

REVIEW

Molecular Cancer Vaccines: Tumor Therapy Using Antigen-Specific Immunizations

Tamás SCHWEIGHOFFER

Department Cell Biology, Boehringer Ingelheim Research and Development, Wien, Austria

Vaccination against tumors promises selective destruction of malignant cells by the host's immune system. Molecular cancer vaccines rely on recently identified tumor antigens as immunogens. Tumor antigens can be applied in many forms, as genes in recombinant vectors, as proteins or peptides representing T cell epitopes.

Key words: vaccines, molecular, immunotherapy, tumor

Analysis of various aspects indicates some advantage for peptide-based vaccines over the other modalities. Further refinements and extensively monitored clinical trials are necessary to advance molecular cancer vaccines from concepts into powerful therapy. (Pathology Oncology Research Vol 3, No 3, 164–176, 1997)

Background: Tumor immunotherapy, and the roots of molecular cancer vaccines

Immune responses are based on making distinctions – self or non-self, danger or no harm,⁷⁸ good or bad. Not surprisingly, almost a century has been spent trying to identify a basis by which the immune system would be capable of distinguishing tumors. That tumor cells indeed can carry a “difference”, at the cellular level at least, was first demonstrated by transplantation experiments with mouse tumors.^{93,107} Cancer cell specific immune sera⁴⁸ and development of monoclonal antibodies⁹⁹ suggested that distinct molecules constitute the basis for an immunologically interpretable difference between normal and malignant cells. Studies of chemically induced tumors identified cytotoxic T cells (CTLs) as the major immune effector arm involved in tumor rejection; in addition,

long before understanding the molecular basis of T cell – target cell interactions, these studies also proved that tumor specific determinants can be used to distinguish independently derived tumors from one another as well as from normal cells.⁸⁶

This gradually increasing knowledge was, however, not readily transformed into useful immunotherapeutic strategies – apart from rare examples, induction of anti-tumor immunity succeeded only in a rather irreproducible fashion. A new wave of interest emerged towards tumor immunotherapy when genetically modified, irradiated tumor cells were used successfully in mouse experiments to induce rejection of fully growth-competent, unmodified parental tumor cells (reviewed in,^{32,106} our implementation in¹¹⁵). This approach, termed cellular cancer vaccines, invariably relies on autologous/syngeneic tumor cells as the source of antigens which are not further defined, and on various highly efficient gene transfer techniques enabling these cells to express immunomodulatory molecules. The repertoire of transfected genes covers cytokines, such as IL-2,³⁶ IL-4,¹²⁶ TNF α , IFN γ ,⁹⁹ GM-CSF,³¹ and cell surface molecules with T cell costimulatory capabilities, usually from the B-7 family (CD80/CD86).¹⁵⁴ In some models mice were not only rendered immune against a subsequent challenge but were also cured from a pre-existing tumor burden. Cytokine-transfected autologous cellular cancer vaccines were thus rapidly adapted for human therapy, resulting in a number of clinical applications.¹²⁵

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Correspondence: Tamás SCHWEIGHOFFER, M.D., Ph.D., Department Cell Biology, Boehringer Ingelheim Research and Development, Dr. Boehringer-Gasse 5, A-1120 Wien, Austria; Tel: (43) 1-80105-258/office, (43) 1-80105-284/lab; Fax: (43) 1-80105-366; e-mail: schweig@bender.co.at

Abbreviations: CTL, cytotoxic T lymphocyte; TA, tumor antigen; TCR, T cell receptor; TIL, tumor infiltrating lymphocyte. Usage of the terms “tumor” and “cancer” does not strictly follow their definition in pathology; rather they serve as labels for various malignancies.

Although details of how autologous cellular vaccines induce anti-tumor immunity still remain elusive, in most cases anti-tumor CTLs have been found, along with a wide spectrum of other cellular and humoral components (reviewed in⁸⁴). Since further experiments also revealed that (re-)presentation of the tumor antigens of the vaccine

cells by host bone marrow-derived antigen presenting cells is required,^{55,73} comparable vaccination effects may be expected when other sources of tumor antigens are substituted for autologous cells. Allogeneic tumor cells are thus a next logical choice, provided they are an equally good source of equally relevant tumor antigens. While allo-

Table 1. Current list of human tumor antigens from which at least one HLA-restricted peptide epitope has been identified.

<i>Tumor antigen</i>	<i>Expression on tumors</i>	<i>Expression on normal cells</i>	<i>HLA restriction</i>	<i>Ref.</i>
<i>Altered gene products</i>				
β-catenin	unique mutation in melanoma	unmutated gene widely expressed	HLA-A24	100
BCR/abl	fusion protein in CML	—		10, 76
CDK4	in 1/28 other melanoma	unmutated gene widely expressed	HLA-A1	8, 150
HLAA2-R170I	mutated HLA class I in LB996-RCC	—	presents itself	12
MUC1	breast, ovarian, pancreatic cc. myeloma	normal colon mucosa	HLA-A11; unrestricted	29
MUM1	mutated exon-intron junction	unmutated gene widely expressed	HLA-B44	21
NA17-A;	epitope derived from an intron whose	fully spliced mRNA widely found	HLA-A2	43
NAc-Glucosaminyl transferase V	promoter is active only in melanomas			
p53 mutants	in many tumors	unmutated gene widely expressed	HLA-A2	127
Ras p21 mutants	in many tumors	unmutated gene widely expressed	HLA-class II	44
<i>Restricted expression in normal tissues</i>				
BAGE	in 10-30% of various tumors	testes / placenta in adults	HLACw*1601	8
GAGE	<25% of melanomas, sarcomas, NSCLL, head-and-neck cc.	testes / placenta in adults	HLACw*0601	138
MAGE family	related genes with 64-85% identity	testes / placenta in adults	HLA-A1	42
MAGE-1		testes / placenta in adults	HLA-A1	140
			HLACw*1601	
MAGE-3	70% of melanomas	testes / placenta in adults	Tbl. 2	140
p15	melanoma	wide expression	HLA-A24	101
PRAME	melanoma, lung, head-and-neck cc.	testes, ovary, low on normal tissues	HLA-A24	57
RAGE1 (family)	renal cancer		HLA-B7	41
<i>Lineage specific</i>				
gp75 / TRP-1	melanoma	retina, melanocytes	HLA-A31	129, 147
gp75 alt. ORF ^a	melanoma	retina, melanocytes	HLA-A31	146
gp100 / Pmel-17	melanoma	retina, melanocytes	HLA-A*0201	22, 67
MART1 / MelanA	melanoma	retina, melanocytes	HLA-A2	66, 68
TRP2	melanoma	retina, melanocytes	HLA-A31	145
Tyrosinase	melanoma, some neuroblastomas	retina, melanocytes	HLA class II	129
			HLA-A2	152
			HLA-A24	64
			HLA-B*4403	
<i>Viral</i>				
HPV16-E6/E7	cervical cc.	—	HLA-A*0201	97

a. product of an alternative open reading frame encoded by the gp75 gene

geneic cellular vaccines can circumvent many technical obstacles of vaccine production associated with the *in vitro* expansion and processing of autologous tumor cells into vaccines, mouse models⁶⁹ and clinical application of this approach⁵ show varying success. It is important to realize that all forms of anti-tumor vaccinations are generally antigen-specific in their nature, independently from the degree of antigen purity. The ultimate goal is to use pure, defined TAs as vaccines, which is the objective of molecular cancer vaccines.

Tumor antigens: the basis for molecular cancer vaccines

In its broadest sense, any molecule of a tumor cell which can be recognized by any component of the immune system is a tumor antigen (TA). Structures recognized by cytotoxic T cells (CTLs) which mediated efficient rejection of experimental tumors were the first antigens designated as tumor (associated) antigens (TAs). With the help of CTL clones, even multiple TAs appearing on a single tumor could be precisely mapped,¹⁵³ but identity of these TAs remained an enigma for a long time. Finally, in 1988 Boon and coworkers cloned the first rejection antigens: they converted by *in vitro* mutagenesis weakly immunogenic mouse tumors into immunogenic ones, and designated these variants as tum-.⁹ Using CTLs reactive against the immunogenic variants as read-out reagents, in an elaborate genetic screen a series of tum- TAs derived from the P815 mouse mastocytoma were identified.^{24,72,119}

In the meantime it became also gradually clear that CTL responses in general are HLA / MHC-restricted¹⁵⁵ and that also a peptide derived from cellular or pathogenic gene products¹³⁰ must be presented by the HLA/MHC restriction elements. Although a wide variety of peptide sequences can be presented, a set of rules (which are influenced by the presenting HLA/MHC allele) could be observed to which most of the peptides obey. Accordingly, chain length must be within certain limits (8-10 AA for most class I alleles), and defined amino acids (designated primary and secondary anchor residues) must occupy certain positions to ensure optimal binding.^{35,95,96}

The tum- antigens are derived from normal cellular genes, but they carry mutations altering single amino acids. As a consequence of these mutations, the tum-derived peptides form immunogenic epitopes while their normal counterparts do not. The single amino acid changes represent novel epitopes for T cell recognition either because the mutation creates an anchor residue which enables binding of a novel peptide to presenting class I molecules,¹²³ or simply by modifying the peptide – T cell receptor recognition interface. Subsequently natural TAs from the parental P815 line have also been cloned.^{70,136} In contrast to the tum- TAs, these antigens

turned out to be products of unmutated genes, whose expression in normal animals is limited to testes and placenta.

Using a similar approach the same group also cloned the first CTL-defined human tumor antigen, MAGE-1,¹⁴¹ and ever since new antigens follow: human TAs which have been cloned and where at least one HLA-restricted T-cell epitope peptide has been identified are summarized in *Table 1*. These human TAs can be classified into four distinct categories: (a) altered gene products, arising mostly from mutations or, like in the case of MUC-1, from incomplete posttranslational modification; (b) the “cancer/testis” group, genes whose expression in adults is restricted to immune privileged tissues, such as testes and placenta; (c) lineage specific genes; derived most prominently from melanin producing cells; (d) viral antigens. Remarkably, this classification appears to be valid also for mouse TAs, with the tum- and natural P815 TAs falling into categories (a) and (b). Others, such as mouse TRP-2⁷ and TAs derived from retroviral products, which are apparently dominating the mouse TA repertoire^{23,50,56} are examples for categories (c) and (d), respectively.

Antigens derived from melanomas are the most frequent ones in the list of human TAs. Malignant melanoma functions as a model disease for cancer immunotherapy, based on numerous observations indicating a strong immune response against the disease: cases of spontaneous remissions, frequently found lymphocytic infiltrates of lesions, and also that vitiligo (resulting from selective autoimmune destruction of melanocytes by the host) correlates with a favorable disease course.^{16,49} Accordingly, a long list of immunotherapeutic modalities have been tried in melanoma, including monoclonal antibodies,⁸⁶ cytokines,¹⁰⁴ *in vitro* expanded LAK/TIL cell infusions¹⁰⁵, cytokine-transfected cancer vaccines.¹¹⁵ These approaches not only resulted in occasional success, but also greatly assisted the development of reagents and technologies critical to the cloning of tumor antigens: a broad collection of melanoma lines, TIL cultures and well-characterized autologous anti-melanoma CTL lines¹⁵¹ were established, along with improved transfection and assay techniques. The accumulated knowledge predicts that TAs from other human tumors will follow, since generation of autologous CTLs against gastric,⁵⁴ ovarian³⁸ and rectal cancer⁶⁰ as well as sarcoma lines⁵¹ have been reported.

Cloning of HLA-restricted peptide TAs has shifted the attention from antibodies that target only cell surface structures *in vivo* towards T cell-mediated immunity. However, studies of antigen processing³⁴ prompt one to view every stretch of amino acids that can be processed and accommodated properly by the presenting HLA molecules as a potential source of antigenicity. Tumor specific products, identified by antibodies or by whatever other means, may be thus integrated into the peptide epitope – TA concept.

Best examples are structures, like CEA and MUC-1, which were defined as being tumor specific by (monoclonal) antibodies; however, CTL responses against HLA restricted or unrestricted peptide epitopes were detected in the peripheral blood of patients upon active immunization.^{13,29,131} On the other hand, the MART-1/Melan-A antigen defined originally by CTLs,⁶⁶ can also be recognized by antibodies.²⁰

SEREX is a further extension of this approach¹⁰⁹: here sera from tumor patients are employed to screen a phage cDNA library derived from the corresponding tumor tissue. Among the increasing number of TAs identified this way belong NY-ESO-1,¹⁹ HOM-MEL-40 (SSX-2)^{109,132} and galectin-9¹³³ to the cancer/testis TA group; but also the ubiquitous eIF4-gamma¹¹ overexpressed in squamous lung cancer has been uncovered by SEREX. No functional epitope peptides derived from these TA transcripts have been reported yet, however. Powerful genetic methods exploring differences between tumor cells and normal tissues, such as differential display and various subtraction methods¹¹⁸ are yielding also promising TA candidates, but validating biological significance of these hits requires even more time and effort.

Converting tumor antigens into effective molecular cancer vaccines

With the cloning of TAs it should be now possible to use these antigens as immunogens to generate a host response against tumor cells that express the particular TA. Two main modes of application can be envisioned: (1) using the whole antigen, either as recombinant DNA vaccine or as protein product; (2) selection of epitope peptides.

Whole-antigen approaches have the advantage that all potential epitopes (including those which may represent a target for antibodies) are included in the immunogen. When injected intramuscularly, plasmid DNA itself can be used by the host to express immunogenic proteins.^{30,134} The achieved expression levels seem to be sufficient to induce an antibody response, but there are but a few reports on effective generation of CTLs⁴⁰ and there is no consensus about the mechanistic principles of this kind of vaccine.⁹⁰ A promising mode of delivery of naked DNA is by the gene gun,⁵⁸ which apparently results in the generation of both antibodies and CTLs. Recombinant viruses or microorganisms that harbor one or more TAs alone or in combination with other genes to augment the immune response represent a very potent alternative.^{18,65,88,98} The amount of expressed immunogen is usually not limiting, but in many cases, like with adeno- and poxviruses or recombinant bacteria the intrinsic immunogenicity of the carrier organism dominates the vaccine. Consequently, the response against the TA may remain suboptimal while the

elicited strong anti-carrier immune response prevents repeated applications. Low-immunogenicity carriers, like retroviruses or adenovirus mutants may solve this problem.¹²² Past experience advocates the use of proteins as vaccines, but lack of effective adjuvants, besides complex and costly manufacturing and application procedures render them unattractive for widespread use.

With all of the whole-antigen vaccines also extra regions of the TA that may have additional immunomodulatory functions are co-delivered. There are speculations (but only sparsely data) that 'T helper' epitopes, i.e. AA stretches which themselves are not necessarily targets of CTLs but can enhance immunogenicity of other (usually class I restricted) epitopes may be present on a whole-antigen vaccine.¹⁴⁹ If this kind of help is required,⁵⁶ peptides derived from commonly used immunogens can be substituted to perform this 'helper' role efficiently.¹⁴³ On the other hand, regions with tolerogenic potential that are deleterious for the vaccine effect may also be present within the full-length antigen. This is best illustrated on the example of HER-2/neu, where synthetic peptides but not whole proteins were capable of eliciting a response.²⁸ Not overcoming the pre-existing tolerance directed towards regions of the whole protein vaccine was inferred to be the reason for its failure.

Since the bulk of accumulated evidence suggests that effective tumor rejection depends on CTL epitopes, and since these epitopes are available for many TAs, nothing is more straightforward than applying these epitope peptides as vaccines. There are already examples of successful application of peptide-based cancer vaccines in animal models: a mutated connexin peptide was shown to be effective against the mouse Lewis lung carcinoma,⁷⁵ while an HPV16-E7 derived peptide prevented outgrowth of an HPV induced tumor.³⁷ Peptide vaccines offer not only the highest degree of specificity, but are also appealing because they represent a minimalist approach, avoiding a plethora of problems associated with cellular or with the more complex whole-antigen vaccines. Development is also aided by the characteristics of peptides resembling conventional pharmaceutical products – well defined structure, stability, ease of production and handling, constant quality.

However, all this cannot obscure the problems still attached to peptide-based vaccines. By definition, this approach is always HLA allele-specific, and requires that the peptide is an immunologically relevant rejection antigen epitope. This means, that on the patients' tumor cells the identical peptide must be expressed, processed and presented essentially in the same conformation, and in quantities that can trigger a responsive T cell. Until now for most TAs (ranging from only a few up to more than a thousand amino acids in length) only one or just a handful of such peptides presented by various HLA restriction elements

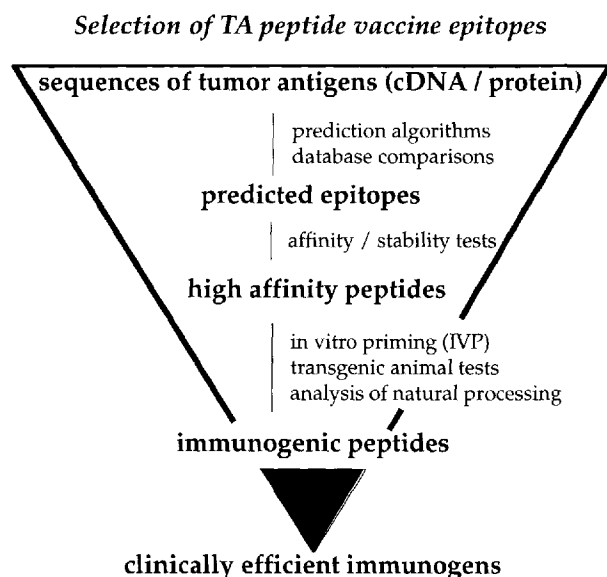


Figure 1. Sequential steps for the identification of epitope peptides starting from the primary sequence of a tumor antigen. Width of the triangle illustrates numbers of candidates decreasing during the selection procedure, yielding one or just a few peptides that may advance into clinical trials.

have been identified based on reactivity of patient-derived CTLs. Thus with the TA peptides only a subset of patients expressing the matching HLA allele can be vaccinated. An added technical complication is that HLA subtypes belonging to the same serological group may differ in their peptide binding preferences, and thus HLA matching has to proceed in many cases beyond that of conventional serological typing. Consequently, a substantial portion of the patient pool may remain untreated, despite of a defined TA being expressed by their tumors. Therefore, it is imperative to derive promising vaccine candidate peptides applicable to as many different HLA alleles as possible starting from the whole protein sequence of cloned TAs.

Definition of immunogenic peptides within a protein product is aided by a series of tools (Fig. 1). First of all, stability and a long half-life of the HLA class I – β 2-microglobulin – peptide trimeric complex was found to be directly proportional to the immunogenicity of the particular peptide.^{45,142} Binding motifs that have to be contained within a peptide to ensure good HLA binding have been defined for most of the human alleles.⁹⁶ Databases harboring experimental data of HLA-peptide interactions are being maintained,¹⁴ and with automated tools available through the world-wide web it is possible to predict high affinity binder sequences.^{63,91} These algorithms may thus yield interesting candidate peptides matched to a given allele, which are worth to be tested more extensively. The predicted stability can be biochemically analyzed by stabilization of cell surface HLA expression,¹³⁷ peptide-

induced in-vitro-refolding of bacterially produced class I heavy chains and Scatchard-type competition assays with isolated natural or recombinant HLA molecules,^{87,116} yielding a set of high-affinity candidates. Tumor cell lines of the appropriate HLA-type ought to be analyzed for the presence of the candidates to ensure that the natural TA processing yields the identical product: posttranslational modifications that markedly impact on immunogenicity are not uncommon.^{82,121}

Good binding alone does not automatically ensure a good anti-tumor immune response, and in fact, reliable data about effectiveness of a particular peptide as immunogen can most likely be only obtained in clinical trials. However, it is important to find out whether the T cell repertoire can respond to the peptide, or, not unlikely, the relevant cells have been deleted or tolerized. Currently this is best done by performing an in vitro priming (IVP) assay, where peripheral CD8+ T cells from several normal donors are cultivated with autologous stimulator cells in the presence of various cytokines and the tested peptide.¹⁷ If CTL lines or clones that are reactive against the peptide presented in appropriate HLA context can not be grown out, the peptide is not considered being a promising immunogen. CTLs should also be able to lyse HLA-matched tumor targets expressing the respective antigen. Transgenic mice harboring human or human/mouse hybrid HLA molecules offer an interesting alternative to efficacy testing in vivo.¹²⁷ The mouse T cell receptor (TCR) repertoire is apparently flexible enough to enable vaccination results comparable to humans.¹⁴⁸

I would like to illustrate the needs, obstacles and potential solutions outlined in the last paragraphs in a literature- and computer analysis on the example of MAGE-3 (Table 2). MAGE-3 has been identified as a gene that cross-hybridizes with MAGE-1. Analysis with patient-derived CTL clones qualified MAGE-3 as a real TA, and the natural epitope EVDPIGHLY presented in HLA-A1 context was identified.⁴² The proportion of melanoma patients displaying this peptide is estimated to be about 18%, with 26% of the population being HLA-A1 positive, and 70% of the melanomas expressing MAGE-3. How can the MAGE-3-positive, but HLA-A1-negative (further 44%) population be vaccinated with MAGE-3 peptides? Essentially the steps outlined above and in Fig. 1 were followed. The MAGE-3 sequence was analyzed for several other HLA alleles; three of them (HLA-A*0201, HLA-A3, HLA-B*4403), which (together with HLA-A1) give a cumulative coverage of about 90% for the Caucasian population, yielded evaluable results. The sequence was first submitted to a prediction algorithm developed by KC Parker.⁹¹ Estimated half-life of the best ranking peptides was retrieved (Table 2, column c). For these peptides data for biochemically measured affinity (d), for stability with less sensitive methods (e), whether clones resulting from

Table 2. Analysis of MAGE-3 derived peptides

Allele	Peptide	$T_{1/2}$ ^c score	Affinity nM ^d	Stability score ^e	IVP ^f peptide	IVP ^g tumor	CTL ^h natural	Ref.
HLA-A1	EVDPIGHLY	250.0	4.6		+	+	+	17,42
	(A)SSLPTTMNY	37.5	9.6		—	—		
	GSVVGWQY	0.7	500.0		—	—		17
<i>RP.S16</i> ^a	MIEPRTLQY	2250.0		NATURAL				96
HLA-A*0201	FLWGPRALV	2655.0		10.2	+	+	—	135,139
	KVAELVHFL	339.0						
	KIWEELSVL	329.0						
	ALSRKVAELV	49.1		7.8	—	—		139
<i>HBc</i>	FLPSDFFPSV	2310.0	3.3		4/4		4/4 ⁱ	148
<i>HBsAg</i>	WLSLLVPFV	4047.0	4.5	NATURAL	2/6		5/9	96
<i>HBpol514</i>	LLAQFTSAI	67.4	50.0		0/5		0/4	148
HLA-A3	ALVETSYVK ^b	135.0						139
	ELVHFLLLK	81.0		2.28	—	—		81
	YFFPVIFSK	1.0		1.98	—	—		81
	FLLKRYAR	0.6		1.23	—	—		81
	ISGGPHISY	0.1		1.92				81
<i>UNKNOWN</i>	KLFKNILYK	900.0		NATURAL				96
<i>INFLUENZA</i>	ILRGSAVHK	90.0			+	+/-	—	27
HLA-B*4403	MEVDPIGHLY	240.0		21.2	+	+	???	52
	WEELSVLEVF	90.0		5.6	—	—		39
	LESEFQAALS	6.0		13.9	—	—		39
<i>EF-2</i>	AEMGKGSFKY	1620.0		NATURAL				87

a, name of protein source for natural ligand is given in italics

b, the peptide ALVETSYVK was tested as a potential HLA-A*0201 ligand, displaying good refolding stability but no CTL generation¹³⁹

c, estimate half-life is expressed as a relative score⁹¹

d, affinity was measured in competition assay; lower values denote stronger binding

e, stability was measured in refolding or cellular stabilization assays by comparing the test peptide to a standard peptide; higher values denote stronger binding. NATURAL stands for peptides that have been identified as natural binders

f, g, successful (+) or unsuccessful (—) lysis of peptide-loaded (f) or natural tumor targets (g). Where available, the success rate was given.

h, activity of patient-derived CTLs against natural tumor targets

i, this block of values denotes rates of successful *in vivo* priming obtained in transgenic mice expressing HLA-A2/H2Kb (AAB) hybrid class I molecules

Entries left blank indicate that corresponding information is not available.

in vitro priming lyse peptide-pulsed targets (f), or HLA matched natural tumor cell targets (g), and whether peptide reactive CTLs can be isolated from patients' samples (i) were retrieved from the indicated references. In addition, naturally processed peptides from irrelevant sources were at least partially analyzed for comparison.

The following results emerged:

(a) In essentially every case examined, there is a remarkable concordance between computer-predicted half-life

values and experimentally measured affinity. Stability values, obtained from various experimental systems with likely quite different sensitivity, are somewhat more heterogeneous – however, the highest half-life score still correspond to the best stability score in each group. Although there are examples of immunogenic epitopes that break the binding rules incorporated in the prediction algorithms, this correlation suggest that for a given allele the top ranking peptides are the most promising candidates.

(b) Predicted scores even for the best MAGE-3 candidates appear to be rather low in most cases when compared to natural (self-protein or pathogen-derived) ligands: the HLA-A1-binding EVDPIGHLY peptide has a score of 250 compared to 2250 assigned to a natural ligand. However, the biochemically measured affinity (4.6 nM,¹⁷) is still way above the estimated threshold (about 50 nM;¹¹⁷) required for good immunogenicity.

(c) High scores, high binding generally translates into positive in vitro priming cultures or good priming of HLA-transgenic mice. On the other hand, self peptides binding with high affinity may also prove to be non-immunogenic, suggesting a preference for epitopes with intermediate affinity;⁹² this may be because of the responsive T cells are tolerized or deleted from the peripheral repertoire.

Another problematic aspect is exemplified by the dominant HLA-A*0201 restricted FLWGPRALV epitope, which qualifies as a good functional immunogen based on favorable scoring, binding and IVP results.¹³⁹ However, follow-up analysis suggests that natural antigen processing does not generate this peptide in amounts which would suffice for melanomas being a good target;¹³⁵ consequently this peptide might not be useful in vaccinating melanoma patients.

(d) Binding motifs partially shared between HLA-A1 and HLA-B*4403 (and, in addition, HLA-A29⁷¹) result in overlapping or identical peptide sequences. The HLA-B44-binding homologue of the natural HLA-A1 peptide scored well in all tests, but the final biological evidence for its usefulness as therapeutic immunogen has yet to be awaited.

The most important conclusion is that only peptide epitopes meeting rigorous testing criteria have a real chance to be useful immunogens – in the case of MAGE-3 this is only to the HLA-A1 epitope (plus its HLA-B*4403-binding variant). The analysis suggests, that there is only little chance that other functional epitopes useful for vaccination are contained in the MAGE-3 sequence (the two HLA-A2 restricted peptides with relatively good computer-derived scores should be tested). Thus there is no point in applying a whole-antigen type vaccine to an HLA-unmatched broad patient population, since T cell responses mediating rejection can only be expected in cases where a functionally meaningful epitope is generated, and this happens only in HLA-A1+ (B*4403+) individuals. As it stands, HLA-A3+ and A*0201+ patients are predicted not to benefit from a vaccination, since likely they do not generate relevant epitopes.

MAGE-3 is undoubtedly one of the best understood TA; and very likely similar results may be expected when other TAs are analyzed for immunogenic epitopes. Larger proteins, such as tyrosinase or HER2/neu may harbor enough natural epitopes restricted by several different HLA alleles (*see Table 1*); in such cases a selection of

these peptides can serve as vaccines. Taken together with the arguments listed before, these results strongly argue for peptide-based vaccines instead of the whole-antigen approach.

Reliable evaluation of performance is the key for the development of a successful vaccine therapy

While in mouse models experimental conditions generally enable one to carry out lengthy in vivo / in vitro tests, such as bulk CTL or limiting dilution assays, mAb depletion and cellular transfer analysis to assess contribution and specificity of T cells, such investigations are only rarely possible with human subjects. The reason for this is partly conceptual (individual patient history, naturally developing tumors of highly variable characteristics positioned at various anatomic locations, different tumor/vaccine/host proportions, outbred population with unaccounted variations), and partly technical (the required amount of patient material is rarely available, individual optimization of tests may be necessary). As a result, there is essentially no reliable method ("surrogate marker") to measure effectiveness of vaccination therapy in humans, apart from the observation of the clinical course. Consequently, many of the past cancer immunotherapy trials relied solely on monitoring clinical responses. With the rapidly increasing number of therapeutic options and clinical trial protocols (and the concomitantly decreasing number of patients available for new trials), there is an increasing need to monitor vaccination efficiency regularly during the therapy. Indeed, effective monitoring is likely the key for improving efficiency of immunotherapies.¹¹⁰

Again I wish to emphasize that the aim of all tumor immunotherapy approaches is the generation of TA-specific responses; and this is explicitly true for molecular cancer vaccines. Since within our current paradigm an antigen (peptide)-specific response is expected solely from T cells, diagnostic methods assessing these T cells are of highest priority. The crudest approach is a DTH-test against tumor antigens.^{6,94} The technical ease of this method offsets its rather cumbersome evaluation, yielding only semi-quantitative results at best. In some cases DTH data seem to correlate reasonably well with other parameters, including clinical responses.

The next group of in vitro assays is based on the biological responses of primed T cells: when these cells see their target antigen in the appropriate context, they release cytokines, kill the target cells and proliferate. A general problem of measuring one or more of these responses is that the precursor frequency of reactive T cells in the peripheral blood may be very low, and assays usually lack the required sensitivity. CTL assays circumvent this problem by in vitro selection and amplification of the responsive T cells, while limiting dilution-based approaches rely

on extensive cultivation of statistically distributed cell populations. Both of these yield only semi-quantitative results, besides being rather irreproducible and labour-intensive. Visualization of cytokine-secretion on a single-cell basis with a modified ELISA approach, termed ELISPOT, seems to be more promising. Tumor-reactive patient-derived cells can be detected directly, without lengthy *in vitro* manipulations, and the low precursor frequency is compensated by the large number of cells that can be efficiently screened. Increased number of Melan-A/MART-1 spots were seen in melanoma patients,⁵³ and presence of ELISPOT positive cells correlated with a beneficial outcome.¹¹⁰

A reagent which can directly detect T cells expressing a TCR with a desired recognition capacity has been recently developed.³ A soluble HLA molecule was refolded *in vitro* in the presence of β 2-microglobulin and a particular HIV-derived peptide and tetramerized with fluorophore-labelled streptavidin. Multimeric peptide/HLA complexes are required because of the low intrinsic affinity of the monomers towards the TCR.⁸⁰ This reagent was then used to detect HIV-peptide-specific T cells in peripheral blood samples by FACS analysis. It should be possible to use similar methods to detect and quantitate tumor antigen-specific T cells. Based on identification and enumeration of responder T cells at the single cell level the effectiveness of various vaccine approaches can be exactly determined.

Critical issues

Despite the steady progress in both identifying novel tumor antigens and building better cancer vaccines, a whole series of critical issues remains to be examined. There are general problems associated also with other forms of immunotherapies which are relevant for molecular cancer vaccines, such as (a) provocation of autoimmunity; (b) immunosuppression caused by tumors; (c) uneven expression and presentation of the targeted tumor antigens. Additional problems connected to the peptide-based approach are (d) MHC / HLA restriction; (e) need for adjuvants because of low immunogenicity; (f) lack of T cell help and tolerance induction; (g) epitope dominance.

Induction of autoimmunity remains an issue of deep scrutinization – many TAs (especially the lineage-specific ones) are also present on some normal tissues, and an effective immunization protocol may tear down the barriers which prevent these tissues from destruction. Quite surprising is therefore, that – except of the noted vitiligo,¹⁰³ autoimmune side-effects do not seem to occur frequently. When compared to cellular-based vaccines, chances for generation of autoimmunity are expected to be even lower with molecular cancer vaccines, since by using selected antigens, the specificity is considerably better controlled.

Far more evidence has been accumulated documenting resistance of tumors to vaccination, which is again a problem pertinent to all immunotherapy approaches. Tumors can directly impair the immune response by secreting suppressive mediators, such as TGF β or IL-10, and by attacking crucial signaling pathways in responsive T cells.^{47,83} Also tumors can find many ways to lose their distinctive antigens – expression of the TA may cease, the processing machinery may be rendered non-functional, presenting HLA alleles can be lost or downregulated.⁷⁴ Cells that can escape immune surveillance enjoy a selective growth advantage and may form new metastases. Such immunoselection *in vivo* was documented in melanoma patients in response of selection pressure imposed on by peptide vaccines.⁶² To circumvent selection one can think of applying several TAs concomitantly or in sequence. This is again much easier done with peptide-based vaccines than with whole proteins or recombinant viruses.

The tumor mass is not expressing its TAs homogeneously at any stage. Strictly interpreted, this would preclude any kind of vaccination approach. However, it is probably not necessary to attack each and every tumor cell by specific T cells. As observed in infection models, once tolerance is broken, the ongoing immune response can provide relatively unrestricted effectors which are sufficient to kill nearby (antigen negative) cells.¹⁰⁸ Destroyed tumor cells themselves may provide novel antigens to the immune system, spreading the immune response also to cells which were not recognized initially (epitope spreading).¹¹⁴ It remains to be tested to what degree these mechanisms are operational in patients, and whether they can contribute to the vaccination effect.

The rigidity of HLA restriction of peptide vaccines is offset by their high specificity and flexibility in many other areas. Attempts to create peptides which can bind to more than one HLA allele led to the definition of HLA “supermotifs”.^{25,120} On the basis of supermotifs peptide-based vaccines have been proposed which could bind not only to a single HLA allele, but at least to a family of related alleles; usefulness of this extension, however, remains to be demonstrated.

Peptides are generally considered as poor immunogens. This notion is supported also by rigorous comparative studies,^{2,144} although the actual reasons for the low immunogenicity remain largely unknown. That is why adjuvants, most often incomplete Freund's adjuvant (IFA) are necessary to coapply. Promising novel candidates for adjuvants are cytokines: IL-12^{58,85} and GM-CSF¹ appear to be the most effective in experimental and clinical⁶¹ applications. Moreover, peptide-induced unresponsiveness¹²⁸ may be reversed by the application of IL-12 in some models.⁴⁶ However, effects can be sometimes seen even if the peptide is applied in buffered salt solutions: the MAGE-3-derived EVDPIGHLY peptide has been applied in a PBS/DMSO

solution to HLA-A1+ melanoma patients. Preliminary results reporting several clinical remissions suggest that this vaccination approach may be a useful one.⁷⁷

Peptides with attached lipid tails represent a rationally designed alternative.²⁶ It has been speculated that such lipopeptides may form liposome-like aggregates of a certain critical size, and thus are taken up and processed more effectively for class I restricted presentation by antigen presenting cells (APCs).¹⁰² A lipopeptide vaccine supplemented with a tetanus toxin-derived peptide epitope has been used to successfully vaccinate against hepatitis B virus.¹⁴³ The class II-restricted tetanus peptide is designed to function as a "helper" epitope: primed tetanus-reactive CD4+ T cells, present in most patients with high probability, should provide a "help" to CD8+ T cells responding to the vaccine. Another novel method utilizes polycations to "transload" peptides.¹¹³ Huge quantities of peptides can reach cytoplasmic compartments of various cells, including APCs.¹⁵ In mouse tumor models, superior protection has been found with transloaded peptides when compared to other delivery methods.¹¹² Composition and chain-length of the polycation is clearly critical for the vaccination efficiency; on the other hand, one may speculate that the polycation itself may perform a "helper" function, like the tetanus-derived peptide, when presented in class II context.¹¹¹ Despite of these initial accomplishments, there is still room and necessity for further improvements of delivery methods. Frustrated by inefficient adjuvants, several groups have used autologous, in vitro expanded APCs coated with the desired peptide as immunogen.^{44,156} Although the obtained results are respectable in some cases, using cellular carriers for the delivery of molecular vaccines has to be viewed as an interim solution at its best.

Unfortunately, there is little known about how immunologically dominant epitopes relate to those mediating rejection. Observations suggest that this relationship may be complex: while lineage-restricted TAs are most frequently recognized by melanoma-specific TILs, and dominant melanoma TAs are apparently not the genetically altered ones,⁸⁹ with other patients TAs not found on normal melanocytes appear to dominate HLA-A2⁴ and -A3⁷⁹ restricted responses. Immunoselection experiments with mouse MCA tumors suggest that dominant 'private' TA epitopes do mask secondary unique and public (shared) ones,³³ suggesting that vaccines applied at different times against the same tumor may have to be composed differently in order to obtain maximum efficiency.

The vaccination procedure itself may also result in inadvertent effects. Findings that antigen presentation by epidermal cells,¹²⁴ and/or inappropriate application of peptide vaccines may induce tolerance instead of immunity¹²⁸ indicate that further exploratory work is needed before

these vaccines can be reliably applied. Carefully chosen vaccination protocols along with selected adjuvants may overcome these problems.

Conclusion

Recent advances in cloning of tumor antigens opened up novel prospects for antigen-specific tumor immunotherapy. At the moment, however, molecular cancer vaccines represent a conceptual framework rather than established procedures. Critical issues, such as selection and application of appropriate immunogens, as well as improved monitoring of the elicited immune responses have to be thoroughly worked out before molecular cancer vaccines can find their place in the anti-cancer armamentarium.

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