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Gene Therapy: Development of Immunostimulatory Treatments for Cancer

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Introduction

Gene therapy encompasses a broad range of strategies, which aim to treat human diseases by the transfer of genetic information. The idea was initially conceived for the treatment of inherited, monogenic disorders such as cystic fibrosis, in which the disease phenotype is due to the lack of a properly functional gene product in certain tissues. In principle, transfer of a functional gene encoding the relevant, wild-type protein could restore the affected cells to normality. It was soon realized that there are many other situations in which the introduction of specific genetic modifications to target cells could confer properties which could be of benefit in many other clinical situations, including cardiovascular, neurological and infectious diseases, and cancer.

There are numerous approaches to cancer gene therapy; the majority are designed to treat patients who have presented with cancer, rather than focusing on pre-emptive treatment of patients with known inherited predisposition to cancer. Approaches include interference with oncogene action within tumour cells; restoration of tumour-suppressor gene function; and expression of enzymes that enable the tumour cells to activate non-toxic prodrugs to cytotoxic species. This review focuses exclusively on cancer gene therapy strategies that are intended to induce immune responses against the malignant cells. The approach is attractive, particularly because the disseminated nature of many cancers at later stages of the disease, and often at presentation, poses

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Abbreviations: TCR, T cell receptor; CTL, cytotoxic T lymphocyte; Th, helper T lymphocyte; MHC, major histocompatibility complex; HLA, human leukocyte antigen; APC, antigen presenting cell; TAA, tumour associated antigen; HPV, human papillomavirus; PCR, polymerase chain reaction; SEREX, serological identification of antigens by recombinant expression cloning; IL-2, interleukin-2; DTH, delayed type hypersensitivity; GM-CSF, granulocyte-macrophage colony stimulating factor; DC, dendritic cell; NK cells, natural killer cells; CHO, Chinese hamster ovary; IFN-γ, interferon-γ; LAK, lymphokine-activated killer; PMA, phorbol myristate acetate; 4-1BBL, 4-1BB ligand; SEA, staphylococcal enterotoxin A; CEA, carcino-embryonic antigen; MUC-1, mucin-1; TIL, tumour-infiltrating lymphocyte; TNF, tumour necrosis factor; FBP, folate binding protein; AAV, adeno-associated virus; HSV, herpes simplex virus; DISC, disabled infectious single cycle; TGF-β, transforming growth factor-β.

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a daunting challenge to therapies which rely upon gene transfer, if not to every cancer cell, at least to a reasonable proportion of cancer cells within each metastasis. The immune system is adapted to survey the entire body and so should be better able to locate remote metastases than current gene therapy vectors. For other reviews of immunotherapeutic cancer gene therapy, see Verbick and Josji (1995), Colombo and Forni (1996), Searle and Young (1996), Vile and Chong (1996), Tuting *et al.* (1997), and Pawelec *et al.* (1999).

We begin this review with a brief overview of key aspects of the immune system relevant to cancer therapy. This is followed by a review of some of the major immunotherapeutic strategies, with selected examples of illustrative studies in animal models and initial human clinical trials. All gene therapy depends upon the ability to achieve efficient gene transfer to the target cells; we also, therefore, briefly review the most important types of viral vectors and non-viral gene transfer methods used for this purpose, and their suitability for immunotherapy.

Cancer and the immune system

CELL MEDIATED IMMUNITY

The immune system is complex and could fill many reviews; for further background information the reader should refer to general reviews and text books such as Roitt *et al.* (1996) and Alam (1998); more detailed reviews of specific aspects include Lehner and Cresswell (1996), Watts (1997), Dutton *et al.* (1998), Lindauer *et al.* (1998), Whitton (1998), Garcia *et al.* (1999), Lanzavecchia *et al.* (1999), and Sebzda *et al.* (1999).

Immune responses are mediated by the cells of the immune system and the soluble molecules they secrete. The B and T lymphocytes are the central players, but other leukocytes also participate in immune responses by signalling to lymphocytes and responding to cytokines released by T lymphocytes and macrophages. The B lymphocytes are responsible for the generation of antibodies; these are important in the fight against infection, particularly in the neutralization and destruction of extracellular infectious agents. However, it is the cell-mediated immunity of T cells which is considered to have greater potential in combating cancer. T lymphocytes use their T cell receptor (TCR) to recognize specific 'foreign' antigenic determinants, which are displayed at the surface of a target cell. The two chains of the TCR are encoded by a multigene family and generate vast diversity during T cell maturation by the combinatorial joining of different segments. Any one T cell displays TCRs of a very precise specificity, but the entire T cell population contains specificities for a vast array of different antigens, giving the body the ability to react to a wide variety of foreign antigens, and so eliminate pathogen-infected cells. There are several distinct types of T lymphocytes; cytotoxic T lymphocytes (CTLs) display the CD8 marker and have the ability to kill cells displaying their cognate antigen. Other helper T lymphocytes (Ths) display the CD4 marker; the Th1 subset helps support the generation of CTLs, while the Th2 subset provides similar help to B cells. It is the aspiration of the cancer immunotherapist to harness these components and make the immune system as effective in eradicating malignant cells as it can be in elimination of infectious disease.

THE BASICS OF ANTIGEN PRESENTATION BY MHC

Antigens are not presented as complete proteins to T cells, but are processed intracellularly through the proteosome complex before presentation as short peptides. These peptides become anchored in the binding groove of specific molecules present on the host cell, encoded by a series of genes known as the major histocompatibility complex (MHC; human MHC is also known as the human leukocyte antigen, or HLA, system). There are several HLA loci, each of which is highly polymorphic; hence each cell expresses several different MHC molecules, the combination of which varies between individuals. The various MHC molecules can each bind different subsets of the many various possible peptides generated during processing, and so the cells of an individual present many different antigenic peptides to be scanned by T cells. Nevertheless, these are only a subset of all the peptides that could be generated from the proteins in the cell, and cells from individuals with different HLA haplotypes will display a different selection of peptides.

Cells that present antigen to T cells may be either specialized (or 'professional') antigen presenting cells (APCs), such as dendritic cells, or other host cells displaying foreign antigens (eg from a pathogen) which thereby become legitimate targets for CTLs. T cells scan the surface of APCs for specific peptides bound to the appropriate MHC molecule; specific engagement of the TCR can then trigger an appropriate response, such as killing of a pathogen-infected target cell (see *Figure 18.1* for a simplified representation of the cellular interactions of T cells).

Two classes of MHC molecules are involved in presenting antigenic peptides to T cells. MHC class I molecules on most cells display endogenous antigens from the host cell allowing $CD8^+$ T cells to monitor the repertoire of proteins expressed by that cell. MHC class II molecules, whose expression is largely restricted to professional APCs, display peptides derived from exogenous antigen taken up and processed by the APC, allowing activation of $CD4^+$ Th cells specific for antigens produced at a remote location. Uniquely, dendritic cells can also display exogenous antigens via class I MHC to assist in the initial activation of CTLs. Whilst each TCR recognizes a specific peptide lodged in the peptide binding groove of the MHC molecule (an interaction which is stabilized by interaction of the CD4 or CD8 molecules with the MHC), other pairs of molecules are known to play additional important roles in T cell/APC interaction. Three such important accessory molecules are CD2, LFA-1 and CD28 present on T cells, which interact with LFA3, ICAM-1 and B7 respectively on APCs. Two homologues of the B7 molecule exist, B7-1 (CD80) and B7-2 (CD86), and these are important co-stimulatory molecules that provide the T cell with a second signal at the time of initial antigen recognition. However, CD80 and CD86 also interact with another receptor known as CTLA-4, expression of which is induced following activation of T lymphocytes. Signalling through CTLA-4 appears to be inhibitory, and plays a role in limiting the duration or magnitude of an immune response. The receptor 4-1BB (CD137) expressed on activated T cells, and its ligand 4-1BBL also appear to have co-stimulatory functions. The absence of appropriate co-stimulation when naïve T cells encounter antigen can result in a form of immunological tolerance known as anergy, in which the ability to respond to the cognate antigen at a later time is profoundly inhibited.

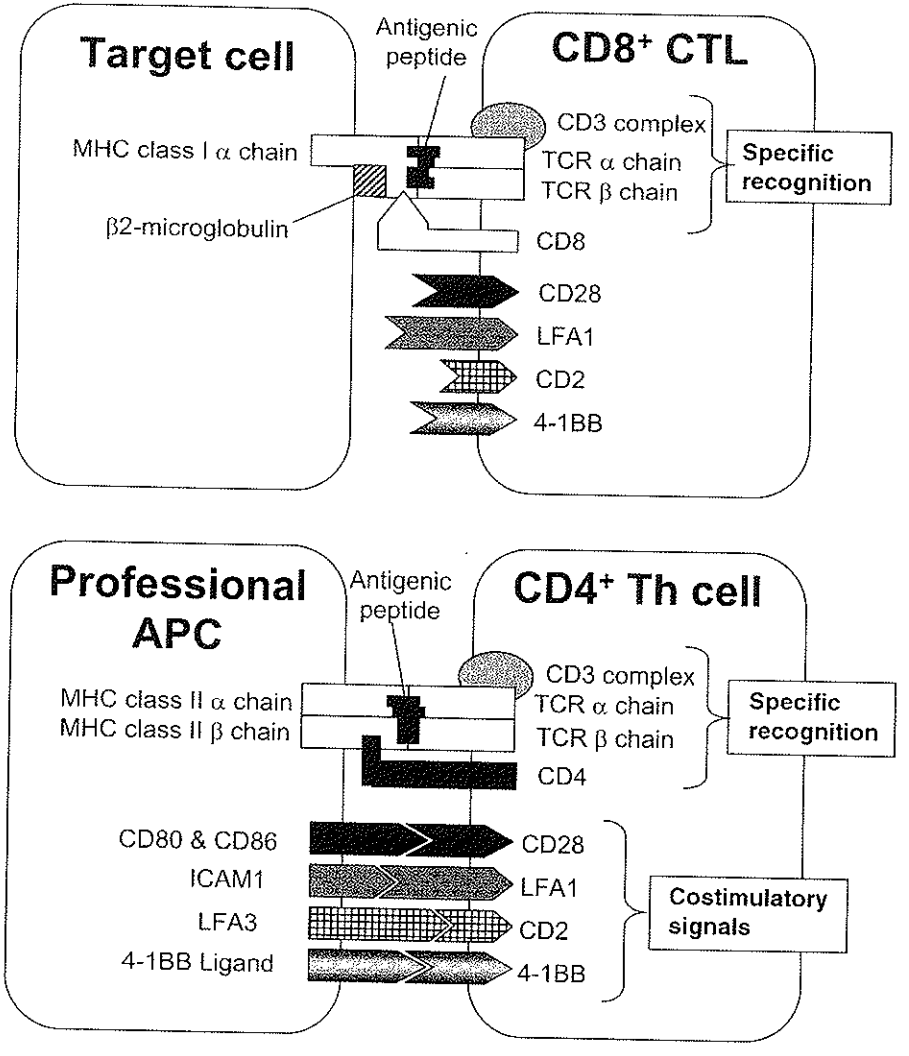

Figure 18.1. Diagram illustrating some of the important proteins involved in cellular interactions of T lymphocytes. The upper part of the figure illustrates a $CD8^+$, cytotoxic T lymphocyte (CTL) interacting with a MHC class I-expressing target cell. MHC class I is a dimer composed of the transmembrane α chain and an extracellular chain, β2-microglobulin. Peptide antigens derived from intracellular proteins are displayed on the MHC, where they may be recognized by the T cell receptor (TCR, made up of α and β chains). CD8 is an accessory molecule which helps to stabilize the interaction by binding to a conserved region of MHC class I. The CD3 complex is made up of several protein chains which interact with the TCR and through which the TCR can signal to the cell that specific binding has occurred. The specific recognition event through the TCR complex can trigger activated CTLs to kill the target cell. CD28, LFA1, CD2 and 4-1BB are non-polymorphic receptors on T cells for receiving co-stimulatory signals, required for activation of naïve or resting T cells. The lower part of the figure illustrates a $CD4^+$ helper T lymphocyte (Th) interacting with a professional antigen-presenting cell (APC). The CD4 molecule stabilizes specific interactions between the TCR and a recognized peptide displayed by the MHC class II molecule, which is made up of α and β chains. Professional APCs can express high levels of the co-stimulatory proteins, which interact with their receptors on Th cells (or CTLs).

TUMOUR ANTIGENS

Many gene therapy approaches to cancer aim to either exploit or increase the existing immunogenicity of the tumour. Gene amplification, or mutation, and chromosome translocation in tumours can lead to expression of altered or amplified proteins which can function as tumour specific, or tumour associated antigens (TAAs). Tumours of viral aetiology often continue to express viral proteins that can be targeted as potential tumour antigens, for example the human papillomavirus (HPV) proteins in cervical carcinoma.

Tumour antigens fall into a number of categories that include antigens shared by many tumours, differentiation antigens, antigens specific to individual tumours, and ubiquitous antigens. Amongst the shared antigens, three families of genes (MAGE, BAGE, GAGE) have been identified that appear to encode highly specific TAAs (van der Bruggen *et al.*, 1991; De Plaen *et al.*, 1994; Boel *et al.*, 1995; Van den Eynde *et al.*, 1995). These antigens were initially identified by analysing the specificity of CTL clones obtained by stimulating lymphocytes with autologous melanoma cell lines. Besides being widely expressed in melanoma, they are frequently present in a wide variety of other tumours, including sarcoma, lung, and bladder carcinoma. There is particular interest in the therapeutic potential of this group as more than 60% of Caucasian melanoma patients bear one of these TAAs on their cancer. Although expression also occurs in the testis, initial studies in mice (Haas *et al.*, 1998) suggest that these antigens could be used for immunization without harmful immunological side effects.

Also, among the shared tumour antigens are epitopes of mucin, a surface protein composed of multiple tandem repeats of 20 amino acids (Barnd *et al.*, 1988; Lancaster *et al.*, 1990; Jerome *et al.*, 1993; Gendler *et al.*, 1998) expressed on breast, ovarian and pancreatic carcinomas. Under-glycosylated mucin antigens appear to be very specific for tumour cells, and they have a potential use as vaccines since the glycosylation status of these multiple repeats permits direct CTL recognition without the normal constraints of HLA restriction (Barnd *et al.*, 1989; Ioannides *et al.*, 1993).

Both normal melanocytes and melanoma cells have previously been used to generate autologous CTL specific for differentiation antigens, such as tyrosinase, Melan-A/MART-1, gp100 and gp75, that are expressed on both cell types (Brichard *et al.*, 1993; Bakker *et al.*, 1994; Coulie *et al.*, 1994; Cox *et al.*, 1994; Kawakami *et al.*, 1994; Wang *et al.*, 1995). Most melanoma patients have differentiation antigen-specific CTL precursors that can be readily re-stimulated *in vitro* with autologous tumour cells (Sensi *et al.*, 1995; Brichard *et al.*, 1996). However, the fact that many of these patients have progressive disease suggests these CTLs are not working effectively *in vivo*, although the mechanisms behind such suppression/tolerance are still unclear. Using new technology (involving fluorescence-labelled tetramers of specific MHC-peptide complexes) to identify T cells of defined specificity directly, a recent study identified $CD8^+$ T cells with TCR specificity for defined TAAs at frequencies of up to 2.2% of $CD8^+$ cells from melanoma patients, and yet these cells showed a profile of other markers atypical of CTLs, and appeared incapable of lysing cells presenting the cognate antigen (Lee *et al.*, 1999). This finding highlights the fact that it is not sufficient to detect or generate TAA-specific $CD8^+$ lymphocytes in significant numbers, but that strategies are required that can prevent, or overcome, such unresponsiveness or anergy.

Also among the differentiation antigens are the oncofoetal antigens, which are expressed during foetal development but are absent, or only present at very low levels, in normal adult life. Tumour development is often associated with re-expression of oncofoetal tumour antigens such as alpha-foetoprotein (expressed by liver tumour cells) and carcino-embryonic antigen (in epithelial tumours).

Autologous CTLs in melanoma have also been found with specificity for uniquely tumour-specific antigens, generated, for example, by random mutations during tumour development. In terms of potential therapy, this type of antigen has the benefit of being absolutely specific for tumour cells. In general, it would appear too technologically complex, time-consuming, and labour-intensive to identify the unique antigens of a patient's tumour and develop these into individualized cancer vaccines. However, in the special case of B cell lineage tumours in which clonally unique antibody gene rearrangements provide predictably unique tumour antigens, it is possible to isolate these rapidly by polymerase chain reaction (PCR) to generate individualized protein or gene-based tumour vaccines which may prove clinically useful (Stevenson *et al.*, 1995; Spellerberg *et al.*, 1997; Whittington *et al.*, 1998).

Culture of lymphocytes with autologous tumour cells can sometimes generate CTLs with specificity for ubiquitously expressed genes (Robbins *et al.*, 1996). However, only those which are often over-expressed in tumours, such as the product of the HER-2/neu proto-oncogene (Ioannides *et al.*, 1993; Fisk *et al.*, 1994), could be considered for candidate vaccines.

Until a few years ago, the majority of tumour antigens were identified by analysis of T cell repertoire against tumours, using either autologous T cell clones or antigenic peptides eluted from MHC molecules on tumour cells. However, the development of a technique involving the serological identification of antigens by recombinant expression cloning (SEREX) has led to a huge increase in the number of new tumour antigens being identified (Sahin *et al.*, 1995; Tureci *et al.*, 1996). Although SEREX identifies immunogenic tumour proteins on the basis of their reaction with antibodies in autologous human sera, the technique has detected antigens (MAGE-1, tyrosinase and NY-ESO-1) that are also recognized by CTLs of cancer patients.

To summarize, it now seems that, in many cases at least, there is not a shortage of tumour antigens with the potential to serve as determinants for recognition and tumour destruction by CTLs. Indeed, tumour-specific T cells can be present at surprisingly high frequency in peripheral blood. Clearly, there must be other factors that restrict the efficacy of anti-tumour immune responses, and a major factor appears to be the manner of antigen presentation to the immune system. As already described, initial encounter of T cells with TAA in the absence of appropriate co-stimulation can lead to anergy rather than activation; thus, transfer of TAA from a relatively 'healthy' tumour to professional APCs may be one limiting step. However, it appears that the manner in which antigens are made available to APCs also has a vital role; antigens must be taken up in the context of an inflammatory, or 'danger' signal in order to be immunogenic (Matzinger, 1994; Matzinger, 1998). Necrotic cell death is pro-inflammatory, and dendritic cells admixed with necrotic cells become activated and able to activate previously activated or naïve T cells; in contrast, admixed healthy or apoptotic cells do not trigger DC activation and may promote tolerance towards antigens taken up from apoptotic cells (Albert *et al.*, 1998; Gallucci *et al.*, 1999). A variety of mediators may be responsible for the 'danger' signal in different circum-

stances, ranging from bacterial cell wall components, DNA containing unmethylated CpG motifs, pro-inflammatory cytokines such as interferon (IFN)-γ, and heat-shock proteins induced in response to a variety of cellular stresses (Melcher *et al.*, 1998; Gallucci *et al.*, 1999; Melcher *et al.*, 1999). An important component of the 'help' provided by $CD4^+$ Th cells in the generation and support of $CD8^+$ CTLs also appears to be mediated via dendritic cells, which are enabled to activate CTLs following CD40 ligation by $CD4^+$ cells (Ridge *et al.*, 1998; Schoenberger *et al.*, 1998).

Genes and strategies for immunostimulation

INTERLEUKIN-2 (IL-2)

The earliest approaches to cancer gene therapy were inspired by previous clinical trials using systemic, high dose IL-2. Previously known as T cell growth factor, the mature form of human IL-2 is a polypeptide of 153 amino acid residues that is secreted primarily by $CD4^+$ Th cells (Callard and Gearing, 1994). It acts predominantly on T cells of all types, for which it is the most potent of all the growth factors at activating and stimulating proliferation. It is produced when $CD4^+$ cells are activated with certain mitogens or following interaction of TCRs with antigen/MHC complexes on the surface of APCs. Activation of Th cells leads to both production of IL-2 and expression of IL-2 receptors leading to autocrine stimulation of clonal expansion of antigen specific cells. Systemic delivery of IL-2 to cancer patients in clinical trials resulted in some clinical responses, but also very toxic side effects (Mule *et al.*, 1984; Rosenberg *et al.*, 1985). Local delivery of IL-2 by gene-modified cells was therefore an attractive option, to avoid the toxic side effects seen with systemic administration.

Subsequently, tumour cells genetically engineered to produce IL-2 have been intensively studied as cancer vaccines, initially in animal models and now also in clinical trials. In one study, an adenovirus expressing the murine IL-2 gene was injected directly into tumours of P815 murine mastocytoma. This resulted in complete regression in 75% of cases, and generation of systemic immunity against subsequent challenge injections (Cordier *et al.*, 1995). In such models, the main action of IL-2 is probably via the replacement of the $CD4^+$ helper T cell function, but another important mechanism of action is the activation of tumour reactive T cells that have signalling defects (probably representing anergized T cells) (Salvadori *et al.*, 1994; Zier and Gansbacher, 1996). Thus, T cells from mice with growing, unmodified tumours exhibited defects in signal transduction following activation through the TCR, whereas those from mice bearing IL-2 secreting tumours exhibited stronger signalling and normal cytokine secretion.

An important consideration in using IL-2 (or indeed, any cytokine) is the dosage effect, whereby levels of cytokine secretion either lower or higher than an optimum level result in reduced anti-tumour efficacy. For example, in the murine S91 melanoma model, the best immunization was obtained using cells secreting 1,000–3,000 units of IL-2 per 10^5 cells per day; at this level, 7 of 8 animals were protected against subsequent challenge. Lower level IL-2 secretion resulted in a reduced proportion of successfully treated animals, and higher levels of IL-2 completely abrogated protection as the production of tumour-specific CTLs was impaired (Schmidt *et al.*, 1995). These results have important implications for the clinical application of cancer

vaccines and most trial designs include a vaccine dose escalation to study this effect.

IL-2 is currently being used in a number of gene therapy clinical trials, which are beginning to be published. In one trial, a patient with glioblastoma developed an anti-tumour immune response resulting in marked tumour necrosis that was mediated in part by $CD8^+$ CTLs (Sobol *et al.*, 1995). In another phase I trial, autologous melanoma cells transfected with IL-2 were injected into the skin of patients with advanced disease. Fifteen patients received 2–8 vaccinations and, although none experienced complete or partial regressions, 5 experienced periods of disease stabilization, including the shrinkage of single metastases. Overall, the vaccine was well tolerated, melanoma-specific delayed-type hypersensitivity (DTH) reactions were evident, and histology showed infiltration of predominantly $CD4^+$ cells around regressing metastases (Schreiber *et al.*, 1999). In a similar phase I study, 12 melanoma patients were vaccinated 1–3 times. Again, treatment was well tolerated, and it was demonstrated that, in 4 patients, the activity of anti-tumour CTLs present pre-vaccination, increased following vaccination. Three patients had stable disease 7–15 months, one of whom had not progressed (15+ months) (Palmer *et al.*, 1999). These studies so far show that vaccination with autologous IL-2 expressing cells is safe, and provide some encouragement that this approach may be of clinical benefit to patients with less advanced disease. However, approaches which rely upon growth of tumour cells *in vitro* can be thwarted by the difficulties of growing the cells in sufficient quantity, of achieving efficient gene transfer, and of doing so on a shorter time scale than that of disease progression and patient death.

GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF)

GM-CSF is a pleiotropic cytokine that can stimulate the proliferation, maturation and function of haematopoietic cells (Callard and Gearing, 1994; Dranoff and Mulligan, 1994). It attracted attention as a cytokine adjuvant because of its important role in the maturation and function of APCs such as dendritic cells (DCs) and macrophages (Caux *et al.*, 1992). In addition, it has been shown to stimulate the rejection of MHC class I negative tumours through activation of natural killer (NK) cells and $CD4^+$ T cells. Dranoff *et al.* (1993) compared murine B16 melanoma tumour cell vaccines genetically engineered to secrete GM-CSF, IL-2, IL-4, IL-5 or IL-6. Only those expressing GM-CSF could induce long-lasting, anti-tumour immunity, and this was found to require the presence of both $CD4^+$ and $CD8^+$ T cells in the recipient. Similar efficacy of GM-CSF has been seen by other groups and in other tumour models (Levitsky *et al.*, 1996; Lee *et al.*, 1997).

In an attempt to use animal models that more closely mimic the treatment of a patient with pre-existing cancer, similar experiments have been conducted using mice with pre-established tumours. In one study, Lewis lung carcinoma (3LL) cells were infected with an adenovirus expressing murine GM-CSF, and then irradiated before injection into mice with pre-established 3LL tumours. Tumour regression was seen in 6 out of 10 cases, compared to only 3 out of 10 regressions in the control group. The GM-CSF mediated regressions were accompanied by accumulation of DCs at the tumour site and low, but significant, levels of tumour specific CTLs (Lee *et al.*, 1997).

Abe *et al.* (1995) compared the efficacy of an irradiated B16 murine melanoma vaccine that expressed murine GM-CSF following transduction using either retrovirus

or adenovirus vectors. Although the retrovirally-modified cells produced far less GM-CSF than those modified by adenovirus, the former resulted in complete protection of the mice, whereas only 40% of mice receiving the adenovirally transduced vaccine were able to reject the tumour. Thus, as with IL-2, it appears that care should be taken to establish the optimal dose of GM-CSF, and that use of a vaccine producing too high a level of cytokine may lead to reduced efficacy. The authors suggested this might be due to suppression of APC or lymphocyte functioning.

One of the first reported clinical trials of vaccination using tumour cells engineered to secrete GM-CSF was published recently (Soiffer *et al.*, 1998). The trial involved patients with metastatic melanoma and involved culturing surgically resected tumour, transducing the expanded tumour cell population with a retrovirus carrying the human GM-CSF gene, irradiating the cells and using them to immunize both intradermally and subcutaneously. Non-transduced cells were used to test for a DTH reaction. In 21 evaluable patients, the immunization sites were intensely infiltrated with T cells, DCs, macrophages and eosinophils. Metastatic lesions resected after vaccination showed extensive T cell and eosinophil infiltrates and extensive tumour destruction (up to 80%). Strong DTH reactions were detected following vaccination, whereas no immune responses were detectable prior to treatment. Antimelanoma CTL and antibody responses were also induced, suggesting that this vaccination protocol stimulated a potent anti-tumour immunity. Further testing of this vaccination procedure is warranted with randomized clinical trials conducted in the setting of minimal residual disease.

OTHER CYTOKINES

Earlier reviews of the use of cytokines in gene therapy include Culver (1994) and Foa *et al.* (1994). A number of other cytokines with diverse functions have shown efficacy in similar models of immunostimulatory gene therapy for cancer. For example, even IL-4 and IL-10, which are associated with antibody-based immune responses via stimulation of Th2 cells rather than with generation of Th1 and CTL, have been reported to help generate systemic immunity against tumours. Thus, an adenoviral vector expressing IL-4 was shown to prevent mammary adenocarcinoma growth in 61% of mice when cells infected *ex vivo* were injected. Direct intratumoural injection of the virus induced complete regression in 50% of mice and, in both cases, mice that had rejected tumours were protected from a challenge with parental cells (Addison *et al.*, 1995). The mechanism of this effect appeared to involve a massive recruitment of eosinophils by IL-4; however, as eosinophils do not possess immune memory, the long-lived anti-tumour effects must be due to other cell types, perhaps secondary to tumour antigen release and uptake by APCs.

IL-10 was shown to have anti-tumour effects in the syngeneic B16 murine melanoma model (Gerard *et al.*, 1996); inhibition of tumour growth appeared to involve T lymphocytes and NK cells, as the IL-10 producing tumours were able to grow in nude mice, or mice depleted of $CD8^+$ or NK cells; and a systemic response able to protect against subsequent challenge with parental cells was induced. Richter *et al.* (1993) reported that IL-10 expression inhibited the growth of Chinese hamster ovary (CHO) cell tumours in immunodeficient nude mice. In this latter example, the parental cells formed tumours which were infiltrated by macrophages; IL-10 expression

appeared to inhibit the macrophage infiltrate and tumour growth, and it was surmised that CHO tumour growth may depend upon factors (possibly angiogenic) secreted by the macrophages.

Interferon-γ (IFN-γ has also been much used in immunotherapy protocols, eg Lollini *et al.* (1993) and Sun *et al.* (1995)) and the results of a phase I study in 17 patients with advanced melanoma has recently been published (Nemunaitis *et al.*, 1999). Seventeen patients were given a course of direct intratumoural injections of IFN-γ retroviral vector. Eight showed elevated anti-tumour antibody responses, 1 of 9 treated with a single injection, and 8 of 8 patients receiving multiple injections subsequently experienced stable disease. At the time of reporting, 4 of the patients treated with multiple injections were still alive, and so the median survival could not be defined. The authors concluded that this was a safe, non-toxic therapy with evidence of anti-tumour activity when administered as multiple injections.

Interleukin-12 (IL-12) is a cytokine with a wide range of biological effects, including induction of IFN-γ production by T and NK cells (Naume *et al.*, 1992), enhancing the lytic activity of CTLs (Trinchieri, 1995), NK and lymphokine-activated killer (LAK) cells (Kobayashi *et al.*, 1989), and promotion of a Th1-like response, thus favouring cell mediated immunity (Hsieh *et al.*, 1993). It has also been reported to have anti-angiogenic properties that might augment its anti-tumour activity (Voest *et al.*, 1995). It has shown therapeutic effects in a number of animal models of cancer (Brunda *et al.*, 1993; Tahara *et al.*, 1994; Meko *et al.*, 1995; Tahara *et al.*, 1995). A phase I clinical trial of an autologous IL-12 secreting tumour cell vaccine in 6 patients with metastatic melanoma was recently published (Sun *et al.*, 1998). The vaccination induced a number of immunological changes including heavy $CD4^+/CD8^+$ T cell infiltration into metastases and an increased number of tumour-reactive proliferative and cytolytic T cells.

USE OF ALLOGENEIC MHC TO STIMULATE CROSS-PRIMING

Rejection of allogeneic tumours (ie with MHC molecules differing from those of the host) in laboratory experiments is a classic finding. The HLA class I molecule HLA B7 expressed in an allogeneic tumour environment is a strong immunostimulator, resulting in local production of interferons and other cytokines. These in turn cause up-regulation of other molecules, such as HLA class I, on the tumour cells, which further enhances the immune response by facilitating tumour antigen expression. HLA-B7 gene-modified cancer cells have been used in clinical trials for melanoma, colorectal, and renal cell carcinoma; so far, the results of three small phase I studies have been published (Nabel *et al.*, 1993; Rubin *et al.*, 1994; Rubin *et al.*, 1997; Stopeck *et al.*, 1997). These studies all involved direct intratumoural injection of DNA complexed with cationic lipid, and demonstrated that this therapy was relatively non-toxic; HLA-B7 expression was achieved *in vivo*, and CTL responses and tumour regression were seen in some patients. Nearly 400 patients have now been treated by cationic lipid-mediated delivery of a HLA-B7 expression plasmid to a variety of tumours (Wiley Genetic Medicine Clinical Trials Database), so the next few years should see the publication of the results from a number of these.

A study by Hui *et al.* (1997) compared direct intratumoural injection of either allogeneic HLA-A2, HLA-B13, or xenogenic (murine) $H\text{-}2K^k$. Results from 19

patients with a variety of types of cancer indicated a better therapeutic effect of HLA-A2 over HLA-B13 and H-2K^k. In 5 of 6 patients with cervical or ovarian cancer, allogeneic HLA-A2 transduction into tumours resulted in local, but significant, size reductions.

CO-STIMULATION

For full and efficient activation, T cells require, in addition to the activation signal provided by engagement of the TCR with a cognate antigenic peptide-MHC complex, a second signal which can be provided by ligation of a co-stimulatory molecule with an appropriate receptor on the T cell. Such co-stimulatory molecules are neither MHC restricted nor antigen specific. However, failure to deliver the second signal can lead to T cell anergy, an unresponsive state in which the T cell will not respond to the subsequent presentation of appropriate antigen (Mueller *et al.*, 1989; Schwartz, 1990). The basis of this anergy was shown to be an inability of these lymphocytes to secrete sufficient IL-2 to drive their proliferation following stimulus. Several cell surface molecules, such as ICAM-1 and LFA-3, have been shown to have co-stimulatory function, but one of the dominant co-stimulatory pathways is provided by signalling through CD28 (Lenshaw *et al.*, 1996; Watts and DeBenedette, 1999). Two ligands for CD28 have been described, B7-1 (CD80) and B7-2 (CD86); these are expressed on the majority of professional APCs such as DCs, macrophages and activated B cells (Freeman *et al.*, 1991; Larsen *et al.*, 1992; Razi-Wolf *et al.*, 1992). The potent co-stimulatory role of CD80 has been demonstrated in a number of *in vitro* studies. Proliferation of T cells in response to phorbol myristate acetate (PMA) or anti-CD3 (which mimics signalling through the TCR) can be increased by the addition of CD80-transfected cells.

An initial antigen encounter without interaction between CD28 and CD80/CD86 can lead to CD4$^+$ and CD8$^+$ T cell anergy (Linsley and Ledbetter, 1993), and this may be a mechanism by which tumours evade destruction by the immune system. The gradual development of tumours may avoid delivery of tumour antigens to professional APCs in a way that could provoke immunogenic antigen presentation to T cells. Many tumours express normal levels of MHC and have intact antigen presentation capacity, but they are not 'professional' APCs and so do not express CD80 or CD86. Hence, if T lymphocytes with appropriate TCR specificity first encounter cognate TAA presented by the tumour cells, the absence of co-stimulatory signals has the potential to result in anergy of the tumour-specific T-cells.

Gene transfer leading to expression of co-stimulatory molecules by tumour cells may be a route to achieve a more immunogenic interaction between T lymphocytes and tumour. There is now a considerable body of evidence to show that CD80 expression in murine tumour cells can mediate tumour regression in immunocompetent mice (Baskar *et al.*, 1993; Townsend and Allison, 1993; Chen *et al.*, 1994b; Townsend *et al.*, 1994; Baskar *et al.*, 1995). Townsend and Allison transfected the K1735 mouse melanoma cell line with murine CD80. The transfected tumour grew very poorly in comparison to control in a syngeneic host and was able to protect the host from a subsequent challenge with the parental cell line. Depletion of CD4$^+$ cells did not diminish the immunogenicity of the CD80-expressing vaccine, suggesting that CD80 expression on the tumour cells may have bypassed the need for CD4$^+$

T cell help. Transduction of human tumour cells to express CD80 has been shown to enhance proliferation of co-cultured T cells (Sule-Suso *et al.*, 1995; Gilligan *et al.*, 1998) and CTL generation (Kaufmann *et al.*, 1997; Gueguen *et al.*, 1998; Zajac *et al.*, 1998). No clinical data is yet available on the efficacy of vaccinating cancer patients with CD80-expressing tumour cells.

Only tumours with some inherent immunogenicity seemed able to induce protective immune responses when modified to express CD80 or CD86 (Chen *et al.*, 1994a). One limiting factor in this system may be the fact that activated T cells express CTLA-4, a second receptor for CD80 and CD86, which transmits an inhibitory signal to the T cell (Walunas *et al.*, 1994; Krummel and Allison, 1995). Indeed, selective blockade of signalling through CTLA-4 can enable mice to reject normally non-immunogenic tumours (Leach *et al.*, 1996). Shrikant *et al.* (1999) showed that, using adoptive transfer of transgenic T cells specific for the model tumour antigen OVA, tumour-specific $CD8^+$ CTLs initially proliferated and developed lytic function upon challenging the mice with the OVA expressing thymoma EG7. However, by day 10 the T cells had all left the tumour site, migrated to the lymph nodes and spleen, and became anergic, without tumour eradication. Monoclonal antibody-mediated blockade of CTLA-4 allowed continued proliferation of the CTLs and control of the tumour. This effect was shown to require $CD4^+$ Th cells, and could be mimicked by administration of IL-2 on days 4 and 5 after tumour challenge. There are certainly interesting therapeutic possibilities here to be explored.

Another co-stimulatory pathway involves the T cell surface molecule 4-1BB, and its ligand 4-1BBL expressed on several types of APC. Expression of 4-1BB is restricted to primed $CD4^+$ and $CD8^+$ T cells, and signalling via interaction with its ligand or by antibody ligation delivers a dual signal to T cells for activation and growth. Administration of anti 4-1BB antibodies can eradicate large established tumours in mice, including the poorly immunogenic Ag104A sarcoma and the highly tumourigenic P815 mastocytoma (Melero *et al.*, 1997). The immune response induced was mediated by both $CD8^+$ and $CD4^+$ T cells, and a marked increase in tumour specific CTLs was seen. Following co-stimulation through CD28, T cells will normally initially undergo clonal expansion, but this is followed by deletion, as the majority of the T cells die by apoptosis. It appears that ligation of 4-1BB may inhibit such peripheral deletion of T cells. Takahashi *et al.* (1999) showed that a similar T cell response was generated initially in mice injected with the superantigen staphylococcal enterotoxin A (SEA), whether or not they were also injected with an agonist anti-4-1BB antibody. Significantly, however, after 21 days, the number of specific $CD8^+$ T cells in mice injected with SEA alone had declined 10-fold from its peak to below the starting level, whereas there was little decline from peak levels in animals treated with the 4-1BB antibody. The 4-1BB antibody treatment also reduced the deletion of activated $CD4^+$ cells, though to a lesser extent. This suggests that ligation of 4-1BB can inhibit activation induced T cell death *in vivo* and act as a long-term survival signal for T cells. This effect may be equivalent to the role of adjuvants in traditional vaccines, and has practical implications for the design of anti-cancer vaccines, though it has yet to be exploited as a modality of gene therapy.

SPECIFIC TUMOUR ANTIGEN VACCINES

By analogy with the many successful vaccines against the agents of infectious diseases, based upon some appropriate means of vaccination with antigen derived from the pathogen, many approaches to cancer immunotherapy involve some form of vaccination with more or less well-defined tumour antigens. The preparations of tumour antigen used can take several forms, including whole tumour cells, tumour cell lysates, purified or synthetic tumour peptides, or various methods of gene-based delivery of antigen. The immunogenicity of an antigen depends on the context in which it is delivered; for example, specific antigenic peptides can either simply be delivered systemically with an adjuvant, or they can be pre-loaded onto professional APCs *ex vivo*.

Almost all vaccine trials before 1990 involved treating melanoma patients with autologous or allogeneic tumour cell preparations. Several fairly large-scale trials were conducted, such as those by Mitchell *et al.* (1988, 1990, 1993) in which melanoma patients were treated with allogeneic cell lysate vaccine administered with DETOX adjuvant. Twenty objective responses were observed among 106 patients and the median duration of response was 21 months. As gene delivery systems developed, this vaccination approach was adapted to utilize cells modified to express TAA, cytokines, or co-stimulatory molecules (see above). However, a major trend in research is now directed towards using defined tumour antigens either as whole protein or as specific peptides known to bind particular MHC class I molecules. Defined peptide epitopes have been used extensively and results from several clinical trials, mostly involving melanoma, are now published (Marchand *et al.*, 1995; Mukherji *et al.*, 1995; Salgaller *et al.*, 1995; Hu *et al.*, 1996; Nestle *et al.*, 1998). For example, immunization of melanoma patients with a MAGE-3-derived peptide induced limited tumour regression in 5 of 17 patients (Marchand *et al.*, 1995). Vaccination with peptides has a number of drawbacks, including poor immunogenicity and the monospecificity of any induced immune response. Thus, in some of the initial trials with peptide vaccines, patients initially showed a partial response but subsequently relapsed with metastatic disease, showing loss of antigen and/or HLA expression (Jager *et al.*, 1996). In principle, simultaneous vaccination against a greater number of TAAs might decrease the likelihood of such immune escape.

Direct TAA vaccination utilizing the entire protein rather than selected peptide epitopes allows the possible display of a number of peptides to the immune system, possibly using different HLA molecules. For example, the *HER2/neu* proto-oncogene encodes a protein containing a number of HLA-A2 restricted moieties that are recognized by T cells from cancer patients (Fisk *et al.*, 1995); another epitope of this protein can be recognized in an HLA-A3 restricted context (Kawashima *et al.*, 1999). HER-2-derived peptides presented by the MHC class II molecule HLA-DR4 and capable of inducing $CD4^+$ T cell proliferation have also been recognized (Tuttle *et al.*, 1998). Thus, vaccination with whole protein has the potential to stimulate both class I and class II restricted responses.

As described above, there are now many proteins that are known to be potential targets for the immune system in cancer, and so the gene-based approaches for the delivery of TAA provide alternative possibilities to induce, restore or augment immune responses against host tumour cells. Many defined tumour antigens have

now been cloned into suitable vectors and tested in clinical trials. Most of these are melanoma antigens, but other examples include carcinoembryonic antigen (CEA), mucin-1 (MUC-1), and the E6 and E7 oncoproteins of HPV types 16 and 18. The main route of delivery for these genes is by using a recombinant virus such as vaccinia or adenovirus, either in direct vaccination or to introduce the gene *ex vivo* into professional APCs, of which dendritic cells are probably the most effective. Vaccinia virus encoding CEA induced both humoral and cellular responses when administered to colorectal cancer patients (Tsang *et al.*, 1995). Recombinant canarypox viruses have been produced encoding CEA and p53; both have been shown to be highly effective at protecting mice from lethal tumour cell challenge (Roth *et al.*, 1996; Hodge *et al.*, 1997). Construction of these different vectors allows the opportunity to immunize with one vector and then boost with the other, a protocol superior to using either alone (Hodge *et al.*, 1997). In a recent phase I/II trial in cervical cancer (Borysiewicz *et al.*, 1996), 8 patients were immunized with a single dose of a live recombinant vaccinia virus encoding modified (non-oncogenic) derivatives of HPV 16 and 18 E6 and E7 protein sequences. No clinically significant side effects were seen, and each patient mounted an anti-vaccinia response. Three patients developed an HPV-specific antibody response that could be ascribed to the vaccination and from one of three evaluable patients an HPV-specific CTL response was detected. Trials are being extended to allow repeat vaccinations, and to treat patients with earlier stages of disease.

Thus, it can be concluded that the various formats of specific TAA-based vaccination can be effective in inducing specific immune responses, although the potential limitations of inducing a monospecific immune response have been noted. There is considerable ongoing effort to identify more TAAs, which could allow the generation of multivalent vaccines and possibly reduce the likelihood of tumour escape by antigen loss, and to develop more immunogenic ways to deliver the antigens, eg by co-expression of cytokines.

DENDRITIC CELL-BASED THERAPIES

DCs are the most efficient APC, especially for the activation of naïve T cells. They are, therefore, becoming widely used in the field of cancer immunotherapy. They express high levels of MHC class I and II, together with the co-stimulatory molecules essential for immunogenic antigen presentation, and have the ability to take up exogenous antigens, which they can process and display on both class I and class II MHC molecules. In recent years, simple methods have been developed to generate large numbers of functionally active DCs *in vitro* by culturing DC precursors from bone marrow or blood in the presence of GM-CSF and/or other cytokines, such as IL-4 and FLT3 ligand (Caux *et al.*, 1992; Reid *et al.*, 1993; Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994; Romani *et al.*, 1996). A number of strategies are being developed to achieve effective presentation of TAA by DCs, including a simple transient incubation (pulse) in medium containing the peptide or peptide-encoding RNA, and infection with recombinant virus to express the TAA within the DCs. The resulting TAA-presenting DCs have been used in both animal models and human phase I clinical trials for the treatment of B cell lymphoma, prostate cancer, and melanoma (Hsu *et al.*, 1996; Murphy *et al.*, 1996; Nestle *et al.*, 1998).

A recent study identified adenovirus as the most promising vectors for gene transfer to DCs with up to 95% positivity achieved (Arthur *et al.*, 1997). Most of the published clinical studies have vaccinated patients with DCs pulsed with antigen in the form of peptide or tumour cell lysate. Four patients with low-grade follicular B cell lymphoma were infused with antigen pulsed DCs (Hsu *et al.*, 1996). Complete remission was observed in 2 patients, one patient had a partial response, and one patient experienced stable disease. Immunization with the antigen (monoclonal surface immunoglobulin) alone or with adjuvant induced no regression (Kwak *et al.*, 1992). In another study, of 16 melanoma patients vaccinated with peptide or tumour lysate-pulsed DCs, 5 showed objective clinical responses with regression of metastases in various organs. Although the number of studies is limited, results so far suggest that the use of crude tumour lysate is more successful than using defined MHC class I binding peptides. This is consistent with the generation of a broader immune response involving both $CD4^+$ and $CD8^+$ T cells specific for a number of epitopes, and a humoral immune response may also be induced (Morton *et al.*, 1992; Morton *et al.*, 1993).

Another exciting aspect of DC vaccination is the ability of these cells to overcome anergy or tolerance, the state of immunological unresponsiveness to antigens. One of the best-studied examples involves the human carcinoma antigen, MUC-1. Transgenic mice that express human MUC-1 are unresponsive to this antigen when immunized with irradiated MUC-1 positive cells. However, immunization of these mice with fusion hybrids of DCs and MUC-1-positive tumour cells induced a potent immune response, which both protected against tumour challenge and also rejected established metastases (Gong *et al.*, 1998). The tumour/DC fusion hybrids stimulated both $CD4^+$ and $CD8^+$ T cells, as well as NK cell anti-tumour responses.

A number of phase I trials are now under way using DCs expressing TAAs (MART-1, gp-100, tyrosinase, MAGE-1 and MAGE-3) and many of these are using adenoviral vectors for gene delivery. This type of approach has been used very successfully to treat mice and to generate CTLs *in vitro* but, as yet, no results are available from any of the clinical trials.

GENETIC MODIFICATION OF LYMPHOCYTES

The first studies of adoptive transfer of T cells to treat cancer were accompanied by severe side effects from the co-administration of high dose recombinant IL-2 (Mule *et al.*, 1984; Rosenberg *et al.*, 1985). Better results were achieved using TILs (tumour infiltrating lymphocytes isolated following surgery and expanded *in vitro*) (Rosenberg *et al.*, 1994). However, as toxicity associated with IL-2 remained a problem, Rosenberg and colleagues have now developed techniques for gene transfer to TILs. The first patients to receive cytokine gene transduced TILs were treated at the NIH in January 1991, in a phase I dose escalation study. Patients received tumour necrosis factor (TNF)-transduced TILs, either with or without IL-2. The first 6 patients showed few side effects, and one patient had a sustained response. Use of this approach is still hampered by the inefficiency of gene transfer to T cells, but in the near future, as vectors are being improved, gene transfer to TILs should become more practicable.

Another approach uses genetically modified T cells to circumvent the usual difficulties of generating tumour-specific CTLs. Rather than rely upon those T cells

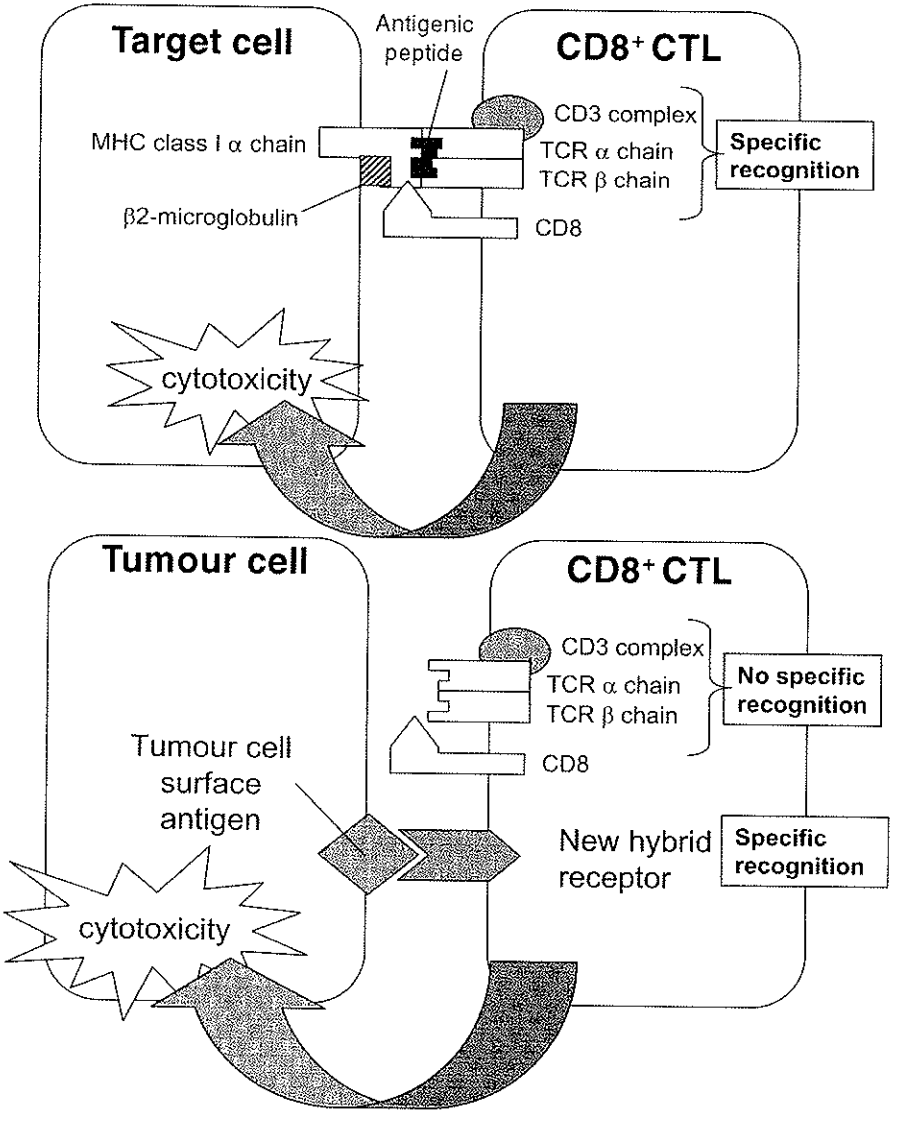

Figure 18.2. Diagram illustrating the use of a new, hybrid receptor to redirect CTLs to a tumour cell antigen. The upper panel illustrates the normal specific recognition event between an antigenic peptide (eg derived from an intracellular pathogen) and a TCR of appropriate specificity carried by an activated $CD8^+$ CTL. The specific recognition event triggers the CTL to kill the target cell. In the lower panel, a tumour cell is depicted that has lost the ability to present peptide antigens via the MHC class I proteins, and so cannot be recognized by CTLs. A tumour cell surface antigen (eg MUC-1 or FBP) can be recognized by a hybrid receptor introduced into T lymphocytes; the hybrid receptor has an intracellular signalling domain and an extracellular domain, eg derived from a single-chain antibody. Binding of the tumour surface antigen by the hybrid receptor can trigger the CTL to lyse the tumour cell.

whose genomically encoded TCR has an appropriate specificity, a new receptor of predetermined specificity for a chosen TAA is introduced. This could be the α and β chains of a suitably characterized TCR, but recently it has been demonstrated that

fusion of extracellular ligand binding domains with the signal-transducing zeta chain of the TCR/CD3 complex results in molecules that can be activated by interaction with intact ligand, thus bypassing the need for appropriate antigen processing and presentation on MHC (see *Figure 18.2*). These approaches have been used successfully to redirect CTL activity against cells displaying the antigen recognized by the surrogate receptor. For example, a chimeric TCR was introduced into naïve T cells enabling them to recognize Neu/erbB-2, erbB-3 and erbB-4 receptors (Altenschmidt *et al.*, 1996). These antigens are over-expressed in a number of tumours, including ovarian and breast carcinomas. A similar approach to redirecting T cells is through the expression of a chimeric receptor formed from fusion of the variable domain of an antibody recognizing a TAA to the zeta chain of the TCR. This approach has been shown to be effective *in vivo*, mediating the regression in nude mice of established human tumours expressing folate binding protein (FBP) (Hwu *et al.*, 1995). This technique is to be tested in a phase I trial involving patients with advanced epithelial ovarian cancer, using anti-CD3 stimulated peripheral blood lymphocytes transduced with a chimeric receptor which recognizes FBP. As FBP is over-expressed in more than 90% of ovarian tumours (Garin-Chesa *et al.*, 1993), this technique could be widely applicable. Another antigen that is being targeted using this technology is TAG-72, a mucin antigen expressed by most adenocarcinomas. A chimeric antibody/TCR gene has been introduced into human T cells, and these can lyse TAG-72 positive tumour cells both *in vitro* and *in vivo* (McGuinness *et al.*, 1999). Again, this approach is being utilized in a phase I clinical trial for the treatment of colon cancer. Attractive aspects of this type of treatment are both the implications for its generalization to treat a variety of human cancers and also that this approach bypasses normal mechanisms of antigen processing and presentation by MHC. Thus, it is independent of haplotype and would be applicable to tumours with down-regulation or loss of MHC.

Gene transfer vectors

It is implicit in the concept of gene therapy that the vector used for gene transfer should allow efficient transfer of the therapeutic gene to the intended target cells and its subsequent expression at an appropriate level for long enough to bring about the intended biological response. No currently available vectors are ideal for all purposes, and different types of gene therapy place somewhat different demands on the vectors. Generally desirable features include the ability to produce the vector at high concentration; and its production, storage and formulation should be convenient and reproducible. It is also generally desirable that the vector itself should not provoke, or be the target of, an immunological response to the detriment of the intended therapy. An area of much current research interest is to improve the target cell specificity of vectors; for example, to allow selective gene delivery to tumour cells *in vivo*. A selection of vectors that have been used in immunotherapeutic gene therapy research is briefly reviewed below.

RETROVIRAL VECTORS

Retroviral vectors based on Moloney murine leukaemia virus were among the first

transfer systems to be used in gene therapy and remain widely used (Miller, 1992). The natural life cycle of retroviruses makes them favourable gene transfer vectors, especially when long-term gene expression is required. The specificity of the amphotropic envelope protein commonly employed allows efficient infection of many types of human cell. In infected cells, the genomic RNA of the virus is reverse transcribed into DNA, which then integrates permanently into host chromosomal DNA, resulting in stable transfer of any included genes. This aspect of retroviral vectors makes them attractive for many uses including *ex vivo* gene therapy, as it provides the opportunity for long-term expression of the therapeutic genes in the target cells and subsequent derived cell generations. Whilst stable integration also brings the risk of insertional mutagenesis of host genes, including activation of cellular oncogenes or inactivation of tumour suppressor genes, in practice this does not appear to have caused harmful consequences in the clinical trials to date.

The modified retrovirus genomes used for gene therapy contain the regulatory signals required for the virus life cycle, including the long terminal repeats and genome packaging signals, but the viral *gag*, *pol* and *env* genes (encoding the viral capsid proteins, the reverse transcriptase/integrase functions, and the envelope protein respectively) are replaced by the therapeutic gene construct. The viral proteins essential for virion assembly and subsequent infection of target cells are provided by specially designed packaging cell lines, into which the therapeutic viral genome is transfected as a plasmid. Recombinant virus particles produced from the packaging cells can be used for a single round of target cell infection (or transduction) but, because the transferred genomes encode no virus proteins, the constructs cannot spread further, ie they are replication-defective. This also ensures that the transduced cells cannot become targets for immune responses against retrovirus antigens.

One drawback to the use of retroviruses is the potential generation of replication-competent retroviruses by recombination between the therapeutic construct and the constructs expressing the *gag*, *pol*, and *env* genes in the packaging cells; this risk can be minimized by improved design of the packaging cells (Miller and Buttimore, 1986; Markowitz *et al.*, 1988; Cosset *et al.*, 1995). Additionally, the rapid inactivation of retroviruses produced in murine cells by complement-mediated mechanisms in human serum limits their potential for *in vivo* use (Takeuchi *et al.*, 1994; Takeuchi *et al.*, 1996), though this has been addressed by the construction of appropriate packaging cells based on human cells (Cosset *et al.*, 1995). A further disadvantage of standard retroviral vectors based on murine leukaemia virus is that they cannot be used to infect non-dividing cells, since replication is necessary for proviral integration to occur (Miller *et al.*, 1990). However, retroviruses of the lentivirus group (which includes HIV) are able to infect non-dividing cells, and so lentiviral vectors and packaging cells are also currently being developed for use in gene therapy (Haselhorst *et al.*, 1998).

Of the immunotherapeutic strategies discussed, retroviruses currently appear to be most suitable for *ex vivo* modification of tumour cells to express immunostimulatory molecules for use as an autologous vaccine and for stable *ex vivo* modifications of lymphocytes, eg to express novel surrogate TCRs. However, in practice, the difficulty of establishing replicating tumour cells *in vitro* to allow efficient retroviral gene transfer has been a limitation, especially when the patients have a limited life span.

ADENOVIRAL VECTORS

Adenoviruses are a family of DNA viruses causing benign respiratory tract infections in humans. Following infection of a target cell, adenoviruses do not integrate into host DNA but are replicated as episomal elements, leading to death of the infected cell and release of the packaged virions. Most of the modified adenoviral vectors used for gene therapy are made replication-defective by removal of the E1A and E1B genes; many also have deletions within the non-essential E3 region. As the E1 proteins are necessary for viral replication, these are provided *in trans* by helper cells such as 293 cells (Graham *et al.*, 1977). The viruses are relatively stable, and can be concentrated to titres > 10^{12} pfu/ml, an attractive feature enabling direct, *in vivo* use (Yeh and Perricaudet, 1997). They can infect a wide range of cells, irrespective of their proliferative state, and can give high levels of transgene expression. However, transgene expression is often transient (5–10 days), either due to loss of the episomal genome from dividing cells, or *in vivo*, because of an immune response against the infected cells. Studies in mice have shown that a far greater duration of transgene expression can be achieved in immunocompromised mice (Dai *et al.*, 1995). In some cases, the immune response may be directed against a foreign transgene product, but leaky, E1-independent expression of other adenoviral proteins encoded within the vectors can also provide significant target antigens for the immune system. To get around this problem, as well as to increase the capacity for non-viral DNA, new adenoviral vectors have recently been produced that lack all viral genes except the elements which define the ends of the viral genome and the viral packaging sequence (Kochanek *et al.*, 1996; Chen *et al.*, 1997; Morsy and Caskey, 1999). Studies so far have suggested that these helper-dependent or 'gut-less' vectors are significantly less 'visible' to the immune system, allowing longer-term persistence of cells expressing the transgene (Engelhardt *et al.*, 1994; Chen *et al.*, 1997; Morsy *et al.*, 1998). A recent paper has suggested that even very low levels of replication-competent adenovirus in virus stocks may make an important contribution to immunogenicity, via E1A induction of heat-shock proteins in the infected cell (Melcher *et al.*, 1999).

Natural adenoviral infections elicit both a cellular and humoral response against the virus, and most humans have pre-existing immunity to adenovirus serotypes 2 or 5, which form the basis for the vectors in current use. The presence of neutralizing antibodies can greatly diminish the efficiency of adenoviral gene transfer, and reactivating such a response by an initial *in vivo* delivery of vector can significantly further reduce the efficacy of subsequent re-administrations of the same vector (Yang *et al.*, 1994; Dai *et al.*, 1995; Harvey *et al.*, 1999). So, whilst the high titre of adenoviral vectors can allow very efficient gene transfer to tumour cells *ex vivo*, and *in vivo* efficacy has also been observed, neutralizing antibodies may reduce the efficiency of *in vivo* delivery, especially on repeated administration. The lack of stable transgene persistence does not appear to be a limitation, as the duration of expression should be sufficient for induction of the intended immune response. It remains to be determined whether the potential cell-mediated immune response against vector-encoded adenoviral antigens hinders or facilitates the generation of anti-tumour immune responses. However, the helper-dependent vectors provide a solution, should this be a problem.

VACCINIA VIRUS

Vaccinia virus, previously used as a live vaccine in the eradication of smallpox, has also been used as a viral vector for gene therapy. The vectors remain replication-competent, leading to lysis of the infected cells and further rounds of cell infection until cleared by an anti-viral immune response. Recombinant vaccinia viruses are best suited to the expression of defined TAA within a highly immunogenic context, and have been constructed to express a wide variety of genes, including HER-2/neu, p53 and a number of cytokines (Paoletti *et al.*, 1993). Vaccinia vectors are of particular interest for immunotherapy, as in animal models the co-presentation of a weak immunogen with the highly immunogenic vaccinia proteins can elicit a strong immune response against the inserted gene product. However, the potent immune response against vaccinia itself means that recombinant vaccinia virus is likely to be unsuitable for multiple administrations, or for use in patients previously immunized against smallpox, as antiviral responses may swamp those against the TAA. The availability of other similar, but antigenically distinct, vectors such as canarypox (which is replication-defective in mammalian cells) may provide a way to circumvent this difficulty; for example, to allow an initial immunization with a recombinant vaccinia, and a subsequent boost with the same TAA expressed by canarypox.

ADENO-ASSOCIATED VIRUS

Adeno-associated virus (AAV) is a simple, single-stranded DNA virus with just two genes encoding the capsid and replication/integration functions. It is non-pathogenic, and naturally dependent upon co-infection, either with adenovirus or herpesvirus for its replication. In recombinant AAV vectors, the viral genes are usually replaced by the transgene, and their functions must instead provided *in trans* for virus production, in addition to the helper virus. AAV infects a wide variety of replicating and non-replicating cell types and, under certain conditions, viral DNA can integrate preferentially into human chromosome 19, which has the potential to allow long-term transgene expression. Disadvantages of the early AAV vector systems are the need to carefully separate any contaminating helper virus and the fact that the helper functions cannot be simply provided by a stable cell line; however, these difficulties are being overcome (Rabinowitz and Samulski, 1998; Xiao *et al.*, 1998; Summerford and Samulski, 1999). AAV can only accommodate small amounts (3.5–4 kb) of foreign DNA and, like adenoviral vectors, existing immune responses may be a problem as 80% of adults have circulating antibodies to AAV.

HERPES SIMPLEX VIRUS

Vectors based on herpes simplex virus (HSV) are also being developed for gene therapy. HSV has a wide tropism for many cell types but it uniquely establishes a lifelong latent infection in cells of the nervous system. A disabled infectious single cycle (DISC) HSV has been developed as a potential prophylactic vaccine for genital herpes (Boursnell *et al.*, 1997). Lacking the gene encoding glycoprotein H that is essential for cell infection, the virus undergoes only one complete lytic cycle after infection in humans or animals, producing non-infectious progeny virions. Recently,

DISC HSV has been used to infect dendritic cells *in vitro* which, *in vivo*, were able to generate CTLs and protective immune responses against the transgene (McLean *et al.*, 1998). Like other potential viral vectors, pre-existing immunity to components of HSV may be a problem in those who have had a previous infection with the naturally occurring virus.

NON-VIRAL DELIVERY SYSTEMS

Most non-viral methods of gene transfer rely on cellular pathways for the uptake and transport of macromolecules. Early attempts at non-viral gene transfer *in vivo* involved injection of naked DNA which gave expression of reporter genes for up to two months after injection in mouse skeletal muscle (Wolff *et al.*, 1990). The ease and low cost of production are obvious benefits of this approach, and a number of clinical trials have used direct injection of plasmid DNA (Roth and Cristiano, 1997).

The 'gene gun' approach for delivery is based on particle bombardment, using gold or tungsten particles coated with plasmid DNA which are fired at tissues by electrical or gas pulse acceleration (Yang *et al.*, 1991). While expression of the transgene may be short-term as the DNA remains extra-chromosomal, gene gun transfer of GM-CSF can lead to a successful anti-tumour response, eg in a mouse myeloma model (Turner *et al.*, 1998).

Synthetic lipid formulations have also been developed to deliver plasmid DNA; the simplest of these are mixtures of DNA and cationic lipids, which form aggregates but cannot target specific cells and deliver the gene only at the site of administration (Lehn *et al.*, 1998). More complex vectors with targeting ability have been developed using DNA binding agents attached to a protein or synthetic ligand (Smith *et al.*, 1997). Immunotherapy trials using lipid/DNA delivery systems are ongoing (Roth and Cristiano, 1997) but, at present, purely synthetic vectors are not as efficient in gene delivery as their viral counterparts. A major limitation of the purely synthetic vectors appears to be the efficiency of DNA transfer across the cytoplasm to the nucleus; greatly improved delivery can be attained by incorporation of inactivated adenovirus particles with the DNA/polycation/ligand complex (Maass *et al.*, 1995; Schmidt *et al.*, 1997).

Final thoughts

It is now 10 years since the first clinical trials of immunomodulatory gene therapy in cancer patients and, at the time of writing, over 380 gene therapy protocols have been approved worldwide, of which 240 involve cancer patients (Wiley Genetic Medicine Clinical Trials Database). More than 3,000 patients have entered gene therapy clinical trials, and it can be concluded that the procedures are generally safe. Safety must, of course, remain high on the agenda, especially for replication-competent viral vectors that have the potential to spread, and for supposedly replication-defective viruses with the potential for inadvertent generation of contaminating, replication-competent virus. Nevertheless, experience suggests that the risks can be suitably controlled by good design of the vectors and packaging cells, and by careful monitoring of production.

It is now firmly established that many tumours do express antigens that can

serve as the basis for specific recognition and killing by CTLs. Despite this, cancers obviously are able to develop within immune-competent individuals. The paradox is probably best explained by the hypothesis that, during tumour development, the TAAs are not presented to the immune system in a suitably immunogenic manner (Antonia *et al.*, 1998). Thus, TAA-reactive T cells are more likely to be anergized by the tumour itself, than to be activated by encounter with TAAs optimally presented by professional APCs, together with all the necessary co-stimulatory signals.

Because of such T cell anergy, we should expect it to be more difficult to induce responses in human cancer patients than in animal models where the immune response can be induced before tumour challenge. However, some experimental protocols have addressed this issue, and it has been possible to 'cure' pre-existing tumours in mice by immunotherapeutic strategies. Human tumours are likely to present a more difficult target because of the time scale over which they have developed and a correspondingly greater degree of heterogeneity, allowing the selection of antigen-loss variants. Other mechanisms for tumour avoidance of immune destruction include the down-regulation of MHC expression or other steps in the antigen presentation pathway (Garrido *et al.*, 1993; Hicklin *et al.*, 1999), or the secretion of immuno-suppressive factors such as transforming growth factor (TGF)-β (Letterio and Roberts, 1998).

In view of these difficulties, we should not be unduly disappointed that clinical trials to date have demonstrated rather little unambiguous therapeutic efficacy. One should bear in mind that the majority has involved patients with advanced disease, and draw encouragement from evidence of induced immunological responses. Our understanding of the immune system is continuing to advance rapidly and the recent development of techniques for the detection of T cells of a given antigenic specificity, based on their ability to bind a specific tetrameric, fluorescently-labelled, soluble MHC-peptide complex or to secrete IFN-γ following appropriate contact with cognate peptide-presenting cells detected *in situ* by the ELISPOT assay, will greatly enhance our ability to monitor T cell responses to TAAs (Romero *et al.*, 1998a; Romero *et al.*, 1998b).

In parallel with improved immunological techniques and understanding, the vectors used for gene transfer are also continually being improved. The modifications aim to achieve more efficient, targeted gene delivery and expression, better control over the level of transgene expression, greater ease of production, and reduction of unwanted side effects.

Clearly, gene therapy for cancer, as for any other disease, has not provided a 'quick fix'; however, there is potential. Gene transfer enables us to alter the biological properties of cells in a very precise manner, and the possibilities are virtually endless. All aspects of the process are amenable to considerable further optimization. Following initial demonstrations of safety in patients with advanced disease, it will be possible to use gene therapy in patients with lower tumour burden, eg with minimal residual disease following surgery, where there is a greater chance of therapeutic benefit. In the medium to long term, the chances are high that some of the immunostimulatory gene therapies for cancer currently under development will be of proven clinical benefit and will become part of routine cancer management.

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PART 7

Polysaccharide Biotechnology