Enzyme-linked Immunospot, Cytokine Flow Cytometry, and Tetramers in the Detection of T-Cell Responses to a Dendritic Cell-based Multipeptide Vaccine in Patients with Melanoma[1]

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ABSTRACT

A new generation of single-cell assays for measuring the frequency of peptide-specific T lymphocytes in cellular populations has become widely available. These assays, enzyme-linked immunospot (ELISPOT), cytokine flow cytometry, and tetramer binding, are used frequently for monitoring of cancer patient responses to vaccination therapies. We concomitantly used these three assays to determine the frequency of $CD8^+$ T cells in the circulation of 8 patients with metastatic melanoma who had received a multiepitope peptide/dendritic cell-based vaccine. Using peripheral blood mononuclear cells harvested before and a week after the last of four vaccines, we observed that the three assays detected a substantially different frequency of peptide-specific $CD8^+$ T cells. The tetramer assay consistently detected the highest numbers of peptide-specific $CD8^+$ T cells, followed by cytokine flow cytometry and then ELISPOT. There was no significant concordance among the assays in measuring the numbers of $CD8^+$ T cells specific for each of the peptides in the peripheral circulation of the patient. No significant pre- to postvaccine changes in the number of $CD8^+$ T cells specific for any of the peptides were observed. Thus, the dendritic cell-based vaccine was observed not to augment immune responses to the peptides in the patients. Because of a low frequency of the peptide-specific T cells in the peripheral circulation of the patients, these sensitive single-cell assays were used to measure values at the lower limit of detection. For this and other reasons, including the issues of tetramer specificity and ELISPOT sensitivity, caution in interpretation and serious attention to quality control are needed in monitoring of immune responses to anticancer vaccines.

INTRODUCTION

The role of immune cells in the control of cancer development and progression has been investigated for many years, and the notion that up-regulation of innate and adaptive immune responses leads to tumor rejection has provided a rationale for therapy of cancer patients with various forms of immunotherapy, including vaccines (1, 2). In view of the recently expanding use of antitumor vaccines, attention has focused on the methods for evaluation of immune responses to the vaccine and on the development of surrogate end points for measuring of vaccine efficacy.

Traditionally, cellular immune responses have been evaluated using methods measuring cytotoxicity, proliferation, or release of cytokines in populations of mononuclear cells (3, 4). In the clinical setting, these assays often require IVS with the relevant antigen or mitogen followed by 3–7-day culture to be able to detect measurable responses. Thus, the assays do not measure the frequency of precursor cells present in the peripheral circulation of patient. Cellular expansion or death, as well as cytokine-mediated amplification of responses ongoing in such cultures are likely to influence results, making the quantification of tumor-specific precursor cells all but impossible. Yet, to evaluate efficacy of vaccines, it would seem necessary to demonstrate the increase in the frequency of tumor-specific precursor T cells and to relate it to clinical endpoints. In this respect, the newer assays, including ELISPOT,[3] CFC, and tetramer binding have the capability to estimate the frequency of antigen-responsive T cells in the population (5–7). Generally referred to as single-cell assays, they offer an advantage of being able to measure not only the ability of the cell to make a cytokine in response to an antigen and to define the phenotype of the cell but also to quantify the number of such cells in the study population.

Of the three single-cell assays, ELISPOT has been used

Received 6/14/02; revised 9/30/02; accepted 9/30/02.
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[1] Supported in part by NIH Grants PO1-DE12321, PO1-CA73743, and RO1-CA82016.
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[3] The abbreviations used are: ELISPOT, enzyme-linked immunospot; CFC, cytokine flow cytometry; TCR, T-cell receptor; DC, dendritic cell; UPCI, University of Pittsburgh Cancer Institute; PBMC, peripheral blood mononuclear cell; GM-CSF, granulocyte macrophage colony-stimulating factor; PE, phycoerythrin; FACS, fluorescence-activated cell sorter; PMA, phorbol 12-myristate 13-acetate; IVS, *in vitro* sensitization; LLD, lower limit of detection.

most frequently for monitoring of vaccination trials both in cancer and other diseases (8–10). More recently, CFC has been used to measure responses to antigens expressed by infectious agents such as cytomegalovirus (11), EBV (12), and HIV (13), to tumor-associated antigens, such as MUC-1 (14), or melanoma differentiation antigens (15). With the introduction of tetrameric MHC-peptide complexes (7, 15) and their wider availability, tetramers are increasingly often used for monitoring of cancer vaccination trials (16). These methodologies, all based on the principle of specific recognition by TCR of a peptide presented on the relevant MHC molecule, have not been compared thus far in the context of a clinical trial. The general perception has been that all three of the assays have sufficiently high sensitivity to be able to detect frequencies of precursor cells as low as 1/100,000 cells, although their specificity as well as reproducibility have been of concern (17).

We were able to concomitantly use all three of the methods to compare responses of patients with metastatic melanoma to a multiepitope peptide/DC-based vaccine. We found considerable differences in the frequency of peptide-specific $CD8^+$ T cells in the peripheral circulation of these patients, depending on the method used for T-cell detection. Such disparate results are likely to complicate interpretation and frustrate attempts at correlation of immunological data with clinical outcome.

MATERIALS AND METHODS

Patients. Melanoma patients were enrolled in a Phase I/II clinical trial of a multi-epitope DC-based vaccine performed at the UPCI (#97–009) and approved by the Institutional Review Board. All of the patients signed a consent form before entry on the protocol. The patients received a vaccine consisting of granulocyte colony-stimulating factor mobilized (Neupogen; Amgen) autologous DCs, which were cultured and then separately pulsed with the following peptides: MART 1/Melan-A_{32-40}, $gp100_{280-288}$, $tyrosinase_{368-376}$, and influenza $matrix_{58-66}$. The protocol was designed to compare vaccination efficacy of DCs derived either from $CD34^+$ peripheral blood precursors or from peripheral blood adherent precursors. The patients were leukapheresed and randomized to receive either: (*a*) monocyte-derived DCs obtained by adherence of PBMCs to plastic and cultured in the presence of GM-CSF+ interleukin 4, as described previously (18); or (*b*) stem cell-derived DCs obtained by positive selection, using the Ceprate system (19) and cultured in GM-CSF+ interleukin 4 or in GM-CSF+ tumor necrosis factor α. Four weekly vaccines were administered s.c. (25% of the vaccine) and i.v. (75% of the vaccine) to each patient. The monitoring of this trial included the ELISPOT assays and CFC for IFN-γ in response to immunizing, and control (HIV*pol*) peptides and tetramer analysis. Monitoring was performed in the Immunological Monitoring and Cellular Products Laboratory at the UPCI. Twelve patients entered the protocol before its termination, and 8 completed the DC-based vaccine therapy. All 8 of the patients received monocyte-derived DC/peptide vaccines. No clinical responses to therapy were observed in this group of patients.

Normal Controls. Lekapheresis products (buffy coats) were obtained from platelet donors through the Central Blood Bank of Pittsburgh. Peripheral blood was also obtained from normal volunteers all of whom signed the Institutional Review Board-approved consent form agreeing to serve as controls for the assays.

PBMCs. Venous blood was obtained from the patients at times designated in the protocol (at 1 week before first vaccine and at 1 week after each of the four vaccines). Heparinized blood samples were delivered to the laboratory and immediately processed to recover PBMCs on Ficoll-Hypaque gradients. The cells were washed in medium, counted in a trypan blue dye, and cryopreserved using Cryomed, according to the standard operating procedure established and used at the Immunological Monitoring and Cellular Products Laboratory. For this study, only pre- and posttherapy (1 week after the last vaccine) specimens of each patient were thawed and tested in the same assays. Each PBMC preparation was split into three parts: one for ELISPOT, one for tetramer analysis, and one for CFC. Leukapheresis products and venous blood obtained from normal volunteers were processed in the same way.

Media, Reagents, and Cell Lines. AIM V and RPMI 1640 media and FCS were purchased from Life Technologies, Inc., Gaithersburg, MD. Peptides used as stimulators in the ELISPOT or CFC assays were synthesized using *N*-(9-fluorenyl)methoxycarbonyl chemistry and the 432 Asynergy peptide synthesizer (Applied Biosystems, Foster City, CA) at the UPCI Peptide Synthesis Core Facility. They were purified to >85% purity by reverse-phase high-performance liquid chromatography, and peptide sequences were confirmed by mass spectrometry before use in the assays. The following peptides were synthesized: melanoma peptides: $MART\text{-}1_{32-40}$ (ILTVILGVL), $gp100_{280-288}$ (YLEPGPVTA), and $tyrosinase_{368-376}$ (YMDGTMSQV), as well as two control peptides: influenza matrix peptide, GILGFVFTL (residues 58 to 66), and HIV-1 reverse transcriptase peptide, ILKEPVHGV (pol 476–484). T2 cells were obtained from American Type Culture Collection, Manassas, VA, and maintained in RPMI 1640 supplemented with 5% FCS and antibiotics. Before their use in ELISPOT and CFC assays as peptide-presenting cells, T2 cells were pulsed with peptides by incubating the cells for 2 h with 10 μg/ml of each peptide.

Antibodies for Surface Staining. The monoclonal antibodies used for flow cytometry in this study were: FITC- or PE-conjugated anti-CD14 (RMO52), ECD-conjugated anti-CD3 (UCHT1), and PC5-conjugated anti-CD8 (SFCI21Thy2D3) purchased from Beckman Coulter (Miami, FL). The respective isotype controls were also purchased from Beckman Coulter. The antibodies (1 μl, undiluted) were added directly to cell pellets, as described below.

Tetramers. The streptavidin-PE-labeled tetramers used in this study were obtained from the core facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA). All of the peptides provided for tetramer synthesis were high-performance liquid chromatography purified. The tetramers were used at a dilution of 1:40 in buffer, based on titrations performed with PBMCs of normal donors who were HLA-A2+ or negative, as described previously (17).

Tetramer Staining. Cryopreserved PBMCs were thawed and washed twice in PBS containing 0.1% BSA and 0.1% NaN_3 (FACS buffer). The cells were divided into aliquots of 3×10^6 cells/well of a 96-well round-bottomed microtiter plate. Aliquots (3 μl) of tetramers diluted to 1:40 in FACS

buffer were added to pelleted cells. The plates were vortexed and incubated at room temperature for 30 min in the dark, followed by a 30-min incubation with antibodies (1 μl of each) at 4°C. After two washes with buffer, the cells were resuspended in 1% (w/v) paraformaldehyde in PBS and examined by flow cytometry.

Intracellular Staining for IFN-γ. PBMCs were stimulated by the peptide-loaded T2 cells (see above) at an effector/stimulator ratio of 10:1 for 24 h at 37°C in the atmosphere of 5% CO_2 in air. The same peptides as those used for vaccination of the patient were used for PBMC stimulation. Monensin A (2 μM; Sigma, St. Louis, MO) was added to the cells for the last 4 h of incubation. After stimulation, the cells were harvested, washed twice in PBS, and stained with labeled monoclonal antibodies to the surface antigens (anti-CD3 and anti-CD8, 1 μl of each), which were added directly to the cell pellets. After washing twice, PBMCs were fixed with 1% (w/v) paraformaldehyde in PBS, and washed once with FACS buffer and once with permeabilization buffer (0.1% saponin in PBS). Then, the cells were stained with FITC-conjugated anti-IFN-γ (PharMingen, San Diego, CA) for 30 min at 4°C. As a negative control, cells were stained with mouse IgG1 isotype control (Immunotech). Cells were washed once with permeabilization buffer and once with FACS buffer, and then analyzed by flow cytometry. As a positive control, we used PBMCs obtained from normal volunteers and incubated with 1 ng/ml of PMA (Sigma) and 1 μM of ionomycin (Sigma) for 24 h at 37°C in 5% CO_2.

Flow Cytometry. Four-color flow cytometry analysis was performed on a Coulter Epics XL cytometer with a single 488-nm argon ion laser. The amplification and compensation were set according to the standard procedure, using negative controls (isotype immunoglobulin-stained cells) and tested cells stained in a single color or combination of colors (FL1, FITC-CD14; FL2, PE-tetramer; FL3, ECD-CD3; and FL4, PC5-CD8). First, a large gate was set on mononuclear cells. After monocytes were excluded as CD14+ cells, the CD3+ gate was set, and the frequency of CD8+ and tetramer+ T cells or CD8+ and IFN-γ T cells was determined by acquiring at least 5×10^5 events. Before cytometry of tetramer-stained cells, the negative cutoff was set using PBMCs of an HLA-A2-negative normal control, and verified by using PBMCs of HLA-A2-positive normal donor stained with an HIV peptide tetramer, as described previously (17). For cytometry of IFN-γ expressing CD8+ T cells, the negative and positive cutoffs were set using PBMCs stained with isotype immunoglobulin control or with PMA/ionomycin, respectively. The flow cytometry data were analyzed in real time using Beckman-Coulter System II software.

ELISPOT Assays for IFN-γ. The PBMCs to be tested in ELISPOT assays were enriched in CD8+ T cells by positive selection using immunobeads (Miltenyi Biotech, Auburn, CA). The procedure recommended by the manufacturer was followed, after it was optimized, using PBMCs obtained from normal donors ($n = 4$). Flow cytometry was performed before and after positive selection on microbeads to determine the percentage of CD8+ T cells in the unenriched and enriched fractions. The median proportion of CD8+T cells was 22% before enrichment and 90% afterward. The median recovery was 84%. The concentration of CD8+ cells was adjusted to 1×10^6/ml in AIM V medium, and a 100-μl aliquot of the cell suspension was added to each well of an ELISPOT plate precoated with the capture antibody (Mabtech, Inc., Cincinnati, OH). Next, T2 cells 1×10^4/well pulsed with the relevant peptide were added to triplicate wells. The plates were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The direct ELISPOT assay was performed exactly as described previously (20). A Zeiss image analyzer (Carl Zeiss, Inc., Chester, VA) was used for determinations of the spot numbers in each well. The HIV*pol* peptide was used as a negative control, and PMA/ionomycin as a positive control. Normal control and patient CD8+T cells were also stimulated with anti-CD3 (OKT-3) antibody added to the cells at the concentration of 10 μg/ml to compare their reactivity. The spots in wells containing responder CD8+ T cells plus unpulsed T2 cells were considered as background spots, and were subtracted from spots in the experimental wells.

Data Interpretation. Tetramer-positive cells and cells expressing IFN-γ in CFC were quantified by flow cytometry and expressed as reciprocal frequencies (*i.e.*, 1000 means that 1/1000 cells was positive) or as numbers of positive $CD8^+$ T cells per 10^5 cells tested. The LLD for tetramer-positive $CD8^+$ T cells was established as 1 positive cell per 7805 based on the upper 99th percentile of tetramer-positive $CD8^+$ T cells in 10 HLA-$A2^-$ individuals (17). Consequently, all of the reciprocal frequency values >7805 are considered as negative. The permutation test was used to determine the significance of response in triplicate ELISPOT wells at $P < 0.05$.

Statistics. With 8 patients to be evaluated for immune responses before and after vaccine administration, descriptive statistics were used. Concordance among the three assays in measuring increases or decreases in responses pre- and post-vaccine therapy was evaluated by comparisons of concomitant measures. Concomitance of immunological responses (*i.e.*, how well the three assays agreed in measuring changes in T-cell responses pre- *versus* post-therapy), was also compared. Finally, a more formal analysis of differences among the assay was performed using the Mack Skillings test of equality applied to all three of the assays, and the Wilcoxon test was used to evaluate differences between pairs of assays.

RESULTS

The three assays were compared using aliquots of the same thawed PBMCs without any previous culture or IVS. For ELISPOT assays, enriched CD8+ cell preparations were plated, whereas in flow cytometry-based assays, the gate was set on CD8+ T cells. In all of the cases, the number of IFN-γ-positive or tetramer-positive CD8+ T cells per total number plated or gated was determined and expressed either as the number of positive cells per 10^5 cells tested or as a reciprocal frequency.

Tetramer Staining *versus* CFC. These two procedures are flow-cytometry based, and their performance in detecting peptide-specific CD8+ T cells in the peripheral circulation of patients with melanoma was compared first. As can be seen from representative results shown in Fig. 1, the percentages of peptide-specific $CD8^+$ T cells detected in PBMCs of the patients using either procedure were generally low. The number of tetramer-positive cells per 10^5 cells tested ranged from zero to 346 (Table 1). In patients with a low number of circulating peptide-specific T cells, it was possible to detect distinct clusters

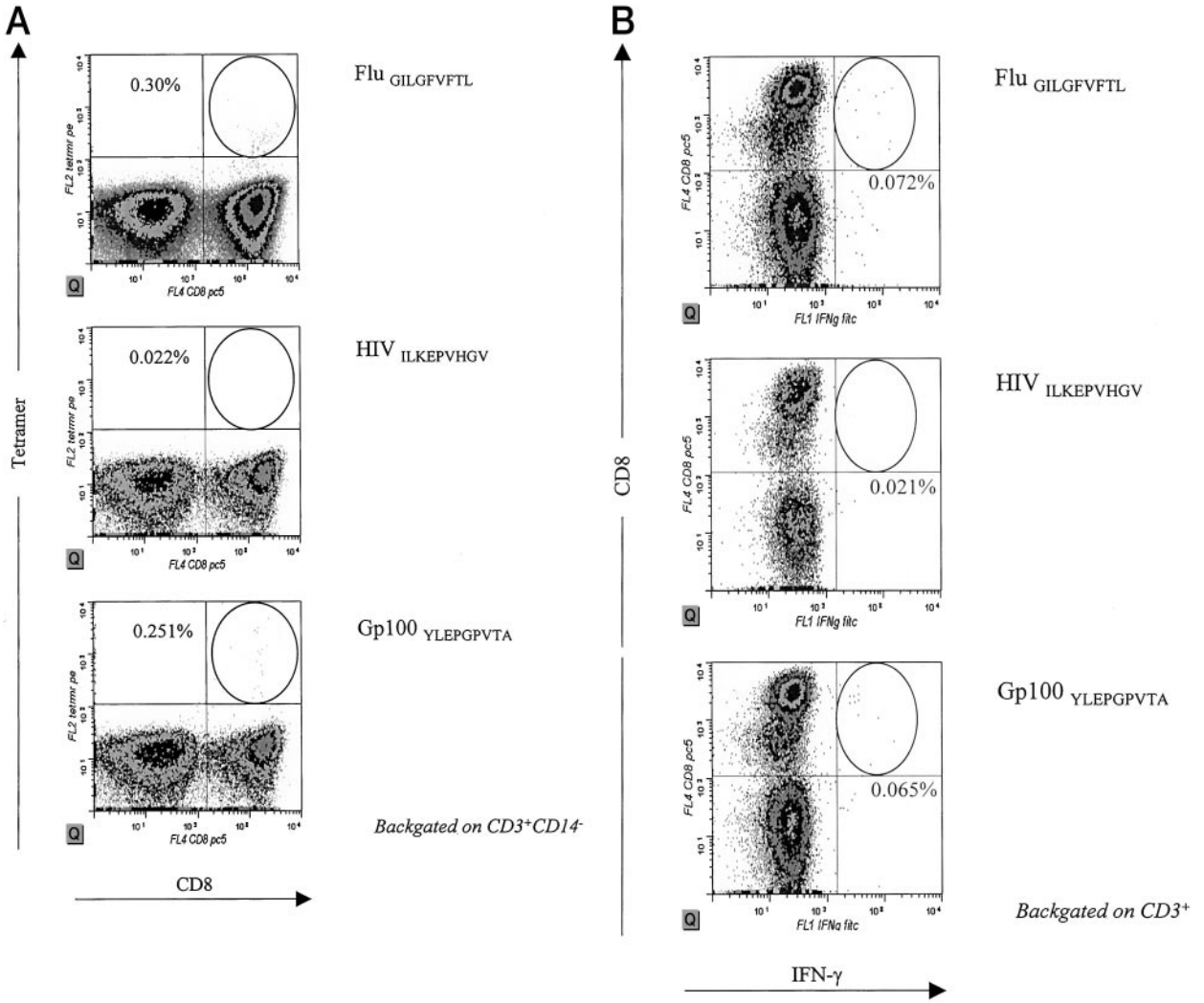

Fig. 1 *A*, tetramer binding to $CD8^+$ T lymphocytes in the peripheral circulation of representative patients with metastatic melanoma (Table 1: #6 for gp100 and HIV tetramers; #7 for the flu tetramer). The proportions of tetramer-binding $CD8^+$ T cells within each circle are indicated. *B*, CFC for detection of IFN-γ-expressing $CD8^+$ T lymphocytes in the peripheral circulation of a patient with melanoma (#6 in Table 1). The proportions of IFN-γ + $CD8^+$ T cells within each circle are indicated. For both *A* and *B* at least 0.5×10^6 cells were acquired in the gate.

of these T cells, provided at least 0.5×10^6 lymphocytes were acquired for analysis (Fig. 1). In general, for MART-1 and gp-100 peptides, the numbers of positive cells detected through tetramer binding were higher than those measured by CFC for the same samples (Table 1), although in some cases, the numbers were comparable. For example, the numbers of gp-100-specific precursor $CD8^+$ T cells were higher by tetramer binding than CFC in 6 of 8 cases before vaccination and in 7 of 8 cases after vaccination. On the other hand, tyrosinase-specific $CD8^+$ T cells were detected by CFC in 6 of 6 cases before the vaccine administration and in 5 of 6 cases afterward, but were not detected by tetramer binding. In Fig. 2, it can be seen that results for measures of gp-100 and MART-1-responsive $CD8^+$ T cells were concordant for the two flow cytometry assays, although they were not for tyrosinase or flu.

Changes in the number of peptide-specific CD8+ T cells from pre- to postvaccine administration were observed in nearly all of the cases (Table 2); however, in many cases, a decrease rather than an increase in these numbers was seen, and the direction of the change (*i.e.*, decreased or increased response) was often not concordant in CFC relative to tetramer staining. The magnitude of responses varied from patient to patient and from one peptide to another, but in many cases, a >2-fold change in either direction was evident (data not shown). Some patients responded to the flu matrix peptide by substantial increase in the frequency of specific precursor cells, whereas others had a lower frequency of flu-specific $CD8^+$ T cells after vaccination. Responses to PMA and ionomycin by patient $CD8^+$ T cells were also measured by CFC as a positive control, and were found to be increased in 3 patients and either unaltered or decreased in others after the administration of all four of the vaccines.

ELISPOT Assay. The results of ELISPOT assays were generally not in agreement with the flow cytometry-based as-

Table 1 Numbers and reciprocal frequencies of peptide-specific $CD8^+$ T Cells in peripheral blood of patients with melanoma[a]

	Number/10^5 cells (reciprocal frequency)	
	Pre	Post
Tetramer[b]		
MART 1-1/ MELAN-A	0–346 (0–289)	22–286 (350–4,598)
gp-100	14–178 (562–621)	0–161 (0–621)
tyr	0–8 (0)	0–8 (0)
flu	0–175 (0–569)	1–300 (0–350)
HIV	0 (0)	0 (0)
CFC		
MART-1/ MELAN-A	0–97 (0–1,032)	0–93 (0–1,078)
gp-100	13–65 (7,547–1,547)	1–52 (36,235–1,937)
tyr	4–34 (8,183–2,482)	7–92 (14,837–1,082)
flu	14–67 (7,051–1,494)	3–72 (31,546–1,395)
HIV	0 (0)	0 (0)
PMA/ Ionomycin	1,160–10,700 (86–9)	900–8,490 (60–11)
ELISPOT		
MART-1/ MELAN-A	0–28 (0–3,571)	0–25 (0–4,000)
gp-100	0–11 (0–9,091)	0–25 (0–6,250)
tyr	0–6 (0–16,667)	0–17 (0–14,286)
Flu	0–20 (0–5,000)	0–32 (0–3,125)
HIV	0	0
PMA/Ionomycin	40–3,900 (2,500–26)	50–2,900 (2,000–34)
OKT-3Ab	20–1,570 (5,000–64)	40–1,400 (2,500–71)

[a] PBMCs were obtained from patients and simultaneously studied by three different methods. The data are ranges in the numbers of positive $CD8^+$ T cells per 10^5 cells tested with ranges in reciprocal frequencies of responsive $CD8^+$ T cells shown in parenthesis. The PBMCs were obtained before and after 4 weekly vaccinations with the DC-based polyepitope vaccines. The numbers and reciprocal frequencies of responsive $CD8^+$ T cells were determined without *in vitro* expansion.

[b] The lower limit of detection for tetramer-positive $CD8^+$ cells was established as 1/7,805 based on testing of 10 HLA-$A2^-$ individuals (17). Therefore, although detectable and measurable, all reciprocal frequency values >7,805 are reported as 0.

says (Table 1; Fig. 2). Overall, substantially fewer peptide-specific $CD8^+$ T cells were being detected in the patient $CD8^+$ T cells by ELISPOT than by the other two assays. The sensitivity of detection in ELISPOT assays in our hands was determined to be 1/100,000 cells (20). Nevertheless, we conservatively designate all frequencies of 1/50,000 or lower as negative. The coefficient of variation for the assay varies from 15 to 25% ($n = 50$) depending on the technician performing the assay, as determined by testing of the same cryopreserved PBMCs, which are thawed and stimulated with PMA/ionomycin every time the assay is performed.

The experimental design used in this study predicated that a loss in $CD8^+$ T cells might occur in the course of the enrichment procedure performed before ELISPOT assays. Thus, we carefully monitored by flow cytometry the recovery of $CD8^+$ T cells after positive selection on immunobeads from PBMCs of each patient. The data indicated that the mean recovery of $CD8^+$ cells from PBMCs of the patients with melanoma was 57.2% ± 5% (SD), compared with 84% recovery of $CD8^+$ T cells from PBMCs of normal controls. Thus, it was possible that some of the peptide-reactive precursors in this subset of T cells were lost during enrichment.

Interestingly, responses of the patient $CD8^+$ T cells to PMA/ionomycin were consistently lower in ELISPOT assays than in CFC (Table 1). However, when we compared PMA responses of the patients to those of normal donors in ELISPOT assays, the median numbers per 10^5 cells plated were comparable: 1230 and 1330, respectively. The numbers of PMA-reactive $CD8^+$ T cells were lowest in patients #4 and 5, who also had no or poor responses to the vaccinating peptides. In four patients, we also measured responses of patient $CD8^+$ T cells to the anti-CD3 (OKT-3) antibody. With the exception of patient #6, the numbers of OKT-3-reactive cells in patients were considerably lower (median = $60/10^5$ cells plated) than those in normal controls, which ranged from 150 to $402/10^5$ cells plated. Also, the frequencies of OKT-3-reactive cells generally decreased after vaccination. In aggregate, these ELISPOT results suggest that this assay detects fewer peptide-responsive precursors or PMA-responsive $CD8^+$ T cells than the flow-based assay, and that signaling via TCR might be impaired in patients with melanoma, as indicated by low frequencies of OKT-3-reactive $CD8^+$ T cells in these patients.

Comparison among the Three Assays. A scatter plot matrix (a 3 × 3 square design) was initially used to obtain an "eye view" of whether the three assays correlated with one another (Fig. 2). This exploratory, graphic display of the data allows for establishing the overall agreement or the lack of it between the assays compared after adjusting the frequencies of positive cells to a common denominator of 10^5 cells plated or tested per assay. In a few instances, as with tetramer and CFC assays for MART-1/MELAN-A or for gp100, convincing evidence for correlation was observed. However, in most cases the three assays were not concordant (Fig. 2).

Next, a statistical analysis of the data was performed. The Mack Skillings test of equality was used to compare the assays for the ability to detect the numbers of $CD8^+$ T cells responding to the melanoma peptides or to the flu peptide. As shown in Table 3, significant and consistent differences were observed regardless of whether the assays were compared together or independently with one another. This is particularly evident when responses to gp-100 and MART-1/MELAN-A peptides are examined. The numbers of these peptide-specific CD8+ T cells were well within the detectable range of the three assays. Although the two flow-cytometry based assays were found to be concordant in their overall direction of measurements of responses to gp-100 or MART-1 peptides, the tetramer assay was able to detect significantly more peptide-specific T cells than CFC.

DISCUSSION

The number of candidate antigens recognized specifically by CD8+ T cells in the context of MHC class I molecules has substantially increased in recent years (reviewed in Ref. 21). Consequently, the numbers of clinical trials evaluating various types of anticancer vaccines have increased as well (reviewed in Ref. 22). Promising clinical outcomes have been reported in some of these trials (22–24) but not in others (25). A desired objective of clinical cancer vaccine trials is to induce vaccine-

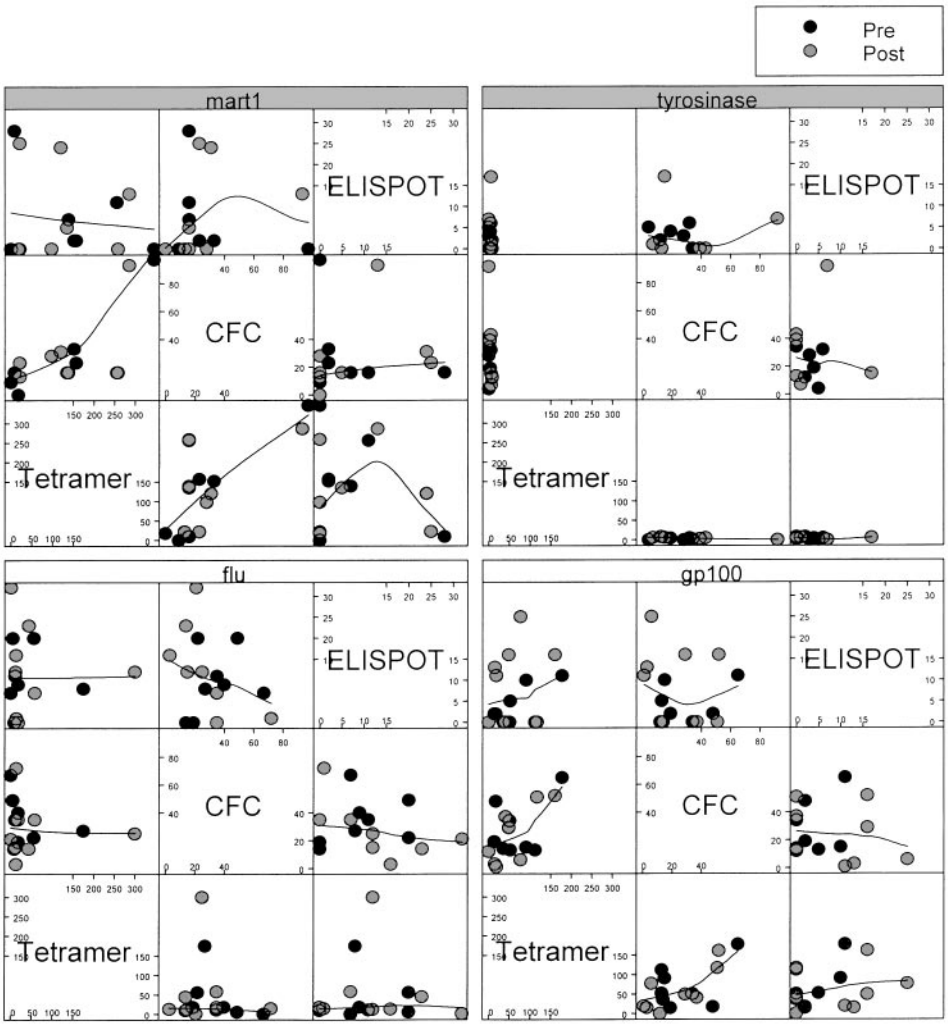

Fig. 2 A scatter plot matrix for comparing the three assays used to detect the number of reactive $CD8^+$ T cells in patient samples obtained pre- and post-vaccine therapy. The figures on the ordinate or the abscissa are numbers of responsive $CD8^+$ T cells per 10^5 cells tested in each assay. All of the possible pairwise plots are shown in the squares of the 3×3 grid (the matrix) for each of the 4 peptides. The stars in squares for MART-1 and gp-100, for example, indicate that CFC and tetramer assays correlate.

specific and tumor-specific responses mediated by CD8+ T cells. Therefore, it is essential to measure the frequency of the responding $CD8^+$ T cells before and after vaccination, and to correlate it with a clinical outcome. It has been pointed out that to achieve this, methods used for measurements of immunological end points have to be optimized, standardized, and quality controlled (26–28). Three types of single-cell assays are currently in use for measuring responses to MHC class I-restricted peptides: two are flow cytometry-based [recombinant MHC class I multimers loaded with the relevant peptide (7) and CFC for IFN-γ expression (29)], whereas the third, an ELISPOT assay, is dependent on the ability of single cells placed on nitrocellulose to secrete IFN-γ in response to the relevant peptide (5). There are indications in the literature that these assays are not equivalent in measuring effector cell numbers and functions (26, 30). Furthermore, there are various formats of these assays in general use, including IVS procedures, which call for culture of effector cells with a vaccinating peptide followed by an assay performed on day 7 or 14 (9, 31, 32). The three assays have not been thus far compared in the context of a clinical trial for their ability to detect and measure the frequency of peptide-specific CD8+ T cells in patient PBMCs. Therefore, we concomitantly performed the assays to determine the frequency of peptide-specific CD8+ T cells in the circulation of 8 patients with metastatic melanoma who participated in a Phase I/II DC-based multiepitope vaccination trial.

The laboratory that performed the described assays is experienced in immunological monitoring of clinical trials. Furthermore, two of the three assays used (ELISPOT and tetramer binding) have been evaluated extensively and their performance described previously by us in detail (17, 20, 32). The CFC assay has been described by others (29), and it has been possible to establish and compare the assay characteristics with those available from the literature (33). The limit of detection for the three assays is thought to vary from 1/10,000 (0.01%) for tetramers to 1/50,000 (0.002%) for CFC and 1/100,000 (0.001%) cells for ELISPOT (20, 33). The nonspecific binding of tetrameric complexes to B cells, non-MHC-restricted T cells, and monocytes has been well documented, and in this study, we adapted the "rare event" gating strategy to avoid such nonspecific staining (17). The specificity of CFC and ELISPOT assays depends on the quality and titer of antibodies used for the detection of

Table 2 The number of patients showing a change in TCRs from pre- to postvaccine

Change[a]	Mart-1/Melan A	gp100	Tyrosinase	Flu
+++	0	0	1	1
++−	1	0	0	1
+−+	0	1	0	2
+−−	1	0	2	1
−++	1	1	1	0
−+−	1	0	1	0
−−+	2	3	0	2
−−−	0	0	0	0
other[b]	2	3	3	1

[a] This is the change from pre- to postvaccination (+ = increase, − = decrease) in 8 patients in the order of: tetramer, CFC, and ELISPOT.
[b] Either no change or one of the values was missing.

Table 3 Differences among the assays in the number of TCRs per 100,000 tested

Antigen	Mack-Skillings[a]	T *vs.* C[b,c]	T *vs.* E[b]	C *vs.* E[b]
Mart1/MelanA	0.0019	0.0024	0.0024	0.0048
gp100	<0.0001	0.0057	0.0005	0.0083
Tyrosinase	0.0019	0.0022	0.8135	0.0024
Flu	0.0284	0.8603	0.0407	0.0041

[a] Mack-Skillings = test of equality of all three assays. Data are *P*s.
[b] Pairwise Wilcoxon tests. C = cytokine flow cytometry.
[c] T, tetramer; C, cytokine flow cytometry; E, ELISPOT.

peptide-reactive T cells, and again, we were careful to select and pretiter these reagents. The reproducibility of the flow cytometry-based assays is strictly governed by the operator's skills of setting the gates and excluding nonspecific events, and is, therefore, open to considerable subjectivity. On the other hand, the reproducibility of ELISPOT is a function of the interassay variability, as described recently (32), and of the necessity for enrichment of CD8+ T cells by positive selection on immunobeads in the experimental format used here. Therefore, it cannot be assumed *a priori* or expected that the three assays will yield comparable results.

Indeed, the comparison of the three assays yielded results that varied considerably for the same specimens. The results of tetramer staining were consistently higher than those of the two other assays, possibly because we did not gate out B cells, which can bind tetramers,[4] or because of the presence of increased proportions of Annexin-binding T cells in the circulation of patients with melanoma (34). It appears that CD8+ T cells are preferentially targeted for apoptosis in patients with cancer (35), and these "preapoptotic" CD8+ T cells could nonspecifically bind tetramer-peptide complexes, resulting in the high frequency estimates. We tentatively concluded that the higher frequencies of peptide-specific T cells we observed using tetramers were probably because of their nonspecific binding rather than greater sensitivity of this assay. In contrast, it is unlikely that the results of CFC, which measures intracytoplasmic expression of IFN-γ in CD8+ T cells based on the use of antibodies, reflected nonspecific events. However, it should be noted that PBMCs were coincubated with T2 cells pulsed with the relevant peptide for 24 h before flow cytometry, largely to keep the stimulation conditions identical to those used in ELISPOT assays. Others have reported 6-h stimulation as optimal for cytokine expression in the cytomegalovirus system (36). It is possible that culture of unseparated PBMCs for 24 h before the assay resulted in the amplification of the responses via the release of cytokines by the peptide-activated CD8+ and CD4+ T cells. We were unable to compare patient samples in 6 h *versus* 24 h assays, because of limited cell numbers. It is also possible that the effector/stimulator ratio of 10:1 used for CFC was not optimal, resulting in underestimated values. Another explanation for the observed discrepancy between tetramer and CFC results could be that not all of the peptide-reactive $CD8^+$ T cells express IFN-γ. This possibility appears to be the most likely explanation in view of results reported by Lee *et al.* (15) and others.

The ELISPOT assays used enriched populations of $CD8^+$ T cells plated at the concentration of 10^5 per well, which distributes cells in a single cell layer on the surface of the nitrocellulose membrane. In this assay, single cells, bound to the membrane and spatially separated from their counterparts, were stimulated with T2 cells pulsed with the peptide. Thus, the basic design of this assay was different from CFC in that responses of single $CD8^+$ T cells to peptide-pulsed antigen-presenting cells and not responses of subpopulations of $CD8^+$ and $CD4^+$ T cells, were measured. In this context, it is informative to consider nonspecific responses of T cells to PMA/ionomycin, which in our study were consistently much higher in CFC than in ELISPOT assays, presumably because of amplification of the response in the population of incubated cells. Thus, only ELISPOT assay detects individual precursor cells capable of responding to the presented peptide by IFN-γ secretion without accompanying secondary cytokines produced when cocultures of cells are used, as in the CFC assay. The ELISPOT assay performed with PBMCs without IVS is the only assay that measures the frequency of peptide-specific precursor cells able to respond to the relevant peptide under the conditions of the assay. The literature suggests that the frequency of such precursor cells is very low in the circulation of patients with cancer (37) and that this frequency may not be substantially increased after DC-based multiepitope vaccine administration, as also indicated by our results.

The results of the three assays compared in this study showed only limited concordance in direction of measurement and no concordance in detecting changes in the number of responders after vaccination. The overall impression is that of poor, if any, responses to the DC-based vaccine in this clinical trial. This might be because of selection of the peptides for the vaccine which, in retrospect, was not optimal but at the time of selection was justified by our preclinical data (38). The lack of robust responses to the vaccine regardless of the detection method used is the most striking feature of this and other immunological monitoring protocols in patients with cancer. In this respect, the observation that ELISPOT responses to OKT-3 antibodies were depressed in the patients relative to normal controls is in agreement with our earlier studies, demonstrating defects in TCR signaling of T cells of patients with cancer (34,

[4] F. Marincola, personal communication.

35, 39). It is possible that these defects are related to tumor-induced immunosuppression or to previous chemotherapy. But because most tumor-associated antigens are self-antigens, it is likely that responses to the peptides derived from these antigens are tolerized. If so, then powerful immunization protocols are needed to break such self-tolerance. The current generation of antitumor vaccines, including autologous DCs pulsed with melanoma peptides, appear to have a limited clinical effectiveness, and it becomes necessary to consider alternative immunogens able to elicit more robust antitumor responses. Clearly, current attempts at establishing a correlation between tumor-specific immunological responses and clinical endpoints are not likely to succeed in the absence of clinical responses.

All three of the methods for measuring responses to peptides at the single-cell level are highly sensitive, with a LLD ranging between 1/50,000 and 1/100,000 peptide-specific T cells in a population. However, the necessity of using these assays at the LLD or of amplifying responses by IVS, as commonly done by many investigators, is neither optimal nor convenient for routine monitoring of responses in patients enrolled in vaccination trials. The true meaning of discordant results obtained by monitoring of this clinical trial using three different assays cannot be appropriately evaluated working at the lower limit of the assay sensitivity. It will be necessary to re-evaluate these assays when improved cancer vaccines capable of inducing robust antitumor responses are available. Only in the context of clinical responses to such antitumor vaccines might it be possible in the future to define those immunological end points that will convincingly serve as surrogates of clinical responses.

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Enzyme-linked Immunospot, Cytokine Flow Cytometry, and Tetramers in the Detection of T-Cell Responses to a Dendritic Cell-based Multipeptide Vaccine in Patients with Melanoma

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Clin Cancer Res 2003;9:641-649.