gp100/pmel 17 Is a Murine Tumor Rejection Antigen: Induction of “Self”-reactive, Tumoricidal T Cells Using High-affinity, Altered Peptide Ligand

By Willem W. Overwijk,* Allan Tsung,* Kari R. Irvine,* Maria R. Parkhurst,* Theresa J. Goletz,‡ Kangla Tsung,§ Miles W. Carroll,‡ Chunlei Liu,* Bernard Moss,‡ Steven A. Rosenberg,* and Nicholas P. Restifo*

From the *Surgery Branch and the ‡Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; the §Department of Surgery, Veterans Administration Medical Center, University of California San Francisco, San Francisco, California 94121; and the †Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Summary

Many tumor-associated antigens are nonmutated, poorly immunogenic tissue differentiation antigens. Their weak immunogenicity may be due to “self”-tolerance. To induce autoreactive T cells, we studied immune responses to gp100/pmel 17, an antigen naturally expressed by both normal melanocytes and melanoma cells. Although a recombinant vaccinia virus (rVV) encoding the mouse homologue of gp100 was nonimmunogenic, immunization of normal C57BL/6 mice with the rVV encoding the human gp100 elicited a specific CD8+ T cell response. These lymphocytes were cross-reactive with mgp100 in vitro and treated established B16 melanoma upon adoptive transfer. To understand the mechanism of the greater immunogenicity of the human version of gp100, we characterized a 9-amino acid (AA) epitope, restricted by H-2Dk, that was recognized by the T cells. The ability to induce specific T cells with human but not mouse gp100 resulted from differences within the major histocompatibility complex (MHC) class I-restricted epitope and not from differences elsewhere in the molecule, as was evidenced by experiments in which mice were immunized with rVV containing minigenes encoding these epitopes. Although the human (hgp10025–33) and mouse (mgp10025–33) epitopes were homologous, differences in the three NH2-terminal AAs resulted in a 2-log increase in the ability of the human peptide to stabilize “empty” Dk on RMA-S cells and a 3-log increase in its ability to trigger interferon γ release by T cells. Thus, the fortuitous existence of a peptide homologue with significantly greater avidity for MHC class I resulted in the generation of self-reactive T cells. High-affinity, altered peptide ligands might be useful in the rational design of recombinant and synthetic vaccines that target tissue differentiation antigens expressed by tumors.

Key words: melanoma • tumor-associated antigen • gp100 • xenoimmunization • CD8+ T lymphocyte

The recent cloning of tumor antigens recognized by T cells has caused considerable interest in the development of antigen-specific cancer vaccines (1–3). Some antigens are especially attractive candidates for use in vaccines due to their shared nature between individuals, including the melanocyte differentiation antigens (MDA)1 gp100, melanoma antigen recognized by T lymphocytes (MART)-1, and tyrosinase (2), as well as several proteins in the MAGE family (1). However, as indicated by results from clinical trials thus far, inducing therapeutic T cells to these antigens has been difficult. One reason for the apparent hyporesponsiveness of the human immune system to many tumor antigens may be that they are normal, nonmutated “self”-proteins, expressed on normal tissues as well as on tumor cells. An incomplete understanding of the processes of central and peripheral tolerance has hampered the development of successful cancer vaccines targeting these autoantigens, limiting the use of the growing number of candidate tumor antigens.
The absence of an immune response to a defined autot antigen can be due to negative selection of self-antigen-specific T cells during maturation in the thymus, termed “central” tolerance (4). A low level of autoreactivity is required for positive selection in the thymus (5, 6).

T cells with low reactivity to autot antigens thus persist. Mature T lymphocytes with reactivity to self-antigens may remain in a functionally tolerant state, termed “ignorance”, if they do not traffic to antigen-bearing cells, or if the target antigen is not processed and presented to a level that can trigger the specific TCR. Mature self-reactive T cells that encounter antigen on normal tissues in the absence of an activating costimulatory microenvironment can be functionally eliminated by anergyization or physically by deletion, thus effecting extrathymic or peripheral tolerance (7, 8).

The mechanisms of breaking tolerance to self-antigens may be relevant for the induction of immune responses to tissue differentiation antigens expressed by tumors. To study requirements for activation of self-reactive, tumor-specific T cells specific for a naturally expressed antigen, we targeted gp100, a normal, nonmutated MDA. In humans, gp100 is expressed both by normal melanocytes and the majority of malignant melanomas tested (9). CD8+ T lymphocytes with reactivity to gp100 have been detected in patients with metastatic melanoma. The mouse homologue for gp100, also known as pmel 17, has been cloned previously (10, 11), and like its human counterpart is normally expressed in melanocytes in an unproduced murine melanoma C57BL/6 mouse as well as in mouse melanomas. We sought to determine the requirements to break T cell tolerance to a naturally expressed self-antigen. Furthermore, we evaluated the functional characteristics of autoreactive T cells in the recognition and destruction of a spontaneous mouse melanoma, B16, in vivo.

Materials and Methods

 Animals and Cell Lines. Female C57BL/6 (H-2b) mice, 6–10 wk old, obtained from Frederick Cancer Research Center (Frederick, MD) and maintained in a barrier facility, were used for all experiments. EL-4 thymoma (H-2k) and the derived β-galactosidase (β-gal)-transfected clone E22 have been described previously (12). B16 (H-2d), hereafter named B16.WT, is a spontaneous murine melanoma expressing gp100, MART-1, tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 by FACS® and Western blot analysis (data not shown). B16.WT-B7-1 is a hyperpigmented clone of B16.WT that was stably transduced using a Moloney mouse leukemia virus encoding the gene for B7-1 driven by a LTR promoter. JB/M/S is a pigmented, chemically induced melanoma expressing gp100, provided by Dr. Vincent Hearing (National Cancer Institute, NIH, Bethesda, MD). 293K and 293Kb of hgp100 DNA have been described previously (15, 16). Cloning of the genes for gp100 and mMART-1/Melan-A has been described previously (11), and the cDNAs for hgp100, mMART-1, and mMART-2 were confirmed by immunostaining (20) as well as by PCR-based viral genome analysis using primers flanking the viral locus encoding the small subunit of ribonucleotide reductase (12). rVVhgp100, rVVmMART-1, rVVmMART-2, rVVmTRP-1, and rVVmTRP-2 were based on the plasmid pS56 (15), containing the completely synthetic early/late promoter pS56, drives expression of the antigen and the early/late promoter p7.5, drives expression of the LacZ gene (15). The rVVSe13-β-gal has been described previously (15, 16). Cloning of the genes for gp100 and mMART-1/Melan-A has been described previously (11), and the cDNAs for hgp100, mMART-1, and mMART-2 were confirmed by Dr. Y. Kawakami (Surgery Branch, NIH; reference 9), Dr. H. Y. Amamoto (Tohoku University, Sendai, Japan; reference 17), Dr. S. Shibahara (Friedrich Miescher Institut, Basel, Switzerland; reference 18), and Dr. V. H. Hearing (Laboratory of Cell Biology, NCI, NIH; reference 19). The rVVhgp100, recombinant fowlpox virus (rFPV)hgp100, rFPVmgp100, and rFPVβ-gal were provided by Therion Biologics Corp. (Boston, MA). Plasmid DNA constructs (pDNA) were based on the pCDNA3 backbone, and encoded hgp100, mgp100, or β-galactosidase under the control of the CMV promoter. Expression of rVV, rFPV, and pDNA were confirmed by immunostaining (20) as well as by Western blot of transfected and infected cells using antiserum (21, 22) provided by Dr. V. H. Hearing. Recombinant adenoviruses were provided by Dr. Bruce Roberts (Genzyme Corp., Framingham, MA), and encoded the genes for hgp100, mgp100, or β-galactosidase under the control of the CMV promoter (11).

Generation of gp100-reactive T Cell Line. Mice were vaccinated twice at 3–wk intervals by a hand-held helium-driven device (Geneva Inc., Middletown, WI) with 1 μg of hgp100 DNA (23), and 3 wk after the second vaccination splenocytes were cultured with rVVhgp100-infected dendritic cells (DCs), generated as previously described (16) in CM with 30 IU/ml rhIL-2 (a gift from Chiron Corp., Emeryville, CA) for 7 d, and were subsequently restimulated every 7–10 d with 2 × 10⁶ B16.B7-1. gp100 is a M urine Tumor R ejection Antigen.
were infected at multiplicities of infection of the manufacturer's protocol. rVV-, rFPV- and rAd-infected targets were prepared using Lipofectamine (GIBCO BRL, Gaithersburg, MD) following manufacturer's guidelines.

Splenocytes from immunized mice were restimulated in vitro using syngeneic DCs infected with appropriate rVV, rFPV, or rAd, and subsequent restimulations were performed in a blinded, randomized fashion.

**Results**

gp100 Is Recognized by Mouse T Cells. To generate gp100-specific T cells, mice were immunized with mgp100 encoded in plasmid DNA or rVV, two vectors with proven efficacy in the generation of specific T cells (3, 23, 24). Splenocytes from immunized mice were restimulated in vitro using syngenic DCs infected with appropriate rVV, rFPV, or rAd, and subsequent restimulations were performed with irradiated splenocytes and irradiated B16.B7-1, a clone of B16 retrovirally transduced to express the costimulatory molecule CD80 (B7-1). None of the cultures exhibited gp100-specific reactivity when tested by IFN-γ release against gp100-positive targets (data not shown). Since xenogeneic antigen can, in some instances, induce immune reactivity where the autologous antigen failed (25–27), mice were immunized with pDNA encoding hgp100.

A CD4+CD8+ lytic T lymphocyte line was generated by gene-gun administration of pDNA encoding hgp100 followed by restimulation of splenocytes ex vivo with DCs that had been infected with rVVhgp100. After two subsequent in vitro restimulations with B16.B7-1 melanoma, the T cells recognized B16 melanoma, as well as the immortalized normal melanocyte line M elan-A, with a high degree of specificity (Fig. 1). The EL-4 thymoma and the M C38 sarcoma were not recognized. However, recognition could be conferred upon 293 K D b cells after infection with rVV encoding either mgp100 or hgp100, but not after infection with rVV containing any of the other known M DAs tyrosinase, TRP-1, or TRP-2.

Antitumor Activity of gp100-specific T Cells. We sought to evaluate the relevance of mgp100 as a tumor rejection antigen. To exclude the potential influence of T cells with a specificity other than gp100, the bulk T cell line was cloned by limiting dilution. 12 clones were evaluated and each had reactivities identical to the bulk T cell line shown in Fig. 1. The gp100-reactive T cell clone (clone no. 9) was tested in vitro (Fig. 2A) and in vivo (Fig. 2B). Mice bearing 3-d-old B16 pulmonary nodules were infused with clone no. 9 followed by rL-2, resulting in a dramatic tumor destruction (P < 0.0001, 4 × 106 β-gal-specific T cells + rhL-2 versus 4 × 106 clone no. 9 T cells + rhL-2), whereas treatment was ineffective using control β-gal-reactive T cells or rL-2 alone (P > 0.5 versus no treatment) (Fig. 2B).

**Figure 1.** B16 melanoma-reactive T cells specifically recognize nonmutated gp100. Splenocytes from hgp100-immunized mice were cultured as described in Materials and Methods, and cocultured for 24 h with various targets shown on the ordinate, including B16 melanoma, the immortalized normal melanocyte line M elan-A, and human 293 kidney cells expressing the mouse restriction elements H-2K b and H-2D b infected with rVV encoding mouse melanocyte differentiation antigens. Supernatants were assayed for IFN-γ by ELISA. Specific IFN-γ release was detected against targets expressing mgp100 or hgp100.
Separate gp100-reactive T cells clones were comparable in their ability to reject B16 in vivo (data not shown). These results suggest that gp100 functions as a true tumor rejection antigen in established murine melanoma.

Identification of a Cross-reactive, MHC Class I-restricted Epitope in gp100. We sought to understand why T cells could only be induced by the xenogeneic, human form of gp100 and not the self sequence. To determine whether the difference in immunogenicity resided in the actual peptides from gp100 that were recognized by T cells, or in differences in sequences outside of those recognized, we characterized the epitopes recognized by the T cell clone. To identify the MHC class I molecule that restricted gp100 recognition, we used the human renal cell carcinoma line, 293, stably transfected with the mouse restriction elements, K\(^b\) and D\(^b\). Both cell lines were transiently transfected with plasmids encoding either mouse or human gp100, or with a control plasmid encoding NP. Only 293 K\(^b\)D\(^b\) cells (and not 293 K\(^b\)) cells expressing mgp100 or hgp100 triggered the release of IFN-\(\gamma\) from gp100-reactive T cells, suggesting that D\(^b\) was the dominant and perhaps only restriction element for gp100 recognition (Fig. 3A).

At 626 AAs in length, many possible epitopes in gp100 could be recognized by gp100-reactive T cells. To reduce the number of candidate peptides, a set of progressively shorter versions of the gene encoding hgp100 was used (28). These shortened forms were generated by exonuclease digestion or PCR amplification of the original hgp100 cDNA. Fragments were then transfected into 293 K\(^b\)D\(^b\) cells that were used as targets for T cell recognition (Fig. 3B). Even the shortest fragment of the hgp100 cDNA, with a length of 300 bp by gel electrophoresis, conferred a high degree of recognition upon the 293 transfectants, implying that a major epitope was located in the first 100 AAs of the molecule.

To identify the sequence of the epitope, 9-AA-long peptides were evaluated for their potential to bind to D\(^b\) using a computer-generated epitope forecast, based on previously published peptide binding data, that is designed to predict binding affinity for a variety of human and mouse MHC class I alleles (29, 30; and http://bimas.dcmrt.nih.gov/molbio/hla_bind). Based on these predictions, sets of synthetic peptides were made from both hgp100 and mgp100, which although similar are not identical. When the peptides were added to cultures of gp100-reactive T cells, one peptide pair, gp100\(^{25–33}\), was recognized with a high degree of specificity in a GM-CSF release assay (Fig. 3C).

T Cell Reactivity to gp100 Can Be Induced by Xenoimmunization. With the identification of a defined peptide epitope, we explored the immunological mechanism underlying the disparate ability of mgp100 and hgp100 to induce gp100-specific T cells. Mice were vaccinated with rVVmgp100, encoding mgp100, or rVVhgp100, encoding hgp100, and splenocytes were isolated after 3 wk and stimulated for 6 d in vitro with synthetic peptides corresponding to hgp100\(^{25–33}\), mgp100\(^{25–33}\), or OVA\(^{257–264}\) and then tested in a GM-CSF release assay (Table 1, first two columns). Splenocytes from
Mice were vaccinated with rVV mgp100, rVVEShgp100, and rVVES mgp100, and restimulated with either mgp100 or hgp100 peptides. These results were not consistent with a major role for differential peptide processing and transport. Instead, the immunodominance of the mouse minigenes gp100 construct was found to be similar to that of the full-length gp100 molecule. The immunogenicity of hgp100 was found to be higher than that of mgp100, which was consistent with the higher degree of recognition observed for hgp100-reactive T cells (Fig. 5). Indeed, a triple-substituted peptide, AAARNQDWL, fully retained recognition by gp100-reactive T cells (Fig. 5). This result suggests that the immunodominance of hgp100 is intrinsic to the MHC Class I–peptide interactions and the immunogenicity of gp100.

To evaluate the relative avidity of the gp100-specific T cells to the mgp100 and hgp100 peptides, we pulsed the peptides onto EL-4 cells (H-2b) (Fig. 4). A striking difference in the relative T cell activities to the mgp100 and hgp100 peptides was observed. The mgp100 peptide was recognized at concentrations as low as $10^{-9} \text{M}$, with half-maximal recognition occurring at about $10^{-8} \text{M}$, whereas the hgp100 peptide was recognized at concentrations as low as $10^{-15} \text{M}$. Alanine substitutions at positions 1, 2, or 3 in either the mouse or human versions of the synthetic peptides did not substantially alter recognition by gp100-reactive T cells (Fig. 5A). Indeed, a triple-substituted peptide, AAARNQDWL, fully retained the ability to sensitize EL-4 for recognition (Fig. 5B). The first three AAs could even be deleted and a substantial degree of recognition remained (Fig. 5B). However, substitution of any of the AAs at positions 4 through 9 abrogated recognition of both the hgp100 and mgp100 peptides.

T cells recognizing mgp100 could be induced exclusively by xenoinmunization with the hgp100 molecule. Increased immunogenicity of hgp100 is intrinsic to the MHC Class I–restricted epitope. Several mechanisms could account for the apparent immunological unresponsiveness to mgp100 and the ability of hgp100 to break it. Nongenetic regions of the full-length hgp100 (which is 76% identical to mgp100 at the AA level; reference 11) could result in intramolecular epitope-spreading (31, 32) or facilitate antibody-mediated antigen capture by APCs (26, 33–35). Alternatively, sequence differences in the relevant epitopes or their flanking sequences could result in differential proteolytic cleavage or transporter associated with antigen processing (TAP)–mediated transport across the endoplasmic reticulum (ER) membrane (36). To explore these possibilities, we constructed a series of rVV-containing minigenes encoding the relevant 9-AA T cell epitopes preceded by the E3/19K adenoviral ER-insertion signal sequence (ES), previously shown to result in TAP-independent transport of antigenic peptides (37), and followed by a double stop codon. These constructs eliminated differences in flanking sequences and other nonhomologous regions of the molecule.

Mice were vaccinated with rVV mgp100, rVVEShgp100, or rVVES mgp100, and restimulated with either mgp100 or hgp100 peptides. These results were not consistent with a major role for differential peptide processing and transport. Instead, the immunodominance of the mouse minigenes gp100 construct was found to be similar to that of the full-length gp100 molecule. The immunogenicity of hgp100 was found to be higher than that of mgp100, which was consistent with the higher degree of recognition observed for hgp100-reactive T cells (Fig. 5). Indeed, a triple-substituted peptide, AAARNQDWL, fully retained recognition by gp100-reactive T cells (Fig. 5). This result suggests that the immunodominance of hgp100 is intrinsic to the MHC Class I–peptide interactions and the immunogenicity of gp100.

To evaluate the relative avidity of the gp100-specific T cells to the mgp100 and hgp100 peptides, we pulsed the peptides onto EL-4 cells (H-2b) (Fig. 4). A striking difference in the relative T cell activities to the mgp100 and hgp100 peptides was observed. The mgp100 peptide was recognized at concentrations as low as $10^{-9} \text{M}$, with half-maximal recognition occurring at about $10^{-8} \text{M}$, whereas the hgp100 peptide was recognized at concentrations as low as $10^{-15} \text{M}$. Alanine substitutions at positions 1, 2, or 3 in either the mouse or human versions of the synthetic peptides did not substantially alter recognition by gp100-reactive T cells (Fig. 5A). Indeed, a triple-substituted peptide, AAARNQDWL, fully retained the ability to sensitize EL-4 for recognition (Fig. 5B). The first three AAs could even be deleted and a substantial degree of recognition remained (Fig. 5B). However, substitution of any of the AAs at positions 4 through 9 abrogated recognition of both the hgp100 and mgp100 peptides.

T cells recognizing mgp100 could be induced exclusively by xenoinmunization with the hgp100 molecule. Increased immunogenicity of hgp100 is intrinsic to the MHC Class I–restricted epitope. Several mechanisms could account for the apparent immunological unresponsiveness to mgp100 and the ability of hgp100 to break it. Nongenetic regions of the full-length hgp100 (which is 76% identical to mgp100 at the AA level; reference 11) could result in intramolecular epitope-spreading (31, 32) or facilitate antibody-mediated antigen capture by APCs (26, 33–35). Alternatively, sequence differences in the relevant epitopes or their flanking sequences could result in differential proteolytic cleavage or transporter associated with antigen processing (TAP)–mediated transport across the endoplasmic reticulum (ER) membrane (36). To explore these possibilities, we constructed a series of rVV-containing minigenes encoding the relevant 9-AA T cell epitopes preceded by the E3/19K adenoviral ER-insertion signal sequence (ES), previously shown to result in TAP-independent transport of antigenic peptides (37), and followed by a double stop codon. These constructs eliminated differences in flanking sequences and other nonhomologous regions of the molecule.

Mice were vaccinated with rVV mgp100, rVVEShgp100, or rVVES mgp100, and restimulated with either mgp100 or hgp100 peptides. These results were not consistent with a major role for differential peptide processing and transport. Instead, the immunodominance of the mouse minigenes gp100 construct was found to be similar to that of the full-length gp100 molecule. The immunogenicity of hgp100 was found to be higher than that of mgp100, which was consistent with the higher degree of recognition observed for hgp100-reactive T cells (Fig. 5). Indeed, a triple-substituted peptide, AAARNQDWL, fully retained recognition by gp100-reactive T cells (Fig. 5). This result suggests that the immunodominance of hgp100 is intrinsic to the MHC Class I–peptide interactions and the immunogenicity of gp100.

To evaluate the relative avidity of the gp100-specific T cells to the mgp100 and hgp100 peptides, we pulsed the peptides onto EL-4 cells (H-2b) (Fig. 4). A striking difference in the relative T cell activities to the mgp100 and hgp100 peptides was observed. The mgp100 peptide was recognized at concentrations as low as $10^{-9} \text{M}$, with half-maximal recognition occurring at about $10^{-8} \text{M}$, whereas the hgp100 peptide was recognized at concentrations as low as $10^{-15} \text{M}$. Alanine substitutions at positions 1, 2, or 3 in either the mouse or human versions of the synthetic peptides did not substantially alter recognition by gp100-reactive T cells (Fig. 5A). Indeed, a triple-substituted peptide, AAARNQDWL, fully retained the ability to sensitize EL-4 for recognition (Fig. 5B). The first three AAs could even be deleted and a substantial degree of recognition remained (Fig. 5B). However, substitution of any of the AAs at positions 4 through 9 abrogated recognition of both the hgp100 and mgp100 peptides.
tide variants. To determine the involvement of MHC class I binding affinity, an MHC class I stabilization assay was done on RMA/S cells, which lack activity of the TAP transporters (13). FACS analysis revealed 50% stabilization of Db by hgp10025–33 peptide at a concentration 100-fold lower than for mgp10025–33 (Fig. 6). This indicated that the apparent avidity of gp100-reactive T cells for the hgp10025–33 peptide could be largely attributed to its greater ability to stabilize the restricting MHC class I molecule, H-2Db.

Self-reactive T Cells Induced with Altered Antigen Function In Vivo. One major potential use for self-reactive T cells is their application in cancer treatment. To evaluate the usefulness of altered antigen in the development of a synthetic cancer vaccine capable of eliciting therapeutic T cells, mice were vaccinated once with rVVhgp100 and restimulated for three rounds with splenocytes pulsed with 1 μM mgp10025–33 peptide. CD8+ T cells were tested for gp100 recognition by IFN-γ release (Fig. 7 A). T cells strongly recognized both mgp10025–33 and hgp10025–33 peptides, but not control OVA 257–264 peptide, and recognized as well melanoma B16.WT, B16.B7-1, and JB/MS, a chemically induced C57BL/6 melanoma. Control H-2b, gp100 tumor lines were not recognized.

The function of T lymphocytes generated using purely recombinant and synthetic forms of gp100 were evaluated by adoptive transfer of T cells to mice bearing tumors established for 3 d in vivo. Mice receiving mgp10025–33-specific T cells experienced a significant reduction in tumor burden compared with mice receiving control T cells (Fig. 7 B). The survival of these cells was evaluated in vivo by transfer 5 d before tumor challenge, with T cells retaining their antitumor activity for this period (Fig. 7 C). This indicated that tumor-specific T cells induced with an antigen-based cancer vaccine can survive in vivo and mediate efficient tumor destruction.

Discussion

The nonhomologous sequences flanking a MHC-restricted epitope could influence the immunogenicity of the epitope through a variety of mechanisms. The full-length hgp100 and mgp100 molecules are 76% identical at the AA level (11). Xenoimmunization could induce antibodies to nonhomologous determinants on the xenoantigen. When expressed on the surface of B cells that produce them, these antibodies could capture the xenoantigen and make it available for B cell processing and presentation on MHC class II to activate CD4+ T cells (35). This mechanism has been postulated to play a role in the initiation of human autoimmune diseases such as SLE, which is largely mediated by CD4+ T cells and autoreactive antibodies (25, 34). Recent data suggest that B cells may cross-present antigen on MHC class I after capture (39). However, there is a vig-

Table 1. IFN-γ Release by Peptide-stimulated Splenocytes from gp100-immunized Mice

<table>
<thead>
<tr>
<th>Immunization</th>
<th>rVVhgp100</th>
<th>rVVmgp100</th>
<th>rVVEShgp10025–33</th>
<th>rVVESmgp10025–33</th>
<th>Naive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide targets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N one</td>
<td>264</td>
<td>584</td>
<td>236</td>
<td>137</td>
<td>0</td>
</tr>
<tr>
<td>hgp</td>
<td>15,120</td>
<td>20,751</td>
<td>250</td>
<td>137</td>
<td>1</td>
</tr>
<tr>
<td>mgp</td>
<td>2,567</td>
<td>19,307</td>
<td>257</td>
<td>94</td>
<td>58</td>
</tr>
<tr>
<td>OVA</td>
<td>165</td>
<td>343</td>
<td>144</td>
<td>137</td>
<td>37</td>
</tr>
</tbody>
</table>

Mice were vaccinated with indicated rVVs, and splenocytes were isolated 3 wk later and restimulated for 6 d with 1 μg/ml peptide. Subsequent stimulation of cultured cells with hgp10025–33 or mgp10025–33 peptide resulted in IFN-γ release only by splenocytes from mice immunized with rVVhgp100 or rVVEShgp10025–33. hgp, hgp100 AA 25–33 (KVPRNQDWL); mgp, mgp100 AA 25–33 (EGSRNQDWL); OVA, ovalbumin, AA 257–264 (SIINFEKL). Four repeat experiments yielded similar results; numbers indicate IFN-γ (pg/ml) secreted by 2 × 10⁶ CTLs in 24 h; numbers in bold indicate secretion 3-fold over control peptide.
orous debate over the ability of B cells to activate "virgin" T cells (i.e., T cells that have not previously been activated by antigen) (33, 40). DCs can also capture immune complexes containing xenoantigen through Fc receptors and present it through MHC class I and class II pathways, inducing de novo activation of autoreactive T cells (41). In this scenario, the completely autologous mgp100 would not induce such antibodies and thus would fail to be captured and presented. The involvement of extra-epitope sequences in the immunogenicity of the human gp100 molecules is not consistent with the results shown in Table 1 where the immunogenicity of human full-length and minimal determinant constructs are compared. In fact, the two constructs elicit comparable CD8+ T cell responses to the 25–33 epitope.

Another mechanism by which xenoimmunization could enhance immunogenicity is by the processing of a given epitope. For example, the hgp10025–33 peptide might be processed more efficiently than the mgp10025–33 peptide, since the two differ in the three NH2-terminal AAs, as well as in the AA sequences surrounding the peptides in the full-length molecule (36, 42). The differences between the processing of the human and mouse sequences is minimized and possibly eliminated by the use of minigene constructs preceded by ER-insertion signal sequences that bypass proteasome-mediated peptide liberation as well as TAP-mediated peptide transport (Table 1).

Indeed, the increased immunogenicity of human gp100 appeared to reside completely within the 9-mer peptide. The hgp10025–33 peptide differed from its mouse counterpart in three NH2-terminal AAs. In fact, these three differences were rather dramatic, with a positively charged lysine (K) replacing a negatively charged glutamic acid (E), a me-
dium-sized valine (V) replacing a small glycine (G), and proline (P), which is a cyclic residue and known to reduce the number of possible conformations due to impaired hydrogen bonding, replacing serine (S), which may not induce such a structural distortion. Despite the presence in both peptides of optimal anchor residues at both dominant anchor positions, 5 (N) and 9 (L), the difference in the three NH2-terminal residues resulted in a dramatically increased affinity of the hgp100 25–33 peptide for the mouse MHC class I allele, H-2Db, compared with the mgp100 25–33 peptide (Fig. 6). Clearly, the ability of the peptide to stabilize molecules on the surface of RMA-S cells could reflect the amount of peptide presented on the surface of APCs, which in turn could determine the activation of T cell precursors. In retrospect, it was fortuitous that the interspecies differences in the gp100 sequences created such a high binder, whereas the similarities preserved sufficient T cell receptor contact residues to allow cross-recognition.

The efficacy of the mechanism described here through which xenoimmunization induced autoreactive T cells required that a number of criteria be satisfied. There must be an MHC-binding epitope in the autologous protein which is naturally processed and presented that can be recognized by the available T cell repertoire. Most importantly, a homologous epitope from the xenogeneic protein must also be naturally processed and presented, but must be presented in the context of MHC at a higher density on the surface of the APCs. The chance that such an epitope will be found in any given xenoantigen may be small. However, an understanding of this mechanism clearly points the way to the rational design of immunogens based on the enhancement of the stability of peptide-MHC complexes.

The data presented here suggest that the main difference between the mgp100 25–33 peptide and its hgp100 25–33 analogue resides in binding to MHC class I. In human in vitro studies, modification of the second AA of the hgp100 pep-
tide epitope hgp100<sub>209–217</sub> to methionine has been shown to significantly increase its affinity to HLA-A2, leading to dramatically increased ability to raise gp100-specific T cells from patient PBLs in vitro (43). The higher affinity peptide is also more effective in vaccinating patients in vivo, increasing gp100-specific T cell precursor levels, and possibly resulting in higher treatment response rates (43a). Similarly, other groups have reported increased immunogenicity of peptide epitopes with enhanced MHC class I binding (26, 44, 45).

Taken together, these data suggest that there is indeed unresponsiveness to gp100 in mice. However, the unresponsiveness is relative and can be broken by using a peptide homologue with higher affinity for MHC class I. One mechanism through which a peptide with higher MHC class I binding can break tolerance is based on the assumption that T cell tolerance exists to a level of antigen rather than to the identity of the antigen (44, 46, 47). In the case of gp100, CD8<sup>+</sup> T cell precursors with the ability to recognize a certain amount of peptide in the context of MHC class I are inactivated, through either thymic and/or peripheral deletion or anergization. The remaining T cells have TCRs with an affinity that is too low to be triggered by the levels of gp100 peptide present on melanocytes or APCs in lymph nodes draining the skin. Therefore, these T cells are never activated, deleted, or anergized. Instead, they remain “ignorant” of MHC class I with mgp100<sub>25–33</sub> peptide. A vaccination with autologous mgp100 will not trigger these T cells unless the vaccine is able to significantly raise the amount of peptide–MHC class I complex on professional APCs to a level high enough to surpass the TCR threshold. Only then do the T cells become activated. We are currently evaluating the clinical efficacy of antitumor effects of gp100-based cancer vaccines that contain epitopes with enhanced stability of peptide–MHC complexes.

The authors wish to thank Deborah R. Surman and Mr. Paul J. Spiess for expert technical assistance; Messrs. Martha Blalock for assistance with graphics; Drs. Dennis Piancati and Linda Gritz (Therion Biologics) for providing rVVs; and Dr. Bruce Roberts for providing recombinant adenoviruses.

Correspondence to Nicholas P. Restifo, Senior Investigator, National Cancer Institute, Bldg. 10, Rm. 2842, Bethesda, MD 20892-1502. Phone: 301-496-4904; Fax: 301-496-0011; E-mail: restifo@nih.gov

Received for publication 17 March 1998.

References


Published July 20, 1998

Downloaded from on April 9, 2017

286 gp100 Is a Murine Tumor Rejection Antigen