Recognition of Multiple Epitopes in the Human Melanoma Antigen gp100 by Peripheral Blood Lymphocytes Stimulated in Vitro with Synthetic Peptides

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ABSTRACT

gp100 is a melanocyte lineage-specific antigen recognized by tumor-infiltrating lymphocytes whose adoptive transfer has been associated with tumor regression in patients with metastatic melanoma. The peripheral blood mononuclear cells of five melanoma patients were sensitized in vitro with synthetic peptides to elicit antigen-specific cytotoxic T lymphocyte (CTL) lines against four gp100 epitopes. These epitope-specific CTL lines were generated following weekly in vitro stimulation with the synthetic decamer G10476 (V-L-Y-R-Y-G-S-F-S-V) or the nonamers G9280 (Y-L-E-P-G-P-V-T-A), G9154 (K-T-W-G-Q-Y-W-Q-V), or G9209 (I-T-D-Q-V-P-F-S-S-V) pulsed onto autologous irradiated peripheral blood mononuclear cells. These lines grew as long as 4 months in culture in low-dose interleukin 2 (30 IU/ml) and exhibited antigen-specific, MHC class I-restricted lysis of peptide-pulsed tumor cells and HLA-A2*, gp100* established melanoma cell lines. G10476 and G9280-specific CTLs demonstrated specific release of granulocyte-macrophage-colony-stimulating factor and tumor necrosis factor α in response to T2 cells pulsed with relevant peptide, as well as to gp100* melanoma cell lines. These results demonstrate that several peptides derived from the gp100 protein are presented on the surface of melanoma cells and are sufficiently immunogenic to generate, in vitro, potent CTLs capable of cytolysis and the secretion of cytokines. Therefore, for HLA-A2* melanoma patients, these and possibly other gp100 peptides could represent good candidates for antigen-specific immunotherapy either singly or in a multivalent regimen.

INTRODUCTION

It is well established that tumor-derived proteins can be processed, presented, and recognized by T cells in the context of MHC molecules (1–4). Recently, several tumor-associated antigens have been identified which can serve as targets for adoptive immunotherapies or as immunogens in a vaccine regimen. The human tumor-associated antigens elucidated within the past few years include the MAGE gene family (5), as well as the melanocyte lineage-specific antigens MART-1/Melan-A (6, 7), gp100 (8), and tyrosinase (9). Cytotoxic T lymphocytes generated against these antigens represent a potential clinical tool as well as reagents to study the cell surface moieties involved in tumor rejection (2).

Adoptive transfer of bulk populations of cytotoxic T cells recognizing tumor antigens can result in tumor regression and increased survival in autologous murine and human hosts (10–13). Cellular immune responses enriched with cells directed against defined immunodominant epitopes may improve the efficacy of these biotherapies. PBLs capable of specific cytolysis and cytokine secretion have now been induced against the gene products of MAGE-1 (14), MAGE-3 (15), and MART-1 (16). Clinical trials are planned using either adoptive transfer of epitope-specific cells or the insertion of these genes into viral vectors for active immunization.

Tumor-derived proteins are intracellularly processed and cleaved into antigenic peptides subsequent to presentation on the cell surface in the context of MHC class I molecules (4, 17). Since multiple peptides may be expressed on the cell surface, it would be desirable to elicit an immune response against a set of shared melanoma antigens (18–20) rather than a single epitope. Among these antigenic proteins, gp100 is a melanocyte lineage-restricted intracellular glycoprotein that can be presented on the cell surface in association with MHC molecules to T lymphocytes (8, 21). gp100 was recognized by four bulk tumor-infiltrating lymphocyte cell lines, all of which were associated with tumor regression in melanoma patients following adoptive transfer in conjunction with IL-2 (8). Several peptides generated from gp100 have recently been isolated and characterized (8, 22, 23). To assess the immune response to gp100 and its potential use as a target of immunotherapy, we evaluated whether the gp100-derived synthetic peptides G10476 (V-L-Y-R-Y-G-S-F-S-V) (23) or the nonamers G9280 (Y-L-E-P-G-P-V-T-A) (22), G9154 (K-T-W-G-Q-Y-W-Q-V), or G9209 (I-T-D-Q-V-P-F-S-V) (23) could elicit in vitro CTLs capable of recognizing melanoma cells. In this investigation, we report the generation of two G10476-specific, three G9280-specific, two G9154-specific, and one G9209-specific CTLs, derived from the PBLs of HLA-A2* melanoma patients, following weekly in vitro stimulation with peptide-pulsed autologous PBMCs. These lines demonstrated specific cytolytic activity and cytokine secretion (TNF-α and GM-CSF) when cocultured with relevant targets. They were successfully propagated in low-dose IL-2 (30 Cetus Units/ml) for over 3 months while maintaining their cytolytic properties. Melanoma-reactive CTLs generated using this approach may be useful in a multivalent regimen for the treatment of patients with melanoma.

MATERIALS AND METHODS

Cultured Cell Lines. All tumor cell lines were initiated in the Surgery Branch, National Cancer Institute, as previously described (24) by overnight incubation of minced, fresh tumor with a mixture of 0.1% collagenase type IV, 0.002% DNase type 1, and 0.01% hyaluronidase type V (Sigma Chemical Corp., St. Louis, MO) at 25°C. These established cultures are those with the designated suffix "mel." They were propagated as monolayers in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, 50 μM HEPES (pH 7.0), 10 μM glutamine, 250 units/ml penicillin-streptomycin, and 0.25 mg/ml gentamicin.

The Daudi lymphoma line served as a LAK cell target in chromium release assays.

LAK cells were harvested from normal buffy coats as described previously (25). They were propagated in RPMI 1640 plus 2% human AB serum, 1000 units/ml recombinant IL-2, plus the supplements listed above.
Generation of gp100 Peptide-specific CTLs. PBMCs were harvested from buffy coats or lymphphocytapheresis specimens of patients with malignant melanoma treated in the Surgery Branch, National Cancer Institute. Cells were isolated by separation over lymphocyte separation medium (Organon-Teknika Corp., Durham, NC), washed with Ca2+- and Mg2+-free HBSS, and used as fresh or cryopreserved samples. HLA typing of PBMCs from tumor-bearing patients was performed by the NIH HLA Typing Laboratory using the modified Amos microcytotoxicity assay (27).

On day 0, CTL cultures were established in 24-well plates from the PBMCs of patients 1, 2, 3, and 4 by plating 1.5 × 106 cells/ml in 2 ml IMEM (Biofluids, Inc.) supplemented with 10% heat-inactivated human AB serum, as well as 50 μM HEPES, 10 mM glutamine, 250 μM penicillin-streptomycin, 50 μg/ml gentamicin, and 30 IU/ml recombinant IL-2 (kind gift from Chiron Corp., Emeryville, CA). All cultures were established in the presence of 1 μg/ml peptide, the exception being 5 μg/ml G9260 added to the PBMCs of patient 3. On day 3, 50 IU/ml IL-2 were added to the cultures. Lymphocytes were harvested weekly for restimulation, washed once in IMEM, then replated at a concentration of 2-3 × 106 cells/ml in 24-well plates. These responders were restimulated with autologous PBMCs that had been thawed, washed twice, and pulsed with 1 μg/ml peptide (or 5 μg/ml G9260, in the case of PBMCs from patient 3) for at least 3 h at 37°C in 15-ml conical tubes (5-ml volume). During this time the cells would be gently resuspended every hour to prevent clumping, and viability was not affected.

Stimulators were subsequently irradiated with 3000 rad using a 137Cs source, washed once with IMEM, and added to each well at stimulator-responder ratios ranging between 2:1 and 10:1 (1-ml volume each). The next day 30 IU/ml IL-2 were added to the cultures, and weekly restimulation followed by addition of cytokines 1 day later was continued weekly. CTL activity was initially assessed of cultured CTL or LAK effectors. Targets exhibiting less than 50% viability served as a negative control.

Phenotypic Analysis. Flow cytometry of CTLs was performed using a FACScan (Becton Dickinson, Mountain View, CA), as described previously (27). Cells were stained with murine antihuman mAbs against CD3 (Leu-4), CD4 (Leu-3), CD8 (Leu-2), TCR-a/β, and CD25 (IL-2 receptor a) (all from Becton Dickinson). Isotype-matched murine antibodies (IgGl and IgG2a) served as a negative control.

Cytotoxicity Assays. Target cells (1 × 106) were labeled with 200 μCi 51Cr (New England Nuclear, Boston, MA) in 0.5 ml RPMI 1640 with 2% human AB serum for 2 h at 37°C; then washed three times before the addition of cultured CTL or LAK effectors. Targets exhibiting less than 50% viability were centrifuged over a lymphocyte separation medium to remove dead cells, and only samples with at least 70% viability were used. Incubation of target and effector cells was carried out for 4 h at 37°C. Supernatants were collected and counted on a gamma counter. Percentage of specific lysis was calculated as: (sample counts – spontaneous counts/maximum counts – spontaneous counts) × 100%. Spontaneous release was calculated from targets incubated with RPMI 1640, maximum release from targets incubated with 2% SDS. All assays were performed in triplicate. Spontaneous counts did not exceed 30% of maximum 51Cr labeling.

Cytokine Release. CTLs prepared for cytokine analysis were cultured for at least 2 days without IL-2, then washed twice to further eliminate IL-2 or other cytokines. CTLs (5 × 104/ml) were incubated with an equivalent number of irradiated tumor stimulators in IMEM plus 10% human AB serum for 24 h at 37°C as described previously (28). Established cell lines were harvested by exposure to 0.05% trypsin plus 0.02% EDTA (trypsin/ Versene; Biofluids), then washed twice. Supernatants were centrifuged at 2000 rpm to remove cells, then stored at -70°C until use. CTLs added to OKT3-coated plates for 24 h served as a positive control for the TNF-α and GM-CSF assays. Targets incubated in the media alone without coculturing with effector cells served as a measure of spontaneous release.

All cytokine determinations were performed using commercially available ELISA kits: TNF-α (Quantikine Human TNF-α Immunoassay; R & D Systems, Minneapolis, MN; minimum detectable concentration = 5 pg/ml) and GM-CSF (Quantikine Human GM-CSF Immunoassay; R & D Systems; minimum detectable concentration = 2 pg/ml).

Peptides. G105-76 (V-L-Y-R-Y-G-G-S-F-S-V), G9260 (Y-I-E-P-G-P-V-T-A), G9144 (K-T-W-G-Q-Y-W-Q-V), or G9260 (I-T-D-Q-V-V-P-F-S-V) peptides were generated with a peptide synthesizer (model AMS 422; Gibco Laboratories, Inc., Worthington, OH; >90% purity) using a solid-phase technique. MART-127-35 peptide (A-A-G-I-G-I-L-T-V) was synthesized by Peptide Technologies, Inc. (Gaithersburg, MD). The influenza matrix protein M1,22-30 (G-I-L-G-F-V-F-T-L), derived from the influenza matrix protein M1, was synthesized by Multiple Peptide Systems (San Diego, CA). All are presented in the context of HLA-A2 (29).

Patient Histories. All patients had metastatic melanoma. Histological diagnoses were confirmed by pathologists at the National Cancer Institute. All protocols were approved by the Clinical Research Committee of the National Cancer Institute. Patients signed an informed consent prior to treatment. All but patient 6 received immunotherapy including IL-2 at the dose of 720,000 IU/kg i.v. every 8 h for up to 5 days (30), either alone or in conjunction with other agents such as IFN-α or chemotherapy. To be eligible for treatment, patients were required to have evaluable metastatic melanoma, good performance status, and no evidence of ischemic heart or brain metastases. Each course of therapy was divided into two cycles separated by approximately a 10-day recovery interval; either cycle treatment was pushed to dose-limiting toxicity. Objective partial response to therapy was categorized as a greater than 50% decrease of the sum of the perpendicular diameters of all lesions lasting at least 1 month without progression of any tumor sites. Complete response consisted of the disappearance of all evaluable disease. Patient 6 underwent one course of chemotherapy consisting of cis-platinum, dacarbazine, tamoxifen, and IFN-α before the harvest of PBMCs used for this study. All of the other patients had at least one course of IL-2-based therapy before the harvest of the PBMCs.

RESULTS

Growth Kinetics and Characterization of CTLs Reactive to gp100-derived Peptides. Growth curves for CTLs from patients 1 to 6 are shown in Fig. 1. Total weeks of stimulation varied because of the
cells were cryopreserved. For some patients, the availability of autologous PBMCs was assessed at 4 weeks of culture, but at that time only PBMCs from patient 1 grew in culture over 120 days, after which all cells were cryopreserved for CD3+/CD8+ and CD3+/CD4+-activated TCR-a/ß-expressing T cells, and that such findings are largely independent of major populations of CD8+ CTLs (66%) in the bulk population. These data suggest that the CTLs generated with such a protocol contain nearly equal populations of CD3+/CD8+ and CD3+/CD4+-activated TCR-a/ß-expressing T cells, and that such findings are largely independent of the experimental conditions, as evidenced by HLA-DR staining. Interestingly, the percentages of CD8+ and CD4+ cells were nearly equivalent. When the peptide used to stimulate bulk cultures was gp100-derived, there was little variation in the cytometric profile of CTLs. In one only instance, PBMCs of patient 2 stimulated with MART-127-35, was there a majority of CD8+ CTLs (66%) in the bulk population. These data suggest that the CTLs generated with such a protocol contain nearly equal populations of CD3+/CD8+ and CD3+/CD4+-activated TCR-a/ß-expressing T cells, and that such findings are largely independent of the PBMCs or gp100-derived peptide used. All cytolytic and cytokine data shown in this report used CTLs harvested within 2 weeks of the phenotypic analysis shown above.

Flow cytometric characterization of bulk CTLs from patients 1, 2, 3, 4, and 5 stimulated by gp100-, MART-1-derived peptides was performed on day 55 (Table 1). For every patient and condition, CTLs generated were at least 94% CD3+, with 89% or more of that population consisting of TCR-a/ß+ T cells. Activation markers were expressed by greater than 83% of cells, regardless of the experimental conditions, as evidenced by HLA-DR staining. Interestingly, the percentages of CD8+ and CD4+ cells were nearly equivalent. When the peptide used to stimulate bulk cultures was gp100-derived, there was little variation in the cytometric profile of CTLs. In one only instance, PBMCs of patient 2 stimulated with MART-127-35, there was a majority of CD8+ CTLs (66%) in the bulk population. These data suggest that the CTLs generated with such a protocol contain nearly equal populations of CD3+/CD8+ and CD3+/CD4+-activated TCR-a/ß-expressing T cells, and that such findings are largely independent of the PBMCs or gp100-derived peptide used. All cytolytic and cytokine data shown in this report used CTLs harvested within 2 weeks of the phenotypic analysis shown above.

**Table 1 Flow cytometric characterization of gp100-specific bulk CTLs**

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</table>

* Percentage of positive cells.

Different supply of autologous PBMCs for weekly restimulations. Maximum expansion for patient CTLs varied widely from less than 10-fold (patient 4) to over 100-fold (patient 1) during 7–10 weeks, and also exhibited differential expansion of the same patient CTLs stimulated with different synthetic epitopes. Expansion of PBMCs stimulated with the M158p65 influenza peptide was in the intermediate range compared to those stimulated with the other synthetic tumor epitopes (data not shown). After 6–10 weeks some bulk CTLs became quiescent, doubling less than once per week, and were cryopreserved at that time. After 10 weeks, those bulk CTLs still expanding were also cryopreserved at that time. At least two separate series of in vitro stimulations of bulk PBMCs were performed for each patient and peptide combination. These demonstrated similar cell growth except in one instance: during a second series of in vitro stimulations, PBMCs from patient 1 grew in culture over 120 days, after which all cells were cryopreserved. For some patients, the availability of autologous PMBCs to serve as peptide-pulsed stimulators limited the duration of culture. Since we were unable to elicit gp100-, MART-1-, or M1-specific CTLs from the PBMCs of patient 6, this patient is not discussed further.

**Flow cytometric characterization of bulk CTLs from patients 1, 2, 3, 4, and 5 stimulated by gp100-, MART-1-derived peptides was performed on day 55 (Table 1). For every patient and condition, CTLs generated were at least 94% CD3+, with 89% or more of that population consisting of TCR-a/ß+ T cells. Activation markers were expressed by greater than 83% of cells, regardless of the experimental conditions, as evidenced by HLA-DR staining. Interestingly, the percentages of CD8+ and CD4+ cells were nearly equivalent. When the peptide used to stimulate bulk cultures was gp100-derived, there was little variation in the cytometric profile of CTLs. In one only instance, PBMCs of patient 2 stimulated with MART-127-35, was there a majority of CD8+ CTLs (66%) in the bulk population. These data suggest that the CTLs generated with such a protocol contain nearly equal populations of CD3+/CD8+ and CD3+/CD4+-activated TCR-a/ß-expressing T cells, and that such findings are largely independent of the PBMCs or gp100-derived peptide used. All cytolytic and cytokine data shown in this report used CTLs harvested within 2 weeks of the phenotypic analysis shown above.

Generation of CTLs Demonstrating HLA-A2-restricted Lysis of Targets Expressing gp100-derived Peptides. After about 7 weeks of culture, CTLs from patients 1, 2, 3, 4, and 5 demonstrated strong specific cytosis against a wide range of gp100-expressing targets. These data are summarized in Figs. 2 and 3. Cytolytic activity of bulk PBMCs stimulated in vitro with synthetic peptides was first assessed at 4 weeks of culture, but at that time only PBMCs from patients 1, 2, 3, 4, and 5 pulsed only with synthetic peptides MART-127-35 or M158p65 demonstrated specific cytotoxic activity (greater than 10% lysis of relevant compared to control targets at all effector:responder ratios tested; data not shown). Initially, we tested reactivity to T2 cells pulsed with various antigens and two cultured melanoma clones (624.38mel and 624.28mel, Fig. 2). CTLs from patient 1 displayed lysis of relevant targets at least 3-fold greater than that observed against nonspecific targets when either G10476, G92800 or MART-127-35 was used to stimulate the cultures (Fig. 2a). Recognition of the cultured melanoma clone 624.38mel (which expresses gp100, MART-1, and HLA-A2) but not clone 624.28mel (which has lost HLA-A2 expression but expresses gp100 and MART-1; Ref. 16) indicated that patient CTLs recognized gp100+ targets in a HLA-A2-restricted manner. CTLs from patient 1 stimulated with M158p65 displayed peptide specificity in a HLA-A2-restricted fashion, yet with higher background that is not uncommonly observed. As expected, M158p66-specific cells from patient 1 did not lyse the (HLA-A2+, M1+) clonal line 624.38mel. Compared to CTLs from patient 1, specific cytolyis was stronger using CTLs from patient 2 stimulated in vitro with any of the four synthetic peptides: percentage lysis of 624.28mel and T2 pulsed with irrelevant peptide was always 3- to 5-fold lower at any E:T ratio (Fig. 2b). Nonspecific lysis against 624.28mel and T2 pulsed with irrelevant peptide was also minimal (never exceeding 19% at the highest E:T ratio) using CTLs from patient 3 (Fig. 2c). Lysis of M158p66-pulsed T2 cells by specific CTLs was evident, although the weakest of the three patient CTLs (Fig. 2c). Anti-G9154 CTLs demonstrating similar specificity could be generated from both patients 4 and 5, and anti-G9280 CTLs were obtained from patient 4 (Fig. 2, d and e). For patients 4 and 5, we used only M158p66 as a control for the ability to generate peptide-specific CTLs from PBMCs, and therefore did not attempt to generate anti-MART-127-35 CTLs. Therefore, for patients 1, 2, and 3, those CTLs stimulated with MART-127-35 lysed only T2 pulsed with relevant peptide as well as the HLA-A2+, MART-1+ melanoma cell line 624.38mel. CTLs from the five patients stimulated with M158p66 only T2 pulsed with relevant peptide. This was expected since none of the established cell lines tested naturally present M158p66.

Our cytotoxicity data were only interpretable if the lysability of nonspecific targets was confirmed. Accordingly, we included LAK cells as a nonspecific effector in all of the assays shown in Fig. 2. LAK cell lysis of nonspecific targets (T2 alone or pulsed with irrelevant peptide, 624.28mel, and Daudi) ranged from 33 to 52% for CTLs from patients 1 and 2, 14–49% for CTLs from patient 3, 31–45% for CTLs from patient 4, and 14–30% for CTLs from patient 5 at a E:T ratio of 25:1 (data not shown).

To further establish the specificity of HLA-A2-restriction of four of the five patient CTLs, we tested them for cytolytic activity against a panel of established cell lines (Fig. 3). These included melanoma lines 526mel and 952mel (gp100+, HLA-A2+), 397mel (gp100+, HLA-A2+), WM115 (gp100+, HLA-A2+), RM (HLA-A2+, breast tumor cell line), and Daudi, the Burkitt’s B cell lymphoma cell line (LAK control for MHC-unrestricted activity). As shown in Fig. 3, CTLs stimulated with G10476 or G92800 displayed significant cytotoxic activity against the gp100+, HLA-A2-expressing cell lines 526mel and 952mel. Patient 5 CTLs stimulated with G9244 displayed significant cytotoxic activity against the gp100+, HLA-A2-expressing cell lines 526mel, but were not tested against

* M. Salgaller and F. Marincola, unpublished observations.
Fig. 2. Cytolytic activity against T2 pulsed with synthetic peptides or melanoma cell lines by CTLs generated by weekly stimulation with gp100-derived, peptide-pulsed autologous PBMCs. Epitopes given above each graph represent the synthetic peptide used to stimulate each bulk of PBMCs. a. CTLs from patient 1 stimulated with G1047, G9, and M1. b. CTLs from patient 2 stimulated with G1047, G9, MART-17-35, and M1. c. CTLs from patient 3 stimulated with G9, MART-17-35, and M1. d. CTLs from patient 4 stimulated with G9, and M1. e. CTLs from patient 5 stimulated with G9 and M1. For patients 1 to 3, targets included T2-pulsed with G1047 (•) and G9 (•). For patients 4 and 5, targets included T2-pulsed with G9 (•) and G9 (•). For patients 1 to 4, targets also included MART-17-35 (Δ); for patients 1 to 5, targets also included M1 (○), or T2 alone (●). Established clonal lines HLA-A2* gp100*624.38mel (○) and HLA-A2* gp100*624.28mel (○), as well as the NK-sensitive Daudi lymphoma line (○) were assayed with all CTLs as well. Results of a second assay were similar.

952mel. For CTLs from patient 1, 2, and 5, cytolytic activity against the gp100* cell lines as well as HLA-mismatched targets was always minimal (less than 13% at any E:T ratio). Even the highest nonspecific lysis observed, that of patient 3 anti-G929p CTLs versus 397mel and WM115 at 25:1, was still 3-fold less than that of relevant targets. Lack of lysis versus RM, an HLA-A2* breast tumor cell line, suggests that the effect was melanoma specific. Sufficient anti-G929p CTLs from patient 5, as well as anti-G9154 and anti-G929p CTLs from patient 4, did not exist to test them against this panel of established cell lines. For CTLs raised against the MART-17-35 or M1 cell lines was always minimal (less than 5% or 7%, respectively; data not shown).

For the data shown in Fig. 3, we again included LAK cells as a nonspecific effector in all experiments to demonstrate lysis activity of nonspecific targets. LAK cell lysis of nonspecific targets (397mel, WM115, RM, and Daudi) ranged from 49 to 65% for CTLs from patients 1 and 2, 21-49% for CTLs from patient 3, and 31-46% for CTLs from patient 5 at an E:T ratio of 25:1 (data not shown).
RECOGNITION OF MELANOMA ANTIGEN BY LYMPHOCYTES

Fig. 3. HLA-A2 and gp100 expression are required for the cytolytic activity of G10476- and G9280-restricted CTLs. Panel 1, CTLs from patient 1 stimulated with G10476 panel 2, CTLs from patient 1 stimulated with G9280; panel 3, CTLs from patient 2 stimulated with G10476 panel 4, CTLs from patient 2 stimulated with G9280 panel 5, CTLs from patient 3 stimulated with G9280 and panel 6, CTLs from patient 3 stimulated with G9154. Established cell line targets included 952mel (●; HLA-A2*, gp100+), 526mel (●; HLA-A2*, gp100+), 397mel (●; HLA-A1*, gp100+), WM115 (●; HLA-A2*, gp100+), RM (●; HLA-A2* breast tumor), and Daudi (●). Results of a second assay were similar.

CTLs Demonstrate Cytokine Secretion in a Peptide-specific, HLA-A2-restricted Manner. Significant titers of the cytokines GM-CSF and TNF-α (Table 2) were released following 24-h cocultivation of G10476- or G9280-specific CTLs with HLA-A2+, gp100* melanoma cell lines. All G10476 CTLs were tested at day 51, and all G9280 CTLs at day 60 of culture. Although the greatest secretion by CTLs was observed when coincubated with peptide-pulsed T2 cells, high levels of cytokine were also released following coincubation with melanoma cell lines, presumably presenting the relevant peptide less abundantly on the cell membrane. As with the cytotoxycity data, there was no cross-reactivity between CTLs stimulated with either of the two gp100-derived peptides. Cytokine release of CTLs stimulated with T2 plus irrelevant peptides was not higher than that caused by T2 alone. Cytokine secretion was not observed in gp100-specific CTLs coincubated with T2 pulsed with the irrelevant peptides MART-127–35 and M138–66. However, bulk CTLs from patients 1, 2, and 3 stimulated with MART-127–35 demonstrated specific cytokine release when cocultured with T2 cells pulsed with MART-127–35 relevant peptide as well as MART-1* established cell lines (data not shown). Also, bulk CTLs from these three patients generated against influenza peptide showed specific cytokine release when cocultured with T2 pulsed with M138–66 (data not shown). Sufficient anti-gp100 CTLs from patients 4 and 5 did not exist to test them for cytokine secretion. All bulk CTLs contained large percentages of both CD8* and CD4* cells, and since we did not separate the cells into subsets it was not possible to determine which population was responsible for the observed effects.

We used the G10476 and G9280 synthetic peptides studied in this

Table 2. Melanoma patient CTLs exhibit gp100-peptide- and HLA-A2-specific GM-CSF and TNF-α secretion (pg/ml/24 h)$^a$

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$^a$ Measurement of GM-CSF in supernatants from CTLstimulator coincubations, as determined by specific double-determinant ELISA.

$^b$ Assays were performed concurrently with control supernatants from targets without CTL cocultivation. Background cytokine secretion resulting from targets alone has been subtracted from experimental values. A second assay using the same supernatant harvests yielded comparable results.

$^c$ Below detection level of assay (<8 pg/ml).

$^d$ Cell lines from HLA-A2-positive patients are designated A2; those with any other allele are designated non-A2. gp$^+$, gp100 expression; gp$^-$, lack thereof, as determined by reverse transcriptase/PCR.

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RECOGNITION OF MELANOMA ANTIGEN BY LYMPHOCYTES

Fig. 4. Lysis by gp100 peptide-specific CTLs of T2 cells pulsed with increasing concentrations of G10476 or G921I. In all cases, the data shown are for the lysis of G10476- or G921I-pulsed targets by CTLs specifically generated against that corresponding peptide. CTLs analyzed include patient 2 anti-G1047 ( ), patient 1 anti-G1047 ( ), patient 2 anti-G92 ( ), patient 1 anti-G92 ( ), and patient 3 anti-G92 ( ). CTLs used were at day 55 of culture.

We used autologous PBMCs as antigen-presenting cells, pulsed with 1 μM synthetic peptide and cultured in low-dose IL-2 to generate G10476-restricted CTLs from the PBMCs of two of four melanoma patients (patients 1 and 2), G92-restricted CTLs in three of four melanoma patients (patients 1–3), G92-restricted CTLs from the PBMCs of two of two melanoma patients (patients 4 and 5), and G92-restricted CTLs from the PBMCs of one of two melanoma patients (patient 4). In only one instance was a higher peptide concentration (5 μM) required to generate peptide-specific responses: G92-specific cells were generated twice from the PBMCs of patient 3 when 5 μM peptide was used, whereas the bulk CTLs remained nonspecific when 1 μM was used (data not shown). Three of four patient CTLs (patients 1–3) developed MART-1-restricted activity when in vitro stimulated with this peptide. MART-1-restricted served as a tumor-associated peptide control, since it is an immunodominant epitope expressed on normal and tumor cells of melanocytic lineage and was recognized by most HLA-A2-restricted tumor-restricted TILs from melanoma patients (16). PBMCs of patients 1–5 also developed influenza matrix peptide M1-restricted bulk CTLs when stimulated in vitro with this peptide. This highly immunogenic viral antigen served as a control for the overall ability of PBMCs to respond to the in vitro sensitization techniques we have used.

The lysis of melanoma cells by gp100 peptide-specific CTLs suggests the presence of these peptides on the surface of melanomas. PBMCs from patient 1 at day 55 of culture were used as antitumor vaccines (35, 36).

PBMCs from patient 1 showed specific cytotoxicity to gp100 at 28 days of culture, 1–2 weeks before this was demonstrated with CTLs of patients 2–5 (data not shown). This is interesting in light of the fact that patient 1 was the only objective responder to immunotherapy. Similar in vitro stimulation studies using MART-1 epitopes indicate that the genesis of MART-1 specificity of CTLs can be reproducibly detected after as early as 2 weeks of culture (16). This is not unexpected since the MART-1 peptide is recognized by most HLA-A2-restricted, melanoma-specific CTLs produced from TILs (6, 8), and thus is likely more abundant on the cell surface. Yet it is possible that if patients are less anergic or tolerized to the less ubiquitous G92 than G10476, MART-1-restricted cell lines presumably expressing G92 and G10476 peptides less abundantly. MART-1-restricted secretion has been associated with tumor regression in humans (38). Because of the lack of sufficient PBMCs from patients 4 and 5, we were not able to culture enough cells for cytokine analysis. Although we wanted to limit this study to an autologous setting, recent work has demonstrated that allogeneic reagents can elicit MART-1-specific CTLs from human PBMCs (39).

The peptide titration data were in agreement with the recent work of Kawakami et al. (23), which demonstrated that G92 and G10476 were intermediate-to-high binding peptides, and thus capable at low concentrations of sensitizing T cells to cytolytic bulk capable of recognizing both epitopes. Recently, it has been confirmed that HLA-peptide interactions demonstrating high affinity strongly correlate with the ability to sensitize PBMCs with synthetic peptides (40). Our findings are in general agreement, but it is difficult to make

DISCUSSION

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Highly immunogenic M1 influenza peptide, which suggests an overall lack of responsiveness. The PBMCs from this patient were collected during (rather than following) a course of chemotherapy, and only patient 6 did not receive an IL-2-based regimen. This suggests that prior treatment should be taken into consideration when deciding when to harvest bulk PBMCs for in vitro sensitization.

With regard to the work of Rivoltini et al. (16) with MART-1 peptides, we have been able to produce peptide-restricted CTLs in most patients analyzed using PBMCs as antigen-presenting cells, precluding the need for enriched antigen-presenting cells such as EBV-B cells (14), superantigen-stimulated B cells (15), or acid-stripped PBLs. This comparatively simple method can more readily be followed by others to investigate the immunogenic potential of the increasing number of tumor-associated molecules being isolated and characterized. Culture conditions like those reported here using low-dose IL-2 (30 IU/ml) reduce the likelihood of activating nonspecific LAK or NK cells, as has been often observed when using the 6000 IU/ml IL-2 concentration used to propagate TILs long term (31). Previously, similar conditions have been used to elicit epitope-specific CTLs against mutated p21 ras (32, 33), chimeric bcrl abl (33), and proto-oncogenes such as HER-2/neu (34). However, unlike these earlier reports, this study demonstrates the capacity to generate peptide-specific CTLs recognizing nonmutated self-epitopes already shown to be recognized by TILs which mediated in vivo tumor regression (23).

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firm conclusions since we did not test a large panel of low-, medium-, and high-affinity peptides. However, it can be noted that in vitro sensitization with the low-affinity gp100-derived peptide G10457 (L—D—G—T—A—L—R—L; 483 nm G104, required for 50% inhibition of a standard radiolabeled peptide) could not elicit antigen-specific CTLs in any of four melanoma patient PBMCs attempted. These data support the use of initial screenings of candidate peptides based on binding motifs and MHC affinity to select candidate peptides whose higher likelihood of generating CTL immunoreactivity makes them more potentially useful for immunotherapy.

Great variation was observed in the degree of expansion of cultures, although all bulk CTLs grew successfully in low-dose IL-2 for up to 4 months. The greatest expansion was observed with PBMCs from patient 1, the only patient in this report to respond to immunotherapy. Currently, we are attempting to optimize growth conditions so that adoptive transfer of both MART-1- and gp100-specific CTLs will be possible. Some groups have reported growth enhancement of cultures using IL-7 (41) or IL-12 (42, 43) in place of or in addition to IL-2. Alternative conditions such as these could be studied if the present methodology is unable to repeatedly produce the $10^{10}$–$10^{11}$ CTLs required for adoptive immunotherapy.

To have relevance for clinical treatment, it is essential that any tumor-associated peptide occur on the surface of tumor cells in vivo in sufficient abundance to serve as a potential immunotherapeutic target. The G92K peptide, isolated from the gp100 protein, was identified and characterized from naturally processed peptides presented by HLA-A2.1 and was recognized as an immunodominant epitope by CTLs derived from five melanoma patients (22). The ability of epitope-specific CTLs, such as those generated against MART-1, to lyse and secrete cytokine when cocultivated with uncultured melanoma cells (16) lends further credence to the possible use of such cells in adoptive transfer.

Finally, it may be important to produce peptide-specific CTLs from more than one epitope of a tumor-associated antigen if the cells are to be used for therapy because of potential HLA- and antigen-loss variants. Diminution or complete loss of HLA class I expression in melanomas has been described (44), and can sometimes be attributed to loss of a genomic fragment (45). Stimulated CTLs might overcome the ability of variants to remain undetected by secreting TNF to lyse in vivo, eradicate disseminated leukemia and provide specific immunologic memory. J. Immunol., 138: 104–108, 1987.


Recognition of Multiple Epitopes in the Human Melanoma Antigen gp100 by Peripheral Blood Lymphocytes Stimulated \textit{in Vitro} with Synthetic Peptides


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