[CANCER RESEARCH 61, 5964–5968, August 15, 2001]

Advances in Brief

Spontaneous Cytotoxic T-Cell Responses against Survivin-derived MHC Class I-restricted T-Cell Epitopes *in Situ* As Well As *ex Vivo* in Cancer Patients[1]

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Abstract

Recent advances in therapeutic tumor vaccinations necessitate the identification of broadly expressed, immunogenic tumor antigens that are not prone to immune selection. To this end, the human inhibitor of apoptosis, survivin, is a prime candidate because it is expressed in most human neoplasms but not in normal, differentiated tissues. Here, we demonstrate spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in breast cancer, leukemia, and melanoma patients both *in situ* as well as *ex vivo*. Moreover, survivin-reactive T cells isolated by magnetic beads coated with MHC/peptide complexes were cytotoxic against HLA-matched tumors of different tissue types. Being a universal tumor antigen, survivin may serve as a widely applicable target for anticancer immunotherapy.

Introduction

It is well established that peptide epitopes derived from human TAAs[3] can be recognized by CTLs in the context of MHC molecules (1). Survivin is a recently described member of the inhibitor of apoptosis proteins gene family, which is undetectable in normal adult tissues but is expressed in several human cancers including lung, colon, breast, pancreas, and prostate cancer as well as hematopoetic malignancies (2, 3). Furthermore, series of melanoma and nonmelanoma skin cancers have also been reported to be invariably survivin positive (4, 5). The overexpression of survivin in most human cancers suggests a general role of apoptosis inhibition during tumor progression. This notion is substantiated by the observation that expression of survivin was associated with an unfavorable prognosis in colorectal and bladder cancers as well as in neuroblastoma (6–8). These characteristics suggest that survivin might be eligible to serve as a universal TAA for both diagnostic and therapeutic purposes. Two groups independently provided initial evidence to sustain this hypothesis. Schmitz *et al.* (9) described survivin as capable of inducing specific CTLs *in vitro* when the protein is processed and presented by dendritic cells. The second group (10) followed a "reverse immunology" approach, *i.e.*, to scan the survivin protein for the presence of HLA-A*0201 (HLA-A2) binding motifs and to use these epitopes to search for specific T-cell responses in cancer patients, to test the notion of survivin being an universal tumor antigen. To this end, spontaneous T-cell reactivity was detected by ELISPOT assay in leukemia and melanoma patients. However, both of these studies were flawed by the lack of evidence proving the capacity of survivin-reactive T cells to lyse tumor cells of different tissue origin. Moreover, the relevance of CTL responses solely detected in the peripheral blood has recently been challenged (11, 12). Thus, in the present study, we took advantage of recent technological advances allowing the *in situ* detection as well as the *ex vivo* isolation and analysis of antigen-specific cytotoxic T cells to validate the natural immunogenicity of survivin.

Materials and Methods

Construction of HLA-Peptide Complexes for T-Cell Staining and T-Cell Sorting. A recognition site for enzymatic biotinylation using biotin protein ligase (BirA) in fusion with the 5′ end of the extracellular domains of HLA A*0201 (residues 1–275) was expressed in *Escherichia coli* BL21 (DE3). The recombinant protein was purified by size-chromatography (Sephadex G25, Pharmacia) and ion-exchange chromatography (mono-Q, Pharmacia) from inclusion bodies solubilized in 8 M urea. The HLA A*0201 was folded *in vitro* by dilution in presence of the modified survivin peptide Sur1M2 (LMLGEFLKL) or the melanoma-associated antigen peptide $gp100_{154-163}$, and subsequently biotinylated as described previously (13, 14). After gel filtration on a Pharmacia Sephadex G25 column to remove unbound biotin, the protein was multimerized with streptavidin-FITC-conjugated dextran molecules (kindly provided by L. Winther, DAKO, Glostrup, Denmark) to generate multivalent HLA-dextran compounds for immunohistochemistry.[4] The HLA A*0201 construct was a kind gift of Dr. Mark M. Davis (Department of Microbiology and Immunology, Stanford University, Palo Alto, CA). Cell separation was performed as previously described (15). Briefly, 5×10^6 streptavidin-conjugated magnetic beads (Dynal, Oslo, Norway) and were washed twice in 200 μl of cold PBS; 0.5 μg of peptide/A*0201 monomers were added, and the mixture was incubated for 15 min at room temperature. After two washes, these beads were mixed with PBL at a ratio of 1:10 and subsequently incubated for 1 h followed by a precipitation of bead-bound cells in a magnetic field. The precipitation step was repeated once.

Immunohistochemistry Stainings. For staining with FITC-conjugated multimeric peptide/MHC complexes, sections were dried overnight and subsequently fixed in cold acetone for 5 min. All of the incubation steps were performed at room temperature and in the dark: (*a*) 45 min of the primary antibody (1:100 diluted); (*b*) Cy 3-conjugated goat antimouse (1:500 diluted; code 115-165-100; Jackson ImmunoResearch, obtained from Dianova, Hamburg, Germany) for 45 min; and finally (*c*) the multimers for 75 min. Between each step, the slides were washed two times for 10 min in PBS/BSA 0.1%. The slides were mounted in vectashield and kept in the refrigerator until observed under the confocal microscope.

Cytotoxicity Assay. Conventional [^{51}Cr] release assays for CTL-mediated cytotoxicity were carried out as described elsewhere (16). Target cells were autologous EBV-transformed B-cell lines, the HLA-A2-positive breast cancer

Received 5/17/01; accepted 7/3/01.

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[1] Supported by grants from the Danish Medical Research Council, Novo Nordisk Foundation, Danish Cancer Society, Danish Cancer Research Foundation, and Deutsche Forschungsgemeinschaft (DFG) Grants Be 1394/5-2 and SFB465.

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[3] The abbreviations used are: TAA, tumor-associated antigen; CLL, chronic lymphatic leukemia; PBL, peripheral blood leukocyte; ELISPOT, enzyme-linked immunospot.

[4] Pedersen, L. O., Winther, L., Kirkin, A. F., Hansen, N. J., Scholler, J., Andersen, M. H., Buus, S., Becker, J. C., and thor Straten, P., Highly efficient and specific detection of tumor specific T cells with polymer MHC class I protein complexes, manuscript in preparation.

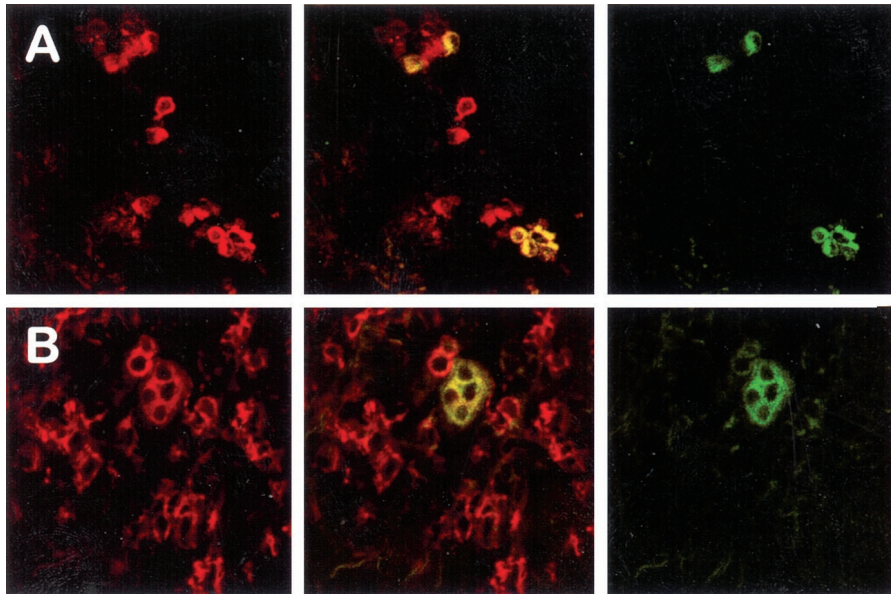

Fig. 1. *In situ* detection of survivin-reactive CTLs. *A*, confocal laser scanning microscopy was used to detect CTLs reacting with a Cy 3-conjugated anti-CD8 antibody (*red channel*) and/or an FITC-conjugated multimeric MHC/survivin-peptide construct (*green channel*) in a primary tumor from a stage III melanoma patient. *B*, staining with an anti-CD8 antibody (*red channel*) and a FITC-conjugated multimeric MHC/survivin-peptide construct (*green channel*) in a sentinel lymph node from the same patient.

cell line MCF-7 [available at American Type Culture Collection (ATCC)], the HLA-A2-positive melanoma cell line FM3 (17), the HLA-A2-negative breast cancer cell line BT-20 (available at ATCC), and the HLA-A2-negative melanoma cell line FM45 (17). All of the cancer cell lines expressed survivin as examined by reverse transcription-PCR (data not shown).

ELISPOT Assay. The ELISPOT assay was used to quantify peptide epitope-specific IFN-γ releasing effector cells and has been described previously (18). Briefly, nitrocellulose-bottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated with an anti-IFN-γ antibody (1-D1K; Mabtech, Stockholm, Sweden), and nonspecific binding was blocked using AIM V (Life Technologies, Inc., Gaithersburg, MD). Lymphocytes were added at different cell concentrations together with the specific peptides and T2 cells and were incubated overnight at 37°C. After two washes, the biotinylated detection antibody (7-B6–1-Biotin; Mabtech) was added. Specific binding was visualized using alkaline phosphatase-avidin together with the respective substrate (Life Technologies, Inc.). The reaction was terminated on the appearance of dark purple spots, which were quantitated using the AlphaImager System (Alpha Innotech, San Leandro, CA). The peptides used for the ELISPOT were Sur1 (survivin$_{96–104}$, LTLGEFLKL), Sur9 (survivin$_{95–104}$, ELTLGEFLKL), and a Sur1 analogue peptide Sur1M2 in which a better anchor residue (methionine) replaced the natural threonine at position 2.

Results

***In Situ* Staining of HLA-A2/Survivin-reactive T-Cells.** Previously, we have identified two survivin-derived peptide epitopes recognized by T cells in leukemia and melanoma, *i.e.*, Sur1, position 96-104, (LTLGEFLKL) and Sur9, position 95-104 (ELTLGEFLKL; 10). The weak binding affinity of Sur1 to HLA-A2 was improved by replacing threonine at position 2 with a better anchor residue (methionine; Sur1M2). This measure enabled the construction of stable HLA-A2/peptide complexes. These complexes were multimerized using dextran molecules, which were conjugated with both streptavidin and FITC. Multimerized MHC-complexes were used to stain acetone-fixed, frozen material. Using a confocal laser microscope, Sur1M2/HLA-A*0201-reactive CTLs could readily be detected *in situ* in the tumor microenvironment. We depicted such cells in the primary tumor and the sentinel lymph node of a stage III melanoma patient as well as in a primary breast cancer lesion (Figs. 1 and 2). To ensure the specificity of the staining, we performed a series of negative controls (exemplified in Fig. 2, *B* and *C*). The use of neither peptide/HLA-dextran multimers with peptides derived from the melanoma differentiation antigen gp100 on the same tumor nor Sur1M2/HLA-dextran multimers in case of a tumor sample obtained from a HLA-A2-negative donor resulted in a positive staining.

Isolated Survivin-reactive CTL Lyse Tumor Cell Lines of Different Origin. To characterize the functional capacity of survivin-reactive CTLs, these cells were isolated by means of magnetic beads coated with HLA-A2/Sur1M2-complexes. Recently, we isolated gp100- and Mart-1-specific T-cells in a similar manner showing that this method is highly efficient to enrich specific peptide/MHC-reactive T cells (14). A freshly resected melanoma-infiltrated lymph node was minced into small fragments and crushed to release cells into culture. Cells were stimulated once with peptide *in vitro* prior to isolation. One day after isolation, interleukin 2 was added, and on day 5, the capacity of these cells to kill tumor cells was tested either by ELISPOT or in standard ^{51}Cr release assays. First, by means of ELISPOT analysis, we were able to establish that CTLs that were isolated using the modified Sur1M2/HLA-A2-complex also reacted against the native Sur1 peptide (data not shown). Second, we tested the cytotoxicity of the survivin-reactive CTLs against the HLA-A2-positive melanoma cell line FM3 (Fig. 3*A*) and the HLA-A2-positive breast-cancer cell line MCF-7 (Fig. 3*B*). The isolated T cells efficiently lysed both of the HLA-A*0201 cell lines. In contrast, no cytotoxicity was observed against the HLA-A2-negative melanoma cell line FM45 (Fig. 3*A*) or the HLA-A2-negative breast cancer cell line BT-20 (Fig. 3*B*).

Survivin Reactivity Measured in PBL by ELISPOT. The presence of survivin-reactive T cells in PBL from 10 HLA-A2-positive breast cancer patients was examined by the ELISPOT. Before analysis, PBLs were stimulated once *in vitro* to extend the sensitivity of the assay. Reactivity to the following survivin peptides was examined: (*a*) Sur1; (*b*) Sur9; and (*c*) Sur1M2. Survivin-specific T cells were detected in 6 of the 10 HLA-A2-positive breast cancer patients. Representative examples are given in Fig. 4. In PBLs from two patients, we detected a response against Sur1 and the modified analogue Sur1M2, but not against Sur9 (Fig. 4, *A* and *B*); in three patients, a response against Sur9 was detected, but not against Sur1 or Sur1M2 (Fig. 4*C*); and one patient responded only against Sur1M2. In contrast, no survivin responses were detected in PBLs from 20 healthy HLA-A2-positive donors. Similarly, PBLs from 14 HLA-A2-positive melanoma patients were examined. Survivin responses were present in seven of these patients (Table 1); two reacted against the Sur9 peptide,

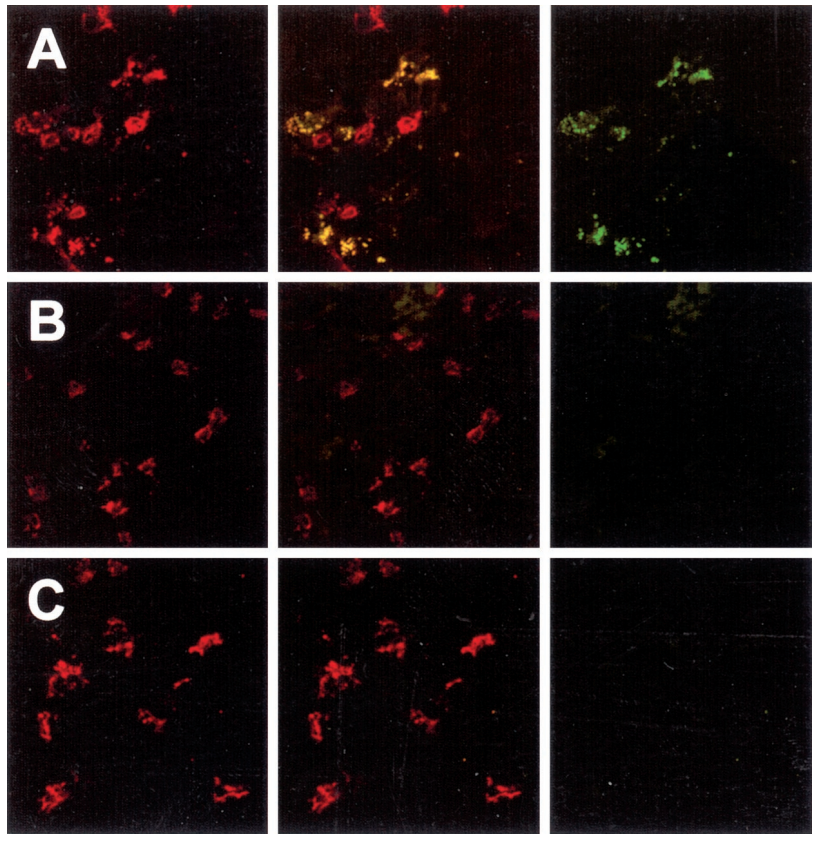

Fig. 2. *In situ* detection of survivin-reactive CTLs. *A*, confocal laser scanning microscopy was used to detect CTLs reacting with a Cy 3-conjugated anti-CD8 antibody (*red channel*) and a FITC-conjugated multimeric MHC/survivin-peptide construct (*green channel*) in a breast cancer metastasis from a HLA-A2-positive patient. *B*, staining with an anti-CD8 antibody (*red channel*) and a FITC-conjugated multimeric MHC/gp100-peptide construct (*green channel*) in a breast cancer metastasis from a HLA-A2-positive patient. *C*, staining with an anti-CD8 antibody (*red channel*) and a FITC-conjugated multimeric MHC/survivin-peptide construct (*green channel*) in a breast cancer metastasis from a HLA-A2-negative patient.

three against the Sur1M2 peptide, one against both Sur1 and SurM2, and one against all three peptides. Recently, we described T-cell response against survivin in three CLL patients (Table 1; CLL1, CLL2, and CLL3; Ref. 10). We have extended these studies with PBLs from three additional CLL patients. Notably, all of the patients harbored a T-cell response against at least one survivin epitope (Table 1; CLL5, CLL6, CLL7). In addition, we examined PBLs from one patient suffering from chronic myeloid leukemia. In this patient, a response against all three peptides was identified (data not shown). The data are summarized in Table 1.

Discussion

Similar to the catalytic subunit of telomerase (19), survivin can be regarded as a universal tumor antigen expressed in most human malignancies but not in normal, differentiated tissue. However, although reactivity of human CTLs against telomerase has to date only been demonstrated in T-cell lines after *in vitro* stimulation, our report is the first to describe spontaneous CTL reactivity against such a TAA in cancer patients by *in situ* and *ex vivo* analysis. The detection of survivin-reactive CTL *in situ* is of particular importance. There is consensus that the induction of efficient and powerful T-cell responses requires proper priming of the T cell. However, it is equally important that the T cells acquire the ability to home to the site of action. The combined detection of survivin-specific T cells in the blood and in the tumor lesions indicate that these cells are capable of circulating and homing to the effector site. Importantly, this was shown both for melanoma and breast cancer. This is a significant finding, because several clinical reports have reported a functional dissociation between local and systemic antimelanoma T-cell responses. Thus, at least in melanoma, the presence of TAA-specific T cells in the blood may not lead to clinically relevant responses, *i.e.*, responses at the tumor site (11, 12, 20). Furthermore, it is worth noting that the stained cells were located in small clusters, which suggested a local expansion of antigen-reactive cells.

The ELISPOT methodology represents a strong tool to monitor peptide-specific T-cell responses. However, although it has been shown that ELISPOT reactivity in most cases correlates with the capacity to lyse the target cell, the formal proof for this notion can be given only directly. We provided such evidence, because survivin-reactive T cells, isolated by means of recombinant HLA/peptide complexes, were shown to possess the capacity of target-cell lysis. Moreover, the isolated T cells were capable of lysing HLA-matched tumor cells of different origin, *i.e.*, melanoma and breast cancer. The important finding that the CTLs of a melanoma patient mediate efficient lysis of both melanoma and breast-cancer cell lines strongly suggests that cancer cells in general process and present the same endogenous survivin peptide. Therefore, a major implication of our

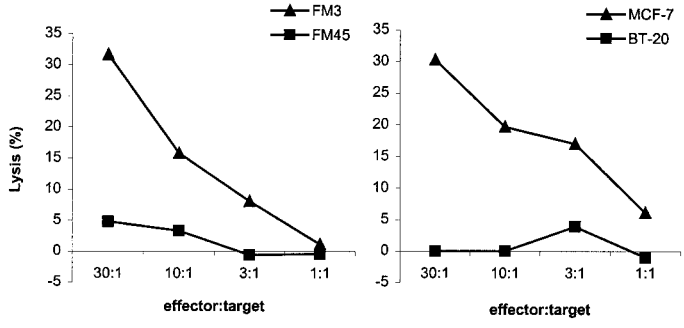

Fig. 3. Functional activity of survivin-specific CTLs. CTLs were isolated from a melanoma-infiltrated lymph node using survivin-coated magnetic beads. *A*, specific lysis of melanoma cell lines; the HLA-A2-positive FM3 (▲) and the HLA-A2-negative FM45 (■). *B*, specific lysis of breast cancer cell lines; the HLA-A2positive MCF-7 (▲) and the HLA-A2-negative BT-20 (■).

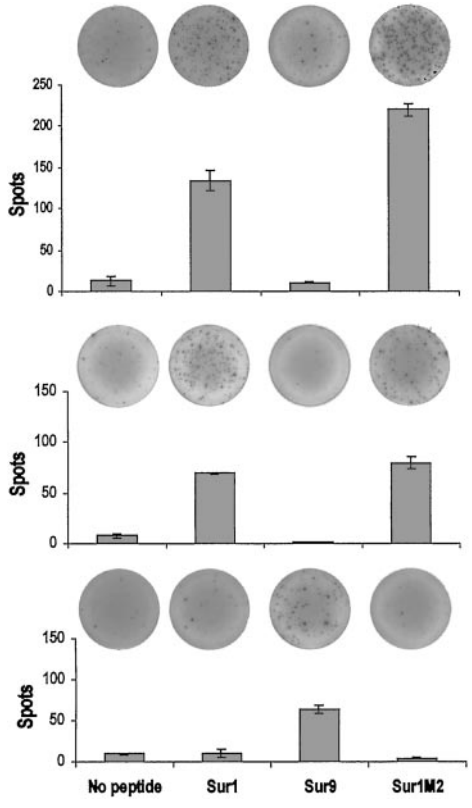

Fig. 4. Frequency of survivin-reactive CTLs in PBL from breast cancer patients. Reactivity was examined in three breast cancer patients (*top*, *middle*, and *bottom panels*) by the ELISPOT. For each patient, the first wells represent assays performed in the absence of peptide, the second wells in the presence of sur1 peptide, the third wells in the presence of sur9, and the fourth wells in the presence of the modified sur1M2 peptide. One $\times$ 10^4 effector cells per well were used. The graph depicts the quantification of reactive cells; *gray columns*, the average number of IFN-γ-producing cells.

study is that the Sur1 and Sur9 peptides are expressed and complexed with HLA-A2 molecules on a variety of cancer cells of different histological origins. This renders them susceptible to destruction by CTLs and emphasizes the potential advantage of survivin immunization, which may possibly control the growth of different neoplasms. The presence of spontaneous CTL responses in PBLs against HLA-A2-restricted survivin-derived peptide epitopes from patients suffering from three unrelated cancer types, *i.e.*, breast cancer, melanoma, and CLL, further substantiates the immunotherapeutic potential of this tumor antigen.

Spontaneous CTL responses were observed against the deca-mer Sur9 and the nona-mer Sur1. Because the sequences of the Sur1 and the Sur9 peptides are very similar, the initial concept was that the T-cells cross-reacted with the two peptides. However, in most patients, we detected a response against either the nona-mer or the deca-mer peptide, which indicated that at least a proportion of the T cells discriminate between the two peptides. In only a few patients did we observe T-cell responses against both peptides, which suggests that both peptides are processed and presented, but that the immunodominance of these peptides may differ from patient to patient.

To increase the affinity of the weak HLA-A2 binding peptide Sur1, we modified the peptide by replacing the natural threonine at position 2 with a methionine (*i.e.*, Sur1M2). This improved the binding affinity to HLA-A2, and in all patients hosting a Sur1 response, we were able to detect a strong response against the modified peptide. Furthermore, in a number of patients, we were able to detect a Sur1M2 response but not a response against the native peptide. The use of modified peptides with improved HLA binding affinities has previously been demonstrated to be suitable for induction of CTL responses (21).

In approximately one-half of the melanoma and breast cancer patients, we were not able to detect a response against the survivin-derived epitopes in PBL. Obviously, this might simply reflect that these patients do not host a spontaneous response. However, responses in these patients may have been present but below the detection limit of our assays. Furthermore, other class I MHC-restricted survivin-derived peptide epitopes may dominate the response in these patients. These epitopes could be presented by other HLA alleles, but the presence of additional peptides processed and presented in the context of HLA-A2 cannot be excluded. In our search for peptides that bind to HLA-A2, we only analyzed for binding. However, procedures in which *in vitro* proteasome digestion is incorporated may reveal additional peptides (22).

Because most human cancers express high levels of survivin, immunotherapeutic strategies aiming at this antigen may have broad clinical applications. The major concern of such an approach would be the induction of autoreactive immune responses. Thus, the future of survivin-based vaccination will depend on both the therapeutic efficacy and on the type of side effects that may follow immunization. When peptides derived from melanocyte differentiation antigens were first used to treat patients with stage IV melanoma, it was envisioned that this might lead to pronounced destruction of melanocytes, which in turn would manifest clinically, *i.e.*, in vitiligo or retinitis. However, clinical experience demonstrated that the incidence of vitiligo in patients receiving vaccinations was not significantly higher than the incidence of melanoma-associated hypopigmentation in patients receiving other forms of therapy (23). For survivin, the odds that no major adverse autoimmune effects will be induced are even better, because overexpression of survivin is largely restricted to neoplastic cells. Importantly, no spontaneous immune reactions against survivin were detected in any of the healthy HLA-A2-positive donors. This finding is substantiated by Rohayem *et al.* (24), who described antibody responses to survivin in up to 20% of tumor patients but not in healthy individuals.

The attractiveness of survivin for vaccination purposes relies not only on the universal expression among tumors of different origin, but also on the fact that down-regulation or loss of its expression as a

Table 1 *Patients with survivin peptide-specific T lymphocytes in PBLs as measured by ELISPOT*

Patient	Sur1	Sur9	Sur1M2
	Melanoma[a]		
P4			97
P11			112
P13			71
P15	61		101
P17		172	
P39		127	
P64	112	70	128
	Breast cancer[b]		
B1	122		208
B2	67		72
B3		54	
B4		45	
B5		19	
B6			24
	CLLs[c]		
CLL1		27	320
CLL2		39	
CLL3	23	127	122
CLL5		100	124
CLL6		121	360
CLL7	68	132	174

[a] Frequency of reactive cells per 10^4; 14 patients examined in total.
[b] Frequency of reactive cells per 10^4; 10 patients examined in total.
[c] Frequency of reactive cells per 10^5; seven patients examined in total.

means of immune escape would hamper the progression of the tumor. Expression of most previously described TAAs is heterogeneous among tumors from different patients and can even vary among metastases obtained from the same patient (25, 26). Furthermore, several TAAs are derived from proteins that are not essential for the survival of the tumor cell. Thus, if CTL responses are induced by therapeutic measures such as vaccinations it is likely that tumor cells lacking the expression of these antigens will have a pronounced growth advantage (27–29). The apparent obligatory expression of survivin and telomerase by cancer cells, implies that these TAAs are not prone to immune selection, because down-regulation or loss of their expression would severely inflict the growth potential of the tumor cell (2, 7, 30). Furthermore, the expression of survivin in tumors is correlated with drug resistance and/or shorter survival of cancer patients (6–8, 31–33). Thus, the combination of a survivin-based immunotherapy with conventional cancer chemotherapy might prove to be an effective modus operandi to fight cancer.

Acknowledgments

We thank Merete Jonassen, Tina Seremet, and Claudia Siedel for their excellent technical assistance.

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