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GENE THERAPY, CONCEPTS, CURRENT TRIALS AND FUTURE DIRECTIONS

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INTRODUCTION

Human gene therapy is now a reality. After almost a decade of expectation that the technical capacity to isolate and purify human genes would rapidly lead to gene therapies, the first sanctioned US clinical trial, involving a retroviral vector carrying a bacterial marker gene, began in May, 1989. In the subsequent three years, there has been an explosion of clinical testing, both to continue using the cell-marking capabilities of gene transfer, as well as to explore actual therapies for genetic disease and cancer.

This review traces the events leading to the first clinical tests, and describes the development of the gene transfer or vector systems used. The concepts underlying the first clinical tests are described, and the initial results obtained in the clinic are reviewed. Recent developments in alternative vector systems are also considered, together with some possible future clinical directions.

CONCEPTS AND HISTORICAL BACKGROUND

EARLY EVENTS The concepts underlying a clinical approach to the treatment of genetic disease by replacement of affected genes by functioning ones evolved long ago, and in fact are almost intuitively obvious. Practical methods of gene transfer began to be considered (1) following understanding of how tumor viruses achieve oncogenic transformation by integration of viral genomes stably into the DNA of target cells. Further speculation (2–4) was fueled by the development of effective ways to transfer DNA into mammalian

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cells (5–7) and by the general advances in recombinant DNA technologies capable of isolating and generating large amounts of pure gene sequences.

Therefore, with pure genes, and at least functional delivery systems, the focus was directed to specific genetic corrections. Initially, the obvious disease targets seemed to be the hemoglobinopathies, since these genetic diseases were the first to be characterized at the molecular level for defects in and around the globin chain genes. Additionally, the target tissue, bone marrow, was accessible both for sampling as well as for reimplantation by bone marrow transplantation (BMT).

In fact, an early, abortive attempt to correct β-thalassemia with gene therapy was conducted by a US clinical investigator on two patients in countries outside the USA. This attempt (8, 9) and subsequent sanctions placed on the investigator (10) led to a major reevaluation of the feasibility and time frames, as well as the available technologies for human gene therapy. It also led to a more serious consideration of ethical issues associated with human gene therapy, and to the development of systems for review and oversight of human clinical trials. This responsibility, for federally funded research and clinical activities, fell upon the already existing National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC).

As a consequence of these reevaluations, the disease targets for human gene therapy (11) switched from the hemoglobinopathies to the Severe Combined Immune Deficiency (SCID) diseases, such as adenosine deaminase (ADA) deficiency. The technical reasons for this switch were that in SCID, unlike the hemoglobinopathies where sophisticated regulation of expression was a requirement to achieve balanced globin chain production, the requirements in regulating gene expression were much less severe. Additionally, after earlier speculation on the use of viruses as gene transfer vectors, it was concluded that the retroviral vector system possessed the properties suitable for the first attempts at gene therapy in humans (12), and this conclusion has had a major influence in directing initial gene therapies towards the use of retroviral vector systems. Even the initial choice of target tissue, the bone marrow stem cells, were changed to differentiated white blood cells, or lymphocytes, in the first clinical protocols.

Nevertheless, two basic concepts have remained constant: the fundamental basis for a gene therapy approach, i.e. the introduction of a functioning copy of a gene into a cell that lacks copies, or contains defective copies; and the "out of the body" or ex vivo approach to gene therapy, i.e. the removal of cells from a patient, the introduction of the gene into the cells (and possibly their expansion), and the reintroduction of the modified cells back to the patient. This ex vivo approach was very important in the development of the first sanctioned clinical protocols, both because of its technical feasibility and

safety features such as the ability to monitor and control the quality of modified cells before their administration to a patient.

FIRST DELIVERY SYSTEMS: RETROVIRAL VECTORS

Advances made in the late 1970s and early 80s in understanding the biology of retroviruses led to the use of these agents as vectors for the efficient transfer of foreign genes into mammalian cells. Retroviruses contain their information in double-stranded RNA genomes, which are copied or reverse-transcribed by the enzyme reverse-transcriptase during the retroviral life cycle (13). The proviral DNA product is stably introduced into the genome of the infected cell. To use retroviruses as gene vectors, the endogenous viral genes, i.e. those concerned with virus replication, are removed, and replaced by the foreign gene(s) of interest. Such manipulations are conducted at the DNA level, using the standard techniques of recombinant DNA.

The resulting vector structure is replication-defective, but since the desired retroviral vector needs to have its RNA transcript packaged, the packaging and envelope functions are provided in *trans,* by a cell line generally called a packaging cell line. Packaging cell lines contain viral genes that lack the signals for packaging, so that, ideally, only replication-defective vector transcripts are packaged. Although the resulting vector particles (which are referred to by the generic term, retroviral vector) can infect and introduce provirus into a target cell, they are not capable of any replication or production of infectious virus particles. They therefore function as one-time, single-hit gene transfer systems.

The most commonly used class of retroviral vectors are derived from a murine retrovirus, Moloney Murine Leukemia Virus (MoMuLV). The development of these vectors, with their myriad of technical features and innovations, has been extensively reviewed recently (14–18), and therefore is not discussed in detail here. A recent review on the development of packaging cell lines (19) gives a good overview of the technical issues surrounding these cells.

The features and properties of retroviral vectors that have made them suitable for the first clinical testing of gene transfer and gene therapy are as follows: (*a*) they are well studied viruses whose life cycle, and molecular biology, are well understood; (*b*) many foreign genes have been introduced into and expressed with retroviral vectors in many different cell types, both in vitro and in vivo; (*c*) good titer stocks of retroviral vectors, free of wild-type replication competent virus, can be produced; (*d*) when vectors integrate into cells, the sites of integration can be limited to one or only a few, but the sites of integration are at random; (*e*) the efficiency of transfer to many cell types

can be very high, sometimes close to 100%; (*f*) substantial safety studies with retroviral vectors have been conducted in rodents and primates; (*g*) the transfer of the provirus is stable and is passed on to progeny cells if the recipient cells divide; and (*h*) cell replication is required to introduce retroviral vectors into cells. Most significant among these features are the efficiency of gene transfer into mammalian cells, the ability to continue to express genes in vivo, and the stable nature of the gene transfer to target cells. The major drawbacks to the use of retroviral vectors are their requirement for dividing cells, and the random nature of the site of integration into the chromosome. This latter feature, highly pertinent in evaluating the safety and risk/benefit of this technology, is discussed in detail in the section on Safety Issues.

FIRST CLINICAL MARKER PROTOCOL

It seemed likely that the first candidate protocol for gene therapy would be the introduction of the ADA gene into the bone marrow of a patient with this form of SCID. Indeed, a preclinical discussion document was considered by the Human Gene Therapy Subcommittee of the NIH RAC, in December 1987, but such a protocol was deemed premature because of the then low efficiency of transfer and expression of the human ADA gene in primate bone marrow (20).

Then an unanticipated opportunity arose for the first clinical testing of gene transfer in the form of a protocol to use retroviral vectors to genetically mark a class of lymphocytes called Tumor Infiltrating Lymphocytes (TIL) and to follow the fate of these cells in humans in an adoptive immunotherapy procedure for cancer called TIL therapy (21, 22). In TIL therapy, developed for metastatic melanoma and renal cell carcinoma, a tumor deposit is excised and a population of the lymphoid cells that had originally infiltrated the tumor is cultured under the influence of recombinant Interleukin-2 (IL-2). Very large numbers of these TIL ($>10^{11}$) are returned to the patient intravenously, together with high doses of IL-2. In a patient cohort that had failed all other forms of therapy, 35–40% of the patients responded to TIL therapy (22). However, most patients still fail to respond, and even among responders, relapse is common within 6 to 12 months. One problem with TIL therapy is the inability to follow or track the fate of autologous cells in the patients, except with very short half-life $Indium^{111}$ labeling (23). A protocol was therefore developed, using retrovirally marked TIL, to monitor trafficking of TIL back to sites of tumors and to look for other clinical correlates. The bacterial antibiotic resistance gene, neomycin phosphotransferase (Neo®) was chosen as the marker gene. This protocol began its review process in June 1988, and finally commenced in May 1989. The details of the review process are discussed below.

A variety of preclinical studies (24) were conducted in support of this protocol (25), and data have been published from the first five of the ten patients treated (26, 27). Although major clinical correlates have not yet been obtained from these data, the protocol has been highly significant in that it demonstrated the safety (no side effects or pathology of any kind have been attributable to the gene transfer) and feasibility of gene transfer (marked cells could be recovered both from the blood stream and from tumors). It also demonstrated that at least some of the TIL have the ability to home to sites of tumors. Other TIL gene-marking protocols have followed the first. In one case (28), effects of TIL "homing" to tumor deposits is being studied under the influence of Interleukin 4, and in another (29), different classes of lymphocytes will be marked with vectors distinguishable at the DNA level by PCR techniques. Finally, a TIL gene-marker protocol similar to the initial US protocol began in late 1991 in Lyon, France.

The use of genes as markers has many advantages over more conventional marking methods, including the stability of the marker gene, which is not metabolized but persists for the life time of the cell and is even passed on to progeny cells should the initial marked cells divide in vivo. Furthermore, in theory, marked cells containing a selectable marker gene (such as the Neo®) gene) can be recovered from biopsies by selection, although this has proven to be exceptionally difficult with TIL. Finally, very sensitive assays (molecular hybridization, PCR analysis) are available to detect marker genes.

REVIEW PROCESS

In the USA, gene therapy protocols must undergo multiple levels of review, part of which, that by the NIH RAC, is open to the public. The initial protocols, submitted from 1988 through 1991, were first required to receive internal institutional approvals, from local Institutional Biosafety Committees (IBCs) and Institution Review Boards (IRBs); the former to deal with safety issues, the latter with ethics, informed consent, and any other patient issues. The NIH RAC, originally established to review protocols involving recombinant DNA experimentation, was subsequently made responsible for conducting a review process of gene therapy protocols. This RAC review, which is conducted in an open public meeting and whose agendas published in the Federal Register 30 days before meetings, is required for any gene therapy protocol where investigators or institutions are in receipt of federal funding.

To acquire the expertise to review gene therapy protocols, the NIH RAC established a Human Gene Therapy Subcommittee (HGT) of the RAC. This subcommittee was an extension of a working group established in 1983 to deliberate on issues concerning gene therapy and current recommendations. For the first several years therefore the review process involved two tiers,

review first by the Subcommittee followed by the RAC itself. Duplication and delays were common, mainly because the HGT subcommittee and the full RAC met infrequently during the year. After extensive debate over reducing overlap, the HGT subcommittee was removed from the formal review process in June 1992. Review is now solely in the hands of the RAC.

For a submission to the RAC, investigators are required to answer a series of questions about the protocol. These questions are formalized in a set of "Points to Consider" (30). The RAC reports its recommendations directly to the Director of the NIH, whose written approval is required before any protocol can be implemented. The main responsibility of the RAC in reviewing human gene therapy protocols is to consider issues of patient and societal safety. However, particularly for the early protocols reviewed, the technical and scientific aspects of the protocols were closely scrutinized, as were the vector systems used, the validity of preclinical data justifying the protocol (with strong emphasis on animal model data), and informed consent issues for patient protection. The RAC consists of 25 members, of whom at least 14 must be knowledgeable in the field of molecular genetics, molecular biology, or recombinant DNA research, and at least 6 must be knowledgeable in applicable law, standards of professional conduct and practice, public attitudes, the environment, public health, occupational health or related fields. Members serve terms of three years.

Final approval for any gene therapy protocol comes from the Food and Drug Administration (FDA). The requirements for FDA submissions are documented in an FDA "Points to Consider" (31). The FDA review, of documents called Investigational New Drug (IND) applications and Drug Master Files for the vector agents used, is not a public process, but typically involves at least one and often more meetings between investigators and the FDA. The FDA is required by law to respond within 30 days of receipt of an IND, and meetings to deal with the issues that arise can be scheduled at any time. Thus, unless exceptional issues arise, the FDA process of review can be, and often has been, more rapid than that of the RAC, particularly when the HGT subcommittee was in operation. Moreover, the FDA review process can be initiated to coincide with, or even precede the RAC review. In one case at least, FDA approval for a protocol was received before that from RAC. A specialist gene therapy journal, *Human Gene Therapy,* publishes every protocol that the RAC committee approves. The current review process for trials of human gene therapy is summarized in Table 1.

SAFETY ISSUES

For retroviral vectors, three major safety issues were identified from the review processes of the first clinical protocols of gene transfer and gene therapy: the

Table 1 Review process for human gene therapy protocols

possible generation of infectious, wild-type or "helper" retrovirus in the vector preparation; the fact that retroviral vectors integrate at random within the genome of the target cell, with the potential consequence of activating an oncogene or inactivating a tumor suppressor gene; the specific therapy itself, and the gene product encoded by the vector used.

Several recent reviews (32–34) have examined these safety issues of retroviral vectors, so the major issues are only summarized here. The generation of wild-type virus occurs as a consequence of recombination events at the RNA level during the retroviral life cycle in the producer cell line. Therefore, the frequency of its occurrence will be primarily related to the degree of homology between the retroviral vector and any viral genes for replication contained within the producer cell line. The initial retroviral vectors used in clinical trials, the L series vectors (35), have been so designed as to minimize this overlap with sequences in the producer cell line, PA317 (36). This combination of vector and packaging cell line has proven remarkably consistent in producing, under carefully controlled conditions, retroviral vector preparations free of helper virus for many preparations of vector in volumes totaling more than one hundred liters (G. McGarrity, personal communication). A modification to further reduce the likelihood of generating wild-type helper involved an alteration to the packaging cell line to separate, or "split" the viral replication genes from each other (37–39).

Several studies have investigated the likely consequence of exposure of primates to wild-type virus. Initial studies in primates (40–42) suggested that the amphotropically packaged retrovirus is not an acute pathogen. However, a more recent study by investigators at NIH (A. W. Nienhuis, personal communication) has resulted in three monkeys developing malignant T cell lymphomas after a bone marrow transplantation of marrow cells transduced with a vector contaminated by significant concentrations of wild-type virus. It seems likely that the wild-type virus was responsible for these lymphomas, underscoring the necessity of maintaining clinical vectors free from contaminating wild-type virus.

Any analysis of the significance and potential consequences of random vector insertion into the genome should note that activation of an oncogene, for example, will not obligatorily lead to the formation of a cancer or tumor. Activation of an oncogene is normally one of a series of events required to generate a malignancy. Thus, the critical factor is the contribution of such events to the already existing background, but any such increase is very difficult to measure directly, due to the extremely low frequency of such insertional activation events. Clearly, the key issue then becomes one of risk/benefit, and significantly elevated risks of subsequent tumors are acceptable risks in conventional therapies such as radiation, chemotherapy, and immunosuppression. Despite numerous safety studies in rodents (34), no event

of vector-related tumor generation has yet been observed with retroviral vectors of the type used clinically.

The third safety issue relates to each specific vector, the encoded product and the protocol. Clearly, certain genes represent no particular hazard, e.g. intracellular products such as neomycin phosphotransferase in marker protocols, or human adenosine deaminase for SCID. For certain secreted products, such as lymphokines and cytokines with known toxic effects, e.g. Tumor Necrosis Factor, Interleukin 2, caution must be exercised to avoid risks of over-production of such substances to the possible detriment of the patient.

FIRST THERAPEUTIC PROTOCOLS

ADA PROTOCOL After the TIL marker gene clinical protocol was successfully and safely implemented, Drs. Anderson, Blaese, and Culver at the NIH proposed a therapeutic protocol for the genetic disease, ADA deficiency (a form of SCID). This protocol proposed the isolation of peripheral lymphocytes from an ADA-deficiency patient, introduction of a human ADA gene via a retroviral vector in vitro, expansion of the modified lymphocytes for a period of a few days, and then their reintroduction into the patient. This regimen would be conducted repetitively, at 6–8 week intervals, on patients already being treated by enzyme replacement with the agent polyethylene glycol (PEG) bovine ADA.

The protocol was so designed because of continuing technical hurdles in modifying bone marrow cells efficiently, and to present a more conservative approach, since it proposed the modification of terminally differentiated cells, with a finite and relatively short life span. The additional rationale came from in vitro studies (43) showing that immortalized lymphocyte cell lines from an ADA patient could be corrected with a retroviral vector expressing the human ADA gene. These and other preclinical studies (44–46) formed the basis of support for such a lymphocyte-based protocol (47).

The potential clinical utility of this protocol was supported by several hypotheses. First, corrected cells may have a selective advantage in vivo over uncorrected cells. The results of matched Bone Marrow Transplantation (matched BMT is curative for ADA deficiency) showed that patients often retain all of their own blood cells except T cells, which are exclusively of donor origin (48). Second, the procedure of repetitively sampling T cell populations for correction increases the likelihood of obtaining a wider immunological repertoire in the corrected cell population. The patients eligible for this protocol were required to be receiving (and to continue to receive) enzyme replacement therapy with PEG-ADA.

The clinical protocol received RAC and FDA approval, and began on a 4-year old girl at the NIH in September 1990, followed by a second patient

in January 1991. The results to date have been extremely encouraging, showing significantly improved immune functions, and the presence of a substantial number of gene-corrected T cells in the circulation of the patients. In the first patient, levels of human ADA activity in circulating white cells have increased steadily to about 50% of that of her father, a heterozygote for ADA deficiency. Clinical results obtained to date have recently been summarized (49, 50). The data are extraordinarily encouraging, particularly as they represent the first patients in the first test of the therapeutic potential of gene therapy. One surprising feature has been the persistence of the corrected T lymphocytes for longer than 6 months after cessation of infusions. This result provides firm evidence for one of the advantages postulated for gene therapy in ADA deficiency (12), namely the selective advantage of corrected cells in such patients. Nonetheless, prudence is still appropriate at this early stage of the study in that gaps will probably exist in the immune repertoire of these patients since only mature T cells are being corrected.

To address this issue, an addendum to the current protocol has been prepared that attempts to correct bone marrow stem cells in addition to circulating lymphocytes. The source of bone marrow stem cells will be the very small numbers known to be present in the circulation and these will be enriched from mature blood cells by the use of an immune selection procedure involving the cell surface marker CD34. Hence, two different populations of cells will be independently transduced, and two different ADA vectors will be used to follow the fates of these cells. Both vectors express the human ADA gene, but have slightly different structures such that the vector integrants can be distinguished in the DNA of recipient cells by PCR techniques. In this way, the fates of cells (and their progeny) transduced by the different vectors can be followed in the one patient, a further illustration of the power of gene transfer as a cell-marking technique. This protocol has received approval from the NIH RAC and is currently undergoing FDA review.

A second therapeutic trial involving ADA deficiency recently started in Milan, Italy. Initially, peripheral lymphocytes were corrected with an ADA vector, but in a subsequent modification, a bone marrow sample was also transduced and returned to the patient. Here too, different ADA vectors were used to distinguish the recipient cells from each other. Finally, an ADA gene therapy protocol involving only the bone marrow will probably soon be initiated in the Netherlands, by a group who have performed extensive preclinical studies in primates (51).

SUBSEQUENT MARKER PROTOCOLS

The marking protocols, using vectors containing a Neo® gene to study TIL therapy, have already been referred to above. Several additional gene-marking

protocols have also been approved, and in some cases conducted. The objective in one class of protocols is to study the origins of relapse in a variety of leukemias and other cancers after BMT. The principle involved requires genetic marking of any residual disease cells in a fraction of the bone marrow used to rescue patients after ablative chemotherapy or radiation therapy. The clinical question is whether a subsequent relapse has its origins, at least in part, in diseased cells transplanted back with the rescue marrow, or in residual systemic disease. The outcome of such studies has considerable clinical significance, since it would determine whether bone marrow purging of residual disease (a procedure that can damage marrow and affect BMT), or more stringent ablative therapy is the more appropriate clinical course. Should marrow purging be needed, then the gene-marking techniques could also provide a sensitive marker for disease cells, often lacking for many forms of cancer. Thus, marked cells could be used to determine the efficiency of bone marrow purging procedures.

The first such clinical protocol (52) was designed to study relapse in acute myeloid leukemia (AML) in children, and began in September 1991, as the first sanctioned gene transfer protocol conducted outside of the NIH. Preclinical studies (53) established the feasibility of taking a fraction of bone marrow (in addition to that normally used for the rescue) and labeling residual disease cells with a retroviral vector. The likelihood of being able to detect a marked relapse was also calculated. This ability depends both on the efficiency of transduction (found to average about 10%) and the number of residual disease cells in the marrow needed to contribute to a relapse. Provided that the latter number is not too low (e.g. 100 cells or more), the chances of detecting a marked relapse were calculated to be very high, even with very few patients. This was an important calculation since a failure to detect any gene-marked cells in the relapse would not provide any useful information. It has now been observed (M. Brenner, in preparation) that the first two patients in this protocol who suffered a relapse had gene-marked cells in the leukemic relapse cells. This first attempt to use gene transfer techniques for this kind of marker study has been a significant success. Efforts may now be focussed on improving bone marrow purging techniques for use in this disease. The marking techniques may also be useful in establishing whether AML involves an early multilineage progenitor, or derives from lineage-restricted cells.

A similar protocol has also been proposed for first remission and relapse-refractory neuroblastoma (54, 55). In this disease, the rate of relapse after intensive chemotherapy, irradiation, and remission-autologous BMT is very high. The origins of relapse after ABMT need to be established, for the same reasons as those described for AML. Preclinical studies conducted for this protocol also showed that clonogenic neuroblastoma cells could be transduced,

albeit with somewhat lower efficiency. This protocol has received regulatory approval, and portions of the rescue marrows of several patients have been marked and cryopreserved awaiting a relapse (M. Brenner, personal communication).

Similar studies have been proposed and initiated for adult chronic myelogenous leukemia (CML) at the M. D. Anderson Cancer Center at Houston, Texas (56), and for adult AML and acute lymphocytic leukemia (ALL) at Indiana University (57). Finally, a series of bone marrow marking protocols to study marrow involvement in multiple myeloma, breast cancer, and CML have been approved at the June 1992 meeting of the NIH RAC (A. W. Nienhuis, personal communication).

Another clinical protocol that makes use of gene-transfer marking properties concerns hepatocellular transplantation (HCT) with heterologous hepatocytes. This procedure is planned for testing as an alternative to orthotopic liver transplantation for patients with end-stage liver disease who are awaiting a liver for whole-organ transplantation. HTC could provide significant short-term support for such patients, since pieces of liver from which hepatocytes can be isolated may be more readily available. Feasibility studies have been conducted in animal models (58) and a protocol (59) has received RAC approval.

A recently approved gene-marking protocol proposes to treat lymphoma in AIDS patients by chemotherapy, followed by ABMT. A component of this treatment involves the isolation and culture in vitro of HIV antigen-specific T cells that are readministered to the patient in addition to the bone marrow cells. The cultured T cells will be marked with a retroviral vector (60), which contains the gene for a fusion protein between a bacterial hygromycin phosphotransferase and the herpes simplex thymidine kinase. This fusion protein confers activities both for positive selection with the antibiotic, hygromycin, together with the possibility of a negative selection with the drugs gancyclovir or acyclovir. The negative selection property represents a safety mechanism to allow the administered cells to be killed should a problem, such as uncontrolled T cell clone growth, occur in the patient.

SUBSEQUENT THERAPEUTIC PROTOCOLS

CANCER The first application of gene therapy to the treatment of cancer grew out of the initial TIL cell-marking protocol. The therapeutic study also provides an excellent illustration of the drug-delivery potential of gene transfer techniques. The therapeutic concept involves the introduction into TIL cells of the gene(s) for agents that can either mediate tumor cell killing directly, or induce a stronger immune response locally and thereby indirectly mediate

an antitumor effect. If a significant proportion of TIL are able to migrate to tumor deposits, then they will represent a means of delivery of such agents to the local environment where they are required. This ability is of particular significance for certain lymphokines and cytokines that have very short half-lives within the circulation and must therefore be administered systemically in very large amounts to reach effective levels at the desired sites of action. The resultant toxicities or side effects may often limit or even prevent the effective use of such agents administered by injection into the bloodstream.

The initial choice of agent to be delivered via gene therapy procedures using TIL was the substance, Tumor Necrosis Factor (TNF). While recombinant TNF can effectively mediate tumor regression in rodents when injected systemically, its use in humans has been limited by a much lower tolerance (up to 50-fold less) of systemic TNF than in rodents. A clinical protocol (61) was approved and began in January 1991, with a phase I toxicity study mandated by the FDA to establish the possible accumulation of toxic levels of TNF in lungs, liver, or other organs. This study has been completed with no observed toxic side effects, and the second phase of the protocol, using appropriate levels of TIL transduced with TNF vectors, is in progress.

An alternative use of gene therapy procedures in the treatment of cancer comes from the observation, in rodent models, that introduction of a lymphokine gene into transplantable tumor cells can ablate the ability of the tumor cells to become established as tumors in the animal. This result has been demonstrated for a variety of lymphokines, including TNF (61), Interleukin-2, Interleukin 4, Interferon γ, and granulocyte-macrophage colony stimulating factor (GMCSF) (for references, see (50)). The mechanism underlying this effect is not yet generally understood. It has been proposed that local expression of TNF somehow inhibits efficient vascularization of the tumor (61, 62). In several studies where tumor regression or ablation has been achieved, the animal may become resistant to subsequent challenge with unmodified tumor cells, suggesting a possible immunological "memory" to the tumor, and that this approach represents a type of tumor vaccination. In only one case (63) where IL-4 was used has an effect on preestablished tumor deposits been claimed. But the overall pattern of these animal modeling studies strongly indicates a potential clinical benefit for humans of using gene modified tumor cells to strengthen the immune response that the body normally mounts against tumors.

An initial clinical study at the NIH, approved and begun in late 1991, is already testing these concepts, and some of the candidate lymphokines and cytokines, in humans. The study represents an extension and modification of the TIL therapy approach, and has the potential of extending this adoptive immunotherapy to a broader set of disease targets beyond the current targets of malignant melanoma and renal cell carcinoma. The protocol involves the

use of TNF-modified (64) or Il-2-modified (65) tumor cells reintroduced both intramuscularly and subcutaneously into patients. After three weeks, inoculation sites and nearby draining lymphocytes are harvested, and any lymphocytes from these samples are grown up and reintroduced back to the patient in a manner analogous to TIL therapy.

The final gene therapy approach initiated as a human trial differs from all previous gene transfer and therapy protocols in two ways: it involves the use of a nonretroviral vector system; and it uses an in vivo, rather than an ex vivo, route of vector delivery. The aim is to transfer into the tumor cells of patients the gene for a class I major histocompatibility antigen (HLA-B7) that is not normally expressed in the tumors. The vector, which is directly injected into tumor masses, is a liposome-encapsulated DNA preparation that contains the DNA and the signals required to express it. This protocol raises issues of transfer of the gene to other cells in addition to the tumor cells, and the possibility of an inadvertent germ-line manipulation. The protocol has therefore been restricted to terminal cancer patients beyond reproductive age, and was initiated in the spring of 1992 at Ann Arbor, Michigan.

GENETIC DISEASES Two other therapeutic protocols are underway, both aimed at the amelioration of a genetic disease. The first is a therapeutic approach to the severe genetic disease, Familial Hypercholesterolemia, characterized by extraordinarily elevated cholesterol levels with the clinical consequence of very early onset vascular disease. The disease is due to defective or absent receptors on hepatocytes of the low-density lipoprotein (LDL) receptor. In the gene therapy protocol, a substantial part of the patient's liver is resected. The hepatocytes obtained from the liver section are corrected in vitro of their defect in the LDL receptor by retroviral-mediated gene transfer. The cells are then returned to the patient by direct infusion into the liver via an in-dwelling catheter retained after the original partial hepatectomy. Preclinical studies in vitro and in animal models, including the animal model of the disease, the Watanabe rabbit, have been encouraging (66–69). A clinical protocol (70) has been approved and performed on a patient in the summer of 1992 (J. M. Wilson, personal communication). The animal model studies promise hope for a clinical response in patients. The major issues surrounding the protocol are its technical and surgical complexity, and the duration of any affect produced by modifying a fully differentiated cell population such as the hepatocytes used.

The second protocol seeks to address the blood coagulation disorder, Hemophilia B. A deficiency or absence of the blood-clotting protein, Factor IX, underlies this disorder. At Fudan University in Shanghai, China, two Hemophilia B patients received implants of autologous fibroblasts modified with a retroviral vector containing the gene for human factor IX. The review

process that this protocol received before implementation is not known at present. Additionally, technical questions surround the longevity of expression in vivo of the Factor IX gene delivered to fibroblasts via retroviral vectors, and also the longevity of survival of implanted fibroblasts.

NEW VECTOR SYSTEMS

VIRAL VECTORS While retroviral vectors are being thought of as the standard system for at least certain types of gene transfer therapy, they have not been fully exploited. The development of viral vectors continues, with improvements in titer, efficiency of transfer, and levels of expression. Although the size of DNA that can be introduced is limited (7-8 kb), nevertheless two and sometimes three genes can be introduced into the vector. Cell- and tissue-specific regulatory elements, such as promoters and enhancers, are being evaluated, and at the level of the packaging cell, different envelopes are being used to alter the host range of the vector. Thus, the evolution and improvement of retroviral vectors continues, and they will certainly remain important tools in the human gene therapy arsenal for the foreseeable future.

Other viral systems, however, are being developed and tested for their clinical utility. Among the most advanced are adenoviral vectors (71), derived from certain strains of human adenoviruses. Replication-deficient adenovirus vectors have been constructed with the capacity at present to accept 7–8 kb of foreign DNA. The advantages of adenoviral vectors are that they can introduce DNA into a wide variety of nondividing cells, do not integrate into the chromosome, and are relatively much more stable than retroviral vectors and can be concentrated to titers of between 10^{11} and 10^{12} plaque-forming units (pfu) per ml. Adenoviral vectors, with a tropism for lung tissue, can also be used as in vivo vectors to deliver genes to cells in the respiratory tract. This delivery has been carried out in animal models for the human genes α-1 antitrypsin (72) and Cystic Fibrosis Transmembrane Regulator (CFTR) (73). The use of adenoviral vector may possibly be restricted by short duration of expression. Thus, chronic protein delivery may require repeated use, and under such circumstances immunological responses to the in vivo administered vector may become a limiting factor. Issues related to the safety of using adenovirus vectors in humans include complementation by other viruses (including normal wild-type adenovirus) that could result in continued production and shedding in the patient of infectious vector particles, and possible recombination with the viruses in vivo. On the other hand, attenuated strains of adenovirus have long been used as vaccines in humans, with an apparently excellent safety record. The novel and attractive features of adenoviral vectors suggest that these agents will be tested in humans in gene

therapy protocols as soon as the major safety issues have been addressed in animal studies. The pulmonary manifestations of cystic fibrosis would seem a likely target for early clinical trial, since gene transfer of the normal gene corrects the chloride transport defect in patients' cells in vitro (74, 75).

Another vector system currently under intensive study is derived from the virus Adeno-Associated Virus (AAV) (76). Although AAV vectors can accept only relatively small pieces of DNA (approximately 4 kb) and are technically still difficult to produce without the risk of contaminating wild-type adenovirus, the ability of AAV to integrate into a specific target site in chromosome 19 is attracting considerable interest (77). While this specific targeting mechanism has not yet been unequivocally shown to be preserved in vectors derived from AAV, the specific integration mechanism potentially represents a solution to the safety issues raised by the random integration of retroviral vectors.

Other viral systems are being developed as vector systems. Most are still at relatively early stages of development, but could well be useful clinically for their distinctive features. For example, vectors can be and have been generated from herpes viruses (78, 79), and their tropism and special properties in neuronal tissue could be beneficial. Overall, researchers have only begun to scratch the surface of the extraordinary wealth of diversity and special properties of natural viral systems as a potential tool for gene transfer. Viruses are, after all, natural gene transfer systems, and have had many millions of years to evolve and find solutions to specificity, expression, and reduction of pathogenicity during infection.

NONVIRAL VECTORS Initially, DNA transfer involved physical means, such as the use of calcium phosphate precipitate of DNA (6), or direct injection (5), but various nonviral means of introducing DNA into cells have subsequently been developed. Direct injection of DNA leads to uptake and expression of DNA, particularly in muscle tissue (80, 81). Studies using liposome encapsulation of DNA for in vivo gene transfer (82) have already led to a human clinical trial, as described above. Other physical methods for DNA transfer include simple electroporation, as well as the more sophisticated use of DNA-coated gold or silver particles, introduced into cells by high-energy bombardment (83).

An innovative approach to the targeted delivery of liver cells in vivo has been the development of conjugates of DNA with a protein complex, asialo ovomucoid-polylysine. This complex has the ability to target in vivo with some specificity to the liver (84). While expression in target cells using this technique is short-lived, it has been extended by the performance of a two-thirds partial hepatectomy shortly after the infusion of the DNA (85). A variation on this theme has been to use a transferrin-DNA complex to target

DNA to cells bearing the transferrin receptor (86). Additionally, complexes involving the use of adenovirus with DNA carried on the outside of the particle have been used in DNA transfer studies (87,88). The adenovirus particle is thought to improve the release of DNA from the endosomes used by the cell to take up these complexes.

FUTURE DIRECTIONS

NEAR TERM New clinical approaches are being developed or are close to clinical implementation. One involves yet another modality of vector delivery, "in situ" gene therapy. Here the producer cells capable of generating a retroviral vector containing a "suicide gene", the thymidine kinase (tk) gene of herpes simplex, are introduced directly into the site of a brain tumor called a glioblastoma. Over several days, the producer cells generate retroviral vector right at the tumor sites where the rapidly dividing tumor cells are transduced with high efficiency. Other cells in the brain, being mitotically inactive, are not affected. Administration of the drug Gancyclovir (an antiviral agent already approved by the FDA) will kill all the cells containing the tk gene, including the producer cells, and will thereby halt further production of vector particles. The concept for this approach, devised some time ago (89), has recently been validated in rat animal model studies with a transplantable glioma (90). Surprisingly, not all tumor cells need to be transduced (as might have been expected) to mediate tumor regression, i.e. there is some kind of "bystander" effect that can destroy neighboring untransduced tumor cells. A clinical protocol to treat primary neuroblastoma in humans received RAC approval in June, 1992.

Another approach using gene transfer techniques in a cancer therapy has involved the use of the multi-drug resistance (MDR) gene. Since the characterization of the phenomenon of drug resistance in tumors mediated by the amplification of the MDR-1 gene and the cloning of the human MDR-1 gene (91), the concept of conferring drug resistance to chemotherapeutic agents in the bone marrow of cancer patients has evolved. Recent animal studies in mice (92) and in primates (A. W. Nienhuis, personal communication) have shown not only that the MDR gene can be introduced into bone marrow stem cells, but also that an in vivo amplification of cells containing it can be obtained in mice by administration of the drug, Taxol. These animal data will likely form the basis of a clinical protocol in cancer patients, in which the bone marrow would be modified to tolerate higher levels of chemotherapeutic drugs.

Various gene therapy approaches towards HIV infection have been proposed. Significant preclinical and animal studies have been conducted in two

of these, and clinical tests are being seriously considered. The immunological approach involves the expression of the HIV envelope glycoprotein in a retroviral vector. The aim is to use the vector to express the gene in an individual's autologous fibroblasts. These cells would be reintroduced into the patient, in the expectation that the envelope, or fragments of it, would be so processed and presented on the surface of the fibroblasts as to elicit a stronger cell-mediated immune response than for the native virus particle. This concept has been supported experimentally in animal models (93), and a clinical protocol, which was not submitted for RAC review, has reportedly received FDA approval. The trial will presumably be conducted in a non-federally funded clinical center.

The second gene therapy strategy falls within the general "decoy" concept, and takes two forms. The first extracellular strategy uses a soluble form of the naturally used receptor for HIV, namely CD4. Although administration of soluble CD4 (94), or fusion derivatives such as SCD4/IgG, has not produced any clinical benefit as injectable recombinant proteins (94), the hope is that continuous expression of these agents through a gene therapy approach will show a clinical response and may reduce the viral burden in patients infected with the AIDS virus (95). The other decoy strategy, termed "intracellular immunization" (96), aims at producing within the cell sufficient copies of a decoy nucleic acid sequence that can compete for a regulatory protein, for example the *tat* transcriptional activator, to interrupt the replication cycle of the virus and so effectively "immunize" the cell. The ideal target cell for such an approach would obviously be the bone marrow stem cell, so that the blood cell lineages produced would all be resistant to productive infection by HIV. However, since technical difficulties remain in efficiently transducing bone marrow stem cells, an initial clinical approach is likely to involve the use of peripheral blood lymphocytes for transduction. Two groups, at the NIH and the Memorial Sloan Kettering Institute, are developing protocols of this kind. Other regulatory target sites in HIV, for example the rev/RRE regulatory system, can be envisaged as similar decoy targets, whereas other researchers have proposed the use of antisense or ribozyme strategies to prevent HIV replication. The complexities of the HIV virus, its target cells, and its variety of pathological effects make it extremely unlikely that any single agent or approach will prove significant. A combination of the approaches outlined above is more likely to succeed.

As discussed above (see Adenovirus Vectors), a clinical study is under development that uses a CFTR adenovirus vector, instilled directly into the lungs, and extensive preclinical and safety studies are likely in progress. Adenovirus vectors are also being evaluated in preclinical and animal models for disease applications (97) in addition to cystic fibrosis.

LONGER TERM The potential applications for gene therapy in genetic or acquired diseases are obviously enormous. However, most current or planned applications involve intensive, individual patient-based therapies. This restricted scope will undoubtedly initially only impact situations of catastrophic disease, where no alternative therapies may exist. Although a significant number of patients may be treated, big strides are needed to make gene therapy straightforward and routine before it can fulfill its promise as a broadly enabling therapeutic technique.

From our current perspective, this goal can best be reached by the development of targetable, injectable vectors (49). Vectors that can injected into a patient's blood stream must be engineered, and specificity or "docking" mechanisms built in so that the vectors will only recognize and enter a desired cell type. That naturally occurring viruses already exist with exquisite cell tropisms—hepatitis B virus, rabies virus, herpes virus, for example—provides encouragement to efforts manipulate and construct a particular specificity into a functional viral envelope. To obtain another layer of cell specificity, signals for gene expression specific for a particular cell or tissue type can be built into vectors to ensure their expression only in the desired cell type. Many of these signals are in fact already known and characterized. Built into these vectors must be the signals required for the physiological regulation of expression of certain proteins, e.g. insulin. A final property will almost certainly be the ability to integrate into a preselected site in the genome, to remove the potential risks from random integration methods.

Some researchers are proposing as a long-term strategy the construction of an artificial mini-chromosome, containing the required gene, its regulatory signals, and the information to maintain and replicate the artificial chromosome. This approach would allow incorporation of very large stretches of DNA around the desired gene, and thereby encompass all of the natural signals involved in specific gene regulation. This approach would also completely circumvent the issues of random integration. The complexity of the technology required to construct such a miniature chromosome is daunting by current standards, as is the development of ways to introduce such structures efficiently into cells. However, for stem cell therapy, perhaps only a limited number of cells would need to be treated in this way.

SUMMARY

Since the initial human clinical trials of retroviral-mediated gene transfer in the USA in 1989, numerous additional protocols are in process or have been proposed. In the first therapeutic protocol, to treat the genetic disease ADA deficiency, encouraging signs of clinical benefit have been observed in the

first two patients. Gene-marking properties are being extensively used in many protocols, particularly in the area of autologous bone marrow transplantation for various cancers. The drug delivery potential of gene therapy is initially being evaluated through delivery of various lymphokines and cytokines in cancer therapy protocols. Testing has also begun for other genetic diseases, Familial Hypercholesterolemia and Hemophilia B.

Vector systems and retroviral vectors are developing rapidly, and a clinical trial using a liposome-based delivery has started. The pace of technical development and clinical application has intensified. Although significant clinical therapies are expected from these initial studies, the full potential of gene therapy for wide applications still requires innovative research programs, directed towards true in vivo vectors.

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