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Review

Generation of cellular immune responses to antigenic tumor peptides

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Abstract. Tumor immunotherapy is currently receiving close scrutiny. However, with the identification of tumor antigens and their production by recombinant means, the use of cytokines and knowledge of major histocompatibility complex (MHC) class I and class II presentation has provided ample reagents for use and clear indications of how they should be used. At this time, much attention is focused on using peptides to be presented by MHC class I molecules to both induce and be targets for $CD8^+$ cytolytic T cells. Many peptides generated endogenously or given exogenously can enter the class I pathway, but a number of other methods of entering this pathway are also known and are discussed in detail herein. While the review concentrates on inducing cytotoxic T cells (CTLs), it is becoming increasingly apparent that other modes of immunotherapy would be desirable, such as class II presentation to induce increased helper activity (for CTL), but also activating macrophages to be effective against tumor cells.

Key words. Tumor antigens; vaccines; class I presentation; class II presentation; peptides; toxins; adjuvants; antigen targeting; clinical trials; CTL epitopes.

Introduction

Every 20 years there is a reemergence of interest in tumor immunotherapy: in the 1970s BCG mixed with tumor cell lysates was used, and while some initial successes were found in melanoma, the procedure was ultimately abandoned. Tumor immunotherapy has again reemerged, but this time there are many different ways of attacking tumors, and optimism is high that these will work. The reason for the recent emergence of tumor immunotherapy is based predominantly on the use of genetic engineering techniques, which have made difficult techniques a reality. The major advances are in several areas (table 1):

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1. Detection of tumor antigens

Tumor antigens have been detected either as unique antigens found on tumors (especially melanoma) and on few normal tissues; or they are present in larger amounts in tumors, so that the relative amount of antigen in a tumor is much greater than in the normal tissue, e.g. mucins are present in 40–100-fold higher concentrations in breast cancer than normal resting breast epithelium [1]. The antigens—either tumor-specific or tumor-associated—can be oncogenic products, expressed uniquely in the tumor after gene mutation or rearrangement, or be overexpressed in cancers to give a differential expression in tumors. It should be noted that particularly in melanoma, the starting point for detecting tumor antigens was the description of cytotoxic lymphocytes (CTLs) against tumor antigens such

as MAGE, gp100 and tyrosinase [2], whereas other antigens, such as mucins, could be suitable targets simply because there is a lot present in the tumor and the description of CTLs followed the identification of mucins as a potential tumor target [3, 4]. In most cases the identification of the tumor antigens was accompanied by the isolation of the complementary DNA (cDNA) or genes encoding the antigens.

2. Production of tumor antigens

The antigen can be produced in large amounts by recombinant techniques, either as fusion proteins in bacterial or other systems, or as soluble molecules in eukaryotic systems. Furthermore, synthetic peptides can be made from the tumor antigen; this must be one of the major technical breakthroughs in tumor immunotherapy—previously whole tumors were irradiated or used as lysates, and such preparations contained only small amounts of antigen which was not reproducible in production, consistency or stability. These problems have been largely overcome by making synthetic peptides or recombinant proteins. In addition, monoclonal antibodies to the tumor antigens can be used in the identification and purification of tumor antigens. Thus, tumor specific and tumor associated antigens have been identified and are available in virtually unlimited amounts.

Table 1. Recent advances in tumor immunotherapy.

	Reference
Identification of tumor antigens	(see table 3)
Production of tumor antigens	157, 158, 159
• prokaryotic expression	
• eukaryotic expression	
• synthetic peptides	
Identification and production of cytokines and chemokines	
• IL-1-18	160
• TNFα, β	160
• IFNα, β, γ	160
• chemokines	5, 6
Knowledge of the immune system	
• presentation of peptides by MHC class I and II	9
• peptide trafficking into cells and into the pathway	161
• affinity vs. tolerance	162
• DC in vitro/in vivo	12
Assays of cellular immunity	
• CTL/CTLp (LDA)	13
• proliferation	14
• DTH biopsy	130
• cytokine production ELISPOT; intracytoplasmic cytokines	15, 16
• tetramer binding	17, 21

3. Cytokines

Cytokines have been described in the last 20 years, and now there are ~ 25 cytokines [IL-1-18, TNF-α, -β, interferon (IFN) and others] and 50 chemokines and their receptors [5, 6]. There is almost an embarrassment of riches here, for to understand the mode of action of each cytokine and their interactions in a clinical setting is an enormous undertaking. These agents will be useful in manipulating immune responses to tumor antigens, particularly in patients whose immune system is suppressed [7, 8].

4. Function of the immune system

There is now more knowledge of how the immune system functions, and how cellular immune responses are generated [9]. Major histocompatibility complex (MHC) restriction is now understood in molecular terms and refers to the restricted repertoire of peptides presented by either class I or class II MHC molecules—the repertoire being different for each MHC allele. This knowledge has led to the peptide approach to tumor immunotherapy. Peptides (8–9mers) can be presented by MHC class I molecules, leading to the generation of $CD8^+$-mediated cellular responses consisting of CTLs and cytokine secretion (predominantly IFN-γ and TNF-α). Class II molecules present longer peptides (10–15mer) to $CD4^+$ cells which can make different cytokine responses—the so-called T1 or T2 responses. Thus far in tumor immunotherapy, most work has concentrated on the presentation of peptides by MHC class I molecules to $CD8^+$ cells, and attempts to generate $CD8^+$ CTLs are currently at the forefront of tumor immunotherapy. Unfortunately, many tumors survive and evade the immune system by having major alterations or deletions of their MHC class I molecules which would decrease CTL activity [10]. Thus far, little has been done on class II presentation of tumor antigens, and as most solid tumors do not express class II molecules, tumor cells cannot be directly attacked by cytokine-secreting CD4 cells. However, infiltrating class II + cells, e.g. macrophages, could lead to the generation of $CD4^+$ helper cytokine secretion to improve the induction of $CD8^+$ CTLs and have direct cytolytic or destructive effects. In addition, there is much knowledge of the intracellular trafficking of peptides—either into the class I or class II pathways, and much of this review will concentrate on 'diverting' peptides from intracellular degradation pathways in lysosomes and late endosomes into the cytoplasm where they can be processed and ultimately presented by MHC class I molecules to induce $CD8^+$ effector cells [11].

5. Antigen-presenting cells (APCs)

The dendritic cell (DC) has emerged as a central player in generating either antibodies after class II presenta-

tion or CD8 CTLs after class I presentation. A recent innovation has been to obtain DCs from patients, isolate and expand in vitro, feeding them peptides and transferring such 'loaded' DCs into the patient [12]. Others are using such loaded DCs to generate specifically activated T cells in vitro prior to transfer.

6. Measuring cellular immunity

Over the last 5 years much time has been expanded on measuring CTLs or their precursors, using tedious limiting dilution (LDA) assays, often after multiple rounds of restimulation in vitro taking up to 5–6 weeks to obtain results [13]. Lymphocyte proliferation of either CD4 or CD8 cells has also been performed, but it is difficult to interpret the meaning of this in patients immunized with complex structures like whole tumors [14]. In vivo delayed type hypersensitivity (DTH) responses have been measured—often unrewarding in immunosuppressed patients, but more recently, the biopsy and histological examination of DTH reaction has been of value; still, it is difficult to quantitate such responses. More recently, cytokine production by cells in culture (the ELISPOT assay) has been introduced, and our recent description of the detection of intracellular cytokines by flow cytometry in cancer is proving useful [15, 16]. Another technique described by Altman et al., particularly in infectious disease and now in cancer, has been the binding of class I MHC plus peptides into a tetrameric complex, which then binds with high avidity to T cells bearing the appropriate receptor for the complex [17, 18]. At times, up to 50% of peripheral T cells can bind tetrameric complexes, although such figures are unusual [19]. Nonetheless, up to 1–2% of circulating cells are often found which bind antigen more sensitively than a CTL precursor frequency of 1:20,000–100,000 [20]. In addition, melanoma-specific HLA-A*0201 tetramers consisting of tyrosinase 368–376 or modified Melan-A 26–35 peptide detected CTL in tumor-infiltrated lymph nodes at frequencies as high as 1/30 $CD3^+CD8^+$ cells [21]. Furthermore, Melan-A-specific CTL isolated from tumor-infiltrated lymph nodes by flow-cytometric sorting were efficient in killing autologous tumor cells.

Thus, there is an enormous amount of information and reagents available to attack tumors. There are now many trials in progress using tumor antigens with or without cytokines, including in vitro sensitization of DCs. As a major focus in tumor immunotherapy is on the induction of CTLs, this review will concentrate on methods of inducing such CTLs for tumor immunotherapy, including peptide antigen processing and how this can lead to peptides being selectively presented by either class I or II molecules.

Antigen-processing pathways

MHC class I presentation pathways

An essential first step in CD8+ T cell generation is the uptake and presentation of peptides by MHC molecules by antigen-presenting cells (APCs). MHC class I proteins consist of three subunits, the heavy chain made up of $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains of $M_r = 45$ kDa and a light chain $\beta 2$-microglobulin (12 kDa); in close association with the $\alpha 1/2$ domain is an 8–9mer peptide in the MHC groove (fig. 1). All three components are crucial for the formation of a stable complex. MHC class I molecules can present endogenous nuclear and cytosolic peptides, and exogenous antigens. For MHC class I presentation, exogeneous antigens are taken up by APC into endosomes and escape into the cytosol (either directly or after degradation) where they enter the proteasome, which is responsible for their further degradation (fig. 2). As will be discussed, the presence of a peptide (of either endogenous or exogenous origin) in the cytosol is required for processing to the class I pathway. In the proteasome are two low molecular weight subunits, LMP2 and LMP7, whose activity increases after induction by IFN-γ, which increases the generation of peptides (fig. 2). These peptides are then transported into the endoplasmic reticulum by a transporter (TAP), where they become available to newly synthesized MHC class I molecules, and in toto are presented on the surface of the APC (figs 1 and 2) [22–24]. Other pathways (including a TAP-independent pathway) have also been discussed.

X-ray crystallography of MHC class I molecules with their bound peptides has provided information on peptide binding. The interaction of peptides with the MHC class I groove is determined by the peptide sequence, with discrete amino acids interacting with pockets in the MHC groove (which have a fixed spacing from each other) and have specificity for anchoring amino acid side chains. Crystallographic studies of different MHC class I molecules have shown that the amino and the carboxy termini of the peptides are anchored at either end of the MHC groove, and in many cases these are anchors in positions P2 or P3, P5 or P7 (although there are exceptions) [4, 25]. The peptide length is determined by the MHC allele: for example, $H2K^b$ preferentially binds 8mers, HLA-A*0201 and $H2D^b$ 9mers, and up to 13mers can be bound by HLA-Aw68; however, most MHC alleles bind 8- or 9mers [24]. The peptides also interact with the T cell receptor, although in general little of the peptide is exposed; low-affinity peptides are more exposed [26]. Recent information indicates that glycosylation of exposed amino acids can be detected by the T cell receptor [27]. Information on

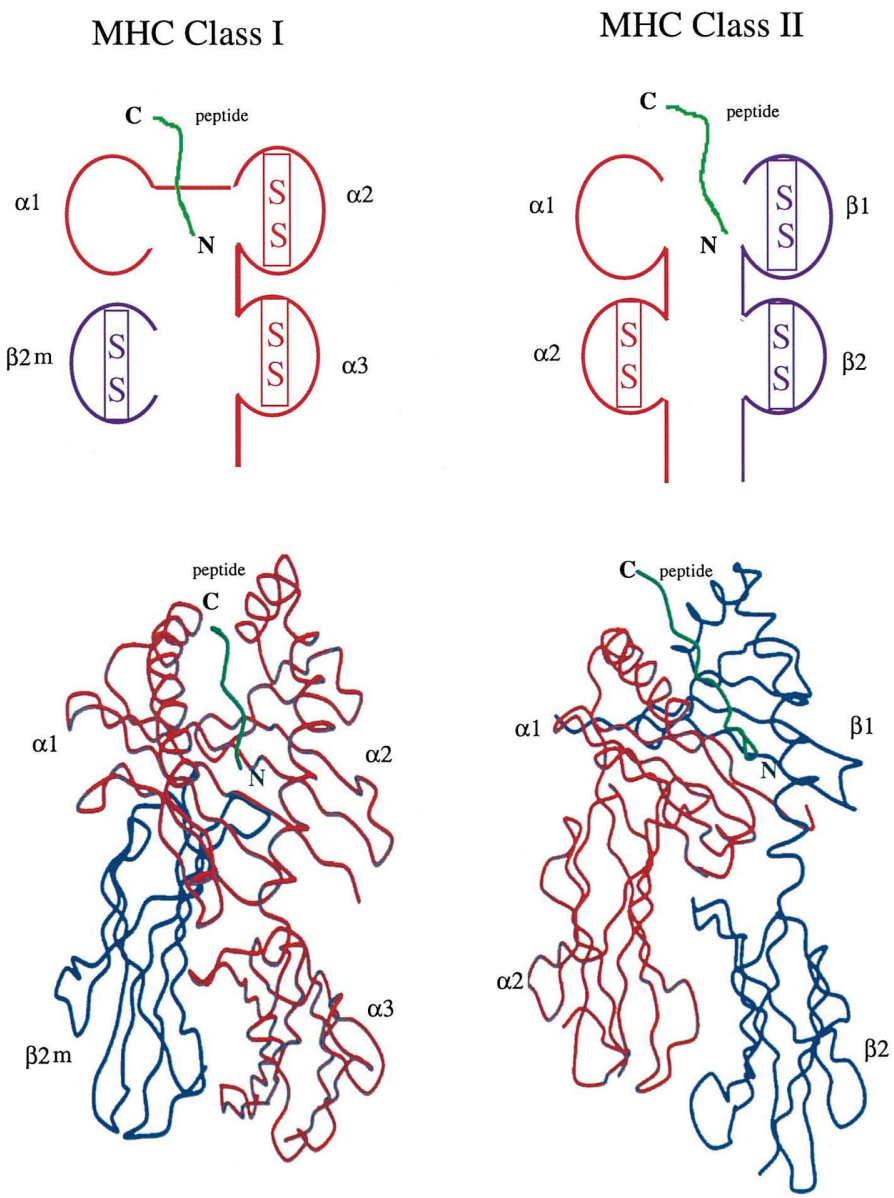

Figure 1. Schematic representation (top) and 3D representation (botttom) of MHC class I and MHC class II molecules. This figure was created using MOLSCRIPT and RASTER3D as described previously [181].

MHC class I peptide and presentation and allele-specific motifs can be used to identify unknown T cell epitopes within a protein of known sequence; however, epitopes can be missed by this method, as can low-affinity binding peptides [28].

MHC class II-processing pathway

MHC class II proteins are heterodimers consisting of α and β chains of similar size (30 kDa), which bind the peptide (fig. 1). Class II molecules are formed in the endoplasmic reticulum, where the two chains are bound by the invariant chain (Ii) [29], which prevents premature loading of peptides and directs the α/β heterodimer to the class II loading compartment. The mode of interaction of the Ii chain with the α/β heterodimer is not clear, but certain Ii peptides called CLIPs (class II-associated invariant chain peptides) have been found with some class II molecules. The peptide loaded α/β heterodimers are presented on the surface of the APC

(figs 1 and 2). The interaction of peptides in the MHC class II groove is also determined by fixed pockets in the MHC groove which have specificity for anchoring side chains of the amino acids in the peptide. Usually the first position of the peptide, P1, is a hydrophobic anchor fitting into a deep pocket; other anchors can be found at P4, P6, P7 and P9 (and occasionally at P3 or at P5). Epitope prediction of class II binding peptides is not as accurate as for class I, as the anchors are more degenerate in their nature.

For a tumor antigen to contain immunogenic epitopes, there must be the appropriate peptides within the protein which can bind to either or both class I/II molecules. Further, the peptide must remain intact after proteolysis of the whole protein in endosomes and proteasome. For this degradation, it is clear that adjacent sequences are important and may alter the processing of the peptides. Clearly the use of small synthetic peptides, especially if they could be taken up, processed and presented by the APCs, has appeal. However, each synthetic peptide can be presented only by a restricted number of MHC alleles—thus mixtures of peptides may be necessary for presentation by a number of alleles.

Peptide-based vaccines

With the identification of peptide tumor antigens and the characterization of T cell epitopes, it is now possible to make peptide antigens synthetically for immunization. Synthetic peptides alone are rarely useful for immunization, as they are too small and are rapidly lost, either in the urine or by proteolysis, and approaches used to increase their utility will now be described.

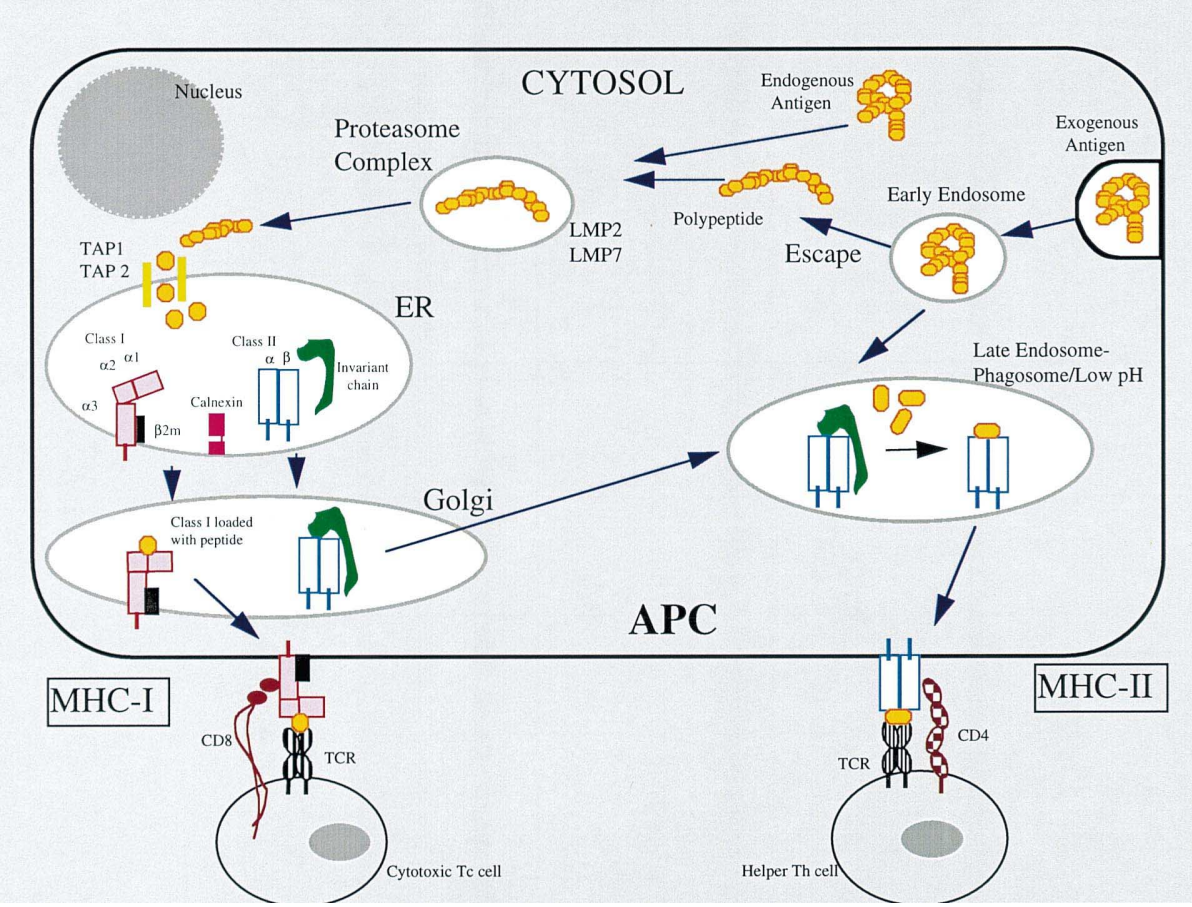

Figure 2. Schematic overview of MHC class I and MHC class II processing pathway.

Increasing the immunogenicity of peptides

The immunogenicity of small peptides can be increased by making the peptides larger, by binding to carriers and also by using adjuvants to activate macrophages and other elements of the immune response. Recombinant cytokines such as IL-2, GM-CSF and IL-12 have also been used to increase the immunogenicity of synthetic peptides [30]. Cytokines can also be directly fused to peptides. For example, (i) a fusion protein containing IL-2 and HSV-1 glycoprotein D-induced strong cellular and humoral immunity to HSV-1 [31]; (ii) a fusion protein consisting of a B cell idiotype to granulocyte macrophage-colony stimulating factor (GM-CSF) induced strong antiidiotypic antibodies and protected mice from a lethal challenge of tumor cells [32]; (iii) IL-4 fused with a B cell tumor idiotype was immunogenic. The IL-2-idiotype fusion protein generated high titres of IgG2a and IgG3 antibodies, whereas IL4 and GM-CSF proteins did not [33].

It should be noted that of the many adjuvants used in preclinical studies [34], few have been approved (e.g. Alum) or used in clinical trials.

Overcoming antigenic heterogeneity and genetic variation

The disadvantage of using a single peptide is that due to MHC restriction, only humans with a particular allele will be able to bind that peptide, compared with the intact protein which may contain multiple epitopes that can be presented by different alleles. To overcome this problem, mixtures of separate peptides can be used, or a number of epitopes maybe attached together as a 'string of beads' using either peptide synthesis or genetic engineering techniques [35, 36]. Infectious viruses such as human immunodeficiency virus and influenza virus continually change the amino acids in the hypervariable regions of their coat proteins; to overcome this antigenic variation of pathogens, combinatorial peptide libraries have been used for immunization [37]. A library of 7.5×10^5 related 22–25mer peptides derived from 180 sequences of the human immunodeficiency virus (HIV) gp120 V3 loop was used to immunize rabbits. The antibodies reacted with closely related isolates but not with a distantly related isolate. The combinatorial peptide strategy could be also used in cancer to increase the binding affinity of class I binding peptides by using different amino acids at the anchor positions.

Peptide stability

Novel strategies are being used to overcome the stability of peptides, including the development of retro-inverso peptides, where the natural amino acids are replaced with unnatural D-amino acids, and assembling the peptide in the reverse order; such peptides are resistant to proteases and are usually immunogenic. For example, immunization using a retro-inverso analog of the 141–159 peptide of the VP1 protein of foot-and-mouth-disease virus gave rise to neutralizing antibodies and protection in guinea pigs similar to using the natural peptide [38]. In another example retro-inversion of a class II binding peptide led to loss of class II binding and the ability to cause T cell stimulation, and the peptide did not bind antibodies raised to the native peptide [39]; in another study, 300-fold more retro-inverso peptide than native peptide was required for IL-2 release [40]. A partial retro-inverso influenza matrix protein peptide, M58–66, retained binding to HLA-A2 molecules [41]. Such retroinverso-peptides could be used in cancer therapy.

Another approach is to use unnatural modified amino acids. For example, a study of the proteolytic degradation pathways of the MAGE-1 HLA-A1 CTL peptide EADPTHGHSY (using high pressure liquid chromatography/mass spectrometry [HPLC/MS]) indicated cleavage between EA followed by cleavage between AD and SY by exopeptidases as the major degradation pathway in serum ($T1/2 \sim 30$ min) [42]. By careful analysis of the anchor positions and the TCR contact residues, a series of nonnatural peptides was synthesized, of which an analog with α-aminoisobutyric acid in the P2 position and *N*-methylserine in the P8 position was stable in serum for more than 24 h, bound HLA-A1 and activated MAGE-1-specific CTL clones.

Modification of CTL peptide to improve immunogenicity

The immunogenicity of CTL peptides is dependent on the binding strength of the 9mer to the MHC complex and subsequent recognition by the T cell receptor. Examination of the binding affinity, rate of dissociation from the MHC and immunogenicity (CTL responses) of a series of high-, medium- and low-affinity CTL peptides indicated that a low disssociation rate (≥ 3 h) was more favorable for immunogenicity than the measured affinity [43], i.e. the longer the peptide remains bound to the MHC complex, the greater the chance it will induce a T cell response. Thus, altering amino acid residues within CTL peptides will help to increase the lifetime of peptides in the MHC on the surface as demonstrated by P2 analogs of the wild-type HLA-A11-presented IVTDFSVIK epitope [44].

In another study a bacterial fusion protein containing the high-affinity D^b-restricted influenza nucleoprotein peptide linked to human β2m enhanced MHC stabilization and immunogenicity [45].

Thus, small peptides alone are unlikely to be of value because of their small size. However, there is at least one report of their effectiveness intradermally [46].

Table 2. Approaches for producing immune responses to peptides.

			Reference
Target receptors on antigen-presenting cells		B cells	47, 48
		T cells	49, 50
		Monocytes	51
	* α2-macroglobin receptor		52, 53
	* transferrin receptor		54
	* mannose receptor		57, 58
	* Fc receptor		59
Microbial toxins	* anthrax toxin		61
	* diphtheria toxin		63
	* *Pseudomonas* exotoxin		64
	* *Bordetella* pertussis toxin		65
Membrane fusing agents	* *Drosophila* transcription factor (*Antennapedia*)		75
	* HIV Tat protein		76
	* measles virus fusion peptide		77
Lipopeptides			78, 79, 80
ISCOMs			83, 84, 86
Heat shock proteins			103, 104

There are several modifications which can be used to make them immunogenic, including carriers, adjuvants and more recently, cytokines and sequence variations to make them resistant to proteases and acceptable to proteasomes; different peptide epitopes can be coupled together to give a more immunogenic complex. Clinical studies with synthetic peptides will be discussed in detail later.

Targeting antigen-presenting cells

As well as entering the cytosol, access to antigen-processing pathways also involves receptor-mediated uptake or phagocytosis by cells. The intracellular delivery of proteins or peptides can also be accomplished using carrier molecules that directly bind a receptor on the surface of antigen-presenting cells and transfer the antigenic peptides to the desired site in the cell for entry to class I and II pathways. The carrier molecules can be either antibodies to cell surface antigens present on APCs, or ligands that bind to a receptor (table 2). As the procedure is highly efficient, the targeted delivery of antigen enables a lower dose of antigen to be used and can target different pathways to generate different types of immune responses.

B cells are efficient APCs due to surface immunoglobulin, which captures antigen for subsequent class II MHC expression. Targeting antigens to B cells by antibody to surface Ig or to Fc receptors results in enhanced antibody responses. A study of the relative efficiency of presentation of hen egg lysozyme to a T cell hybridoma indicated that HEL antigen targeted to sIgD, sIgM or to class I or class II molecules was presented efficiently by normal splenic B cells but not at all when targeted to FcγRII or B220 (CD45) [47]. The covalent binding of tetanus toxoid to complement C3b also resulted in an increased stimulation of specific T cell proliferation when exposed to B cells [48]. Thus for antibody production, targeting B cells has appeal.

T cells are capable of antigen presentation, but they lack the phagocytic capabilities of dendritic cells and macrophages, or the antigen-capturing capacity of B cells. Nevertheless, antigens targeted to CD4, CD8 or transferrin receptor molecules on T cells can be effectively presented. It is interesting that the gp120 protein of HIV, which binds CD4 on T cells and monocytes, can be presented effectively to gp120-specific human T cells [49]. In addition, a hybrid particle containing the hepatitis B envelope protein coupled to the HIV gp 120 was internalized via CD4 and presented to hepatitis B envelope peptide S193–207 recognizing T cell clone [50]. Tumor antigens have not been used as yet for such studies.

Monocytes. Targeting of peptide epitopes to monocyte FcγRI receptors was done with a recombinant monovalent anti-FcγRI Fab containing a T helper epitope of tetanus toxoid TT830; the fusion protein was 1000-fold more effective than the free peptide in stimulating tetanus toxoid-specific class II-restricted T cell lines [51].

Natural ligands to cell surface receptors have also be used for targeting antigens to APC. The endogeneous plasma proteinase α2-macroglobin has also been used as a vehicle to carry an HIV-1 peptide to macrophages via an α2-macroglobin receptor, resulting in high-titre anti-HIV-1 antibodies [52]. In addition, it was also shown that the use of the mouse α2-macroglobin rather

than human α2-macroglobin further increases antibody production [53].

Transferrin-mediated targeting of the HLA-A2-restricted 57–68 influenza virus matrix protein-derived peptide was internalized by the transferrin receptor and presented to class I via a TAP-independent pathway; however, the concentration of conjugated peptide required for sensitization was 5–10 times higher than free peptide [54].

Mannose receptor. Peptides linked to mannose have been used to target APCs by mannose receptor-mediated uptake, and such mannosylated antigens have 200–10,000-fold enhanced capacity to stimulate class II-restricted peptide-specific T cell clones [55]. When mice were immunized with a mannosylated HPV E7-glutathione-*S*-transferase fusion protein, CTLs were generated and mice were protected from a lethal dose of E7-expressing EL-4 tumor cells [G. Pietersz, unpublished]. The mannose-containing carbohydrate mannan, isolated from the yeast ceast cell wall, when chemically conjugated to a mucin-1-glutathione-*S*-transferase fusion protein generated strong CTL responses in mice and protected mice from MUC1$^+$ tumors [3]. The type of immune response was dependent on the type of chemical linkage, where the oxidized form of the conjugate generated a T1 type of response and the reduced form a T2 response. Studies on the mechanism of action indicated that the oxidized conjugate binds the mannose receptor and internalizes with concomitant escape from the endosomal compartment due to the presence of aldehydes [56].

Scavenger receptor. Endotoxins, oxidized low-density lipoprotein and other negatively charged proteins are internalized by the scavenger receptor family and degraded in macrophages. Maleylated ovalbumin binds the scavenger receptor, which enhances its presentation to ovalbumin-specific MHC class I-restricted CTL by macrophages and B cells [57]. In addition, maleylated diphtheria toxoid was also more immunogenic than nonmaleylated diphtheria toxoid and generated enhanced antibody and T cell proliferative responses [58].

Fc Receptor. An alternative method of targeting antigens to APC is to use 'antigenized' antibodies. Here the potential immunogenic peptides of 11–15 amino acids are inserted into the complementarity-determining regions (CDRs) of the antibody by genetic engineering techniques. The antigenized antibody binds the Fc receptors of the APC, is internalized, processed and presented to the T cell receptor. Immunoglobulins containing both T-helper cell and B cell epitopes have been engineered [59]. When such antibodies are exposed to dendritic cells, presentation to to a class II-restricted T cell was 1000 times more efficient that presentation to a class I-restricted T cell.

The targeting approach has inherent advantages in that no adjuvants are needed. Antigens maybe targeted to any cell surface receptor either by using a ligand or an antibody to the receptor, and using genetic engineering techniques fusion proteins may be synthesized with tumor antigen, antigen-binding capacity and fusion peptide sequences to direct antigen into the cytoplasm. Very few of these techniques have been applied to tumors but will be used in the future to induce tumor-specific responses.

Cytoplasmic delivery for MHC class I presentation

As described, it was thought that extracellular antigens are endocytosed, degraded in lysosomes and presented by class II molecules, whereas peptides from intracellular or nuclear proteins are presented by class I molecules. There are now many exceptions to this, and using these exceptions strategies have been designed to introduce extraneous peptides directly into the cytoplasm to enable class I-restricted cellular immune responses to be generated; such methods include microbial toxins, membrane-fusing agents, lipopeptides, immune-stimulating complexes and liposomes.

Microbial toxins

Microbial toxins enter cells by receptor-mediated endocytosis and deliver cellular toxins into the cytoplasm, i.e. they have a cytoplasmic delivery system, which can be used to carry other peptides. Most toxins consist of (i) a binding domain which binds to a specific receptor on the cell surface; the complex internalizes where (ii) the toxic domain is released from the endosome into the cytoplasm. Fusing foreign peptides to the toxic domain enables the delivery of such peptides directly to the cytoplasm for subsequent presentation by the class I pathway. Examples of this are:

Shiga toxin. A recombinant fusion protein composed of the receptor-binding nontoxic B chain of Shiga toxin fused to MAGE 1 melanoma antigen generated class I-restricted MAGE 1-specific CTL [60].

Anthrax toxin. A protein containing the amino terminal 254 amino acids of the toxic domain of anthrax fused to HIV gp120 was produced, and when the protein was exposed to cells in the presence of the internalizing domain, presented class I peptide to gp120-specific CTL [61]. The N-terminal domain of the anthrax lethal factor was also used to deliver OVA (257–264) and Listeriolysin (91–99) peptides to induce CTLs and protection from *Listeria* in mice [62].

Diphtheria toxin. The toxic domain of the diphtheria toxin can translocate 12–30 amino acid peptides across cell membranes and may also be used for the translocation of CTL inducing peptides [63].

Pseudomonas exotoxin. A fusion protein of the binding domain of *Pseudomonas* exotoxin and influenza A matrix protein or nucleoprotein was internalized and processed [64].

Bordetella pertussis toxin. This toxin enters cells by direct translocation across the cell membrane without the need for a specific receptor. *Bordetella pertussis* toxin-containing peptides from the V3 region of HIV-1 gp120 or lymphochoriomeningitis virus (LCMV) were capable of eliciting CTL responses [65]. In addition presentation of the ovalbumin SIINFEKL peptide by APCs could occur when fused to a nontoxic derivative of pertussis toxin (PT9K/129G). It was of interest that the processing of this complex was inhibited by brefeldin A (which inhibits trafficking of pertussis toxin in cells and secretion of class I/peptide complexes), but not by the inhibitors lactacystin or LLnL (which inhibit proteasome activity) and not affected in TAP-ve cells, suggesting an alternative route to the proteasome TAP-dependent route for antigen presentation [66]. However, in a more recent study a nontoxic mutant *Bordetella pertussis* toxin (CyaA-OVAE5) with inserted ovalbumin SIINFEKL peptide when exposed to APC presented ovalbumin CTL epitopes using a TAP-dependent pathway [67].

Live bacteria [e.g. *Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus Calmette-Guérin* (BCG)] can also be used to translocate CTL peptides from viruses and bacteria into the cytoplasm to generate cellular responses and can be applied to tumors [68–72]. Thus microbial toxins can be readily utilized to translocate peptides or proteins into the cytosol of cells; however, the value of such constructs in humans remains to be seen, due to the presence of immunity to some toxins.

Membrane fusing agents

The previous section described methods of introducing antigens into the cytoplasm by toxins; in this section peptides that directly access the cytoplasm by membrane fusion will be discussed. Special peptides gain entry into the cytoplasm by membrane fusion and can be used to carry tumor or other peptides into the cytoplasm.

Antennapedia. The DNA binding domain (homeodomain) of the *Drosophila* transcription factor *Antennapedia* is spontaneously internalized by all cells by a nonreceptor-dependent mechanism by forming an inverted micelle [73, 74]. This peptide is 60 amino acids long and consists of 3 α-helices, the region responsible for internalization being mapped to a 16-amino acid peptide within the third helix; this 16mer peptide can be used to directly internalize peptides or proteins into the cytoplasm. For example, (i) a fusion protein consisting of the 60-amino acid homeodomain with 170–179 HLA-Cw or with an influenza nucleoprotein 147–156 peptide could prime for the different class I restricted CTLs in DBA/2 mice [75]; (ii) a 24mer peptide consisting of the 16-amino acid fusion peptide (RQIKIWTQNRRMKWKK) of *Antennapedia* and the SIINFEKL epitope of ovalbumin generated ovalbumin-specific CTLs in mice and protected mice from a lethal challenge of ovalbumin-expressing EL4 tumor cells [G. Pietersz, unpublished].

Tat protein. The 86-amino acid protein Tat from HIV-1 has cell membrane translocation properties which have been mapped to a 9-amino acid peptide (RKKRRQRRR). Ovalbumin covalently linked with the 9-amino acid peptide was presented by class I molecules. Immunization of mice with dendritic cells pulsed with this complex led to the production of antigen-specific CTLs [76].

Measles virus fusion peptide. A membrane-fusing peptide from the amino-terminal sequence of measles virus was fused to a peptide of the measles virus nucleoprotein; mice immunized intraperitoneally with this chimeric peptide induced CTLs which could lyse measles virus-infected target cells [77].

Such hybrid peptides using a fuseogenic peptide with the peptide of interest are attractive in that they can be easily synthesized and could be useful for the ex vivo pulsing of dendritic cells studies as an alternative to surface loading for use as cancer vaccines.

Lipopeptides

Lipopeptides directly enter the cytoplasm by insertion into the lipophilic cell membrane and contain both a lipid and peptide. The lipid component is a long aliphatic hydrocarbon chain such as palmitoyl coupled to the peptide. For example, (i) a palmitoylated 26-amino acid peptide from *Plasmodium falciparum* liver stage antigen-3 was found to be highly immunogenic in mice and chimpanzees, leading to long-lasting T and B cell responses [78]; (ii) the C-terminal 11-amino acid peptide of the SV40 large tumor antigen was covalently linked to *S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmityl-*R*-cysteine (Pam3Cys) and used to immunize BALB/c mice, which were protected from a challenge of a SV40-transformed cell line [79]; (iii) CTL responses were also generated in mice immunized with HIV-1 envelope peptides 312–327 and 302–335 modified at the C-terminal with *N*-ε-palmitoyl-L-lysylamide [80]. Recently, it was found that S-palmitoylated peptides are more immunogenic than N-palmitoylated peptides, possibly due to the lability of the thioester bond to thioesterases in the cell, leading to more efficient release of the peptide fragments [81].

Lipopeptides are appealing in that they are easy to synthesize, nontoxic and overcome the need for carrier molecules; their application in human vaccines remains to be seen.

Immune-stimulating complexes (ISCOMs)

ISCOMS are ordered structures 40 nm in diameter consisting of Quill A, cholesterol, phospholipid and proteins. Quill A is a mixture of several related saponins isolated from the bark of the South American tree *Quillaja saponaria* Molina; saponins are adjuvants which bind to cholesterol on cell membranes resulting in pore formation, which causes local inflammatory effects. Quill A has been shown to aid both antibody and cellular responses to a variety of antigens. ISCOM formation requires an antigen with a hydrophobic component; the protein composition in ISCOM may vary with a particular antigen, but the molar ratio of cholesterol:phospholipid:Quill A must remain the same. Since ISCOMs are only formed with hydrophobic antigens, some antigens have to be denatured with detergents, urea or low pH to expose the hydrophobic region [82]; peptide antigens may be made hydrophobic by the addition of palmitic or myristic acid tails. Hydrophilic peptides can be incorporated into ISCOMs by forming longer peptides incorporating the fusion peptides of viruses—the fusion peptide from measles virus linked to the measles nucleoprotein peptide 281–290 sensitized target cells to lysis by NP-specific CTL and also induced NP-specific CTL in mice [83]. The adjuvant activity of ISCOMs could be due to a number of mechanisms: penetration into cells by fusion with membranes, or endocytosis or phagocytosis—the two mechanisms resulting in both class I/II responses. For example, two ISCOM preparations containing ovalbumin were able to induce enhanced levels of ovalbumin-specific IgG1 and IgG2a antibodies and increased levels of IFN-γ and IL-4 compared with ovalbumin alone [84]. It has also been shown that influenza envelope protein ISCOM-pulsed APCs stimulated primed T cells to proliferate and secrete cytokines IL-2, IFN-γ and IL-4 in vitro; DCs were superior to other APCs (Peritoneal exudate cells [PEC], unfractionated splenocytes, B cells) in producing IFN-γ [85]. ISCOMs containing a variety of antigens have been used to generate immune responses to tumors, viruses and bacteria. ISCOMs containing the E6 and E7 early proteins from the human papillomavirus type 11 generate specific CTL responses in mice [86]. Cynomolgous monkeys immunized intramuscularly with an influenza virus glycoprotein ISCOM generated high titres of IgM, IgA, IgG and proliferative T cell responses and were completely protected from a intratracheal challenge with the virus, making it likely that CTLs were produced [87].

Despite the good T1 or T2 responses attainable with ISCOMs, toxicity of weakly immunogenic preparations due to larger amounts of Quill A used has been a major concern for human use.

Liposomes

Liposomes are closed concentric bilayer membranes formed when phospholipids or other polar amphiphiles are mixed with water, resulting in entrapment of water, solutes and proteins. Both membrane fluidity and the size of liposomes can be controlled by lipid composition. Foreign proteins or peptides can be incorporated inside the liposome by entrapment, or by chemical modification within the lipid layer bilayer where charges or reactive groups can be introduced on the liposome surface [88–90]. The adjuvant activity of liposomes is considered to be due to a slow release of antigen, increased antigen presentation by macrophages or by delivery of antigen directly into the cytoplasm by membrane fusion due to the lipid components [91]. The immunogenicity of peptides incorporated into liposomes is dependent on the size, charge, lipid composition of the liposome and the location of antigen (i.e. entrapped within the liposome or on the surface). For example, using ovalbumin-loaded liposomes, it was shown that positively charged liposomes were taken up effectively by macrophages, and could induce antigen-specific CTLs, whereas negatively charged or neutral liposomes were not [92]. Animals immunized with liposomes encapsulated with a truncated human T lymphotropic virus gag and env fusion protein coated with mannan were internalized by the mannose receptor and induced killer cells specific for HTLV-1$^+$ cells and rejected HTLV-1$^+$ tumors [93]. Similarly, cellular responses were generated when a mannose coated liposomes with entrapped HIV-1 gp120 peptide [94]. A human MUC1 peptide incorporated into liposomes induced MUC1-specific proliferative responses and high levels of IFN-γ in mice [95].

In this section we have described agents which primarily take exogenous antigens into the cytoplasm—a site necessary for subsequent presentation by class I molecules for CD8$^+$ CTL induction and function (table 2). Cytoplasmic transfer can occur in two ways; (i) directly through the cell membrane (membrane-fusing agents, liposomes, ISCOMs) or (ii) via endosomes (toxins, oxidized mannan); it should be noted that both ISCOM and liposomes have multiple effects. Which of these methods will be most suitable for cancer antigens remains to be seen.

Directing antigens to cellular compartments

Other than to the cytoplsm, peptides can be targeted to various intracellular compartments by incorporating cellular 'sorting signals' introduced as fusion proteins or as recombinant DNA constructs. For example:

Class II LAMP-1. Endogenously expressed HIV-1 envelope protein (which binds to CD4) was targeted to the

specialized class II processing endosomal compartment by a DNA construct coding for a fusion protein of the extracellular domain of CD4 and the transmembrane and cytoplasmic domains of the lysosomal membrane protein LAMP-1; when envelope protein and the CD4-LAMP-1 fusion protein were coexpressed, proliferative responses of envelope-specific CD4+ clones to infected cells were greatly enhanced compared with the wild-type envelope protein [96]. A similar strategy used a vaccinia construct containing a chimeric human papilloma virus E7/LAMP-1 gene; mice immunized with this construct yielded lymphocytes which proliferated specifically on exposure to E7 [97].

ER targeting peptide. Peptides may also be targeted to the endoplasmic reticulum (ER) for class I presentation using a peptide containing a T cell epitope and an ER retention sequence, e.g. ER-ovalbumin CTL epitope or P815 mastosarcoma P1A CTL epitope protected mice from tumor challenge [98].

CLIP. The invariant chain of class II blocks premature peptide loading of class II molecules in the ER and prior to loading is degraded to invariant peptide. CLIP binds class II molecules in a similar way to antigenic peptides. When recombinant class II proteins where the CLIP region was replaced with potential T cell peptides from tetanus toxin or the acetylcholine receptor were exposed to peripheral blood mononuclear cells (PBMCs), CLIP led to increased stimulation of specific T cell clones [99].

Heat shock proteins (hsp) are involved in the folding of polypeptide chains and transport to intracellular compartments; such complexes dissociate in the presence of ATP [100]. Hsp-peptide complexes are potent stimulators of T cell responses [101]. Hsp binds peptides by hydrophobic interactions with the peptide side chain, and recently the crystal structure for one such complex was solved [102]. The hsp-peptide complexes are taken up by macrophages and enter the class I processing pathway. Thus, a fusion protein of a fragment of ovalbumin and mycobacteria hsp 70 generated strong CTL responses and tumor protection [103], and LCMV CTL-inducing peptides complexed with hsp 70 were used to immunize mice and eradicate the virus [104]. Hsp-tumor peptides are currently in clinical trials.

Preclinical and clinical use of peptides for immunotherapy in cancer

Much of the foregoing refers to a range of methods to convey peptides into the class I processing pathways for MHC class I presentation—for induction or to be a target for CTLs. Many of the methods are being used in tumor models or available for use as such. Many are in advanced preclinical or, indeed, clinical trials.

Melanoma

Melanoma is unique as more tumor specific peptide epitopes have been described for it than for any other tumor. Peptides derived from melanoma used to stimulate T cells include gp100, MART-1/MelanA, tyrosinase, TRP-1, TRP-2, MAGE-1–3 and BAGE (table 3). Some of these can be classed as 'tumor-specific' (BAGE, GAGE and MAGE) as they are expressed predominantly on melanoma cells and not on normal cells (except for the testis), whereas others are also found in normal cells (gp100, MART-1/MelanA, tyrosinase, TRP-1, TRP-2).

MAGE weakly stimulates peripheral blood lymphocytes (PBL) in vitro, whereas the peptide AAGIGILTV MART-1/MelanA strongly stimulates PBL (table 3) [105]. TRP-2, expressed in most melanomas, is recognized by TIL in the context of HLA-A31 and HLA-A33. In another study, by screening the sequence of TRP-2, 41 peptides were selected based on motifs which could bind HLA-A*0201$^+$ and were used to stimulate PBL from HLA-A*0201+ melanoma patients [106]. The peptide TRP-2(180–188) (SVYDFFVWL), was able to induce CTLs from the PBL of 3/4 melanoma patients. Tumor-infiltrating cells (TILs) recognizing tyrosinase and gp100 recognize an HLA-A1-binding tyrosinase peptide (SSDYVIPIGTY) and an HLA-A3-binding gp100 peptide (LIYRRRLMK). T cells restricted to HLA-A2 (TIL1383) also recognized a gp100 peptide (RLPRIFCSC) (table 3) [107]. In an interesting study, a slight mutation in the native MART-1 peptide by substituting the amino acid alanine from MART1(27–35) with a Leu in position 1 (LAGIGILTV) results in a peptide that acts as a 'superagonist' and induced T cells in vitro which are IL-specific from PBLs of HLA-A2$^+$ melanoma patients; these T cells also secreted high levels of IFN-γ and IL-2 [105].

Further in a clinical trial 45 HLA-A*0201 patients produced CTL responses to different combinations of 22 (47.8%) peptides when treated with a vaccine containing $\sim$50 peptides derived from MAGE-3, Melan-A/MART-1, gp100, tyrosinase, melanocortin receptor and dopachrome tautomerase (TRP-2) [108], i.e. patients develop a T cell response to different combinations of peptides [108]. The heterogeneity of the response to peptides in patients with the same HLA type points to the neccessity to construct vaccines containing multiple peptides to increase the number of patients responding to therapy. In another study, the HLA-A2*0201 binding gp100 peptides were mutated to

improve HLA binding affinity and used in incomplete Freund's adjuvant to immunize patients with advanced melanoma [16]. Peptides used were g209 (ITDQVPFSV), g280 (YLEPGPVTA), modified g209 (g209-2M: IMDQVPFSV) or modified g280 (g280-9V: YLEPGPVTV) peptide. The ELISPOT assay for γ-IFN showed 6/7 patients responded to g209-2M and 5/7 patients to native g209 peptide, 5/6 to g209, 1/3 patients to g280-9V and 4/7 to g280.

In a separate study 17 patients were immunized intradermally (10^5–10^7 cells/injection) monthly for 4 months using tumor lysate-pulsed autologous APCs [109]. One patient had a partial response of the tumor, and 9 had a delayed type hypersensitivity (DTH) response; no CTLs were evident, but after expanding T cells in vitro with autologous loaded APCs, $CD8^+$ CTLs were found in 5/9 [109].

In other studies DCs generated with GM-CSF and IL-4 were pulsed with a tumor lysate or a mixture of peptides and injected into patients together with KLH [110]. Sixteen patients with advanced melanoma were immunized; all developed a DTH response to keyhole limpet hemocyanin (KLH) and 11/16 to the melanoma peptides; tumor responses were found in 5/16 patients with regression of metastases in organs of the skin, lung and pancreas (two complete responses, three partial responses) [110]. In a different study, gp100 peptides binding to HLA-A*0201 were used to treat patients with metastatic melanoma; most patients developed an immune response, and 13/31 patients receiving the peptide + IL-2 had objective tumor responses; 4 patients had minor responses [111]. Although all these immunization protocols are promising, further studies are necessary.

Table 3. Tumor-associated peptides recognised by CTL.

	Peptide	MHC restriction	Reference
Melanoma			
MAGE-1	EADPTGHSY	HLA-A1	163
	SAYGEPRKL	HLA-Cw*1601	163
MAGE-2	KMVELVHFL	HLA-A*0201	164
	YLQLVFGIEV	HLA-A*0201	164
MAGE-3	FLWGPRALV	HLA-A*0201	165
BAGE	AARAVFLAL	HLA-Cw*1601	166
GAGE	YRPRPRRY	HLA-Cw6	167
RAGE-1	SPSSNRIRNT	HLA-B7	168
gp100/Pmel17	YLEPGPVTA	HLA-A*0201	169
	LLDGTATLRL	HLA-A*0201	170
	KTWGQYWQV	HLA-A*0201	171
	ITDQVPFSV	HLA-A*0201	169
	RLPRIFCSC	HLA-A*0201	172
	LIYRRRLMK	HLA-A3	172
MART-1/ Melan-A	ILTVILGVL	HLA-A*0201	173
	AAGIGILTV	HLA-A*0201	174
Tyrosinase	MLLAVLYLL	HLA-A*0201	175
	YMDGTMSQV	HLA-A*0201	175
	SSDYVIPIGTY	HLA-A1	172
	AFLPWHRLF	HLA-A24	176
TRP-2	SVYDFFVWL	HLA-A*0201	106
Breast Cancer			
MUC1	STAPPAHGV	HLA-A*0201, HLA-A11	25, 177
	TSAPDTRPA	HLA-A*0201	4
	SAPDTRPAP	$H2K^b$	4
	PAPGSTAPP	$H2D^b$	4
	SAPDTRPAP	$H2D^d$	4
	APDTRPAPG	$H2L^d$	4
	PDTRPAPGS	$H2K^k$	4
p53	LLPENNVLSPL	HLA-A*0201	178
	GLAPPQHLIRV	HLA-A*0201	178
	RMPEAAPPV	HLA-A*0201	178
	KTCPVQLWV	HLA-A*0201	178
	LLGRNSFEV	HLA-A*0201	179
	STPPPGTRV	HLA-A*0201	139
Her-2/neu	KIFGSLAFL	HLA-A*0201	145
	IISAVVGIL	HLA-A*0201	146
	VMAGVGSPYV	HLA-A*0201	180
	VLRENTSPK	HLA-A3	147
Colon Cancer			
CEA	YLSGANLNL	HLA-A*0201	155
	YLSGADLNL	HLA-A*0201	155
	HLFGYSWYK	HLA-A3	147
	QYSWFVNGTF	HLA-A24	156
	TYACFVSNL	HLA-A24	156

Breast cancer

MUC1. More studies have been done on MUC1 peptides in breast cancer than on other peptides, but in contrast to melanoma peptides (where the peptide epitopes were defined by CTLs) MUC1 peptides were used, as MUC1 is greatly increased in breast cancer—and could therefore provide a possible target for CTLs [112]. MUC1 is a large cell surface molecule which contains a 20-amino acid repeat in the extracellular region (the VNTR), which is repeated ~ 40 times in different alleles [112]. MUC1 is highly immunogenic in mice, initially demonstrated by immunizing mice with cancer cells or peptides to make monoclonal antibodies, most of which reacted with the amino acids APDTR within the VNTR [113, 114]. At the same time, Finn's group demonstrated that lymph nodes of patients with breast, ovarian or pancreatic cancer contained T cells, were directed against the same VNTR amino acids as those of the monoclonal antibodies (APDTR, region) and were non-MHC restricted [115]. These findings led the way for immunotherapeutic approaches of MUC1 peptides using peptides from the VNTR region.

Human MUC1 is immunogenic in mice, as $MUC1^+$ tumor immunization induced protection in mice against a tumor challenge and the induction of CTLs [116]. Fusion proteins or synthetic peptides from the VNTR,

linked to carriers (KLH or DT) and mixed with Freund's, Ribi, QS21 or BCG adjuvants, were able to induce strong antibody and DTH responses, but no CTLs and only weak antitumor effects [116–118]. By using mannan-MUC1, CTLs were induced; later, a number of MUC1 constructs were developed to induce CTL and tumor protection, including vaccinia and retroviral vectors, liposomes, DNA and dendritic cell fusions [3, 95, 119–126]. A further study demostrated that the CTLs in different strains could recognize MUC1 presented by five different H2 alleles (K^b, D^b, D^d, L^d and K^k) and by HLA-A*0201 (table 3) [4, 25]. In addition, there have been examples of immune responses in inbred mice and MUC1 transgenic mice with no signs of autoimmunity [127–129]. CTL responses were enhanced by using mannan-MUC1 with cyclophosphamide [130]. In addition, adjuvants such as GMDP, MDP and incomplete Freund's adjuvant can also increase the CTLp frequency [125], as did the cytokines IL-4 + γ-IFN, IL-2 + γ-IFN and IL-12 [131, 132]. However, one of the most potent modes of immunization is the in vitro targeting of mannose receptor-bearing cells [133]. Poor responses were noted in clinical trials where patients were injected with doses of 100–1000 μg of synthetic VNTR peptide conjugated to DT [134]. In another study MUC1 synthetic peptide (containing 105 amino acids = 5 VNTR repeats) mixed with BCG was used to inject 63 patients with adenocarcinoma; 37/55 biopsies showed intense T cell infiltration, and 7/22 patients had a two- to fourfold increase in MUC1 CTLp; however, there were no antibodies or T cell proliferation detected [135]. In a separate study, 16 patients were immunized with a 16-amino acid MUC1 peptide conjugated to KLH and mixed with DETOX adjuvant; 3/16 generated MUC1 antibody responses, and 7/11 patients showed CTL activity at higher E:T ratios (100:1) [136]. Patients with advanced adenocarcinoma were immunized with mannan-MUC1, where 3/10 (HLA-A*0201) patients made CTLs, 4/15 (HLA-A1.B8, DR3) patients a T cell proliferative response and 13/25 patients a strong antibody response [137]. Other clinical trials currently in progress are using native and mutated MUC1 peptides, a MUC1 mimic peptide, the addition of cytokines, in vivo targeted APC, whole mucin conjugated to mannan, MUC1 in liposomes, MUC1 cDNA, and vaccinia virus or peptides with various adjuvants. All of these clinical studies are in early phase I/II, using patients with advanced disease, and thus far convincing antitumor responses have yet to be seen.

p53. p53, a tumor suppressor molecule, is found in ~40% of human breast cancers. p53 gene mutations occur in most human carcinomas, and altered protein products accumulate within the cancer cell. The sera from cancer patients contain anti-p53 antibodies directed to the N- and C-terminal regions of p53 and in general do not distinguish the difference between wild-type and mutated p53. These antibodies have not been found in normal individuals or cancer patients who do not express p53 mutations [138]. In addition, CTLs have been obtained from patients with $p53^+$ cancers; these also proliferate to the wild-type p53 protein [138]. Mutated p53 or wild-type p53 overexpression could be targets for tumor immunotherapy. An HLA-A*0201-binding p53 peptide has been identified (LLGRNSFEV) which is detected by CTLs from patients, and a p53 peptide from HLA-A*0201 transgenic mice (STPPPGTRV) has also been identified (table 3) [139]. Naturally occurring CTL responses to p53 are weak, but transferring human p53-specific CTLs (p53 p149–157) from HLA-A*0201 transgenic mice to *scid* mice suppressed the growth of the human $p53^+$ pancreatic carcinoma cell line Panc-1 [140]. In another study the full-length wild-type human p53, expressed in a recombinant canarypox vector, induced CTL that lysed tumors expressing human mutant p53 [141]. In mice, immunization with plasmid full-length wild-type p53 DNA also induced CTL responses and protection against mastocytoma cells transfected with mutant p53 [141]. In another study, mice received injections of dendritic cells pulsed with mutant p53 peptide and reduced tumor growth was evident, but only during treatment; tumors resumed growth after cessation of DC-peptide injections [142]. IL-2 appeared to increase the immunogenicity of the DC-peptide [142].

Although results are promising, an increase in CTL activity is required; the problem does not appear to be with the peptide, as the affinity of p53 binding peptides for class I is high. However, the CTLs induced are of low avidity.

Her-2/neu. Her-2/neu is part of the EGF receptor family and functions as a growth factor receptor. In humans it is expressed during fetal development; in adults it is very weakly expressed in normal epithelial cells, but in cancer there is an upregulation of the Her-2/neu gene in ~20–40% of breast and 30% of ovarian carcinomas, and this is associated with a poor prognosis. Some patients with cancer have natural humoral and cellular immune responses to Her-2/neu, and the aim of using Her-2/neu as a target for immunotherapy is to boost this preexisting immunity [143]. Initially, CTLs were identified in HLA-A*0201 patients which recognized the peptide p971–980; this peptide could stimulate CTLs obtained from PBMCs of patients with ovarian carcinoma (table 3) [144]. Other CTL epitopes have also been identified: p369–377 from the extracellular domain and p654–662 from the transmembrane region of Her-2/neu (table 3) [145, 146]. Recently, an HLA-A3-restricted CTL epitope (VLRENTSPK) was identified, and CTLs could lyse HLA-A3- and Her-2/neu-positive

tumor cell lines (table 3) [147]. Because of the cellular responses in patients, rats were immunized with vaccinia virus expressing whole rat neu, but although the construct was immunogenic in mice, it failed to elicit immune responses in rats [148]. This resistance could be overcome by immunizing rats with peptide fragments of Her-2/neu, where $CD4^+$ T cells and antibodies were detected [148]. The peptides which induce immune responses in rats were identical to those identified in humans. It is of interest that GM-CSF was found to increase the immune response to Her2/neu peptides [143]. Phase I clinical trials have now begun using Her-2/neu peptides [143].

Colon cancer

CEA. The common target for immunotherapy is carcinoembryonic antigen (CEA), a glycoprotein of M_r 180 kDa and it is present on $>90\%$ gastrointestinal carcinomas and is also found in the serum of patients with cancer. Recombinant vaccinia virus CEA (rV-CEA) constructs injected in mice induce antibodies, reduction in tumor growth, DTH, T cell proliferation and CTL responses [149]. In addition, Rhesus monkeys immunized with rV-CEA developed anti-CEA antibodies and DTH responses [150]. In a phase I clinical trial, 26 patients with gastrointestinal, breast and lung carcinomas were injected with 10^7 pfu of rV-CEA at monthly intervals for 3 months. Although T cell responses to vaccinia virus were noted, there were no responses to CEA. However, in another study CTLs were obtained from peripheral blood lymphocytes (PBLs) after rV-CEA vaccinations and T cell lines obtained from PBLs pulsed with an HLA-A*A0201 binding peptide and were able to lyse CEA HLA-A2 cell lines (table 3) [151]. Vaccinia virus is limited for immunization, and canarypox has also been engineered to express CEA (ALVAC-CEA) and could be used for booster immunizations in these patients. In mice ALVAC-CEA induces antibodies, CTL and tumor inhibition; however, using rV-CEA as the first injection followed by ALVAC-CEA was superior to using either alone in inducing T cell responses [152].

Full-length CEA complementary DNA (cDNA) with a CMV promoter has been injected in mice which developed antibodies and T cell proliferation, and protection noted with intramuscular injection. Furthermore, plasmid DNA encoding CEA and HBsAg (as an internal positive control $\sim$ pCEA/HBsAg), was injected intramuscularly into pig-tailed macaques who developed antibody, T cell proliferation, IL-2 secretion and DTH responses [153]. On the basis of these findings, patients with metastatic colorectal carcinoma are receiving pCEA/HBsAg intramuscularly. In other studies CEA transgenic mice have been produced, but in these the whole CEA was unable to generate immune responses unless given as rV-CEA, which generated strong antibodies, CD4 T cells, CTLs and protection against a challenge with CEA-expressing tumor cells [154]. No autoimmune responses were noted.

CEA peptides have also being used. The peptide YLSGANLNL is a CEA CTL (HLA-A*A0201) epitope recognized by CTLs from rV-CEA-vaccinated colon cancer patients (table 3). A single amino acid substitution N6D (YLSGADLNL) sensitized CTL 100–1000 times more efficiently than the native peptide (table 3). The enhanced recognition of the analog was not due to increased binding to HLA-A*0201, and therefore the analogue, YLSGADLNL, is a CTL enhancer agonist peptide [155]. Recently, HLA-A3 (HLFGYSWYK) and HLA-A24 (QYSWFVNGTF, TYACFVSNL) restricted CTL epitopes were identified in in vitro PBMC cultures, and the CTL so produced lysed CEA^+ tumor cell lines (table 3) [147, 156]. The identification of such CTL peptide epitopes offers a greater opportunity for the design of peptide-based immunotherapy of patients.

Conclusion

Tumor immunotherapy is clearly receiving much attention, and there are many extensive preclinical and clinical trials in progress with tumor cell lysates, peptides and cytokines—used in the hope that peptides will be presented by MHC class I molecules leading to the induction of CTLs. Cytokines aid in this process either in the induction or effector phase. However, it is becoming increasingly clear that peptides alone, even with cytokine, often do not induce an appropriate CTL response, and in this review we have described various ways of ensuring that peptides can enter the class I pathway. Thus, the peptides can be presented in various formulations to be attracted initially to the APCs, e.g. the mannose or scavenger receptors, or having a hydrophobic content so they are attracted to cell membranes. There are other peptides which cause rapid transfer of such tumor peptides into the cytoplasm and into the class I pathway, such as that derived from the *Drosophila* transcription factor, *Antennapedia*. Thus, peptides can now be very effectively targeted to APCs. Such targeting can be improved by directly loading the peptide on to APCs such as dendritic cells and, with the foregoing methods, should give even more efficient localization and targeting to the appropriate cells. Once inside the APC, there are other methods to ensure that class I presentation ultimately occurs by targeting to various subcellular compartments such as the endoplasmic reticulum and the cytosol. For example, aldehydes

cause rapid and effective transfer to the class I pathway by unknown means, and various microbial toxins have the same effects. Heat shock protein is of interest in that it not only targets APCs (presumably there is a discrete receptor for this on the cell surface), but also ensures rapid translocation into the cytoplasm for class I presentation. There are clearly more than enough methods to obtain presentation of peptides by class I molecules.

Originally it was thought that the ideal peptides had to have several anchors and adhere to a rigid formula; it is now apparent that this is not the case. Indeed, MUC1 peptides can be highly immunogenic: they lack the known anchors and bind to MHC class I molecules in an unusual way. With this in mind, it is difficult to predict whether a peptide is likely to be immunogenic or not—clearly the presence of anchors is helpful, but it doesn't always indicate whether that peptide will be functional and presented in an immunogenic way by the class I molecule. Thus, biological studies have to be performed by loading APCs with peptides and seeing if they can be lysed by CTLs already present in patients or induced. HLA transgenic mice have been inordinately helpful in this regard, bearing in mind that the T cell repertoire of mice and humans is different. Thus, peptides from tumors can be identified and readily prepared by recombinant methods or by peptide synthesis. This is an enormous advance over 10–20 years ago when simple tumor cell lysates were made and it was hoped that the appropriate peptides would be contained within the lysates. However, can effective CTLs be induced, and will these suffice to eradicate tumors? In some cases the use of peptides is misleading in that CTLs can lyse target cells loaded with the peptide but are not effective against the tumor expressing the native peptides. Thus, studies with synthetic peptides should be accompanied by studies where the native molecules are expressed—either by transfection in experimental models or using real tumors obtained from patients and converted into cell lines. Ideally, the patients' own tumor cells should be tested, but as cells obtained from solid tumors often do not grow in culture, and particularly not in a way that can be used in a CTL assay, these studies are extremely difficult to do.

If a large number of CTLs of high affinity could be induced, would they eradicate tumors entirely? At present this is still a theoretical possibility. There are cases, particularly in melanoma, where the best CTL responses are seen in patients with rapidly advancing tumors; however, this may be a reflection of the type of patients seen. Unfortunately, the practice for using new approaches to therapy, such as vaccination, relies on patients with advanced disease who are not the ideal patients to study in vaccine programs, where a healthy immune system is required. Perhaps in vitro treatment of cells and infusion into the patients may overcome this to some extent. At this time, it is really too early to make any firm conclusions; clinical trials are underway at present, and it will be at least another 2–5 years before firm conclusions could be made concerning the role of CTLs in eradicating solid tumors. It is clear, however, that the best tumors that could be eradicated are single-cell metastases or small clumps of cells rather than the large tumors often present in patients with advanced disease. Another problem, not described herein, but well known in the literature is the mutations that occur in class I molecules leading to the absence or decrease on the cell surface—in the absence of any class I molecules, there can clearly be no CTL effect, and a decrease in the number of HLA molecules would make a less ideal target.

To our mind, it is slowly becoming apparent that CTLs may not be effective for all cells of all cancers, and that other arms of the immune system will need to be investigated and activated for the successful eradication of tumors. In this review, we have concentrated on class I peptide presentation, and sufficent to say, there are now a number of studies on the important role of CD4 cells—either to act as helper cells to induce greater CTL effectiveness or as effector cells using the Fas ligand pathway (as opposed to the perforin granzyme pathway of $CD8^+$ CTL). Furthermore, $CD4^+$ cells are a crucial component of DTH-type reactions, and there is now increasing evidence that the central role of these cells in induction of cytolytic macrophages are the cells in the immune system. It is likely that all these components will be required to successfully eradicate solid tumors.

While it is apparent we have many reagents to use, it is going to take much time and effort to determine the appropriate combination of such reagents and even longer to achieve successful clinical trials. However, real progress is being made, and we should be optimistic that immunotherapy will in the future play an important role in the treatment of solid tumors. It is now more a question of time; however, it is unlikely that the existing and planned studies will be completed within the next 5–10 years.

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