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Identification of Endogenous HLA-A2–Restricted Reactivity Against Shared Melanoma Antigens in Patients Using the Quantitative Real-Time Polymerase Chain Reaction

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Summary

This study was conducted to determine whether reactivity to melanoma cells of pretreatment peripheral blood mononuclear cells (PBMCs) from patients with metastatic melanoma correlated with subsequent response to treatment with interleukin-2 (IL-2). The sensitivity of the quantitative real-time polymerase chain reaction (PCR) assay was optimized, including the total number of cells used (3×10^6 in 1 mL), the responder-to-stimulator cell ratio (5:1), the optimal time to incubate PBMCs with tumor (2 h), the appropriate tumor stimulators (melanoma cell lines differing only in the expression of histocompatibility leukocyte antigen [HLA-A2]), the duration of recovery in the culture of PBMCs after cryopreservation (18–24 h), and the medium used (Iscove, 10% human AB serum). Using this optimized assay to detect HLA-A2–restricted antitumor reactivity in the pretreatment PBMCs from patients with melanoma, positive reactive responses were detected in 7 of 28 patients with an objective clinical response to IL-2 therapy compared with 6 of 21 positive reactive responses in nonresponding patients. None of 12 healthy donors were positive in this study. Thus, there was no significant difference in the reactivity of pretreatment PBMCs when responders were compared with nonresponders, although the melanoma patients had an increased incidence of response compared with healthy donors ($p = 0.05$). The PBMCs from 11 of the 13 melanoma patients with pretreatment HLA-A2–restricted antimelanoma reactivity were tested against a panel of transfectants expressing known shared melanoma antigens. Anti-MART-1 reactivity was detected in the pretreatment PBMCs of three patients. It thus appears that some melanoma patients are immunologically primed to antigens expressed on the tumor surface, although the HLA-A2–restricted antimelanoma activity detected in this real-time PCR assay was not predictive of patients' responses to IL-2 therapy.

Keywords

Melanoma antigens; Histocompatibility leukocyte antigen; Immunotherapy; Quantitative real-time polymerase chain reaction

Approximately 15% of patients with metastatic melanoma have objective clinical responses to the administration of intravenous high-dose bolus interleukin (IL)-2 (1). The administration of IL-2 results in the development of vitiligo in patients with melanoma but not in those with metastatic renal cell carcinoma, suggesting that T cells from melanoma patients are presensitized to the antigens expressed by their tumors and their normal melanocytes (2). T cells that can recognize these melanoma–melanocyte antigens have been detected in the peripheral circulation of melanoma patients (3,4) and can be isolated from tumor-infiltrating lymphocytes. Several clinical studies have shown that melanoma patients can be immunized

with these antigens; 42% of melanoma patients achieved a clinical response to IL-2 administered in combination with an anchor-fixed, modified peptide from the melanoma antigen gp100 (5). Although IL-2 has been administered safely to thousands of persons, patients must be monitored in the hospital for IL-2-related toxicity (6).

No method exists to predict which patients will respond to treatment. We hypothesized that patients pre-sensitized to melanoma antigens might have an increased likelihood of responding to IL-2. To address this question, we designed a system to evaluate the ex vivo tumor reactivity of pretreatment peripheral blood mononuclear cells (PBMCs) from melanoma patients to shared melanoma antigens using the quantitative real-time polymerase chain reaction (PCR).

Several limitations made the study of pretreatment PBMCs problematic. Because it was unclear which antigens pretreatment lymphocytes might react against, we stimulated PBMCs with intact tumor cells rather than individual known melanoma antigens to broaden the repertoire of antigens tested. Because autologous tumor was often unavailable, we used allogeneic tumor lines that could enable the detection of shared antigens. Because tumor cells are relatively poor stimulators and express low levels of antigens and costimulatory molecules (7), and because the antigen-specific T-cell precursor frequency in naïve PBMCs was likely to be low, we needed to develop a sensitive and efficient assay that could be used to detect putative tumor reactivity. Most assays designed to address these questions require extended periods of in vitro stimulation, during which allogeneic reactivity can develop in the presence of tumor stimulators. For these reasons, we first optimized a rapid molecular assay for the quantitative real-time PCR to harness its amplification capability. The pretreatment PBMCs of clinical responders and nonresponders were cultured with allogeneic histocompatibility leukocyte antigen (HLA-A2)-matched tumor cell lines to determine whether a correlation existed between the pretreatment reactivity against tumor and a response to treatment.

MATERIALS AND METHODS

Cell Lines

Tumor cell lines 624.38 Mel (HLA-A*0201), 624.28 Mel (HLA-A2⁻), 888 A2 Mel (HLA-A*0201), and 888 Mel (HLA-A2⁻) were established in the Surgery Branch, National Cancer Institute (Bethesda, MD, U.S.A.). Cell lines 624.28 and 624.38 were derived from the same parental cell line 624 Mel and share a similar pattern of antigen and HLA allele expression, except for HLA-A*0201, which is expressed in 624.38 but not in 624.28 because of aberrant pre-mRNA splicing (8). Cell line 888A2 Mel was obtained by stable transfection of 888 Mel with HLA*A0201 cDNA (pCDNA3 plasmid; Invitrogen, Carlsbad, CA, U.S.A.). The 293A2 cell line was obtained by stable transfection of 293 cells, derived from primary human embryonal kidney cells, with the HLA*A0201 gene. All cell lines were maintained in continuous culture in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM nonessential amino acids, 10 mM Hepes, 100 U/mL penicillin-streptomycin, 2 mM L-glutamine, 50 µg/mL gentamycin sulfate, 250 µg/mL amphotericin, and 50 µM 2-mercaptoethanol. All reagents were obtained from Biofluids (Rockville, MD, U.S.A.), except for fetal calf serum and 2-mercaptoethanol, which were from Gibco-BRL (Rockville, MD, U.S.A.).

Clinical Protocols

All patients were HLA-A*0201 as determined by high-resolution nested sequence PCR subtyping, and all had histologically confirmed metastatic melanoma. All patients were treated in institutional review board-approved clinical trials in the Surgery Branch, National Cancer Institute using 720,000 IU/kg IL-2 administered as an intravenous bolus every 8 hours to tolerance, as previously described (1,5,6). IL-2 was administered in conjunction with various

vaccines in different trials, including recombinant viruses or peptides (5,9). Before treatment, all patients gave written informed consent and underwent a complete clinical evaluation, including measurement and radiographic imaging of all evaluable tumor sites. The patients had received no other treatment in the month before beginning immunotherapy, and no patients were receiving any immunosuppressive drugs, including steroids.

All known sites of disease were reevaluated 3–4 weeks after two cycles of IL-2 given at 3–4 weekly intervals. If patients had stable or regressing disease, additional treatments were administered and similar criteria were used to determine subsequent courses of treatment. If patients had progression of disease, no further therapy was administered. An objective clinical response was defined as a 50% or greater decrease in the sum of the products of the greatest perpendicular diameters of all known lesions, lasting at least 1 month and without increase in any tumor or the appearance of any new disease. Any patient who did not have an objective response was considered a nonresponder.

Before treatment, patients underwent leukapheresis. PBMCs were isolated by Ficoll-Hypaque separation and were cryopreserved at 10^8 cells/vial and stored at -180°C . Cryopreserved PBMCs from 28 melanoma patients with objective clinical responses to high-dose IL-2, from 21 clinical nonresponders, and from 12 healthy volunteers comprised the study material.

RNA Isolation and Complementary DNA Synthesis

RNA was isolated from preimmunization PBMCs stimulated with melanoma cells using RNeasy mini kits (Qiagen, Santa Clarita, CA, U.S.A.) according to the manufacturer's instructions. The RNA was eluted with water and stored at -70°C . For complementary DNA (cDNA) synthesis, approximately 1 μg total RNA was transcribed with cDNA transcription reagents (PE Bio-systems, Foster City, CA, U.S.A.) using random hexamers, according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction

Gene expression was measured in real-time with the GeneAmp 5700 Sequence Detection System (PE Biosystems). Primers and *TaqMan* probes (Custom Oligo Synthesis Service, Foster City, CA, U.S.A.) were designed to span intronic junctions to avoid amplification of genomic DNA and also to generate amplicons of fewer than 150 base pairs to enhance the efficiency of PCR amplification. Probes were labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein; emission $\lambda_{\text{max}} = 518 \text{ nm}$) and at the 3' end with the quencher dye molecule TAMRA (6-carboxytetramethyl-rhodamine; emission $\lambda_{\text{max}} = 582 \text{ nm}$). On amplification, sequence-specific probes annealed to the template were cleaved by the 5' nuclease activity of the *Taq* polymerase reaction. Real-time monitoring of fluorescent emission from cleavage of probes allowed definition of the cycle threshold during the exponential phase of the amplification. DNA standards were generated by PCR amplification of the gene product and quantitation by spectrophotometry. The number of copies was calculated based on the molecular weight of each gene amplicon. Real-time PCRs of cDNA samples and DNA standards were performed in a total volume of 25 μL with 1x *TaqMan* Master Mix (PE Biosystems), primers at 800 nM, and probes at 200 nM. Primer and probe sequences were as follows: interferon- γ (IFN- γ forward) (5')-AGCTCTGCATC-GTTTGGGTT; IFN- γ (reverse) (5')-GTTCCAT-TATCCGCTACATCTGAA; IFN- γ (probe) FAM-TCTTGGCTGTACTGCCAGGACCCA-TAMRA; CD8 (forward) (5')-CCCTGAGCAACTCCATCATGT; CD8 (reverse) (5')-GTGGGCTTCGCTGGCA; and CD8 (probe) FAM-TCAGCCACTTCGTGCCGGTCTTC-TAMRA. Thermal cycler parameters included 2 min at 50°C , 10 min at 95°C , and 40 cycles involving denaturation at 95°C for 15 seconds and annealing–extension at 60°C for 1 minute. Standard curves were generated for the gene of interest, IFN- γ and the endogenous reference gene, CD8. Standard curve

extrapolation of the copy number was performed for both. Sample data were normalized by dividing the number of copies of IFN- γ transcripts by the number of copies of CD8 transcripts.

Direct Molecular Assessment of Pretreatment Peripheral Blood Mononuclear Cell HLA-A2 Restricted Reactivity Against Intact Melanoma Cells

Cryopreserved preimmunization PBMCs were thawed and suspended in complete medium containing Iscove medium supplemented with 10% human AB serum (Biochemed, Winchester, VA, U.S.A.), 10 mM nonessential amino acids, 10 mM Hepes, 100 U/mL penicillin–streptomycin, 2 mM L-glutamine, 50 μ g/mL gentamycin sulfate, 250 μ g/mL amphotericin, and 50 μ M 2-mercaptoethanol. To optimize the conditions for assessing direct HLA-A*0201–restricted reactivity of PBMCs to melanoma cell lines, experiments were conducted using PBMCs obtained from patients that were shown, after immunization with an HLA-A*0201–restricted peptide, to have high levels of antimelanoma precursors by in vitro sensitization assays previously described (5). Responder-to-stimulator ratios were evaluated, as were total cell numbers, culture plate surface areas, harvest times, recovery time after cryopreservation, serum type, and tumor stimulators. Based on the results from the optimization assays, PBMCs were prepared for *TaqMan* in the following manner: 3×10^6 PBMCs were plated in 48-well plates in 500 μ L complete medium and incubated overnight. The next day, 6×10^5 melanoma cells were added to the PBMCs in 500 μ L complete medium and incubated for 2 h. The cells were harvested for RNA extraction and cDNA transcription. The quantitative real-time PCR was performed for IFN- γ mRNA expression normalized to CD8 mRNA expression from the same sample. The normalized IFN- γ mRNA value obtained by PBMC stimulation with an HLA-A*0201⁺ melanoma line was controlled for by comparison with that obtained by PBMC stimulation with a matched HLA-A2[−] melanoma control line.

Direct Molecular Assessment of Pretreatment Peripheral Blood Mononuclear Cell Reactivity Against 293A2 Cells Transfected With Known Melanoma-Associated Antigens

Plasmid DNA (2 μ g) encoding one of 10 of the following antigens, GFP, MART-1, gp100, tyrosinase, TRP-1, TRP-2–6b (a TRP-2 isoform), OA-1, P. polypeptide, SOX-10, or NY-ESO-1, were transfected into 293A2 cells with Lipofectamine 2000 (LF2000) reagent (Invitrogen). Briefly, 1.5×10^6 cells per well were plated in six-well plates on the day before transfection in 3 mL complete medium containing Iscove media supplemented with 10% human AB serum (Biochemed, Winchester, VA, U.S.A.), 100 U/mL penicillin–streptomycin, 2 mM L-glutamine, and 50 μ g/mL gentamycin sulfate. On the day of transfection, each plasmid DNA (2 μ g) was diluted into 250 μ L OPTI-MEM I medium (Life Technologies). For each well of cells, 10 μ L LF2000 reagent was diluted into 240 μ L OPTI-MEM I medium and incubated for 5 min at room temperature. After the LF2000 was diluted, it was combined with the diluted DNA and allowed to incubate for 20 min. During this time, old medium from 293A2 plates was removed and replaced with 2.5 mL fresh complete medium. After the incubation, the DNA-LF2000 mixture was added to the cells. The plates were incubated at 37°C in 5% carbon dioxide for 24 hours. The next day, the cells were harvested and added at 6×10^5 cells per well to 48-well plates containing 3×10^6 pretreatment PBMCs per well that had been plated on the preceding day and allowed to incubate at 37°C in 5% carbon dioxide overnight. The plates were centrifuged at 500g for 5 minutes before a 2-hour incubation at 37°C in 5% carbon dioxide. After incubation, the cells were harvested for an RNA extraction followed by reverse transcription and a quantitative real-time PCR assay to quantify IFN- γ mRNA normalized to CD8 mRNA. Transfection efficiency was assessed by visualizing the green fluorescent protein transfectants under the microscope, and positive controls were obtained using MART-1 reactive tumor-infiltrating lymphocytes and a gp100-specific T-cell clone against the MART-1 and gp100 transfectants, respectively.

Statistical Analyses

To determine specific responses to stimulation, mRNA for IFN- γ from PBMCs stimulated with HLA-A*0201 melanoma cell lines versus HLA-A2⁻ melanoma cell lines was detected by quantitative PCR. The IFN- γ mRNA copy number was first corrected for CD8 mRNA copy number. Data were adjusted for CD8 mRNA copies based on the assumption that stimulation with HLA-A2⁺ versus HLA-A2⁻ tumor cells limited CD8⁺ T cells as the only relevant population. A stimulation index was defined as the ratio of IFN- γ mRNA (corrected for CD8 mRNA) obtained for PBMCs stimulated with an HLA-A*0201 melanoma cell line to that obtained from PBMCs stimulated with a corresponding HLA-A2⁻ melanoma cell line. A stimulation index value ≥ 2 that was reproducible was considered evidence of preimmunization HLA-A2–restricted reactivity against shared melanoma antigens. The Fisher exact test was used to identify differences in results between groups.

RESULTS

Optimization of a Quantitative Real-Time Polymerase Chain Reaction Assay for Direct Assessment of Peripheral Blood Lymphocyte Reactivity

Gene expression was measured using the GeneAmp 5700 sequence detection system. This system allows real-time PCR monitoring of fluorescent emission from the cleavage of sequence-specific probes by the 5'-nuclease activity of *Taq* polymerase. At any given cycle within the exponential phase of amplification, defined as the cycle threshold (C_T), the amount of PCR product is proportional to the number of initial template copies. Using 10-fold dilutions of IFN- γ DNA standards of known copy number, the typical kinetics of PCR amplification with its defined C_T are shown in Figure 1A. The C_T was chosen arbitrarily as a value that corresponded to the linear portion of the PCR amplification plots of both the IFN- γ and CD8 standards. The IFN- γ and CD8 standard curves (plotted as copies of DNA standards versus C_T) had an excellent PCR amplification efficiency (90%–100%, where 100% indicates that, in each cycle, the amount of template is doubled) as determined by the slope of the standard curves (Fig. 1B). Linear regression analysis of all standard curves had a coefficient of determination (r^2) of 0.99 or more.

For the measurement of IFN- γ mRNA expression for the experimental samples, IFN- γ mRNA and CD8 mRNA amplifications were performed synchronously on each sample (Figs. 1C and D). C_T values were obtained for each gene, and the mRNA copy numbers for both genes were determined by extrapolation from standard curves. To control for the variability in the number of CD8⁺ cells in the samples and the variability in reverse transcription efficiency during cDNA preparation, the IFN- γ mRNA copy number was calculated relative to the CD8 mRNA copy number. To demonstrate the data calculations, Table 1 presents a typical experiment using PBMCs from a known clinical responder to immunotherapy who consistently demonstrated pretreatment HLA-A2–restricted reactivity to melanoma cells. The cDNA obtained from each PBMC stimulation reaction was run in duplicate for the PCR amplification, and the initial copy number obtained for each gene represented an average of the two duplicates. After a 2-hour incubation with melanoma cells, PBMCs stimulated with the HLA-A*0201 624.38 cell line had a 2.8-fold greater IFN- γ mRNA expression level (normalized to CD8 mRNA expression) than the PBMCs stimulated with the matched HLA-A2⁻ line.

Optimization experiments were performed using PBMCs from a patient who had been immunized with a modified HLA-A2–restricted peptide, 209–2M. For the purposes of the time course assay, we also used pretreatment PBMCs from a clinical responder to control for the possibility that the kinetics of IFN- γ mRNA production might differ between pretreatment and posttreatment PBMCs. Optimization experiments were conducted by exposing bulk PBMCs *ex vivo* to the melanoma cells. For times ranging from 2–30 hours after coincubation, IFN- γ

mRNA expression for both pretreatment and posttreatment PBMCs peaked at 2 hours (Fig. 2). Because the quantitative expression of IFN- γ mRNA had been shown to be several times greater than other commonly studied cytokines (10), we chose this gene as the most sensitive marker of immune reactivity. Further optimization experiments compared total cell number, responder-to-stimulator ratio, optimal media and sera, optimal surface area for the reaction, optimal time for physiologic recovery after thawing for PBMCs, and pretreatment of tumor cells with IFN- γ for major histocompatibility complex class I upregulation. Table 2 summarizes the optimized parameters as determined by these studies.

Comparison of Immunologic Reactivity in Melanoma Patients Who Responded to Immunotherapy and Those Who Did Not

The optimized quantitative real-time PCR assay for measuring induction of IFN- γ mRNA was performed on pretreatment PBMCs from melanoma patients who had received IL-2 treatment. Of 28 patients who had objective clinical responses to immunotherapy, 7 had HLA-A2–restricted pretreatment reactivity against shared melanoma antigens (Table 3A). A stimulation index of ≥ 2 against either or both of the paired melanoma cell lines that was reproducible was considered positive. Six of 21 patients who did not respond to immunotherapy had HLA-A2–restricted pretreatment reactivity against one or both of the paired melanoma cell lines (Table 3B). Thus, no significant difference was observed using this assay between responding and nonresponding patients.

Immunologic Reactivity Against Melanoma in Healthy Volunteers

The PBMCs from 12 healthy HLA-A*0201 volunteers were examined for their reactivity against melanoma cell lines. As shown in Table 4, none of the volunteers had HLA-A2–restricted reactivity against shared melanoma antigens as measured by IFN- γ mRNA expression monitored by the quantitative real-time PCR. In contrast, the melanoma patients as a group showed pretreatment HLA-A2–restricted antimelanoma reactivity in 13 of 49 cases ($p = 0.05$).

Immunologic Reactivity of Melanoma Patients With Pretreatment Histocompatibility Leukocyte Antigen-A2–Restricted Melanoma Reactivity When Tested Against 293A2 Transfected With Known Melanoma-Associated Antigens

The pretreatment PBMCs of 11 of 13 melanoma patients who showed pretreatment HLA-A2–restricted reactivity against shared melanoma antigens were cocultured in a single experiment with 293A2 cells transfected with DNA encoding known melanoma-associated antigens. One clinical responder and two nonresponding patients showed reactivity against MART-1 (Table 5). Reactivity was not detected against any of the other melanoma antigens in these pretreatment samples. The remaining 2 of the 13 patients could not be tested against the panel of transfectants because of the limited availability of pretreatment PBMCs.

DISCUSSION

In this investigation, we established a model to study pretreatment PBMC reactivity against melanoma cells using the quantitative real-time PCR and found that pre-treatment PBMC reactivity could be detected against intact melanoma cells. This assay has several potential benefits. It can accurately reflect the presence and magnitude of in vivo reactivity of PBMCs against a target. Measurement is not influenced by the inherent variability and potential changes involved in the culturing of lymphocytes, because fresh lymphocytes were used without the need for in vitro stimulation. Measurement with this assay is rapid, and reactivity against a stimulator can be assessed in a few hours. This permits allogeneic tumors to be used as stimulators, because the development of alloreactivity is circumvented with such a short incubation period.

In previous studies, we found that a 12-day in vitro stimulation assay of the reactivity of postimmunized PBMCs to a single peptide was more sensitive than a 2-hour quantitative real-time PCR assay (11). We could not use peptide stimulators in the current study, because the tumor-antigen specificity of pretreatment lymphocytes was unknown. To test for pretreatment reactivity against shared melanoma antigens, we used intact allogeneic tumor cells expressing a broad array of antigens. Although the use of autologous tumor lines would have increased the array of potential antigens detected, autologous lines were not available for most patients. We screened for HLA-A2–restricted antimelanoma reactivity because HLA-A2 is the most common HLA type in our melanoma patients. Using this approach, any preexisting reactivity against melanoma antigens presented on molecules other than HLA-A*0201 would not be detected.

Although we saw no difference in the melanoma reactivity in pretreatment samples when we compared responding and nonresponding patients using this assay, we did find, using intact melanoma cells, that patients with metastatic melanoma had greater pretreatment HLA-A2–restricted reactivity against shared melanoma antigens than did healthy volunteers. Thirteen of 49 melanoma patients had pretreatment HLA-A2–restricted reactivity against melanoma cell lines (7 of 28 clinical responders and 6 of 21 nonresponders) compared with none of 12 healthy volunteers ($p = 0.05$). This suggests that the long-term tumor-bearing state sensitizes some patients against melanoma-associated antigens. Although normal melanocytes share most of the melanoma-associated antigens so far described (12), a preferential sensitization against these antigens might occur in patients with metastatic melanoma because of the different conditions of inflammation, antigen distribution, and antigen presentation at the tumor site or nodal groups draining the tumor. By comparing clinically responding and nonresponding patients, however, we could not demonstrate that the enhanced sensitization of PBMCs from melanoma patients against shared melanoma antigens played a role in the recognition and rejection of metastatic lesions in response to the systemic administration of IL-2.

To study further the nature of the antigens detected in pretreatment PBMCs that recognized melanoma cells in an HLA-A*0201–restricted manner, PBMCs from five of the seven clinical responders and all six nonresponders were tested for pretreatment reactivity against known shared melanoma antigens (Table 5). In screening against a panel of 293A2 transfectants incorporating nine melanoma-associated antigens, only anti-MART-1 reactivity could be detected in one clinical responder and two nonresponding patients. The PBMCs from the other melanoma patients that were reactive against entire melanoma cells may have recognized antigens not in our test panel. Alternatively, the reactivity against intact melanoma cells (as measured by IFN- γ mRNA expression in this assay) may represent the sum of all individual reactive responses against multiple antigens on the cells, and this assay may not be sensitive enough to detect specific antimelanoma reactivity against an individual antigen.

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FIG. 1.

A quantitative, real-time PCR assay was developed for the direct assessment of peripheral blood lymphocyte reactivity against melanoma cell lines. **(A)** A sample PCR amplification plot of IFN- γ DNA standards assayed in duplicate is shown. Similar plots were seen for CD8 standards. **(B)** Standard curves for the measurement of IFN- γ and CD8 copy numbers are shown. The equations represent the regression lines for IFN- γ and CD8. **(C)** A PCR amplification plot is shown for IFN- γ from samples taken from a patient. **(D)** A PCR amplification plot of CD8 from the same patient was generated concurrently with that for IFN- γ .

FIG. 2.

Pretreatment PBMCs from a clinical responder and the posttreatment PBMCs from a patient immunized with an HLA-A2–restricted epitope from gp100 were stimulated with melanoma cells for 2 to 30 h. The 2-h point was optimal in both cases. Each time point was run in duplicate and standard errors were generated.

Quantitative real-time polymerase chain reaction (PCR) data and calculations generated for a pretreatment peripheral blood mononuclear cells (PBMC) sample*

TABLE 1

	IFN C _T	IFN CN	CD8 C _T	CD8 CN	IFN avg CN	CD8 avg CN	IFN/CD8CN	624.38/624.28 SI [†]
624.28 Mel (HLA-A2 ⁻)	33.47	2043	26.19	282378	2281	271667	0.0084	2.8
	33.13	2518	26.31	260956				
624.38 Mel (HLA-A2 ⁺)	31.45	7028	26.22	276773	6407	275834	0.0232	
	31.77	5786	26.23	274895				

CT, PCR cycle at which the amplification plot crossed cycle threshold; CN, copy number; SI, stimulation index.

* Stimulation of pretreatment PBMC of a clinical responder by 624.38 and 624.28 melanoma cell lines. Cocultured cells were evaluated by quantitative real-time PCR for both IFN- γ and CD8 mRNA expression.

[†] Values represent copies of interferon-gamma (IFN- γ) messenger RNA (mRNA) per copies of CD8 mRNA obtained from PBMC stimulation with 624.38 divided by the same ratio obtained by PBMC stimulation with 624.28.

TABLE 2

Optimized conditions for the direct molecular assessment of melanoma specific cytotoxic T lymphocytes (CTL) reactivity

Parameters	Optimal condition
Incubation time of PBMC with tumor cells (2–30 h)	2 h
Responder to stimulator ratio (10:1–1:1)	5:1
Total PBMC number (1×10^7 – 3×10^5)	3×10^6
Surface area for reaction (96 vs. 48 vs. 24 well plate)	48 well plate
Physiological recovery time for thawed PBMC (0 h vs. overnight)	overnight
Serum (10% Human AB vs. 10% FCS)	10% Human AB serum
Tumor targets	624.38 vs. 624.28; 888(A2) vs. 888

PBMC, peripheral blood mononuclear cells; vs., versus.

TABLE 3

Pretreatment HLA-A2-restricted anti-melanoma reactivity of patients with melanoma*

A. Patient [†]	SI 624.38/624.28 [§]	SI 888A2/888
1	<u>3.1</u> //	nt [#]
2	0.71	0.81
3	1.3	1.4
4	1.2	0.8
5	0.8	1.2
6	<u>2.1</u>	1.2
7	0.5	1.5
8	0.9	1.0
9	1.3	1.1
10	1.2	1.6
11	1.4	0.9
12	0.8	1.0
13	1.3	1.2
14	1.2	1.0
15	0.5	<u>3.0</u>
16	0.7	<u>2.0</u>
17	1.3	1.1
18	<u>2.1</u>	1.7
19	<u>2.3</u>	1.4
20	1.0	1.0
21	1.2	1.0
22	1.6	0.9
23	1.6	0.7
24	1.1	0.6
25	1.1	1.8
26	1.5	1.3
27	1.0	1.4
28	<u>2.0</u>	0.7
B. Patient [‡]		
1	1.2	1.2
2	1.3	1.0
3	1.1	1.4
4	1.4	1
5	1.4	0.9
6	0.9	1.7
7	1.0	<u>2.5</u>
8	1.0	1.0
9	<u>2.2</u>	1.3
10	1.5	1.0
11	0.4	1.9
12	1.5	<u>2.5</u>
13	<u>3.2</u>	<u>2.8</u>
14	2.2	1.4
15	1.1	0.6
16	1.6	1.4
17	1.5	2.4
18	0.6	3.2
19	<u>5.3</u>	0.6
20	0.7	1.3
21	1.4	<u>2.5</u>

PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction.

* Pretreatment PBMC were incubated for 2 hours with melanoma cell lines (HLA-A2⁺ vs. HLA-A2⁻) and assayed for IFN- γ mRNA expression using quantitative real-time PCR. The first experiment is shown for each melanoma patient tested.

[†] Responders to immunotherapy.

[‡] Non-responders to immunotherapy.

[§] SI: Stimulation index which represents copies of interferon gamma (IFN- γ) messenger RNA (mRNA) per copies of CD8 mRNA obtained by stimulation with HLA-A2⁺ melanoma divided by the same ratio obtained for stimulation with HLA-A2⁻ melanoma.

// Underlined values represent stimulation indices ≥ 2.0 that were repeatable.

nt: not tested.

TABLE 4

Summary of pretreatment reactivity in melanoma patients and normal volunteers*

Patient category	Total	SI < 2.0	SI ≥ 2.0
Responders	28	21	7
Non-responders	21	15	6
Normal volunteers	12	12	0

* Three cohorts were screened for pretreatment HLA-A2-restricted melanoma-specific cytotoxic T lymphocytes (CTL) reactivity. Stimulation indices ≥ 2.0 that were repeatable were regarded as demonstrating pretreatment reactivity against intact melanoma cells.

TABLE 5
Evaluation of reactivity of pretreatment peripheral blood mononuclear cells (PBMC) against known melanoma associated antigens*

Patient no.	Experiment I				Experiment II				Experiment III			
	12 [‡]	19 [‡]	9 [‡]	7 [‡]	1 [‡]	13 [‡]	15 [‡]	21 [‡]	19 [‡]	18 [‡]	6 [‡]	
GFP	357	491	1652	596	3516	839	746	752	826	4490	3358	
MART-1	346	1181 [§]	1880	650	5624	1789 [§]	659	593	897	12354 [§]	3757	
gp100	284	531	1633	470	2721	989	1357	513	688	2282	3416	
Tyrosinase	421	391	918	580	2623	811	675	545	737	3532	2627	
TRP-1	330	441	1129	585	2334	576	891	544	668	1928	3613	
RP-2 Iso	553	398	2118	559	2913	763	572	545	1274	1514	2801	
OA-1	252	380	1261	394	2267	671	588	477	726	nt ^{//}	3174	
P. polypeptide	237	324	920	549	1552	537	419	667	660	nt	2409	(Copies IFN- γ mRNA/Copies CD8 mRNA) × 10 ⁻⁴
Sox-10	377	430	1170	518	3955	511	479	464	621	nt	1808	
NY-ESO	274	659	1190	563	2531	533	383	657	745	nt	2640	
624.28 (A2-)	nt	188	716	nt	162	498	nt	nt	117	8551	10551	
624.38 (A2+)	nt	457 [§]	3554 [§]	nt	473 [§]	1226 [§]	nt	nt	267 [§]	21340 [§]	38046 [§]	
888	nt	nt	nt	nt	nt	nt	91	184	nt	nt	nt	
888(A2)	nt	nt	nt	nt	nt	nt	883 [§]	473 [§]	nt	nt	nt	
GFP	gp100 clone [#]				MART-1 TIL [#]				gp100 clone			
MART-1	157	nt	749	nt	100	nt	252	302	nt	nt	nt	
gp100	2690	11631	nt	nt	512	6590	nt	722	nt	nt	nt	

* Pretreatment PBMC were incubated for 2 hours with 293A2 cells transfected with melanoma antigens and assayed for IFN-γ mRNA expression via quantitative real-time PCR

[‡] Responders to immunotherapy

[‡] Non-responders to immunotherapy.

[§] Underlined values represent ratios of (copies of IFN-γ mRNA/copies of CD8 mRNA) × 10⁴ that were twice background.

^{//} nt: not tested.

[#] T-cells with known specificity against gp100 and MART-1 were used to assess the adequacy of the transfections.