Cancer vaccines and **immunotherapy**

Rapid progress in the definition of tumour antigens, and improved immunization methods, bring effective cancer vaccines within reach. In this wide-ranging survey, clinicians and scientists at the forefront of these developments review therapeutic cancer vaccine strategies against a variety of diseases and molecular targets.

Intended for an interdisciplinary readership, chapters cover the rationale, development and implementation of vaccines in human cancers generally, and with specific reference to cancer of the cervix, breast, colon, bladder and prostate, and to melanoma and lymphoma. Target identification, delivery vectors and clinical trial design are reviewed, and the book begins and ends with lucid overviews from the editors, including the most recent developments.

Encapsulating recent scientific progress and the likely clinical potential of cancer vaccines, this book provides an essential introduction and guide for oncologists, immunologists and, indeed, all clinicians treating cancer patients.

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1

Immunity and cancer

Peter C.L. Beverley, Miles W. Carroll and Peter L. Stern

Introduction

As early as the turn of the century, Paul Erhlich suggested that 'aberrant germs' (tumours) occurred at a high frequency in all humans but were kept in check by the immune system. Developments in understanding of the protective roles of antibodies and phagocytes in infectious disease in the early years of the century led to attempts to stimulate the immune system to reject tumours. The New York surgeon, Coley, used bacterial vaccines to cause a 'commotion in the blood' and occasional regressions following treatment or occurring spontaneously were taken as evidence of an effective immune response.

Early experimental work demonstrated that transplanted (allogeneic) tumours usually regressed. However, it was soon realized that this was a consequence of the genetic disparity of host and tumour and was revealing immune responses to foreign tissue transplants, not tumour antigens. However, what these early studies did show was that a strong immune response could prevent the growth of a tumour and cure the animal.

Immune surveillance

In the 1950s, Burnett[1] and Thomas[2] restated Erhlich's idea as the theory of 'immune surveillance'. It was proposed that the immune system was able to recognize abnormal cells, which were destroyed before they could develop into a tumour. Since tumours do develop in many individuals it was also suggested that the immune system played a role in delaying growth or causing regression of established tumours.

The strongest evidence for an effect of the immune system on tumours derives from the association between immunosuppression and increased tumour incidence. In kidney transplant recipients, many of whom have been followed for over 20 years, there is quite clearly a greatly increased frequency of tumours. On closer examination this data is not quite so straightforward as it at first appears. On the

Hepatitis B	Carcinoma of the liver
Human papillomaviruses (HPV) 16, 18 and other oncogenic types	Carcinoma of the cervix
Papillomaviruses	Carcinoma of the skin
Epstein–Barr virus (EBV)	Burkitt's lymphoma, nasopharyngeal carcinoma. Possibly Hodgkin's disease
Human herpes virus 8	Kaposi's sarcoma
Human T cell leukaemia virus-1 (HTLV-1)	Adult T cell leukaemia

Box 1.1. Viruses and human tumours

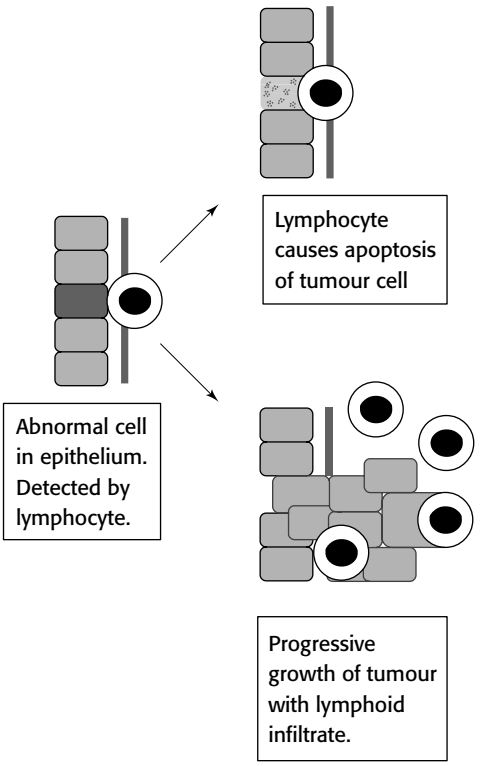
one hand, there is a large increase in the frequency of several tumours in which viruses are known to be involved (see Box 1.1); on the other, there is also a slight but definite increased risk for many other cancers in which viruses are not known to play a role[3].

These data strongly suggest that the immune response may be most effective in preventing the spread of potentially oncogenic viruses. Recent evidence that the incidence of hepatic carcinoma decreases following the institution of mass hepatitis B vaccination campaigns strongly supports the view that the immune system can be highly effective in preventing cancer, in this case by preventing infection with oncogene hepatitis B virus[4].

Experiments in immunosuppressed animals support the view that immune surveillance is largely directed towards viruses rather than tumours[5]. Many experiments have subsequently shown that cellular immune responses, mediated by thymus-derived (T) lymphocytes, are the key protective responses against viruses. These experimental data do not imply that there is *no* immune response to the majority of tumours but suggest that, for the majority of tumours, the immune response may be *relatively* ineffective (Figure 1.1).

The immune system and cancer

Although the evidence discussed above implies that the immune response against most nonviral tumours is ineffective, underlying the work discussed in the following chapters of this book is the assumption that antigen-specific immune responses against tumours *are* relevant. This assumption rests, first, on the idea that tumours are sufficiently distinct from other host cells that the immune response can distinguish between them; and, second, that an appropriate tumour-specific response



Tumours often have a lymphoid infiltrate, which may be associated with a good prognosis.

Spontaneous remissions occur.

Tumours occur mainly in the young and old when the immune system functions less well.

Tumours occur in immunosuppressed or genetically immunocompromised individuals.

Figure 1.1 Immune surveillance and failure of surveillance

can cause tumour regression or elimination. A list of tumour antigen types and their potential immune recognition is given in Box 1.2.

Many tumour cells are distinguishable from corresponding normal cells using antibodies. First polyclonal antibodies then murine mAbs were used to identify tumour-associated antigens[6]. Not all aberrantly expressed molecules provoke an immune response by the host but passive immunotherapy may be directed at antigens which are well expressed on tumours, so long as side effects due to targeting of any normal cells expressing the antigen are acceptable. This principle underlies the use of most antibodies in immunotherapy and many trials have been carried out with mAbs which are known to target some normal cells as well as tumours[7].

There is also abundant evidence that nonviral tumours express antigens to which the host immune system can respond. Recently, host antibodies have been used to clone a number of antigens[8] (see Chapter 8) and pioneering work by Boon and his colleagues has firmly established that melanomas and other tumours express antigens recognized by T lymphocytes. They carried out in vitro mixed lymphocyte–tumour cultures to restimulate cytotoxic T lymphocyte precursors (CTLp).

Mechanism	Detection	Example
Point mutations, deletions and translocations generate new amino acid sequences	Host T cells	ras, p53, bcr-abl, etc.
Increased expression of highly tissue specific gene products	Host T cells	Mage-1, tyrosinase, prostate-specific antigen (PSA)
Expression of oncofetal antigens	Antibody	Carcinoembryonic antigen (CEA), α-fetoprotein
Aberrant glycosylation	Antibody Possibly host T cells	MUC-1, T and Tn antigens
Expression of normally inaccessible antigens	Antibody	CEA, α-fetoprotein
Viral antigens	Host T cells Antibody	HPV-16, EBV, HTLV-1 HHV-8, HepB
Expression of single cell specific antigens	Antibody Host T cells	Idiotypes of B- and T-cell tumours

Box 1.2. Origin and detection of tumour antigens

The resulting CTLs were used to define and clone many antigens of melanoma cells recognized by host T cells[9] (see Chapter 11).

That immune mechanisms can contain or eliminate tumours is also no longer in doubt. The data from animal experiments with allogeneic tumours showed that a tumour *could* be eliminated if completely foreign to the host. Later experiments showed that a small number of antigen-specific CTLs can cause complete regression of a tumour[10]. Similarly, in human posttransplant EBV lymphoma patients, infusion of immune T cells can cause complete tumour regression[11] and antibody-mediated therapy of a lymphoma caused regression of large tumour masses and a very long remission in the first patient treated[12]. The undoubted effects of IL-2 in some melanoma and renal cell carcinoma patients[13], and of BCG in bladder cancer (see Chapter 2), is also strongly suggestive of an effective cellular immune response even when induced nonspecifically.

Nevertheless, despite the undoubted existence of tumour-associated or tumour-specific antigens and the encouraging precedents for therapeutic effects described above, tumours do arise, grow and frequently kill patients. The remainder of this chapter attempts to illuminate this paradox by discussing the mechanisms of immune responses and how these might influence immunotherapeutic strategies.

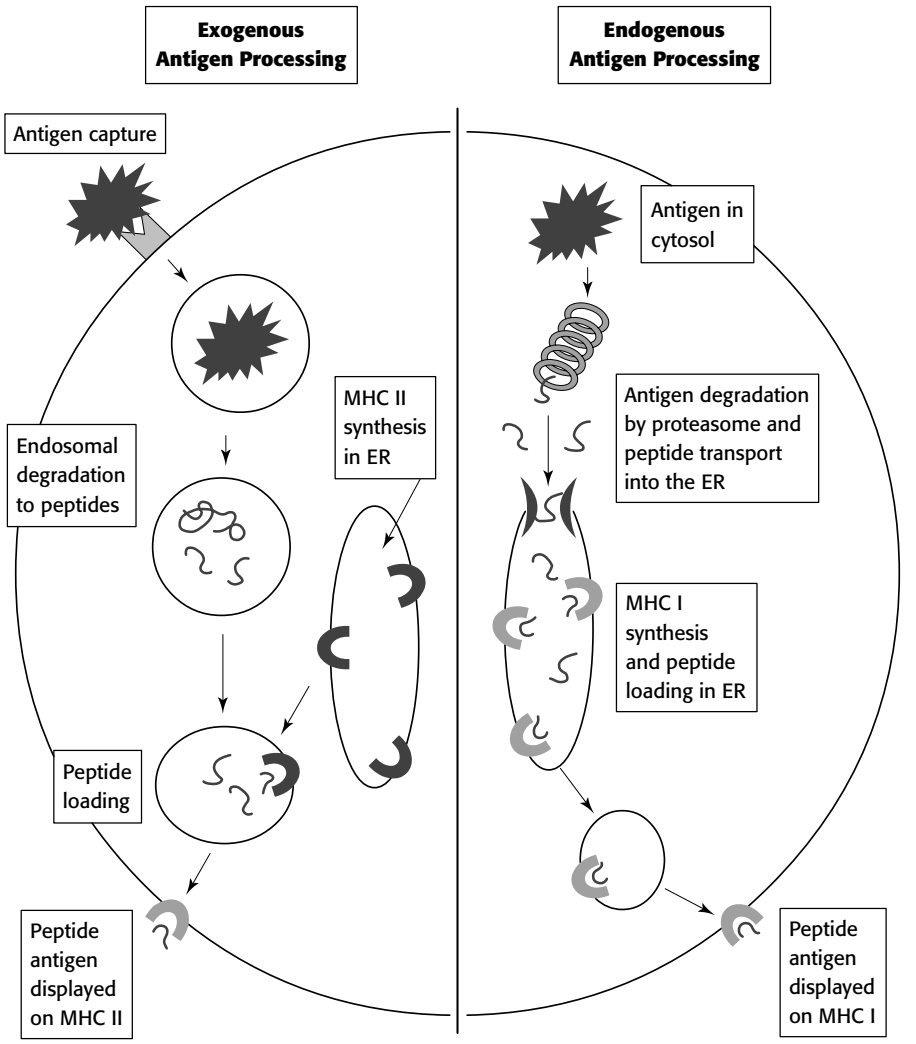


Figure 1.2 Antigen processing

Antigen recognition

Antigen processing

Antigen recognition by antibody occurs through the interaction of the binding site of an antibody molecule with a complementary three-dimensional structure (an epitope) on another molecule, the antigen. Although this may be complicated because the antigen is fixed in an array (for example in a cell surface) and because of the multivalent nature of antibodies, it is essentially a simple interaction. This is not the case for recognition of antigen by T cells. The key features of this process are illustrated in Figure 1.2.

Exogenous processing	Endogenous processing
The antigen must be taken up by specialized antigen-presenting cells. Danger signals are needed to initiate processing in APC.	Antigen must be synthesized in the cell. Processing can occur in any MHC-1 positive cell. Danger signals upregulate processing.
The peptides generated are dependent on: the specificity of processing enzymes, the glycosylation of the protein, the flanking sequences of the epitope, the cytokine microenvironment of the cell.	
	Peptides generated in the cytosol must be recognized by peptide transporters to enter the ER.
Peptides need to bind to the MHC I or II alleles of the processing cell in order to be displayed at the cell surface. Different alleles bind peptides with different motifs.	

Box 1.3. Factors influencing antigen presentation

Antigen processing for presentation on either major histocompatibility class I or II antigens (MHC I or II) is a complex process and the selection of peptides to be displayed is governed by factors which operate at each level of the processing mechanism (see Box 1.3). Processing of antigens is inefficient in the absence of 'danger signals'. These are nonantigen-specific signals, which indicate to the immune system that it has encountered a foreign material[14]; examples are bacterial lipopolysaccharide or specific sequence motifs of the DNA and RNA of micro-organisms. Danger signals are recognized by evolutionarily conserved receptors and are particularly effective in activating specialized antigen-presenting cells (APC) to process and present antigen.

The last step in the process, the binding of processed peptides to MHC molecules, is a critical step. The MHC is a highly polymorphic system and each allele binds a different set of peptides. For MHC class I the peptides are generally 8–10 amino acids long and binding is greatly influenced by one or two key 'anchor' residues, which fit into pockets in the MHC binding groove. The nature (charged, hydrophobic, etc.) and positions of the anchor residues in the peptide sequence make up a peptide binding motif which differs for each MHC allele. For MHC class II the peptides are generally 12–15 amino acids long but sequence motifs again influence peptide binding and the motifs may be allele specific. The consequence

of this specificity of binding is that not all new protein sequences may be recognized by T cells as foreign. For this to happen it is essential that some peptides generated from the new sequence by processing, bind with sufficient affinity to host MHC molecules to stabilize them and allow their transport to the cell surface[15,16].

As a rule, viral infection has been thought to be controlled by CTLs restricted through MHC I following endogenous processing of, for example, viral antigens. Recently it has become apparent that exogenous presentation is critical for induction of an immune response during viral infection of peripheral tissues[17]. This is perhaps not so surprising, since it would make little sense for the dendritic cells critical to the cross priming events necessary for subsequent CTL development, to be susceptible to various viral escape mechanisms apparent in other types of infected cells.

Self-tolerance

Since MHC molecules are unstable at the cell surface in the absence of bound peptides, the fact that most tissue cells express low levels of MHC class I molecules and a variety of APCs express MHC class I and II, implies that antigen processing proceeds in the absence of danger. Elution and sequencing of peptides from cells has shown that many of the peptides displayed are derived from normal self-proteins. Since, in general, the immune system does not respond to these self-molecules there must be mechanisms to prevent this.

Early experiments suggested that the thymus plays a key role in the development of T lymphocytes, including the selection of 'useful' T cells and the deletion of 'harmful' self-reactive cells[18]. Positive and negative selection are complex mechanisms but involve the interaction of the T cell receptor (TCR) of thymocytes with MHC–self-peptide complexes on APC. Depending on the affinity of this interaction and the presence or absence of other signals (co-stimuli), the developing thymocyte may survive and proliferate or die. In the bone marrow, similar selective mechanisms operate on developing B lymphocytes. Negative selection is not a foolproof mechanism so that autoreactive T and B cells exist in the periphery. In general, only B cells with relatively low affinity for self-antigens are present in peripheral lymphoid tissue. Development of high affinity antiself-reactive antibody requires somatic mutation in activated B cells, a process needing T cell help. Autoreactivity of B cells is therefore controlled by T cells.

After developing T cells leave the thymus to seed the periphery, the repertoire of available T cells continues to be shaped by a variety of mechanisms. These are either dependent on death or functional inactivation of self-reactive T cells by a variety of mechanisms, but these usually come into play when lymphocytes encounter antigen in the absence of adequate co-stimulation[19] (see Box 1.4).

Thymocytes with high affinity for self-MHC + self-peptide deleted by apoptosis.

Peripheral T cells encountering MHC + peptide in absence of co-stimuli may be deleted or anergized.

T cells may ignore antigens presented without co-stimuli.

Activation of T cells is dependent on concentration of MHC-peptide and amount of co-stimulation.

T-cell responses may be suppressed.

Box 1.4. Mechanisms influencing the peripheral T-cell repertoire

Co-stimulation and initiation of responses

T lymphocytes are the key regulators of the immune system. Activation of T cells to become effector cells, requires another signal (signal 2) in addition to that delivered through the TCR (signal 1). The nature of signal 2 has been the subject of intense investigation over the last few years and it has become clear that many different ligand–receptor pairs on the antigen-presenting cell and the T cell play a role (Figure 1.3). Some of these are listed in Box 1.5.

An important point in considering this cellular interaction is that it is a two-way process. As well as receiving signals from the APC, the T cell delivers signals to it and the consequence is activation and differentiation of both cell types. There is abundant evidence that the key antigen-presenting cell type in primary activation of T cells is the dendritic cell (DC)[20].

Recent evidence suggests that the sequence of events requires, first, that the DC is activated by 'danger' signals. Following this a process of maturation occurs, with up-regulation of key co-stimulatory molecules on the DC surface including CD80 and 86. In turn this initiates T cell activation and up-regulation of T cell surface molecules such as the IL-2 receptor, which is essential for growth of T cells. At the same time T cell CD154 (the ligand for CD40) is expressed and this delivers a very strong signal for further activation to dendritic cells. Very recently it has been demonstrated that ligation of DC–CD40 enables DC to acquire the ability to activate naive cytotoxic T cell precursors (CTLp) without the necessity for further signals delivered by T helper (Th) cells[21–23]. CD40–CD154 interaction is therefore a key stage in the DC–T cell interaction.

Cytokines produced by both cell types have effects on growth and differentiation of the cells. IL-12 and IL-4/10 have been shown to be particularly important in directing the production of Th-1 and Th-2 effector cells[24]. Chemokines control the migration of both DC and lymphocytes during the initiation of an immune response and its effector phase[25] (see Box 1.5).

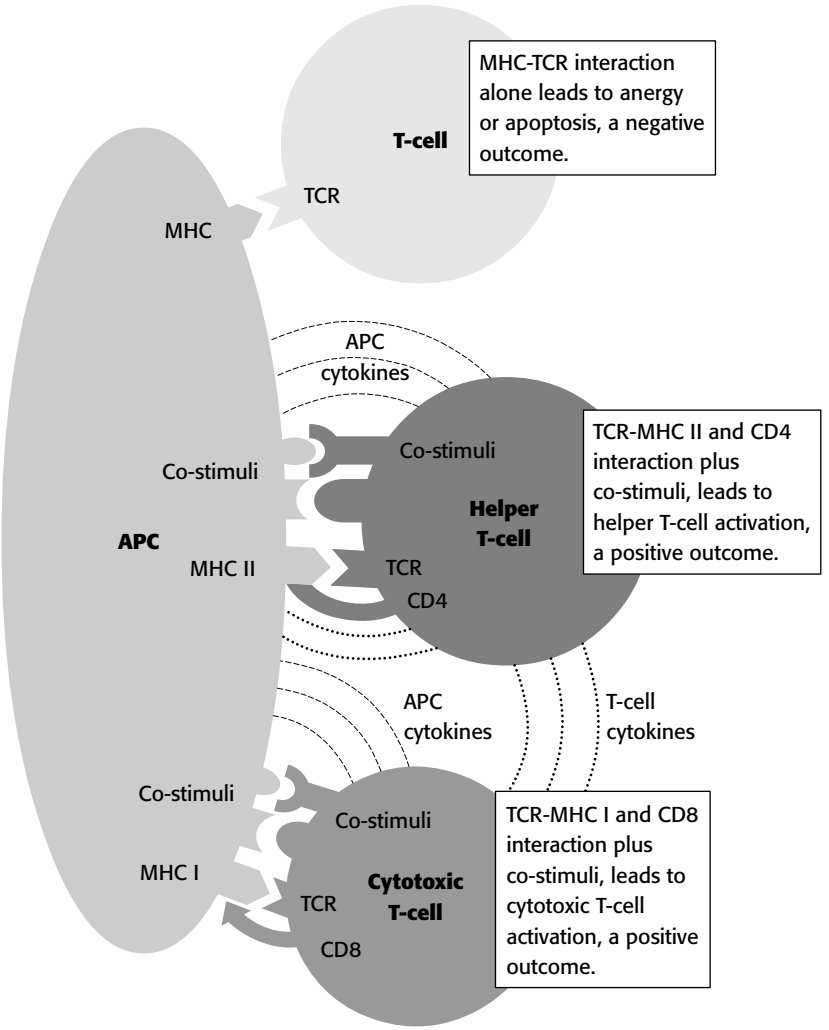


Figure 1.3 Co-stimulation

Priming of antitumour responses

In most immune responses to micro-organisms, priming is thought to occur in lymph nodes draining the site of infection. Initiation of the immune response requires a danger signal to alert the system. Without this DC will not be activated to process antigen, up-regulate co-stimulatory molecules and leave peripheral tissues to migrate to lymph nodes, where potentially responsive naive T cells encounter the antigen.

A small tumour may not initiate a response because it fails to deliver a danger signal. In contrast, once inflammation occurs in the tumour, perhaps through

APC	T cell	Outcome of interaction
MHC I	TCR	Delivery of Signal 1
MHC II	TCR	Delivery of Signal 1
CD4	MHC II	Facilitation of MHC II-TCR interaction
CD8	MHC I	Facilitation of MHC I-TCR interaction
CD56	CD2	Adhesion
CD11a/CD18	CD54	Adhesion and T-cell activation
CD80/86	CD28/CD152	Co-stimulation of T cells and activation of APC
CD40	CD154	Activation of DC and co-stimulation of T cells
	Cytokines in DC–T-cell interaction	
GM-CSFR	GM-CSF	DC growth and chemoattraction
TNFαR	TNFα	DC growth and maturation
IL-4R	IL-4	Bias to production of Th-2 effector cell sIL-6R
IL-10R	IL-10	Bias to production of Th-2 effector cells
IL-12	IL-12R	Bias to production of Th-1 effectors and CTL
IFNαR	IFNγ	Bias to production of Th-1 effectors and CTL
	Both DC and T cells express several chemokine receptors and chemokines	

Box 1.5. Cell-surface interactions between DC and T cells

breakdown of the epithelial barrier and entry of micro-organisms if the tumour is at a superficial epithelial site, or through tumour necrosis if it outgrows its blood supply, there will be an influx of inflammatory cells including DC. Necrotic or apoptotic tumour cells may provide a source of tumour antigen[26]. DC are stimulated and leave the tumour to migrate to draining nodes. Experimental evidence suggests that this is the main route for priming against tumour cells rather than direct priming by the tumour cells themselves[27]. Surprisingly this is the case for both the exogenous MHC class II and endogenous class I pathways, suggesting that in DC exogenous antigen can enter both processing routes[17].

While tumour antigen will eventually reach the draining node is there likely to be a high frequency of potential responder T cells? In theory, since many tumour antigens are unaltered self-molecules, high affinity responsive cells should have been deleted in the thymus, but in practice deletion is incomplete and T cells reactive to self-antigens including tumour-associated molecules, have been repeatedly demonstrated. Whether they are present at lower frequency or have lower affinity than T cells capable of responding to exogenous antigens, is currently unclear. In any case by the time patients present for immunotherapy, it is likely that tumour-

reactive cells will have been primed and that effector cells may have re-circulated to enter the tumour site. Studies of tumour-infiltrating lymphocytes (TILs) provide support for this concept[28]. Strategies for immunization against a growing tumour may therefore aim to prime naive lymphocytes or to boost pre-existing immunity.

Immunotherapeutic immunization strategies

The key factors in any attempt to generate or boost antitumour immunity is the delivery of relevant and immunogenic tumour antigens to professional antigen-presenting cells. This critical step is fundamental in the generation of any primary specific cellular or humoral immunity. This may result from a nonspecific activation induced locally by delivery of BCG (see Chapter 2) or the use of irradiated allogeneic tumour cells with cytokines and/or co-stimulatory molecules. Adjuvants (including cytokines) are usually utilized when immunizing with protein or peptides (Chapters 5, 6, 7 and 11) whereas pox viruses encoding tumour target antigens act as a potent inducer of the danger signals associated with APC activation etc. (see Chapters 3, 4 and 5). DNA vaccines must eventually lead to expression of tumour antigens and their processing by APCs (Chapter 12), whereas direct delivery of tumour antigens as proteins, by cell fusion or by cDNA to dendritic cells represents the most direct approach to attempt to generate antitumour immunity (see Chapter 13). These approaches are frequently biased by the prejudice that specific T cell immunity is likely to be of greater relevance in tumour therapy. However, the role of antibodies directly (e.g. Chapter 7) or indirectly (see Chapter 8) and generally in tumour immunity is probably being underestimated (see Chapter 10) and may be of critical importance in some virally associated tumours (Chapter 9).

One group of immunization strategies uses tumour cells as the immunogen on the assumption that many tumour antigens may not yet be defined. It is often assumed that the tumour cell presents its own antigens but since many tumours exhibit MHC class I down-regulation and lack MHC class II as well, they are unlikely to be optimal APC even if this does occur. However, many attempts have been made to remedy this by transduction of the tumour cells with genes for some of the missing molecules. The logic of this is obscure if immunization occurs, not through presentation of antigens by tumour cells themselves, but by processing of tumour-derived antigen in host antigen presenting cells. Additionally, although one or two co-stimulatory or MHC molecules can be inserted into a tumour cell, it is highly unlikely that this will make it present antigen as efficiently as a 'professional' antigen-presenting cell (usually a dendritic cell).

An alternative type of strategy attempts to ensure that tumour antigen reaches antigen-presenting cells. This can be achieved by transducing tumour cells in vivo with genes for cytokines or chemokines (e.g. GM-CSF), which might attract

Aim	Strategy
Prime naïve T cells	Present tumour antigen with adjuvant (danger signal) to initiate a response and clonal expansion
Boost memory T cells	Present tumour antigen with adjuvant to induce activation and further clonal expansion
Activate pre-existing specific effector cells	Local or systemic cytokines (IL-2)
Activate nonspecific effector mechanisms	Local or systemic cytokines
Relieve immunosuppression or modulate the immune response	Local or systemic cytokines, immunomodulatory agents

Box 1.6. Active immunotherapy

antigen-presenting cells to the lesion[29]. Alternatively, in vitro grown tumour cells may be transduced, inactivated and used as an immunogen. A logical extension is to use DC directly loaded with tumour antigens in vitro as the immunogen[26]. There are now many approaches focused on specific or nonspecific immunization using DC (see Chapter 13).

The antigen need not be in the form of tumour cells since tumour antigens are rapidly being defined. Subunit vaccines of various types have the advantage that they remove irrelevant molecules and potentially interfering or immunosuppressive ones. The down-side is that if few T cell epitopes are included in the vaccine there may be no epitopes, which bind with high affinity to the MHC alleles of some vaccinees, since each allele binds epitopes with a particular sequence motif[30]. Various strategies are summarized in Box 1.7.

Strategies aimed at DC have the advantage that ultimately they target lymph nodes, mimicking the physiology of a normal immune response (Figure 1.4). Another possible advantage of methods employing in vitro transduced cells is that they may be injected at a site distant from the tumour, avoiding the problem that the tumour itself may produce immunosuppressive substances such as the cytokine TGFβ[31] and that these may reach tumour-draining nodes. Unfortunately, tumour patients' T cells sometimes exhibit poor responsiveness in vitro, suggesting a systemic immunosuppressive effect of tumours[32]. This has been attributed to abnormalities in expression of the CD3ζ chain, which is involved in signal transduction. What causes this is a matter of debate and how specifically related to cancer is the defect is not established, but the functional abnormality can sometimes be reversed in vitro and possibly in vivo by IL-2[33]. This is discussed in Chapter 5.

Problems

The tumour antigens need to be defined.
Single T-cell epitopes are often MHC allele specific.
Subunits lack danger signals.

Advantages/solutions

Removal of irrelevant but competing or suppressive antigens.
Adjuvants, helper antigens, cytokines or co-stimuli can be easily combined with subunits.
Multiple epitopes from different antigens can be combined in epitope strings to overcome the allele problem.
The vaccine can be designed to generate appropriate immune responses.
Vectors can be tailored to achieve optimal immunization.

Methods for administration

Peptides with or without adjuvant.
Recombinant proteins with or without adjuvant.
Glycoconjugates with helper epitope and adjuvant.
Recombinant viruses (e.g. vaccinia-MUC-1 or Vac-HPV-16 E6 and E7, with or without cytokines).
DNA, combining antigen with co-stimuli or cytokines.

Box 1.7. Subunit vaccine strategies for tumours

Effector function

The immune system has multiple effector mechanisms for combating invading micro-organisms (see Box 1.8) but it remains unclear which of these are most effective against tumours. Complicating the issue is the enormous variation in the behaviour of different tumour types, so the most important effector mechanism may well differ depending on the tumour type.

The available animal experimental data is not particularly helpful. Evidence for some mechanisms is mainly based on in vitro experiments and extrapolation from immunohistology. For example, macrophages are abundant in many tumours and can be shown in vitro to inhibit the growth of tumour cells, but it is less clear what role they play in vivo. The role of antibodies produced by the host itself is also controversial, although such antibodies have proved to be an important tool for definition of tumour-associated molecules (Chapter 10). On the other hand, monoclonal antibodies (mAbs) have been shown in humans to be able to localize tumours and have been demonstrated convincingly to delay the onset of tumour progression and increase survival in a randomized trial of a mAb as adjuvant

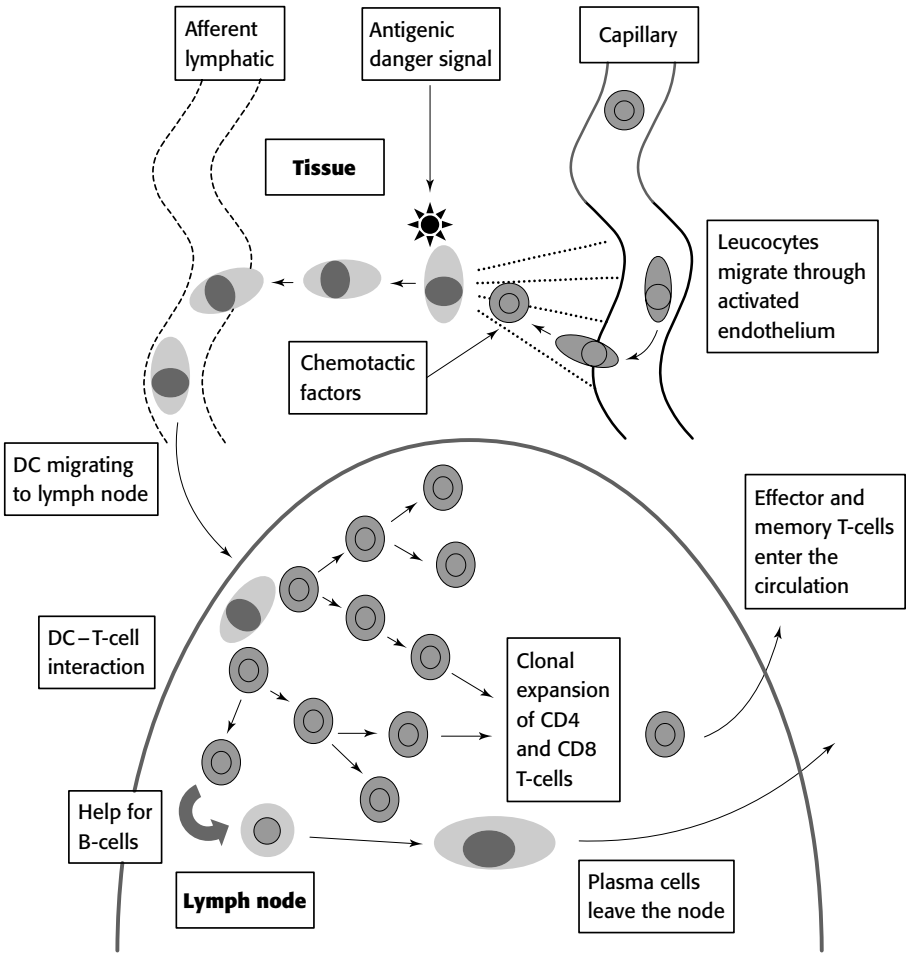


Figure 1.4 The anatomy of an immune response

therapy for colon cancer[7]. The mechanism of this effect has not been elucidated, nor are the mechanisms which have occasionally led to remissions in lymphoma patients treated with anti-idiotypes or to tumour dormancy in experiments in a murine model[34] (see Chapter 8). As yet there have been few attempts to generate high titre antibodies to tumour-associated antigens in humans except in trials targeted to idiotypes of B cell tumours[35] (see Chapter 12). Whether antibodies to other surface antigens, generated by active immunization of the host, might be effective particularly against small metastases, remains to be properly investigated.

The evidence that allograft rejection is mediated by T cells has led many investigators to focus on T lymphocytes as antitumour effectors. In mouse experimental models, there is evidence for the participation of T cells in protection against

Humoral

Antibody blocking (for example, of growth factor receptors).
Antibody-induced apoptosis.
Antibody- and complement-mediated lysis.
Cytokine-mediated cytostasis or cytotoxicity (e.g. cytostatic effects or interferons or cytotoxicity of TNFα).

Humoral and cellular

IgE-mediated allergic reactions involving basophils and mast cells.
Antibody-mediated cellular cytotoxicity by natural killer cells and macrophages.

Cellular

Natural killer cell cytotoxicity.
Cytostasis and cytotoxicity mediated by activated macrophages.
T cell cytotoxicity
  by $\alpha\beta$ T cells
  by $\gamma\delta$ T cells

Box 1.8. Immune effector mechanisms

tumour challenge (an artificial situation in which the animal is first immunized against the tumour and then challenged with viable tumour cells) and in rejection of established tumours. Evidence described earlier indicates that small numbers of activated cytotoxic T lymphocytes (CTL) can certainly eliminate relatively large tumour masses under optimal circumstances, and many human tumour antigens have been defined using CTL, so that there continues to be a concentration of effort on immunization against MHC class I binding epitopes.

The overwhelming problem of this strategy is the loss of MHC class I molecules, which is such a prominent feature of human tumours. This may be allele specific or global and several molecular mechanisms have been defined, including mutations in the peptide transporters, in MHC molecules themselves and in β2-microglobulin[35]. Loss of MHC molecules suggests that the T cell immune response applies selective pressure to tumour cell populations, but it also implies that by the time a tumour is detectable it may already have been selected for resistance to the T cell antitumour immune response. Although natural killer (NK) cells may recognize better the cells which express low levels of MHC[36], few NK cells can be demonstrated in most tumours and infusions of lymphokine activated killer (LAK) cells have not been notably successful. All this suggests that MHC loss is likely to be a major bar to immunotherapy aimed at stimulating CTL.

Conclusions

Immunotherapy has undergone many ups and downs during this century. What is unarguable is that the immune system can destroy large tissue masses if it can be brought to bear on them. Recent data suggest that human tumours do differ from their hosts sufficiently for them to be recognized as foreign, although the frequency and affinity of the responding cells is not clear. Tumours may be initially poor immunogens because they lack danger signals and produce immunosuppressive substances, which interfere with immune responses. Once an immune response is generated, there is evidence for escape through down-regulation of MHC molecules.

All this makes it clear that therapeutic active immunization may be difficult. Early institution of immunotherapy is likely to be more effective, when the tumour has had less chance to escape and the immune system has not been damaged by chemotherapy. It also makes sense to target as many antigens as possible, making escape more difficult. Rapid progress in definition of tumour antigens and improvements in methods for immunization, will mean that it will at least be possible to test whether optimal immunization to obtain a large and broadly targeted response, will be an effective therapeutic anticancer modality. This volume details the present state of the art, although as yet this goal has not been reached.

Historically, immunization has been most effective when administered prophylactically. Definition of more and more tumour antigens may open the way to prophylactic immunization for nonviral as well as viral tumours, at least in high-risk groups. The problem of immunoselection may in the future be overcome by using T cells engineered to recognize antigen through an introduced antibody receptor. Antibody itself can be effective. A conservative view is therefore that, in the next decade, some forms of immunotherapy will take their place as standard cancer treatment.

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2

Immunotherapy of bladder cancer

Anton B. Alexandroff and Keith James

Introduction

During recent years there have been major strides in our understanding of both the molecular basis of malignant disease and the cellular and molecular immunological response thereto. Nevertheless, despite these exciting developments, arguably the most successful immunotherapeutic approach for the treatment of any malignancy is the relatively unsophisticated approach, involving the intravesical administration of BCG in transitional cell carcinoma of the bladder. In this chapter we briefly describe the natural history of bladder cancer, the conventional treatments for this malignancy, the effects of BCG on the immune system and bladder cancer in vitro and in vivo, and speculate on its possible mode of action. Finally, we comment on the problems associated with this treatment and highlight current and future developments aimed at further improving the antitumour potential of BCG and other mycobacteria. For other excellent reviews in this area we would recommend Jackson and James[1], O'Donnell and DeWolf[2], Martinez-Pineiro and Martinez-Pineiro[3], Lamm[4–6], and Kurth[7].

Bladder cancer as a disease

Bladder cancer is the fourth most common cancer among men and the eighth most common among women[8]. The annual incidence of bladder cancer in the USA is 54 000 patients and the total number is currently estimated at 300 000 (O'Donnell, personal communication). This results in over 11 000 deaths a year[8]. Females are relatively spared with a ratio of 1 to 3, possibly due to the different occupations and environments to which they are exposed[9]. In general, the same picture is true for the European Union with over 53 000 new cases of bladder cancer diagnosed every year and its being responsible for 4% of cancer deaths[10] (Figure 2.1A) with the highest incidence observed in Denmark, Belgium, Italy and the UK. In the UK there are over 9000 new cases detected every year, according to the Cancer Research Campaign Yearbook 1995/96. Once again it is the fourth most common malignancy, only exceeded by skin, lung and prostate cancer (Figure 2.1B). It is also the

sixth most common cause of cancer deaths (it shares this place with pancreatic cancer) (Figure 2.1C). Furthermore, the incidence of bladder cancer continues to increase[10–13]. It should also be noted that although bladder cancer mortality is declining in the USA and Canada, it continues to increase in males in many European countries[14].

In common with many cancers, bladder cancer is a disease of the old and peaks after the sixth decade. It is also more prevalent in industrialized countries. Interestingly, however, Asian or New Zealand immigrants to Britain retain a lower risk of bladder cancer[15–17]. Furthermore, even within the same country Afro-Caribbeans are relatively spared compared to Caucasians[18].

Bladder cancer was one of the first cancers to be recognized as being associated with certain occupations. It is more common in workers in the rubber, petrol, dye, leather, printing and aluminium refining industries and among hairdressers[19,20]. The relatively higher prevalence in lower social classes may be partly due to their comprising a greater proportion of the industrial work force[21]. Some of the causative agents are likely to be aniline dyes and benzedrine compounds[22]. There is also a strong association with smoking[12,13,23]. In developing countries bladder cancer is frequently associated with schistosomiasis[12,24]. As a secondary cancer it may be associated with irradiation of the pelvis[25].

Most cases present as superficial cancer with symptoms of haematuria and dysuria[5,26]. Of these, about 90% are transitional cell carcinoma, with the remainder comprising squamous cell carcinoma and adenocarcinoma. Depending on its histological appearance transitional cell carcinoma is divided into grades I–III. It is also staged depending on the depth of invasion. Lesions which do not invade the lamina propria are classified as superficial forms of bladder cancer (pTa, PT1) and are susceptible to treatment by BCG immunotherapy. Advanced bladder cancer invades muscle layers and there is a high risk of its developing into system disease (pT2 and greater). Carcinoma in situ (Cis) is special in that it is diffusely spread, preventing complete surgical resection, and is also a high grade form. Left untreated it is likely to progress within five years to muscle invasive stage in up to 80% of patients[4]. Classification of bladder cancer is discussed in detail in an extended review by Lamm et al. [27]. In transitional cell carcinomas stage and grade remain the most important prognostic factors[28–31].

Conventional therapy

Superficial bladder cancer (pTa-pT1) can be successfully treated by surgical resection alone. However, 40–80% of tumours will subsequently recur and 10% of these will progress to the invasive stage or metastasize within three years[7,32] with overall progression averaging 12% per year[33]. Consequently, life-long follow-up is

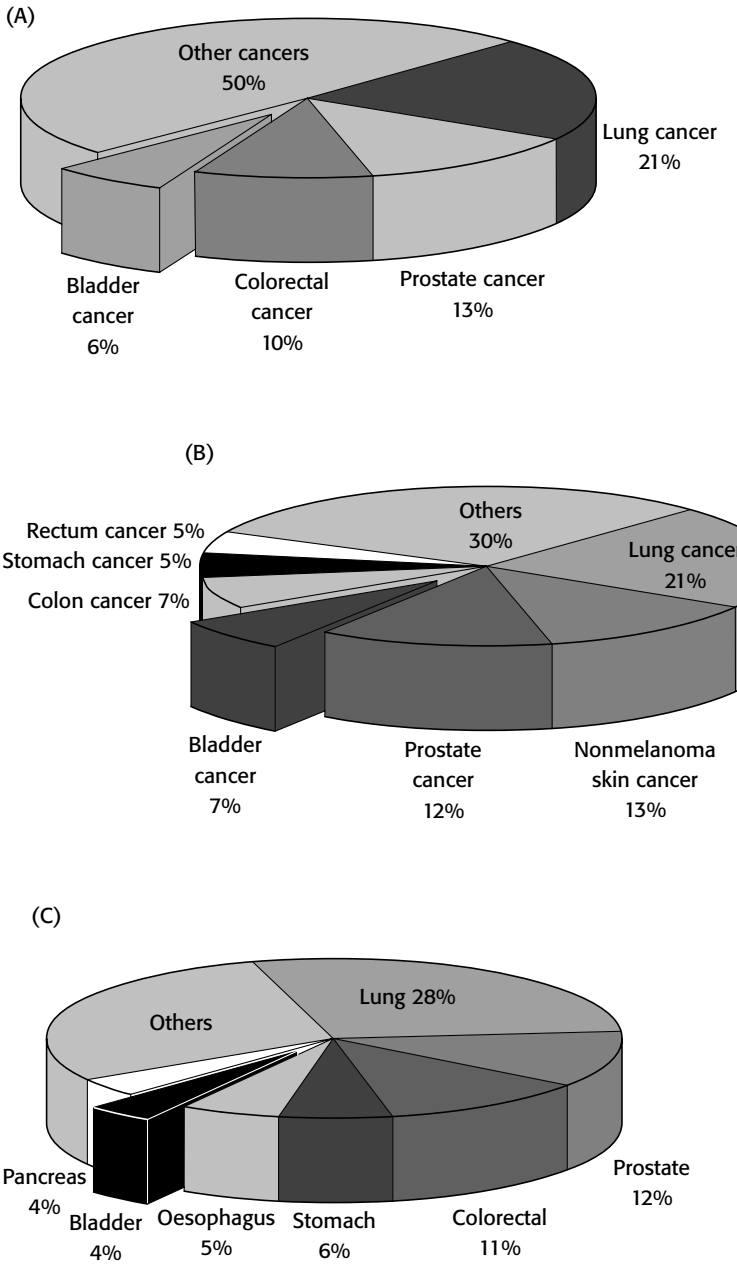

Figure 2.1 (A) Bladder cancer is the fourth leading cancer in the European Union and is inferior to only lung, prostate and colorectal cancers (based on Jensen et al.[10]). (B) Bladder cancer is the fourth most common cancer in the UK. Please note that in this figure colon and rectal cancers are split, otherwise bladder cancer would take the fifth place. (C) Bladder cancer also shares sixth place with pancreatic cancer as the most common cancer cause of death in the UK

Table 2.1. *Guidelines on therapy for superficial bladder cancer are outlined (based on Harris and Sarosdy[34]). Note that different approaches exist presently in different clinics. Advanced bladder cancer (pT2 and greater) is treated with partial or total cystectomy or palliatively and is out of the scope of the present review. BCG therapy is not usually employed for advanced stages except as a palliative treatment.*

Risks	Clinical characteristics	First line therapy	Special notes
Low risk of progression and recurrence	Primary, solitary and small pTa or pT1 stage and any grade	TUR ± single instillation of thiotepa	
Low progression risk, high recurrence risk	Large solitary tumours or primary multiple or any recurrent tumours of pTa stage and any grade	TUR + 6 months' course of intravesical mitomycin C or thiotepa	Should be given within 24 hours of TUR, or time of therapy should be increased
High progression risk	As above but pT1 stage and grade 2–3 or CIS	Intravesical BCG immunotherapy	Not for immuno-compromised patients; and should not be given soon after TUR

recommended for these patients. The highest progression is shown for pT1 G3 tumours[32]. It is more difficult to eliminate Cis only by surgical means due to its diffuse spread.

The treatment rationales are based on weighing risks and benefits[7,34]. Low risk tumours (primary, solitary, pTa G2–3, pT1 G1–3) have a less than 2% risk of progression and can be treated by transurethral resection (TUR) and a single instillation of thiotepa (Table 2.1). In patients with high recurrence but low progression risks (e.g. pTa G1–3 multiple primary or recurrent tumours or large tumours) instillation of either thiotepa or mitomycin C is necessary. Intravesical therapy should be given within 6–24 hours of TUR and, in this case, a six-month course is sufficient, otherwise 12 months' therapy should be given. Other chemotherapeutic agents used include Doxorubicin Epodyl and Epirubicin. They have a similar efficacy to thiotepa but are more expensive and have a higher level of side effects. Mitomycin C is the chemotherapeutic agent of choice for Cis treatment. Although it is regarded as inferior to BCG[34,35], a recent study has failed to find a difference between them[36]. High progression risk patients (pT1 G2–3, Cis) should be treated with intravesical BCG immunotherapy.

As might be expected, the survival of patients depends on the stage of the tumour at the time of presentation. For example, patients presenting with superficial cancer (pTa, pT1) had 80% and 69% five-year survival, while those with muscle invasive disease (pT2 or greater) showed only 23% five-year survival[37]. However, a more recent study suggests that bladder-sparing radical transurethral resection for muscle infiltrating carcinoma may yield five- and ten-year cancer-specific survival of 80% and 74%, respectively, with bladder preservation rates of 82% and 79%[38]. Similar results were reported in another study where a combination of methotrexate, vinblastine, adriamycin and cisplatin (MVAC) chemotherapy, followed by bladder-sparing surgery, led to a 74% ten-year survival[39]. It should be noted that 56% of patients developed recurrence during the eight years following treatment. Of those who required cystectomy it was life saving in 65%. It is important to stress that BCG immunotherapy is not effective for invasive bladder cancer (pT2 and greater) and can be used only as a palliative treatment[40].

BCG immunotherapy

BCG immunotherapy is superior to chemotherapy in the treatment of bladder cancers with a high risk of progression. This conclusion was drawn from the analysis of results from multiple clinical trials with over 4000 patients[41]. These trials compared transurethral resection alone with a combination of transurethral resection with either chemotherapy or BCG instillations. The average benefit of adding adjuvant intravesical agents was 14% for chemotherapy and 44% for BCG. Furthermore, combined data for four EORTC and two MRC trials (a total of 2535 patients) revealed a significantly prolonged duration of the disease-free interval in BCG-treated patients[42]. Chemotherapy failed to prevent progression and long-term recurrence (over five years) although it demonstrated short benefit (14%) in another analysis of over 2000 patients[43]. Given the carcinogenic effects of chemotherapy Lamm and associates have questioned the rationale for routine chemoprophylaxis[43]. Response rates for BCG vary between 50 and 60% for papillary tumours and between 70 and 80% for Cis[4,5]. These are sustained with remission averaging 70% for five years. Recent findings suggest that complete response for recurrent superficial transitional cell carcinoma (CIS, Ta, T1) treated with optimal BCG therapy can be as high as 87% (Schenkman and Lamm, unpublished). Importantly, a combination of induction and maintenance instillations increases complete response (from 73% to 87%), long-term disease-free status (from 65% to 83%) and survival at four years (from 86% to 92%).

In addition to preventing recurrence, BCG immunotherapy also reduces tumour progression[44]. Importantly, BCG immunotherapy can be given as a second-line agent, should initial chemotherapy fail[7].

Although highly efficient, BCG immunotherapy is not without problems. Immunotherapy works by activating the host immune system and therefore is not suitable for immunocompromised patients (including those with HIV, congenital immunodeficiency syndromes, secondary malignancy, treatment with immunosuppressive drugs, patients with tuberculosis, and pregnant or lactating women[3]. It is generally accepted that it also has a higher level of side effects than chemotherapy. Fortunately, most of them are mild and over 95% of patients tolerate therapy well. Up to 90% of patients may experience cystitis (frequency, urgency and painful urination) which may be associated with malaise and mild fever (see detailed analysis in Lamm[6]). These symptoms usually occur after the third instillation and may increase in severity as the course of therapy progresses; they can be treated symptomatically. Haematuria occurs in about a third of patients and may require a delay in instillations if severe. If symptoms persist over 48 hours or are very severe, daily prophylaxis of isoniazid is recommended. Isoniazid should not be routinely used as it is toxic for the liver and may reduce the effectiveness of BCG[6]. The most common severe symptom is high fever seen in 3% of patients (combined data of 2602 patients treated with different strains of BCG)[6]. In most of these patients symptoms resolve within two days, but some of them may progress to anaphylaxis or sepsis whereupon treatment in hospital is recommended. Other less common side effects (under 1% of patients) include granulomatous prostatitis, hepatitis, pneumatitis, arthritis, skin rashes, abscesses and urethral obstruction. Sepsis occurs in 0.4% of treated patients and may lead to a fatal outcome with at least ten deaths having been recorded to date. It is thought that inappropriate timing of instillation (during cystitis or soon after tumour resection) might have been responsible for most of these cases. In contrast to chemotherapy, BCG should not be administered immediately after tumour resection.

The association between mycobacterial disease and low incidence of cancer was first noted 100 years ago in pathological studies (reviewed in Jackson and James[1]). The first attempts to treat cancer by mycobacteria were attempted by Centanni and Rezzesi[45] at the beginning of this century. However, it was only with the development of the avirulent BCG strain (Bacillus Calmette Guerin) that mycobacterial immunotherapy became plausible. In the 1960s there were a lot of attempts to treat different human malignancies with BCG. Most of these failed to produce significant and/or reproducible results. The most promising results were obtained in the treatment of lymphoblastic leukaemia by Mathe et al.[46]. This group reported that as long as ten years after treatment a third of patients receiving BCG maintenance therapy were in remission, while there were zero survivors in the placebo group[47]. However, with subsequent development of better chemotherapeutic protocols BCG immunotherapy was forgotten. It should be noted though that many protocols used in the past killed either BCG or BCG derivatives.

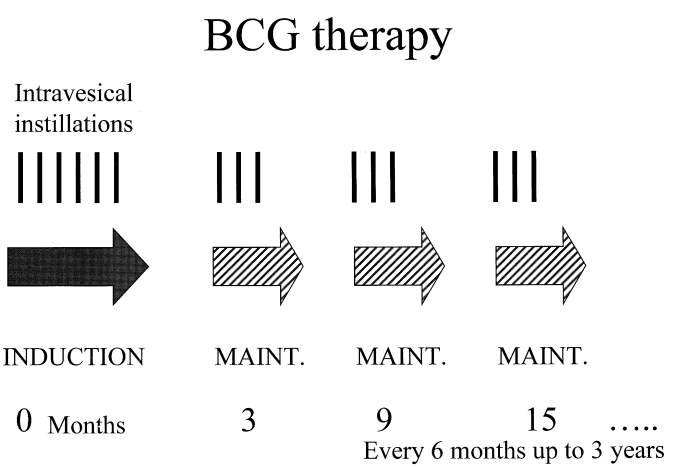

Figure 2.2 Induction course consists of six weekly instillations of BCG. After three months another three weekly instillations are given as a maintenance course. Maintenance courses are repeated thereafter every six months for three years. Note that the second and third maintenance treatment is given only if the preceding instillation was without increased side effects (based on a review of Schenkman and Lamm, 1998, unpublished)

Morales was the first to successfully use BCG for the treatment of bladder cancer[48]. His original protocol of six weekly instillations has survived almost unchanged until today. The previous practice of simultaneous percutaneous scarifications is no longer followed[49]. If patients have not responded, a second course of six weekly instillations is advised[3]. For responders the benefits of maintenance instillations are now widely accepted. These are given either as a thrice weekly treatment every 3–6 months or are continued on a monthly basis[2] (Figure 2.2). One of the recommended protocols consists of an induction course of six weekly instillations followed by three weekly instillations at three months and then every six months for three years (Figure 2.2).

The long-term effects of BCG immunotherapy of high risk superficial bladder cancer have recently been reported[50]. Despite the success achieved to date with BCG it is estimated that currently only 8% of patients in the USA are treated with BCG, while elsewhere its application may be even less (O'Donnell, personal communication).

Other biological therapies have been suggested and tried. They include interferon alpha, *Lactobacillus casei*, bropirimine and keyhole-limpet haemocyanin (KLH). All of them are currently inferior to BCG immunotherapy and are reviewed elsewhere[7,51]. A recent report suggests that photodynamic therapy may be safe and effective for treatment of resistant superficial bladder cancer[52]. However, further studies are required to evaluate its potential.

Table 2.2. *Direct effects of BCG on both immune system and tumour are listed in the order of decreasing significance and/or probability. Induction or expression of cross-reactive antigens on cancer cells has been suggested but such cross-reactive or induced antigens have never been characterized.*

(1)	Effects on tumour
	Cytotoxic and cytostatic effects
	Production of cytokines and chemokines (TNF alpha, GM-CSF, IL-6, IL-8)
	Induction of adhesion molecules (e.g. ICAM-1)
	Induction of MHC Class I and II antigens*
	Induction of CD40, FAS and other co-stimulatory molecules*
	Induction of putative cross-reactive antigens†
(2)	Effects on host immune system
	Production of pro-inflammatory cytokines (mostly TH1 spectre, e.g. IFN gamma, TNF beta and alpha, IL-2)
	Induction of BAK cells
	Stimulation of tumoricidal activity of macrophages
	Expansion and stimulation of tumoricidal activity of gamma/delta T lymphocytes
	Recruitment/activation of lymphocytes, monocytes and granulocytes*
	Induction of BCG-tumour cross-reactive CTLs†

Notes:

* These effects are secondary to cytokine production by tumour cells or the host immune system.

† These effects may occur but have still to be demonstrated.

Mechanisms: effects on host immune system in vitro

A number of studies have clearly demonstrated that mycobacteria can exert many effects on immune cells such as T lymphocytes and macrophages. These include inducing cytokine production, cell proliferation and, importantly, stimulation of their antitumour activity. In addition they may also induce or enhance the expression of other molecules playing a key role in host tumour cell interactions including adhesion and co-stimulatory molecules. The effects may be achieved with whole live mycobacteria, killed bacilli, conditioned media, mycobacterial heat shock proteins and low-molecular components found in mycobacteria, e.g. isopentyl pyrophosphates[53–55]. These may affect various cell types in different ways. For example, low molecular weight glycopyrophosphates directly elicit proliferative effects on T cells bearing the gamma-delta T-cell receptor[56]. On the other hand, stimulatory effects of live bacilli on conventional T lymphocytes require the active participation of macrophages[57]. Primary effects of BCG on different cell types are summarized in Table 2.2.

At the present time evidence for the induction of specific antitumour activity in immune cells by BCG is rather limited. There is evidence, however, for the induction of a unique antitumour immune cell population designated BCG activated killer cells or BAK cells. This population expresses markers of both T- and NK-cell lineage, namely CD8 and CD56[58]. However, whether or not BAK cells represent a homogenous group or a combination of T and NK cells working in close co-operation remains to be established. Like NK cells they recognize different tumour cell types in a nonMHC restricted manner. To date the mycobacterial antigens responsible for the induction of BAK cells remain to be identified but possible candidates include heat shock proteins and glycopyrophosphates[59]. It is apparent that macrophages and IFN gamma are necessary for the induction of BAK cells but not for their lytic activity[57]. Finally, the presence of BAK cells in vivo has yet to be confirmed although recent progress in the isolation and characterization of urinary lymphocytes, shed following intravesical instillation, may facilitate this[60].

There is also evidence that mycobacterial heat shock proteins can induce potent antitumour activity in gamma-delta T lymphocytes in vitro[61]. T lymphocytes bearing the gamma or delta T cell receptor are attractive candidates as possible effector cells as they readily respond to mycobacterial stimulation and are relatively prevalent at mucosal surfaces (at least in mouse) and possess antitumour activity[62]. Furthermore, we have shown that patients may shed an increased number of gamma or delta T lymphocytes into the urine following intravesical BCG instillations[60]. Due to their presence in cutaneous areas and the likelihood of first contact with mycobacteria, gamma-delta T cells may influence the type of response following intravesical therapy[63]. This is discussed in detail below.

In addition to influencing the induction of BAK cell activity macrophages are also able to exert direct antitumour effects themselves, and these can be augmented by incubation with BCG micro-organisms[64]. They are also likely to contribute to the production of cytokines detected following immunotherapy (e.g. IFN gamma, TNF alpha, IL-12) and at present they are arguably the most potent antigen-presenting cells in situ (as the presence of dendritic cells following BCG immunotherapy is as yet unconfirmed).

The role of neutrophils in BCG immunotherapy of bladder cancer is at present unclear. They have been suggested to be responsible for the toxicity and side effects associated with intravesical therapy. However, for a number of reasons we feel they may play an important role in the successful clinical outcome. In brief they are the most abundant immune cell type detected locally following immunotherapy, produce appreciable amounts of important cytokines (e.g. GM-CSF, IFN alpha, MIP1, IP-10, TNF alpha, IL-1, IL-8, IL-12), may express FAS-ligand (FAS-L), can adhere to human bladder tumour cells in vitro and have been

shown to exert antitumour effects in other models[65–67]. Obviously, the overproduction of pro-inflammatory cytokines may lead to increased toxicity. Nevertheless, we feel that the moderate recruitment of neutrophils associated with their 'nonspecific' reduction of tumour burden may be essential for optimal clinical effect.

The effects that BCG and other related mycobacteria have on the expression of cytokines and other key immunoregulatory molecules by the host immune system, and indeed by tumours themselves (see later), are obviously crucial to its mode of action. In vitro studies on both whole blood mononuclear cells and T cell clones have clearly shown that mycobacteria readily elicit TH1 and pro-inflammatory cytokines including IL-2, IFN gamma and TNF alpha[68–70]. Such cytokines can in turn influence the activity of other cells including lymphocyte activated killer (LAK) cells[71] and rescue neutrophils from apoptosis[72]. In addition to this they may directly exert antiproliferative effects on tumour cells[73].

On the basis of the above and other observations it was originally believed that the effectiveness of BCG in the treatment of bladder cancer was largely due to its ability to preferentially induce TH1 type cell-mediated immune responses[57,74]. However, as will be discussed below the picture is less clear-cut than originally believed for a vast array of both TH1 and TH2 cytokines are now known to be produced following the intravesical administration of BCG.

While the possible benefit to the host of certain BCG-induced T cell and macrophage-derived cytokines may be readily explained, we are less sure about the value of others. For example, on the basis of both in vitro and in vivo evidence it is apparent that IL-6 may either enhance[75,76] or inhibit tumour growth directly or through activation of the immune system[77–79]. Whether or not this is due to the production of different isoforms of IL-6 remains to be established[80]. Furthermore, IL- 8 may, on the one hand, act as a potent chemoattractant for neutrophils and T lymphocytes and rescue neutrophils from apoptosis[81,82] while, on the other hand, it may theoretically serve as a growth factor for tumour cells and may stimulate angiogenesis[83]. We would anticipate, however, that the GM-CSF produced is likely to promote the maturation of both macrophages and dendritic cells[84,85], thus enhancing antitumour responses. In contrast, the overproduction of the anti-inflammatory cytokines IL-10 and TGF beta is likely to have an adverse effect. We would speculate, however, that the clinical outcome depends on the amounts of pro-inflammatory cytokines (e.g. IFN gamma, TNF alpha, IL-2, IL-12) produced relative to those with 'inhibitory' activity (such as IL-10, TGF beta). If there is a relative overproduction of cytokines with 'negative regulation' then the antitumour response is likely to fail. Nevertheless, overproduction of pro-inflammatory cytokines can lead to side effects which would necessitate discontinuing therapy, so resulting in a suboptimal response (Figure 2.3).

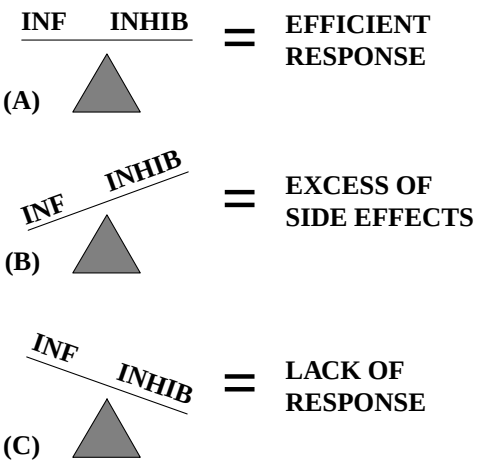

Figure 2.3 For an optimal antitumour response a balance in the production of inflammatory (e.g. IFN gamma, TNF alpha, IL-8) and inhibitory factors (TGF beta, IL-10) may be required (A). However, excess production of inflammatory cytokines (B) may lead to intolerable side effects, premature ending of therapy and suboptimal response. On the other hand, an initial excess of inhibitory factors may lead to incomplete eradication of tumour (C)

Effects on tumour in vitro

As previously postulated[1] we believe that the success of BCG immunotherapy in bladder cancer is most probably due to its effects on both the host immune system and the tumour itself. There is now strong evidence, at least in vitro, that the interaction of BCG with bladder tumour cell lines may induce or enhance the synthesis of a variety of molecules including cell adhesion, MHC, chemokine and pro-inflammatory cytokines. If such events occur in vivo they should promote the attraction, localization and antitumour activity of a number of cells of the immune system. These and other effects are discussed in greater detail below.

It is now readily apparent that BCG is a potent modulator of the survival and function of bladder tumour cell lines. First of all, BCG (and indeed other mycobacteria) can exert direct antiproliferative and cytotoxic effects on human bladder tumour cells which are as potent as the effects observed with IFN gamma[86,87]. Secondly, it induces their expression of adhesion molecules (ICAM-1) and production of chemokines and cytokines (IL-6, IL-8, TNF alpha, GM-CSF, IP-10)[75,88,89]. ICAM-1 facilitates interaction between T lymphocytes and tumour cells and plays an important role in the destruction of the latter[71]. IL-8 may be important for recruitment and activation of T lymphocytes and neutrophils. TNF alpha may exert a number of effects including the activation of T lymphocytes, the induction of adhesion and MHC class II molecules on tumour cells, or a direct antiproliferative effect on malignant cells[71,73,90]. Furthermore, in combination with GM-CSF it may

stimulate maturation of dendritic cells[85]. The antiangiogenic chemokine interferon-inducible protein 10 (IP-10) may be important by inhibiting development of new blood vessels in tumour[89]. In this regard it is interesting to note that the angiogenic characteristics and expression of vascular endothelial growth factor (VEGF) by bladder tumours significantly correlated with recurrence and progression[91,92].

BCG has been shown to be endocytosed by bladder cancer cells both in vivo and in vitro[93]. This may lead to the expression by tumour of antigens that can be recognized by the host's immune system. The likely candidates are heat shock proteins and indeed they can be detected in bladder cancer cells at least in vitro[59,94]. In this regard it is interesting to note that murine bladder carcinoma cells may present BCG antigens to autologous T lymphocytes in an MHC-restricted manner[95]. This antigen-presenting ability may be due to the enhanced production and expression by BCG-treated bladder cells of a number of important molecules known to be involved, directly or indirectly, in antigen presentation and subsequent effector cell activation. This includes MHC class II, cell adhesion (ICAM-1), co-stimulatory (CD40, FAS) and cytokine molecules (TNF alpha, GM-CSF)[73,86,96]. Such induction can be caused directly by mycobacteria or maybe indirectly through production of TNF alpha or other cytokines.

The expression of accessory molecules is also likely to affect tumour cells in other ways. The interaction of CD40 and FAS-expressing tumour cells with cell-bound or cell-free ligands may induce their apoptosis or suppress their proliferation (Taub, unpublished data). In addition, bladder cancer cells stimulated with recombinant CD40 ligand readily respond by increasing ICAM-1, FAS expression and production of stimulatory (IL-6, IL-8, GM-CSF and TNF alpha) but not immunosuppressive cytokines (IL-4, IL-10 or TGF beta) (Alexandroff et al., unpublished). Both CD40 and FAS expression can be augmented on bladder cancer cells by treatment with IFN-gamma. However, the increase of FAS expression on tumour cells may not necessarily result in more effective induction of apoptosis. This may be due to a number of reasons including parallel co-induction of soluble FAS molecules or induction of its truncated splice-variant, which may antagonize the function of cell-bound FAS[97]. Soluble CD40 may also be produced and serve as a negative regulator of antitumour responses[96].

Possible sequence of events in vivo

It is difficult to predict the exact chain of events in vivo following intravesical BCG therapy. The following is an approximation and it is possible that the timing and/or sequence of events may well be different. Following instillation BCG micro-organisms adhere to and are endocytosed by malignant and nontransformed epithelium (through fibronectin-dependent mechanism) and possibly by residual tissue macrophages. This stimulates the production of chemokines (e.g. IL-8), cytokines (IL-6, TNF alpha, GM-CSF) and augments the expression of ICAM-adhesion

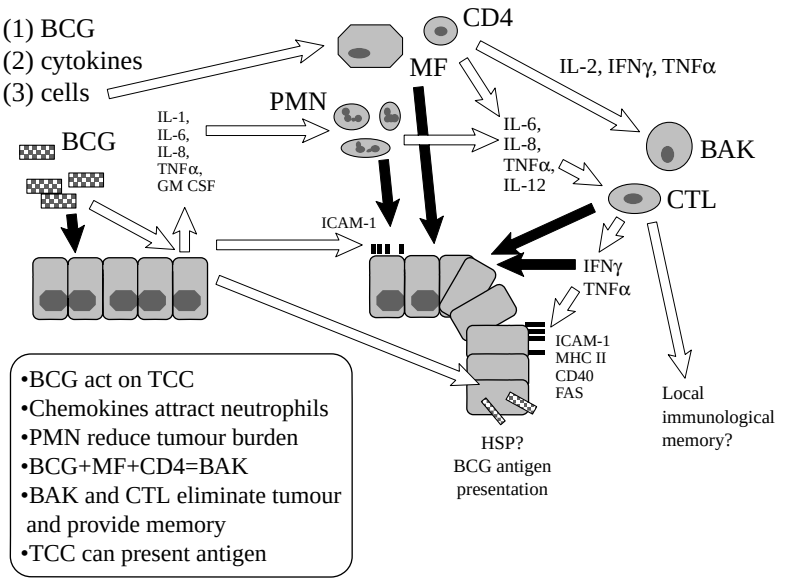

Figure 2.4 Possible antitumour effects elicited by intravesical BCG immunotherapy in bladder cancer. In this figure the white arrows depict the development of the immune response and the black arrows the three main mechanisms of tumour destruction, namely directly through BCG by BCG-induced cell-mediated mechanisms or via cytokines and other soluble factors. Following instillation, mycobacteria interacts with urothelium and is endocytosed. This may result in direct antiproliferative effects, the release of inflammatory mediators (such as IL-1, IL-8, etc.) and upregulation of adhesion molecules such as ICAM-1. The inflammatory mediators released attract and activate neutrophils and later macrophages and T cells. The cellular infiltrate produces more cytokines which, in turn, recruit and activate cytotoxic T cells (CTL) and BAK cells which recognize and kill the tumour. This recognition may be via unique antigens presented more effectively by the MHC class II antigens induced on the tumour surface or via BCG-induced heat shock proteins themselves. Further killing of the tumour may be effected by locally released immune cell derived cytokines (e.g. TNFα, IFNγ) or soluble ligands (e.g. FAS-L, CD40–L). Circulating CTLs may give rise to local immunological memory. Throughout this process the interaction between effector cells and tumour is enhanced by the increased expression on the tumour cell of adhesion molecules such as ICAM-1

molecules on tumour and possibly other cells (Figure 2.4). It has also been suggested that BCG may either express their own or induce the expression of endogenous heat show proteins in or on tumour cells and such may serve as tumour cross-reactive antigens[59]. We postulate that BCG or heat shock proteins may be recognized by gamma or delta T lymphocytes in the bladder wall. Their capacity to produce either IFN gamma or IL-4 may determine the T helper type polarization of the immune response (either TH1 or TH2)[63]. BCG, TNF alpha and potentially gamma or delta T lymphocytes may reduce tumour burden in the initial stages. The

cytokines produced at this stage attract and activate other immunocompetent cells, especially neutrophils and, to a lesser extent, macrophages. Neutrophils and macrophages further boost immune responses by secreting the same and new cytokines (IFN gamma, IL-12) and may also reduce tumour burden through nonspecific mechanisms. For example, neutrophils may use their FAS ligand to induce tumour cell apoptosis[98]. IFN gamma in turn may induce ICAM-1, FAS, MHC Class I and II, CD40 and probably a number of other, as yet unidentified molecules on tumour cells and possibly normal urothelium and macrophages[73,83,99]. As stressed above, this may facilitate the localization and activation of effector lymphocytes. As suggested earlier, the local accumulation of neutrophils may be responsible for some of the side effects associated with intravesical BCG therapy.

Towards the end of the course of immunotherapy (instillations 4–5 onwards) this immunopotent cocktail recruits and activates T lymphocytes. T cells produce IL-2 which acts as an autocrine growth factor. Macrophages and CD4 lymphocytes 'educate' BAK cells and classic cytotoxic T lymphocytes (CTL). Although therapy is accompanied by a massive leucocyte infiltration[100] neither BAK cells nor CTL have yet been demonstrated in situ following immunotherapy. However, a recent study has identified and characterized a bladder cancer antigen recognized by autologous CTL in patients[101]. Furthermore, both CD4 and CD8 T lymphocytes have been shown to be vitally important for anticancer response as their neutralization in mice abolished tumour destruction, and patients' urine frequently contains conjugates between bladder cancer and T cells[1,102]. The main difference between BAK and CTL lies in MHC restriction for CTL and the requirement for low amounts of live BCG for the induction of BAK cells. From in vitro data T lymphocytes are likely to kill tumour cells through cytolysis and apoptosis[90,103]. They can also produce soluble or cell-bound FAS ligand, CD40 ligand, IFN gamma and TNF alpha, all of which have been shown, or suggested, to exert cytotoxic or antiproliferative effects on tumour cells. The maximum recruitment of T cells at the end of the therapy also coincides with the maximum shedding of urinary gamma or delta T lymphocytes[60]. Finally, T lymphocytes may also provide local immunological memory, which may be responsible for the reduction in tumour recurrences seen in immunotherapy but not chemotherapy.

Following completion of therapy the expression of MHC molecules on urothelium and its infiltration by lymphocytes gradually subsides reaching a minimum some 3–6 months later[100]. Further BCG maintenance instillations at this time lead to a boost in cytokine production (Esuvaranathan, unpublished data).

Interesting but unanswered questions

One of the major questions relating to bladder cancer immunotherapy is whether or not antigen-specific mechanisms are responsible in any way for the observed

clinical effects. An answer to this question should help clarify the possible potential of this therapy to other malignancies. At present there is limited evidence that it is antigen-specific. MAGE antigens and heat shock proteins have been detected respectively in bladder neoplasms or on bladder cancer cell lines in vitro, but no corresponding specific T lymphocytes have been shown in situ[104]. However, the recent demonstration and characterization of a human bladder antigen recognized by autologous CTL holds the possibility that antigen-specific CTLs are induced following BCG immunotherapy in bladder cancer[101]. Furthermore, the development of fluorescent soluble antigen-specific MHC complexes may facilitate isolation from patients of such antigen-specific CTLs if they exist, e.g. to MAGE[96]. Whether these tumour antigen-specific T cells can be isolated, expanded and used for adoptive immunotherapy in nonresponding patients, or indeed patients with advanced bladder cancer, is a challenging question. On the other hand, a number of immune factors generated by BCG can clearly exert antigen-independent antitumour effects including cytokines, soluble FAS-L and CD40-L and even FAS-L positive CD4 T lymphocytes. It may be that both nonantigen-specific tumour and antigen-specific mechanisms are equally important to a successful outcome by respectively reducing the bulk of the tumour and eliminating scattered residual tumour cells.

Secondly, it remains to be established whether the T helper type response generated has anything to do with clinical outcome. To date both TH1 and TH2 associated cytokines have been identified in the urine of patients receiving immunotherapy[105–107]. It is of note that no urinary IL-4 has ever been detected, although in one study IL-4 was found in situ[108]. Both TH1 and TH2 cytokine show the same time course of urinary production, increasing throughout the course of therapy and reaching maximum levels at instillations 5 and 6. Although in one recent study a correlation between induction of humoral (and thus TH2) immune response and poor clinical outcome was shown[109], in general no correlation has been noted between production of either type of cytokines in urine and favourable outcome[107]. The possible reasons for this are not clear but may include the simultaneous production of biological modifiers of cytokine activity such as, for example, neutralizing soluble TNF receptors, IL-12 homodimers or even anticytokine antibodies[110,111]. This matter is discussed further later. Of course, a simple explanation is that the immunoassays used to measure cytokines do not reflect their bioactivity. Furthermore, many cytokines formally classified as TH1 or TH2 cell response could be produced by other cell types (e.g. IL-10 can be produced by macrophages) thus masking a 'real' TH response. A way round this would be to isolate T lymphocytes directly from the urine of patients using MACS technology and then stain them for intracellular expression of cytokines and demonstrate the presence of corresponding message RNA[60]. Thus, by confirming both protein and mRNA expression of certain cytokines in highly purified T cells obtained directly from patients we should be in a position to resolve the TH dilemma once and for all.

It has been suggested that spontaneous production by bladder cancer cells of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and GM-CSF may be important for effective anticancer response[75,112–114]. In this regard, however, it is interesting to note that high grade bladder cancer cell lines seem to produce more cytokines spontaneously, more readily express adhesion molecules such as ICAM-1 and respond better to exogenous stimuli by producing cytokines[75,112,115]. It has been suggested that this may benefit the tumour through a number of mechanisms, including autocrine growth loops, enhancement of metastasis through activation or upregulation adhesion molecules, increased resistance to antineoplastic antibiotics and by elimination of gamma-delta T lymphocytes[116–118]. In this context ICAM-1 expression, which is involved in LAK cell killing, may serve as an indirect 'marker' product of cytokine production. It is also of interest that IL-6 and TNF alpha have been implicated in tumorigenesis of bladder cancer[76,119].

It is quite embarrassing to admit that we still do not know whether dendritic cells play any role in the immunotherapy of bladder cancer. Dendritic cells are the most potent antigen-presenting cells described to date and in other tumour models have proved to be most attractive candidates for augmentation of anticancer responses. Furthermore, it has been demonstrated that BCG can directly activate dendritic cells[120].

In the light of multiple antitumour functions of the CD40 ligand it is also of importance to see if this soluble molecule is produced following therapy and may serve as a ready marker of successful immune response[96]. However, in measuring this protein one has to remember that soluble CD40 with antagonizing functions may also be produced[96]. Finally, at the present time we know of only one chemokine (IL-8) and some co-stimulatory molecules which can be expressed by bladder cancer cells. The possible expression of other important chemokines and cytokines by BCG-treated tumour cells and their relevance to the initiation of antitumour responses remains to be investigated.

Remaining problems, developments and prospects

It would be premature to conclude that BCG therapy is optimal and does not require further improvement. Two of the major drawbacks are the level of toxicity and the side effects. Although the side effects experienced are low or moderate the incidence is high compared to chemotherapy. Undoubtedly a reduction in side effects would make BCG immunotherapy more attractive to both clinicians and patients. One possible approach is to reduce the dose of BCG. Recent studies indicate that this can be readily achieved without affecting antitumour response[121,122].

There is still a very low, yet definite, risk of life-threatening complications, including systemic dissemination of BCG. In the majority of cases this has been

observed after traumatic catheterization facilitating blood-borne spreading of mycobacteria. Although this is potentially preventable, once it occurs it should be readily recognized and aggressively treated. The current development of recombinant BCG permanently incorporating suicide genes or genes responsible for increasing mycobacteria sensitivity to antibiotics and/or lacking virulent features, should eliminate this problem in the future[123].

Unfortunately, at the present time there are no simple, affordable methods for determining which patients will respond to BCG immunotherapy. The availability of such a test would improve patient treatment by enabling the prompter implementation of alternative treatment strategies and also avoid prolonged courses of administration of BCG with their consequent side effects. On both theoretical and practical grounds the most appealing approach to date has been to monitor urinary TH1 or pro-inflammatory cytokine production. Following intravesical therapy, urine is collected, dialysed, aliquoted and cytokines measured using commercially available ELISAs. This is noninvasive and theoretically should measure local as distinct from systemic immune responses. A great number of immunomodulating molecules (both of theoretically positive and negative importance) have been detected including IL-1, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, GM-CSF, TNF alpha and beta, TGF beta, IFN gamma, IP-10, soluble ICAM-1 and soluble CD14[105,107,124]. Unfortunately most cytokine studies have shown similar pictures of production which generally increased throughout therapy, reaching a maximum at the fourth to the sixth instillation. Different patients varied markedly in this picture and it was impossible to identify clearly groups of patients with either TH1 or TH2 response[105]. Thus some patients fail to produce some cytokines at all, while others produce maximum cytokines at the beginning and smaller amounts towards the end of the therapy course. Furthermore, only limited correlation was found between individual cytokine production and clinical outcome[107]. The explanation is not known but, as previously mentioned, may be due to concurrent production of cytokine antagonists (soluble TNF receptors, IL-2 receptors) and modifiers (IL-10) which could complicate the overall picture[110]. For example, soluble receptors may bind and impair the bioactivity of the corresponding cytokine, IL-10 may antagonize the bioactivity of IFN gamma through inhibiting induction of antigen-presenting and accessory molecules (MHC, B7, ICAM-1)[125]. Interestingly, soluble CD14 showed a negative correlation with outcome[124]. CD14 provides signalling into macrophages upon interaction with bacterial lipopolysaccharide, which may result in the production of pro-inflammatory cytokines[126]. Whether soluble CD14 in this setting may act as an antagonist to its membrane-bound form remains to be studied.

Recently, an alternative approach to identifying those bladder cancer patients who will respond to BCG immunotherapy has been suggested[127]. This involves

determining IL-2 mRNA production by peripheral blood mononuclear cells from patients undergoing immunotherapy. While this method looks promising it still remains to be verified.

An even better situation would be where one could predict responders prior to treatment. Recent studies in mice suggest that this may be feasible in the near future[128]. These studies revealed that the response of bladder cancer to BCG was genetically predetermined. It depended on the expression of a natural resistance-associated protein (N ramp1) necessary for the activation of macrophages and fast destruction of intracellular infective agents. The identification of homologous genes in humans would also open up the possibility of genetic counselling of patients prior to BCG therapy.

Recent progress in recombinant technology opens alluring prospects for the development of more effective and less toxic BCG vaccines for the treatment of bladder cancer and possibly other malignancies[123]. Although the effectiveness of currently available strains of BCG has been verified on thousands of patients, it is believed that it can be improved. For example, can BCG be engineered to express human cytokine genes which are understood to be important for anticancer response such as IL-2, IFN gamma or TNF alpha? This would not only ensure that, following instillation, all patients would produce the necessary cytokines but might also permit a reduction in BCG dose with a consequent reduction in side effects and risks of complications. Such an approach is obviously feasible as recombinant-producing active human and mouse cytokines have been obtained[129,130]. We have also recently produced recombinant *Mycobacteria smegmatis* expressing human TNF alpha[114]. *M. smegmatis* has the advantage of being nonpathogenic to humans and having a faster proliferation cycle which allows easier manipulations. In addition, it appears to be vastly superior to BCG in the destruction of human bladder cancer cells, at least in vitro, and in the induction of cytokines and adhesion molecules on tumour cells. Unfortunately, none of the recombinant vaccines have yet been tested in in vivo human bladder cancer models.

With the complete sequence of *M. tuberculosis* now known and the development of homologous recombination technology it should be possible to incorporate multiple genes of interest permanently into mycobacteria and, at the same time, delete virulent genes. This should facilitate the development of safer and possibly cheaper vaccines for immunotherapy of malignancies. Further details on the production, properties and potential of recombinant mycobacterium may be found in the excellent review of O'Donnell[123].

For unknown reasons BCG immunotherapy seems to be ineffective in the treatment of advanced invasive disease, although it can still be used as a palliative measure. One possible explanation is that in advanced invasive disease the host immune system is suppressed, so that the immunological responses noted above

are not adequately manifested[96], and therefore it may be necessary first to debulk tumour mass and then activate the immune system. In this respect immunostimulation with fused autologous BCG-treated tumour cells and dendritic cells may prove particularly effective[131,132].

Conclusion

Twenty years later and thousands of patients after the first successful use of BCG immunotherapy in bladder cancer patients, we are closer to the understanding of how it works. Nevertheless, we still have a lot to learn if we are to improve our present rates of success in bladder cancer and possibly extend it to other malignancies. The following questions will present main challenges during the coming years:

(1) Can we predict responders prior to therapy?
(2) Is IL-2 mRNA production by peripheral blood and mononuclear cells a reliable method of predicting the clinical outcome and can we make it more simple?
(3) Can more bladder cancer-specific antigens capable of triggering effective CTL be identified?
(4) Can nonpathogenic mycobacteria species be superior to BCG in the treatment of bladder cancer patients?
(5) Can we decrease side effects and improve the rate of response by using stable recombinant mycobacteria lacking 'virulent' genes and producing human immunomodulatory molecules (e.g. IL-2)?
(6) Can we increase efficacy and further minimize effects by combining BCG and other treatments, such as IFN alpha or soluble CD40 ligand?
(7) Can we adopt immunotherapy for advanced bladder cancer (e.g. by debulking tumour burden and combining BCG and tumour–dendritic cell fusion hybrids)?
(8) When we understand the main mechanisms of BCG immunotherapy and are able to predict responders, would we be able to adapt or substitute BCG immunotherapy for treatment of malignancies other than bladder cancer?

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3

Poxviruses as vectors for cancer immunotherapy

Miles W. Carroll and Nicholas P. Restifo

Introduction

While a widely efficacious tumour vaccine is not yet available, a great deal of progress has been made in the development of effective cancer vaccines. Vaccines designed to treat patients with metastatic cancer have shown the first evidence of efficacy in the clinic[1–3]. In this chapter, we will focus on efforts in which recombinant poxviruses have been used in the clinical and preclinical treatment of cancer. This work is based on a 'reductionistic' approach which has made possible an understanding of the interactions between the immune system and tumour cells on a molecular level[4]. The thrust of this work comes from observations, discussed at greater length below, that infection of a tumour-bearing animal with a recombinant poxvirus encoding a tumour-associated antigen can result in tumour destruction and prolong the survival of the animal.

While viruses are demonstrably immunogenic, tumour cells have notoriously poor immunogenicity. The reasons for this apparent lack of immunogenicity (as discussed in Chapter 1) may be that cancer antigens are generally not presented to the immune system in a micro-environment that favours the activation of immune cells. Although no single known mechanism can explain poor tumour immunogenicity in all experimental models studied, the molecular bases can be separated conceptually into four distinct groupings: (1) lack of expression of co-stimulatory molecules; (2) production of immuno-inhibitory substances; (3) poor antigen processing and presentation; and (4) variability in the expression of antigen by tumours. While the first two groups are mechanisms of poor immune activation shared by normal cells in the body, the latter two concern the inherent genetic instability of cancer cells. This instability leads to much heterogeneity among tumour cells, making the immune destruction of all tumour cells in the body an extremely difficult task[5].

Identification of tumour antigens

As discussed in Chapter 1, much progress has been made in the identification of tumour-associated antigens that are potentially useful in the development

of recombinant anticancer vaccines[6–8]. Many of the tumour antigens that have been identified are tissue differentiation antigens in melanocytes and include gp100, MART-1/MelanA, tyrosinase and tyrosinase-related proteins (TRP) 1/gp75 and TRP-2. Interestingly, these antigens are involved in the synthesis of melanin and give both melanocytes and deposits of melanoma tumour their dark pigment.

The fact that differentiation antigens are nonmutated in most tumours has two important implications. First, expression of these tissue differentiation enzymes are shared by the great majority of melanoma nodules from the majority of patients, and thus an 'off the shelf' vaccine strategy targeting these antigens is possible (a strategy that targets a mutated antigen may have to be individualized for every mutation). Secondly, the nonmutated nature of these antigens suggests that immunotherapies that target these antigens could elicit auto-reactivity. One consequence of this 'auto-reactivity' may be vitiligo, the patchy and permanent loss of pigment from the skin and hair thought to result from the auto-immune destruction of pigment cells. Vitiligo has been correlated with objective shrinkage of deposits of metastatic melanoma in patients receiving high dose interleukin-2 (IL-2), a cytokine known to activate and expand T lymphocytes[9].

Thus, there is evidence that vitiligo can be coupled with tumour regression, and that adoptive transfer of antitumour T cells recognizing differentiation antigens is associated with objective shrinkage of melanoma deposits. Another important group of target antigens are those that are specific to tumour cells and testis, and are encoded by genes with family names such as MAGE and BAGE. NY-ESO-1 and several other antigens identified by SEREX also fall into this group and are expressed in a significant proportion of human melanoma cells as well as other tumour histologies including breast, ovary, bladder, prostate and liver[6,10].

Tumour protection studies using experimental animals have shown that protective responses are generally tumour specific, leading to the conclusion that, at least in mice, tumour rejection antigens are often unique[11]. These antigens are thought to be the result of mutations in the genome[12]. Human tumours also express mutated antigens that can be processed and presented for recognition by T cells. Mutated antigens may not lend themselves easily to 'off the shelf' vaccines consisting of purely recombinant and synthetic components, since each neo-antigen for each patient must be checked for sequence and that sequence must be verified to be present on the surface of a tumour cell. Some workers have asserted that mutated tumour antigens are superior targets for vaccine design because immune cells will not be 'tolerized' to these antigens[13]. However, recent work has shown that even the most immunogenic 'foreign' antigen, such as the haemagglutonin (HA) antigen from the influenza A virus can be tolerizing when expressed peripherally (i.e. outside the thymus) either in normal cells[14] or in tumour cells[15]. Thus, mutated or

otherwise 'foreign' antigens may also induce peripheral tolerance when expressed by tumour cells.

The development of recombinant poxvirus-based vaccines for cancer

Essentials of an efficient delivery vector

In addition to the tumour antigen target, the antigen delivery system is equally as important for the development of a successful cancer vaccine. As mentioned previously, two reasons why tumour cells do not induce therapeutic immune responses include the absence of co-stimulation and the existence of immunological tolerance. Additionally, tumour cells commonly lyse via apoptosis which is thought to be an ineffectual route for immune stimulation[16]. However, some viruses such as vaccinia (VV), and other members of the poxviridae, cause widespread cellular lysis and also express genes that block apoptosis during infection. It is evident, therefore, that an effective, versatile vector will be required to deliver the tumour antigen to the host immune system for the induction of a therapeutic anticancer response. A variety of vectors have been evaluated in a spectrum of murine tumour models; however, recombinant VV (rVV) and other poxviruses appear to offer superior properties in relation to the induction of efficacious immune responses to tumour antigens.

Recombinant vaccinia virus

Since 1982, when rVV were first constructed[17,18], VV and other members of the poxvirus family have been used extensively to induce both antibody[19] and CTL[20] responses to a broad spectrum of recombinant proteins. Although VV was initially used as a nonspecific adjuvant in melanoma treatment several decades ago, it was not until the mid-1980s that rVV was employed in targeted cancer immunotherapy. Recombinant VV expressing the melanoma antigen p97 was used successfully in the therapy of melanoma in a murine tumour model. Additionally, this construct induced both antibody and cell-mediated responses to p97 in macaques[21]. Subsequently, rVV expressing a wide range of tumour-associated antigens have been constructed and evaluated in murine models. These constructs include rVV expressing: nonself viral antigens, e.g. human papilloma virus (HPV) E6 and E7[22], mutated self-antigens, e.g. murine p53[23] and nonmutated self-antigens, e.g. human MUC-1 (see Chapter 7) and murine gp100[24]. More recently, rVV expressing murine melanoma antigens have been shown to induce immune responses in mice. Inoculation of mice with a rVV expressing murine TRP-1 induced vitiligo as seen in melanoma patients who respond favourably to IL-2 treatment. More importantly these animals were resistant to challenge with the synergeneic melanoma tumour line B16[25]. In many cases evaluation of cancer therapies in murine and nonhuman primate models has led to phase I and II clinical trials.

Nonreplicating vaccinia virus vectors

Vaccinia virus is replication competent in mammalian and human cells and during the smallpox eradication programme, in which many millions of doses were given, a relatively high incidence of vaccination complication was seen[26]. The potential dangers of administering live VV, especially to immunocompromised subjects, has been raised on many occasions by critics of recombinant poxviruses (see also Chapter 4). To neutralize such criticism several attenuated VV strains have been developed that are unable to replicate effectively in human cells, e.g. NYVAC[27], MVA[28] and a VV lacking an essential gene for replication in all mammalian cells[29]. NYVAC was created by the deletion of some 18 genes that restrict its replication in human cells and results in a vector which has been shown to be nontoxic and safe in the clinic[30].

MVA (modified vaccinia virus Ankara) was developed by passaging a smallpox vaccine strain over 500 times in chick embryo fibroblast cells, after which time it was shown to be replication restricted in human and most mammalian cells[28,31–34] due to defects in several genes[35]. MVA was initially used to vaccinate over 120 000 people against smallpox, many of whom were at risk from the contraindications associated with the replication-competent vaccine, without reports of complications. MVA has been compared to replication-competent VV strains in several infectious diseases[36,37] and tumour immunotherapy models[38]. In all cases MVA, though nonreplicating, appeared to be as efficacious at inducing therapeutic or protective immune responses as replicating VV strains. In some cases MVA appeared to be more efficient than the replication-competent viral vectors. The reasons for such superior properties are thought to be due to the reduced lytic activity of MVA in mammalian cells[33,36]. Additionally, MVA's lack of immune evasion molecules[39], that are thought to shield the viral proteins from the host immune system[40], may also render recombinant proteins more antigenic. Due to these advantageous properties recombinant MVA constructs are scheduled to enter the clinic in late 1999 for cervical and breast cancer immunotherapy trials.

Nonvaccinia poxvirus vectors

A large proportion of the population >30 years of age were immunized with VV in the smallpox eradication campaign (as discussed also in Chapter 4). Such pre-existing immunity may have a detrimental effect on the induction of an immune response to recombinant proteins expressed by rVV.[41] To overcome this obstacle several viruses within the poxviridae family, of which VV is the prototypic member[42], especially those from the avian poxvirus genus, have been developed that are not affected by pre-existing immunity to VV[43]. Both fowlpox virus (FPV) and canarypox (commercially referred to as ALVAC) are from the avipox genus and have been developed as recombinant virus vaccine vectors[30,44]. Both have been used

in humans with no signs of toxicity. Interestingly, animal models show that ALVAC appears more effective than FPV at inducing protective immune responses to some antigens[30].

Although the problems of pre-existing immunity to VV can be obviated by using an immunologically distinct poxvirus vector as discussed above, a recent study using a murine model suggests that the obstacle of pre-existing immunity can also be overcome if the rVV is delivered by the mucosal route[45].

Enhancing the efficacy of poxvirus vectors

Poxviruses have been shown to be the vector of choice in a number of murine tumour models, but there are several ways to further enhance their efficacy. Improvements to poxvirus efficiency fall into four general categories: (1) promoter kinetics and strength; (2) replication–lytic properties; (3) co-expression of immune co-factors; and (4) inoculation regimes.

Promoter kinetics and lytic properties

Poxvirus protein expression is regulated by viral specific promoters that are classified into two general categories: early (before DNA replication) and late (after DNA replication). It has long been accepted that, due to VV interference of host protein synthesis and with the cellular antigen processing machinery[47], the requirement for early promoters driving expression of the recombinant protein is beneficial for the induction of optimal CTL responses. However, a recent study using a panel of synthetic early and late promoters[48] driving expression of the model TAA LacZ indicate that early and late promoters both induce CTL responses, although early promoters are drastically more effective at inducing CTL-mediated tumour therapy in a LacZ murine model[49]. Furthermore, it was illustrated that only dendritic cells (DC) infected with a rVV containing LacZ under an early promoter was able to prime murine β-gal specific CTL. This phenomenon is most likely due to the inability of VV to express proteins under late promoters in DC. Based on VV promoter mutagenesis studies highly efficient vaccinia virus promoters have been constructed[50]. These findings will be crucial to the success of poxviruses in the clinic.

A nonreplicating VV derivative that is defective in the D4 gene, which is essential for VV DNA replication, has been developed[29]. This vector and a replication competent VV, expressing tick-borne encephalitis virus antigens (TBE), have been evaluated in an infectious disease model. It was found that a tenth of the defective rVV was required to induce protection to a lethal challenge dose of TBE (Holzer et al., unpublished data). As in the case of MVA[38] the superior efficacy may be due to the less lytic activity of the vector for the induction of a more optimal immune response.

Expression of immune co-factors

Recombinant poxviruses are able to induce effective immune responses to exogenous proteins; however, in the case of an anticancer response the induction of effective TH-1 type response is often vital. The co-expression of immune co-factors that steer the immune system towards a TH-1 type response may clearly be beneficial. Due to the large capacity of the VV genome for foreign DNA this is an option for recombinant poxviruses. IL-2 was the first immune co-factor to be expressed by vaccinia virus and was shown to have an attenuating effect in nude mice[51,52]. Subsequently, rVV expressing a spectrum of immune enhancers have been constructed with the ultimate aim of manipulating the immune response by creating a localized immunological micro-environment in which the host immune system can interact with the recombinant protein. Many cytokines, e.g. IL-2 and IL-12, have clear antitumour activity, but their toxic side effects can be severe when delivered systemically. Reports show that co-expression of a model TAA and IL-12 by a rVV can obviate the requirement of systemic delivery of toxic levels of IL-12[53]. Additionally, rVV co-expression of IL-2 and a model TAA has also been shown to enhance the therapeutic activity of a rVV in a murine tumour model[54]. Furthermore, co-stimulatory molecules, e.g. B7.1, essential for effective induction of CD8 responses co-expressed by a rVV, have enhanced the tumour therapeutic activity of these viruses.

Recombinant poxviruses have also been utilized to evaluate the potential synergistic effects of co-stimulatory molecules and cytokines, e.g. B7.1 and IL-12[53] in an in vivo environment. Such recombinant poxviruses have the potential of providing some of the essential properties of antigen-presenting cells. It is clear that the co-expression of immune co-factors offers a further dimension to the enhancement of therapeutic vaccines. In Chapter 6, by Kauffman and Schlom, the use of B7.1 within a recombinant poxvirus co-expressing CEA in a colon cancer clinical trial is described. Initial data indicate that there are no signs of autoimmune toxicity associated with such an approach. Additionally, co-expression of IL-2 and MUC-1 within a rVV appears to be nontoxic[69].

On many occasions a suitable TAA is unknown for a specific tumour. However, theoretically this problem can be overcome if one could supply the tumour cell with necessary immune signals to initiate an antitumour immune response itself. The original concept of oncolysates involved taking melanoma cells and infecting them with a lytic virus such as VV. These infected cells were then inoculated back into the patient and on some occasions induced an anticancer response. An improvement of this concept involves the infection of tumour cells in vitro with rVV expressing immune co-factors to initiate an effective antitumour response[55].

Inoculation regimes

Repeated inoculation with rVV has led to enhanced immune responses to the recombinant gene product both at the antibody and CD8 + level[37,57]. However, due

to the immunogenicity of the vector proteins it is likely that the efficacy of the secondary inoculation may be diminished due to rapid clearance of the recombinant virus. Additionally, boosting of the recombinant protein response will be competing against several hundred vector-derived antigens. This problem has been circumvented by using heterologous booster protocols which employ two immunologically noncross reacting poxvirus vectors, e.g. FPV and MVA[38] and ALVAC and VV[58]. These regimens appear to improve therapeutic and cell-mediated responses in animal models. Several reports show that the use of naked DNA as a priming vector and recombinant poxvirus to boost the response[57,59,70] induces significantly more efficacious antibody and CTL responses to the recombinant protein. Interestingly, the order of the regime is vital. It is likely that these vaccination regimes will be adapted to the clinic in the near future.

An alternative approach to enhancing the effectiveness of poxvirus-induced immune responses involves infection of key immunological cells, e.g. DC directly with rVV in vitro[60]. It has been shown that human DC infected with rVV, expressing melanoma antigens, are able to induce melanoma-specific CTL. The in vitro induced CTLs recognize the same HLA restricted peptides as do CTL derived from melanoma patients. However, in the case of poxviruses, it remains to be seen if such an approach is beneficial compared to conventional inoculation methods.

Recombinant poxviruses in the clinic

Recombinant poxviruses have been evaluated in numerous infectious disease clinical trials without reports of complications, and have induced both antibody and cell-mediated immune responses to the various expressed recombinant proteins[61].

The anticancer properties of VV were first noted after its use in treatment of malignant melanoma in the form of VV infected irradiated melanoma cells. Such oncolysates were initially thought to induce significant clinical responses, but more recent phase III studies show that, over all, this approach may not offer significant clinical benefit[71].

With the identification of specific antigens associated with tumour cells, several groups have constructed rVV and evaluated their antitumour properties in murine and subsequently in clinical settings (see Table 3.1). An early trial involved the use of a rVV expressing E6 and E7 of HPV 16 and 18. E6/E7 are nonself-antigens and are highly associated with cervical cancer, therefore making them excellent targets for cancer immunotherapy. An initial Phase I trial involved a group of eight late stage cervical cancer patients[72]. Vaccination resulted in antibody responses in three of the eight patients and of the three who were able to be evaluated, one showed a significant E6/E7 CTL response. The CTL induction also correlated with the one patient who showed a clinical response, although spontaneous remission and not vaccination may have been responsible. Importantly, there were no reports of

Table 3.1. *Recombinant poxvirus-based cancer immunotherapy clinical trials*

Poxvirus	Antigen	Clinical trial	Toxicity	Immune response	Clinical outcome
[71]Vaccinia	Whole tumour cell	Melanoma	No	Ab	Variable*
[72]Vaccinia	HPV E6/E7	Cervical cancer	No	Ab 3 of 8 CTL 1 of 3	1 CR†
[69]Vaccinia	MUC-1	Breast cancer	No	CD4 proliferation	—
[67]Vaccinia	PSA	Prostate cancer	No	Ab	—
[63,73]Vaccinia	CEA	Advanced metastatic CEA expressing carcinomas	No	CEA specific CTL	—
[64]Canarypox ALVAC	CEA	Advanced metastatic CEA expressing carcinomas	No	T-cell enhancement in 7 of 9 patients	—
‡Canarypox ALVAC	CEA and B7.1	Colorectal cancer	No	Ongoing	Ongoing

Notes:

* Specific groups of patients did show significant survival improvements.

† One patient showed complete remission but this may have been a spontaneous event.

‡ Kaufman et al., unpublished data.

vaccinia-associated side effects or environmental contamination due to shedding of the rVV (see Chapter 4).

Vaccinia viruses expressing the TAA carcinoembryonic antigen (CEA) were constructed in the early 1990s[62] and were shown to protect mice against challenge with syngeneic tumour cells expressing human CEA. Phase I trials with patients suffering from CEA positive adenocarcinomas revealed that patients inoculated with a rVV-CEA were able to mount a CEA-specific CTL response[63] (see Chapter 6). Fortunately, such a response did not induce autoimmune cytotoxicity as may have been expected when mounting an immune response to a self antigen whose expression is not restricted to tumour cells. Due to the initial encouraging results additional trials have been carried out using an avian poxvirus, ALVAC, which is nonreplicating in human cells and is immunologically distinct from VV. Seven of

the nine patients vaccinated showed significant improvements in their CEA-specific T cell responses[64]. It is hoped that prime boost regimes using VV and ALVAC will improve the efficacy of the vaccine as was illustrated in murine models[58]. An ongoing trial has been designed to evaluate the benefits of co-expression of the co-stimulatory molecule B7.1 in an ALVAC–CEA construct (Kauffman et al., unpublished data) (see Chapter 6). Initial results indicate that the virus does not induce autoimmune toxicity. Unfortunately, no clinical improvement was noted in the above CEA studies which is perhaps not surprising as the patients had advanced disease. Perhaps future trials will include early-stage patients so that clinical efficacy may be a more realistic outcome.

MUC-1 is a highly glycosylated mucin, normally found on the surface of many cell types (see Chapter 7). A glycosylated form of MUC-1, associated with the onset of breast and ovarian cancer, holds promise as a putative target for cancer immunotherapy[65]. In a rat model inoculation with rVV expressing MUC-1 was able to protect against challenge with syngeneic tumour cells expressing MUC-1. In transgenic mice expressing human MUC-1, it was shown that the co-expression of MUC-1 and IL-2 improved the efficacy of the antitumour response in a self-antigen environment[66]. These encouraging results led to a clinical trial of a rVV expressing MUC-1 and IL-2 in patients with advanced inoperable breast cancer. Nine patients received varying doses of rVV, those patients responding favourably immunologically received a booster inoculation. There were no significant clinical side effects. One patient showed a proliferative response to a MUC-1 peptide and in two patients there was evidence of MUC-1 specific cytotoxic T cells. Additionally, all patients showed an increase in T memory cells in tumour biopsies, following vaccination[66,69].

Prostate-specific antigen (PSA) expression is highly associated with prostate cancer cells and is a potential TAA for use in cancer immunotherapy. Initial evaluation of a rVV expressing PSA was carried out in nonhuman primates in which the PSA gene shares 94% homology with the human homologue. All monkeys that received the rVV showed a short-lived antiPSA IgM response and those which received a higher dose of virus showed PSA-specific T-cell responses. More recently a rVV-PSA has been evaluated in prostate cancer patients, who had recently undergone radical prostatectomy, in order to prevent prostate cancer recurrence[67]. Of the eight patients included in the trial, one developed an IgG PSA-specific antibody response which was above baseline. Additionally, one patient showed decreased levels of serum PSA, but this did not correlate with an increased PSA antibody response, suggesting that the clinical effect may be due to T cell-mediated mechanisms.

To date, recombinant poxviruses have been evaluated in several cancer immunotherapy clinical trials. In summary such trials have established poxviruses as a safe delivery mechanism for TAA that are able to induce both CTL and antibody

responses in a self-antigen environment. Unfortunately, little clinical effect has been seen in these trials; however, this is perhaps not surprising considering the late-stage cancer patients recruited. With the wealth of knowledge gained from murine models and the apparent safety of recombinant poxviruses in humans, perhaps future clinical trials will be carried out in early-stage cancers employing recombinant viruses engineered to express multiple TAA and immune co-factors. Additionally, heterologous prime boost regimes may also aid in improvements of clinical efficacy.

Conclusion

In this chapter, we have attempted to describe the basic immunological principles that may be useful in the design of recombinant poxvirus-based vaccines. These principles are derived in large part from a deeper understanding of immune function that has resulted from a 'reductionistic' approach, characterized by understanding the interactions between the immune system and tumour cells on a molecular level[68]. We have described the weak immunogenicity of human tumours, perhaps due to their lack of expression of important immune-activating signals in vivo. Specifically, tumours may lack expression of appropriate co-stimulatory molecules, tumour antigens and MHC molecules and they may directly suppress immune function through the expression of suppressive molecules such as TGF and IL-10.

Nevertheless, tumour-associated antigens can be identified, making possible the design of recombinant and synthetic vaccines for cancer. Potential targets include the products of mutations in tumour cells as well as normal (not mutated) differentiation antigens. Work with tumour-associated antigens in animal models and, preliminarily, in human clinical trials has shown that immunizations which are most effective in generating reactive T cells are also the most therapeutically effective. Independent and collaborative roles for the CD8+ and CD4+ T cell subsets are now being elucidated. Following cancer immunotherapy the activation of T cells is likely to be effected largely through DC. Thus, the function of promoters for recombinant vaccines must be optimized in these 'professional' antigen-presenting cells. Further, adjuvants might best be designed to 'super-activate' DC. Finally, the inclusion of early-stage cancer patients in future immunotherapy clinical trials may illustrate the efficacy of recombinant poxvirus cancer therapies.

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Vaccinia-based human papillomavirus vaccines in cervical cancer

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Introduction

The *Poxviridae* are a family of double-stranded DNA viruses that characteristically replicate in the cytoplasm of infected cells and, by encoding a wide range of host-independent enzymes, are able to express gene products and replicate DNA, even in enucleated cells. They are further subclassified but only two members of the subfamily *Chordopoxviridae* (poxviruses of vertebrates), molluscum contagiosum virus and variola (smallpox), are specifically human viruses. Infection with poxviruses of other species can occur with cowpox, vaccinia[1] and monkeypox, sometimes resulting in localized outbreaks[2]. In addition, skin infections from inadvertent local inoculation with orf (a virus of sheep and goats), pseudocowpox (producing milker's nodules), tannapox and yabapoxvirus occur. The most notorious member of this family – variola virus – was historically the cause of widespread pandemics of smallpox[3]. It is estimated that 400 000 people died each year in Europe at the turn of the eighteenth century[4] with a consequent major impact on communities[5]. Clinically, it has been recognized that immunity was lifelong following exposure to variola, resulting in the practice of variolation (direct inoculation of infected scabs from patients into healthy recipients[4]). Although immunity to variola and a significant reduction in mortality was achieved, a substantial morbidity and mortality associated with the procedure remained[6]. This provided the background to the classical experiments of Jenner, who used direct inoculation of cowpox to induce protective immunity to experimental exposure to variola.

The relative safety of this procedure saw it widely accepted, but in the middle of the nineteenth century difficulties arose. First, it was recognized that repeat vaccination may be necessary as occasional subjects acquired smallpox after vaccination, and this reduced confidence in the procedure. Secondly, supply of vaccine was a perennial problem. Initially arm-to-arm inoculation was used and it was recognized that dried material could be used, although its effectiveness was variable. Orthopox infections in cows were not common, therefore fresh source material was

limited and the use of such material was opposed by antivaccinationist societies[7]. Despite these problems vaccination was probably the major factor in the hundredfold reduction in smallpox mortality in Sweden between 1800 and 1821[6]. To overcome the problem of uniformity of supply, deliberate inoculation of the skin of calves was practised in Italy as early as 1805, although this did not spread to the rest of Europe until the mid-nineteenth century. Compulsory vaccination was introduced in the UK in 1853 and the continuation of the practice of arm-to-arm vaccination banned in 1898. With increasing use in Europe much of the continent was free of smallpox shortly after World War I and smallpox transmission in Europe and North America stopped after World War II.

However, the source of vaccine remained a problem, as the calf-derived stocks were inactive after 48 hours. An industrial method for preserving viability by freeze drying was developed[8], and this enabled large-scale batches and stocks of vaccine to be prepared. This set the stage for international co-operation in the eradication of a virus with no known reservoir outside man, heralded by the Pan American Sanitary Organization programme to eliminate smallpox in the Americas in 1950 (successful with the exception of Brazil by 1967). In 1958, the USSR proposed a global eradication programme, which commenced in 1959. However, in 1967 an Intensified Global Eradication Programme was established by the World Health Assembly based on: (*a*) a mass vaccination campaign to reach 80% of a community; (*b*) use of vaccines of known potency and stability; (*c*) independent monitoring; and (*d*) outbreak control. From an estimated 10 million cases worldwide in 1967 the final case of naturally acquired smallpox was recorded in October 1976 in Somalia[6].

Vaccinia virus and strains

The nature of the agent used in this remarkable eradication campaign – vaccinia – has remained a mystery. It had been widely assumed that it was derived from cowpox despite passage of the 'virus' in a range of hosts from cows, sheep and water buffalo but also occasionally rabbits, horses and man. Even in 1928 it was being suggested that horsepox, sheeppox or goatpox could be used as a seed lymph[7]. Since 1960 two main strains have been used in vaccination: the Lister and Wyeth strains, although other local strains remained in use throughout the eradication programme. With this background it is perhaps not surprising that the precise provenance of vaccinia remains unknown. Poxviruses are adept at recombining with other poxviruses[9] and subgenomic DNA fragments. Restriction endonuclease maps of poxviruses suggest that vaccinia was entirely separate from cowpox[10], and it has even been suggested that it was a laboratory remnant of an extinct orthopoxvirus[11]. However, a number of poxviruses have been sequenced including vaccinia

virus Copenhagen strain (192kbp)[12], variola strain Bangladesh (189kbp)[13], variola strain India (189kbp)[13], molluscum contagiosum virus (190kbp)[14,15] and modified vaccinia strain Ankara (178kbp)[16]; sequence comparison suggests that a DNA insert in Ankara codes for a cluster of genes found in vaccinia WR strain and cowpox implying a cowpox origin for the virus[16]. In addition to the natural strains of vaccinia that have arisen either opportunistically or as a direct result of attempted in vitro attenuation, targeted modifications are being introduced into the virus to retain its ability to induce effective immune response yet reduce the potential for side effects. These agents are considered below with other aspects of the safety of use of these agents.

Despite uncertainties concerning its origins, vaccinia is the prototype orthopoxvirus and its life cycle has been examined in detail. The virus particles are 300–400 nm surrounded by lipid envelopes containing a linear 200kbp DNA, together with a number of viral enzymes. These initiate 'early' virus gene transcription which encode a number of functions to affect host cell function, evade immune responses and induce transcription of 'intermediate' viral genes, and viral DNA replication. This is followed by 'late' gene expression – largely the structural proteins of progeny virus particles[17]. This temporally regulated cascade of virus gene expression is maintained by interaction of specific viral proteins with sequence motifs in promoter regions. The specificity of these promoters for viral proteins as opposed to host proteins provides an important regulatory check when recombinant vaccinia viruses are constructed. Once structural genes are expressed the virions are assembled and surrounded by one or two (Golgi-derived) lipid membranes. The virus buds from the cell surface or may remain associated with the cell membrane and allow cell-to-cell spread.

The virus has a large potential capacity for foreign genes, which together with its stability and the ability to regulate expression through use of viral promoters made it an attractive vector. The recognition of homologous recombination as a frequent naturally occurring event among poxviruses[9] allowed the development of a direct recombination into a specific virus site with an inherent selection marker[18]. This ease of manipulation and the high levels of expression of inserted genes have made vaccinia a powerful research tool[18,19]. In combination with its established success as a vaccine, it has been promoted not just as a vector for experimental vaccines in models but as a vector for human single and multiple vaccines[20,21]. This is supported by the ability of vaccinia recombinants to induce protective immunity, especially CTL, to a variety of inserted genes[22]. It also has a broad tropism, is relatively species nonspecific and is a suitable vector for veterinary vaccines[19] including immunization in the wild to prevent spread of rabies[23]. Natural poxviruses of a range of commercial stock animals have been used, e.g. fowlpox and avian influenza[22]. These observations have combined to drive the use

of recombinant poxviruses as live vaccines against infection and for immunotherapy of cancer (see also Chapter 3), yet despite preclinical evidence of protection against 20 pathogens, in 15 species and 15 years of experimentation, no product is yet in clinical practice[24].

However, if the primary aim of immunization is to induce CD8 + CTL immune responses, in experimental rodent models recombinant vaccinia is established as a potent inducer of such CTL to viral- and tumour-associated antigens[22]. In particular, these include viral oncogenic proteins including HPV 16 E6[25] and E6/E7[26] and the protective immunity induced was mediated by CD8 + CTL[27].

Human papillomavirus and cervical cancer (see also Chapter 5)

Cervical cancer remains the second most common cancer of women worldwide with an annual incidence of approximately 400 000 cases[28–30]. The burden of this disease falls disproportionately on developing countries[29,30] and is strongly linked to local socio-economic deprivation[31]. Improved socio-economic status and effective treatment of pre-invasive disease detected by cytological screening are probably responsible for the falling incidence and mortality in Western Europe and North America[31–33]. Early detection and presentation may be an important factor in improving survival with this disease in developing countries[34], yet it is widely held that implementing cytological screening may be ineffective because of compliance, costs and an ability to maintain quality control of cervical cytology[35]. Cervical cancer is associated with HPV infection; invasive disease in eight countries is associated with a 96% prevalence of HPV in invasive cervical cancer[28], and with further refinement of detection techniques this has increased to 99.8% (Walboomers, personal communication). Specific HPV types, particularly 16 and 18, have the capacity to induce and sustain cellular transformation, largely requiring continuous expression of the nonstructural E6 and E7 genes[36].

These observations have led to the development of two nonmutually exclusive immunological approaches to disease control. First, the continued nuclear expression of HPV E6 and E7 genes in malignant cells raised the possibility of generating CD8 cytotoxic T lymphocytes (CTL) against these antigens as an immunotherapy for established disease. We have identified such effector cells at sites of disease[37] and in peripheral blood mononuclear cells (PBMC)[38]. Secondly, the HPV capsid is made up of a major 55kD (L1) protein and minor (L2) 70kD protein and expression of the structural L1 (+/− L2) genes can be used to generate virus-like particles (VLP), which are serologically indistinguishable from the natural homologous virus capsid[39]. VLP protect against HPV transmission in dog and rabbit papillomavirus infection[40] and specific antibodies are induced in primates[41]. These VLP are currently in phase I human volunteer studies and will be subject to wider clinical

efficacy studies[42]. However, it is recognized that there may be problems with induction and longevity of local mucosal immunity[43–45] and, as these viral proteins are not expressed in malignant cells, it is unlikely that L1 VLPs will be effective against established malignant disease.

There are no suitable small animal models which mimic the progression of HPV-induced genital lesions to invasive cancer, as observed in man[46]. Nevertheless, HPV16 E6 and E7 gene products are immunogenic to rodents, inducing CTL in mice[25,27] and rats[26]. Furthermore, the HPV-specific CTL generated could mediate specific rejection of tumour cells transfected with HPV16 antigens[26,27]. Vaccinia virus recombinants also induced DTH reactions in mice bearing transplanted mouse keratinocytes expressing HPV16 E7 proteins[47], establishing that HPV16 E6 and E7 gene products were immunogenic to murine CD4 and CD8 T cells.

Induction of HPV-specific cytotoxic T lymphocytes in vivo

The observations in murine models suggested that HPV E6 and E7 were immunogenic and provided preliminary evidence that specific CD8 + CTL could be generated. However, evidence up until our later observations suggested that few, if any, human HPV E6 and E7 specific CTL were present in the context of HPV-associated malignancy[48]. The nature of HPV infection required an appropriate vector to present linear epitopes from the whole protein and yet maintain an appropriate safety profile both in terms of the insert and the patient population that was to be targeted.

Development of recombinant HPV vaccine

The initial selection of a vaccinia-based vector was based on the following considerations:

(1) Vaccinia has a lytic life cycle, which ensures that any risk associated with an expression of a potentially oncogenic insert would be restricted to the lytically infected target cell. HPV E6 and E7 genes from HPV 16 and 18 immortalize cells in culture[49,50]. Consequently, there is a risk that introduction of these genes into human cells in vivo could lead to long-term expression which could result in cell transformation. Quantification of this risk is extremely difficult.

(2) Expression of inserted genes is regulated by vaccinia virus promoters, which differ substantially from mammalian promoters and rely on vaccinia-encoded transcription components[19]. It is therefore unlikely that DNA released from vaccinia virus infected cells could be taken up by healthy cells and expressed.

(3) There is evidence of widespread use and an established safety profile in man. Wyeth strain vaccinia was chosen, because of its low rate of complications among the various strains used during the smallpox eradication campaign[51].

(4) There is an absence of vaccinia virus persistence or latency[1,6,17]. This further reduces the risk of a cell infected with a recombinant expressing E6 and E7 surviving and transforming locally, even in the face of probable pharmacological immunosuppression in this patient group.

To further reduce potential risks the HPV sequences inserted in the vaccinia were modified. The E7 genes of both HPV16 and 18 were modified by site-directed mutagenesis to the Rb binding site. Reduced Rb binding following this modification was demonstrated and co-transformation activity abolished in rat fibroblasts[52]. The possibility of natural recombination with any locally present HPVs that may be present at the site of vaccination[53–55] was reduced by the reorientation of E6 and E7 relative to each other and the organization of these two genes as a fusion protein. Although these modifications could have ablated or even created artefactual CTL epitopes, it was considered an appropriate modification bearing in mind the linear nature of CTL epitopes and the excess of primary structure made available by the inclusion of whole proteins rather than subunits of E6/E7. The modified HPV16 and HPV18 E6 and E7 genes were inserted in a head-to-head orientation under the control of the p7.5 and H6 promoters at a neutral site in the vaccinia virus Wyeth strain genome (TA-HPV). In preclinical testing, immunization of C56BL/6 mice with TA-HPV induced CTL responses against HPV16 E7[52].

Phase I/II trial of TA-HPV in patients with advanced or recurrent cervical cancer

The safety and ability of TA-HPV to induce CTL was investigated in a phase I/II trial of eight patients with advanced or recurrent cervical cancer; 58 patients were originally screened for immunocompetence as we and others had previously reported that patients with cancer of the cervix, especially in advanced stages, had reduced specific and nonspecific immune responses, often irrespective of prior therapies received[56] (see also Chapter 5). This was a particularly important consideration as complications with vaccinia have been reported when vaccination had been performed with congenitally reduced immune responses[6,51,57] as well as advanced HIV infection[58]. The screen used: total leucocyte count, complement profile, immunoglobulin levels, T-cell subsets and the ability to respond to pneumococcal vaccine in vivo, as well as PHA transformation. This was to ensure comparability with older studies that employed lymphocyte proliferation as a measure of immunodeficiency during the smallpox eradication campaign[6,51,57]. The description of a case of disseminated vaccinia in a patient with a very low CD4 count (20 per μl) with HIV infection[58], led to a stringent criterion of CD4 count >400 per μl being adopted in this study. The majority of patients excluded were as a result of this criterion.

All patients in the vaccination group had initial biopsies that were HPV16

positive and negative for HPV18, 31 and 33 by PCR. Vaccination was by multipuncture intradermal scarification through a 20 μl drop of TA-HPV containing 10^8 pfu/ml [7]. To prevent environmental release of a novel recombinant agent, patients were isolated in the infectious disease unit for two weeks after vaccination. Subsequent testing revealed that live virus was recovered from the vaccination site and the dressing in contact with it but not the bed linen, room, bathroom or toilet areas. There was no evidence of systemic spread by direct virus culture. No short- or medium-term complications of vaccination were observed. When re-isolated virus was examined by PCR of material from the inoculation site or the scab formed after 10–14 days, all isolates contained the inserted sequences in appropriate orientation, confirming stability of the construct even after in vivo passage[59].

All patients had a successful 'take' of the vaccine by clinical criteria and this was confirmed by the presence of antivaccinia IgG antibodies after vaccination. Pre-existing HPV16 E6 or E7 antibody was present in most patients but new HPV18-specific antibody responses could be detected in three patients after vaccination[59]. Attempts to generate HPV-specific CTL using TA-HPV infected cells for secondary in vitro re-stimulation resulted in nonspecific killing (S. Graham, personal communication) and vaccinia-specific lysis[60–62]. When adenovirus recombinants were used[38], HPV-specific CTL responses were detected in PBMC from one of three patients who responded to control stimuli such as mixed lymphocyte culture. These CTL were detected nine weeks after vaccination, following re-stimulation with HPV18 E6/E7 adenoviruses in vitro. Interestingly, both the operationally defined HPV18- and 16-specific CTL responses were transient, as no CTL responses could be detected at 14 or 20 weeks postvaccination. This may reflect a loss of HPV-specific CTL activity and/or an increase in nonspecific lysis masking HPV-specific lysis. The tumour biopsy from this patient was HPV16 positive and HPV18 negative and HPV18-specific CTL were not present before vaccination, suggesting that this response was a result of immunization with TA-HPV.

In such a small and selected study no therapeutic effect can be ascribed to vaccination. At follow-up, seven of the eight patients have died as a result of disease progression. In the discussion of the original report we commented on the clinical improvement in one of the subjects, patient no. 6[59]. This patient presented in 1986 and was treated by radical hysterectomy and radiotherapy for FIGO stage 1b disease. Radiological recurrence was noted in 1991 and there was partial response to further treatment. However, at the time of vaccination (July 1994) a CT scan suggested her recurrent pelvic tumour was still present, although reduced in size following single-agent cisplatin treatment in 1992. Interestingly, this patient was initially excluded from this study due to a CD4 count below the exclusion limit ($<$400 CD4 cells/μl); however, ethical permission for vaccination to proceed was granted when the CD4 rose to 320 CD4 cells/μl. In addition, this patient did not

produce an antiHPV antibody response, had pre-existing antivaccinia antibodies and was the subject who developed an HPV-specific CTL response. She continues to remain well and tumour free at follow-up, some four years after vaccination (April, 1999).

This study, the first European study to use a recombinant viral vaccine in man, suggested that TA-HPV was capable of inducing both antiviral antibody and CTL responses. Further studies to determine appropriate dosage schedules and applicability to patients with earlier stages of cervical disease were warranted and necessary before proceeding to a full clinical effectiveness trial.

Immunogenicity of TA-HPV in CIN3 patients

A further trial involving 12 CIN3 patients has now been completed. This trial investigated the immunogenicity of TA-HPV by delivering two doses of vaccine (eight weeks apart) to an immunocompetent patient group. The method of vaccine delivery was also changed to a bifurcated needle to allow a more standard dose of vaccine to be administered. The two tines of the needle allow the adherence of 0.0025 ml of vaccine by capillary action when dipped into the vaccinia suspension[7]. The needle is then positioned vertically above the inoculation site on the upper arm and 5–15 strokes of the needle applied to the skin such that erythema with occasional capillary bleeding is observed on completion. Clinical efficacy was not a primary endpoint as all vaccinated patients had standard surgical treatment of their CIN3 lesions. All patients had antivaccinia antibody responses as well as a clinical 'take' of the vaccine, as observed in the earlier phase I/II study[59]. In contrast to the previous trial with TA-HPV, no antiHPV antibodies were detected in this group either pre- or postvaccination. Preliminary results of CTL studies confirm our observations in CIN3 patients[38] that HPV E6/E7-specific CTL can be detected in blood prior to immunization. However, new HPV-specific CTL responses could also be detected after immunization in three further patients who did not have pre-existing CTL responses, confirming the single observations made in patient no. 6[59]. No effect of the additional dose of vaccine or any relationship between prior exposure to vaccinia and detection or development of HPV CTL responses postvaccination was observed (S. Man, manuscript submitted).

Current studies

The completed clinical studies with TA-HPV have not assessed clinical efficacy but confirm that this vaccinia recombinant is capable of inducing specific humoral and CTL immunity to inserted HPV E6 and E7 genes. These responses were obtained despite the late stage of the disease, preceding immunosuppressive therapy and in the face of prior vaccination. The result of a further immunogenicity study in a phase I, US-based trial of TA-HPV on 12 patients with late-stage cervical cancer at

NCI is awaited. We are engaged in further studies to evaluate TA-HPV, with a shift in emphasis to clinical effect alongside CTL induction:

(1) A small European Organization for the Research and Treatment of Cancer (EORTC) phase II trial has completed recruitment in the UK and five other European centres (including Sweden, Holland and Germany). A total of 29 cervical cancer patients with FIGO stage IIa (or earlier) cervical cancer have been recruited. Each patient received two vaccinations with TA-HPV using a bifurcated needle, one prior to and one following surgery. Direct effect on primary disease is unknown because of surgical treatment. Patients are being monitored for immunological, including CTL, responses at a central laboratory to minimize experimental variation. Initial results indicate that the immunological endpoint (HPV-specific immune response in >10%) had been achieved. The frequency of disease recurrence in this patient group is being compared with nonvaccinated controls. However, these results will not be available for 5–8 years. Further phase II trials are under consideration (http://www.cantab.co.uk).

(2) We are undertaking a small study of CIN3 patients to evaluate regression in CIN3 in the short term (less than three months) following TA-HPV vaccination. This study will monitor local disease colposcopically and with biopsy, as well as making a fragment of the final excision biopsy available for further immunological evaluation. This will permit evaluation of migration of vaccination-induced CTL to sites of disease.

(3) A large study investigating the role of HPV-specific CTL in the natural history of HPV-associated disease in unselected rural women in sub-Saharan Africa is being undertaken in collaboration with the MRC Reproductive Biology Programme, MRC Laboratories, The Gambia. A pilot project has been completed (Borysiewicz et al., unpublished). Although in its early phases, this study is of particular importance as it addresses the issue of the role of CTL in patient populations where an immunotherapeutic or prophylactic approach might offer the greatest clinical benefit.

In the context of these clinical studies it has become apparent that there is a requirement to simplify HPV CTL assays if they are to be useful for monitoring immune responses in the field. HPV type 16 and 18, E6 and E7 presented by TA-HPV or in hybrid VLPs (incorporating HPV type 16 E7)[63–65] are capable of inducing cytotoxic T lymphocyte responses in man. This will allow the definition of peptide epitopes recognized by such CTL and assessment of T cell memory after vaccination. However, current approaches to refining CTL assays for CTL require the identity of a peptide epitope. We have adapted novel technologies such as fluoresceinated MHC–peptide complexes to label HPV type 16 $E7_{11–20}$ specific CD8+

T cells (Youde et al., unpublished) and ELISPOT assays to measure cytokine release[66]. These approaches have increased the sensitivity as well as simplicity of detection of human immune effector cells, although important issues relating to their comparative sensitivity with conventional approaches, as well as their functional specificity, remain to be addressed. Incorporation of these technologies into future clinical trials may allow simultaneous examination of both CD4 and CD8 T cells from limited clinical samples. Most studies of HPV cell-mediated immunity focus on the CD8 + CTL response but this often reflects current expertise and does not obviate an important role for the CD4 + T cells. There is increasing evidence that CD4 responses are important in HPV-associated cervical neoplasia[67,68], and assays to measure DTH or T cell proliferation assays against HPV antigens must be incorporated into future studies.

Is recombinant vaccinia the optimum vector for HPV-associated cervical disease?

The choice of vaccinia as a vector was pragmatic and related to specific features which enabled their rapid utilization in clinical studies in man. This was based on:

(1) The requirement for a large insert size.
(2) Lytic replicative cycle and use of virus-specific promoters limiting expression to cells which have already triggered the lytic replicative cycle.
(3) No virus persistence in the host.
(4) The documented safety of vaccinia in the smallpox eradication campaign.
(5) The lack of animal models of cervical cancer requiring a rapid implementation of any vector in man.

All of these factors are relative rather than absolute and, whilst valid in the first phase of investigations, they may be superseded by developments in vector technology. An important consideration is the nature of the insert that will be used and the type of immune response it is proposed to develop in vivo following immunization. However, certain aspects of poxvirus-based strategies warrant further examination in the context of induction of HPV-specific immunity.

Safety

The adverse reaction rates for vaccinia viruses in the smallpox vaccination campaign were about 1 in 50 000, and higher for primary as opposed to repeated exposure[51]. Initially this risk was acceptable, as was historically the risk associated with variolation in the light of the severe morbidity and mortality associated with smallpox. However, even in the later stages of the eradication campaign the issue of vaccination-induced morbidity was raised, and often considered unacceptable when

there was a low prevalence of smallpox in the community being vaccinated[6]. These arguments are always raised when the possible use of recombinant vaccinia virus vaccine is being considered. Often, especially in cancer immunotherapy for late-stage disease, or if a disease represents a major international health hazard, e.g. HIV infection[69], potential benefits are deemed to outweigh the risks. The relative ease of production, low costs, the advantages of a live vector in induction of both humoral and cell-mediated immunity and previous successful application in developing countries, makes poxviruses attractive candidate vectors in a global setting. Nevertheless, recombinant vaccinia virus vaccines are live viruses capable of replication and spread. Spread of the agent can occur both to the environment and in the individual patient (including his or her close contacts). This is an important issue in those receiving immunosuppressive therapies. Not only is this suppression pharmacologically induced, but is often apparent because of the nature of the malignancy; this is the case with cervical cancer[56] and limits recruitment into clinical trials[59]. Two approaches may be adopted to deal with these issues. First, the rigorous screening of the patient population (as in our studies[56,59] and, secondly, to modify the vector to reduce its pathogenicity.

This drive to improve safety has led to the development of attenuated strains such as modified vaccinia virus Ankara (MVA) and NYVAC, as well as poxviruses from other species which replicate to a limited extent in human cells, e.g. avipox. MVA was attenuated by passage in chicken fibroblasts and continues to grow well in these cells but has lost its ability to replicate in mammalian cells[70] and is not pathogenic even in immunodeficient mice[71,72]. Recent studies in mouse models suggest that this vaccinia strain is as effective as replication-competent vaccinia in inducing murine CTL responses[72]. The attenuation of this virus is due to multiple deletions resulting in a failure of late-stage morphogenesis which inhibits release of infectious virus from mammalian cells, whilst allowing the expression of viral proteins in the infected cell[16,73]. Furthermore, MVA was used to vaccinate 120 000 subjects at high risk of side effects from vaccinia without untoward effect.

NYVAC was developed by the deliberate deletion of 18 open reading frames in the fully sequenced genome of Copenhagen strain vaccinia[74]. This virus has a debilitated replication phenotype in mammalian cells yet replicates in chick fibroblasts and, similarly to MVA, has reduced pathogenicity in immunocompromised animals. However, although it has been used in a number of veterinary vaccines, most notably in Japanese B encephalitis in swine and some small trials of human vaccines, it lacks the established safety profile of MVA and other vaccinia strains[21].

Avipox viruses are restricted in their host range to avian species, yet expression of inserted genes will occur in mammalian cells in the absence of virus replication[21]. This vector has also been used in veterinary vaccines in a number of species. HIV vaccination of seronegative subjects using a canarypox vector (with adjuvant

recombinant *env* subunit) has been studied with an enhanced ability to generate CTL (either CD4+ or CD8+) over subunit alone[75].

These modifications to conventional vaccinia strains (WR, Wyeth and Copenhagen) have allowed longer expression of antigen in the host and improved their safety profile for immunocompromised subjects. However, this has been achieved at the cost of the full lytic replicative cycle of the virus and reduced cytopathology in the infected cell. When considering a vector for immunotherapy in man the nature of the insert often dictates the choice of vector[52,59]. HPV E6 and E7 are potentially oncogenic and, whilst site-directed mutagenesis can block Rb binding, it is essential to prevent long-term expression and avoid any persistence of these gene products in the host. These considerations dominated the original choice of vector and might limit the use of less well characterized vaccinia variants. It has to be remembered that the choice of vaccinia limits the duration of antigen expression in the host and, whilst this is necessary to reduce risks to the patient to a minimum, it is undoubtedly suboptimal for efficient antigen presentation. Therefore, the choice of vaccinia was appropriate for initial investigations but as other vectors, including poxviruses, are better characterized and experience of their use in man grows, alternative delivery systems may become more attractive.

Route of inoculation and vaccination schedules

The selected route of inoculation in these studies was by dermal scarification with the object of establishing a poxvirus infection in the lower layers of the epidermis. Conventionally, the risks of pyrexia and malaise following such inoculation have been held to be a consequence of systemic inoculation by inexperienced vaccinators[6]. However, this route is restricting because the specific dose of inoculum delivered is unknown and frequently clinical 'take' is often related to operator experience. Furthermore, the presence of cutaneous lesions results in a greater likelihood of person-to-person transmission[7] and environmental spread. In a systematic study of 91 volunteers intradermal or even intramuscular inoculation did not result in a higher incidence of side effects nor was it less immunogenic, but cutaneous lesions were reduced[76]. This may be of importance, particularly if the more attenuated vaccinia viruses are used as vectors.

The frequency of vaccination and requirement for booster vaccination was established historically and was widely practised for those at risk during the Smallpox Global Eradication Programme. However, the optimum frequency of vaccination is unknown. It has been argued that frequent vaccination will reduce the relative potency of the vaccine because of pre-existing immunity, as well as potentially enhancing local inflammation at the vaccination site. To date, we have observed a reduced second lesion in most vaccinees given a second scarification eight weeks after the first. As noted above, no boost to HPV-specific CTL responses

has been observed, although assays employed to date are nonquantitative. These failures have resulted in many investigators employing the concept of a 'prime-boost' approach using two different vectors in order to avoid antivector immunity. This approach has been most widely promulgated in HIV infection, and in most regimes a poxvirus vector has been one of the agents employed[69,77–79]. These approaches have been extended to other antigens, including malaria[80–82], and may represent an attractive option for inducing antitumour immunity in vivo.

Role of prior antivaccinia immunity

One of the desirable qualities of vaccinia as a vector is its potent induction of humoral and cellular (including CD8 + CTL) immune responses. In general, these are stronger than those generated using peptide, protein or DNA immunization, although combinations in prime-boost strategies may be more effective[78–82]. It has been argued that pre-existing immunity to vaccinia in those vaccinated against smallpox may limit subsequent responses against a recombinant vaccinia virus vaccine[83]. While this has been shown in some animal models[84], there is no consensus that this is the case in man. In a study of 18 HIV seronegative individuals immunized with a recombinant HIVgp160-vaccinia, the authors suggested that vaccinia virus vaccines might be more efficacious in vaccinia-naive individuals[83]. However, all recombinant vaccinated individuals produced both antibody and T cell proliferative responses against HIVgp160. Antibody and proliferative responses were reduced on average in the vaccinia-primed group compared with the vaccinia-naive group, but the sample size of two respective groups (16 naive versus 2 vaccinia immune) was insufficient to suggest this as a general rule. Results from our recent clinical trials, although limited in the same way by numbers, suggest that previous exposure to vaccinia did not limit the induction of CTL responses by a recombinant vaccinia-based vaccine.

In mice, antivaccinia CTL can be detected directly from the freshly isolated splenocytes of vaccinia-immunized animals[84]. Such CTL could restrict CTL responses after revaccination. However, despite smallpox vaccination, antivaccinia CTL are difficult to detect in many[60–62,85]. This may be a technical problem, as distinguishing vaccinia-specific CTL from nonspecific killing in PBMC is difficult and further compounded by the lytic replicative cycle of the virus making in vitro restimulation difficult.

Antigen processing and immunogenicity

Vaccinia encodes numerous genes, which contribute to virulence, whose deletion may produce a safer poxvirus vector[86]. These genes often block immune responses or inhibit inflammation[87–89]. Vaccinia virus inhibits antigen processing and presen-

tation[90], via a mechanism unrelated to vaccinia virus-encoded serine protease inhibitors[91]. While there have been no further reports of such inhibition, another concern expressed about using vaccinia virus recombinants expressing full-length gene products is that the viruses may not produce the relevant CTL epitopes, either qualitatively or quantitatively. To overcome this perceived limitation some have used minigene constructs in which only the epitope of interest is encoded. This removes the rate-limiting step of antigen processing, and should result in more efficient delivery of the peptide to nascent class I molecules in the endoplasmic reticulum. Multiple epitopes could be incorporated in a 'string of beads' fashion to induce CTL of multiple specificities[92]. Alternatively, selection of MHC class I or class II restricted responses could be accomplished by targeting antigens to different pathways: ubiquitination for rapid degradation/MHC class I presentation[90] or towards the endosomes for class II responses.

Immunogenicity of vaccinia recombinants may be improved by the incorporation of cytokine genes[93]. In animal models these promote tumour rejection either through enhancement of antigen processing[94] or through promoting activation or growth of CTL. Enhanced induction of immune responses by vaccinia could be achieved by maximizing gene expression in the most potent APCs. Recently, it has been found that the type of vaccinia virus promoter utilized in the recombinant will determine the levels of antigen expression in dendritic cells and other APCs[95].

Conclusions

The use of poxvirus vectors has been an important initial step in the demonstration of the ability of HPV nonstructural genes, particularly HPV type 16 and 18, E6 and E7, to induce specific T cell immunity in man. The versatility and scope for large inserts, as well as their well established safety profile, make them attractive vectors in the first instance. Paradoxically, the lytic nature of the infection and limited transcription of inserted genes, regulated through vaccinia-specific promoters, makes them even more attractive in a setting where the inserted gene products may themselves be oncogenic. Vector technology has improved as well as our understanding of antigen presentation to selectively enhance individual immune responses to protein or subunit, and these developments raise the question as to whether these vectors are still the most relevant for future use. However, developments in poxvirus technology have also modified the vector such that it is substantially improved as an antigen delivery system, although issues of safety and application in man remain. This, together with improvements in vaccination schedules, may well point to combined approaches utilizing several vectors to ensure optimum generation of antitumour immunity, but it is also probable that one of these will still be poxvirus-based in the foreseeable future.

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5

Vaccine delivery and immunosuppression in cervical cancer

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Introduction

A correlation between human papillomavirus (HPV) infection of cervical epithelial cells and cervical cancer has been unequivocally established. A significant proportion of early-stage precancerous lesions, cervical intraepithelial neoplasia (CIN) and essentially all cervical carcinomas are positive for certain high-risk types of HPV. The potential for immunotherapeutic intervention directed against viral targets expressed in cervical neoplasia is discussed in this chapter.

Human papillomaviruses and cervical neoplasia

Epidemiology

Human papillomaviruses (HPVs) are the most common sexually transmitted viruses[1] and there are over 90 different HPV types described so far, of which about 35 types infect the anogenital tract[2]. The risk of infection is approximately the same for men as for women, but in general the infection rate for HPV is greater at a younger age (below 25 years) than later in life[3]. Most HPV infections are subclinical and go unnoticed[4], although some infections may progress into benign lesions, like the common genital warts in the anogenital region. HPV genomic DNA is detected in approximately 10–15% of the sexually active adults in the United States, whereas approximately only 1% of the population show clinical manifestations of infection, cervical intraepithelial neoplasia CIN[5]. Only a minority of these CIN lesions will later progress into cervical carcinomas and adenocarcinomas.

Analysis of biopsy material of CIN lesions and cervical carcinomas showed the prevalence of HPV 6 or HPV 11 in low-grade CIN lesions, whereas HPV 16 and HPV 18 dominated in high-grade CIN and cervical carcinoma samples. Therefore, HPV 6 and 11 are usually referred to as low-risk types; meanwhile, HPV 16 and 18, as well as the rarely seen types 31, 33 and 45, are referred to as high-risk types for the progression to cervical cancer. Of those HPV 16 can be found in almost 50% of all cervical carcinomas and HPV 18 in about 14% of the cases[6]. Nevertheless, HPV

18 predominates in third world countries like Indonesia and is mainly found in adenosquamous carcinomas. Moreover, HPV 18 causes more aggressive growing tumours than HPV 16[7]. Overall, the high-risk types of HPV are clearly correlated to the induction of cervical cancer and PCR technologies in diagnostics revealed the presence of DNA of high-risk types of HPV in more than 99% of all examined cervical carcinoma specimens (J.M. Walboomers, personal communication).

Transforming activities of HPV proteins

The early proteins E6 and E7 of high risk papillomaviruses have transforming capabilities and are expressed in all stages of the carcinomas[8,9]. The interaction of E6 with the cell cycle control protein p53[10] leads to rapid ubiquitation of p53 and subsequent degradation[11]. Moreover, the abrogation of the normal function of p53 also protects the cells from undergoing apoptosis[12,13]. The E7 protein interacts with the retinoblastoma protein pRb and other members of the Rb family[14,15] and this interaction results in loss of the growth inhibitory function in the cell cycle control in transformed cells. Although the expression of both viral proteins is needed for the maintenance of the transformed state, E6 and E7 transformed cells are non-malignant and co-operation with a second activated oncogene is required for malignancy. For example, co-transfection of HPV 16 transformed cells with activated *ras* oncogene does render the E6/E7 expressing cells malignant[16]. Reducing the translation of E6 and E7 mRNA in malignant carcinoma cells with E6 and E7 antisense RNA can reduce the cell growth and tumorigenicity of the transformed cells[17]. Additionally, for efficient transformation and malignancy the integration of the viral genome into the host genome is required. In fact, tumour cell lines from cervical carcinomas only retain integrated viral DNA and in only very few samples extrachromosomal viral genome can be found[18]. Integration of viral DNA involves the disruption of the E2 gene, which leads to the upregulation of E6 and E7 expression and efficient transformation of the host cell[19]. Taken together, the requirement for a second event like *ras* activation, as well as the necessity for integration of the viral DNA into the host genome, might explain the low frequency of cervical carcinoma amongst high-risk HPV-infected women. Another explanation could be that the infection with HPV is controlled and eliminated by the immune system. The transforming proteins E6 and E7 are of particular interest since their expression is needed for the maintenance of the transformed state of the tumour cells and thus are potential immunological targets. Therefore, animal models were designed to study the influence of the immune system on the control of tumour growth.

Immune responses against HPV in animal models

The high-risk types of human papillomavirus have the advantage that they also transform rodent cells, in particular murine cells, which allows immune responses

to high risk HPVs to be studied in animal models. The immunogenicity of HPV-derived transforming proteins E6 or E7 can be demonstrated by vaccination with either irradiated cells transfected with the HPV genome or the E6 and E7 genes. For example, immunization experiments with HPV 16 E7 gene transfected fibroblasts protected mice from a subsequent challenge with tumorigenic mouse melanoma cells also transfected with the HPV 16 E7 gene[20]. The protection was abrogated by depletion of the $CD8^+$ cells in these mice, indicating that the tumour growth was controlled by an HPV 16 E7 specific $CD8^+$ CTL response induced by vaccination with E7 transfected cells. A similar experiment with E6 transfected fibroblast cells induced regression of transplanted E6 expressing tumours in vaccinated mice[21]. Spleen cells isolated from vaccinated mice could specifically kill E6 transfected tumour cells in vitro, confirming the specificity of the antitumour response. Overall, these experiments indicated the ability of a specific immune response to control the growth of tumour cells expressing the HPV 16 antigens E6 or E7 and, moreover, that these two proteins can serve as tumour rejection antigens. Subsequent studies focused on the E6 and E7 derived peptides presented by tumour cells and the specificity of the induced immune response, in particular which epitopes were recognized by specific T lymphocytes.

In 1974 the restriction for cellular immune responses to certain MHC class I molecules was published[22] and since then the detailed mechanisms of the interaction between T cell receptor and MHC class I molecules in antigen recognition have been unravelled. MHC class I molecules are located in the endoplasmic reticulum with intracellular protein-derived peptides fulfilling certain criteria in length (8 to 11 amino acids in length) and amino acid composition (anchor residues specific for the haplotype)[23–25]. Upon transportation of peptide-loaded MHC class I molecules on the cell surface, specific $CD8^+$ T cells can recognize the peptides presented in the context of a specific MHC class I molecule via their T cell receptor (TCR) and thereby get activated. Therefore, the interaction between the MHC class I molecule and the peptide, as well as the T cell repertoire, are of crucial importance in mounting and also shaping a specific immune response against intracellular pathogens and viruses.

In order to analyse the MHC-presented peptides, acid elution of naturally presented peptides from the MHC class I molecules of tumour cells has been widely used[26], allowing the identification of naturally processed peptides[16,27–30]. Alternatively, sets of peptides spanning the entire length of the protein to be analysed can be tested for the capacity to bind to the MHC class I molecule of interest[31]. This approach circumvents the laborious acid elution and subsequent identification process by HPLC of the first method, but requires additional testing for natural processing and presentation by target cells. So far, it has been possible to identify a series of MHC class I binding peptides derived from HPV capable of

inducing a specific immune response in vivo in mice and in vitro in human PBL (Table 5.1).

The principle of peptide vaccination was established in 1991 by Schulz et al. who studied a lymphocytic choriomeningitis virus (LCMV)-derived peptide[32], and Kast et al. who studied a Sendai virus-derived peptide[33]. The specific CTL epitopes were emulsified in incomplete Freund's adjuvant (IFA) and applied subcutaneously to mice. In both cases a cellular immune response in vivo was induced, which protected the vaccinated mice against a challenge with a lethal dose of virus. These results proved the possibility of inducing protective immune responses with single CTL epitope-based vaccines. In order to test peptide vaccines in an HPV 16-induced tumour model, mouse embryo cells were transformed with activated E-J *ras* and the HPV 16 genome[16]. The resulting C3 cell line was tumorigenic in C57BL/6 mice and expressed the E6 and E7 proteins of HPV 16. After immunization of C57BL/6 mice with an HPV 16 E7-derived epitope RAHYNIVTF (aa49–57), which binds the murine MHC class I H-2D^b molecule with high affinity[16,30], a strong CTL response against the C3 tumour cell line was observed[16,34]. Moreover, the immunized mice were also protected from developing a tumour after an otherwise lethal dose of tumour cells and a long-term memory persisted in these vaccinated mice[34]. This clearly demonstrated the versatility of peptide-based vaccines for the prevention of HPV-induced tumour cell outgrowth. Unfortunately, the same peptide failed to induce an effective antitumour immune response upon injection into tumour-bearing mice and no tumour regression could be observed. Nevertheless, CTLs specific for the immunogenic peptide were able to eliminate established tumour masses as has been shown by adoptive transfer experiments[34]. Infusing a CTL clone specific for a H-2D^b-restricted immunogenic HPV 16 E7-derived CTL epitope into C3 tumour-bearing mice led to total tumour regression and survival of the mice, thereby demonstrating the ability of highly specific CTL to recognize and destroy established tumours in vivo.

The inability of the HPV 16 E7-derived peptide RAHYNIVTF to induce a CTL response in vivo in the situation of an already established tumour mass could be reversed upon intravenous infusion of dendritic cells (DC) loaded with the HPV 16 E7 peptide. In vitro-generated DCs were loaded with HPV 16 E7-derived peptide (aa49–57) and infused intravenously in C57BL/6 mice bearing day 14 subcutaneous C3 tumour masses (approximately 1 cm^3)[35]. This treatment resulted in total tumour regression and survival of 95% of the treated mice. Even when tumour growth was extended to 21 days prior to therapy, 60% of the treated mice showed total tumour regression, whereas vaccination with emulsified peptides in IFA or DCs loaded with irrelevant peptide did not induce tumour regression. These experiments clearly demonstrated that the HPV 16 E7 peptide RAHYNIVTF (aa49–57) can not only induce a protective immune response upon vaccination,

Table 5.1. *MHC class I restricted CTL epitopes of human papillomavirus*

	Restriction	Peptide sequence	Protein and position of peptide	Reference
Human	HLA-A*0201	RLVTLKDIV	HPV 11 E7 (4–12)	124
		YMLDLQPETT	HPV 16 E7 (11–20)	53
		TLGIVCPI	HPV 16 E7 (86–93)	53
		LLMGTLGIV	HPV 16 E7 (82–90)	53
		THIDIILECV	HPV 16 E6 (29–38)	53
		FAFRDLCIV	HPV 16 E6 (52–60)	53
		KLPQLCTEL	HPV 16 E6 (18–26)	125
		ALQAIELQL	HPV 16 E2 (69–77)	54
		TLLQQYCLYL	HPV 16 E1 (253–262)	54
		LQDIEITCV	HPV 18 E6 (25–33)	55
		ELTEVFEFA	HPV 18 E6 (40–48)	55
		KLTNTGLYNL	HPV 18 E6 (92–101)	55
		KLPDLCTEL	HPV 18 E6 (13–21)	55
	HLA-B*0702	ERPRKLPQL	HPV 16 E6 (7–15)	126
		RPRKLPQLC	HPV 16 E6 (8–16)	126
Mouse	H-2D^b	RAHYNIVTF	HPV 16 E7 (49–57)	30,16
	H-2K^b	RWTGRCMSCC	HPV 16 E6 (131–140)	127
		GRWTGRCM	HPV 16 E6 (130–137)	128
		KQQLLRREVY	HPV 16 E6 (41–50)	128
		QLLRREVYDF	HPV 16 E6 (36–45)	129
		YSLYGTTLEQ	HPV 16 E6 (81–90)	129
		DLYCYEQLND	HPV 16 E7 (21–30)	129
		STHVDIRTLE	HPV 16 E7 (71–80)	129
		DLYCYEQL	HPV 16 E7 (21–28)	130
		DRAHYNIV	HPV 16 E7 (48–55)	129
		YHAGTSRLLAVGHPY	HPV 16 L1 (61–75)	129
		VGHPYFPIKKPNNNK	HPV 16 L1 (71–85)	129
		VSGLQYRVFRIHLPD	HPV 16 L1 (91–105)	129
		VGISGHPLINKLDDT	HPV 16 L1 (141–155)	129
		YAANAGVDNRECISM	HPV 16 L1 (161–175)	129
		CTNVAVNPGDCPPLE	HPV 16 L1 (201–215)	129
		CTSICKYPDYIKMVS	HPV 16 L1 (251–265)	129
		YKNTNFKEYLRHGEE	HPV 16 L1 (381–395)	129
		TFWEVNLKEKFSADL	HPV 16 L1 (471–485)	129
		KAKPKFTLGKRKATP	HPV 16 L1 (501–515)	129
	n.d.	TTLEQQYNKP	HPV 16 E6 (86–95)	127
		QRHLDKKQRF	HPV 16 E6 (116–125)	127

Note:
n.d. = not determined.

but also induce an antitumour response upon presentation by professional antigen-presenting cells such as DC in therapeutic settings. These findings emphasize that the appropriate presentation of immunogenic CTL epitopes is of crucial importance for the induction of an effective CTL response.

The destruction of tumour cells by CTL requires the expression of MHC class I molecules with appropriate peptides by the tumour cell itself. Failure to express either the peptide or class I-restricting element would allow tumour escape. For example, in an Adenovirus tumour model with mouse embryo cell transfected with the viral proteins E1A and E1B and activated *ras*, the presentation of an E1B-encoded immunogenic CTL epitope was inhibited by the activated *ras* oncogene. This downmodulation of peptide presentation resulted in failure of an E1B epitope specific CTL clone upon adoptive transfer to eradicate tumour cells in a tumour-bearing host[36]. Nevertheless, intravenous injection of an E1A peptide-specific CTL clone resulted in elimination of large subcutaneous tumour masses and the CTL activity persisted for months after tumour regression[37]. Obviously, some tumour cells can selectively downregulate the presentation of immunogenic peptides in order to escape immune responses. Therefore, the use of more than one CTL epitope for vaccination could help to overcome escape mechanism by tumour cells.

Overall, the animal experiments demonstrated that it is possible to induce a highly effective and specific CTL response in vivo upon vaccination with MHC class I restricted peptides. The cellular immune response specific for the peptides is capable of killing cells expressing the corresponding tumour-rejection antigens such as HPV 16 E6 or E7 genes, thereby preventing the outgrowth of HPV-induced tumours. An established tumour mass may obviate the induction of an effective CTL response following vaccination. It is possible to overcome such unresponsiveness and induce a specific CTL response which is capable of eradicating established tumour masses by manipulation of the peptide delivery system.

Defining immunogenic CTL epitopes in humans

Evidence of human T-cell responses to HPV

Various approaches have provided evidence of active immunity to HPV in cervical neoplasia. For example: (*a*) A higher incidence for the development of HPV-related malignant lesions in the anogenital tract of patients with impaired immune systems (e.g. AIDS); drug-induced immune suppression after transplantation; and patients suffering from epidermodysplasia verruciformis[38,39]. (*b*) In patients with high-grade cervical intraepithelial neoplasia, or CIN III, T cell (CD4 and CD8) responses can be measured against HPV E6 and E7 proteins[40–43], as well as the L1 capsid protein[44]. (*c*) Cellular immune responses against HPV-induced cancer is supported by the observation that a large proportion of HPV-related cervical cancers show

MHC class I expression downregulation[45,46] which presumably might be selected to allow the tumour to escape immune attack. Moreover, patients with downregulation of MHC class I have an overall poor prognosis compared to patients with normal MHC class I expression[47,48]. (*d*) Regression of CIN is accompanied by massive infiltration of $CD4^+$ and $CD8^+$ T cells and macrophages, although the specificity of these cells is unknown[49,50]. It is reasonable to believe that the proof principle of peptide vaccinations in animal models can be translated into clinical settings, namely defining immunogenic peptides and subsequently designing vaccines for the treatment or prevention of HPV-induced cervical cancer in humans.

Design of HPV peptides for CTL induction

For the identification of immunogenic CTL epitopes derived from HPV 16 proteins, a set of overlapping synthetic peptides of predefined size, spanning the entire E6 and E7 has been screened for peptides binding to distinct HLA class I molecules. Since the human MHC class I molecule HLA-A*0201 is the most abundant in the world[51], research has focused primarily on epitopes presented in the context of this allele. Subsequent testing of peptides binding with high affinity to HLA-A*0201 for immunogenicity in vitro on human PBLs allowed the definition of immunogenic CTL epitopes in the E6 and E7 proteins[52,53]. For the E7 protein, three highly immunogenic peptides could be defined (position 11–20, 86–93 and 82–90). Similar strategies have been successful also for the E2 protein of HPV 16, as well as for the E6 protein of HPV 18[54,55]. A list of published immunogenic CTL epitopes of different HPV types in humans as defined by in vitro assays is summarized in Table 5.1. However, the immunogenicity of the peptides in vitro may not reflect the situation in vivo. Therefore, the CTL epitopes were also tested in vivo in a transgenic mouse model expressing the human HLA-A*0201 molecule[53]. For example, the HPV 16 E7 protein-derived human HLA-A*0201-restricted peptides 11–20 and 86–93 showed a high immunogenicity upon injection in transgenic mice expressing the chimeric MHC class I molecule $A2K^b$. For HPV 16, as well as for hepatitis B virus, it was shown that infected patients occasionally develop CTLs against one of the immunogenic peptides identified in $A2K^b$ transgenic animals[56–60]. These in vivo tests of immunogenicity allowed the selection of peptides to be included in a vaccine for treatment of cervical cancer patients.

The induction of good CTL responses induced by peptide vaccines is dependent on the simultaneous stimulation of specific T helper cells. In general, it can be assumed that the activation of $CD4^+$ T cells directed to a tumour-specific antigen enhances the maturation of tumour-specific $CD8^+$ T cells by providing an optimal cytokine profile of a Th1 type cell. This cytokine profile, mainly characterized by the production of significant amounts of IFN, IL2 and TNF, but not IL4 and IL10[61,62], mediates a cellular immune response. Therefore, a Th1-like cytokine

profile at the site of an antitumour immune reaction is favourable for the induction of an effective CTL response[63]. The importance of T helper cells in vaccination approaches is further demonstrated by vaccination experiments with E7 in vivo. Wu et al. fused the sorting signals of the lysosome-associated membrane protein LAMP-1 to the E7 protein of HPV 16 (termed Sig/E7/LAMP-1), which targets the E7 protein efficiently into the lysosomal pathway and therefore into the MHC class II processing pathway[64]. Immunization with a recombinant vaccinia virus construct expressing the Sig/E7/LAMP-1 construct induced a pronounced cytotoxic T lymphocyte (CTL) response specific for the E7 protein in vivo, protecting the vaccinated mice from a subsequent challenge with E7 protein expressing tumour cells. In contrast, immunization of mice with vaccinia virus expressing the E7 protein alone induced a less pronounced immune response, which was not protective against tumour challenge[65]. However, the enhanced antitumour CTL response could also have been due to an enhanced MHC class I presentation of peptides through the use of a signal peptide directing the fusion protein also into the class I pathway. This signal peptide was not present in the control construct and so the enhanced antitumour response may not have been only mediated by the activation of the CD4 + T cell population. More direct evidence of the importance of CD4 + T helper cell activation in the induction of CTL responses by peptide vaccination was demonstrated in a murine leukaemia virus model. A protective CTL response was achieved only when a specific CTL epitope was injected emulsified in IFA simultaneously with a specific helper peptide[66], indicating a strong synergistic effect of the helper peptide. In a similar experiment, Ossendorp et al. showed that vaccination with a specific viral T helper cell epitope induced a protective, CD8-mediated immune response against MHC class II negative tumour cells[67]. A control peptide, representing an unrelated T helper epitope, did not induce a protective immune response; meanwhile, simultaneous injection of a specific CTL epitope together with the viral T helper epitope resulted in strong synergistic effect. Overall, the presence of a helper epitope in a peptide vaccine is not absolutely necessary for the induction of a CTL response but the synergistic effects lead to a more effective and long-lasting immunity.

In recent years many different helper epitopes in HPV proteins have been described, which activate CD4 T helper cells in vivo (Table 5.2). However, it is difficult to match the HLA-A*0201-restricted CTL epitopes with the particular helper epitopes restricted to the MHC class II haplotypes of the patients to be vaccinated. To overcome these problems, a pan DR-helper epitope (PADRE)[68] has been designed, which binds to a broad spectrum of human MHC class II molecules and is capable of inducing T helper cell proliferation and cytokine production. Thus, the PADRE epitope overcomes the HLA class II restriction and was chosen to be included in the first clinical trials with an HPV 16-derived peptide-based vaccine.

Table 5.2. *MHC class II restricted helper epitopes of HPV 16*

	Responder MHC haplotype	Peptide sequence	Protein and position of peptide	Reference
Human	DR4, Drw13	TPTLHEYMLDLQPE	E7 (5–18)	131
	DR1, Drw11	PETTDLYCYEQLNDSSEEEDEI	E7 (17–38)	131
		DLYCYEQLNDSSEE	E7 (21–34)	131
		YEQLNDSSEEEDEI	E7 (25–38)	131
		VQSTHVDIRTLEDLLMGT	E7 (69–86)	131
		HVDIRTLEDLLMGT	E7 (73–86)	131
	DR7, Drw7	VPVSKVVSTDEYVART	E6 (45–57)	132
		EQMFVRHLFNRAGTVG	L1 (279–294)	132
	DR4, Dw4	PPVPVSKVVSTDEYVARTNIYYHA	L1 (40–63)	132
		TVIQDGDMVHTGFGAMDFTTLQANKS	L1 (219–244)	132
	DR3	VSGLQYRVFRIHLPDP	L1 (91–106)	132,136
	n.d.	THVDIRTLEDLLMGTLGIVCPICSQ	E7 (72–97)	42
		PETTDLYCYEQLNDSSEEEDE	E7 (17–37)	42
		EIDGPAGQAEPDRAHYNI	E7 (37–54)	42
		DSTLRLCVQSTHVDIRTLE	E7 (62–80)	42
Mouse	IA^k, IE^k	TDLYCYEQLN	E7 (20–29)	133
		AEPDRAHYNI	E7 (45–54)	133
		STLRLCVQSTHVIRTL	E7 (60–79)	133
		GTLGIVCPIC	E7 (85–94)	133
	IA^a, IA^b, IA^d IA^k, IA^s, IE^a, IE^d IE^k, IE^s	DRAHYNI	E7 (48–54)	134
	IA^d, IE^d	AHYNIVTFCCK	E7 (50–60)	135
	IA^d, IE^d, IA^k, IE^k	PPVPVSKVVSTDEYVARTNIYYHA	L1 (40–63)	136
		VSGLQYRVFRIHLPDP	L1 (91–106)	136
		EQMFVRHLFNRAGTVG	L1 (279–294)	136
	IA^b	EVYDFAFRDL	E6 (41–50)	127
		QYNKPLCDLL	E6 (91–100)	127
		RRQTQL	E6 (146–151)	127

Note:
n.d. = not determined.

Peptide-based vaccines in clinical trials

A clinical trial with peptide-based vaccination for HPV-induced carcinoma has been performed in the Netherlands[69,70]. In this phase I/II trial 15 patients suffering from recurrent cervical carcinoma received two HPV 16 E7-derived HLA-A*0201-restricted CTL epitopes coupled to the PADRE helper epitope. The patients received three to four vaccinations with either 100, 300 or 1000 μg peptides emulsified in Montanide ISA51 (a pure form of IFA) every three weeks. After completion of the vaccination protocol, two patients showed stable disease and the other patients had progression of tumour growth. In short-term cultures of PBLs from these patients no peptide-specific CTL response could be detected, although local lymphocyte infiltration at the site of injection could be observed and also occasionally PADRE specific proliferation could be demonstrated. An important issue is that the patients immunized were at a late stage of disease and this does not represent an optimal setting for vaccination. This is supported by the overall down-regulation of the immunity in these patients as evidenced from examining HLA-A*0201-restricted CTL responses to influenza. Further follow-up of these patients showed that one patient had a partial clinical response and another patient a complete clinical response after triple chemotherapy following the vaccine trial. Such results may indicate the activation of the immune system by the peptide vaccine, allowing the immune system to attack the tumour after reducing the tumour load in vivo with chemotherapy. It seems feasible that HPV peptide vaccination is likely to be more beneficial in patients with less far progressed disease. An ongoing clinical trial with the HPV 16 E7-derived E7 peptide (11–20) in CIN III patients has documented CTL responses after vaccination (J. Weber, personal communication).

In another phase I clinical trial[71] 12 women with refractory cervical carcinoma were immunized four times with a lipopeptide vaccine, consisting of the HLA-A*0201-restricted, E7 (86–93) CTL epitope coupled to palmitic acid tails. Similar lipopeptide approaches in murine models are reported to be very efficient[72]. The CTL responses were tested using purified $CD8^+$ cells stimulated with autologous peptide-loaded dendritic cells in vitro before and after vaccination, and specific cellular immune responses were assessed by measuring IFNγ release upon interaction with an HLA-A*0201 positive peptide-loaded target cell. All patients showed a specific response against an influenza control peptide, and 4 out of 12 patients had an HPV–peptide specific response prior to vaccination. Six patients were unable to go through the full cycle of four vaccinations, while two patients declined the blood donation after two inoculations. After the second vaccination, five out of seven evaluable patients showed an HPV 16 E7 (86–93)-specific immune response and a specific response was detected in two out of three remaining evaluable patients after

the full cycle of four vaccinations (two additional patients declined blood donation). No clinical responses were seen following four inoculations; two patients had stable disease and three had progressive disease. In summary, this trial has shown that it is possible to administer safely a peptide vaccine without serious side effects, and that the immune system is capable of reacting to the vaccine. It is important to note that the patient group has progressed disease and the lack of clinical responses may reflect the advanced stage of the disease.

Several other clinical trials with peptide-based vaccines have been completed. For example, HBV negative volunteers were immunized with an immunogenic hepatitis B virus CTL epitope binding to the HLA-A*0201 with the peptide coupled to palmitic acid tails together with the PADRE helper epitope[73,74]. Vaccination of humans with a high dose (up to 5 mg/injection) showed induction of specific cellular immune responses[75]. Isolated $CD8^+$ CTLs were specific for HBV-infected target cells in vitro and killed the target cells efficiently. In normal donors the magnitude of the immune response was as good as observed in patients with an acute hepatitis. By contrast, in patients with chronic hepatitis infection, the immunization led to only marginal and low T cell responses and the magnitude and lytic activity of the CTL responses were indistinguishable from those observed in patients with a spontaneous clearance of the virus (A. Sette, personal communication). Overall, this phase I clinical trial showed that it is possible to induce an immune response in healthy volunteers and also, to a certain extent, in virus-infected patients.

Promising results from a clinical trial with CTL epitope-based antitumour vaccines have also been reported for melanoma-associated antigen gp100-derived HLA-A*0201-restricted CTL epitopes[76]. The gp100 protein is expressed only in pigmented cells and is a nonmutated differentiation antigen, similar to MART-1[77,78]. Nevertheless, for clinical trials a mutated form of an HLA-A2 binding peptide was used because this mutated CTL epitope showed greater affinity to HLA-A2 and higher immunogenicity. Moreover, CTL specific for the mutated epitope still recognized the nonmutated peptide[79,80]. Therefore, 11 melanoma patients were immunized in a phase I/II clinical study with the mutated peptide g209–218M in IFA, and subsequently PBLs were tested for reactivity against target cells loaded with either the natural or the mutated peptide[76]. Ten out of 11 patients showed high and consistent reactivity against both peptides. Several patients also showed reactivity against HLA-A2-expressing tumour cell lines, although less efficiently than against peptide-loaded target cells. None of the patients showed objective cancer regression, but three patients showed minor responses and partial regression of some lesions. The same therapy was administered to 19 patients with metastatic melanoma together with systemic IL-2 at high dose[76]. Only three patients showed measurable CTL responses, but eight patients showed objective cancer regression

and six more patients showed mixed responses and stable disease. This study is the first to link the induction of measurable clinical responses with the generation of specific MHC class I-restricted CTLs by epitope vaccination. It is noteworthy that all patients with clinical responses showed few specific CTLs in the PBL, indicating that the overall response to the vaccine might be better than estimated by traditional T cell culturing systems based on PBL isolation and in vitro restimulation.

Monitoring and stimulation of patient immune function in clinical trials

New detection methods for CTL responses

The monitoring of the efficacy of peptide vaccines in clinical trials by detection of peptide-specific $CD8^+$ T cells in PBL from normal or immunized donors is hampered by the low frequency of the peptide-specific T cells in PBLs. In order to overcome the limitations of cell culture in limiting dilutions, several new techniques have been developed, which would allow a more direct assessment of specific T cells than by traditional cell culturing methods such as limiting dilution assays. For example, FACS analysis of cytokine production by intracellular staining of in vitro activated T cells (six hours of in vitro activation) allows the detection of very low frequencies of responding cells[81–83]. The limit has been reported to be as low as one cell in 10^5 PBL cells. Another technique based on measurement of cytokine production at a single cell level is the ELISPOT method[84,85]. The method is based on detection of IFNγ secretion by activated cells, visualized as spots revealed by using enzyme-labelled anti-IFNγ monoclonal antibodies. Each spot represents a single cell producing detectable amounts of IFNγ after specific activation and simple counting of spots allows the estimation of the total number of responder cells in the culture system. ELISPOT is more sensitive than the commonly used cytotoxicity assays for detection of specific CTLs and can detect up to 95% of all responder cells as estimated with specific T cell clones[86]. ELISPOT methods have already been used for the precursor frequency analysis of influenza-specific CTL responses in human PBL cells[87], as well as measuring specific CTL responses after peptide vaccination in an animal model[79]. A more direct approach for detection of peptide-specific $CD8^+$ T cells is a FACS analysis using recombinant MHC class I molecules loaded with peptide[88,89]. Complete HLA-A*0201 molecules can be produced in *Escherichia coli* and multimerized via biotin to fluorescent-labelled Streptavidin in order to form tetramers. These tetrameric MHC class I molecules can be loaded with peptides and are recognized by peptide-specific T cells, allowing the direct visualization of specific T cells in FACS analysis before and after peptide vaccination[90–92]. The advantage of the system is the direct measurement of specific T cells independent of their activation status and the ability to monitor more precisely any peptide vaccine-induced alteration in CTL frequencies. Using this methodology

will facilitate the analysis of lymphocytes from the draining lymph nodes of the site of vaccination, allowing direct monitoring of the induction of an immune response before the specific CTLs are released into the bloodstream.

Immunosuppression in cervical cancer

Cancer patients in general have a downregulation of immune functions. For example, we found that in all patients with CIN and invasive cervical carcinoma PBLs showed a reduced TCR zeta and CD16 zeta expression. Additionally, the IFNγ production after antiCD3 stimulation was significantly downregulated[93]. Comparable results have also been reported in prostate cancer and colorectal cancer[94,95], as well as in a variety of other cancers[96]. Together these studies suggest that downregulation of the expression of the zeta chain in PBL cells may be a common cause of progressive immune suppression in advanced cancer patients. The downregulation of immune functions in cancer patients is correlated with the stage of the disease but is also affected by radiation or chemotherapy[97–99]. In the various peptide vaccine clinical trials for cervical cancer described here, all patients were selected following failure of standard therapies: all had received surgery, radiation or chemotherapy treatments or combinations of each. Thus, their immune system was likely to be downregulated by disease and previous treatments.

Clearly, immunotherapy is likely to be more effective in the early stages of the disease, cervical neoplasia, rather than in advanced cancer patients with metastatic tumours.

Improved delivery of CTL epitope-based vaccines

The downregulation of immune function in cancer can be circumvented by the method of delivery of the vaccine. As outlined above, the delivery of peptide vaccines by loading peptides on professional antigen-presenting cells, rather than simple injection, have been shown to induce antitumour responses in vivo even in the presence of established tumours. The type of delivery of peptide vaccines can have dramatically different effects when mice are immunized with E1A- or E1B-derived, H-2D^b-restricted CTL epitopes emulsified in IFA, CTL responses are tolerized and tumour growth is enhanced[100,101]. This tolerizing effect was probably due to pharmacokinetic behaviour of the peptide. Experiments with adoptive transfer of a specific CTL clone into nude animals prior to administration of the peptide vaccine resulted in quick death of the animals due to lung damage which is consistent with a rapid spread of the peptide from the depot[101]. Upon loading the E1B peptide onto autologous dendritic cells prior to injection into mice, the tolerizing effects could be overcome and immunity could be induced[102].

Large quantities of autologous dendritic cells can be obtained from patient PBL upon incubation of adherent cells with IL-4 and GM-CSF[103]. DCs pulsed in vitro

with specific peptides and infused into animals as carriers for peptides have been shown to induce a strong and highly effective CTL response, capable of eradicating even established tumours in vivo[104,105]. Other studies highlight the ability of DC to induce CTL responses via activation of CD40, even in the absence of a specific $CD4^+$ T helper response. DCs have also been shown to be able to induce a protective CTL response in vivo upon incubation with complete proteins, thereby overcoming the MHC restriction of individual epitope vaccines. Thus, loading of DC with HPV 16 E7 proteins and subsequent infusion into syngeneic animals induced a protective CD8-mediated cellular immune response against an otherwise lethal challenge with tumour cells expressing the E7 protein[106]. The same effect could be accomplished by injection of complete E7 protein emulsified in IFA, indicating an uptake of exogenous protein and subsequent presentation via the MHC class I presentation pathway by DC in vivo. This effect, known as cross-priming, has been shown to be an important mechanism in the induction of cellular immune responses against intracellular pathogens and tumour cells[107,108] as seen, for example, upon vaccination with irradiated tumour cells[109,110]. In fact, DCs loaded with tumour lysate or peptides have already been shown to induce a highly specific cellular immune response towards tumour cells in melanoma patients[111]. A group of 16 patients with advanced melanoma were immunized with DC loaded with autologous tumour-cell lysate or several melanoma-specific peptides mixed with Keyhole Limpet Haemocyanin (KLH), which served as a T helper cell stimulus. All patients showed delayed-type hypersensitivity (DTH) reactions toward KLH, 11 showed DTH against peptide-pulsed DC and five patients showed partial or complete clinical responses, with regression of metastases in various organs. This study has clearly demonstrated the ability of tumour-cell lysate-pulsed or peptide-pulsed DC to induce an efficient immune response against established tumours in humans in vivo.

Despite the fact that only a small amount of DC is required for the induction of efficient antiviral immunity[112], the generation of dendritic cells for routine clinical use is likely to be logistically difficult. Other, easier methods of antigen delivery to the immune system have therefore been explored. A versatile approach is the use of adenoviral vectors containing several CTL epitopes in a string-of-bead fashion[113]. For example, MHC class I CTL epitopes derived from the adenoviral genes E1A and E1B, as well as HPV 16 E7 oncoproteins, have been investigated and vaccinated mice were protected against lethal doses of tumour cells expressing either E1A or HPV 16 E7 proteins. By including HPV 16 E7-derived immunogenic CTL epitopes binding either to human HLA-A1, -A2, -A3, -A11 or -A24 into the recombinant adenovirus construct, the vaccine could be applicable to more than 90% of the Caucasian population. Moreover, adenoviral vectors can be applied safely to humans, since they rarely integrate into host genome and have already

been used safely in vaccination in North American army recruits without side effects[114,115].

Another vaccination strategy against cervical carcinoma in humans is based on virus-like particles as carrier for vaccines. VLPs are formed by HPV capsid proteins L1 and L2, indistinguishable from normal viral capsid[116,117]. Chimeric VLPs can be generated by fusing the capsid protein L2 with any other protein[118], which can therefore gain entry into host cells and be processed and presented in the immune system via the TAP-dependent MHC class I presentation pathway[119]. For example, after vaccination with chimeric L1L2–(HPV 16)-E7 VLP particles, immunized mice were protected in a CD8-restricted and perforin-dependent manner against subsequent tumour challenge with an HPV 17 E7-expressing tumour-cell line[118]. Although not formally demonstrated, these results indicate the induction of peptide-specific CTL response against E7-derived peptides. Moreover, the chimeric L1L2–(HPV 16)-E7 VLP particles were capable of inducing an immune response in mice in the presence of established tumours, resulting in survival of the vaccinated mice (J. Nieland et al., unpublished). Additionally, we have shown a specific CTL response against HPV 16 VLPs in vitro in human PBL[120], indicating that human PBLs can react to VLPs and mount a specific immune response. These results open the possibility of using chimeric VLPs as therapeutic vaccines for human patients with established tumours. Moreover, we have been able to show that chimeric VLPs can overcome the tolerizing effects of certain peptides in vivo[121]. The H-2D^b-restricted CTL epitope derived from the mouse mastocytoma tumour cell line P815-associated protein P1A led to tolerance against P815 tumour growth upon vaccination of DBA mice with emulsified peptide in IFA. In contrast to the above-mentioned experiments the use of peptide-loaded DC as APCs did not reverse this tolerizing effect. Nevertheless, by delivering the peptide to the immune system by virus-like particles, the tolerizing effect could be overcome and the vaccinated mice were protected against a subsequent lethal challenge with P815 tumour cells.

The use of chimeric VLPs as carriers for vaccines has several advantages over CTL epitope vaccines. First, vaccination by chimeric VLPs is not restricted to patients with a particular MHC type and is therefore suitable for the whole population. Secondly, the capsomer proteins of the VLPs induce a strong T helper response and B cell response, obviating the need for additional stimuli such as helper peptides or even adjuvant[112]. Thirdly, chimeric VLPs are safe, with no documented adverse side effects reported. Taken together, clinical trials with chimeric VLPs carrying the HPV 16 E7 are feasible within the near future.

Several other alternative vaccination protocols for vaccines against cervical cancer have been described, including a recombinant polyepitope vaccinia virus vaccine[122,123], as discussed in Chapter 4.

Conclusions

Clinical trials, including trials for treatment of cervical cancer induced by HPVs, have shown the versatility of peptide-based vaccines. The delivery of the CTL epitopes to the immune system is of crucial importance for the induction of an effective CTL response in vivo. In the last few years a better understanding of cellular immune responses has emerged with the help of CTL epitope-based vaccinations in animals. Nevertheless, the translation of results from animal experiments into clinical settings has yet to overcome several obstacles before effective treatment of patients by immunotherapy based on CTL epitopes is achieved. It has been shown that the application of peptides emulsified in an adjuvant is not always the optimal way of delivering CTL epitopes to the immune system in order to stimulate a specific T cell response. Several new and improved delivery systems have been proposed and successfully tested in animal models and these results will soon be translated into clinical trials.

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6

Vaccines for colon cancer

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Introduction

The realistic prospect of developing a vaccine for the treatment or prevention of colon cancer derives from recent advances in molecular biology and a better understanding of the host–tumour immune response. Although the concept of vaccination is not new, the concept that T-cell responses are critical to achieving tumour rejection, coupled with the identification of novel tumour antigens that contain T-cell-specific epitopes, has led to new strategies in the application of tumour vaccines.

Colon cancer

Cancer of the colon or rectum is one of the most common neoplasms in the Western world. The highest incidence is reported in the United States, but rates are especially high in Australia, New Zealand and areas of northern and western Europe[1]. Estimates suggest that over 131 000 new cases are diagnosed annually in the United States and that about 5% of the population will develop colorectal cancer before the age of 75 years[2]. When diagnosed in its earliest stages colorectal cancer is highly curable with surgical treatment. However, once the disease has spread to regional lymph nodes or elsewhere, the cure rates decrease dramatically. Mortality from advanced disease has remained largely unchanged over the last 50 years with over 56 000 deaths annually in the United States[3]. Recent advances in colorectal cancer research have focused on understanding the genetic changes associated with disease progression, defining methods for earlier diagnosis, and improving therapy for advanced disease.

While the exact cause of colorectal cancer remains uncertain, studies have found both genetic and environmental factors that predispose to or promote the disease. Chief among the environmental factors is an association between increased consumption of animal fats and decreased intake of dietary fibre in high-risk populations[4]. Although significant environmental factors have been identified recently,

more attention has been focused on the role of genetic influences in the development and progression of colorectal cancer. Interest in the genetics of colon cancer was spurred by the identification of several unique hereditary forms of the disease. Familial adenomatous polyposis (FAP) is an autosomal dominant trait with over 90% penetrance with affected individuals developing multiple colonic adenomatous polyps by late adolescence. These patients often have over 1000 polyps by 20 years of age and nearly all patients develop carcinoma by the age of 42. The disorder has been associated with a number of other clinical syndromes characterized by the development of colorectal tumours along with other gastrointestinal tract adenomas, dermoid tumours, lipomas, sebaceous cysts, fibromas and osteomas (Gardner's syndrome), multiple sebaceous cysts (Oldfield's syndrome) and malignant CNS tumours (Turcot's syndrome). Genetic linkage and restriction fragment length polymorphism (RFLP) studies identified the familial adenomatous polyposis coli (Apc) gene on the long arm of chromosome 5[5–6]. Truncations in the Apc gene lead to an increased risk of colorectal cancer by allowing more rapid accumulation of other genetic alterations, thus acting as a 'gatekeeper' gene[7].

Another autosomal dominant familial syndrome, termed hereditary nonpolyposis colorectal cancer (HNPCC), has been described by Lynch and may account for 2–4% of all colorectal cancers in the Western world[8]. This syndrome is characterized by the development of colorectal cancer at an early age, disease in several generations, predominance of proximal colon lesions (Lynch syndrome I) and the presence of other adenocarcinomas at an early age, such as endometrial, ovarian, pancreatic, breast, gastric and occasionally other tumours (Lynch syndrome II). Linkage analysis and identification of genome-wide instability (microsatellite instability) in HNPCC kindred resulted in the isolation of novel genetic mutations involving genes coding for DNA mismatch repair functions[7]. Studies of these familial syndromes have suggested that colorectal cancer develops by the accumulation of multiple genetic changes and the sequence of these changes may be critical to the natural history of a given cancer[7]. Thus, the genetic defect in FAP affects the rate of tumour initiation allowing further genetic changes, while the defects in HNPCC affect the rate of tumour progression by targeting the function of DNA mismatch repair genes. The net effect of these genetic changes is the generation of multiple gene abnormalities in the colorectal cancer cell. Among the genes affected are the *ras* oncogene, deleted in colon cancer gene (DCC), mutated in colon cancer gene (MCC) and the p53 tumour suppressor gene[9–10].

Despite the advances in understanding the genetic and environmental influences on the development of colorectal cancer, treatment for patients with advanced disease remains unsatisfactory. Adjuvant chemotherapy with 5-fluorouracil (5–FU) and levamisole results in a 30–35% increase in disease-free and overall survival at five years for patients with Duke's C disease[11]. However, chemotherapy for

patients with metastatic colorectal cancer has thus far failed to show any significant advantage in overall five-year survival statistics.

Carcinoembryonic antigen (CEA) is an oncofetal antigen that is over-expressed in nearly 95% of patients with colorectal cancers and can also be measured in the serum. Although CEA is not sensitive as a screening method, it has been used to follow patients after colon resections as a measure of early recurrence. Patients with elevated serum CEA levels and no evidence of disease by routine radiological imaging may represent a subset of patients with a more favourable prognosis, provided that treatment is initiated as soon as the elevated CEA level is detected[12]. However, other reports have failed to confirm any advantage for this cohort of patients[13]. Radiolabelled monoclonal antibodies directed against CEA have been used for radioimmunodetection of occult lesions via gamma scanning and during surgery to identify sites of microscopic disease using an intra-operative hand-held gamma probe[14]. It is still too early to tell if this will alter the prognosis for these patients.

The poor prognosis for patients with advanced disease, and the limited treatment options available for patients with less advanced disease, led to a search for novel treatments. The use of immunotherapy for advanced colorectal cancer has been encouraging and several lines of evidence suggest that the disease may respond to such therapy. Promising approaches have included the modulation of fluorouracil with other agents, such as interferon-α, monoclonal antibody therapy directed against CEA and vaccine therapy[15–17]. In an early clinical trial using admixtures of irradiated colon cancer cells with the Bacillus Calmette Geurin (BCG), some benefit in disease-free survival for colon cancer patients with Duke's B2 and C lesions were reported, but no difference in overall survival was seen. In a recent study involving this vaccine, significant clinical benefit was seen in stage III colon cancer[18]. Recent advances in colon cancer genetics and tumour antigen biology have identified specific molecular targets in colorectal cancer cells. These findings, coupled with an increased understanding of how immune responses are generated in cancer patients, have resulted in intense interest in the development of new vaccine strategies. Additionally, progress in the development of vaccine therapy for the treatment of malignant melanoma has paved the way for similar approaches in colorectal cancer[19–20].

Colon cancer antigens

Tumour-associated antigens (TAA) can be defined based on their recognition by T cells. While numerous antigens have been isolated with T cell-specific epitopes, the ideal antigen for targeting colon cancer cells has not been found. Ideally, the perfect antigen would be uniquely expressed by all tumour cells in a given patient,

be recognized as foreign by the appropriate immune effector cells and be able to elicit effective, antigen-specific CTL responses resulting in lysis of tumour cells. Potential colorectal TAA gene products that can serve as vaccine targets can be placed into two major categories: (*a*) mutated gene products; and (*b*) those gene products that are overexpressed in colorectal tumours as compared to normal tissues. The two most prevalent and well studied of the mutated gene products are *ras* and p53. The *ras* gene contains point mutations principally at codon 12 in a wide range of human carcinomas, including 38% of all colorectal tumours; three amino acid changes at codon 12 represent over 80% of these mutations[21]. The point mutated p53 tumour suppressor gene also represents an excellent target for colorectal carcinoma[22–24]. However, p53 is mutated at numerous codons spanning several exons and each codon can express a range of mutations. Thus, multiple vaccines would have to be created to cover the array of potential targets. More recently, strategies have been developed that target wild-type p53 since this molecule is accumulated in tissues where a point mutation is present[25–29].

Several antigens have been found overexpressed, but not mutated, in colorectal carcinomas. The most widely studied are CEA and the mucin, MUC (see Chapter 7). CEA represents an attractive vaccine target because it is overexpressed in more than 95% of colorectal, gastric and pancreatic cancers in a homogeneous fashion; it is also overexpressed in approximately 70% of nonsmall cell lung cancers and about 50% of breast carcinomas[30]. CEA is thought to function as a homotypic adhesion molecule in the fetal colon, and is also expressed, to a lesser extent, on the luminal portion of normal adult colonic mucosa[31–34]. Studies in murine tumour models have shown that a certain degree of overexpression is necessary for T cells to kill a tumour target. In several cases this differential expression between tumour and normal tissue has been successful for immunotherapeutic approaches[26,29,35]. However, the differential expression of any TAA on tumour versus normal tissue must be considered as a concern in terms of induction of autoimmunity. Another concern with CEA as a vaccine target is the fact that CEA is a member of the immunoglobulin gene superfamily and shares homology with certain normal tissue antigens such as NCA (found on normal granulocytes) and biliary glycoprotein (BGP)[36–38]. Thus, selection and design of immunodominant epitopes should ideally involve regions of extremely poor homology between CEA and these related proteins to avoid potential cross-reactivity.

The use of any TAA gene product to induce T-cell responses for tumour immunotherapy may require the identification of immunodominant or subdominant epitopes capable of eliciting potent CTL responses. Most of the studies to date have centred on CTL epitopes of the human class I HLA-A2 allele. This is because the HLA-A2 binding motifs have been characterized and the HLA-A2 allele is the most common class I allele, represented in approximately 40–50% of the human

population. Assays for identification of HLA-A2 peptides include binding to and cytotoxicity for human T2 and C1R:A2 cells, ELISPOT for cytokine release and the generation of CTL in vitro using peripheral blood mononuclear cells (PBMCs) pulsed with peptide. More recently, similar studies have been conducted with MHC class I A3 alleles which are represented in another 25% of the population. Peptides reflecting immunodominant and subdominant epitopes can be used not only in immunization protocols, but also for the in vitro generation of epitope-specific CTL and $CD4^+$ T cells for further study and/or adoptive transfer. Many of these targeted antigens are shared by tumours of a given histology, but some have been shown to exhibit differential expression in different patients and by different tumour cells within the same patient[39].

Several experimental approaches have been used to identify unique T-cell-restricted epitopes from both human and murine tumour antigens. The isolation and culture of tumour-infiltrating lymphocytes (TILs) from tumour deposits provide a basis for the identification of tumour-specific antigens expressed by the infiltrated tumour. TILs recognize immunoselected tumour lines in an MHC-restricted manner, allowing a readout for antigen selection[40]. Thus, using a genetic approach, a tumour-derived cDNA library transfected into HLA-expressing, antigen-negative cells, such as COS-7, can be used to test for stimulation of the appropriate tumour-derived TIL line. The gene coding for the antigen of interest can be retrieved from the transfected cell line by subtraction from transfected cell lines that are not recognized by reactive TILs[41–43]. Using a biochemical approach, antigenic tumour cell lines have been used to directly elute tumour antigen peptides bound to the MHC class I molecules[44]. The eluted peptides present in the positive fractions can be further separated, re-evaluated in CTL assays and, finally, characterized by analysis of their amino acid sequence[45]. More recently, potential therapeutic epitopes have been identified using computer-generated predictions of the peptide-binding affinity to specific HLA alleles[46,47]. These predictions are based on the interaction of the HLA molecule with the amino acid sequences and the consensus anchor positions of all potential peptides derived from a known tumour antigen[48]. The deduced peptides can be synthesized and tested in vitro for their actual HLA binding affinity. T-cell recognition of these epitopes can be confirmed by pulsing the candidate peptide onto target cells expressing 'empty', restricted HLA alleles (e.g. T2 cells) and testing these against known CTL clones that recognize the original tumour antigen[49–51].

These techniques were used to identify putative CEA-specific T cell epitopes. Since the entire amino acid sequence of the human CEA gene is known, and putative human class I HLA-A2 consensus motifs have been described, studies were undertaken to identify a series of CEA peptides that could potentially bind human class I HLA-A2 molecules[52]. CEA peptides were selected for further study only if

their sequence diverged appreciably from the CEA-related molecules NCA and BGP. Patients immunized with a recombinant vaccinia virus expressing CEA (rV-CEA) were used as a source for CEA-reactive CTL. PBMCs derived from vaccinated patients were alternately pulsed with CEA peptides and IL-2 in the presence of autologous peripheral blood mononuclear cells (PBMCs) as antigen-presenting cells (APCs). T-cell lines could be established from postvaccination PBMCs from five out of five patients who were cytotoxic for T2 cells when pulsed with one of these peptides (YLSGANLNL, i.e. amino acids 571–579 of CEA); this 9-mer peptide was later given the designation CAP-1 (Carcinoembryonic Antigen Peptide-1). T-cell lines from these patients were primarily $CD8^+$ or $CD4^+/CD8^+$ double positive, whereas T-cell lines could not be established using PBMCs from the same patients before vaccination.

At the time, no studies had demonstrated that human APCs could endogenously process CEA to bind HLA-A2 molecules for presentation at the cell surface. In order to evaluate this, Epstein–Barr virus (EBV)-transformed B cells of patients were transduced with the human CEA gene using a retroviral vector. These cells, and not the control cells, transduced only with retrovirus, could now serve as a target for the T cells from vaccinated patients, resulting in lysis. The question remained as to whether human carcinoma cells could serve as potential targets for T-cell lysis. Subsequent studies revealed that CEA-positive and HLA-A2-positive carcinoma cells could be lysed by such T cells, while tumour cells that were either HLA-A2 negative or CEA negative could not be lysed[52]. These studies demonstrated for the first time that CEA could be processed by professional APCs and, more importantly, by human carcinoma cells for lysis by epitope-specific T cells. To further show the MHC-restricted nature of this lysis, nonHLA-A2 human carcinoma cells were infected with either wild-type vaccinia virus or a recombinant vaccinia virus containing the human HLA-A2 gene. Only the cells infected with the HLA-A2 transgene were susceptible to lysis. Other studies have also demonstrated that an anti-idiotype monoclonal antibody (directed against a monoclonal antibody to CEA) and recombinant CEA can elicit CEA-specific antibody and proliferative T-cell responses in patients[53].

A CTL line (designated V8T) from one patient was subsequently established from rV-CEA postvaccination PBMCs by alternate pulsing of PBMCs with CAP-1 peptide and IL-2[54]. No T-cell line could be established using prerV-CEA-immunized PBMCs from the same patient. V8T cells were shown to be cytotoxic for C1R:A2 cells when pulsed with CAP-1 at E:T ratios as low as 1:1. Studies were undertaken to establish long-term cultures of V8T in anticipation of potential adoptive immunotherapy studies of epitope-specific T cells. It has been hypothesized by some that homing and other T-cell adhesion molecules could be lost in long-term cultures of T cells. Thus, the expression of these molecules, as well as

cytotoxic ability of V8T, was analysed at various in vitro stimulation (IVS) cycles (using CAP-1 and IL-2 for each cycle). The expression of some of these molecules has been implicated in CTL function. These molecules include CD8, CD4, LFA-1, CD2, CD58, CD45RO and CD49d. All of these were analysed by flow cytometry on V8T cells at IVS cycles 9 through 20. Expression of all these molecules and cytolytic activity were not altered during long-term culture. These studies demonstrated the ability to propagate, in long-term culture, T-cell lines capable of tumour–cell lysis without losing necessary homing-associated molecules.

The success of any cancer vaccine ultimately depends not only on the selection of an appropriate target antigen, but also on intact antigen processing and presentation pathways. Tumour antigens are presented to the cellular immune system as peptide complexes by the MHC class I and II molecules, which are recognized by cytotoxic $CD8^+$ and helper $CD4^+$ T cells, respectively (see Figure 6.1). The optimal signalling pathway for vaccine-presented antigens, through class I or class II, is currently unclear. Furthermore, antigen presentation may be defective in some cancer patients because of decreased MHC expression[55], lack of β2-microglobulin[56] or because presented epitopes are only weakly immunogenic[57].

Several schemes have evolved so that vaccines can overcome potential deficiencies in antigen processing and presentation. The class I pathway generally processes antigens by proteosomal degradation of intracellular cytosolic proteins into short 8–10 mer peptide fragments. These fragments are then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP), where they are associated with class I alpha chains and β2-microglobulin via several intermediate complexes[58]. The entire MHC class I–peptide complex is then transported to the cell surface where the peptide is presented for sampling by circulating T cells. If the class I pathway is defective, an ER signal sequence can be added to the 5′ end of a peptide vaccine, allowing it to bypass the TAP transporter and insert directly into the ER[59]. This has been demonstrated by the addition of a leader sequence to the minimal determinant epitope of the P815 antigen expressed in a recombinant vaccinia virus. Immunogenicity was increased using this vaccine and was correlated with increased concentrations of MHC class I–peptide complexes in the ER lumen and subsequently on the cell surface[60].

The class II pathway involves a different sequence of events for antigen presentation. First, antigens must be delivered to APCs by endosomal engulfment and then enter a lysosomal compartment where they are degraded by acidic proteases into 12–24 mer peptides[61]. Similar to using leader sequences that target peptides into the class I pathway, the C-terminal domain of the lysosome-associated membrane protein (LAMP)-1 or LAMP-2 can be added to improperly processed peptides. The fusion product localizes to the lysosomal compartment and results in increased MHC class II binding[62].

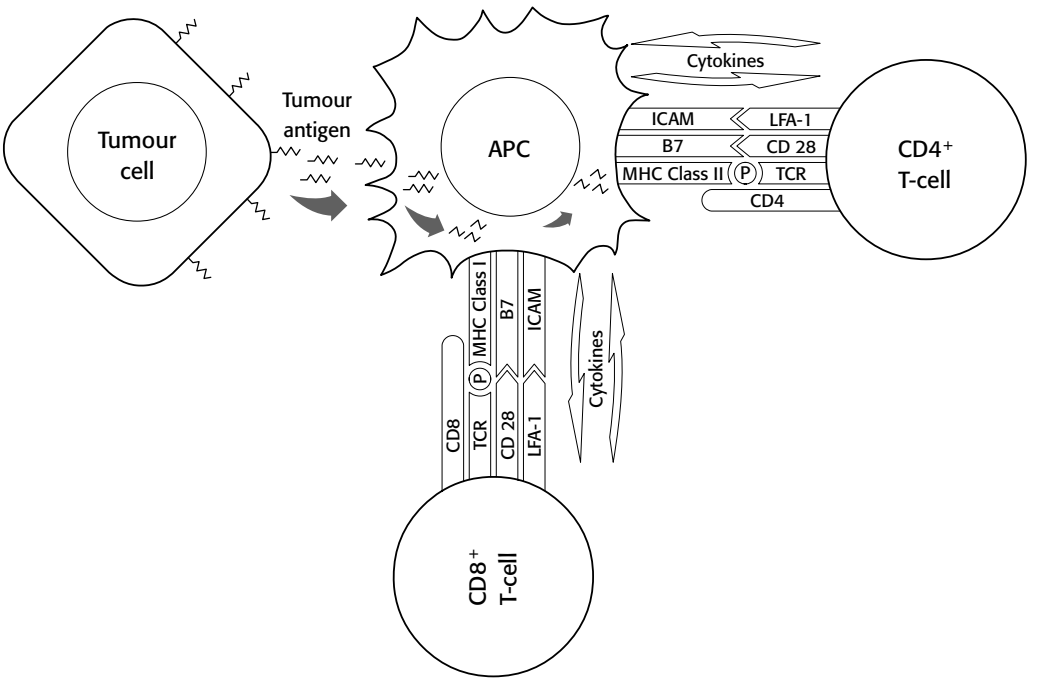

Figure 6.1 Schematic drawing of how cellular immune responses are generated against tumour antigens. Tumour antigen or particulate matter derived from tumour cells is engulfed by antigen-presenting cells (APC). These antigens are processed into peptides for presentation to CD8+ T cells via the MHC class I pathway, or to CD4+ T cells via the MHC class II pathway. The peptide–MHC complex is recognized by the corresponding T-cell receptor (TCR) of responding T cells. Activation occurs when antigen-independent signals, such as binding of B7 to CD28 and ICAM to LFA-1, occur. The local cytokine milieu is also an important factor in the type of immunity that results after antigen presentation

Current evidence suggests that, while the effector response following vaccination requires $CD8^+$ T cells, $CD4^+$ T cells may provide necessary helper functions and may also directly recognize specific tumour antigen epitopes[63]. Activated $CD4^+$ T cells can be classified into two distinct subsets based on their specific patterns of cytokine release after exposure to antigen[64]. Th1 cells secrete IL-2 and IFN-γ, and are thought to help induce cell-mediated immunity and delayed-type hypersensitivity responses. On the other hand, Th2 cells release IL-4, IL-5, IL-6, IL-10 and IL-13, which are critical for activation of B cells and humoral immunity. While the exact mechanism of immune-mediated tumour rejection is unknown, the induction of antigen-specific $CD4^+$ and $CD8^+$ T cells, and the local cytokine environment, may be critical determinants of the ultimate outcome after vaccination. The final outcome may also depend on the involvement of other cells, such as natural killer (NK) cells[65] and the generation of antibody[66].

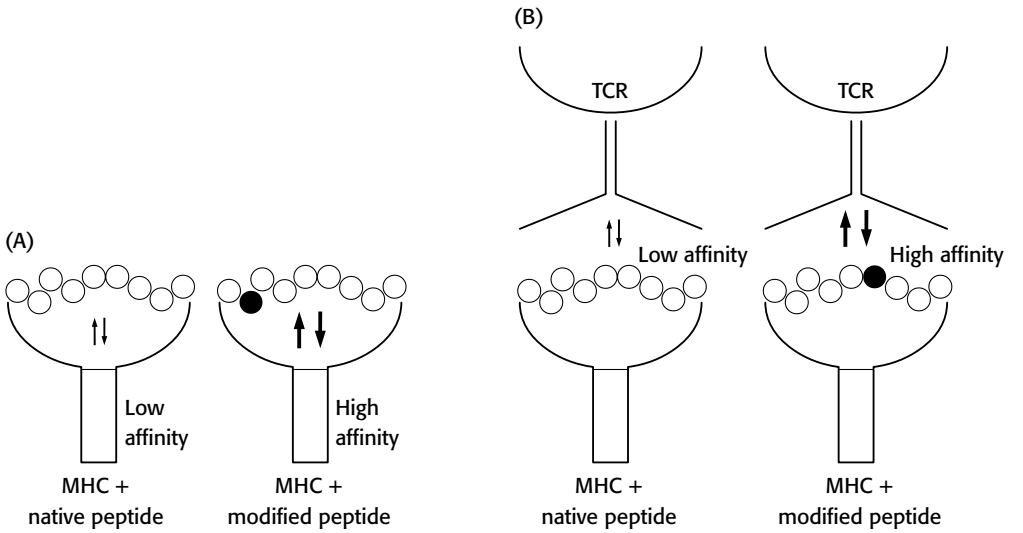

Figure 6.2 Graphic representation of how peptides can be modified to achieve higher affinity binding to the MHC molecules (A) or better recognition by the T-cell receptor (B). Modification of anchor residues, such as position 2 (as in A) can enhance the binding efficiency to the MHC complex. Modifications of TCR recognition sites, such as the CAP1–6D agonist peptide that contains a single amino acid substitution at position 6 (as in B), can significantly alter the TCR recognition of the epitope. Such modified peptides have been proposed as more potent vaccine agents for the treatment of cancer

Defects in effector cell recognition of potential tumour antigens may also occur in the cancer patient. Studies in mice bearing a colon adenocarcinoma were found to have a loss of both the TCR zeta chain and p56(lck) with a corresponding decrease in cytotoxic activity[67]. Similar findings have been reported in humans with primary colorectal cancer[68]. Zeta chain defects have been reversed by retroviral transduction of a chimeric zeta chain construct containing both the TCR zeta chain and a single-chain antibody domain through *ex vivo* gene therapy of tumour-specific T cells[69].

Agonist and antagonist epitopes

The problem of weakly immunogenic antigens that may not be easily recognized by a TCR can be dealt with by modifying the amino acid sequences of potential peptide epitopes (see Figure 6.2). Substitution of key anchor residues can increase the affinity of peptide-MHC binding to the TCR (Figure 6.2). It is essential to ensure that the resultant CTLs derived from such an analogue retain specificity for the native antigen as presented by MHC on tumour cells. Recent studies have shown enhanced immunogenicity in vitro after modification of anchor sequences to MHC. These studies were intended to increase peptide binding to MHC since

anchor residues of those peptides were not optimal[70–73]. Furthermore, substitution of a methionine for the native threonine at position 2 in the melanoma antigen $gp100_{209–217}$, a known HLA-A2-restricted epitope, was recently shown to have greater clinical effects when given to melanoma patients along with IL-2 compared to the native $gp100_{209–217}$ peptide[19]. This strategy can be applied to any known putative peptide epitope and may particularly enhance the immunogenicity of a 'self-antigen' such as CEA. In the case of CAP-1, however, anchor residues conformed to optimal binding motifs[74].

Another approach to improve the immunogenicity of a CTL peptide is to alter nonanchor amino acid residues expected to contact the TCRs (Figure 6.2). This system could generate a T-cell 'agonist' which contains amino acid substitutions of nonMHC anchor positions that stimulates CTLs more efficiently than the native peptide[74]. The rationale for this approach was derived from previous findings involving the identification of peptide antagonists[75–81]. In these studies, inhibition of the T-cell response by modified peptides was shown to be TCR-mediated and could not be explained by MHC competitive binding. By analogy, the strictest definition of a peptide TCR agonist would be an analogue that increased effector function without accompanying increases in MHC binding.

This approach has been applied to the CAP-1 peptide and several factors were considered in deciding which positions to examine for effects on TCR interactions. Sequencing and mapping experiments defined a binding motif in which position 2 and the C terminal (position 9 or 10) are critical for peptide presentation by HLA-A2[82]. In addition, tyrosine at position 1 has been identified as an effective secondary anchor. Therefore, CAP-1 residues at these positions were not altered since they were already optimal. X-ray crystallography of several peptides bound to soluble HLA-A2 suggested that all binding peptides assume a common conformation in the peptide binding groove[83]. When five model peptides were examined, residues 5 through 8 bulge away from the binding groove and are potentially available for contact with a TCR. Therefore, focus was placed on modifying these residues in an attempt to define CAP-1 analogues that would more efficiently stimulate human CEA-specific CTL. A panel of 80 CAP-1 analogue peptides was produced by pin technology in which the residues at positions 5 through 8 were synthesized with each of the 20 natural amino acids. The effects of these amino acid substitutions on TCR recognition was studied using the CAP-1-specific, HLA-A2-restricted human CTL line derived from a patient vaccinated with rV-CEA and designated V8T[52]. For initial screening, V8T was used in a cytotoxicity assay employing C1R:A2 target cells incubated with each member of the peptide panel (at three peptide concentrations). Of the 80 single amino acid substitutions, all but six failed to activate cytotoxicity of V8T[54].

Subsequent studies revealed one peptide, designated CAP1–6D, that was the best candidate agonist. This peptide contained a substitution of asparagine by aspartic

acid (D) at position 6. CAP1–6D was compared to native CAP-1 in a CTL assay over a more extended range of peptide concentrations, using two different cell lines as targets. Analogue CAP1–6D was over 100 times more effective in mediating lysis by V8T than native CAP-1[74] (Figure 6.3A and B). CAP-1 and the CAP1–6D analogue were tested for binding to HLA-A2 by measuring cell surface HLA-A2 in the transport-defective human cell line T2; there were no differences in binding (Figure 6.3C). Thus, the improved effectiveness of CAP1–6D in the CTL assay suggested better engagement with the TCR. The CAP1–6D agonist could potentially be useful in both experimental and clinical applications if it could stimulate growth of CEA-specific CTLs from patients with established carcinomas more efficiently than CAP-1. The postimmunization PBMCs from a cancer patient (designated Vac8) were stimulated in vitro with CAP1–6D and were assayed for CTL activity against targets coated with CAP-1 or CAP1–6D. This new line demonstrated peptide-dependent cytotoxic activity against target cells coated with either CAP1–6D or the native CAP-1 peptide.

PBMCs from patients immunized with rV-CEA were shown to produce CTL activity when stimulated with CAP-1, while pre-immunization PBMCs were negative. New attempts to stimulate CTL activity from healthy, nonimmunized donors using CAP-1, using this protocol, were also unsuccessful. In controlled studies, however, CTLs could be generated from healthy, nonimmunized donors with the agonist peptide CAP1–6D and interleukin-2 (IL-2). Several peptide-specific CTL lines were obtained only when generated with CAP1–6D, not CAP-1. The CAP1–6D-derived CTL line was tested against a panel of human tumour cells and killed only tumour targets expressing endogenous CEA and HLA-A2[74]. All human tumour lines negative for either HLA- A2 or for CEA were not lysed[74].

Animal models

Appropriate model systems for determining the immunogenicity of a given antigen or epitope for vaccine development in humans do not exist. At first glance, rodent models may appear attractive. However, since human class I and class II MHC alleles are distinct from those of any other species, it is inappropriate to try to define whether immunogenicity of a human TAA in a murine model will predict immunogenicity in a human. This is also true in the use of transgenic mice containing a human TAA transgene since transgenic mice still have murine MHC class I and II alleles as well as murine TCRs and thus cannot predict immunogenicity in humans. Certain transgenic models are very useful to help define the principles of the immunogenicity of a 'self-antigen'[84,85]. However, one is never certain how the specific transgenic murine model reflects the human in terms of degree of expression of the gene in question during embryonic development and its actual level of

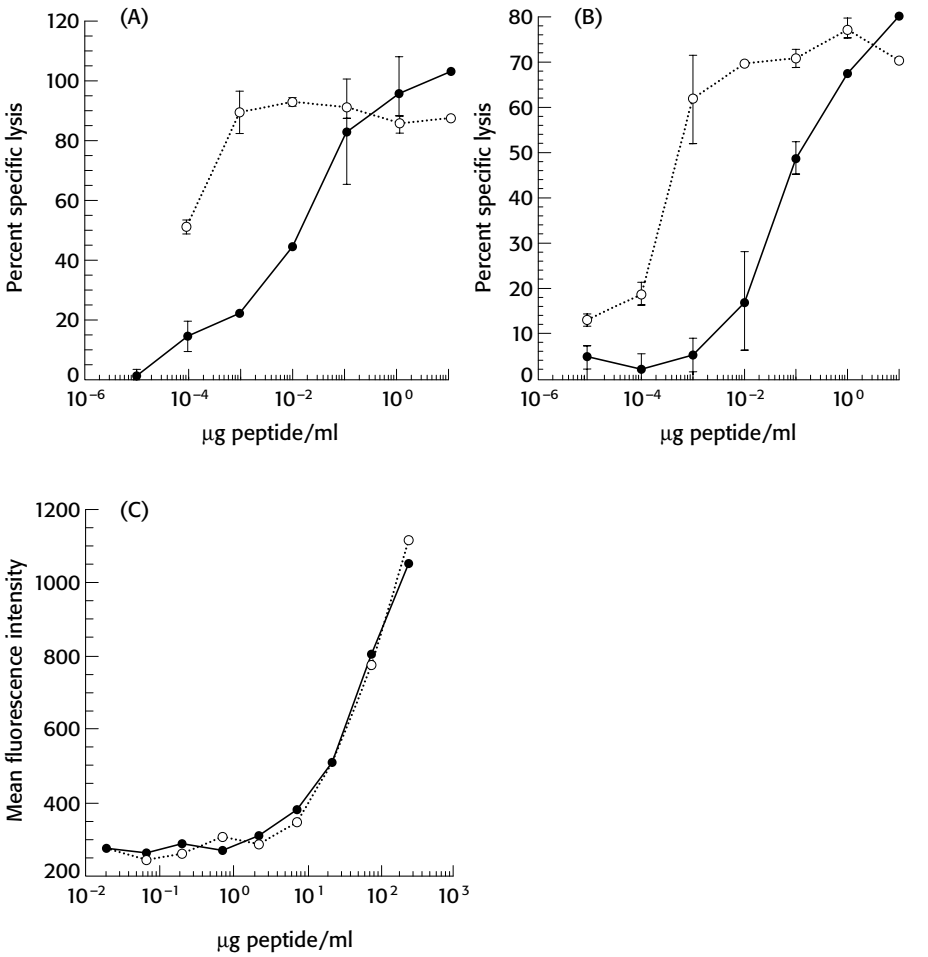

Figure 6.3 Comparative studies of CEA-specific CTL epitope CAP-1 (●) and its analogue CAP1–6D (○). Panel A: CEA-specific CTL (designated T-Vac8) was used as an effector to lyse A2 + T2 cells incubated with CAP-1 or CAP1–6D. T-Vac8 was derived from PBMCs of a patient immunized with rV-CEA; the CTL was derived by incubation with CAP-1 and IL-2[51]. Panel B: Same as panel A except C1R-A2 + cells are targets. Results of panels A and B demonstrate $\Delta 2$ logs more sensitivity to lysis when cells are incubated with analogue vs. CAP-1. Panel C: Demonstration of identical binding to CAP-1 and CAP1–6D to HLA-A2 + T cells

expression in normal versus tumour tissues[86]. In transgenic mice bearing the human MHC class I HLA-A2 transgene, there is not always a correlation between an immune response in the HLA-A2 transgenic mouse as compared to human in vitro immune responses for some epitopes[26]. Thus, one is faced with the dilemma of finding an appropriate in vivo model to determine the immunogenicity of a given human antigen and/or epitope. It has been our experience that a small phase

I clinical trial to define immunogenicity of a given antigen or epitope is without equal. This, however, should not diminish the extensive use of animal models to determine basic concepts of antitumour vaccination.

A number of potentially useful murine models have, nonetheless, been described for evaluating the effectiveness of potential colon cancer vaccines. These models may provide insights into the role of pre-existing tolerance against tumour antigens and allow an evaluation of the role of autoimmunity in tumour rejection. Transgenic mice expressing the human CEA gene have been generated and represent one potentially useful model[87]. These mice express CEA in a spatiotemporal pattern that approximates that of normal human CEA expression, except that the level of expression appears somewhat higher in the transgenic animals[87]. These mice provide a model for evaluation of CEA-directed vaccines in a system where CEA is truly represented as a 'self-antigen'. Strategies to break tolerance can be examined using this mouse which will be more relevant to the patient with a CEA-expressing cancer. However, this model is still limited by the presence of murine MHC and possible differences in antigen presentation as compared to humans.

Studies of familial polyposis, the heritable colon cancer syndrome, led to the isolation of the Apc gene on human chromosome 5[88]. Identification of a murine analogue of the human Apc gene has resulted in the generation of mice carrying a mutated Apc gene[89]. These mice develop multiple intestinal neoplasias and were, hence, termed Min. More recently another set of mice were constructed that contain a truncated Apc gene product resulting in the translation of a 1660 amino acid protein, of which the first 1638 amino acids correspond to the normal Apc gene product. Mice heterozygous for this modification, termed Apc1638, are similar to the Min mice, in that they both have germline mutations in exon 15 of the Apc gene and both develop gastrointestinal tumours and colonic polyps[89]. The Apc1638 mice differ from the Min mice in having a longer life span (one year as against 120 days), developing fewer tumours (an average of three compared with 30), and exhibiting polypoid hyperplasia that more closely represents the pathological changes seen in the human adenomatous polyp. These mice represent good models for evaluating the pathogenesis of gastrointestinal tumours and agents that may inhibit formation of such tumours. Cross-breeding of the CEA transgenic mouse with the Min mouse resulted in a CEA-expressing tumour-prone mouse model[90]. This hybrid mouse represents a superior model for the evaluation of antiCEA therapeutic agents. It is likely that future research will lead to the development of transgenic and knock-out mice representing both tumour-prone phenotypes and human tumour antigen-expressing animals. These models should provide a better system for evaluating novel immunotherapeutic strategies and increase our understanding of how the immune system can be manipulated into rejecting established tumours using 'self-antigens'.

Vectors for vaccine development

Once an appropriate tumour antigen has been selected, there are numerous ways the antigenic epitope(s) can be presented to the host immune system. Perhaps the easiest method of immunization is to simply use a specific peptide composed of the antigenic epitope as a vaccine agent. Peptide vaccines are usually administered as soluble preparations using nonspecific adjuvants, such as incomplete Freund's adjuvant (IFA), or various oil-in-water emulsions. Vaccination-induced immunity depends in large measure on the effectiveness of the antigen-presenting cell that initially processes and presents antigen. Dendritic cells (DC) are among the most effective APC for tumour antigen presentation, which has led to experiments using isolated DC in adoptive transfer studies[91]. Antigen specificity can be conferred to the DC by pulsing the cells with specific peptides[92].

Although peptides or even whole cell preparations can be used, the ability to isolate and clone tumour antigens provides a way to increase the specificity of tumour vaccines. Once a specific antigenic epitope has been isolated, a recombinant or synthetic vector can be constructed using the DNA, RNA or amino acid sequence coding for the antigen, or even the specific epitope of interest. Vaccinia virus has been especially useful for expression of TAA since the genome accepts a large amount of foreign DNA (up to 25 kb), exhibits competent replication, provides accurate posttranslational modification of proteins, is easily administered, remains stable, and elicits potent CD8 + CTL responses against inserted antigens[93]. Studies have also shown that weakly immunogenic antigens may become more immunogenic in the context of vaccinia transgene expression, most likely due to the enormous inflammatory response directed against the replicating vaccinia virus[94].

However, patients exposed to one or two doses of vaccinia virus develop strong neutralizing antibody responses that may prevent subsequent immunizations with vaccinia vectors. One solution to this problem has been the development of nonreplicating vaccinia viruses and the use of other poxvirus species, such as the avian poxviruses[95] (see Chapter 3). The attenuated vaccinia strains, such as NYVAC and modified vaccinia virus Ankara (MVA), contain multiple gene deletions which render virus replication defective in mammalian cells[96]. Fowlpox virus and canarypox virus (ALVAC) are avipoxviruses that infect both avian and mammalian cells, but are unable to replicate in mammalian cells. Since these viruses are nonreplicating, induction of immunity to avipox proteins should have minimal effect on potency and allow multiple administrations. However, these viruses have been able to elicit strong immune responses in nonavian populations, including humans, without the development of neutralizing responses to the virus itself.

Primary and booster treatment regimens have been compared using a single

vector: a recombinant vaccinia virus expressing CEA (homologous boosting); or using two different vectors: vaccinia and canarypox viruses (heterologous boosting). Mice immunized with rV-CEA and then ALVAC-CEA exhibited at least a four-fold greater antiCEA CTL response than mice boosted with the same vector[97]. Priming of antitumour antigen (CEA) responses with naked DNA[98] or using RNA transduced into DC[99] offer additional possibilities for optimal prime/boost vector sequence, but the timing schedules require further study and, ultimately, clinical trials to define the role of these factors in cancer vaccination protocols[100].

As summarized in Figure 6.1, the release of cytokines into the local environment greatly influences the activation of responsive T cells. Thus, cytokines may provide an important adjuvant for enhancing antigen-specific immunity and clinical responses to colorectal cancer vaccines. For example, the administration of low-dose systemic vaccinia virus expressing IL-2 approximately one week following rV-CEA administration was shown to result in enhanced CEA-specific T-cell responses and enhanced antitumour effects[101]. As the mechanisms of antitumour immunity are understood it is likely that other cytokines may also play a role as vaccine adjuvants.

Likewise, T-cell co-stimulation is central to the induction of T-cell responses and antitumour immunity. The most studied co-stimulatory molecule is B7.1 (CD80), which is the ligand for the T-cell surface antigens CD28 and CTLA-4.

The vast majority of studies to date have involved the use of recombinant retroviruses expressing co-stimulatory molecules that infect murine tumour cells in vitro to enhance their immunogenicity. The insertion of co-stimulatory molecules into poxviruses (vaccinia or avipox) represents an attractive alternative for both gene-modified tumour cells and for recombinant vaccine approaches. Vaccine studies can be carried out by: (*a*) inserting the co-stimulatory transgene and TAA gene into vaccinia as one construct; or (*b*) admixing the two recombinant vectors prior to immunization. Preclinical studies have shown that when rV-CEA is admixed with rV-B7.1 and used as immunogen, this combination vaccine results in enhanced specific CTL responses, enhanced T-cell lymphoproliferative responses and enhanced antitumour activity[102]. The use of an admixture affords the flexibility of mixing different recombinant vectors containing co-stimulatory molecules or cytokine genes with different recombinant vectors containing tumour antigen genes.

Clinical trials

Vaccinia-CEA

Experimental studies involving rV-CEA have been described, including murine and monkey toxicology studies that demonstrated minimal toxicity[103–105]. This agent was used in two clinical phase I trials designed to assess toxicity and dosing

in patients with advanced colorectal cancer[52,106]. This, of course, is the least desirable population to examine the efficacy of a vaccine in initiating T-cell responses. One obvious reason for this is that defects in the TCR zeta chains have been observed in patients with colorectal cancer[107]. The study design for rV-CEA consisted of three cohorts of patients with metastatic CEA-expressing carcinomas. The first cohort received 2 x 10^5 pfu three times at monthly intervals. The second cohort received 2 x 10^6 pfu at monthly intervals, and the third cohort received 1 x 10^7 pfu three times at monthly intervals. The degree of erythema following skin scarification of rV-CEA after each dose was also measured. This was analysed because it was anticipated that antivaccinia antibody responses could reduce the degree of replication of vaccinia and thus reduce the expression of the transgene (i.e. CEA).

Initial immunization with rV-CEA resulted in a clinical 'take' as measured by the local erythema reaction in 23 out of 26 patients. Four of seven patients at the lowest dose level (cohort 1) demonstrated erythema, while all 19 patients in cohorts 2 and 3 demonstrated erythema[52]. Thus, despite the fact that all patients had previously received smallpox vaccinations, most patients displayed the classical acute manifestations of the vaccinia immunization, confirming the ability to be reimmunized. Lesion size correlated with the dose of rV-CEA given but was reduced with subsequent vaccinations at the same dose level. No objective responses were observed in these patients with advanced disease. At all doses, no toxicity was observed other than that normally associated with the administration of a live vaccinia virus.

The phase I study employing rV-CEA demonstrated both the strength and weakness of using a recombinant vaccinia vector. A low precursor frequency of CEA-reactive CTL was observed, which may have been a consequence of the administration of a live vector in which the immune response to the vector itself limited transgene expression during boost administrations. On the positive side, the generation of CEA-specific CTLs was demonstrated after vaccination with rV-CEA. In addition, the phase I trials were successful in demonstrating that advanced cancer patients could tolerate high doses of live viral vaccines with minimal toxicity. Further trials are being conducted to improve the clinical effectiveness of this vaccine by using nonreplicating poxvirus vectors, adding treatment with cytokines (i.e. IL-2 and GM-CSF) and cyclophosphamide, and boosting with the HLA-A2-restricted CAP peptides[108].

ALVAC-CEA vaccine trial

In order to avoid the neutralizing antibody responses that may limit vaccinia administration, a recombinant ALVAC virus-expressing human CEA was constructed. This vaccine was studied in a phase I clinical trial with increasing doses of virus given to patients with advanced CEA-expressing tumours[108]. The study accrued 20 patients with primary colorectal cancer ($n = 13$), pancreatic cancer

($n=2$), gastric cancer ($n=2$), cervical cancer ($n=1$) and unknown primary adenocarcinoma ($n=2$). The median age of the patients was 64 (range 22–81) with 13 females and 7 males. Vaccine was administered intramuscularly every 28 days for a total of three immunizations. There was no significant toxicity at doses up to 2.5 x 10^7 pfu. None of the treated patients experienced a clinical response. However, in vitro assays provided evidence for induction of CEA-specific CTL responses after vaccination. T lymphocytes from peripheral blood were collected from nine HLA-A2 patients before and after vaccination and placed in culture. After exposure to irradiated PBMCs pulsed with the HLA-A2-restricted CAP-1 peptide and IL-2 limiting dilution assay was used to determine the precursor frequency of CEA-reactive CTL. Significant increases in CEA-reactive T cells were observed in seven of the nine patients. Furthermore, a CTL line was generated from one patient after vaccination that could recognize CAP-1 pulsed C1R:A2 cells and autologous tumour[108]. These results suggest that ALVAC-CEA is safe, can be administered at doses up to 10^7 pfu, and may be able to generate CEA-specific immunity. Future studies may target patients with earlier stage disease when clinical responses are more likely.

ALVAC-CEA-B7 vaccine trial

In order to improve the clinical effectiveness of antiCEA vaccination, other 'second generation' vaccine strategies have been developed for clinical trials. One such approach is the use of a recombinant ALVAC virus that expresses both human CEA and the B7.1 co-stimulatory molecule (Figure 6.4). The addition of the B7.1 co-stimulatory molecule provides an essential 'second signal' for T-cell activation. This vaccine construct has been generated for clinical use and is currently being administered to patients with advanced CEA-expressing adenocarcinomas.

The objectives of this phase I clinical trial are to determine the optimal dose of virus and define any side effects. Since B7.1 has not previously been given to humans by an ALVAC vector, the potential for autoimmune phenomenon needs to be seriously considered. Secondary objectives for the initial trial of the ALVAC-CEA-B7 vaccine include assessment of clinical responses and evidence of antiCEA immunity as measured by in vitro T-cell assays. The study is designed to enrol six patients at three different doses of virus, 4.5 x 10^6 pfu, 4.5 x 10^7 pfu and 4.5 x 10^8 pfu. The virus is given by intramuscular injection every four weeks for three total immunizations and computed tomography (CT) scans are obtained before treatment and four weeks after the last vaccination. Although this trial is still in progress, preliminary results suggest that there is minimal toxicity associated with the ALVAC-CEA-B7 vaccine, including the absence of autoimmune phenomenon. Patients tolerated all dose levels without difficulty. Clinical information and cellular immune responses are being collected and should be available shortly. Results from this trial should provide insight into the co-administration of tumour antigen

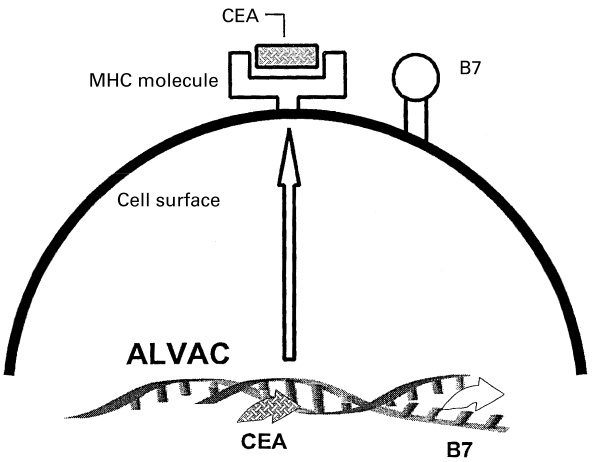

Figure 6.4 Graphic representation of the recombinant ALVAC virus genetically engineered to express human CEA and B7.1 co-stimulatory molecule. CEA expression should result in presentation of MHC-restricted peptide epitopes on the surface of infected cells. These cells should also exhibit increased cell surface expression of B7.1, enhancing the probability of generating CEA-specific T-cell responses

and co-stimulatory genes in a single vector. Although it is unlikely to achieve complete clinical responses in patients with advanced disease, this trial will document the safety and feasibility of using such a vaccine. If evidence for enhanced antiCEA immunity can be confirmed, this agent may be appropriate for further clinical trials in patients with earlier stage disease.

Point mutated *ras* peptide vaccine trials

In the United States alone, there are approximately 140 000 cases of cancer each year with *ras* point mutations[21]. At present, there are approximately 840 000 patients in the USA with diagnosed cancers that contain point mutated *ras*. Whereas the majority of point mutations occur at codon 61 in rodent tumours, the overwhelming majority (>90%) of point mutations in human tumours are at codon 12. A review of the literature has shown that more than 80% of these codon 12 mutations involve three amino acid substitutions (i.e. glycine at position 12 is substituted by either valine, cysteine or aspartic acid). Thus, one can envision the development of only three vaccines to cover the majority of human *ras* mutations. Additional advantages of considering *ras* as a target for immunotherapy are that it is expressed early in preneoplastic lesions and it is exquisitely specific. One potentially negative aspect of employing point mutated *ras* as a target may be its level of expression and presentation by MHC on tumours (i.e. it might not be sufficient to enable efficient T-cell mediated lysis). Another potential concern involves the

insertion of an oncogene into a live vector; for this reason, initial studies have involved the design and development of immunodominant point mutated *ras* peptides. Preclinical studies involving peptides reflecting *ras* point mutations in murine models have been described[21,109–111].

A phase I trial involving the use of 13-mer peptides reflecting the point mutations of *ras* is in progress. The three 13-mer peptides employed contain amino acids 5–17 and represent the most common *ras* position 12 mutations. Thus far, *ras*-specific $CD4^+$ T cell responses have been observed in some patients. Using a lymphoproliferation assay with the 13-mer peptide reflecting the point mutation in the tumour as immunogen, no response was seen to the normal proto-*ras* or to 13-mer peptides, reflecting different position 12 point mutations. Moreover, no response to the immunizing peptide was seen employing PBMCs prior to immunization. These $CD4^+$ T cells could be propagated by the use of the specific peptide and IL-2 through numerous IVS cycles.

Some of the patients who received the *ras* vaccine possessed the class I HLA-A2 allele. It has been demonstrated that some 13-mer peptides used as immunogens also possess a class I HLA-A2 binding site nested within the class II peptide[110,112]. Interestingly, this phenomenon of a class I HLA-A2 binding peptide 'nested' within a class II peptide has also been shown to be present in the murine system. Two of the three HLA-A2 patients who were vaccinated demonstrated *ras*-specific CTL responses. One patient demonstrated CTL responses to the glycine→aspartic acid mutation while the other responded to a glycine→valine mutation. In both cases, the mutant peptide reflected the immunogen and the mutation found in the tumour by PCR. In both cases, $CD8^+$ T cells could be propagated by IVS with a nested 10-mer *ras* peptide and IL-2. Other studies have recently demonstrated that specific T-cell responses can be achieved by vaccinating cancer patients with *ras* peptides. In those studies, immune responses to mutations at codons 13 and 61 were evaluated and a CTL response to a codon 12 mutation was described for the human B35 allele[113].

Adoptive transfer

The identification of immunodominant or subdominant epitopes within molecules such as CEA and point-mutated *ras* has led to the long-term in vitro propagation of epitope-specific human CTL lines. Not only can these lines serve as a source of basic knowledge of the properties of human CTLs against human self-antigens and tumour-specific antigens, but they can also potentially be used in adoptive transfer immunotherapy. It should be emphasized that the use of epitope-specific CTLs is quite distinct from the use of LAK or TIL cells for adoptive transfer. For example, TIL cells are usually heterogeneous and are derived from tumour masses which may, for some tumours, be a source of anergized T cells. Moreover,

epitope-specific T cells can be amplified in vitro and potentially in vivo by defined peptides. Recently, two studies have demonstrated the efficacy of antigen-specific T cells in adoptive immunotherapy. In one case the elimination of CMV infection was achieved[114]; the other case involved the successful treatment of EBV lymphoproliferative disease[115].

Conclusions and future directions

The identification of tumour-associated antigens and epitopes based on their ability to elicit specific T cell responses has increased the pool of potential antigens for vaccine development. Advances in molecular biology have provided unique ways to present these antigens to the immune system by constructing synthetic and recombinant vaccines. Preliminary studies suggest that the selection of antigen, vector, routes of administration, and boosting schedule – as well as the addition of cytokines, co-stimulatory molecules and other adjuvants – can influence vaccine efficacy. The optimal combination of these factors in designing the most effective vaccination strategy for the treatment and prevention of colon cancer is an area of active investigation. Animal models have also suggested that vaccination may be more potent as a means of prevention rather than treatment, similar to the role of vaccines in the prevention of infectious diseases. However, colon cancer vaccines are currently being used in clinical trials for patients with established and often quite advanced disease. These patients may be very difficult to treat since their tumours are often widespread and their immune systems may be weakened or defective. A concentrated effort is being made to monitor the development of immunity in current clinical vaccine trials so that potentially effective vaccines can be identified for future study in patients with less advanced disease. Vaccination remains a powerful tool in the treatment of human disease and may well play a role in the future treatment and prevention of human colon cancer.

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7

MUC1 vaccines and breast cancer

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Features of breast cancer

Breast cancer is the most common malignancy in women in the 'developed' world, with the number of new cases per year in the UK and USA being 34 000 and 180 000, respectively. Breast cancer is rare below the age of 30 but the incidence increases up to the age of 50. Beyond the age of 50, the incidence continues to rise but more slowly compared with premenopausal women. Although the incidence of breast cancer is increasing, recent data suggest that the mortality rate is decreasing. Nevertheless, with a death rate of 20 to 25 per 100 000 in the West and a prevalence over three times the incidence, breast cancer continues to be a significant public health problem. Factors conferring an increased risk of breast cancer include mutations in the breast cancer susceptibility genes BRCA-1 and BRCA-2. While mutations in these genes account for the majority of familial breast cancers, they account for only 5 to 10% of all breast cancers. Other factors conferring increased risk include the presence of atypical hyperplasia and carcinoma in situ (cytologically malignant cells confined within a duct or lobule). Obesity, high alcohol consumption, prolonged use of oestrogens and previous radiotherapy to the breast are thought to confer an increase in risk of less than twice the background risk.

The majority of cases of breast cancer present with a lump in the breast which may be associated with nipple discharge and/or deformity of the breast. Screening women for the development of breast cancer is done with the aim of increasing the proportion of women whose disease at presentation is truly localized. Breast examination may enable earlier detection by increasing awareness of a clinically apparent lump. However, radiological investigations, predominantly mammography, are capable of identifying disease which has not become apparent clinically and has been effective in reducing breast cancer mortality in women over the age of 50. Strategies to increase detection by increasing the frequency of mammography and extending the screened population are being studied.

Most patients with breast cancer present with disease which is apparently localized to the breast (stage I) or breast and regional axillary lymph nodes (stage II),

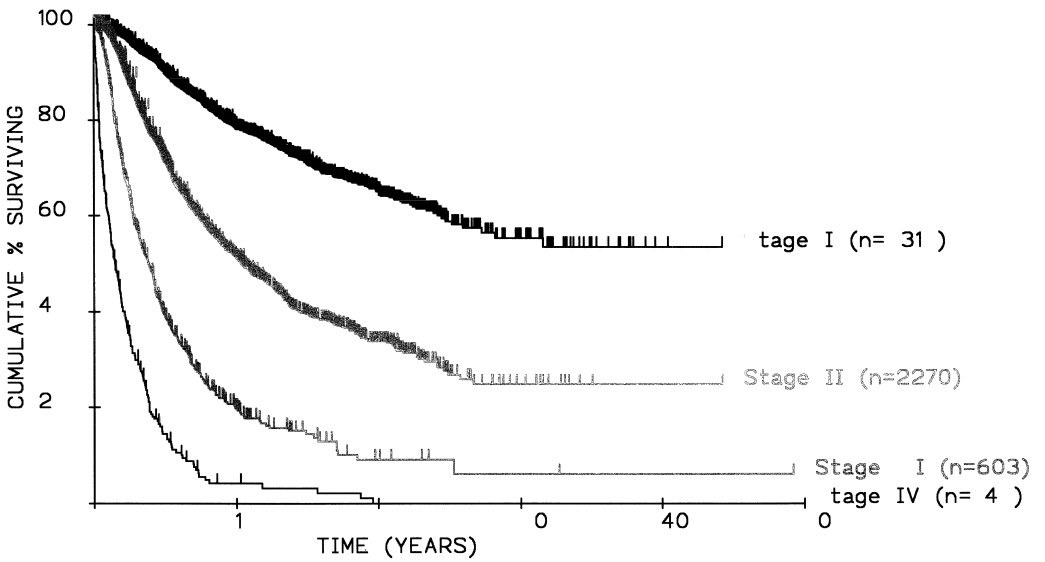

Figure 7.1 Survival of patients with breast cancer by stage at presentation (data from the Breast Unit, Guy's Hospital, London)

involvement of which is most commonly diagnosed by pathological examination following their removal at the time of surgery. In some cases, the extent of disease in the breast makes surgical removal of the entire tumour impossible (stage III). In a small number of cases, patients present with disease which has spread beyond the breast and regional lymph nodes, to other organs such as lung, liver and bone. Survival at presentation (Figure 7.1) depends upon the extent of the tumour, determined by the above staging system. As illustrated in Figure 7.1, many women with disease which is apparently localized to the breast at the time of diagnosis develop metastatic disease and ultimately die of it. The likelihood of the presence of micrometastatic disease at the time of initial surgery is related to features of the primary tumour (size and degree of differentiation) and the number of lymph nodes involved. Such features represent prognostic variables which can be used to predict patient outcome. Therapeutic interventions which are known to reduce tumour burden in patients with advanced breast cancer have been applied to women with operable disease who are at risk of developing metastatic disease subsequently. Such treatments which are adjuncts to surgical removal of the primary tumour and lymph nodes, include hormonal therapy, cytotoxic chemotherapy and radiotherapy. Such strategies reduce the risk of relapse and death in women with operable disease by 20 to 30%[1]. More widespread use of adjuvant treatment may have contributed to the observed reduction in death rates in women with breast cancer. Despite these interventions, many women with apparently localized disease at the

time of diagnosis ultimately develop metastatic disease, the prognosis of which is similar to women presenting with stage IV disease. In patients with metastatic disease treatments, which may include hormonal therapy, chemotherapy and radiotherapy, as well as surgical interventions, are not carried out with curative intent but rather aimed at palliating symptoms and may prolong life. It is apparent, therefore, that more effective agents are required for the treatment of breast cancer. While newer chemotherapy agents have been introduced and dose intensification of chemotherapy drugs has been made more feasible, their impact on survival of women with breast cancer has been disappointing. Novel treatment strategies are evidently required.

Target antigens in breast cancer

This chapter will explore the use of formulations based on the MUC1 mucin in the immunotherapy of breast cancer, but first it seems pertinent to mention briefly some of the other molecules that can be used as target antigens. As well as the non-specific approaches to immunotherapy, for example the introduction of cytokines, MHC class II molecules or co-stimulatory molecules into tumour cells[2–4], specific antigens can also be targeted. These can be antigens with a highly restricted pattern of expression, e.g. MAGE, or can be antigens that are present on normal cells but show increased expression on tumour cells, e.g. c-erbB-2. In addition, some molecules, e.g. MUC1, may also be altered in their posttranslational modifications when expressed by tumour cells (see below).

c-erbB-2 or neu is a member of the EGFR family of receptors that can form homo- and heterodimeric receptor complexes leading to tyrosine phosphorylation and signal transduction. c-erbB-2 is overexpressed in about 30% of breast carcinomas and overexpression has been correlated with poor prognosis[5]. It appears that this association may define a causal relationship as transgenic mice expressing wild type neu under the MMTV promoter develop focal mammary tumours[6]. Recently, results of clinical trials have been reported using a humanized antibody to c-erbB-2 (known as Herceptin) either alone or in combination with chemotherapy[7,8]. In phase II and III trials in patients with metastatic breast cancer, Herceptin induced objective responses with a favourable toxicity profile[7,9]. The use of Herceptin in combination with chemotherapeutic drugs led to increased response rates[8], although Herceptin in combination with doxorubicin did lead to increased cardiac toxicity. The results of these trials has led to Genentech being granted a licence by the FDA to market Herceptin.

The use of c-erbB-2 in active specific immunotherapy using preclinical models has also been described[10]. Here, vaccination of mice with full length or truncated cDNA encoding c-erbB-2 protected mice from subsequent challenge with neu

expressing tumours. Interestingly, the truncated cDNA which coded for secreted c-erbB-2 was as effective as the full length. This is important as the in vivo transfection of cells with full length c-erbB-2 capable of signal transduction could conceivably lead to their transformation.

The MAGE antigens, originally identified as tumour-associated antigens expressed by melanomas, are expressed by a percentage of breast cancers[11]. These antigens have been shown to contain CTL epitopes [12] and breast cancer patients of a defined HLA class I type have been entered into clinical trials using peptides containing class I restricted CTL epitopes[13]. Several clinical trials are in progress using different MUC1 based formulations. Since the molecule is complex, some background on its structure and function is warranted in order to evaluate the different approaches.

The MUC1 mucin gene and its product

The epithelial mucin which is coded for by the MUC1 gene is not a classic extracellular complex mucin such as those found as major components of the mucous layers covering the gastrointestinal and respiratory tracts, but is a transmembrane molecule, expressed by most glandular epithelial cells. The molecule was first identified in human milk (where it is shed from the milk-secreting epithelial cells), as a large molecular weight glycoprotein rich in serines, threonines and prolines carrying a high percentage of O-linked carbohydrate[14]. Interest in MUC1 was dramatically enhanced, however, when monoclonal antibodies developed to surface molecules on epithelial cells and carcinomas were found to react with the mucin. In particular, the membrane of the lactating mammary epithelial cell which surrounds the fat globules shed into milk, was widely used as an immunogen, and the mucin appears to dominate antibody responses in the mouse. The MUC1 reactive mouse monoclonal antibodies have been analysed in two 'wet' workshops[15,16] as well as by individual investigators[17–19] so that the epitopes which they recognize have been well characterized.

From immunohistochemical staining of tissue sections with the antibodies it became clear that although MUC1 is widely expressed by normal glandular epithelial cells[20], the expression is dramatically increased when the cells became malignant. This has been well documented in breast and ovarian cancer[21] and available data suggest that this is also true in some lung, pancreatic and prostate cancers[21–23]. The antibody studies also have the first indications that not only is the mucin overexpressed in carcinomas, but the pattern of glycosylation is altered. Thus, in the breast cancer mucin, glycosylation changes result in certain epitopes in the core protein being exposed which are masked in the mucin produced by the lactating mammary gland[24]. The antibody SM3[25] has been particularly important in defining this difference, the epitope in the tandem repeat (see Figure 7.2) being

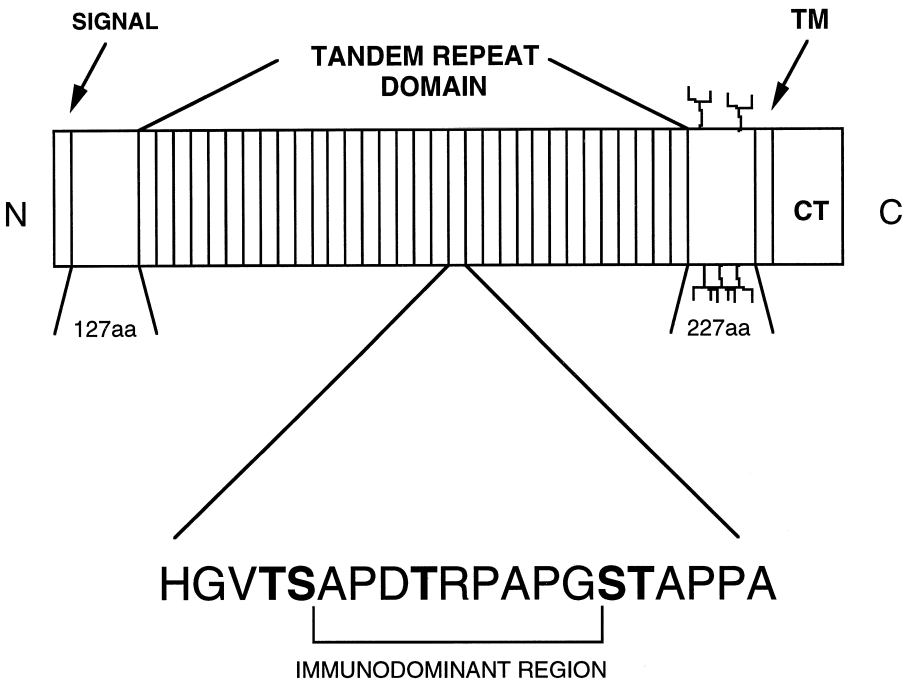

Figure 7.2 Core protein of the MUC1 mucin. The amino acids in bold refer to potential O-linked glycosylation sites. -[= potential N-linked glycans; TM = transmembrane region; CT = cytoplasmic tail

accessible in breast cancers, but not in normal breast[21]. Changes in glycosylation patterns also occur in other malignancies, as in the colon[26]. However, the changes are imposed on the normal pattern of glycosylation, which is very different in different tissues.

Because of the availability of antibodies to the core protein, the gene coding for this protein was the first mucin gene to be cloned[27,28]. The extracellular domain was found to be made up of tandem repeats (TRs) of 20 amino acids each, of which five contain potential glycosylation sites, and many of the antibodies to MUC1 map to a region between pairs of serines and threonines within the tandem repeat (see Figure 7.2). A similar strategy of raising antibodies to the core protein stripped of carbohydrate was used to clone the cDNAs encoding the core protein of other mucins, and TR domains containing O-glycosylation sites are found to be a common feature of all the mucin core proteins for which sequence has been obtained. Clearly these domains provide the scaffold for the attachment of the multiple O-glycans, which are the defining feature of mucins.

The TR domain of MUC1 is flanked by degenerate tandem repeats which also contain sites for O-lined glycosylation, and there are five potential N glycosylation

sites closer to the transmembrane domain[27,28]. Comparison of the human MUC1 with the homologous gene product in other species shows a high conservation of sequence in the transmembrane and cytoplasmic domains, while within the tandem repeats conservation is limited to retaining the serines and threonines for the addition of O-glycans[29]. The function of the highly conserved regions has not been defined, but it is suggested that these domains may play a role in signal transduction and in protein interactions.

The antibodies to the MUC1 mucin have been available since the early 1980s and clinical studies with these antibodies are beginning to mature. With the availability of the cDNA coding for the core protein of the MUC1 mucin, and the characterization of the changes in glycosylation which can occur in cancer, focus is now also being given to the use of antigens based on MUC1 in 'active specific immunotherapy' of cancer patients.

MUC1 as a target antigen in breast cancer

While the increased expression and changed glycosylation of MUC1 in breast cancer provoked interest in the molecule as a target antigen for immunotherapeutic approaches, there are other factors which have played a role in focusing on MUC1 based immunogens (see Box 7.1). In particular, the report that cytotoxic T cells (CTL) could be isolated from breast and other cancer patients which could kill MUC1 expressing cells in a nonMHC restricted fashion[30,31] suggested that MUC1 formulations could be widely used as immunogens. Antibodies to MUC1 have also been detected in cancer patients[32] and, more recently, MHC restricted CTL responses have been described[33]. The repetitive structure of MUC1 at the level of both the protein core and the O-glycans may enhance the immunogenicity of the molecule and the glycoform will determine any lectin interactions which might occur. It is therefore important to consider the changes in glycosylation which occur in breast cancer, as these may influence the choice of immunogen.

Changes in glycosylation of the MUC1 mucin in breast cancer

The different glycosylation of MUC1 produced by breast cancers was suggested by the reactivity of the SM3 antibody which detects a core protein epitope on the cancer mucin which is masked in the normal. What these changes are, and the underlying mechanisms leading to the aberrant glycosylation, are now being clarified. This is partly because the pathways of mucin-type O-glycosylation are now being elucidated in more detail with the cloning of the genes coding for the glycosyltransferases involved in the synthesis of O-glycans. To put in context the changes in glycosylation of the MUC1 mucine which occur in breast cancer it is appropriate to briefly describe these pathways.

Immunogens based on MUC1

- cell lines expressing MUC1
- peptide epitopes
- monoclonal antibodies to MUC1
- cDNA encoding MUC1 including viral vectors
- carbohydrate antigens

Box 7.1.

O-glycosylation in mammalian cells

The addition of N-acetyl galactosamine to serine or threonine initiates O-glycosylation in mammalian cells[34]. It is now clear that this reaction is catalysed by not one, but by a family of enzymes (polypeptide N-acetylgalactosaminyltransferases) which show overlapping but different specificities with regard to peptide sequence[35]. The three GalNAcTs (GalNAcT1, T2 and T3) which have been specifically localized by immunoelectromicroscopy in cells transfected with the tagged genes, are found distributed throughout the Golgi apparatus[36], indicating that chain initiation is not restricted to the cis compartment. O-glycans are then synthesized by the addition of sugars individually and sequentially as the mucin passes through the Golgi. Chains are generally extended by the addition of polylactosamine side chains and terminated by the addition of sialic acid, fucose or galactose. Again, it is becoming clear that more than one enzyme can catalyse the same specific reaction, and that the same substrate may be acted on by enzymes which produce different products. Thus, the sites of glycosylation of the same core protein could vary depending on the profile of expression of GalNAcTs, while the composition of the O-glycan will be influenced both by the level of activity of a specific glycosyltransferase, and its position in the Golgi pathway relative to other enzymes which can compete for the same substrate. The early work with antibodies indicated that there were differences in the pattern of O-glycosylation of MUC1 in breast cancer. These changes could involve differences in sites of glycosylation or in O-glycan structure, or both.

Changes in the sites of O-glycosylation of MUC1 in breast cancer

The techniques for analysing specifically the sites in proteins which are O-glycosylated in vivo have only recently been developed to the point where they can be applied to mucin molecules which may contain hundreds or even thousands of O-glycans[37]. There are five potential O-glycosylation sites in each tandem repeat of MUC1 and the use of these sites in the normal and malignant mammary gland has recently been analysed. Analysis of the mucin produced by the normal lactating

mammary gland has shown that while all of these sites can be glycosylated, the average number of O-glycans added to each tandem repeat is around 2.5[38]. Analysis of the sites of glycosylation of the MUC1 mucin produced by the breast cancer cell line T47D, however, suggests that all five sites are occupied in each tandem repeat (Hanisch, personal communication). This difference could be attributable to a different profile of expression of the GalNAcTs in normal and malignant mammary tissue. However, initial analysis of expression of four GalNAcTs in sections of breast cancers by immunohistochemistry has not given a clear answer to this point (Burchell and Mandel, unpublished observations). Another possible explanation is that the O-glycans on the T47D mucin which are shorter (see over) allow initiation of glycosylation to proceed throughout the Golgi apparatus, while the longer side chains which are built on to the normal mucin block access of the GalNAcTs at a certain point.

Changes in the composition of O-glycans added to MUC1 in breast cancer

In the normal human mammary gland, the addition of GalNac to serines or threonines is followed by the addition of galactose to form core 1 which then acts as a substrate for the core 2 β1, 6GlcNAc T (C2GnT) enzyme, leading to the formation of core 2 (see Figure 7.3). Type II polyactosamine side chains are then formed and terminated by sialic acid or fucose[39,40]. Thus, the O-glycans on the normally glycosylated mucin are core 2 based. Direct analysis of MUC1 produced by breast cancer cell lines has, however, shown the structure of the O-glycans to be core 1 and not core 2 based, i.e. the side chains are shorter and less complex on the cancer mucin.[41,42] These direct analyses confirm the earlier work of the late George Springer, who demonstrated the appearance of the T epitope in breast cancer[43] and used a preparation of erythrocytes expressing this antigen in the treatment of breast cancer patients. The sialyl Tn epitope (NeuAcα2,6 GalNAc) has been reported to be specifically expressed in several cancers, including gastric and breast, and has been used, coupled to a carrier protein, as an immunogen for cancer patients (see below).

Correlation in changes of glycosyltransferase activities with changes in O-glycan structure in breast cancer

An examination of Figure 7.3 shows that core 1 can be a substrate for an enzyme leading to chain extension via core 2 or for an enzyme which, by adding sialic acid to core 1 to form sialyl T, terminates chain extension. Analysis of the activity of the enzymes catalysing these reactions in extracts of normal and malignant breast epithelial cell lines showed that the α2,3 sialyl-transferase activity was increased 8–10 fold in three breast cancer cell lines relative to the normal cell line (MTSV1–7),

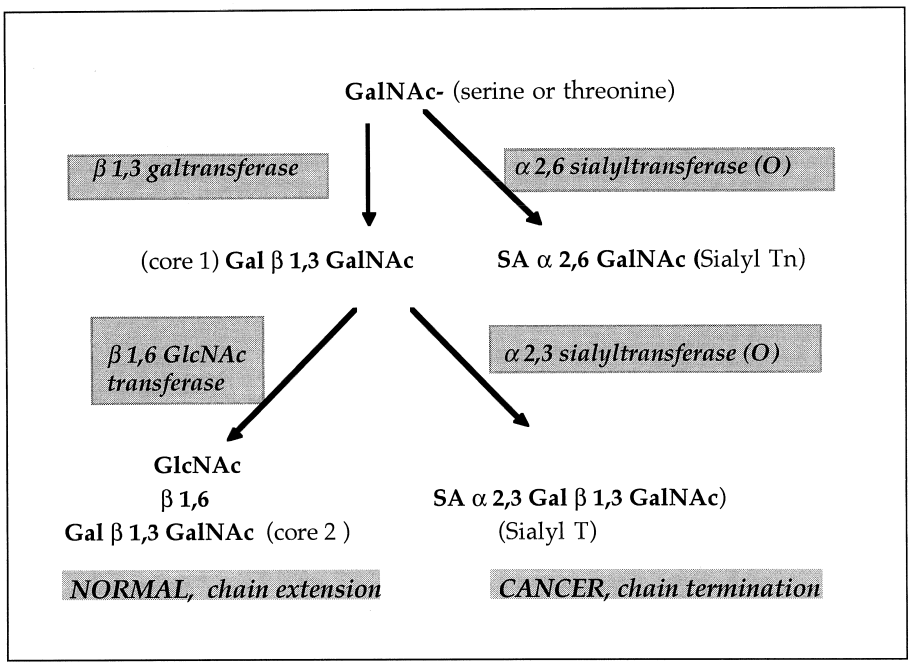

Figure 7.3 Pathways of O-glycosylation of MUC1 in breast tissue. In normal mammary epithelial cells core 1 glycans are converted to core 2 by the action of core 2β1, 6 GlcNAcT. In breast carcinomas this conversion is reduced, resulting in core 1-based structures being found on MUC1

while the C2GnT enzyme activity was absent in two of the breast cancer cell lines (T47D and BT20) and reduced by 50% in a third[44].

Clearly in measuring the activity of a cell extract several enzymes may be involved in the same reaction. In the case of the activity catalysing the formation of core 2 from core 1, it is clear that it is the L enzyme[45] which is being measured. In the case of the sialyltransferase, there are at least three enzymes which could theoretically catalyse the formation of the addition of sialic acid in α2,3 linkage to Galβ1,3–GalNAc[46,47]. However, in situ analysis of tumour sections with a specific ST3Gal I probe[48] shows a marked increase in expression of this specific sialyltransferase in breast cancers. Significantly, the increased expression appeared to be correlated with the invasiveness of the tumour, and correlated with the expression of the SM3 epitope.

Changes in glycosylation of MUC1 have been noted in other cancers[49–51], but these are in the context of the profile of enzymes expressed in a particular tissue. It should be noted, of course, that the changes in expression of glycosyltransferases seen in breast cancer will affect the glycosylation not only of MUC1 but of any other molecule undergoing mucin-type O-glycosylation.

Effects of MUC1 expression on the behavioural properties of cancer cells

The changes in expression and posttranslational modification of MUC1 has stimulated investigations into whether this affects the behaviour of cancer cells, particularly relating to interactions with other cells and with the extracellular matrix. In the normal glandular epithelial cell, MUC1 expression is limited to the apical surface bordering a lumen. In cancer cells, however, which have lost polarity, the mucin is expressed all over the surface. Because of its rod-like structure, the molecule extends more than 100–200 nm above the surface, which is 5–10 fold the length of most membrane molecules. By virtue of the high level of sialic acid, MUC1 is also negatively charged and cells expressing high levels may repel each other. Such repulsive effects have been demonstrated by showing that MUC1 transfectants show reduced aggregation as compared to the nonexpressing parental cells[52] and interactions with the extracellular matrix are also inhibited[53]. With E-cadherin mediated cell interactions, MUC1 has been reported to be inhibitory (in L cells transfected with E-cadherin and MUC1)[54] and to enhance adhesion by interacting with β-catenin[55].

In considering the effects of MUC1 on cell–cell interactions, it is clear that without specific interactions, for example with a lectin molecule, the long highly charged molecule can easily result in repulsion between cells. These inhibitory effects on cell interactions appear to depend on both the large size of the molecule and the negative charge[53,54]. However, where a specific interaction is possible – for example, a particular carbohydrate epitope binding to a lectin – then cell interactions may be enhanced. Thus, MUC1 has been reported to be a ligand for ICAM1 expressed by endothelial cells[56]. Moreover, MUC1 has been reported to enhance antigen presentation to T cells, possibly operating through a lectin interaction[57]. Furthermore, MUC1 has been shown to be a ligand for sialoadhesin, a macrophage restricted adhesion molecule, which specifically binds Neu5Acα2,3Gal which is highly expressed on MUC1 produced by breast cancer cells. The MUC1 sialoadhesin interaction may therefore be involved in recruiting macrophages into the tumour site[58]. How the surface MUC1 on cancer cells may influence metastatic progression is not clear, although in MUC1 null mice, mammary tumour progression has been reported to be delayed[59].

MUC1 glycoforms and the immune response

In considering MUC1 as an antigen, it is reasonable to ask whether the immune response will be affected by the pattern of glycosylation of the mucin. In this context it is important to consider the whole repertoire of the immune system which in fact developed to reject invading pathogens, and which, in vertebrates,

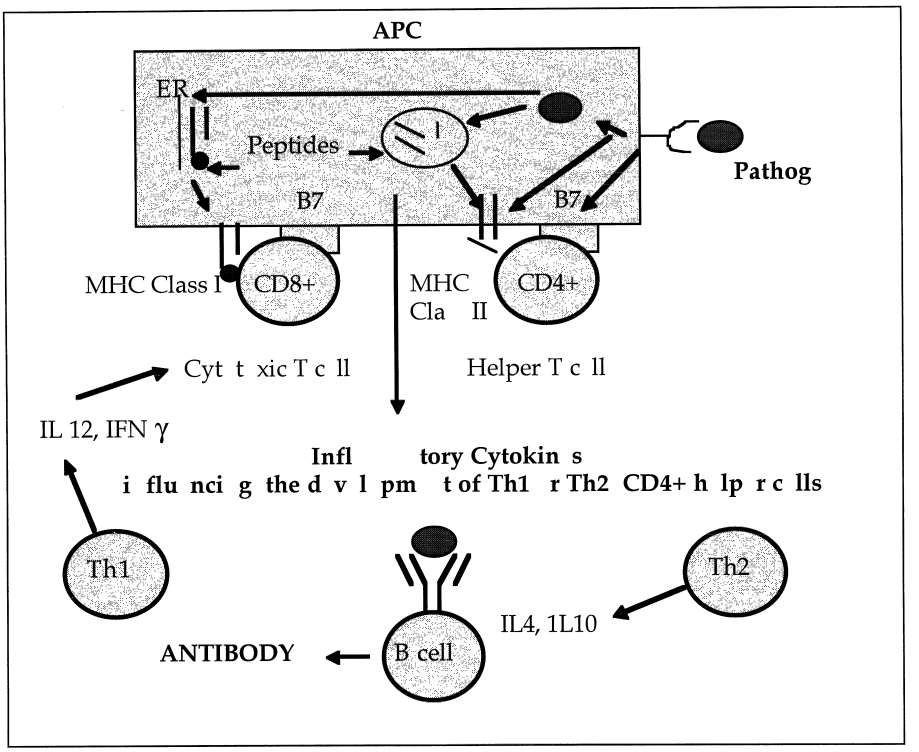

Figure 7.4 Recognition of a pathogen by APCs through innate immunity leads to activation of adaptive immunity

includes the 'innate' and 'adaptive' immune response. There is considerable evidence to suggest that, without the activation of APCs induced by the innate immune response to pathogens, the adaptive response, i.e. the recruitment of T cells and B cells, does not occur, but rather anergy ensues[60] (see Figure 7.4). Activation of APCs can be initiated by various molecules expressed by pathogens, some of which, in view of this function, are used as adjuvants. Members of a particular class of pathogens express similar but not identical molecules which share a particular pattern (pathogen associated molecular patterns or PAMPs), and many of these PAMPs have a high carbohydrate content and can interact with surface lectins. An example of such a class of molecules are the mannans of yeast which interact with the mannose receptor.

As we have seen, interactions of MUC1 with surface lectins have been reported and these interactions will depend on the composition and clustering of the O-glycans attached to the mucin core protein. Since MUC1 is a highly repetitive molecule with a specific structural pattern, it is possible that the interaction of MUC1 with a surface lectin on an APC may mimic the activation of this cell induced by

certain pathogens. Certainly, wherever the whole mucin MUC1 molecule plays a role in the immune response, the glycoform will be of paramount importance. This will apply to the antibody response as well as to the lectin mediated interactions of the immune system. The nonHLA-restricted killing of MUC1 expressing cells by CTL is also dependent on the glycoform. Thus, the CTL recognize the cancer-associated mucin carrying the shorter O-glycans, while with MUC1 expressing B cells, inhibition of glycosylation with analogues of GalNAc (which inhibit chain extension) is required for target recognition. Whether the presentation of peptides by MHC molecules is occurring and whether this is affected by glycosylation remains to be clarified. Theoretically, class II presentation could be affected since exogenous antigen is taken up and degraded by APCs. Interactions with surface lectins on APCs will depend on the glycoform, as will uptake, breakdown and presentation by class II molecules[61]. Class I molecules on the other hand are loaded in the ER before O-glycosylation is initiated, and it would seem unlikely that peptides carrying O-glycans can be presented. The exception may be presentation by dendritic cells (DCs) which can direct antigen taken up exogenously to the cytoplasm[62].

Cell–cell interactions may also be inhibited by certain glycoforms of the membrane-associated MUC1 and such an effect has been repeated for T cell interactions[63]. Clearly, the possibility of the dual function of repelling cells by the highly charged extended mucin or enhancing adhesion via lectin interactions bring a complexity to the function of the molecule in the context of cell interactions which is not easily resolved.

In the clinical studies which have been initiated with MUC1-based immunogens, the question of the importance of the pattern of glycosylation has not really been addressed. The trial using a carbohydrate epitope (sialyl Tn) coupled to KLH (see below) addresses in a more general way the importance of specific O-glycans in the immune response to tumours, but not in the context of MUC1. Peptide-based vaccines have so far been based on the unglycosylated tandem repeat sequence, and the glycoform of the MUC1 expressed from DNA based vaccines in vivo is uncharacterized. It would now be possible to modify the glycosylation pattern, e.g. by co-expressing a specific glycosyltransferase in a virus or co-injecting the relevant cDNA. Preclinical studies directed towards analysing the relevance of MUC1 glycosylation to its efficacy as an immunogen are therefore we believe of crucial importance.

Immunotherapy with antibodies to MUC1

The monoclonal antibodies which have been raised to MUC1 have been available since the early 1980s and have been used in the clinic mainly as carriers of radioactive elements. The HMFG1 antibody (originally called 1.10.F3)[64] was developed in 1981 and has been used mainly in ovarian cancer patients to deliver a high dose of

yttrium to the peritoneum[65]. In patients with minimal residual disease after chemotherapy, a significant survival benefit was seen as compared to historical controls. The results are of sufficient interest to have prompted a phase III multicentre trial in these patients which is now ongoing. Antibody treatment in the context of breast cancer has also been examined in the MUC1 transgenic mouse where treatment with unlabelled murine SM3 antibody inhibited the development of MMTV-induced tumours[66]. A phase I study of humanized HMFG1 in patients with primary breast cancer who have received definitive treatment is now in progress at the ICRF group at Guy's Hospital. Another MUC1-reactive monoclonal antibody, BrE-3, labelled with ^{111}In has been used to target breast carcinomas[67] and more recently ^{111}In and ^{90}Y labelled BrE-3 has also been used in the treatment of breast cancer[68]. In this study of six patients, three showed transient clinical response, thus warranting further studies.

Imaging studies with radiolabelled antibodies, particularly ovarian cancer patients with the SM3 antibody have shown that malignant tumours can be distinguished from benign lesions. This form of screening could be implemented in high-risk patients, for example, those with a strong family background of disease. Some success has also been observed in detecting breast cancer cells in involved lymph nodes in breast cancer patients[69]. Finally, antibodies to MUC1 are also used in breast cancer management to detect MUC1 in serum in an attempt to detect relapse before clinical symptoms appear.

Active specific immunotherapy based on MUC1

Before clinical studies can be initiated, some form of preclinical testing in animal models is necessary. To this end, syngeneic and transgenic mouse models have been developed[66,70–72]. Syngeneic models have been used to investigate the efficacy of immunogens based on MUC1, including naked DNA[70], viral vectors[73], peptides[74] and liposome encapsulation of peptides[75]. Using MUC1 transgenic mice it has been shown that tolerance to the mucin can be overcome without inducing autoimmunity[76,77]. However, even when using MUC1 transgenic mice the immune response observed is representative of the murine repertoire. Studies using HLA-A2 transgenic mice have shown that there are functional CTL A2 epitopes within MUC1[78] and probably the next generation of preclinical testing should involve the use of MUC1, HLA-A2 double transgenic mice. However, even in this model other compartments of the immune response, e.g. receptors on APCs, are still murine and so no animal model can truly predict the response that will be observed in humans. It is therefore important to initiate clinical studies and to obtain as much data as possible relating to immune responses in the patients entered into the trials.

Several immunogens based on MUC1 have been investigated in the clinic (see Box 7.1). Early work examining the nature of the immune response in animal

models utilized MUC1 expressing cell lines. Although this work continues, particularly in the preclinical setting, the complex ex vivo manipulation of autologous lymphoid and/or tumour cells is likely to raise problems of feasibility in common epithelial malignancies. Consequently, immunogens designed to target both the peptide backbone (including peptides, cDNA constructs and antibodies) and carbohydrate side chains, are being developed.

Cell lines expressing MUC1

The use of cell lines expressing MUC1 as an immunogen was first tested in syngeneic mouse models. The human MUC1 gene was transfected into a mouse mammary tumour cell line 410.4, which has the same MHC haplotype as Balb/C mice[79]. Injection of a low dose of MUC1-expressing transfectants resulted in a lower tumour incidence and delayed tumour growth compared to injection of 410.4 cells that did not express MUC1. Preimmunization with a low dose of MUC1-expressing tumour cells or with peptides derived from the tandem repeat sequence also inhibited tumour growth[74].

These findings suggested that an immune response to MUC1-expressing tumour cells may result in inhibition of tumour growth. More recently, transgenic mice expressing the human MUC1 gene in the same epithelial cells as express MUC1 in humans have been used to examine MUC1-induced tumour rejection. Modification of the MUC1-expressing tumour cells either by fusion with dendritic cells[77] or by overexpression of the co-stimulatory molecule B7.1[76] resulted in a dramatic reduction in tumourigenicity. In the case of the B7.1/MUC1 expressing 410.4 cells, the effect of B7.1 expression on survival was lost in mice depleted of T cells, indicating that a T-cell response was involved in tumour rejection. Most importantly, these experiments demonstrate that tolerance can be broken without inducing autoimmunity.

EBV-immortalized B cells can be effective antigen presenting cells (APC) and B cells from patients with cancer, immortalized and transfected with MUC1, become effective targets for autologous cytotoxic T cells in vitro when chain extension of O-glycans is inhibited[80]. In view of this observation, chimpanzees have been immunized subcutaneously with MUC1 transfected immortalized autologous B cells[81]. Before immunization, no MUC1-specific CTL were detectable, but following a single injection, MUC1-specific CTL activity was demonstrable in peripheral blood. The CTLs were expanded in vitro and shown to lyse cells from a MUC1-expressing human breast cancer cell line and two MUC1-expressing pancreatic cancer cell lines, suggesting that killing was not MHC restricted. No antibody response was detectable in these animals. Again there was no evidence of an autoimmune response, chimpanzee and human MUC1 genes being similar in having homology of the tandem repeat sequences.

Peptide vaccines

Some of the humoral and cytotoxic immune responses to MUC1 in cancer patients have been shown to be directed to specific sequences in the tandem repeat. As a result peptide sequences derived from MUC1 have been used in animal and human studies of immunotherapy. Mice immunized with a 20 amino acid synthetic peptide (derived from the MUC1 tandem repeat region) coupled to KLH in Ribi™ adjuvant[74] induced antibodies specific for MUC1 and a delayed type hypersensitivity (DTH) reaction in response to rechallenge with antigen. Following immunization, the growth of implanted MUC1-expressing tumours was impaired and the survival of tumour-bearing mice was prolonged. Subsequent studies using a liposomal form of the 24mer have also demonstrated a predominantly cellular response and tumour regression correlated with the degree of cellular rather than humoral immunity[75].

Other groups have demonstrated that immunization with synthetic peptide epitope antigens resulted in a predominantly humoral response with little anti-tumour activity[82]. Attempts to augment the cellular response to MUC1 have included conjugation of peptides from the tandem repeat region to diphtheria toxoid which contains helper T-cell epitopes[83]. In a phase I trial of this construct, six of thirteen patients generated antibodies reactive with both peptide and MUC1, two developed a DTH response on rechallenge, and in three patients proliferative T cell responses to MUC1 were detected. Stable disease was reported in six of twelve evaluable patients during the study period although no long-term follow-up data are available. The same group have also attempted to target MUC1 peptide to antigen presenting cells by coupling to mannan which binds to mannose receptors on APCs[84]. Mannans are expressed by most yeasts and can initiate the innate immune response and activate APCs[60]. The strategy of using mannan-coupled MUC1 as an immunogen is therefore logical. However, although the approach has proved effective in the mouse, clinical trial data have been disappointing[85]. The oxidization state of mannan appears to modulate the immune response: oxidized mannan resulting in a predominantly cytotoxic response in mice and production of IFN-γ (a Th1 response), whereas reduced mannan in the fusion protein resulted in a strong antibody response and secretion of IL-4 (Th2 response)[84]. However, the reasons for this apparent difference are unclear. In mice, oxidized mannan linked to a peptide of five MUC1 tandem repeats was found to be a potent and effective immunogen, inducing tumour rejection and an increase in the frequency of CTL precursors specific for MUC1[84].

In contrast to the mouse data, the results from clinical trials using mannan-MUC1 in patients with advanced breast and colorectal cancer[85] showed that the predominant response was humoral with few CTL responses being observed. This apparent discrepancy highlights the limitations of translating studies in animal models into

the clinical setting. The different response in humans is attributed to the presence of cross reactive antibodies present in humans (but not in mice) which form an immune complex with the antigen, which in turn induces a Th2 type response[86].

Other investigators have used a 105 amino acid peptide (corresponding to five tandem repeats) mixed with BCG as an adjuvant in cancer patients[87]. Most patients developed a DTH reaction in response to re-challenge with the peptide; biopsy from these sites showed variable degrees of T-cell infiltration. In one third of patients tested, the frequency of cytotoxic T-cell precursors specific for MUC1 rose more than twofold. In this study an attempt was made to measure parameters reflective of the immune status of the patient. Before immunization most T cells in these patients lacked the CD3 ζ chain, a key component for T-cell activation. Following administration of multiple injections of the vaccine, this defect was reversed in 20% of patients, suggesting that vaccination could be associated with restoration of normal T-cell function in a proportion of cases.

More recent data have suggested a predominantly cellular response following immunization of patients with metastatic breast cancer with a 16 amino acid sequence from the tandem repeat of MUC1 coupled to KLH[33]. The 16 amino acid sequence stimulated CTL in A1, A11 and A2 patients, but killing was MHC restricted. In this study multiple re-stimulations with antigen were not required for generation of CTLs in vitro.

Although the data from clinical trials suggest that the cytotoxic T-cell response in patients to both peptides and MUC1 expressing cells is MHC restricted, in non-immunized cancer patients the MUC1-specific cytotoxic T cells were reported to be MHC unrestricted. The importance of MHC restriction in the cellular responses which may be relevant in tumour rejection is not clear. Certainly, any cytotoxic T-cell responses to MUC1 seen in mouse models have been MHC restricted[78,88]. Whatever the mechanisms, the limited clinical studies done so far suggest that peptide vaccination in man can result in specific cellular and humoral immune responses. Importantly, immunization has not been associated with significant toxicity or evidence of autoimmunity.

Use of cDNA coding for the MUC1 mucin

The use of cDNA coding for an antigen aims to target the endogenous pathway by which intracellular pathogens stimulate an immune response. This form of delivery is attractive for several reasons, not least of which is the ease with which DNA can be prepared and manipulated. Studies with plasmid DNA in general use either the intramuscular route of injection[89] or DNA is delivered intradermally coated on gold particles using a gene gun[90].

Intramuscular injection of MUC1 DNA has been tested in a syngeneic mouse

model using C57 mice and RMA tumour cells expressing MUC1[70]. In this system, multiple injections of MUC1 cDNA [at >50 μg per injection) prior to injection of tumour cells resulted in significant protection, with 82% of mice remaining tumour free compared to 41% of controls injected with the empty vector. The presence of CpG islands in the plasmid apparently recruits the innate immune response when relatively high levels of DNA are used – an advantage to this form of antigen delivery[91]. Although antiMUC1 antibodies were detected after immunization, indicating that the injected cDNA was expressed, the humoral response did not correlate with tumour rejection. MUC1-specific CTL were detected, but only following tumour challenge.

Therefore, in this mouse model, immunization with naked MUC1 cDNA resulted in a humoral response and may have augmented the cellular response to MUC1. Following cDNA vaccination the mice were capable of resisting tumour growth. In studies with vaccines for infectious disease, successful protection against the pathogen has been achieved using initial injections of cDNA followed by other forms of antigen delivery such as protein or a recombinant virus[92].

Viral vectors

The use of viral vectors for intracellular delivery of cDNA coding for a tumour antigen would seem to be a logical way of utilizing the immune response which has developed to reject intracellular pathogens. MUC1 cDNA has been cloned into the vaccinia virus (VV) genome (VV-MUC1) and tested as an immunogen in several animal models[73,93]. In murine and human cells the MUC1 expressed from VV-MUC1 shows reactivities with antibodies detecting tumour-associated epitopes[73]. In a rat model VV-MUC1 was used for immunization prior to tumour challenge with a fibroblast cell line transfected with MUC1[93]. Immunization inhibited tumour growth in 82% of animals. Although there were high titres of MUC1-specific antibody, no cytotoxic T-cell responses were detected. In DBA mice, immunization with VV-MUC1 resulted in 30% rejection of tumourigenic P815 cells transfected with MUC1. In Balb/c mice immunization with VV-MUC1 delayed tumour growth of MUC1 transfected 3T3 cells rather than eliciting complete rejection. No MUC1-specific CTL were detected in either of these model systems. AntiMUC1 antibodies were observed, but the titres did not correlate with antitumour response.

The immune response to viral vaccines can be increased by the co-expression of tumour-associated antigen and an adjuvant cytokine. In view of this, a recombinant VV carrying cDNA for MUC1 and for IL-2 (VV-MUC1/IL-2) has been constructed[94]. This construct showed increased efficacy in rejecting MUC1 expressing tumours and inducing CTL in mouse models. VV-MUC1/IL-2 has therefore been

used in a phase I study of patients with advanced breast cancer. Immunization was not associated with significant toxicity and immune responses were detected in some patients[95]. A phase II multicentre trial using the VV-MUC1/IL-2 construct in patients with metastatic breast cancer is in progress.

In recombinant viruses because of the exact conservation of sequence, tandem repeats are lost by recombination and the VV/MUC1/IL-2 used in these studies contains a reduced number of repeats, estimated at 3–7 (wild type contains 25–100). One way to avoid this problem would be to restructure the gene so that the nucleotide sequence in each repeat is different but still codes for the same amino acid sequence, and such constructs are under development.

Carbohydrate antigens

MUC1 expressed by malignant cells is aberrantly glycosylated and novel carbohydrate residues, such as the T antigen, Tn and their sialylated derivatives are preferentially expressed in epithelial malignancies (see Figure 7.3).

The potential importance of carbohydrate antigens in tumour rejection was initially investigated in a mouse model using a murine metastatic breast cancer cell line TA3–Ha. This cell line expresses a glycoform of the mucin epiglycanin which carries short O-glycans and forms tumours rapidly in CAF1/J mice. Two synthetic carbohydrate epitopes, thought to be exposed by aberrant glycosylation, were tested as immunogens in this system[96,97]. The survival of mice with TA3–Ha tumours was prolonged by treatment with synthetic T conjugated to keyhole limpet haemocyanin (KLH) and emulsified in DETOX adjuvant. Approximately 30% of the animals were able to resist and sustain long-term survival when re-challenged with tumour cells[96]. Immunization of mice with desialylated ovine submaxillary mucin, which expresses large amounts of Tn, protected mice against TA3–Ha tumour challenge and produced a high antibody titre to Tn[97].

A prospective, randomized clinical trial using sTn as a target for active specific immunotherapy in patients with breast cancer has recently been reported[98]. The patients had histologically proven breast cancer, with locoregional relapse after appropriate primary therapy, or metastatic disease. They were Eastern Co-operative Oncology Group Performance Status 0–2, and their disease did not require conventional therapy and they did not require management of symptoms caused by the cancer.

All patients were immunized subcutaneously with sTn conjugated to keyhole limpet haemocyanin (KLH) with DETOX adjuvant on weeks 0, 2, 5 and 9. sTn has been detected in the circulation, and soluble antigens have been demonstrated to induce tolerance or anergy, rather than an effective immune response[99]. In mice this apparent 'suppressor' activity can be overcome by pretreatment with cyclophos-

phamide to allow immunotherapy to be effective[96]. To determine its effectiveness in man, patients were randomized to receive before the first immunization either cyclophosphamide 300 mg intravenously on day −3 or cyclophosphamide 50 mg orally on days −14 to −3, or no pretreatment with cyclophosphamide[100].

The treatment had minimal toxicity and all patients generated an antibody response to sTn, sTn-positive mucin and KLH. The highest antibody titres were in patients pretreated with intravenous cyclophosphamide. The median survival for the group pretreated with i.v. cyclophosphamide was significantly longer than that for the other groups (19.7 months versus 12.6 months, $p = 0.0176$). The patients receiving i.v. cyclophosphamide were less likely to have progressive disease, and there was a negative correlation between the growth of measurable tumours and antibody titre to sTn. Significantly, there was no correlation between progression and antibody titres to KLH.

As there were no differences between the groups in terms of the natural history of their disease or the number and type of previous treatments, the results suggest a therapeutic effect for pretreatment with i.v. cyclophosphamide followed by immunization with sTn-KLH. A large multicentre trial comparing i.v. cyclophosphamide and sTn-KLH/DETOX-B with i.v. cyclophosphamide and KLH/DETOX-B in the treatment of patients with breast cancer is planned.

Preclinical studies have suggested that sialyl-Tn may be present at the cell surface in two distinct configurations, namely as clustered and nonclustered arrays[101]. Immunization of colorectal cancer patients with synthetic sTn resulted in high IgM and IgG titres when measured against the synthetic epitope, but only weak to moderate reactivity to clustered epitopes[102]. This group continues clinical studies utilizing clustered sTn rather than synthetic hapten in epithelial malignancies[103].

Conclusions

The MUC1 molecule is a large transmembrane molecule which is upregulated and aberrantly glycosylated in many carcinomas. It is useful as a model glycoprotein in the study of the changes in O-glycosylation associated with breast malignancies as changes in activities of glycosyltransferases have been directly correlated with changes in the structure of O-glycans attached to MUC1. The glycoform of the membrane mucin can influence whether cell–cell interactions are inhibited or enhanced, while interactions of the soluble mucin with surface lectins on APCs could affect their activity as well as regulating uptake and breakdown of the antigen. MUC1 has many characteristics that make it an excellent candidate molecule for active specific immunotherapy, and this is reflected by the number and scope of early clinical studies that have been initiated.

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8

Anti-idiotypic vaccination

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Introduction

The ideal cancer vaccine should stimulate cytotoxic T cells (CTL), helper T cells and antibodies. The CTLs will efficiently kill all tumour cells expressing target antigen and MHC. Helper T cells will help in the production of CTLs but will also migrate to tissues expressing the target antigen. Once they have localized within the tissues they will release the cytotoxic cytokines (TNFβ, IFNγ) and recruit nonspecific effector cells such as macrophages. Both of these cytotoxic effects will result in tumour cell death of antigen positive or negative cells. They are therefore synergistic with CTL killing. T helper cells can also recruit natural killer (NK) cells that will kill any tumour cells that have lost MHC expression. As this is a common mechanism for tumours to evade CTL killing it is an important component of any immune response induced by a cancer vaccine. The potential of antibody responses to contribute to antitumour effects is less clear. The 'type 1' T cells that help in the activation of CTLs can also help in the production of specific subclasses of antibodies (IgG2a in mice and IgG1 in humans). These antibodies will kill any tumour cell expressing target antigen by antibody-dependent cellular cytotoxicity that is mediated by Fc receptor expressing leucocytes, including NK cells. T helper cell recruitment of NK cells into tumour tissues is therefore also essential for antibody-mediated tumour killing. Complement fixation could also play a role, either as a lytic effector mechanism, or as a trigger for activating local immune responses. The remainder of this chapter will now consider how an anti-idiotypic antibody can fulfil the requirements of an 'ideal vaccine'.

Anti-idiotypic antibodies

Anti-idiotypic antibodies are produced in response to unique features of the binding site of an antibody, and anti-idiotypic antibodies can themselves stimulate immune responses; this sequence of relationships is described as an anti-idiotypic network. In the terminology of these networks an antibody which recognizes an antigen is described as an Ab1. Antibodies recognize antigens via the interaction of

hypervariable loops (complementarity determining regions, CDRs). These loops are constrained by the framework regions of the antibody. The consequence of these features is the positioning of the CDRs in prominent surface locations that allow them to interact with specific antigens. This overall conformation, termed the idiotype, is itself immunogenic and can result in the generation of anti-idiotypic antibodies or Ab2s. Anti-idiotypic antibodies can also induce a third antibody (Ab3). Since Ab1 binds to both antigen and Ab2 these molecules may show sequence of structural similarity. Hence Ab3 that is stimulated by Ab2 might also recognize the original antigen. Thus, anti-idiotypic antibodies can mimic antigen and be used as surrogate antigens. The ease of production of anti-idiotypic antibodies makes them attractive candidates for cancer vaccines.

Can anti-idiotypic antibodies stimulate antitumour T-cell responses?

Naïve T cells can only be activated by antigen that has been processed and presented on MHC molecules of antigen-presenting cells in the appropriate stage of differentiation–activation, including expression of the co-stimulatory molecules CD80 and CD86. These co-stimulatory molecules provide a vital second signal via CD28 on the T cells. In contrast T cells encountering signal 1 (peptide–MHC) on a cell that does not express the appropriate co-stimulatory environment, receives a negative signal and is anergized. The only cells thought to stimulate naïve T cells are dendritic cells that constantly survey tissues then migrate to lymph nodes. If they have been activated they express co-stimulatory molecules and activate naïve T cells. In contrast, if they have not been activated they fail to provide co-stimulation and anergize naïve T cells. An effective cancer vaccine must therefore target activated dendritic cells. The signal that activates dendritic cells is poorly defined although bacterial products such as LPS or cytokines such as TNFα can elicit the response. More recently, Matzinger[1] has proposed her danger hypothesis which states that antigen-presenting cells are activated by 'danger'. This could be unprogrammed cell death or necrosis that results in the release of intracellular molecules such as mitochondria or heat shock proteins.

Human anti-idiotypic antibodies may be particularly effective at targeting activated antigen-presenting cells. Dendritic cells constantly survey tissues where they can either endocytose antigen by fluid phase macropinocytosis or by receptor-mediated endocytosis. The latter process is more efficient and can result in a 1000-fold greater accumulation of antigen than pinocytosis[2]. Antigen-presenting cells express both the high (CD64) and low affinity (CD32) Fc receptor for internalization of either monomeric or complexed human IgG, respectively[3]. A human monoclonal antibody can therefore be rapidly internalized by antigen-presenting cells by Fc mediated endocytosis. Interestingly, resting dendritic cells do not express the

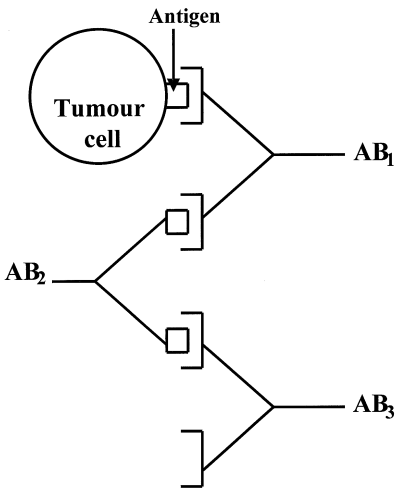

Figure 8.1 An antibody (anti-idiotypic antibody; Ab2) which binds at the combining site of an antibody (Ab1) which recognizes that an antigen can be a mimic of the antigen and induce immune responses that also recognize antigen

high affinity receptor but can be induced to express it by inflammatory cytokines[4]. Thus, monomeric human anti-idiotypic antibodies can target activated dendritic cells. Mouse Fc binds poorly to CD64 and therefore mouse anti-idiotypes are very poor at stimulating unprimed human T cells. A mouse anti-idiotypic antibody, 708, that mimics CEA could stimulate T cell responses in vitro with lymphocytes from cancer patients[5] but could not stimulate unprimed T cells from healthy donors. However, when this anti-idiotypic antibody was chimerized to a human IgG1 antibody it stimulated strong proliferative responses in unprimed T cells[6].

There are two types of antigens which can be targeted on tumour cells by T cells, either tumour-specific antigen or tumour-associated antigen. Tumour-specific antigens are novel proteins only expressed in tumours as a result of mutations. They include the products of both oncogenes and tumour suppressor genes. T cells recognizing these antigens have the advantage that they will only attack tumour cells and normal cells will be unaffected. Evidence that anti-idiotypic networks may be stimulated to tumour suppressor genes was provided by the elegant studies of Ruiz et al.[7]. Resistance to tumour challenge was induced by induction of an anti-idiotypic network by immunization of mice with a monoclonal antibody which was specific for mutated p53. The immunized mice produced IgG antibodies to p53 (Ab3) and mounted a cytotoxic reaction to a tumour line bearing mutated p53.

Tumour-associated antigens are differentiation antigens that are highly over-expressed on tumour cells as compared to normal tissues. Many peptides can be

selected from these antigens that can bind to a wide range of MHC antigens and stimulate effective T-cell responses. The main disadvantage of this approach is that T cells which recognize these antigens may also attack normal cells. The solution may be to select epitopes from these antigens which bind with moderate affinity to MHC. Peptides with highest affinity will occupy more of the available MHC than peptides of a lower affinity and the prevalence of any of these peptides will change in relation to the amounts of antigen processed. This will have several consequences. T cells recognizing moderate affinity epitopes may avoid negative selection in the thymus[8]. Secondly, dendritic cells surveying normal tissues will express too low a level of a moderate affinity epitope to activate T cells. As there is also no danger signal in normal tissues, these dendritic cells will fail to provide either signal 1 or 2 to the T cells specific for moderate affinity epitopes. The result will be that these T cells will be neither anergized nor activated by normal tissues. However, if a cancer vaccine presents large amounts of the moderate affinity epitope in the context of danger, then the dendritic cells will present both signal 1 and signal 2 and activate T cells. These activated T cells will leave the lymph node and migrate to tissues expressing antigen and MHC. This will include both normal and tumour tissues. The normal tissues will still only present moderate affinity epitopes that would be below the threshold for T-cell killing. However, tumour cells overexpressing tumour-associated antigens would result in increased expression of even the moderate affinity epitopes to above the threshold for T-cell killing. A cancer vaccine targeting a tumour-associated antigen must therefore efficiently present the moderate affinity epitope in the context of 'danger'. This can be achieved by immunizing with the moderate affinity epitope alone to prevent competition for MHC occupancy with higher affinity epitopes. One approach is to use peptides but these are rapidly degraded in vivo and are poorly taken up by antigen-presenting cells. In contrast, a human anti-idiotypic antibody is very stable in vivo, is efficiently internalized by antigen-presenting cells by receptor-mediated endocytosis and is unlikely to contain any competing high affinity T-cell epitopes. Therefore, a human antibody can be used to effectively present a selected moderate affinity epitope from a tumour-associated antigen. This can be achieved by either cloning a human anti-idiotypic antibody from a cancer patient[9] or by directly inserting a T-cell epitope into the CDR region of a human antibody[10].

The human anti-idiotypic monoclonal antibody 105AD7 was isolated from a cancer patient receiving the antiCD55 monoclonal antibody, 791T/36 for diagnostic imaging[9]. He made a very strong anti-idiotypic response that was cloned and characterized. 105AD7 bound specifically to the binding site of 791T/36 and could induce antibodies in mice that bound to CD55 and could prime DTH responses in mice and rats to human tumour cells expressing the CD55 antigen[11]. Over 200 patients have now received this human anti-idiotypic vaccine with no associated

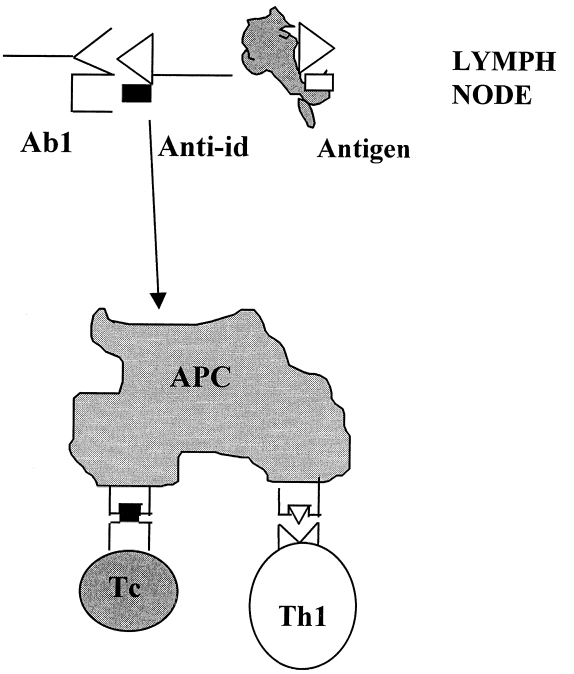

Figure 8.2 An antigen or an anti-idiotypic antibody is taken up and presented by antigen-presenting cells (APC). Similar T-cell epitopes are presented to helper and cytotoxic T cells

toxicity. Inflammatory T-cell responses were measured both in the blood[12–14] and at the tumour site of immunized patients[15]. For an anti-idiotypic antibody to stimulate T cells that also recognize antigen, a similar T-cell epitope must be processed and presented on MHC from both anti-idiotype and antigen. An anti-idiotypic antibody that mimics hepatitis B surface antigen showed significant amino acid homology between antigen and its CDRH3. The minimal B- and T-cell epitope was defined as a 6-amino acid peptide expressed by both the anti-idiotypic antibody and the antigen[16]. Similarly, a mouse anti-idiotypic antibody which mimics CEA (3H1) showed significant homology between its CDRL1 region and the N terminus of CEA. Peptide based upon either of these regions could stimulate T cell from patients who had been immunized with the 3H1 anti-idiotype[17].

The human anti-idiotype 105AD7 is unusual in that it shows three areas of homology between three of its CDR regions and three regions of CD55[18]. All three regions on both anti-idiotype and antigen define the binding site for the Ab1, 791T/36 as it binds to peptides based upon these amino acid sequences. However, only the CDRH3 region stimulates T-cell responses. T-cell epitope analysis of the CDRH3 region identifies peptides that could bind to HLAA1, A3 and A24 and

HLA DR1, 3, 7[19]. Ninety-six per cent of all patients who show a T-cell response to 105AD7 vaccination are of the predicted phenotype. Interestingly, the epitopes in 105AD7 have a higher predicted dissociation rate than the homologous epitopes in CD55. The epitopes in CD55 are of moderate to low affinity and therefore should not be expressed sufficiently on normal cells for T-cell recognition. This is important as CD55 is a complement regulatory protein that is expressed by a wide range of normal cells. However, it is over-expressed 10–1000 fold on tumour cells[20]. If the T-cell epitope presented from 105AD7 is of higher affinity than the homologous epitope presented from antigen then this would make 105AD7 a more effective immunogen than antigen. Rosenberg et al[21] demonstrated the principle using T-cell epitopes from melanoma antigens. When the anchor residues of these epitopes were mutated they bound with higher affinity to MHC but still stimulated T cells that recognized the unmutated epitope. A clinical trial with these mutated peptides resulted in tumour responses in melanoma patients. Similarly, mutating amino acids within a T-cell epitope of CEA produced a peptide that did not bind better to MHC but was more effective at stimulating T cells[22]. These T cells could also still recognize the unmutated epitope on target cells. This approach could easily be extended to anti-idiotypic immunization as antibodies can be cloned and residues mutated by site-directed mutagenesis. Anti-idiotypic antibodies could be genetically engineered to express any T-cell epitope of choice. Zaghouani et al.[10] compared the immunogenicity of an influenza T-cell epitope when presented as either antigen, a peptide or an anti-idiotypic antibody engineered to express the T-cell epitope within its CDRH3. Anti-idiotypic antibody was the most effective immunogen stimulating stronger proliferative responses than either peptide or antigen.

There are two potential limitations in using anti-idiotypic antibodies as immunogens. The first is the limited number of T-cell epitopes which anti-idiotypic antibodies can present. Like peptides they usually only present one or two epitopes. However, recent results have suggested that the immune response to viruses which contain hundreds of potential epitopes is usually restricted to one or two immunodominant epitopes. Furthermore, in acute viral infections, up to 44% of CD8 cells can be selectively activated by a single epitope[23]. Therefore, the number of epitopes presented is unlikely to be a limitation for an effective vaccine. An important caveat is the possibility of the tumour-losing expression of the target antigen. This is less likely in a vaccine that stimulates all arms of the immune response as recruitment of nonspecific effector cells or secretion of cytokines would also kill antigen-negative cells. Their second limitation is as to whether anti-idiotypic antibodies can stimulate CTL responses. Two recent pieces of evidence suggest that anti-idiotypes can indeed induce cytotoxic T-cell responses. The 708

chimeric but not the mouse anti-idiotype that mimics CEA-stimulated primary in vitro CTL responses, showed MHC-restricted CEA-specific tumour-cell killing. This implies that Fc uptake by dendritic cells may allow cross priming of CTL responses by exogenous antigen. The second piece of evidence was provided by the study that stimulated an anti-idiotypic network to p53. Lymphocytes were induced that specifically killed a tumour cell line expressing mutated p53[7].

Can anti-idiotypic antibodies stimulate antitumour antibody responses?

In contrast to T cells which recognize linear peptides, antibodies predominantly recognize conformational determinants. If an anti-idiotypic antibody is therefore going to mimic a B-cell epitope on antigen it will have to share some structural similarity with antigen. One excellent example of conformational mimicry between an anti-idiotypic antibody and antigen was provided by the elegant study of Bentley et al.[24]. They compared the crystal structure of an Ab1-anti-idiotypic antibody complex with the crystal structure of Ab1 complexed to antigen. Although there was no evidence of sequence homology both complexes showed similar interactions. Thus, anti-idiotype can differ greatly from antigen in structure and still be recognized as similar by the same antibody. Indeed anti-idiotypic antibodies can effectively mimic nonprotein antigens such as carbohydrates. An anti-idiotypic antibody mimicking the ganglioside GD2 has been shown to induce antitumour antibodies resulting in long-term survival of small-cell lung cancer patients. Recent studies by Magliani et al. showed that an anti-idiotypic scFv could induce memory IgG responses of sufficient titre to confer passive immunity to newborns[25].

105AD7 not only displays amino acid homology between three of its CDRs and three separate regions of CD55 but also shows structural similarity[18]. Analysis of molecular models of 105AD7 and CD55 highlights this similarity. The model of 105AD7 shows that the three CDRs that show homology with CD55 are brought into close proximity with each other. Molecular modelling of CD55, based on the NMR solved structure of factor H complement control protein, shows that the regions homologous to 105AD7 are arranged in similar positions within the antigen as the homologous CDRs are within the antibody. This concept is supported by the results showing that 105AD7 Ab3s shows identical binding specificities against CD55, 105AD7 and its peptides as 791T/36. As conformation is very important in the mimicry of CD55 by 105AD7 it is unlikely that any linear peptide would be as effective an immunogen as the human anti-idiotype. In contrast, it is possible for B cells to recognize linear peptides as was clearly demonstrated with an anti-idiotypic antibody which mimics the type 3 reovirus receptor[26]. An area of homology was observed between antigen and the CDR2 of both heavy and light

chains. When the sequences were linked together VL/VH peptide could induce virus neutralizing antibodies.

Numerous other anti-idiotypic antibodies have been shown to recognize tumour-associated antigens although the precise molecular mimicry has not been elucidated. Unfortunately, however, even with anti-idiotypic antibodies which show good molecular mimicry and induce antibodies which recognize antigen this is only a small component of the polyclonal response. Most of the antibodies recognize components of the anti-idiotype that do not mimic antigen[27]. Mouse serum raised against the human anti-idiotype 105AD7 shows titres of 1/100 against CD55, 1/1000 against the 105AD7 binding site and 1/10 000 against whole 105AD7 antibody (includes mouse antihuman antibodies). If only an antibody response is required (virus neutralization) it will be more effective to immunize with antigen than anti-idiotypic antibody. However, as previously discussed, to effectively treat cancer it will be necessary to stimulate both B- and T-cell responses. As human anti-idiotypic antibodies can effectively stimulate all arms of the immune system they may indeed be the 'ideal vaccine'.

Clinical trials

Several clinical trials with anti-idiotypic antibodies have been reported. The earliest was immunization of colorectal cancer patients with a goat anti-idiotypic polyclonal antiserum that mimicked the tumour-associated antigen 17–1A. Antitumour antibody responses were induced in 15 of 18 patients[28]. The survival of these patients was difficult to assess as half of them had also received chemotherapy. A follow-up trial by the same group used a different goat polyclonal antibody in twelve patients who had undergone resection of their primary tumour[29]. Six of these patients developed antibodies against the anti-idiotypic antibody and two had antigen-specific T cells. In addition, seven of the original twelve showed tumour remissions which lasted between 1.1 and 4.1 years postimmunization. More recently animal studies with the mouse homologue of 17–1A have shown that it is only possible to cause tumour regression if animals are immunized with whole antigen expressed within adenovirus and not with antigen alone or anti-idiotypic antibodies. This correlated with the induction of CTLs. The viral construct allowed intracellular production of the antigen that was efficiently processed and presented by class I MHC[30].

A mouse anti-idiotypic antibody, 3H1 that mimics CEA has also been administered to colorectal cancer patients. Thirteen of 23 patients showed an antiCEA antibody response and 5 of 23 showed a CEA-specific T-cell proliferation response. However, there was no detectable clinical benefit[31]. This was attributed to the

extensive disease of these immunized patients. In a new study treating patients with minimal residual disease, 10 of 21 patients showed T-cell responses to both the anti-idiotypic antibody and CEA[17]. A randomized adjuvant trial with this anti-idiotypic antibody is now in progress. Following resection of their tumours colorectal cancer patients are randomized to receive chemotherapy with or without anti-idiotypic vaccination.

Two human anti-idiotypic antibodies have been administered to colorectal cancer patients: a human anti-idiotypic antibody which mimics 17–1A and the human monoclonal anti-idiotypic antibody that mimics CD55. The human anti-idiotypic antibody mimicking 17–1A induced both antibody and helper T-cell responses in seven of ten patients[32]. 105AD7 anti-idiotype induces both helper and cytotoxic T-cell responses in cancer patients. In advanced colorectal cancer patients this was not associated with any survival benefit. However, in a neoadjuvant trial there was a highly significant increase in apoptosis of tumour cells in 105AD7 immunized as compared to control patients.

One of the main problems in evaluating not only anti-idiotypic immunization but all vaccine approaches is the clinical evaluation of the tumour and immune response. Tumour apoptosis as exemplified by the 105AD7 studies may be a way of measuring tumour response. However, current in vitro cell culture assays such as T-cell blastogenesis or chromium release cytotoxicity assays are at best insensitive and at worst difficult to interpret. Recent developments in methods to enumerate antigen-specific T cells by tetramer or intracellular cytokine staining could be the solution to this problem[33].

Apart from providing an accurate assessment of the number and phenotype (CD4 or CD8) of cells responding to vaccination it may allow optimization of immunization protocols. Thus, it may be possible to compare antigen and anti-idiotype vaccines or to compare a range of immune adjuvants. It may also be possible to determine the frequency and number of immunizations that are necessary for successful treatment. These measurements could also address perhaps the most important questions of all: Is there synergy between vaccines which target different antigens? Is there synergy between vaccines and chemotherapy?

Conclusion

Cancer vaccines are still in their infancy, and developments in the understanding of the molecular basis of immune responses have enabled the design of many new approaches. A similar development in our ability to measure both tumour and immune responses in patients will allow selection of the most effective vaccines. Human monoclonal anti-idiotypic antibodies offer a real possibility of fulfilling the role of the 'ideal cancer vaccine'.

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Immunotherapy and vaccination against Epstein–Barr virus-associated cancer

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Introduction

Epstein–Barr virus (EBV) is a member of the Herpes family of viruses and infects the human population worldwide. In the 35 years since its discovery, EBV has attracted ever-increasing attention from medical, molecular biological, virological and epidemiological viewpoints. It is one of the most efficient cellular growth-transforming viruses known and yet, following natural primary infection during childhood, it generally coexists within its host completely asymptomatically and, in common with other herpesviruses, establishes a persistent infection which is maintained lifelong[1]. In Western communities about 85–90% of all adults carry the virus, whereas in developing countries the infection level approaches 100% by the age of two. At any given time, about 20% of virus-positive individuals shed infectious virus in saliva which is believed to form the primary route of transmission. However, under certain circumstances its pathogenic potential is unleashed and EBV is associated with a wide spectrum of clinical conditions, many of which are malignant.

Clinical significance of EBV

In developed countries primary infection is often delayed for several years. Following first time infection during adolescence or young adulthood, clinical infectious mononucleosis (IM) develops in about half of the instances. In the United States alone, it is estimated that there are about 100 000 new cases per year. However, despite the significant morbidity which is directly attributable to the aetiological role of EBV in the causation of IM, it is more the virus' associations with various human cancers which are the prime considerations behind the need for vaccination and/or therapy. The virus has long been associated with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC), but recently has been linked to an increasing variety of tumours of various cellular origins, many of which

appear to manifest themselves in the setting of immunosuppression, be that iatrogenic e.g. in transplant recipients or acquired via infection as in AIDS.

Burkitt's lymphoma is perhaps the classic virus-associated human cancer. It occurs predominantly in children and is endemic in the malarial belts of Africa and New Guinea. Nasopharyngeal carcinoma is a malignant epithelial tumour of the postnasal space. Like BL, the geographical distribution of NPC is well defined, in this instance being most prevalent in parts of South East Asia (15–30 cases per 100 000 population) where, for example, in some areas of southern China, such as the region around Wuzhou in Guangxi Province, it is the most prevalent malignancy of males and the second most common in women. Given the large population density of China, this high incidence makes NPC a very significant cancer in global numerical terms. Although rare throughout the rest of the world, small areas of high prevalence are found in some Inuit populations and in parts of Central and North Africa.

Whilst BL and NPC are geographically restricted in their principal high incidence areas, the lymphoma known as Hodgkin's disease (HD) is much more widespread and has an incidence of 2–4 per 100 000 in Western Europe and the USA[2]. Observations[3] suggest an involvement of EBV in up to 50% of cases of this common tumour.

As the number of worldwide organ transplants (kidney, heart, liver, thymus or allogeneic bone marrow) increases, so too does a significant complication in the management of such immunocompromised patients. This problem is the development of EBV-driven immunoblastic lymphomas[4]. Lymph nodes and other tissues such as the central nervous system may become infiltrated with mono-, oligo- or polyclonal, EBV genome positive, immature B lymphocytes and the tumours are often refractory to conventional treatment.

Other, numerically less notable but nevertheless important, EBV-associated clinical problems occur in the form of nasal or other lymphomas of T- or NK-cell origin[5,6], an inherited condition known as X-linked lymphoproliferative syndrome or Duncan's disease[7] in which afflicted individuals fail to mount an effective immune response to EBV with eventually fatal consequences, and as a variety of AIDS-associated complications including lymphoma[8], smooth muscle tumours[9] and hairy oral leukoplakia[10]. More recently an apparent association of EBV with gastric carcinoma has been observed[11]. This cancer is relatively frequent and is of particular importance in Japan where it is the most common cancer (approximately 95 000 new cases per annum). Studies reveal the presence of EBV in about 7% of the total cases in Japanese patients. Clearly then, the worldwide number of cases of EBV-associated acute, chronic and malignant diseases is very large.

From a clinical point of view one advantage of virus-associated cancers is that

the virus leaves characteristic footprints which provide a defined target at which the research clinician can aim strategies for novel, specific preventive or treatment regimes. As with all cancers the need for new therapies to combat EBV-associated malignancies is pressing whilst effective prophylactic vaccination against EBV itself would have a very significant effect on worldwide morbidity and mortality. Here we examine the progress which is currently being made in these areas.

Genome organization of EBV

The DNA genome extracted from EB virus particles is a linear, double stranded molecule. The prototype strain, B95–8, has been completely sequenced and the 'standard' genome for the purpose of points of reference is deemed to be 172 281 nucleotide pairs long . However, the genome is not a unique structure. It contains a variable number of terminal tandem repeats and a short and a long unique region about 15 and 150 kilobase (Kb) in size, respectively. These are separated by a variable number (5–12) of internal tandem repeats with a nonintegral number of copies. In addition EBV contains other smaller repeated sequences. Although EB virion DNA is linear, the predominant intracellular form of the viral DNA is episomal and circular.

The organization of the linear virion genome is shown schematically in Figure 9.1A.

The overall extent of genetic variation between different isolates of EBV is uncertain. However, despite the overall similarity, two distinct EBV types (type 1 and type 2, alternatively known as types A and B), have been defined on the basis of specific sequence variation within the EBNA2 gene encoding antigenically distinct forms of EBNA2. Further studies have revealed that such type-specific differences extend to the EBNA3 a,b,c genes and to the transcription units of the EBER RNAs.

The two types of virus differ in biological properties in an in vitro transformation assay, type 1 being more efficient, although in the wild both types seem to contribute with equal efficiency to the pathogenesis of BL and NPC.

Latent infection

EBV, in its so-called latent or persistent state, maintains its genome within the cell and expresses only a limited set of genes. These latent gene products are important in cellular immortalization by EBV and have particular relevance to immunotherapy and vaccination since they are the principal targets of cell killing by EBV-specific cytotoxic T lymphocytes (CTL). The combinations of these latent genes which are expressed in different cellular or pathological situations varies and for convenience have been classified into three latency states. In EBV-associated cancers the virus remains in one or other of these states of latency although other

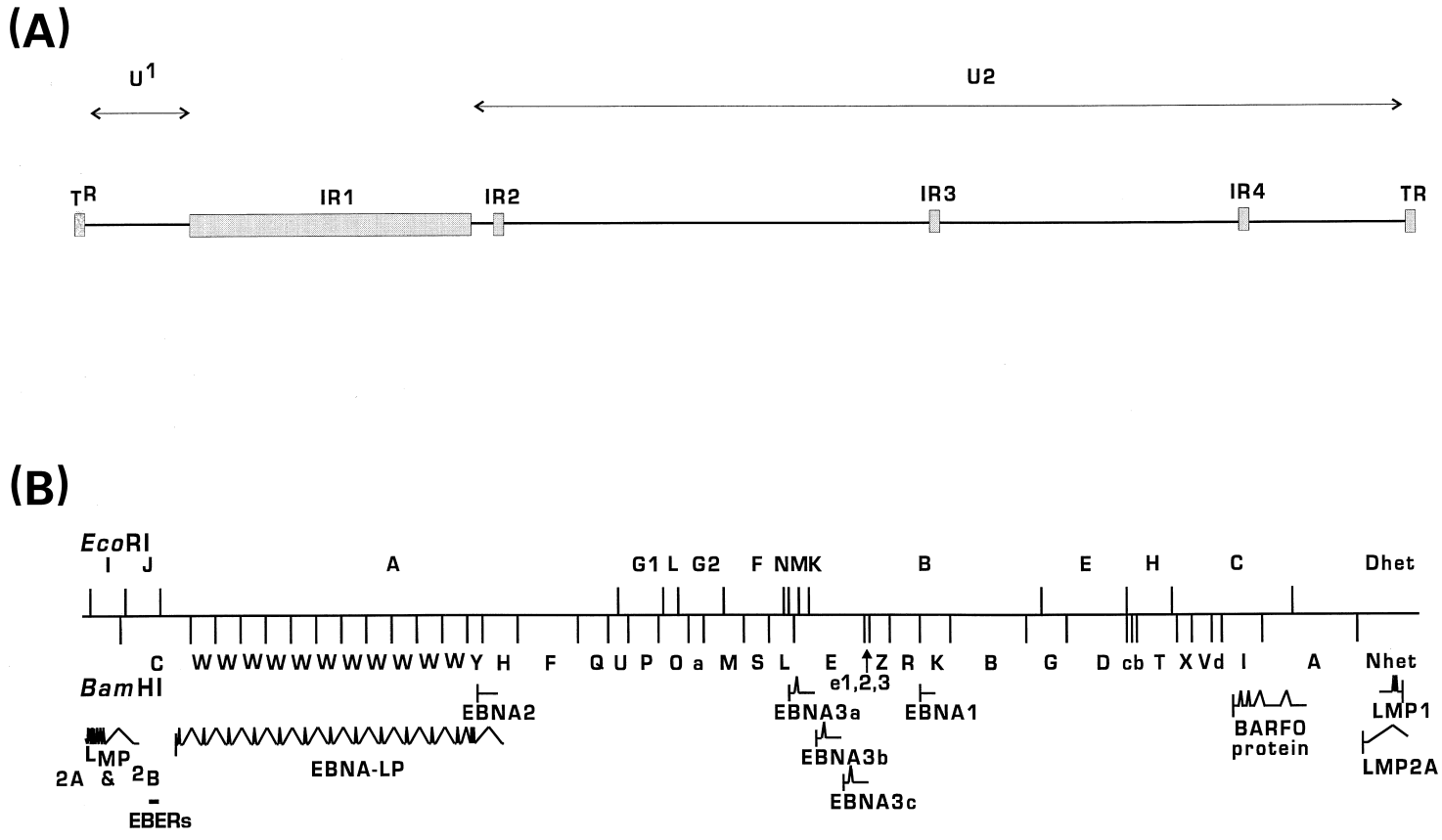

Figure 9.1 (A) General organization of linear EBV DNA. U1 and U2 refer to the unique seqence regions of the genome which are interspersed with four internal repeat regions IR1–4. IR3 is the glycine-alanine repeat within EBNA1. TR are the terminal repeats. (B) *Eco* RI and *Bam* HI restriction endonuclease maps of EBV B95–8 DNA and the positions of the latent genes. The splicing patterns of the latent mRNAs through the coding regions are indicated. The complete mRNA structures of the EBNA genes are more complex than shown here

states may be found in the natural, asymptomatic life cycle of EBV[12]. The state now termed 'latency III' is genetically the most complex and defines the full set of latent genes.

Latency III

In vitro infection of primary, resting B cells by EBV immortalizes the cells to permanently growing lymphoblastoid cell lines (LCLs) which generally carry several copies of the viral genome in an episomal form and express only a restricted number of gene products (Figure 9.1B). These consist of six nuclear antigens (EBNAs 1, 2, 3a, 3b and 3c, and the leader protein, EBNA-LP), three latent membrane proteins (LMPs 1, 2a and 2b) and two small RNAs known as the EBERs. In addition, a family of highly spliced RNAs which span the *Bam*HI-I/A region and which appear to encode a membrane-associated protein(s) from the BARF0 open reading frame, are expressed from a promoter within *Bam*HI-I. This pattern of latent gene expression (Figure 9.1B) is known as 'latency III' and is also found in immunoblastic lymphomas of immunocompromised patients such as those suffering from AIDS or recipients of kidney, heart, liver, thymus or allogeneic bone marrow transplants.

Latency I

The most restricted pattern of latent gene expression, known as latency I, is found in Burkitt's lymphoma (BL) tumour biopsies and early passage BL cell lines in vitro. Here, along with the ubiquitous, abundant EBER expression, the only viral protein products are EBNA1 and probably the BARF0 protein.

Latency II

A different form of latency, latency II, is seen in nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD). In this case, EBNA1 is accompanied by the LMPs, EBERs and the BARF0 protein.

T-cell responses to persistent infection

EBV is an extremely efficient agent for the growth transformation of B cells and yet it is carried by the vast majority of immunocompetent people in a totally asymptomatic fashion. The virus–host balance is believed to be struck in the following way. Discrete sites of virus production provide a source of virions which in turn infect B cells. These potentially growth-transformed circulating B cells exhibit a latency III phenotype and are recognized and destroyed in an HLA class I restricted context by $CD8^+$ cytotoxic T cells. Such T cells recognize degraded forms of viral

proteins which are presented at the cell surface as HLA class I:peptide complexes (see Chapter 1). Thus, in circulating EBV-positive B cells, any or all of the latent proteins are potential sources of appropriate target peptides.

It appears that all the latent proteins, with the notable exception of EBNA1, can be targets for CTL responses and that HLA class I type is a key determinant in the choice of target antigen: a particular allele tends to focus the response to a single viral protein, e.g. HLA-A11 tends to target EBNA3b. The EBNA3a,b,c family seems to be the most frequent immunodominant target whereas LMP2 provides a range of subdominant targets.

The nonimmunogenicity of EBNA1 is due to a curious property of the internal glycine–alanine repeat domain of this protein. It has been found that inclusion of this domain within a protein renders that molecule refractory to ubiquitin or proteasome-dependent protein degradation[13]. Since it is this pathway that is responsible for the generation of peptides for presentation via the HLA class I route, EBNA1 can generally evade such immune surveillance. This protection does not extend to the HLA class II processing pathway, and class II-restricted LCLs directed against an EBNA1 epitope have been obtained[14]. In addition, very rare HLA class I-restricted CTLs have been detected which indicates that the glycine–alanine repeat-mediated evasive mechanism may occasionally be bypassed. This appears to involve exogenous processing and presentation by a TAP-independent pathway[15].

For a more extensive discussion of CTL recognition see Rickinson and Moss[16].

Defined CTL epitopes from the EBV latent proteins are shown in Table 9.1.

Immunotherapy

In this chapter the oft-used term 'therapeutic vaccine' is not recognized and a distinction is made between 'immunotherapy' and 'vaccination'. In the former the treatment involves infusion of cells of the immune system and may be prophylactic or therapeutic, whereas 'vaccination' is defined as being prophylactic and involves the administration of antigen(s).

Immunotherapeutic strategies, at different stages of development, are currently aimed at three EBV-associated malignancies: immunoblastic lymphoma in immunosuppressed patients, NPC and Hodgkin's disease. In all cases the aim is to deliver EBV-specific CTL to target and destroy the tumour cells.

In the context of employing EBV-specific CTL responses in the development of new therapeutic or prophylactic strategies, four key issues may be identified:

(1) CD8-positive cytotoxic T cells are important in recognition and killing of EBV-positive cells.
(2) The virus latent proteins are the targets for such T-cell attack.

Table 9.1. *CTL epitopes from EBV latent antigens*

Latent antigen	Epitope sequence	HLA restriction Class I	HLA restriction Class II	EBV type specificity
EBNA1	HPVGEADYFEY	B35.01		n.d.
	VLKDAIKDL	A2.03		n.d.
	TSLYNLRRGTALA		DR1	1 & 2
EBNA2	DTPLIPLTIF	A2/B51		1
	TVFYNIPPMPL	B18*	DQ2*	1
EBNA3A	QAKWRLQTL	B8		1
	AYSSWMYSY	A30.02		1 & 2
	RYSIFFDY	A24		1
	FLRGRAYGL	B8		1
	RPPIFIRRL	B7		1
	LEKARGSTY	B62		1
	HLAAQGMAY	?		1
	YPLHEQHGM	B35.01		1
	VFSDGRVAC	A29		1
	VPAPAGPIV	B7		1
	SVRDRLARL	A2		1 & 2
	RLRAEAQVK	A3		1 & 2
EBNA3B	NPTQAPVIQLVHAVY	A11		1
	HRCQAIRKK	B27.05		n.d.
	TYSAGIVQI	A24.02		1
	RRARSLSAERY	B27.02		1
	AVFDRKSDAK	A11		1
	IVTDFSVIK	A11		1
	LPGPQVTAVLLHEES	A11		1
	AVLLHEESM	B35.01		1
	DEPASTEPVHDQLL	A11		1
	VEITPYKPTW	B44		1
	GQGGSPTAM	B62		1
EBNA3C	EGGVGWRHW	B44.03		1 & 2
	QNGALAINTF	B62		2
	LRGKWQRRYR	B27.05		1
	RRIYDLIEL	B27.02/.04/.05		1
	HHIWQNLL	B39		1 & 2
	EENLLDFVRF	B44.02		1 & 2
	LLDFVRFMGV	A2.01		1 & 2
	KEHVIQNAF	B44.02		1 & 2
	FRKAQIQGL	B27.05		1
	QPRAPIRPI	B7		1 & 2

Table 9.1. (*cont.*)

Latent antigen	Epitope sequence	HLA restriction Class I	Class II	EBV type specificity
EBNA-LP	Occasional responses identified, no epitopes defined			
LMP1	YLLEMLWRL	A2		n.d.
	YLQQNWWTL	A2		n.d.
LMP2	PYLFWLAAI	A23		1 & 2
	IEDPPFNSL	B40		1 & 2
	RRRWRRLTV	B27.04		1 & 2
	LLWTLVVLL	A2.01		1 & 2
	SSCSSCPLSKI	A11		1 & 2
	TYGPVFMCL	A24		1 & 2
	CLGGLLTMV	A2.01		1 & 2
	VMSNTLLSAW	A25		1 & 2
	LTAGFLIFL	A2.06		1 & 2
BARF0 protein	LLWAARPRL	A2.01		n.d.

Notes:

Data from Blake et al.[15], Rickinson and Moss[16], Khanna et al.[24], Moss, Suhrbier and Elliott[44], Kienzle et al.[48].

n.d. = not determined.

* This sequence contains overlapping class I and class II epitopes.

(3) The pattern of latent protein expression is not identical in all EBV-associated diseases.
(4) Many of the CTL target peptide epitopes have been defined.

Immunoblastic lymphoma

In the normal asymptomatic host, there exists a balance between potentially proliferative EBV-positive B cells which express the full latency III repertoire of gene products and CTL which eliminate those cells via latent protein targets. The post-transplant lymphoproliferative disease (PTLD) which may arise in immunocompromised patients is classified as immunoblastic lymphoma (IBL) which also exhibits a latency III phenotype. In some cases the lymphoma may regress following reduction of immunosuppression but this may lead to rejection of the graft. However, this observation suggests that if the EBV-specific CTL response could be restored in these patients the outgrowth of EBV-transformed cells may be reversed.

In bone marrow transplant patients the complicating IBL is usually of donor origin and may be either polyclonal or monoclonal. Two early studies were successful in demonstrating the principle and potential of treatment by infusing

EBV-specific CTLs into the patient. Papadopoulos et al.[17] used unseparated leukocytes from EBV-seropositive donors on the hypothesis that such cell preparations would contain sufficient numbers of cytotoxic T-cell precursors presensitized to EBV. Five patients were treated with infusions of donor leukocytes at doses calculated to contain around 10^6 CD3-positive T cells per kilogram of body weight. All five patients exhibited complete pathological or clinical responses but unfortunately pulmonary complications led to the deaths of two patients and graft-versus-host disease was observed in the three survivors.

It was surmised that alloreactive T cells present in infusions of unmanipulated cells could be the cause of graft-versus-host disease. In the second early trial Rooney et al.[18] investigated ten patients and, rather than using unseparated leukocytes, EBV-specific CTL were prepared from donors and infused into the patients. Prior to treatment three patients showed evidence of EBV reactivation as estimated by PCR analysis of EBV genome load in peripheral blood and one of these three had IBL. Following infusion of EBV-specific CTL the EBV genome load rapidly fell back to normal levels. In addition the patient with lymphoma showed complete remission of disease. The remaining seven patients received CTL prophylactically and remained disease-free. This protocol did not result in complicating graft-versus-host disease. Genetic marking of the transferred cells has shown that the infusion established populations of CTL precursors which could respond to EBV challenge up to 18 months after administration[19].

In a recent extension of their study, Rooney et al.[20] reported on 39 bone marrow transplant patients who received prophylactic infusions of donor-derived CTL. Six of these patients had high EBV genome load at entry and, as before, this was rapidly diminished following CTL treatment. None of the 39 patients developed lymphoma, as compared with an 11.5% incidence in the first year following transplantation in an untreated control group. In two additional patients who did not receive prophylactic therapy, T-cell infusion was shown to result in complete response of overt IBL.

Thus, although at present a relatively specialized treatment regime, the adoptive transfer of CTLs has been demonstrated to be an effective procedure in at least a proportion of patients, for the prophylaxis and treatment of immunoblastic lymphoma in bone marrow transplant recipients. It is argued that although the time required to generate CTL lines is a drawback of this approach, the cost of prophylactic treatment is significantly less than the cost of conventional treatment of established IBL. The approach is probably justified in patients deemed to be at high risk of IBL.

In contrast to bone marrow transplant patients in whom the IBL is of donor B-cell origin, PTLD in solid organ transplant patients is of recipient B-cell origin. Immunotherapeutic interventions in these patients have lagged behind those in

bone marrow recipients even though the incidence of lymphoma is higher. In many cases the donors are unavailable and the tumour has to be targeted by in vitro reactivated recipient CTLs. Recently it has proved possible to activate an EBV-specific CTL response in solid organ transplant recipients and to demonstrate reduction in EBV genome load following infusion[21].

Similar CTLs have been generated from heart and heart–lung transplant recipients who were seronegative at the time of engraftment and received an EBV-seropositive graft. In an initial trial in Australia, these CTLs have been infused into a single solid organ transplant patient and very good PTLD regression was observed. Unfortunately the patient eventually died, not of PTLD but from a haemorrhage apparently unrelated to the CTL infusion (D.J. Moss, personal communication).

Nasopharyngeal carcinoma

As mentioned earlier the EBNA3 family of latent proteins provide the immunodominant target epitopes in most individuals and therefore polyclonal CTL lines derived from donors are directed mostly at these. Few, if any, clones will be directed at subdominant epitopes such as those provided by the other EBNAs or the LMPs. Since the tumour cells in NPC exhibit a latency II pattern of expression, the LMPs, BARF0 protein (and EBNA1) are the only EBV target antigens. Evidence for the existence of the BARF0 protein has only recently emerged. Consequently little work has been done to explore the potential of this as a target protein.

Any immunotherapeutic strategy is dependent upon the efficient expression of HLA class I on the surface of the tumour cell; a criterion that is not always obtained in carcinomas (see Chapter 1). Examination of NPC biopsies reveals that the expression of HLA class I and II[22] and the TAP1 and TAP2 transporters[23] is not compromised in the tumour cells. In addition, NPC cells passaged in nude mice were shown to be capable of processing and presenting antigens to virus-specific LCLs[23]. These data suggest that NPC cells (latency II) should be able effectively to process LMP epitopes and that these could form the basis of prophylactic or therapeutic strategies.

CTL responses to LMP1 are generally weak and target epitopes have only very recently been defined[24], whereas nine target epitopes to LMP2 have been identified (Table 9.1). Three of these epitopes are associated with HLA types that are present at high frequency in the southern Chinese population which is most at risk for NPC. The epitope SSCSSCPLSK is A1101-restricted and present in around 50% of the susceptible population; TYGPVFMCL is A2402-restricted and found in about 30%, whilst IEDPPFNSL is B40011-restricted, and present in approximately 32% of the population[25].

On the basis of the above considerations, a clinical trial has been initiated in

Hong Kong with the aim of eliciting an effective LMP2-directed CTL response in NPC patients (A.B. Rickinson, personal communication). Patients with metastatic or locally recurrent NPC and who have the appropriate A11, A24 or B40 HLA subtype, are infused with autologous dendritic cells which have been pulsed with the appropriate LMP2 epitope peptide. Dendritic cells are the professional antigen-presenting cells of the immune system and are able to generate primary T-cell responses[26]. The dendritic cells are additionally pulsed with tetanus toxoid in order to induce a CD4-positive 'helper' T-cell response which is necessary for the induction of an effective, long-lasting CD8-positive cytotoxic T-cell response[27]. The study seeks to assess any treatment-related toxicity, generation or boosting of LMP2-specific CTL responses and any clinical effects. No adverse reactions to the treatment have so far been observed in the first few patients. The eventual outcome is awaited with great interest.

Hodgkin's disease

Like the malignant epithelial cells of NPC the Reed–Sternberg cells of EBV-positive Hodgkin's disease (HD) also exhibit an EBV latency II phenotype. In many cases they also express TAP1/2 and HLA class I and have the ability to process and present EBV antigens to EBV-specific CTL clones[28].

In initial studies to assess the feasibility of generating EBV-specific CTL from HD patients, lines were successfully generated from 9 of 13 patients (5 active disease, 4 remission). Three patients were infused with CTL lines and it was demonstrated that this led to a reduction in EBV genetic load in PBMC and persistence of the infused cells similar to that observed in bone marrow transplant patients. Encouragingly, in vitro studies on the CTL from one patient demonstrated LMP2 specificity. However, it was not clear whether any positive clinical effect could be directly attributable to the CTL infusion[29].

Unfortunately the expansion rate and proliferative potential of the CTL lines were much lower than those obtained from normal donors. In order to address this problem along with the difficulty of requiring a response primarily to subdominant epitopes, it is proposed, as in the NPC trial, to use dendritic cells to present LMP2. Antigen-loaded dendritic cells can be generated in 7–10 days rather than the four weeks required for normal LCLs and longer in the case of HD patients[20].

Vaccine studies

The potential importance of a vaccination programme to prevent infection by Epstein–Barr virus (EBV) was first proposed by Epstein in the 1970s[30,31]. The emerging evidence from the Taiwanese major vaccination campaign against hepatitis B virus, which indicates a resultant marked reduction in hepatocellular

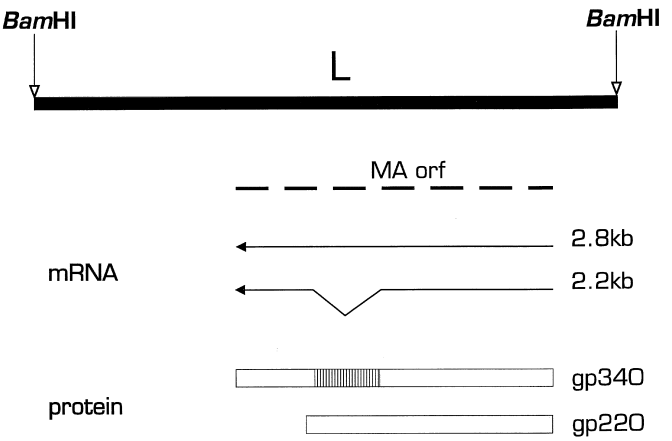

Figure 9.2 Relationship of gp340 and gp220. The *Bam*HI-L fragment of EBV is depicted by the line at the top (see Figure 9.1B for the complete *Bam*HI restriction map of EBV DNA). Beneath this is shown the position of the major membrane antigen open reading frame (MA orf) within *Bam*HI-L which is transcribed as 2 mRNAs (arrows), one of which is spliced. The boxes at the bottom indicate the protein products gp340 (encoded by the unspliced message) and the spliced transcript-encoded gp220 which is devoid of the repeated region (shown by the shading)

carcinoma[32] points to the clear cancer-preventive effect of such a strategy. In the case of EBV two basic approaches have been explored: first, a vaccine based on the major membrane antigen glycoprotein which elicits a strong virus neutralizing antibody response; and, secondly, vaccination with peptide epitopes designed to induce a CTL response. Clinical trials are now underway using both strategies.

EBV membrane antigen

The first approach to the development of an EBV vaccine concentrated on the virus membrane antigen (MA) which can be detected on the envelopes of intact virions and on the plasma membrane of virus producer cell lines. MA consists of at least three glycoproteins of molecular weights 340–350 kD (gp340), 220–270 kD (gp220) and about 85 kD (gp85). The first two glycoproteins are the major components of MA and were shown to share antigenic determinants, a property which was subsequently demonstrated to be due to their structural relationship. The two proteins are derived from a single gene by splicing, without a change in reading frame (Figure 9.2). Antibodies raised against the plasma membranes of EBV-producer cell lines or EBV envelopes have virus-neutralizing activity. In addition, monoclonal antibodies or monospecific antisera which recognize EBV MA will also neutralize the virus. These data suggested that gp340/220 would form a good basis for the development of an antiEBV vaccine.

A potential subunit vaccine

With this in mind, Epstein's group purified gp340/220 from B95–8 cells and obtained sufficient material for an assessment of the feasibility of the basic approach[33]. Earlier studies on the oncogenic potential of EBV had shown that the virus could cause tumours in two species of New World primates: owl monkeys and cottontop tamarins. For various reasons the latter was chosen as the test species in which to assess the efficacy of the putative vaccine preparation. It was soon shown that vaccination of the animals with purified gp340/220 would indeed prevent the establishment of massive, fatal lymphomas following challenge with a tumourigenic dose of EBV[33].

The foregoing work established the principle that, at least in the tamarin lymphoma-induction model, a subunit vaccine based on gp340 had the appropriate potential. However, the yields of protein that could be obtained from available cell lines were totally inadequate for any large-scale vaccine intervention initiative such as may be required in South-East Asia as a putative antiNPC measure. In addition, even to achieve these levels, the potentially oncogenic phorbol ester 12–*O*-tetradecanoyl-phorbol-13-acetate (TPA) had to be added to the cell cultures as an inducing agent. Omission of this inducer would reduce the already low levels by an order of magnitude.

Recombinant gp340

In order to overcome this problem, a number of different laboratories used genetic manipulation techniques in an effort to produce a biologically relevant, recombinant gp340/220 in viable amounts. Attempts using *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris* or recombinant baculovirus were unsatisfactory for a variety of reasons.

Expression systems in cultured mammalian cells have greater potential to yield authentic products due to their capacity to perform appropriate posttranslational modifications and several attempts at gp340/220 expression have been reported. In early work two such cell lines were extensively studied as potential sources of material on which to base a subunit vaccine. The first used an expression plasmid driven by the SV40 early promoter and *dhfr* selection in a Chinese hamster ovary (CHO) cell line[34] whilst in the second system gp340/220 expression was driven using the mouse metallothionine gene promoter carried in a bovine papillomavirus vector in mouse C127 cells. An easily usable level of expression of the product was obtained which was stable for at least 20 passages of the cells in culture, the product could be effectively purified by a simple procedure, appeared to be of similar size (i.e. was probably similarly glycosylated) to the authentic proteins, raised virus-neutralizing antibodies in experimental animals, was recognized by human EBV-specific T cells, and was protective in the cottontop tamarin challenge system[35–37]

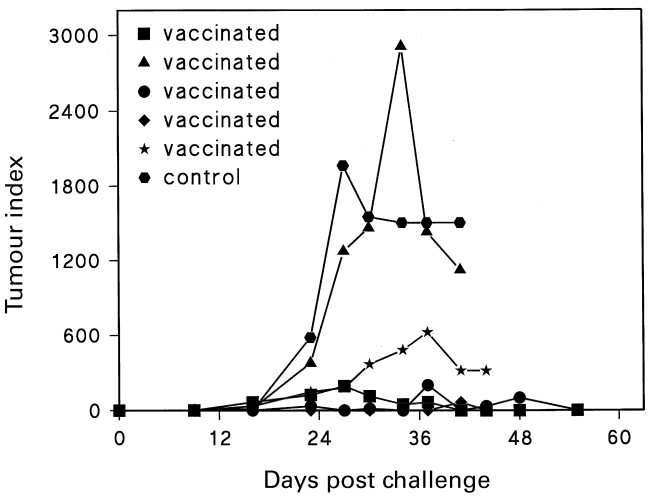

Figure 9.3 EBV challenge experiment in tamarins. Animals were vaccinated with recombinant gp340/220 in alhydrogel adjuvant (vaccinated) or with adjuvant alone (control). They were subsequently challenged with a tumorigenic dose of EBV. In this experiment four of the five vaccinated animals were protected. Tumour index is the sum of the volumes of palpable tumours in cubic millimetres. With permission, from J.R. Arrand et al. (1998). Vaccination and virus-mediated therapy. In: *Viruses and Human Cancer*, eds. J.R. Arrand and D.R. Harper. Oxford: Bios Scientific Publishers, pp.145–178

(Figure 9.3). In addition, experiments in the common marmoset model system demonstrated a reduction in virus load in vaccinated versus nonvaccinated animals following subsequent challenge with EBV[38].

The ratio of gp340:gp220 varies considerably in different cell lines. It is not clear whether such variation occurs within a single line, perhaps in response to slight variations in growth conditions. If this were to happen it could pose problems with regard to the consistency of formulation of different batches of vaccine preparations. With this in mind new gp340-expressing cell lines have been engineered in which the splicing signals have been removed by specific mutagenesis to ensure the production of homogeneous gp340 in the absence of gp220. Recently a phase I randomized, double-blind clinical trial using such material has been initiated[39].

Alternative recombinant delivery systems

The EBV gp340/220 coding sequences have been incorporated in several different recombinant delivery systems with various degrees of subsequent characterization. Vaccinia virus recombinants have been shown to produce gp340 (vaccinia does not have an RNA splicing mechanism and therefore cannot produce gp220) which was highly glycosylated, could be detected on the cell surface of infected cells and had a

molecular weight of about 340 kD, all of which are properties of the authentic gp340[40]. Sera from rabbits immunized with the recombinants, neutralized EBV in a cord blood immortalization assay and vaccination with the recombinant protected cottontop tamarins in the challenge assay[41]. Interestingly, the protected animals did not possess any detectable serum antibodies against gp340, suggesting that the principal protective mechanism may be via cell-mediated rather than humoral responses.

More recently, Gu et al.[42] reported the construction of a similar recombinant virus using the Chinese vaccine strain of vaccinia, Tian Tan. The recombinant was shown to express a product which was recognized in tissue culture by sera from NPC patients and when injected into rabbits raised antisera which reacted with the surface of B95–8 cells. The recombinant has subsequently been used in a small-scale trial in groups of adults and children. An appropriate antiMA antibody response was generated in children who were serologically MA-negative prior to vaccination. Sixteen months after vaccination only three out of nine vaccinated infants had seroconverted to EBV positivity whereas all ten controls were seropositive[42].

Because of the potential complications in, for example, individuals harbouring a silent HIV infection or those who are immunocompromised for other reasons it seems unlikely that a live virus-vectored vaccine will be licenced for widespread use. Nevertheless the result obtained by Gu et al. is most encouraging for the trials of a gp340 subunit vaccine since it suggests that determinants within this molecule may be able to elicit sterile immunity against EBV infection. Even if sterile immunity is not achieved, these data and those from the cottontop tamarin and common marmoset systems indicate that a gp340-based vaccine should certainly be effective in lowering virus load. This suggests positive potential for the prevention of IM and probably also for IBL in seronegative transplant recipients. Whether it will be effective in the prevention of later-onset disease such as BL, HD and particularly NPC, remains to be seen.

Vaccines based on CTL induction

The mechanism of antigen processing and HLA-restricted peptide recognition (see Chapter 1) leads to the concept of vaccination with CTL target epitope peptides which should in turn stimulate the generation of the appropriate population of CTL and confer protection via elimination of cells expressing the appropriate viral protein. This concept has been validated in, for example, Sendai virus infection of mice, where it was known that a single Sendai virus nucleoprotein peptide epitope was recognized by CTL. Following vaccination with a peptide containing this epitope the mice were protected from a fivefold lethal dose of Sendai virus[43].

The immunotherapy trials described earlier are a vivid example of the impor-

tance of the CTL response in controlling EBV-associated disease. The elucidation of the major immunodominant target epitopes for CTL control (Table 9.1) enables the principle of peptide vaccination to be explored. The precedent of CTL infusion suggests that this approach would have obvious utility in the setting of organ transplantation to seronegative recipients. In contrast to the preclinical development work using gp340-based vaccines no animal model using EBV challenge has been evaluated. Nevertheless, a phase I clinical trial is currently under way in Australia in which healthy, EBV-negative volunteers of HLA type B8 are being vaccinated with the peptide FLRGRAYGL formulated with tetanus toxoid in the water in oil adjuvant Montanide ISA 720. This peptide is the major B8-restricted determinant of EBNA3a (Table 9.1) and appears to be conserved in type 1 EBV found in Western populations. In addition HLA B8 is common and effectively nonpolymorphic and most EBV seropositive, HLA B8 individuals are reactive against this epitope. The vaccine has so far been well tolerated and no significant adverse reactions have been encountered[44].

It is obvious from the earlier description of EBV-specific CTL target epitopes that many different peptides will be required in order to generate appropriate responses in the population as a whole, given the need to encompass the variety of susceptible targets dictated by HLA type, EBV type and virus variation. Although the major CTL epitopes have been described (Table 9.1), the definition of the complete array of potential target epitopes still requires more effort.

Delivery of the complete array of epitopes could be achieved merely by mixing peptides but this would involve a complex formulation. However, a potentially simpler approach is suggested by experiments demonstrating that a synthetic polyepitope consisting of a continuous string of nine minimal epitopes from the EBNA proteins, delivered and expressed by recombinant vaccinia virus, is effective in eliciting appropriate presentation of each individual epitope by its restricting allele[45]. The polyepitope-expressing vaccinia virus was used to infect a panel of target cells which expressed the HLA alleles that were known to restrict each epitope. Autologous CTL clones specific for each epitope were used as effector cells in chromium release assays. As positive controls the target cells were infected with recombinant vaccinia viruses expressing the appropriate EBNA protein, whilst cells infected with a thymidine kinase-negative vaccinia served as negative controls. In all cases the CTL clones recognized and killed the appropriate target cell infected with the polyepitope vaccinia (Figure 9.4).

It seems likely that both $CD4^+$ and $CD8^+$ T cells are required in order to generate the most effective immune response. The vaccinia–polytope approach has proved effective in carrying class II epitopes and eliciting the appropriate $CD4^+$ response[46].

As mentioned above, it seems unlikely that live virus vaccines will be licenced for

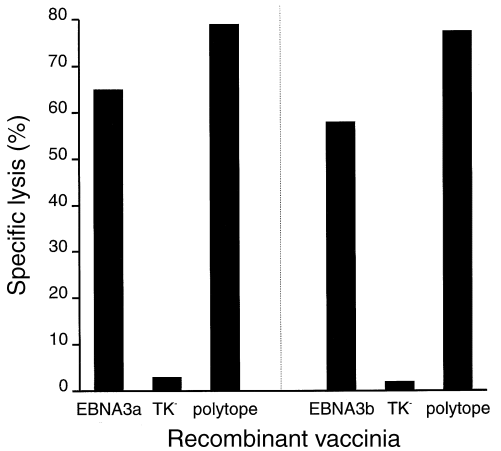

Figure 9.4 CTL recognition of individual epitopes within a polyepitope protein. Chromium release assay to demonstrate CTL recognition of EBNA3a and EBNA3b epitopes expressed in a vaccinia virus polyepitope recombinant (polytope). Recombinants expressing the intact EBNA3a or EBNA3b serve as positive controls. A thymidine kinase-negative vaccinia virus (TK^-) is negative control. (Data from Thomson et al.[45])

widespread use. An attractive alternative system is vaccination with pure DNA. In a mouse model system it has recently been demonstrated that vaccination with plasmid DNA encoding a polyepitope protein elicited MHC-restricted CTL responses to each of the epitopes and that these CTL were protective in various challenge systems. In addition, the CTL response lasted for at least a year following vaccination, could be enhanced by covaccination with GM–CSF and appeared to be generated in the absence of CD4 T-cell-mediated help[47].

Conclusions

These are exciting times for the treatment of EBV-associated diseases. In the field of immunotherapy there have been some most significant results in the treatment and prophylaxis of posttransplant lymphoma in bone marrow transplant patients. The outcome of this experience gives great impetus to efforts to develop similar treatment regimes for other EBV- (and other viral-) associated cancers. Similar treatments for solid organ transplant patients appear promising but are currently lagging behind. It is anticipated that the number of trials in this area will imminently increase and that there will be similarly encouraging outcomes. In the case of other EBV-associated malignancies, experience is very limited. Nevertheless, trials for Hodgkin's disease may be anticipated to be initiated in the foreseeable future whilst the outcome of the current NPC trial is eagerly awaited. Success in the

treatment of NPC would be an enormous breakthrough given the large numbers of fatalities attributable to this tumour.

On the vaccine front, phase 1 clinical trials are under way using both gp340-based and CTL epitope-based strategies. It is generally anticipated that these vaccines will most likely be effective prophylactic measures against both infectious mononucleosis and PTLD in seronegative recipients. It is conceivable that some form of dual approach, i.e. simultaneous administration of gp340 and CTL epitopes against latent antigens, may be even more effective. One may envisage gp340 fused to a 'polytope tail'. Whether such vaccines will ever be able to prevent the development of late-onset malignancies such as NPC is questionable and can only be answered definitively by long-term trials. However, the Taiwan experience of reduction in the frequency of early-onset, childhood liver cancer following anti-hepatitis B virus vaccination provides grounds for optimism.

Currently, immunotherapy is having the greater clinical impact. We must be hopeful that preventive vaccination will ultimately prove effective. Prophylaxis is better (and cheaper) than therapy.

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10

Serologically identified tumour antigens as cancer vaccines

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Introduction

Vaccination strategies for the treatment of human cancer depend on the existence of tumour antigens which are able to elicit specific immune responses in the tumour-bearing host. The specific recognition of antigens by the immune system is accomplished by two targeting systems: CD4+ and CD8+ T lymphocytes recognize processed antigens presented on MHC class II and class I molecules, respectively, while B lymphocytes produce antibody molecules that bind specifically to unprocessed antigens. The analysis of humoral and cellular immune responses in cancer patients had indicated for a long time that cancer-specific antigens do indeed exist and are recognized by the immune system of the tumour-bearing host[1]. However, the molecular nature of these antigens remained unclear until cloning techniques were developed that used established cytotoxic T lymphocyte (CTL) clones[2] or circulating antibodies[3] as probes for screening of tumour-derived expression libraries. The CTL approach and the antigens identified by it are reviewed elsewhere in this book (Chapter 11). This chapter is intended to give an introduction to the serological approach, to summarize the current status of antigens identified and to provide a perspective for the use of these antigens for cancer immunotherapy.

Rationale for using the antibody repertoire of cancer patients for the identification of tumour antigens

A variety of in vitro studies and animal tumour models demonstrated that CTLs are the protagonists of an effective cytotoxic antitumoural immune response and motivated the search for antigens recognized by CD8+ T lymphocytes. The necessity of established precharacterized CTL clones with tumour-cell restricted reactivity is the major obstacle of the CTL-based cloning approach and was the main reason why the majority of antigens defined hitherto were identified in malignant melanoma[4]. Even though tumour immunology has been CTL-centric in the last decade, it is common knowledge that antitumour immune recognition is a

concerted action. A large body of evidence points to a coordinated recruitment of CD4+, CD8+ and B-cell responses to the same tumour antigen, and suggests that once immune recognition of an antigen is elicited, it is not restricted to merely one effector system. Furthermore, it is frequently argued that the CTL repertoire of cancer patients is deleted for many relevant CTL precursors. However, it is quite unlikely that a concomitant antibody response towards antigens (in particular intracellular ones) for which respective CTLs have been deleted, would also be erased[5]. Thus, specific antibodies may be the persisting hallmark of a substantial tumour–immune system confrontation and may help to trace back to deleted CTL specificities. Based on these rationales, we designed a novel strategy using the antibody repertoire of cancer patients for the molecular definition of antigens. Once identified serologically, these tumour antigens were analysed subsequently for T-lymphocyte-recognized epitopes presented by MHC class I or II molecules.

The SEREX approach

For the systematic and unbiased cloning of tumour antigens recognized by the antibody repertoire of cancer patients, we developed a serological cloning approach, termed the SEREX (serological analysis of tumour antigens by recombinant cDNA expression cloning) approach[6,7]. For SEREX, cDNA expression libraries are constructed from fresh tumour specimens, packaged into lambda-phage vectors and expressed recombinantly in *Escherichia coli*. Recombinant proteins expressed during the lytic infection of bacteria are transferred onto nitrocellulose membranes. These are incubated with diluted and extensively preabsorbed autologous patient serum. Clones reactive with high-titred IgG antibodies are visualized using an enzyme-conjugated secondary antibody specific for human IgG. Positive clones are subcloned to monoclonality, thus allowing the direct molecular characterization. The SEREX approach is technically characterized by several features:

(1) There is no need for established tumour cell lines and precharacterized CTL clones.
(2) The use of fresh tumour specimens restricts the analysis to genes that are expressed by the tumour cells in vivo and circumvents in vitro artefacts associated with short- and long-term tumour cell culture.
(3) The use of the polyclonal (polyspecific) patient's serum as a probe for immunoscreening allow for the identification of multiple antigens with one screening course.
(4) The screening is restricted to clones against which the patient's immune system has raised high-titred IgG and/or IgA antibody responses indicating the presence of a concomitant T-helper lymphocyte response in vivo.
(5) As both the expressed antigenic protein and the coding cDNA are present in the same plaque of the phage immunoscreening assay, identified antigens can

be sequenced immediately. Sequence information and excised cDNA inserts can be directly used to determine the expression spectrum of identified transcripts by Northern blot and reverse transcription polymerase chain reaction (RT-PCR).

(6) The release of periplasmic proteins involved in protein folding during phage-induced bacterial lysis allows at least partial folding of recombinant proteins and provides the basis for the identification of linear as well as nonlinear epitopes. This has been confirmed by the expression of transcripts which code for enzymatically active proteins (our unpublished results). In contrast, epitopes derived from eucaryotic posttranslational modification (e.g. glycosylation) are not detected by the phage immunoscreening assay.

Antigens identified by SEREX

As stated above, the SEREX approach allows for the simultaneous identification of multiple antigens using the antibody repertoire of a single cancer patient. The analysis of a variety of neoplasms demonstrated that all hitherto investigated neoplasms are immunogenic in the tumour-bearing host and that immunogenicity is conferred by multiple antigens. As the antitumour antibody repertoires from individual cancer patients vary considerably, a large number of antigens could be identified by SEREX. The proliferation of the technology to, and the cooperation with, many other laboratories in the coordinated analysis of different types of human cancers will provide the systematic typing of the expressed immunogenic human cancer genome. For the systematic documentation and archivation of sequence data and immunological characteristics of identified antigens, an electronic SEREX database was initiated by Dr Lloyd Old and implemented by Victor Jongeneel and colleagues of the Ludwig Cancer Research Institute. It provides a variety of means for sequence analysis and homology searches.

Classification of tumour antigens

By December 1998 more than 900 entries have been made in the SEREX database, the majority of them representing independent antigens. These include known tumour antigens such as the melanoma antigens MAGE-1, MAGE-4a and tyrosinase, which demonstrates that at least some of the serologically identified antigens are also targets for CTL. A second group of antigens is comprised of transcripts that are either identical or highly homologous to known genes which have not been known to elicit immune responses in humans, e.g. kinectin, a microtubule-associated transporter of golgi vesicles. The third group of serologically defined antigens consists of previously unknown genes, such as HOM-HD-21, a new galectin from a tissue affected by Hodgkin's disease[8]. The abundance of antigens and the fact that

Table 10.1. *Categories of tumour-associated antigens identified by SEREX*

Class	Antigen	Homology/identity	Source
Cancer testis antigens	HOM-MEL-40	SSX-2	Melanoma
Differentiation antigens	HOM-MEL-55	Tyrosinase	Melanoma
Overexpressed gene products	HOM-HD-21	Galectin-9	Hodgkin
Mutated gene products	NY-COL-2	p53	Colon cancer
Splice variants	HOM-HD-397	Restin	Hodgkin
Gene amplification products	HOM-NSCLC-11	eIF-4g	Lung cancer
Cancer-related autoantigens	HOM-MEL-2.4	CEBPgamma	Melanoma

a large portion of them is encoded by previously unknown genes, calls for a precise procedure to assess the role of the identified transcripts and observed immune reactions in the course of the malignant disease. This is performed by a three-step analysis which comprises a sequence analysis (search for tumour-associated sequence alterations) with subsequent homology search, expression studies in neoplastic and normal tissues and the determination of the immunogenic spectrum as assessed by the frequency of antibodies in sera from cancer patients and healthy controls. Based on the results of this basic analysis the SEREX antigens can be assigned to different groups (Table 10.1).

Expression studies are performed by Northern blot analysis using labelled cDNA as probes and by RT-PCR with transcript-specific primers. These investigations identify transcripts with a tumour-associated expression. Tumour antigens with high or frequent expression in tumour cells or tissues are restricted (i.e. no or very low expression) in normal tissues and are of special interest for cancer immunotherapy. Antigens with such a type of expression fall into several expression categories.

Cancer testis antigens (CTA) are selectively expressed in a variety of neoplasms (in a lineage-independent manner), but not in normal tissues except for testis. Examples are members of the MAGE gene family, which had already been defined by CTL approaches, and several new antigens such as HOM-MEL-40 and NY-ESO-1 which will be discussed in more detail below.

Differentiation antigens demonstrate a lineage-specific expression in tumours, but also in normal cells of the same origin; examples are tyrosinase and GFAP (glial fibrillary acidic protein) which are antigenic in malignant melanoma and glioma, but are also expressed in melanocytes or brain cells, respectively.

Overexpressed genes code for many tumour antigens identified by SEREX. The members of this class are expressed in low levels in normal tissues (usually detectable by RT-PCR), but are up to 100-fold overexpressed in tumours. An example is

HOM-RCC-3.1.3, a new carbonic anhydrase which is overexpressed in a fraction of renal cell cancers[9]. The overexpression of a transcript may result from gene amplification as demonstrated for the translation initiation factor eIF-4g in a squamous cell lung cancer[10].

Antigens encoded by mutated genes have been demonstrated only rarely by the serological approach, with mutated p53 being one example[11].

Tumour-specific splice variants of otherwise ubiquitously expressed genes can also result in tumour-associated immunogenicity. Similar to CTA, these splice variants can display an expression pattern that is restricted to tumours and testis (unpublished data).

Virus-encoded antigens that elicit an autologous antibody response have also been detected by SEREX, e.g. the env protein of the human endogenous retrovirus HERV-K10[12] which was found in a renal cell cancer.

Cancer-related autoantigens elicit antibody responses in patients with different types of cancer, but not in individuals without malignant diseases. Examples for this class are p53[13] and HOM-TS-64/kinectin (unpublished data).

Cancer-independent autoantigens elicit autoimmunity that is not related to neoplastic disease. An example is HOM-MEL-23 which is identical to the proliferating cell nuclear antigen (PCNA), a known nuclear autoantigen.

The designation of an antigen to a particular category validate a newly identified molecule for different fields of cancer research. The identification of a multitude of new genes (including mutated products) with tumour-associated expression provides new information and new targets for a better understanding of cancer biology. The transcripts derived from these genes and the proteins encoded by them as well as the associated immune responses may be clinically useful as novel diagnostic or prognostic cancer markers. Ongoing studies with large groups of patients will show which subpopulations of tumour patients develop antibodies to particular antigens and whether these immune reactions may be useful as early markers for the serodiagnosis of cancer.

Optimization of cancer vaccines

From the therapeutic point of view, the main application for cancer antigens is cancer vaccination. For this purpose it is important to choose the right targets. For clinical as well as for technical and economical considerations, it is apparent that only a few antigens can be chosen as targets for controlled clinical cancer immunotherapy studies. It will therefore be important to define rationales for the selection of suitable antigens. In this regard a number of requirements for the rational selection of vaccine candidates can be listed. The main idea of cancer vaccination is to induce an effective specific cytolytic immune activity against tumour cells. To avoid

major side-effects by destruction of nonneoplastic cells the molecular targets of the induced cytolytic activity should not be expressed or at least not recognized on tissues which are essential for the health of the vaccinated individual. With respect to specificity several classes including CTA, differentiation antigens, tumour-associated overexpressed gene products, mutated gene products and tumour-specific splice variants may be useful as targets. In this review we will focus on the features of cancer testis antigens.

Cancer testis antigens as targets for cancer vaccination

The search for tumour-specific antigens revealed a novel class of antigens with an intriguing expression pattern. CT antigens are expressed by a variable proportion, ranging from 10 to 70% of a wide range of different human tumour types. In normal tissues, cancer testis antigens (CTA) and their respective encoding genes, the so-called cancer testis genes (CTG) are not expressed, except for testis. Interestingly, the prototypes of this category, MAGE[2], BAGE[14] and GAGE[15], were initially identified as targets for cytotoxic T cells. Several new members which have been added by SEREX to this category will be discussed in more detail below.

HOM-MEL-40/SSX-2

The HOM-MEL-40 antigen which was detected in a melanoma library is the first cancer testis antigen identified by SEREX. It is encoded by the SSX-2 gene. The members of the SSX genes, SSX1 and SSX2, have been shown to be involved in the t(X;18)(p11.2; q11.2) translocation which is found in the majority of human synovial sarcomas[16]. By this translocation the SSX genes fuse with the SYT gene from chromosome 18 resulting in the hybrid transcript -5' SYT-SSX 3'- which codes for a fusion protein. We observed that the SSX genes are silenced in normal tissues except for testis but are expressed in a wide variety of human tumours. Interestingly, the transcripts expressed in neoplasms other than synovial sarcoma are all derived from nonmutated, nontranslocated genes. Using homology cloning, additional members of the SSX family were identified[17] revealing at least five genes, of which four (SSX-1, 2, 4 and 5) demonstrate a CT-type expression[18,19]. In the meantime we have identified several antigenic peptides derived from SSX gene products (unpublished results).

NY-ESO-1

By applying the SEREX methodology to oesophageal squamous cell carcinoma, Chen et al.[20] identified NY-ESO-1 as a new CTA. NY-ESO-1 mRNA expression is detectable in a variable proportion of a wide array of human cancers, including melanomas, breast cancer, bladder cancer and prostate cancer. A homologous gene, named LAGE-1, was subsequently isolated by a subtractive cloning approach[21]

demonstrating that NY-ESO-1 belongs to a gene family with at least two members. Interestingly, NY-ESO-1 was recently also identified by the CTL cloning approach using melanoma-derived tumour infiltrating lymphocytes[22]. That NY-ESO-1 may be simultaneously an immune target for both antibody and CTL responses in the same patient was demonstrated by studies of Jäger et al.[23]. Stockert et al.[24] observed that IgG antibody responses directed against NY-ESO-1 are present in up to 50% of antigen-expressing patients, indicating that this antigen may also be an important target for CD4+ T lymphocytes.

HOM-TES-14/SCP-1

The expression of CT antigens in tumours and testis prompted our group to modify the original SEREX technique in order to bias for the detection of members of the CT class. For this intention, testis expression libraries were enriched for testis-specific transcripts by subtractive techniques and immunoscreened with allogeneic sera from cancer patients. SEREX screening using such testis-specific surrogate libraries proved to be a successful strategy for the identification of additional CTA[25]. One of the identified new CTAs was shown to be encoded by the gene coding for the synaptonemal complex protein-1 (SCP-1). SCP-1 is known to be selectively expressed during the meiotic prophase of spermatocytes and is involved in the pairing of homologous chromosomes[26], an essential step for the generation of haploid cells in meiosis I. Investigation of a broad spectrum of normal and malignant tissues revealed expression of SCP-1 transcripts and antigen selectively in a variety of neoplastic tissues and tumour cell lines. Immunofluorescence microscopy analysis with specific antiserum showed a cell cycle phase-independent nuclear expression of SCP-1 protein in cancer cells. SCP-1 is hitherto the only CTA with a known function. It is therefore intriguing to speculate which role aberrant expression of a meiotic protein in a somatic cell plays for the genomic instability of cancer cells.

To cope with the rapidly growing number of CTAs, a new nomenclature has been suggested. According to the order of this initial identification the individual genes are designated by enumeration. Thus CT-1 (CT-1.1 – CT-1.13) represents MAGE members, CT-2 BAGE, CT-3 GAGE, CT-4 stands for the SSX-family members, etc. Since individual CTAs are expressed only in a variable proportion of tumours, only the availability of several CTAs could significantly enlarge the proportion of patients eligible for vaccination studies. In this regard it is interesting that members of a given gene family tend to be expressed in a co-regulated fashion, whereas different gene families are preferentially expressed in other sets of tumours[27]. It is therefore reasonable to choose antigens from different CT families to cover as many tumours as possible. Despite the fact that SEREX enlarged the pool of available tumour antigens, the proportion of antigen-negative tumours is still high,

particularly in frequent neoplasms such as colon and prostate cancer. Moreover, immunohistological investigations for MAGE antigens have demonstrated a heterogeneity of antigen expression even in the same tumour specimen[28]. Thus, the combined or sequential use of a whole set of several antigens in a patient would have the potential of reducing or even preventing the in vivo selection of antigen loss tumour cell variants and would also address the problem of a heterogeneous expression of a given antigen in an individual tumour specimen.

Recognition of SEREX antigens by T lymphocytes

As stated above, antitumour immune responses result from a concerted immunological action which involves both cellular and humoral effector mechanisms. Since the isotype switching and the development of high-titred IgG in vivo requires cognate CD4+ T-cell help, SEREX can be instrumentalized to analyse the CD4+ T-cell repertoire against tumour antigens. With regard to CD8+ T lymphocytes that are recognizing SEREX antigens, it is noteworthy that MAGE-1, MAGE-4a and tyrosinase originally described as CTL targets have also shown up during the SEREX immunoscreening of several tumours, suggesting that at least some of the serologically identified antigens may bear epitopes that are recognized by CTL. Moreover, CTL responses have been demonstrated by two independent groups for the SEREX-defined NY-ESO-1 antigen and resulted in the identification of three HLA-A201 and three HLA-A31 restricted epitopes. Interestingly, antigenic peptides for two of the three HLA-A31 restricted epitopes are encoded by an alternative open reading frame indicating that CTLs may respond to two different gene products translated from alternative reading frames of the same gene.

Identification of T-cell epitopes of SEREX antigens

The search for T-lymphocyte-recognized epitopes of defined molecules is an important new field of molecular tumour immunology and has been titled 'reverse T-cell immunology' by Thierry Boon. Because of the diversity of peptides presented by the highly polymorphic HLA alleles this objective means an enormous challenge for each individual antigen. Our group is also addressing this question in detail for members of the CTA class identified by SEREX. Several strategies have been created and used in recent years for this purpose.

The antigenic peptide approach

Using different algorithms, the sequences of serologically identified antigens are scanned for peptides containing binding motifs for MHC I or MHC II alleles[29,30]. Due to the polymorphism of HLA we focus on well characterized and frequent

MHC alleles. The predicted peptides are synthesized and tested for binding to the respective MHC molecules. Peptides with affinity to MHC molecules are loaded onto dendritic cells or other professional antigen presenters and used to stimulate autologous T lymphocytes. T cells expanded by repeated stimulation are tested for HLA-restricted reactivity to antigen-positive target cells. Using this approach we have identified several HLA-A201-presented antigenic peptides for different SEREX antigens.

The whole-protein approach

Another strategy is based on the utilization of the full-length antigen which is either expressed in professional antigen-presenting cells by polynucleotide transfection or is fed as recombinant protein. Successful transfection of polynucleotides in dendritic cells has been described using in vitro translated RNA[31] or recombinant viral delivery systems, such as vaccinia or adenovirus[32]. Dendritic cells presenting antigenic peptides after processing of endogenously expressed or exogenously loaded antigens are used for repeated stimulation of autologous T lymphocytes. Similar to the peptide approach expanded T lymphocytes are tested for antigen-specific reactivity and MHC restriction.

The pre-existing CTL approach

A multitude of CTL clones with tumour cell specific reactivity has been established worldwide and in many cases the identification of the respective target antigens has resisted scrutiny. Using COS cells co-transfected with cDNA coding for serologically identified antigens together with the restriction element, the reactivity of these T cells against SEREX antigens can be easily tested.

Despite the fact that reverse T-cell immunology is a new terrain in tumour immunology the elaboration of technical advances provides rapid progress in this field. The analysis of peptides eluted from MHC molecules by mass spectroscopy is becoming more and more sensitive and will assist in the direct identification of naturally processed peptides derived from particular antigens. Together with the typing of the immunogenic genome provided by SEREX this will shape an ever more complete picture of the repertoire of cancer-associated antigenic peptides. The knowledge derived from these studies will form the basis for a rational immunotherapy, the success and failure of which could be analysed at the molecular level.

Conclusions and prospects for the future

The multitude of tumour-specific antigens identified by the SEREX technique has revealed that the immune recognition of human tumours by the autologous host's

immune system is not impaired and opens the perspective for depicting an antigenic profile for each individual tumour. With the identification and molecular definition of multiple antigens expressed by a given tumour, which elicit an immune response in the autologous cancer patient, it has now become evident that the recognition of tumour antigens is not the limiting step in immune responses against tumours. Rather, it is more likely that it is the effector arm of the immune system that contributes to the failure of the cancer patient's immune system to prevent or control cancer. The availability of molecular defined genes, which are specifically expressed or overexpressed in many (and possibly all) human cancers, now provides a tool for the redirection or upregulation of the effector arm of the immune response towards an efficient cytotoxic response against malignant cells using various cancer vaccine strategies. The study and long-term follow-up of large numbers of patients will help to determine the diagnostic and prognostic relevance of tumour-specific antibodies in patients' sera and of antigen expression in tumours, as well as CTL responses. The abundance of human tumour antigens will enable us to proceed with the development of polyvalent vaccines for a wide spectrum of human cancers using pure preparations of molecular defined antigens or antigenic peptide fragments.

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11

CTL-defined cancer vaccines in melanoma and other epithelial cancers

Elke Jäger, Dirk Jäger and Alexander Knuth

Introduction

Spontaneous regressions of human tumours have been reported in different types of cancer, especially in melanoma and renal cell carcinoma[1,2], but also in other types of cancer, such as nonsmall cell lung cancer, bladder carcinoma and breast cancer. These observations suggest the interaction of the immune system with antigenic determinants presented by the tumour. Early attempts to activate the immune system against tumour growth were based on observations made in cultured melanoma cells which were shown to be lysed by autologous CD8 + T lymphocytes in vitro. The clinical translation of this observation was applied to single patients with metastatic melanoma, who received irradiated autologous tumour cells as a vaccine. Two patients (SK-29 and MZ-2) with recurrent metastatic melanoma have been observed by our group since 1978 and 1982, respectively[3,4]. Both patients received intradermal immunization with irradiated autologous tumour cells for an extended period of time. Complete regression of tumour manifestations was documented after prolonged immunization with autologous tumour cells. The patients have remained free of disease until now for 19 and 14 years, respectively. Based on these favourable clinical evolutions, a systematic search was initiated to identify and characterize the cancer antigens and immune effector mechanisms mediating these tumour regressions in vivo[4,5].

Human tumour antigens recognized by the immune system

Cancer testis antigens

Cytotoxic T lymphocytes (CTL) have been isolated first from melanoma patients that effectively lyse melanoma cells in vitro[4]. Antigenic peptides have been found to be presented by MHC class I and II molecules. The first antigen recognized by CTL in an HLA-A1 restricted fashion was isolated from the melanoma tissue of patient MZ-2, designated as MAGE-1[6]. Later, a family of MAGE-1 related genes (MAGE-1, MAGE-3, BAGE, GAGE) was identified, encoding antigens that are expressed in melanomas and several other tumours, but not in normal tissues

except testis[6–10]. Therefore, antigens with this pattern of expression are designated as 'cancer testis' (CT) antigens. Most recently, a new CT antigen, NY-ESO-1 was identified from oesophageal cancer by a serological approach (SEREX), based on the screening of recombinant tumour cDNA libraries for specific interactions with autologous serum antibodies[11,12]. HLA-A2 binding peptides derived from NY-ESO-1 were characterized that elicit strong CTL reactions in vitro[13]. Since NY-ESO-1 and other members of the MAGE gene family are frequently expressed in different cancers, these antigens represent attractive targets for specific immunotherapy in cancer patients.

Melanocyte differentiation antigens

A second category of antigens derived from melanocyte differentiation antigens was identified to be recognized by autologous CTL in melanomas, which are also expressed in normal melanocytes[14–18]. Several epitopes derived from these self-antigens, such as Melan A/MART-1, tyrosinase, gp100/Pmel17 and gp75/TRP-1, have been found to be targets for CTL and tumour-infiltrating lymphocytes (TIL) in the context of HLA-A2.1 and other MHC molecules[19–21], resulting in objective tumour regressions in some patients. Phase I clinical trials in melanoma patients with peptides derived from these antigens have shown that specific DTH reactions can be elicited after intradermal peptide injection[22]. GM-CSF used as a systemic adjuvant strongly enhanced peptide-related DTH reactions in single patients[23]. In contrast to phase I clinical trials with MAGE-derived peptides, the induction of peptide-specific CTL was observed after immunization with peptides derived from Melan A and tyrosinase[22,24]. Furthermore, objective tumour regressions were observed in single patients under continued immunization[22,24,25].

Point mutations

Another group of potential cancer antigens is created by point mutations. These point mutations may induce strong CTL responses against tumour cells in cancer patients or experimental animals[26–28]. In breast cancer, mutations of the oncogenic proteins p53 and Ras have been reported. Humoral immune responses occurring spontaneously in patients with breast, lung and gastrointestinal cancer to the mutated and to the wild type proteins have been detected[29,30]. In women with a family history of breast cancer, antibody responses to p53 occur with a higher incidence compared to controls (11 vs 1%)[29]. Since the majority of p53 antibodies detected are IgG, a preceding CD4+ T cell response to p53 can be predicted. In single patients with breast cancer showing accumulation of p53 in primary tumours, a lymphoproliferative CD4+ T cell response to wild type p53 was demonstrated[31]. From these observations made in a limited number of patients it may be concluded that immune responses occur after mutation of oncoproteins. These

may be directed against nonmutated portions of the proteins. To date it is unknown whether p53 as an intracellular protein is available in the extracellular cancer environment to serve as an immunotherapeutic target for humoral and/or cellular effectors to mediate tumour regression.

In animal models, mutant p53 has been shown to elicit specific CTL responses that mediate lysis of the transformed cells. In a murine sarcoma model, it was demonstrated that vaccination with p53 peptides combined with IL-12 led to regression of p53 expressing advanced Meth A sarcomas[32]. In many human cancers, accumulation of wild type p53 in the cytosol can be detected. It is assumed that accumulated p53 is effectively presented by MHC class I molecules in amounts sufficient to elicit specific CTL responses. Therefore, immune responses against wild type p53 may be useful in the defence of cancers with p53 accumulation.

Ras mutations described so far involve single amino acid substitutions, mostly at positions 12 and 61. These are less complex than in p53 and thus easier to evaluate. CD4+ and CD8+ T cell responses leading to tumour lysis can be elicited by immunization with Ras peptides containing the mutant segment in animal models[33]. In humans, it remains to be determined whether wild type or mutant Ras protein is a useful target for active or passive therapeutic immune interventions. In a limited number of patients with pancreatic cancer, immunization with MHC class I restricted Ras peptides led to proliferative T-cell responses[34].

Other mutation-based antigens, which have been defined primarily via CTL recognition, i.e. MUM-1 and mutated CDK4, have been shown to generate new peptide epitopes that are presented by MHC class I molecules. It is unknown whether these antigens are useful targets for CTL-based vaccines in a larger patient population[26,28].

Overexpressed antigens

Some tumour types constitutively express normal 'self' proteins in abundance. The most extensively studied 'self' antigens that serve as targets for active and passive immunotherapy are Melan A, a melanocyte differentiation antigen present in melanoma and normal melanocytes, and HER-2/neu, a growth factor receptor overexpressed in 30% of breast and ovarian cancers and a variety of other adenocarcinomas[29]. Immune reactions directed against this type of antigen theoretically result in destruction of normal tissues. However, preliminary experiences with peptide immunization in patients with Melan A-expressing melanomas have not shown prominent toxicity except the development of vitiligo in single patients[22–25]. Patients with HER-2/neu-expressing tumours have been shown to produce spontaneous humoral and cellular immune responses that may be amplified by appropriate routes of immunization towards a therapeutic response leading to tumour regression[29].

Viral antigens

Some human malignancies are associated with defined viral diseases, i.e. Burkitt's lymphoma (BL) and Epstein–Barr virus (EBV)[35], hepatocellular carcinoma and hepatitis B and C virus (HBV, HCV)[36,37], cervical and anal carcinoma and human papilloma virus (HPV)[38], human T lymphotropic virus (HTLV) and T-cell leukaemia. Independent of whether the viral infection becomes the oncogenic agent, it was shown that viral antigens are expressed in the associated tumours and can be used as targets for preventive or therapeutic vaccination[39].

Development of immunotherapeutic strategies

Peptides derived from CT antigens as active immunogens?

MAGE-1- and MAGE-3-derived peptides have been used as a vaccine in HLA-A1-positive patients with tumours expressing the respective antigens to assess toxicity and immunological responses. Tumour regression responses have been observed in 7 of 25 melanoma patients after immunization with the MAGE-3-derived, HLA-A1-restricted peptide[40]. CTL against MAGE-3, however, could not be identified in response to the vaccine in these patients[41]. In a subsequent study using systemic GM-CSF to improve antigen presentation by enhancement of CD1a+ dermal Langerhans' cells followed by intradermal administration of MAGE-1 and MAGE-3 peptides, a partial regression of liver and lung metastases was achieved in a melanoma patient within three months of immunization (E. Jäger et al., unpublished data). In parallel to this remarkable clinical development, MAGE-1- and MAGE-3-specific CTL were detected that showed an increase in frequency subsequent to immunization. Based on these promising results, phase I studies are currently being initiated to evaluate immune reactions to peptide vaccination in patients with other MAGE-expressing carcinomas.

CTL against the HLA-A1-restricted MAGE-1- and MAGE-3-derived peptides were repeatedly isolated from the peripheral blood of patient MZ2, the patient from whom the MAGE-1 and MAGE-3 genes were cloned[6,8]. This observation strongly suggests that CTL responses are effective mediators of tumour regression, since this patient experienced a complete regression of metastatic MAGE-1- and MAGE-3-positive melanoma metastases after repeated immunization with autologous MAGE-1- and MAGE-3-positive tumour cells. During the course of repeated tumour cell vaccination, an increased frequency of CTL-recognizing autologous tumour cells were detected in the peripheral blood of this patient[42]. The specificity of CTL responses, however, could not be assessed at that time, since the structure of the autologous tumour-associated antigens was not known. The rare detection of CTL against MAGE genes in patients with MAGE-positive melanoma may be attributed to either a low immunogenicity of MAGE genes, or to a low

frequency of CTL precursors. Different methods for the assessment of MAGE-specific CTL responses are being evaluated. One of the promising approaches appears to be the ELI-spot assay, an ELISA assay that visualizes the direct antigen–T-cell receptor interaction by staining of the spot-like liberation of gamma-interferon or other cytokines by the T cell reacting to a defined antigen.

Lessons from targeting differentiation antigens in melanoma

Objective tumour responses in single melanoma patients have been observed after adoptive transfer of tumour-infiltrating lymphocyte (TIL) lines recognizing gp100/Pmel17–, tyrosinase- and gp75-derived epitopes, suggesting that differentiation antigens can serve as tumour rejection antigens[14,17,43]. To further study the effects of T-cell interactions with melanocyte differentiation antigens in vitro and in vivo, we determined: (*a*) the spontaneous CTL reactivity against HLA-A2-restricted peptides derived from the differentiation antigens Melan A/MART-1, tyrosinase and gp100/Pmel17 in HLA-A2 positive melanoma patients and healthy individuals[22–25]; (*b*) cellular immune responses to melanoma-associated peptides administered intradermally as a vaccine to HLA-A2 positive melanoma patients[22–25]; and (*c*) changes of expression of melanoma-associated antigens and peptide-presenting MHC class I molecules in melanoma tissues showing regression or progression in the presence or absence of antigen-specific CTL responses in vivo[44].

The baseline CTL reactivity against melanoma-associated peptides was determined in melanoma patients and healthy individuals as a basis for the development of active immunotherapeutic strategies using antigenic peptides. Spontaneous CTL reactivity against the differentiation antigens Melan A/MART-1, tyrosinase and gp100/Pmel17 is frequently detected in melanoma patients and healthy individuals, without providing differences in intensity and frequency of CTL responses[22–25, 45,46]. These findings suggest that CTL responses against 'self' antigens occur spontaneously in individuals and may be amplified by appropriate vaccination.

Antigenic peptides derived from Melan A/MART-1, tyrosinase or gp100/Pmel17 were shown to induce delayed-type hypersensitivity (DTH) reactions and specific CD8 + CTL responses after intradermal immunization. The induction of objective clinical responses was associated with measurable CTL responses to the vaccine. Toxic side-effects of the vaccine were not observed. Some patients with beneficial clinical development, however, developed reversible vitiligo[22–25].

Dermal APC, such as dendritic Langerhans cells, can be stimulated by granulocyte macrophage colony-stimulating factor (GM–CSF) in vivo[47]. Combined administration of melanoma-associated peptides and GM–CSF resulted in enhanced DTH reactions CD8 + CTL responses. Immunohistochemical characterization of

DTH constituting elements showed infiltrates of CD4+ and CD8+ T lymphocytes and a strong expression of IL-2 and gamma-interferon, suggesting the activation of CD4+ Th1 and CD8+ CTL by peptides presented by MHC class I molecules of dermal APC[22–25].

Immunoselection in vivo mediated by peptide-specific CTL

The development of monoclonal antibodies used for immunohistochemical staining of melanocyte differentiation antigens expressed in melanoma tissues has set the basis for studying the microheterogeneity of defined antigens in tumour lesions under specific immunotherapy[48,49]. In HLA-A2-positive melanoma patients immunized with Melan A-, tyrosinase- and gp100-derived peptides combined with GM-CSF, we observed after an initial phase of tumour regression in some patients progressive disease in the presence of detectable peptide-specific CTL, that efficiently lysed HLA-matched melanoma cell lines in vitro[44]. Biopsies were obtained from lesions in the phase of progressive tumour growth and compared to the initially described homogenous antigen expression, a highly heterogenous distribution of antigens was observed in response to increased peptide-specific CTL responses. Furthermore, a loss of expression of MHC class I molecules as detected by immunohistochemistry was found in single cases, which represents an additional mechanism of immune escape from antigen-specific T-cell recognition.

Future clinical studies involving antigen-specific T-cell reactions in cancer patients should analyse the prognostic implication of the heterogeneity of MHC class I- and tumour-associated antigen expression in tumour tissues for T cell-based immunotherapy. Cytokines, i.e. interferon gamma, will be of interest in future trials to show whether these can mediate the upregulation of antigens and antigen-presenting molecules in tumour tissues.

Perspectives for active immunotherapy in cancer patients

Different types of cancer-expressing defined tumour-associated antigens may become targets for immunotherapeutic interventions. The growing number of tumour antigens detected and the lessons learned from immunotherapy in malignant melanoma, have set a solid basis for the development of immunotherapeutic strategies in cancer patients. CT antigens appear to be most promising targets for specific CTL responses induced by peptide or protein vaccines. The detection of spontaneous antibody responses to CT antigens in sera of cancer patients[50] suggests also the spontaneous stimulation of CD4+ T cells against peptides presented by MHC class II molecules on the surface of tumour cells. The characterization of these antigens as targets for CD4+ T-cell responses will allow concurrent immunization with MHC class I and class II epitopes to potentially mount more effective immune responses.

Future perspectives of tumour vaccine development are focused on more potent strategies of immunization. Vaccination with whole proteins containing multiple possibly relevant antigenic epitopes may increase the chance of multidirectional B- and T-cell activation. Adjuvants may enhance the immunogenicity of peptides and proteins by activating co-stimulatory factors and mediating the production of cytokines[51]. Autologous dendritic cells pulsed with peptides or proteins in vitro, or transfected with the relevant genes, may effectively activate both class I- and class II-restricted T lymphocytes in vivo[52,53]. Cytokines have been identified to play a key role in T-cell activation. GM-CSF has been shown to induce long-lasting Th1 and CD8+ T-cell responses by efficient activation of dendritic cells in vivo[54]. Interleukin-12 (IL-12) is a potent activator of Th1 and CD8+ T lymphocytes. At low dose levels it has been shown to mediate complete tumour regressions when used as an adjuvant to immunization with a mutant peptide of p53 in an animal model[32]. The identification of further tumour antigens will give a broader basis for polyvalent immunization strategies to prevent the escape of antigen loss variants[22–25]. Once the clinical effectiveness of cancer vaccination is more established, immunotherapy may become another modality for the adjuvant treatment of cancer patients at high risk of recurrence.

Conclusions

The characterization of tumour-associated antigens recognized by cellular or humoral effectors of the immune system has opened new perspectives for cancer therapy. Several categories of cancer-associated antigens have been described as targets for cytotoxic T lymphocytes (CTL) in vitro and in vivo: (1) 'cancer testis' (CT) antigens expressed in different tumours and normal testis; (2) melanocyte differentiation antigens; (3) point mutations of normal genes; (4) antigens that are overexpressed in malignant tissues; and (5) viral antigens. Clinical studies with peptides derived from these antigens have been initiated to induce specific CTL responses in vivo. Immunological and clinical parameters for the assessment of peptide-specific reactions have been defined, i.e. induction of DTH, CTL, autoimmune and tumour regression responses. Preliminary results demonstrate that tumour-associated peptides alone elicit specific DTH and CTL responses leading to tumour regression after intradermal injection. GM-CSF was proven effective to enhance peptide-specific immune reactions by amplification of dermal peptide-presenting dendritic cells. Long-lasting complete tumour regressions have been observed after induction of CTL by peptide immunization. However, in single cases with disease progression after an initial tumour response either a loss of the respective tumour antigen targeted by CTL or of the presenting MHC class I molecule was detected as a mechanism of immune escape under immunization in vivo. Based on these observations, cytokines to enhance antigen and MHC class I expression in

vivo are being evaluated to prevent immunoselection. Recently, a strategy utilizing spontaneous antibody responses to tumour-associated antigens (SEREX) has led to the identification of a new CT antigen, NY-ESO-1. In a melanoma patient with high titre antibody against NY-ESO-1 a strong HLA-A2 restricted CTL reactivity against the same antigen was found. Clinical studies involving tumour antigens that induce both antibody and CTL responses will show whether these are better candidates for immunotherapy of cancer.

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12

DNA vaccines against B-cell tumours

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Introduction

There are tantalizing glimpses of the intrinsic ability of the human immune system to control emerging cancer cells. This is seen most clearly in EBV-associated B-cell tumours which emerge in patients undergoing immunosuppression following organ transplantation[1]. Humoral immune responses against tumour antigens of melanoma and of epithelial malignancies have also been detected in patients[2]. However, when patients have cancer, the tumour cells have obviously evaded any immunological weapons. Vaccination as a treatment has to be able to activate a defeated immune system, in a situation where tumour antigens may still be present, and where the patient may be debilitated by disease or treatment.

Tumours of B lymphocytes are attractive for study because of the availability of cells, and because of an increasing understanding of the array of potential target molecules expressed at the cell surface. There is also a clinical need to develop new treatments for the many categories for which chemotherapy has not improved survival. However, these tumours exist despite exposure to the full power of the immune system. If we can succeed in activating an antitumour response against B-cell tumours, therefore, we should be in a strong position to attack other cancers which are less exposed. Molecular technology is facilitating vaccine development in three ways: first, by revealing novel tumour antigens; second, by aiding our understanding of immune mechanisms; and third, by facilitating new delivery systems to mobilize those mechanisms.

This review will focus on malignancies arising from B lymphocytes. The nature of these malignancies, and the clinical need to activate the immune system against tumours in remission, will be discussed. We shall concentrate on the idiotypic determinants of the clonal immunoglobulin (Ig) expressed by these tumours as the target antigens. The vaccines we are developing are DNA vaccines, designed so that the tumour antigens are synthesized by the host and presented directly to the immune system. One problem for cancer vaccines is that tumour antigens often represent small structural modifications of self proteins, and may therefore be poor

at priming the immune system. Novel aspects of the strategies we are using to alert and activate antitumour immunity will be described.

B-cell malignancies and the clinical need for vaccines

In the Western world, B-cell malignancies comprise the majority of tumours of the lymphoid system. Tumours may arise at every stage of lymphoid differentiation from the pre-B cell to the plasma cell, and the pattern of expression of Ig generally reflects that of the normal cell counterpart. The cardinal feature of B cells is the rearrangement of the Ig genes which culminates in expression of Ig protein. During the rearrangement process, the lability of this region of the genome may contribute to the genesis of lymphomas. Chromosomal translocations which can relocate proto-oncogenes to sites subject to the influence of the Ig enhancer are clearly implicated in the pathogenesis of several B-cell tumours (reviewed in Rabbitts[3]). Clinical classifications have separated low grade tumours, which are usually indolent, responsive to chemotherapy, but likely to relapse and are hence incurable, from intermediate and high grade tumours which are aggressive, but respond to chemotherapy and are hence potentially curable. This distinction has served well enough for the rather indiscriminate attack on disease by chemotherapy, but lacks the precision required for immunological strategies. The 'real' classification has defined clinical entities rather than histological patterns[4]. In many cases it has been possible, by morphology and immunophenotypic analysis, to recognize where tumours arise in the differentiation pathway, but this assignment has been greatly facilitated by immunogenetic analysis (see below).

Although B-cell lymphomas are viewed as one of the success areas for chemotherapy in oncology, particularly if we include acute lymphoblastic leukaemia (ALL) in children and Hodgkin's disease in young adults, the majority of patients die from their disease. Some, such as chronic lymphocytic leukaemia (CLL) and follicle centre lymphoma (FCL), are incurable by current treatments. Others, such as myeloma and mantle cell lymphoma (MCL), are both incurable and almost universally fatal. Even diffuse large cell lymphoma (DLCL), which is often cured by standard chemotherapy, proves lethal in 60% of cases[5]. Most B-cell lymphomas are initially chemosensitive, with a majority achieving clinical remission, although disease may be detectable by molecular means. Relapse remains a risk in most cases, and treatments to prolong remission have yet to fulfil their promise. High dose consolidation with stem cell support add an advantage in some forms of DLCL, and is under investigation for FCL, CLL and myeloma. Outcomes are eagerly awaited, but already late relapses in myeloma are evident. Prolongation of remission by immunological means is being explored, and interferon may have a modest effect in myeloma[6] and FCL[7]. The graft-versus-tumour effect of bone marrow allografting

is real, but accompanied by excessive toxicity and high mortality. So-called 'Lite' transplants, aimed at inducing graft-versus-tumour effect without marrow ablation by using fludaribine-based chemotherapy, are the immunotherapy techniques of the moment[8].

During remission there is a window of opportunity for vaccination. Relapse is usually delayed long enough for a vaccination schedule which may take three months to complete. However, even with an ideal vaccine, potential problems remain, including the fact that patients may be immunosuppressed by either disease or by treatment. In fact, major immunosuppression is rare except in CLL and possibly myeloma, or when the patient has been treated with fludaribine or cladribine. Treatment of B-cell tumours with monoclonal antibodies is also an option, and the effect of this on the immune response obviously depends on the target antigen. The rate of recovery of normal B cells following treatment with antiCD20 is awaited with interest[9]. Apart from general immunosuppression, there remains the possibility that the tumour has induced specific immune tolerance to tumour antigens, in the T-cell population. It is not yet clear whether this occurs or whether T cells can recover, although the clinical trial of idiotypic protein vaccines in patients with low grade FCL indicates that responses to this antigen can be induced[10]. It is also possible that autologous transplantation can restore response. However, strategies to overcome this potential problem need to be explored.

Idiotypic determinants as B-cell tumour antigens

There has been a long history of the use of idiotypic determinants of B-cell tumours as target antigens. The first models were mouse myelomas where idiotypic protein could be readily purified and tested for its ability to induce protective immunity[11]. Some success was reported, but the mechanism of protection was unclear, particularly as mouse plasmacytomas tended to both express and secrete idiotypic Ig[11]. The situation with murine B-cell lymphomas was more straightforward, and idiotypic protein has been shown in several models to provide a highly effective tumour antigen[12,13]. The results have been sufficiently convincing to allow the clinical trial of patients with low grade lymphoma, with promising results[10]. Mouse lymphoma models have implicated anti-idiotypic antibody as a major mediator of protection[12,13], and it appears that antibody may induce apoptosis in target B cells[14]. Intriguingly, there is evidence in several models that tumour cells are not completely eliminated but that there is a reservoir of dormant tumour[15].

However, there are several problems with idiotypic protein vaccines. The first is that the vaccine for each patient has to be made on an individual basis, usually by generating heterohybridomas secreting tumour Ig, which is tedious and expensive. The second is that the idiotypic protein has, in most cases, to be chemically coupled

to KLH to be effective[10,13]. The third is that induction of antibody is unlikely to be useful for other B-cell tumours such as myeloma, where the idiotypic Ig is secreted but not expressed at the cell surface. An additional problem is that adjuvant is required, and there are few effective adjuvants available for clinical use. Advances in our understanding of immunoglobulin variable region genes, which encode idiotypic determinants, have offered an opportunity to develop alternative approaches to producing individual vaccines. This knowledge has led, first, to expression of recombinant Ig protein and, subsequently, to DNA vaccines.

Tumour-derived variable region genes

We now have a clear view of the molecular gymnastics performed by the immunoglobulin genes in order to create a functional Ig molecule. Recombinatorial events occurring in the heavy chain genes bring together the V_H–D–J_H elements to generate a transcriptional unit. The repertoire of V_H and D-segment genes available for recombination is now known due to mapping of the germ line genes[16]. Imprecision at the V_H–D and D–J_H joints leads to the use of alternative reading frames of the D-segment genes. Together with the addition of nucleotides by terminal deoxynucleotide transferase, this produces an amino acid sequence in the third complementarity-determining region (CDR3) which is unique for each B cell. Since the CDR3 is at the centre of the antibody recognition site, the diversity has obvious implications for antigen recognition. A similar recombinatorial event occurs in the light chain, although diversity is limited since it involves only V_L and J_L. The germ line repertoires of V_κ and V_λ genes have also been mapped[17,18].

Following antigen encounter, B cells in germinal centres acquire another level of sequence heterogeneity by somatic mutation[19]. This process tends to focus amino acid changes in the CDR1 and CDR2 sites[20], which, together with CDR3, form the contact points for conventional antigen binding. Antigen held on follicular dendritic cells then selects optimal sequences for further maturation to generate memory B cells and antibody-secreting plasma cells.

Analysis of V-genes in B-cell tumours has revealed that neoplastic transformation can occur at many points of normal B-cell differentiation (Figure 12.1) (reviewed in Stevenson et al.[21]). The neoplastic B cells therefore carry evidence of their clonal history in the sequences. Tumours of less mature B cells, such as acute lymphoblastic leukaemia (ALL) and subsets of CLL and mantle cell lymphoma, do not undergo somatic mutation. However, some cases of CLL appear to have undergone somatic mutation and therefore have arisen from cells which have traversed the germinal centre site[21]. Prolymphocytic leukaemia (PLL) is also in this category (Figure 12.1). In tumours arising from B cells which have encountered the germinal centre, somatic mutations are likely to have introduced amino acid changes. These can create new idiotopes which provide further targets for immune attack.

In tumours remaining in the germinal centre environment, such as FCL, Burkitt's lymphoma (BL), DLCL and mucosa-associated lymphoid tissue (MALT) lymphoma (Figure 12.1), malignant B cells frequently continue to accumulate mutations post transformation, and this can generate sequence heterogeneity within the clone. Although individual idiotopes could be affected, this is not likely to present a major problem, since active vaccination should induce a polyclonal response against the full range of determinants. Escape from immune pressure would require deletion of Ig which is a rare event in B-cell tumours, but may occur in Hodgkin's disease (HD)[12]. The rarity may reflect the importance of Ig expression in maintaining survival of B cells[22]. A large cohort of tumours develop from B cells which have traversed the germinal centre site, undergone somatic mutation, and then exited from the site. The mutational pattern is therefore stable, and examples (Figure 12.1) include lymphoplasmacytoid lymphoma (LPL), marginal zone lymphoma (MZL), splenic lymphoma with villous lymphocytes (SLVL), hairy cell leukaemia (HCL) and multiple myeloma (MM)[21]. Curiously, some cases of monoclonal gammopathy of undetermined significance (MGUS) show ongoing mutation, possibly indicative of some influence of the germinal centre environment[21].

For B-cell tumours, identification of V_H and V_L sequences used to encode idiotypic determinants is relatively simple by PCR cloning and sequencing[23]. It is then possible to express idiotypic protein in vitro to produce a protein vaccine. We opted to express V-region genes in a single chain Fv (scFv) format[23], initially using bacterial expression. However, in models for which we had MoAbs against idiotypic determinants, we found that only low levels of folded protein were produced[23]. Preliminary tests also indicated that poorly folded scFv did not induce protective immunity. Since this approach did not lend itself to wide clinical application, we did not pursue it further. Alternative constructs have been used to express recombinant whole Ig molecules in eukaryotic cells. One design incorporated V regions from a mouse lymphoma fused to human constant regions[24]. Although the protein alone was poorly immunogenic, fusion of cytokines to the C terminus of the heavy chain did promote anti-idiotypic immunity, with some protection against lymphoma[24]. However, such recombinant proteins are difficult to make on a routine basis. The attractions of DNA vaccines, where the vaccinated individual synthesizes the idiotypic protein in situ, were obvious.

DNA vaccines

General

The discovery that injection of DNA containing a gene-encoding β-galactosidase into mouse muscle led to expression of functional enzyme, was a surprise[25]. The

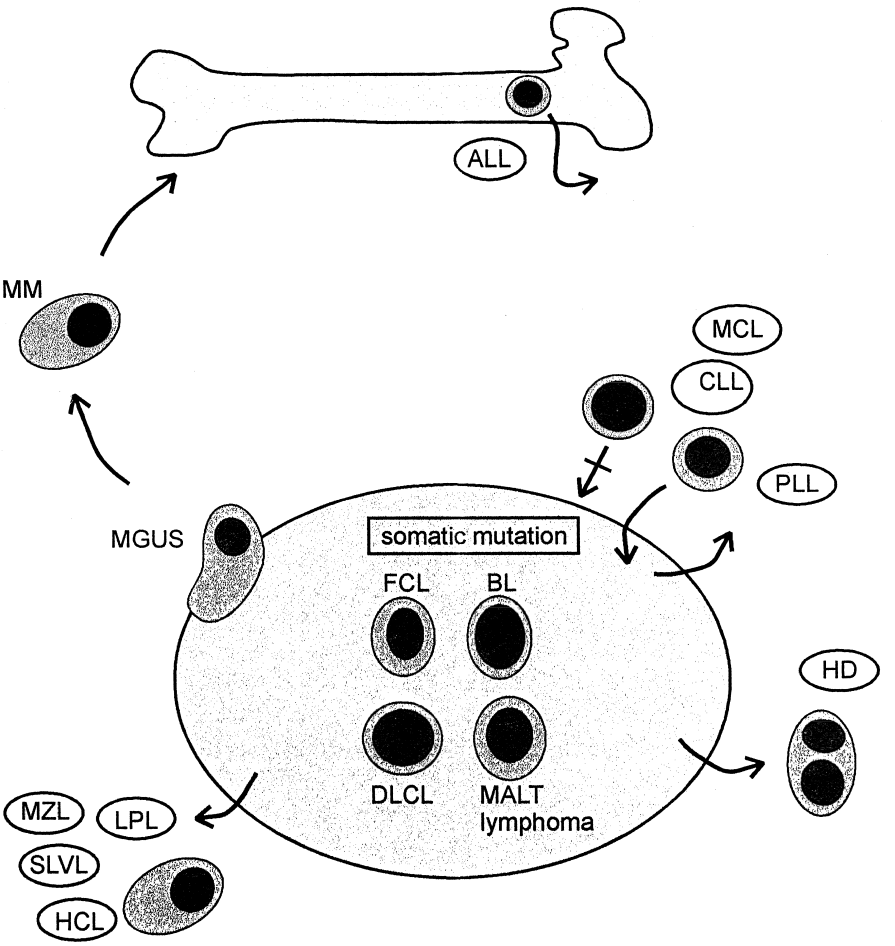

Figure 12.1 Origin of B-cell tumours in relation to the site of somatic mutation. Tumours which have unmutated V genes are considered not to have encountered the site where somatic mutation is activated (ALL, and subsets of CLL and MCL). Those with mutated V genes and evidence of continuing mutational activity are still under the influence of the mutator (FCL, BL, DLCL, MALT lymphoma and a subset of MGUS). Those with mutated V genes but no ongoing mutation have exited the site (LPL, MZL, SLVL, HCL, MM and a subset of MGUS). (Key: ALL: acute lymphoblastic leukaemia; CLL: chronic lymphocytic leukaemia; MCL: mantle cell lymphoma; PLL: prolymphocytic leukaemia; HD: Hodgkin's disease; FCL: follicle centre lymphoma; BL: Burkitt's lymphoma; DLCL: diffuse large cell lymphoma; MALT: mucosa-associated lymphoid tissue; LPL: lymphoplasmacytoid lymphoma; SLVL: splenic lymphoma with villous lymphocytes; MZL: mantle zone lymphoma; HCL: hairy cell leukaemia; MGUS: monoclonal gammopathy of undetermined significance; MM: multiple myeloma.)

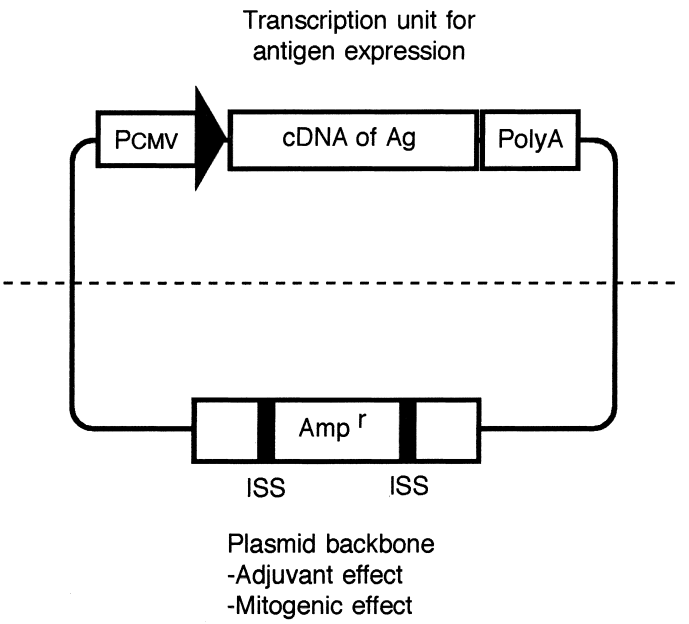

Figure 12.2 Active components of DNA plasmid vaccines. The plasmid contains the cDNA sequence of the candidate tumour antigen under the influence of the CMV promoter. The plasmid backbone also contains ISS (immunostimulatory) sequences derived from unmethylated CpG dinucleotide repeats, located mainly in the ampicillin resistance gene

subsequent observation that encoded proteins from influenza virus, delivered by this route, could induce antibody, cytotoxic T cells (CTLs) and protection against challenge, has set the scene for development of novel vaccines[26]. Currently, there is immense interest in DNA vaccines against infectious organisms, particularly for those where current vaccines are inadequate or unavailable.

The basic principles involved in the operation of these vaccines are being unravelled. Transcription of the cDNA encoding the antigen is usually driven by the powerful CMV promoter, and a popular choice of plasmid is pcDNA3 which contains the poly-A terminator derived from the bovine growth hormone gene (Figure 12.2). One factor which assists activation of the immune system is the presence of immunostimulatory sequences (ISSs) within the DNA backbone of the plasmid[27] (Figure 12.2). The ISSs contain unmethylated CpG dinucleotide repeats which are common in bacteria but not in mammalian cells[27]. The effect of these sequences is to induce production of IFNγ, IFNα, IFNβ, IL-12 and IL-18, and direct antibody responses to the encoded protein down a T_H1-dominated pathway[28].

Following intramuscular injection, terminally differentiated muscle cells can take up DNA via caveloae and T tubules without requiring a carrier[25]. It appears that the DNA does not integrate into the genome, but persists for long periods[29].

Immunological evidence indicates that muscle cells act as a depot for antigen, with entry of protein into the blood[29]. Transfer to antigen-presenting cells (APCs) for induction of cytotoxic T cells (CTLs) appears to occur mainly via 'cross-priming'[30]. This process is available for certain proteins, possibly delivered in membrane-bound form[31], or via heat shock proteins [32]. It may be a crucial pathway by which APCs acquire antigens from apoptotic cells following either infection or other tissue-damaging processes[33]. There is a possibility that direct transfection of a small number of APCs may also occur[34]. Evidence for this is particularly clear following injection by the intradermal route[35]. This route has the apparent advantage of requiring only a small amount of DNA vaccine for induction of a response, with delivery of DNA-coated gold bead particles via the 'gene gun' being particularly efficient[36]. The immunological outcome of DNA injection by either route appears to mimic that induced by certain viruses, with efficient induction of CTLs, even against proteins such as ovalbumin, which usually have an extracellular location[37].

DNA vaccines against lymphoma

Identification and assembly of tumour-derived variable region genes

The procedure for identification of V_H and V_L genes used to encode tumour Ig from patients or from mouse models has been described[23]. Since B cells may have biallelic rearrangements, the preferred starting material is RNA, which favours identification of the functionally rearranged genes. By using mixed family-specific 5'-primers in V_H together with mixes of primers complementary to J_H sequences, sequences from all B cells in the biopsy should be amplified. To identify tumour-derived sequences, we clone the amplified genes into bacteria and sequence random colonies. Repeated sequences with similar CDR3 stretches reveal the presence of tumour[21,23]. Although there can be problems with somatic mutations at primer sites, particularly in sequences from cases of tumours which have high levels of somatic mutations such as multiple myeloma, alternative primer sets can be used, and the success rate is high[23]. A similar PCR method can be applied to obtain the V_L product, with either κ or λ primers used dependent on the phenotype of the tumour. A preliminary check for a clonal population in a biopsy can be made by using a 5'-primer based in the third framework region of V_H and amplifying across the CDR3 sequence. This short product is the most variable between B cells and dominant clonal sequences can be identified by gel electrophoresis or in the automatic sequencer.

Prior to assembly, the V_H and V_L genes sequences require checking for stop codons or frame shifts to confirm functionality. In tumours derived from the germinal centre, such as follicle centre lymphoma, intraclonal sequence heterogeneity may be evident in both V_H and V_L, but there is usually a dominant sequence. This

should be selected for vaccine assembly, although there is no theoretical reason to prevent all variant sequences from being included in the vaccine. Assembly as scFv is by a simple two-step PCR procedure using overlapping primers[23]. A linker encoding 15 amino acids $(Gly_4Ser)_3$ is incorporated and full length scFv is cut and cloned into a pcDNA3 vector for vaccination. A leader sequence is included which can be either the natural 5'-V_H leader or a human V_H1 leader[23].

Requirement for an alert signal

Early experiments in mouse lymphoma models soon revealed that the presence of scFv sequence alone was insufficient to activate protective immunity against challenge[38]. In retrospect, this is not surprising given that the transfected cell would be secreting low levels of scFv protein, which is unlikely to attract the attention of the immune system. Similar weak responses using constructs encoding whole Ig were reported[39], making it unlikely that the scFv format was the problem. However, some improvement in immunogenicity of whole Ig was observed when the mouse constant regions were replaced by human constant regions[39]. A further interesting finding was that attachment of a peptide derived from IL-1β to scFv sequence increased anti-idiotypic antibody production and provided protection against tumour[40].

In view of the poor performance of scFv alone, we opted to attach a sequence encoding a highly immunogenic carrier protein to the scFv sequence. We chose the fragment C (FrC) of tetanus toxin (TT), a non-toxic C-terminal polypeptide of 50KD. The ability of the gene-encoding FrC to induce antiTT antibodies and protective immunity against challenge with *Clostridium tetani* had already been demonstrated[41]. We therefore simply attached the FrC gene to the 3′-end of a scFv sequence derived from a patient with lymphoma, leaving a short peptide (GlyProGlyPro) as a spacer[42]. One advantage of the fusion protein is that expression in vitro can be assessed and compared for different scFv constructs by measuring levels of FrC secreted by transfected cells[43].

Induction of antibodies by DNA scFv-FrC fusion vaccines

The first question was whether the scFv-FrC constructs could induce an immune response in mice. To investigate this, we chose three random scFv sequences from patients with lymphoma. We then injected 50 μg of DNA, containing either scFv sequence alone or the scFv-FrC fusion gene, into two sites in the quadriceps muscles of mice. The results showed a dramatic promotion of antibody against the patient's IgM by the use of the fusion gene construct (Figure 12.3). In fact, the scFv constructs alone induced negligible responses. Antibodies against FrC were also induced by the fusion genes as expected. The other point was that antibodies appeared to be specific for the patient's IgM. This is illustrated in Figure 12.4 where

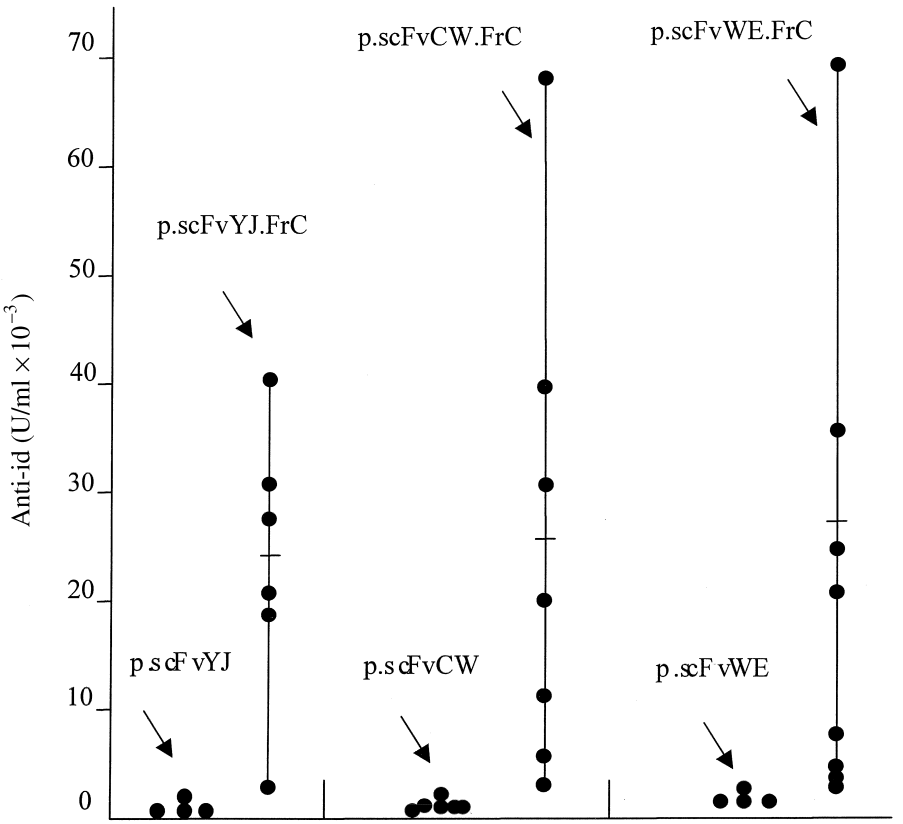

Figure 12.3 Antibody responses against patients' tumour-derived IgM induced by DNA scFv vaccines. DNA scFv vaccines were assembled using V_H and V_L sequences from lymphoma patients YJ, CW, and WE. In each case, plasmids contained either scFv sequence alone (p.scFv) or fused to fragment C sequence (p.scFv.FrC). Mice were injected in intramuscular sites with 50 μg of DNA on days 0, 21 and 42, and bled on day 63. Antibodies against tumour IgM were measured by ELISA, and each point represents a single mouse

it is clear that, even though the V_H genes were all derived from V_H2 and, for PR and WD, were from the same germ line gene, the induced antibodies were highly specific for the parental IgM. The apparent induction of anti-idiotypic antibodies is an important result since it shows that the scFv protein is able to fold optimally in the fusion format.

Mechanism of promotional effect of FrC fusion

Tetanus toxoid has often been used as a carrier protein for enhancing immune responses against peptides[44]. The assumption is that the carrier protein induces T-cell help for the antibody response by the classical hapten-carrier effect[45]. To test if

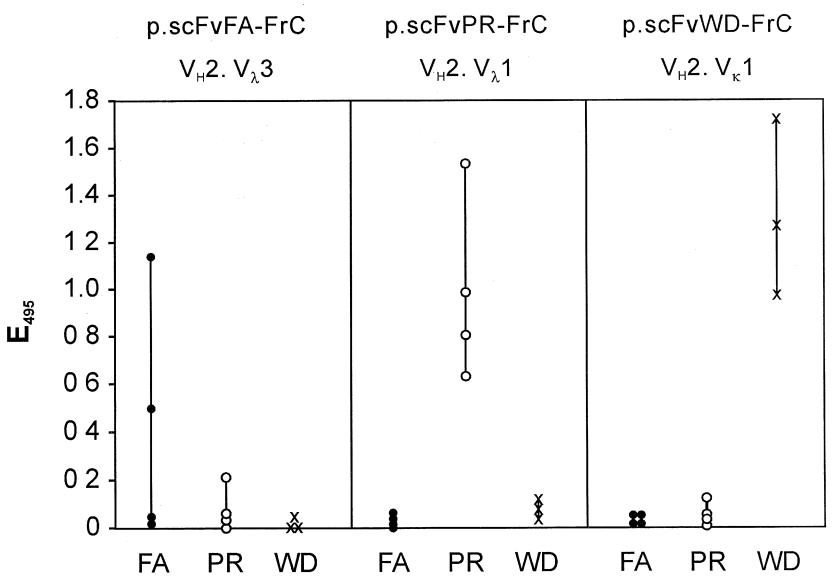

Figure 12.4 Induction of anti-idiotypic antibodies by DNAscFv-FrC vaccines. Sera from mice vaccinated with fusion constructs (p.scFv-FrC) containing scFv from three different patients (FA, PR and WD) were tested for reactivity with IgM from each patient's tumour by ELISA. All IgMs were derived from V_H2 and therefore shared some V sequences, but the majority of reactivity appeared specific for the original tumour IgM, consistent with an anti-idiotypic response

this was the mechanism operating for the scFv-FrC fusion gene, we compared antibody responses induced by separate vectors containing scFv and FrC to those induced by the fused scFv-FrC gene. The results showed clearly that FrC delivered on a separate vector could induce antiFrC antibodies, but completely failed to promote the antibody response to the scFv[43]. This was the case even when the two proteins were encoded within the same plasmid (unpublished data). Linkage is evidently an absolute requirement, as expected for cognate T-cell help.

Induction of protective immunity against a mouse B-cell lymphoma

The ability of the DNA scFv-FrC fusion design to induce protective immunity against a syngeneic lymphoma was tested using the A31 lymphoma. Induction of syngeneic anti-idiotypic antibody (anti-Id) with scFvA31–FrC followed the same pattern as for the human scFv-FrC with good levels of antibody detectable (Figure 12.5A)[43]. As before, scFvA31 alone in the construct was completely ineffective. The regime of vaccination which appeared optimal for induction was three intramuscular injections of 50 μg of plasmid. Challenge of vaccinated mice with A31

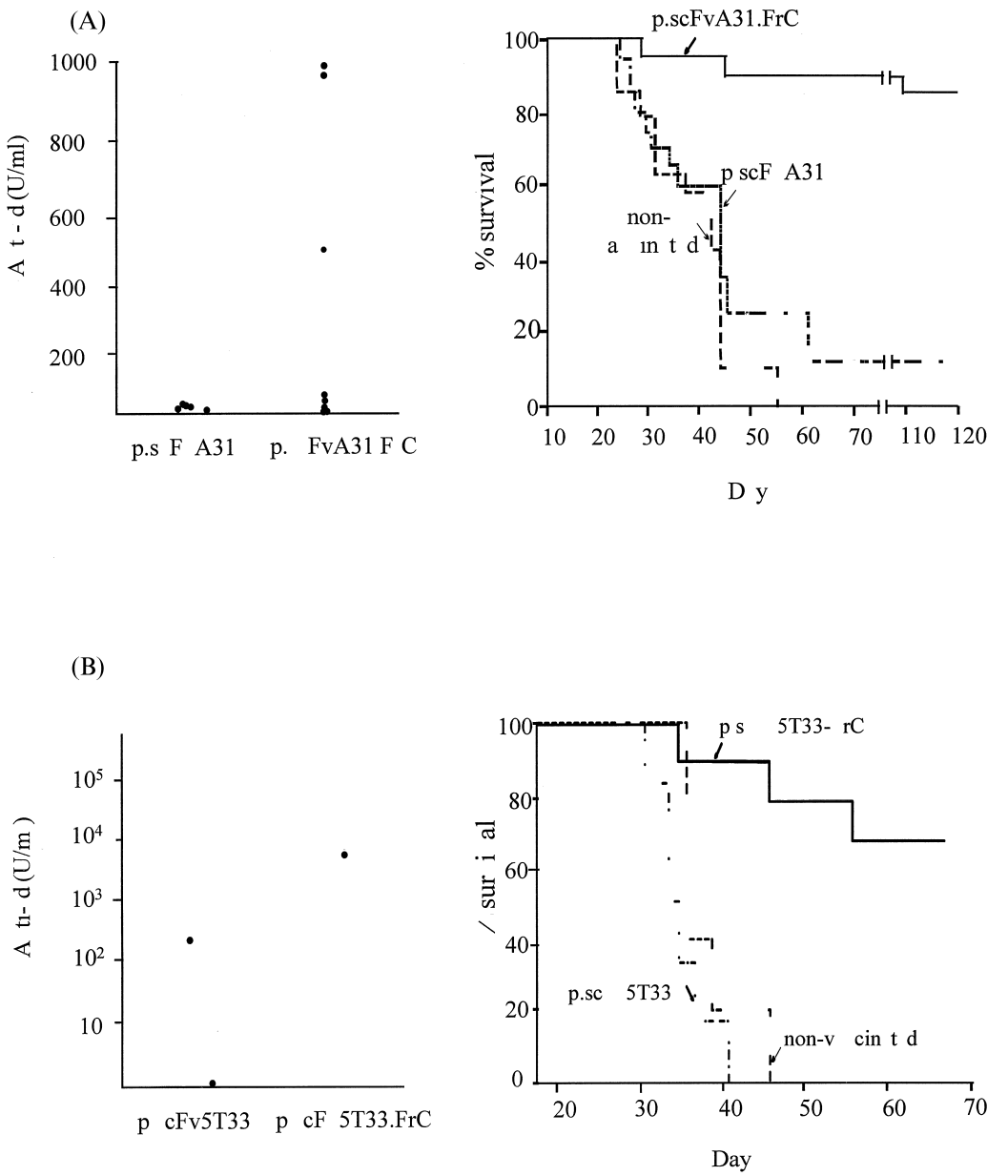

Figure 12.5 Induction of protective immunity against B-cell tumours by DNAscFv- FrC vaccines. Mice were vaccinated with DNAscFv-FrC or DNAscFv derived from either: (A) a mouse lymphoma (A31); or (B) a mouse myeloma (5T33), at days 0, 21 and 42. Anti-idiotypic antibody levels against the tumour Ig were measured, and mice were then challenged with tumour. In each case, anti-idiotypic antibodies and protection were induced by the DNAscFv-FrC fusion vaccine but not significantly by the DNAscFv alone

tumour showed protection in mice vaccinated with scFv-FrC construct, but not with scFv alone, as expected (Figure 12.5A)[43]. Since we know from data from vaccination with idiotypic protein that anti-Id is a strong mediator of protection[12,13], it is likely that antibody is involved in protection after DNA vaccination.

DNA vaccines against multiple myeloma

There has been a great deal of interest in using idiotypic vaccines against multiple myeloma[11]. The challenge is to induce an immune response capable of killing neoplastic plasma cells which do not express surface Ig, but secrete large amounts. Idiotypic protein vaccines have been developed[11], but these may be limited in scope since exogenous Ig protein tends to induce T_H2-dominated responses[43]. Anti-Id antibodies could have a role in eliminating residual disease if there were 'precursor' or less mature cells in the neoplastic clone continually differentiating into the plasma cell compartment. However, recent immunogenetic studies do not support the existence of such cells in the malignant population. The V-genes of the myeloma cell are somatically mutated with no intraclonal variation, a pattern typical of a mature postfollicular cell[46,47]. One complication is that transcripts of the tumour-derived V_H sequence linked to C_μ have been detected in a few cases of isotype-switched myeloma[48,49], but these cells do not appear to contribute to malignant behaviour and their origin and status remain unclear.

Vaccination therefore needs to be aimed at the surface Ig-negative plasma cell. These cells are secreting idiotypic protein and it is likely that they process idiotypic Ig and express peptides in the MHC Class I. In a mouse model, idiotypic peptides expressed by myeloma cells could be recognized by CD8+ CTLs[50]. However, one problem with mouse models is that, in contrast to human myeloma, the V-genes tend to have few or no somatic mutations[51]. This means that opportunities for generating mutated peptide motifs which can bind to the MHC class I molecule are limited. Encouraging evidence from transgenic mice has shown that anti-idiotypic CD4+ T cells can also attack myeloma cells[52]. The process appears to involve processing of secreted Ig by APCs, followed by presentation of peptides to CD4+ T cells. The T cells then release cytokines which attack the plasma cells in the vicinity[52].

Induction of protective immunity against a mouse myeloma

To test the ability of DNA vaccines to suppress myeloma, we used the myeloma model developed by Dr J. Radl[53]. The 5T series of tumours arose spontaneously in aging C57BL/KaLwRij mice, and the myelomas have all the features of the human disease, including osteolytic lesions[53]. The 5T33 myeloma exists as both an in vivo and in vitro line, and secretes an IgG2b monoclonal protein. We identified the V_H[51]

and V_κ genes used to encode the idiotypic determinants, and assembled them as scFv. We then tested the ability of constructs containing scFv alone or the scFv-FrC fusion gene, to induce anti-Id in syngeneic mice. The results were exactly parallel to those obtained in the lymphoma model, with induction of anti-Id only by using the fusion gene (Figure 12.5B). Following challenge with myeloma cells, we also observed strong protection against tumour (Figure 12.5B).

Since we could not detect any surface Ig on the myeloma cells[43], it seemed unlikely that anti-Id was mediating protection. To check this, we vaccinated mice with the idiotypic protein in CFA, a procedure which raised high levels of anti-Id[43]. These mice were not protected against challenge, confirming that anti-Id was not involved. At present, it is unclear what cellular mechanism is responsible for suppressing the myeloma. It is Id-specific and again relies on fusion between scFv and FrC. The likely mediator is a CD4+ T cell, analogous to those described previously[52], and experiments are in progress to define these cells.

Clinical trials of DNA vaccines

A phase 1 clinical trial of idiotypic DNA scFv vaccines in low grade FCL has been carried out in seven patients in Cambridge and Bournemouth. The clinical protocol has been published[54], and was essentially to test any toxicity from escalating doses of DNA. All the patients had end-stage disease, and it was a condition of entry that they should have received no chemotherapy, including steroids, for ten weeks prior to vaccination. They also were required to have a life expectancy of six months after beginning vaccination, and both these criteria restricted entry. All the patients were profoundly immunosuppressed, and typically, blood lymphocytes failed to respond to stimulation with Concanavalin A. The schedule was for six intramuscular injections over 12 weeks. Doses began at 100 μg per injection, and increased every second patient to 400 μg. The only treatment-related toxicity was of minor redness of the skin and muscle ache. No antiDNA antibodies were detected, and there was no evidence of muscle damage. Not unexpectedly, there was no detectable specific immune response to the idiotypic antigen.

A second trial is now undergoing final preparation. It will be similar in design to the first trial in using escalating doses of DNA, beginning at 300 μg and increasing to 2400 μg. The vaccine will incorporate DNA encoding the idiotypic scFv fused to the gene encoding FrC of tetanus toxin (see above). Subjects will be patients with FCL in first remission, and treatment with purine analogues will be an exclusion criterion. There will be a further assessment of any toxicity due to this modified vaccine construct, and the endpoint will be detection of antibody against both FrC and against the patient's idiotypic protein.

Concluding remarks

It took ~200 years before the early attempts to vaccinate against smallpox led to total eradication of the disease. Although progress may be faster now, we shall need time to develop effective vaccines against cancer. DNA vaccines offer opportunities to activate all pathways of the immune response. Importantly, the ease of construction and manipulation means that new designs can be tested quickly. DNA vaccines are already in clinical trials for prevention of certain infectious diseases[55], and we shall learn a great deal from those.

For cancer, where immunity has to be induced in patients with disease, success is likely to depend first on attaining clinical remission. Ideally, this will allow recovery of immune capacity without resurgent tumour. The potential problem of tolerance may be overcome by bone marrow transplantation, but provision of additional CD4+ T-cell help by adjuvant sequences in the vaccine will be necessary. The principle of activating immunity against cell surface or secreted tumour antigens by DNA vaccines containing antigen linked to FrC should apply to a range of tumour antigens, and many are being defined. For intracellular antigens, such as mutated proto-oncogenes, a similar principle may be applicable but detailed strategy will differ. There are exciting prospects ahead, and the distance between the laboratory and the clinic is diminishing.

Acknowledgments

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13

Dendritic cell approaches to immunotherapy

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Introduction

While the term 'magic bullet' has resulted in the association of cancer immunotherapy with the use of monoclonal antibodies to target tumours, it is clear that the most effective way to exploit the immune system to clear tumours is by generating tumour-specific cytotoxic T cells (CTLs)[1,2]. The major question for cancer immunotherapy must thus be, how can an effective antitumour CTL response best be elicited? The surprisingly universal answer that has emerged from a number of different studies is that this requires the stimulation of T cells by a specific antigen-presenting cell (APC), the dendritic cell (DC)[2–4]. DCs were first described as the morphologically distinct Langerhans cells in the skin and have since been shown to be the most efficient APC for the activation of naïve T cells[1,5]. The main impetus for their study as APCs was the development of simple methods for the isolation of DC-precursors from blood and the expansion of these cells in vitro to yield potent APCs[2,3,5,6]. For clinical researchers, DCs have the promise of providing a vehicle for effective anticancer immunotherapy. However, many questions remain. Is a defect in APC function a significant component of the failure of the immune system to eradicate cancer? Is the restoration of competent APC function sufficient to permit the execution of an effective CTL response (or is the augmentation of DC function sufficient to overcome other defects in a cell-mediated immune response to cancer)? What are the optimum schedules for the preparation and administration of DC? To answer these questions requires meticulously designed clinical trials, which urgently need to be undertaken before the therapeutic use of DC, prepared by a host of different schedules, become accepted dogma haphazardly applied. Despite these uncertainties, there are compelling reasons why the manipulation of DC hold great promise for the future of anticancer immunotherapy, as we will now discuss.

Evidence that DCs can induce a specific antitumour CTL response

A number of diverse approaches have been used to load DCs, ex vivo, with tumour antigens[3,4,7–15]. These antigen-loaded APCs are then given to the patient in the hope

that they will elicit a specific antitumour response. These approaches have been tested in both animal studies and human phase I clinical studies. Initial work involved loading the DCs with peptides eluted from class I MHC molecules[7,8], tumour-specific idiotypic protein[10] or RNA derived from neoplastic cells[9]. However, more recent work has shown that DCs can also be loaded with tumour antigens by simply fusing them to tumour cells[4,11]. These cell fusion studies, though presently only described in animal models, are particularly exciting as they show effective responses not just against primary tumours but also against metastatic disease. Moreover, they stimulate both CD4+ and CD8+ T cells, as well as NK cell, antitumour responses.

Clinical trials of tumour-antigen-loaded DCs have been initiated for the treatment of B-cell lymphoma[10], prostate cancer[12] and melanoma[13]. These initial studies used DCs pulsed with idiotypic protein, peptides derived from prostate-specific membrane antigen, multiple known melanoma-specific antigen peptides or simply with crude tumour lysates derived from biopsy material, respectively. These studies virtually establish 'proof of principle' although, in the case of melanomas, the response rates were not substantially different from those seen after systemic cytokine therapy; thus, 'virtually' because to what degree the responses seen are due to a nonspecific cytokine effect remains unclear. Nonetheless, the successful use of uncharacterized tumour lysate to load DCs with tumour-specific antigens showed that the use of defined tumour antigens may not be required and suggests that DC-based cancer immunotherapy should be widely applicable in most types of cancers where biopsy material is available. Clinical trials using crude tumour lysates to load DCs with uncharacterized tumour antigens have shown a clinical antitumour response, consistent with the presentation of a number of tumour antigens, on both class I and II MHC molecules, by the tumour lysate-loaded DCs which would result in the stimulation of both CD8+ and CD4+ effector T cells and larger numbers of CTLs[13].

The most encouraging results from the various studies on the use of DC-based cancer immunotherapy are those showing that the presentation of tumour antigens by DCs can override the 'immunological unresponsiveness' that has been reported to be a feature of some tumour antigens[14,15]. This inability to mount an immune response to certain tumour antigens is thought to reflect the induction of 'tolerance' to these tumour antigens and is generally accepted as the manner by which tumours evade 'immune surveillance', the mechanism by which the immune system is currently believed to deal with cells that develop potentially neoplastic mutations[2,16]. The most widely studied 'tolerogenic' tumour antigen is the human carcinoma antigen MUC1 and a tumour model for 'immunological unresponsiveness' has been established with the development of transgenic mice expressing human MUC1[14,17]. These transgenic mice are unresponsive to MUC1 antigen even

when immunized with irradiated MUC1-carrying tumour cells. However, recent work has shown that immunization of MUC1 transgenic mice with fusion hybrids of DCs and MUC1-carrying tumour cells induces a potent immune response against MUC1 that not only protects against tumour challenge, but also results in the rejection of established metastases[14]. This reversal of 'tolerance' to MUC1 by the use of DC-tumour hybrids expressing the MUC1 antigen is further supported by the demonstration of MUC1 specific immune responses in chimpanzees immunized with DCs loaded with an MUC1-derived peptide[15]. Both these studies mirror previous studies showing that immunization with donor DCs as APCs can override neonatal tolerance[16,18] and underline the central role of the DC in the generation of the immune response.

Optimal source of autologous DC

Dendritic cells represent a heterogeneous group of cells varying widely in their antigen presentation potential according to their precursor cell of origin, the mode of their in vitro preparation, and the state of their maturation and activation[19]. For optimal preparation of DC, the following characteristics have been considered as predictive of their immunotherapeutic efficacy: well developed dendritic processes and abundant expression of MHC class I and class II and co-stimulatory molecules required for efficient antigen presentation; motility and the capacity to home into the T-cell zones of the draining lymph nodes; and the potential to stimulate and expand quiescent T cells into proliferatively active, antigen-specific T-cell clones[6]. A number of studies have been conducted in an attempt to define 'stand alone' morphological and immunophenotypic characteristics of DC accurately predictive of their antigen presentation potential[19–23]. The clinical studies completed so far have relied on empirical approaches principally directed at producing the required large numbers of DC in a practically reliable and ethically permissible way[6,24]. It is anticipated, however, as discussed below, that future studies will adopt more scientific approaches based on new evidence, using DC of a prescribed high level of clinical efficacy.

Current methods of autologous DC preparation

Methods adopted for preparation of autologous DC in clinical studies include isolation of preformed dendritic cells from peripheral blood mononuclear cells using density gradient centrifugation[10], or in vitro expansion of DC from purified monocyte precursor cells using a mixture of GM-CSF and IL-4 applied for 7–9 days[13,25]. Leukaphoresis may be the preferred method for obtaining the relatively large amount (100–200 ml) of blood needed to yield approximately 10 million DC per patient required for the therapy.

Characterization of in vitro-generated DC

In most studies, morphological characterization of DC has been focused on recognition of dendritic processes using phase contrast or confocal laser scanning microscopy. Flow cytometry has been used as the preferred method for immunophenotyping of DC, with CD1a and MHC class I and class II expression used as an index of the antigen presentation capacity of the DC, and CD80 (or CD86) and CD83 expression as the respective markers of their co-stimulatory capacity and state of maturation.

Antigen loading of DC

To date, the methodologies for antigen loading have been empirical; clearly, the state of DC maturation is an important variable to be considered, and is discussed later. Synthetic peptide(s) representative of T-cell specific antigenic epitope(s), soluble purified tumour-specific antigen, or crude tumour lysate, have all been used for antigen loading for DC for clinical application. The overall approach once again has been largely empirical, with little or no data available on the relative efficacy of the alternative methods used. The dose of antigen and the length of exposure of DC to the antigen, have similarly been empirically derived, with the antigen dose varying between 10 and 100 μg/ml, and the time of DC exposure varying from a few hours to a day, with the incubation conducted either at room temperature or at 37°C. As discussed above, future approaches might include cell fusion, heat shock protein, or liposomal antigen preparation[26].

Clinical testing of DC vaccines: lessons from animal models and progress to date

Animal models consistently demonstrate that DC vaccines are critically dependent on functioning T cells, B7 co-stimulation and T-helper cell Th1 associated cytokines[27], and that specific T-lymphocyte responses induced are powerful enough to eradicate established tumour. In three well characterized tumour models, it has been demonstrated that naïve mice injected with bone marrow-derived dendritic cells, prepulsed with tumour-associated, haplotype-specific peptides, developed an MHC-restricted specific T-lymphocyte response, and were protected against subsequent lethal tumour challenge[28]. Specific CTL response was induced in tumour-bearing mice powerful enough to eradicate established tumour nodules of up to 1 cm^2 in size in more than 80% of mice. Similar results have been achieved in other animal models using a range of antigen sources including specific synthetic peptide, proteins, tumour lysate and tumour RNA[9,11,27–30]. To mediate an anti-tumour effect, specific T cells need to migrate across endothelial barriers and the

extent to which DCs play a part in this is not known. T cells need to remain effective at the tumour site where they may meet a very hostile environment – including tumour cells expressing the Fas receptor. Fas-mediated apoptotic death may occur in the T cell or even the DC, expressing Fas-ligand. A wide range of tumours, including lung cancer, melanoma and colorectal cancers[31–33], have been shown to express the Fas receptor. The environment in the vicinity of the tumour may also be hostile to DC function and contain a variety of influences including IL10[34], VEGF[35] and prostaglandins[36], all of which may interfere with DC antigen-presenting function and promote tumour tolerance. Gabrilovich et al.[37] found that peptide-pulsed DC isolated from tumour-bearing mice showed a significantly reduced ability to induce specific CTL in control animals. Re-stimulation of T cells from immunized tumour-bearing mice with DC from normal mice significantly increased CTL responses to control levels.

It remains to be seen to what extent DCs, with their unique antigen-presenting ability, expression of co-stimulatory molecules CD80 and CD86 and production of Th1 lymphokines can overcome such tumour-induced tolerogenic influences. This is the key question for clinicians treating cancer. To be therapeutically useful, the CTL response needs to be of sufficient duration and power to eradicate tumour effectively. Unfortunately, early clinical studies inevitably tend to include patients whose cellular immune system is likely to be compromised by previous immunosuppressive therapy, as well as the immunosuppressive effects of the tumour itself. This makes assessment of vaccine efficacy difficult. Nevertheless, the demonstration of objective antitumour effect with immune response in early clinical DC studies to date is encouraging.

Nestle et al.[13], in a key study in patients with advanced melanoma, prepared autologous DC from peripheral blood precursors cultured ex vivo with GMCSF and IL-4[6]. The DCs were antigen-loaded with a cocktail of specific tumour peptides or tumour lysate. Objective antitumour responses (two complete and three partial) were seen in 5 of 16 patients after DC vaccination. These patients had relatively low tumour load but metastases were present in multiple organs in most patients. Vaccination was well tolerated with occasional mild fever or swelling of the injected lymph node lasting 1–2 days with no physical signs of autoimmune disease. However, antiTSH antibodies became detectable without clinical effect on thyroid function. An immune response was demonstrated by a positive delayed-type hypersensitivity reaction (DTH) to keyhole limpet haemocyanin (KLH) helper protein in all patients as well as a positive DTH reaction to peptide-pulsed DCs in 11 patients. Significant DTH reactivity ($>$10 mm in diameter) against DCs was observed in five patients of whom four had a major clinical response, two of which were long-lasting. A positive DTH response was observed in two clinical responders lasting more than six months after the last DC vaccine, suggesting that

a sustained cellular immune response had been induced. Two HLA-A0201 patients who developed DTH reactivity toward DC tumour lysate were demonstrated to have peptide-specific CTLs in the DTH biopsy, which induced specific lysis against targets expressing two of the tumour-associated peptides, Melan-A and gp100. Thus, there appeared to be a clear relationship between DTH reactivity and clinical response. Staining of cryosections of the DTH biopsy site revealed large numbers of infiltrating CD8 cells.

In another study, objective tumour regression has been observed using vaccination with DC prepared directly from peripheral blood[10]. Of four patients with follicular lymphoma who received intravenous DC pulsed with tumour-specific idiotypic protein, one patient developed a clinical complete remission, one had a partial response, a third patient was documented as having resolution of all evidence of disease (as shown by a sensitive tumour-specific molecular analysis) and the fourth had stable disease. Again, treatments were well tolerated by all patients and no significant side effects were seen. All patients developed measurable cellular proliferative responses against a control protein (KLH) and the idiotypic protein following vaccination. The degree of cellular immune response was similar in all patients and was sustained for several months. In one patient, peripheral lymphocytes were shown to induce specific lysis in vitro against autologous hybridoma tumour targets.

Lymphoma and melanoma are two tumours where responses to nonspecific immunotherapies are well described. However, other diseases may also be amenable to such an approach. For example, hormone-resistant prostate cancer is a disease where immunotherapy has never previously been associated with a clinical response[12]. Nevertheless, using a DC vaccine pulsed with prostate-specific membrane antigen peptides, it was possible to demonstrate decreased PSA levels in seven patients (which met defined criteria for partial response) as well as enhanced cellular response to one of the peptides. Again, no significant toxicity was observed and there was no antitumour or immune response in those patients who received the peptides alone.

Therefore, clinical studies to date have shown no major toxicities for such DC vaccines and provide evidence of antitumour responses associated with evidence of a specific CTL response against a range of tumour-associated antigens. These antitumour responses have been observed when immature DCs are pulsed with antigen in the form of specific peptide, pure idiotypic protein antigen or antigen derived from tumour lysate.

Which tumours to study for further proof of principle?

In order to demonstrate that a specific CTL response correlates with an antitumour effect in man, tumours with a defined tumour antigen may be preferred. Cervical

cancer provides a good model for investigation because of its well characterized tumour antigens, the gene products of human papilloma virus (HPV). In fact, HPV, particularly HPV 16 and 18, have been demonstrated in 93% of cervical cancers of all cell types[38]. Continued expression of HPV oncogenic proteins E6 and E7 has been demonstrated to be constitutive in an HPV-positive tumour, and is required for the maintenance of the malignant phenotype[39]. This, together with its high frequency of occurrence, makes HPV an attractive 'foreign' antigenic target for immunotherapy of cervical cancer. The presence of HPV-specific CTL has been demonstrated at the site of cervical cancers and draining lymph nodes[40] but, by contrast, is not demonstrable in normal subjects. The potential for DC therapy in this disease is further supported by the sustained remission of established murine C3 tumours which expresses HPV 16 E7 antigen, when animals are treated with DC pulsed with HPV 16 E7 peptides[28]. Furthermore, human dendritic cells pulsed with HPV antigens have been shown to stimulate an MHC-restricted specific T-cell response which kills autologous targets expressing those antigens in vitro[41]. Borysiewicz et al.[42] have shown that vaccination with live vaccinia virus engineered to express HPV 16 and 18 E6/7 antigens (TA-HPV) resulted in a specific CTL response in one of three immunologically evaluable patients with advanced cervical cancer. This approach is currently being further explored in an EORTC phase II study in operable cervical cancer patients. It remains to be seen whether a DC vaccine loaded with HPV antigens offers an advantage over vaccinia-based vaccines in such patients.

As discussed above, the demonstration that immunization with donor DCs as APCs can override neonatal tolerance of the MUC-1 peptide[17,18] supports clinical studies in ovarian and breast cancer. The recent demonstration of CD8 + and CD4 + T-cell responses against the cancer testis antigen NY-ESO-1[43], shown to be expressed in a variety of cancer types, may offer a further antigenic target for such a therapeutic approach. Ultimately, however, it is likely that the patients who may benefit most from immunotherapy will be those who are immunized as an adjuvant treatment to primary surgery. Patients who have had a complete resection of their primary tumour, but who are known to have a poor prognosis as determined by histological factors such as nodal status, may yet prove to be the ideal candidates for clinical studies.

Which patients are immunocompetent for immunotherapy?

Some patients with advanced cancer who may be considered for phase I immunotherapy trials are likely to have disturbed immunological function as a result of the immunosuppressive influences of the cancer itself. Fiander et al.[44], whilst recruiting patients for a phase Ib study of a vaccinia-based immunotherapy, found that T-cell numbers and proliferative responses to the mitogens PHA, Con A and OKT3,

were significantly reduced in patients with established invasive disease as compared with normal controls. Both CD4+ and CD8+ T-cell subsets were affected, even in early stage cervical cancer, and the CD4 and CD8 counts fell significantly with increasing tumour volume to an extent that was independent of treatment. In nine of ten patients (seven of whom had advanced, recurrent cancer), lymphocyte transformation studies, whilst within the normal range, were nonetheless depressed as compared with normal volunteers. It was noted that the patient with the lowest lymphocyte proliferation response also had a low CD4 count. In contrast, B-cell number, immunoglobulins and complement levels were normal, as were in vivo responses to polysaccharide vaccine. Fiander (personal communication) has also noted that 55% of patients with invasive cervical cancer had elevated peripheral levels of IL-10 as compared with normal volunteers. Similarly, Fortis et al.[45] reported increased serum IL-10 levels in 40 of 99 patients with a range of solid tumours. Such immunological profiles, which may reflect tolerogenic influences, may result in the inability to mount a DTH response to a range of recall antigens at skin testing, e.g. using the Pasteur–Merieux multitest skin test, a largely CD4+ T-cell mediated effect. There must be a critical tumour burden beyond which cancer-associated immunosuppressive influences ensure that a response to immunotherapeutic approaches is highly unlikely. However, to what extent DC vaccines can break such tolerogenic influences and mount a significant CTL response needs to be demonstrated. For future studies it is important that indices of cellular immune status are recorded, although it must be said that selection criteria based on immune indices such as the ability to mount a DTH response, remain arbitrary.

Which clinical endpoints should be measured?

While most of the focus in tumour immunology is on the CD8+ MHC-restricted CTL response, recent evidence supports a critical role for the CD4+ T cell in antitumour immune response[46]. To mediate a specific CD8+ T-cell response, activated DCs must travel to draining lymph nodes where they can present MHC class I peptides to CD8+ T cells. However, full activation of DC to induce a CD8+ T cell response requires CD4+ T-cell help, which in turn involves the interaction between CD40 on the DC and CD40L on the CD4+ T cell[47]. The importance of the CD4+ T cell and class 2 peptide presentation has recently been demonstrated in cervical cancer patients. A pool of 14 overlapping 20-*mer* peptides covering the entire sequence of HPV 16 E7 was used to stimulate peripheral blood lymphocytes from cervical cancer patients at different stages of cervical cancer. It was shown that peptide-specific IL-2 secretion depended on CD4+ T cells that were restricted to MHC class 2. IL-2 secretion occurred more frequently in cancer patients with persistent HPV infection, compared to uninfected cancer patients or those without

neoplasia[48] Therefore, it is clear that, ideally, CD4 + and CD8 + responses need to be incorporated in immune endpoints for DC vaccine assessment.

CD8 + MHC-restricted specific response

The key aim of a DC antitumour vaccine has to be the induction of an MHC class I-restricted T-cell response. Consequently, the reliable quantification of antigen-specific MHC class I-restricted T lymphocytes, which may be of low frequency, is a key end point for the monitoring of cancer vaccine responses. The interferon-gamma ELISPOT is able to detect low-frequency, influenza peptide specific CD8 + T cells from peripheral blood with good sensitivity and specificity, as compared with more cumbersome serial dilution experiments[49]. Thus, the IFN-gamma ELISPOT can reliably demonstrate peptide CD8 + T cells from peripheral blood as a means of monitoring vaccination if the class I peptide sequence is known. More recently, Dunbar et al.[50] have described a method which uses MHC class I tetramers to isolate specific CTLs recognizing an HLA-A0201 influenza matrix peptide complex directly from human peripheral blood, sufficient to allow phenotypic characterization and cloning of these CTLs. As a demonstration of the potential of this method to analyse specific CTL responses, an HLA-A0201 melanoma antigen-specific tetramer was successfully synthesized and used to enrich melanoma-specific CTL derived from a tumour-infiltrated lymph node. However, both methods depend on a knowledge of the sequence of the specific MHC class I tumour antigen peptide which tends to be restricted to selected haplotypes, particularly HLA-A0201 individuals. When the antigen is known but the specific peptide is not available, less sensitive methods based on chromium release assays can be successfully employed. Autologous targets for specific lysis engineered to express the relevant antigen, e.g. HPV gene products, have also been used as a 'model' target for in vitro assessment of a CTL response to DC.

CD4 response and T-cell polarization

The above argues for the importance of the CD4 + response, and hence for its place in the immunological endpoint in DC vaccine studies. The delayed hypersensitivity reaction is mediated largely by specific Th1 cells, which release lymphokines and chemokines locally at the site of injection. Such reactions can also involve CD8 + cells, and biopsy of a positive DTH skin reaction may be a source of specific CD8 + as well as CD4 + T lymphocytes, and the former may be expanded in response to specific peptides. Nestle et al.[13] successfully demonstrated the relationship between DTH reactivity and clinical response, and expanded specific CD8 + cells from two melanoma patients who had had a clinical and DTH response to tumour lysate-pulsed DC. DTH reactivity may be a clinical means of monitoring a change

in polarization in response to a DC vaccine. Gabrilovich et al.[37], in his animal tumour model, noted that tumour progression was associated with a change of Th1–Th2 balance in favour of the Th2-like cytokine profile. Effective immunization was associated with a shift to the Th1 phenotype. Therefore, monitoring such changes may represent an important endpoint in examining the efficacy of a DC vaccine in a cancer-bearing individual.

What is the optimum way to prepare and antigen load the DC vaccine?

Most clinical and animal studies to date have prepared DC from peripheral blood CD14+ progenitors with the use of cytokines IL-4 and GM-CSF[6]. Adequate numbers may be efficiently prepared by leukaphoresis. The DC so produced, whilst capable of inducing antitumour immune response, are functionally and phenotypically immature. Maturation with agents such as TNF-α or CD40 ligand produces a phenotype which expresses CD83 and which has been suggested to represent the most allo-stimulatory DC for use in vaccination studies[51]. However, Lotze[52], in his murine tumour models, has found that DC tumour vaccines matured with TNF-α are not good at eliciting an antitumour response. However, Morse et al.[53] have more recently suggested that optimal stimulation is dependent on the appropriate sequence of antigen loading and maturation with CD40L, and that the sequence may vary with the type of antigen since a reduction in antigen uptake with maturation may particularly affect large molecules.

A further consideration is the polarization of the mature DC phenotype, which may be influenced towards a Th2 phenotype by tolerogenic factors such as prostaglandin E2 and IL-10, both of which may be increased in amounts in the presence of invasive cancer. Kalinski et al.[22] found that DC matured in the presence of PGE_2 showed impaired production of cytokines, and bias Th cell development towards a Th2 phenotype. They have proposed that the capacity of DC to produce IL-12 in response to their contact with T cells in the T-cell zone of lymph node, is critical to the generation of a Th1 response. These data imply that the character of the local inflammatory reaction may instruct DC towards priming a Th1 or Th2 response. Polarization towards a Th1 phenotype may be critical to promote a cellular response and could significantly impact on the antitumour efficacy of a DC vaccine.

Determining the optimum practical means of antigen loading DCs is another key issue. Most tumour models have used synthetic MHC-specific peptides which can only be used when the immunogenic peptide sequence is known. Alternative approaches include using tumour lysates, messenger RNA from the tumour, and DC-tumour-cell fusion, and all have proved effective in animal tumour models. The use of tumour lysate as a source of tumour-associated antigen may have several

practical advantages. It avoids the need for viable fresh tumour cells, as well as avoiding the necessity of molecular characterization of haplotype-restricted tumour antigens. However, perhaps the most important advantage is that a potentially broader spectrum of CTL responses to a range of tumour antigens could be induced, rather than to a single well defined antigen. Such an approach has been shown to work in animal models and may reduce the possibility of tumour escape from a single antigen. It may also increase the chance of simultaneous loading of MHC class I as well as class II antigens for presentation by DC[54]. However, there is a need to improve the efficiency of protein antigen loading in such a vaccine. One way to resolve this issue is the use of DC-derived exosomes, which express class I and class II MHC, as well as T-cell co-stimulatory molecules, as a cell-free vaccine. Zitvogel et al. reported the first study to demonstrate that tumour peptide-pulsed DC-derived exosomes were capable of successfully eradicating established murine tumours in a T-cell dependent manner[55]. Furthermore, exosomes may provide a practical alternative means of repeated and prolonged vaccination, and this needs to be explored.

A further approach might be to exploit the stimulatory effects of exogenously administered FLT-3 ligand, which in an animal model has been shown to result in the accumulation of functionally active DC, which in turn may stimulate an effective antitumour response[56]. This approach is currently under preliminary clinical investigation.

Thus, key questions related to optimum antigen presentation and antigen loading need to be carefully addressed in future clinical trials. In view of the scope for major variation in the preparation of such DC vaccines, it is important that considerable attention is given to the precise details and conditions of DC preparation, that the means of antigen loading, maturation and lymphokine polarization are clearly documented for such clinical studies and are correlated with any antitumour effect which may be observed.

An integrated view of why dendritic cells may be central to cancer immunology

The important role of DCs in immunology is highlighted by the emerging concept that, contrary to the conventional view that the immune system exists primarily to distinguish 'self' from 'nonself', the immune system functions primarily to distinguish dangerous from nondangerous antigens[57–59]. This change is in part due to the recent reassertion of the importance of so-called 'innate immunity', which consists of nonspecific components of the immune system, in controlling the antigen-specific acquired immune responses[57,61,62]. This new paradigm of immunity is also suggested by the 'danger model' of the immune system[59], which provides a

conceptual mechanism by which the immune system might distinguish dangerous from nondangerous antigens[57,58].

Thus, current thinking suggests that the immune system essentially makes a 'value judgement' of what is 'dangerous', be it self or nonself, and that cells of the DC lineage make this distinction[57–59]. This central role for DCs is consistent with promising clinical trials using DC-based cancer immunotherapy and poses an interesting question of how DCs recognize antigen as 'dangerous' in the normal functioning of the immune system. The danger model proposes that the most likely signal for 'danger' are signals of cell distress that would be released only by cells undergoing necrosis (and not apoptotic) cell death[59]. It is suggested that this is most likely to be a passive (e.g. an essential cytoplasmic housekeeping gene product) rather than an active signal (e.g. a cytokine), as the production of the latter could be suppressed by a pathogen. However, there is an interesting group of proteins that may be classed as both active and passive signals as defined by the danger model, the heat shock proteins (hsps)[60]. The hsps are a family of stress-induced proteins which are not only the major proteins induced by heat stress, but also contain a number of constitutively expressed members[30,63]. These proteins are not only functionally essential cytoplasmic proteins as required for passive distress signals but are also strongly induced by stresses such as heat shock and, more interestingly for active distress signals, by inflammation members. The suggestion that the hsps may act as danger signals is strongly supported by two lines of evidence from studies on tumour immunology. First, tumour-derived heat shock proteins have been successfully used as cancer vaccines in a number of tumour models in mice[2,64] and also in a preliminary human phase I clinical trial[65]. Secondly, the immunogenicity of tumour cell lines has been shown to be directly related to the expression and release of the stress protein hsp70 from these cells[66]. The latter studies also showed that the amount of hsp70 release from tumour cell lines is related to the amount of necrotic versus apoptotic cell death in these cultures, leaving open the possibility that hsp70 may function as a distress signal as in the danger model[59,66].

Do hsp-bound peptides provide more than just a nonspecific danger signal?

Although the hsps are clearly the best candidates for the danger signals that may trigger the immune response, the specificity of the antitumour response induced by tumour-derived hsp vaccines[64,67] suggests a more important role for hsps than just a nonspecific danger signal[68,69]. An intriguing possibility is that the uptake of hsps by DCs also provides an active mechanism for the efficient capture of specific antigen by these cells[60,70] (Figure 13.1). Indeed, the tumour-specific immunity observed is mediated by the tumour-specific peptides 'chaperoned' by the hsps and it is against these antigens that the resulting immune response is directed[64,65,67]. These cancer vaccine studies have shown that the APCs of the macrophage–DC

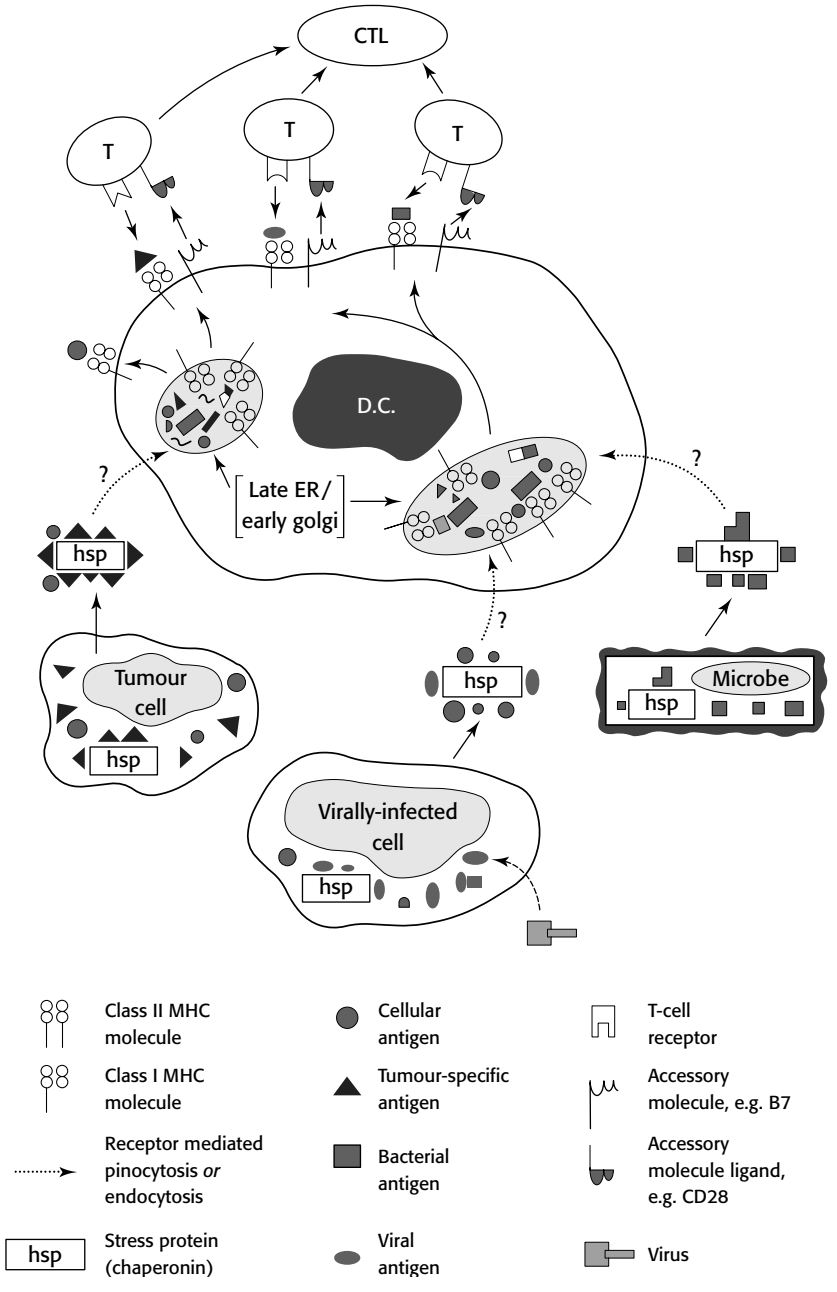

Figure 13.1 Diagram illustrating how DCs utilize stress proteins (hsps) to efficiently capture chaperoned antigens for presentation to T cells in an MHC-restricted manner. Antigens from cells undergoing necrosis, such as irradiated tumour cells or virally infected cells, are bound to cellular hsps and thus taken up by DCs that recognize hsps (antigens from extracellular pathogens such as bacteria are bound to endogenous hsps and thus taken up directly by DCs). The chaperoned peptides–hsp complexes are internalized and the captured peptides stripped off and loaded onto MHC class I molecules in the endoplasmic reticulum/Golgi compartment of the DC. Expression of these MHC–peptide complexes on the DC surface enables the activation of CD8+ CTL precursors bearing antigen receptors of the relevant specificity to the MHC-restricted peptide presented

lineage can take up hsp-tumour peptide complexes and present efficiently these chaperoned peptides to CD8+ T lymphocytes, yielding tumour-specific CTLs. Most importantly, these studies have shown that the hsp-chaperoned peptides are independent of the MHC type of the tumours from which they are derived, whereas their presentation to the CTLs is MHC class I-restricted and is defined by the MHC phenotype of the APC used. This suggests that the hsp-chaperoned tumour peptides feed into the normal antigen–MHC class I loading pathway, a conclusion supported by the sensitivity of this presentation to intracellular ATP concentrations and brefeldin A, which both inhibit the intracellular loading of class I MHC molecules with antigen[64,67].

Tumour immunological surveillance or blindness?

If the normal mechanism by which the DC picks up antigens is via hsps as proposed[60] (Figure 13.1), it is interesting to consider the implications of this hypothesis on tumour immunology. As it seems unlikely that tumours would produce and release hsps unless subjected to exogenous stresses such as radiation or chemotherapy, it follows that the immune system may normally be 'blind' to tumours and, contrary to historical dogma, 'immune surveillance' as a physiological response[16] does not exist. This suggestion is indirectly supported by the studies demonstrating the efficacy of tumour-derived hsps as cancer vaccines[64,65] and more directly by the studies showing unambiguously that the in vivo immunogenicity of tumour-cell lines is dependent on the expression and release of the stress protein hsp70 from these cells[66]. Furthermore, it could also be argued that the extremely high incidence of cancer and the correlation of the mathematically predicted genetic mutation rate with the incidence of colorectal cancers[71] are both more consistent with the suggestion that the immune system is blind to tumours than with the dogma of immune surveillance. From a therapeutic viewpoint, however, the theory of 'immune blindness' gives one confidence that the approach of DC-based cancer immunotherapy is the way forward as the antitumour CTLs are present in the patient and only need to be activated to mount an effective antitumour response.

The future – a plea for well designed clinical trials

The therapeutic use of DC in the immunotherapy of cancer is one of the most promising developments in tumour immunology in the last 30 years. Nonetheless, progress in this complex field requires that the same rigour be applied to clinical studies as has been applied to basic scientific studies. It is clear that there are many phenotypic variations on the theme of the 'dendritic cell'. Should we be treating patients with mature or immature DC? CD34+ cell-derived or CD14 (monocyte–macrophage)-derived? How should DC be primed? Should we add exogenous

heat shock proteins to the antigen preparation (or induce endogenous hsp)? Should we use incubation with tumour lysate or fusion to tumour cells to load DCs with tumour antigens? Unless these different approaches are compared in preclinical studies, it is difficult to envisage how an optimum clinical protocol can be developed.

However, let us assume that such studies are undertaken, and that we have a strategy, or series of strategies, that are promising enough to be considered as 'standard' protocols for DC preparation. Adequate phase I or II studies should define the best protocol (if necessary, using randomized phase II studies to distinguish between competing protocols). Most importantly, the successful protocols should be adequately tested in a phase III setting against other immunological – or non-immunological – treatments to define carefully the place of DC-based immunotherapy. For example, in the case of HPV-associated cervical cancer, randomized trials should compare the use of DC with TA-HPV, with fusion peptide vaccines, or with ex vivo expanded HPV-specific CTLs, as well as with systemic cytokines such as IL-2.

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14

Overview

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Introduction

The volume has covered aspects of the development of therapeutic cancer vaccine strategies against a variety of molecular targets and diseases with a strong bias to the generation of specific cell-mediated (CTL) responses. This brief overview will consider some common lessons with respect to: (1) target molecules; (2) delivery systems; and (3) evaluation methodology relevant to success of immunotherapy for cancer. A summary of the rationale, optimism, limitations and further keys for development for the various cancer vaccine approaches outlined in this volume is given in Table 14.1.

Target molecules

Viral targets

When there is an established viral aetiology for particular malignancies such as HPV with cancer of the uterine cervix or EBV with nasopharyngeal carcinoma, virally encoded tumour-associated molecules offer exogenous cancer vaccine targets where there is unlikely to be immunological tolerance at the immune repertoire level. However, prevention will always be better than cure, so immunization to reduce infection is likely to be more efficacious and cost-effective than immunotherapeutic approaches. This is clearly shown by the example of the association of hepatitis B virus with hepatocellular carcinoma, where classical prophylactic vaccination programmes have dramatically influenced the incidence of the cancer in at risk populations[1]. A similar strategy for the high risk papillomaviruses associated with cervical neoplasia is also planned[2].

Overall, the implementation of worldwide immunization against viruses such as HPV, where the malignant disease is a late complication of the viral infection, may be difficult. This is because the virus involvement is necessary but not sufficient for cancer development, and the malignancy develops following a multistep process and involves several genetic events. Nevertheless, the consistent and obligate

Table 14.1 *Summary*

Cancer vaccine approach	Rationale and optimism	Further development or limitations
Chapter 2: Immunotherapy of bladder cancer	Long-standing approach which works, but we do not know why	Key question: is it specific or nonspecific, or both? Will further knowledge of the above improve morbidity and efficacy?
Chapter 3: Poxviruses as vectors for cancer immunotherapy	Mimic natural danger but some evoke strong vector responses which might subvert required specific immunity	Need to know the target, rather few definitely identified, and none yet shown to be therapeutic May show greater efficacy in combination approaches with heterologous vectors
Chapter 4: Vaccinia-based human papillomavirus vaccines in cervical cancer	Clear targets since HPV causes cervical cancer but necessary but not sufficient. Other genetic changes may provide idiotypic targets in individual cancers	Natural history of immunity does not show whether E6 and E7 T-cell immunity will necessarily be effective in treatment Are other viral proteins important targets? What types of immunity are most important? No suitable models of this disease Immune escape by HLA dysregulation and HPV variants
Chapter 5: Vaccine delivery and immunosuppression in cervical cancer		Peptides which can be good targets in vitro may not be naturally processed in vivo Individual HLA-type vaccines may not be very efficient in the light of immune selection Patient immunity may be specifically or nonspecifically compromised

Table 14.1 (*cont.*)

Cancer vaccine approach	Rationale and optimism	Further development or limitations
Chapter 6: Vaccines for colon cancer	CEA target has impressive variety of preclinical evidence and potential multiplicity of approaches for cancer vaccine therapy	Peptide design can improve things but need to consider restricted usefulness Different pox-related vectors may offer some advantages, such as reduced potential toxicity Immune escape important in determining who to vaccinate
Chapter 7: MUC1 vaccines and breast cancer	Not all cancer targets are necessarily protein epitopes. Antibodies can have a direct effect	What are the relative contributions of antibody, nonspecific or specific T-cell immunity?
Chapter 8: Anti-idiotypic vaccination	Indirect method of targeting cancers	Substitute for knowing real target – does it help when you do? Does this mimic a natural process? Is this practical on a larger scale?
Chapter 9: Immunotherapy and vaccination against Epstein–Barr virus-associated cancer	EBV aetiology helps to focus on potential immune targets but virus is essentially commensal	Is the problem related to immune suppression or immunogentics? Cannot prevent exposure so no catch-all prophylactic strategy, identify people at risk and reduce the likelihood of malignancy
Chapter 10: Serologically identified tumour antigens as cancer vaccines	Important in further understanding of the natural history of cancer but as yet no clear evidence that the target antigens will be useful	Are these targets the evidence of failure or misdirection of the immune response and part of the positive selection of tumours? How important is humoral immunity in cancer?

Table 14.1 (*cont.*)

Cancer vaccine approach	Rationale and optimism	Further development or limitations
Chapter 11: CTL-defined cancer vaccines in melanoma and other epithelial cancers	First clear evidence in melanoma of useful induced tumour immunity using peptides to differentiation antigens, easy to prepare and, with appropriate adjuvants or cytokines, can work	Problems of immune escape at HLA and epitope expression levels Strong argument for polyclonal responses
Chapter 12: DNA vaccines against B-cell tumours	Unique targets but individual antibody purification from patients can work and may be simpler with DNA with built-in adjuvant from bacterial sequences and helper type epitopes	Logistics of preparing pure DNA easier than protein. No clear evidence of how to optimally deliver DNA vaccines. Oral routes provide an exciting possibility but regulatory and safety issues barely addressed Combinations with pox vaccines work well Nucleic acid approaches can be used to address polyclonal vaccines
Chapter 13: Dendritic cell approaches to immunotherapy	All vaccines probably work in some way through this route and may therefore be the most important centre for successful immunization	Can be used with or without a knowledge of specific target using tumour protein or nucleic acid Autoimmune problems not yet properly considered

expression of viral oncogenic proteins in the malignant cells offers defined candidate targets for therapeutic immunization. A priori, the evolution of these genes, to ensure the virus production, must have included selection to avoid the attention of the cellular immune response both by oncogene sequence and general virus life cycle stealth strategies. The first generation of high risk HPV16 oncogene vaccines (peptide, protein or vaccinia based) are already in clinical trial but there is evidence that specific T-cell responses to HPV 16 E7 can be associated with progression to

malignancy in the natural history of cervical neoplasia[3]. Just because a molecule should be a good target it does not follow that it will be. In addition, it is possible that patients with a virally-associated cancer could have been 'selected' from the immunogenetically disadvantaged, although this is difficult to establish[4]. On the other hand, the demonstration of high frequency and complex patterns of down-regulation of HLA class I molecules in progressing cervical intraepithelial lesions and cancers is consistent with immune selection of the tumours to avoid specific CTL or other effector populations, such as NK cells[5]. HPV therapeutic vaccines for cervical cancer are at the forefront of the tide of optimism surrounding tumour immunotherapy and it is important that clear evidence of success emerges in the next few years. The rational selection of immunocompetent patients whose tumour will be suitable for immunotherapy (residual HLA expression) and treatment with multiepitope vaccines will be of central importance to the demonstration of measurable clinical efficacy.

Self antigens

Mutated antigens

Most tumours are not associated with exogenous pathogens but much progress has been made in defining other tumour-associated antigens suitable as immunotherapeutic targets. The knowledge that particular cellular genes are mutated in the process of carcinogenesis in different tissues has provided a category of targets analogous to viral oncogenes. Thus, CTL to mutated p53, ras and Cdk4 have been demonstrated. Many of the cellular tumour suppressor genes associated with human cancer have functions which regulate the cell cycle and DNA repair. Their dysfunction leads to accumulation of further genetic changes which are an integral part of the process of malignant transformation. Many of these genetic changes (deletions, frame shifts, point mutations) or altered posttranslational modifications, may also generate potential tumour-associated antigens. These may play some role in, and be typical of, a particular type of cancer or could be totally unique to an individual tumour (idiotypic) and not necessarily of any functional significance. The immune response should not be innately tolerized to such antigens but immune selection of the tumour in individuals may favour, for example, a particular p53 mutation in their malignant cells and thus influence the natural history of the malignancy[6].

Nonmutated antigens

A much championed group of tumour-associated antigens with potential as cancer vaccine targets has emerged which are not altered by mutation of the coding sequence. These tumour antigens may be expressed inappropriately by virtue of altered developmental control (oncofetal antigens), overexpression compared to

normal tissues (quantitative antigens) or show restricted tissue-specific expression (differentiation antigens). The advantage of these self-antigens is that they may be useful to treat a broad range of different common cancers. However, there may be a requirement to break tolerance and this carries a risk of inducing autoimmunity. Clearly, if the side effect is tolerable, for example vitiligo associated with some CTL-based immunotherapies of metastatic melanoma, compared with the potential therapeutic effect and the certainty of death, the choice is simple. This principle has focused much attention on the so-called cancer testis (CT) antigens since the potential for autoimmunity versus testis can be balanced with the relative proportions of several common cancers expressing these targets.

Initial attempts to define tumour antigens were driven by the advent of monoclonal antibodies and focused on tumour-cell surface molecules with very restricted normal tissue expression. Now, 25 years later, it is the recognition that human sera from cancer patients can contain specific antibodies to a plethora of potential tumour-associated antigens expressed from cDNA libraries made from tumour cells, which is driving an explosion of activity in defining SEREX targets. An important limitation to these screens is that they ignore any changes that may depend on posttranslational modifications. As a note of caution for too much euphoria concerning the multiplicity of new targets, there are as yet few examples of SEREX antigens which have been analysed even to the level of preclinical models. One aspect of the optimism associated with SEREX approaches is the likely amelioration of regulatory issues accompanying the approval of such new therapies because a natural humoral response occurs in cancer patients with no obvious toxicity. However, the natural history and immunological significance of these antibodies in human cancer is virtually completely unknown. Thus, one might conclude that since they are found in patients with cancer they are not necessarily a positive attribute and could reflect a type of immune enhancement of malignant growth.

The list of antigens defined by CTL–autologous tumour pairs in vitro is biased by the definition of the MAGE and other related families (CT antigens) where the original patient was highly immunized with melanoma tumour cells. In other cancers this approach has identified mainly idiotypic type tumour antigens. The generation of human CTL versus specific peptides derived from a putative tumour antigen sequence by multiple re-stimulations in vitro is no proof that such effectors will be useful or even exist in vivo. A critical question is to what extent tumour-associated antigens of any origin are recognized naturally in patients since there are very few examples where such CTLs have been documented in unimmunized patients. This might be because the antigens are: (1) insufficiently immunogenic (thus immunological intervention may be helpful); (2) naturally immunogenic but the repertoire of potential T cells has been eliminated or suppressed during the

natural history of the cancer (will immunization make any difference?); or (3) not in the peripheral blood (where are they and how will we know that vaccination does anything?).

Delivery

At the fulcrum of many cancer vaccine strategies is the belief that the various tumour-associated targets can be delivered in an immunogenic fashion and generate useful and safe antitumour cell-mediated immunity. There is some substance to the focus for generating specific CTL effectors based on evidence from animal models of tumour immunity. However, it is not clear how valuable these models of tumour protection are compared to the natural history of human cancer. Indeed, it is fair to say that there is virtually no data concerning any specific immunity causally related to disease outcome in patients with cancer. Our ideas about cancer vaccine strategies may be over-influenced by the many principal investigators who agree the best approach is to generate CTLs. The demonstration of interference with tumour surveillance in knockout mice with defective cytokine receptor–signal transduction pathways emphasizes the possibility that other types of specific T-cell effectors and their downstream consequences may be equally important, or even more important, in the development of effective immunotherapeutic strategies[7].

At present, the delivery vehicles for the tumour antigens are mostly aimed at generating CTLs. There are basically three approaches whereby tumour-associated antigen (TAA) is delivered either as protein, encoded by the recombinant virus or by DNA encoding the TAA. The goal is to establish appropriate preclinical studies, the proof of principle of tumour immunity and to maximize the immunogenicity and minimize any toxicity. The protein vaccines, if delivered as specific HLA-binding peptides, have the advantage of being cheap to make for clinical practice and their inherent simplicity supports the prejudice that they are likely to be safe. On the other hand, they can only be used in patients with particular HLA types, such single epitope vaccines (possibly modified to improve immunogenicity) may fall foul of the frequent downregulation of HLA class I in human cancer and they probably require addition of adjuvants to elicit any type of long-lived immune response. All things being equal in most cases, the use of the whole TAA molecule or the means to express it offers more options for the immune response provided it is delivered in such a way as to elicit a useful tumour immune response. By definition, it is likely that this has already failed to occur or be sustained in cancer patients. However, providing there is an immune repertoire with sufficient residual potential for TAA-reactivity delivery of the protein TAA or the encoding nucleic acid to the antigen-presenting cells directly or indirectly must occur in any vaccine therapy. These events must seek to elicit the activation of T cells so as to maximize

the ability to deal with the tumour while balancing the potential for generation of autoimmunity.

Live recombinant vectors based on various pox viruses can induce both CTL and antibodies to tumour-associated antigens. They take advantage of the host response to the virus infection which elicits the appropriate danger signals but suffer from delivering many viral proteins which can distract the desired specificity in responses and minimize the ability to boost immunity by multiple vaccinations. Other viral vectors not discussed in this volume (adenovirus, herpes virus, Semilki forest virus, influenza) may offer advantages in safety but are potentially limited in efficacy by pre-existing immunity in patients[8]. However, successive vaccination with different vectors encoding tumour-associated antigens is an attractive proposition. Indeed, the use of DNA vaccines followed by recombinant viral vectors may offer additional advantages[9]. Using naked DNA directly, eliminates the problem of responses to viral vector antigens and there are several physical delivery systems which have been shown to induce CTL responses. Oral delivery, which may allow for the mass vaccination of at risk populations in some viral associated cancers with deliverable logistics, is an exciting prospect and studies of microencapsulated DNA vaccines[10] or recombinant bacterial vectors[11,12] are very encouraging. The immunogenicity of chimeric proteins engineered as virus-like particles[2] or yeast Ty particle[13] have also recently been shown as effective in generating CTLs and may offer additional opportunities for antitumour immunization.

Discussion of the vaccine type and delivery method and site of immunization highlights the requirement of the induced immunity not only to be of the correct type and specificity, but also in the appropriate location[14]. In general, activation of specific T cells is controlled by the restricted distribution of specialized antigen-presenting cells which are only activated at a site of infection or damage by the danger signals. Following the subsequent processing, transport and delivery of relevant antigens to secondary lymphoid tissue by activated DCs, specific T cells are activated, expanded and subsequently migrate to the site of danger or damage. Tumour cells cannot directly activate T cells even if they do express appropriate HLA–peptide complexes because they cannot provide the necessary second signals. There have been several cancer vaccine strategies based on modifying tumour cells to deliver such second signals with co-stimulatory and/or cytokine modifications of autologous[15] or allogeneic[16] tumour cells. There are often severe logistical limitations to such approaches and ultimately the activation of specific T cells probably depends on cross priming through host dendritic cells. A more important issue is how will tumour-specific T cells know where to go and what is the best route to induce relevant cellular immunity? Although we are aware of the problem we do not necessarily know how to produce the most effective protocol to deliver the solution.

One of the major advances in the potential for manipulating antitumour immunity is the capacity to manipulate human dendritic cells. All of the delivery vehicles for TAA available can be used ex vivo with isolated human dendritic cells derived from either CD34 stem cells or monocytes. In almost all cases of antitumour vaccines, a target antigen has been chosen reflecting the expression patterns of a particular malignancy or its viral aetiology. An important novel opportunity offered by direct DC vaccine approaches is the concept of utilizing all potential tumour antigens (multiple neoantigens resulting from cumulative genetic changes in natural history of individual cancer) by DC–tumour fusion or DC transfection with tumour biopsy RNA[17]. Thus, while every vaccination would be individually specific there would be many potential targets and since DC can elicit primary activation of T cells, exploitation of a different part of the repertoire than previously possible in the patients. Thus, direct activation by DC vaccines might overcome existing anergies in T-cell responses to tumour targets or override elements of generalized immune dysfunction such as downregulation T cell CD3 zeta. The downside is the individualistic nature of the treatment and the potential for autoimmunity.

Evaluation

The plethora of cancer vaccine strategies available with good preclinical study credentials will generate champions for many and varied clinical trials in different types of cancer patients. The challenge is to optimize the trial series design to ensure safety, immunogenicity and measurable (relevant) clinical efficacy. This will require the development of better and more specific tools for evaluation of both immunological and clinical aspects of cancer vaccine treatments. The choice of malignant target and stage of disease are probably critical to the latter but less so to safety issues. While most preclinical tumour models can provide protection, truly therapeutic situations are usually not studied in animals and are never comparable to late-stage human disease. In order to measure a clinical effect one needs to recruit patients for trials with greater than a poor prognosis.

The concept of stimulation of antitumour immunity is biased in many approaches to cell-mediated therapies working through cytotoxic T cells. There is often no easy way to monitor the induction of these effectors following in vivo vaccination except by in vitro re-stimulations requiring lengthy and logistically difficult assays. The ELISPOT and tetravalent HLA methodologies can suffer from requiring predictions about the target epitopes for tumour antigens and the functional relationship of these populations of T cells to active tumour immunity is not established as compared to those documented for virus infections[18,19]. Measurement of T-cell subpopulation activity by proliferation assay or specific

cytokine–chemokine release or by intracellular FACs staining[20] can also provide measures of tumour immunity which may be of direct significance. In the final analysis it is the induction of specific immunity at tumour sites which will be important and there is no a priori reason why these will be accessible by sampling from the peripheral blood. Isolation of tumour-infiltrating T cells may give a better idea of the relevant and useful specificities to stimulate for successful antitumour immunity[21]. Delivery of ex vivo stimulated and expanded CTLs may help to study the importance of the kinetics and migration of effector cells to tumours in patients[22]. Antibodies may be more important in tumour immunity than most tumour immunologists realize but again local immunity (e.g. mucosal IgA) may be critical in relation to any therapeutic effect. Despite the sophistication of our immunological insights and molecular biological skills in developing tumour vaccines we still do not know in humans whether they will really work. It is ironic that the cancer vaccine with most evidence of clinical efficacy (BCG in bladder carcinoma) is based on the oldest and nonspecific approaches. However, there is enough anecdotal evidence of clinical responses from recent trials with specific vaccines for lymphomas, colon cancer and melanoma which can be explained by the proposed immunological rationale. A major problem resulting from tumour heterogeneity is selection of immunological escape variants which are analogous to the development of chemoresistances. It seems most reasonable to see cancer vaccines as part of the armoury of cancer treatments which are available to oncologists. The future may see the use of immunotherapies immediately following surgery and prior to any potentially immunosuppressive treatments. The momentum for possible adjuvant use will depend on continued improvements in our ability to diagnose cancer at an early stage.

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15

Recent developments

Peter L. Stern

This final chapter aims to update the reader with recent developments in the field of cancer vaccines.

Dendritic cells (DCs) and the control of T-cell activation

A key area of basic research likely to influence the development of cancer vaccines is the increasing understanding of the processes whereby T cells are activated or anergized by interaction with DCs.

An important new concept is of early events which cause immature DCs to sense and relay information about the nature of the danger which subsequently influences the character of the responses[1]. The first step in the activation process for DCs derives from the local microenvironment (pathogen-induced or -derived, or constitutively produced tissue factors) when encountering a specific damage or danger. Following DC maturation and differentiation with presentation of processed antigen and acquisition of co-stimulatory potential, there is a polarization of the T-helper type of response selected in the draining lymph node (LN). Thus, control of the development of naïve T-helper cells into T-helper type 1 (CTL, NK etc.) or type 2 (B-cell activation, isotype switching, etc.) subsets is biased by the first experiences of the DC. Therefore, specific T cells are presented not only with the antigen structure but also the nature of the pathogenicity (danger). Since the window of sampling for such DC-mediated polarization is short it may not necessarily be the same in every LN, allowing for independent regulation.

DC production of the cytokine IL-12 is very important in the subsequent development of Th1 responses but production or responsiveness to other well recognized Th1 or Th2 driving factors (IL-4, IL-10, prostaglandin E2 and IFNγ or α) are also regulated by IL-12. The early activation and later maturation of the DC under the influence of the integrated cytokine milieu produces a population of stable DCs with different levels of IL-12 production and co-stimulatory ability which controls the ratio of Th1–Th2 response. Investigations in animal models to optimize the priming of T cells and effectiveness of cancer vaccines has shown that local pro-

duction of IL-12 by irradiated tumour cells can cure mice with a considerable leukaemic burden[2].

DCs are quiescent in most tissues and control of their activation is critical to balancing the requirement to stimulate protective immunity to pathogens without generating autoimmunity. A recent study has shown that DCs can be activated by endogenous signals received from cells that are stressed, virally infected or killed necrotically but not by healthy cells or those undergoing apoptosis[3]. Resting DCs seem to be able to respond to prepackaged and inducible signals from damaged cells in the absence of foreign antigens. It is argued that DCs can capture both necrotic and apoptotic cells but only the former can generate DCs with co-stimulatory ability. Thus, DCs can discriminate between tissues that are damaged by stress or infection and those undergoing tissue remodelling as a normal process. In a pathogen infection, damage is done, necrotic cells are generated with a cascade of other factors, which might also include those of microbial origin not normally produced by vertebrates (LPS, teiochic acid, nonmethylated CpG or double stranded RNA), and the adaptive immune response is brought into play and orchestrated by the activated DCs.

It is instructive to consider what types of priming of DCs occur in the natural history of cancer, because these events appear to lead to anergy rather than activation. In early cancer, like normal tissue homeostasis, there may be no damage and cell loss is principally by apoptosis. If unstimulated DCs operate to capture apoptotic cells and present the captured antigens, without co-stimulation this may induce tolerance in autoreactive or tumour-associated antigen (TAA)-specific T cells. This concept is supported by reports that show that dying allogeneic apoptotic T cells producing IL-10 can promote allograft acceptance by tolerogenizing reactive T cells by deletion and regulation[4]. However, under other circumstances, it appears that apoptotic cells phagocytosed by APCs together with IL-2 can also act as potent tumour vaccines[5]. The recent demonstration of a key role for indolamine 2,3 dioxygenase (IDO), which catabolizes tryptophan, in protection from maternal T-cell attack of the mammalian fetus, may also have wider implications for control of T-cell responses in cancer, with tumour-associated macrophages making IDO-enforcing cell tolerance to TAA[6]. Clearly, there is a major challenge ahead to further characterize and exploit these various activation and anergic signals to create natural adjuvants with which to elicit effective anti-tumour immunity without autoreactivity. A series of papers has now clearly shown the benefits of using CD40 activation to overcome peripheral T-cell tolerance. Antibodies that activate the CD40 receptor can alter otherwise tolerogenizing immunogens and/or enhance antitumour vaccine efficacy[7–9]. The likely mechanism is the bypassing of a requirement for T cell help with CD40–CD40-ligand interaction on the DC leading to priming of naïve tumour-specific T cells. This

supports the clinical use of CD40 stimulating agents as components of anticancer vaccines.

If relevant T-cell memory of TAA exists then it would be important to activate this optimally. A recent report[10] has shown that T-cell memory consists of two populations that differ in their expression of the CCR7 chemokine receptor which controls homing to secondary organs. CCR7−ve effector cells express receptors for migration to inflamed tissues and display immediate effector function, whereas central memory T cells are CCR7+ve, lack immediate effector function but efficiently stimulate DC and differentiate into CCR7−ve effector cells upon secondary stimulation. In the context of immunotherapy, the prime goal must be to activate the specific central memory T cells in the LN and to convert them to T cells that could enter the tissues. Is this a case for immune activation prior to any surgery to remove local nodes?

The studies reviewed here provide an important experimental framework for the development of rational vaccine and clinical trial protocol design which is likely to be the major growth area in tumour immunotherapy in the next few years.

Progress in the clinic

One important target for therapeutic intervention which has not been dealt with in the previous chapters is prostate cancer. It is one of the leading causes of cancer deaths in the Western Hemisphere. Men with rising prostate-specific antigen (PSA) serum levels after primary therapies such as prostatectomy or radiotherapy are a significant patient group for whom no standard therapy option exists. PSA and prostate-specific membrane antigen (PSMA) show selective expression by the prostate and their use as immune targets is analogous to the cancer-testes (CT) type antigens[11]. It has been shown that peptides derived from these proteins can invoke CTLs and T-helper type responses[12,13] and some cases lyse tumour cell lines[14]. Recently, clinical trials have been initiated using HLA-A2 restricted PSMA peptide loaded DC[15] or the latter with GM-CSF[16,17]. The preliminary results show positive immune responses in about 30% of patients with no demonstrable additional influence from GM-CSF. These studies suggest that DC-based cancer vaccines may provide an alternative therapy for prostate cancer patients whose primary treatment failed. Another clinical trial has used irradiated autologous prostate tumour cells engineered to secrete GM-CSF with induction of both T- and B-cell immune responses[18]. It is sobering to consider, however, that in prostate cancer 85% of primary tumours show HLA class I downregulation[19] and virtually all metastases[20].

In bladder cancer, long-term follow-up of intravesical BCG treatment for stage T1 grade 3 bladder carcinoma continues to support its use for conservative treatment in this disease[21]. Indeed, a subsequent three month bladder biopsy is not nec-

essary in patients with normal orifice cystoscopy or an erythematous bladder and normal urine cytology[22].

In HPV-associated malignancy there is a plethora of activity to refine therapeutic vaccine approaches. The potential advantage of direct delivery of HPV oncogene proteins in human DCs for generating HPV-specific CTL responses[23,24] and the recognition by CD4 tumour infiltrating lymphocytes of HPV oncogene HLA class II restricted peptides[25] have been demonstrated. Interestingly, chimaeric HPV 16 L1E7 virus-like particles appear to be immunogenic in the absence of adjuvant[26]. With the overwhelming evidence that high risk HPV types cause most anogenital malignancies, the opportunity for prophylaxis is also being explored. A key factor of relevance to both prevention and treatment is how to stimulate the appropriate local immunity. Model systems have now shown that mucosal immunization appears to offer some advantages[27] including overcoming pre-existing poxvirus immunity[28].

The multicentre European study of TA-HPV, a live recombinant virus expressing the human papillomavirus 16 and 18 E6 and E7 proteins, in patients with early cervical cancer, has now closed. However, because of the overall good prognosis for this group of patients any clinical outcomes will only be manifest after a long time (five to eight years). HPV (mostly type 16) is also found in a high proportion of vulval intraepithelial neoplasia (VIN) cases. The incidence of the high grade lesions (VIN3) appears to be increasing and its multifocal presentation and uncertain risk of progression to cancer disease makes it a difficult disease to treat and there is a high recurrence rate. Previous clinical data have shown that TA-HPV is capable of producing an immunological response with evidence of a B-cell reaction and, perhaps more importantly, a specific cytotoxic T-cell response, even in patients with advanced HPV-related disease. This approach is now to be further studied in women with VIN3 in a clinical trial centred in Manchester, UK. As well as studying the safety, tolerability and immunogenicity of the vaccination, the trial design can provide for evidence of clinical efficacy by comparing the extent of VIN3 and HPV status of the lesions before and after TA-HPV vaccination. The progress of the various HPV oncogene-based therapeutic vaccines is likely to be an important barometer of cancer vaccine progress in general and the current trial outcomes are awaited with eager anticipation.

Results from phase I clinical trial of patients with colon cancer given recombinant vaccinia virus (rVV) encoding carcinoembryonic antigen (CEA)[29] or autologous tumour cells with IL-2 expressing fibroblasts[30], found either no evidence of specific immune stimulation or reported pre-existing CTLp at low frequency which may be increased by the protocol. These studies highlight the logistical difficulties of measuring tumour-specific cell-mediated immunity following immunotherapeutic vaccinations.

Several trials using anti-idiotypic approaches have been reported. CeaVac, representing an internal image of CEA, induced both cellular and humoral immune responses in all patients and the use of 5-FU in Dukes' C cases did not affect the immune responses[31]. With SCV 106, mimicking 17-1A glycoprotein, it has been shown that vaccination of immunologically responding metastatic colorectal carcinoma patients leads to slowed disease progression, decreased tumour dissemination and significantly longer survival time[32]. Finally, 105AD7 which represents decay accelerating factor[33], can increase the level of activated lymphocyte infiltration to the tumour areas, suggesting potential use in adjuvant therapy in early disease[34].

Melanoma is the clinical target with a multiplicity of peptide-, vector- or tumour-based vaccine approaches in the clinic. Several reports on melanoma tumour cell-based approaches, in some cases with cytokine delivery, have all been shown as safe. However, the studies present a range of attempts to either evaluate or demonstrate immunogenicity (specific or nonspecific) but, throughout, all objective clinical responses are rare[35–38].

Peptide-based vaccines using MART-1 delivered with adjuvant, generated peptide-specific T-cell responses in about half of the patients as detected by ELISPOT, with some correlation with improved survival[39]. In contrast, gp100 peptide given with IL-2, reduced the number of circulating specific T cells in patients, but these appeared to have a better clinical response[40]. These studies emphasize the difficulties of rational measurement of immune consequences in the peripheral blood of patients. The important site is the tumour, which is difficult to access for routine monitoring of immunogenicity of vaccination in a clinical trial. This problem is further illuminated by using HLA–peptide tetramers to enumerate melanoma antigen-specific T cells with the demonstration that increased vaccine-specific T-cell frequency following immunization can correlate with in vitro stimulation but may not lead to tumour regression[41]. In another study, circulating T cells specific for tumour-associated antigens in melanoma patients were shown to be functionally unresponsive[42]. Combination of MAGE-3 HLA-A1 peptides with DCs for vaccination of advanced melanoma patients produced significant expansion of CTL in 8 out of 11 patients which subsequently declined. However, individual metastases showed regression in 6 of 11 patients and significant CD8+ T-cell infiltration[43].

Peptide-based vaccines using ras oncogene mutations in advanced cancers[44] or HER-2/neu with GM-CSF in patients with breast or ovarian cancer[45], have also been reported. A review of various peptide trials has been presented by Bellone et al.[46].

Perhaps the best cases for clinical efficacy of immunotherapies have been shown in the treatment of various lymphomas. This may result partly from the availabil-

ity of unique target antigens and patients with relatively good performance indicators following conventional therapy-induced remissions. In follicular lymphoma, residual t(14;18) positive lymphoma cells can be detected in some patients even following chemotherapy and being in complete remission. This allows the effects of treatment to be monitored with great sensitivity using PCR-based assessment of the lymphoma-associated translocations in the peripheral blood. Thus, Bendandi et al.[47] immunized follicular lymphoma (FL) patients in remission with a vaccine combining an FL-associated idiotype IgG with keyhole lympet haemocyanin (KLH), the latter to provide for additional T-helper recognition and generation of optimal CTL as well as to provide for a positive control to monitor T-cell priming in the patients. The vaccine was administered with GM-SCF. Eight out of eleven patients with previously molecularly detectable disease subsequently developed sustained molecular remissions. In all, 19 of 20 patients developed CD8 and CD4 specific tumour T cells, whereas antibodies were detected but not required for clinical responses. The clear message from this study is that cancer vaccines can work and be seen to work by expected mechanisms when the target and trial design is appropriate. The possibilities for further lymphoma approaches based on using DC are now being actively explored[48,49].

The future

Cancer vaccines are only one aspect of the harnessing of basic molecular and cellular studies which underwrite current cancer research. The advantages of understanding the molecular basis of cancer, particularly with respect to cell cycle control, is of enormous potential importance for the development of new cancer therapies. But the natural history of cancer often involves genetic changes which in themselves offer opportunity for immune recognition. Immune recognition may be the basis for further selection and tumour progression. In a clinical context, the real challenge is the treatment of metastatic disease. Efficacious cancer treatments work quickly (complete surgical and/or chemo- and/or radiotherapeutic 'excision') and presumably allow little time for selection of resistant variants. The successful use of immunotherapies based on cancer vaccines is likely to be similar. The use of therapeutic vaccines may find an effective niche with likely clinical efficacy in stimulating antitumour immunity with multiple target specificity in patients with minimal residual disease to prevent recurrence. Vaccine treatment of some more advanced tumours may also show important clinical value when set against the relative ineffectiveness of some current treatments with their concomitant influence on quality of life. Those working in the field of immunotherapy translational research are optimistic that immune approaches will deliver real treatments for malignant disease in the next few years.

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Index