Fine Specificity of High Molecular Weight-Melanoma-Associated Antigen-Specific Cytotoxic T Lymphocytes Elicited by Anti-Idiotypic Monoclonal Antibodies in Patients with Melanoma

James L. Murray,1 Michael Gillogly,1 Kouichiro Kawano,2 Clay L. Efferson,2 Jeffrey E. Lee,3 Merrick Ross,3 Xinhu Wang,3 Soldano Ferrone,3 and Constantin G. Ioannides2,4

Departments of 1Bioimmunotherapy, 2Gynecologic Oncology, 3Surgical Oncology, and 4Immunology, M.D. Anderson Cancer Center, Houston, Texas, and 5Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York

ABSTRACT

HLA-A2-restricted CTLs, which lysed high molecular weight (HMW)-melanoma-associated antigen (MAA)3 melanoma cells, were induced in patients with melanoma immunized with MELIMMUNE, a combination of the murine anti-idiotypic (anti-id) monoclonal antibodies (mAb) MEL-2 and MF11–30 (MW Pride et al., Clin Cancer Res 1998;4:2363). In the present study we investigated whether CTL epitopes are present in anti-id mAb MF-11–30 and activate T cells to recognize HMW-MAA on melanoma cells. One candidate epitope in the mAb MF11–30 VH chain, VH (3–11), was selected based on the presence of HLA-A2 anchor residues and partial homology with the HMW-MAA epitope, HMW-MAA (76 – 84). This epitope and to a variant HMW-MAA peptide to a significantly greater extent than autologous lymphocytes stimulated with an irrelevant peptide and lymphocytes from nonimmunized patients. No proliferative response was detected to the wild-type HMW-MAA peptide (76 – 84). Significant increase in IFN-γ production but not in interleukin 10 production in response to VH (3–11) and to variant HMW-MAA peptide (76 – 84) was observed in lymphocytes from the immunized patients. Stimulation of lymphocytes from HLA-A2+ patients with the two peptides induced CTLs, which lysed HMW-MAA +/HLA-A2 + A375SM melanoma cells. This is the first report documenting the presence of immunogenic peptides in a murine anti-id mAb for a defined epitope expressed by a human melanoma-associated antigen. These results may be relevant for development of novel vaccines based on homology between anti-id mAb and tumor-associated antigen amino acid sequences.

INTRODUCTION

The treatment of malignant melanoma with chemotherapy and radiotherapy has shown little progress in the past 20 years (1). As a result, alternative therapeutic strategies are being developed and applied. In recent years, active specific immunotherapy has been emphasized because immunological events appear to play a role in the pathogenesis and in the clinical course of melanoma. A broad spectrum of immunogens has been and is being used in clinical trials, which have been performed or are in progress. They range from cultured human melanoma cells or their lysates (2, 3) to well defined moieties. The latter include melanoma-associated antigen (MAA) derived peptides with HLA class I antigen binding motifs, which are targets of CTLs (4, 5), gangliosides (GD2; 6), and anti-idiotypic (anti-id) monoclonal antibodies (mAb), which mimic antigenic determinants expressed on MAA (7–10). Anti-id mAbs, which mimic human GD2 ganglioside, and high molecular weight (HMW)-melanoma-associated antigen have been used as immunogens in clinical trials (7, 8, 10). A humoral and/or a cellular immune response to the nominal antigen has been elicited in a variable percentage of patients with melanoma immunized with anti-id mAbs, which mimic the HMW-MAA (7–9, 11–14). This immune response has been found to be associated with regression of metastases in a few patients (11) and with disease-free interval and/or survival prolongation (7, 8, 12, 13). The mechanism underlying the latter association in the case of HMW-MAA has been suggested to be an inhibition of its role in melanoma cell biology because this antigen has been shown to influence adhesion, migration, and proliferation of melanoma cells by acting as a receptor for components of the extracellular matrix and various circulating molecules. Furthermore, anti-HMW-MAA antibodies have been shown to inhibit melanoma cell spreading on basement membrane synthesized by endothelial cells (15–18).

There is only scant knowledge about CTL responses elicited by anti-id mAbs that mimic tumor-associated antigen (TAA). Furthermore, whether anti-id mAbs contain epitopes recognized by T cells and whether these epitopes are homologous with the amino acid sequences in the nominal TAA remains to be determined.

The basis of structural mimicry between an antigen (Ag) and the corresponding anti-id antibody is unclear. One possibility is represented by the structural basis of the interactions of a TAA and corresponding anti-id antibody with the same area of the Ag combining site of the anti-TAA antibody. These interactions are likely to involve side chains of amino acids, which can form strong H-bonds (involving OH groups) and even stronger electrostatic interactions between positively/negatively charged terminal groups of the side chains and reversely charged groups in Ag-binding regions of antibodies and of T-cell receptors (TCR).

Peptides derived from TAA, including the HMW-MAA, may be presented by HLA class I molecules, but they are all weak inducers of CTLs (19). Peptides from xenogeneic proteins in the vaccine can enhance the existent CTL responses to tumor cells. Alternatively, if peptides presented by melanoma cells are tolerogenic (20), then anti-id mAb peptides in the vaccine can induce CTLs, which can cross-recognize melanoma cells. CTL precursors of unknown specificity are present during induction of the id/anti-id cascade (21). Little attention has been paid to such CTLs, because the T-helper (Th2) environment suitable for induction of humoral anti-anti-id responses was not considered to be favorable to effector CTL development (22).

The question is whether this pool of potential effectors, when activated, can mediate an antitumor effector response. To address this question we used as a model ex vivo isolated lymphocytes from high-risk melanoma patients treated with MELIMMUNE (14). The latter is a combination of the anti-id mAb MEL-2 and MF11–30, which mimic the antigenic determinants of the HMW-MAA defined by mAbs MEM136 and 225.28, respectively (23, 24). MELIMMUNE was shown to induce HLA-A2-restricted CTLs, which lyse melanoma cells expressing both HLA-A2 Ag and HMW-MAA. However, the epitope(s) recognized by those CTLs was not characterized.
In the present study we found that the amino acid sequence 3–11 of the heavy chain variable region (VH) of the anti-id mAb MF 11–30 is partially homologous with that of HMW-MAA core protein (76–84). We designed a variant (v)HMW-MAA (76–84) peptide to align the shifted Tyr3Ser4 in HMW-MAA sequence with Tyr3Ser4 in VH (3–11) sequence. In vitro stimulation with peptides VH (3–11) and vHMW-MAA (76–84) but not with wild-type (wt)HMW-MAA (76–84) peptide generated HMW-MAA-specific CTLs from peripheral blood mononuclear cells (PBMC) from HLA-A2* patients who had been immunized with MELIMUMUNE. Melanoma cell lysis by VH (3–11) and vHMW-MAA peptide-elicited CTLs was inhibited by VH (3–11) peptide indicating structural similarities between these epitopes. These results suggest an evolutionary mechanism of tumor escape from immunosurveillance, which can be counteracted by CTL antigenic diversity for epitopes generated from VH anti-id antibodies during induction of a humoral response.

MATERIALS AND METHODS

Patients. Of the 10 studied patients, 5 are free of disease 5–7 years after vaccination with MELIMUMUNE, and 5 are currently being followed in the melanoma outpatient clinic at M.D. Anderson Cancer Center. All of the patients had disease resected a minimum of 2 months before study and had an Eastern Cooperative Oncology Group performance status of 0. None were currently receiving and/or had received chemotherapy and/or biological therapy within 2 months from study.

Antibodies. The anti-HLA-A2.1 mAbs MA2.1 and BB7.2 (25, 26) were purified from the corresponding hybridoma supernatants by ammonium sulfate precipitation followed by column chromatography. Both anti-HLA-A2 mAbs secreting hybridomas were obtained from American Type Culture Collection (Rockville, MD). FITC-conjugated isotype-specific antihuman IgG and all of the other antibodies used in the present study were purchased from BD PharMingen (La Jolla, CA.)

Cells. The hybridoma secreting the anti-id mAb MF11–30 was developed as described (23). It was cultured in medium RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma Chemical Co., St. Louis, MO). The melanoma cell line A375SM (HMW-MAA*/HLA-A2*; donated by Dr. Michael Rosenblum, M. D. Anderson Cancer Center, Houston, TX) and the lymphoblastoid T2 cells (donated by Dr. Peter Cresswell, Yale University, New Haven, CT) were grown in DMEM containing 10% heat-inactivated fetal bovine serum (Sigma Chemical Co.), 5 mM l-glutamine, 5 mM nonessential amino acids, 5 mM sodium pyruvate, and 0.1% gentamicin (Life Technologies Inc., Grand Island, NY). PBMCs were obtained from patients with resected melanoma (American Joint Committee on Cancer) using Ficoll gradients (Sigma Chemical Co.).

Synthetic Peptides. Peptide I-100 (LVLLYLSSKL) is from the anti-id mAb MF11–30 residues 3–11. Peptide I-101 (LLLLYSGLR) corresponds to the wild-type amino acid sequence of HMW-MAA residues 76–84. Peptide I-102 (LLLLQYSGRL) has the amino acid sequence of the HMW-MAA stretch 76–84 modified by inclusion of Gly at position 80. Peptide F119 (AA-AAGILTV) corresponds to the CTL epitope from MAA MART-1 (amino acids 27–35). Peptide E71, HER-2 (828–836) corresponds to an inactivating HLA-A2 epitope from HER-2/neu protein. All of the peptides were synthesized in the M.D. Anderson Cancer Center Peptide Core Laboratory and purified by high-performance liquid chromatography. Their identity was determined by amino acid analysis. The purity of these peptides was >95%.

Identification of VH and VL Gene Sequences Used by Anti-Id mAb MF11–30. The VHg and VLg nucleotide and deduced amino acid sequences of anti-id mAb MF11–30 are shown in Table 1. The VH gene segment of the mAb MF11–30 shows high homology with the QS2 VH gene family. The D gene segment is part of the DSP2 mini-gene family. The JH gene segment is derived from the JH3 family. The Vk gene segment shows homology with subgroup III of the VK family. The Kk gene segment is 100% homologous with the Jk2 mini-gene.

RESULTS

Identification of VH and VL Gene Sequences in HMW-MAA and in Anti-Id mAb MF11–30. The amino acid sequences of the mAb MF11–30 VHg and VLg were compared with the published amino acid sequence of the HMW-MAA core protein (31) using the program BLOSUM62 from the ExPasy website.6 Partial (between 20% and 40%) homology between these two proteins was observed only on short stretches of 10–17 amino acids. However, these areas of homology are unlikely to contain HLA-A2 binding peptides, because HLA-A2 binding motifs were not identified in these areas of homology.

We then searched the amino acid sequence of mAb MF11–30 VH and VL for HLA-A2 binding motifs, using the program by Taylor et al. available online.7 The sequence VH (3–11) LLVLLYSL in the framework region 1 met the criteria of an HLA-A2 binding peptide, i.e., hydrophilic aliphatic residues at positions 2 and 9, as well as hydrophilic aliphatic residue at position 1 and aromatic residue at position 6. This peptide had the highest binding affinity for HLA-A2 Ag of all VH peptides (Table 2). Analysis of the VH amino acid sequence with proteasome digestion programs (32, 33) indicated that this peptide is protected from proteasome digestion (data not shown). A search in the databases using the FASTA3 program identified only few partially homologous sequences with the VH (3–11) in immuno-
On the basis of the nucleotide sequence, the Leu 5 residue is less protected from proteasome destruction than VH (3–11) residues. Arg 8, which is most likely to form H bonds with the TCR, is intercalated before the COOH-terminal group Leu 9 in HMW-MAA. The HMW-MAA (76–84) peptide indicates positions at which insertions occur.

Arg 8. The HMW-MAA (76–84) sequence was used for homology search in HMW-MAA and Lys 8 Leu 9 in VH (3–11), respectively. On the basis of the nucleotide sequence, the change Lys–Arg is the result of a point mutation.

Because the shift of Tyr-Ser group may affect TCR recognition and Ag immunogenicity, Gly, which lacks side chain and allows flexible positioning, was introduced at position 5 of HMW-MAA (76–84) to align the Tyr and Ser in both VH (3–11) and HMW-MAA peptides. The resulting sequence was LLQLYGSRLL. In this decapetide, the amino acid positions 1, 2, 4, 6, and 7 are aligned with VH (3–11) peptide. Because this decapetide contains the same COOH-terminal anchors as the non-apeptide LLQLYGSRLL, the same TCR is likely to interact with both peptides, although with different affinities. VH3–11 and variant globulins and other proteins (data not shown) suggesting that this peptide sequence was “idiotypic” for mAb MF11–30.

The VH3 (3–11) sequence was used for homology search in HMW-MAA. We found a partially homologous peptide (five of nine residues) in the HMW-MAA sequence 76–84. The first four amino acids of VH (3–11) sequence, YSKL, with the differences in polarity. The last five amino acids, YSGRL, are similar with the last five amino acids of VH (3–11) sequence. The change V→Q is neither a significant structural change nor a change in charge but results in a change in binding affinity to HLA-A2 Ag.

Table 1  Anti-id mAb MF11-30 VH and VL nucleotide and deduced amino acid sequences

The numbering of residues and positioning of complementarity determining regions (CDRs) in anti-id mAb MF11-30 VH and VL sequences are based on the Kabat Numbering Scheme and the Kabat definition of CDRs at the following website: http://www.bioinf.org.uk/abs/. Bolded letter codes of amino acids represent CDR. Underlined letter codes of amino acids indicate positions at which insertions occur.

Table 2  Alignment of anti-id mAb and HMW-MAA amino acid sequences in homologous areas

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<table>
<thead>
<tr>
<th>(Est.1/2)</th>
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<th>9</th>
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<td>S</td>
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<td>S</td>
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<td>R</td>
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<td>L</td>
<td>K</td>
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<td>HMW-MAA, 76–84, variant</td>
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<td>L</td>
<td>L</td>
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<td>G</td>
<td>S</td>
<td>Y</td>
<td>R</td>
<td>b</td>
<td>L</td>
<td>1-102</td>
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</table>

* anti-id, anti-idiotypic; mAb, monoclonal antibody; HMW-MAA, high molecular weight-melanoma-associated antigen; wt, wild-type.

a, indicate gaps introduced to illustrate alignment.
b, Binding affinity to HLA-A2 Ag.

globulins and other proteins (data not shown) suggesting that this peptide sequence was “idiotypic” for mAb MF11–30.

The VH3 (3–11) sequence was used for homology search in HMW-MAA. We found a partially homologous peptide (five of nine residues) in the HMW-MAA sequence 76–84. The first four amino acids of VH (3–11) sequence, YSKL, with the differences in polarity. The last five amino acids, YSGRL, are similar with the last five amino acids of VH (3–11) sequence. The change V→Q is neither a significant structural change nor a change in charge but results in a change in binding affinity to HLA-A2 Ag.

Table 3  Proliferation of melanoma patients PBMCs stimulated with peptides I-100 and I-102

<table>
<thead>
<tr>
<th>Patient number</th>
<th>HLA-A2 phenotype/immunization</th>
<th>NP</th>
<th>I-100 (S.I.)</th>
<th>I-102 (S.I.)</th>
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<tr>
<td>1</td>
<td>+/+</td>
<td>456 ± 62</td>
<td>1123 ± 240</td>
<td>5191 ± 92</td>
</tr>
<tr>
<td>2</td>
<td>+/+</td>
<td>256 ± 63</td>
<td>853±138</td>
<td>766±90</td>
</tr>
<tr>
<td>3</td>
<td>-/-</td>
<td>586 ± 47</td>
<td>635 ± 51</td>
<td>821±45</td>
</tr>
<tr>
<td>4</td>
<td>-/-</td>
<td>380 ± 30</td>
<td>583±97</td>
<td>640±62</td>
</tr>
<tr>
<td>5</td>
<td>-/-</td>
<td>373 ± 55</td>
<td>636±37</td>
<td>745±61</td>
</tr>
<tr>
<td>6</td>
<td>+/+</td>
<td>518 ± 32</td>
<td>1793±58</td>
<td>1464±53</td>
</tr>
<tr>
<td>7</td>
<td>+/-</td>
<td>618 ± 58</td>
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<td>755±56</td>
</tr>
<tr>
<td>8</td>
<td>-/+</td>
<td>560 ± 22</td>
<td>490 ± 54</td>
<td>650 ± 31</td>
</tr>
<tr>
<td>9</td>
<td>-/+</td>
<td>455 ± 39</td>
<td>334 ± 26</td>
<td>425 ± 40</td>
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</tbody>
</table>

* PBMC, peripheral blood mononuclear cell; NP, no peptide.

b, S.I., stimulation index. Values >2 are considered significant.

c, d P < 0.001.
d, P < 0.01.
e, significant increase over no peptide (NP).

f, P < 0.05.
HMW-MAA (vHMW) peptides have the identical amino acids at positions 6 and 7. Their homology is seven of nine residues (77%). The binding affinities to HLA-A2 Ag of both wild-type (wt) and variant (v) HMW-MAA peptides are similar. The corresponding peptides were designated as I-100, VH: LLVLLYSKL; as I-101, wtHMW-MAA: LLQLYGSGRL; and as I-102, vHMW-MAA: LLQLGYSGR( Table 2).

Increased Proliferative Responses to Both VH3–11 and vHMW-MAA Peptides in Lymphocytes from Patients Immunized with MELIMMUNE. We tested first whether patients immunized with MELIMMUNE had T cells that recognized peptides from the anti-id mAb used as an immunogen as well as from the HMW-MAA. Thus, we determined whether PBMCs isolated from immunized patients responded to peptides I-100 and I-101 better than PBMCs from nonimmunized patients. In addition, we tested whether PBMCs contained T cells that recognized wtHMW-MAA and vHMW-MAA peptides. In preliminary experiments we observed that peptide I-101 did not stimulate the proliferation as well as IFN-γ and IL-10 secretion by PBMCs from HLA-A2+ patient 6 and from four HLA-A2- patients, because these parameters were similar to those found in cultures of autologous PBMCs incubated without peptide. At reactivation with peptide I-101, the PBMCs of patient 6 became apoptotic, suggesting that the wtHMW-MAA was tolerogenic by deletion mechanisms (data not shown). Therefore, we focused our subsequent investigations on the analysis of VH3–11 and vHMW-MAA peptides, which appeared to be stimulatory.

As shown in Table 3 when incubated with I-100 and I-102 peptides for up to 6 days (1° stimulation; STIM), PBMCs from patients 1, 2, 4, and 6 proliferated to a markedly higher extent than autologous PBMCs incubated with no peptide (NP). The extent of proliferation induced by I-100 and I-102 peptides was similar.

Lymphocytes from the HLA-A2+ patients 1, 2, and 6 who had been immunized with MELIMMUNE (Table 3) responded with significantly stronger proliferation to both I-100 and I-102 peptides and had significantly higher stimulation index (2.7 ± 0.7) than HLA-A2- patients 3, 5, 9, and 10 who had not been immunized (1.2 ± 0.4; *P < 0.001). Thus, induction of proliferation by I-100 and I-102 peptides was associated with HLA-A2 Ag expression. These results also indicate that immunization activated a large population of T cells capable of responding to both VH3–11 and vHMW-MAA peptides. The mitogenic effect of VH3–11 and vHMW-MAA peptides was specific, because irrelevant HER-2/neu-specific peptide, E71, was not mitogenic for lymphocytes from HLA-A2+ immunized patients 1, 2, and 6 (NP = 353 ± 84 cpm versus E71 = 411 ± 190 cpm; *P = 0.65) and HLA-A2- patient 8 (NP = 560 ± 22 versus E71 = 685 ± 103; *P = 0.06). Like the E71 peptide, the MART-1 peptide F119 was not mitogenic for PBMCs from patients 1 (330 ± 36 cpm; stimulation index = 0.7) and 10 (215 ± 26 cpm; stimulation index = 1.1).

Cytokine Induction by VH3–11 and vHMW-MAA Peptides in PBMCs from Immunized Patients. Interaction of T cells with Ag can induce a few cycles of division resulting in Ag-primed uncommitted T cells or can induce T cells to differentiate and gain effector function. The effector function is best characterized by the ability of Ag-stimulated T cells to secrete Th1 cytokines and to mediate lysis of specific targets. To determine whether T cells from immunized patients acquired cytokine effector function, IFN-γ and IL-10 were measured in supernatants harvested from PBMC cultures stimulated with I-100 and I-102 peptides. IL-10 was assayed instead of IL-4 as a representative Th2 cytokine, because the PBMCs used as responders were from patients with melanoma. Therefore, they might contain regulatory cells. Patient 6 had supernatants saved from two separate experiments. This afforded validation of conclusions in replicated
analyses performed at separated time points. Because after priming, all of the stimulated PBMCs were expanded in IL-2, we determined the effect of culturing PBMCs in IL-2 on IFN-γ induction. Representative individual patient examples of IFN-γ and IL-10 induction are shown in Fig. 1. Both IFN-γ and IL-10 levels from peptide-stimulated PBMCs from HLA-A2+ patient 6 and HLA-A2+ patient 7 were low after 1° STIM with peptide I-100 (Fig. 1, A and B).

To verify the significance of cytokine levels induced by I-100 and I-102 peptides, the cytokine responses induced by the dominant MART-1 epitope, represented by peptide F119, were determined in I-100 peptides, the cytokine responses induced by the dominant I-100 peptide than with I-102 peptide. The IFN-γ:I-10 ratios were low after 1° STIM than with 2° STIM, whereas IL-10 levels did not differ (Fig. 2B). After restimulation, IFN-γ levels in patients 2 and 6 increased by >1 log. The average IFN-γ levels after 2° STIM were significantly higher (616.1 ± 86.3) for PBMCs from HLA-A2+ patients than for those for PBMCs from HLA-A2- patients (59.5 ± 24.4; P < 0.007; Fig. 2C). A similar analysis for IL-10 did not detect significant differences (pg/ml IL-10: HLA-A2+ = 155.7 ± 36.3 versus HLA-A2- = 61.1 ± 25; P = 0.06). The IFN-γ:IL-10 ratios for HLA-A2+ patients were 4-fold higher (3.96) than those for HLA-A2- patients (0.97) indicating a trend toward Th1 responses in HLA-A2+ patients. Therefore, immunization with anti-id mAb MF11–30 recruited and activated T cells to acquire the potential of IFN-γ production. Demonstration of activation of effector cytokines required either priming and restimulation in vitro with peptide or culture of PBMCs in IL-2 before stimulation with peptide.

Induction of VH3–11 and vHMW-MAA Peptide-Specific CTLs by Immunization with MELIMMUNE. To determine the effects of the immunization with anti-id mAb on activation of melanoma cell-lytic CTLs, we compared the responses of immunized HLA-A2+ patients 1 and 6 with that of nonimmunized HLA-A2+ patient 4. As specificity and restriction control we analyzed the responses of nonimmunized HLA-A2+ patients 3 and 5. All of the CTLs were tested for their ability to lyse T2 cells pulsed with the stimulating peptide and A375sm melanoma cells. I-100-CTL and I-102-CTL from the HLA-A2+ patients 1 and 6 lysed I-100 pulsed T2 cells (T2-I-100) to a significantly greater extent than control targets T2-NP, i.e., T2 cells that had not been pulsed with peptide (Fig. 2, A and B). CTLs induced by the HMW-MAA peptide I-102 also recognized the VH (3–11) peptide I-100 (data not shown). The HLA-A2+ patient 4 I-100-CTL did not lyse T2-I-100, yet autologous I-102-CTL lysed T2-I-102 (Fig. 2C). These results indicate that immunization with MELIMMUNE activated CTL precursors to both the VH3–11 peptide and to the HMW-MAA peptide, whereas in vitro stimulation with the same peptides of HLA-A2+ PBMCs from the nonimmunized patient activated only the existent CTL precursors for HMW-MAA.

PBMCs from the HLA-A2+ patients 3 and 5 that had not been immunized with MELIMMUNE but were only stimulated in vitro with VH3–11 and HMW-MAA peptides did not develop CTLs recognizing the stimulating peptide. As shown in Fig. 3, D and E, there was no specific lysis of T2-I-102 targets by CTL from patient 3 stimulated repetitively with I-102 peptide compared with targets incubated without peptide (T2-NP). Lymphocytes from patient 5 were also unable to specifically lyse T2-I-100 and T2-I-102 targets (Fig. 3E). This indicates that CTL activation by I-100 and I-102 peptides was restricted by HLA-2 Ag.

Identification of HMW-MAA-Specific CTLs by Immunization with MELIMMUNE. To determine whether in vitro stimulation with peptides generated CTLs that recognized epitopes on HMW-MAA+ HLA-A2+ melanoma cells, PBMCs from immunized HLA-A2+ patients 1 and 6 were stimulated with I-100 and I-102 peptides and then tested for their ability to lyse A375SM melanoma cells. As shown in Fig. 4A, the I-100-stimulated PBMCs and I-102-stimulated PBMCs of patient 1 effectively lysed A375SM cells, demonstrating that CTLs were present in peptide-stimulated PBMCs. Therefore, these effectors were designated as I-100 CTL and I-102 CTL, respectively. Lysis was effectively blocked by “cold” T2-I-100. This result demonstrated both the specificity of CTLs for the VH3–11 peptide and expression of a structurally similar peptide-HLA-A2 epitope on melanoma cells. The results confirmed that the significant degree of homology in the sequence of epitopes formed by I-100 and I-102 peptides (>67%) resulted in CTLs cross-recognizing the HMW-MAA epitope presented by tumor cells. In addition, lysis was inhibited by HLA-A2-specific mAb MA2.1 by >50%, confirming that this molecule is the restricting element for CTL (Fig. 4B). Similar results were obtained with CTLs from patient 6. Fig. 4C shows the kinetics of lysis of A375SM cells by I-100-CTL and I-102-CTL. Effectors used as controls were generated from autologous PBMCs by
stimulation in the same experiment and under the same experimental conditions with T2 cells, which had been pulsed with HMW-MAA peptide or incubated with IL-2 as a growth factor. The latter were designated as NP-CTL. NP-CTLs were used as effectors to determine whether memory CTLs induced by the vaccine were present in this patient and up-regulated their cytolytic function after stimulation with cytokines and HLA-A2 Ag, which was not presenting cognate Ag. The results show that NP-CTLs (“memory-like”) specifically recognized an epitope on tumor cells, which is structurally similar to the one formed by T2-I-100. However, their lytic activity was weak and did not increase over time. In contrast, I-100-CTL recognized the same epitope with faster kinetics in the first 5 h than I-102-CTL. Their kinetics of lysis slowed over time. I-102-CTL recognized the same epitope on tumor cells with apparently slower kinetics than I-100-CTL, during the first 5 h, but the kinetics of lysis became somewhat faster over time compared with that of I-100-CTL (59% increase from 5 h to 20 h). We could not determine the exact number of effectors in each population bearing specific TCRs for each peptide, because the necessary reagents were not available. Nevertheless, the differences in kinetics of lysis between I-100-CTL and I-102-CTL suggest small quantitative differences in their functional avidity for tumor cells.

**DISCUSSION**

To the best of our knowledge, this is the first study to identify a peptide from an anti-id mAb that induces CTLs recognizing HMW-MAA+ melanoma cells in a HLA-A2-restricted fashion. PBMCs used to generate CTLs were obtained from patients with melanoma who had been immunized with anti-id mAb MEL-2 and MF11–30. The two anti-id mAb mimic two distinct determinants of HMW-MAA. The peptide VH3–11 derived from anti-id mAb MF11–30 induced proliferation and cytokine production by T cells and induced HMW-MAA-specific CTLs. The CTLs elicited by VH3–11 peptide were specific for a peptide structurally similar to that expressed on tumor cells because tumor cell lysis was inhibited by T2 cells pulsed with I-100 peptide. Lysis was restricted by HLA-A2 Ag because it required HLA-A2 Ag matching of effector and target cells and was markedly inhibited by anti-HLA-A2 mAb MA2.1. Induction of CTL by anti-id mAb MF11–30 derived peptide and by the variant HMW-MAA peptide demonstrated the existence in the immunized patients of populations of T cells bearing TCRs, which can be activated by the vaccine and which cross-recognize wtHMW-MAA epitopes. wtHMW-MAA (76–84) peptide, I-101, did not stimulate proliferation or IFN-γ secretion in PBMCs. Peptide I-101 might delete or inactivate CTLs induced by peptide I-100.

A significant increase in IFN-γ secretion compared with IL-10 was observed at restimulation with peptides I-100 and I-102 suggesting that both peptides have the potential to induce a Th1 polarizing environment. Their Th1 potential may be even stronger, because culture of patient lymphocytes in IL-2 before peptide stimulation resulted in >10-fold higher levels of IFN-γ than of IL-10. This finding raises the possibility that γ-chain cytokines such as IL-2, if administered after vaccination, may synergize with immunogens such as anti-id mAb in inducing inflammatory cytokines.

Our results are novel with regard to induction of adoptive CTL immunity to tumor cells by immunization with anti-id mAb. A target CTL epitope from Hmw-Maa on tumor cells and the homologous epitope on the anti-id mAb used as an immunogen have been identified for the first time. Cellular responses elicited by immunization with anti-id antibodies, which mimic TAA, have been reported in preclinical models (34–36). Anti-id-specific T cells have been proposed to be involved in tumor regression, but their specificity and effector mechanisms have not been identified (37). In patients with malignant diseases, Durrant et al. (38) described tumor cell-specific killing by lymphocytes derived from lymph nodes adjacent to tumors and from PBMCs from three patients with rectal cancer after immunization with the human anti-id mAb 105AD7. The target epitopes on the Ag and the anti-id mAb have not been described yet. Furthermore, Saha et al. (36) reported that murine dendritic cells pulsed with an anti-id mAb induce Ag-specific protective antitumor immunity. In agreement with our results, the same authors (39) found that peptides from the VH and VL of the anti-id mAb 3H1, which mimics carcinoembryonic antigen, induced in vitro proliferation of PBMCs from patients with colon cancer who had been immunized with this anti-id mAb. Similarly, anti-id mAb 11D10 induced T-cell proliferation in PBMCs from breast cancer patients immunized with this antibody (40). Analysis of the subsets of T cells stimulated revealed that CD4+ cells that secrete IFN-γ were predominant. The carcinoembryonic antigen peptides used by Chatterjee et al. (39) were mostly 12–14 amino acids long and induced strong HLA class II Ag-restricted responses involving CD4+ as well as CD8+ T cells. Proliferative T-cell responses to anti-id mAb GA733 were also observed in colon cancer patients (41) indicating the existence of a large T-cell repertoire capable of response to various anti-id mAbs.

Several mechanisms may be envisioned for the generation of HMW-MAA-specific CTLs after immunizations with anti-id mAb. First, peptides with HLA class I Ag binding motifs from anti-id mAb VH or VL when bound to particular HLA-A, B, or C molecules may form complexes that structurally resemble HMW-MAA epitopes expressed by the same HLA class I specificities. The VH peptides are more immunogenic than the HMW-MAA peptides because they can induce CTLs, which recognize the wt epitope on tumor cells. This possibility is supported by the recently reported ability of myeloma immunoglobulins to elicit in an
autologous system CTL effectors capable of lysing the corresponding myeloma secreting tumor cells (42). An alternative, although not exclusive, possibility is that anti-anti-id antibodies generated by immunization with anti-id antibodies, such as the one used in this study, bind HMW-MAA present in plasma. The complexes taken up by dendritic cells induce HMW-MAA-specific CTLs. This possibility is supported by the high immunogenicity of Ag-antibody complexes, which has been shown in a number of antigenic systems (43). An additional possibility that needs future investigations is the genetic association between expression of HLA-A2 Ag and induction of CTLs by HLA-A2-presented peptides derived from anti-id mAb and HMW-MAA in patients with melanoma (44).

The significance of the sequence shift of Tyr<sup>6</sup>Ser<sup>7</sup> in the HMW-MAA sequence compared with the VH3–11 sequence deserves additional investigation. The shift is induced by deletion of Leu<sup>1</sup> in HMW-MAA and is then “repaired” by introduction of Gly<sup>3</sup>. Thus, the last two amino acids in both VH3–11 and HMW-MAA 76–84 sequences are similar. In CTL epitopes, amino acids in the central area, position 4–7 from the HLA-A2 plane, are more likely to contact the TCR CDR3 and activate its signaling (45). Is the Leu<sup>1</sup> codon deletion an evolutionary response to CTL pressure to protect HMW-MAA-expressing cells? Additional studies are required to address this question.

In summary, the peptides we have identified may be useful alone or in combination with other MAA-derived peptides in the development of immunogens for immunotherapy of melanoma. The HMW-MAA is homogeneously and stably expressed in &gt;80% of primary and metastatic melanoma lesions (46). Induction of anti-HMW-MAA antibodies by immunization with anti-id antibodies, with consequent expression of CTL-activating peptides, followed by proinflammatory cytokines such as IL-12 and T-cell growth promoting γ-chain cytokines, may provide a novel approach to cancer therapy.

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James L. Murray, Michael Gillogly, Kouichiro Kawano, et al.

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