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Future Perspectives in Specific Immunotherapy of Melanoma

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With the discovery of T-cell recognised tumour-associated antigens (TAAs), interest in specific immunotherapy for treatment of malignancies has increased substantially. The majority of studies investigating TAAs have focused on melanoma-associated antigens because of evidence that the immune system influences the pathogenesis of melanoma. This paper reviews the different types of melanoma antigens, their *in vitro* and *in vivo* immunogenicity and clinical data regarding the use of specific immunotherapy in patients with stage I–IV melanoma. Results of clinical studies are highly variable but encourage further research in these patients. Developing and perfecting laboratory and clinical correlates of response to these specific immunotherapies are vital to determining their role in clinical practice. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

THE DISCOVERY of T-cell recognised tumour-associated antigens (TAAs) and their molecular definition has paved the way for new approaches in specific tumour immunotherapy [1]. Theoretically these TAAs can be used to develop tumour-specific T-lymphocytes *in vitro* for adoptive transfer to cancer patients with neoplasms expressing the appropriate epitopes as determined prior to immunotherapy. TAAs also may be used to actively vaccinate cancer patients. Melanoma is the human tumour for which the largest number of T-cell defined TAAs is known. Therefore, melanoma patients are most frequently treated with this new generation of peptide-based vaccines in clinical trials.

Melanoma antigens

TAAs that are recognised by cytotoxic T cells and expressed by human melanomas are summarised in Table 1. A first group of TAAs includes proteins that are predominantly, though not exclusively, expressed by melanomas and other histologically distinct neoplasms, but not by normal tissues except for placenta, spermatogonia and spermatocytes (e.g. MAGE, BAGE and GAGE families). However, since these latter tissues do not express significant amounts of HLA-A, -B, or -C class I molecules, even if a cytotoxic T-lymphocyte (CTL) reaction is elicited in immunised patients, spermatogonia and spermatocytes will be spared due to the lack of target complex formed by the peptide and MHC molecule on their cell surface.

A second group of TAAs includes numerous normal, melanocyte-related differentiation proteins, such as tyrosinase, gp100, Melan-A/MART-1, that are found in normal melanocytes of the skin and retina but not in other tissues. A CTL reaction against these TAAs may kill normal melanocytes when they are reached by CTL effectors. Melanocyte destruction has occurred in several melanoma patients responding to immunological therapies but only in the skin as vitiligo and not in the eye or other organs [2]. However, proof that vitiligo in these patients is caused by a T-cell reaction is lacking.

A third interesting group of melanoma TAAs encompasses a still limited number of proteins that are derived from point gene mutations (e.g. CDK4, MUM-1, or β-catenin) [3, 4] or from transcription or translation alterations in tumour cells that cause a new gene sequence, generating a new epitope recognisable by T cells.

As shown in Table 1, however, the majority of antigenic epitopes are recognised in the context of few HLA-A, -B, -C alleles and some important, relatively frequent, alleles have not been studied. To this aim, we have generated CTL clones that recognise autologous melanomas in an HLA-A3.1-restricted fashion. These clones had a pattern of lysis of normal (melanocytes) and melanoma cells that suggested the recognition of both differentiation-like antigens and melanoma-restricted antigens [5]. A cDNA library was then constructed from the HLA-A3.1 tumour and screened using the COS-7/CTL assay based on the release of tumour necrosis

Table 1. *Human melanoma antigens recognised by T-cells* [1]

Antigen	Expressed by	Peptide sequence	HLA-A, -B, -C restriction
MAGE-1	Various tumours and testis	EADPTGHSY	A1
		SAYGEPRKL	Cw1601
MAGE-2	Various tumours and testis	EVVPISHLY	A1
MAGE-3	Various tumours and testis	EVDPGHLY	A1, B44
		FLWGPRALV	A2.1
BAGE	Various tumours and testis	AALAVFLAL	Cw1601
GAGE	Various tumours and testis	YRPRPRRY	Cw6
NA-17	Melanoma	VLPDVFIRCV	A2.1
Tyrosinase	Melanoma and melanocytes	MLLAVLYCLY	A2.1
		YMNGTMSOV	A2.1
		YMDGTMSQV	
		AFLPWHRLF	A24
		SEIWRDIDF	B44
gp100	Melanoma and melanocytes	YLEPGPVTA	A2.1
		LLDGTATLRL	A2.1
		KTWGQYWQV	A2.1
		ITDQVPFSV	A2.1
		VLYRYGSFSV	A2.1
		ALLAVGATK	A3.1
gp75 (TRP-1) TRP-2	Melanoma and melanocytes	MSLQRQFLR	A31
		LLPGGRPYR	A31
Melan-A/MART1	Melanoma and melanocytes	AAGIGILTV	A2.1
		ILTVILGVL	A2.1
		AEEAAGIGIL	B45
Mutated CDK4	Melanoma	ACDPHSGHFV	A2.1
MUM-1	Melanoma	EEKLIVVLF	B44
β-catenin	Melanoma	SYLDSGIHF	A24

factor α (TNF-α). This allows identification of subgenic fragments containing the DNA that encodes the antigen recognised by the CTL clone. Subsequently, sequencing the appropriate cDNA fragment and peptide synthesis of the putative epitope lead to the identification of a new peptide derived from the already known antigen gp100, which can be recognised within HLA-A3.1 context [6] Castelli and colleagues (Istituto Nazionale Tumori, Milan). In addition to the expanding number of antigenic epitopes derived from known melanocyte-related TAAs, a reservoir of tumour-restricted antigens may exist. By analysing more than 100 HLA-A2.1-restricted CTL clones, half were shown to recognise autologous or allogeneic HLA-A2.1-positive melanomas but not HLA-A2.1 melanocytes and none recognised presently known melanoma TAAs [7].

Overall, currently available epitopes would allow their use in a large proportion of melanoma patients. In fact, most of the differentiation TAAs are expressed in a high percentage of metastatic melanomas (80–90%) and, as shown in Table 1, can provide epitopes for patients bearing the HLA-A2.1 allele (40% of population), HLA-A3.1 (20%), HLA-A24 (18%) and HLA-A31 (6%). Tumour-restricted antigens (MAGE, BAGE, GAGE, NA-17) show a lower frequency of expression in melanomas (30–70%) and generate epitopes recognised by several HLA alleles (see Table 1). The availability of these peptides theoretically allow the vaccination of the majority (approximately 80%) of melanoma patients.

Immunogenicity of melanoma antigens

In vitro *immunogenicity.* In order to be used *in vivo*, these antigens should demonstrate their ability to stimulate *in vitro* the T-cell component of the immune system. In fact, most of the epitopes are actually part of normal proteins that may not be recognised by the immune system because of anergy, antigenic ignorance, or other form of tolerance [8]. Several studies that have addressed *in vitro* response [5, 9–13] are summarised in Table 2. Although results from different experiments in different laboratories cannot be directly compared, it is clear that most tumour-restricted antigens like MAGE do not appear to be strongly immunogenic. In contrast, Melan-A/MART-1 appears to possess the highest stimulatory activity of peripheral blood T lymphocytes as determined by the frequency of melanoma patients from whom it was possible to generate anti-Melan-A/MART-1 activity [10]. However, with a lower frequency or a longer period of stimulation, anti-Melan-A/MART-1 CTLs could be obtained also from peripheral blood lymphocytes of normal individuals. This indicates that CTL precursors recognising this antigen are circulating in the blood. Tumour growth may

Table 2. In vitro *induction of antitumour CTL in melanoma patients or healthy donors* [5, 9–13]

	n responders/*n* individuals tested	
Antigen	Melanoma patients	Healthy donors
Unknown*	15/16	ND
MAGE-1.A1	3/5	ND
MAGE-3.A1	1/4	1/2
Tyrosinase	4/36	6/16
Melan-A/MART1	24/25	9/16
gp75.A31	0/10	ND
gp100.A2	10/20	9/23
gp100.A3	2/3	ND

ND, not determined. *Experiments in which tumour cells were used as stimulators.

stimulate an expansion of such precursors as evidenced indirectly by the shorter kinetics of these effectors obtained *in vitro* in melanoma patients as compared to normal donors upon stimulation with the Melan-A/MART-1_{27-35} immunodominant peptide pulsed to autologous antigen-presenting cells (APCs) [11]. This conclusion has been confirmed under different experimental conditions by the Knuth group [12]. It is of note that CTL precursors recognising other normal differentiation antigens (e.g. tyrosinase, gp100) have been detected in normal individuals as well [12, 13], an observation which remains of unclear biological significance.

The mechanism for the apparently high immunostimulatory activity of Melan-A/MART-1 immunodominant peptide is unknown. We tested the hypothesis that this is due to a cross-reaction with other, unrelated proteins containing peptide motifs that are recognised by Melan-A/MART-1-specific CTL clones. This molecular mimicry mechanism has been observed in autoimmune diseases such as multiple sclerosis (i.e. cross-reactions between myelin basic protein and viruses) [14]. We then searched for proteins that contain possible binding motifs based on the presence of anchor residues at positions 2 and 9 of the HLA-A2.1 molecule. Among the many proteins detected with this feature, few were selected that are present in pathogens known to come in contact with the human body. In particular, glycoprotein III (gpIII) of the herpes simplex virus (HSV) was evaluated for its ability to stimulate anti-Melan-A/MART-1 CTL clones. APCs pulsed with the appropriate protein peptide were able to specifically stimulate anti-Melan-A/MART-1 CTLs. A similar result was obtained by stimulating CTLs with cells infected with a virus vaccine construct bearing the cassette containing the gpIII HSV minigene [15]. It was concluded that molecular mimicry with natural peptides like that present in a common human pathogen (e.g. HSV) may explain the high stimulatory activity of Melan-A/MART-1.

In vivo *immunogenicity.* Melan-A/MART-1 and other melanoma epitopes have been evaluated in early trials of *in vivo* immunisation (vaccination) to obtain information on their immunogenicity and toxicity. Several different studies have been carried out using either naked peptides or peptides admixed with adjuvants and different routes of administration. The immunological responses identified in these studies are summarised in Table 3. The results are quite heterogeneous, but they indicate that under some circumstances, a significant immunological response was achieved after subcutaneous or intradermal injection of melanoma peptides. By *in vivo* administration, Melan-A/MART-1 appears to be the most potent antigen as melanoma patients had increased CTL activity in their blood after vaccination in comparison with pre-vaccination [17, 19]. The stimulatory activity was particularly frequent when incomplete Freund's adjuvant was used [19]. No CTL-specific activity was found in the blood of patients receiving MAGE-1 or MAGE-3 peptides without adjuvants, even in those patients vaccinated with the naked MAGE-3 peptide that showed clinical responses (see below) (Table 3). Importantly, limited knowledge regarding factors that may influence peptide vaccination of humans lead to the use of different administration schedules and adjuvants that render comparison of these results problematic.

Most of the negative data may be due to many variables that are now being clearly identified. In addition to the possible lack of effective immunisation, it is possible that in tumour-bearing patients the most effective CTLs may be sequestered within neoplastic lesions where recognisable antigens are found. Therefore, CTLs would be difficult to detect in blood where they can be diluted by the entire T-cell population. This hypothesis was based on our previous studies of T cell receptor (TCR) usage by T cells that infiltrate primary or metastatic melanoma lesions, including metastases of vaccinated patients. The results of these studies indicate that T cells infiltrating primary or metastatic nodules include oligoclonal and often monoclonal populations of lymphocytes that are defined by the analysis of their TCRBV repertoire [21]. Metastatic melanoma patients vaccinated with dinitrophenyl-modified, autologous, irradiated tumour cells admixed with bacille Calmette-Guérin (BCG) by Berd and coworkers achieved a clinical response rate of 10% (5/46) [22]. The TCR repertoire was analysed in the responding patients by examining peripheral blood lymphocytes, melanoma lesions and normal skin before and after vaccination. In 4 patients, multiple lesions were evaluated over approximately 1 year of subsequent vaccine administration. While no significant changes were found in the TCR repertoire of peripheral blood lymphocytes after vaccination, an oligoclonal expansion and a monoclonal overexpression of a few TCR subsets was detected in lesions analysed after vaccination [23]. Identical TCRBV were found in asynchronous metastases of the same patient [23]. Moreover, T cells of the infiltrate, which were expanded *in vitro* with antibodies directed to the overexpressed TCRBV and to the CD28 molecule, contained CTL-recognising melanoma antigens on autologous neoplasms (Table 4). This provides a strong indication that, as a result of vaccination, antigen-specific antitumour T cells may be overexpressed within tumour lesions without being detectable in peripheral blood lymphocytes.

Active immunotherapy or vaccination

Vaccination of cancer patients, particularly melanoma patients, has a long history and has involved several cancer centres throughout the world, particularly in the U.S. and Australia. Overall, the results of these studies have been negative despite phase I and II evidence of clinical responses. However, Morton and coworkers [24] concluded that multicellular vaccines may provide a significant increase in overall

Table 3. In vivo *immunogenicity of melanoma peptides*

Peptides	Adjuvant	T-cell response in PBL *n* responding patients/ *n* patients tested	Reference
MAGE-1.A1	–	0/3	Parmiani*
MAGE-1.A1	APC	2/3	[16]
MAGE-3.A1	–	0/7	Parmiani/ Coulie†
Melan-A/MART1	–	3/6	[17]
Melan-A/MART1	GM-CSF	3/3	[18]
Melan-A/MART1	IFA	13/18	[19]
Tyrosinase	–	2/5	[17]
gp100	–	1/6	[17]
gp100	IFA	3/7	[20]

APC, antigen-presenting cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFA, incomplete Freund's adjuvant; PBL, peripheral blood lymphocytes. *G. Parmiani, Istituto Nazionale Tumori, Milan. †G. Parmiani and P. Coulie, Istituto Nazionale Tumori, Milan and Ludwig Institute, Brussels.

survival for certain patient subsets, such as stage IIIA patients with regional soft tissue metastases. Prospective phase III clinical trials are needed to validate these conclusions. More recently, these authors found that prolonged survival time may be explained by a reduction in brain metastases [25]. Because of a lack of knowledge regarding the molecular nature of melanoma TAAs, the TAAs administered to patients and the specific T-cell immune response were unknown in most of these studies.

The availability of melanoma peptides prompted the initiation of several clinical trials to assess the feasibility, toxicity and immune-response induction of the peptides. Table 5 summarises the available results from these studies. MAGE-1 and MAGE-3 peptides without adjuvants were first administered subcutaneously to metastatic melanoma patients. No major toxicity was observed. No clinical responses were detected in patients vaccinated with MAGE-1. However, partial and complete durable responses were observed in 3 and 2 patients, respectively, of 17 evaluable patients treated with 100 to 300 μg 4 weeks apart for 3–5 times with the MAGE-3.A1 peptide [26]. Of the responding patients, only one received 100 μg (partial response) while the others were all treated with 300 μg. Rosenberg and coworkers focused on the epitopes of differentiation TAAs, using different vaccine formulations in an attempt to increase the immunogenicity of these normal peptides [27]. Clinical response rates have been negligible although data are not yet fully available.

It is evident that at least two clinical studies conducted by Rosenberg and colleagues achieved a high frequency of T-cell immune response in melanoma patients given either gp100 or Melan-A/MART-1 peptides admixed with the incomplete Freund's adjuvant (IFA) [19, 20]. In a smaller number of patients and using a different immunisation schedule that included granulocyte-macrophage colony-stimulating factor (GM-CSF) administration, a T-cell-specific immune response and clinical response was confirmed by Jager and coworkers [18]. However, despite increased immune reactivity elicited by vaccination, no major clinical responses were obtained with Melan-A/MART-1 or gp100 peptide-based vaccines [19, 20]. In contrast, five clinical responses, two of which were complete and durable, were produced after administration of MAGE-3 peptides in advanced melanoma patients that did not have detectable increases in T-cell responses in blood [26]. This paradox is difficult to explain but it is possible that a clinical response occurs only when specific T cells can massively infiltrate neoplastic lesions, as it was shown in patients responding clinically to MAGE-3, Sensi and Parmiani, data not shown (Istituto Nazionale Tumori, Milan). If this is the case, detection of CTL activity in blood would suggest that the effectors are in the wrong place because they are unable to target melanoma lesions. It is clear, therefore, that further studies are needed to better understand the kinetics and biology of the immune response induced by peptide-based vaccines in cancer patients.

Several other factors may impact the efficacy of immunological therapies. T cells have been described as functionally altered in tumour-bearing hosts although such impairment may be overcome after *in vitro* exposure to interleuken-2 (IL-2) [28]. Vaccinated patients who respond with an increase in lytic or cytokine-release activity should be studied to determine whether peptide immunisation restores CTL function. As for tumour cells, the major defect reported involved down regulation of MHC class I expression [29] or lack of melanoma epitopes [30] that could be selected by the immune system during tumour progression. Melanoma cells are also known to release immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and, with a lower frequency, IL-4 or IL-10 [31]. More recently, another potential T-cell inhibitory activity has been described involving the expression of Fas L by melanoma cells, which could block or cause apoptotic death of incoming activated T lymphocytes [32]. Although these mechanisms may operate simultaneously only rarely, it is likely that even one of them may compromise either the activation of a T-cell response or its effectiveness against the tumour. In any event, the occurrence of one of these escape mechanisms will drastically reduce the likelihood of significant tumour killing and thus a clinical response.

Table 4. Autologous tumor cytotoxicity by BV14-positive CTL lines obtained from postvaccine metastatic lesions of a patient given DNP-modified autologous vaccine

	% Specific lysis on autologous melanoma in the presence of		
CTL lines*	No MAb	Anti-HLA class I	Anti-HLA-A2
TIL-1	13	1	0
TIL-2	21	0	0
LAK	60	51	53

DNP, dynitrophenyl; CTL, cytotoxic T lymphocytes; TIL, tumour infiltrating lymphocytes; LAK, lymphokine-activated killer; MAb, monoclonal antibody. *Lines were obtained after 5 weeks of *in vitro* stimulation of anti-βV14 and anti-CD28 monoclonal antibodies. CTL were tested in 4 hour ^{51}Cr-release assay at E:T ratio of 20:1. Inhibition of lysis was tested after pre-incubation of target cells with anti-HLA monoclonal antibody. LAK cells were used as control of target lysability. Adapted and reproduced with permission from *J Clin Invest*, 1997 [23], by copyright permission of The American Society for Clinical Investigation.

Future directions for specific immunotherapy of melanoma

Peptide-based vaccination holds promise as an effective treatment approach for melanoma patients. More than 30 different epitopes that would theoretically allow vaccination of up to 80% of patients belonging to a variety of HLA class I alleles are now available. Furthermore, the list of epitopes resulting from mutations or altered transcription events is increasing. These latter peptides, which are unique, may generate T-cell responses that are stronger and biologically

Table 5. Summary of early clinical results from vaccination of melanoma patients with peptides

Peptide	*n* of responding patients/ *n* of patients entered	References
MAGE-1.A1	0/15	Boon and colleagues*
MAGE-3.A1	5/17	26†
gp100	1/20	20
Melan-A/MART1 + IFA	0/18	19
Melan-A/MART1 + tyrosinase + gp100	3/6	18

IFA, incomplete Freund's adjuvant.
*T. Boon, Ludwig Institute, Brussels. †Ludwig Institute, Brussels.

more relevant than peptide epitopes derived from normal proteins, including the MAGE, BAGE and GAGE families, or differentiation TAAs. Several types of adjuvants are also being explored to identify more appropriate ones for different peptides.

Important progress also has been made in understanding the mechanism of T-cell recognition of melanoma antigens and the repertoire of T cells involved [21]. We have recently shown that the composition of lymphocyte TCR directed at specific melanoma TAAs allows their isolation and expansion in quantities close to those necessary for effectively targeting melanoma lesions *in vivo* [33]. Thus, *in vitro* expansion of melanoma antigen-specific T cells can be optimised by using antibodies for the generation of autologous T-cell populations that are used for adoptive immunotherapy of melanoma patients. This therapeutic approach also can be facilitated by the use of T cells engineered to express a TCR molecule that recognises and kills melanoma cells [34] or that bears a chimeric TCR/scFv that will provide antibody-specific lymphocytes directed against a tumour cell-surface target [35].

These various approaches are currently being tested in cancer patients and additional future trials are planned. Vaccination or adoptive therapy with lymphocytes or the combination of these immunotherapies may ultimately provide new tools for more effective therapy of advanced melanoma patients.

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