Development of Cancer Immunotherapies Based on Identification of the Genes Encoding Cancer Regression Antigens

Steven A. Rosenberg*

Tumor-infiltrating lymphocytes (TILs) have been grown from patients with metastatic melanoma and administered to the autologous patients to identify those TIL populations capable of mediating tumor regression. These TILs have been used to clone the genes that encode melanoma antigens. With the use of this strategy, we have identified six different genes encoding antigens restricted by multiple HLA alleles that appear to be related to tumor regression in patients. These antigens are now being used to develop immunization approaches for the treatment of patients with metastatic melanoma. The availability of genes encoding unique cancer antigens is opening new possibilities for the development of immunotherapies for the treatment of patients with cancer. [J Natl Cancer Inst 1996;88:1635-44]

Human cancers can stimulate immunologic reactions directed against antigens selectively expressed on tumor cells. In vitro, it has been possible to generate T lymphocytes that are capable of selectively recognizing autologous tumor from cancer patients bearing a variety of histologic types of cancer, including melanoma, lymphoma, and cancers of the ovary, breast, colon, and kidney (1-9). Compelling evidence that immune reactions can result in tumor destruction in vivo comes from human studies of the administration of interleukin 2 (IL-2), a cytokine with no direct effect on cancer cells, but with potent immunomodulatory activity. Durable cancer regressions have been induced by treatment with IL-2 in humans with metastatic melanomas and renal cancers (10,11). The results of the treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer treated with high-dose IL-2 alone in the Surgery Branch, National Cancer Institute (NCI), are shown in Table 1 (11). Twenty-four patients achieved a complete regression and 29 patients achieved a partial regression of metastatic cancer; 19 of 24 patients achieving a complete regression have not shown recurrence when evaluated at 39-114 months after treatment.

The molecular identification of the cancer antigens involved in the immune system-mediated destruction of cancer cells could be useful for the development of specific active immunization strategies against cancer (e.g., cancer vaccines) as well as for the in vitro generation of lymphocytes for use in adoptive immunotherapy. Using lymphocytes reactive against human cancer antigens in vitro, it has been possible to screen cDNA or genomic libraries to identify the genes encoding these antigens (12,13). All antigens identified by lymphocytes, in vitro, however, may not serve as targets for immune destruction in vivo. To maximize the chances of identifying the cancer antigens involved in effective therapeutic immune reactions in cancer patients, we have used the strategy outlined in Table 2.

Tumor-infiltrating lymphocytes (TILs) are lymphocytes infiltrating into the stroma of cancer nodules (14). TILs grown in vitro from about one half of patients with metastatic melanoma can recognize cancer associated antigens in a major histocompatibility complex (MHC)-restricted fashion using assays of specific lysis or cytokine release by TILs coincubated with autologous tumor cells. TILs with specific reactivity against colon, breast, and ovarian cancers and lymphomas are seen with lesser frequency. In a pilot clinical trial, the adoptive transfer of TILs plus IL-2 could mediate objective cancer regression in 29 (34%) of 86 patients with metastatic melanoma; an equal response rate was seen in patients who had previously failed treatment with IL-2 alone (Table 1) (15,16). TILs capable of recognizing tumor antigens in vitro and capable of mediating cancer regression in vivo when adoptively transferred to the autologous cancer patient were used to screen complementary DNA (cDNA) libraries from the autologous tumor in an attempt to identify the genes encoding the antigens responsible for the in vivo therapeutic effect (13). The genes isolated using this strategy were in many instances different from the genes isolated by others using peripheral blood lymphocytes sensitized in vitro to tumor cells. As a final test of the therapeutic relevance of these antigens, the genes or gene products recognized by

*Affiliation of author: Surgery Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, MD.

Correspondence to: Steven A. Rosenberg, M.D., Ph.D., National Institutes of Health, Bldg. 10, Rm. 2B42, Bethesda, MD 20892.

See “Note” section following “References.”

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Table 1. Immunotherapy for patients with metastatic melanoma in the Surgery Branch, National Cancer Institute

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Treatment*</th>
<th>Total No. of patients</th>
<th>No. of patients with objective response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal cancer</td>
<td>IL-2</td>
<td>149</td>
<td>30 (20)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>IL-2</td>
<td>134</td>
<td>23 (17)</td>
</tr>
<tr>
<td>TILs + IL-2</td>
<td>86</td>
<td>29 (34)</td>
<td></td>
</tr>
<tr>
<td>Prior IL-2</td>
<td>28</td>
<td>9 (32)</td>
<td></td>
</tr>
<tr>
<td>No prior IL-2</td>
<td>58</td>
<td>20 (34)</td>
<td></td>
</tr>
</tbody>
</table>

*All IL-2 administered at 720 000 IU/kg intravenously every 8 hours. IL-2 = interleukin 2; TILs = tumor-infiltrating lymphocytes.
TILs are being used to treat patients to evaluate whether they can induce cancer regression.

**Identification of the Genes Encoding Cancer Regression Antigens Recognized by TILs**

To identify the genes encoding the tumor antigens recognized by TILs, cDNA libraries were prepared from tumor cells and transfected into target cells bearing the appropriate MHC restriction element. These transfected target cells were then tested for the ability to be recognized by TILs, previously shown to be associated with tumor regression in vivo (Fig. 1). Melanoma antigens were identified using both stable and transient transfectant systems. Initial studies were directed at identifying tumor antigens restricted by HLA-A2, since this is the most common HLA antigen in humans. Thus, to isolate the genes encoding antigens recognized by human leukocyte antigen (HLA)-A2 TILs, a cDNA library was transfected into the HLA-A2* breast cancer cell line MDA-231, stable transfecnt clones were isolated, and each clone was individually tested for its susceptibility to recognition by HLA-A2* TIL 1200 or TIL 1235 (17,18). In subsequent studies, this labor-intensive strategy was supplanted by the use of transient transfection systems in which pools of cDNA were transiently transfected into a highly transfectable cell line, such as the COS monkey kidney cell line or the 293 human embryonic kidney cell line expressing the appropriate MHC restriction element (19,20). After screening of the transient transfectants, subclones of positive pools were made until a single gene capable of conferring TIL reactivity was identified.

Using these approaches, six genes encoding antigens recognized by TILs have been identified (17-22). The general characteristics of these antigens are presented in Table 3.

**MART-1 and gp100**

The MART-1 (Melanoma Antigen Recognized by T cells) and gp100 antigens are melanocyte differentiation antigens recognized by HLA-A2 restricted TIL 1235 and TIL 1200, respectively (17,18). The MART-1 gene encodes a 118 amino acid protein and contains a 21 amino acid putative transmembrane domain. The gp100 gene encodes a 661 amino acid protein containing a leader sequence and a single transmembrane domain. The MART-1 gene was independently cloned by Coulie et al. (23) and was termed Melan-A. The gp100 gene had previously been known to react with monoclonal antibodies, such as HMB-45 and NKJ/betab, although it had not been appreciated that gp100 could also serve as an antigen recognized by T lymphocytes (24).

Northern blot analyses revealed that both the MART-1 and gp100 genes were expressed in the majority of melanomas, normal melanocyte cell lines, and in retina, but not in a wide variety of nonmelanomas or normal tissues (17,18). The exact functions of MART-1 and gp100 are unknown, although their distribution and subcellular localization suggest that they are involved in melanin synthesis. Thus, both genes appear to be normal, nonmutated melanocyte differentiation antigens.

By screening multiple peptides that have the appropriate HLA-A2 binding motifs, the immunodominant peptides from these proteins were identified (25,26). MART-1 appeared to contain a single immunodominant nonapeptide (AAGIGILTV), although two 10 amino acid peptides were also recognized that contained a single amino acid at either the amino or carboxyl terminal end of the immunodominant nonapeptide (25). This MART-1 peptide appears to be the immunodominant antigen recognized by TILs from most HLA-A2* patients with melanoma. Of 18 HLA-A2-restricted TILs administered to cancer patients, 15 recognized the MART-1 antigen and all 15 recognized the same nonapeptide.

Using multiple TILs, five different peptides from the gp100 protein were recognized (26). Three of these peptides, gp100aa154-162 (GTWGQYWQV), gp100aa209-217 (ITDQVFPFSV), and gp100aa230-238 (YLEPGPVTA), appeared to be immunodominant and were most widely recognized by TILs. The gp100aa230-238 peptide was independently isolated from melanomas by elution from HLA-A2 molecules (27). Of the 18 HLA-A2-restricted TILs, 11 recognized gp100; three recognized gp100 alone and eight recognized both gp100 and MART-1. Thus, although there may be other antigens recognized by HLA-A2-restricted TILs, all of the reactivity of the HLA-A2-restricted TILs we have tested thus far can be accounted for by reactivity with either MART-1 or gp100. With use of peripheral blood lymphocytes stimulated in vitro with melanoma cells, Brichard et al. (28) isolated lymphocytes that could recognize tyrosinase in an HLA-A2-restricted fashion. However, none of the HLA-A2-restricted TILs that we have obtained from the tumors of cancer patients recognized tyrosinase.

**Tyrosinase**

The gene encoding tyrosinase was isolated using TIL 888 that recognized melanomas in an HLA-A24-restricted fashion (19).
Adoptive transfer of this TIL mediated complete regression of metastases to the lung, subcutaneous tissues, and mucosa in a patient with metastatic melanoma (19,29).

Tyrosinase is the only tumor antigen that has also been identified as recognized by CD4+ TILs in a class II-restricted fashion (30). To identify antigens recognized by CD4+ cells, we incubated cell extracts with Epstein-Barr virus (EBV)-transformed B cells, which were then coincubated with TILs. TIL 1088 recognized autologous EBV-transformed B cells incubated with cell extracts from a variety of melanoma and melanocyte cell lines but not with extracts from normal cells. Using extracts of COS cells transfected with tyrosinase cDNA, tyrosinase was identified as the antigen recognized by TIL 1088 in a class II, HLA-DR0401-restricted fashion.

Tyrosinase is a 529 amino acid membrane-associated glycoprotein with enzymatic activity involved in melanin synthesis. Like the genes encoding MART-1 and gp100, this gene encodes a nonmutated normal product present in melanomas, melanocytes, and retina, but not other normal tissues. A nine amino acid peptide from tyrosinase recognized by TIL 1413 has been identified (AFLPWHRLF), although the peptide recognized by TIL 888 is still unknown (31).

Thus, tyrosinase is an antigen that appears to be capable of presenting immunogenic peptides on at least two class I molecules.
(HLA-A24 and HLA-A2) as well as on the class II (HLA-DRD0401) molecule. However, only TILs restricted by HLA-A24 have been shown to mediate tumor regression in patients with metastatic cancer.

**Tyrosinase-Related Protein 1 (TRP1)**

The adoptive transfer of TIL 586 mediated the regression of a large apical lung metastasis in a patient with melanoma and recognized shared melanoma antigens in an HLA-A31-restricted fashion (20,32). The gene encoding the antigen recognized by TIL 586 was shown to be identical to the gene encoding TRP1, a molecule previously shown to be recognized by antibodies in the serum of a patient with melanoma (33). The TRP1 protein (also called gp75) contains 527 amino acids with enzymatic activity involved in melanin biosynthesis. This gene also is a normal, nonmutated gene encoding a protein present in melanomas, normal melanocytes, and retina but not other tissues. Examination of multiple peptides from the TRP1 protein failed to reveal any that could be recognized by TIL 586 (32). However, by examining a variety of peptides from alternative open reading frames of the TRP1 gene, a single nine amino acid peptide (MSLQRQFLR) initiated by an ATG in the third open reading frame of the TRP1 gene was identified that was capable of sensitizing HLA-A31 positive 586EBV (EBV-transformed) cells to lysis by TIL 586. Although it was known that some viruses could translate functional proteins from different alternative open reading frames of the same gene sequence, it had not been previously recognized that eukaryotic cells could also translate in this fashion. Thus, the TRP1 gene gives rise to two completely different functional polypeptide chains, one has enzymatic activity and the other has immunogenic activity in patients with melanoma. It thus will be important to examine alternative open reading frames of other tumor antigens as they are isolated to determine whether they contain the tumor antigenic peptide. Quelle et al. (34) recently showed that two different polypeptide chains encoded by alternative open reading frames of the p16INK4a gene could play a role in cell cycle control.

**p15**

HLA-A24-restricted TIL 1290 that recognized melanomas in an HLA-A24-restricted fashion was used to isolate the gene encoding an antigen we have called p15 (22). The gene encoding p15 was a normal, nonmutated gene; but it differed from those previously described because it appeared to be transcribed in a wide variety of normal tissues, including autologous fibroblasts and EBV-carrying cell lines, even though these tissues were not recognized by TIL 1290. Thus, it appeared that post-transcriptional mechanisms were involved in the expression of this gene product on the surface of cells. A nine amino acid peptide (AYGLDFYIL) was identified in p15 that could be recognized by TIL 1290 (22).

**Beta-Catenin**

TILs are oligoclonal and are capable of recognizing more than one antigen. Thus, TIL 1290, which recognized the p15 antigen, also recognized the product of another gene that encoded a molecule almost identical to beta-catenin. Beta-catenin is a cytoplasmic protein that binds to the intracellular adhesion molecule, E-cadherin, and plays an important role in cell adhesion (21). The APC tumor suppressor gene product binds to beta-catenin (35).

The cDNA clone encoding the protein recognized by TIL 1290 differed from the normal beta-catenin gene sequence by a single C to T nucleotide mutation that resulted in an amino acid change from a serine to a phenylalanine (21). The peptide epitope from the beta-catenin molecule that conferred recognition to TIL 1290 was a nine amino acid peptide that contained this mutated phenylalanine in the ninth position. Previous studies had shown that phenylalanine was a dominant anchor residue at position nine for HLA-A24-binding peptides. Mutated peptide could sensitize target cells for lysis by TIL 1290 at a concentration of 1 picomolar, a million-fold lower than the concentration required of the native peptide. Mutated beta-catenin is the only tumor antigen recognized by TILs that results from a mutated normal gene. The mutation was found only in tumor from patient 1290 but was not found in normal cells from the autologous patient or in any of 11 allogeneic

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**Table 3. Identification of melanoma antigens recognized by TILs associated with in vivo tumor rejection***

<table>
<thead>
<tr>
<th>Name of antigen (reference No.)</th>
<th>Alternate name(s)</th>
<th>TILs used for identification</th>
<th>No. of amino acids</th>
<th>HLA restriction</th>
<th>Immunodominant epitopes</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>MART-1 (18)</td>
<td>MelanA</td>
<td>1235 and others</td>
<td>118</td>
<td>A2</td>
<td>AAGIGILTV</td>
<td>Normal differentiation antigen</td>
</tr>
<tr>
<td>gp100 (17)</td>
<td>HMB-45</td>
<td>1200 and others</td>
<td>661</td>
<td>A2</td>
<td>KTWQQQQWQV</td>
<td>Normal differentiation antigen</td>
</tr>
<tr>
<td></td>
<td>HMB-50</td>
<td></td>
<td></td>
<td></td>
<td>ITDQVPFSV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NKF/beta</td>
<td></td>
<td></td>
<td></td>
<td>YLEPGPVPYTA</td>
<td></td>
</tr>
<tr>
<td>Tyrosinase (19)</td>
<td>—</td>
<td>888</td>
<td>529</td>
<td>A24</td>
<td>AFLPWHRLF</td>
<td>Normal differentiation antigen</td>
</tr>
<tr>
<td>p15 (22)</td>
<td>—</td>
<td>1290</td>
<td>128</td>
<td>A24</td>
<td>AYGLDFYIL</td>
<td>Post-transcriptional control</td>
</tr>
<tr>
<td>TRP-1 (20)</td>
<td>gp75</td>
<td>586</td>
<td>527</td>
<td>A31</td>
<td>MSLQRQFLR</td>
<td>Translated from alternative open reading frame</td>
</tr>
<tr>
<td>beta-catenin (21)</td>
<td>—</td>
<td>1290</td>
<td>781</td>
<td>A24</td>
<td>SYLDQGHHF</td>
<td>Single base mutation</td>
</tr>
</tbody>
</table>

*TILs = tumor-infiltrating lymphocytes; HLA = human leukocyte antigen.
melanomas tested. Using peripheral blood lymphocytes sensitized in vitro to tumor, others have identified two genes in melanomas that encode antigens arising from mutated normal proteins, MUM-1 and CDK4. We have not yet found TILs that recognize MUM-1 or CDK4 (36,37).

Cancer Therapies Based on the Genes Encoding Cancer Regression Antigens

The molecular identification of tumor antigens and the genes encoding these antigens have opened new possibilities for the development of immunization strategies against cancer antigens (13).

In the absence of clearly defined tumor regression antigens, attempts to develop cancer vaccines have used intact irradiated tumor cells or subcellular fractions of tumor cells either alone or mixed with adjuvants. More recently, tumor cells genetically modified to increase their immunogenicity have been used. Although these attempts at immunization could result in protection of experimental animals against a tumor challenge, they were largely ineffective in eradicating established growing tumors. These minimal therapeutic effects have been mirrored in clinical efforts using this approach.

The strategy underlying the use of TILs to identify cancer regression antigens was based on the ability of TILs to mediate tumor regression on adoptive transfer to patients with metastatic cancer. Although IL-2 was administered along with TILs, the effectiveness of TILs in tumor regression was evidenced by the higher response rate seen when TILs plus IL-2 were used and the ability of TILs plus IL-2 to cause tumor regression in patients who had previously failed treatment with IL-2 alone (15,16).

The approaches used to immunize against the characterized cancer antigens are influenced by the nature of the antigens themselves. Five of the six antigens recognized by TILs are the products of normal, nonmutated genes. Similarly, the MAGE, BAGE, and GAGE antigens identified by Van den Bruggen et al. (38), Boel et al. (39), and Van den Eynde et al. (40) also represent the products of normal, nonmutated genes expressed on some normal cells such as those of testis.

Evidence that normal melanocyte differentiation proteins recognized by TILs can serve as tumor regression antigens comes from studies of vitiligo in patients treated with IL-2-based immunotherapies (41). A prospective analysis of patients treated in the Surgery Branch, NCI, with IL-2 revealed no cases of vitiligo in 104 patients with metastatic renal cell cancer compared with 12 (16%) of 73 patients with metastatic melanoma who developed vitiligo (two-sided P < 0.0001). None of 27 nonresponding patients compared with 12 (29%) of 42 patients who underwent tumor regression following IL-2 therapy developed vitiligo (two-sided P < 0.002). The association of clinical response with vitiligo suggested that the targets involved in the regression of cancer were the same targets associated with the destruction of normal melanocytes. It thus appears that the development of a strong immune response against normal differentiation antigens highly expressed on melanoma cells and on normal melanocytes can result in the regression of metastatic melanoma. On the basis of this principle, a variety of other nonessential organs that give rise to cancers can potentially be targeted through immunization with tissue-specific differentiation antigens, such as cancers of the thyroid, prostate, breast, ovary, and testis. The epithelial cells of each of these organs contain proteins unique to that tissue.

The mechanism by which tolerance to normal tissue antigens is broken by the growing cancer is currently unknown. The overexpression of selected proteins by the cancer as well as the unique inflammatory cytokines secreted at the tumor site may give rise to immune reactions capable of breaking tolerance to normal antigens. It is of interest that none of the six epitopes in the MART-1 or gp100 antigens recognized by TILs from HLA-A2+ patients are high-affinity binding peptides (25,26). Each of these peptides lacks at least one dominant residue at an anchor position required for high binding to HLA-A2. This finding suggests that lymphocytes reactive to high-affinity peptides have been eliminated or tolerized and that peptides with low binding affinity (or "cryptic peptides") evade tolerizing mechanisms and can serve as immunogens. Thus, the administration or endogenous production of very large amounts of these lower affinity peptides by immunization of the cancer patient may be an effective strategy for the development of cancer immunotherapies.

Cancer immunotherapies can be categorized as either passive or active approaches. In passive immunotherapy, immune cells (or antibodies) with anticancer activity are generated and adoptively transferred to the tumor-bearing host. Active immunotherapy (cancer vaccines) refers to approaches in which tumor antigens are utilized to directly immunize the tumor-bearing host with the intent of endogenously generating immune reactions capable of tumor destruction. Opportunities for the development of cancer immunotherapies based on the identification of the genes encoding cancer antigens are summarized in Table 4.

Passive Immunotherapy: Generation of Antitumor Effector Cells

Identification of the specific molecules recognized as tumor antigens has enabled the development of improved means for generating cells in vitro with specific antitumor reactivity. The adoptive transfer of TILs results in tumor regression in only about one third of patients with metastatic melanoma (16) and these in vivo antitumor effects correlate with the ability of TILs to specifically lyse tumor cells in vitro as well as to specifically release cytokines such as granulocyte–monocyte colony-stimulating factor (GM-CSF) when coincubated with tumor cells (42,43). Although TILs can be grown from approximately 90% of patients with melanoma, only about one half of TIL cultures are capable of specifically recognizing tumor antigens. Rivoltini et al. (44) and Salgaller et al. (45) demonstrated that peripheral blood lymphocytes (PBLs) from virtually all patients with melanoma could be specifically sensitized in vitro by repeated exposure to antigen-presenting cells pulsed with the immunodominant MART-1 or gp100 tumor-antigen peptides. These cells could be expanded over 10,000-fold during 2 months and had specific lytic activity 50-100 times greater on a per cell basis than did the TILs originally used to identify these tumor antigens. PBLs generated by repeated in vitro sensitization were able to lyse tumor and to secrete a variety of cytokines such as...
Thus, these cells may be more effective than TILs when adoptively transferred into cancer patients, and clinical protocols to utilize these TILs are now being developed.

Immunodominant peptides modified at dominant anchor positions to increase their binding affinity (see below) have been identified, and in vitro generation with these modified immunodominant peptides can further improve the rapidity with which specific antitumor effector cells can be generated in vitro (46).

The genes encoding the alpha and beta chains of the T-cell receptors capable of recognizing the MART-1 tumor antigen have now been isolated and sequenced (47,48). There appears to be significant diversity in the T-cell receptor repertoire capable of recognizing the same nine amino acid immunodominant epitope from MART-1 since different alpha and beta chains have been identified as being capable of recognizing this single nonapeptide bound to HLA-A2. Transfection of the genes encoding the T-cell receptor chains into Jurkat cells conferred reactivity to the specific tumor antigen (49). These experiments have suggested two possibilities for the development of passive immunotherapies for the treatment of cancer patients. These T-cell receptor genes could be transduced into effector T cells such as OKT3-stimulated peripheral blood mononuclear cells that could be expanded in vitro and used for adoptive therapy. Of greater interest, however, is the possibility of genetically modifying bone marrow-derived hematopoietic stem cells with the genes encoding these T-cell receptors. The transplantation of transduced autologous marrow could then give rise to large numbers of endogenous lymphocytes capable of recognizing and destroying tumor cells.

Active Immunotherapy: Cancer Vaccines

Identification of the genes encoding cancer antigens has enabled the development of new immunization strategies for patients with cancer (Table 4). These strategies include immunization with immunodominant peptides, peptides modified to increase immunogenicity, proteins, “naked” DNA encoding cancer antigens, antigen-presenting cells expressing the antigen, or recombinant viruses or bacteria containing the genes encoding cancer antigens.

In animal models, it has been clearly shown that model tumor antigens presented on tumor cells are not as immunogenic as these same antigens presented by immunizing viruses capable of infecting and expressing the tumor antigen on normal cells (50). Thus, whereas tumors bearing the model beta-galactosidase tumor antigen grew as well as unmodified tumor cells, the presentation of this antigen by immunization with naked DNA (51-53) or by recombinant viruses encoding the gene for beta-galactosidase led to the successful treatment of established tumors bearing this antigen. In these animal models, immunization could be enhanced by the administration of cytokines such as IL-2 or IL-12 or by incorporating the genes encoding immunostimulatory cytokines or costimulatory molecules along with the tumor antigen into recombinant viruses (52,54,55).

GM-CSF and gamma-interferon when coincubated with tumor. Thus, these cells may be more effective than TILs when adoptively transferred into cancer patients, and clinical protocols to utilize these TILs are now being developed.

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These and other murine tumor models have served as the basis of a series of clinical trials that are being conducted in the Surgery Branch, NCI, in patients with metastatic melanoma. In these trials, we are attempting to immunize the patients against the MART-1 and gp100 melanoma antigens. A summary of the status of these trials is shown in Table 5.

An essential part of these clinical trials was the development of reliable assays for monitoring the state of immunization of cancer patients against these tumor antigens. The goal of these immunization strategies was to increase the frequency of precursor cells capable of recognizing melanoma antigens to a level sufficient to mediate antitumor effects. The development of reliable quantitative assessments of precursor cell frequency using limiting dilution assays has been difficult, and thus the assays used to monitor these trials have relied on assessments of the potential reactivity of bulk peripheral lymphocyte populations against these melanoma antigens. The ability to reproducibly generate lymphocytes with specific reactivity against tumor an-
Table 5. Immunization of patients with metastatic melanoma against cancer antigens (status as of 9/1/96)*

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Immunization</th>
<th>Dose range</th>
<th>Schedule</th>
<th>Entry dates</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>95-C-71</td>
<td>MART-1 peptide in IFA</td>
<td>0.1-10 mg</td>
<td>Every 3 wk x 4</td>
<td>2/2/95-9/14/95</td>
<td>23</td>
</tr>
<tr>
<td>95-C-145</td>
<td>gp100 peptides in IFA: aa154-162, aa280-288, aa209-217</td>
<td>1-10 mg</td>
<td>Every 3 wk x 4</td>
<td>6/20/95-11/3/95</td>
<td>28</td>
</tr>
<tr>
<td>95-C-145-C</td>
<td>Modified gp100 peptides aa280-9V, aa209-2M</td>
<td>1 mg</td>
<td>Every 3 wk x 4</td>
<td>11/21/95-present</td>
<td>15</td>
</tr>
<tr>
<td>95-C-79</td>
<td>Adenovirus-MART-1 (± IL-2)</td>
<td>$10^7-10^{11}$ pfu</td>
<td>Every 4 wk x 2</td>
<td>1/2/95-present</td>
<td>35</td>
</tr>
<tr>
<td>95-C-143</td>
<td>MART-1 peptide in IFA (± IL-12)</td>
<td>1 mg (10-300 ng/kg)</td>
<td>Every 3 wk x 4</td>
<td>1/1/96-present</td>
<td>12</td>
</tr>
<tr>
<td>95-C-78</td>
<td>Adenovirus gp100 (± IL-2)</td>
<td>$10^5-10^{11}$ pfu</td>
<td>Every 4 wk x 2</td>
<td>4/23/96-present</td>
<td>14</td>
</tr>
<tr>
<td>95-C-32</td>
<td>Fowlpox-MART (± IL-2)</td>
<td>$10^7-3 \times 10^8$ pfu</td>
<td>Every 4 wk x 2</td>
<td>6/6/96-present</td>
<td>10</td>
</tr>
<tr>
<td>96-C-107</td>
<td>Adoptive transfer of lymphocytes in vitro sensitized to gp100 aa209-210M-217</td>
<td>$10^8-10^{11}$ cells</td>
<td>Single infusion</td>
<td>7/9/96-present</td>
<td>4</td>
</tr>
</tbody>
</table>

*IFA = incomplete Freund's adjuvant; pfu = plaque-forming units.

Tigens by repeated in vitro sensitization using peptides presented on antigen-presenting cells has provided the basis for a semi-quantitative assessment of the potential reactivity of the circulating lymphocytes toward the tumor cells of melanoma patients (44,56). The number of repeated in vitro sensitizations required to generate specific antitumor reactivity, as well as the quantitation of lymphocyte reactivity generated after a specified number of in vitro stimulations, have provided means for assessing the antitumor response (57,58). An example of one such assay is shown in Fig. 2.

Because of the ease of preparation of peptide and the safety of administration, the initial clinical trials using MART-1 and gp100 attempted to immunize against the immunodominant peptides from these antigens emulsified in incomplete Freund's adjuvant (IFA). In these trials, patients with metastatic melanoma were immunized with either the MART-1 immunodominant peptide, MART$_{27-35}$, or one of three gp100 immunodominant peptides, gp100$_{154-162}$, gp100$_{209-217}$, or gp100$_{280-288}$ adjuvant given subcutaneously every 3 weeks for a total of four doses. As of September 1996, a total of 23 patients were immunized with the MART-1 peptide, and 28 patients were immunized with one of the gp100 peptides. These immunizations were administered as outpatient treatments, and with the exception of mild transient erythema and induration at the injection site, there were no side effects of these immunizations.

The native level of precursors capable of responding to these different peptides appeared to be different. Although reproducible anti-MART-1 peptide reactivity could be seen after three in vitro sensitizations, it often took four or five in vitro sensitizations before any reactivity was seen against the three gp100 immunodominant peptides. The ability to shorten the number of in vitro sensitizations required to detect specific antitumor reactivity as well as the ability to generate increased

Fig. 2. In vitro generation of lymphocytes with specific anti-MART-1 lytic activity from the peripheral blood of a patient prior to and after two and four vaccinations with the MART-1 peptide (1 mg) in incomplete Freund's adjuvant.
reactivity provided a reproducible basis for assessing the efficacy of these peptide immunizations (57). It appeared that immunization with the MART-1 peptide could increase the frequency of anti-MART-1 precursors in circulating peripheral blood of approximately one half of patients. More potent immunization, however, resulted from administration of the gp100_{209-217} immunodominant peptide, and virtually all patients immunized with this peptide showed evidence of increased antitumor reactivity in vitro (58). We could not detect increased reactivity in patients immunized with the gp100_{154-162} peptide. A summary of some of these results is shown in Table 6.

Because each of these immunodominant peptides had a relatively weak binding affinity to the HLA-A2 molecule, studies were undertaken to explore individual amino acid substitutions within these peptides aimed at increasing their affinity without compromising recognition by TILs. Amino acid substitutions at positions two and nine of the peptides were tested because these were the anchor positions known to be optimal for binding to HLA-A2 on the basis of known peptide binding motifs (46). Although many substitutions were identified that resulted in increased binding affinity to HLA-A2, many of these substitutions abrogated immune recognition. However, modification of the gp100_{209-217} peptide with a methionine substituted at position two (called gp100_{209-217M}) and the gp100_{280-288} peptide with a valine substituted at position nine (called gp100_{280-288v}) resulted in a greater than 10-fold increase in binding affinity to HLA-A2 as well as increased recognition by TILs capable of recognizing the native epitope (46). In vitro immunization utilizing these peptides resulted in a dramatic increase in the ability to sensitize peripheral blood lymphocytes from melanoma patients to the modified as well as the native peptide. Because of this increase in in vitro immunogenicity, we have initiated a clinical study in which patients are being immunized with one of these two high-affinity binding synthetic peptides. Initial results indicated that these modified peptides are more effective at immunizing against native gp100 epitopes than are the native peptides themselves. These studies are continuing.

The most effective means for immunizing experimental animals against model tumor antigens utilized recombinant viruses containing the genes encoding these antigens, such as adenovirus, fowlpox, and vaccinia (50,52,53). The concomitant administration of IL-2 or IL-12 immediately following immunization increased the antitumor effects of this approach in tumor-bearing mice. We have thus recently begun a clinical trial in which patients with advanced melanoma are immunized with recombinant adenovirus encoding the MART-1 or gp100 tumor antigen (59). Two vaccinations are given subcutaneously or intramuscularly, separated by 4 weeks, and some patients receive IL-2 systemically following each immunization. We also have recently begun clinical trials using the MART-1 immunodominant peptide administered with IFA, followed by the subcutaneous administration of IL-12, again based on the potency of this combination in animal models (34).

The clinical impact of these immunizations is currently under evaluation. Although three patients showed evidence of tumor shrinkage following immunization with the MART-1 peptide, none achieved the full criteria for an objective antitumor response. One of the patients receiving the gp100_{209-217} peptide achieved a complete clinical regression of cancer that lasted 5 months. These trials, however, represent initial efforts to immunize against cancer regression antigens recognized by TILs, and continued optimization based on in vitro analysis of the specific immune reactivity to these melanoma antigens is ongoing.

### Table 6. Protocol 95-C-145: metastatic melanoma gp100 peptides in incomplete Freund’s adjuvant (28 patients treated between 6/20/95 and 11/3/95)

<table>
<thead>
<tr>
<th>Peptide*</th>
<th>Specific cytokine release after two in vitro stimulations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preimmunization (pg interferon-γ/mL/24 h)</td>
</tr>
<tr>
<td>gp100_{154-162}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>gp100_{209-217}</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>880</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>gp100_{280-288}</td>
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*1- to 10-mg peptide every 3 weeks x 4 doses.
†In vitro sensitized versus modified peptide; tested versus native peptide (58).

### Concluding Comments

The strategy outlined in Table 2 represents an attempt to identify cancer regression antigens in patients with melanoma. The ability to utilize T cells that can cause tumor regression in patients for the in vitro identification of these antigens is a major advantage in attempting to identify those antigens that are clinically relevant. The availability of these genes and gene products has opened new possibilities for the development of cancer vaccines, and initial attempts at clinical trials using a variety of immunization strategies based on these tumor antigen genes have recently begun. Although these studies have thus far concerned only patients with melanoma, techniques to identify tumor antigens are being continually improved. If specific T cells reactive with tumor antigens can be identified, then identification of the genes encoding these antigens becomes increasingly feasible. We have recently demonstrated that CD4+ TILs can be identified from selected patients with breast cancer that are capable of recognizing unique antigens on those tumors (8,60). We and others have also identified both CD4+ or CD8+ cells capable of recognizing unique antigens on autologous ovarian cancers (5,6,67). The use of techniques similar to those described in this paper to identify genes encoding these antigens may allow for the extension of the immunization strategies.

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described in patients with melanoma to patients with a variety of epithelial cancers.

References


Note
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