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Tumor Antigens Recognized by T Cells

The Use of Melanosomal Proteins in the Immunotherapy of Melanoma

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Summary: Clinical observations in the interleukin (IL) 2-based immunotherapies suggest that T cells play a central role in the rejection of melanoma. Using cDNA expression cloning, we have isolated genes encoding melanoma antigens recognized by tumor-infiltrating T lymphocytes. These antigens are categorized as (a) melanocyte-specific melanosomal proteins (MART-1/melan A, gp100, tyrosinase, TRP-1, and TRP-2), (b) tumor-specific mutated proteins (β-catenin), and (c) others (p15). A variety of mechanisms has been identified for the generation of T cell epitopes on tumor cells. Some of the HLA-A2 binding epitopes from the melanosomal antigens appear to be subdominant self-determinants with relatively low major histocompatibility complex binding affinity. The effectiveness of adoptive transfer into patients of cytotoxic T lymphocytes recognizing the melanosomal antigens, the significant correlation between vitiligo development and clinical response in patients receiving IL-2-based immunotherapies, and the sporadic tumor regressions observed in some patients following immunization with the MART-1 or gp100 peptides in incomplete Freund's adjuvant or recombinant viruses expressing the MART-1 antigen suggest that these epitopes may represent tumor rejection antigens. Phase I immunization trials using peptides or recombinant viruses containing genes encoding the melanosomal antigens MART-1 or gp100, with or without co-administration of cytokines such as IL-2, IL-12, or granulocyte-macrophage colony-stimulating factor, are being conducted in the Surgery Branch of the National Cancer Institute. These studies may demonstrate the feasibility of using melanosomal proteins for the immunotherapy of patients with melanoma. **Key Words:** Melanoma antigens—MART-1—gp100—Subdominant epitopes—Immunotherapy.

T cells play an important role in in vivo tumor regression in many animal tumor models. Adoptive transfer of cultured cytotoxic T lymphocytes (CTLs) derived from tumor-infiltrating T lymphocytes (TILs) along with interleukin (IL) 2 resulted in tumor regression in 30–40% of patients with metastatic melanoma (1,2). The presence of T cell infiltrates in regressing tumors after IL-2-based immunotherapy as well as the correlation between the accumulation of injected T cells in tumor sites and clinical response to TIL therapy suggested that T cells play an important role in the in vivo rejection of melanoma (3). Identification of antigens recognized by these T cells may provide insight into tumor recognition by autolo-

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gous T cells as well as the development of new immunotherapies for patients with cancer. Many antigens recognized by T cells have recently been identified (4), but a number of issues still need to be resolved concerning their optimal use in immunotherapy (Table 1).

MOLECULAR IDENTIFICATION OF ANTIGENS RECOGNIZED BY TIL

In the Surgery Branch of the National Cancer Institute, several human melanoma antigens recognized by TILs have been identified by either testing candidate molecules or by using TILs to screen cDNA expression libraries (3). Methods for isolation of antigens presented by major histocompatibility complex (MHC) class I have now been well established; however, efficient methods for the isolation of MHC class II-restricted antigens remain to be developed. The majority of antigens isolated in our laboratory represent melanocyte lineage-specific melanosomal proteins (MART-1/melan A, gp100, tyrosinase, TRP-1, and TRP-2). A tumor-specific mutated protein (β-catenin) as well as a nonmutated protein that is expressed in a variety of tissues (p15) have also been isolated (Table 2).

Melanosomal Proteins

Many cultured TILs recognize autologous melanoma cells in vitro as well as allogeneic melanoma cells and cultured melanocytes that share MHC class I molecules, but do not recognize cells derived from other tissues (5–7). A correlation between the development of vitiligo and favorable prognosis or responses of melanoma patients to chemoimmunotherapy has previously been reported (8,9). In addition, a correlation between vitiligo development and melanoma regression after IL-2-based immunotherapies has been observed in our institute (10). These in vitro and in vivo observations suggest that autoreactive T cells specific for nonmutated peptides derived from melanocyte lineage-specific proteins may be involved in in vivo tumor regression. To date, five melanocyte-specific proteins (MART-1/melan A, gp100, tyrosinase, TRP-1, and TRP-2) have been identified as antigens recognized by TILs (11–16). These proteins are present in melanosomes where melanin is synthesized, and a number of them have been shown to be enzymes directly involved in the synthesis of melanin. These melanosomal proteins have been found to be presented by a variety of HLA alleles, suggesting high immunogenicity in a broad range of patients with diverse HLA types. Tyrosinase and gp100 epitopes have been found to be presented by HLA-A1, -A2, -A24, -B44, -DR4, or HLA-A1, -A2, -A3, respectively (11,13,17–25; Y. Kawakami, unpublished results) (Table 2).

TABLE 1. *Important questions in tumor antigen research*

A. Molecular identification of tumor antigens
   Recognition by autologous or allogeneic cells
   Development of methods to identify tumor antigens ($CD8^+$ T cells, $CD4^+$ T cells, antibodies)
   Nature of immunogenic tumor antigens (mechanisms involved in generation of T cell epitopes)
B. Roles of identified antigens in in vivo tumor rejection
   Development of animal models (mutated peptides, tissue-specific proteins)
   Clinical trials (immune-monitoring methods, passive and active immunotherapy)
C. Frequency and mechanisms of tumor escape
   Loss of recognition molecules (antigen, MHC, β_2-microglobulin, TAP, LMP)
   Negative immune regulation against antitumor immune responses [tolerance (deletion, anergy), suppressive factors, altered T cell signaling, Fas ligand expression, Th1/Th2 shift]

MHC, major histocompatibility complex.

TABLE 2. *Human melanoma antigens recognized by T lymphocytes*

Melanoma	Presenting MHC	No. of identified epitopes
A. Melanosomal proteins		
gp100[a]	HLA-A1	1
	HLA-A2	10
	HLA-A3	2
	HLA-A24	1[b]
Tyrosinase[a]	HLA-A1	1
	HLA-A2	2
	HLA-A24	2
	HLA-B44	1
	HLA-DR4	2
MART-1/melan-A[a]	HLA-A2	2
TRP-1[a]	HLA-A31	1
TRP-2[a]	HLA-A31	1
	HLA-A33	1
B. Tumor-specific mutated antigens		
β-Catenin[a]	HLA-A24	1
CDK4	HLA-A2	1
MUM-1	HLA-B44	1[b]
C. Tumor-specific shared antigens		
a. Proteins expressed in testis and other cancers		
MAGE1	HLA-A1	1
	HLA-Cw16	1
MAGE3	HLA-A1	1
	HLA-A2	1
BAGE	HLA-Cw16	1
GAGE	HLA-Cw6	1
b. GnT-V	HLA-A2	1
D. Others		
p15[a]	HLA-A24	1

MHC, major histocompatibility complex.
[a] Antigens were recognized by tumor-infiltrating T lymphocytes in the Surgery Branch (National Cancer Institute).
[b] Epitopes encoded by sequences derived from introns.

Tumor-Specific Antigens

The variety of genetic alterations found in tumors would be expected to lead, in at least some cases, to the generation of an immunogenic T cell epitope. Some T cell clones were shown to recognize only autologous melanoma cells in vitro. We isolated a mutated β-catenin cDNA following the screening of a cDNA library with HLA-A24-restricted T cells (26). A single C-to-T transition in this product that may be the result of UV-induced DNA damage generated a peptide that conformed to the optimal HLA-A24 binding motif. A dramatic regression of tumor was found following adoptive immunotherapy treatment of the autologous patient, suggesting that this may represent a tumor regression antigen.

MECHANISMS INVOLVED IN GENERATING T CELL EPITOPES ON TUMOR CELLS

The identification of epitopes from tumor antigens revealed a variety of mechanisms capable of generating T cell epitopes on tumor cells (Table 3). Many melanoma-reactive T cells have been shown to recognize nonmutated self-peptides derived from melanosomal proteins, indicating that these represent highly immunogenic antigens (3). It is not clear why melanosomal proteins are highly immunogenic, although melanosomal proteins may be transferred to Langerhans cells, which may enhance their immunogenicity. In addition, melanosomes and lysosomes may be derived from a similar intracellular pathway, which may also enhance the immunogenicity of these proteins.

To characterize these immunogenic nonmutated self-epitopes, we determined the affinity of the HLA-A2 binding epitopes that had been identified from melanosomal antigens (23). In comparison with peptides that have previously been isolated directly from cell surface HLA-A2 molecules as well as viral peptide epitopes, a number of the melanoma epitopes appeared to possess relatively low HLA-A2 binding affinities. This may result from the presence of nondominant anchor residues such as the alanine or threonine residues found at P2 or the alanine residues found at P9 in certain peptides. MHC binding affinity is one of the important factors that influence the density of peptides on the cell surface. Thus, these melanoma epitopes may represent subdominant determinants expressed at low levels on the surface of melanocytes or even cryptic epitopes that are not present at detectable levels on the surface of normal melanocytes in situ (23,27,28).

TABLE 3. *Mechanisms involved in generating T cell epitopes on melanoma cells*

Mechanism	Antigen
A. Translation of normal nonmutated genes	MART-1/melan-A,[a] gp100,[a] tyrosinase,[a] TRP-2,[a] p15,[a] MAGE1, MAGE3, BAGE, GAGE
B. Translation of alternative ORF	TRP-1[a]
C. Mutation of widely expressed genes	β-Catenin,[a] CDK4, MUM-1
D. Incomplete splicing	gp100,[a] MUM-1
E. Posttranslational modification	Tyrosinase
F. Transcription from cryptic promoter	*N*-Acetylglucosaminyl transferase-V

[a] Antigens were recognized by tumor-infiltrating T lymphocytes in the Surgery Branch (National Cancer Institute).

When 10 HLA-A2 binding peptides from MART-1 were examined for their ability to induce responses in patient peripheral blood lymphocytes (PBLs), cytotoxic T lymphocytes (CTLs) could be induced with peptides that bind with intermediate affinity but not with peptides that bind with high affinity (29). One possible explanation for these findings is that peptides that are efficiently processed and presented on the surface of normal cells at high levels can induce T cell tolerance, whereas subdominant or cryptic determinants may not be capable of efficiently inducing T cell tolerance. Thus, CTLs that recognize subdominant or cryptic determinants and are not activated in a normal individual might be activated in patients bearing tumors. This could be a result of the increased expression of antigen, MHC, or accessory molecules on the tumor cell surface. In addition, tissue destruction present at the tumor site and the resulting inflammatory cytokines might activate T cells directly or act indirectly to augment antigen or MHC expression in the tumor. Tumor-reactive T cells that have been activated in vivo can then be further expanded by in vitro culture with IL-2 and used for adoptive immunotherapy.

Nonmutated self-epitopes derived from the MAGE, BAGE, and GAGE families of proteins have been found by Boon and colleagues to be expressed in normal adult testis as well as in a variety of cancers (30). These were identified using T cells from a single patient; however, TILs reactive with these proteins have not as yet been identified in the Surgery Branch. In contrast to the peptides derived from melanosomal proteins, these epitopes may not represent autoreactive or subdominant epitopes, since spermatocytes and spermatogonia do not express MHC class I molecules and therefore should not be able to present these peptides on the cell surface.

Tumor-reactive T cells have now been shown to recognize mutated peptide epitopes derived from β-catenin,

MUM-1, and CDK4 (26,31,32). Although mutated antigens appear to represent ideal targets for immunotherapy in that they are truly tumor specific, it is unclear how often mutations that give rise to immunogenic peptides can be identified. Most tumors have acquired a large number of mutations, at least some of which would be expected to be immunogenic. Nevertheless, for most tumor types, it has been difficult to induce T cell responses against autologous tumor cells. In melanoma, although the relatively strong response against melanosomal proteins may mask responses to mutated antigens when bulk melanoma-reactive CTLs have been used for analysis, autologous tumor-specific CTLs could be detected (33) and more mutated epitopes are likely to be identified in the future. A number of factors such as the binding to MHC molecules and the efficiency of antigen processing have been shown to influence expression of epitopes on the cell surface, and thus the chances that any particular mutated peptide would actually get expressed on the cell surface may be fairly low. In addition, it has been reported that p53 mutations found in lung tumors derived from HLA-A2 patients were not located in regions that contain potential HLA-A2 binding peptides, suggesting that tumor cells that express highly immunogenic mutated peptides might be eliminated before the tumors grew to a clinically detectable size (34). The mutated β-catenin and CDK4 proteins may play a role in tumorigenesis, however, and thus these might represent exceptional cases where there is a selective advantage conferred by the mutation. Tumor cells might not easily lose expression of these mutations, so that these mutated peptides might have been detected as tumor antigens recognized by autologous T cells.

During the isolation of T cell epitopes in melanosomal proteins, other unexpected mechanisms involved in the generation of T cell epitopes have been found. One epitope was found to be derived from the protein encoded by an alternative open reading frame of the known melanosomal protein TRP-1 (35). It is not yet clear whether this peptide was derived from a nonfunctional translated product or from an alternatively spliced product. Another epitope was found to be derived from proteins encoded by an intron of gp100 because of incomplete splicing (36). This incomplete gp100 protein seems to be present at a low level in tumor cells and normal melanocytes, since the mRNA containing the intron is present at low levels. An epitope derived from an intron sequence has also been reported in the antigen MUM-1 (31). In another case, we could not identify any epitope in tyrosinase recognized by one TIL even after screening overlapping synthetic peptides spanning the region containing the T cell epitope based on the analysis of truncated tyrosinase cDNA fragments, suggesting that posttranslational modification may be necessary to generate this T cell epitope (13). Recently, deamidation of an asparagine residue has been shown to be involved with generation of a tyrosinase epitope (37).

ROLE OF IDENTIFIED ANTIGENS IN IN VIVO TUMOR REJECTION AND IMPLICATIONS FOR IMMUNOTHERAPY

Possibly Autoreactive Antigens (Melanosomal Proteins)

Melanoma-reactive T cells specific for nonmutated peptides of melanosomal proteins can be induced from many patients of diverse HLA types, suggesting that it will be possible to use these antigens for the development of widely applicable tumor vaccine therapies. T cells reactive with these antigens have generally been found to recognize peptides in the picomolar range and, in addition, appear to efficiently recognize cultured as well as uncultured tumor cells (23,38).

A significant correlation has been observed between vitiligo development and tumor regression in patients receiving IL-2-based immunotherapies, suggesting that autoreactive T cells may be involved in in vivo melanoma regression (10). In particular, a significant correlation between gp100 reactivity and tumor regression in the adoptive transfer of HLA-A2-restricted melanoma-reactive CTLs has been observed (23). In addition, tumor regression has been observed in some patients in active immunization protocols using MART-1, gp100, or tyrosinase (39,40). The observation that melanoma metastases that have lost expression of melanosomal antigens grew progressively, while other multiple metastases regressed, suggests that immune responses against these melanosomal proteins may be involved in the tumor regression seen in those patients (40).

The role of tissue-specific proteins in tumor rejection has not been well studied in animal tumor models. However, recent murine studies targeting the melanosomal proteins including TRP-1, TRP-2, and gp100 suggested that responses to nonmutated peptides from these proteins may be capable of leading to tumor rejection (41–43). Model systems have utilized administration of antibody or T cells specific for these antigens as well as immunization with recombinant viruses expressing these antigens. These in vitro and in vivo observations suggest that nonmutated melanosomal proteins may be useful for the development of immunotherapies applicable in a broad range of patients.

There are several potential limitations to the use of self-peptides derived from tissue-specific proteins (27).

Subdominant epitopes may not be efficient immunogens because of relatively low MHC binding affinity or inefficient antigen processing. However, more immunogeneic form of the antigens can be generated by modification of epitopes and the use of high protein expression systems as described below.

These self-antigens may only induce low-avidity T cells in some patients or may be expressed at only relatively low densities on surface of tumor cells in vivo (44). As a result, these T cells may not efficiently recognize tumor cells. A significant correlation was found between tumor regression following adoptive transfer of TILs and the ability of these cells to respond to gp100, but clinical responses did not correlate with responses to MART-1 (23). However, it has been more difficult to induce responses in vitro against the gp100 epitopes that possess higher affinities to HLA-A2 than the MART-1 epitope (29,45). Again, it is possible that the gp100 epitopes may be present at higher densities on the cell surface than the MART-1 epitope and therefore may more readily induce T cell tolerance, making it more difficult to induce responses against these peptides. However, T cells that recognize the gp100 epitopes, when they are induced, may recognize tumors more efficiently. The possible inverse relationship between immunogenicity and tumor recognition in nonmutated self-peptides on tumor cells may indicate some difficulties when self-peptides are used in the immunotherapy.

Augmentation of immune responses has not been observed in patients that had been immunized with $gp100_{154\text{-}162}$, the peptide with the highest affinity of all of the HLA-A2 binding gp100 peptides identified to date, suggesting that this peptide may induce T cell tolerance in many patients (46). Some peptides may be expressed at sufficient levels on the surface of normal melanocytes to lead to the deletion of high-affinity T cells, leaving only T cells with low-affinity antigen receptors. CTLs that recognized peptide-pulsed target cells, but not tumor cells, were induced from PBLs of some patients by in vitro stimulation with peptides. The tolerance status against each peptide may be different among patients. Low-avidity T cells may be preferentially expanded in vitro in some patients in whom tolerance is induced in high-avidity T cells.

Tumors may escape recognition through loss of expression of these apparently nonessential gene products for tumor growth. Conversely, the induction of more potent immune responses could potentially lead to induction of autoimmune problems similar to those found in autoimmune diseases directed against melanocytes such as Vogt-Koyanagi-Harada syndrome or sympathetic ophthalmia. Patients may develop symptoms such as vitiligo, poliosis, uveitis, dysacusis, or meningismus as a result of melanocyte destruction in skin, uvea, retina, internal ear, and choroid plexus. However, dramatic tumor regression was observed in some patients who received IL-2-based immunotherapies here with only occasional vitiligo (2,11,47), and no ophthalmic problems have been observed in these patients. Since the susceptibility of normal cells and tumor cells to immune responses may be different because of tissue structure or inflammatory status (48), autoimmune side effects may be minimal.

Tumor-Specific Shared Antigens (MAGE, BAGE, GAGE Family)

MAGE1, MAGE3, BAGE, and GAGE antigens have been isolated from a patient with prolonged survival after treatment (30). Although it appears to be difficult to induce T cells specific for the MAGE antigens from PBLs of patients (49), immunization with the MAGE3 HLA-A1 binding peptide has been reported to result in tumor regression in some melanoma patients (50). These antigens are expressed on a variety of tumor cells other than melanoma and may be useful for the immunotherapy of these cancers.

Tumor-Specific Unique Antigens

In murine models, mutated unique peptides appear to be strong rejection antigens (51–53). In the human, mutated peptides were found to be derived from molecules such as β-catenin, MUM-1, and CDK4, which were isolated using T cells from patients who were disease-free after treatment, suggesting that the responses to these mutated peptides may be directly responsible for tumor regression seen in these patients (26,31,32). In addition, antigen loss might not occur, since these mutated molecules may play a role in the growth or metastasis of these tumors. However, it is not clear whether immunogenic mutated epitopes are generally presented by MHC molecules on the cell surface of human tumor cells and mutated peptides are strong rejection antigens in human tumors, since tumor cells that expressed highly immunogenic peptides may already have been eliminated. The general role of mutated epitopes in human tumor regression needs to be further evaluated.

IMMUNO-GENE THERAPY OF CANCER USING MELANOSOMAL PROTEINS

Development of Immunotherapy

Mutated epitopes that are expressed in tumors from only a small percentage of patients do not represent widely applicable targets for immunotherapy unless

TABLE 4. *Clinical immunization protocols against melanoma in Surgery Branch (National Cancer Institute)*

Protocol	Dose	Schedule	Date started
MART-1 27-35 peptide in incomplete Freund's adjuvant	0.1, 0.3, 1, 3, 10 mg	q3W × 4	February 2, 1995
gp100 peptides (154–162, 209–217, 280–288) in incomplete Freund's adjuvant	1, 3, 10 mg	q3W × 4	June 20, 1995
Modified gp100 peptides (209-2M, 280-9V) in incomplete Freund's adjuvant	1 mg	q3W × 4	November 21, 1995
MART-1 27-35 peptide in incomplete Freund's adjuvant plus systemic IL-12	1 mg	q3W × 4	January 14, 1996
Recombinant adenovirus encoding MART-1 (alone or with systemic IL-2)	10^7–10^{11} pfu	q4W × 2	December 8, 1995
Recombinant adenovirus encoding gp100 (alone or with systemic IL-2)	10^9–10^{11} pfu	q4W × 2	April 23, 1996
Recombinant fowlpox virus encoding MART-1 (alone or with systemic IL-2)	10^7–10^9 pfu	q4W × 4	June 6, 1996
Recombinant fowlpox virus encoding gp100 (alone or with systemic IL-2)	10^7–3×10^8 pfu	q4W × 4	
Recombinant vaccinia virus encoding gp100 (alone or with systemic IL-2)	10^7–10^9 pfu	q4W × 4	September 5, 1996
Recombinant vaccinia virus encoding MART-1 (alone or with systemic IL-2)	10^7–3×10^8 pfu	q4W × 4	
Adoptive transfer of PBLs sensitized in vitro to peptide			July 9, 1996

See text for abbreviations.

more efficient techniques can be developed for the identification of tumor antigens. Alternatively, strategies that do not require the identification of specific antigens can be utilized. Patients can be immunized with modified autologous tumor cells (54), dendritic cells pulsed with peptide mixtures eluted from autologous tumor cells (55), or heat shock protein (gp96)-peptide complexes extracted from autologous tumor cells (56). Although nonmutated self-peptides may not represent strong immunogens or tumor rejection antigens, techniques that utilize these antigens are readily applicable in a broad range of patients. The HLA-A2 allele is expressed in ~50% of patients, and to date, 10 epitopes in gp100 and 2 epitopes in MART-1 presented by HLA-A2 have been identified (11,23,24,38,57). In the Surgery Branch of the National Cancer Institute we have begun testing a variety of immunotherapy strategies using the melanosomal proteins MART-1 and gp100 (Table 4).

A variety of reagents may be considered for cancer immunotherapy (Table 5). To efficiently induce T cells specific for subdominant or cryptic self-epitopes, it may be important to utilize methods that allow high expression of these epitopes on the cell surface of antigen-presenting cells (APCs). This may be accomplished through the use of methods that result in high-level expression of proteins such as use of recombinant viral vectors containing tumor antigen genes (58) or professional APCs such as dendritic cells exogenously pulsed with peptides (28,59) (Table 6). Recombinant vaccinia virus, fowlpox virus, and adenovirus containing MART-1 or gp100 have been constructed, and clinical immunization trials using these recombinant viruses have begun in the Surgery Branch of the National Cancer Institute (42). In addition to the high-level expression of antigens, strong antiviral response may facilitate activation of relatively weak antitumor T cell responses. Viral vectors have potential risks of pathogenic infection and cannot be used repeatedly to boost immune responses because antiviral T cell responses decrease the efficacy of subsequent immunizations. Immunization with recombinant vaccinia virus may not be effective in patients who were immunized in their childhood. High titers of neutralizing antibodies against adenoviruses have been detected in patients, which may reduce the efficacy of immunization with recombinant adenovirus. In contrast to recombinant viruses, immunization with "naked DNA" is safe, and multiple injections can be performed but usually result in less efficient induction of T cell responses (60). This method may be useful to boost immune responses.

Peptides may be used for immunization in conjunction with adjuvants or lipids. Clinical trials using MART-1 or gp100 peptides with incomplete Freund's adjuvant are being conducted in melanoma patients in the Surgery Branch of the National Cancer Institute (39,61). Evidence of minor regression of some tumor nodules has been observed in 3 of 23 melanoma patients who were immunized with the MART-$1_{27\text{-}35}$ peptide. Professional APCs such as dendritic cells pulsed with the peptides or transfected with DNA-encoding tumor antigens (62) may

TABLE 5. *Reagents for active immunization of cancer patients*

A. Antigenic peptides or whole proteins (with adjuvants, combined with lipids or liposomes, with gp96, Hsp70, or Hsp90)
B. Recombinant viruses containing tumor antigen genes (adenovirus, fowlpox virus, vaccinia virus)
C. Naked DNA-encoding tumor antigen genes (intramuscular or by "gene gun")
D. Recombinant bacteria containing tumor antigen genes (Bacillus Culmette-Guérin, *Salmonella, Listeria*)
E. Cells expressing tumor antigens (dendritic cells pulsed with epitopes, cells transfected with tumor antigens, HLA and B7 genes, cells transfected with epitope/HLA fusion genes)
F. Cytokines that may augment efficacy of immunization and tumor recognition (IL-2, IL-12, GM-CSF, interferon)

IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor.

TABLE 6. *Methods for induction of T cells specific for subdominant self-determinants*

A. Use of high-protein expression system (recombinant viral vectors)
B. Cells pulsed with epitopes exogenously (epitope-pulsed dendritic cells)
C. Use of epitopes modified to have high MHC binding affinity
D. Use of cells expressing epitope-linked MHC molecules

MHC, major histocompatibility complex.

be used as immunogens, and heat shock proteins may be useful as peptide delivery vehicles for APCs (63). Potent melanoma-reactive T cells can be induced from the PBLs of some patients by repeated in vitro stimulation with the melanoma epitopes (29,45); however, these CTLs may be more efficiently induced from patients who are immunized with peptides (61) or recombinant antigens and may be useful in adoptive transfer protocols.

Many melanoma epitopes have relatively low MHC binding affinities; therefore, we have attempted to increase their immunogenicity by increasing the MHC binding affinity of these peptides. By changing the nondominant anchor amino acids to dominant amino acids at the primary anchor positions of the gp100 epitopes, peptides with higher HLA-A2 binding affinity could be created. These modified epitopes were also found, in some cases, to induce melanoma-reactive CTLs in vitro more efficiently than the native epitopes (64). These modified peptides may be useful for induction of melanoma-reactive T cells in vitro and in vivo. Immunization trials using these modified peptides have begun in the Surgery Branch of the National Cancer Institute, and preliminary results suggest that the gp100 209-2M peptide in which a methionine residue was substituted for threonine at the P2 primary anchor position could immunize patients more effectively than did the native peptide. These amino acid changes have also been incorporated into the gp100 cDNA, which will be used for the construction of recombinant viruses or naked DNA vaccines. Use of these modified reagents in vitro and in vivo may induce low-avidity T cells that do not recognize tumor cells efficiently, and conversely immunization with these modified antigens may result in autoimmune problems. In another attempt to express melanoma epitopes at high density on the cell surface, melanoma epitopes have been covalently linked to HLA-A2.1 molecules with a spacer to promote association of peptides in the groove of HLA-A2 molecules. B cells transfected with the epitope-HLA-A2 fusion DNA expressed fusion proteins at high density on the cell surface. Melanoma-reactive CTLs could be efficiently induced in vitro from PBLs of patients by stimulation with the B cells expressing the MART-1 or gp100 epitope-HLA-A2 fusion protein (65).

Administration of cytokines such as IL-2, IL-12, granulocyte-macrophage colony-stimulating factor, or interferon may increase the efficacy of immunotherapies through improving immunization as well as enhancing tumor recognition at the effector phase by increasing the level of expression of epitope/MHC complexes on the tumor cell surface (40,66). These cytokines may be produced locally by incorporating cytokine genes in the same recombinant viral vectors containing tumor antigen genes.

Since melanocytes and melanoma cells may induce tolerance in T cells in vivo, repeated immunizations may be necessary until tumors completely regress (67,68). To maintain antitumor T cell responses, sequential immunizations with multiple antigens using different immunization methods including multiple recombinant viral vectors are planned in the Surgery Branch.

Immune Monitoring of Patients Immunized with Tumor Antigens

It is important to monitor the efficiency of immunization with specific antigens in Phase I protocols (69). However, it has been difficult to quantitatively assess $CD8^+$ T cell immune responses specific for melanoma antigens. CTL precursor frequencies are too low ($<1/10^5$) to measure using standard limiting dilution techniques in most cases even after immunization. However, after in vitro expansion of specific T cells by one to three restimulations with antigenic peptides, immune augmentation has been detected in most patients immunized with MART-1 or gp100 peptides (39,46,61). It is important to distinguish high-avidity T cells that recognize melanoma cells expressing endogenously processed epitopes at a low density on the cell surface from low-avidity T cells that may be induced during in vitro stimulation with relatively high concentrations of peptides. Elispot assays and analysis of T cell receptor variable regions have also been investigated for monitoring immunization efficacy (70,71) (Table 7).

Tumor Escape from T Cell Immune Responses

Heterogeneous expression of melanosomal proteins and HLA molecules has been observed in metastatic

TABLE 7. *Immune monitoring of patients immunized with tumor antigens*

In vivo	Delayed-type hypersensitivity
	Tumor regression
In vitro	Tumor-reactive T cell precursor frequency (Elispot, limiting dilution)
	Tumor-reactive T cell induction
	T cell receptor analysis

melanoma, and tumor cells that lost expression of antigens, HLA, or β_2-microglobulin have been found in some patients (40,72,73). Tumor cells may escape from T cell responses through loss of other molecules involved in antigen processing. Antitumor immune responses may be down-regulated through the induction of tolerance, a shift from Th1 to TH2, altered T cell signaling, Fas ligand expression by tumor cells, as well as suppressive factors released from tumor cells (74,75). The frequency and mechanisms of tumor escape need to be carefully evaluated in clinical protocols as well as animal tumor models. It may be important to select patients eligible for the antigen-specific immunotherapy by screening expression of antigens and MHC molecules in tumor cells. It may also be necessary in many cases to combine other types of treatment such as chemotherapy with immunotherapy to completely eradicate tumors.

CONCLUDING REMARKS

During the last several years, many tumor epitopes recognized by autologous $CD8^+$ T cells, as well as a variety of mechanisms involved in generating these epitopes, have been identified in patients with melanoma. However, the role of T cell responses to these antigens in tumor rejection in vivo as well as their usefulness in cancer immunotherapy are not yet clear. In addition, the role of $CD4^+$ T cells in melanoma rejection has not been evaluated, although autologous tumor-specific and shared antigens have been observed in MHC class II-restricted melanoma-reactive TILs in the Surgery Branch of the National Cancer Institute. Clinical trials and analysis using relevant animal tumor models may give us clearer insights into the role of the identified antigens, including mutated and nonmutated peptides, in in vivo tumor rejection, and should lead to the development of more effective cancer immunotherapies.

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