Evaluation of the Modified ELISPOT Assay for Gamma Interferon Production in Cancer Patients Receiving Antitumor Vaccines

TADAO ASAI, WALTER J. STORKUS, AND THERESA L. WHITESIDE

University of Pittsburgh Cancer Institute and Departments of Pathology and Surgery

Received 28 June 1999/Returned for modification 25 August 1999/Accepted 5 October 1999

Frequencies of vaccine-responsive T-lymphocyte precursors in peripheral blood mononuclear cells (PBMC) prior to and after administration of peptide-based vaccines in patients with cancer can be measured by limiting-dilution assays (LDA) or by ELISPOT assays. We have used a modified version of the ELISPOT assay to monitor changes in the frequency of gamma interferon (IFN-γ)-producing T cells in a population of lymphocytes responding to a relevant peptide or a nonspecific stimulator, such as phorbol myristate acetate-ionomycin. Prior to its use for monitoring of patient samples, the assay was validated and found to be comparable to the LDA performed in parallel, using tumor-reactive cytolytic T-lymphocyte (CTL) lines. The sensitivity of the ELISPOT assay was found to be 1/100,000 cells, with an interassay coefficient of variation of 15%, indicating that it could be reliably used for monitoring of changes in the frequency of IFN-γ-secreting responder cells in noncultured or cultured lymphocyte populations. To establish that the assay is able to detect the T-cell precursor cells responsive to the vaccine, we used CD8+ T-cell populations positively selected from PBMC of HLA-A2+ patients with metastatic melanoma, who were treated with dendritic cell-based vaccines containing gp100, MELAN-A/MART-1, tyrosinase, and influenza virus matrix peptides. The frequency of peptide-specific responder T cells ranged from 0 to 1/2,600 before vaccination and increased by at least 1 log unit after vaccination in two patients, one of whom had a clinical response to the vaccine. However, no increases in the frequency of peptide-responsive T cells were observed in noncultured PBMC or PBMC cultured in the presence of the relevant peptides after the melanoma patients enrolled in another trial were treated with the intramuscular peptide vaccine plus MF59 adjuvant. Thus, while the ELISPOT assay was found to be readily applicable to assessments of frequencies of CTL precursors of established CTL lines and ex vivo-amplified PBMC, its usefulness for monitoring of fresh PBMC in patients with cancer was limited. In many of these patients antitumor effector T cells are present at frequencies of lower than 1/100,000 in the peripheral circulation. Serial monitoring of such patients may require prior ex vivo amplification of specific precursor cells.

The ELISPOT assay has been described as a method which can measure the frequency in a clonal population of T cells capable of responding to the antigen by secretion of cytokines (5, 9, 10, 28, 29). While the assay has been extensively evaluated for its ability to estimate the frequencies of antiviral effector cells, only a few studies used ELISPOT for the assessment of antitumor responses (22, 29). With the recent introduction of antitumor vaccines, a great deal of interest has developed in ELISPOT and its utilization for monitoring of antigen- or peptide-specific responses to tumor vaccines in patients with cancer. A number of vaccine trials have been in progress, mainly with patients with metastatic melanoma, as a result of recent successes in the identification of a rapidly increasing number of unique HLA-restricted melanoma peptides (2, 33, 34, 40). In contrast to the case for viral infections, however, it has been difficult to demonstrate the presence of tumor-specific cytotoxic T lymphocytes (CTL) (4, 11) or their generation as a result of vaccine administration to patients with advanced cancer (12, 23). Even in patients with metastatic melanoma who had complete or partial clinical responses following vaccination with MAGE-3, the presence of MAGE-3-specific CTL circulating in the peripheral blood could not be demonstrated (16). In other vaccination trials, CTL responses were detectable only after several cycles of in vitro stimulation of peripheral blood mononuclear cells (PBMC) with the immunizing peptides (26). This is in contrast to vaccinations with viral peptides, e.g., influenza virus peptides, where the ELISPOT assay is able to detect peptide-specific memory CD8+ T cells in freshly isolated PBMC (6, 15). It is reasonable to anticipate that, unlike T cells mediating antiviral immune responses (1, 19), T cells with specificity for self or differentiation epitopes (which are potentially tolerogenic) might be infrequent or absent. Therefore, a sensitive and reliable assay that allows for accurate detection of frequencies, and particularly for demonstration of increased postvaccination frequencies, of T cells responsive to the peptides or proteins used in the vaccine is essential for monitoring patient responses or for confirming their absence.

The only assay known to reliably measure frequencies of single-antigen-responding T cells is the limiting-dilution assay (LDA), which has been extensively utilized in human tumor antigen studies to estimate the numbers of proliferating T-lymphocyte precursors or CTL precursors (CTL-p) in various effector cell populations (32). However, with immune cells obtained from cancer patients, LDA has generally detected low CTL-p frequencies (17, 37). Furthermore, LDA does not...
lend itself to routine clinical monitoring, largely because of its technical complexity, and efforts to replace it with a more practical but equally sensitive method have been undertaken in a number of different laboratories (7, 9, 10, 13, 14, 20, 28, 29).

We describe here the development, preclinical assessment, and application to cancer patient monitoring of a modified ELISPOT assay for individual T cells which secrete gamma interferon (IFN-γ) in response to specific, major histocompatibility complex (MHC)-restricted stimulating antigens or anti- genic peptides. The assay is applicable to frequency measurements of T cells with ex vivo-activated lymphocyte populations on established CTL lines. However, its utility may be limited for a routine evaluation of patient samples such as fresh PBMC obtained from patients with cancer.

MATERIALS AND METHODS

Tumor and lymphocyte cell lines. The HLA-A2* human tumor cell lines PCI-13, a squamous carcinoma of the head and neck (SCCHN), and HR, a gastric carcinoma, were established from tumor biopsies and maintained in culture as previously described (8, 30). A human melanoma cell line, Mel 526, was established from a tumor biopsy obtained from Steven A. Rosenberg. CTL line 1520 is specific for the gp100209-217 peptides, and CTL line 1088 is specific for the MELAN-A/MART-1 146-154 peptide. Both lines are restricted by HLA-A2. The CTL lines were cultured in heparinized tubes. PBMC were isolated by Ficoll-Hypaque gradient centrifugation. PBMC were separated into CD8+ T cells by negative selection performed with each CTL line to enrich for CD8+ T cells. Frequency determinations were performed on day 31 of culture with CTL line 4 and on day 39 with CTL line 5.

Generation and culture of PCI-13-specific CTL lines. The CTL lines were induced from leukopaks obtained from HLA-A2* platelet donors through the Central Blood Bank of Pittsburgh, Pittsburgh, Pa. Mononuclear cells were separated from Ficol-Hypaque gradients, washed, and used for induction of CTL lines as follows.

(i) In the case of CTL lines 1 to 3, PBMC (106) were incubated with 105 irradiated (100 Gy) PCI-13 cells (a ratio of 10 responder cells to 1 stimulator cell) in wells of 24-well tissue culture plates containing 2 ml of AIM-V medium (Gibco) supplemented with 5% human AB serum (NABI, Miami, Fla.), 100 IU of IL-2 (Chiron, Emeryville, Calif.) per ml, 10 U of IL-1β (Genzyme Corp., Cambridge, Mass.) per ml, 50 IU of IL-4 (Schering Plough, Kenilworth, N.J.) per ml, and 12.5 U of IL-7 (Sandoz, Vianen, The Netherlands). As a stimulator, the PCI-13 cell line was treated with 1000 IU of IFN-γ (Roosel UCLAF, Romainville, France) per ml for 48 h to increase expression of MHC class I molecules. Responder T lymphocytes were cultured at 37°C in an atmosphere of 5% CO2 in air and were restimulated every 7 to 10 days with irradiated, HLA-A2-restricted, allogeneic PBMC obtained from a healthy donor used as feeder cells. The culture medium was AIM-V containing 5% (vol/vol) heat-inactivated human AB serum, IL-2 (10 IU/ml), IL-1β (0.2 ng/ml), and IL-7 (0.2 ng/ml). At 2 to 3 weeks later, wells containing proliferating lymphocytes were quantitated in order to determine the frequency of proliferating T-lymphocyte precursors.

(ii) In the case of CTL lines 4 and 5, first and second ex vivo stimulations were performed with autologous dendritic cells (DC) pulsed with a peptide preparation obtained from PCI-13 cells. After the third stimulation, irradiated PCI-13 cells were used as stimulators, exactly as described above.

The CTL lines were tested for specificity in 4-h 3HCr release assays against PCI-13 cells and a panel of HLA-A2* and HLA-A2* tumor cell lines and normal cell targets. Blocking with anti-MHC class I and anti-HLA-A2 antibodies (Abs) was used to confirm that the CTL lines were HLA-A2 restricted.

PCI-13-derived peptide preparation. PCI-13 cells were grown in a cell factory (Nunc, Fisher Scientific) until they were 80% confluent in culture medium consisting of Dulbecco modified Eagle medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 IU of penicillin and streptomycin per ml, 2 mM l-glutamine, and 50 μg of gentamicin per ml (all from Gibco). Prior to trypsination, the monolayers were washed twice with Hank’s balanced salt solution (HBSS). Trypsin-EDTA solution was added, and a single-cell suspension was obtained. All cell cultures were harvested, washed extensively in 0.05% Tween 20–PBS, and blocked with 1% (vol/vol) bovine serum albumin in PBS for 2 h at 37°C. PBMC, purified T cells, or cultured T cells resuspended in AIM-V medium supplemented with 10% (vol/vol) AB human serum were then added at various numbers, e.g., from 103 to 500 cells per well in triplicate wells, and the plates were span at 200 × g for <1 min. The stimulatory peptides pulsed onto irradiated presenting cells (e.g., T2 or C1R.A2) or irradiated tumor cells were then added to each well, and the plates were incubated for 24 h (for CTL lines or T cells cultured in the presence of IL-2) or 48 h (for noncultured PBMC plus stimulators) at 37°C. Next, the plates were vigorously washed six times with the solution of 0.05% Tween 20–PBS, and the biotinylated detection anti-IFN-γ Ab (Pharmingen catalog no. 18891D) per ml in 50 μl of the diluent (phosphate-buffered saline [PBS], pH 7.2) per well. The plates were incubated overnight in a moist chamber, washed extensively in 0.05% Tween 20–PBS, and blocked with 1% (vol/vol) bovine serum albumin in PBS for 2 h at 37°C. PBMC, purified T cells, or cultured T cells resuspended in AIM-V medium supplemented with 10% (vol/vol) AB human serum were then added at various numbers, e.g., from 103 to 500 cells per well in triplicate wells, and the plates were span at 200 × g for <1 min. The stimulatory peptides pulsed onto irradiated presenting cells (e.g., T2 or C1R.A2) or irradiated tumor cells were then added to each well, and the plates were incubated for 24 h (for CTL lines or T cells cultured in the presence of IL-2) or 48 h (for noncultured PBMC plus stimulators) at 37°C. Next, the plates were vigorously washed six times with the solution of 0.05% Tween 20–PBS, and the biotinylated detection anti-IFN-γ Ab (Pharmingen catalog no. 18902D) was added at 5 μg/ml. The plates were then incubated for 2 h at 37°C, washed, and developed with peroxidase-conjugated streptavidin. The plates were washed again to remove excess conjugate, the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and an additional 20 to 30 min of incubation, the plates were prepared for counting of blue spots, microscopically or using image analysis. The number of blue spots per well was determined by correlated phase-contrast microscope (Nikon model SZH10), or with a computer-assisted image analysis system (KS ELISPOT; Carl Zeiss, Hallbergmoos, Germany). The frequency of positive
(IFN-γ-producing) cells per the total number of plated cells was calculated after the number of spots in control wells had been subtracted from that in experimental wells. These control wells contained T2 cells and responding lymphocytes but no peptide(s). As control wells for the assay included reagent (blank), nonstimulated effector cells (spontaneous IFN-γ production), or normal PBMC stimulated with phorbol myristate acetate (PMA) at 1 ng/ml and ionomycin at 1 μg/ml and IFN-γ production), or normal PBMC stimulated with phorbol myristate acetate (PMA) at 1 ng/ml and ionomycin at 1 μg/ml and IFN-γ production), or normal PBMC stimulated with phorbol myristate acetate (PMA) at 1 ng/ml and ionomycin at 1 μg/ml and IFN-γ production), or normal PBMC stimulated with phorbol myristate acetate (PMA) at 1 ng/ml and ionomycin at 1 μg/ml and ionomycin at 1 μg/ml and ionomycin at 1 μg/ml and ionomycin at 1 μg/ml and ionomycin.

**Synthetic peptides.** Peptides were synthesized using 9-fluorenylmethoxy carbonyl chemistry by the Peptide Synthesis Facility at the University of Pittsburgh Cancer Institute (UPCI). Each peptide was purified to >95% homogeneity by reverse-phase high-pressure liquid chromatography, and the identity of each peptide was confirmed by mass spectrometry. The following peptides were synthesized and used in the present study: MART-1-27-35 (AAGIGILTV) (recognized by TIL 1088), gp100-209-217 (ITDQVPFSV) (recognized by TIL 1520), tyrosinase (YMDGTMSOV), and an influenza virus matrix peptide, flu M1 65-70 (GILGFVFTL).

**Cytotoxicity assays.** Four-hour 51Cr release assays were performed in triplicate, as previously described (35), using tumor cells (P13-15 or Mel 562) as targets. The percent specific lysis was calculated as ([experimental cpm - spontaneous cpm]/[spontaneous cpm] × 100). The data were expressed as lytic units (LU/L)106 effector cells, using a computer program, as previously described (35). Unlabeled K562 cells were used as cold targets in all cytotoxicity experiments (35).

**Flow cytometry.** Bulk or cultured lymphocytes were stained with fluoresein- or phycoerythrin-labeled monoclonal Abs to surface antigens CD3, CD8, CD4, and HLA-A2 (Becton Dickinson, San Jose, Calif.) and examined in a flow cytometer as described earlier (39). Isotype control Abs were included in all experiments.

**Patient specimens.** Blood specimens from patients with metastatic melanoma who participated in one phase I vaccination trials at the UPCI were obtained pre- and postvaccination. In one trial, 24 patients with metastatic melanoma were randomized to receive a vaccine of either MELAN-A/MART-1, gp100, or tyrosinase peptide given intramuscularly with MF59 as an adjuvant. Patients received between 1 and 5 weekly courses of a vaccine, except for one patient who received 11 courses. In the second, phase I-II clinical trial, 25 patients with high-risk stage III or IV metastatic melanoma received one to four courses of intravenous vaccine with autologous DC pulsed with five different peptides: MELAN-A/MART-1 (ILTVGLVIL), MELAN-A/MART-1 (AAGIGILTV), gp100 (YLGPGPVTA), tyrosinase (YMDGTMSOV), or flu matrix (GILGF VFTL).

Patients’ PBMC were recovered on Ficoll-Hypaque gradients and cryopreserved to allow for testing of pre- and postvaccination samples in the same ELISPOT assay. Two approaches were used to enrich PBMC in T cells responsive to the immunizing peptides. One used PBMC which were thawed, washed, and placed in culture with the peptide(s) (10 μM) used for vaccinations for 14 days. Conditions for expansion of bulk T-cell cultures were the same as those described for PMA-13-specific CTL lines above. The second approach utilized PBMC which were thawed, washed, and placed in culture on the day of the assay, and following enrichment in CD8+ T cells, were tested in ELISPOT assays without ex vivo expansion.

**Statistical analysis.** Differences between paired groups of values were tested using the Wilcoxon test. Whenever applicable, Student’s t test was also used. Differences with a P value of <0.05 were considered significant.

## RESULTS

### Selection and titration of anti-IFN-γ Abs. The ELISPOT assay used two monoclonal Abs directed against different determinants of human IFN-γ. To determine the optimal concentrations of these capture and detection Abs, either a gp100-specific CTL line or normal human PBMC activated with PMA-ionomycin were plated in triplicate wells of 96-well plates coated with various dilutions of the capture Ab. After 24 or 48 h of incubation, supernatants were removed, and after extensive washing, the detection Ab was added at various dilutions. These checkerboard titrations were performed in at least three independent experiments. As shown in Table 1, the optimal Ab dilutions were determined to be 5 μg/ml for capture Abs and 2.5 μg/ml for detection Abs. The lots of Abs titrated as described above were reserved and purchased in bulk to ensure the reproducibility of the assay.

**TABLE 1. Titration of the capture and detection Abs for use in ELISPOT**

| Detection Ab concn (μg/ml) | Spots counted¹/¹⁰⁵ CTL plated with capture antibody concn (μg/ml) of: |
|---------------------------|---------------------------------------------------------------|
|                           | 1                          | 2.5                        | 5                          | 10                         |
|                           | 1                          | 26,000                     | 35,000                     | 49,000                     |
|                           | 2.5                        | 38,000                     | 60,000                     | 46,000                     |
|                           | 5                          | 25,000                     | 50,000                     | 45,000                     |
|                           | 10                         | 44,000                     | 46,000                     | 52,000                     |

*Checkboard titrations were performed to determine the optimal number of spots per well in an ELISPOT assay for IFN-γ production. Melanoma gp100-reactive CTL (line 1520), used as responders, were titrated into wells and stimulated with gp100 (1 μg/ml)-pulsed T2 cells for 24 h. The capture and detection Abs described in Materials and Methods were used at various concentrations, as indicated. The numbers shown are extrapolated. Results from a representative experiment of three performed is shown.

1. NS, no spots detected.
2. Largest number of spots detected.
3. * Statistical analysis.

**Counting of spots.** Spots associated with IFN-γ secretion by single cells were evaluated microscopically using an inverted phase-contrast microscope or a computer-assisted image analysis system. To avoid bias, microscopic counts were independently determined by two technologists, and the results were averaged. Comparisons between the two scoring methods indicated that the numbers of spots counted were comparable for wells containing the same number of plated cells. In 20 individual comparisons of the two scoring methods at various cell concentrations per well, the mean values ± standard deviations (SD) were 164 ± 146 spots counted microscopically and 144 ± 129 spots counted by image analysis (P > 0.05). Spot counts could be performed faster and with fewer dilutions of the plated cells in the image analysis system than by eye. The advantage of the image analysis system is that wells containing large numbers of spots can be accurately scored. Also, for monitoring of large numbers of wells, computer-assisted analysis is more practical.

**Frequency of IFN-γ-producing cells in populations of PMA-ionomycin-stimulated normal PBMC.** Using the ELISPOT assay, we first determined the frequency of T lymphocytes able to secrete IFN-γ after stimulation with PMA-ionomycin in PBMC obtained from normal donors. As indicated in Table 2, this frequency was found to range from 1/2 to 1/500 in PBMC obtained from 20 normal donors, with 47 distinct assays performed. The determined mean frequency (± SD) of 10% ± 11.8% of IFN-γ-producing cells in response to the nonspecific T-cell activators PMA and ionomycin (Table 2) was used as a reference normal control value. In all ELISPOT assays, PBMC of at least one normal donor were routinely used as a positive assay control.

**Optimal conditions for ELISPOT assays with different types of effector cells.** In initial experiments, attempts were made to optimize the assay for different types of effector cells, e.g., fresh PBMC or antigen-specific CTL lines. We observed that the quality of the spots was markedly different depending on the effector cells used. The quality of spots obtained in an ELISPOT assay performed with a PMA-13-specific CTL line is shown in Fig. 1. Large, diffuse spots signify the secretion of considerable levels of IFN-γ. In contrast to spots seen with CTL lines cultured in the presence of IL-2, the quality of spots seen with fresh PBMC stimulated with PMA-ionomycin was notably different (Fig. 2). Intense well-defined small or large spots were seen in this system (Fig. 2), with the sizes of spots reflecting the variable quantities of IFN-γ secreted by individual T-cell clones.
An advantage of ELISPOT assays performed in conventional plastic plates without nitrocellulose inserts is that supernatants can be easily recovered from each well, without disturbing the spots, and tested for levels of IFN-γ by ELISA. We were therefore able to confirm that ELISPOT supernatants from wells with diffuse, large spots formed by CTL (Fig. 1) contained \(610 \pm 192\) pg of IFN-γ per ml (mean ± SD from four determinations), while wells with the compact, small spots seen with PMA-ionomycin-activated PBMC (Fig. 2) contained only \(34 \pm 20\) pg of IFN-γ per ml. This observation suggested that the conditions of the assay may have to be very carefully adjusted in order to achieve optimal spot formation. To this end, we performed ELISPOT assays with PCI-13-specific CTL lines and fresh or frozen and thawed PBMC activated with PMA-ionomycin under various experimental conditions (data not shown). Comparisons of fresh with cryopreserved PBMC \((n = 10)\) showed no significant differences \((P > 0.5)\) in the frequencies of IFN-γ-producing cells stimulated with PMA-ionomycin. These experiments allowed for the following conclusions to be made. (i) With fresh or frozen and thawed PBMC, the ELISPOT assay should be performed in the presence of 10% (vol/vol) AB serum and 20 IU of IL-2 per ml and the effector cells need to be incubated with a stimulator (antigen) for 24 to 48 h, with small, intense, pin-like spots to be expected. (ii) With CTL lines or PBMC sensitized in vitro with antigens and IL-2, the assay should be performed in the absence of serum or exogenous IL-2 for 20 to 24 h, with large, diffuse spots to be expected. In either case, it is necessary to select the optimal antigen concentration in preliminary experiments or to perform the ELISPOT assay at several different antigen concentrations.

Reproducibility and sensitivity of ELISPOT assays. In order to assess the performance characteristics of the ELISPOT assay for IFN-γ, we first determined its intra- and interassay variabilities, using PMA-ionomycin as a nonspecific T-cell stimulator. As shown in Table 3, both the interassay reproducibility and that of assays performed on the same day but plated in different plates were excellent. In general, the frequency of

### TABLE 2. ELISPOT assays for IFN-γ production by PBMC obtained from normal donors

| Assay conditions                                      | Frequency | IFN-γ-producing cells (%) |
|-------------------------------------------------------|-----------|---------------------------|
| Medium (spontaneous release)                          | \(<1/100,000^b\) | \(<0.001^b\) |
| Medium + PMA (1 ng/ml) + ionomycin (1 μM)             | \(1/10 (1/500–1/2)^c\) | \(10 \pm 11.8 (0.2–50)^d\) |

*PBMC were obtained from 20 normal volunteers and were tested in 47 distinct assays over a period of 6 months. PBMC were titrated into wells of ELISPOT plates \((10^3\) to \(10^7\) cells/well) in triplicate and incubated in medium alone or with the stimulating agents for 48 h at 37°C. Spots were counted to determine frequencies of responding cells in each population.

^b Theoretical sensitivity of the ELISPOT assay with 100,000 cells plated per well.

^c Mean (range).

^d Mean ± SD (range).
The presented data are spot counts obtained with titrated PBMC of the same normal individual, which were cryopreserved, thawed, stimulated with PMA-ionomycin, and tested in ELISPOT assays performed on different days. Limiting dilution of the samples was performed (Table 4), and the samples plated in triplicate were scored for the number of spots at each cell concentration. The mean numbers of spots in assays run on different days were compared and found to differ minimally (<1%). The representative limiting-dilution plots for cryopreserved PBMC obtained from another normal individual and tested in five independent ELISPOT assays are shown in Fig. 3. They confirm the excellent reproducibility of the assay.

To determine the sensitivity of the assay, a melanoma-specific CTL line, with a predetermined frequency of IFN-γ-producing cells, was titrated into wells containing nonactivated PBMC of the same donors. As shown in Table 5, the frequency of IFN-γ-producing cells was 1/3 in the CTL line, and the ELISPOT assay detected even 1 IFN-γ-producing CTL in 100,000 PBL. Thus, the theoretical and practical sensitivities of the ELISPOT assay were found to be the same at 1 positive cell per 100,000 cells plated.

Frequencies of melanoma peptide-specific T cells in cultured CTL lines. We next applied the ELISPOT assay to determine the frequencies of peptide-specific T cells among CTL lines (no. 1520 and 1088) with known specificity for melanoma targets, such as Mel 526, which express gp100 and MELAN-A/MART-1. The CTL lines were maintained in culture in the presence of 100 IU of IL-2 per ml. Line 1520 recognizes an HLA-A2-binding gp100,209-217 epitope, while line 1088 recogn-

### TABLE 3. Reproducibility of ELISPOT assays

| Comparison                      | Assay date (mo/day/yr) | Frequency (%) of IFN-γ-producing cells |
|---------------------------------|------------------------|----------------------------------------|
| Assays performed on different days | 10/14/97               | 1/20 (5)                               |
|                                  | 3/10/98                | 1/15 (7)                               |
|                                  | 10/27/97               | 1/25 (4)                               |
|                                  | 12/2/97                | 1/25 (4)                               |
|                                  | 11/27/97               | 1/30 (3)                               |
|                                  | 1/6/98                 | 1/40 (2.5)                             |
|                                  | 10/27/97               | 1/25 (4)                               |
|                                  | 3/24/98                | 1/25 (4)                               |
|                                  | 4/21/98                | 1/16 (4)                               |
|                                  | 4/8/98                 | 1/6 (17)                               |
|                                  | 5/12/98                | 1/7 (15)                               |
| Assays performed on the same day | 1/21/98                | 1/6 (17)                               |
|                                  | 1/21/98                | 1/6 (17)                               |
|                                  | 12/29/97               | 1/21 (5)                               |
|                                  | 12/29/97               | 1/36 (3)                               |
|                                  | 4/15/98                | 1/10 (10)                              |
|                                  | 4/15/98                | 1/9 (11)                               |
|                                  | 1/28/98                | 1/20 (5)                               |
|                                  | 1/28/98                | 1/18 (6)                               |
|                                  | 4/1/98                 | 1/10 (10)                              |
|                                  | 4/19/98                | 1/12 (8)                               |

* ELISPOT assays were performed with PBMC obtained from normal donors. Cells were stimulated with PMA (1 ng/ml) plus ionomycin (1 μM) for 48 h. Assays were performed on the same or different days with PBMC of the same donors. Assays performed on the same day were set up on different plates in parallel. Spot counts in triplicate wells containing the same numbers of cells were not significantly different ($P > 0.05$). The mean of the difference ± SD for all paired values was $1\% \pm 1\%$.

### TABLE 4. Limiting dilution of samples and interassay reproducibility in ELISPOT

| Day | Cells/well | Spot count | Spots/10^6 cells | Frequency (1/100,000) |
|-----|------------|------------|------------------|-----------------------|
|     |            | Well 1     | Well 2           | Well 3                | Mean    | SD      |                |
| A   | 10,000     | 400        | 400              | 400                   | 400     | 0       | 40,000          | 25       |
|     | 2,000      | 73         | 82               | 78                    | 78      | 3.7     | 39,000          | 26       |
|     | 400        | 16         | 18               | 17                    | 17      | 0.8     | 42,500          | 24       |
|     | 80         | 4          | 4                | 2                     | 3.3     | 1.1     | 41,250          | 25       |
| B   | 10,000     | 381        | 352              | 368                   | 367     | 11.9    | 36,700          | 27       |
|     | 2,000      | 99         | 95               | 97                    | 97      | 1.6     | 48,500          | 21       |
|     | 400        | 15         | 21               | 18                    | 18      | 2.4     | 45,000          | 22       |
|     | 80         | 4          | 3                | 5                     | 4       | 1.0     | 50,000          | 20       |
| C   | 10,000     | 500        | 425              | 410                   | 445     | 48.2    | 48,200          | 21       |
|     | 2,000      | 108        | 80               | 94                    | 94      | 11.4    | 47,000          | 21       |
|     | 400        | 20         | 21               | 15                    | 19      | 3.2     | 47,500          | 21       |
|     | 80         | 5          | 4                | 5                     | 4.6     | 0.6     | 57,500          | 17       |

* Representative ELISPOT results selected from over 50 distinct assays performed with cryopreserved PBMC of the same normal individual are shown. The PBMC were thawed, titrated into wells of ELISPOT plates (10^5 to 80 cells/well) in triplicate, and activated with PMA-ionomycin (see Materials and Methods). Triplicate control wells containing cells incubated in medium alone were also plated. Following 48 h of incubation at 37°C, all control wells were negative (no spots), while the wells plated with 10^6 cells stimulated with PMA plus ionomycin contained too many spots to be counted. The presented data are spot counts obtained with titrated PBMC of the same individual tested by ELISPOT on three different days (A, B, and C). The mean difference ± SD in the frequency of IFN-γ-secreting cells was not significant between the assays performed on different days (<1% ± 0.2%).
nizes the MELAN-A/MART-127-35 peptide (personal communication). The frequency of T cells able to respond to the gp100 peptide (1 μg/ml) presented on T2 cells was 1/2 in CTL line 1520. T-cell line 1520 had a frequency of 1/260 for T cells specific for the MELAN-A/MART-1 peptide (Table 6). When irradiated Mel 526 targets were used as stimulators, the frequency was 1/33 for the gp100-specific and 1/434 for the MELAN-A/MART-1-specific T-cell lines. These results suggest that the assay is able to effectively detect and quantitate tumor antigen-specific T cells in bulk cultured T-cell lines.

To further show that the production of IFN-γ by human tumor-specific CTL upon stimulation with the autologous tumor depends on MHC class I-restricted antigen presentation, we have evaluated a series of CTL lines which recognize a shared antigen expressed on HLA-A2+ SCCHNs but not on other HLA-A2+ human carcinomas (our unpublished data).

### TABLE 5. Sensitivity of the ELISPOT assay

| Assay conditions | No. of expected spots/well | No. of counted spots/well | Frequency (%) of IFN-γ-producing cells |
|------------------|-----------------------------|---------------------------|--------------------------------------|
| CTL alone (100)  | 33                          | 33                        | 1/3                                  |
| PBL alone (10^3) | 0                           | 0                         | <1/100,000                           |
| PBL (10^3) + CTL (100) | 33                      | 32                        | 1/3,128                              |
| PBL (10^3) + CTL (50) | 16                       | 16                        | 1/6,253                              |
| PBL (10^3) + CTL (25) | 8                         | 9                         | 1/11,113                             |
| PBL (10^3) + CTL (5) | 1                          | 1                         | 1/100,000                            |
| PBL (10^3) + CTL (3) | 1                          | 1                         | 1/100,000                            |

* CTL line 1520 (gp100 peptide specific) was used in titration experiments. This line was pretested in ELISPOT for the frequency of IFN-γ-producing T cells in response to gp100 peptide on the day preceding the titration experiment. The frequency was 1/3. Decreasing numbers of CTL were mixed with normal nonactivated PBL (10^3/well), and ELISPOT assays were performed, using the gp100 peptide (1 μg/ml) for T-cell stimulation in triplicate wells. Results are from one of two titration experiments performed.

These lines were established and characterized in our laboratory (21, 36). As shown in Table 7, preincubation of PCI-13 tumor cells with anti-HLA class I Ab (0.4 μg of w6/32) completely eliminated tumor-specific IFN-γ production by the CTL, reducing the frequency of detectable spots from 1/32 to 1/1,000, which is equal to the frequency observed for T cells alone. These data show that the modified ELISPOT assay could be readily used to quantitate the frequency of tumor-specific T cells in cultures of lymphocytes obtained from patients with melanoma, head and neck cancer, or other malignancies. It should be noted, however, that this frequency varied widely even in the same CTL line tested at different time points of ex vivo culture.

### Comparison of ELISPOT assay with LDA.

To further confirm that ELISPOT assay can be reliably performed in lieu of LDA, we directly compared the two assays, using CTL lines generated from normal PBMC sensitized with PCI-13 cells or PCI-13-derived peptides in vitro. The bulk CTL lines generated as described in Materials and Methods were first tested in cytotoxicity assays against PCI-13 targets, in the presence or absence of anti-class I (w6/32) or anti-HLA-A2 (BB7.2) block-

### TABLE 7. ELISPOT assay for IFN-γ production by PCI-13-reactive HLA-A2-restricted CTL

| Assay conditions | Frequency (%) of IFN-γ-producing cells |
|------------------|--------------------------------------|
| CTL 1 (spontaneous release) | 1/1,000 (0.1) |
| CTL 1 + Ir PCI-13 - | 1/5 (12.5) |
| CTL 2 (spontaneous release) | 1/1,000 (0.1) |
| CTL 2 + Ir PCI-13 - | 1/32 (3.1) |
| CTL 2 + Ir PCI-13 | 1/50 (2.0) |
| CTL 2 + Ir PCI-13 + W6/32 Ab* | 1/1000 (0.1) |
| CTL 2 + Ir HR* | 1/1000 (0.1) |

* Two different PCI-13 (SCCHN)-reactive CTL lines were tested. T cells were titrated into wells of ELISPOT plates (6 × 10^4 to 2 × 10^5 cells) in triplicate and coincubated with irradiated (Ir) PCI-13 cells (10 lymphocytes/tumor cell) for 48 h at 37°C. Spots were counted to determine the frequency of IFN-γ-producing T cells in each coculture.

* The CTL 2 line was tested on different days 2 weeks apart.

* PCI-13 cells were preincubated with anti-MHC class I Ab (w6/32) for 30 min.

* CTL 2 cells were coincubated with the HLA-A2+ gastric carcinoma cell line used as a control.
TABLE 8. Comparison of ELISPOT with LDA for the ability to measure the frequency of T cells in CTL lines responding to PCI-13 by IFN-γ productiona

| CTL line | ELISPOT assay [frequency (%) of cells secreting IFN-γ] | LDA [no. of clones with specific lysis of >10%/no. of wells] |
|----------|--------------------------------------------------------|------------------------------------------------------------|
| CTL.3    | 1/71 (1.41)                                            | 5/480 (1.04)                                               |
| CTL.4    | 1/179 (0.56)                                           | 3/480 (0.63)                                               |
| CTL.5    | 1/91 (1.10)                                            | 4/464 (0.86)                                               |

a PCI-13-specific bulk CTL lines were established from PBMC of normal donors by multiple rounds of in vitro sensitization with irradiated PCI-13 (CTL.3) or with the peptide preparations derived from PCI-13 and presented on DC (CTL.4 and CTL.5). The cells were plated in LDA at 1 cell/well. All proliferating microcultures were studied for the ability to kill PCI-13 targets in 4-h 51Cr release assays. The ELISPOT assays are described in Materials and Methods.

TABLE 9. ELISPOT assay for melanoma peptide-specific T lymphocytes in the peripheral blood of patients with melanoma vaccinated with peptide-based tumor vaccinea

| Patient | Time         | Frequency of IFN-γ-secreting T cells |
|---------|--------------|-------------------------------------|
|         |              |          |                        |
| 1       | Prevaccination | 1/3,300 |
|         | Postvaccination | 1/1,500 |
| 2       | Prevaccination | 1/727   |
|         | Postvaccination | 1/800   |
| 3       | Prevaccination | 1/4,000 |
|         | Postvaccination | 1/3,333 |

a The patients with metastatic melanoma were participants in a phase I vaccination trial performed at the UPCI (see Materials and Methods). The data shown are for three patients randomized to receive the MELAN-A/MART-1 peptide vaccine plus MF59 as adjuvant. Their PBMC were obtained prior to and after intramuscular vaccine administration, expanded in culture during two cycles of stimulation with a mixture of melanoma peptides (including the MELAN-A/MART-1 peptide [AAGGLILTV] used for vaccination), and on day 17 tested in ELISPOT assays for IFN-γ production in response to the same MELAN-A/MART-1 peptide (1 μg/ml) pulsed onto CIR.A2 cells. No MELAN-A/MART-1-responsive lymphocytes were detected in fresh, noncultured PBMC of the three patients. These patients’ data were randomly selected to illustrate ELISPOT responses obtained pre- and postvaccination in this clinical trial.

TABLE 10. ELISPOT responses of positively selected CD8+ T cells to melanoma peptides in peripheral blood of patients with metastatic melanoma vaccinated with peptide-based tumor vaccinea

| Patient | Stimulating peptide | Frequency of IFN-γ-secreting T cells: |
|---------|---------------------|-------------------------------------|
|         |                     | Prevaccination | Postvaccination |
| 1       | gp100               | 0 | 0 |
|         | Tyrosinase          | 0 | 0 |
|         | MELAN-A/MART-127-35 | 1/10,000 | 1/1,000 |
|         | Flu matrix36-66     | 1/7,000 | 1/600 |
| 2       | gp100               | 0 | 1/10,000 |
|         | Tyrosinase          | 0 | 1/2,500 |
|         | MELAN-A/MART-127-35 | 1/2,600 | 1/200 |
|         | Flu matrix36-66     | 1/14,000 | 1/1,500 |
| 3       | gp100               | 0 | 0 |
|         | Tyrosinase          | 0 | 0 |
|         | MELAN-A/MART-127-35 | 0 | 0 |
|         | Flu matrix36-66     | 1/6,250 | 1/16,600 |

a The patients with metastatic melanoma were participating in a DC-based multipipeptide melanoma peptide vaccine at the UPCI. Their PBMC were obtained prior to and after vaccine administration and cryopreserved. Prior to ELISPOT assay, PBMC were thawed, and positively selected CD8+ T cells were tested in ELISPOT assay for IFN-γ secretion in response to individual peptides pulsed on T2 cells at a concentration of 1 μg/ml.
freshly harvested PBMC in most patients with metastatic melanoma vaccinated in our two phase I clinical trials.

**DISCUSSION**

Cytokine release determined by ELISA or \(^{51}\)Cr release cytotoxicity assays, which are MHC restricted, has been frequently used to measure T-cell responses to antigenic epitopes (3, 19, 33). However, these assays estimate bulk effector responses, without providing an estimate of the number of cells which are functionally responsive to a given stimulus. In situations when a comparison of the frequencies of responding T cells in two populations, e.g., prior to and after vaccination, is necessary, these assays are particularly uninformative. On the other hand, the only assay available for the analysis of frequencies of specific T cells, the LDA, is not applicable to serial monitoring of patient responses, largely owing to its complexity and a labor-intense format. The ELISPOT assay for secretion of cytokines (TNF-\(\alpha\), IFN-\(\gamma\), or granulocyte-macrophage colony-stimulating factor) by single responders is widely considered to be the best replacement for LDA (10, 24, 28, 29), although few direct comparisons between the two types of assays have been reported so far (15, 18). Studies comparing the two assays have utilized viral antigens, which induce robust memory responses (see, e.g., reference 15). Human tumor-specific responses, on the other hand, are always difficult to quantitate, especially by LDA, perhaps because of the self nature of epitopes involved or tumor-induced immunosuppression which impairs lymphocyte proliferation (17, 37). A great need exists for a clinically applicable assay, such as ELISPOT, to measure frequencies of antigen-responsive T cells, preferably without ex vivo amplification, which could introduce in vitro artifacts.

To be able to utilize the ELISPOT assay for monitoring of those patients with cancer who receive cancer vaccines, it was first necessary to establish its feasibility, sensitivity, and reliability. We performed these studies with PBMC obtained from normal donors stimulated with nonspecific activators (such as PMA and ionomycin), with tumor antigen-specific CTL lines, or with bulk T-cell lines generated by in vitro sensitization of normal or patient-derived lymphocytes with tumor peptides or irradiated tumor cells. Our results allowed us to determine performance characteristics and make recommendations for the optimal utilization of this assay, depending on the type of effector cell tested. As the assay is antibody based, the quality and titer of the capture and detection antibodies are of particular importance. Also, effector cells are plated in a single cell layer in this assay, and, thus background effects, levels of the cytokine produced by individual cells, interactions between the plated cells, and the presence or absence of exogenous cytokines are likely to influence the assay results. Similarly, conditions used for in vitro sensitization, including the concentration and presentation of peptides, the effector/target cell ratio, the presence of AB serum, and time of incubation of effector cells with the stimulating agents were found to be critically important for the number and appearance (intensity and size) of spots. For these various reasons, ELISPOT assays have to be performed under carefully defined and strictly quality-controlled conditions. Nevertheless, the assay, when established and routinely executed by an experienced laboratory, was found to be highly reproducible, with an interassay coefficient of variation of 15%. Its sensitivity was found to be 1/100,000 cells.

The question arises as to the rationale for selection of IFN-\(\gamma\) as the cytokine of choice for the ELISPOT assay. While activated T cells produce a variety of cytokines, both TNF-\(\alpha\) and IFN-\(\gamma\) have been reported to correlate with specific antitumor cytotoxicity in clonal as well as nonclonal ex vivo assays (9, 10, 13, 28, 33). In our hands, the IFN-\(\gamma\) ELISPOT assay gave lower nonspecific background and discriminated better between low and high cytokine secretion (as judged by the quality of small, dense spots versus large, diffuse spots) than the TNF-\(\alpha\) ELISPOT assay. In addition, IFN-\(\gamma\) secretion was shown to be MHC class I restricted, using CTL lines with specificity for SCCHN. However, the most convincing result was the positive correlation observed between the IFN-\(\gamma\) secretion by a tumor antigen-specific CTL line and the LDA (Table 8). It is necessary to realize, however, that this comparison was performed with an established CTL line which proliferated well and generated numerous clones. Nevertheless, we were reassured that the ELISPOT assay measured the frequency of individual antigen-specific effector T cells in bulk lymphocyte populations responding to the tumor as accurately as the LDA.

Having established the optimal conditions for the ELISPOT assay and having confirmed its reproducibility, we next applied this assay to the assessment of patients’ specimens obtained as a part of two different peptide-based vaccination protocols open to patients with metastatic melanoma at our institution. While the final results of these studies will be reported separately, it is interesting that we were unable to detect melanoma peptide-responsive T cells in the blood of several of these patients (Table 9) prior to or after vaccinations, using nonenriched PBMC for ELISPOT assays. These results are in agreement with reports by others (22, 26). On the other hand, following positive selection of CD8\(^{+}\) T lymphocytes, a procedure designed to enrich CTL-p in this fraction, ELISPOT assays were positive in some but not all patients with metastatic melanoma tested as a part of the DC-based vaccine trial (Table 10). For example, two of three patients immunized with the DC-based multiepitope vaccine showed increased postvaccination frequencies of T cells specific to MELAN-A/MART-1 and influenza virus peptides or to all vaccinating peptides. Patient 1 in Table 10 was a clinical responder to the vaccine.

Because the ELISPOT assay was negative in nonenriched PBMC of the majority of patients with metastatic melanoma initially tested, we next used rounds of in vitro stimulation with the relevant peptides presented on autologous PBMC to expand and amplify T-cell responses. With this ex vivo amplification, high frequencies of CTL were detected by ELISPOT assay both prior to and after vaccination in PBMC of patients with melanoma. In examples presented in Table 9, the prevaccination frequency of MELAN-A/MART-1-specific T cells as assessed in ELISPOT assays in three HLA-A2\(^{+}\) patients with melanoma ranged between 1/727 and 1/3,333. The frequencies of T cells responsive to the flu M155-56 peptide ranged between 1/500 and 1/10,000 (not shown). However, these frequencies did not change appreciably following vaccinations.

Overall, our results indicate that the ELISPOT assay is able to detect relatively low frequencies (i.e., 1/10\(^3\)) of antitumor-specific T cells and thus is about 2 log units more sensitive than cytotoxicity assays estimated to be able to detect 1/1,000 specific effector cells (24). However, even this sensitive assay cannot detect antitumor reactive T cells if their frequency is lower than 1/10\(^5\) in the peripheral blood of patients with cancer. Two strategies are presently available to determine the frequency of such rare CTL-p: (i) enrichment in CD8\(^{+}\) T cells prior to ELISPOT assay or (ii) culture of PBMC with at least two cycles of in vitro sensitization with relevant peptides to expand CTL-p. Both of these strategies increase the assay complexity, but in the presence of appropriate controls they can reliably
ELISPOT ASSAY FOR IFN-γ IN PATIENTS WITH CANCER

13. Kabilan, L., G. Andersson, F. Lolli, H.-P. Ekre, T. Olsson, and M. Troye-Blomberg. 1990. Detection of intra-cellular expression and secretion of interferon-γ at the single-cell level after activation of human T cells with tetanus toxoid in vitro. Eur. J. Immunol. 20:1085-1089.

14. Klännar, D. M. 1994. ELISPOT assay to detect cytokine-secreting murine and human cells. Curr. Protocols Immunol. 1:6–18.

15. Lalvani, A., R. Brooks, S. Hambleton, W. J. Briton, A. V. S. Hill, and A. J. McMichael. 1997. Rapid effector function in CD8+ memory T cells. J. Exp. Med. 186:595–604.

16. Marchand, M., P. Weynants, E. Rankin, F. Arietti, F. Belli, G. Parmiani, N. Cassinelli, A. Bourin, R. Vanwijk, Y. Humbel, J.-L. Canon, C. Laurent, J. J. Feyerta, R. Plague, H. J. Schumacher, A. Kauffman, J. Herman, P. G. Coulie, and T. Boon. 1995. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. Int. J. Cancer 60:803–815.

17. Mischler, S. T., L. Whiteside, L. Moretta, and V. Von Fieandt. 1987. Clonal and frequent analyses of tumor-specific lymphocytes from human solid tumors. J. Immunol. 138:4004–4011.

18. Miyahira, Y., K. Murata, D. Rodriguez, J. R. Rodriguez, M. Esteban, M. M. Rodriguez, and F. Vazquez. 1995. Quantification of antigen-specific CD4+ T cells using an ELISPOT assay. J. Immunol. Methods 181:45–54.

19. Morris, A. G., Y. L. Lin, and B. A. Askonas. 1982. Immune interferon release when a cloned cytotoxic T cell line meets its correct influenza-infected target cell. Nature 292:150–152.

20. Nordstrom, L., and B. Ferrua. 1992. Reverse elispot assay for clonal analysis of cytokine production. II. Enumeration of interleukin-1-secreting cells by amplified (avidin-biotin antiperoxidase) assay. J. Immunol. Methods 158:199–206.

21. Otsuki, K., S. Yasumura, I. Muller-Fleckenstein, B. Fleckenstein, S. Talib, U. Koldovsky, and T. L. Whiteside. 1997. Interactions between autologous CD4+ and CD8+ T lymphocytes and human squamous cell carcinoma of the head and neck. Cell. Immunol. 177:35–48.

22. Pass, H. A., S. L. Schwarz, J. E. Wolchok, and S. A. Rosenberg. 1995. Immunization of patients with melanoma peptide vaccines: immunologic assessment using ELISPOT assay. Cancer J. Sci. Am. 4:316–323.

23. Rivoltini, L., D. J. Loftus, P. Squarcina, C. Castelli, F. Rini, F. Arietti, F. Belli, F. M. Marincola, C. G. Greider, A. Burns, E. Appella, and G. Parmiani. 1998. Recognition of melanoma-derived antigens by CTL: possible mechanisms in downregulating anti-tumor T-cell reactivity. Crit. Rev. Immunol. 18:45–63.

24. Romero, P., J.-C. Cerottini, and G. Waanders. 1998. Novel methods to monitor antigen-specific cytokine T-cell responses in cancer immunotherapy. Mol. Med. Today 4:305–312.

25. Ronnelid, J., and L. Klareskog. 1997. A comparison between ELISPOT methods for the detection of cytokine-producing cells: graded sensitivity and specificity using ELISA plates as compared to nitrocellulose membranes. J. Immunol. Methods 206:727–727.

26. Rosenberg, S. A., J. C. Yang, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, P. J. Rest, M. M. Dudley, S. L. Schwartz, P. J. Spies, J. R. Wunderlich, M. R. Parkhurst, Y. Kawakami, C. A. Seipp, J. H. Emborn, and D. E. White. 1998. Immunologic and therapeutic evaluation of a specific peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med. 4:321–327.

27. Rotzschke, O., K. Fulk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H. G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature 348:252–254.

28. Scheibenbogen, C., K.-H. Lee, S. Mayer, S. Stevanovic, U. Mebus, W. Herr, H.-G. Rammensee, and U. Keilholz. 1997. A sensitive ELISPOT assay for detection of CD8+ T lymphocytes specific for HLA class I-binding peptide epitopes derived from influenza proteins in the blood of healthy donors and melanoma patients. Clin. Cancer Res. 3:221–229.

29. Scheibenbogen, C., K.-H. Lee, S. Stevanovic, M. Witzens, M. Willhauck, V. Waldmann, H. Naether, H.-G. Rammensee, and U. Keilholz. 1997. Analysis of the T-cell response to tumor and viral peptide antigens by an IFN-γ ELISPOT assay. Int. J. Cancer 75:1–5.

30. Shimizu, Y., S. Isozaki, R. B. Herberman, and T. L. Whiteside. 1997. CD8+ T-cell analysis of tumor-infiltrating lymphocytes from human primary and metastatic liver tumors. Int. J. Cancer 46:378–393.

31. Tang, S., and J. Killian. 1994. Direct comparison of ELISPOT and ELISA-based assays for detection of individual cytokine-secreting cells. Lymphokine Cytokine Res. 13:259–263.

32. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J. Immunol. 126:614–619.

33. Van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. de Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1995. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254:1647–1647.

34. Van der Bruggen, B., and P. van der Bruggen. 1997. T-cell defined tumor antigens. Curr. Opin. Immunol. 9:684–693.

35. Whiteside, T. L., J. Bryant, R. Day, and R. B. Herberman. 1995. Natural killer cell reactivity in the diagnosis of immune dysfunction: criteria for a reproducible assay. J. Clin. Lab. Anal. 4:102–114.

36. Whiteside, T. L., K. Chikamatsu, S. Nagashima, and K. Okada. 1996. An-
titumor effects of cytolytic T lymphocytes (CTL) and natural killer (NK) cells in head and neck cancer. Anticancer Res. 16:2357–2364.

37. Whiteside, T. L., S. Miescher, J. Hurtlimann, and V. von Fliedner. 1986. Separation, phenotyping and limiting-dilution analysis of T-lymphocytes infiltrating human solid tumors. Int. J. Cancer 37:803–811.

38. Whiteside, T. L., and H. Rabinowich. 1998. The role of Fas/FasL in immunosuppression induced by human tumors. Cancer Immunol. Immunother. 46:175–184.

39. Whiteside, T. L., M.-W. Sung, S. Nagashima, K. Chikamatsu, K. Okada, and N. L. Vujanovic. 1998. Human tumor antigen-specific T lymphocytes and IL2-activated natural killer (A-NK) cells: comparisons of antitumor effects in vitro and in vivo. Clin. Cancer Res. 4:1135–1145.

40. Wolfel, T., A. van Pel, V. Brichard, J. Schneider, B. Selinger, K. H. Meyer zum Buschenfelde, and T. Boon. 1994. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. Eur. J. Immunol. 24:759–764.