Gene therapy: potential applications in clinical transplantation

Jeremy W. Fry and Kathryn J. Wood

Gene therapy continues to offer much hope for the future treatment of a variety of clinical conditions. The development of tailored, novel gene transfer vectors will improve the efficiency and stability of therapeutic gene expression in the many settings of gene therapy. In the context of tissue and organ transplantation, gene therapy is being harnessed to prevent the acute and chronic rejection of transplanted tissues by introducing either new genes that are important in preventing rejection (e.g. co-stimulatory blocking molecules or immunosuppressive cytokines) or antisense nucleic acids to block the production of rejection-associated molecules such as adhesion molecules. The delivery of genes by gene therapy vectors that encode foreign donor antigens (alloantigens) might also be an effective means of inducing donor-specific unresponsiveness (immunological tolerance) in the recipient, perhaps eliminating the requirement for potentially harmful whole-body immunosuppression.

Study of diseases over several hundreds of years has resulted in the wide range of sophisticated techniques that is used today for diagnosis, treatment and research. During the 1960s, research to determine the underlying cause of diseases was limited to analysing the biochemistry of diseased cells and investigating various protein interactions. Although this research was valuable, scientists at the time lacked the specific technology and reagents to break down disease processes into their constituent parts in order to understand them more thoroughly. During the 1970s, restriction enzymes that cut DNA at specific sequences were first discovered and then used in molecular biology. Using these restriction enzymes to cut, remove and join genes together, researchers were able to start to understand the important roles that genetic factors play in disease. Currently, as the Human Genome Project nears completion, we can attempt to interpret the

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wealth of data available to us and thus make new links between particular genes and diseases. Once established, this information should accelerate the progress in the use of gene therapy as a therapeutic strategy.

In this review, we have discussed the basic principles of gene therapy and described the various vectors that are currently used both in research and in the clinic to deliver introduced genes (transgenes) to target tissues. The implications of gene transfer strategies to transplantation and potential future applications have also been reviewed.

Allograft rejection and immunological tolerance

For the foreseeable future, patients will continue to be transplanted with allogeneic organs which, by definition, are not identical at the major histocompatibility complex (MHC) locus. Without any other treatment, such as the administration of immunosuppressive drugs, the immune response generated by the recipient, which is mainly T-cell mediated, will reject such a graft (Fig. 1). Self tolerance (the inability of self T cells to react against self tissue) is acquired as the immature T cells develop and pass through the thymus. This occurs because the majority of any potentially autoreactive T cells are 'negatively selected' by the process of clonal deletion, although clonal anergy (the existence of viable T cells that are unresponsive to antigen) and the generation of a population of regulatory T cells might have a role to play (Ref. 1). The ultimate aim of transplant immunologists is to induce long-term transplantation tolerance to alloantigens in graft recipients. Such an immunological state would allow patients to continue to respond as normal to foreign antigens (e.g. bacteria, viruses and arising malignant cells), but tolerate, and not reject, a transplanted graft. In such an 'ideal' situation, there would be no need for systemic immunosuppressive drugs (with all the disadvantages that they bring), and transplanted patients would have a fully functioning, healthy immune system.

What is gene therapy?

A gene is a linear sequence of DNA that codes for a particular protein. On rare occasions, usually during the division of the cell, the nucleotide sequence (the order of the DNA base pairs) of a gene can get jumbled up and mutated, so that the resultant protein is faulty. Such a mutation event is the root cause of genetic diseases such as cystic fibrosis, adenosine deaminase (ADA) deficiency and sickle-cell anaemia. For example, people who suffer from cystic fibrosis produce a faulty cellular transport protein called cystic fibrosis transmembrane conductance regulator, which results in the build-up of mucous in their lungs.

The earliest applications of gene therapy were based on the principle that a disease is caused by a faulty gene (or combination of genes), and if such genes can be replaced with 'correct' versions, the disease might be controlled, prevented or cured. Gene therapy is being applied to many different genetic diseases, both congenital (since birth) and acquired. However, most diseases involve multiple genetic factors (they are polygenic). Until the precise involvement of different genes (their regulation and expression) in the disease process and the proteins they encode is established, gene therapy is most likely to be clinically effective as a preventative or curative treatment for single-gene defects such as ADA deficiency, familial hypercholesterolaemia (Ref. 2) and cystic fibrosis. Several clinical trials employing gene therapy protocols have already been completed, with some success in patients who have cystic fibrosis and ADA deficiency, although the effectiveness of the protocols was not as dramatic as first envisaged, mainly owing to the inefficiency of the gene transfer vectors that were used.

Originally known as 'genetic replacement therapy' during the early 1980s, 'gene therapy' has now outgrown its original definition and is applied to all manner of protocols that involve an element of gene transfer, either in vivo or ex vivo, and not necessarily a gene that is known to cause a disease. In vivo gene transfer is the introduction of genes to cells at the site they are found in the body, for example to skin cells on an arm, or to lung epithelial cells following inhalation of the gene transfer vector. Ex vivo gene transfer is the transfer of genes into viable cells that have been temporarily removed from the patient and are then returned following treatment (e.g. bone marrow cells). Gene therapy can be subdivided into somatic cell gene transfer (that is transfer to normal diploid cells), which is the focus of this review, and germline gene transfer (transfer to haploid sperm or egg cells of the reproductive system). The ethical issues associated with germline gene therapy are far more complex than

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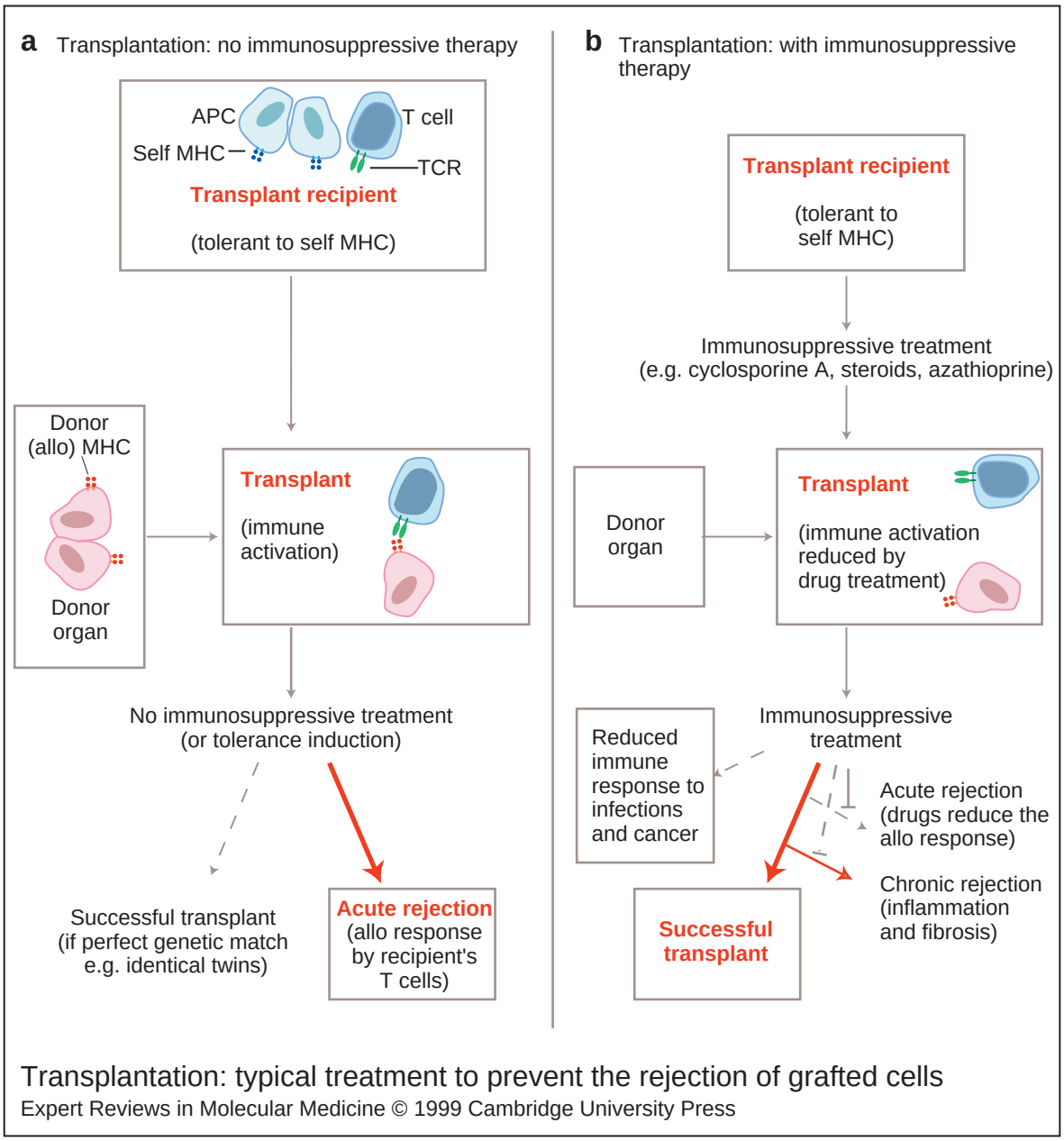

Figure 1. Transplantation: typical treatment to prevent the rejection of grafted cells. Possible outcomes of graft transplantation with or without the administration of immunosuppressive drugs. Abbreviations used: APC = antigen-presenting cells; MHC = major histocompatibility complex; TCR = T-cell receptor; T cell = T lymphocytes **(fig001jfo).**

those surrounding somatic cell gene transfer, because the genes are transferred not only to treated individuals but also to their progeny. Germline gene therapy is being widely used for the production of transgenic animals for research, and increasingly for agriculture and biotechnology, but the long-term effects of each transferred gene in animals will need to be carefully monitored and analysed, as well as the significance of any residual vector DNA if applicable. The benefits that the use of germline gene therapy in humans could bring are significant. The development of serious and distressing inherited genetic diseases could be prevented before birth and eliminated in subsequent generations. However, because of the potential for abuse and eugenics, gene therapy in humans needs to be widely discussed and the

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associated safety issues evaluated before this approach can be used for the treatment of diseases.

Gene therapy applied to transplantation

In 1958, the first recorded attempts to use DNA in transplantation research were carried out by Haskova and colleagues (Ref. 3) and Medawar (Ref. 4), who were investigating whether the administration of DNA from a donor strain could result in immunisation to a subsequent transplant (that is its rapid rejection). In the experiments carried out by Medawar, DNA from the spleen of donor mouse strain A was purified and 5 mg injected into the peritoneal cavity of a previously unmanipulated recipient mouse (CBA strain). The recipient mice were transplanted 3–5 days later with skin from donor A strain mice and the grafts monitored over time. The grafts were rejected at the same rate as that in mice that had not received DNA, and no heightened response was measured. In another experiment by Medawar (Ref. 4), following on from his success in injecting donor strain cells into neonates to induce transplantation tolerance, newborn mice were repeatedly injected with 'high doses' of donor strain DNA in an attempt to induce transplantation tolerance; however, this approach did not prolong the acceptance of skin grafts after subsequent transplantatation. The negative results of these early experiments were attributed by Medawar to impure DNA preparations and contamination with polysaccharides, although, with hindsight, quite different results could probably have been achieved if a different route of injection, such as intramuscular, had been employed, as was recently demonstrated by Geissler and colleagues (Ref. 5).

Organ transplantation is now well established as a therapy for end-stage organ failure. Remarkable advances in the development of immunosuppressive drugs (e.g. cyclosporin, corticosteroids and rapamycin) have increased the survival rate of 1-year and 5-year renal grafts to 85% and 70%, respectively (Ref. 6). Although this is an impressive achievement, healthy grafts can still be rejected, and the use of systemic immunosuppressive drugs is associated with significant risks, such as an increased risk of the development of cancers, infections and ischaemic heart disease, even in patients with long-term functioning grafts (Ref. 6).

Gene therapy is a good strategy for approaching existing problems associated with transplantation, but will most likely be used only as a complementary approach (Fig. 2). For example, grafts themselves could be targeted to reduce their immunogenicity by the introduction of genes to block T-cell activation, or donor-specific MHC antigens could be introduced into the recipient before transplantation to induce transplantation tolerance. Both strategies are potentially powerful and their prospects are discussed later.

Genes of interest in transplantation

MHC

The MHC is a highly conserved yet polymorphic gene locus. MHC molecules are surface proteins that present intracellularly processed peptides in a helical groove to their ligand, the T-cell receptor (TCR). Cognate interaction between an MHC molecule presenting peptide on an antigen-presenting cell and a specific TCR on a T cell can result in T-cell activation if the appropriate co-stimulatory molecules are present on the antigen-presenting cell. MHC class I molecules consist of three alpha domains and a β2 microglobulin chain, which is not encoded by the MHC gene locus. MHC class II molecules consist of two alpha domains and two beta domains. Peptides that are presented on the class I molecule are usually derived from intracellular proteins, whereas class II molecules present extracellularly derived peptides. The mechanism by which these peptides are transported to the immature MHC molecule is also very different for class I and class II MHC molecules, and has been recently reviewed in this journal (Ref. 7). The MHC is the major identification molecule that triggers allograft rejection, because it determines the difference between self (syngeneic) and non-self (allogeneic). When searching for a suitable organ donor, it is the MHC antigens that are matched between donor and recipient, to give the graft as good a chance as possible of functioning. In defined situations, this potency of the MHC has been exploited to tip the balance of the immune system from immunity to tolerance. The exposure of the recipient of a graft to donor MHC antigens before transplantation to induce tolerance was first investigated in a mouse model by Billingham and colleagues in 1953, when cells from a donor strain were introduced into a recipient mouse in utero (Ref. 8).

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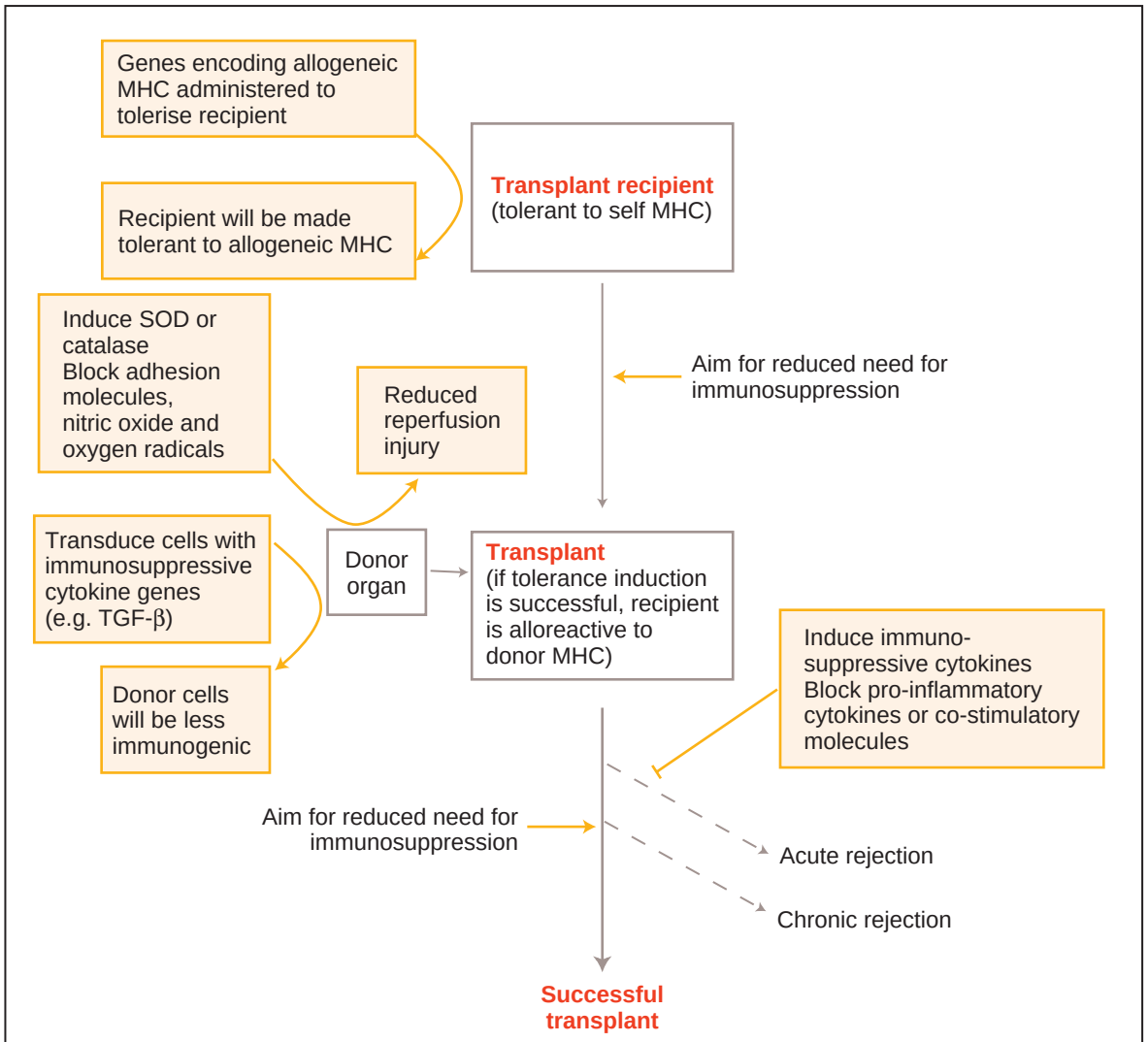

Figure 2. Transplantation: gene therapy approaches to prevent rejection of grafted cells. Flow diagram to describe the possible outcomes of graft transplantation following the induction of donor-specific immunological tolerance or the reduction of the immunogenicity of the graft itself using gene therapy approaches. Abbreviations used: MHC = major histocompatibility complex; SOD = superoxide dismutase; TGF-β = transforming growth factor beta **(fig002jfo).**

Following this first attempt, and further studies, pre-transplantation blood transfusions (although not necessarily from graft donors) have been used in the clinic as a means of delivering MHC alloantigens before transplantation, but with limited success. However, the use of blood products also carries inherent risks, such as infections and transfusion reactions; thus, a novel therapy using a more specific approach would eliminate the risks of sensitising transplant recipients to alloantigens that are present in the blood. The delivery of donor genes to cells or tissues in a recipient would offer a highly specific therapy, one that is free from the risks associated with foreign cells and allows transplant recipients to be pre-treated with foreign genes before donor tissue becomes available.

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The transfer of MHC genes is also useful in animal models to study the effects of allogeneic MHC antigens on the immune cells of a recipient without the influence of other alloantigens. Such an approach was first carried out by Madsen and colleagues (Ref. 9), when a single MHC class I gene from a donor was transfected into a recipient-type mouse cell line and administered to a recipient. Not only was unresponsiveness to a subsequent cardiac allograft achieved in this study, but it showed that the recipient did not need to be exposed to all of the mismatched donor MHC molecules. Although these experiments proved that this strategy could work, transfected recipient cells are not a practical choice in a clinical setting. The next step was made by Wong and colleagues (Ref. 10); bone marrow cells from recipient mice were transduced (infected with virus) ex vivo with an MHC class I gene using a retroviral gene therapy vector. This approach also resulted in long-term unresponsiveness to a fully allogeneic cardiac allograft, but rejection of a third-party graft, which had MHC class I genes that the recipient had not previously been exposed to.

Another interesting characteristic of MHC molecules is their ability to modify the immune response, depending on whether they are soluble or membrane bound. Observations following human liver transplantation have demonstrated that soluble donor human leukocyte antigens (HLA; human MHC antigens) are present post-transplant at high concentrations (Ref. 11). It has been hypothesised that microchimerism (the co-existence of donor cells a very low levels within the recipient) of donor leukocytes alone causes the tolerant state; however, another equally valid explanation is that tolerance is caused by the large quantities of soluble MHC molecules that the liver naturally produces. Soluble donor-specific MHC class I molecules can have immunosuppressive effects, which can be used to promote graft survival in transplantation. Geissler and colleagues (Ref. 12) used a mouse model in which hepatocytes from a recipient strain were transfected using lipofectin with a plasmid encoding either membrane-bound or soluble allogeneic MHC class I molecules. Hepatocytes that expressed membrane-bound MHC class I molecules were found to prime cytotoxic T-lymphocyte (CTL) precursor cells, whereas exposure to soluble MHC class I molecules reduced the number (frequency) of CTL precursors. This idea has been reinforced by in vitro data from Zavazava and colleagues (Ref. 13), which showed that soluble HLA class I molecules can induce apoptosis in human alloreactive CTLs.

Immunosuppressive cytokines

The delivery of genes that encode immunomodulatory molecules to the site of the graft, or to the graft itself, has much scope for reducing the harmful local immune response against foreign tissue that occurs in acute and chronic rejection.

Cytokines are soluble mediators of the immune system, and some of them have immunosuppressive effects. The viral form of interleukin 10 (vIL-10) is a protein that is encoded by the Epstein–Barr virus; it is structurally homologous to mouse and human IL-10 but does not possess the T-cell co-stimulatory properties that IL-10 does. Thus, it is a useful tool in gene transfer to tissue where T-cell activation needs to be switched off or downregulated. DeBruyne and colleagues (Ref. 14) have demonstrated that gene transfer of vIL-10 to a murine cardiac allograft via vasculature perfusion using DNA–liposome complexes prolonged graft survival (16 days compared with 8 days for untreated grafts). The result was attributed to the vIL-10 gene, because treatment with either an antisense plasmid to vIL-10 or a monoclonal antibody targeted against vIL-10 reversed the graft-prolongation effect. Other cytokine genes, such as transforming growth factor beta (TGF-β), have also been shown to have a significant immunosuppressive effect (Ref. 15). This type of approach is not intended to induce immunological tolerance, but might be useful for the delivery of local immunosuppression.

Blockage of co-stimulatory signal

In addition to the production of an intracellular first signal following specific TCR–MHC interaction, full activation of a T cell requires a second co-stimulatory signal, which can be provided by the interaction of CD28 and B7-1 or B7-2 (CD80 or CD86, respectively). Cytotoxic T-lymphocyte antigen 4 (CTLA-4; also called CD152) is an alternative ligand for CD80 and CD86, and is homologous with CD28. CTLA-4 is believed to play a role in the negative regulation of T-cell activation. The blockage of this co-stimulatory signal, for example using a fusion protein, has been shown in many murine and

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primate studies to inhibit cell-mediated and humoral immune responses in vivo. In one such study that used an adenoviral vector to deliver a CTLA-4Ig gene [a fusion protein comprising CTLA-4 and an immunoglobulin (Ig)] intravenously following cardiac allograft transplantation, the median survival time was increased from 6 days in the control group to 23 days in the group treated with the adenoviral vector expressing the CTLA-4Ig transgene (Ref. 16). In another investigation by Chahine and colleagues (Ref. 17), a transgene for CTLA-4Ig was transfected to both syngeneic and allogeneic mouse muscle precursor cells (myoblasts) and co-transplanted with allogeneic pancreatic islet cells under the kidney capsule of diabetic mice. Syngeneic myoblasts significantly prolonged the survival of the islets from 11 days to 31.7 days; no beneficial effect was observed for the transfected allogeneic myoblasts. The syngeneic myoblasts actively secreted CTLA-4Ig, to create local immunosuppression in the environment of the allogeneic islets, and thus allow them to function. When the myoblasts were allogeneic themselves, the MHC disparity with the recipient was enough to destroy them, thus preventing any CTLA-4Ig from being produced.

Genes associated with chronic rejection

Damage to an allograft can continue for years after transplantation, and despite improvements in immunosuppressive drugs and organ preservation, chronic rejection is still the most important factor in the failure of transplanted grafts (Ref. 18). Histologically, during chronic rejection, smooth muscle cells are seen to proliferate in the vasculature of the transplanted organ, sometimes resulting in transplant atherosclerosis; several factors can contribute to this end point. Adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1 or CD54; Ref. 19) and growth factors such as vascular endothelial-cell growth factor are upregulated, and inducible nitric oxide synthase is imbalanced (Ref. 20).

ICAM-1

ICAM-1 is a member of the Ig superfamily and is very important in both cellular adhesion and T-cell co-stimulation. Strategies to reduce T-cell activation by eliminating the effects of ICAM-1 have been carried out successfully in clinical trials involving renal allograft patients and the use of antibodies targeted against the ICAM-1 molecule (Ref. 19). In a study of 18 patients, the anti-ICAM-1 antibody (BIRR1) was given to those patients who had received renal grafts from cadaver donors and were at a high risk for delayed graft function. An adequate level of BIRR1 in the serum (>10 μg/ml) was found to significantly reduce the incidence of both delayed graft function ($p<0.01$) and rejection episodes ($p<0.01$). This therapy has been developed further in murine models to use antisense oligonucleotides that are targeted against the messenger RNA (mRNA) for ICAM-1 (Refs 21, 22).

Nitric oxide

The intimal proliferation of vessels in transplanted organs is another indication of chronic rejection. To test the hypothesis that endothelium-derived nitric oxide is an endogenous inhibitor of vascular lesion formation, a Sendai virus virosome (see later section entitled 'Fusigenic virosomes') was used to deliver the endothelial-cell nitric oxide synthase gene in vivo. Von der Leyen and colleagues demonstrated that following disruption of the endothelium of rat carotid arteries, using a balloon-injury model, neointimal proliferation was reduced by 70% after transfer of the endothelial-cell nitric oxide synthase gene (Ref. 23). A similar approach using a recombinant adenovirus to deliver inducible nitric oxide synthase to sites of arterial injury in vivo also demonstrated that the delivery of this gene could increase local levels of nitric oxide and thus prevent intimal hyperplasia (Ref. 20).

Oxygen free radicals

Before transplantation, most solid organs are kept cold and without a full blood supply, which results in the effects of cold ischaemia. This, combined with reperfusion of the newly restored blood supply, can induce cell damage mediated by oxygen free radicals, which is thought to increase the chances of chronic rejection. To prevent serious damage, soluble superoxide dismutase (SOD) has been delivered to the graft ex vivo in perfusion solutions to 'mop up' free radicals. To date, few studies have been carried out using SOD in gene delivery. One investigation used recombinant adenoviruses encoding SOD (or catalase, which has similar effects) for use in oxidant-injury-related diseases (Ref. 24). In this lung-perfusion model of rats, ischaemia-reperfusion injury was evaluated; surprisingly, the

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overexpression of SOD worsened ischaemia-reperfusion injury. The expression of both SOD and catalase transgenes prevented this increase in ischaemia-reperfusion injury but did not protect from it.

Inhibition of the proto-oncogene c-myb

The proto-oncogene c-*myb* is involved in the mitogen-induced proliferation of vascular smooth muscle cells, which constitutes a major pathway of atherogenesis. An increase in c-*myb* mRNA is seen after smooth muscle cell growth. The inhibition of c-*myb* has been reported to prevent the proliferation and migration of smooth muscle cells that are associated with the intima of blood vessels. Several studies have used antisense strategies (Refs 25, 26) or ribozymes (see below; Ref. 27) to inhibit the proliferation of smooth muscle cells; however, these data are controversial, following reports that the effect might be non-specific and due to four contiguous guanosine nucleotides in the antisense oligonucleotide (Ref. 28).

Ribozymes as enzymes to target mRNA

In 1981, Cech and colleagues revealed that proteins were not the only molecules capable of catalysing cellular reactions (Ref. 29). Ribozymes are naturally occurring RNA molecules that act as enzymes and catalyse reactions on themselves or on other RNA molecules (for reviews and information, see Refs 30, 31, 32, 33 and http://www.rpi.com/sinfo.htm). Ribozymes were first isolated from the ciliated protozoan *Tetrahymena thermophila,* and subsequently became known as group I introns. The precursor ribosomal RNA (rRNA) molecule that is transcribed from the rRNA gene was found to be self-splicing. To initiate splicing, free guanosine nucleotide is bound to the specific site on the RNA molecule, the intron folds, cuts itself out and splices the exons together. Group II introns (which are found predominantly in fungal mitochondria) use extremely reactive adenine nucleotides within the intron sequence itself to initiate splicing. It soon became apparent that the modification of ribozymes could result in a powerful new molecular tool. Once the original group I introns had been modified to act on an external RNA substrate, ribozymes were then engineered to recognise specific nucleic acid sequences, so that they would cleave in a sequence-specific manner. Many different ribozymes are now in use, the most common being hepatitis delta virus (HDV) ribozyme, hammerhead (HH) ribozyme and hairpin (HP) ribozyme. The HH, HP and HDV ribozymes were all originally isolated in their self-cleaving forms and then modified to act on external substrates. The HP, HH and HDV ribozymes are relatively small, and thus easy to manipulate for gene therapy purposes. Delivery into target cells has been through (1) the direct injection of ribozyme RNA (the ribozymes were modified to increase their half-life), (2) the direct injection of oligodeoxynucleotides encoding ribozymes or (3) the delivery of ribozyme genes using a viral vector such as adenovirus.

The potential disease targets for ribozymes are abundant, including those conditions in which a gene product is overproduced, such as in cardiovascular disease (the genes encoding platelet-activating factor and fibrinogen), arthritis, cancer (oncogene function can be targeted) and many other immunological diseases.

Ribozymes targeting xenoantigens

Ribozymes also have a role to play in targeting genes that are involved in transplantation. In the xenogeneic setting (the transplantation of cells or tissues between species), the sugar epitope galα(1,3)gal is a major xenoantigen, which contributes greatly to the hyperacute rejection response that is characteristic of xenotransplantation. The enzyme that is involved in the production of this sugar, namely α(1,3)galactosyltransferase, has been targeted using a ribozyme delivered by an adenoviral vector (Ref. 34); complement-dependent cytotoxicity was significantly reduced, and would be expected to inhibit hyperacute rejection in subsequent studies, for example in vivo xenotransplantation.

Ribozymes targeting Fas ligand and perforin

Du and colleagues (Ref. 35) have characterised the ability of HH ribozymes to target perforin and Fas ligand (FasL) mRNAs in vitro as a therapy to prevent graft versus host disease (GvHD), which can follow allogeneic bone marrow transplantation. Fas ligand is a cell-surface molecule that belongs to the tumour necrosis factor (TNF) receptor family. When it binds with Fas, a 'death signal' is sent to the Fas-bearing cell, resulting in apoptosis (also

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known as programmed cell death). Perforin, on the other hand, is a protein that can polymerise to form membrane pores; it is an important part of the killing mechanism used both by CTLs and by natural killer cells. Mice that have been transplanted with bone marrow from genetically engineered 'knockout' mice that do not express perforin or FasL do not develop GvHD. Such a genetic knockout approach cannot be applied to humans, thus the ability to destroy the perforin and FasL mRNAs in donor bone marrow using ribozymes could prove to be a useful method of reducing GvHD.

Ribozymes targeting inflammatory cytokines and chemokines

Another potentially useful target of ribozymes in the transplant setting is inflammatory cytokines, such as interferon gamma (IFN-γ) or tumour necrosis factor alpha (TNF-α), which are upregulated during allograft rejection. Chemokines, which can play a role in directing inflammatory cells to the site of a transplant and amplifying their effects, including RANTES (regulated upon activation, normal T-cell expressed and secreted), interferon-gamma-inducible protein (IP-10), macrophage-inflammatory protein 1 alpha (MIP-1α), macrophage-inflammatory protein 1 beta (MIP-1β) and monocyte chemoattractant protein (MCP-1), are all potent future targets for ribozyme gene therapy in transplantation.

Routes of administration and cell targets for gene therapy vectors

Intrathymic administration

The application of the process of intrathymic T-cell development to transplantation and tolerance induction was first described by Posselt and colleagues (Ref. 36). Self tolerance (the failure to respond to antigen borne on self tissue) develops as T-lymphocyte precursor cells that are $CD4^-$ and $CD8^-$ ('double negative') pass through the thymus. Because the T cells are exposed to antigen on thymic epithelial cells, any T cells that have a high-affinity interaction with antigen in the thymus, and are therefore potentially autoreactive cells, are 'negatively selected' by the process of clonal deletion. Cells that have TCRs that have no (or an extremely low) affinity for intrathymic antigen, yet still have a high-affinity interaction with self MHC, are 'positively selected'; they can thus mature and go on to populate and expand into larger clonal populations in the periphery. For a recent review of the mechanisms of the induction of intrathymic tolerance, see Turvey and colleagues (Ref. 1).

Knechtle and colleagues (Ref. 37) showed it was possible to induce tolerance using a gene therapy strategy in a rat model. First, they took syngeneic recipient muscle cells and then transfected them in vitro with an MHC class I gene derived from the donor. These cells were then injected into the thymus of the recipient. Next, the peripheral immune system of the recipient was depleted of potentially alloreactive T cells using anti-lymphocyte serum. This was then followed by a liver transplant from the donor, to which the recipient was found to be unresponsive. In a subsequent study (Ref. 38), the MHC class I complementary DNA (cDNA) from the donor strain rat was introduced directly into the thymus to transfect recipient thymic cells in situ; analysis using the polymerase chain reaction (PCR) detected the transient expression of donor DNA in the thymus (and later in the spleen, which was probably due to the export of transfected thymocytes out of the thymus).

The above approaches have used either live cells transfected with DNA or naked DNA itself to deliver donor MHC genes. Adenovirus could be used to improve the efficiency of gene therapy that has been achieved using DNA transfection. Adenovirus vectors (as discussed later in the section entitled 'Adenovirus') are ideal for use in intrathymic applications because they can be generated at high titres and can transduce a range of cell types. Not only can genes be transferred to the antigen-presenting thymic epithelial cells and possibly to the developing thymocytes, but central tolerance (tolerance that is established in lymphocytes developing in central lymphoid organs such as the thymus, spleen and bone marrow) to the immunogenic adenoviral antigens can be induced, as shown by Ilan and colleagues (Ref. 39). Their work demonstrated that intrathymic inoculation of the recombinant adenovirus inhibited the appearance of neutralising antibodies and CTLs against the recombinant adenovirus.

Liver

The liver has many interesting properties with respect to gene transfer and transplantation. Recipients of liver grafts have been shown in

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some situations to accept MHC-mismatched grafts spontaneously, without the requirement for post-transplant systemic immunosuppression. It has been hypothesised that this observation was due to the post-transplant solubilisation and release into the circulation of donor MHC molecules, which subsequently downregulated the alloreactive CTL response (Refs 12, 13).

The portal vein, hepatic artery or both offer the best routes of access to the liver in vivo for delivering either a perfusate of a viral or non-viral gene therapy vector (Ref. 40). In one study, Chia and colleagues (Ref. 40) demonstrated that efficient gene transfer could be achieved after perfusion with an adenovirus encoding a reporter gene via both the portal vein and the hepatic artery into clamped, cold-preserved liver grafts. This efficiency was said to have been due, in part, to the improved access to the hepatic microcirculation and thus virus–cell contact.

Retroviral vectors have also been used in mouse models for hepatic gene transfer; however, because these vectors are efficient at transducing only actively dividing cells, a partial hepatectomy must be carried out in most cases before retroviral transduction, to encourage the hepatocytes to divide (Ref. 41).

Bone marrow cells

The importance of bone marrow cells, particularly the sub-population of haematopoietic stem cells (HSCs), has never been underestimated in the context of gene therapy. Their potential for self renewal and differentiation into all haematopoietic lineages makes them very attractive as targets for gene transfer, especially in those situations (such as genetic disorders) where long-term transgene expression is required. Unfortunately, because HSCs occur at extremely low frequencies in the bone marrow and peripheral blood, it is difficult to obtain sufficient numbers of cells to transduce ex vivo for a subsequent in vivo biological effect. The purification of HSCs for gene therapy relies mainly on mobilising the stem cells from the bone marrow into the peripheral circulation, using an agent such as granulocyte macrophage colony-stimulating factor; cells are then selected for, using methods such as fluorescent-activated cell sorting or antibody-coated magnetic beads. This type of positive-enrichment method requires the existence of cell-surface markers that are potentially specific for stem cells, such as c-kit (the receptor for stem-cell factor in the mouse) and CD38 in humans (although this is more controversial; Ref. 42). Negative depletion (which excludes those cells that are definitely not stem cells) is another method, and is often used in combination with positive selection. The search for new stem-cell-specific markers is an extremely active area of research at present.

With regards to clinical transplantation, bone marrow itself is a frequently transplanted tissue, for example in patients who have undergone radical cytotoxic therapy for leukaemias and other haematological malignancies. The infusion of donor bone marrow has often been used to expose recipients before transplantation to the alloantigens of a mismatched organ. This approach is thought to be worthwhile despite the possibility that GvHD could occur. The exposure of recipients to MHC transgenes that have been derived from donors is a more specific and safer method; furthermore, it does not require the introduction of live donor lymphocytes and therefore poses no risk of the transfer of cells that induce GvHD. The transfer of MHC genes to syngeneic bone marrow ex vivo or in vivo could be carried out as a means of exposing recipients to alloantigen genes. Gene transfer to bone marrow could also be used to introduce genes that encode immunoregulatory molecules (such as cytokines) that would modulate the immune function in haematopoietic cells (Ref. 43).

Sykes and colleagues (Ref. 44) demonstrated in irradiated mice, using a retroviral gene therapy vector, that the pre-transplantation transfer of a donor MHC class I gene to recipient strain bone marrow cells ex vivo prolonged the survival of mismatched skin grafts that were mismatched by a single alloantigen; however, fully allogeneic skin grafts with multiple mismatches were rejected.

Wong and colleagues (Ref. 10) studied a similar system that also used a retroviral vector encoding a donor MHC class I molecule. This time, fully allogeneic CBA mice with the MHC haplotype $H2^k$ were used as transplant recipients. First, they were pre-treated 28 days before transplant with two doses of an anti-CD4 monoclonal antibody and 5×10^6 recipient bone marrow cells. These cells had been transduced ex vivo with a retroviral vector carrying the donor-specific MHC class I gene K^b. As a result of this tolerisation regime, the mice were able to accept indefinitely a subsequent donor-specific [C57BL/10 ($H2^b$)] cardiac allograft.

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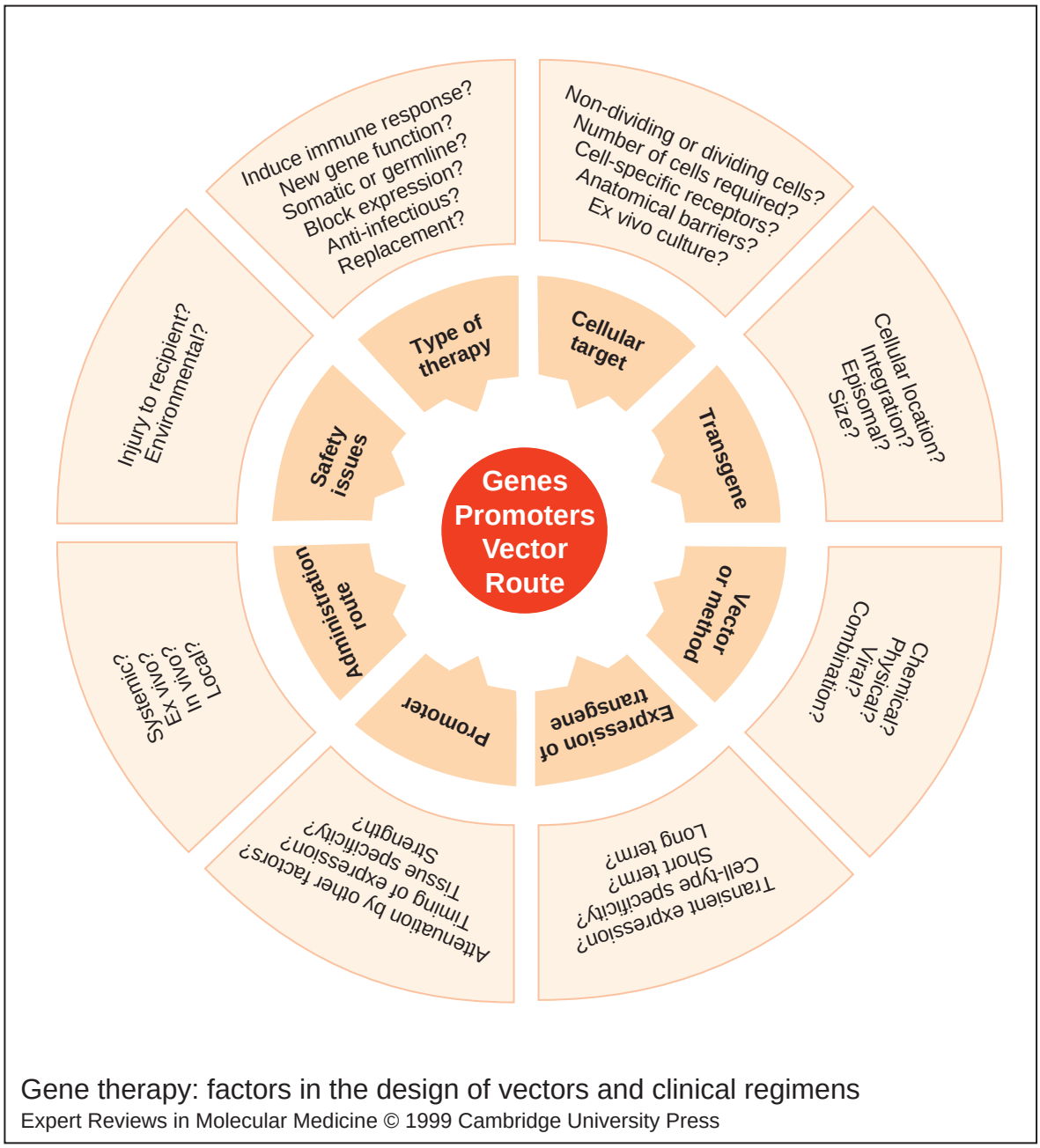

Gene therapy: factors in the design of vectors and clinical regimens

Figure 3. Gene therapy: factors in the design of vectors and clinical regimens (fig003jfo).

This study has important clinical implications, because it demonstrated that recipients do not need to be exposed to the full range of donor MHC molecules present on a graft for that graft to be accepted long term. This tolerogenic (or unresponsive) state does not reduce the potency of the immune system; it still reacts with full vigour against any other foreign or third-party antigen.

Gene transfer vectors

Vectors are the vehicles that are used in gene therapy to transfer the gene(s) of interest [transgene(s)] to the target cells, which will then go on to express the therapeutic protein encoded by the transgene(s). The most important factor in any gene transfer protocol (Fig. 3), apart from the gene of interest, is the choice of vector, which can result in either success or failure. Unfortunately,

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Table 1. A comparison of vectors in use for clinical gene transfer (tab001jfo)

Vector classification	Application for human use: Ex vivo	In vivo	Expression	Advantages	Disadvantages
Viral					
Recombinant Moloney murine leukaemia virus (MMLV; retrovirus)	+++	++	Stable	No immune response against the viral vector; integrates	Only low viral titres achieved; transduces only dividing cells
Recombinant lentivirus (retrovirus)	+	+	Stable	Transduces non-dividing cells; can target $CD4^+$ T cells	Potential safety risk
Recombinant adenovirus	+++	++	Transient	High viral titre; wide host-cell range	Immunogenic; does not integrate; short-term transgene expression
Recombinant adeno-associated virus	+++	++	Stable	Little immunogenicity; integrates	Lower transduction efficiency than adenovirus
Recombinant herpes simplex virus (HSV)	++ (research)	++	Not known	Can target neuronal tissue	Safety concerns
Recombinant vaccinia virus	+/–	+++	Transient	Suitable for cancer gene therapy	Safety concerns
Non viral					
DNA–ligand conjugates	–	++	Transient	Cell-specific targeting	
Liposomes and virosomes	++	+++	Stable or transient	Cell-specific targeting; efficient transfection	
Direct DNA injection	–	++	Transient	Simple	Only short-term expression achieved
Ballistic delivery (gene gun)	++ (research)	+/–	Transient	Simple	Requires 'exposed' tissues or cells

Abbreviations used: '+++' = major application; '++' = some application; '+/–' = limited application; '–' = no application.

there is no such thing as a 'good universal vector'; all of the vectors that are currently available have both advantages and disadvantages. For example, one vector might be able to enter target cells very efficiently but once there invokes a strong immune response, resulting in that cell being killed by the immune system. Many factors must be taken into consideration when choosing a vector. The most import ones are: (1) the length of time that the transgene needs to be expressed, (2) the dividing state of the target cells, (3) the type of target cell, (4) the size of the transgene, (5) the potential for an immune response against the vector to be induced, and whether this is deleterious, (6) the ability to administer the vector more than once, (7) the ease of production of the vector, (8) the facilities available, (9) safety issues and (10) regulatory issues. Table 1 outlines the advantages, disadvantages and major differences of the gene delivery vectors that are currently in research and clinical use.

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Viral gene delivery

For millions of years, viruses have been transferring genes into all types of cells, including plant, animal and human cells. The experimental technique of viral gene delivery was developed from this natural ability, which offers many intrinsic advantages to scientists and clinicians: (1) specific cell-binding and entry properties, (2) efficient targeting of the transgene to the nucleus of the cell and (3) the ability to avoid intracellular degradation. The general principle involved in the development of most viral vector systems is that an intact wild-type virus is modified for safe use and effective gene transfer; for example, the specific genes that are involved in viral replication can be modified or deleted, thus rendering the new recombinant virus 'replication defective' and safer for use in gene therapy protocols (Fig. 4). Usually, the transgene that is to be delivered by the virus must be inserted into the viral genome, using molecular biological techniques; transgenes are often inserted into the space created by the removal of viral replication genes. In general, the more severely attenuated the viral vector is from its wild-type state (i.e. the greater the number of virulence-associated genes that have been removed), the safer the virus is for use in gene therapy protocols. The size of the transgene has to be matched to the potential space in the viral genome; if the new viral genome is too large, it cannot be packaged into an infectious particle. Because many of the viruses that are used as vectors lack replication genes and therefore cannot replicate in normal cells, the recombinant virus with its transgene must be grown up to higher titres in a packaging cell line. This is a cell line that contains all of the complementary genes that the virus requires to replicate (i.e. those that were previously removed). The recombinant viral particles can then be purified as live infectious virus from the packaging cell line and used to infect (transduce) cells or tissues in vivo or ex vivo.

Retroviral vectors

The Retroviridae is a large family of RNA viruses including spumavirus (foamy viruses), Moloney-murine-lentivirus-related viruses [e.g. Moloney murine leukaemia virus (MMLV) and the C family of human endogenous retroviruses (HERV-C)] and lentivirus [e.g. human immunodeficiency virus type 1 (HIV-1) and human immunodeficiency virus type 2 (HIV-2)]. The diameter of retroviral virions ranges from 80 nm to 130 nm, and their genomes consist of two identical positive-sense single-stranded RNA molecules, in the size range of 3.5–10 kb. The genomes are encased in a capsid, along with the integrase and reverse transcriptase enzymes (Ref. 45). For an excellent review of these vectors, see http://www.mc.vanderbilt.edu/gcrc/gene/retro.htm. Retroviral vectors are the most widely used viral vectors in clinical trials at present.

Retroviruses will only transduce cells that are actively undergoing mitosis, and are therefore well suited for gene transfer protocols to pluripotent (i.e. able to differentiate into several different final differentiated cell types) HSCs (Refs 46, 47, 48, 49, 50). Retroviral vectors give good gene expression over the long term and are technically easy to produce. However, low viral titres are yielded (generally up to 1×10^7 colony-forming units per ml) and, although very rare, contamination with helper virus is a possibility, which needs to be monitored.

MMLV

Most of the retroviral vectors that are being used for gene therapy applications are based on the MMLV, such as the LNSX series of vectors from Miller's laboratory (Ref. 51). Replication was prevented by removing the *gag*, *pol* and *env* gene regions. The *gag* region encodes the capsid proteins, the *pol* region encodes the reverse transcriptase and the integrase, and the *env* region encodes the proteins that are required for receptor recognition and envelope anchoring. The genome also includes long terminal repeats (LTRs) at either end, which play vital roles in initiating DNA synthesis and regulating transcription of the viral genes. For example, in the LNSX vector, the LTR drives the transcription of a neomycin-resistance marker gene (which is used to select transduced cells), and an internal Simian virus 40 (SV40) promoter drives the transcription of the transgene. The *gag*, *pol* and *env* gene products have to be supplied by a complementary packaging cell line, into which these genes have been transferred and are stably expressed. When a retroviral vector plasmid is introduced into a packaging cell line (such as pA317), viral RNA is produced, packaged into virions and secreted into the medium. Viral titres of up to 1×10^7 colony-forming units per ml can be obtained in this way. Because the resultant viral particles lack the *gag*, *pol* and *env* genes, each particle is only able to integrate itself into the

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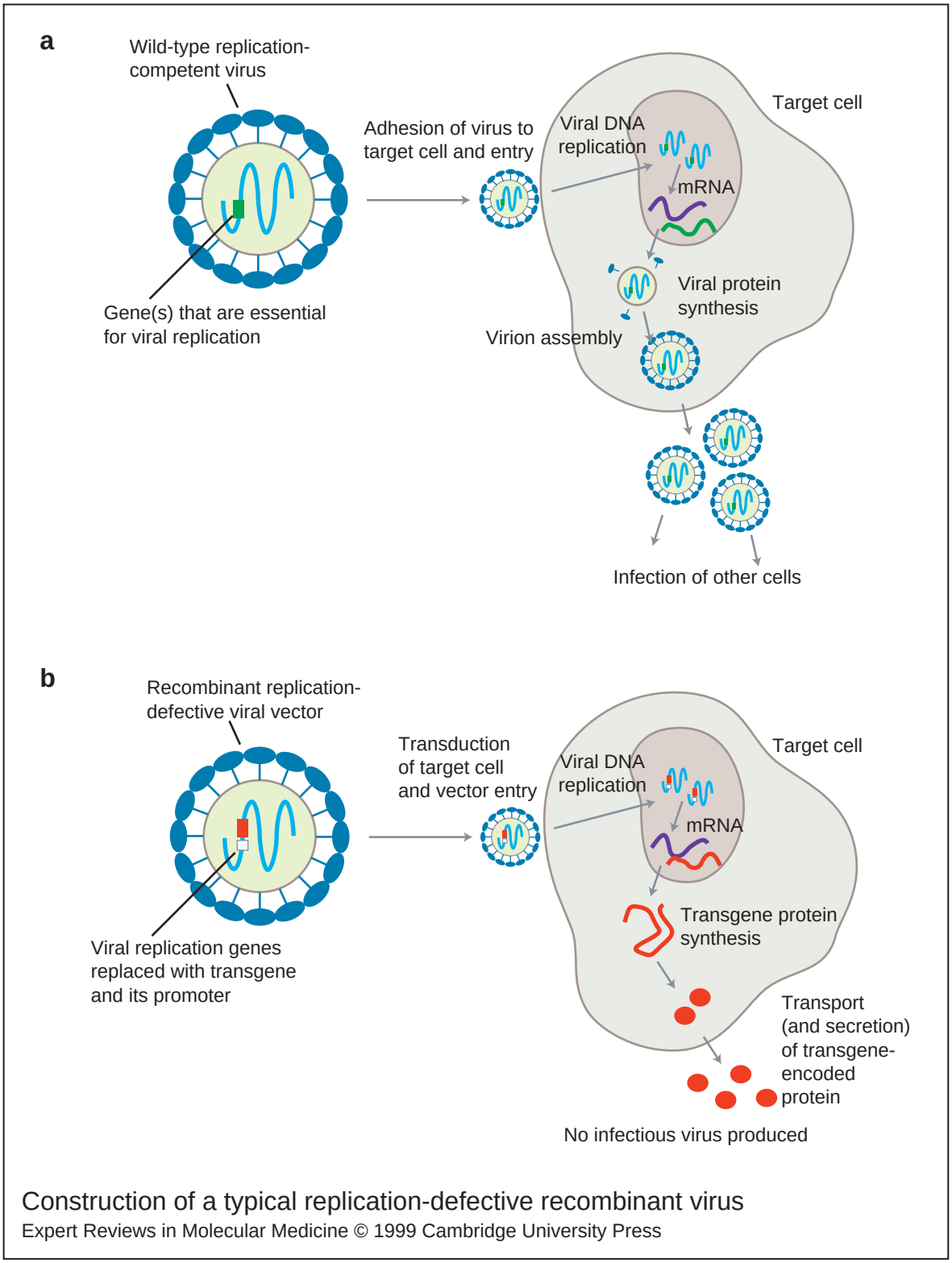

Construction of a typical replication-defective recombinant virus

Figure 4. Construction of a typical replication-defective recombinant virus *(see next page for legend)* **(fig004jfo).**

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Figure 4. Construction of a typical replication-defective recombinant virus. (a) On reaching the nucleus of the target cell, the nucleic acid of the wild-type virus initiates the transcription and translation of all of its viral gene products, resulting in new viral particle (virion) assembly in the cytoplasm. The new infectious virus particles are then released from the host cell, sometimes causing lysis of the cell and ultimately the death of that cell. (b) The nucleic acid of recombinant replication-defective viral vectors also reaches the nucleus of the target cell in the same manner as that of the wild-type viruses, but because essential viral replication genes have been removed, the virus cannot replicate. The transgene, together with its regulatory elements such as the promoter, is carried into the nucleus of the target cell and transcription and translation of the transgene-encoded protein follows this, resulting in biologically active protein that can potentially modulate the function of that cell and also other cells or organs if it is secreted **(fig004jfo).**

genome of the host cell and is unable to produce more viral particles. The transduced DNA sequences are integrated stably into the chromosomal DNA of the target cells and thus transferred to the progeny of transduced cells (Refs 52, 53).

Lentivirus

The most recently discovered members of the retrovirus family are the human immunodeficiency viruses (HIVs), which belong to a subclass of retroviruses known as lentiviruses. Gene therapy vectors that have been derived from HIVs have several advantages over MMLV retrovirus vectors. Lentivirus vectors are able to transduce non-dividing cells, as well as those that are actively dividing, thereby considerably broadening their usefulness as gene transfer vehicles. Because they integrate their genetic material into the genome of the host cell, lentivirus vectors have the potential to result in the long-term, stable gene expression of transgenes (Ref. 54). The prospects for using lentivirus vectors as gene therapy vectors for immunological purposes are very exciting because of their inherent tropism (affinity) for $CD4^+$ T cells, macrophages and HSCs; this makes them useful vehicles for gene therapeutic approaches to prevent or treat HIV infection and acquired immunodeficiency syndrome (AIDS). Genetic modifications, such as the introduction of vesticular stomatitis virus G protein into the lentiviral envelope, have widened the tropism of this vector. These vectors can now be used to target airway epithelial cells for the gene therapy of cystic fibrosis (Ref. 55), or potentially to target fully differentiated neurones for the gene therapy of Parkinson's disease (Ref. 56). Although this development is exciting, in practice, the use of an HIV-based vector for therapies targeting diseases other than HIV might be extremely difficult to introduce clinically, until it has been established that such a vector is safe to use.

Adenovirus

Adenoviruses are non-enveloped, icosahedral, double-stranded DNA viruses with a capsid diameter of 70–100 nm, and comprise 252 capsomeres (240 hexons and 12 pentons). They are not incorporated into the genome of the target cell (non-integrating) but remain as an extrachromosomal entity in the nucleus of the host cell. Replication-defective recombinant adenoviruses are the second most commonly used viral vectors in clinical trials today. Adenoviruses commonly infect humans, and were first isolated in 1953 by Rowe and colleagues (Ref. 57) from US army recruits who had acute respiratory symptoms. Primary (untransformed) cell cultures derived from the adenoids of these recruits were established, and the cells were seen to degenerate spontaneously in culture owing to the presence of the virus. To date, 47 serotypes of adenovirus have been characterised, and have been associated with a variety of symptoms ranging from a mild cold to acute febrile pharyngitis. Ad2 and Ad5 have been subjected to the most studies and are the main serotypes in use for gene therapy applications; they are not associated with severe disease, causing mainly only mild cold symptoms (Ref. 58). The structure and replication cycle of adenoviruses have been recently reviewed by Lee (Ref. 58) and Shenk (Ref. 59).

The 36-kb genome of adenoviruses can be divided into two main regions, early (E) and late (L), according to the time at which their genes are expressed during the replication cycle of the virus. There are four regions of early genes, which are termed E1, E2, E3 and E4, and one region of late genes, which comprises the five coding units termed L1, L2, L3, L4 and L5 (Fig. 5).

The E1 region of adenoviruses has been subdivided into E1A and E1B. The E1A gene product is a viral transcription unit, which activates the expression of other adenoviral transcription units by binding to viral promoters (Ref. 60). The E1B region codes for a 55-kD protein

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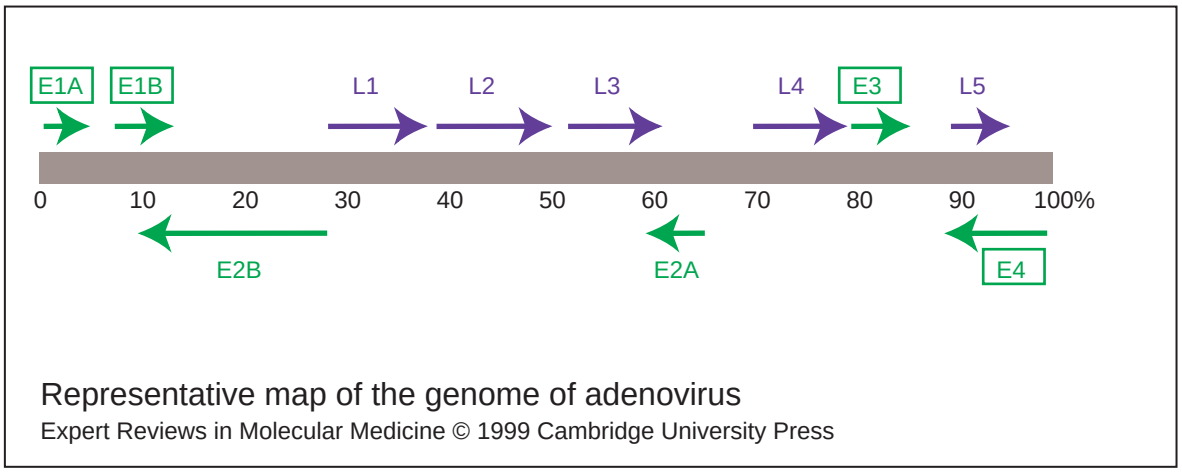

Figure 5. Representative map of the genome of adenovirus. The genes in boxes are those that can be removed during the production of a replication-defective virus for gene therapy protocols. The E1A gene (which encodes the initial viral transcription unit) must be removed to prevent the recombinant virus from replicating. Other genes can be deleted to make more space for the insertion of larger transgenes and, in the case of E4-deletion recombinant adenoviruses, the immunogenicity has been reported to be significantly reduced **(fig005jfo).**

that interacts with the cellular p53 tumour-suppressor protein. p53 regulates the progression of the host cells' cycle from the G_1 phase to the S phase; this phase is optimal for viral replication. E1B also binds to viral E4 proteins as well as p53, which together act to shut down host protein synthesis. The E2 region codes for viral DNA polymerase and the adenovirus single-stranded DNA-binding protein. The E3 region is not required by the adenovirus for in vitro replication but does, however, offer the virus some protection against host defence mechanisms by inhibiting infected cells being killed by CTLs or TNF-α. The E4 region codes for proteins that are known to be involved in (1) the regulation of viral and cellular protein expression, (2) viral DNA replication and (3) the switching off of the synthesis of host proteins (Ref. 61). The late genes (L1–L5) are expressed at the onset of viral DNA replication, and code for structural polypeptides that are required for virion assembly. Disruption of the cellular cytoskeleton and membrane, owing to the accumulation of newly synthesised viral particles, results in the collapse of the cell and release of the virus.

The E1 region is essential for viral replication; thus, those adenoviruses that artificially lack the E1 region are considered replication defective. In a replication-defective adenovirus, the E1 region can be replaced with the transgene that is to be expressed. Further removal of genetic material from the vector, such as the deletion of the E3 region and even the E4 region (Ref. 62), has been carried out to allow larger genes to be inserted in their place and also to reduce the immunogenicity of the virus; such recombinant viruses are usually referred to as 'gutless'.

For gene therapy in almost all cell types both in vivo and ex vivo, the transduction efficiency of adenoviruses is high compared with that of other viral vectors. For transplantation, adenovirus has the distinct advantage that it can bind to the surface of its target cells at low temperatures (e.g. 4°C). This allows pre-transplant gene transfer to be carried out during the process of cold preservation (Ref. 63). Because of the structural stability of the capsid polypeptides of adenovirus, viral particles can be purified and concentrated to a very high titre of 1×10^{13} plaque-forming units (pfu) per ml, although a titre of 1×10^{10} pfu per ml is more usual. Retroviral titres are much lower ($\sim 1 \times 10^{7}$ pfu per ml) because their capsid is structurally more unstable and cannot be purified and concentrated on a caesium chloride gradient. Another advantage of adenoviruses for use in gene therapy is that the adenovirus genome does not integrate into the human genome, but remains in the nucleus of target cells as a non-replicating extrachromosomal entity; however, this means that there is a very low chance of activating human oncogenes or interrupting a human tumour-suppressor gene.

A major disadvantage of using adenovirus as a vector in vivo is the CTL response that is

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induced against capsid-derived peptides; this response can cause the destruction of vector-transduced cells and also leads to local tissue damage and inflammation (Refs 64, 65). Host cells presenting peptides of adenovirus-encoded foreign transgene products have also been shown to target the host cell for CTL-mediated destruction (Ref. 66).

Because adenovirus is a commonly occurring virus, most humans are pre-primed to at least one specific serotype. Using the same serotype in a gene therapy context will almost certainly result in a rapid and vigorous memory immune response, such that high levels of anti-adenovirus antibody become measurable in the sera of the recipients within days of administration of the adenovirus vector. It might help to screen recipients of such vectors to detect which serotypes they have previously encountered, and then use a different serotype as the vector. However, this approach would require the availability of a very large panel of recombinant vectors with different serotypes. Another potential problem is the strong secondary immune response that would be induced by the re-administration of a vector of the same serotype.

The period of expression of an adenovirus-encoded transgene is also relatively short. Expression is reported to last at a 'reasonable' level for ~14 days in vivo; however, manipulation of the immune response has resulted in expression for longer periods (Refs 67, 68, 69, 70, 71). This short expression time is mainly due to the expression of viral polypeptides inducing a CTL response, and to some extent the transgene itself, especially if it is not normally expressed in that individual (i.e. it is foreign). Because the adenoviral genome does not integrate into the genome of the target cell, only one of the daughter cells (if the target cells are dividing) will contain the transgene, therefore halving the total number of cells that contains the transgene.

Adenoviral gene delivery is ideally suited to those situations that require only a one-off delivery of a transgene, for example in growth factor therapy, in which transient, as opposed to long-term, expression of growth factor is required. In protocols that are aimed at inducing transplantation tolerance, the delivery of an adenoviral vector to a recipient before transplantation should be sufficient to induce a regulatory population of T lymphocytes that will impart long-term immunological tolerance to that recipient.

Adeno-associated virus

The adeno-associated virus (AAV) vectors offer many of the same advantages as adenovirus vectors, including a wide host-cell range and, in some situations, a relatively high transduction efficiency (Ref. 72). In addition, unlike adenovirus, which can cause a high degree of cell death (cytopathogenicity), AAV causes little damage in target cells. AAV also stably, and at specific sites, integrates into the host-cell genome (in chromosome 19 of humans; Ref. 73), which has the beneficial effect of a longer-lasting transgene expression. However, there is evidence to suggest that AAVs are significantly less efficient than retroviral vectors at transducing primary-cell cultures (Refs 56, 74). In primary-cell transductions, most of the AAV vector DNA does not integrate into the host genome but remains extrachromosomal, and this inefficiency might limit its use for in vivo application.

Herpes simplex virus

Herpes simplex virus (HSV) vectors are being developed for several applications, including for use in gene transfer protocols that target neuronal tissue, such as the treatment of Parkinson's disease, malignant gliomas (a type of brain tumour) and cerebral ischaemia (starving of brain tissue from essential nutrients). HSV is maintained as an extrachromosomal DNA element in the nucleus of host cells, and has an excellent ability to establish long-lived asymptomatic infections in the sensory neurones of the peripheral nervous system and in some central nervous tissue. This provides the opportunity for long-term gene expression in neuronal target tissue. HSV vectors also have a wide host range, can accept large gene inserts, and have had multiple deletions of immediate-early (IE) genes that are essential for replication to make the vectors less cytotoxic to target cells (Ref. 75) and reduce safety concerns. The main problem associated with the use of HSV as a gene therapy vector at present is concern about its safety for clinical use, owing to reports of the wild-type virus replicating lytically in the human brain and resulting in potentially serious encephalitis (Ref. 76).

Vaccinia virus

Although vaccinia virus vectors are not currently

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in use for transplantation studies, they are in development for cancer gene therapy (Ref. 77). Vaccinia virus has been used worldwide to eradicate smallpox and has been shown to be a safe live vaccine. Vaccinia virus vectors do not integrate into the genome of the host cell; they can, however, accommodate large transgenes and are extremely immunogenic. Vaccinia virus can be used to immunise patients against tumour antigens by cloning (inserting) into its large genome tumour antigen genes or genes encoding proteins that enhance the immune response (e.g. cytokine genes; Ref. 78). Most transgenes are expressed at a high level in vivo, eliciting a specific immune response against the tumour antigen, which otherwise would not have occurred at levels that are sufficient to kill cancer cells. If required, more than one gene can be cloned into the vector, owing to its large capacity.

Non-viral gene delivery

Non-essential genes can be removed from viral vectors to make more room for transgenes, to reduce inflammatory responses or to increase their safety; this involves the virus being simplified, sometimes to an extreme. What remains can be an artificial 'vector shell', which has been designed to allow the gene of interest to be expressed at high levels, in a highly regulated specific manner and for a controlled period of time (either short term or long term). Another approach to achieve the same result is to produce a system that can simply introduce genetic material to the nucleus of cells. This has been the focus of intensive research over the past few years, and has resulted in the development of several non-viral vector systems.

Liposomes

In their most basic form, liposomes consist of two lipid species, a cationic amphiphile and a neutral phospholipid (typically, dioleoyl-phosphatidylethanolamine; Ref. 79) both of which are commercially available. Liposomes bind to and condense DNA spontaneously to form complexes that have a high affinity for the plasma membranes of cells; this results in the uptake of liposomes to the cytoplasm by the process of endocytosis (Ref. 80). Many adaptations of this basic protocol have been tested and have resulted in varying levels of gene expression.

Fusigenic virosomes

More recently, some of the advantages of viral delivery vectors have been combined with the safety and 'simplicity' of the liposome to produce fusigenic virosomes (Ref. 81). Virosomes have been engineered by complexing the membrane fusion proteins of haemagglutinating virus of Japan (HVJ, which is also known as Sendai virus) with either liposomes that already encapsulate plasmid DNA or oligodeoxynucleotides for antisense applications. The inherent ability of the viral proteins in virosomes to cause fusion with cell membranes means that these hybrid vectors can be very efficient at introducing their nucleic acid to the target cell, resulting in good gene expression. Each viral vector has a limit on the size of transgene that can be incorporated into its genome; no such limit exists for virosome or liposome technology. Genes of up to 100 kilobase pairs have been delivered by fusigenic virosomes to cells both ex vivo and in vivo (Ref. 81).

DNA–ligand conjugates

DNA–ligand conjugates have two main components: a DNA-binding domain and a ligand for cell-surface receptors. The transgene can therefore be guided specifically to the target cell, where it is internalised via receptor-mediated endocytosis. Once the DNA–ligand complex is in the endocytic pathway, the conjugate is likely to be destroyed when the endosome fuses with a lysosome. Curiel and colleagues (Ref. 82) have developed a method to avoid this, which incorporates an adenovirus-derived domain into the cell-surface receptor part of the ligand. The conjugates then have the same specificity as adenoviruses, binding to a wide host-cell range; they also possess an adenovirus characteristic that allows the conjugate to leave the endosome and enter the cytoplasm (by a process known as endosomolysis) before the endosome is destroyed by a lysosome.

Naked DNA

One of the simplest ideas for non-viral gene delivery techniques is the use of purified DNA in the form of plasmids. This approach is being used for DNA vaccines, among other protocols, and has been tried in many situations for gene therapy. However, despite the simplicity of this approach, studies have revealed that the transfection efficiency is very low and will therefore limit its application. The injection of plasmid DNA

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encoding an MHC class I antigen derived from a donor rat strain into the thymus of a recipient rat strain, in conjunction with a dose of anti-lymphocyte serum, induced donor-specific tolerance to subsequently transplanted liver grafts (Ref. 38). Donor DNA was detected in the thymus up to 4 days after injection and in the spleen 7 days after injection.

Ballistic gene delivery

This physical method involves the use of microcarriers (usually gold particles or another inert substance), which are coated with DNA and 'fired' at high velocity using an explosive or gas-powered ballistic device called a gene gun. Once the particles are inside the target cell, the DNA is slowly released from the microcarriers, and can result in useful levels of gene transcription and translation. This technique has been widely used experimentally, but its clinical use is restricted to exposable surfaces because the fired particles do not penetrate deeply into tissues. Possible clinical applications are gene transfer to the bladder urothelium, the cornea and skin epithelial cells.

$CaPO_4$ transfection

$CaPO_4$ transfection is a chemical method that has been successfully used by molecular biologists for many years to introduce transgenes into cells in vitro with a relatively good efficiency (10%). Although it is an integral part of protocols to produce many viral vectors for subsequent experimental and clinical use, this method is not suitable for in vivo application.

Promoter attenuation

It is essential for the success of gene therapy vectors that an appropriate promoter is linked to the gene of interest. A promoter is a regulatory sequence of DNA that is located upstream of a gene and to which proteins (transcription factors and RNA polymerase) bind, to initiate the synthesis of mRNA and, subsequently, protein. Most experimental expression vectors and gene therapy vectors use promoter elements that have been derived from pathogenic viruses, because of the high level of constitutive (constant) gene expression that they will induce.

Various gene transfer studies have used promoter and enhancer elements from cytomegalovirus (CMV), Rous sarcoma virus (RSV) and SV40, and have reported encouraging success; however, the levels of expression do depend on many factors including the vector used, its route of administration and the type of cell transduced.

One of the common problems encountered by investigators is that transgenes are expressed only at a low level and only transiently. Although the molecular mechanisms that are responsible for this poor expression are poorly defined, it could be mainly due to attenuation of the promoter.

Considering the importance of promoter attenuation to the field of gene therapy, remarkably few studies have been carried out to look at this problem directly. It has been shown in experimental systems that the application of adenoviral vectors in vivo induces cytokine production through specific or non-specific immune responses. These cytokines can then act on the adenovirus-infected cell that is carrying the transgene, and initiate cytokine-mediated cellular signals, which will modulate transgene expression (Ref. 83). Qin and colleagues showed that transgene expression, controlled by many viral promoters, was inhibited by IFN-γ and TNF-α, and that both of these cytokines had synergistic effects. Promoters derived from CMV and RSV were found to be the most sensitive to this cytokine treatment.

In a different mouse model that also used recombinant adenovirus, Harms and Splitter (Ref. 84) demonstrated that the administration of a neutralising anti-IFN-γ monoclonal antibody in vivo resulted in enhanced expression of the transgene.

At the molecular level, promoters derived from SV40, CMV and RSV all have an interferon response sequence. Nuclear factors, produced as a result of IFN-γ interacting at the cell surface, bind to elements in these viral promoters, which inhibits transcription of the transgene (Ref. 85). Strong constitutive viral promoters have been used (and work successfully) in vitro in mammalian expression vectors where there are no inflammatory cytokines present. The use of these strong viral promoters was naturally assumed to be ideal for the development of clinical gene therapy protocols. However, low levels of transgene expression are usually seen, and this is thought to result from the design of the vector as a whole, as opposed to a particular component of the vector. Common to most gene therapy expression systems are the viral promoter and enhancer elements. Viruses and isolated viral promoters that are used in both in vitro expression

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vectors and in vivo gene therapy vectors can be adversely affected by cytokines produced by infected cells. It therefore makes sense to choose promoters for gene transfer that can be upregulated by factors in the environment that will be present where the vector is delivered and when and where transgene expression is desired. For example, the MHC class I promoter might be a better choice as a promoter for immunoregulatory gene therapy applications because inflammatory cytokines, such as IFN-γ, actually act on this promoter to enhance transcription.

Clinical implications/applications

To date, a total of 3134 patients have been enrolled in 373 human gene transfer protocols worldwide (see http://www.wiley.co.uk/wileychi/genmed). Of these, 234 are for cancer therapy, and 53 for monogenic diseases, such as cystic fibrosis and ADA deficiency. Most of the gene therapy studies in the transplantation setting are still in the relatively early stages of development. That is to say that they have only been assessed in animal models or have used human tissue in vitro. It has been demonstrated, for example, that vIL-10 can be expressed in human islets in vitro (Ref. 86). This success might lead to ex vivo gene transfer of grafted tissues and organs before transplantation, as a means of producing local immunosuppression at the site of the graft.

Research in progress and outstanding research questions

Most of the studies discussed in this review have been published recently; moreover, work in many of these areas is still being actively pursued. Although the ultimate aim in transplantation, the induction of donor-specific tolerance in clinical patients, is still a long way off, significant hope for the future is offered by advances in the induction of tolerance in animal models of allogeneic and xenogeneic transplantation. The development of pre-transplant microchimerism in transplant recipients, by the infusion of syngeneic bone marrow transduced with donor MHC class I or MHC class II genes, is an exciting and relevant approach, and advances in HSC isolation should improve the problem of GvHD.

With regards to gene therapy, the main focus of research is the development of new gene transfer vectors that are more efficient and safer, in an effort to overcome the major hurdles that are currently associated with clinical gene therapy. As discussed earlier, these new vectors are hybrids, incorporating the qualities of different vectors, such as targeting capability, strength and duration of transgene expression. The results of these studies are eagerly anticipated so that they can be applied in clinical gene therapy protocols.

Summary

On reviewing the literature on gene therapy vectors that are currently under development, it is clear that the previously distinct boundaries between viral and non-viral vectors are becoming increasingly blurred. The next generation of vectors is likely to offer highly efficient, targeted and long-lasting gene transfer; they will need to be safe and raise none of the concerns that have been associated with the first few uses of viral gene delivery in clinical trials. New and more advanced vectors will continue to be designed to achieve reliable and efficient gene transfer, particularly with regard to modulation of the immune system.

In the context of transplantation, gene transfer will be invaluable in seeking means to induce donor-specific immune unresponsiveness and prevent the longer-term effects of chronic graft rejection.

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Further reading, resources and contacts

All the Virology on the WWW: Viral Vectors and Gene Therapy is an excellent starting point, providing good links to detailed information on all aspects of viral gene therapy vectors.
http://www.tulane.edu/~dmsander/garryfavwebgenether.html

The Journal of Gene Medicine is a new print and electronic journal resource, which focuses on the clinical aspects of gene therapy; it also includes more basic biological research pertaining to gene transfer vectors.
http://www.wiley.co.uk/wileychi/genmed

The Introduction to Gene Therapy website is an excellent educational resource in a tutorial format, providing useful information on all aspects of gene transfer technology.
http://www.mc.vanderbilt.edu/gcrc/gene/index.html

Features associated with this article

Table
Table 1. A comparison of vectors in use for clinical gene transfer (tab001jfo).

Schematic figures
Figure 1. Transplantation: typical treatment to prevent the rejection of grafted cells (fig001jfo).
Figure 2. Transplantation: gene therapy approaches to prevent rejection of grafted cells (fig002jfo).
Figure 3. Gene therapy: factors in the design of vectors and clinical regimens (fig003jfo).
Figure 4. Construction of a typical replication-defective recombinant virus (fig004jfo).
Figure 5. Representative map of the genome of adenovirus (fig005jfo).

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