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Review Article

Rational Design of Peptide-Based Tumor Vaccines

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Administration of synthetic peptides derived from proteins uniquely or overexpressed in tumor cells (tumor-associated antigens) can elicit tumor-specific immune responses *in vivo*. This is because cytotoxic T lymphocytes can recognize and lyse tumor cells that display peptides derived from tumor-associated antigens (TAAs) in the context of class I major histocompatibility complex (MHC) molecules. TAA peptides, in contrast to peptides of viral origin, generally bind weakly to the MHC molecule. In many cases, this explains the poor magnitude of T cell response directed at the tumor *in vivo*. Improving MHC binding as a strategy to upregulate antigen recognition can convert low affinity TAA peptides into useful tools in clinical trial settings. High-resolution structures of class I MHC molecules reported over the past two decades provided the framework for designing peptides that can induce optimal T cell response. This review will discuss the basic and clinical aspects of modifying native TAA peptides as tumor vaccines.

KEY WORDS: class I MHC; antigenic peptides; tumor-associated antigens; cytotoxic T cells and tumor immunotherapy.

INTRODUCTION

It has been known for decades that natural antitumor immunity exists in vertebrates. Only recently, specific molecular targets of tumor immunotherapy were identified (1). The primary participants in immune rejection of tumors, antigen-specific CD8+ T cells, are stimulated by presentation of peptide epitopes on antigen presenting cells, together with "help" provided by activated CD4+ T cells. This leads to clonal expansion of CD8+ cytotoxic T lymphocytes (CTLs) that will specifically lyse target cells expressing the same major histocompatibility complex (MHC)/peptide complex. Studies in mouse models and in humans have demonstrated that vaccination with synthetic peptides corresponding to T cell epitopes in tumor-associated antigens (TAAs) can bolster antitumor immunity (reviewed recently in ref. 2).

There is a growing list of TAA that are expressed at low levels in normal tissues but are overexpressed in tumor cells (reviewed recently in ref. 3). Because the immune system has been continuously exposed to these "self" antigens, high-affinity MHC-associated immunodominant epitopes may be deleted during lymphocyte development in the thymus. T cells that are specific for low-affinity subdominant epitopes, however, remain available for recruitment by vaccination (4). The difficulty in vaccination with low-affinity epitopes is that they are in general not capable of mobilizing their precursors CD8+ T cells (5). In the following sections, we will first describe the rationale for modifying native TAA peptides, follow with a summary of the results from clinical studies involving modified peptides, and conclude with a discussion of the future directions of peptide vaccine design.

Whether a particular MHC/peptide complex can trigger a T cell response *in vivo* depends on the T cell receptors (TCRs) available in the host. Minor changes in a given peptide sequence can alter or abolish a T cell response. The X-ray structures of TCR and MHC/peptide complexes reported thus far, however, indicate a lack of correlation between structural changes and the type of T cell signals induced (6–8). This is evident in the structures of TCR/peptide/HLA-A2 complexes of three variants (V7R, P6A, and Y8A) of the HTLV-1 Tax peptide (LLFGYPVAV) with the A6 T cell receptor. With respect to their ability to activate A6 T cells, the variant V7R is a weak agonist, and the variants P6A and Y8A are antagonists (8,9). The X-ray structures of each of the variant, however, show only slight readjustments of the TCR/MHC/peptide interface to accommodate the different side-chain conformations.

It also has been demonstrated that binding affinity and/or stability of a peptide with the MHC molecule are correlated with immunogenicity (10–12). By increasing the number of copies of a given MHC/peptide complex on the cell surface, the likelihood of engaging its corresponding TCR over a required threshold for activation is enhanced. Thus, a logical step toward eliciting higher magnitude of antigen-specific T cell response is to optimize the MHC/peptide contacts.

The principal method to improve peptide binding to MHC molecules is to replace amino acids in the native sequence with "preferred" ones. The preferences are based on the observation that most class I molecules bind peptides pos-

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sess two key residues (anchors) at specific locations to achieve high-affinity binding (13,14). For the human class I MHC molecule HLA-A2, an important target of cancer immunotherapy, the anchor residues are leucine, valine, or methionine at position 2 and valine or leucine at position 9 (13). *In vitro* experiments have clearly established that introducing these "anchors" into native class-I bound peptides can enhance binding.

CLINICAL APPLICATIONS OF MODIFIED TAA EPITOPES

The sequence-modification approach has clear clinical implications. The most widely investigated modified peptide to date is $gp100_{209–217}$-2M (IMDQVPFSV; from hereon refer as g209-2M), an analogue of the native epitope $gp100_{209–217}$ mutated at the 2 position (T→M). This peptide was originally described in 1996 (15). The gp100 antigen was identified as a potential target for immunotherapy during the analysis of melanoma patient tumor-infiltrating lymphocyte (TIL) reactivity to peptides from melanoma-derived cDNAs by the Rosenberg group from the Surgery Branch, NCI (16). A systematic analysis of all possible substitutions in the native epitope indicated that g209-2M bound with greater affinity, crossreacted with the native $gp100_{209–217}$ peptide, and was more efficient at stimulating gp100-specific T cell cultures than the native peptide (15). This led to the prediction that g209-2M was more immunogenic *in vivo* and may induce significant antitumor immunity.

Subsequently, advanced melanoma patients were treated in a phase I clinical trial with the native or g209-2M in adjuvant alone or in conjunction with repeated cycles of systemic administration of high dose bolus interleukin (IL-2). Early clinical results from this study included the highest response rates for a melanoma immunotherapy trial to date, with 13 of 31 (42%) patients achieving objective tumor regressions (17) in the cohort treated with the peptide plus IL-2. Patients receiving g209-2M had stronger immunologic responses (10/11) than those vaccinated with the native peptide (2/8). These initial promising clinical results, however, were not seen in subsequent studies with high-dose IL-2 combined with peptides (18,19).

An unexpected finding of this study was that immunologic responses in peripheral blood mononuclear cells (PBMCs) of treated patients were mostly positive in the peptide/adjuvant group, but negative for the peptide plus IL-2 group (the group with the high clinical responses). This counter-intuitive finding was examined in five subsequent studies (19–23).

The first two studies examined T cell cultures and "cloids" (bulk T cells that have undergone a cloning step without having clonality demonstrated (21) expanded from both PBMCs and TIL (20,21)). The T cells were found to have a great deal of functional heterogeneity with differing levels of recognition of peptide-pulsed targets and melanoma cell lines, with some T cells only recognizing peptide-pulsed targets and not melanoma cells. Importantly, crossreactivity of the g209-2M-immunized patient PBMCs with the native gp100 peptide continued to be observed. In 11 peptide/adjuvant patient samples studied, 10 of 11 had increased peptide-specific T cell activity post-treatment but no objective tumor responses, confirming the initial lack of correlation.

Serial analysis of gene expression was used to examine genes up-regulated in the PBMCs and TIL samples from the same groups of patients (22). This analysis found 8 of 11 peptide/adjuvant-treated patients had increased levels of interferon (IFN)γ-producing T cells in their metastatic lesions post-treatment whereas no increase in INFγ was observed among samples from other patients not treated with the peptide or from patients given high-dose IL-2. Two other reports (19,23) used other immunologic monitoring techniques: MHC tetramer, FastImmune intracellular cytokine staining, and real-time reverse-transcription PCR analysis to investigate the same group of patients plus those treated with the modified peptide and other cytokine adjuvants, IL-12, or granulocyte macrophage-colony stimulating factor (GM-CSF). These reports demonstrated that with MHC tetramer, 7 of 7 g209-2M-treated patients and 4 of 5 peptide plus IL-12-treated patients had increased frequencies of peptide-specific cells detected but only 1 of 11 peptide plus IL-2 patients did. In the other report, 6 of 8 peptide samples had increased tetramer-positive cells and 5 of 8 had increased IFNγ production, indicating that these peptide-specific T cells detected by tetramer were functional for IFNγ production upon specific target recognition. Hence, the conclusion drawn was that the peptide/adjuvant vaccine clearly resulted in expansion of functional peptide-specific T cells that could be detected by a wide variety of assays in both peripheral blood and TIL but that this cellular response was insufficient to result in clinical response.

The variant g209-2M has also been loaded directly onto dendritic cells (DCs) and used as a vaccine (24). This strategy provides for peptide presentation on the most potent antigen-presenting cells known. DC differentiated in GM-CSF and IL-4 were loaded with both g209-2M and $MART\text{-}1_{27–35}$ and delivered intravenously. The doses escalated from 6×10^7, to 2×10^8 to 2×10^8, plus high-dose IL-2. Clinical results were that only 1 of 7 evaluated patients experienced a partial tumor response, and limited immunologic responses evaluated were that 1 of 5 patients had increased CTL activity. Because of this meager response level, the trial was stopped early. The authors suggested possible reasons for the response level included the route of DC administration, the type of DC administered, or the doses. Subsequently, intradermal, intranodal, and subcutaneous injections have been found to be superior routes of DC vaccine administration (25), and maturation treatment of DC may give superior results (26,27).

A recent study also using DC pulsed with g209-2M found very encouraging clinical and immunologic responses (28). These DC were differentiated from CD34+ precursor cells and pulsed with the gp100 peptide as well as the $MART\text{-}1_{27}$, $tyrosinase_{368}$, and $MAGE\text{-}3_{271}$ melanoma peptides and injected subcutaneously. The Flu M1 58–66 peptide (a two-anchor, strong binding recall antigen) and keyhole limpet hemocyanin (KLH; a xenoantigen) were also administered as markers of successful immunization. Of the 18 patients treated, 16 had immunologic responses to KLH and Flu, indicating successful immunization; 10 showed positive immunologic responses to more than 2 melanoma peptides; and of those 10, 3 had stabilization of disease (SD), 4 had mixed responses (MR), and 3 experienced complete responses (CR). Hence, in this clinical trial, there was a very good correlation between clinical and immunologic response.

Two additional reports of trials of patients immunized

with the same g209-2M peptide (29,30), either pulsed onto GM-CSF/IL-4 DC (with the tyrosinase$_{368}$ peptide) or in adjuvant with or without IL-12, have recently been published. Of the 16 stage IV melanoma patients treated with peptides on DC delivered intravenously, one had a CR, two had MR, and two SD. Five of the 16 had positive IFNγ ELISA immunologic results, post-treatment compared to pretreatment. In the peptide/adjuvant trial, 34 of 40 developed a positive skin test toward g209-2M but none to the tyrosinase peptide. Most patients had positive tetramer and peptide-specific IFNγ production (detected by ELISA). These studies with g209-2M clearly demonstrated its *in vivo* immunogenicity. The stronger immunologic response seen with the modified peptide suggests that the increased binding affinity has real biologic impact. Whether the modified peptides have been beneficial or not clinically, however, cannot be ascertained until they are compared head-to-head with the native peptides in clinical trial settings

Anchor-substituted variants of the MART-1$_{27–35}$ or MART-1$_{26–35}$ melanoma differentiation antigen peptides have also been tested *in vitro*. The LAGIGILTV peptide was found to bind better and acted as a superagonist peptide for CTL stimulation compared to the native peptide AAGIGILTV (31). The other report tested the 9-mer peptides without success, but found that the 10-mer variant ELAGIGILTV both bound better and acted as a superagonist (32)

Optimizing T-Cell Receptor Contacts

The lack of structural correlation in T cell activation does not mean that the TCR and MHC/peptide interfaces cannot be optimized. Amino acids in the native peptide can be modified to increase the affinity of the MHC/peptide complex for TCR rather than the affinity of peptide for MHC. Higher TCR affinity and improved cytolytic activity were observed for the murine H-2L^d-restricted peptide antigen derived from gp70 (SPSYVYHQF; amino acids 423–431) with the valine at position 5 mutated to an alanine (33).

This concept has been tested in humans. A variant of the carcinoembryonic antigen (CEA) epitope CEA$_{605–613}$ (YLSGANLNL) modified at a TCR-contact site has shown to induce tumor regression in patients with colorectal cancer (34). Serum levels of CEA are increased in patients with colon cancer, and CEA has been investigated as a tumor rejection antigen. The immunodominant peptide "CAP-1" (YLSGANLNL), which is restricted by HLA-A2, has been analyzed. This peptide already contains appropriate anchor residues for MHC binding but is not very immunogenic. A substituted variant, 6D (YLSGADLNL), was identified. Although, it did not bind more efficiently, it sensitized T cells more efficiently (35), possibly through stronger Zap-70 phosphorylation in responding T cells (36). These T cells cross-reacted with native CEA peptide. In a recent clinical trial (34), 12 patients with either CEA-positive colon or non-small cell lung cancer were immunized with Flt-3 ligand-expanded DC pulsed with KLH and the CEA-6D peptide in a phase I dose-escalation study. The clinical responses were as follows: one CR ongoing, one CR lasting 10 months, one MR, and two SD. The immunologic responses detected were 10 of 12 patients showing increased tetramer positive cells post-treatment and 8 of 12 with increased CTL responses. This is a demonstration of successful use of a modified peptide in a clinical trial where significant immunologic response and clinical efficacy were found. The native peptide, however, was not tested in the trial that would allow for direct comparison.

FUTURE DIRECTIONS

Structure-Based Sequence Modifications

Peptides presented in class I molecules generally are derived from intracellular proteins after being digested by the proteasome complex, transported to the endoplasmic reticulum by the specialized peptide transporters (TAP1 and TAP2) and finally bound to MHC class I molecules before being displayed on the cell surface (reviewed in ref. 37). The precise sequences of the peptides being presented are determined by selective molecular interactions at each step in the presentation process, with the MHC/peptide interaction characterized most extensively at the structural level.

All class I molecules consist of a membrane-inserted heavy chain (α) and a non-covalently associated light chain (β-2-microglobulin) (38). The part of the MHC molecule that interacts with antigenic peptides consists of approximately 180 amino acid residues at the amino terminus of the α chain. The peptide-binding groove is formed by a platform of an eight-stranded, β-pleated sheet supporting two parallel strands of α-helix (α1 and α2).

Although a major portion of the MHC/peptide binding energy comes from the backbone of the peptide interacting with MHC residues in the binding groove (39), the peptide side chains account for the observed binding selectivity. This is because MHC residues in the binding groove form "pockets" to accommodate side chains of the peptide. X-ray structures of HLA-A2 show that regions of the binding groove underneath peptide positions 2 and 9 have physicochemical environments well suited to the binding of hydrophobic side chains (40,41), providing the structural basis for the anchor residue requirements.

Side chains at positions 1, 3, and 7 can also interact with MHC residues and influence binding (42–45). These peptide side chains have been shown to be important for binding via interactions with additional MHC "pockets" along the binding cleft (41). Hydrophobic side chains (e.g., leucine or isoleucine) are preferred at position 3, and phenylalanine is preferred at position 7 (43,44). Placing a phenylalanine or tyrosine at position 1 can increase binding via aromatic stacking that involves Trp-167 of the MHC molecule (42,46,47). In general, non-anchor positions are more tolerant of amino acid variations than the anchor residues. Together, the anchor and non-anchor residues form the basis for modifying peptide sequences aiming at improving binding and stability (44).

Side Chain Interactions in the MHC Binding Groove

Despite the advances described above, prediction of MHC binding is not straightforward (48,49). Many HLA-A2-restricted epitopes, including those derived from TAAs, bind poorly despite having the correct MHC binding motif (48). Furthermore, it is often not possible to accurately predict the effects of single amino acid substitutions (48). The amino acids in a given bound peptide are not necessarily additive in their influence on binding and stability; sequence context can strongly influence the final observed effects.

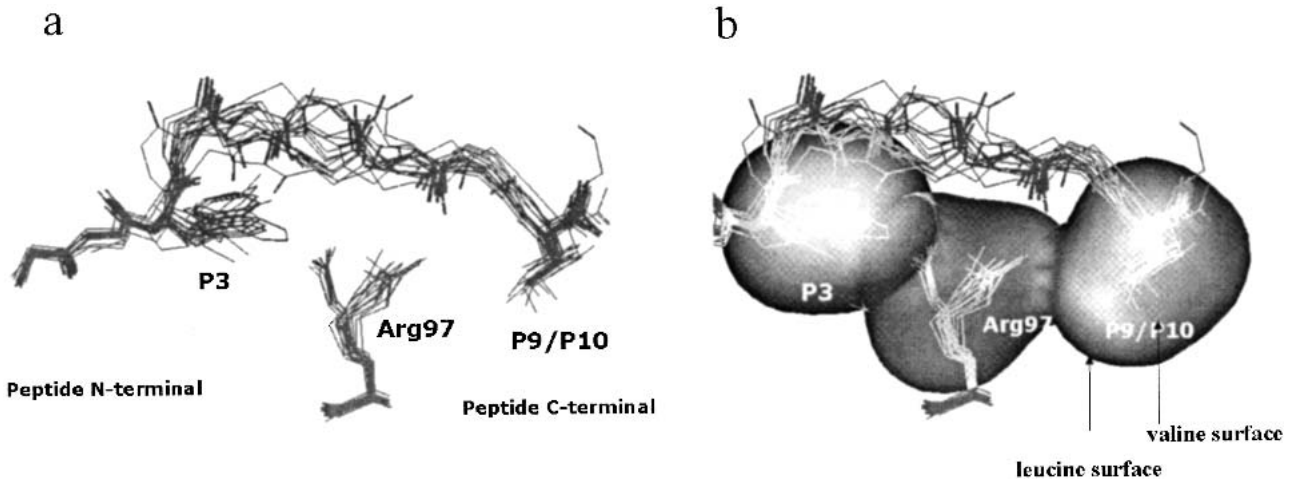

Fig. 1. Overlay of X-ray conformations of the peptides and Arg97 in 18 HLA-A2/peptide complexes (pdb entries: ihhg, 1hhi, 1hhk, 1hhj, 1hhk, 2clr, 1qr1, 1jht, 1b0g, 1ilf, 1ily, 1qse, 1akj, 1a07, 1bdz, 1duy, 1duz, and 1qrn). The peptides are viewed from the side with their N-terminal on the left. The structures were superimposed by minimizing the root-mean square deviations of the Cα atoms of the MHC molecules using the "superpose" module in the molecular modeling program MOE (version 2001.01, Chemical Computing Group, Toronto, Canada). Note that the side chain of Arg97 is either oriented toward the peptide N-terminal or the C-terminal. Panel (a) shows the close proximity of the P3 side chains with a N-terminal oriented Arg97. In panel (b), the interactions of these residues are illustrated with calculated molecular surfaces (using the Gauss/Connolly module in MOE) of the side chains showing that a bulky peptide side chain at the C-terminal position can "push" the Arg97 side chain toward the N-terminal direction.

The importance of pairwise interactions between peptide residues has been shown in MHC binding (50–52) and T cell activation (53,54). Our collective analysis of 18 HLA-A2 X-ray structures available in the Brookhaven Protein Data Bank (8,46,52,55–57, and references reviewed in 58) reveal that the side chains at position (P3) and the C-terminal position of the peptide are interdependent.

The side chain at P3 generally is oriented toward the central part of the binding groove (Fig. 1), where a number of polymorphic MHC residues are located. Of these, the centrally located Arg97 side chain is flexible in its orientation: It can orient toward the N-terminal or C-terminal of the bound peptide (Fig. 1). Madden *et al.* (41) observed that the Arg97 side chain tended to orient toward the C-terminal of the peptide when a small side chain (e.g., valine) is at the peptide C-terminal position (e.g., TLTSCNTS<u>V</u>). Because of steric hindrance, when a larger leucine is at the same position (e.g., GILGFVFT<u>L</u>), the Arg97 side chain tends to orient toward the N-terminal of the peptide. Crystallized HLA-A2 structures thus far indicate that their prediction was generally correct (Table I). A structure-based motif for binding can therefore be postulated such that a large hydrophobic side chain at the C-terminal will facilitate binding when a hydrogen bonding side chain is placed at P3. The rationale is that the N-terminal oriented Arg97 side chain is in closer proximity with the P3 side chain.

Water at the MHC/Peptide Binding Interface

Another level of complexity in the design of optimal MHC ligands is that the peptide-binding groove is flexible. Evidence is accumulating that water is present at the MHC/peptide interface (50,51,56,59), and in some cases, involved in the selection of peptide side chains. A specific example of this was shown in the murine class I molecule H-2K^b complexed with RGYVYQGL or SIINFEKL (50). In RGYVYQGL, the small glycine at position 2 allowed a water molecule to fit into the binding groove and mediate hydrogen bonding between residues of the MHC molecule and the hydroxyl group of Y5. With SIINFEKL, this water cannot be similarly positioned because of the larger isoleucine side chain at position 2. Thus, a phenylalanine, instead of a tyrosine, is favored at position 5 in SIINFEKL. This was the first evidence that water is involved in the selection of side chains in class I bound peptides. Similar functions of water have also been shown in an X-ray structure of the human class I molecule HLA-B53 (51).

Theoretical analyses of the HLA-A2 structures have provided insights into the dynamics of water at the binding interface (60–62). Because the backbone in class I-bound peptides bulges out between residues at position 3 and position 8, a significant "gap" is seen between the peptide and MHC residues in the central region of the binding groove (61). This region is lined with His70, Thr73, His74, Asp77, Arg97, Tyr99, His114, and Tyr116, all with charged or hydrogen bonding side chains oriented into the binding groove. Early X-ray structures of HLA-A2 have shown this "gap" to be empty. Molecular dynamics simulations led to the prediction that as many as 12 water molecules could fit in this region, forming a network of hydrogen bonds involving the peptide and the side chains of the MHC molecule (60,61). The water molecules were flexible in their positions over time and were seen to

Table I. Average Distances of Arg97 and the Peptide Side Chain at Position 3[a]

	Peptides with a leucine at the C-terminal position	Peptides with a valine at the C-terminal position
Average distance ($P3_{C\beta}$-$R97_{C\zeta}$)	6.6 Å	7.8 Å

[a] Analysis of 18 x-ray structures of HLA-A2 (pdb entries: ihhg, 1hhi, 1hhk, 1hhj, 1hhk, 2clr, 1qr1, 1jht, 1b0g, 1ilf, 1ily, 1qse, 1akj, 1a07, 1bdz, 1duy, 1duz, and 1qrn).

"adjust" to different peptide sequences (GILGFVFTL, TLTSCNTSV, ILKEPVHGTV, and LLFGYPVYV) (61). This theoretical water model provided the conceptual basis for designing a variant of Flu (GIhsGFVFTL; hs = homoserine) that has improved MHC binding over the native peptide (63).

The functional role of water at the MHC/peptide binding interface in TAA peptides has been confirmed recently. X-ray structures of HLA-A2 complexed with epitopes derived from MAGE-4 (59) and Melan A/MART-1 (56) showed 5–8 water molecules in the proposed "gap" region. The positions of the water in these structures strongly suggested they mediate MHC/peptide interactions. Interestingly, different patterns of water clusters were seen in the two closely related Melan A/MART-1 peptides, ELAGIGILTV and ALGIGILTV (56), supporting the notion that water at the binding interface can "adjust" to different peptides.

MHC/peptide interaction is only one of several parameters that are important for peptide-induced tumor-specific CD8+ T cell responses. The nature of the adjuvant used and the target TAA are also critical in generating effective antitumor immune response *in vivo*. Significant impact on clinical outcome will most likely be realized when multiple factors are optimized simultaneously. DCs, with their high densities of MHC and costimulatory molecules, are promising vehicles for the *in vivo* delivery of TAA-related peptides.

An essential requirement of any epitope modification approach is the preservation of T cell recognition toward the native epitope. It is almost always impossible to predict if the substitutions will cause gross conformational changes (52) and result in altered or lost of T cell activation. Computational modeling techniques can nicely complement crystallographic studies in this regard (61,64–66).

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