JPP
Journal of
Pharmacy and Pharmacology

The challenge of gene therapy and DNA delivery

Anthony J. Phillips

Abstract

It is 10 years since the first gene therapeutic was administered to patients. Since that time approximately 3500 patients have been treated and although some evidence of gene transfer has been seen it has generally been inadequate for a meaningful clinical response. The major challenges have been delivery of DNA to the target cells and duration of expression. A variety of gene transfer systems have been evaluated and these are summarized with their advantages and disadvantages. Encouraging data have been obtained recently by a number of workers and there is some optimism that the enormous potential of gene therapy will be realized in the second decade of its development.

Introduction

Gene therapy may be described as "the ultimate application of genomics", and the availability of the human genome and the linkage between DNA and expressed proteins should provide a wealth of potential therapeutic opportunities. In addition the determination of the relationships between genotype and disease using pharmacogenetics will provide the opportunity for the development of gene therapeutics in a variety of disease states. It is a broad-based platform technology that may be applied to a wide range of diseases depending on the gene (or more accurately the DNA) being delivered and has the potential to revolutionize their treatment by affecting the causes rather than the symptoms.

It is now 10 years since French Anderson and co-workers undertook the first gene therapy trial to treat a four-year-old patient for a severe combined immunodeficiency Deficiency disorder (Culver et al 1991). Her disease was caused by a genetic defect that resulted in the inability to produce the enzyme adenosine deaminase (ADA) and prevented her immune system from combating infection. In this trial her white cells were removed and mature T-cells were genetically modified ex-vivo with retrovirus encoding the ADA gene before infusion into her bloodstream. The combination therapy with polyethyleneglycol (PEG)-ADA was demonstrated to result in an improvement to her immune system and she was able to return to a more normal life. A number of other children have been enrolled into similar studies since then but, whilst they have shown some clinical improvement, they have not been cured. This apparent early success generated enormous "hype" around the possible opportunities to treat intractable diseases. Unfortunately, as a consequence, much of the fundamental science required to underpin the technology was overlooked, resulting in significant disappointment and scepticism of the approach.

Since then in excess of 3500 patients have been treated in over 300 studies with limited success but with some evidence of generating clinical benefit. In these studies

Albany, Arkesden, Essex CB11
4HB, UK

Anthony J. Phillips

Table 1 DNA transfer to target cells in current clinical studies.

Inherited monogenic disorders	Lung epithelial cells, antigen presenting cells (APC), T-cells, fibroblasts, muscle cells, blood progenitor cells
Infectious diseases	T-cells, APC, muscle cells, blood progenitor cells
Cancer	Tumour cells, APC, T-cells, muscle cells, fibroblasts, blood progenitor cells
Cardiovascular disease	Endothelial cells, muscle cells
Rheumatoid arthritis	Synovial lining cells
Cubital tunnel syndrome	Nerve cells

a range of diseases and cells have been targeted (see Table 1), but in order for gene therapy to reach its potential, the development of a safe, efficient and consistent system of DNA delivery to the target is required. Many techniques have been investigated, including ex-vivo and in-vivo administration and viral and non-viral vectors. The major challenge is to deliver DNA to the target tissues and to transport it to the cell nucleus to enable the required protein to be expressed. The problem is twofold. A system must be designed to deliver DNA to a specific target and to prevent degradation within the body, and an expression system must be built into the DNA construct to allow the target cell to express the protein at therapeutic levels for the desired length of time.

This paper will review the various approaches that have been followed with appropriate examples. A comprehensive review of the progress of gene therapy over the last 10 years has been published recently by Mountain (2000).

DNA delivery and transfer systems

There are three main types of DNA-delivery systems, all of which have their own advantages and disadvantages. There is viral, predominantly retrovirus, adenovirus and adeno-associated virus but also lentivirus, herpes, vaccinia, sendai and several RNA viruses. There is non-viral, based on naked DNA, cationic lipid–DNA complexes and cationic polymeric condensates often used in association with lipids. Finally, there is physical, including electroporation and needle-free injectors of naked or complexed DNA.

At the present stage of development, viral vectors have been the most widely studied and used in approximately 80 % of the clinical protocols submitted in Europe or the USA (Table 2), predominantly because of their ability to give significantly greater transfection.

Viral delivery vectors
The main viral vectors and their advantages and disadvantages are shown in Table 3. Although there are no

Table 2 Gene transfer studies approved by or submitted to US and European regulatory authorities.

Disease	Number of protocols
Cancer	216
Monogenic diseases	49
Infectious diseases	24
Cardiovascular diseases	8
Rheumatoid arthritis	2
Cubital tunnel syndrome	1

Vector	Number of protocols
Retrovirus	159
Adenovirus	58
Adeno-associated virus	4
Other viruses	20
Lipids	40
Other non-viral (e.g. naked DNA, gene gun)	20

generalizations the principal disadvantages are safety concerns (immunogenicity and insertional mutagenesis), the size of DNA constructs that can be incorporated, and their manufacture and quality control. The magnitude of these disadvantages is virus-dependent.

Retroviral vectors transfect dividing cells by integrating the transgene into the target-cell chromosome and therefore this generally leads to more prolonged, but lower, expression. The vector gives inefficient gene transfer for most cell types in-vivo because of inactivation by the human complement system and thus their most extensive use has been ex-vivo. Due to integration into the chromosome there is the risk of insertional mutagenesis. Predominantly they have been used in cancer trials. Another example of the use of adenoviral and retroviral vectors has been in gene delivered enzyme prodrug therapy (GDEPT) as described by Li et al (1997). This is a novel application in which the DNA that codes for an enzyme is delivered and causes the conversion of an inactive pro-drug into the active form at the intended site of action. Studies have been undertaken in animals where DNA coding

Table 3 The advantages and disadvantages of viral vectors.

Vector	Advantages	Disadvantages
Adenovirus	High transfection efficiency Transfects proliferating and non-proliferating cells Substantial clinical experience	Strong immune responses Insert size limit of 7.5 kb Difficult to manufacture and quality control Poor storage characteristics Short duration of expression
Retrovirus	Fairly prolonged expression High transfection efficiency Substantial clinical experience Low immunogenicity	Low transfection efficiency in-vivo Insert size limit of 8 kb ex-vivo Transfects only proliferating cells Difficult manufacture and quality control Safety concerns (mutagenesis)
Lentivirus	Transfects proliferating and non-cells Transfects haematopoietic stem cells	Very difficult manufacture and quality control Poor storage characteristics Insert size limit of 8 kb No clinical experience Safety concerns (origins in HIV)
Adeno-associated virus	Efficient transfection of wide variety of cell types in-vivo Prolonged expression Low immunogenicity	Difficult manufacture and quality control Insert size limit of 4.5 kb Limited clinical experience Safety concerns (mutagenesis)

for cytosine deaminase is administered into the hepatic artery with 5-fluorocytosine (5-FC) given orally. In the rapidly dividing tumour cells 5-FC is converted into the cytotoxic 5-fluorouracil at high localized concentrations and adjacent cells are killed by a bystander effect. As hepatocytes are not rapidly dividing they are unaffected by this treatment.

Adenoviral vectors have the benefit of transfecting dividing and non-dividing cells and give more efficient gene transfer in-vivo than other systems. Transgene expression occurs at a high level but is of short duration. The most serious limitation of these vectors is their ability to elicit strong immune inflammatory responses and this was the cause of the unfortunate death of Jesse Gelsinger in the US last year. They are being engineered to produce second and third generation vectors which reduce immunogenicity or involve immunosuppression, of which the following are examples:

- To reduce immunogenicity (Benihoud et al 1998);
- To reduce the effects of macrophage depletion and anti-CD40 ligand on transgene expression and redosing with recombinant adenovirus (Stein et al 1998);
- To modify cellular and humoral immune responses to viral antigens which create barriers to lung-directed gene therapy with recombinant adenoviruses (Yang et al 1995); and
- To blunt immune responses to adenoviral vectors in mouse liver and lung with CTLA4Ig (Joos et al 1998).

PEGylation has been used to increase the duration of transgene expression by providing a degree of protection from the immune system (O'Riordan et al 1999).

As can be seen from Table 2 a large number of clinical studies have been undertaken with adenoviral vectors. As an example they were used in the early cystic fibrosis trials, but their utility is reduced by the necessity to repeat-dose and the immunological responses obtained (see Alton et al (1998) for a review).

Encouraging animal and clinical data have been obtained in peripheral vascular and coronary artery diseases where DNA coding for vascular endothelial growth factor has been injected around the site of occlusion. New blood vessels created by angiogenesis have partially restored blood flow and the function of diseased tissue as measured by physical and biochemical means (Lee & Feldman 1998).

Adeno-associated virus is also capable of integration into the target-cell chromosome and the limited number of clinical protocols that have been carried out, including in cystic fibrosis (Wagner 1998), have shown that sustained transgene expression can be obtained. However, the virus is small which limits the size of the DNA of interest that may be inserted. In addition, the immunological difficulties seen with adenovirus remain.

In summary, there are a number of areas for improvement of viral vectors. These are:

- Specificity and efficiency of DNA transfer;

Table 4 The advantages and disadvantages of non-viral vectors.

Advantages	Disadvantages
Manufacture and quality control relatively simple	Short duration of expression
Good storage characteristics	Repeat therapy required as plasmid does not replicate with host cells
Low immunogenicity	
Good safety profile	Inefficient transfection in-vivo
Efficient transfection ex-vivo	
Delivery to any somatic cell	
Non-infectious	
No limit on size of plasmid	

- Specificity, magnitude and duration of expression;
- Reduction or elimination of immunogenicity and any other safety concerns;
- Ease of manufacture and control.

Non-viral delivery vectors

Non-viral vectors utilize plasmid DNA produced by bacterial fermentation and this results in more efficient manufacture of the biological and easier characterization and control. In the simplest form, naked DNA can be directly injected into tissue but under most circumstances the transfection efficiency is low and high doses of DNA are required. However, non-viral vector systems have a number of advantages and disadvantages that are shown in Table 4.

Cationic lipids condense the DNA structure and bind by electrostatic attraction thus protecting them from degradation in blood and other biological fluids and enhancing their uptake by endocytosis. Similar protection to product degradation is also obtained. Some of these lipid systems are fusogenic and facilitate transport into the cell. On endocytosis the complex is taken up into a primary endosome and then needs to be released into the cytosol to enable nuclear uptake. During this process, loss of DNA occurs by a variety of routes including non-productive extracellular interactions, entrapment in the primary or secondary endosome, lysosomal degradation and poor nuclear uptake. All of these factors combine to give generally poor transfection rates for these DNA–lipid complexes. There is a rapidly increasing range of cationic lipids based on quaternary ammonium compounds (e.g. DOTMA, DMRIE, DLRIE, DOGS), cholesterol and lecithin derivatives (e.g. dimethylaminoethane carbamoyl cholesterol and ethyl dimyristoyl phosphatidylcholine), and long chain polyamines (e.g. Transfectam). These lipids are generally used with co-lipids e.g. cholesterol or DOPE, and their structure determines transfection efficiency and

their safety and tolerability. To produce these complexes, buffered DNA solution and liposomes prepared from the lipid/co-lipid mixture are mixed under controlled conditions. As the electrostatic binding is essentially an instantaneous process occurring when the components come together, good control is required to prevent the formation of complexes with a range of DNA ratios and, therefore, different charges and sizes that will affect their ability to transfect. There are a number of factors that affect transfection and the subsequent extent and duration of protein expression:

- Regulatory factors in the DNA construct e.g. enhancer/promoter system, and the nature of the intron and the polyA sites chosen;
- The cationic lipid;
- Choice of the co-lipid; and
- Inclusion of any other factors e.g. PEG, targeting ligands, viral coat proteins/peptides.

In addition, there are a number of pharmaceutical factors that affect the nature of the DNA complex and its ability to transfect:

- Lipid/DNA ratio and zeta potential;
- DNA concentration required to deliver the dose;
- Composition of the vehicle e.g. pH, buffer salts, osmotic agents;
- DNA and lipid purities;
- Manufacturing process and the temperature during the complexation; and
- Cationic liposome size.

Numerous studies have been published based on DNA–lipid complexes. Examples are intranasal and inhaled studies on patients with cystic fibrosis (Caplen et al 1995; Alton et al 1997; Noone et al 2000). All of these studies demonstrated that DNA was transfected as shown by physiological, molecular and biochemical tests, but that it was inadequate to obtain any clinical benefit. This is typical for studies carried out with these systems in a variety of animal and human models.

An alternative method of condensing DNA is to use polymeric or protein based cations e.g. polyethylene imine (Boussif et al 1996), chitosan (Illum 1998), oligopeptides (Gottschalk et al 1960) and polylysine (Wagner et al 1990) to produce particles for uptake by endocytosis. Although there are reports of uptake in immortalized cell lines there is only limited evidence of uptake in primary cell lines (Philips 1998) and few examples of in-vivo transfection giving superior results to naked DNA. No clinical studies are reported on these systems.

In summary, non-viral systems have the benefit of comparative ease of manufacture and good safety and tolerability. Significant improvement in the efficiency of gene transfer and the magnitude, specificity and dur-

ation of expression is required if they are to become clinically usable DNA delivery vehicles.

Physical systems

The skin has been seen as an attractive target for the administration of DNA to obtain an immuno-therapeutic response for the treatment of viral diseases or some cancers. In these systems, DNA coding for the chosen antigen or antigens is administered and stimulates the production of an immune response. The approach has a number of advantages over conventional vaccination of which the most important is the ability to stimulate both arms of the immune system to produce both humoral and cellular responses, including the production of cytotoxic T lymphocytes. Two physical methods of delivering DNA by this route have been evaluated.

Increasing cell permeability by the transient application of an electric current has been used for a number of years for small molecules, proteins and peptides. There has been only limited use of this approach for DNA administration but there is some evidence in mice that improved transfection of skin and tumours can be obtained (Nishi et al 1997).

Needle-free injectors based on different technologies are being evaluated as "non-invasive" approaches to the administration of DNA by the dermal route. The Intraject system administers liquids under high pressure to the subcutaneous layer and has been reported to give greater immune responses in mice than by intramuscular injection (Mathei et al 1997). In contrast, particle mediated delivery with the PowderJect system uses high-pressure helium to fire gold particles coated with DNA into the epidermis and importantly into the professional antigen presenting cells. These then migrate to the lymph nodes where the expressed antigen stimulates the immune system. Due to direct intracellular delivery, much lower doses of DNA are required and this has the significant benefits of facilitating manufacture and reducing the amount of DNA available for biodistribution and potential safety concerns. A number of encouraging clinical studies have been reported in which both humoral and cellular responses have been demonstrated (Tacket et al 1999; Roy et al 2000).

Prospects for the Future

Many of the practical issues associated with gene therapy have now been overcome. Processes are available for the production of plasmid DNA and viral systems in sufficient quantity and purity to support the development of delivery systems that may be used with confidence in animals and man. There are encouraging studies that have been reported in which gene transfer has been demonstrated for viral systems and using biolistic delivery. To date the administration using non-viral vectors e.g. DNA–lipid complexes has failed to produce clinically meaningful levels of transgene expression, although they are greater than obtained with the original systems.

There are ethical considerations in the use of gene therapy. To carry out any gene therapy studies in man, approval of the protocol is required by the Gene Therapy Advisory Committee (GTAC) in the UK and the Recombinant DNA Advisory Committee (RAC) in the US, in addition to the Medicines Control Agency and Food and Drug Administration. The GTAC and RAC review ethical and safety considerations, particularly the risk that patients may be subjected to in conditions where the benefit may be minimal or non-existent. Particular areas of concern are the possibility of insertion into the patient genome and any possibility of passing on the modified gene in the germ line. At this time modifying genotype in-utero or of the germ line for inherited diseases is not permitted. In European countries the ethical control of gene therapy studies is normally undertaken by the ethics review committee of the testing centre.

There have been many disappointments over the first 10 years of gene therapy but its development has moved into a phase where steady progress is being made in vector design and the practicalities of manufacture. It is hoped that the second decade will see the attainment of the enormous potential it has to offer in the treatment of disease by modifying the underlying causal molecular processes.

References

Alton, E. W. F. W., Stern, M., Farley, R., Jaffe, A., Chadwick, S. L., Phillips, J., Davies, J., Smith, S. N., Browning, J., Hodsdon, M. E., Durham, S. R., Li, D., Jeffrey, P. C., Scallon, M., Balfour, R., Eastman, S. J., Cheng, S. H., Smith, A. E., Meeker, D., Geddes, D. M. (1997) Cationic lipid-mediated CFTR gene transfer to the lungs and noses of patients with cystic fibrosis: a double-blinded placebo-controlled study. *Lancet* **353**: 947–954

Alton, E. W. F. W., Geddes, D. M., Gill, D. R., Higgins, C. F., Hyde, S. C., Innes, J. A., Porteus, D. J. (1998) Towards gene therapy for cystic fibrosis: a clinical progress report. *Gene Ther.* **5**: 291–292

Benihoud, K., Saggio, I., Opolon, P., Salone, B., Amiot, F., Connault, E., Chianale, C., Dautry, F., Yeh, P., Perricaudet, M. (1998) Efficient repeated adenovirus-mediated gene transfer in mice lacking both tumour necrosis factor alpha and lymphotoxin alpha. *J. Virol.* **72**: 9514–9525

Boussif, O., Zanta, M. A., Behr, J. P. (1996) Optimised galenics improve *in vitro* gene transfer by cationic molecules up to 1000 fold. *Gene Ther*. **3**: 1074–1080

Caplen, N. J., Alton, E. F. W. F., Middleton, P. G., Dorin, J. R., Stevenson, B. J., Gao, X., Durham, S. R., Jeffrey, P. K., Hodson, M. E., Coutelle, C., Huang, L., Porteus, D. J., Williamson, R., Geddes, D. M. (1995) Liposome-mediated *CFTR* gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Med*. **1**: 39–46

Culver, K. W., Osborne, W. R., Miller, A. D., Fleisher, T. A., Berger, M., Anderson, W. F., Blaese, R. M. (1991) Correction of ADA deficiency in human T lymphocytes using retroviral-mediated gene transfer. *Transplant. Proc*. **23**: 170–171

Gottschalk, S., Sparrow, J. T, Hauer, J., Mims, M. P., Leland, F. E., Woo, S. L., Smith, L. C. (1996) A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther*. **3**: 448–457

Illum, L. (1998) Chitosan and its use as a pharmaceutical excipient. *Pharm. Res*. **15**: 1326–1331

Joos, K., Turka, L. A., Wilson, J. M. (1998) Blunting of immune responses to adenoviral vectors in mouse liver and lung with CTLA4Ig. *Gene Ther*. **5**: 309–319

Lee, J. S., Feldman, A. M. (1998) Gene therapy for therapeutic myocardial angiogenesis: a promising synthesis of two emerging technologies. *Nature Med*. **4**: 739–742

Li, Z., Shanmugam, N., Katayose, D., Huber, B., Srivasta, S., Cowan, K., Seth, P., (1997) Enzyme/prodrug gene therapy approach for breast cancer using a recombinant adenovirus expressing *Escherichia coli* cytosine deaminase. *Cancer Gene Ther*. **4**: 113–117

Mathei, C., Van Damme, P., Meheus, A. (1997) Hepatitis B vaccine administration: comparison between jet-gun and syringe and needle. *Vaccine* **15**: 402–404

Mountain, A. (2000) Gene therapy: the first decade. *Trends Biotechnol*. **18**: 119–128

Nishi, T., Dev, S. B., Yoshizato, K., Kuratsu, J., Ushio, Y. (1997) Treatment of cancer using pulsed electric field in combination with chemotherapeutic agents or genes. *Hum. Cell* **10**: 81–86

Noone, P. G., Hohneker, K. W., Zhaoqing, Z., Johnson, L. G., Foy, F., Gipson, C., Jones, K., Noah, T. L., Leigh, M. W., Schwartzbach, C., Efthimiou, J., Pearlman, R., Boucher, R. C., Knowles, M. R. (2000) Safety and biological efficacy of a lipid-CFTR complex for gene transfer in the nasal epithelium of adult patients with cystic fibrosis. *Mol. Ther*. **1**: 105–114

O'Rioridan, C. R., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S. C., Smith, A. E., Francis, G. E. (1999) PEGylation of adenovirus with retention of infectivity and protection from neutralising antibody in vitro and in vivo. *Hum. Gene Ther*. **10**: 1349–1358

Philips, R. (1998) International Patent Application PCT/GB98/00424

Roy, M. J., Wu, M. S., Barr, L. J., Fuller, J. T., Tussey, L. G., Speller, S., Culp, J., Burkholder, J. K., Swain, W. F., Dixon, R. M., Widera, G., Vessey, R., King, A., Ogg, G., Gallimore, A., Haynes, J. R., Fuller, D. H. (2000) Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. *Vaccine* **19**: 764–778

Stein, C. S., Pemberton, J. L., van Rooijen, N., Davidson, B. L. (1998) Effects of macrophage depletion and anti-CD40 ligand on transgene expression and redosing with recombinant adenovirus. *Gene Ther*. **5**: 431–439

Tacket, C. O., Roy, M. J., Widera, G., Swain, W. F., Broome, S., Edelman, R. (1999) Phase 1 safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. *Vaccine* **17**: 2826–2829

Wagner, J. A. (1998) Efficient and persistent gene transfer of AAV-CFTR in maxillary sinus. *Lancet* **351**: 1702–1703

Wagner, E., Zenke, M., Cotton, M., Beug, H., Birnstiel, M. L. (1990) Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc. Natl. Acad. Sci. USA* **87**: 3410–3414

Yang, Y., Li, Q., Ertl, H. C., Wilson, J. M. (1995) Cellular and tumoral responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol*. **69**: 2004–2015