Evaluation of Pre-existent Immunity in Patients with Primary Breast Cancer: Molecular and Cellular Assays to Quantify Antigen-Specific T Lymphocytes in Peripheral Blood Mononuclear Cells

Christine Rentzsch, Simone Kayser, Susanne Stumm, Iris Watermann, Steffen Walter, Stefan Stevanovic, Diethelm Wallwiener, and Brigitte Gückel

Department of Obstetrics and Gynecology [C. R., S. K., I. W., D. W., B. G.] and Department of Immunology, Institute for Cell Biology [S. W., S. Ste.], University of Tübingen, 72076 Tübingen, Germany, and Institute of Immunology, University of Heidelberg, Heidelberg, Germany [S. Stu.]

ABSTRACT

Purpose: Breast cancers are known to frequently (over)express several well-characterized tumor-associated antigens (TAAs) such as carcinoembryonic antigen, MUC-1, Her-2/neu, and cancer/testis antigens such as NY-ESO-1, SSX-2, and members of the MAGE family. Whereas in melanoma patients, the detection of pre-existing T cell responses to tumor-associated differentiation antigens was a rationale to initiate several vaccination strategies, little is known thus far concerning tumor-specific immunity in breast cancer patients. The objectives of our study were (a) to modify and compare different immunodiagnostic T cell assays with regard to their suitability for clinical applications and (b) to determine endogenous TAA-specific T cell immunity of breast cancer patients at the time point of primary diagnosis.

Experimental Design: Using MUC-1- and Her-2/neu-derived HLA-A*0201-restricted peptides as model antigens, we analyzed antigen-dependent IFN-γ release of T cells by enzyme-linked immunospot (ELISpot) assay, intracellular cytokine flow cytometry (CytoSpot), and quantitative real-time PCR. As an assay independent of T cell function, we performed tetramer staining.

Results: In our hands, the quantitative real-time PCR method is most sensitive and a feasible screening test to perform an “immunological staging” of cancer patients. By doing this, we detected in 7 of 13 (54%) of HLA-A*0201 breast cancer patients a pre-existent specific cellular immune response to at least one of the investigated TAAs (MUC-1, Her-2/neu, carcinoembryonic antigen, NY-ESO-1, and SSX-2). Forty of 21 patients (19%) were found to have a significant Her-2/neu-specific T cell response as defined by a stimulation index ≥ 2 (range, 10–88).

Conclusions: Although the clinical relevance of endogenous TAA-specific immunity remains unclear, our findings suggest that patients with primary breast cancer can mount a T cell immune response to their tumor that might be beneficially enhanced by TAA-dependent vaccination strategies in the adjuvant situation.

INTRODUCTION

The characterization of TAAs recognized by cellular or humoral effectors of the immune system has opened new perspectives for cancer therapy. Immunomodulating strategies such as peptide-based approaches or cellular cancer vaccines are considered as potential adjuvant therapies in breast cancer patients either to treat minimal residual disease or to prevent relapse (1, 2). These strategies are based on the assumption that the T cell repertoire of individuals contains TAA-specific CTL precursors or tumor-primed memory T cells and that the patient’s immune system can be sensitized to TAAs of the patient’s own tumor. However, little is known about the incidence and magnitude of a pre-existing tumor-specific cellular immune response in patients with primary breast cancer.

Breast cancer cells frequently overexpress TAAs such as CEA, MUC-1, and Her-2/neu known to elicit HLA-restricted CTLs (3–10). Furthermore, Her-2/neu proves a target for therapeutic antibodies used in clinical studies (11, 12). The family of C/T antigens, first identified in malignant melanoma, includes the most promising candidates for clinical vaccination approaches due to their tumor-restricted expression pattern (13). Multiple C/T antigens, for example, members of the MAGE and BAGE family, SSX-2, and NY-ESO-1, are ubiquitously ex-

Received 3/25/03; revised 6/9/03; accepted 6/13/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by Deutsche Forschungsgemeinschaft Grants GU 511/1-1 (to B. G.) and SFB510 (S. Ste.), Deutsche Krebshilfe Grant 10-1529 Gü I (to B. G. and C. R.), and the Angewante Klinische Forschung-Program of the University of Tübingen (S. K.).

1 Both authors contributed equally to this work.

2 To whom requests for reprints should be addressed, at Department of Obstetrics and Gynecology, University of Tübingen, Calwerstrasse 7, 72076 Tübingen, Germany. Phone: 49-7071-29-77626; Fax: 49-7071-29-5653; E-mail: brigitte.gueckel@uni-tuebingen.de.
pressed in breast cancer as demonstrated by PCR and immunohistochemistry (14).

In several studies investigating PBMCs of melanoma patients, it was demonstrated that the T cell repertoire frequently contains precursors against tumor differentiation antigens such as Melan-A, tyrosinase, or C/T antigens such as NY-ESO-1 (15–19). These findings were the rationale to initiate several TAA-specific vaccination strategies in melanoma and renal cell carcinoma (13, 20). Recently, it was demonstrated that in contrast to healthy donors, melanoma patients show a non-naive phenotype in fractions of Melan-A-specific T cells revealing tumor-dependent immune activation. Furthermore, vaccinations using Melan-A-derived peptides were able to increase the frequency of specific T cells and changed their differentiation stage from naïve to memory cells (21).

In breast cancer patients, little information exists about endogenous TAA-specific cellular immune responses. A study analyzing patients with advanced, Her-2/neu-overexpressing breast or ovarian cancer for Her-2/neu-specific immunity showed proliferative PBMC responses to the recombinant antigen in only 10% of the cases. However, in case of HLA-A*02 patients, no CD8+ T cell response to a defined Her-2/neu HLA-A*02 epitope (E75, Her-2/neu_369–377) could be detected (22). Another study analyzing breast cancer patient T cells from the bone marrow demonstrated tumor-reactive memory T cells by ELISpot in 10 of 17 patients (23). Interestingly, approximately 70% of CD3+ T cells in the bone marrow of breast cancer patients were CD45RO+ memory T cells (24). Among those, Her-2/neu- and MUC-1-specific HLA-tetramer-binding CD8+ T cells were detectable in a few cases that were able to mount a cytotoxic response, whereas T cells isolated from the blood of the same patients were not able to do so (23).

MUC-1 potentially represents an ideal antigen for immunotherapy because it is overexpressed on a variety of human epithelial tumor cells in an underglycosylated form and proved a target for humoral and cellular immune responses. MUC-1-specific CTL lines stimulated in vitro were shown to lyse MUC-1-expressing tumor cells (including breast cancer cells) in a HLA-restricted manner (6, 7, 25). Using MUC-1-derived HLA-A*0201-restricted peptides together with viral peptides (HCMV, EBV, and influenza) as model antigens, we compared different assays to monitor antigen-specific T cells. As functional tests, we analyzed antigen-dependent IFN-γ release of T cells by either ELISpot assay, intracellular cytokine flow cytometry (CytoSpot), or quantification of mRNA encoding IFN-γ using qRT-PCR. Using tetramers, we were able to detect specific T cells independent of their functionality.

Analyzing the pre-existent cellular immune response to frequently expressed breast cancer-associated TAAAs, we questioned whether breast cancer patients could have evidence of TAA-specific cellular immunity at the time point of primary diagnosis. In parallel, we investigated blood samples of healthy donors. In approximately 54% of HLA-A*0201+ breast cancer patients, a pre-existing specific cellular immune response to at least one of the investigated TAAAs (MUC-1, Her-2/neu, CEA, NY-ESO-1, or SSX-2) could be detected. Nineteen percent of the patients showed pre-existent CD8+ T cell reactions related to Her-2/neu_369–377 and Her-2/neu_654–662. However, the clinical significance of this level of tumor-associated immunity is currently unknown.

MATERIALS AND METHODS

Patients and Blood Samples. Healthy donors and patients were selected on the basis of HLA-A*02 antigen expression. Blood samples were obtained after informed consent from patients with primary breast cancer before surgery. Patients enrolled in this study had not received any neoadjuvant or adjuvant chemotherapy, hormone therapy, or radiotherapy before sample collection. PBMCs were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation, washed twice with PBS (Life Technologies, Inc.), and cryopreserved in FCS and 10% DMSO. Vials contained 1 × 10^7 cells/ml at maximum and were frozen gradually to −80 °C before storage in liquid nitrogen.

Synthetic Peptides. Peptides were synthesized by solid-phase Fmoc chemistry using a peptide synthesizer 432A (Applied Biosystems, Foster City, CA). Identity and purity (>90%) of the peptides were analyzed by reversed-phase high-performance liquid chromatography and matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry. Peptides were dissolved in DMSO at 10 mg/ml, further diluted in H_2O before a final concentration of 1 mg/ml, and stored at −20 °C. The following HLA-A*0201-binding peptides were synthesized: CEA_371–379 (YLSGANLNL); MAGE-1_78–296 (KVLEY-VIK); NY-ESO-1_108–116 (SLAQDAPPL); NY-ESO-1_86–94 (RLLFYLYAM); NY-ESO-1_159–167 (LMWITQCFI); SSX-2_103–111 (RLQGISPKI); MUC-1_50–595 (STAPPHVNY); MUC-1_12–20 (LLLLTMLTV); Her-2/neu_369–377 (E75; KIFGSLAFL); Her-2/neu_654–662 (GP2; IISAVVGL); HCMV pp56_495–503 (NLVPMVATV); influenza matrix protein flank_6–66 (GILGFVFTL); EBV EBNA-2_284–293 (LDDLFVRFMGV); EBV IE63_229–267 (GLCTLVAML); and RNA-dependent helicase 6PH_146–154 (YLLPAIVHI).

Peptide Presensitization of PBMCs. Cryopreserved PBMCs were thawed in RPMI 1640 supplemented with 10% heat-inactivated HS (ccPro, Neustadt, Germany), 1% penicillin/streptomycin, and 4 mM L-glutamine (all from Life Technologies, Inc., Eggenstein, Germany). PBMCs were seeded into round-bottomed 96-well plates (Costar, Bodenheim, Germany) at a concentration of 1 × 10^5 cells/well and cultured overnight for reconstitution. Thereafter, PBMCs were harvested, washed, and used for functional assays (ex vivo testing). For peptide presensitization, PBMCs containing CD8+ effector T cells as well as subsets of antigen-presenting cells were stimulated directly with peptide antigens added in final concentrations of 5 μg/ml. Presensitized PBMCs were harvested after 7 days of peptide stimulation and either stained directly using tetramers or restimulated for an additional 2 h using peptide-pulsed T2 cells as antigen-presenting cells before further functional analysis (peptide presensitization). The same antigens were used for the peptide presensitization as well as the restimulation period.

As positive controls, PBMCs were activated nonspecifically by incubation with a combination of the calcium ionophore A 23187 (ionomycin, 250 nM; Sigma, Taufkirchen, Germany) and PMA (60 nM; Sigma).
ELISpot Assay. ELISpot assay was carried out as described previously (26). In brief, multiscreen 96-well nitrocellulose plates (Millipore, Bedford, the Netherlands) were coated overnight at 4°C with anti-human IFN-γ mAb (1-D1K; 2 μg/ml; Mabtech, Stockholm, Sweden) diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6). Plates were blocked with RPMI 1640 containing 10% HS for 2 h at 37°C. Different numbers of PBMCs (6.25 × 10^4 to 10 × 10^5) presensitized with peptide for 7 days or not (see above) were added to each well and coincubated with 3.5 × 10^4 peptide-pulsed T2 cells for 18 h in RPMI 1640 and 10% HS. T2 is an antigen-presenting-defective cell line that does express HLA-B/C at rather low levels and expresses higher levels of HLA-A*0201 (27). The antigen pulse was performed in serum-free X-Vivo medium (Biowhittaker, Verviers, Belgium) at a peptide concentration of 5 μg/ml for 2 h. Background controls were performed using p68-pulsed T2 cells as stimulator cells and shown in comparison to TAA and viral antigen stimulations, thereby excluding natural killer and unspecific cellular responses. As a positive control, PBMCs were stimulated with a combination of 250 μM immunogen and 60 μM PMA. After incubation, plates were washed extensively with PBS and 0.05% Tween 20 (Sigma), and further incubated with 100 μl/well anti-IFN-γ Ab (7-B6-1-biotin; 0.2 μg/ml; Mabtech). After incubation for 3 h at room temperature, plates were washed and developed for additional 2 h with streptavidin-alkaline phosphatase (1 μg/ml; Mabtech). Spots were visualized by adding the substrate [5-bromo-4-chloro-3-indoly1-phosphate/ nitroblue tetrazolium; Sigma] and counted automatically using an automated ELISpot reader (Zeiss Vision, Göttingen, Germany).

Intracellular Fluorescence-activated Cell Sorter Staining for IFN-γ (CytoSpot). PBMCs were presensitized with antigens or not as described above. Cells were harvested and resuspended at 2 × 10^6 cells/ml in RPMI 1640 and 10% HS. The cultures were restimulated with 2 × 10^6 cells/ml peptide-pulsed T2 cells (see above). As a positive control, PBMCs were stimulated with a combination of immunogen and PMA. After 2 h of incubation, 0.7 μl/ml Golgi-Stop-Solution (PharMingen, San Jose, CA) was added, and the incubation period was extended to 12 h at 37°C, 5% CO2. Cells were then labeled with PE-, FITC-, or Tricolor-conjugated Abs by incubation on ice for 30 min. For intracellular labeling, cells were fixed and permeabilized for 30 min on ice using Cytoperm/Cytotix solution followed by washing steps with Washing Buffer (all from PharMingen). Nonspecific binding was measured using PE-conjugated isotype-matched mouse Ig. The following Abs were used: anti-CD8-Tricolor (Caltac, Burlingame, CA); anti-IFN-γ-PE (PharMingen); and anti-CD4-FITC (Becton Dickinson, Heidelberg, Germany). After washing, stained cells were analyzed by flow cytometry (FACS-Calibur; Becton Dickinson).

Tetramer Staining. HLA-A*0201 tetramer complexes were produced as described previously, with minor modifications (28, 29). The HLA-A*0201-binding peptide used for the refolding was KIFGSLAFL from Her-2/neu. Tetramers were assembled by mixing biotinylated monomers with streptavidin-PE ( Molecular Probes, Eugene, OR) at a 4:1 ratio. PBMCs were presensitized with peptides as described above, except that they were collected after 6 days of culture. Staining of the PBMCs was performed using 10 μg/ml tetrameric complex in 50 μl of PBS, 0.01% NaN3, 2 mM EDTA, and 2% FCS for 30 min at 4°C in the dark. Cells were counterstained using mAbs CD8-Tricolor (Caltac) and mAb CD4-FITC (Becton Dickinson) for 30 min at 4°C. After extensive washing, stained cells were analyzed by flow cytometry (FACS-Calibur; Becton Dickinson) by appropriate gating on CD8+ cells and excluding CD4+ cells.

RNA Isolation and cDNA Synthesis. PBMCs were presensitized with antigens or not as described above. PBMCs that experienced a presensitization were restimulated with 1 × 10^4 peptide-loaded T2 cells per well. Unspecific stimulations with ionomycin/PMA were used as positive controls. After 2 h of restimulation, 1–3 × 10^5 cells were harvested, lysed in 800 μl of Trizol (Invitrogen, Karlsruhe, Germany), and stored at −80°C for a maximum of 30 days before RNA extraction. Total RNA was isolated using Trizol according to the manufacturer’s instructions for small cell numbers (Invitrogen). After extraction, RNA was resuspended in 10 μl of RNase-free water (Promega, Madison, WI). 5 μl of RNA were reverse transcribed into cDNA using the Omniscript kit (Qiagen, Hilden, Germany) and 20 units of RNasin (Promega) according to the manufacturer’s instructions and stored at −20°C until use.

Quantification of IFN-γ-specific mRNA Expression by Real-Time PCR. Quantification of IFN-γ gene expression was performed by using the ABI Prism 7700 sequence detection system (Perkin-Elmer, Foster City, CA) with primers and probes as described elsewhere (30–32). Amplification of a CDS sequence for normalization was performed in separate tubes with primers and probes as described previously (31). Probes were labeled at the 5′ end with the reporter dye molecule FAM (emission λmax = 518 nm) and at the 3′ end with the quencher dye molecule TAMRA (emission λmax = 582 nm). The qRT-PCR was performed with the TaqMan Universal Master Mix (Applied Biosystems) using 5 μl of 1:10 diluted cDNA, fluorescence-labeled probe at a final concentration of 150 nM, and primers at final concentrations of 400 nM in a reaction volume of 20 μl. Cycling conditions were as follows: one cycle (50°C, 2 min; 95°C, 10 min) followed by 40 cycles (95°C, 15 s; 60°C, 1 min).

Cycle threshold values of IFN-γ were normalized to cycle threshold values of CDS. The relative expression was defined as relative value in comparison with the arbitrary expression value 1 achieved with T cells stimulated with the self-antigen p68.

qRT-PCR primers and probes were as follows: IFN-γ, 5′-AGCTCTGACGTGTTTGGTTGTT-3′ (forward), 5′-GTCTT-CATTATCCGCTACATCTGAA-TAMRA (probe); and CD8, 5′-CCCTGACCCTCACTCCATGT-3′ (reverse), 5′-GTGG-GCTTCGCGGTTA-3′ (reverse), and FAM-TGACCCACTTT-GTCGGGTTCA TMA R A (probe).

RESULTS

Assessment of Antigen-specific T Cells by ELISpot, CytoSpot, and IFN-γ-specific mRNA Quantification in the Blood of Healthy Donors. The quantification of mRNA encoding T cell activation markers for immune assessment was described to be rather sensitive but is still investigational. Therefore, we first performed IFN-γ-specific qRT-PCR using PBMCs ex vivo or after short-time in vitro stimulation to detect antigen-specific T cells in the peripheral blood. In a second step, we try
to confirm qRT-PCR results using additional functional assays such as ELISpot and CytoSpot or function-independent tetramer staining. To do so, a mixture of four HLA-A*0201-restricted viral peptides (positive control; see “Materials and Methods”) and tested after 2 h (ex vivo) for increase of IFN-γ-specific mRNA by qRT-PCR. IFN-γ-specific mRNA expression was normalized to CD8 expression and given as an x-fold increase (stimulation index) in comparison with p68 stimulations. B, PBMCs were tested in parallel in an ELISpot assay. PBMCs were stimulated ex vivo for 18 h on ELISpot plates, thereby analyzing their IFN-γ secretion. The diagram shows the number of spots in 10^6 PBMCs. C, in parallel, PBMCs of HD5 were stimulated ex vivo for 10 h using peptide-loaded T2 cells and stained for intracellular IFN-γ (CytoSpot). A p68 stimulation served as background control; antigen-independent activation using ionomycin and PMA served as positive control. Cells were double-stained using PE-labeled IFN-γ antibodies and anti-CD8-Tricolor.

Methods to Detect T Cells Specific for Her-2/neu in the Blood of Breast Cancer Patients. Patient blood samples are usually limited and have to be cryopreserved to allow parallel testing of samples obtained at different time points under immunotherapy. Furthermore, the frequencies of TAA-specific T cells are expected to be rather low. Therefore, we evaluated the functional T cell assays with regard to their feasibility and transferability to the needs of the clinic using Her-2/neu as a model antigen.

Figs. 2 and 3 show the results of two breast cancer patients (BCP8 and BCP9) with a pre-existing cellular immune response specific for Her-2/neu. PBMCs of BCP8 were analyzed either ex vivo or after peptide presensitization with Her-2/neu_369–377 and viral antigens, respectively. The importance of presensitization
with peptides for demonstrating TAA-specific reactivity is illustrated in Fig. 2A. T lymphocytes obtained directly from the blood of BCP8 did not respond within 2 h of antigen exposure to Her-2/neu 369–377 but to viral peptides by mRNA synthesis encoding IFN-γ. Testing 20 breast cancer patients, no or minimal TAA-specific T cell reactivity was observed in the absence of peptide presensitization (data not shown). In contrast, examples shown in Figs. 2 and 3 demonstrated strong Her-2/neu 369–377 reactivity after Her-2/neu 369–377 presensitization of the PBMCs, in comparison with the background controls stimulated with p68 (BCP8, 88-fold; BCP9, 23-fold). Similarly, the reactions against the viral peptide mix were augmented by specific peptide presensitization (Fig. 2A).

**Fig. 2** Assessment of Her-2/neu 369–377-specific immunity in breast cancer patient BCP8 using IFN-γ mRNA quantification, ELISpot, and tetramer staining. A, 1 × 10^6 PBMCs of a breast cancer patient (BCP8) were cocultured with 5 μg/ml Her-2/neu 369–377, p68 (negative control; expression value set to 1), or a mix of four HLA-A*0201-restricted viral peptides (positive control; see “Materials and Methods”). T cells were tested for increase of IFN-γ-specific mRNA by qRT-PCR either after 2 h (ex vivo) or after peptide presensitization for 7 days followed by an additional 2-h restimulation with 1 × 10^6 peptide-loaded T2 cells. IFN-γ-specific mRNA expression was normalized to CD8 expression and given as an x-fold increase (stimulation index) in comparison with p68 stimulations. B, PBMCs of patient BCP8 were tested in parallel in an ELISpot assay. The indicated peptides were used for in vitro presensitization. PBMCs were restimulated in varying concentrations for 18 h on ELISpot plates using p68-loaded (●), Her-2/neu 369–377-loaded (▲), or viral antigen-loaded (■) T2 cells, thereby analyzing their IFN-γ secretion. The diagram shows the number of spots for different cell concentrations. Inset, photographs illustrate ELISpot wells at the highest PBMC concentration (1.2 × 10^6). C, in parallel, PBMCs of patient BCP8 were harvested after 6 days of peptide presensitization and stained directly using Her-2/neu 369–377-loaded HLA-A*0201-specific tetramers. A p68 stimulation served as negative control. Cells were triple-stained with PE-labeled tetramers, anti-CD8-Tricolor, and anti-CD4-FITC mAbs.

**Fig. 3** Analysis of Her-2/neu 369–377-specific T lymphocytes of breast cancer patients BCP9 after peptide presensitization. Example of functional and phenotypic analyses using PBMCs of another breast cancer patient (BCP9). Assays were performed as described in the Fig. 2 legend.
In accordance with our data analyzing MUC-1-specific T cell responses, Her-2/neu-specific reactions shown by increase of IFN-γ-specific mRNA could be confirmed by the ELISpot assay. Figs. 2B and 3B show the number of spots obtained after 1 week of peptide presensitization. In the mRNA quantification as well as in the ELISpot assay, Her-2/neu369-specific responses were lower (although clearly above the p68 background) than the viral antigen-induced reactions. Tetramer staining as a nonfunctional assay revealed a distinct population of Her-2/neu369/HLA-A*0201-tetramer+- and CD8+ T cells in both patients (Figs. 2B and 3B). These populations are likely to be specific because far fewer cells were detected after p68 stimulation. After TAA presensitization, negative results of the IFN-γ qRT-PCT could be confirmed using ELISpot as well as tetramer analyses (data not shown).

In general, analyzing T cell reactions toward one antigen together with the proper control reactions needed approximately $0.9 \times 10^6$ PBMCs in case of IFN-γ-specific qRT-PCR (triplicates), $1.2 \times 10^6$ PBMCs for the CytoSpot or tetramer staining, and $1.8 \times 10^6$ PBMCs for the ELISpot assay (triplicates).

Pre-existing Immunity to Her-2/neu in Patients with Breast Cancer and Healthy Donors. Having confirmed the sensitivity and feasibility of the qRT-PCR-based T cell assay, we screened PBMCs of HLA-A*0201** healthy donors and breast cancer patients, respectively, to compare Her-2/neu-specific T cell frequencies. Although low levels of reactivity were detected in a few patients, the presensitization step appeared essential to warrant detection of TAA-specific T cells at rather low frequencies. Blood samples of patients were taken before surgery. To exclude a potentially therapy-based immunosuppression, none of the patients included in this study received any neoadjuvant chemotherapy or hormone therapy. In some cases, the tumor’s Her-2/neu status could be evaluated using the DAKO classification.

PBMCs of 20 breast cancer patients and 10 healthy donors were analyzed for Her-2/neu-dependent IFN-γ-specific mRNA increase after in vitro presensitization (Table 1). Her-2/neu369–377 (E75) and Her-2/neu654–662 (GP2), both described to be immunodominant epitopes of Her-2/neu, were used for stimulation.

Table 1 summarizes the results of Her-2/neu-dependent IFN-γ mRNA quantification, normalized to the CD8 expression. A reaction was considered positive if it was 2-fold above the background control (p68 stimulation) plus 2-fold SD. All individuals tested showed a clear increase of IFN-γ-mRNA in response to ionomycin/PMA stimulation (data not shown). Four of 20 patients (19%) and none of the healthy donors (0 of 10 donors) showed an up-regulation of IFN-γ-mRNA after stimulation with Her-2/neu epitopes. The quantity of the reactions was rather high, with stimulation indices of 10.0, 23.9, 78.2, and 88.0, respectively. Comparable high stimulation indices were only shown for virus antigen-dependent reactions (see Table 2).

Due to limited knowledge of antigen expression in the patient tumors, it was not possible to correlate Her-2/neu expression and T cell response. However, it is noteworthy that responders could either overexpress Her-2/neu at the primary tumor (BCP8, DAKO score 2+) or not (BCP9, DAKO score 0).
MAGE-1, NY-ESO-1, and SSX-2 were detected in breast cancer lesions, although at lower frequencies [5–25% (35–39)]. In this context, it was of interest to look for T cells specific for one of these TAAs after peptide presensitization.

Table 2 summarizes the results of TAA-dependent IFN-γ mRNA quantification, normalized to CD8 expression. A reaction was considered as positive if it was 2-fold above the background control (p68 stimulation) plus 2-fold SD. Again, all individuals tested showed a clear increase of IFN-γ mRNA in response to ionomycin/PMA stimulation (data not shown). In addition, IFN-γ mRNA quantification was performed after peptide presensitization using a mixture of HLA-A*0201-restricted viral antigens (cytomegalovirus, EBV, and flu) to get an impression of the functional immune status of tested individuals.

Thirteen of 21 (62%) breast cancer patients (Table 2A) and 6 of 11 (54%) healthy donors (Table 2B) did show a significant reaction toward viral antigens. Positive reactions ranged from 5.1–417.3, demonstrating a high interindividual heterogeneity in mounting a virus-specific T cell memory response. However, patients with no detectable virus-specific T cell activation did not show TAA-specific responses either.

In contrast to MUC-1_12–20 MUC-1_958–958-specific T cell reactions were commonly detected in patients (46%) as well as in healthy donors (28%). CEA- and NY-ESO-1-specific responses were more limited and restricted to breast cancer patients (each was seen in 1 of 10 responders). SSX-2-related T cell responses were shown for one breast cancer patient (of 10 cases). Surprisingly, we could also see an IFN-γ mRNA increase in response to these C/T antigens in one of the healthy donors (HD8). No T cell reactivity at all could be observed toward a MAGE-1 epitope.

**DISCUSSION**

There is a considerable interest to validate T cell detection assays as surrogates for immune competence and vaccine potency. Besides sensitivity and robustness, several aspects have to be taken into account with regard to their clinical usefulness, i.e., the need for use of frozen/thawed PBMC samples to analyze batches gained during therapy in parallel and the avoidance of collecting large blood samples. Therefore, antigen-dependent cytokine release assays, on either the protein or mRNA level, and tetramer staining represent powerful tools to assess the specificity and magnitude of T cell responses.

In this study, we first tested the feasibility of the qRT-PCR method to assess antigen-specific T cell reactions in the peripheral blood. PBMCs were directly stimulated *ex vivo* using HLA-A*0201-restricted epitopes derived from influenza, EBV, and cytomegalovirus, allowing antigen presentation among the sample cells. In response to the given peptides, IFN-γ-specific mRNA was quantified after 2 h of stimulation. The signal was normalized using CD8-specific mRNA as reference, which is not likely to be sensitive to the peptide stimulus applied in the time frame of the assay (31,32). In contrast to ELISPOT and CytoSpot assays, IFN-γ mRNA quantification does not take into account the variations in the frequency of the cell subset targeted by the stimulus. Therefore, one of the major problems in the interpretation of qRT-PCR results is discriminating a positive from a negative result (40). We considered a positive result an at least 2-fold IFN-γ increase above the mean expression obtained in control stimulations plus a 2-fold SD. By doing so, we were able to detect *ex vivo* virus-specific T cell responses in the majority of tested individuals. More importantly, we could confirm positive and negative qRT-PCR results by additional independent functional assays such as ELISPOT and CytoSpot or by tetramer staining, irrespective of T cell function. Due to the assay conditions using PBMCs, the magnitude of positive T cell responses correlated in a rather relative but not absolute manner: qRT-PCR-, CytoSpot-, and tetramer-reacting T cells could be restricted to the CD8+ subset; whereas ELISPOT-reacting T cells could not. In addition, T cell frequencies defined by tetramer staining include functional as well as anergic CD8+ T cells.

In comparison with cellular immunity directed against viral recall antigens, tumor-specific T cell reactions represent another quality of immune responses due to the restricted immunogenicity of self-antigens and the tolerizing mechanisms provided by the tumor itself. Therefore, the expected frequency of TAA-reacting T cells is limited and might lie beyond the threshold level of successful detection. Although low levels of reactivity have been *ex vivo* with a few patients using the qRT-PCR assay in our study, the presensitization step appeared essential to warrant a certain detection of TAA-specific T cells in unimmunized volunteers.

In our hands, the combination of peptide presensitization with the qRT-PCR technique is practicable, most sensitive, and a suitable screening test for immunological staging that needs only small blood samples. This method was previously shown to represent a useful tool for the monitoring of patients with cancer undergoing immune manipulation. Kammula et al. (31) identified T cell reactivity toward epitopes used for active-specific vaccination of melanoma patients by quantifying IFN-γ transcript levels in PBMCs obtained before and after treatment. The evidence of vaccine-induced immune responses obtained with qRT-PCR correlated with results obtained with classic *in vitro* sensitization assays, intracellular cytokine detection by fluorescence-activated cell-sorting analysis, and tetramer staining (32,41). However, evaluating TAA-specific T cell reactions directly *ex vivo* in individuals not receiving immunomodulating therapies seemed to be rather difficult at times (42,43). Confirming our data, Jäger and co-workers (19,43,44) demonstrated that in the case of patients with advanced tumors overexpressing NY-ESO-1, strong antigen-specific reactivity was best seen when combining *in vitro* peptide presensitization of T cells together with ELISPOT or CytoSpot techniques. Additionally, a more recent publication (45) described the complete absence of *ex vivo* T cell responses in breast cancer patients toward a small panel of TAAs such as Her-2/neu, CEA, and Ep-CAM using the ELISPOT assay without TAA presensitization.

Several studies analyzing immune responses to melanoma-associated differentiation antigens and MUC-1 revealed that cancer patients as well as healthy donors can show high frequencies of Melan-A-specific naive CD8+ T cells or MUC-1-specific memory T cells (17,46,47). Thus, we performed an evaluation of volunteers without cancer together with breast cancer patients to assess the prevalence of immunity in an unimmunized breast cancer population. We further decided to analyze blood samples before surgery and adjuvant therapy because of (a) the suggestion that pre-existing antitumoral im-

Downloaded from clincancerres.aacrjournals.org on April 15, 2017. © 2003 American Association for Cancer Research.
<table>
<thead>
<tr>
<th>Breast cancer patient</th>
<th>p68</th>
<th>NY-ESO-1</th>
<th>NY-ESO-1</th>
<th>NY-ESO-1</th>
<th>NY-ESO-1</th>
<th>MAGE-1</th>
<th>MUC-1</th>
<th>MUC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.45</td>
<td>6.59±2.30</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.67±0.14</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.24</td>
<td>7.7±1.44</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.74±0.18</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.38</td>
<td>122.36±32.92</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.77±0.97</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.19</td>
<td>11.96±2.22</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.03±0.04</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.09</td>
<td>9.13±0.72</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.41±0.23</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.43</td>
<td>0.66±0.35</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.27±1.24</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.37</td>
<td>27.28±7.62</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.34±0.45</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.31</td>
<td>417.32±109.40</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.95±0.90</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.23</td>
<td>78.79±21.15</td>
<td>n.a.</td>
<td>n.a.</td>
<td>2.35±0.47</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.17</td>
<td>121.2±20.26</td>
<td>n.a.</td>
<td>0.18±0.02</td>
<td>0.20±0.02</td>
<td>0.17±0.04</td>
<td>0.22±0.03</td>
<td></td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.11</td>
<td>21.78±4.42</td>
<td>0.56±0.16</td>
<td>0.46±0.04</td>
<td>1.32±0.11</td>
<td>1.77±0.91</td>
<td>n.a.</td>
<td>18.38±2.08</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.11</td>
<td>21.78±4.42</td>
<td>0.56±0.16</td>
<td>0.46±0.04</td>
<td>1.32±0.11</td>
<td>1.77±0.91</td>
<td>n.a.</td>
<td>18.38±2.08</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.54</td>
<td>21.78±4.42</td>
<td>0.56±0.16</td>
<td>0.46±0.04</td>
<td>1.32±0.11</td>
<td>1.77±0.91</td>
<td>n.a.</td>
<td>18.38±2.08</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.04</td>
<td>66.95±20.76</td>
<td>0.94±0.14</td>
<td>n.a.</td>
<td>1.09±1.05</td>
<td>n.a.</td>
<td>8.14±1.40</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.37</td>
<td>0.54±0.16</td>
<td>0.39±0.03</td>
<td>0.82±0.29</td>
<td>0.38±0.16</td>
<td>0.95±0.31</td>
<td>n.a.</td>
<td>0.47±0.14</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.28</td>
<td>0.36±0.12</td>
<td>0.67±0.14</td>
<td>1.06±0.47</td>
<td>0.70±0.18</td>
<td>0.60±0.20</td>
<td>n.a.</td>
<td>0.78±0.34</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.57</td>
<td>0.73±0.34</td>
<td>0.52±0.33</td>
<td>2.07±2.19</td>
<td>0.83±0.29</td>
<td>0.59±0.28</td>
<td>n.a.</td>
<td>0.71±0.28</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.11</td>
<td>17.39±1.51</td>
<td>0.37±0.09</td>
<td>0.34±0.04</td>
<td>0.91±0.30</td>
<td>0.76±0.08</td>
<td>n.a.</td>
<td>22.01±2.52</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.43</td>
<td>43.22±2.47</td>
<td>17.51±39.2</td>
<td>1.44±0.53</td>
<td>0.70±0.36</td>
<td>0.70±0.27</td>
<td>n.a.</td>
<td>3.84±1.47</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.05</td>
<td>13.64±4.76</td>
<td>12.08±0.73</td>
<td>51.45±4.93</td>
<td>15.45±1.07</td>
<td>n.a.</td>
<td>106.38±37.24</td>
<td>n.a.</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.11</td>
<td>7.09±3.69</td>
<td>1.41±0.75</td>
<td>6.15±2.08</td>
<td>6.85±2.09</td>
<td>n.a.</td>
<td>3.72±0.75</td>
<td>5.15±3.27</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.46</td>
<td>27.10±9.21</td>
<td>1.40±0.51</td>
<td>1.31±0.73</td>
<td>0.42±0.20</td>
<td>0.50±0.37</td>
<td>n.a.</td>
<td>1.99±1.12</td>
</tr>
<tr>
<td>BCPI</td>
<td>1±0.41</td>
<td>18.13±5.24</td>
<td>2.06±0.83</td>
<td>6.19±2.56</td>
<td>5.01±2.08</td>
<td>2.48±1.69</td>
<td>n.a.</td>
<td>3.73±1.14</td>
</tr>
</tbody>
</table>

### Table 2. TAA- and viral antigen-stimulated increase of IFN-γ-specific mRNA in T lymphocytes of (A) breast cancer patients and (B) healthy volunteers

**A. Breast cancer patients**

<table>
<thead>
<tr>
<th>Healthy donor</th>
<th>p68</th>
<th>NY-ESO-1</th>
<th>NY-ESO-1</th>
<th>NY-ESO-1</th>
<th>MAGE-1</th>
<th>MUC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD1</td>
<td>1±0.31</td>
<td>4.52±1.38</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.34±0.22</td>
<td>n.a.</td>
</tr>
<tr>
<td>HD2</td>
<td>1±0.17</td>
<td>5.13±0.65</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.67±0.15</td>
<td>n.a.</td>
</tr>
<tr>
<td>HD3</td>
<td>1±0.21</td>
<td>41.36±7.14</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.39±0.27</td>
<td>n.a.</td>
</tr>
<tr>
<td>HD4</td>
<td>1±0.17</td>
<td>23.18±3.99</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.15±0.23</td>
<td>n.a.</td>
</tr>
<tr>
<td>HD5</td>
<td>1±0.27</td>
<td>22.39±4.44</td>
<td>n.a.</td>
<td>n.a.</td>
<td>61.0±16.14</td>
<td>1.89±0.4</td>
</tr>
<tr>
<td>HD6</td>
<td>1±0.86</td>
<td>20.1±1.28</td>
<td>1.60±0.33</td>
<td>3.15±0.64</td>
<td>1.69±0.33</td>
<td>n.a.</td>
</tr>
<tr>
<td>HD7</td>
<td>1±0.03</td>
<td>37.92±1.45</td>
<td>n.a.</td>
<td>n.a.</td>
<td>6.4±3.98</td>
<td>0.8±0.47</td>
</tr>
<tr>
<td>HD8</td>
<td>1±0.03</td>
<td>18.93±5.62</td>
<td>n.a.</td>
<td>n.a.</td>
<td>11.7±2.53</td>
<td>15.2±10.0</td>
</tr>
<tr>
<td>HD9</td>
<td>1±0.17</td>
<td>10.38±0.38</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.12±0.56</td>
<td>n.a.</td>
</tr>
<tr>
<td>HD10</td>
<td>1±0.27</td>
<td>0.81±0.45</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.46±0.41</td>
<td>3.2±3.1</td>
</tr>
</tbody>
</table>

---

* PBMCs (3 × 10⁶) of breast cancer patients before therapy or of healthy donors were cocultured with 5 µg/mL of different TAA-peptides, p68, or a mix of viral peptides. PBMCs were tested for IFN-γ-specific mRNA by qRT-PCR after peptide presensitization for 7 days followed by an additional 2-h restimulation with 3 × 10⁴ peptide-loaded T2 cells.

* Mix of four HLA-A*0201-restricted viral peptides: H1u26, EBNA-6-EBV-284-293, IEF3-EBV-292-267, and pp65 HCMV A95_993.

* IFN-γ-specific mRNA expression was normalized to CD8 expression and expressed as a x-fold increase (stimulation index) in comparison to the p68 stimulations. SDs are given in parentheses. A reaction was considered as positive (bold letters) lying 2-fold above the background control (p68 stimulation) plus 2-fold SDs.

* n.a., not analyzed.
munity depends on antigen persistence, (b) the unpredictable influence of therapeutic interventions, and (c) our former failure in detecting CEA-, MAGE-1, or Her-2/neu-specific T cells using tetramers in breast cancer patients receiving chemotherapy.\(^4\)

Her-2/neu appears to be the most prominent and best analyzed tumor antigen associated with breast cancer. However, most of the studies defining Her-2/neu as an immunogenic antigen in patients whose tumors overexpress this oncogene represent individual case reports (9, 48, 49). To our knowledge, our study is the first extensive evaluation of pre-existing immunity directed against HLA-A*0201-restricted epitopes of Her-2/neu. We could find Her-2/neu-specific CD8\(^+\) T cell responses in 4 of 22 HLA-A*0201\(^+\) breast cancer patients and in none of the investigated healthy blood donors. This is in contrast to a study by Disis et al. (22), who could not detect (despite peptide presensitization before ELISPOT analysis) pre-existent T cells specific for Her-2/neu\(_{690–377}\) in eight breast cancer patients overexpressing Her-2/neu. As expected, we saw the highest Her-2/neu\(_{654–662}\)-specific reactivity in a patient overexpressing Her-2/neu (BCP8; DAKO score, 2+). Surprisingly, strong Her-2/neu-specific T cell reaction was demonstrated in another patient without Her-2/neu overexpression (BCP9; DAKO score, 0), probably representing an example for the selective pressure of Her-2/neu immunity and emergence of antigen loss variants. In addition, strong Her-2/neu overexpression did not necessarily provoke a T cell response (BCP13; DAKO score, 3+), probably due to gaps in the patient’s T cell repertoire, tumor-mediated tolerance, or lack of appropriate diagnostic tools. However, due to our limited knowledge of Her-2/neu expression patterns in the patients analyzed here, it is not possible to make a clear correlation between antigen expression and T cell response. A comprehensive study analyzing cellular and humoral immunity pointed out that Ab and T cell response to NY-ESO-1 occurred only in patients with NY-ESO-1-expressing tumors (43). However, a study is ongoing to determine whether there is a correlation between the antigen expression pattern in tumors and the presence of tumor-specific immunity. Furthermore, it would be of interest to analyze the status of activation of circulating tumor-related T cells to explain their paradoxical coexistence with unimpaired tumor growth.

Because of its ubiquitous expression pattern in breast cancer, we were also interested in MUC-1-specific immunity (34). MUC-1-derived epitopes were already included in clinical studies, and several investigators demonstrated evidence for only MUC-1-specific T cell responses after MUC-1-based vaccinations (7, 50, 51). In contrast to these findings, we could frequently detect MUC-1\(_{950–958}\)-specific T cells in unimmunized healthy volunteers (28%) as well as in breast cancer patients (46%). Because MUC-1-specific T\(_h\) responses could be detected in multiparous women, it was speculated that there is a natural immunization against MUC-1 epitopes during pregnancy (47). Although our collective of patients includes multiparous women, this was not the case for the healthy volunteers included in our study. However, it is of interest that we could not detect any pre-existing immunity toward MUC-1\(_{12–20}\) which turned out to be immunodominant in vaccinations using peptide-pulsed dendritic cells (7). At the moment, we cannot explain the high magnitude of MUC-1\(_{950–958}\)-specific T cell responses in healthy donors and have started investigations comparing the functional state of these T cell populations and their capacity to lyse MUC-1-expressing tumor cells.

In our study, positive reactions toward cancer-associated antigens such as CEA, SSX-2, NY-ESO-1, and MAGE-1 seemed to be exceptional. Despite its limited expression pattern to tumors, testis, or placenta, we were surprised to find one healthy donor reacting to SSX-2.

In summary, with exception of Her-2/neu, our study revealed no distinct differences in TAA-related cellular immunity between cancer patients and a control group consisting of healthy volunteers. However, in approximately 54% of HLA-A*0201\(^+\) breast cancer patients, a pre-existent specific cellular immune response to at least one of the investigated TAA s MUC-1, Her-2/neu, CEA, NY-ESO-1, or SSX-2 could be detected. Nineteen percent of the patients showed pre-existent CD8\(^+\) T cell reactions related to Her-2/neu\(_{369–377}\) and Her-2/neu\(_{654–662}\). Although the clinical relevance of an endogenous TAA-specific immunity remains unclear, our findings suggest that patients with primary breast cancer can mount a T cell immune response to their tumor that might be beneficially enhanced by TAA-dependent vaccination strategies in the adjuvant situation.

ACKNOWLEDGMENTS

We thank Dr. Cecile Gouttefangeas (Department of Immunology, Institute for Cell Biology, University of Tübingen) for helping us with tetramer analyses, and we acknowledge the expert technical assistance of Beatrice Puttkammer. We thank all of the patients and volunteers who agreed to participate in this study.

REFERENCES


Evaluation of Pre-existent Immunity in Patients with Primary Breast Cancer: Molecular and Cellular Assays to Quantify Antigen-Specific T Lymphocytes in Peripheral Blood Mononuclear Cells

Christine Rentzsch, Simone Kayser, Susanne Stumm, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/12/4376

Cited articles
This article cites 49 articles, 23 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/12/4376.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/9/12/4376.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.