Enhancement of Cell-mediated Immunity in Melanoma Patients Immunized with Murine Anti-Idiotypic Monoclonal Antibodies (MELIMMUNE®) That Mimic the High Molecular Weight Proteoglycan Antigen

Michael W. Pride, Steve Shuey, Antonio Grillo-Lopez, Gary Braslawsky, Merrick Ross, Sewa S. Legha, Omar Eton, Antonio Buzaid, Constantine Ioannides, and James L. Murray

Departments of Bioimmunotherapy [M. W. P., J. L. M.], Immunology [M. W. P., C. I.], Surgery [M. R.], Melanoma-Sarcoma [S. S. L., O. E., A. B.], and Gynecologic Oncology [C. I.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030; and IDEC Pharmaceuticals, San Diego, California 92121 [S. S., A. G-L., G. B.]

ABSTRACT

The purpose of this study was to determine whether a combination of two anti-idiotypic antibodies that mimic the high molecular weight proteoglycan antigen found on most melanoma tumors was capable of enhancing cellular immunity in vaccinated high-risk patients with melanoma. Twenty-eight stage I-IV high-risk patients with melanoma were immunized with a mixture of variable concentrations of MELIMMUNE-1 and MELIMMUNE-2, along with the adjuvant SAF-m, using two immunization schedules. Peripheral blood mononuclear cells were collected before the first immunization and 4 weeks after the final immunization and tested for in vitro proliferation to MELIMMUNE-1 and MELIMMUNE-2 and for cytotoxicity against 51Cr-labeled target cell lines.

Additionally, supernatants from in vitro proliferation cultures were tested for interleukin 10 and IFN-γ levels. Significant in vitro proliferation to MELIMMUNE-1 and MELIMMUNE-2 were observed in postimmunization samples but not in prevaccination samples. The mean stimulation index for MELIMMUNE-2 (33.7 ± 0.6) was significantly higher than that for MELIMMUNE-1 (13.9 ± 0.3; P < 0.025). Supernatants obtained from 78% of the in vitro stimulated cultures pre- or postvaccination contained significant levels of interleukin 10 (range, 0.43–142 pg/ml), whereas IFN-γ levels were elevated in 53% of postvaccination samples (range, 3–245 pg/ml) but not prevaccination samples. More importantly, we were able to generate specific CTL responses in 43% of the patients, which correlated with elevated IFN-γ levels. These results indicate that MELIMMUNE enhances cell-mediated immunity in patients with melanoma.

INTRODUCTION

Active specific immunotherapy is being used more frequently in treatment of patients with malignant melanoma, with the intent of stimulating an endogenous immune response against melanoma-associated antigens on the tumor cells. Melanoma cancers are excellent candidates for this type of vaccine development because these tumors express well-characterized differentiated antigens and peptides in association with specific class I epitopes. Many patients with melanoma have immune reactions to their tumors, and some of these responses are to shared antigens [1, 2]. Many of the antigens described to date are restricted by the class I allele HLA-A2, which is found in 30–40% of Caucasians.

One immunotherapeutic approach uses tumor-derived material (whole cell or cell lysate) as the immunogen. In some cases, the tumor-associated antigen is unknown or extremely difficult to obtain and purify in sufficient quantities. An alternative approach to the use of tumor antigens or tumor cells is anti-id Abs. The immune network theory proposed by Jerne (3) predicts the appearance of several types of anti-id Abs during the immune response to a given antigen. The subset of “internal image” anti-id Abs (Ab2s) has been proposed to be antiparasitic and to mimic the molecular features of the original antigen (4, 5). This working hypothesis is based on the concept that certain homologous or analogous molecular motifs of the anti-id sequence can mimic specific immunogenic epitopes of the infectious organism, thereby inducing a protective immune response (4, 5). Ab2s have been used in various experimental systems as surrogate vaccines against specific tumors, as well as bacterial, viral, and parasitic organisms (reviewed in Refs. 6–8).

A clinical trial has been completed at M. D. Anderson...
Cancer Center and several other institutions in which patients were treated with a mixture of two monoclonal anti-id Abs [MELIMMUNE-1 (Mel-1) and MELIMMUNE-2 (Mel-2); the mixture is referred to as MELIMMUNE] that mimic epitopes on the melanoma-associated high molecular weight proteoglycan, also referred to as HMPG (9). HMPG is present on 80–95% of all malignant melanomas, both primary and metastatic, and is only minimally expressed on normal tissue (10–11). HMPG is important because it may influence the biology of the tumor (adhesion, migration, and proliferation) by acting as a receptor for components of the extracellular matrix (collagen, fibrinogen, laminin, and other proteoglycans) and various circulating molecules (12–16). Therefore, it seems reasonable to assume that tumor growth could be inhibited or impaired if HMPG function were hindered. This notion was supported by Reisfeld et al. (12), who demonstrated that Abs to proteoglycan antigens have inhibitory effects on the early events of cell spreading on a basement membrane synthesized by endothelial cells.

Until recently, attempts to purify or produce HMPG for use as an immunogen from cell culture or by recombinant DNA technology have been largely unsuccessful. Therefore, an anti-id strategy was adopted to induce an anti-HMPG immune response in humans. In previous preclinical studies, injection of MELIMMUNE into animals was shown to induce Abs (anti-anti-id Abs; Ab3s) that were capable of binding HMPG tumor cells. More importantly, these anti-anti-id Abs significantly reduced lung metastases when they were administered to severe combined immunodeficient mice that were inoculated iv. with human melanoma cells.5 In Phase I trials, it was shown that administration of MELIMMUNE at doses of 2 mg, administered with the adjuvant SAF-m, were well tolerated (17). A pilot comparative Phase Ib trial of Mel-2 at three different doses (without adjuvant) suggested that a lower dose of 0.5 mg may be as effective as higher doses.5 Objectives of the current Phase Ib clinical trial were: to compare different immunization schedules of a 1:1 composition of Mel-1 and Mel-2, administered with SAF-m, in patients with resected melanoma who had no evidence of metastatic disease; to determine whether toxicity occurred; and to determine whether MELIMMUNE could induce cellular immune responses and whether these responses were dose dependent. In this study, we demonstrate that PBMCs obtained from patients immunized with MELIMMUNE could proliferate in vitro in response to Mel-1 and Mel-2 and could additionally generate CTL responses in 43% of patients who appeared to express the major histocompatibility complex HLA-A2 allele.

### MATERIALS AND METHODS

#### Patient Selection. This study was an open-label randomized Phase Ib multicenter trial. Peripheral blood was obtained from 32 patients with melanoma who met the following criteria: resected American Joint Committee on Cancer stage III or IV disease, stage II disease with primary tumor being ≥4 mm thick, or recurrent metastatic disease; surgery within 4 months of treatment; Karnofsky performance of ≥70%; adequate hepatic, renal, and bone marrow function; and expected survival of >4 months. All subjects gave written, informed consent for participation, and all details of this study were approved by the clinical investigation committees of M. D. Anderson Cancer Center and all other participating centers. Patients had to have undergone surgical resection at least 2 weeks before study entry and could not be on any concomitant adjuvant treatment program.

#### Treatment Plan. The vaccination schema consisted of two schedules (Table 1). Schedule A patients were subdivided into three groups. Patients in this group received escalating concentrations of a 1:1 ratio of Mel-1 and Mel-2, mixed with a single concentration of SAF-m administered as five separate i.m. injections. The SAF-m adjuvant consisted of temurtide mixed with an emulsion solution containing squalene, polysorbate 80, and copolymer 401. Group B patients received six injections of vaccine containing 2 mg each of Mel-1 and Mel-2, combined with SAF-m. The final injection volume for all groups was 1 ml.

#### Study Parameters. Routine prescreening studies and blood sampling to assess cellular immune response were performed before the first immunization and 4 weeks after the last injection. Blood counts and chemistries were performed before every other vaccination.

#### Abs and Cell Lines. Mel-1 and Mel-2 are murine monoclonal anti-id Abs (Ab2s; IgG1) that bind to the murine anti-HMGP monoclonal Abs 225.28 and MEM136, respectively (Ab1s), and mimic separate epitopes on HMPG (18). The anti-id Abs were developed and purified from ascites fluid by sequential precipitation and caprylic acid and ammonium sulfate, as described (19). Ab purity was monitored by SDS-PAGE. An anti-id Ab reactive against a B-cell lymphoma idiotype (IgG1) was used as an isotype control throughout this study (supplied by IDEC Pharmaceuticals, San Diego, CA). F(ab')2 fragments were produced using a commercially available kit (Pierce, Rockford, IL). BB7.2, an Ab that binds to HLA-A2 (American

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\(^5\) J. Merritt, unpublished data.

### Table 1: Vaccination schedule

<table>
<thead>
<tr>
<th>Treatment group no.</th>
<th>No. of Patients</th>
<th>Mel-1 (mg)</th>
<th>Mel-2 (mg)</th>
<th>Termurtide (mg)</th>
<th>Total no. of doses</th>
<th>Schedule</th>
<th>Treatment days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>5</td>
<td>A</td>
<td>1, 8, 43, 99, 155</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
<td>5</td>
<td>A</td>
<td>1, 8, 43, 99, 155</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>2.0</td>
<td>2.0</td>
<td>0.1</td>
<td>5</td>
<td>A</td>
<td>1, 8, 43, 99, 155</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>2.0</td>
<td>2.0</td>
<td>0.1</td>
<td>5</td>
<td>B</td>
<td>1, 15, 29, 43, 99, 155</td>
</tr>
</tbody>
</table>
Type Culture Collection, Manassas, VA), was purified from culture supernatants.

Due to the lack of autologous tumor cell lines for use as targets in CTL assays, donors were screened for HLA-A2 positivity, and A375sm (HMPG+/HLA-A2+; donated by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, TX), a highly metastatic melanoma cell line, served as our HLA-A2+ melanoma tumor target. SKOV3-A2 (donated by Dr. I. G. Iannides), an HLA-A2-transfected ovarian cell line, served as a specificity control. HS294t is a HMPG+/HLA-A2- melanoma cell line (American Type Culture Collection). K562 is an NK-sensitive cell line. All tumor cell lines were cultured in complete RPMI 1640 containing 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 10% heat-inactivated fetal bovine serum (CELLect Gold; ICN Biomedicals, Aurora, OH).

Preparation of Human PBMCs. PBMCs were obtained from patients before the first immunization and 4 weeks after their last immunization (day 185). Blood samples were drawn in heparinized tubes and sent from the various study centers to Houston by overnight Federal Express service at ambient temperature. Upon delivery, PBMCs were separated from heparinized whole blood by Ficoll-metrizoate Histopaque-1077, (Sigma Chemical Co., St. Louis, MO). Interface cells were collected and washed in Dulbecco’s PBS (magnesium- and calcium-free; Life Technologies, Inc., Grand Island, NY) three to five times to remove excess platelets. The cells were resuspended in 10% DMSO-90% fetal bovine serum and stored in calcium-free; Life Technologies, Inc., Grand Island, NY) three to five times to remove excess platelets. The cells were resuspended in 10% DMSO-90% fetal bovine serum and stored in liquid nitrogen until all samples were collected. Upon thawing, cell viability ranged from 70 to 90%.

Proliferation Assay. PBMCs were cultured in 96-well flat-bottomed plates (Costar, Cambridge, MA) at a concentration of 2.5 × 10^5 cells/well per 100 μl. All cells were cultured in complete RPMI 1640 containing 10 μg/well F(ab’)2 fragments of Mel-1 or Mel-2, control Ab, or 1 μg/ml PHA. PBMCs were cultured for 6 days in a 37°C, 5.5% CO2, 94.5% air incubator. The results were expressed as mean ± SE percentage fluorescence and mean fluorescence intensity.

Statistical Methods. Statistical comparisons were made between preimmunization and postimmunization values using the paired t test. Differences between patient subsets were examined using Student’s t test for independent means as well as χ^2 analysis.

RESULTS

Patients. Thirty-two of 36 patients entered on this study received either five or six injections of MELIMMUNE. Four patients failed to complete all injections and were excluded from analysis. Three patients were classified as stage I by American Joint Committee on Cancer criteria, 19 were classified as stage II, 7 were classified as stage III, and 2 were classified as stage IV. One patient was unclassified. All had had tumors resected 2–8 weeks before treatment. Six patients received injections of MELIMMUNE at 0.1 mg each, 10 received 0.5 mg, and 16 received 2.0 mg (Table 1).

Binding of Abs to Tumor Cell Lines. Both A375 and HS294t melanoma cell lines stained positive for HMPG (91 ± 3.6% and 62 ± 2%, respectively). In addition, A375 was highly positive for HLA-A2 (100 ± 0.1%), whereas HS294t was negative (<1%). Fifty-six ± 13% of SKOV3-A2 cells stained positively for HLA-A2 but were negative for HMPG.

Lymphocyte Proliferation to Mel-1 and Mel-2. In 29 of 32 patients, pre- and postimmunization PBMC samples were collected for various assays. Three samples had inadequate numbers of viable PBMCs for all studies. Lymphocyte proliferation to PHA from patients prior to vaccination ranged from 37,308 to 216,285 cpm. Results of the lymphocyte proliferation assay demonstrated that the mean proliferative response of PBMCs to F(ab’)2 fragments of Mel-1 or Mel-2 from prevaccination samples (1416 ± 42 and 2309 ± 56, respectively) was not significantly different from background (1206 ± 62) or PBMCs incubated with control anti-id F(ab’)2 (1060 ± 51). In
contrast, proliferative responses of PBMCs obtained 4 weeks after patients’ last immunization that were cultured in vitro with Mel-1 (15,206 ± 346) or Mel-2 (34,755 ± 888) were significantly higher than the medium alone (1,036 ± 25), negative control anti-id Ab (1,700 ± 46.6; $P \leq 0.0001$), or preimmunization samples. When expressed as a SI, Mel-2 had a significantly higher mean SI (33.7 ± 0.6) than that with Mel-1 (13.9 ± 0.3; $P \leq 0.025$; Fig. 1). The SI for control anti-id Ab was 1.6 ± 0.02 (Fig. 1).

We were interested in determining whether there were any differences in the proliferative responses to Mel-1 or Mel-2 between patients who remained disease free 4–6 months after the last vaccination versus those who were alive with recurrent disease and those who had subsequently died on study. Information on 26 patients was available; three patients were lost to follow-up. Of these 26 patients, 14 remained in continuous disease-free remission, 6 were alive with recurrent disease, and 6 had subsequently died. Although there were no differences in proliferative responses of PBMCs obtained from patients with either no evidence of disease or those who were alive with recurrent disease to either MEL-1 (16.8 ± 0.5 versus 13.2 ± 0.3) or Mel-2 (37.5 ± 1.1 versus 32.3 ± 0.7; $P > 0.05$), there was a significantly lower response to each Ab in PBMCs from patients who subsequently died compared to the other two groups (Mel-1 = 5.5 ± 1.2, $P \leq 0.01$; Mel-2 = 18 ± 3.2, $P \leq 0.025$). There were no significant differences in lymphocyte proliferation based on initial patient stage or the amount of MELIMMUNE administered ($P > 0.05$).

Cytokine Levels. IFN-γ and IL-10 levels (Th1- and Th2-derived cytokines, respectively) were measured in supernatants from PBMCs obtained from patients before and after immunization that had been incubated in vitro 48 h with Mel-1, Mel-2, or control Ab. Values that were $\geq 1.2$ pg/ml were considered significant. Seventy-seven % of patient’s PBMCs produced elevated IL-10 levels in response to any in vitro stimuli, including irrelevant control anti-id Ab. Levels varied considerably (range, 1.7–127), resulting in large SEs. Due to this variation, no significant differences were seen between IL-10 levels in cultures stimulated with Mel-1, Mel-2, or control anti-id Abs ($P > 0.05$).

Data analyzed for IFN-γ secretion were more homogeneous (Fig. 2). Fifty-three % of all samples obtained postvaccination, regardless of subgroup, had IFN-γ levels that were $\geq 1.2$ pg/ml. Postvaccination levels of IFN-γ were significantly higher than prevaccination levels for both Mel-1 (33.9 ± 3.0 pg/ml postvaccination versus 1.8 ± 0.3 pg/ml prevaccination; $P \leq 0.001$) and Mel-2 (30.3 ± 2.3 pg/ml postvaccination versus 1.2 ± 0.1 pg/ml prevaccination; $P \leq 0.025$; Fig. 2). There were no significant differences in IFN-γ levels from postvaccination samples stimulated with either anti-id Ab (Fig. 2). Eleven of 24 patients, who had no evidence of relapse (7 patients) or who were alive with either recurrent disease (4 patients), had elevated IFN-γ levels after vaccination, whereas only 1 of 6 deceased patients had elevated levels ($P \leq 0.005$, $\chi^2$ analysis; data not shown).

Cell-mediated Cytotoxicity. Because autologous tumor tissue was unavailable for this trial, we tested the ability of PBMCs from 28 patients to lyse the melanoma cell line A375. Several previously described melanoma peptides have been demonstrated to be recognized in association with the class I HLA-A2 allele (20, 21). We analyzed patient PBMCs and tumor cells for HLA-A2 positivity.

Patients were divided into three groups based on their characteristic CTL responses. Group 1 ($n = 11$) had virtually no cytotoxicity (i.e., $<1\%$) against A375 or the control cell lines SKOV3-A2 or K562 (data not shown). Three of these 11 patients were HLA-A2+. Group 2 ($n = 8$) demonstrated significant killing of A375 cells after vaccination but not before and no
ate nonspecific killing was also seen against SKOV3-A2 and tumor cells both before and after vaccination (Fig. 3B). The two patients in group 3 who were HLA-A2 had cytotoxicity against control cell lines (Fig. 2). As shown in Fig. 4, lysis of A375 cells was significantly higher for HLA-A2 patients than HLA-A2 patients (P < 0.005, χ²). The remaining patients had no measurable levels of either cytokine.

### DISCUSSION

Human trials using anti-id Abs to stimulate immunity against tumors have shown promising results (reviewed in Refs. 7 and 8). Anti-id Abs also offer the advantage of being a pure and abundant supply of the immunogen and of possibly being more immunogenic than the original antigen. There is, however, a paucity of data in human systems that demonstrate the induction of antigen-specific cytotoxic T cells by anti-id Abs.

The major findings of this study were: (a) both Mel-1 and Mel-2 were capable of stimulating in vitro proliferative responses in vaccinated patients; (b) cytotoxicity against a HMPG⁺/HLA-A2⁺ melanoma cell line was greater in patients who were HLA-A2 matched; and (c) there was a significant correlation between IFN-γ production and specific cytotoxicity. The proliferative responses observed in our studies were specific and not due to Fc receptor cross-linking because no responses were elicited using an isotype matched control anti-id and (Fab')₂ fragments of the MELIMMUNE anti-id Abs were used as in vitro stimuli.

This study represents one of the first to examine T-cell cytotoxicity in patients with melanoma who were immunized with anti-id Abs. Several studies using anti-id Abs as vaccines in patients have demonstrated lymphocyte proliferative responses to anti-id Abs and, in the case of carcinoembryonic antigen, purified antigen (22, 23). Specific antitumor responses that correlated with T-cell infiltrates in tumors, and enhanced CTL and NK activity against autologous tumors have been observed in colorectal cancer anti-id Abs (24, 25). Several studies demonstrating Ab responses to MELIMMUNE and anti-id Abs that mimic HMPG have been published (17, 26, 27). Mel-2 has also been shown to induce higher Ab3 Ab titers than Mel-1 (28). An interesting finding in our study was that the proliferative response to Mel-2 was significantly higher than that to Mel-1. Patients were also examined to determine whether there was any correlation between lymphocyte SIIs and/or proliferation versus Ab titers. Unfortunately, no correlation was seen.

We also demonstrated that patients who subsequently died at 4–6 weeks after vaccination had significantly lower proliferative responses to both anti-id Abs. This finding could simply represent the development of anergy among a subset of patients, although these patients were generally healthy adults before the study because responses to PHA were within normal range. Unfortunately, PHA responses were not consistently measured posttreatment. Hence, whether this finding has prognostic significance can only be definitively answered in a randomized trial.

In over 75% of patients, IL-10 levels were elevated in...
supernatants from both pre- and postimmunization PBMCs and in samples incubated with control anti-id. The meaning of these findings is uncertain; the data imply that spontaneous release of IL-10 is occurring in PBMCs from unvaccinated patients. Becker et al. (29) described two T-cell clones that produced high amounts of IL-10, after being rendered anergic by autologous melanoma cells. Because IL-10 is a Th2 cytokine that can inhibit antigen-specific Th1 and Th2 responses, as well as promote Ab production (30–32), these findings may have relevance with respect to this and other vaccine trials. It is conceivable that the ratio of IFN-γ to IL-10 may be a determinant of Th1- or Th2-predominant responses, which might explain why HLA-A2 patients had higher or lower CTL activity due to higher or lower ratios of IFN-γ to IL-10.

The finding that elevated IFN-γ levels after vaccination correlated with MHC-class I specific cytotoxicity is relevant because the release of IFN-γ by Th1 helper lymphocytes enhances CTL responses. Hence, elevated levels of IFN-γ may help to induce T cell-restricted killing of melanoma cells.

Specific T-cell cytotoxicity against A375 was observed in
~12 of 28 (43%) of patients after treatment, and 9 of the 12 donors were HLA-A2+. Caution must be used in interpreting the data in our study. Other studies in patients with melanoma have demonstrated that several melanoma peptides (MART-1 and gp100) can induce specific killing of melanoma in association with HLA-A2 (20, 21). Mel-2 may stimulate T cells, which can recognize distinct peptide sequences within the Ab V-region that mimic HMPG-specific HLA-class I-specific recognition, as has been seen as with anti-id Abs against carcinoembryonic antigen (33), or Mel-2 may induce T-cell precursors that recognize other melanoma-associated peptides/antigens on A375. CTL experiments against a variety of melanoma cells expressing well-known melanoma antigens need to be performed. At this juncture, there is no firm evidence that a peptide derived from the idiotype is actually stimulating a class I response. Additional studies are ongoing in this regard.

Preliminary data from our laboratory in two HLA-A2+ patients demonstrate partial blocking (40–50%) of CTL activity by Abs to MHC class I and class II (data not shown). In these experiments, cells were cultured and restimulated with Mel-2 over several weeks, resulting in complete elimination of NK activity. PBMCs from these patients were also incapable of lysing HLA-A2+ melanoma cells. These data may be supportive of target recognition in association with both MHC class I and class II molecules. Although we have not definitively identified the CTL target antigen, cytolytic CD4+ cells restricted by HLA-DR15 have recently been described in melanoma (34). Studies using model antigens have demonstrated that immunization with soluble antigen can induce both proliferative and CTL responses (35). The CTL recognizes processed peptides through a phagosome-to-cytosol presentation pathway (35). Because the sequence of HMPG has recently been published (36), additional studies need to be done using purified antigen or relevant peptides selected for class I and class II epitopes.

In summary, MELIMMUNE immunization resulted in enhancement of specific effector mechanisms in 43% of high-risk patients with melanoma. Additional randomized Phase III studies are required to prove whether immunization with MELIMMUNE results in specific T-cell induction against autologous tumors and whether vaccination improves relapse-free and overall survival in these patients.

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2370 Cell-mediated Responses to Anti-Idiotype Antibodies


Enhancement of cell-mediated immunity in melanoma patients immunized with murine anti-idiotypic monoclonal antibodies (MELIMMUNE) that mimic the high molecular weight proteoglycan antigen.


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