HUMAN GENE THERAPY

INTERNAL MEDICINE GRAND ROUNDS

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INTRODUCTION

In recent months, much has been written in the popular press as well as the scientific literature about the experiments currently underway to correct human genetic disease by gene therapy. Although the results of these investigations will not be known for some months, it seems appropriate to review the scientific basis of this rapidly changing technology for this medical audience. Even if these first protocols are unsuccessful or only transiently successful in correcting the underlying disease, simply establishing the safety and low morbidity of the gene therapy approach will undoubtedly lead to government approval for further attempts in other centers to obtain long-term success.

I. Germ-line versus somatic cell gene therapy

What is not under discussion today is the controversial topic of introducing genes into the human germ-line, which would alter the genetic make-up of subsequent generations. Although the production of such transgenic strains of animals carrying foreign genes is now a relatively routine technique in many laboratories, it is unlikely that such methods will be extended to human embryos, both for moral and technical reasons. Indeed, since the efficiency of transfer of foreign genes into mammalian zygotes is not uniform, only a fraction of eggs microinjected with DNA produce transgenic animals with high levels of gene expression. Moreover, there is currently no way to screen the embryos for successful gene therapy, i.e. both integration into the genome and correct expression of the gene, prior to implantation in the uterus.

In addition, to allow gene therapy for recessive disorders to be applied to preimplantation embryos, methods would have to be developed to determine first which fertilized eggs were normal, heterozygous, or homozygous recessive defective. In fact, the polymerase chain reaction technique could conceivably be applied to the molecular diagnosis of preimplantation embryos. For example, DNA isolated from a single cell biopsied from a single blastomere could be amplified in vitro and then tested for the presence of normal and mutant alleles of the gene in question. However, were such an analysis performed, it would be far simpler both technically and ethically to implant only the normal or heterozygous embryos in the mother rather than to attempt to carry out gene transfer on the recessive mutant embryos. Thus, it seems unlikely that gene therapy for recessive diseases will ever be applied to human preimplantation embryos.

II. Allogeneic bone marrow transplantation versus somatic gene therapy

One question immediately raised by the prospect of gene therapy into bone marrow cells is why not simply do a bone marrow transplant to replace the hematopoietic system with normal cells from a compatible donor? Indeed, Table 1 contains a list of the disease that respond completely or partially to bone marrow transplantation. An additional advantage of transplantation is that one does not have to have identified and cloned the gene responsible for the disease in question, an obvious necessity for gene therapy.

Table 1

Genetic Diseases Which May Be Responsive to Allogeneic Bone Marrow Transplantation

Immunologic disorders:
ADA-deficient SCID
Non-ADA-deficient SCID
Purine nucleotide phosphorylase (PNP) deficiency
Wiskott–Aldrich syndrome
Chediak-Higachi syndrome
Chronic granulomatous disease
Reticular dysgenesis
Hematologic disorders:
Thalassemia
Sickle cell disease
Fanconi's anemia
Metabolic disorders
Mucopolysaccharidosis
Gaucher's disease
Adrenoleukodystrophy
Metachromatic leukodystrophy
Osteopetrosis
Lesch–Nyhan syndrome

1 Kohn, Anderson, Blaese, 1989

However, allogeneic bone marrow transplantation is a difficult procedure with a high degree of morbidity and significant early mortality, probably 10-20%. In addition, only about 30% of potential candidates for a bone marrow transplant prove have a genetically compatible sibling who is matched at the major histocompatibility locus. Even with immunologic matching, the transplants may be complicated by long-term graft versus host disease due to other antigenic differences between donor and recipient. Thus, many patients with genetic diseases theoretically correctable by an allogeneic transplant do not undergo this therapy. On the other hand, the knowledge that a given disease can be cured by a bone marrow transplant is an important predictor that gene therapy applied to hematopoietic cells also has the potential for cure.

III. What diseases are good candidates for somatic cell gene therapy?

As mentioned above, the most obvious requirement for gene therapy is identification and cloning of the gene causing the disease. A corollary to this point is that the disease must be a simple genetic disorder that results from defects at a single gene. At the current time the disease must also be recessive, rather than dominant, so that gene addition or supplementation with a single normal gene copy should correct the disorder. Although there are experimental approaches to gene replacement for dominant disorders such techniques are still far too inefficient to be applied to human disease.

In addition to the above requirements, several additional considerations are important in determining the likelihood of success for gene therapy in a given disease. First, the genetic defect should be expressed in a tissue that is easily manipulated in vitro and capable of long-term survival in vivo after transplantation back into the recipient. Although hematopoietic stem cells and skin fibroblasts have been the most frequent tissues considered for gene therapy, as we shall see even the survival of these cells in vivo remains a major problem. Moreover, other tissues, such as endothelial cells, respiratory epithelium, hepatocytes, and even neurons are being studied as potential vehicles for gene therapy in other diseases that are not likely to be cured by gene expression in bone marrow cells. Table 2 lists a number of diseases that are candidates for human gene therapy and indicates the ideal target tissue for expression of the transferred gene.

TABLE 2. Candidate Disorders for Somatic Gene Therapy

Disease	Gene	Cell affected	Tissue specificity	Expression level needed	Other therapy
SCID	Adenosine deaminase	Lymphocytes	Bone Marrow	1-5% normal	Marrow transplant Enzyme replacement
SCID	Purine nucleoside phosphorylase	Lymphocytes	Bone Marrow	1-5% normal	Marrow transplant Enzyme replacement
Gaucher's I	Glucocerebrosidase	Macrophages	No	?	Marrow transplant ?Splenectomy
Phenylketonuria	Phenylalanine hydroxylase	Hepatocytes	?	1-5% normal	Special diet
Chronic granulomatous disease	cytochrom b	Granulocytes	Bone marrow	?	Interferon, Bone marrow transplant
Thalassemia	α- and β-globin genes	Red blood cells	Erythroid required	Precise regulation needed	Blood transfusion
Hemophilia	Factor VIII Factor XI	Hepatocytes	No	10% normal	Exogenous factor
Lesch-Nyhan	HG phosphoribosyl transferase	Many, esp. basal ganglia	Basal ganglia?	?	None
Cystic Fibrosis	CFTR	Respiratory Exocrine	?	?	Supportive ?Amiloride
α_1-antitrypsin	α_1-antitrypsin	Lung, Liver	?Liver	?	?Liver Transplant

Refs: Gaucher's: Correll et al, 1989; Fink et al, 1990; Nolta et al, 1990; α_1-antitrypsin: Crystal, 1989; CGD: Gallin and Malech, 1990; Factor VIII: Israel and Kaufman, 1990; Factor IX: Palmer TD et al, 1989; Lesch-Nyhan: Palella et al, 1989; Wolff and Friedmann, 1988

Finally, the diseases most likely to respond initially to gene therapy are those in which the gene does not require very high levels of expression or in which expression of the gene in certain tissues might be deleterious or in which the level of gene expression is very closely regulated. For example, it appears that diseases such as the hemophilias and the immunodeficiencies due to the enzyme defects in adenosine deaminase and purine nucleoside phosphorylase would probably only require 5-10% of normal expression levels to achieve cure or significant amelioration of the disease. Moreover, these genes are either normally expressed in nearly all cell types or probably would not be deleterious if they were expressed in other cell types. Indeed, in part because of these consideration, probably the most effort in developing gene therapy has been directed at the correction of human ADA deficiency (Belmont et al, 1988; Germann et al, 1989, 1990; Hantzopoulos et al, 1989; Hock et al, 1989; Kaleko et al, 1990; Lim et al, 1989; Van Beusechem et al, 1989; Wilson et al, 1990).

On the other hand, complete cures of diseases such as the thalassemias would require not only very specific levels of gene expression to obtain balanced production of the globin chains, but also might be harmful if globin chain synthesis occurred in cell types other than the red blood cell. Indeed, although a great deal of work has been directed toward gene therapy with the human β-globin gene, obtaining adequate and specific gene expression in vivo remains a major problem (Bank et al, 1989; Gale, 1989; Miller et al, 1988; Bender et al, 1988, 1989; Gelinas et al, 1989; Dzierzak et al, 1988; Grosveld et al, 1987; Novak et al., 1990).

Additional diseases that may be amenable to gene therapy that are not listed in the Table include leukocyte adhesion defects (CD11/CD18) (Hibbs et al, 1990), myeloperoxidase deficiency, specific granule deficiencies (i.e. defensin), argininiosuccinic aciduria (arginosuccinase), citrullenemia (arginosuccinate synthetase) (Herman et al, 1989), elliptocytosis type 1 (protein 4.1), elliptocytosis type 2 (spectrin), granulocyte actin deficiency.

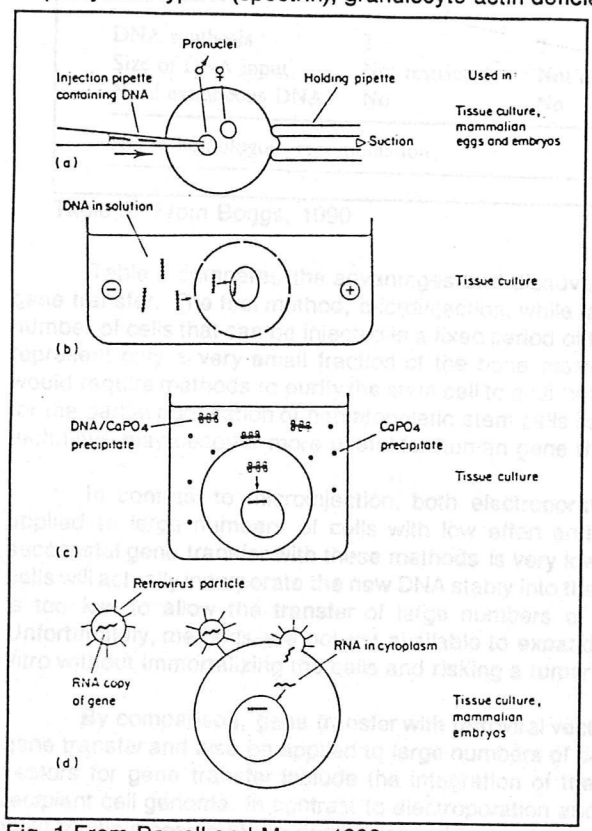


Fig. 1 From Powell and Moor, 1989

Diseases with more complex gene regulation include complement factor deficiencies (C2, C4, C9), and hereditary angioneurotic edema (C1 inhibitor). Other diseases in which the tissue targeting is a major problem include Fabry's disease (alpha galactosidase), fucosidosis (alpha fucosidase), infantile forms of Gaucher's disease, Tay-Sachs disease (hexosaminidase A), and familial hypercholesterolemia (LDL receptor) (Miyanohara et al, 1988; Wilson et al, 1988).

IV. Methods for Gene Transfer into Cells

In the last 10-15 years, a number of methods for gene transfer have been developed. As shown in Figure 1, foreign DNA can be introduced into cells by (a) direct microinjection into the cell nucleus (Gordon, 1990), (b) electroporation (electrophoretic transfer across the cell membrane which is reversibly damaged by the electric field) (Potter, 1988; Keating and Toneguzzo, 1990), (c) transfection, usually performed layering cells with calcium phosphate precipitates of DNA (Hogan et al, 1986), and (d) the use of retroviral vectors (Miller, 1990).

Comparison of DNA transfer methods

	Microinjection	Electroporation	Retrovirus
Efficiency	10-100%	0.0001-1%	1-100% (depends on titre)
Effort	High	Low	Intermediate
Expense	High	Low	Intermediate
HR frequency	High	10^{-2}-10^{-7}	Low
Stability	Good	10^{-2}-10^{-4} Good	May be inactivated or become infective
DNA synthesis	?	?	Required
Size of DNA input	Not restricted	Not restricted	Limited ($\leq$8 kb)
Need extraneous DNA	No	No	Yes

HR = homologous recombination

Table 3. From Boggs, 1990

Table 3 compares the advantages and disadvantages of these different techniques for gene transfer. The first method, microinjection, while fairly efficient, is limited in practice by the number of cells that can be injected in a fixed period of time. Because hematopoietic stem cells represent only a very small fraction of the bone marrow cells, the success of microinjection would require methods to purify the stem cell to near homogeneity. If methods already available for the partial purification of hematopoietic stem cells can be further perfected (see below), this technique may become more useful for human gene therapy.

In contrast to microinjection, both electroporation and transfection methods can be applied to large numbers of cells with low effort and expense. However, the efficiency of successful gene transfer with these methods is very low. Probably 1 in 10,000 to 1 in 100,000 cells will actually incorporate the new DNA stably into their genome. Clearly this level of success is too low to allow the transfer of large numbers of corrected cells back into the patient. Unfortunately, methods are not yet available to expand the rare cell carrying the new gene in vitro without immortalizing the cells and risking a tumor after transfer into the patient.

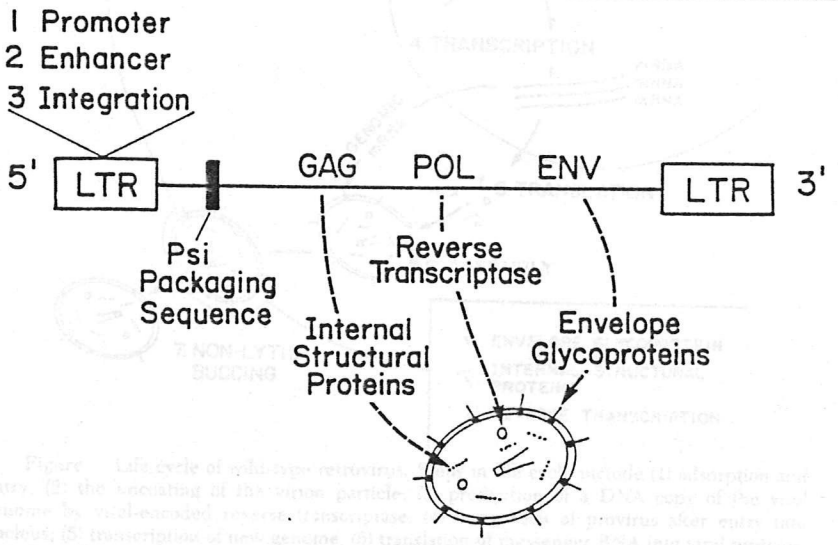
By comparison, gene transfer with retroviral vectors can achieve both high efficiency of gene transfer and also be applied to large numbers of cells. Additional advantages to retroviral vectors for gene transfer include the integration of the DNA usually as a single copy in the recipient cell genome, in contrast to electroporation and transfection techniques which usually yield multiple copies of the gene arranged as tandem repeats.

On the other hand, retroviral vectors are limited in the size of the gene that can be incorporated into the virion, usually about 8 kilobases of DNA. Thus, some very large genes or genes that require large flanking sequences of DNA promoters and/or enhancers in order to obtain normal tissue specific expression and regulation may not be compatible with the retroviral

system. Other disadvantages to retroviral vectors are (1) integration of the viral DNA is dependent on active DNA replication by the host cell (Miller et al, 1990), (2) the risk of inducing an active viral infection in the recipient of the gene-modified cells, and (3) the risk, albeit low, that the random insertion of the foreign gene into the host cell genome may occur at the site of a cellular oncogene, leading to its activation and the potential for malignant transformation of the cell. As we shall see in the next section, methods have been developed to minimize several of these potential problems.

V. Packaging cell lines and retroviral vectors for gene transfer

Figure 2 is a schematic representation of a typical wild-type retrovirus. A critical feature to note is the presence of a viral sequence, called the ψ (Psi) region, that was identified several years ago by Richard Mulligan and David Baltimore (Mann et al, 1983). This region is required in the viral life cycle so that the viral RNA can be successfully packaged into the budding viral particles along with the internal structural proteins derived from the *gag* gene. Without this sequence the result will be the production of "empty" viral particles because the viral RNA cannot enter the virion without the critical ψ encapsidation sequence.



Genome of wild-type retrovirus. Long terminal repeats (LTR) including the viral promoter and enhancer and sequences directing integration; Psi = packaging sequence required *in cis* for packaging of genomic RNA into virion particles; GAG = gene encoding internal structural proteins of virion core; POL = gene encoding reverse transcriptase; ENV = gene encoding envelope glycoproteins present on virion membrane.

Fig. 2 From Williams, 1988

Another feature to note is that the *env* gene determines the viral coat glycoproteins and thus controls the species specificity of the viral infection. For example, some viral *env* genes are ecotropic, which means that they can infect only their native host species. An ecotropic mouse virus would therefore be of little use for the infection of human cells. Fortunately, other viruses encode env glycoproteins that are amphotropic, and are capable of infecting both mouse and human cells (Danos and Mulligan, 1988).

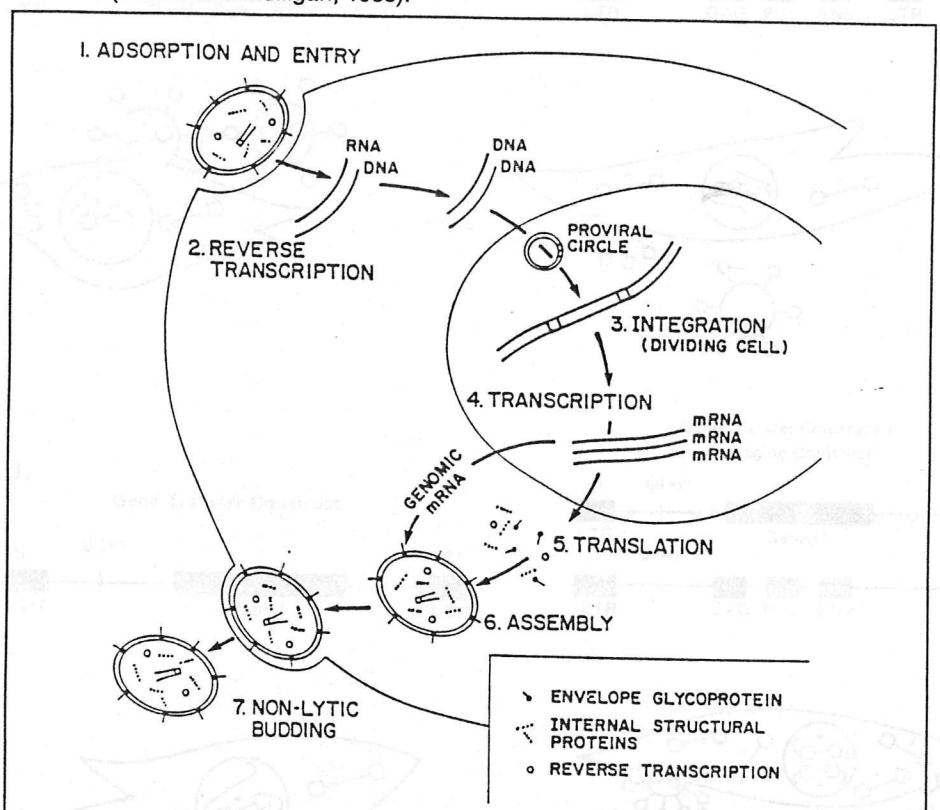


Figure Life cycle of wild-type retrovirus. Steps in life cycle include (1) adsorption and entry; (2) the uncoating of the virion particle; (3) production of a DNA copy of the viral genome by viral-encoded reverse transcriptase; (4) integration of provirus after entry into nucleus; (5) transcription of new genome; (6) translation of messenger RNA into viral proteins; and (7) nonlytic budding of new particles assembly.

Fig. 3 From Williams, 1988

Figure 3 is a review of the normal life-cycle of a wild-type retrovirus, showing how the various viral genes are required for reproduction in the host cell. It is important to note that in contrast to other viruses, the retrovirus does not usually cause the death of the host cell, as the viral budding and release of infectious virions is not lytic.

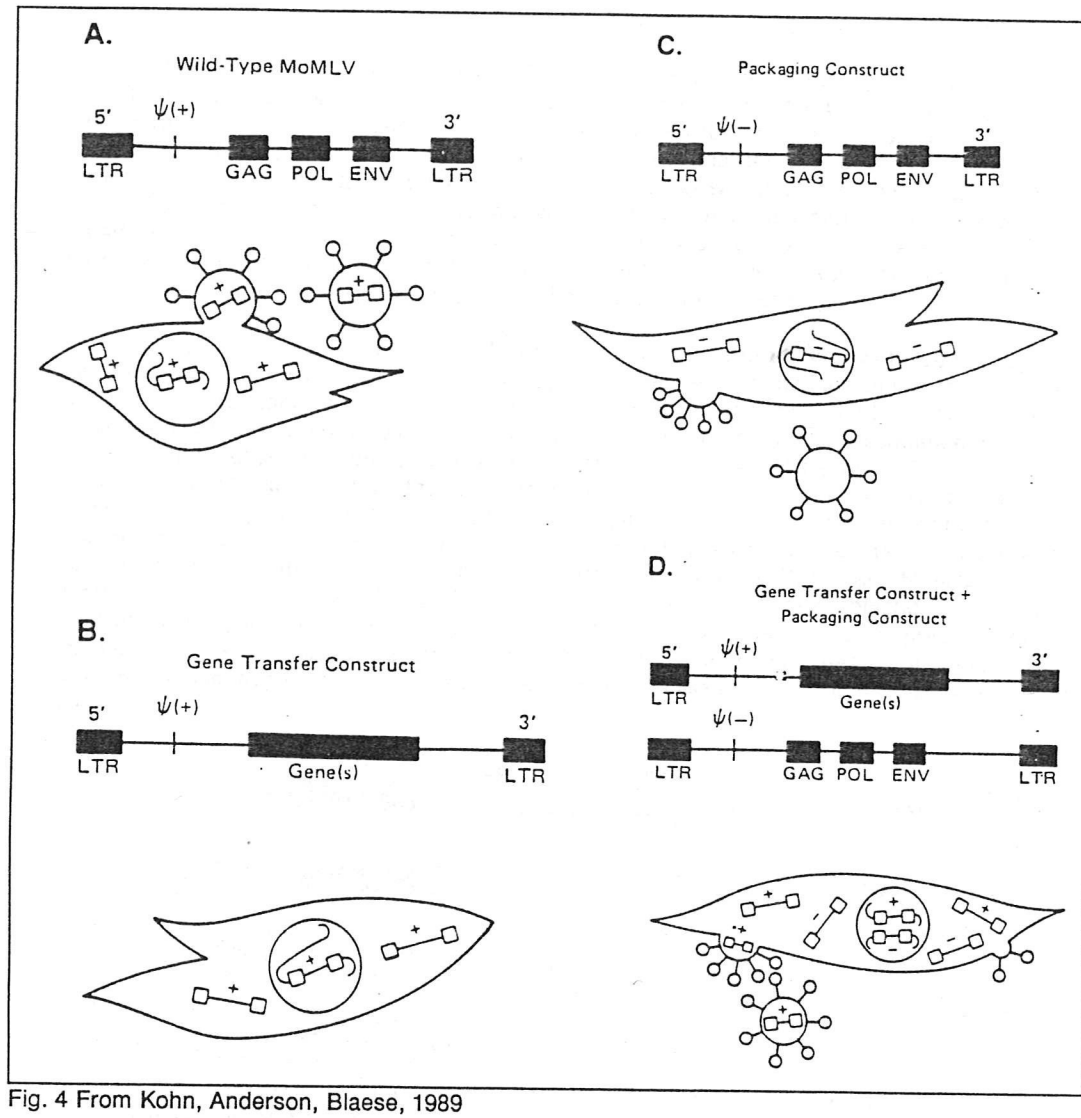


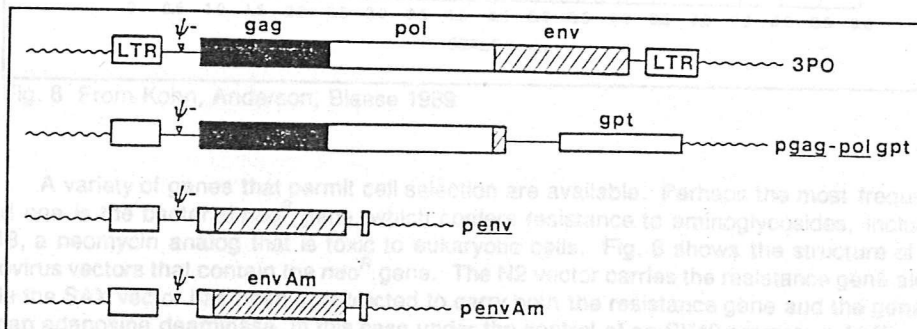
Fig. 4 From Kohn, Anderson, Blaese, 1989

With the identification of the ψ region, Mulligan and colleagues were further able to develop cell lines, called packaging cell lines, that produce replication-defective retroviral vectors containing a specific gene of choice (Cone and Mulligan, 1984). As shown in Figure 4B, the molecule constructed for gene transfer needs to contain the gene of choice, an intact ψ sequence so that the molecule can be packaged into viral particles, and the viral sequences at the 5' and 3' ends called LTR (for long terminal repeat), which are required as both promoters

and enhancers for gene expression, as well as integration of the viral genome into the host DNA.

Introduction of this gene construct by transfection into a packaging cell line (Fig. 4C) leads to the desired production of viral particles that contain the gene transfer construct, but no intact viral *gag*, *pol*, or *env* genes that would be required to establish further rounds of viral infection and viremia in the host. As shown in Fig. 4D., the final cell line contains both the gene transfer construct which can be packaged into viral particles which are then unable to replicate further, and the encapsidation-defective retrovirus which can make viral particles but cannot itself be efficiently packaged into the virions. The resulting viruses are capable of infecting the target cells for gene transfer only once, but cannot produce further virus because the information for making new virus coats and internal proteins has not been transferred.

Recently, several important modifications have been made to improve these packaging cell lines (Miller and Buttimore, 1986). Although the ψ sequence mutations should theoretically prevent the production of wild-type retrovirus, a single recombination event between the gene transfer construct and the ψ-defective virus could lead to the correction of ψ sequence defect in the virus, and subsequent production of replication competent wild-type virus. To reduce this possibility, which has been found to occur not infrequently in practice, several laboratories have produced packaging cell lines in which the viral genes have been separated onto different plasmid molecules, as shown in Fig. 5. Thus one ψ defective molecule contains the *gag* and *pol* genes, while another ψ defective molecule carries the *env* gene (Danos and Mulligan, 1988; Markowitz et al, 1988; Dougherty et al, 1989;. Thus, at least 2 recombination events are theoretically required in these lines to produce replication competent virus. In practice, these modifications, as well as several others that involve introducing complementary mutations into the virus genes, appear to have essentially eliminated the likelihood any infectious virus.



Plasmids containing retroviral DNA sequences. The top line shows 3PO, a plasmid containing most of the Moloney virus retroviral genome including all the viral components shown. The second line shows the construct p*gag-pol*gt that contains the Moloney *gag* and *pol* genes and the *gpt* gene. Line 3 shows a plasmid with the Moloney ecotropic *env* gene, p*env*, and line 4, a similar plasmid with an amphotropic *env* gene, p*env*Am.

Fig. 5 From Bank et al, 1989

VI. Selectable viruses

An additional feature of many retroviral vectors, as well as other gene transfer methods, is the co-transfer of a second gene which confers on the recipient cell resistance to an inhibitory or toxic compound. The drug can then be used in appropriate doses to selectively suppress the growth of uninfected cells while allowing for outgrowth of cells which have taken up both the "therapeutic gene" as well as the gene for drug resistance. The selection process could be performed either ex vivo, before the bone marrow or other cells are introduced into the patient, or in vivo, by systemic administration of the drug to the patient after cell transplantation into the patient.

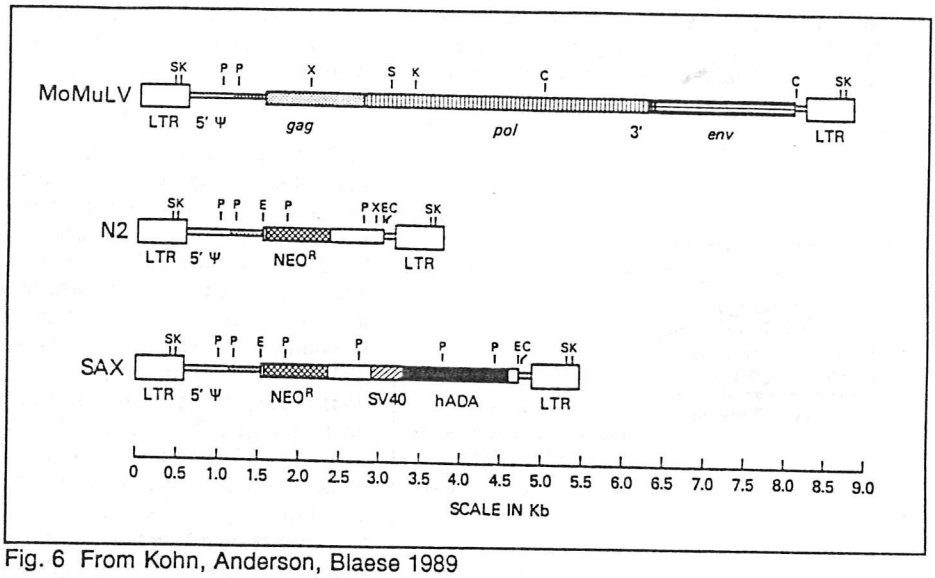


Fig. 6 From Kohn, Anderson, Blaese 1989

A variety of genes that permit cell selection are available. Perhaps the most frequently used one is the bacterial *neo*^{R} gene, which confers resistance to aminoglycosides, including G418, a neomycin analog that is toxic to eukaryotic cells. Fig. 6 shows the structure of two retrovirus vectors that contain the *neo*^{R} gene. The N2 vector carries the resistance gene alone, while the SAX vector has been constructed to carry both the resistance gene and the gene for human adenosine deaminase, in this case under the control of an SV40 promoter. In Fig. 7, a typical experimental protocol is diagrammed to show how the presence of the *neo*^{R} gene allows both selection of the packaging cells which contain the construct, and then in a second step, allows selection of the cells from the 3T3 cell line which have successfully been infected by the retroviral vector, integrated the viral genome, and expressed the resistance gene.

Other genes which have been used for selection include a mutant form of the dihydrofolate reductase gene (DHFR) isolated from cells resistant to methotrexate (Miller et al, 1985; Corey et al, 1990). Cells containing this altered DHFR are thus able to grow in methotrexate concentrations that are toxic to cells that have not taken up the foreign gene.

Similarly the CAT gene, which inactivates chloramphenicol, the eukaryotic multidrug resistance gene (MDR), which is responsible for resistance to several chemotherapy agents (Guild et al, 1988; Kane et al, 1989), and the gpt gene, which confers resistance to the toxic compound mycophenolic acid, have been used by different laboratories.

VII. In Vivo Gene Transfer in Animal Models - Mouse models

In the initial experiments that explored gene transfer into bone marrow, vectors carrying only the *neo* resistance gene were used by some groups, while in other labs vectors were used that contained both *neo* and either β-globin genes or the human gene for adenosine deaminase. Because the results of these early experiments were generally similar and somewhat independent of the vector used, I will discuss the overall experience with selected examples from all three of these approaches.

Beginning about 5 years ago, a number of investigators published the first papers showing that it was possible to infect mouse bone marrow cells in vitro with a variety of different retroviruses, transfer these cells back into irradiated or stem-cell deficient mice, and detect the foreign gene in the donor cells in vivo in the first weeks after the transplant (Keller et al, 1985; Dick et al, 1985; Eglitis et al, 1985; Miller et al, 1984). The protocol employed in many of these experiments is illustrated in Fig. 7. In many of these cases it was also shown that the foreign gene was expressed, and with the right promoter in the construct, expression could even be shown to be tissue-specific.

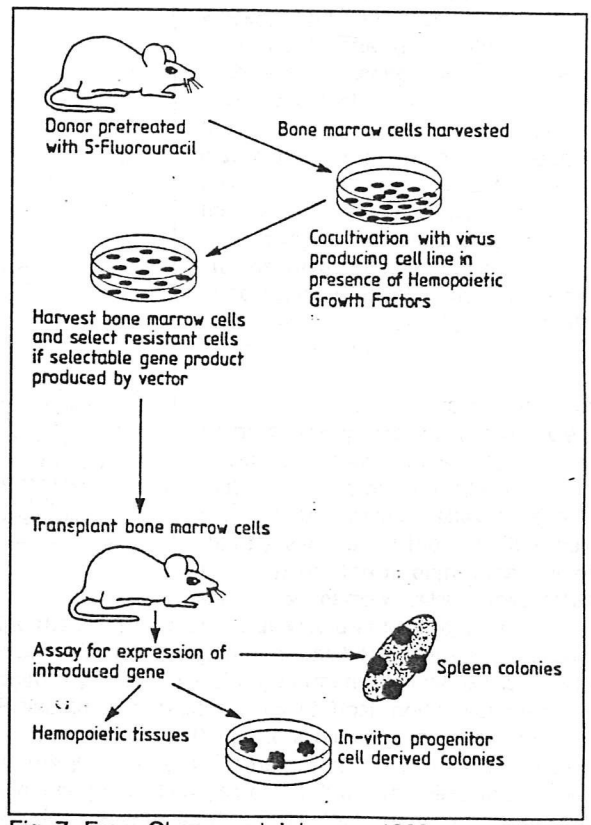


Fig. 7 From Chang and Johnson, 1989

However, the early enthusiasm soon diminished as experimenters began to perform longer term analyses and follow the recipient mice for longer periods of time. What became clear to all the groups working in the field is illustrated in the Figure from the work of Nienhuis and colleagues on transferring the human β-globin gene into mouse bone marrow (Fig. 8) (Bodine et al, 1989; Karlsson et al, 1988)

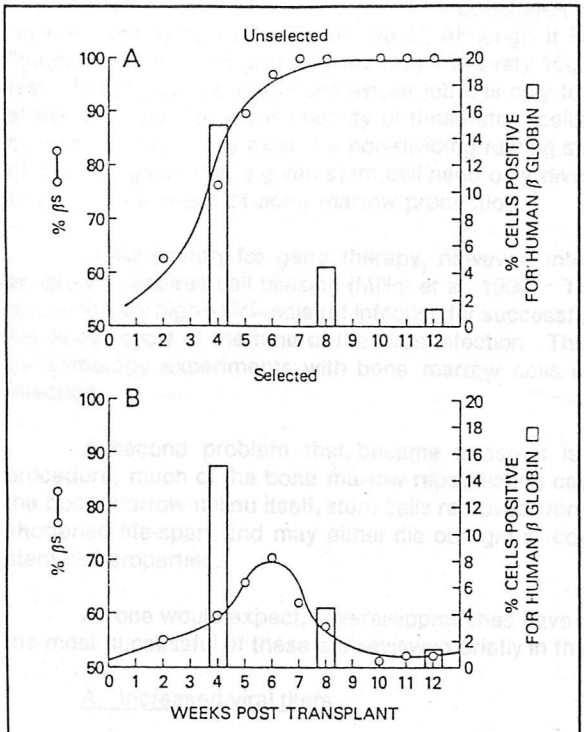


FIG. 5. Time course of human β-globin chain production in transplanted mice. The donor mice are homozygous for the β^s gene, but the recipients contain β^s, β^{major}, and β^{minor} hemoglobins. A recipient with 100% β^s has 100% reconstitution of donor bone marrow. The solid lines show the % donor hemoglobin (β^s) and the bars indicate the % cells stained with the anti-human β-globin monoclonal antibody. (*A*) The mouse whose cells are shown in Fig. 4*C* (injected with unselected cells). (*B*) The mouse whose cells are shown in Fig. 4 *A* and *B* (injected with G418-selected cells).

Fig. 8 From Karlsson et al, 1988

As shown in the upper panel of Fig. 8, bone marrow cells that were simply infected with the retrovirus carrying the β-globin construct and transferred into recipient irradiated mice showed a progressive replacement of peripheral blood red cells by cells of donor origin, reaching 100% by 8 weeks after transplantation and persisting for at least a total of 3 months. However, the presence of cells positive for the human β-globin gene, while very high at 4 weeks, dropped dramatically by 8 and 12 weeks, and, in fact, disappeared entirely thereafter (not shown). Thus despite achieving a permanent engraftment of donor bone marrow, the foreign protein was rapidly disappearing from the surviving donor cells. Although one possibility was that the gene was still present, but was simply not expressed, DNA studies revealed that the number of gene copies present in the donor cells also decreased rapidly, eventually reaching undetectable levels.

In an attempt to resolve this problem, this group also tried to select in vitro only those bone marrow cells that were carrying both the *neo*^{R} and the β-globin gene. They reasoned that by eliminating the cells that had not acquired the foreign genes, all of the bone marrow cells transplanted into the recipient mice would be carrying the construct, and thus would not be replaced by the uninfected cells. However, as shown in panel B, in this experiment, the bone marrow graft itself, independent of the foreign gene, reached a partial level of engraftment at 6 weeks and then totally disappeared by 12 weeks. Presumably sufficient cells of host origin survived the irradiation procedure to repopulate the bone marrow in the period following the initial abortive engraftment of donor cells. Of course, the β-globin gene construct also rapidly disappeared from the circulation with the loss of the donor graft. Thus, selection had the unexpected effect of reducing the donor marrow's repopulating capacity so drastically that insufficient stem cells remained to cause permanent engraftment of the host.

The explanation for these problems in gene therapy using bone marrow lies in the structure of the hematopoietic system that was alluded to earlier in this talk. True hematopoietic

stem cells, i.e. cells with the capacity to completely repopulate a recipient animal with both myeloid and lymphoid cells are rare. Although it is difficult to give an exact number, the "totipotent"stem cells probably are only 1 in every 100,000 bone marrow cells, or possibly even less. In addition, as stem cells whose job it is only to function in times of very severe marrow stress or injury, the great majority of these stem cells are not undergoing cell division or cell cycling. Instead, they exist in a non-dividing resting stage usually termed G0. In fact, a variety of data suggests that a given stem cell need only divide at very infrequent intervals in order to supply all the needs of bone marrow production.

Unfortunately for gene therapy, however, integration of the retroviral DNA construct absolutely requires cell division (Miller et al, 1990). Thus, not only are stem cells rare, *a prior* requiring very high efficiencies of infection for successful transfers, but most of the stem cells are not in cell cycle at the time of the virus infection. Thus, much of the difficulty encountered in gene therapy experiments with bone marrow cells can be related to a failure of stem cell infection.

A second problem that became apparent is that during the 24-48 hours infection procedure, much of the bone marrow repopulating capacity is lost. Although long-lived within the bone marrow milieu itself, stem cells removed from this normal environment have a greatly shortened life-span, and may either die outright or committ to differentiation, thus losing their stem cell properties.

As one would expect, several approaches have been taken to solve these dilemmas, and the most successful of these are reviewed briefly in the next section.

A. Increased viral titers

A number of groups have focused on improving the packaging cell lines and viral constructs in order to achieve the highest possible titers of virus. Although 10^4 to 10^5 virions per ml of culture media can frequently be achieved, recently Mulligan and colleagues have recently described experiments carried out with viral titers as high as 10^6 to 10^7 per ml (Wilson et al, 1990). Indeed, their data show that with such high titer preparations, it is possible to obtain expression of the human ADA gene for at least 6 months after transplantation in the great majority of the transplanted mice. With lower titers of virus, however, the same group was only able to obtain 18 of 104 mice with long-term persistence of the viral sequences (Dzierak et al, 1988). Thus, the authors suggest that the major factor limiting the success of gene therapy is not the low rate of stem cell cycling but inadequate numbers of virus particles to ensure a large number of stem cells will carry the gene transfer construct. Similar results have recently been obtained in a primate model, although the presence of some infectious viral recombinants in the preparations complicate the interpretation of these results (Bodine et al, 1990).

B. Growth factors

As an alternative approach to improving the efficiency of gene transfer into the stem cell, a number of groups have studied the use of hematopoietic growth factors to stimulate the stem cell to go through at least one round of cell division. Recently, a combination of IL-3 and IL-6 have been reported to increase the frequency of gene transfer into hematopoietic stem cells in vitro (Bodine et al, 1989). However, evidence that this approach actually improves the results

of in vivo transfers has not been reported. In addition, some investigators think that the available growth factors actually may be deleterious to stem cell maintenance. These scientists suggest that known growth factors may simply cause the stem cell to committ to differentiation and thereby lose much of its stem cell properties.

Recently, however, a novel factor that may be specific for the hematopoietic stem cell has been cloned and purified. Although the results of this work are just now in press and about to be published in the Octboer 5 issue of Cell, this factor, or one like it, offers the promise of promoting stem cell division without causing committment to differentiation. What is known is that this new factor, which has been termed "stem cell growth factor", is the ligand for a proto-oncogene membrane receptor, *c-kit*, that is normally present on hematopoietic stem cells in the mouse. Because defects in either this receptor or the growth factor are known to produce an aplastic anemia in the mouse, it appears possible that this new factor may allow manipulation of the earliest bone marrow stem cells. Ideally this factor will prove to be capable of causing stem cell division and "self-renewal"without causing differentiation into a committed cell pathway.

Lending credence to this idea is the recent discovery of a known factor has just this property for another type of stem cell, embryonic stem cells (Smith et al, 1988). Although it has been possible for a number of years to maintain these embryonic stem cells in culture, the cells had to be grown on feeder layers of cells or they immediately underwent spontaneous differentiation and loss of their "stemness". However, a known factor, called leukemia inhibitory factor or LIF, has now been shown to prevent the spontaneous differentiation that occurs in culture, eliminating the need for growth on feeder layers. The availability of a similar regulatory molecule for the hematopoietic stem cell would greatly facilitate attempts at gene therapy with bone marrow.

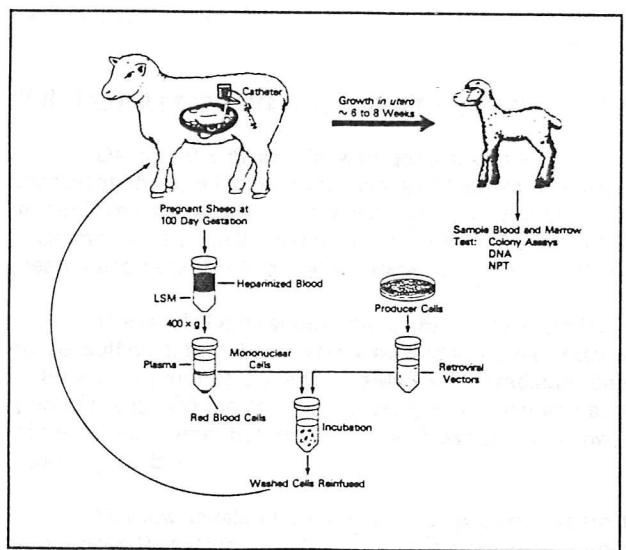


Fig. 9 From Karson, 1990

C. Fetal stem cells

Although adult hematopoietic stem cells are non-cycling, during fetal life the developing hematopoietic system's demands are so great that nearly every cell, stem cells included, are thought to be undergoing rapid cell division. In an attempt to take advantage of this property, Zanjani and his colleagues have performed a number of experiments in a sheep model system (Kantoff et al, 1989; Ekhterae et al, 1990). As shown in Figure 9, these investigators actually remove circulating cells from the fetus 7 weeks before birth, infect these cells in vitro, and then return the infected cells to the fetus. Using this method, this

group was able to show that the *neo* gene was detectable after birth in the bone marrow and hematopoietic organs of several of these lambs. In one case, drug-resistant hematopoietic cells were still present 2 years after birth, providing indirect evidence that the *neo* gene had persisted in a small number of hematopoietic cells. As expected, the same procedure was unsuccessful when applied to adult sheep, however, recent data suggests that neonatal hematopoietic cells may also be superior to adult cells and as least as useful as fetal ones (al-Lebban et al, 1990).

D. Long-term bone marrow culture, stem cell purification

Other approaches to improving gene transfer into hematopoietic stem cells include the development of culture systems that reproduce the environment of the bone marrow and thus provide a long-term system to maintain hematopoietic stem cells in vitro. Limited evidence exists to suggest that bone marrow grown in these specialized cultures can reconstitute human hematopoiesis in vivo (Spooncer and Dexter, 1983). Moreover, efficient infection of hematopoietic cells in vitro has been demonstrated in mouse (Anklesaria et al, 1987; Nolta et al, 1990), canine (Schuening et al, 1989), and human cultures (Holland et al, 1989; Hughes et al, 1989).

Significant progress has also been achieved in isolating and purifying the hematopoietic stem cell from mouse (Spangrude et al, 1988), primate (Berenson et al, 1988), and human bone marrow (Andrews et al, 1989). Using highly purified mouse stem cells, Eaves and colleagues have recently shown that persistance and function of the *neo* gene for up to 6 months in the majority of mice transplanted with 150-2000 of these purified, virus-infected stem cells (Szilvassy et al, 1989). Clearly, the advantages of working with purified cells include the need to infect fewer cells and the ability to more closely control the culture conditions and addition of recombinant growth factors.

VIII. In vivo gene therapy in animals - Recent mouse results

Despite the problems with gene transfer into hematopoieitc stem cells, with the latest modifications to infection protocols and increases in virus titer, several groups have been able to achieve long-term engraftments in mice with vectors carrying both the human β-globin gene (Bender et al, 1989; Bodine et al, 1989; Dzierak et al, 1988) and the human adenosine deaminase gene (Belmont et al, 1988; Lim et al, 1989; Wilson et al, 1990; Kaleko et al, 1990).

The level of expression of the adenosine deaminase gene achieved in these mice seems to be sufficient to achieve some benefit in human recipients, as judged by the levels of ADA achieved in patients currently treated with irradiated cell transfusions and stabilized enzyme preparations. On the other hand, the β-globin experiments have achieved long-term expression of the human gene, but the level of expression is still low, only 1-2% of the endogenous normal mouse β-globin.

The low levels of expression for β-globin seem to result from the lack of regulatory sequences that strongly enhance β-globin expression, but which are located at a considerable distance from the gene itself (Grosveld et al, 1987). Clearly, inclusion of these enhancer sequences in the gene transfer construct will present possibly insurmountable problems,

because of the roughly 8 kb limit on the size of the viral genome. Moreover, even within this limit, larger molecules seem to be associated with lower viral titers, further complicating stem cell infection. Current research on the β-globin gene has thus been directed to improvements in the promoter region that will allow higher levels of expression in future mouse model experiments (Miller et al, 1988; Guild et al, 1988; Valerio et al, 1989).

IX. In vivo gene therapy in animals - Large animal models

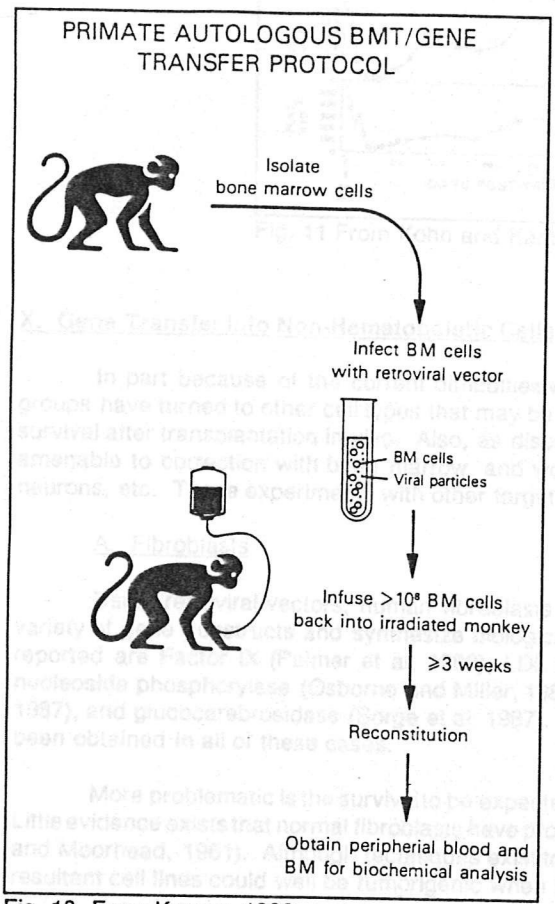


Fig. 10 From Karson, 1990

Although the most recent results with the ADA gene and β-globin in the mouse demonstrate unequivocal long-term engraftments and gene expression, translation of these results to larger animals presents additional difficulties. For example, engraftment success is still highly variable from mouse to mouse, probably reflecting the fact that only a small number of stem cells actually account for each successful engraftment. In addition, large losses of marrow cell number and repopulating capacity are still inevitable in the in vitro steps accompanying the retroviral infection.

Of course, in the mouse the number of donor bone marrow cells obtainable is not really limited by the number of available donors animals. Indeed, many of the most successful mouse protocols have actually used multiple mouse donors for each mouse recipient. Thus, "scaling up" experimental numbers is an option with mice that will not be feasible in primate experiments and eventual human experiments.

Because of these difficulties, only a small number of experiments with large animals, primarily dogs and monkeys, have been published (Kantoff et al, 1987; Stead et al, 1988; Eglitis et al, 1988; Bodine et al, 1990). One protocol used to attempt gene transfer of the human ADA gene in primates is shown in Figure 10. Although short-term expression of the ADA gene was demonstrated in a number of the primate recipients, long-term expression was not seen. As shown in Figure 11, in the most successful case, human ADA activity was detected at 2-3 months after transfer and then became undetectable thereafter.

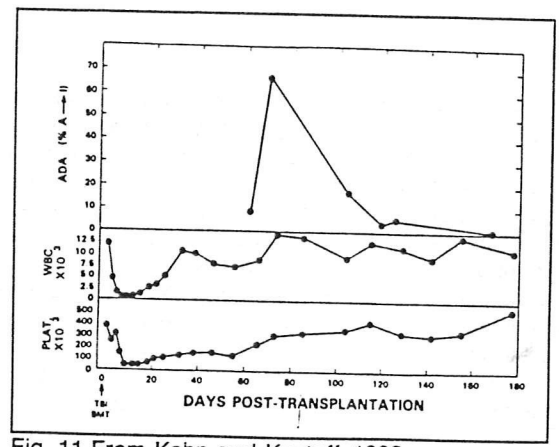


Fig. 11 From Kohn and Kantoff, 1989

X. Gene Transfer into Non-Hematopoietic Cells

In part because of the current difficulties with hematopoietic stem cells, a number of groups have turned to other cell types that may be more readily infected, or may have a longer survival after transplantation in vivo. Also, as discussed earlier, a number of diseases are not amenable to correction with bone marrow, and would require gene transfer into hepatocytes, neurons, etc. These experiments with other target cell types will be briefly reviewed here.

A. Fibroblasts

Using retroviral vectors, human fibroblasts have been demonstrated to incorporate a variety of gene constructs and synthesize biologically active proteins. Among the constructs reported are Factor IX (Palmer et al, 1989), LDL receptors (Miyanohara et al, 1988), purine nucleoside phosphorylase (Osborne and Miller, 1988) and adenosine deaminase (Palmer et al, 1987), and glucocerebrosidase (Sorge et al, 1987). Very high efficiencies of gene transfer have been obtained in all of these cases.

More problematic is the survival to be expected for fibroblasts transplanted into recipients. Little evidence exists that normal fibroblasts have prolonged survival and function in vivo (Hayflick and Moorhead, 1961). Although techniques exist to immortalize human fibroblasts in vitro, the resultant cell lines could well be tumorigenic when implanted into the host.

What has been shown is that fibroblasts contained in collagen matrices and implanted under the skin, or grown on collagen beads and then injected intraperitioneally, can secrete active human factor IX in animals (Palmer et al, 1989; St. Louis and Verma, 1988). Although the levels of factor IX detected in some of these animals was close to the levels that would be required for benefit in factor IX-deficient hemophiliacs, expression was not observed for longer than one month in any of the animals.

B. Keratinocytes

Another cell that has been investigated as a target for gene therapy is the keratinocyte. Because keratinocytes have been successfully grown in culture into large sheets and used for replacement of damaged skin in burn patients (Gallico et al, 1984), they could prove to be useful reservoirs for synthesis of a variety of proteins. Furthermore, in vitro experiments have shown that human keratinocytes are capable of secreting biologically active growth hormone after gene transfer (Morgan et al, 1987). Although keratinocytes are separated from the circulation by the basement membrane that divides the epidermis from the dermis, one study has shown that a protein as large as 90 kilodaltons (apolipoprotein E) can gain access to the circulation after transplanted of keratinocytes (Fenjves et al, 1989). In another study, dog keratinocytes infected with a neo^R vector persisted for more than 120 days in a subdermal site and continued to express the drug resistance gene throughout this period (Flowers et al, 1990). Clearly, keratinocytes may prove to be useful for human gene therapy in the near future, especially for low molecular weight proteins.

C. Hepatocytes

Retroviral vectors have also been used to transfer genes into hepatocytes from a variety of species (Ledley et al, 1987; Peng et al, 1988; Wolff et al, 1987; Wilson et al, 1987; Anderson et al, 1989; Wilson et al, 1988). Several studies have also shown that genetically altered hepatocytes can persist for several months in the peritoneal cavity of rats after attachment to a variety of beads or solid support systems (Dementriou et al, 1986, 1988; Thompson et al, 1988, 1989). Clearly additional work, particularly on methods for hepatocyte transplantation, needs to be done to establish the value of this approach for human therapy.

E. Endothelial cells

Using dogs (Wilson et al, 1989) and pigs (Nabel et al, 1989), two different groups have demonstrated that endothelial cells could be infected with a retroviral vector carrying a bacterial β-galactosidase gene at high efficiency. Following seeding on a Dacron graft or directly onto denuded arterial segments, endothelial cells expressing the bacterial gene persisted for more than a month in both systems.

Recently, in the pig model, Nabel and colleagues have gone on to show that a 30 minute in vivo incubation with a viral supernatant introduced directly into a segment of artery isolated by a double lumen catheter can lead to expression of the bacterial gene for up to 5 months (Nabel et al, 1990). A third group has similarly demonstrated that rabbit endothelial cells can be efficiently transduced by retroviral vectors and that intravascular stents can be seeded with the genetically altered endothelial cells and implanted into animals (Zeibel et al, 1989; Dichek et al, 1989)

F. Muscle cells, respiratory cells, and neurons

Limited numbers of papers suggest that these neurons (Palella et al, 1989) and respiratory cells (Iannuzzi et al, 1988) can also be infected with retroviral vectors. In addition, direct microinjection of individual cells has been used to introduce genes into muscle cells directly (Wolff et al, 1990). Obviously, substantially more work will have to be done to adapt these cell types to human gene therapy. The recent report of successful correction of the cystic fibrosis defect by in vitro gene transfer with the cloned CF gene (Drumm et al, 1990), however, may hasten proposals to use aerosols of retroviral particles to infect the respiratory epithelium.

XI. Gene Transfer into Human Lymphocytes

Because of the very limited results obtained to date with hematopoietic stem cells in large animal models, two groups of investigators at the NIH have turned to another hematopoietic cell, the lymphocyte, for the first trials of gene transfer techniques in humans. Although the life-span of a lymphocyte stem cell is unclear, there is good data to suggest that T cells may circulate for many years. As discussed below, the first of these investigations has introduced the *neo*^{R} gene into a specialized class of lymphocyte, the tumor infiltrating lymphocyte or TIL cell. The results of this first attempt has just been reported. Also in progress, and thus far reported only in TIME, Newsweek, and major newspapers is a second study that is attempting to introduce the human ADA gene into the lymphocytes of a child with ADA-deficient SCID (severe combined immunodeficiency).

A. Tumor Infiltrating Lymphocytes (TIL) cells

In the last few years, a number of well publicized studies by Rosenberg and his colleagues have been published regarding the use of IL-2 and natural killer lymphocytes, and more recently the TIL cells, to obtain tumor remissions in patients with melanoma and renal cell carcinoma (Rosenberg et al, 1986, 1988). As part of these studies, these authors recently conducted an initial trial of gene transfer with retroviral vectors into human lymphocytes obtained from biopsies of tumor specimens. The plan of this study is shown in Figure 12. Following tumor biopsy and in vitro expansion of TIL cells with IL-2, a fraction of the cells are incubated with the N2 retroviral vector carrying the *neo*^{R} gene, selected in G418, and mixed back with uninfected TIL cells for infusion into the patient. The idea of the study was not only to demonstrate the safety of the retroviral techniques for human use, but also to provide a marker by which to analyze the survival and homing ability

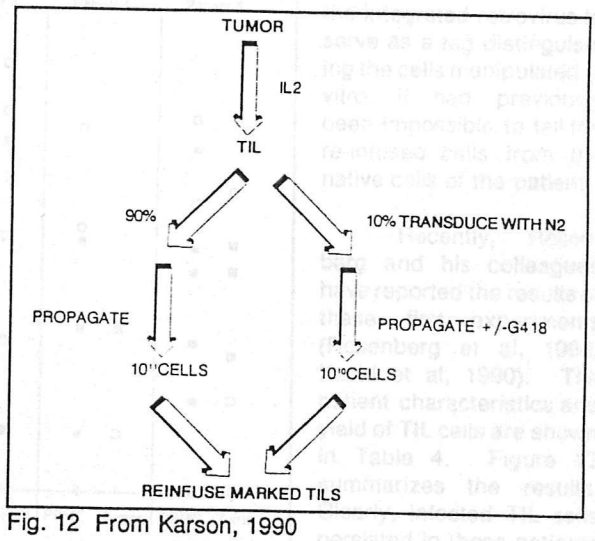


Fig. 12 From Karson, 1990

Table 1. Characteristics of the Patients and Their Tumors.

Patient No.	Age/Sex	Primary Tumor Site	Previous Treatment	Resected Tumors					Sites of Disease That Could Be Evaluated
				Site	Size	Total Cells Obtained	Cells Used to Start Culture*	Percent Lympho-cytes	Percent Tumor Cells	
					cm	$\times 10^{-7}$				
1	52/M	Neck	Wide local excision	Lymph node	4×4×2	33	12	15	85	Lung, liver, spleen
2	46/F	Finger	Amputation of finger Lymph-node dissection	Lymph node	5×5×3	157	50	70	30	Lymph nodes, intramuscular
3	42/M	Back	Wide local excision Lymph-node dissection Melanoma "vaccine" Interleukin-2 and interferon alfa	Lymph node Subcutaneous (2 tumors)	6×5×4 2×2×2	205	120	31	69	Lung, subcutaneous
4	41/M	Chest	Wide local excision Lymph-node dissection Interleukin-2 and interferon alfa	Subcutaneous (2 tumors)	2×1×1 and 5×4×4	41	4	39	61	Lung, liver, lymph nodes, subcutaneous, brain
5	26/F	Arm	Wide local excision	Subcutaneous (6 tumors)	2×2×2 to 5×4×2	71	15	16	84	Lung, lymph nodes, subcutaneous

*Denotes the number of cells used to start the cultures that were ultimately administered to the patient.

Fig. 13 From Rosenberg et al, 1990

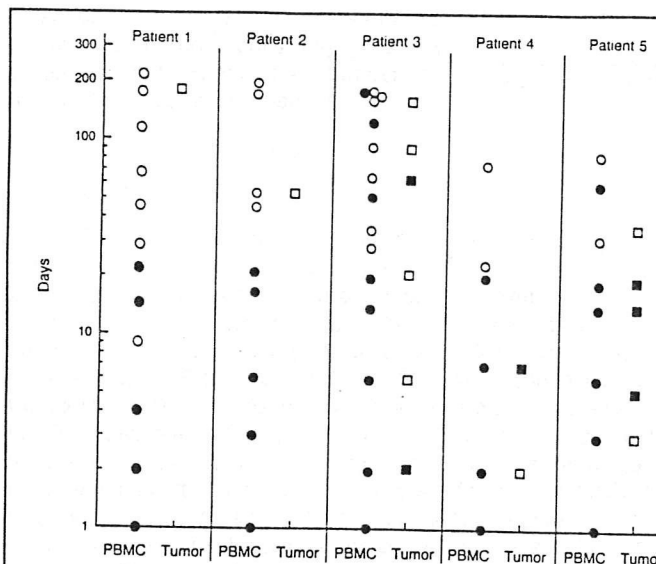


Figure 4. Results of Polymerase-Chain-Reaction Assays of Peripheral-Blood Mononuclear Cells (PBMC, Circles) and Tumor-Biopsy Specimens (Squares) Obtained from Patients at Various Intervals after the Infusion of the Gene-Transduced TIL. Open symbols denote negative results, and solid symbols positive results.

Fig. 14 From Rosenberg et al, 1990

of the TIL cells. Without the integrated retrovirus to serve as a tag distinguishing the cells manipulated in vitro, it had previously been impossible to tell the re-infused cells from the native cells of the patient.

Recently, Rosenberg and his colleagues have reported the results of these first experiments (Rosenberg et al, 1990; Kasid et al, 1990). The patient characteristics and yield of TIL cells are shown in Table 4. Figure 13 summarizes the results. Clearly, infected TIL cells persisted in these patients in both blood and tumor, in one case for 2 months after transplantation. None of the patients showed evidence of viremia.

B. ADA-deficient lymphocytes

Currently in progress is the first trial of gene transfer with actual therapeutic intent. Although no results have been reported, the protocol apparently involves taking T cells from ADA-deficient patients, infecting them with a retroviral vector encoding the human ADA gene, and infusing the cells back into the patient. Clearly, the two major questions to be answered in this study are (1) will sufficient numbers of cells incorporate the ADA gene and express the gene with a high enough efficiency to obtain a therapeutic benefit?, and (2) how long will the genetically altered T cells persist in the lymph nodes and circulation?

Table 5 presents these investigators rationale for this study. A key point that may be relatively unique for ADA deficiency is that lymphocytes with increase ADA activity should have a growth advantage in vivo over the ADA-deficient ones. Indeed, we know that SCID patients given bone marrow transplantations are engrafted with donor lymphoid cells without the need for pre-transplant irradiation or drug conditioning. Thus, without the need for toxic drug selections, even small numbers of genetically altered T cells may gradually proliferate and expand their numbers to obtain more significant benefit with time.

Rationale for Considering ADA-Deficiency SCID for Initial Trials of Human Gene Therapy

1. The normal human ADA gene has been cloned
2. A single gene defect leads to the loss of the enzyme
3. HLA-matched allogeneic bone marrow transplantation completely corrects the disease
4. Treatment for those patients who lack HLA-matched bone marrow donors is less than ideal
5. Corrected cells should have selective growth advantage in vivo
6. A broad range of ADA activity should correct disease and not be harmful

Table 5 From Kohn, Anderson, Blaese, 1989

XII. Conclusion

The rapid progress in the field of gene therapy is surprising and at the same time impressive. It seems clear that successful protocols for a variety of different target cells and diseases will be developed in the not too distant future. On a more distant horizon are proposals to use retroviral vectors and other techniques for gene transfer to treat cancer (Huang et al, 1988; Moolten et al, 1990; Sinkovics, 1989; Dumenco et al, 1989; Bookstein et al, 1990; Jelinek et al, 1988), AIDS (Cann and Karn, 1989; Anderson, 1989), and other more complex diseases. For example, Rosenberg and his colleagues plan to introduce the gene for tumor necrosis factor into TIL cells in an effort to improve tumor cell killing. Others have proposed introducing strong foreign histocompatibility antigens into a patient's tumor cells in order to stimulate anti-tumor host defenses after re-infusion of the altered cells. For AIDS, it is conceivable that large amounts of CD4 produced be engineered cells might compete with the native CD4 on helper T cells and prevent the HIV virus from infecting and killing these cells. Alternatively, genes for molecules that interfere with required HIV regulatory proteins (i.e. *tat* and *rev* genes) might be introduced into CD4 cells early in the course of the disease and render them immune to killing by the virus. Although these ideas may seem unlikely now, just a few short years ago, even the idea of introducing genes into patients was considered improbable.

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