Review

Challenges in the Development of Effective Peptide Vaccines for Cancer

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The ability of the immune system to recognize malignant cells has opened the door to development of tumor vaccines to treat or prevent various types of cancer. In the era of molecular biology, the tumor antigens recognized by the immune system have been identified, allowing the generation of subunit vaccines that may improve safety and efficacy compared with more crude vaccines such as irradiated tumor cells and tumor cell lysates. Synthetic peptides corresponding to defined antigenic epitopes for tumor-reactive lymphocytes represent one of the new types of vaccines currently being developed to treat or prevent various types of malignant disorders. The design of peptide-based vaccines to stimulate antitumor T-cell responses has many attractive features such as ease of manufacturing and characterization (ie, quality control), as well as an excellent safety profile in past clinical studies. However, ambiguous results from initial clinical trials indicate that these vaccines are far from optimal and that considerable efforts for their optimization lie ahead. We attempt to address the 8 most important challenges we currently face for developing peptide-based vaccines that would effectively induce immune responses leading to antitumor effects.

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APC = antigen-presenting cell; CEA = carcinoembryonic antigen; CpG = cytosine guanine; CTL = cytotoxic T lymphocyte; DC = dendritic cell; GM-CSF = granulocyte-macrophage colony-stimulating factor; HTL = helper T lymphocyte; IFA = incomplete Freund adjuvant; IL = interleukin; MHC = major histocompatibility complex; PAMP = pathogen-associated molecular pattern; TAA = tumor-associated antigen; TCR = T-cell receptor; TGF = transforming growth factor; TNF = tumor necrosis factor

The immune system has evolved in all species mainly to fight and prevent the invasion of infectious pathogens. The immune system may also respond to internal attacks such as those resulting from malignant transformation. For many years scientists and clinicians have sought to harness the power of the immune system to treat patients suffering with cancer. More than a century ago, William B. Coley was the first to report the clinical observation in humans of tumor regression at the time the immune system was activated by infection. At the end of the 19th century, observant physicians noticed that tumors sometimes regressed in cancer patients in the presence of systemic bacterial infections. Coley went on to administer bacterial extracts (Coley toxins) to cancer patients in an attempt to stimulate their immune systems and to prompt a tumor-killing response.[1] Unfortunately, the results, although successful in some individuals, were unpredictable and in many cases were accompanied with severe toxic effects. Although these treatments, which would now be considered a form of immunotherapy, were not broadly accepted, the link between immunity and cancer and the possibility of improving cancer immunotherapy stirred considerable interest.

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Extensive laboratory research in animals (mostly in mice) has clearly demonstrated the existence of tumor-specific transplantation antigens, which are responsible for the rejection of tumors mediated by the immune system.[2-4] Mice that either were treated surgically to remove a tumor or were vaccinated with killed tumor cells were shown to reject a rechallenge with the original tumor, but not with an irrelevant tumor. The observations by Coley and the work in animal models have given birth to the field of tumor immunology and immunotherapy.

Among the various elements of the immune system, T lymphocytes are probably the most adept to recognize and eliminate cells expressing foreign or tumor-associated antigens (TAAs). Cytotoxic T lymphocytes (CTLs) express the CD8 cell surface marker and are specialized at inducing lysis of the target cells with which they react via the perforin/granzyme and/or the Fas/Fas-L pathways.[5-8] The T-cell receptor (TCR) for antigen of CTLs binds to a molecular complex on the surface of the target cell formed by small peptides (8-11 residues) derived from processed viral antigens or TAAs, which associate with major histocompatibility complex (MHC) class I molecules.[9] The other major T-cell subset, helper T lymphocytes (HTLs), is characterized by the expression of CD4 surface marker. The HTLs recognize slightly larger peptides (12-20 residues), also derived from foreign antigens or TAAs, but in the context of MHC class II molecules, which are only expressed by specialized antigen-presenting cells (APCs) such as B lymphocytes, macrophages, and dendritic cells (DCs).[10]

The DCs are stellate leukocytes generated in bone marrow and are considered the most potent APCs.[11] As a consequence of TCR stimulation of naive CTLs and HTLs by peptide/MHC complexes on APCs, the CTLs mature into effector killer cells capable of lysing tumor cells that express the corresponding peptide/MHC class I complex. The model depicted in Figure 1 exemplifies how T-cell responses to TAA may be elicited in a tumor-bearing individual. Activated HTLs amplify CTL responses by making the APCs more effective at stimulating the naive CTLs and by producing lymphokines that stimulate the maturation and proliferation of CTLs. The potentiating effect of HTLs occurs both in secondary lymphoid organs where the immune response is initiated and at the tumor site where CTL responses need to be sustained until the tumor cells are eliminated. Thus, one would predict that vaccines should stimulate both tumor-reactive CTLs and HTLs to generate effective antitumor immunity.

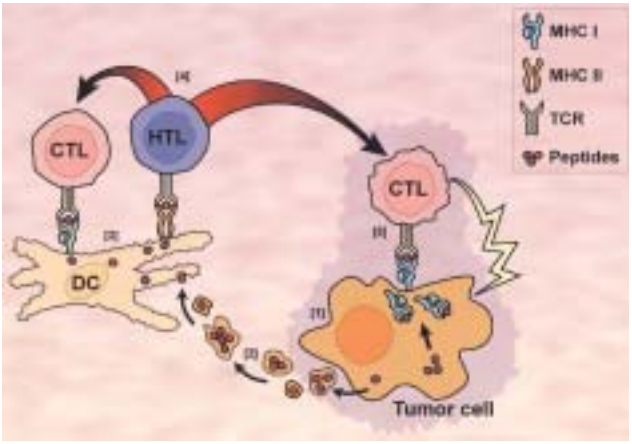

Figure 1. Events that lead to natural induction of T-cell responses against tumor-associated antigen (TAA). [1] Cell fragments containing TAA are produced through numerous mechanisms (necrosis, shedding). [2] Tissue-resident dendritic cells (DCs) capture the TAA and travel to secondary lymphoid organs where [3] they present peptide epitopes to naive cytotoxic T lymphocytes (CTLs) and helper T lymphocytes (HTLs) in the context of major histocompatibility complex (MHC) class I and class II molecules. [4] Activated HTLs provide "help" (lymphokines, costimulation) to enhance CTL reactivity. [5] Activated CTLs journey to the tumor site where they may recognize the peptide epitope presented by the tumor cells triggering their effector function (cytolysis, lymphokine production). TCR = T-cell receptor.

THE 8 MAJOR CHALLENGES FOR PEPTIDE VACCINE DEVELOPMENT

First Challenge: Peptide Epitope Identification for Tumor-Reactive T Lymphocytes

Over the past 12 years, numerous peptide epitopes recognized by tumor-reactive CTLs and HTLs have been identified by molecular and biochemical methods, which has opened a new door for the development of synthetic peptide vaccines to treat and prevent various types of malignancies.[12,13] One of the methods used for identifying these T-cell epitopes relies on the use of tumor-reactive T cells that are isolated from cancer patients, which are used for the screening of tumor-derived complementary DNA expression libraries.[14,15] Another approach, also requiring the use of patient-derived T cells, is based on screening of peptide fractions eluted from purified MHC molecules from tumor cells, which are subsequently sequenced by tandem mass spectroscopy.[16]

A third technique for identifying the peptide epitopes recognized by tumor-reactive T lymphocytes is the predictive or reverse immunology approach.[17,18] In contrast to the other approaches, this method does not require the use of patient-derived tumor-reactive CTLs or HTLs, which in many cases are difficult to isolate. The predictive approach starts with the selection of potential TAA, which may (or may not) bear T-cell epitopes for CTLs and HTLs.

The criteria for the selection of these potential TAAs are 2: First, the selected protein must be preferentially expressed or overexpressed by tumor cells compared with normal tissues. For example, many commonly known tumor markers such as carcinoembryonic antigen (CEA), HER2/*neu* (c-*erb*B2), oncofetal antigen, and p53 fall into this category. Proteins that are tissue specific and continue to be expressed by the malignant cells such as prostate-specific antigen and the melanocyte antigens gp100 and MART-1/Melan-A also fall into this category. Second, the amino acid sequence of the putative TAA for T cells must be known. The predictive approach basically looks for small peptide sequences within the tumor marker proteins, which may potentially bind to MHC class I or class II molecules. The capacity of a peptide to bind to a particular MHC allele is associated with specific amino acid residues situated at precise positions, which allow the peptide to interact with the peptide-binding pockets of the MHC molecules (Figure 2).[20,21]

The MHC binding motifs for most common human MHC class I and II alleles have been described.[13] These motifs itemize the amino acid residues that serve as MHC binding anchors for specific class I and class II MHC alleles. For example, peptides of 9 or 10 residues containing a leucine or methionine at position 2 and a valine or a leucine at the carboxyl-terminus end have a high likelihood of binding to HLA-A2 (Figure 2), the most frequently found human MHC class I allele.

More sophisticated computer-based algorithms that take into account not only the MHC binding anchors but also all the amino acids of the peptide and attempt to predict and quantify the binding affinity of the peptide/MHC interaction have been developed and are available on the World Wide Web (www.bmi-heidelberg.com/scripts/MHCServer.dll/home.htm and bimas.dcrt.nih.gov/molbio/hla_bind). Thus,

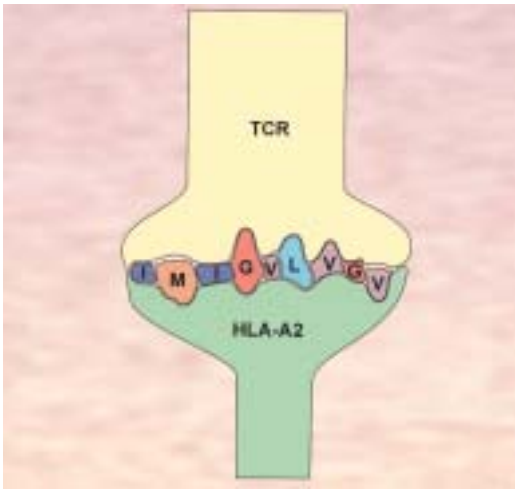

Figure 2. Hypothetical model explaining how a peptide epitope from carcinoembryonic antigen[19] associates with HLA-A2 major histocompatibility complex (MHC) molecules and is presented to the T-cell receptor (TCR). The M residue at position 2 (M2) and the V at position 9 (V9) serve as primary MHC binding anchors, burying themselves in "pockets" of the HLA-A2 molecule. Other residues of the peptide such as G4, L6, and V7 may serve as TCR contact residues endowing the specificity of the T-cell reaction. I = isoleucine; G = glycine; L = leucine; M = methionine; V = valine.

from the input of the known sequence of a tumor marker, which could potentially serve as a TAA for T cells, these algorithms list all potential T-cell epitopes, each with its corresponding predictive binding score. The peptide sequences with highest scores can then be selected and synthesized to be tested for their capacity to stimulate antitumor T-cell responses in vitro. Test tube vaccination of T cells from peripheral blood of normal volunteers with use of synthetic peptides is a valuable method that allows identification of novel CTL and HTL epitopes.[18] The most critical issue in this approach is demonstrating that the peptide-induced T cells are capable of reacting with tumor cells that express the same TAA, from which the peptide was originally selected. The steps involved in the reverse immunology strategy for the identification of T-cell epitopes from TAA, which ultimately lead to vaccine development, are shown in Figure 3. Our laboratory has been successful in identifying numerous peptides derived from various TAAs, all capable of inducing tumor-reactive CTLs and HTLs.[19,22-29] However, we are well aware that this is only the first step toward developing vaccines that can be tested in humans for immunogenicity and for their capacity to limit tumor growth.

Second Challenge: Selection of the Most Appropriate T-Cell Epitopes as Vaccine Candidates

A large number of CTL epitopes from various TAAs have been identified using 1 of the 3 methods described previously.[12,13] The list of T-cell epitopes grows daily, and as a result, some groups have made this information available on the Internet (www.bmi-heidelberg.com/scripts/MHCServer.dll/home.htm; sdmc.krdl.org.sg:8080/fimm; and www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm).

Although most of these epitopes are relevant only for malignant melanoma, several epitopes for epithelial tumors, sarcomas, and hematologic malignancies have also been described. With long lists of potential vaccine epitope candidates, the decision of which ones should be tested in the clinic may be difficult. It is obvious that most researchers are predisposed to conduct clinical studies using the epitopes identified by their own laboratories. However, other parameters should be considered if a rational, scientifically based research program is to be implemented. Issues such as the frequency of expression of a particular TAA in the malignancy that is targeted, whether the TAA is also expressed in normal tissues and potential repercussions (see fourth challenge), and the potential population coverage afforded by the MHC-restricting allele (see fifth challenge) should play a major role in the decision process.

Another decision related to this challenge is whether to include in a vaccine peptide epitopes that stimulate HTL responses. While most peptide vaccine efforts have focused on the induction of antitumor CTLs, better results might be obtained by the concurrent stimulation of HTLs, which would amplify the CTL responses.[30] During the induction of CTL responses, which occurs normally in the lymph nodes, HTLs participate in the activation of DCs via the CD40/CD40L, which primes these APCs to stimulate the naive CTLs.[31,32] Activated HTLs will also enhance the expansion of the stimulated CTLs via secretion of lymphokines such as interleukin (IL) 2. For these reasons, some clinical studies have incorporated into their vaccines MHC class II–restricted peptides to stimulate HTL. In most of these cases, the HTL-stimulating peptides were not derived from TAA sequences but from highly immunogenic antigens such as tetanus toxoid or nonnatural epitopes proven to be strong antigens for HTLs.[33-36] In theory, the HTLs involved at this stage of the immune response do not necessarily have to be specific for the tumor, as long as they are activated and become functional at the same site where the tumor-reactive naive CTLs reside. Nevertheless, emerging information suggests that HTLs may play an important role, not only during the induction of CTLs but also during the effector phase of CTL responses, which occurs at the tumor site.

We recently found that activated HTLs potentiate the proliferation, survival, and effector function of CTLs, allowing them to increase their antitumor activity.[37]

Although the exact mechanism of HTL enhancement of CTL function is still under study, activated CTLs and

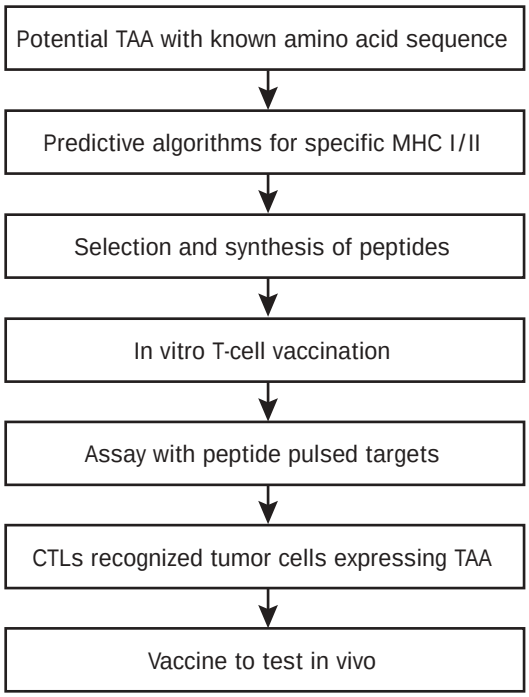

Figure 3. Critical path of the "reverse immunology" approach toward the identification of tumor-associated antigen (TAA) T-cell epitopes for the development of peptide vaccines. CTL = cytotoxic T lymphocyte; MHC = major histocompatibility complex.

HTLs can directly interact with each other through costimulatory molecules such as CD27/CD70 and 4-1BB/4-1BBL. Under this scenario, only tumor-reactive HTLs can enhance the function of the antitumor CTLs because both sets of T lymphocytes require activation by antigen. While tumor-reactive HTLs could be stimulated at the tumor site by APCs that have managed to capture and process TAA (or dead tumor cells), HTLs reactive to irrelevant antigens (eg, tetanus toxoid) have less chance of becoming activated by their corresponding antigen at this site.

Aware of these possibilities, several groups, including ours, are addressing the challenge of identifying HTL epitopes from TAA, with the purpose of increasing vaccine effectiveness by including them in peptide-based vaccines designed to elicit antitumor CTLs.

Third Challenge: Immunogenicity or the Lack Thereof (The Role of Adjuvants)

With this knowledge in hand, clinical studies have been initiated in cancer patients with the goal of inducing T-cell immunity, with the hope that it would lead to antitumor responses and increased survival. The first generation of peptide-based vaccines consisted of administration of purified synthetic peptides via subcutaneous or epidermal injections.[33,35,36,38,39] Although some tumor responses were reported anecdotally, no clear evidence of the induction of T-cell activity to the immunizing peptides could be substantiated. The lack of induction of T-cell responses by these vaccines is not surprising since the immune system has evolved throughout millions of years in most species to respond to threats posed by invading infectious agents and not against aseptic synthetic peptides.

To initiate an immune response, the injected peptide must be preferentially delivered to professional APCs such as DCs because, if the peptide is presented to naive T cells by non-APCs, it most likely will be ignored. In addition, even if the injected peptide has found its way to the DCs, an immune response will not occur unless these cells also receive signals that activate them and induce them to migrate into secondary lymphoid organs, where their job is to present the peptide to the naive T cells. Presentation of peptide/MHC complexes to naive T cells by non-APCs or by nonactivated APCs could actually induce a nonresponsiveness state (anergy) or may even result in elimination of the T cells, which could lead to enhanced tumor growth.[40-42]

The types of signals that induce DCs to become stimulatory APCs are usually derived from components of microorganisms that are recognized by the innate immune system as foreign threats, such as bacterial DNA, double-stranded viral RNA, and bacterial lipopolysaccharides, which have been termed the *pathogen-associated molecular pattern* or PAMP.[43] Another type of signal that activates the DCs to become functional APCs are "danger signals" that cells in distress send out to the immune system to inform it of an imminent problem.[44] These danger signals can be proinflammatory cytokines such as IL-1, tumor necrosis factor α (TNF-α), and type I interferons, which tend to be produced as a consequence of infections. Other danger signals are derived from necrotic cells that release some of their components, such as DNA, heat shock proteins, and mitochondria, which act as a warning sign that the organism is under attack.[45] Interestingly, the use of antibodies that react with the CD40 molecule expressed on DCs are capable of mimicking the danger signals, activating these APCs to become potent stimulators for CTLs.[31,32]

Animal model systems have been valuable in the study of the requirements to produce immunogenic peptide vaccines. Several studies have demonstrated that peptide antigens must be prepared (formulated) with appropriate adjuvants to facilitate their delivery to DCs and at the same time to activate these APCs to trigger marked T-cell responses. In most instances an injected peptide in solution is rapidly degraded and eliminated before it reaches an APC. Thus, peptides have been incorporated or conjugated into microscopic particles such as alum precipitates, microspheres, liposomes, and immunostimulating complexes or in oil-

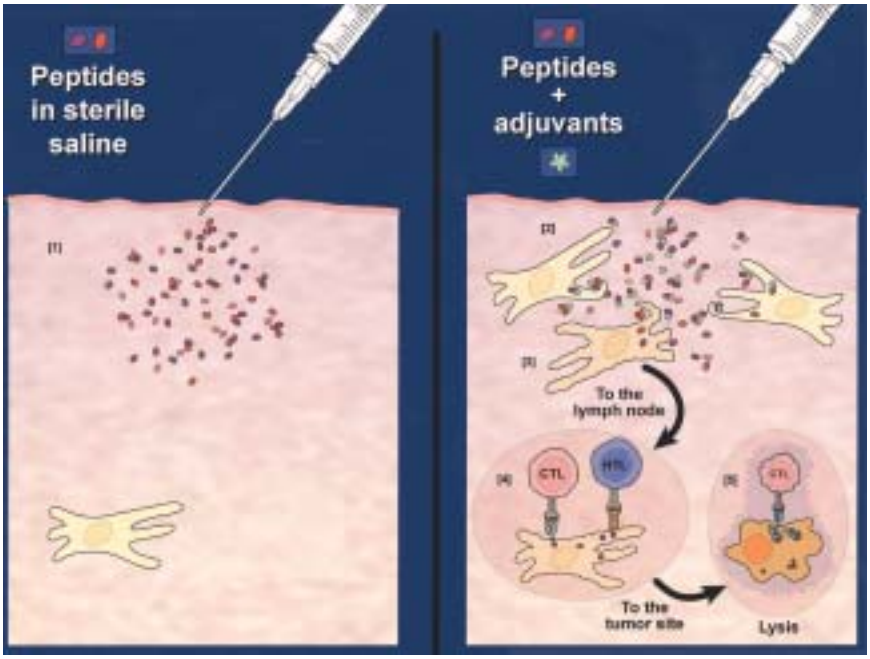

Figure 4. The 2 potential outcomes of peptide vaccination. [1] Peptides prepared in sterile endotoxin- and pathogen-free formulations are usually ignored by antigen-presenting cells (APCs) such as dendritic cells (DCs) because they do not represent a threat. [2] On the other hand, peptides formulated with the appropriate adjuvants will attract DCs to the injection site, allowing the capture of the antigen by these APCs. [3] The adjuvants, mimicking "danger signals" derived from infectious pathogens, will activate the DCs and induce them to travel to secondary lymphoid organs where [4] they will present peptide/major histocompatibility complexes to naïve cytotoxic T lymphocytes (CTLs) and helper T lymphocytes (HTLs). [5] Activated CTLs will migrate to the tumor site to recognize the antigen presented by the tumor cell, triggering their effector function.

water emulsions (eg, incomplete Freund adjuvant [IFA]), all of which function as antigen deposits, protecting the peptide from rapid clearance and allowing slow release into the surrounding tissues.[46] These antigen deposits also function as foreign bodies creating a local inflammatory response that may attract APCs to the injection site (Figure 4). Besides the depot effect, the particulate antigens have the advantage of targeting the peptides to DCs because these APCs are highly phagocytic and have the capacity to ingest large amounts of particles and deliver the peptides to MHC molecules for their presentation to T cells. Since in many cases the particulate peptide formulations do not provide sufficient activation signals to the APCs, researchers have added compounds to their vaccines to persuade the DCs that the injected peptide represents a threat. The types of compounds that have been used to increase the vaccine's potency include PAMP and proinflammatory cytokines. The type of PAMP used in vaccine formulations ranges from dead mycobacteria (found in Freund complete adjuvant) to purified bacterial components or their synthetic counterparts such as lipopolysaccharide, muramyl dipeptide, or bacterial DNA, which is rich in unmethylated cytosine guanine (CpG) motifs. Our laboratory and other groups have demonstrated that peptide vaccines containing synthetic oligodeoxynucleotides with CpG motifs are strongly immunogenic in mice and have the capacity to elicit antitumor responses and prolong survival.[47-49] The cytokines that have added to peptide vaccines have been numerous, but those most effective at increasing T-cell responses have been IL-2, IL-12, interferons, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Although the information gathered from animal models for peptide vaccine development points us in the right direction for improving vaccines for cancer patients, it also raises concerns regarding the safety of vaccines that contain the danger signals that activate APCs and the vaccine may cause serious adverse events. In our view, lack of immunogenicity constitutes one of the most significant challenges in peptide vaccine development. It must be accepted that, to generate a potent immune response to any vaccine, some level of toxicity in the form of local inflammatory reaction, sometimes spreading to the draining lymph nodes, must occur to awaken the immune response. For example, in a recent clinical study at our institution

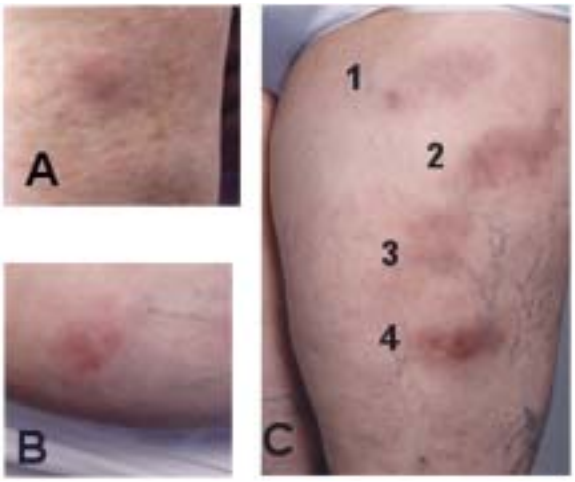

Figure 5. Local inflammatory skin reactions (painless) resulting from vaccination with peptides and adjuvants. A, Skin reaction observed 7 days after injection of peptide emulsified in incomplete Freund adjuvant (IFA). B, Skin reaction observed 7 days after injection of peptide and granulocyte-macrophage colony-stimulating factor (GM-CSF) mixture, emulsified in IFA. C, Long-lasting skin reactions derived from vaccination with peptide, GM-CSF mixtures in IFA. Melanoma patient was vaccinated every 3 weeks, for 4 consecutive times with peptide and GM-CSF emulsified in IFA. Photo was taken 7 days after the fourth injection (numbers represent order of injections).

(unpublished data), we observed that peptides formulated as an emulsion with GM-CSF in IFA induced long-lasting local skin reactions at the injection site, which could be an indication of T-cell reactivity against the peptide epitope (Figure 5). It is possible that more serious systemic effects such as fever, myalgia, headache, nausea, and lymphadenopathy, all commonly observed in acute infections, may also be generated by such immunogenic vaccines. In cancer patients, these symptoms may be a small price to pay particularly compared with severe toxic effects produced by more aggressive chemotherapy and radiation. Even with these potential problems in mind, clinical studies in cancer patients using a second generation of peptide-based vaccines, which included some of the above-mentioned APC-activating compounds, have been evaluated. Rosenberg et al[50] reported that vaccination of melanoma patients with synthetic peptides emulsified in IFA in combination with systemic administration of IL-2 produced tumor responses in 42% of the patients, whereas in the absence of IL-2, no notable responses were observed. Other attempts have used GM-CSF or IL-12 in peptide vaccination protocols, administered either systemically close to the vaccination site or mixed together with the peptides.[51-55] Although the consensus is that these vaccines are superior to similar ones lacking cytokines, statistically significant data are insufficient to substantiate these assumptions. Furthermore, it is probable that mixtures of APC-activating compounds will yield better results than will use of a single agent, but proving this in a clinical setting will be a challenge.

Another approach to improve the immunogenicity of peptide immunization in cancer patients has been the use of DC-based vaccines, which are prepared by ex vivo loading of these APCs with peptides corresponding to T-cell epitopes. In animal model systems, this strategy showed the induction of strong antitumor immunity, which correlated with remarkable antitumor effects in established disease.[56] As a consequence of these studies, several clinical trials have been performed in cancer patients with use of vaccines consisting of autologous DCs, which are pulsed with T-cell peptide epitopes.[57-62] Although the results appear promising, data are insufficient to indicate that these vaccines are better than peptide vaccination alone. Moreover, because it is cumbersome and costly to prepare DCs from cancer patients to produce these vaccines, this approach may not be feasible for the general patient population. Nevertheless, if DC-based vaccines prove to be the most effective way of treating cancer patients, the challenge will be to develop simpler and cost-effective methods to produce these vaccines.

Fourth Challenge: Immune Tolerance vs Autoimmunity

As stated previously, in many cases TAAs that will be targeted for immune intervention are also expressed in some normal tissues. As a consequence of this, many of the T lymphocytes reactive with epitopes derived from these TAAs may have been eliminated through various mechanisms involved in the development of immune tolerance. However, the existence of autoimmune disorders is clear evidence that immune tolerance is not always perfect. Nevertheless, it becomes evident that the magnitude and quality of T-cell responses to most "self" TAA will not be as high as those induced by foreign pathogen–associated antigens, in which tolerance is not an issue. The quality of the T-cell response will be reflected by the affinity of the TCR–peptide/MHC interaction, and in many cases peptide epitopes from TAA are only capable of stimulating low-affinity CTLs and HTLs because tolerance is likely to be developed to the high-affinity ones. Nonetheless, researchers hope that low-affinity T cells may still exhibit antitumor activity in those cases in which the TAA is overexpressed on the tumor cells, as occurs with HER2/*neu* and CEA, and that normal cells, which express lower amounts of the TAA, will be spared (ignored) by the T cells. Because most TAA-derived T-cell epitopes will be generally weaker immunogens than the typical infectious agent–derived epitopes, other strategies besides the ones addressed in the third challenge are being explored to increase the potency of these epitopes. Most notably, the substitution of

some of the amino acid residues of the peptide epitope can increase their antigenicity and immunogenicity.

There are 2 ways by which these reengineered peptide analogues can become more potent than the original counterparts. In many cases substitutions of residues at MHC binding anchor positions can improve dramatically the binding affinity of the peptide for the MHC molecule, which can turn a poorly immunogenic peptide into one capable of inducing strong antitumor responses.[19,63] Animal model systems have shown that the immunogenicity of peptides is directly correlated to their MHC binding affinity.[64] The other approach has been to create heteroclitic epitopes by substituting residues that interact with the TCR in order to improve the capacity of the peptide/MHC complex to activate the T-cell response.[65-67] One caveat of both approaches is that the modified peptides may trigger T-cell responses that fail to cross-react with the original unmodified epitope, which will be the one expressed by the tumor cells, making these responses therapeutically useless.[68]

Ironically, the triumph in breaking tolerance and eliciting strong T-cell responses to some TAAs may have detrimental consequences in the overall health of the vaccinated patient. One of the major concerns of tumor immunotherapy is whether a pathological autoimmune response may be triggered by the vaccine, resulting in the destruction of normal cells. It is well documented that melanoma patients receiving immunotherapy based on the use of TAAs also expressed in normal melanocytes often develop vitiligo.[58,69,70] In the case of melanoma, this level of toxicity would be deemed acceptable if the therapy was successful in slowing down or eliminating the tumor. However, more serious toxic effects could be produced such as those observed in paraneoplastic syndrome in which antigens commonly found in lung tumors, also expressed by cells from the nervous system, elicit immune responses that can cause severe neurologic pathology.[71-73]

Fifth Challenge: Implications of MHC Restriction in Patient Population Coverage

Besides immunogenicity, another major challenge for the development of effective peptide-based vaccines relates to their limitations on patient population coverage imposed by the rules of MHC restriction.[9] In most cases, a single-peptide epitope will be useful only for treating a small subset of patients who express the MHC allele product that is capable of binding that specific peptide. For that reason, researchers have focused their attention on identifying peptide epitopes restricted by the most common MHC class I alleles, in particular HLA-A2, which is found in approximately 40% of the human population. Nevertheless, it is clear that, sooner rather than later, additional CTL epitopes will need to be identified to design vaccines that can be used in a broad patient population. It has been calculated that vaccines containing CTL epitopes restricted by HLA-A1, -A2, -A3, -A24, and -B7 would offer coverage to approximately 80% of individuals of most ethnic backgrounds.[74] Furthermore, population coverage is probably greater since some diverse MHC alleles such as HLA-A3, -A11, -A31, -A33, and -A68 can bind similar sets of peptides.[75,76] A parallel problem exists with regard to the use of MHC class II–restricted HTL epitopes in vaccines with broad applicability for the cancer patient population. Researchers are focusing on identifying HTL epitopes restricted by HLA-DR1, -DR3, -DR4, and -DR7, which are the most frequently found MHC class II alleles.[77] In some cases it has been observed that the same peptide may bind to several of these alleles. Thus, the identification of these "promiscuous" MHC binding peptides for a TAA would certainly simplify development of a vaccine. Our group has been successful in identifying several promiscuous HTL epitopes from TAA such as HER2/*neu*, CEA, gp100, and MAGE3.[26,28,29]

Sixth Challenge: The Requirement for Multiepitope Vaccines

Paradoxically, the successful identification of the necessary CTL and HTL epitopes to ensure population coverage creates another challenge for vaccine development. Furthermore, to allow disease coverage and prevent the emergence of tumor escape mutants more than 1 TAA per malignancy type should be included in each vaccine. Thus, because there is no universal TAA, only by including epitopes from various TAAs will it be possible to surmount the problem imposed by the antigenic heterogeneity of each tumor type. For example, for breast cancer a vaccine containing CTL and HTL epitopes restricted by the most common HLA alleles, for TAA such as HER2/*neu* (expressed on approximately 30% of tumors), CEA (approximately 50% of tumors), and MAGE3 (approximately 30%) would be most desirable. This additional requirement creates the following dilemma: Should vaccines containing multiple epitopes be composed of peptide mixtures or a single peptide consisting of linked epitopes? For those familiar with the field of drug development, it is evident that a peptide mixture would create numerous nightmares since each peptide would have to undergo separate manufacturing, quality control, and safety testing. Then the peptide mixture itself would have to undergo extensive testing to ensure that each component is present at the stated concentration and that interactions between the components do not affect their solubility, biological activity, and stability.

On the other hand, the use of a single construct containing multiple epitopes would avoid these problems but would create other uncertainties such as those related to the

limitations in size of peptides produced by organic chemistry synthesis, whether the epitopes should be directly joined to each other or through linkers and whether the order the epitopes occupy within the peptide construct may influence the strength of immune response to the individual epitopes. Another uncertainty is whether large peptide constructs will be processed correctly by APCs to produce all the T-cell epitopes contained within the construct.

One approach to address some of the potential barriers of the multiepitope constructs could be through the use of "Trojan antigens." Our laboratory recently reported that it is possible to deliver peptide constructs from the extracellular milieu directly into the endoplasmic reticulum and the *trans*-Golgi apparatus of APCs, where antigen processing to create T-cell epitopes can occur.[78] These Trojan antigens are made by linking a positively charged amino acid segment into either end of a peptide construct containing a single T-cell epitope or multiple T-cell epitopes. The processing in these cellular compartments does not require the usual cytoplasmic proteasomal degradation and transport to the endoplasmic reticulum, which could limit the production of some of the epitopes contained in a multiple-epitope construct that would require conventional processing.

Seventh Challenge: Clinical Evaluation of the Vaccine

Thus far we have addressed what we consider the most important challenges related to the design of peptide-based vaccines for cancer. However, there are also numerous challenges related to testing the safety, immunogenicity, and antitumor effectiveness of these vaccines in clinical trials (Figure 6). Most of these challenges are not only related to peptide-based vaccines but also are applicable to other kinds of tumor vaccines and types of immune-based therapies.

First, any attempt to induce an immune response, especially against weak antigens such as those TAAs that represent "self-components," will be futile if the immune system of the cancer patient has been compromised. It is unfortunate that many of the conventional second-line anticancer treatments such as chemotherapy and radiation can be highly immunosuppressive; thus, it becomes important to administer any kind of vaccine once the immune system has recuperated from the effects caused by these treatments. Even in the absence of chemotherapy and radiation and mostly in hematologic malignancies, many patients with advanced cancer become hyporesponsive to immune challenges, possibly because of their poor general state of health or because their tumors may produce immune suppressor factors such as transforming growth factor β and IL-10 (see eighth challenge). Thus, therapeutic antitumor vaccines are likely not to be as effective in these patients compared with cancer patients in early stages of disease. This creates a serious challenge in the design of clinical

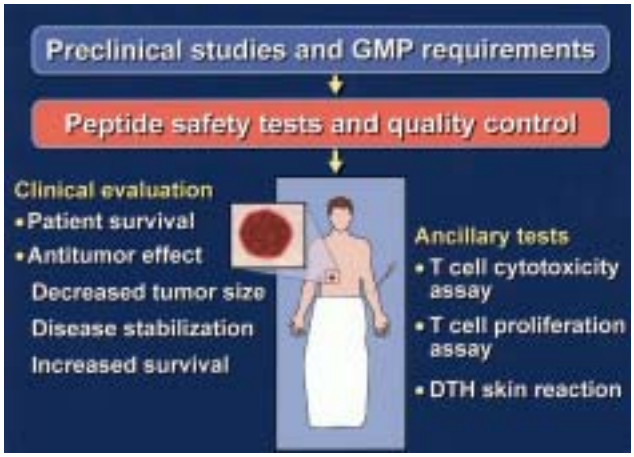

Figure 6. Later stages in the development of peptide-based vaccines. Subsequent to the identification and selection of T-cell epitopes from tumor-associated antigens, synthetic peptides must be prepared and finished (vialed) under Good Manufacturing Practices (GMP) before they can be administered to humans. These products must also undergo extensive quality control testing and safety evaluation to ensure identity, purity, quantity, sterility, and lack of endotoxin. The approved released product can then be tested in patients for its safety (toxicity) in phase 1 trials and at later time points for its efficacy (antitumor effects) in phase 2 and phase 3 studies. Ultimately, a vaccine will be evaluated clinically for its ability to improve patient survival and for its capacity to reduce or stabilize disease. Laboratory and ancillary tests are valuable for determining the immunogenicity of the vaccine, which should correlate with its clinical effectiveness. DTH = delayed-type hypersensitivity.

studies since most experimental therapies are usually tested first in patients suffering from advanced disease. Unfortunately in these patients, the tumor vaccines are not only evaluated for their safety but also are expected to demonstrate some sort of biological activity such as immunogenicity and sometimes even antitumor effects. We believe that immunotherapy trials in patients with advanced cancer will provide little valuable information and may even sway researchers to abandon promising treatments that could be effective in patients with early-stage disease. Conversely, clinical studies in patients with early-stage disease or in the adjuvant setting (after surgery) in the absence of measurable disease will be conducive to determine the immunogenicity of the vaccines but may not allow critical evaluation of objective antitumor responses. In these circumstances, other clinical end points such as time to recurrence and survival would be ideal to determine the effectiveness of the vaccines, but it is obvious that these studies could become lengthy and expensive.

Demonstrating that a vaccine is immunogenic constitutes one of the major challenges for clinical researchers. Since peptide-based epitope vaccines are designed to elicit

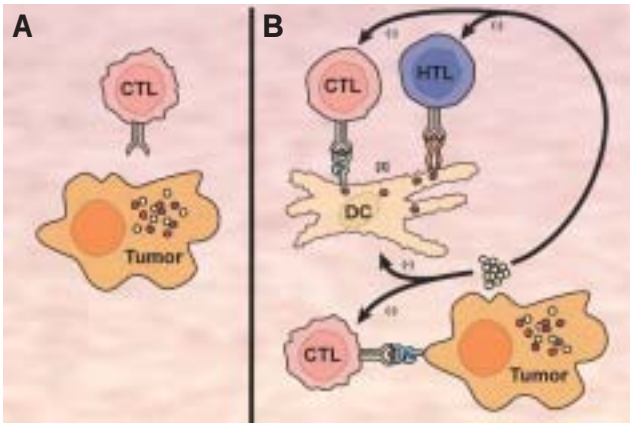

Figure 7. Tumors can evade immune destruction. A, Frequently, mutations result in tumors ceasing to express cell surface major histocompatibility complex/peptide complexes, escaping immune detection and destruction by cytotoxic T lymphocytes (CTLs). B, Tumors are capable of producing immune suppressor factors such as transforming growth factor β, interleukin 10, and Fas-ligand, which can block the induction of immune responses by affecting antigen-presenting cells and naive T cells or may also inhibit the effector function of mature CTLs. DC = dendritic cell; HTL = helper T lymphocyte.

CTL and HTL responses to TAA, the goal has been to prove that such responses are indeed induced or augmented as a consequence of vaccination. Thus, quantifying the numbers of antigen-specific T cells and assessing their function for each of the epitopes in the vaccine is important. Typically, blood lymphocytes are sampled before vaccination and at various time points after vaccination and are then evaluated in vitro for CTL and HTL reactivity against the TAA-derived epitopes. Many conventional laboratory tests have been used in vaccine studies to measure antigen-specific CTL and HTL function such as the cytotoxicity (^{51}Cr release), antigen-induced cell proliferation (^{3}H-thymidine incorporation), and lymphokine release assays. More recently, the enumeration of antigen-specific T-cell frequencies has been done with use of MHC tetramer staining, intracellular cytokine staining, and enzyme-linked immunospot assays.[60,79] Regardless of the type of assay used, the main problem is that these methods rely on the presumption that the antigen-reactive T cells will be present in the blood at the time of sampling. The reality is that T lymphocytes, once activated by antigen, leave the circulation and travel to the peripheral tissue sites where they are meant to perform their function. For this reason, some researchers have decided to evaluate delayed-type hypersensitivity skin reactions induced by injection of small amounts of antigen.[51,54,57,80-82]

Table 1. **The 8 Challenges for Developing Peptide Vaccines for Cancer**

1. Epitope identification
2. Selection of most relevant epitopes
3. Lack of immunogenicity
4. Immune tolerance vs autoimmunity
5. Major histocompatibility complex restriction
6. Multiepitope vaccines
7. Clinical evaluation
8. Immune evasion

Eighth Challenge: Immune Evasion by Tumor Cells

Tumor escape mechanisms from immune destruction constitute another type of challenge for the development of effective vaccines (Figure 7). As mentioned previously, some tumors produce immunosuppressor factors that either paralyze the function of antitumor CTLs or may even induce their death. Among these factors, TGF-β and IL-10 are potent inhibitors of the induction and maintenance of CTL responses.[83-86] In addition, some tumors have been reported to produce Fas-ligand, which is capable of inducing death of CTLs and APCs.

Another major mechanism of immune evasion used by tumors is decreasing or eliminating their expression of the antigenic epitopes recognized by the CTLs (Figure 7). Tumor cells can escape from CTL attack by decreasing the expression of cell surface MHC molecules, by decreasing the production of the tumor peptide epitope through an alteration of the antigen-processing pathway, or simply by ceasing to express the TAA protein, if it is not required to maintain tumor cell growth.[87,88] It is worrisome that the selection of tumor escape mutants that cease to express specific TAAs has been observed in several clinical studies using peptide vaccines corresponding to CTL epitopes.[89,90] This problem could be minimized to some degree by incorporating multiple T-cell epitopes to more than 1 TAA in each vaccine. In addition, because the emergence of tumor escape mutants probably correlates with the tumor burden (number of tumor cells), this problem should be minimal in early stages of disease.

CONCLUSION

In this article we have enumerated some of the challenges that we currently face in the development of peptide-based vaccines for treating cancer patients (Table 1). At first glance these challenges may appear to be insurmountable and could even discourage some researchers working in the field. However, the indisputable evidence that the immune system is capable of recognizing and fighting tumors and the prospect of developing an alternative therapeutic approach to radiation and chemotherapy that would not compromise so severely the patient's quality of life provide us with a strong incentive to succeed in our mission.

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