Impact of Cytokine Administration on the Generation of Antitumor Reactivity in Patients with Metastatic Melanoma Receiving a Peptide Vaccine

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Patients with metastatic melanoma were immunized with an immunodominant peptide derived from the gp100 melanoma-melanocyte differentiation Ag that was modified to increase binding to HLA-A*0201. A total of 10 of 11 patients who received the g209–2M peptide alone developed precursors reactive with the native g209 peptide, compared with only 5 of 16 patients who received g209–2M peptide plus IL-2 (p = 0.005). Peptide reactivity closely correlated with the recognition of HLA-A*0201 melanoma cells (p < 0.001). The decrease in immune reactivity when peptide was administered with IL-2 appeared specific for the immunizing peptide, since reactivity to an influenza peptide resulting from prior exposure was not affected. Preexisting antitumor precursors did not decrease when peptide plus IL-2 was administered. The administration of GM-CSF or IL-12 also resulted in a decrease in circulating precursors compared with the administration of peptide alone, though not as great a decrease as that seen with IL-2. Immunization with peptide plus IL-2 did, however, appear to have clinical impact since 6 of the 16 patients (38%) that received peptide plus IL-2 had objective cancer regressions. It thus appeared possible that immunization with peptide plus IL-2 resulted in sequestering or apoptotic destruction of newly activated immune cells at the tumor site. These represent the first detailed studies of the impact of immunization with tumor peptides in conjunction with a variety of cytokines in patients with metastatic cancer. The Journal of Immunology, 1999, 163: 1690–1695.

In murine tumor models, the stimulation of potent T cell responses against tumor Ags is often capable of protecting animals against tumor challenge and can sometimes mediate the regression of established experimental cancers. Thus, attempts to develop effective immunotherapies for the treatment of patients with cancer have centered on developing methods for generating high levels of T lymphocyte responses against Ags presented on tumor cells.

The recent identification of the genes encoding human tumor Ags has opened new possibilities for the development of immunization approaches capable of stimulating T lymphocyte reactivity against tumors (1, 2). Tumor infiltrating lymphocytes, whose administration was associated with tumor regression in vivo, were used to identify the genes that encoded Ags present on autologous melanomas (3, 4). The two predominant Ags identified in melanomas from HLA-A*0201+ patients were MART-1 and gp100, both melanoma-melanocyte nonmutated differentiation proteins (5, 6). The immunodominant peptides from these proteins were also identified (7, 8). Many of these peptides were of low to intermediate binding affinity to HLA-A*0201, and several synthetic peptides were identified containing substituted amino acids at anchor residues that bound more strongly to the HLA-A*0201 molecule. Replacement of threonine with methionine at the second position of the immunodominant peptide, gp100:209–217 (hereafter referred to as g209), appeared to be more immunogenic in vitro than the native peptide (9). Thus, this modified peptide, gp100:209–217(210M) (hereafter referred to as g209–2M) was selected for in vivo immunization studies in patients with metastatic melanoma.

We recently reported that the immunization of patients with the g209–2M peptide could generate potent T cell responses in patients against the native peptide and melanoma cells (10). The administration of this peptide, along with IL-2, in a pilot clinical trial mediated cancer regression in 13 of 31 (42%) of patients with metastatic melanoma. We have now extended these studies to perform a detailed immunologic analysis of the cellular immune reaction of patients receiving immunization with the g209–2M peptide plus IL-2. In addition, many animal models have suggested that the administration of IL-12 and GM-CSF could substantially increase the antitumor impact of immunization using vaccinia virus, adenovirus, or DNA-encoding model tumor Ags, presumably by increasing T cell–mediated immune reactions (11–14). IL-12 has been shown to enhance immunization with a p53 peptide in mice bearing the Meth A tumor expressing the p53 mutation (14). Similarly, in mouse models, tumors transduced to secrete GM-CSF are more immunogenic than nonsecreting tumors (15), and Jaeger et al. (16) have reported that GM-CSF administration in conjunction with peptide immunization can increase immune reactions in humans. We thus studied the clinical and immunologic consequences of immunization with the g209–2M peptide in conjunction with the systemic administration of IL-12 or GM-CSF in patients with metastatic melanoma.
Materials and Methods

Clinical protocol

All patients immunized in this study had biopsy-proven metastatic melanoma. The majority of patients were between the ages of 30 and 60 years, and all underwent clinical evaluation of tumor sites by physical examination and radiologic studies. All patients were confirmed to be HLA-A*0201-positive by high resolution nested sequence PCR subtyping, and all signed an informed consent before treatment began.

The modified peptide, gp100:209–217 (210M) with the sequence IMDQVPFSV, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA). The identity of the peptide was confirmed by mass spectral analysis, and the peptide was shown to be >98% pure as assessed by high pressure liquid chromatography analysis. A total of 1.5 mg peptide in 1.5 ml water was mixed with an equal volume of IFA (Montanide ISA-51, Seppic, France) and vortexed vigorously on a vortex mixer for 12 min to form an emulsion. Two aliquots of 1 ml each were injected in the s.c. tissue of the anterior thigh for a total peptide injection of 1 mg. Injections of peptide in IFA was given every 3 wk. Before the initial peptide injection and following two injections, all patients were leukopheresed, and PBMC were cryopreserved. At 6 wk and at regular intervals thereafter, the clinical status of patients was assessed by repeat physical and radiologic examination. In this analysis, 11 patients were treated with the g209–2M peptide alone (these patients were reported in Ref. 10). Sixteen consecutive patients were treated with I.IL-2, 14 consecutive patients were treated with peptide plus I.IL-12, and 13 consecutive patients were treated with peptide plus s.c. GM-CSF.

Patients received IL-2 (Cetus-Oncology Division, Chiron, Emeryville, CA) at a dose of 720,000 IU/kg (corresponding to 120,000 Cetus U/kg) administered in 50 ml of normal saline containing 5% human serum albumin as an i.v. bolus over 15 min, starting either 1 day (11 patients) or 5 days (5 patients) after the peptide injection. Patients received IL-2 every 8 h until grade 3 or 4 toxicity was reached that could not be easily reversed by standard supportive measures (generally, 6–10 doses). After the second consecutive cycles of peptide plus IL-2, some subsequent cycles involved peptide alone if additional time for recovery from IL-2 side effects was required. All patients received concomitant medications, including acetaminophen (650 mg every 4 h), indomethacin (50 mg every 8 h), and ranitidine (150 mg every 12 h), to prevent some of the side effects associated with IL-2 administration.

Recombinant IL-12 was supplied by Genetics Institute (Cambridge, MA) and was given i.v. at a dose of 250 ng/kg over 10–20 s. For the first four patients, a test dose of IL-12 was given and was followed 2 wk later by the s.c. injection of 1 mg peptide in IFA followed by five daily i.v. doses of 250 ng/kg IL-12. These five IL-12 doses were then repeated after each subsequent peptide injection. The next 10 patients received 1 mg peptide in IFA along with the test dose of IL-12, and then, 2 wk later, began two cycles of 1 mg peptide in IFA followed by five daily doses of IL-12 at 250 ng/kg i.v. All patients received acetaminophen 650 mg orally every 4–6 h while on therapy, and indomethacin 50 mg orally every 6 h was used to treat persistent (severe) symptoms.

GM-CSF was supplied by Immunix (Seattle, WA) and was injected immediately distal to the site of the peptide injection at a dose of 100 mcg (seven patients) or 500 mcg (six patients) daily for 6 days, starting 3 days before the peptide injection and ending 2 days after the peptide injection.

In vitro assessment of immunologic reactivity to the g209–2M peptide and melanoma

Cryopreserved PBMCs obtained before and 3 wk after two immunizations were thawed and simultaneously tested. A total of 3 × 106 cells were suspended in 2 ml complete medium consisting of IMDM with 25 mM HEPES buffer, 10% heat-inactivated human AB serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biofluids, Rockville, MD; Sigma, St. Louis, MO; Pel-Freez, Brown Deer, WI), and 1 μM g209–2M peptide was added to the culture. Two days later, IL-2 was added to the medium, and every 3–4 thereafter, half of the medium was withdrawn and replaced with fresh complete medium containing IL-2. To determine the optimal conditions for this assay, two different concentrations of IL-2 were evaluated: 30 IU IL-2/ml and 300 IU IL-2/ml. Cultures were harvested at varying times between days 4 and 18, and lymphocyte reactivity was tested in a final volume of 0.2 ml by mixing 105 harvested PBMC incubated with g209-2M peptide in either 30 or 300 IU IL-2/ml; cells harvested at varying days and assayed vs peptide-pulsed T2 cells or tumor cells.

Abbreviation used in this paper: S.I., stimulation index.
Results

Immunization with g209–2M peptide in IFA resulted in the generation of circulating immune precursors that was decreased by the administration of IL-2

We previously reported that 10 of 11 patients immunized with the g209–2M peptide developed circulating lymphocytes capable of recognizing the native g209 peptide (10). Table II provides a characteristic experiment of three patients (1–3) who received the peptide alone and three patients (4–6) who received the peptide plus high-dose IL-2. None of the patients exhibited reactivity to the 209 peptide before immunization. All three patients who received peptide alone developed reactivity to the native g209 peptide following immunization, and none of the three patients who received i.v. IL-2 following the peptide developed this reactivity. A summary of the development of specific immune reaction against the native g209 peptide after immunization for all patients in this trial is shown in Fig. 1. The S.I. (specific reactivity as measured by IFN-γ secretion against T2 cells pulsed with the 209 peptide, compared with T2 cells pulsed with the 280 peptide) was 2 or greater for 10 of 11 patients receiving the g209–2M peptide in IFA, compared with an S.I. of 2 or greater in only 4 of 11 patients who received the g209–2M peptide in conjunction with IL-2, starting 1 day after peptide injection ($p_1 = 0.02$). The highest S.I. of 84 in a patient receiving peptide plus IL-2 occurred in the only patient in the entire series who exhibited specific reactivity to the native g209 peptide prior to any immunization and thus is probably not representative of the other patients in this study. Because of the possibility that the administration of IL-2 shortly after peptide injection might result in activation-induced apoptosis of lymphocytes, a second cohort of five patients was treated with peptide immunization, followed 5 days later by the administration of IL-2. Only one of five of these patients developed significant reactivity (S.I. ≥ 2) against the native g209 peptide. Thus, compared with 10 of 11 patients developing reactivity following injection of the g209–2M peptide alone, only 5 of 16 patients developed significant reactivity when IL-2 was administered ($p_2 = 0.005$). PBMC that did not react to the 209 peptide, however, did contain cellular immune reactivity against an influenza peptide, demonstrating that these patients were immunocompetent and had cells capable of responding to a prior natural challenge with influenza (examples in Table III, patients 2, 4, and 5).

Patients who demonstrated reactivity against the native g209 peptide also exhibited reactivity against HLA-A2*+, but not HLA-A2−, melanomas (examples in Table III, patients 1 and 3). The reactivity of PBMC against peptide was closely correlated with the ability of these cells to respond to HLA-A2+ tumors in 41 independent cultures from multiple patients ($p < 0.001$) (Fig. 2).

The marked decrease in the generation of circulating immune precursors when peptide injections were administered with IL-2 appeared to be directly due to the IL-2 administration and not to the innate immune competence of the patients treated. Thus, five patients who were treated with peptide injection in conjunction with IL-2 who did not develop immune precursors were subsequently treated with peptide alone without IL-2 and developed significant recognition of the g209 peptide (examples in Table IV, patients 1 and 2).

One possible explanation for the decrease in immune precursors when IL-2 was administered with peptide was the migration of immune cells from the peripheral blood into lymph node or other

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Table II. Immunization with 209-2M peptide in IFA either alone or followed by the administration of IL-2

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<th>Patient</th>
<th>IL-2a</th>
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<th>(0.01 μM)</th>
<th>(.0001 μM)</th>
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<td>40</td>
<td>62</td>
<td>98</td>
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</table>

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FIGURE 1. Patients were immunized with g209–2M peptide in IFA either alone or with IL-2, IL-12, or GM-CSF, as described in the text. Each dot represents a single patient. PBMC were obtained after two immunizations and assayed for reactivity as assessed by IFN-γ secretion against the native g209 peptide and a control g280 peptide. Data are presented as the ratio of reactivity to g209 peptide compared with g280 peptide.

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tissues following the administration of IL-2. To test this hypothesis, a series of patients were treated with peptide alone to develop immune precursors and then received the subsequent injection of peptide in conjunction with IL-2. These patients developed immune precursors capable of recognizing the g209 peptide after the administration of peptide in the absence of IL-2, and, when they subsequently received the peptide with IL-2, there was no diminution in the ability to detect circulating precursors, indicating that IL-2 did not mediate the loss of these specific memory cells from the circulation. An example of two patients so treated is shown in Table IV, patients 3 and 4.

The administration of IL-12 or s.c. GM-CSF also led to a decrease in immune precursors when administered in conjunction with the g209–2M peptide

Immune reactivity in PBMCs was also tested before and after immunization with the g209–2M peptide administered with either i.v. IL-12 or s.c. GM-CSF (Fig. 1). IL-12 was administered in conjunction with the g209–2M peptide, and no diminution in antipeptide precursors was seen. Patients then received at least two injections of peptide alone, and no immune precursors were easily detected in PBMC 21 days later. This was a lower percentage of patients successfully immunized, compared with patients receiving 209–2M peptide alone (10 of 11; p = 0.04). Patients receiving IL-12 had a median S.I. of 2, compared with a median S.I. of 26 for patients receiving the 209–2M peptide alone. Similarly, of 13 patients immunized with g209–2M peptide in GM-CSF, only 4 achieved an S.I. of 2 or greater (p = 0.005, compared with g209–2M peptide alone) with a median S.I. of 5.

Clinical response to treatment

No objective clinical responses (partial or complete) were seen in the 11 patients treated with the g209–2M peptide alone. Of the 16 patients in this analysis that received peptide plus IL-2, 6 (38%) responded to peptide injection plus IL-2. There were no objective clinical responses in the 14 patients treated with peptide plus IL-12 or the 13 patients treated with peptide plus GM-CSF.

Table IV. Sequential immunization of patients with g209-2M peptide with or without IL-2

![FIGURE 2](image-url)

**FIGURE 2.** Patients were immunized with g209–2M peptide in IFA. Reactivity of PBMC from four patients were tested multiple times for specific reactivity against the g209 peptide and the HLA-A*0201 501 mel cell line. Reactivity against peptide and tumor as highly correlated (p < 0.001).
Discussion

In our prior studies, we demonstrated that immunization with the g209–2M peptide, a gp100 peptide modified to increase binding to HLA-A*0201, could mediate the generation of antipeptide and antitumor T cell precursors in the circulation of patients with metastatic melanoma (10). These precursors manifested both cytolytic and cytokine-secreting activity after brief reexposure to peptide in vitro culture. Although no objective clinical responses were seen when the g209–2M peptide alone was administered, 42% of 31 patients with metastatic melanoma treated with peptide plus the administration of IL-2 either primarily or after prior exposure to peptide alone exhibited objective tumor regression. The 42% response rate compares favorably with the anti-tumor effects of peptide alone (no responses) or with the response rate of high-dose IL-2 alone (15%) in a cohort of 182 similar patients treated in the Surgery Branch (17) or the response rate (15%) in a separate cohort of 62 similar melanoma patients treated with high-dose IL-2 plus viral vaccines during the same interval as the study reported here. While the actual contribution of the peptide to the antitumor activity of IL-2 remains to be determined in a randomized trial, the preliminary data generated in our trial strongly suggests that peptide immunization increased the effectiveness of IL-2. To understand the mechanisms underlying the clinical observations, in the present study, we have examined the immunologic consequences of IL-2 administration on the generation of antitumor T cell precursors and have extended our observations to the treatment of patients with the g209–2M peptide plus either IL-12 or GM-CSF, two additional cytokines reported to enhance T cell activity in animal models (11–14).

While the administration of IL-2 in addition to peptide was capable of mediating clinical tumor regression in many patients, the addition of IL-2 beginning 1 or 5 days after peptide administration significantly reduced the ability to detect antipeptide precursors in the peripheral circulation, compared with patients who received peptide alone (and had no clinical responses). This discordance between the development of antitumor precursors and clinical response is not clearly understood. The antitumor immune precursors detected in the in vitro assay from patients who received peptide alone were apparently not in an activation state sufficient to recognize tumor, since these patients exhibited no tumor regression and PBMC freshly isolated and uncultured did not recognize tumor in this assay or in a sensitive enzyme-linked immunospot assay (data not shown). The in vivo exposure of sensitized cells to tumor or normal cells expressing the gp100 Ag in the absence of costimulatory signals may have induced an anergic state in these lymphocytes (18). Incubation with peptide for at least 4 days in vitro, presumably to allow for peptide presentation on APC that expressed appropriate costimulatory molecules, was required to activate the cells to manifest peptide and tumor recognition. The administration of IL-2 may have supplied the necessary signals to activate cells newly sensitized by peptide in IFA to recognize tumor. But why then were immune precursors not detected in PBMC of patients receiving peptide plus IL-2?

The administration of IL-2 did not lead to the general elimination of the ability to detect memory T cells in the circulation, since the peripheral cells of patients receiving peptide plus IL-2 that had no activity against the g209 peptide were fully capable of generating immune reactivities to influenza peptide, which presumably were present from prior environmental exposure to influenza (Table III, patients 2, 4, and 5). The inability to develop peptide reactivity in patients given peptide plus IL-2 was also not an accident of patient selection, since the same patients who could not develop precursor reactivity when given peptide plus IL-2 were capable of generating normal antipeptide reactivity when subsequently administered peptide alone (Table IV). Thus, these patients appeared capable of generating new, as well as memory responses, though specific peptide reactivity was not detectable when peptide was given in conjunction with IL-2.

It also did not appear that the failure to detect precursors was due to the redistribution of antitumor memory cells from the circulation, since precursors could easily be detected in patients who received peptide plus IL-2 following the induction of detectable precursors due to the administration of peptide alone (Table IV). In addition, it did not appear likely that the administration of IL-2 inhibited the initial generation of antitumor precursors, since the administration of IL-2 beginning at day 1 or day 5 after peptide injection equally abrogated circulating precursors. Further, it appears likely that the administration of peptide plus IL-2 led to a substantial increase in objective clinical responses, compared with that expected from IL-2 alone, and the likelihood exists that this increase in responses was due to the enhancement of T cell reactivity to the peptide due to IL-2 administration.

Thus, since the administration of peptide plus IL-2 appears capable of mediating the generation of T cell precursors as evidenced by their ability to mediate antitumor responses and yet eliminates these precursors from the peripheral circulation, it appears likely that the combination of peptide administration plus IL-2 leads either to the destruction of T cells with antitumor reactivity at the tumor site once their effector function has been realized or to a sequestration of newly generated but not memory effector cells at the tumor site or elsewhere.

The apoptotic death of highly activated or IL-2-exposed lymphocytes upon engagement of their TCR has been described in several model systems and may be an important regulatory mechanism of active immune responses (19, 20). In mice with acute viral infection, stimulation of lymphocytes by specific Ag at the height of activation can result in the induction of apoptosis (20). In studies using the β-galactosidase model tumor Ag, restimulation with specific β-galactosidase peptide either in vivo or in vitro at the height of immunization can result in apoptotic elimination of specifically reactive cells (Bronte et al., submitted for publication). The timing of lymphocyte stimulation and restimulation, as well as the timing of exposure to IL-2, can have profound impact on the activation or suppression of specific immune reactions. Thus, in our clinical trial, lymphocytes that were sensitized to peptide in IFA and encountered Ag at the tumor site in the presence of IL-2 may be capable of manifesting their effector function (tumor recognition and destruction) but undergo apoptosis in the process. This hypothesis would explain why antitumor effects are seen in patients receiving peptide immunization plus IL-2 but that precursors cannot be detected in the circulation. It should be emphasized, however, that the explanation for a decrease in circulating precursors when IL-2 is administered after peptide vaccination remains speculative.

In our studies, contrary to the predictions resulting from studies of murine tumors (11–14), the administration of GM-CSF or IL-12 did not result in clinical antitumor responses, but also appeared to reduce the level of T cell precursors, although perhaps not as profoundly as seen with IL-2. In other clinical trials of the administration of IL-12 or GM-CSF alone to cancer patients, little, if any, antitumor effects have been seen. It is possible that the exact dose, schedule, and routes of administration of these cytokines may be critical, and it is possible that alterations in these parameters could lead to different results. The mechanism by which the administration of IL-12 and GM-CSF decreased lymphocyte precursors in the circulation is unclear.
It should be emphasized that the studies reported in this paper deal with immunization against nonmutated self-Ags. The presentation of these Ags on normal cells in the absence of costimulatory molecules might represent a profound difference in immune reactivity, compared with the immune reactivity generated against mutated Ags present only on tumors, as is the case in most animal models used to demonstrate the efficacy of IL-12 or GM-CSF administration. A critical need exists for the development of animal models directed against normal nonmutated differentiation Ags to explore the mechanisms involved in immunization, as well as to optimize the immunizing vectors, their modes of administration and adjuvant cytokine administration to help design effective immunotherapy strategies for patients with cancer.

References