Identification of Helper T-Cell Epitopes That Encompass or Lie Proximal to Cytotoxic T-Cell Epitopes in the gp100 Melanoma Tumor Antigen

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ABSTRACT

The melanocyte-associated antigen gp100 constitutes one of the most attractive targets for T-cell-based immunotherapy against malignant melanoma. Although several MHC class I-restricted epitopes have been identified for CTLs, thus far, only one MHC class II helper epitope (restricted by HLA-DR4) has been described in the literature. Using an algorithm to identify promiscuous helper T-cell epitopes, here we describe three additional MHC class II-restricted epitopes from gp100. Whereas one T helper epitope, gp100<sub>175–189</sub>, was restricted by the HLA-DR53 and DQ<sub>6</sub> alleles, the T-cell responses to two other epitopes, gp100<sub>54–68</sub> and gp100<sub>576–590</sub>, were restricted by HLA-DR7. Most interestingly, the newly identified helper T lymphocyte epitopes encompass or lie proximal to previously described CTL epitopes for this tumor-associated antigen. Together with the previously described HLA-DR4-restricted epitope, these T helper epitopes offer coverage for the majority of the human population. Moreover, the use of peptide vaccines containing both CTLs and T helper epitopes could offer therapeutic advantages over current approaches that focus solely on eliciting antitumor CTL responses.

INTRODUCTION

Because HTLs<sup>3</sup> play an essential role in the generation and maintenance of CTLs, the design of effective vaccines for cancer should attempt the stimulation of antigen-specific immunity in both of these types of T cells. For some time, many groups have been actively identifying CTL epitopes for various TAAs, and synthetic peptides representing these epitopes have been used to prepare therapeutic vaccines to treat cancer patients (1–5). However, the therapeutic effects of these vaccines are far from optimal. One of the reasons that could account for the lack of success of these vaccines may be their inability to elicit tumor-specific HTL responses, which may be critical for the induction and persistence of antitumor CTLs (6–8). In view of this, much attention has been recently devoted to the identification of peptide epitopes that trigger antitumor responses in HTLs that could be used for the improvement of vaccines designed to induce antitumor CTLs (9). As a result of these efforts, several HTL epitopes for TAAs such as MAGE-A3, p53, HER2/neu, NY-ESO-1, Melan-A/MART-1 and tyrosinase have been described (10–20). Unfortunately, in many instances, HTL peptide epitopes may be of limited use for the general population because they are restricted to specific MHC class II alleles. Nonetheless, in some cases, promiscuous HTL epitopes capable of binding to several MHC class II alleles have been identified, which broadens their therapeutic use (13, 14, 20).

Several TAAs constitute possible targets for developing T-cell immunotherapy against malignant melanoma (9, 21). Of these, the melanocyte-related antigen gp100 is probably the most studied and is currently being evaluated in the clinic (4, 22). Numerous CTL epitopes restricted by the MHC class I alleles HLA-A2, HLA-A3, HLA-A11, HLA-A24, and HLA-Cw8 have been reported (22–29). On the other hand, only one HTL epitope, which is restricted by the HLA-DR*0401 and HLA-DRB1*0401 MHC class II allele expressed in approximately one-fourth of the population, has been described (30, 31). Thus, to extend population coverage for melanoma patients, it becomes necessary to identify additional HTL epitopes, preferably of the promiscuous MHC class II type.

In the present study, we used a computer-based algorithm to identify peptide sequences from gp100 with potential promiscuous MHC class II binding characteristics (32). Three synthetic peptides corresponding to potential promiscuous HTL epitopes were selected, synthesized, and tested for their capacity to stimulate gp100-specific CD4<sup>+</sup> T cells in vitro using blood cells from healthy volunteers. The results show that three of the predicted epitopes were able to trigger HTL responses in individuals expressing diverse HLA-DR alleles. The T-cell response to one of these peptides, gp100<sub>175–189</sub>, was found to be restricted by either the HLA-DR53 or the DQ<sub>6</sub> alleles, and the responses to the other peptide epitopes (gp100<sub>54–68</sub> and gp100<sub>576–590</sub>) were restricted by HLA-DR7. In all cases, the positions of the newly identified HTL epitopes in the gp100 sequence were found to lie within or close to previously described CTL epitopes. Some of the therapeutic implications of the physical proximity between HTL and CTL epitopes are addressed in the “Discussion.”

MATERIALS AND METHODS

Cell Lines. Melanoma tumor lines 697mel (gp100<sup>+</sup>, DR<sub>4</sub>/15, DR51/53, DQ<sub>6</sub>), 624mel (gp100<sup>+</sup>, DR<sub>4</sub>/7, DR53, DQ<sub>2</sub>), 888mel (gp100<sup>+</sup>, DR<sub>15</sub>/16, DR51, DQ<sub>5</sub>/6), and 1102mel (gp100<sup>+</sup>, DR<sub>4</sub>/15, DR51/53, DQ<sub>6</sub>) were kindly provided by Drs. Y. Kawakami and S. Topalian (Surgery Branch, National Cancer Institute, NIH, Bethesda, MD). The melanoma lines HT-144 (gp100<sup>+</sup>, DR<sub>4</sub>/7, DR53, DQ<sub>8</sub>) and SKmel-28 (gp100<sup>+</sup>, DR4, DR53, DQ<sub>8</sub>) and the Jurkat T-cell lymphoma (gp100<sup>+</sup>–, DR<sup>+</sup> and DQ<sup>+</sup>) were purchased from the American Type Culture Collection (Manassas, VA). EBV-LCLs were produced from PBMCs of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection). Mouse fibroblast cell lines (L cells) transfected and expressing individual human HLA class II molecules were kindly provided by R. W. Karr (Park-Davis, Ann Arbor, MI).

Synthetic Peptides and Algorithm Analysis. Potential MHC class II promiscuous helper T-cell epitopes were predicted from the amino acid sequence of the gp100 antigen using the algorithm tables for three HLA-DR alleles (DBR<sub>1</sub>*0101, DBR<sub>1</sub>*0401, and DBR<sub>1</sub>*0701) published by Southwood et al. (32). A computer program based on Microsoft Excel was fashioned for the three HLA-DR alleles. For each position of a 9-mer amino acid sequence-binding core, a value was assigned for each possible specific amino acid using the tables published by Southwood et al. (32). The computer program calculated the ARB values for each possible 9-mer of the gp100 sequence. The rationale for this approach is that the higher the ARB value of a peptide, the higher the probability that the peptide will bind to the corresponding HLA-DR allele. Peptides that displayed high ARB values and were proximal to known CTL epitopes were synthesized and purified as described. The purity (>95%) and identity of peptides were determined by high-performance liquid chromatography and mass spectrometry.

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<sup>2</sup>The abbreviations used are: HTL, helper T lymphocyte; TAA, tumor-associated antigen; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; APC, antigen-presenting cell; MR<sub>max</sub>, 50% maximal response; EBV-LCL, EBV-transformed lymphoblastoid cell; ARB, average relative binding; GM-CSF, granulocyte macrophage colony-stimulating factor; Mab, monoclonal antibody.

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**In Vitro Induction of Antigen-specific HTLs Using Synthetic Peptides.** The method used for generating tumor antigen-reactive HTL lines and clones using peptide-stimulated PBMCs has been described in detail previously (13, 14). Briefly, DCs were generated in tissue culture from adherent monocytes that were cultured for 7 days in the presence of GM-CSF and interleukin 4. A total of 1 x 10^6 peptide-pulsed DCs (10 μg/ml for 2 h) were irradiated (4200 rad) and cocultured with 3 x 10^6 autologous purified CD4+ T cells (using antibody-coated magnetic beads from Miltenyi Biotec) in each well of a 96-well round-bottomed culture plate. Seven days later, the cultures were restimulated with peptide-pulsed irradiated autologous PBMCs, and 2 days later, human recombinant interleukin 2 was added at a final concentration of 10 IU/ml. After 7 days, the microcultures were tested for their proliferative response to peptides as described below. Those microwells showing a proliferative response to peptide of at least 2.5-fold over background were expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous PBMCs. In some instances, T-cell lines were cloned by limiting dilution and used for additional studies. Culture medium for all procedures consisted of RPMI 1640 supplemented with 5% human male AB serum, 0.1 mM MEM, nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 μg/ml gentamicin. The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all volunteers.

**Antigen-specific Proliferative Response of T Cells.** T cells (3 x 10^4 cells/well) were mixed with irradiated APCs in the presence of various concentrations of antigen (peptides, tumors, or tumor lysates) in 96-well culture plates. APCs consisted of either PBMCs (1 x 10^5 cells/well), HLA-DR-expressing L cells (3 x 10^5 cells/well), or EBV-LCLs or melanoma tumor cells (3 x 10^5 cells/well) pretreated with IFN-γ (500 units/ml for 48 h) to enhance MHC expression. Tumor cell lysates were prepared by three freeze/thaw cycles of 1 x 10^6 tumor cells resuspended in 1 ml of serum-free RPMI 1640. Lysates were used as a source of antigen at 5 x 10^5 cell equivalents/ml. Cell proliferation assays were incubated at 37°C in a 5% CO_2 incubator for 72 h, and during the last 16 h, each well was pulsed with 0.5 μCi [3H]thymidine (Amersham Pharmacia Biotec, Piscataway, NJ). In some cases, culture supernatants were collected before the addition of [3H]thymidine for the demonstration of lymphokine production using ELISA kits (PharMingen, San Diego, CA). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a scintillation counter. The percentage of cytotoxicity was calculated by the ratio of cpm obtained in the absence of T cells:cpm obtained in the presence of T cells:cpm obtained in the presence of different amounts of T cells x 100. All determinations were done in triplicate.

**RESULTS**

**Selection of Potential HTL Epitopes for gp100.** Our initial goal was to identify promiscuous MHC class II HTL epitopes from gp100 that could be used to improve T-cell therapy for melanoma. Thus, we analyzed the amino acid sequence of the product of the gp100 gene for the presence of peptide sequences containing binding motifs for DRB1*0101, DRB1*0401, and DRB1*0701 using the algorithm tables described by Southwood et al. (32). These algorithms calculate the potential (predicted) binding interactions of primary and secondary anchors of a 9-amino acid core region with each MHC allele. Peptide sequences were ranked according to their ARB scores, and rank numbers for the three alleles were added. To identify promiscuous MHC class II binding peptides, we selected the top 10 ranking peptides bearing the lowest rank sum value, which in theory should be inversely proportional to the probability of a particular peptide to bind to all three alleles. The data in Table 1 show the sequences, the ARB values for each allele, and the ranks and rank sum values for the top 10 candidate peptides. When examining the position that these peptides occupy within the gp100 molecule, it became evident that several of these peptides were proximal to or overlapped with known MHC class I-restricted CTL epitopes (Table 1). Because it would be attractive to use relatively small peptides (<25 residues) containing both CTL and HTL epitopes as therapeutic vaccines, we decided to focus on those peptide sequences that were proximal to the known CTL epitopes (marked with an asterisk in Table 1). Another factor that we took into account to narrow down the number of peptide candi-

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**Table 1 Peptides predicted to be promiscuous HTL epitopes**

<table>
<thead>
<tr>
<th>Sequence position (9-mer core)</th>
<th>Position of 1st core residue</th>
<th>DR1 rank</th>
<th>DR1 rank sum</th>
<th>DR4 rank</th>
<th>DR4 rank sum</th>
<th>DR7 rank</th>
<th>DR7 rank sum</th>
<th>Rank sum</th>
<th>HTL peptide</th>
<th>Peptide sequence</th>
<th>Adjoining CTL epitope(s)</th>
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<tbody>
<tr>
<td>MSTPVEATGM</td>
<td>392</td>
<td>399.59</td>
<td>5</td>
<td>44.61</td>
<td>270.83</td>
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<td>29</td>
<td>9</td>
<td>gp100^a^490-401</td>
<td>LAEMSTPVEATGM</td>
<td></td>
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<tr>
<td>ISTAVPQMA</td>
<td>359</td>
<td>36.35</td>
<td>36.35</td>
<td>25</td>
<td>250.38</td>
<td>14</td>
<td>1</td>
<td>29</td>
<td>gp100^b^56-570</td>
<td>TVETSTAVPQMA</td>
<td></td>
</tr>
<tr>
<td>VSTQLIMPG</td>
<td>580</td>
<td>85.89</td>
<td>15</td>
<td>23.81</td>
<td>21</td>
<td>22.86</td>
<td>14</td>
<td>30</td>
<td>gp100^c^76-790</td>
<td>SLAVVSTQLIMPG</td>
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<td>LVLAVMVALA</td>
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<td>23.68</td>
<td>14.81</td>
<td>41.48</td>
<td>21</td>
<td>33</td>
<td>gp100^d^61-615</td>
<td>GILLLVLMAXVLANL</td>
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<td>22.86</td>
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<tr>
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<td>4.42</td>
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<td>26.86</td>
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<td>1</td>
<td>58</td>
<td>gp100^j^380-384</td>
<td>KHRLEQNQDPTLA</td>
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*Rank sum = DR1 rank + DR4 rank + DR7 rank.

Peptide sequences in italic were selected for synthesis for T-cell studies. Peptides marked with an asterisk are situated near a known CTL epitope. Underlined residues correspond to the 9-mer core sequence.

Subdominant CTL epitopes restricted by HLA-A2 identified by Tsai et al. (24).

CTL epitope restricted by HLA-A3 identified by Kawakami et al. (27).

CTL epitope restricted by HLA-A2 identified by Kawakami et al. (27).

HLA epitope restricted by HLA-A2 identified by Castelli et al. (27).

CTL epitope restricted by HLA-A3 identified by Kawashima et al. (27).

CTL epitope restricted by HLA-A2 identified by Kawakami et al. (27) and by Cox et al. (23).
dates was the potential problem that these peptides could pose regarding their synthesis, purification, solubility, and stability, all which could be important for developing a suitable vaccine. With these parameters in mind, we decided for the time being to eliminate peptides gp100175–189 (which contains a C residue that could form disulfide bonds) and gp100515–615 (which is extremely hydrophobic) from our T-cell epitope analysis. We thus selected three peptides (shown in italics in Table 1) to evaluate whether they could induce antigen-specific HTL responses after in vitro immunization.

T-Cell Responses to Promiscuous MHC Class II-binding Candidates from gp100. The three peptides that were selected using the above-mentioned criteria were tested for their capacity to stimulate CD4+ T cells obtained from four healthy, MHC-typed individuals in primary in vitro cultures using peptide-pulsed autologous DCs as APCs. All three peptides were effective in inducing proliferative T-cell responses in at least two blood donors. Peptides gp10074–89 and gp100576–590 induced antigen-specific responses in CD4+ T cells that were restricted by the HLA-DR7 DRB1*0701 allele (Fig. 1). In both cases, the T cells responded to peptide presented by autologous PBMCs in a dose-dependent manner (Fig. 1, a and b). However, the affinity of T-cell response to peptide gp100576–590 appeared to be higher (~15-fold) than the response to peptide gp10074–89 as determined by the amount of peptide required to achieve 50% of maximal proliferative response. The responses of both T-cell lines were restricted by HLA-DR7 molecules as determined by the capacity of DR7-transfected L cells to present peptide to the T cells and by the capacity of anti-DR antibodies (but not anti-DQ antibodies) to block the T-cell proliferative responses (Fig. 1, c and d). T-cell responses to an irrelevant DR7-binding peptide derived from the MAGE-A3 antigen (30) were not observed (data not shown), indicating that these T-cell lines were highly specific to the gp100 peptides. Two separate T-cell clones reactive with peptide gp100175–189 were isolated from two normal individuals and studied in more detail for their proliferative response to antigen. The data presented in Fig. 2 shows that both of the T-cell clones recognized peptide in a dose-dependent manner with similar levels of affinity (Fig. 2, a and b). Interestingly, studies performed to determine the HLA restriction elements for these clones indicated that in one case, the response to peptide gp100175–189 was restricted by HLA-DR53 (Fig. 2c), and in the other case, the response to peptide gp100175–189 was restricted by HLA-DQw6 (Fig. 2d).

We attempted on various occasions to induce T-cell responses restricted by HLA-DR1 (DRB1*0101) and HLA-DR4 (DRB1*0401) with peptides gp10074–89, gp100576–590, and gp100175–189. Although these experiments were performed using PBMCs from two separate donors, we were unable to isolate any T-cell line (or clone) that recognized these peptides in the context of these MHC class II alleles (data not shown).

Recognition of Processed Antigen by Peptide-reactive HTLs. The data presented above demonstrate that all three candidate peptides from gp100 were indeed capable of inducing CD4+ T-cell responses. Nonetheless, it is critical to determine whether these peptides represent true T-cell epitopes that would be relevant for the development of tumor immunotherapy. Thus, it became important to determine whether APCs that naturally capture and process the gp100 antigen (which bears the putative T-cell epitopes) were capable of stimulating the peptide-reactive T cells. It would be more significant to assess whether APCs could capture antigen derived from dead tumor cells (i.e., freeze/thaw cell lysates) expressing gp100 and process this antigen appropriately to stimulate HTLs. In addition, some melanoma tumor cell lines are known to express surface MHC class II molecules, which may present naturally processed peptides to HTLs. The results presented in Fig. 3a demonstrate that the HLA-DR7-restricted T-cell line (10B1) induced with peptide gp10074–89 was efficient at recog-

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Fig. 1. HLA-DR7-restricted T-cell proliferative responses to gp100 peptides. HTL lines were selected by weekly stimulation using peptides gp10074–89 (a and c) and gp100576–590 (b and d). Peptide dose-response curves were created to estimate the overall avidity of the HTLs for their respective antigens (a and b). Dashed lines represent the peptide concentration required to obtain 50% of the maximal proliferation (~0.3 μM for peptide gp10074–89 and ~0.02 μM for peptide gp100576–590). MHC restriction analyses show that these HTLs recognize their respective peptides (tested at 3 μg/ml) in the context of the HLA-DR7 allele (c and d). In both cases, the T-cell proliferative responses to peptide presented by autologous PBMCs were significantly inhibited by monoclonal anti-HLA-DR antibody (μDR) but not by anti-HLA-DQ (μDQ) antibody. * numbers in parentheses represent the percentage inhibition of proliferation induced by the antibodies. Mouse fibroblast lines (L cells) transfected with human HLA-DR7 (but not those transfected with HLA-DR53) were efficient in presenting peptide to both HTL lines. Values shown are the means of triplicate determinations; bars, SD.
nizing tumor cell lysates from a gp100/H11001 melanoma tumor (697mel), but not control tumor lysates (Jurkat), presented by autologous APCs. For these experiments, we prepared the lysates using a DR7/H11002 melanoma cell line (697mel) to ensure that the autologous APCs were indeed capturing the gp100 antigen from the lysate to process and present the epitope to the helper T-cells. In addition, in a separate experiment, lysates prepared from gp100/H11002 melanoma cells (A375) did not stimulate the response of the T cells (data not shown).

These HTLs were also effective in recognizing antigen presented directly by HLA-DR7/H11001 melanoma tumors (HT-144), and this recognition could be inhibited with anti-HLA-DR antibodies (Fig. 3b). These results indicate that the T-cell epitope represented by peptide

Fig. 2. Peptide gp100_176-189 can be recognized by HTLs in the context of HLA-DR53 (a and c) or HLA-DQw6 (b and d). Peptide dose-response curves were created to estimate the overall avidity of these HTLs for peptide gp100_176-189 (a and b). Dashed lines represent the peptide concentration required to obtain 50% of the maximal proliferation (~1 μM for both HTL lines). MHC restriction analyses show that one of the HTLs recognizes peptide gp100_176-189 in the context of the HLA-DR53 allele (c) and the other one in the context of HLA-DQw6 (d). In both cases, the T-cell proliferative responses to peptide presented by autologous PBMCs were significantly inhibited by monoclonal anti-HLA-DR (c) or anti-HLA-DQ (d) antibodies. Numbers in parentheses represent the percentage inhibition of proliferation. Mouse fibroblast lines (L cells) transfected with human HLA-DR53 but not with HLA-DR7 were efficient in presenting peptide gp100_176-189 to one of the HTL lines (c). In addition, an allogeneic lymphoblastoid cell line (LCL) sharing only HLA-DR53 with the T cells was also efficient in stimulating this cell line (c). Allogeneic EBV-LCLs that only share HLA-DQw6 can present peptide gp100_176-189 to the second HTL line (d).

Fig. 3. HLA-DR7-restricted HTLs specific for peptide gp100_75-89 can recognize naturally processed antigen. A, autologous (DR7/H11001) PBMCs are efficient in stimulating HTL responses (assessed by the production of GM-CSF, as measured by ELISA) by presenting peptide gp100_75-89 or a lysate from gp100+/melanoma cells (697mel, DR7−) but not a lysate from Jurkat cells. PBMCs were preincubated overnight with lysates (at a 1 PBMC:1 cell equivalent ratio) before the T cells were added to the assays. Production of GM-CSF by HTLs is also observed with peptide-pulsed APCs (autologous PBMCs or L-DR7 cells) or with intact DR7+/melanoma cells (HT-144), but not with a DR7−/melanoma (888mel). These responses are inhibited by anti-DR Mabs (αDR). Values shown are the means of triplicate determinations; bars, SD. These experiments were repeated at least three times with similar results.
Values shown are the means of triplicate determinations; bars, SD.

**DISCUSSION**

We originally set out to identify promiscuous MHC class II-restricted HTL epitopes with the goal of using this information to improve peptide-based vaccines that have been designed to elicit antitumor CTL responses. The strategy we followed, based on the combination of algorithms to predict binding peptides for DR1 (DRB1*0101), DR4 (DRB1*0401), and DR7 (DRB1*0701) has recently proven to be effective in our hands for identifying promiscuous HTL epitopes from the HER2/new and MAGE-A3 antigens (13, 14). One of the peptides derived from these studies, HER2/s, was able to induce HTL responses restricted by HLA-DR1, DR4, DR5, and DR53 (13). In the case of MAGE-A3, peptide MAGE-A3/146–160 induced HTL responses restricted by DR4 and DR7 (14). As reported by Southwood et al. (32), some peptides that score high for the DR1, DR4, and DR7 algorithms also have the capacity to bind to additional alleles such as DRB1*1501, DRB1*0901, DRB1*1302, and DRB5*0101. This would explain why some of the peptides that we have predicted for HER2/new and for gp100 are presented by other alleles besides DR1, DR4, and DR7. On the other hand, a high algorithm binding score for a particular MHC allele does not necessarily imply that a peptide will actually bind to that allele because these predictive algorithms are not perfect and contain a degree of uncertainty (32). In the present studies, we succeeded in identifying one promiscuous HTL epitope capable of eliciting HTL responses restricted by HLA-DQw6 and DR53 (the DR53 molecule encoded by DRB4*0101) is expressed by all DR4+ and DR7+ individuals. Because approximately one-half of the population will express one or both of these MHC alleles, this peptide could be useful for eliciting
antitumor HTL responses to melanoma in a large number of patients. The other two peptides studied here, gp100 175–189 and gp100 576–590, were found to trigger HTLs restricted by the HLA-DR7 allele, which is expressed in approximately 26% of Caucasians. Although both of these peptides were selected as potential promiscuous epitopes, we were not able to induce HTLs restricted by other alleles such as HLA-DRB1*0101 or DRB1*0104. Nevertheless, from the limited number of attempts to induce these responses (two experiments with cells from two donors), we cannot exclude here that these epitopes may have some degree of promiscuity.

The recognition of naturally processed antigen by peptide-reactive T cells is an important parameter to determine whether a predicted T-cell epitope is indeed present on tumor cells or APCs, which in our view is critical for determining whether the peptide epitope will be useful for immunotherapy. HTLs may recognize peptide-MHC class II complexes directly on tumor cells that are MHC class II+, and as a consequence, the T cells will become activated and produce cytokines that provide help to CTLs or cytokines that may inhibit tumor cell growth. In some cases, HTLs exhibit cytotoxicity toward antigenic MHC class II tumors (11, 12, 17, 18, 31, 34). Our results show that HLA-DQw6-restricted HTLs that react with peptide gp100 175–189 displayed high levels of cytotoxicity against a DQw6+ melanoma cell line (1102mel; Fig. 6). However, cytotoxicity was not observed toward another DQw6+ tumor cell line (697mel; data not shown), suggesting that various tumor cell lines may display different levels of susceptibility to lysis by the T cells. The cytotoxic activity of this HTL toward the 1102mel line was not apparent using standard 4-h 51Cr release assays and required the lengthier JAM assay (33), suggesting that the cytolytic activity of these cells is different rather than the same as conventional CD8+ CTLs. Most importantly, it remains to be determined whether antitumor cytotoxicity by MHC class II-restricted T lymphocytes takes place in vivo and whether it results in tumor responses.

Tumor antigen-reactive HTLs will also recognize peptide-MHC complexes on APCs that have captured and processed tumor antigen, apoptotic cells, or tumor cell debris (lysates). Our results show that HTLs reactive to two of the three predicted peptide epitopes (gp100 175–189 and gp100 576–590) were also capable of recognizing naturally processed antigen in MHC class II+ melanomas or in APCs fed with tumor lysates. On the other hand, HTLs that were induced using peptide gp100 576–590 failed to proliferate or produce lymphokines when interacting with MHC class II+ melanomas or tumor lysates presented by APCs. The capacity of a HTL to become activated (i.e., proliferate and produce lymphokines) when challenged with APCs that have naturally processed the antigen will depend on two major factors. First, and most important, the peptide epitope must be processed adequately to produce the stimulatory peptide-MHC complexes on the surface of the APCs. It is likely that not all cells have the same capacity to process antigens into MHC class II-binding peptides. For example, a melanoma HLA-DR13-restricted MAGE-A3 HTL epitope was produced by APCs pulsed with either protein or tumor lysates but was not detected on DR13+ MAGE-A3+ melanoma cells, suggesting that this epitope could not be produced via the endogenous pathway (10). Our results indicate the absence of responses to APCs pulsed with tumor lysates, but direct recognition on melanoma by the DR53-restricted HTL clone 5D10, specific for peptide gp100 175–189 (Fig. 4; data not shown), suggests that this epitope may not be effectively processed by APCs. However, the same peptide epitope (gp100 175–189) was effectively presented by APCs to the HLA-DQw6-restricted HTL clone (Fig. 5a). Major differences in affinities of the binding of peptide gp100 175–189 to DR53 or DQw6 or differences in the avidities of the HTL clones to their respective peptide-MHC complexes could account for these conflicting results. However, the data presented in Fig. 2, a and b, suggest that this may not be the case because the peptide titration curves, which assess both the affinity of the peptide to MHC class II and the avidity of the HTLs, can almost be superimposed. One possible explanation for the lack of recognition of tumor lysate-pulsed APCs by the DR53-restricted HTLs could be that antigen processing by these cells does not necessarily result in the exact production of peptide gp100 175–189 but may result in the production of a close variant such as gp100 176–189 or gp100 175–189 that could bind to DQw6 but not to DR53. Studies are under way to determine whether residue truncations at either the NH2- or COOH-terminal ends of peptide gp100 175–189 have the same effect or a different effect on the recognition of the epitope by the DR53- and DQw6-restricted HTL clones.

The second parameter that is likely to affect the recognition of processed antigen is the overall affinity (avidity) of the HTL for its peptide epitope. The factors that influence the avidity of T-cell-APC interaction are multiple and rather complex. Most important perhaps are the intrinsic affinity of the T-cell receptor for the peptide-MHC complex and the amounts (or density) of specific peptide-MHC complexes expressed on the APCs. Other factors that may influence the avidity of T cells for their APCs are the presence of adhesion/costimulatory molecules that help stabilize cellular interactions or may even lower the threshold for T-cell receptor activation. Peptide dose responses can provide a rough estimate of the avidity of T cells for their antigen. However, results obtained using peptide titrations can also be affected by the stability of the peptide-MHC complexes on the surface of the APCs. Surprisingly, our results suggest that the HTLs that recognized naturally processed antigen displayed significantly lower avidity (~10-fold) for their peptides (gp100 175–189 and gp100 576–590) than the HTLs (specific for peptide gp100 175–189) that failed to respond to natural antigen. In general, we have observed that tumor-reactive HTLs have an intermediate avidity level for their peptides. These HTLs require a concentration of approximately 1 μM peptide to achieve MR 50. In contrast, HTLs directed to foreign antigens (viral and bacterial) have a much higher affinity for their ligands, requiring approximately 10–100 times less peptide to reach their MR 50 (14, 35). The most likely explanation for the lower avidity of tumor-reactive T cells is that the majority of TAAs are expressed to some extent in normal cells, and as a result, high affinity HTLs may be deleted or tolerized. In accordance with this assumption, a tyrosinase-specific DR4-restricted HTL isolated from a melanoma patient required >10 μM peptide to achieve its MR 50 (19). However, there are also antitumor HTLs that may have relatively high avidity for their epitopes, as reported by Zarour et al. (18), who found that a MART-1-reactive HTL required ~0.1 μM peptide to attain its MR 50. Thus, our observations that peptide gp100 576–590 induced very high avidity...
HTLs (MR_{\text{HLA-A2}} \sim 0.02 \mu\text{M}; Fig. 2b) and that these cells were incapable of recognizing naturally processed antigen suggest that this epitope may constitute a cryptic epitope and may not be produced as such by the APCs that process the gp100 protein. Alternatively, we cannot eliminate the possibility that a minute contaminant in our peptide preparation (peptide with unprotected residues) may be highly immunogenic.

Lastly, another parameter that we consider important for the selection of HTL epitopes for use in improving CTL vaccines is the possibility of using a single peptide of relatively small size (<25 residues) containing epitopes for both CTLs and HTLs. Although this approach may also be achieved by simply linking a CTL epitope with a T helper epitope, we believe that using the antigen’s natural sequence may have significant advantages. First, it is evident that peptides containing more than one T-cell epitope will have to be processed adequately by APCs to produce the separate distinct entities that need to bind effectively to the MHC molecules. Chimeric peptides that are formed by linking two (or more) epitopes found on distant parts of a protein may not be processed adequately to form the exact MHC-binding peptides because the natural prototypic cleavage sites may have been modified. Furthermore, introducing polylinkers such as AAA or KKK sequences between the epitopes may not necessarily overcome this problem because this also modifies the natural protease cleavage sites. The second major advantage of using peptides of natural sequences containing multiple T-cell epitopes over the use of their linked counterparts is that the latter may contain artificially created sequences that could produce new and potent but irrelevant epitopes that would not be found in the tumor antigen. For example, the new peptide sequence formed from the fusion of CTL epitope with the HTL epitope could constitute a high affinity MHC-binding peptide that may elicit a strong T-cell response, which would not be beneficial but would in fact could be detrimental to the generation of antitumor immunity. Because some degree of peripheral immune tolerance exists to many TAAs, the new artificial epitopes have the potential of being more immunogenic than those corresponding to the TAAs, which could result in the inhibition of the induction of antitumor T cells.

Taking into account the above-mentioned criteria, we focused on potential HTL candidates that were situated in proximity to known CTL epitopes. Notably, peptide gp100_{175-189}, which generated DR53- and DQw6-restricted HTLs capable of recognizing naturally processed antigen, includes a potent HLA-A2-restricted CTL epitope (gp100_{217-221}). Previous studies by our laboratory indicated that peptide gp100_{177-186} is very effective in inducing antitumor CTL responses by in vitro immunization of PBMCs from normal individuals using peptide-pulsed DCs (24). Our results show that peptide gp100_{274-289}, which stimulated a HLA-DR7-restricted HTL response, partially overlaps (in its COOH-terminal end) with another CTL epitope, but this epitope is restricted by the HLA-A3 and HLA-A11 alleles (gp100_{67-95}, ALNPFGSQK) (Ref. 25). Thus, a synthetic peptide of 22 residues (gp100_{74-95}) could be capable of eliciting antitumor CTL and HTL responses in patients expressing HLA-A3 (or HLA-A11) and HLA-DR7. Interestingly, the same HTL epitope (gp100_{274-289}), also overlaps in its NH2-terminal end with another CTL epitope (gp100_{270-284}), which is restricted by HLA-Cw8 (29).

In summary, we report here the identification two novel HTL epitopes for the melanoma-associated antigen gp100 that are restricted by MHC class II alleles expressed in a large proportion of the population. These peptides lie proximal to or overlap with previously characterized tumor-reactive CTL epitopes. The present findings suggest that synthetic peptides of relatively small size could be used as therapeutic vaccines that would effectively stimulate both HTL and CTL antitumor responses in melanoma patients. The approach of using peptide vaccines containing both CTL and HTL epitopes was recently described in the HER2/neu antigen system in breast and ovarian cancer patients (36). These vaccines were shown to increase the CTL precursor frequency to the HER2/neu peptides contained in these peptides that lasted for more than a year after vaccination, indicating that antigen-specific HTLs may prolong CTL responses.

REFERENCES

Identification of Helper T-Cell Epitopes That Encompass or Lie Proximal to Cytotoxic T-Cell Epitopes in the gp100 Melanoma Tumor Antigen

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