Gene therapy: the first decade

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Gene therapy promises to revolutionize medicine by treating the causes of disease rather than the symptoms. We are nearing the end of the first decade of gene therapy, and this article summarizes the approaches taken, results achieved, lessons learned and important recent developments. The early results on the clinical efficacy of gene therapies were disappointing, largely because the available gene-transfer vectors proved to be inadequate. Recently, however, clinical benefit has been clearly demonstrated and great progress made in selecting and improving vectors. There is now every prospect that the second decade will see gene therapy live up to its enormous potential.

Gene therapy, the treatment or prevention of disease by gene transfer (see Glossary), is regarded by many as a potential revolution in medicine. This is because gene therapies are aimed at treating or eliminating the causes of disease, whereas most current drugs treat the symptoms. This radical improvement is possible because the gene-based approach can provide superior targeting and prolonged duration of action. Hence, in comparison with other forms of therapy, it will permit biological effects that are more subtle and better localized to the most appropriate cells. Ultimately, this will translate into substantial improvements in therapeutic ratio and cure-rate for diseases that are presently untreatable or poorly managed. Gene therapy also has the important advantage of being a broad platform technology, applicable to a wide range of diseases.

The first clinical studies involving gene transfer began in 1990 (Ref. 1) and since then gene therapy has become the focus of a whole new industry. This review highlights the main approaches to gene therapy, the lessons learned from the first decade of clinical experience and the recent developments that suggest gene therapy might indeed revolutionize medicine in the next decade.

Table 1 indicates the wide range of diseases and target cells for which clinical studies are ongoing. Thus far, all clinical studies have involved gene addition rather than the correction or replacement of defective genes, which is technically more difficult. All clinical protocols approved to date involve gene transfer only to somatic cells rather than germ-line cells, the latter being the subject of considerable ethical debate at present[2]. Gene transfer to these somatic cells can take place either *ex vivo* or *in vivo*. In the *ex vivo* approach, cells are removed from the patient for transfection, and the therapeutic entity comprises engineered cells. This offers the advantage of more-efficient gene transfer and the possibility of cell propagation to generate higher cell doses. However, it has the notable disadvantages of being largely patient-specific as a result of cell immunogenicity and more costly because cell manipulation adds manufacturing and quality-control difficulties. The *in vivo* approach involves direct administration of the gene-transfer vector to patients. It is therefore not patient-specific, thus conferring advantages of reduced cost, logistics and infrastructure requirements.

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Gene-transfer systems

There are three main types of gene-delivery vector: viral, non-viral and physical. Many different viruses are being adapted as vectors[3,4], but the most advanced are retrovirus (Rv), adenovirus (Ad) and adeno-associated virus (AAV). Substantial effort has also gone into developing poxviruses (especially vaccinia) for genetic vaccines[5,6] and herpes simplex virus[7]. Non-viral approaches[8,9] fall into three main categories involving: (1) naked DNA; (2) DNA complexed with cationic lipids; and (3) particles comprising DNA condensed with cationic polymers (in some cases contained in liposomes). The most important physical methods involve needle-free injectors and electroporation. At the present stage of development, the leading viral vectors generally give the most efficient transfection. Their main disadvantages concern insert-size limitation, immunogenicity and manufacture. Non-viral vectors give less efficient transfection (especially *in vivo*) and

Glossary

Adjuvant An entity that enhances immune responses
Angiogenesis The process of new blood vessel formation
Chromatin The complex of DNA with proteins of which mammalian genomes consist
Endothelial cells The cells that line blood vessels
Gene therapy The treatment or prevention of disease by gene transfer
Haematopoietic stem cells The cells from which blood cells are derived
Heterologous transgene Gene delivered that is not derived from the same species as the target cells
Homologous transgene Gene delivered that is derived from the same species as the target cells
Insert The DNA sequences carried by the vector but not derived from the vector
Promoter The short region of DNA at which transcription initiates
Stem cells The earliest progenitor cells from which differentiated cell types are derived
Transcription The production of RNA from the DNA template
Transfection The process of successful gene transfer and expression
Transfection efficiency The proportion of cells that when exposed to the gene-delivery system become transfected
Transgene The gene delivered by the vector
Vector The gene-delivery vehicle

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Table 1. Target cells for gene transfer in ongoing clinical studies

Disease	Target cells
Cancer	Tumour cells, antigen-presenting cells, blood progenitor cells, T cells, fibroblasts, muscle cells
Inherited monogenic disorders	Lung epithelial cells, macrophages, T cells, blood progenitor cells, hepatocytes, muscle cells
Infectious diseases	T cells, blood progenitor cells, antigen-presenting cells, muscle cells
Cardiovascular diseases	Endothelial cells, muscle cells
Rheumatoid arthritis	Sinovial lining cells
Cubital tunnel syndrome	Nerve cells

Table 2. Distribution of gene-transfer clinical protocols approved by or submitted to regulatory authorities in North America and Europe[a]

Disease	Number of protocols	Vector	Number of protocols
Cancer	216	Rv	159
Infectious diseases	24	Ad	58
Monogenic diseases	49	AAV	4
Cardiovascular diseases	8	Poxviruses	19
Rheumatoid arthritis	2	HSV	1
Cubital tunnel syndrome	1	Naked DNA	16
Total	300	Lipids	40
In vivo transfer	144	Gene gun	1
Ex vivo transfer	156[b]	Electroporation	1
		Naked RNA	1
		Total	300

[a]Table compiled largely from Refs 86,87.
[b]Of the 156 *ex vivo* protocols, 32 involved gene marking with no therapeutic intent.
Abbreviations: Rv, retrovirus; Ad, adenovirus; AAV, adeno-associated virus; HSV, herpes simplex virus.

more-transient expression, but have no insert-size limitation, are less immunogenic and easier to manufacture. Physical methods give inefficient transfection and have a limited range of applications, but some of these applications are promising and important.

Clinical studies – targets, results and lessons

Disease targets

Approximately 300 clinical protocols involving gene transfer have been approved, mostly in the USA, but such trials are now being conducted in all five continents. As indicated in Table 2, approximately two-thirds of trials are directed at cancer, and most of the remainder at inherited monogenic disorders (especially cystic fibrosis) or infectious diseases (particularly HIV). This distribution partly reflects the lack of effective alternative therapies and hence the likelihood of obtaining regulatory approval. Most of the early clinical studies involved cells transfected *ex vivo* with Rv and reporter transgenes to determine the pharmacokinetics and biodistribution of the engineered cells in patients with cancer or inherited monogenic diseases. Now, however, these gene-marking studies are uncommon and virtually all trials have therapeutic intent. Most protocols involve killing disease-related cells (by direct or immunological means) rather than long-term restoration of missing or defective proteins. This is because the vectors available, until recently, gave inefficient gene transfer and short-lived expression. Table 2 gives a breakdown of the gene-therapy clinical protocols in North America and Europe according to disease targets and vectors. It shows that ~75% of these protocols involve viral vectors; these give the most efficient gene transfer.

Clinical trials

More than 3500 patients have been administered with experimental gene therapies. Most clinical trials have been small Phase I/II studies with the main objectives of demonstrating safety and gene transfer, and obtaining information to guide dose selection for Phase II and III efficacy studies. In general, a good safety profile has been observed in these Phase I/II studies and this has led to the submission and approval of protocols, in the past three years, for a wider range of diseases. It has been widely reported that the outcome of clinical trials in terms of gene transfer, gene expression, biological consequences and clinical benefit (especially) has been disappointing. This partly reflects the inordinately high profile of the early clinical studies and the unrealistic expectations of the public and, in particular, of the investment community. This has led to a similar credibility problem to that which afflicted biotechnology more generally in the 1980s. However, it is clear from the first decade of clinical experience that developing effective gene therapies is technically much more demanding than originally anticipated, and that the first generation of vectors gave inadequate performance in several respects that are important for achieving clinical benefit with most diseases.

There are some notable exceptions, and Table 3 indicates the therapies that have progressed to Phase II, and for which there are encouraging data on biological activity in terms of surrogate markers and signs of clinical benefit. It is notable that this short list spans a wide range of therapeutic concepts. Like the majority of Phase I/II studies, the *in vivo* approaches all involve local injection to encourage gene transfer to target cells rather than systemic administration. This is partly for safety reasons and partly because vectors allowing targeted delivery and expression have not been available.

Clinical and technical implications

Within the past year, clinical benefit from gene therapy has been clearly demonstrated for the first time. In these studies, naked DNA encoding the angiogenic

Table 3. Gene therapies in Phase II clinical studies

Disease	Vector	Gene transfer	Gene	Therapeutic strategy	Refs
Cancer	Cationic lipid	Tumour cells *in vivo*	HLA B7	Immunogenicity enhancement	55
Cancer	Cationic lipid	Tumour cells *in vivo*	IL-2	Immunogenicity enhancement	86
Cancer	Cationic lipid	Tumour cells *in vivo*	Ad E1A	Apoptosis induction	59
Cancer	Ad	Tumour cells *in vivo*	P53	Apoptosis induction	19
Cancer	Ad	Tumour cells *in vivo*	Ad death genes	Killing by lytic virus	88
Cancer	Rv	Tumour cells *in vivo*	TK	Killing by enzyme and/or prodrug	89
Cancer	Rv	Fibroblasts *ex vivo*	IL-12	Immunogenicity enhancement	86
Limb ischaemia	Naked DNA	Muscle cells *in vivo*	VEGF	Angiogenesis stimulation	10
Cystic fibrosis	AAV	Airway cells *in vivo*	CFTR	Provision of functional protein	39
HIV	Rv	T cells *ex vivo*	Chimeric TCR	Retargeting killer T cells	86

Abbreviations: Ad, adenovirus; Rv, retrovirus; AAV, adeno-associated virus; HLA B7, histocompatibility antigen B7; IL-2, interleukin-2; Ad E1A, multifunctional E1A protein of adenovirus; P53, tumour suppressor P53; TK, thymidine kinase; IL-12, interleukin-12; VEGF, vascular endothelial growth factor; CFTR, cystic fibrosis transmembrane receptor; TCR, T cell receptor.

protein vascular endothelial growth factor (VEGF) was injected into the skeletal muscles of patients with critical limb ischaemia resulting from an inadequate blood supply. The results are remarkable, with dramatic and long-lasting benefit observed in a large proportion of patients, including those who would otherwise have faced amputation[10]. Injection of the same therapy into the heart muscles of patients with ischaemic heart disease is now giving encouraging efficacy data[11].

The outlook for gene therapy is now much brighter than it was in 1995, when lack of efficacy data in clinical studies sponsored by the National Institutes of Health (USA) prompted it to appoint an expert committee to review the available information. The committee recommended that resources should be diverted away from premature clinical studies with inadequate vectors and into more-basic studies on gene transfer, gene expression and more-relevant preclinical testing[12]. This adversely affected investor confidence but led to a more-productive use of resources for progressing gene therapy and to real progress in improving vectors.

Since 1996, it has been clear that the main aspects in which vector improvement is required are: (1) specificity and efficiency of gene transfer; (2) specificity, magnitude and duration of expression; (3) immunogenicity; and (4) manufacturing. Each gene-transfer system has its own combination of advantages and limitations. Table 4 summarizes the basic advantages and disadvantages of the main vector systems.

Gene-transfer systems

Adenovirus

It is clear that for a wide variety of cell types, Ad gives more-efficient gene transfer compared with other systems, especially *in vivo*. Ad vectors can transfer genes to both proliferating and quiescent cells. Following delivery, transgene expression is at a high level but is transient, being low or undetectable in most tissues after two weeks. This is because Ad vectors do not integrate and for safety reasons are disabled for replication. The expression profile with first-generation Ad vectors is not suitable for the long-term correction of chronic diseases but is adequate for direct cell killing, most immunotherapy strategies and some acute diseases. The first-generation vectors have an insert-size limit of ~7.5 kb.

The promoter used most frequently with Ad (and indeed all other vectors) is derived from cytomegalus virus (CMV), which gives strong expression in many cell types. Ad gives particularly efficient gene transfer to the liver, such that dissemination from the site of local injection (such as tumours) and consequent liver transfection, is the most serious safety concern. This is being addressed by attempts to target both viral infection and transgene expression following delivery. Recent work has established that Ad infection can be efficiently retargeted *in vitro* through several cell-surface proteins[13,14] and that many promoter systems giving preferential transcription in certain cell types or tumours retain reasonable specificity in the context of Ad vectors[15,16]. Major improvements in specificity, with retention of efficiency, therefore appear to be feasible with this vector.

The most serious limitation of Ad vectors stems from their tendency to elicit strong immune and (at high doses) inflammatory responses. Single, large doses of Ad provoke neutralizing antibody responses directed to proteins of the viral particle, which prevent binding to target cells and abrogate gene transfer upon repeat dosing by systemic administration routes in animals[14,17]. Most Ad vectors are derived from the Ad5 serotype. Recent studies[18] indicate that 55% of adult humans have pre-existing anti-Ad antibodies capable of neutralizing *in vitro* infection by Ad5. It is not yet clear whether these pre-existing (low titre) antibodies will interfere with gene transfer upon a first systemic Ad administration, but it appears that even high titre, neutralizing antibodies in the blood do not reduce gene transfer by repeated intra-tumoural injections of Ad vectors[19]. It remains to be seen whether such repeat dosing by localized injection into other solid tissues is effective. Attempts to circumvent the neutralizing antibody response to Ad by oral tolerization[20], shielding key epitopes with hydrophilic polymers[21,22] or blockade of the co-stimulatory molecule CD40 ligand[23] are at an early stage. First-generation Ad vectors deliver the transgene along with many residual viral genes; expression of these leads to cytotoxic-T-lymphocyte (CTL) responses directed to the transfected cells. In certain tissues, CTL-mediated destruction of the expressing cells contributes to transgene silencing[14,17,24]. Progress is being made towards circumventing this problem, with strategies that involve immunosuppression by

Table 4. Advantages and disadvantages of gene-transfer vectors

Vector	Advantages	Disadvantages
Adenovirus	Very high transfection efficiency *ex vivo* and *in vivo* Transfects proliferating and non-proliferating cells Substantial clinical experience acquired Efficient retargeted transfection demonstrated	Repeat dosing ineffective owing to strong immune responses Insert-size limit of 7.5 kb Manufacture, storage, QC are moderately difficult Short duration of expression
Retrovirus	Fairly prolonged expression High transfection efficiency *ex vivo* Substantial clinical experience *ex vivo* Low immunogenicity	Low transfection efficiency *in vivo* Insert-size limit of 8 kb Transfects only proliferating cells Safety concern of insertional mutagenesis Manufacture, storage, QC are extremely difficult
Lentivirus	Transfects proliferating and non-proliferating cells Transfects haematopoietic stem cells	Safety concerns from immunodeficiency virus origins Manufacturing, storage, QC are extremely difficult Insert-size limit of 8 kb No clinical experience
AAV	Efficiently transfects a wide variety of cells *in vivo* Very prolonged expression *in vivo* Low immunogenicity	Insert-size limit of 4.5 kb Manufacture, QC are very difficult Little clinical experience Safety concern of insertional mutagenesis Repeat dosing affected by neutralizing antibody responses
Naked DNA	Manufacturing, storage, QC are simple and cheap Very low immunogenicity Clinical efficacy demonstrated in critical limb ischaemia Very good safety profile	Very short duration of expression in most tissues Very inefficient transfection *ex vivo* and *in vivo* Retargeting transfection very difficult
Cationic lipids	Relatively simple manufacturing, storage, QC Efficient transfection *ex vivo* Low immunogenicity Good safety profile	Inefficient transfection *in vivo* Very short duration of expression Little clinical experience Retargeting transfection difficult
Condensed DNA particles	Relatively simple manufacturing, storage, QC Efficient transfection *ex vivo* Low immunogenicity Good safety profile Retargeted transfection demonstrated	Inefficient transfection *in vivo* Very short duration of expression No clinical experience

All viral vectors share the disadvantage of safety concerns arising from the generation of replication-competent virus during manufacturing or after administration to patients.
Abbreviation: QC, quality control.

macrophage depletion[23], blockade of co-stimulatory molecules[24,25] or inflammatory cytokines[26], and deletion of further Ad genes[16,27]. Ad vectors have now been developed, with most or virtually all residual Ad genes deleted, which can accommodate inserts up to 30 kb[16]. Although these 'gutted' vectors (otherwise termed gutless or helper-dependent) appear to show greatly reduced immunogenicity and much more prolonged expression of homologous transgenes in mice[16], they present additional problems in recombinant virus construction and manufacture.

Overall, Ad is the easiest viral vector from the manufacturing viewpoint, allowing the production of large quantities with high titre and in relatively robust formulations. Nevertheless, Ad shares with other viral vectors the problem that first-generation vector preparations are contaminated with replication-competent virus (RCV), which arises through recombination between viral sequences in the vector and in the chromosome of the producer cells. The presence of RCV has limited the maximum dose that can be administered in some clinical studies with Ad viruses. However, a new combination of Ad vector and producer cells has recently been developed that eliminates RCV and is suitable for large-scale manufacture[28].

Many of the clinical studies giving encouraging signs of efficacy use Ad vectors. The most advanced of these delivers the wild-type gene for the tumour suppressor P53 for induction of tumour-cell killing[19]. A series of Phase II studies is under way, testing this recombinant virus alone and in combination with chemotherapies for the local management of various cancers.

Retrovirus

Rv vectors, unlike Ad, transfect by integrating the transgene into the target-cell chromosome. This usually

leads to more-prolonged transgene expression but at reduced levels. Transgene expression typically ceases within days to weeks and tends to be shorter *in vivo*. This silencing phenomenon is not well characterized, but is likely to result from methylation in the vicinity of the promoter[29] and incorporation of the insertion site into condensed chromatin, in which the transgene is inaccessible to the transcription machinery. The Rv provirus penetrates the nucleus only at mitosis, so transfection is restricted to proliferating cells; this has been a major limitation of Rv vectors.

Rv vectors have an insert-size limit of 8 kb. Several tissue-specific promoters work reasonably well in Rv[30], but retargeting transfection is proving to be much more difficult to achieve than with Ad. It is clear that Rv can be retargeted to bind cell-surface proteins other than its natural receptor, but efficient retargeted transfection has not yet been reported. Most first-generation Rvs do not express residual viral genes, which is partly why Rv is much less immunogenic than Ad. Nonetheless, it is clear that the expression of heterologous transgenes leads to CTL responses that eliminate the expressing cells and reduce the effectiveness of repeat dosing. The observation that this occurs even in symptomatic AIDS patients, who are seriously immunocompromised, indicates that this is a serious and general problem for gene therapy[31]. Rv gives inefficient gene transfer for most cell types *in vivo*, partly because it is rapidly inactivated by the human complement system. The possibilities of integrative inactivation of tumour suppressor genes and RCV generation through recombination with cryptic Rvs that are resident in the human genome have contributed to serious safety reservations about Rv vectors, especially for non-cancer indications, even though extensive studies have revealed no evidence that these events occur at unacceptable frequencies.

Given these properties, it is not surprising that the most extensive use of Rv vectors has been with *ex vivo* applications, for which cells can be cultured to allow efficient transfection and for which sustained expression is generally required. *Ex vivo* applications also require smaller quantities of virus; this is an important consideration because Rv has presented serious manufacturing challenges, with difficulties in achieving high titres and retaining infectivity through concentration and storage. Despite these problems, more clinical experience has been obtained so far with Rv than any other vector, largely due to its capability for longer expression; the importance of this experience should not be underestimated. Recent improvements in manufacturing processes and the use of envelope proteins from other viruses, notably vesicular stomatitis virus, to package the recombinant Rv genomes promise to allow higher viral titres, greater complement resistance, transfection of a wider range of cell types and more-efficient transfection *in vivo*[32]. Good progress has been made recently in developing vectors from lentiviruses (Lv) such as HIV and SIV, which are capable of integrative transfection of several quiescent and post-mitotic cell types as well as proliferating cells. Experience with Lv vectors is still limited but recent reports[33,34] suggest that these Lv vectors can transfect true, quiescent haematopoietic stem cells, and might give more-efficient *in vivo* transfection and greater resistance to transcriptional silencing than their Rv predecessors.

Adeno-associated virus

AAV is also capable of integrating into the target-cell chromosome. The integration process is not well characterized but wild-type AAV integrates exclusively into a single site on human chromosome 19. It appears, however, that AAV recombinants integrate much less efficiently and more randomly[35], and many show long-term persistence in unintegrated forms. AAV appears to give sustained transgene expression upon *in vivo* administration, compared with other vectors; expression of homologous genes has been detected two years after injection in mice[36] and several months after injection in dogs[37], primates[38] and man[39]. Long-term correction of haemophilia has been achieved in a dog model of the disease with a single injection of an AAV vector expressing a gene for clotting factor IX (Ref. 37). Although the molecular events responsible and the requirement for integration are not established, this sustained expression is a major advantage in treating chronic diseases, for which AAV shows great promise.

The limited data available concerning immunogenicity of AAV vectors suggest 32% of adult humans possess pre-existing antibodies that neutralize transfection *in vitro* by the AAV2 serotype from which most AAV vectors are derived[18]. Studies in mice suggest injection with doses giving therapeutic levels of transgene expression leads to the generation of neutralizing antibodies that will reduce the effectiveness of repeat dosing[40]. CTL responses to AAV-transfected cells tend to be weak or undetectable, partly because in addition to the transgene the vectors contain only short viral sequences required for packaging. This undoubtedly contributes to prolonged expression, which has been observed in lung, muscle, liver and the central nervous system, in mice. AAV can transfer genes efficiently to both quiescent and proliferating cells. Some promoters with cell-type restriction retain their specificity in AAV, and preliminary data suggest efficient retargeted transfection will prove to be feasible for this vector[41]. The resilience of AAV particles is a major advantage for *in vivo* applications. Indeed, substantial transfection and long-lasting transgene expression in the epithelial and sub-epithelial cells of the upper intestinal tract have recently been reported following oral administration of an AAV vector[42].

The main disadvantages of AAV concern insert size and manufacturing; the virus can only accommodate inserts up to 4.5 kb, and manufacturing processes have required the use of helper viruses (usually Ad), which present problems of low titre, contamination and costly purification procedures. Only a few clinical protocols involving AAV have been initiated so far, largely because of manufacturing difficulties, but the results to date suggest transgene expression with this vector is sustained in man, as it is in animals[39]. Now that improved manufacturing processes allowing high-titre production without a helper virus have been developed[43,44], many more AAV-based therapies are certain to reach the clinic in the next few years.

Naked DNA

Although naked DNA gives virtually no transfection for cells *ex vivo*, it gives surprisingly efficient gene transfer in several tissues following local injection *in vivo*, notably in muscle[45] and skin[46]. The great majority of non-viral formulations fail to give more-efficient gene

transfer *in vivo* than naked DNA. The mechanism of naked DNA uptake by cells *in vivo* is unknown. There are many reports of transgene expression lasting several months, following injection of plasmid DNA into skeletal muscles in mice[45], but expression in skin is only detectable for a few days[46]. The observation that injection of plasmids encoding protein antigens into muscle or skin in mice leads to humoral and cellular immune responses directed to the antigen has led to enormous interest in naked DNA vaccines. In a recent Phase I clinical study involving intramuscular injection of plasmid DNA encoding a gene of *Plasmodium falciparum* (the causative agent of malaria), CTL responses directed to epitopes of the parasite were detected in most of the vaccinees[47]. Although the molecular events leading to effective antigen presentation remain to be clarified, such vaccination is one of the most promising and important applications of naked DNA as a gene-transfer agent. The other important application is the injection of naked DNA encoding angiogenic factors into muscles of patients with ischaemic vascular diseases[10,11].

Naked DNA can be manufactured simply and cheaply in bacteria, an advantage that is magnified in strategies that require co-delivery of several genes. Its disadvantages include: (1) a gene-delivery efficiency that is much lower than Ad or AAV; (2) very brief expression in most tissues; and (3) unsuitability for targeting. Naked DNA does not provoke specific immune responses, but it contains dinucleotide sequences comprising cytosine followed by guanine (CpG sequences); these sequences are unmethylated when produced in bacteria and elicit immune stimulatory and inflammatory cytokines in animals. This is an advantage for vaccine applications (indeed, these CpG sequences are now regarded as the most powerful adjuvant known[48]) but a disadvantage for chronic-disease therapy.

Cationic lipids

Cationic lipids bind DNA tightly by electrostatic interaction and afford substantial protection from degradation. Several cationic lipid–DNA complexes give fairly efficient transfection *ex vivo*. Several give reasonable transfection of endothelial cells in the lungs following injection into the tail vein in mice[49], and of airway epithelial cells following direct installation into the lungs[50]. Such formulations have been extensively tested for delivery of the cystic fibrosis transmembrane receptor gene following administration into the nose and lungs of cystic fibrosis patients. Gene expression and appropriate electrophysiological effects were observed, but the efficiency of gene transfer and the level and duration of expression were too low for clinical benefit[51,52].

The main disadvantages of cationic lipids are formulation instability and heterogeneity, inactivation in blood, relatively low transfection efficiency and poor targeting. Some improvement has been achieved recently by using cationic polymers to compact the DNA before mixing it with the lipid component[53]. Transfection efficiency with cationic lipids appears to be limited by poor uptake and inefficient trafficking of DNA to the site of gene expression in the nucleus.

Two therapeutic strategies involving cationic lipid delivery have recently progressed to Phase II clinical studies (Table 3). These require only brief expression of transgenes in order to effect direct or immunological killing of tumour cells[54,55].

Condensed DNA particles

Many cationic polymers have been used to condense DNA by electrostatic interaction into small particles, with a view to protecting the DNA from degradation and enhancing uptake via endocytosis. The most important polymers are heterogeneous polylysine[56], defined-length oligopeptides[57,58] and polyethylene imine (PEI; Ref. 59). There are many reports of reasonably efficient transfection for each of these with immortalized cell lines *in vitro*, but only one or two with primary cells *ex vivo*[58]. There are very few examples of such formulations giving transfection *in vivo* that is superior to that given by naked DNA. In general, such formulations aggregate in physiological conditions to form entities that are too large to penetrate effectively through solid tissues, and too large to be efficiently taken up by most cell types. Following uptake, they become trapped in the endosome and require the addition of an exogenous endosome-disruption agent (typically chloroquine) to give significant transfection *in vitro*. Use of such exogenous components is not feasible *in vivo*. Nuclear uptake of the DNA also probably limits transfection efficiency, as with most non-viral approaches. The main advantages of this approach are robust manufacture and suitability for improving specificity and efficiency of transfection by attaching targeting ligands or intracellular trafficking enhancers to the DNA condensing peptide. At present, PEI appears to be the most advanced delivery system of this type, giving significant transfection around blood vessels in the lung after injection into the tail vein in mice[60], with targeted transfection appearing feasible[61]. To date, though, no clinical studies have been undertaken with this approach.

Needle-free injection

Two devices have been developed that allow gene delivery by injection without needles. The first and more advanced device uses a high-pressure helium stream to deliver DNA, coated onto gold particles, directly into the cytoplasm, and is often referred to as the 'gene gun'[62]. The other, called the Intraject or Jet-gun[63] uses liquid under high pressure for delivery into interstitial spaces. The gene gun gives a moderate transfection efficiency for a variety of cell types *ex vivo*, and *in vivo* a moderate transfection efficiency for cells in the skin. These skin cells include Langerhans cells, which are antigen-presenting cells that normally capture foreign antigens from invading organisms and present them to naïve T cells to elicit primary immune responses. Gene-gun delivery to the skin with plasmids encoding protein antigens is a promising alternative to the injection of naked plasmid DNA into muscle for genetic vaccination[62]. Both approaches are reported to give an encouraging frequency of immune responses in initial clinical studies, but the gene gun requires much smaller DNA doses[64]. The liquid-based injectors are at an earlier stage of development, but are reported to give stronger immune responses than needle injection, in mice[63].

Electroporation

The application of an electric field to effect transfection of cells *in vitro* has been an important research

method for many years, and is believed to work by inducing areas of transient membrane breakdown through which the DNA enters the cytoplasm. It has not yet been used extensively for gene-therapy applications, partly because procedures giving transfection render the majority of cells inviable. More recently, devices and procedures have been developed that transfect with less cell damage *in vitro*, and there are reports of this approach substantially improving transfection in skin and tumours in mice[65,66]. A potentially very important area, yet to be fully explored, concerns the use of electroporation to stably transfect stem cells for *ex vivo* applications, with chromosomal insertion of the transgene.

Recent developments and future prospects

Over the past two years, improved manufacturing processes have been developed with reduced RCV and increased yield for Ad, Rv and AAV. This is an important development because it means the feasibility of manufacturing these vectors in adequate quality and quantity for clinical studies need no longer be a major factor in vector selection.

The most promising gene-therapy concepts, at the present time, concern the following:

- direct killing of tumour cells with genes delivered by Ad vectors for local management of cancer;
- delivery of naked DNA by injection or by the gene gun for preventative vaccination against infectious diseases;
- naked DNA delivery of genes promoting angiogenesis for cardiovascular disorders; and
- AAV delivery for chronic disorders, such as haemophilia and anaemia.

There is no single vector, at present, with generic utility for all types of diseases and it is unlikely that such a universal vector will emerge in the next few years. Over this period, Ad is likely to remain the most suitable vector for *in vivo* therapeutic concepts that require only short-term expression, especially where a single dose will suffice for clinical benefit. The incorporation of elements for targeted cell uptake and expression, and reduced immunogenicity, will no doubt extend the utility of Ad, but probably not to the long-term treatment of chronic diseases. Over the next few years, AAV is likely to be the most commonly used vector for long-term replacement of defective proteins, at least in cases requiring only a small transgene.

As indicated in Table 3, most of the gene therapies that have progressed into Phase II clinical studies involve gene transfer *in vivo*. The trend observed over the past three years towards an increasing proportion of *in vivo* strategies will continue now that vectors giving improved gene transfer *in vivo* (Ad, AAV) are available. Although, in the medium term, it is likely that there will be a renewal of interest in *ex vivo* therapy with stem cells. Preparation of haematopoietic progenitor cells is becoming increasingly routine in medical centres. Considerable advances are being made in identifying and propagating new types of human stem cell, including totipotent stem cells derived from embryonic or foetal tissue[67]. Newly developed Lv vectors, unlike their Rv predecessors, appear to be capable of giving integrative transfection of true, quiescent haematopoietic stem cells[34]. Following inoculation of such Lv-transfected cells into mice, transgene expression has been demonstrated in several daughter-cell lineages[34]. These vectors represent a significant development, but safety concerns arising from their origins in immunodeficiency viruses will lead to severe regulatory barriers. To address these issues, vector–producer cell combinations are being developed that incorporate multiple safeguards for completely avoiding RCV formation and that render the virus self-inactivating.

For all integrating vectors, and perhaps for most non-integrating vectors, transgene expression appears to be limited and usually silenced by incorporation into condensed and transcriptionally inactive chromatin, both *in vivo* and *ex vivo*. Chromatin opening elements, which resist transcriptional silencing by modifying the structure of chromatin, offer a solution to this problem. These elements are of two types: (1) locus control regions (LCRs); and (2) ubiquitously acting chromatin opening elements (UCOEs). LCRs open chromatin and confer sustained expression in a tissue-specific manner; UCOEs confer the same benefits in a wide range of tissues. The existence of LCRs was established some years ago and LCRs giving specific expression in several different tissues have since been identified[68]. They represent a promising approach to achieving sustained expression in the cell lineage most appropriate for clinical benefit following gene delivery to stem cells. UCOEs were discovered recently (R. Crombie *et al.*, unpublished) and are likely to improve transgene expression for a wide range of vectors.

Combining chromatin opening elements with the inducible promoter systems developed recently should allow full exploitation of the latter. These elegant promoters allow induction of transgene expression in response to exogenous substances[69]. The most advanced system can be induced by an orally active small molecule. It has been shown to enable low baseline expression and repeated cycles of induction to high levels over several months for the erythropoietin gene delivered using AAV to mice or monkeys[70]. These results represent proof of principle for one of the long-standing technical objectives of gene therapy: long-term persistence of a silent transgene with expression induced at will.

Non-viral vectors giving efficient gene transfer *in vivo* are not yet available. However, there is every prospect that non-viral vectors with broad utility will be developed in the next few years, particularly with flexible approaches such as condensed DNA particles. In the long term, non-viral approaches are more attractive for commercial products because they offer crucial advantages over viruses of superior targeting, low immunogenicity and reliable, large-scale manufacture at an acceptable cost. For the wide utility of non-viral vectors, however, it will be necessary to prolong transgene expression substantially, because this (typically) lasts only a few days. This is partly because present non-viral systems lead to neither integration nor extrachromosomal replication and maintenance. Progress, albeit slow, is being made in the development of replicating episomal vectors (REVs) based on oncogenic viruses, such as EBV (Ref. 71) and SV40 (Ref. 72), and on mammalian artificial chromosomes (MACs; Ref. 73). At present, REVs give inadequate stability and safety; MACs are too large (several Mbs) for efficient manipulation, manufacture and delivery.

The approach of developing a non-viral vector with large insert capacity, which exploits the site-specific integration machinery of AAV for safe and sustained transgene expression is very attractive, but is at an early stage of development[74]. This is a good example of a growing trend towards developing systems that combine the advantages of different transfer systems in hybrid vectors. Two further examples are noteworthy: the first uses Ad to deliver both the transgene in a Rv backbone and the Rv packaging genes to target cells. This vector converts the initially transfected cells into transiently active Rv producer cells, allowing stable Rv transfection of neighbouring cells[75]. The vector thus combines the Ad benefits of easier manufacture and more-efficient gene delivery *in vivo* with the more stable transgene expression capability of Rv. The second example uses Ad to deliver a pro-REV that can be released from the viral genome in transfected cells by site-specific recombination. This process yields daughter cells after cell division that display more-stable expression of the transgene, but do not express the residual Ad genes that provoke CTL responses[76]. Considerable development is needed to make these model vectors suitable for human gene therapy, but they illustrate the great potential of hybrid vectors.

Over the next few years, no doubt, most new clinical protocols will involve gene-addition therapy, but substantial progress has been reported recently towards gene-replacement and gene-correction therapy. AAV has been shown to give significantly greater efficiency of DNA replacement by homologous recombination between flanking sequences than any other vector, perhaps as a result of its ability to transfer large numbers of single-stranded DNA molecules to the nucleus. Heritable and accurate replacement in 1% of human fibroblasts *in vitro* has been observed[77]. Thus far, such correction has been confined to very short target sequences *in vitro*, but the results are highly encouraging. An alternative approach uses delivery of double-stranded DNA–RNA chimeric oligonucleotides, with RNA sections to provide targeting homology for a DNA region with a single-base mismatch to the target[78]. This chimeroplasty approach leads to gene correction through mismatched repair, which might give greater efficiencies than homologous recombination. Approximately 10% correction in primary blood cells has been reported[79], and ~40% correction in liver cells following *in vivo* administration in the rat[80]. This technology is not yet robust but, when fully developed, gene-replacement or gene-correction therapy will prove to be preferable to gene-addition therapy for many diseases. This is because it promises to avoid expression and safety problems arising from insertion into inappropriate chromosomal locations.

The substantial improvements in gene transfer and expression technology, in the past few years, have led to much ethical debate concerning the extension of experimental gene therapy to prenatal and germ-line protocols[2,81–83]. Some inherited monogenic disorders are lethal, or become permanently disabling, before or shortly after birth. The prospects of achieving a complete cure for cystic fibrosis, for example, are much greater at the foetal stage than in childhood or beyond, and success has been achieved in treating this disease in transgenic knockout mice using an *in utero* approach[84].

In the USA, gene-therapy clinical protocols that involve unprecedented aspects require approval from the Recombinant Advisory Committee (RAC) as well as the Food and Drug Administration. In March 1999, the RAC refused to approve two protocols involving gene transfer *in utero*, indicating that such approval would require significant additional preclinical and clinical studies on gene transfer, biodistribution and toxicology. Gene-therapy regulatory bodies now demand extensive animal safety data addressing the possible risk of inadvertent gene transfer to gonadal and germ cells in somatic postnatal gene-therapy trials. It is likely, therefore, that attempts at germ-line gene therapy, the step beyond *in utero* intervention, will continue to be prohibited on a worldwide basis for several years. The debate about whether or not a distinction is required between germ-line engineering to fight disease and that for enhancement purposes and, if so, how to enforce it, is presently exercising gene therapists, regulators and activists, but is likely to extend to the political arena in the near future. This has already happened with a related issue, the use of totipotent stem cells derived from embryos as a source of tissues and organs for transplant[85].

In conclusion, the most important recent development in gene therapy is the clear demonstration of efficacy in clinical studies, which might lead to a restoration of public and investor confidence in gene therapy. Substantial progress has been made in the past four years in overcoming the major inadequacies of first-generation gene-transfer vectors. No doubt the genomics revolution will provide an ever-increasing range of genes and information on their causal relationship with diseases, rendering more and more of these amenable to gene therapy. The first decade has held many disappointments, as was the case for recombinant therapeutic proteins, but the field of gene therapy has now settled into a phase of real and steady progress. There is every prospect that, in its second decade, it will fulfil its enormous potential.

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Antibodies beyond the year 2000

Antibody Fusion Proteins

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The timing of this book is indeed auspicious. In the past 12 months, antibodies have hit the headlines (almost weekly) as a result of their approval by the US Food and Drug Administration (FDA), and massive commercial sales of anticancer and antiviral antibodies. The most spectacular examples include Rituxan (IDEC) and Bexxar (Beckman Coulter), both targeting CD20 for therapy of non-Hodgkins lymphoma, and Herceptin (anti-Her2) for breast cancer (Genentech, San Francisco, CA, USA). The potential sales of these examples for use in cancer therapy are over US$1 billion during the year 2001, and they have revitalized the interest of the biotechnology industry in antibodies. These humanized recombinant antibodies are based on primitive protein designs that were developed at least ten years ago. A large number of second-generation recombinant antibodies are now being developed, which represent >30% of all the biological proteins in application for FDA approval.

This book reviews the latest developments in a specific, but rapidly progressing, field – that of fusing antibodies either chemically or genetically to a second functional domain. These bifunctional and bispecific reagents have huge potential application in clinical diagnosis and therapy. *Antibody Fusion Proteins* has two parts, which segregate nine individual mini-review articles that are each written by experts who are currently active in their particular speciality area. The main focus (Part I) clearly evokes the message that the original 'Magic Bullet' concept is still alive; especially that cancer-specific antibodies (Fab-type fragments) can be linked to the delivery of cytotoxic components to a tumour site. The overall tenet is therefore that recombinant antibodies provide the preferred targeting reagent, and that their activity can be significantly modified or manipulated by fusion to other molecules.

Indeed, Chapters 2–4 adequately cover the 'obvious' fusions to cytotoxic enzymes or toxins for cancer killing, radioisotopes for tumour imaging, and IL-2 for immune stimulation. Innovative improvements to the immunotoxin concept are described that include ADEPT, which is a strategy that involves using antibody–enzyme fusion proteins to activate a prodrug into a cytotoxic agent specifically at the target-cell site. Antibody fusions are described that target transferrin receptors and then cross the blood–brain barrier.

Chapters 5 and 7 encompass good reviews on bispecific antibodies and related fusion proteins that can effectively enhance the human immune response via T-cell recruitment strategies. Oddly, there are some

