to the subject. The target cells for gene therapy only include somatic cells and not germline cells due to the ethical dilemma germline therapy imposes. This paper begins with examples of gene therapy under investigation now. Then some of the methods being tried will be presented.

THE TARGET CONDITIONS

Currently, there are approximately 175 active worldwide human gene therapy clinical protocols involving more than 1500 patients.[2,3] There has yet to be conclusive evidence that any of these patients have benefited significantly from gene therapy. These trials have been of two types: therapeutic and gene marking. Gene-marking studies were the subjects of initial gene transfer protocols, and by design were not intended to treat patients directly, but rather to help understand basic cell biology following specific medical procedures. This type accounts for 25% of the 175 protocols and is carried out by ex vivo marking cells with recombinant viruses prior to reinfusion into patients. Transplantation of cells genetically "tagged" with unique markers have helped to show that ineffective purging of tumor cells from the bone marrow prior to transplantation is often the source for recurrent cancer cells in relapsed patients. Although these studies continue to provide insight into basic cell biology, the remainder of this review focuses on gene therapy for treating disease.

The remaining 75% percent of the clinical trials are directed at developing novel therapies. A wide spectrum of diseases are being targeted for gene therapy. These include monogenic conditions involving the loss of a single functioning gene. They also include more complex treatments for infectious, rheumatic, cardiovascular, and malignant diseases.

Gene replacement therapies have been attempted for a number of genetic diseases resulting from the deficiency of a single gene product. Examples include familial hypercholesterolemia, SCID-ADA (severe combined immune deficiency from adenosine deaminase deficiency), human alpha-1 antitrypsin deficiency, glycogen storage diseases, and cystic fibrosis. All of these conditions have been shown to have beneficial outcomes when the defective proteins are supplemented or produced from transplanted organs in a timely fashion. Thus, they seem to be logical targets for gene transfer trials. Although the most desirable way to accomplish this would be to "repair" the defective DNA in the normal source organ(s), our current knowledge of DNA repair and recombination is insufficient to accomplish this. Thus, the standard strategy for these conditions is a gene addition therapy, which involves the introduction of a functioning copy of the defective gene into cells that normally express that gene product. Additionally, it is theoretically possible

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to produce missing proteins from exogenous tissues. A significant obstacle in the treatment of genetic conditions is that these genes generally will need to be replaced for a patient's entire life. In some situations, this may require repeated delivery on a periodic basis.

The development of gene therapy schemes for rare genetic conditions is a logical first step for several reasons: (1) the conditions are generally well characterized and the therapeutic genes cloned, (2) replacement with normal functioning proteins have been shown to favorably alter disease progression, (3) the precise intracellular level of the replaced protein in the target tissue is generally not critical, and (4) excess production in non-native tissues is usually not toxic. It is generally felt that the lessons learned from developing gene therapy techniques for these rare conditions will be directly applicable to the development of therapies for other, more common diseases.

One example of a single gene deficiency condition targeted for gene therapy is hemophilia B, a relatively well-understood bleeding disorder involving the deficiency of plasma factor IX. Factor IX is produced in the liver naturally, so the liver is the preferred organ of choice for this gene therapy. Animal models, however, demonstrate that factor IX gene expression from other exogenous tissues may represent a viable alternative. Like in many genetic diseases, reconstitution of only a small percentage of the normal human serum levels of factor IX restores normal blood clotting ability in affected patients.

Cystic fibrosis, the most common lethal genetic disorder in the white population, is also targeted for gene therapy. Although there are more than a half dozen clinical trails for this condition, to date, no therapeutic benefit has been reported. Nevertheless, we have learned a significant amount about the natural barriers that limit successful gene transfer into the pulmonary tract—information vital to developing successful future trials.

In contrast to genetic conditions, gene therapy for infectious diseases may only need to persist long enough to "clear" the unwanted infectious agents. Human immunodeficency virus (HIV), hepatitis B, and hepatitis C are three viral infectious diseases with poor therapeutic options. Novel gene therapy strategies are now being devised for these conditions. One

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such strategy involves the delivery of a therapeutic molecule known as a ribozyme, which can be designed to specifically cleave and thus destroy specific infectious virus RNA molecules. Delivery of these ribozymes into hepatitis C virus (HCV)-infected hepatocytes has eliminated the HCV RNA in these cells. Clinical trials using HIV-directed ribozymes are currently in progress. Although the usefulness of gene therapy in the treatment of infectious diseases remains to be shown, these strategies will almost certainly play an important role in the future.

Clinical trials for malignant diseases represent the largest group of clinical gene therapy trials. Three different approaches have been undertaken. First, we continue to characterize the genetic events leading to malignant transformation; the inactivation of tumor-suppressing genes is an important predisposing factor. Attempts are being made to replace these defective genes directly in tumors. One potential obstacle for this approach is that a cure will require that essentially all the tumor cells will have to receive effective replacement therapy.

A second scheme for cancer gene therapy involves the delivery of genes that express cytotoxic proteins or proteins that convert prodrugs into cytotoxic agents. One example shown to work successfully in mouse model systems is the expression of the herpes virus thymidine kinase (TK) gene in the tumor cells, which then can convert the prodrug gancyclovir into a toxic intracellular thymidine analog. This then leads to the death of the tumor cells. Both recombinant retrovirus and adenovirus vectors expressing thymidine kinase have been prepared, and have been shown to work in these mouse model systems following in vitro or in vivo transduction and treatment with gancyclovir. Interestingly, significant tumor cell killing has been demonstrated in the transduced cells as well as some of the nontransduced neighboring tumor cells due to a phenomenon known as the bystander effect.

Finally, a third approach to cancer gene therapy has been to increase the immunogenicity of the tumor cells and thereby facilitate recognition by the host immune system. This has been accomplished in two ways. One approach is to express a neoantigen in tumor cells that would then make them appear foreign, and thus elicit a robust immunologic response against the tumor. A second approach is to express single or different groups of cytokines or chemicals that attract cells. These cytokines would function to signal and target the patient's own cancer-fighting cytotoxic cells to the tumor (eg, MHC class I molecule). Vectors expressing cytokines or tumor antigens have been prepared and used in model animal systems as "cancer vaccines" with encouraging success. Translation and demonstration of efficacy into human clinical trials has been more difficult and not yet effective in vivo. As our understanding of oncogenesis and host immune-mediated tumor cell clearance advances, more potent and hopefully more effective therapies will evolve utilizing a combination of strategies.

Many diseases afflicting people are complex conditions resulting from a combination of more than one gene (polygenic) and/or environmental factors (multifactorial). Examples of polygenic/multifactorial conditions include peripheral artery disease, hypertension, hyperlipidemia, diabetes, and rheumatoid arthritis. For many of these conditions, it is still unclear which genes or detoxification steps are at fault. Thus, there has been only limited development of gene therapy schemes. However, a few attempts have been made to use gene therapy to reduce destructive processes that are part of these diseases. One involves the use of gene therapy to modulate immune responses by expressing anti-inflammatory proteins. Another uses anti-sense and/or ribozymes directed against cytokines or genes involved in the regulation of the disease-causing cell cycle. Example target diseases for these therapies include severe rheumatoid arthritis and peripheral vascular disease. Clearly, a better understanding of the precise molecular mechanisms of disease pathogenesis in these complex conditions will facilitate the development of more novel and effective genetic therapies.

Gene transfer is also being developed as a means of vaccination. Antigens produced within an antigen-presenting cell may offer better immunity than infusion of a soluble antigen. Because relatively small amounts of protein are needed for a short period of time, it is likely that some of the early clinical success stories in gene therapy may actually be related to vaccine development.

THE GENE DELIVERY SCHEMES

The major hurdle of gene therapy continues to be the development of a scheme to express a desirable amount of the therapeutic protein within the appropriate target cells. A number of natural barriers limiting the ability for efficient introduction and maintenance of extraneous DNA have evolved in mammals as a defense against the introduction of a myriad of unwanted DNA. It is now clear that for gene therapy to be successful, vectors must be able to traverse these

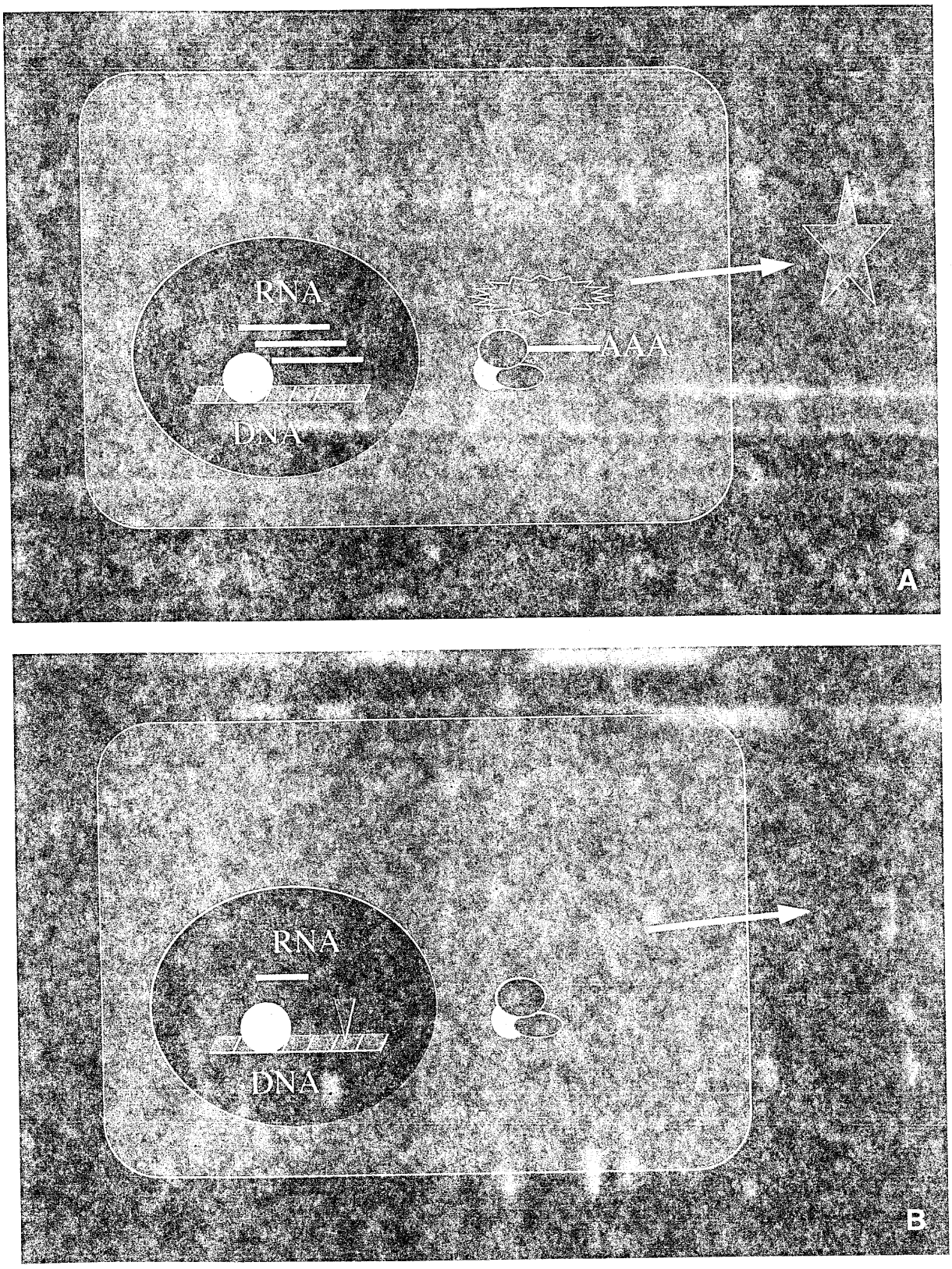
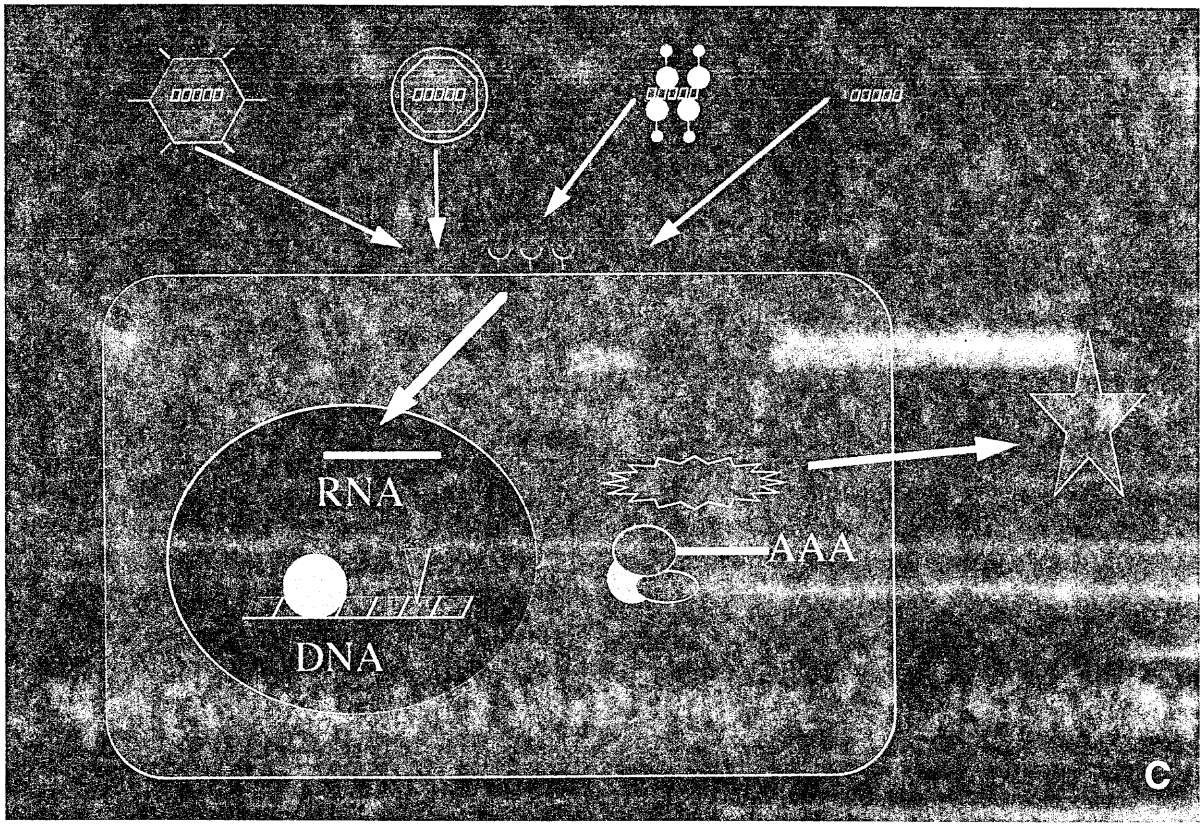


Figure. Schematic diagram of gene-addition strategies used in gene therapy protocols. In panel A, a target cell is shown producing an appropriate RNA molecule in the cell nucleus, which is modified, moved to the cytoplasm, and translated into a functional protein that can be secreted. (continued on 566)



(continued from 565) A mutation at the DNA level (panel B) generally results in a faulty or no RNA produced. Gene addition therapy using adenovirus, retroviruses, DNA/protein complexes, or even naked DNA (going left to right in panel C) are all schemes to add an additional functional copy of the non-functioning gene to the cell. (Reprinted with permission from Gene therapy in hemophilia and hepatitis: update from the 1996 NHF Annual Meeting. *Hemaware.* New York, NY: National Hemophilia Foundation; 1997.)

defenses and efficiently deliver the therapeutic gene to the target cell nucleus. As mentioned previously, most gene therapy protocols have involved a gene addition rather than a gene replacement strategy as outlined in the Figure. A normal cell contains the coded message for a particular protein in the DNA within the cell's nucleus (panel A). This gene or coded DNA is first transcribed into mRNA, and then transferred into the cytoplasm. In the cytoplasm, a large complex of proteins translates the mRNA into a protein, which is then modified and delivered to an appropriate functional site such as the bloodstream. A DNA mutation or alteration (Figure, panel B) results in the production of no normal mRNA (either absent or altered RNA) and consequently no functional protein. Gene addition therapy, as shown in panel C of the Figure, involves the introduction of a new functional gene into the cell nucleus. The current clinical gene therapy protocols use either viral or non-viral-based delivery systems.[4,5] The virus-based vectors, having evolved very sophisticated methods to transfer genetic material into the nucleus of cells, are more efficient at transducing target cells than non-viral vectors. However, each of the viral vectors developed to date still have significant limitations.

Preclinical and clinical trials using non-viral gene delivery schemes have demonstrated very low and transient gene transfer into target organs. Non-viral vectors include (1) liposomes (DNA molecules encapsulated in artificial lipid vesicles that can fuse with cells); (2) naked DNA molecules infused into a target tissue; or (3) DNA molecules complexed with specific proteins to help compact the DNA as well as target it to a specific ligand or cell type. One major obstacle for all of these non-viral approaches is difficulty transporting the DNA across the cytoplasm and into the nucleus. In addition, what DNA makes it into the nucleus often does not persist. Getting gene therapy into the nucleus and keeping it there turns out to be quite important because only in the nucleus can the DNA be transcribed into the mRNA necessary to make a new functional protein.

Viruses have elegant methods of carrying nucleic acid sequences from the outside environment into the nucleus of cells. In some cases, a copy of this gene is permanently inserted or integrated into the nuclear DNA. Several different viral vectors have been developed as vectors for human gene therapy. Four exam-

TABLE

Delivery Systems for Gene Therapy

Delivery System	Obstacles	Further Research
Retrovirus	Random integration Requires mitosis Low titers Complement inactivation	Transduction of nondividing cells Transient induction of mitosis Produce concentrated virus stocks
Adenovirus	Multifaceted immune response Neutralizing abs Lack of transgene persistence	Immune evasion schemes Produce minimal "stable" virus
Adeno-associated (AAV)	Small genome Inefficient cell transduction Neutralizing abs Low titers	Improving purification Preparing hybrid viruses
Nonviral (DNA/complexes)	Poor transduction efficiency Endosome entrapment	Cell-specific targeting Cytoplasmic transport (eg, endosomal lysis) Nuclear localization factors Requirements for DNA persistence

ples are recombinant retrovirus, adenovirus, adeno-associated virus (AAV), and herpes virus. All except herpes viruses have been used in clinical trials.

Murine retrovirus vectors have been the delivery scheme used by the majority of the human clinical trials. Although the genome size of this virus somewhat limits the size of a therapeutic gene that can be carried, the ability of recombinant retrovirus vectors to stably transduce dividing cells by insertion into the host cell genome makes them quite attractive for gene therapy, especially for genetic conditions that require lifelong replacement therapy. Unfortunately, the requirement of cell replication for efficient viral integration into the host genome makes them less attractive for in vivo gene therapy because at any given time only a small percentage of cells within an organ are dividing. Indeed, many studies now confirm that following transduction with retrovirus vectors (particularly using an in vivo protocol), only low amounts of therapeutic protein are produced because of poor transduction efficiency. Compounding this problem is our inability to produce high titer concentrated stocks of recombinant retroviruses. Modification of murine retrovirus vectors or alteration of the route of delivery may improve their therapeutic efficacy. For example, several groups have modified the retrovirus envelope proteins so as to produce a new vector that will only recognize a specific target tissue. In addition, several groups have worked to concentrate retrovirus stocks by also altering envelope proteins so as to make them more stable to conventional concentration schemes. Both of these schemes appear to be effective, but the requirement for cell division in the target organ still limits their usefulness. Finally, the development of a chimeric recombinant murine and human lentivirus (HIV) vector that has the ability to infect nondividing cells has been reported. Although additional studies addressing safety issues remain to be performed before this vector system will be used clinically, this recombinant human retrovirus system could prove to be a significant advance for the field of gene therapy.

An important step in the field of gene therapy was the demonstration that administration of recombinant adenovirus vectors could be used to transfer genetic information into most tissues at high efficiency in the absence of cell division. As a result, a number of genetic animal disease models that included hemophilia B, LDL receptor deficiency (familial hypercholesterolemia), and ornithine transcarbamylase deficiency were used to show that, in principle, these vectors could transiently cure the genetic defect. Since these studies, it has been determined that a multifaceted host immune response directed toward the adenovirus, cells infected with adenovirus, and perhaps even the transgene product are responsible for the limited duration of transgene expression as well as the ability to readminister the recombinant adenovirus. Thus, even though recombinant adenovirus vectors can be produced in sufficient quantities and effectively transduce nondividing cells to achieve a "cure," the resultant array of host immune responses remains an obstacle for gene delivery by recombinant adenovirus.

Adenovirus vector development is currently focusing on ways to effectively evade these host immune responses. Adenovirus is a large (36 000 basepair) DNA virus that contains many genes that are encoded into proteins that are required for replication of the virus as part of the life cycle. The first generation recombinant viruses still have many of these genes but are lacking the E1a gene products required for all other gene expression. This serves two functions: first, to cripple the ability of the virus to replicate, and second, to make room for therapeu-

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tic genes. Recent work has determined that even viruses with E1 deletions produce a small amount of the late gene products and are thus potent targets for the host immune system. So additional deletions are being placed into the vector with the hopes that removing these particularly immunogenic proteins would lead to long-term therapeutic gene expression.

Another approach to evade the host immune response to adenovirus vectors involves the co-administration of newer, less toxic immunoregulatory agents. These are not cytoablative, yet provide transient immunosuppression and perhaps even tolerance to the adenovirus infection. Two such compounds, CTLA4Ig and MR1 (anti-CD40 antibody) each block unique T-cell co-stimulatory pathways necessary for full activation of T-cells. These have produced impressive results when administered with adenovirus in experimental models. Administration at or around the time of adenovirus infection leads to a transient immunosuppression, which allows long-term transgene expression beyond the time the immunoregulatory agents are still detectable. Although the future of immunoregulatory agents in clinical trials remains unclear, it appears that time-specific immunomodulatory schemes will be developed that can facilitate the use of adenovirus or other vectors that stimulate an immune response against the vector, vector-transduced cells, or transgene product.

Adeno-associated virus (AAV) is a small, single-stranded DNA virus that requires wildtype adenovirus for production. It can be produced at relatively pure moderately high titers. Unfortunately, AAV vectors can only accommodate small genes. Several recent studies have shown that AAV can transduce cells from mouse liver, muscle, or brain efficiently and result in the long-term production of therapeutic proteins. It remains unclear whether these viruses are incorporated into the host genome or not. Additionally, a host-neutralizing antibody response to AAV limits our ability to successfully administer the recombinant virus. Also, because AAV is found in the general population, methods may have to be developed to evade pre-existing neutralizing antibodies or to use less common strains.

Aside from more "standard" types of gene therapy, the development of neo-organs or bio-artificial organs could be a significant therapeutic option for many conditions. These organs are actually structures containing genetically modified cells that secrete specific proteins directly into the bloodstream. These structures are still being developed, but they hold great potential for the future.

THE OBSTACLES WE FACE

A wealth of knowledge has been acquired and a number of obstacles have been defined during the first years of the developing field of gene therapy. These obstacles are unique to the type of gene delivery system (ie, vector), the organ targeted for therapy, and possibly the particular disease targeted. Included in the Table is a list of vectors and some of the obstacles faced by each. The third column includes some of the current research directions aimed at removing these obstacles. The Table illustrates that a significant amount of work remains to be done to overcome the numerous obstacles. Yet, excitement from limited successes continues to drive the field ever closer to true success.

How close are we to having gene therapy as a tool in our clinical practices? Although the precise timeframe remains unclear, continued research in developing new vector systems, modifying old vector designs, and understanding better the host immune response toward these vectors will eventually lead to a successful method for human gene therapy.

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