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Invited Review

Perspectives in gene therapy

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Summary. Gene therapy is understood to be both the restitution of genetic alterations caused by mutation or deletion and the control of overexpressed genes. The concept of gene therapy can also encompass molecular strategies to induce cell death in tumor cell by either the so-called "suicided genes" or by certain viral genes that induce a more selective cell death among the transformed cells.

The prospect for the clinical application of gene therapy are enormous and, at least theoretically, its utilization can be extended to a number of diseases known to have a genetic basis, and to neoplastic processes. This review summarizes some of the projects that are currently underway involving neoplastic diseases, liver diseases, hematopoietic cells and respiratory tract cells. The results of most of the ongoing protocols are not yet conclusive, and presumibly, their clinical application is still some years away. One of the major limitations is the method of introducing the genetic sequences into the cells and achieving their constitutive expression by the cells. For ethical reasons, this approach should not be done in germ cells, but at the level of the tissue or cells most closely involved in the development of each gene-based disease. The methods employed in gene therapy are discussed, focusing on those mediated by the application of viral vectors, as well as those requiring the use of liposomes and others.

Key words: Gene therapy, Viral vectors, Non-viral vectors

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1. The concept of gene therapy

The term gene therapy is understood to mean the use of molecular methods to replace defective or absent genes, or to counteract those that are overexpressed. This approach to medicine, which appeared to be a matter of science fiction only a few years ago, is now a completely plausible reality owing to, among other things, the rapid progress made in recombinant DNA techniques and the processes of gene transfer that a number of scientific teams are employing with quite encouraging results (Anderson, 1992; Miller, 1992).

The use of this molecular approach will make it possible to set aside some of the present standard treatments, such as the repeated administration of parenteral proteins (as in the case of hemophilia) or drugs (as in hereditary hypercholesterolemia and tumor processes) (Friedman, 1992).

"Genetic" manipulations involve a series of basic scientific methods that range from coprecipitation of DNA by means of calcium phosphate (according to the classical method that led to the initial transfections) and electroporation (which can only be employed outside the organism), to methods that can be applied *in vivo*, such as direct DNA injection, liposomal lipofection or receptor-mediated transfer (Mulligan, 1993). Advances in the field of virology have shown that the use of

recombinant viral vectors would produce more stable and specific transfections, which can be employed both within and outside the organism (Mitani and Caskey, 1993). It is for this reason that the use of adenoviruses, retroviruses and adeno-associated viruses is rapidly being introduced.

2. Background

The first experiences that could be related to gene therapy date back to 1944 when Avery chanced upon DNA-mediated gene transfer while investigating the substances that transformed pneumococcos (Avery et al., 1944). Twenty years ago, DNA incorporation became possible through calcium phosphate-mediated intracellular transport in cultured cells; in this way, primary cells were transformed by SV40 and polyoma papovavirus, showing that gene transfer in that case viral, was capable of inducing tumor development (Sambrook et al., 1968; Temin, 1971). But it was the development of recombinant DNA that made it possible to clone genes and achieve adequate amounts of them. This was a spectacular advance and has opened enormous perspectives for their use, for example, in genetic diseases such as hemoglobinopathies, Lesch-Nyhan syndrome, cancer and other processes that may respond to a specific molecular treatment (Maniatis et al., 1976; Mulligan and Berg, 1981).

In 1988, the guidelines for human gene therapy trials were approved by the Recombinant DNA Advisory Committee of the National Institutes of Health in Bethesda, where it was concluded, after a separate series of preliminary experiments, that it would be necessary to evaluate the safety of performing this type of transfer in humans.

3. Common targets in gene therapy

With the rapid advances being made in the isolation of the genes responsible for different diseases, most of the genetic changes that have no definitive treatment find an open door in this type of therapy, the results of which, while still somewhat futuristic, are clearly foreseeable.

3.1. Cancer and therapeutic perspectives

Cancer, or the malignant transformation of tumors, is one of the most common causes of death, with overall prospects for cure of 50%. The incidence of cancer has grown enormously since 1900 when it was responsible for 4% of all deaths. In 1994, it was the causative factor in 24% of deaths, taking second place only to coronary diseases. In terms of the prognosis and prospects for cure, the limited clinical progress that has been made toward resolving most epithelial solid tumors is truly singular. The rate of cure of these tumors, which are among the most prevalent, is similar to that reported 15 years ago. The only exception to this observation is the excellent response to antitumor treatment now being obtained in germ-cell tumors and Hodgkin's lymphomas.

With regard to cancer, there are at least two basic premises that must be taken into account in order to understand the different studies that are underway and put them into perspective: 1) the great morphological and prognostic heterogeneity and variability of these lesions, with more than 250 different types of malignant tumors; and 2) the large number of oncogenic molecular changes reported to date (Bos, 1989), of which, including oncogenes and suppressor genes, there will be more than one hundred within a few years (Lattine and Gerson, 1996). Basically, it is admissible that all those genes,whose constitutive activation/inactivation lead to increased cell proliferation, alter the control of the cell cycle, interfere with cellular differentiation or is implicated in programmed cell death, cell suicide or apoptosis, are potentially oncogenic.

The therapeutic perspectives in cancer are linked to advances in the study of the molecular changes occurring in malignant tumors and to the development of methods of inhibition or suppression of said molecular changes in the case of the oncogenes or in the restitution of the "inactivated" suppressor genes (Table 1). In this respect, there are several different lines of research, some of them already being tested in clinical trials, including a few that are particularly interesting (Nabel et al., 1994). Among these, the study of inhibitors of oncogenic activity, discussed in a number of promising works that deal with the inhibitors of the enzymatic pathways involved in their synthesis (e.g. farnesyl transferase and the ras genes), or, for example, the use of specific inhibitors like the tyrosine kinase inhibitor, K252, for the trk oncogene activity which is associated with many types of thyroid cancer should be pointed out. Some approaches deal with the use of chemical substances whose substrate is involved in malignant transformation (e.g. the administration of retinoic acid to patients with promyelocytic acute leukemia to induce neutrophil maturation and disease remission) (Toma et al., 1994), while others are based on the introduction of suppressor genes which are absent or mutated in tumors. Suppressor gene mutations, especially those involving p53 and pRb, have been shown to be the most prevalent oncogenic alterations reported to date in human cancer. The use of different methods to transfer these genes to the malignant cells is one of the most promising paths in gene therapy. Still another strategy involves the inhibition of oncogenic activity by antisense molecules or nucleotide sequences complementary to the mRNA of different oncogenes that hybridize with said RNA, abolishing its activity and cellular expression (Wagnes, 1994). This approach is already being carried out in some cases; it works well *in vitro*, but proves very expensive *in vivo* and, in general, induces a transitory and independent effect. It is currently being tested in certain leukemias with anti-myc molecules. Finally, a great deal of effort is being put into the design of new chemotherapy and radiation therapy protocols. In this branch of research, it is necessary to distinguish between

the study of new antitumor drugs and the use of molecular methods to increase the efficacy of chemotherapy and radiation therapy, such as the transfer of genes resistant to MDR drugs to bone marrow progenitor cells so that chemotherapy doses can be incremented without noxious collateral effects, such as induced myelosuppression (Mastrangelo et al., 1996). Moreover, it should prove possible to apply more specific chemotherapy and radiation therapy protocols for each tumor and each patient depending on the particular oncogenic alterations of the tumor (Harris et al., 1994). It is now known that the cellular response to the different antitumor drugs that are administered to treat most cancers varies according to the oncogenic alterations present; the chemoresistance induced by the ras oncogenes, p53 mutations, raf mutations and radiation therapy resistance should be pointed out, as well as the fact that some oncogenes induce resistance to certain antitumor agents and sensitivity to others.

However, the majority of the methods described are based on specific molecular changes and, as was mentioned before, despite the numerous oncogenic alterations presented by the tumors, their *in vivo* "statute of transformational hierarchy" remains unknown. To eliminate some of these problems, the gene therapy methods that involve transferring genes with generic cytotoxic effects, such as suicide genes, or those capable of inducing apoptosis in tumor cells are highly promising (Table 3). The first group includes thymidine kinase, herpes virus and VZV genes, which can be transferred to malignant cells by means of different vectors and that, following the administration of antivirus drugs such as ganciclovir, produce the death of the tumor cells (Levitzki and Gazit, 1959; Shimotono and Temin, 1981; Culver et al., 1992). Likewise, the overexpression of genes like p53 can induce apoptosis in certain cells, and there are other viral genes such as the adenovirus gene E1A, which induces a marked susceptibility to cell apoptosis after DNA damage (Sánchez-Prieto et al., 1995; Sánchez et al., 1996), suggesting that it can be employed as a coadjuvant antitumor therapeutic agent, as well as to enhance sensitivity to antineoplastic agents that act upon the DNA, such as cisplatin, doxorubicin and radiation therapy. All these efforts and assays "work" well at the experimental level *in vitro* and in laboratory animals, but their projection to use in humans is still controversial. For example, the use of suicide genes to treat brain tumors works spectacularly in animals but is much less effective in humans. In this respect, one of the keys is the development and design of selective viral vectors that are highly efficient in human tumor cells.

Finally, the prospects for advances in antitumor treatment based on immunological methods and mechanisms should be mentioned (Table 1) (Cournoyer and Caskey, 1993; Colombo and Forni, 1994). These methods have been under development for the past several years to potentiate the cytotoxic effect of T lymphocytes by means of the *in vivo* isolation and growth and the transfer of different genes such as interleukin 2 and tumor necrosis factor (Old, 1996) (Table 3). This approach, which is employed in certain clinical protocols such as those dealing with cutaneous melanomas, represents a promising line because, theoretically, it may be complementary to other methods; moreover, it may permit the "killing" of disseminated cells and those that prove resistant to other more classical methods (Goldenberg, 1993).The attempt is also being made to increase the immunogenicity of the tumor cells by transferring genes such as interleukins and HLA-B7 to them, and the use of antitumor vaccines, some of them against certain oncogene antigens or mutated versions of the p53 protein, are recently being assessed. Finally, one of the several highly promising lines of research that are just getting underway involves the use of mimetic peptides from the active protein regions of certain suppressor genes such as p53, which may have an antitumor suppressor effect (Olif et al., 1996).

In summary, it could be said that there is a certain degree of optimism and that the future prospects for cancer therapy are promising because of the rapid progress of molecular studies of malignant tumors, the design of agents that inhibit the different oncogenes, the control of apoptosis, which can prove to be extremely important in terms of gene therapy and the response to chemotherapy and radiation therapy, and the development of vectors or methods of transferring those genes capable of reversing the malignant phenotype or selectively of destroying tumor cells. Nevertheless, in the undertaking of these projects, the definitive leap is their application in human tumor pathology. We must be aware of the enormous frustration often associated with them due to the wide heterogeneity of human tumors and because many of the underlying molecular changes occurring in cancer are yet to be fully understood. The application of these approaches in monogenic or metabolic diseases, certain immunodeficiencies and other diseases is very promising, but the results are just starting to yield the necessary clues.

3.2. Gene therapy in the liver

Ornithine-carbamoyltransferase (OCT) deficiency, an X-linked disorder that affects only males, is the most common human inborn genetic error involving urea metabolism; it has a high rate of new mutations. Owing to hyperammonemia and the symptoms associated with it, it is easily recognized within days of birth although most of the boys who survive are brain damaged due to recurrent catabolic seizures. The two transgenic models in mouse, spf and pf-ash, are associated with substantially reduced OCT activity (by 20% and by 5% to 10%, respectively (Hodges and Rosenberg, 1989)). In terms of biochemistry, both models are associated with orotic aciduria, reduced serum citrulline and elevated serum glutamine levels, all symptoms that characterize OCT-deficient patients.

The phenotypic and biochemical abnormalities associated with the spf model were corrected by injection of complementary DNA (cDNA) from human OCT into the oocytes of homozygous females (Jones et al., 1990; Morsy et al., 1993). Subsequently, the therapeutic efficacy of somatic gene transfer for OCT deficiency was assessed. Adenoviral vectors with defective replication were then constructed, encoded for the reporter gene, ß-galactosidase, and the cDNA from OCT. These adenoviral vectors were shown to be highly efficient for gene transfer in hepatocytes both *in vitro* and *in vivo*, where nearly 100% of the infected hepatocytes expressed the reporter gene (Morsy et al., 1993). Moreover, the treatment of primary cell cultures derived from liver tissue taken from spf-ash mice with the recombinant adenovirus bearing the cDNA from OCT was found to reconstitute more than 100% of the OCT activity. The reconstitution of OCT activity in human primary hepatocytes obtained from an OCT-deficient patient was coincidental. Stratford-Perricaudet also demonstrated the phenotypic reversal using an adenoviral vector bearing cDNA from rat OCT (Stratford-Perricaudet et al., 1990). The duration of the correction in spf mice and the safety of the adenoviruses used *in vivo* are being studied before being tested in humans.

Ponder et al. (1991) evaluated the *ex vivo* method of gene transfer to hepatocytes. Isolated hepatocytes from the two transgenic models, one expressing ß-galactosidase and the other expressing human α1-antitrypsin, were transplanted into nontransgenic mouse livers of the same branch. A large fraction of these cells was identified two months after transplantation and human α1-antitrypsin was detected for more than six months; findings that established the duration of the reversal.

Other studies dealt with the treatment of Watanabe rabbits presenting a deficient LDL receptor. After partial hepatectomy (30%) and intrasplenic injection of retrovirally-transduced hepatocytes expressing the human LDL receptor, the serum total cholesterol concentration was reduced by between 30% and 50% during the last 4 months (Chowdhury et al., 1991). Given the technical difficulties involved in the *ex vivo* development of the cells and the short duration of the therapeutic expression, two clinical protocols have been approved for ex vivo gene transfer. A protocol designed by Ledley et al. (1991) for labeling hematopoietic cells to study the utility of hepatocellular transplantation in liver-damaged patients and a second protocol by Wilson et al. (1992) aimed at the treatment of familial hypercholesterolemia have also been approved.

3.3. Gene therapy in hematopoietic cells

The hematopoietic system is a suitable target for gene therapy for two reasons: first, there are well-defined procedures for bone marrow transplantation (Karlsson, 1991); and, second, hematopoietic cells (HSCs) are capable of totally reconstructing the bone marrow, with which, theoretically, the correction of HSCs, which would represent a small fraction (0.01% to 0.1%) of the bone marrow cells, would be sufficient to correct the damage. Thus, severe combined immunodeficiencies (eg. adenosine deanimase ADA deficiency), hemoglobinopathies (eg. thalassemia), leukocyte adhesion deficiency and lisosomal storage disorders (eg. Gaucher's disease) are candidates for gene therapy (Karlsson, 1991). Recently, the genes responsible for three additional immune-mediated diseases have been identified: agammaglobulinemia, hyper IgM and severe combined immunodeficiency (SCID), all three of which are linked to chromosome 10. To date, the retroviral vectors appear to be the most efficient vectors for gene transfer in HSCs.

ADA deficiency, a rare autosomal recessive disease, makes up 25% of SCID. The gene is predominantly expressed in thymus and other lymphoid tissues, and produces an enzyme that catalyzes the irreversible deamination of adenosine and deoxyinosine, respectively (Kredich and Hershfield, 1989). In the absence of ADA, dAdo accumulates in many tissues. The accumulation of dAdo and its metabolites, in particular deoxyadenosine triphosphate, inhibits DNA synthesis, resulting in a severe dysfunction of T cells and, eventually, of B cells. ADA-deficient children who are untreated normally die of infection before the age of 2 years (Mitani et al., 1993). Bone marrow transplantation is often the chosen treatment and can lead to a complete cure. The replacement of the enzyme would be another option, although this new therapeutic approach does not produce total immune reconstitution, and antipolyethyleneglycol-ADA antibodies, generated in response to replacement of the enzyme, are detected in some patients.The results of bone marrow transplantation suggest that adequate treatment can be achieved with only 20% of the normal blood ADA levels. Thus, the *in vivo* selection of a few corrected HSCs would be sufficient. The transduction of rat and monkey HSCs by retroviral vectors has been demonstrated, together with that of primary T cells and hematopoietic progenitors from ADA-deficient patients (Mitani et al., 1993).

However, repeated doses pf-transduced T cells are necessary, leading to serious doubts about the possibility of insertional mutagenesis of genes related to cancer after repeated retroviral transfections, although these questions were recently ignored when protocols for HSC gene therapy to treat ADA deficiency were approved in Italy (Bordignon, 1993) and Norway (Hoogerbrugge et al., 1992).

Leukocyte adhesion deficiency is a rare autosomal recessive disease characterized by peripheral blood agranulocytosis, recurrent bacterial infections and unmanageable pus formation (Anderson et al., 1989). The genetic product is a cell adhesion molecule (CD18) that is one of the two components of lymphocytic integrins. The retroviral transduction of human CD18 in mouse hematopoietic progenitor cells and human

Table 1. Therapeutic perspective in cancer.

BASED ON ONCOGENIC ALTERATIONS	BASED ON IMMUNOLOGIC MECHANISMS	BASED ON OTHER MECHANISMS
Inhibitors of genetic alterations Myelocytic leukemia (15:17)	Antitumor vaccines Specific antigens Antigens of oncogenes (p53) or mutated suppressor genes	Gene transfer Capable of inducing apoptosis p53 wt Adenovirus E1A
Inhibitors of oncogenic activity (farnesyl transferase) Tyrosine kinase K252	Viruses expressing tumor antigen	Introduction of "suicide" genes TK from HV and GANCICLOVIR TK from VZV and ARA-M
Antisense strategies Leukemias c-Myc	Increased immunogenicity Interleukins (IL2, 4 and 7) HLA-B7	Modulation of cell differentiation Retinoic acid and leukemias
Introduction of suppresor genes	Increased levels of cytotoxic activity IL-2 TNF	

lymphoblasts has resulted in reduced levels of expression (Wilson et al., 1993). This human genetic disease is an excellent candidate for gene therapy based on strategies similar to those used in the correction of ADA deficiency.

Gaucher's disease is an autosomal recessive, lysosomal disease that is characterized by episodic splenomegaly, pancytopenia, hepatomegaly and skeletal osteolytic and osteopenic degeneration. Moreover, two of the three forms of this disease show progressive neurovisceral storage (Barrangel and Ginns, 1989).

The protein product of the erroneous gene is the enzyme, glucocerebrosidase which metabolizes glucocerebroside, a sphingolipid. The retroviral gene transfer of a normal recombinant glucocerebrosidase gene in mouse HSCs, followed by expression of a protein in differentiated macrophages from transduced HSCs, has been confirmed in certain series (Correl et al., 1992).

Hemoglobinopathy, a disease more common in humans, is caused by a single gene. Hemoglobin has a tetrameric structure, consisting of two pairs of chains. Globin genes have very high levels of expression, but only in erythroid cells. Thus, most gene therapy experiments focus on increasing the level of expression, and the globin gene is regulated by means of retroviral vectors (Steinberg, 1991).

3.4. Gene therapy in respiratory cells

Cystic fibrosis (CF) is the most common autosomal recessive disease in white-skinned humans, affecting 1 of 2,500 newborns. The recent identification of a gene related to CF, regulating cystic fibrosis transmembrane conductance (CFTR), enhances the prospects for gene therapy for this disease (Kerem et al., 1989; Rommens et al., 1989). CFTR encodes for a chlorhydride channel and is expressed in the salivary glands, lungs and pancreas. Gene therapy targets the lung epithelial cells; predominantly undividing cells that are difficult to isolate and reimplant. Thus, for CF, *in vivo* gene transfer strategies are preferred to *ex vivo* methods. Tracheal instillation of an adenovirus with defective replication, encoding for human CFTC, was administered to hamster respiratory epithelium, although the expression lasted only 42 days (Rosenfeld et al., 1992). As an alternative, the DNA-liposome complex was also instilled for *in vivo* gene transfer into rat respiratory epithelium in a rat CF model (Snowaert et al., 1992; Hyde et al., 1993). These achievements in CF gene therapy in animals suggest that analogous therapy would be beneficial in humans with CF. Approval for its use has been given to certain centers in the U.S. and another DNA-liposome complex has been approved in Great Britain. Studies will be necessary to determine whether an adenoviral vector will be capable of transducing sufficient toxicity-free genetic product to achieve a long-term therapeutic effect in humans. The marked antigenicity of adenovirus may also induce a host immune response after repeated vector administration, representing an additional potential limitation for vectors with a known short-term expression. The DNA-liposome alternative could eliminate these impediments.

4. General methods

There are two different ways to accomplish gene transfer: either directly, by *in vivo* inoculation; or by development of a graft outside the organism, into which it is later introduced. A clear example of the process of *ex vivo* gene therapy can be seen in the gene transfer performed in bone marrow transplantation, in which a sample can easily be obtained and subsequently be reintroduced into the patient.

Most types of gene therapy carried out to date involve the use of viruses to transport the gene. These viral vectors include retroviruses, adenoviruses, adeno-associated viruses and herpes simplex virus, among others. There are other, nonviral methods of gene transfer of conjugated DNA-ligands (eg. asialoglycoproteins, polylysine) and lipid or liposomal fusogenic vesicles, the study of which is just getting underway to promote localized gene delivery. On other occasions, DNA may be made to penetrate the cell by simple injection (Wolff et al., 1990) or by aiming it, with a

covering of particles, at the cell (the gene gun) (Yang et al., 1990); the latter, although possible, appears to function primarily in heart and skeletal muscle.

Viral vectors should show defective replication. This is achieved by deletion of one of their essential genes, with the exception of those required to infect the primary cells. In fact, properly prepared, these viruses are so defective that once the target cells have been infected, they are unable to replicate or infect other cells. For this reason, they should be propagated in cell lines that complement the essential functions that have been deleted from them. The therapeutic genes, together with their regulatory elements (promoters) and the poly-adenylation signal, are inserted into the manipulated viruses and thus can be transferred to the target cells, and the desired gene introduced.

A retroviral vector would be capable of integrating it into the host genome, which would result in the permanent expression of the therapeutic gene. The opposite occurs with the adenoviruses, which are the second most commonly used viral vectors. They remain independent of the nucleus and are gradually lost through cell division, thus limiting the duration of their expression. This is an important aspect in some types of diseases in which gene regulation is desirable since the expression of the introduced gene may be harmful (although in other cases, such as hemophilia, it is not). To overcome this problem, regulatory systems involving yeast or bacterial genes have been adapted.

5. Viral methods

5.1. Double-chain DNA viruses

5.1.a. Papovirus-mediated.

The viruses belonging to this family were the earliest vectors to be designed; specifically, the first was SV40, which is one of the most thoroughly studied owing to the fact that it was the first animal virus to be sequenced. This virus has a circular DNA of 5.2 kbp. It encodes for few proteins and when it infects permissive cells (such as monkey cells), it produces a lytic cycle with an early and a late phase.

The most widely employed strategy for the use of this virus as an expression vector in mammalian cells is to attempt to maintain the early zone and eliminate the late zone of the virus, replacing it with a cassette containing the gene considered therapeutic. The problem is that, in selecting this region, the virus will no longer be capable of encapsidating. To solve this problem, several strategies have been applied, such as adding wild-type SV40 to the infected cells so they can overcome this deficiency and become capable of encapsidating (Sambrook et al., 1968).

Another option would be to use cells that are able to express the early zone, the COS cells, with a SV40 integrated in their genome expressing the T antigen. Vectors could be achived by replacing the early zone of the virus with the desired gene, which would be capable of replication and encapsidation. These vectors would reach the nucleus of the COS cell, making thousands of copies and expressing great amounts of proteins, although the latter would die.

5.1.b. Adenovirus-mediated

Adenoviruses are medium-sized DNA viruses having a genome consisting of linear molecules of double-chain DNA of approximately 36,000 bp. The virion is an icosahedron measuring 700 nm in diameter exclusively made up of protein and DNA. Adenoviruses have been isolated in many species, 43 in humans, and more than 100 different serotypes have been identified; the most thoroughly characterized adenoviruses are numbers 2, 5 and 12.

For many reasons, adenoviruses are attracting a great deal of attention as potential vectors with expression in primary cells and as recombinant vaccines. In the first place, not only are the viral particles very stable, but in the serotypes most commonly employed as vectors, the insertion of foreign genes is generally maintained throughout successive rounds of viral replication (Graham and Prevec, 1991). Moreover, the adenovirus genome is relatively easy to manipulate using recombinant DNA techniques, and the virus replicates efficiently in permissive cells.

The replication cycle of the virus can be divided into two phases: early, corresponding to events occurring prior to the replication of the viral DNA; and late, corresponding to the period following the start of viral DNA replication. During the early phase, four regions (early regions E) are expressed: E1 (E1A and E1B), E2, E3 and E4. After DNA replication, the major late promoter (MLP), located at 16 mu, carries out most of the viral transcription (Horwitz, 1985). Transcription by the MPL ends near the right end of the genome and the late transcriptions are processed in a complex of different mRNAs that encode most of the structural proteins of the virion.

Among the regions of the viral genome, there are three that are capable of accepting DNA insertions or substitutions for the generation of a recombinant virus: E1, E3 and a small region located between E4 and the end of the genome.

The E1 region is not necessary for viral replication in human cell line 293 (which is transformed by Ad5 DNA and expresses the left end of the genome), although it would be for the remainder of the cell lines. E3 is not required for adenovirus replication in human cultured cells. The maximum amount of DNA that can be packaged in virions is limited to nearly 105% of the weight of the genome for an extra capacity of about 2 kb of extra DNA. To incorporate larger DNA segments, it would be necessary to compensate by deleting the appropriate amounts of viral DNA.

One of the most widely used deletions is that performed by collapsing the two Xbal sites without E3,

to remove 1.9 kb of viral DNA. This results in vectors with a capacity for approximately 4 kb of foreign DNA with the ability to replicate in any cell line commonly used for adenovirus propagation, such as HeLa or KB (Horwitz, 1990). Approximately 3 kb of E1 can be deleted to generate vectors with growth restricted to 293 cells and capable of accepting insertions of 5 kb.

5.1.c. Poxvirus-mediated

The poxvirus family is subdivided into two subfamilies: Entomopoxvirinae (insect poxvirus) and Chordopoxvirinae (vertebrate poxvirus). The latter share a group-specific antigen and are capable of producing nongenetic reactivation of other viruses belonging to the same group (Dales and Pago, 1981).

Poxviruses are the largest-sized animal viruses, and can be seen by light microscopy. The virions are oval or brick-shaped and measure 200 to 400 nm in length. The virion contains a linear DNA molecule, rich in A+T, which can range from 130 to 300 kb, depending on the species of poxvirus. The prototype of the group, the vaccine virus, is 185 kb; the virion contains more than 100 polypeptides that are arranged in four different structures (nucleoid, lateral bodies, membrane and coating) (Dadvison and Moss, 1989).

In recent years, numerous genes that encode important proteins from viruses and other microorganisms have been inserted into the vaccine virus (Mackett et al., 1984). The resulting recombinants have been employed in basic studies dealing with gene expression, transcription, protein processing, transport and secretion of proteins. They have also been used in immunological studies (Moss and Flexner, 1989), especially in the production of target cells expressing surface epitopes, which are useful for cellular immunity reactions, as will be discussed later on in reference to immunotherapy for the treatment of cancer. In any case, the most important application has been the development of infectious vaccines (Buller et al., 1988). The number of diseases in which this approach has been employed is growing continually.

5.2. Single-chain, negatively-charged RNA viruses

5.2.a. Retrovirus-mediated

Retroviruses have been used as gene vectors throughout the past decade. They are RNA viruses of 10 kb, with the single-chain RNA genome which is converted to DNA by means of the reverse transcriptase enzyme characteristic of this type of virus.

Retroviruses must be manipulated to make their replication systems defective by replacing the viral genes (the gag-pol-env genes) with those that are to be introduced. The construction of a transmissible retroviral vector that expresses the therapeutic gene is quite similar to the case of other viruses; it implies the introduction of a plasmid containing the transgene in place of the viral genome but conserving the viral packaging, promoting and polyadenylation sequences (LTRs) in producer or packager cells that also contain a complete viral genoma which, however, is essentially lacking in the aforementioned sequences. The latter, referred to as a cooperater or packager virus, produces the capsid proteins that lead to empty particles unless they combine with the genome constructed with the therapeutic gene, which is defective in terms of replication (Mann et al., 1983). This ensures the first wave of infection and the incorporation by the genome of the cell to be transferred, with no risk of secondary reinfections. The presence of viral promoter sequences and any added sequences of mammaliam or human origin, together with the therapeutic gene, can confer substantial cell line specificity to the gene.

The use of retroviral vectors to transfer suicide genes in selective chemotherapy is advantageous because of their ability to infect only those cells that are undergoing proliferation (Shimotono and Temin, 1981; Culver et al., 1992). This is evident in the case of the glioma, which has been the subject of clinical trials with thymidine kinase of HSV, involving the intracerebral implantation of producer and packager cells and the administration of ganciclovir. Retrovirus-mediated gene transfer can also serve to attain selectivity by means of transcription promoters that are specifically expressed only in given cell lines (Moolten, 1994). Gene therapy can be employed in the treatment of certain diseases requiring the inhibition of gene expression or therapy to replace lost suppressor genes for genetic immunomodulation.

5.3. Single-chain, positively-charged RNA viruses

5.3.a. Togavirus-mediated

The family of the togaviruses includes two types of animal RNA viruses, the alphaviruses and the rubiviruses. The two most widely studied alphaviruses are the Sindbis virus and the Semliki Foset virus (SFV), which have been used for heterologous gene expression (Xiong et al., 1989).

In nature, alphaviruses are transmitted by mosquitos to vertebrate hosts (usually birds or mammals); in culture, they can infect cells from a large variety of birds, mammals, reptiles, amphibians and insects. The infection of cultured vertebrate cells is characterized by a dramatic cytopathic effect and rapid cell death, while their growth in mosquito cells allows them to establish chronic or persistent infections (Berglund et al., 1996).

The genome of the alphaviruses consists of a single-chain RNA with positive polarity. The genome is divided in such a way that replication is encoded by an open-read fragment (ORF) in the genomic DNA, while the structural proteins are encoded by a second separate ORF. This permits the development of subgenomic sequences that can be manipulated with no impact on the replication capacity of the system (Schlesinger, 1993). The most effective method of transferring this RNA

virus is by electroporation or lipofection.

Although some alphaviruses are pathogenic, the two that are used as vectors, SFV and SIN, are nonvirulent in humans. For reasons of biosafety, and because the large scale production of RNA *in vitro* would be costly, some laboratories have developed a new strategy with a DNA-RNA vector system. This system, which is independent of the helper vectors, has the cassette of recombinant alphavirus cDNA expression controlled by a eukaryotic promoter, such as the early cytomegalovirus promoter. This complex is introduced into the cell by conventional DNA transfection methods. In the cell nucleus, the polymerase RNA transcribes the complete unit into RNA, which is transported to the cytoplasm. (Berdenbeek and Rice, 1992; Schlesinger, 1993). As a result of its positive polarity, the RNA is converted to viral replicase, which eliminates the replication of the molecule itself, just like during normal replication of the alphavirus RNA molecule.

5.3.b. Picornavirus-mediated.

The picornavirus family includes some important human pathogens (polioviruses, hepatitis A virus and rhinoviruses). The polioviruses are being used as vectors expressing heterologous sequences because of certain advantages, such as their rapid replication cycle, high-level RNA amplification and the efficient eliminationof host mRNA translation (Kuhn and Wimmer, 1987).

Although the range of hosts of the polioviruses is fairly limited, it appears to be determined more by the level of virus-receptor bonds than by RNA replication. The determination of the structure of the poliovirion (which is unenveloped and presents icosahedric symmetry) and knowledge of its antigenic properties has permitted the manipulation of viable antigenic chimeras using chains of the Sabin poliovirus vaccine type 1 as a possible novel vaccine (Rossenberg, 1992). The recombinants expressing heterologous short peptides as part of the surface of the virion curvature have been constructed on the basis of antigenic domains from poliovirus type 3 and other human pathogens such as HIV-1 and human papillomavirus (HPV) type 1.

6. Non viral methods

6.1. System mediated by cationic liposomes

Cationic liposomes are negatively-charged complexes, transporting DNA by simply mixing with it in such a way that the net charge of the complex is negative. Thus, this complex is easily prepared and transported to the cells. The inefficiency of certain conventional liposomic forms has made the liposomes as a whole unpopular as transporters. However, currently, there are models of cationic liposomes that make them highly versatile and attractive for DNA transport.

6.1.a. The course of their development

The first cationic liposome to be used was lipofectin. It was prepared by sonication of equal weights of a synthetic cationic lipid (DOTMA) and a fusogenic lipid (DOPE). The sonication produced a large number of vesicles ranging in diameter from 50 to 200 nm. The cationic charge of the liposomes partially neutralized the negative charge of the DNA, giving the complex a net positive charge. Because of this charge, when the liposome arrives at the cell surface, which is negatively charged, the two bind efficiently (Felgner et al., 1987). Then, the DNA is most likely transported to the cytoplasm by means of endocytosis, although possible mediation by other mechanisms is also considered.

Fundamentally, all cationic liposomes are based on lipofectin, and all of them were cationic amphipathic lipids, although the final proportions of DOPE range from 0 to 50%.

In the design of novel formulations of cationic liposomes to increase the efficiency of gene transport, a series of derivatives have been synthesized from cationic cholesterols. The DC-Col liposomes are sonicated, resulting in small vesicles that remain stable for at least 6 months when stored at 4 °C. Although they are still in the experimental phase, they have already been tested in a large number of cell lines, some of which are primary cell lines (Farhood et al., 1994).

6.2. Other methods

Other methods that should be mentioned, although their applications may be limited, include electroporation, which can only be employed *in vitro*, despite it being highly effective in gene transport. The purpose of this method is to pass DNA, denuded of liposomes or any sort of coating, into the cells by means of an electric shock that the cells themselves produce, and which causes membrane pores to open up momentarily, allowing the DNA to enter. Once inside, in the cytoplasm, the DNA bonds to the cell genome, introducing the desired gene or genes.

Another method involves transfer-infection, which consists of coating the plasmid with a layer of polylysine to which the transferrin receptor binds. The transferrins protrude from the complex and, thus, are capable of binding to the membrane receptor which, being nearly ubiquitous, can be found almost anywhere on the organism. Other approaches have involved the use of adenovirus particles to facilitate passage by means of endocytosis, and the latest trend is to use other viral proteins such as influenza virus hemagglutinin.

7. The differences among methods

The most frequently viruses used for gene therapy are retroviruses and adenoviruses. Togavirus as vectors is becoming increasingly widespread. A comparison of

Table 2. Differences among methods.

VIRAL METHODS			NON VIRAL METHODS
Retrovirus advantages	Adenovirus advantages	Togavirus advantages	Liposome advantage
High efficiency	High production	Very high titers	Complex, in vitro, with DNA
Stable integration	Extrachromosommal, avoiding alterations	rRNA packaging in infectious particles	Simple to use and prepare
Amphotropic viruses for a wide variety of tissues	Large insertion size and great stability	Variety of hosts	Use of any DNA and RNA, no limit to size
Sequences related to LTR		Immune reponse (vaccines)	
Retrovirus disadvantages	Adenovirus disadvantages	Togavirus disadvantages	Liposome disadvantages
Only enter on cells undergoing proliferation	Adverse effects	Recombines rapidly during packaging	Limited effect
Limited size	Systemic infections	Limited immunological memory	
Integration on the host genome	Immunogenic (requiaring repeated doses)		

Table 3. Example of some gene therapy protocols accepted since 1993.

DISEASE OF INTEREST	GENE OF INTEREST	CELL TARGET	VECTOR
Advanced cancer	Neo(marker)	TIL cellsRVV	
Melanoma	IL-2	Allogenic tumor lines	TF
ADA-SCID	ADA	BMC+PBL	RVVx2
ADA-SCID	ADA	CD34+BMC	RVV
Allo-BMT/Immunomodul	HSV-tk	PBL	RVV
BMT-EBV in LPD		PBL	RVV
Cystic fibrosis	CFTR	Nasal epithelium in vivo	Cat-Liposome
Cystic fibrosis	CFTR	Nasal epithelium in vivo	Cat-Liposome
Melanoma	IL-2	Allogeneic tumor lines	RVV
Melanoma	IL-4	Allogeneic tumor lines	RVV

the advantages and disadvantages of these methods and nonviral approaches is summarized in Table 2.

8. Objectives in the search for the ideal vector

Perhaps the ideal vector is entirely synthetic. The search focuses on a composition of DNA sequences, derived from a number of existing viral vectors and certain plasmid vectors. It should be capable of incorporating large-sized genes and be devoid of immunogenicity. Moreover, it should be possible to direct the vector specifically towards given cell populations (possibly to incorporate elements that recognize components present only on the cell membrane). This, together with the capacity to incorporate elements that limit the expression of these genes to particular cell types, the ability to increase, decrease and modulate the levels of gene expression in response to endogenous regulators or markers such as hormones or glucose, would result in the ideal vector for gene transfer. However, at the present time, there remains a great deal of work to be done in this effort.

9. Ethical considerations

As was mentioned above, in 1988, the Recombinant DNA Advisory Committee of the NIH in Bethesda, Maryland, approved the guidelines for human gene therapy trials (Freese, 1972). The first approach to a genetic disease is to isolate the causative gene and to demonstrate that the gene in question is really the one responsible for the disease by assessing its expression in cultured cells (Blau and Springer, 1995). The safest and most effective vector for use in humans cannot be established until it has been found for cell cultures and animal models (Colledge et al., 1992). Recently, the design of animal models by means of the Knockout technology is providing working models of certain human diseases in mice, thus expanding the capacity to test new genetic strategies for these diseases occurring in mutant mice (McDonald et al., 1990). However, mouse models, created by the rupture of the responsible genes, often do not mimic the same type of damage in humans presenting an equivalent genetic defect. For example, the mdx mouse used as a model of Duchenne's muscular dystrophy (Clemens and Caskey, 1992) has the same

defective gene as the human patient, but only exhibits transient muscle injury. Thus, these models show at least some of the properties of the human disorder, leading to an understanding of how to approach to the problems encountered and providing information with respect to their human counterparts. There have been a multitude of conferences concerning ethics with regard to research in human gene transfer therapy. It is necessary to assess the risk/benefit ratio as compared to conventional medicine.

The birth of gene therapy, as occurred with that of recombinant DNA, appeared to be a Pandora's box, full of potential complications and possible toxic effects, although at the present time, most of the early apprehensions seem to be unjustified. In fact, it has been suggested that the evaluation of clinical gene therapy experiments should be no more rigorous than that applied to other new medical treatments. In any case, gene therapy involving embryos has been totally banned. All these matters are regulated by the Recombinant DNA Advisory Committee of the NIH and by the U.S. Food and Drug Administration.

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