Clonal Expansion of T Lymphocytes in Human Melanoma Metastases after Treatment With a Hapten-modified Autologous Tumor Vaccine

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Abstract

Metastatic melanoma patients treated with an autologous DNP-modified tumor cell vaccine develop inflammatory responses in metastatic tumors characterized by infiltration of CD8+ T cells. To further define this immune response, we analyzed T cell receptor β-chain variable (TCRBV) region repertoire in biopsy specimens and peripheral blood lymphocytes of six patients. After administration of DNP vaccine, a restricted set of TCRBV gene families was found to be expanded compared with prevaccine metastases. In several postvaccine lesions of one patient, obtained over a 2-yr period, TCRBV14+ T cells were clonally expanded and identical T cell clonotypes could be detected. Two major recurring clones were biased toward the use of TCRBJ1S5. Furthermore, T cell lines derived from two such infiltrated skin lesions and, enriched in TCRBV14+ T cells, displayed HLA–class I–restricted lysis of the autologous melanoma cells. Clonal expansion of T cells was demonstrated in the T cell–infiltrated, postvaccine metastasis of a second patient as well. These results indicate that vaccination with autologous, DNP-modified melanoma cells can expand selected clones of T cells at the tumor site and that such clones are potentially destructive to the tumor. (J. Clin. Invest. 1997. 99:710–717.) Key words: melanoma • vaccine • T cell receptor • tumor-infiltrating lymphocytes • cytotoxic T cells

Introduction

In patients with surgically incurable metastatic melanoma, administration of a vaccine consisting of cryopreserved, autologous, irradiated tumor cells modified with DNP can induce clinically evident inflammatory responses in superficial metastases (1, 2). Histopathological examination of the inflamed lesions demonstrated intense infiltration of T lymphocytes, mostly CD8+ (1, 2) which are ordinarily not seen in metastases from nonvaccinated patients (1). The same type of treatment administered to patients with stage III melanoma, free of disease after resection of palpable, large (>3 cm) regional lymph node metastases, has resulted in 50 and 60% 4-yr relapse-free and overall survival, respectively (3).

The molecular definition of melanoma antigens recognized by T cells (4) and the possibility of studying the composition of the T cell receptor (TCR) involved in the MHC-restricted recognition of such epitopes, including epitopes expressed within the tumor mass (5), now allow a more precise evaluation of the immune response in vaccinated patients. Several groups of investigators have documented overexpression or clonal expansions of T cells expressing peculiar TCR β-chain variable (TCRBV) (thereafter BV)-gene families in primary or, less frequently, metastatic tumors of different histologies; in some cases, such clonal expansions have been shown to be driven by the recognition of specific tumor antigens (for review see Sensi et al., reference 5).

The effect of therapy with biological response modifiers on the TCR repertoire in tumor lesions has also been assessed in melanoma or renal carcinoma patients treated with IL-2 alone, IL-2 with IFN-α or IL-2, or cisplatin with/without IFN-α (6–9). Oligoclonal infiltrations in metastases responding to cytokine treatment but not in progressing lesions of the same patient have been described. In contrast, analysis of the effect of tumor cell vaccination has been limited to a single renal carcinoma patient (10). This patient was first injected with irradiated neoplastic cells and subsequently adoptively transferred with in vitro-sensitized T lymphocytes. A responding lung metastasis exhibited an oligoclonal population of T cells expressing BV13, which was overexpressed compared with both pre- and postvaccine peripheral blood lymphocytes and to a nonresponding renal tumor of the same patient. However, clonal expansion of T cells was not demonstrated. In all of these studies, direct evidence that BV repertoire changes occur as a response to therapy has been limited because prevaccination tumor specimens were not analyzed. Furthermore, it has not been determined whether T cells overexpressing peculiar BV gene families in responsive lesions could mediate specific antitumor immunity.

In this paper, we evaluated BV gene family usage by T cells obtained from the metastatic lesions of six patients who developed tumor inflammatory responses after treatment with autologous, irradiated DNP-modified tumor cells. For all patients, pre- and postvaccinations metastases and PBL also were studied. The availability of a series of specimens taken at different times after beginning vaccination from a clinically responding patient rendered possible the identification of dominant T cell clonotypes and the search for antitumor cytotoxic effectors.

Our data provide the first evidence that selectively expanded T cell clones, including HLA–class I–restricted effectors.

1. Abbreviations used in this paper: BV, β chain variable; CDR3, complementarity-determining region; CTL, cytotoxic T lymphocytes; sc, subcutaneous; TIL, tumor-infiltrating lymphocytes; SSCP, single strand conformational polymorphism; TCR, T cell receptor.
tors recognizing melanoma antigens, infiltrate the tumor site after immunization with whole autologous tumor cells.

Methods

Patients and clinical specimens. The patients studied and the characteristics of their melanoma samples are shown in Table I. The clinical protocol for the DNP-vaccine administration has been previously described (1, 2). Briefly, patients were contact sensitized to the hapten by topical application of dinitrofluorobenzene 3 d after low dose (300 mg/M²) cyclophosphamide. 2 wk later, patients were injected intradermally on the upper arm with irradiated, DNP-modified melanoma cells mixed with BCG as adjuvant. Two schedules of administration were tested: (a) DNP-vaccine administered every 28 d with Cy administered 3 d before the first two vaccine injections (patient ED); and (b) DNP-vaccine administered weekly for 6 wk with Cy given 3 d before the 6-wk series (patients FC, CB, JB, RS, LC).

The clinical course of the six patients was as follows: CB, the inflamed metastasis was excised and the patient remained tumor free until he died suddenly of a hemorrhagic brain metastasis at 12 mo; ED, this patient had a mixed response (regression of some subcutaneous tumors simultaneously with growth of others), and she survived 24 mo; RS, despite histologic evidence of tumor inflammation, there was no evidence of tumor regression, but the patient survived for 22 months; LC, despite histologic evidence of tumor inflammation, there was no evidence of tumor regression, and the patient died at 11 mo.

Quantification of the cDNA and TCR repertoire analysis. Semiquantitative PCR was used as recently described (11). Briefly, total RNA was prepared, with the use of RNAzolB (Cinna/Biotecx, Friendswood, TX), from thawed cryopreserved PBL and enzymatically digested tumor samples (without in vitro culture). First-strand cDNA was synthesized with oligo-dT and reverse transcriptase (Superscript; Gibco BRL, Gaithersburg, MD). Serially diluted cDNA from all samples was amplified using TCR constant-region (BC)-specific primers (11). PCR products were electrophoresed on agarose gels, transferred to nylon membranes (Hybond N++; Amersham International, Little Chalfont, UK), hybridized with an internal 32P-labeled BC oligonucleotide and scanned with a PhosphorImager (No. 425; Molecular Dynamics, Sunnyvale, CA). Individual bands were digitized and integrated using the Imagequant software package provided by the manufacturer. BV repertoire analysis was then performed on the same amount of TCR β-chain–specific cDNA template from PBL and tumor samples using a panel of described oligonucleotide primers (12). All experiments were performed at least twice. The levels of specific amplification were measured by densitometry as described above and each BV spot was expressed as a percentage of the sum of all BV signals detected in the repertoire analysis.

Clonality within selected BV gene families was assessed by high resolution–polyacrylamide gel electrophoresis and single strand conformational polymorphism (SSCP) analysis as described (13, 14). Briefly, BV PCRs were performed by 32P endlabeling the BC-specific antisense primer (0.08 μM). PCR products (5 μl) were heat denatured, separated both on 5% denaturing sequencing gel and on 6% nondenaturing polyacrylamide gels containing 10% glycerol and visualized by autoradiography. The official nomenclature proposed by the International Union of Immunological Societies Subcommittee on nomenclature has been adopted throughout this paper (15). Designation of BV gene subfamilies is according to Arden et al. (16).

Table I. Melanoma Patients and Specimens: Identification and Description

<table>
<thead>
<tr>
<th>Patients (HLA-A type)</th>
<th>Before DNP vaccine</th>
<th>After DNP vaccine</th>
<th>Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue Code</td>
<td>Tissue Code</td>
<td>Inflammation</td>
</tr>
<tr>
<td>ED (A1)</td>
<td>PBL PBL-0</td>
<td>PBL PBL-1</td>
<td>Intense</td>
</tr>
<tr>
<td>FC (A1/A1)</td>
<td>PBL-0 S-0</td>
<td>Subcutaneous S-1</td>
<td>Intense</td>
</tr>
<tr>
<td>CB (A1)</td>
<td>PBL PBL-0</td>
<td>PBL PBL-1</td>
<td>Intense</td>
</tr>
<tr>
<td>JB (A1/A28)</td>
<td>PBL PBL-0</td>
<td>Subcutaneous S-1</td>
<td>Intense (regressing tumor)</td>
</tr>
<tr>
<td>RS (A2)</td>
<td>PBL PBL-0</td>
<td>PBL PBL-1</td>
<td>Intense</td>
</tr>
<tr>
<td>LC (A2/A29)</td>
<td>PBL PBL-0</td>
<td>Subcutaneous S-1</td>
<td>Intense</td>
</tr>
</tbody>
</table>

*Except for peripheral blood lymphocytes, all tissues are metastatic melanomas. †No inflammation was observed in prevaccination specimens.
§Interval between the beginning of DNP-vaccine program and the excision of metastases or collection of blood.
**Cloning and sequencing of BV transcripts.** PCR products derived from at least two different sets of amplifications were cloned into the pCR-Script™ SK(+) vector (Stratagene Inc., La Jolla, CA) and random clones sequenced with Sequenase 2.0 (United States Biochemicals, Cleveland, OH). The complementarity-determining region (CDR3) has been defined according to Moss and Bell (17). Their boundaries include one of the serine residues encoded by the 3' end of the BV region up to but not including the phenylalanine residue of the conserved joining region (BJ) motif FGXGT. Germline BJ sequences were obtained from Toyonaga et al. (18).

**HLA typing.** HLA class I typing was available for patients JB (A1/28, B8/51), LC (A2/29, B44/51), and FC (A1/A1, B8/60), and was determined by serology. Patients ED and CB were typed as HLA-A1, whereas patient LC was HLA-A2 by PCR on cDNA from prevaccine PBL using the method and primers described by Browning et al. (19).

**Cell lines.** Melanoma lines from subcutaneous (sc) metastases were established in vitro according to described methods (20).

**In vitro expansion of BV14+ tumor-infiltrating lymphocytes.** Tumor-infiltrating lymphocytes (TIL) from postvaccine sc metastases of patient JB were expanded ex vivo in presence of mAbs to BV14 (CAS.1.1.3; Immunotech, Marseille, France) and to CD28 (L293; Becton Dickinson & Co., Mountainview, CA). Briefly, anti-BV14 (0.2 μg/ml) and anti-CD28 (1 μg/ml) were added in 0.5 ml of RPMI-1640 (Bio-Whitaker, Verviers, Belgium) to culture wells (24-well culture plates, 3524; Costar Corp., Cambridge, MA) precoated with 10 μg/ml of affinity-purified goat anti–mouse IgG (Sigma Chemical Co., St. Louis, MO) and incubated for 40 min at 4°C. A cell suspension consisting of T lymphocytes and tumor cells, recovered from tumor sites and depleted of monocytes after a 2-h adherence to plastic, was panned over the coated wells in a final volume of 2 ml RPMI-1640 supplemented with 10% pooled human serum (complete medium, CM) and cultured for 1 wk. Cells were then restimulated for an additional week on anti-BV14 mAb–coated wells in CM plus 20 U/ml recombinant IL-2, and then cultured in CM plus 50 U/ml recombinant IL-2. Cytotoxic activity on the autologous tumor was tested by a standard 51Cr-release assay as described (20), after 4 and 5 wk of in vitro stimulation. Inhibition of lysis was performed by preincubating tumor targets with 10 μg/ml of purified W6/32 (anti–HLA class-I, HB95; American Type Culture Collection, Rockville, MD) (21) or CR11.351 (22) mAbs for 40 min at 37°C.

**Phenotypic analysis.** Phenotype of lymphocytes was assessed by indirect immunofluorescence followed by flow cytometric analysis with a FACScan® cytometer (Becton Dickinson & Co., Sunnyvale, CA) as described (20). The following mAbs were used: CAS.1.1.3 (anti-BV14); OKT3 (anti-CD3; CRL3001; American Type Culture Collection), OKT8 (anti-CD8, CRL8014; American Type Culture Collection).

**Results**

**Presence of overexpressed BV gene families in postvaccine metastatic lesions.** We obtained from six patients a series of metastatic melanomas that developed an inflammatory response after administration of DNP vaccine and compared their BV repertoire with that of metastases excised before vaccine and with pre- and postvaccine PBL (Table I). For patient JB, five postvaccine biopsy specimens (S-1, L-1, S-2, S-3, S-4) were collected over a 2-yr period. The last sample (S-4) was obtained when the patient had ceased to clinically respond to the vaccine, and that lesion displayed minimal inflammation. Multiple postvaccine metastases were also analyzed for patient LC.

Fig. 1 shows the histology of sc metastases excised from patient FC before and after DNP-vaccine treatment. It is representative of the histologies of inflamed tumors observed in other patients. The postvaccine tumor shows marked infiltration with lymphocytes (shown by flow cytometry to be CD3+ CD8+ T cells), while in the prevaccine lesion lymphocytes are present, but sparse. The development of tumor inflammation following DNP vaccine in an sc metastasis (S-1) of a second patient (JB) has been previously described (2). It should be noted that the prevaccine tumors from patients ED, CB, JB, and RS were lymph node metastases and therefore contained an abundance of T cells.

Fig. 2 shows, for each patient, the relative representation of BV gene families, which, in at least one of the studied specimens, was overexpressed (i.e., increased more than 1.8-fold over prevaccine [either PBL or tumor] samples). This threshold was chosen because it was always above the mean + 2 SD of each corresponding prevaccination sample. Some prevaccine metastases displayed differences in the expression of several BV gene families when compared with matched prevaccine PBL. These include: BV3 in patients LC, JB, and CB; BV5 in patients ED, LC, and CB; BV14 in patients ED, FC, and LC; BV7 in patients ED, FC, LC, and JB. Several other overexpressed BV gene families were peculiar to particular prevaccine tumors (patient ED: BV13, BV15; patient JB: BV20; patient CB: BV16; patient LC: BV4, BV8, BV20; pa-
tient RS: BV4, BV8). These results extend to metastatic lesions the observation previously made in primary tumors: T cells infiltrating metastases of untreated patients may be biased in their BV usage (5). Several of these BV gene families maintained high levels of expression in postvaccine lesions; ie., they were not further modified by vaccination (Fig. 2).

Next, we compared BV expression in postvaccine metastases of each patient with the corresponding prevaccine tumors (Fig. 2 and Table II). BV14 was the only BV gene subfamily overexpressed in postvaccine sc metastases of patients FC and RS. In patient FC, BV14 accounted for > 30% of the total repertoire in the postvaccine metastasis having undergone a fourfold expansion compared with the prevaccine tumor, and a sevenfold expansion compared with prevaccine PBL. In patient RS, BV14 was the only BV gene family expressed by T cells infiltrating the postvaccine metastasis. In patient JB there was an increase in BV1 and BV14 expression in multiple post-vaccine sc metastasis and in the lung metastasis L-1 (Fig. 2 and Table II). BV3, one of the families overexpressed in the prevaccine tumor, was not increased further in the postvaccine specimens (Fig. 2).

The postvaccine tumor of patient ED displayed an overexpression of BV5, which accounted for > 30% of the BV repertoire; this represented a threefold expansion of BV5 compared with prevaccine PBL.

### Table II. BV Gene Families Overexpressed in Postvaccine Tumors Compared With Prevaccine Tumors

<table>
<thead>
<tr>
<th>Patients</th>
<th>Postvaccine specimens</th>
<th>TCRBV families overexpressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>S-1</td>
<td>5 (30.6)*; 23 (2.2); 24 (2.5)</td>
</tr>
<tr>
<td>FC</td>
<td>S-1</td>
<td>14 (31.6)</td>
</tr>
<tr>
<td>CB</td>
<td>LN-1</td>
<td>23 (2.1)</td>
</tr>
<tr>
<td>JB</td>
<td>L-1</td>
<td>1 (12.5); 14 (6.7)</td>
</tr>
<tr>
<td></td>
<td>S-1</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td></td>
<td>S-2</td>
<td>14 (7.2)</td>
</tr>
<tr>
<td></td>
<td>S-3</td>
<td>1 (9.73); 14 (8.2)</td>
</tr>
<tr>
<td></td>
<td>S-4</td>
<td>1 (12.6)</td>
</tr>
<tr>
<td>HLA-A2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>S-1</td>
<td>14 (100)</td>
</tr>
<tr>
<td>LC</td>
<td>S-1</td>
<td>5 (32.9); 15 (3.2)</td>
</tr>
<tr>
<td>B-1</td>
<td>4 (10.4); 15 (5.6)</td>
<td></td>
</tr>
<tr>
<td>B-2</td>
<td>7 (8.4); 15 (3.4)</td>
<td></td>
</tr>
</tbody>
</table>

*In parentheses are indicated the relative percentages of expression. All these BV gene families were also overexpressed when compared with prevaccine PBL, the exception being BV1 in patient JB, specimens S-1 and S-3.
with the prevaccine metastasis and a sevenfold expansion when compared with PBL. BV23 and 24 were also overexpressed in patient ED compared with prevaccine tumor, although to a much lesser extent.

Changes in the BV repertoire of the remaining two patients, LC and CB, were more complex. In patient LC, from whom multiple metastases were available, BV gene families involved were different from lesion to lesion, with the exception of BV15. In the postvaccine metastasis of patient CB, BV23 was overexpressed compared with the prevaccine tumor, although its level accounted for only 2% of the total TCR repertoire. BV5, highly represented in the prevaccine tumor, did not increase after vaccination. These results could be attributed to the histories of postvaccine tumors in these two patients: both showed extensive tumor necrosis, which could also be associated with a nonspecific (polyclonal) infiltration of mononuclear cells that would mask a more restricted response.

In general, differences observed in BV expression of PBL before and during vaccination were minimal even for BV gene families whose levels increased at the tumor site after vaccination (Fig. 2). Patient LC displayed the highest number of BV gene family variation in postvaccine PBL (BV4, BV7, BV8, BV15, BV20).

Overexpressed BV gene subfamilies are oligoclonal and include dominant rearranged transcripts, identical in asynchronous tumor lesions. Since vaccination with DNP-modified autologous tumor cells elicited an expansion of specific BV gene families in metastatic lesions, we determined whether such expansions were due to T cell clonal subpopulations and whether identical clonotypes could be found in multiple lesions. To screen the extent of heterogeneity of the T cell infiltrate, molecular analysis of length and sequence composition of the CDR3 region, which is encoded by the V-D-J junctions and is important for MHC/peptide recognition (23), was performed for the BV14 transcripts that were overexpressed in 3/5 postvaccine lesions from patient JB. To score for any possible intratumoral clonal expansions, all of the JB specimens were included in this analysis.

In polyclonal populations, CDR3 lengths for individual BV gene subfamilies are visualized as a ladder pattern of 3-bp–spaced bands on a sequencing gel. In contrast, the postvaccine metastases, but not the prevaccine metastasis or the PBL, exhibited predominance of particular CDR3-size bands (Fig. 3). This finding could have been due to the expansion of either single clones or of several clones with the same CDR3 size. To distinguish between these alternatives, we performed SSCP analysis. By this technique, each single band represents a specific T cell clonotype and can be detected on a smear if it is expanded in a heterogeneous population. As shown in Fig. 3, while prevaccine metastases and pre- and postvaccine PBL of patient JB taken at different time points exhibited a polyclonal pattern, in all postvaccine lesions, limited numbers of expanded clonotypes could be resolved. In particular, two different bands were common to all of the postvaccine samples.

Nucleotide sequences of random BV14 amplification clones derived from postvaccine sc metastases S-1, S-2, S-3, and S-4 of patient JB confirmed the oligoclonal nature of the expansions (Table III). In fact, 6/8 clones in tumor S-1 had an identical V-D-J rearrangement, which was identical to that found in 2/12, 6/9, and 2/9 clones in tumors S-2, S-3, and S-4, respectively. When evaluated by SSCP, it corresponded to one of the two recurring clonotypes of Fig. 3. A second rearranged transcript was found in 1/8, 1/12, and 1/9 recombinant clones sequenced in S-1, S-2, and S-3, respectively. These two clones were biased toward the use of BJ1S5 and displayed a conserved, nongermine-encoded basic arginine residue (R, in single letter amino acid nomenclature) at the same position within their junctional regions. A third transcript was shared by S-2 and S-3. All of the remaining sequences occurred only once or twice in single postvaccine lesions (Table III). None of these sequences could be found in a similar number of random BV14 clones obtained from the prevaccine lymph node metastasis (LN-0) or pre- and postvaccine PBL (PBL-0, 1, 2) and their sequences were never repeated (data not shown).

The nature of BV14 expansion was also studied in patient FC. This BV region was already overexpressed in the prevaccine tumor, which included several distinct clonotypes (Fig. 3). The pattern observed in the postvaccine lesion, both in CDR3 length and SSCP analysis, was consistent with the emergence of two new major clonotypes.

The presence of these clonotypic changes, their frequency, and their persistence in different metastases from the same patient indicate that an immune response elicited by the autologous vaccine was occurring in these tumors.

BV14-enriched T cell lines isolated from multiple specimens of patient JB display HLA-restricted lysis of the autologous tumor. The availability of a mAb directed to the single member of the BV14 gene family gave us the opportunity to determine whether such T cells could mediate antitumor activity. TIL were recovered from three different sc metastatic lesions (S-2, S-3, S-4) of patient JB.
Clonal Expansion of T Cells After Hapten-modified Autologous Melanoma Vaccine

Table III. Analysis of BV14 Transcripts Expressed in Asynchronous Metastatic Lesions of Patient JB

<table>
<thead>
<tr>
<th>BJC</th>
<th>CDR3*</th>
<th>S-1</th>
<th>S-2</th>
<th>S-3</th>
<th>S-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequences occurring identically in multiple postvaccine metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1S5C1</td>
<td>SPRDLNLOPOH 6/8</td>
<td>2/12</td>
<td>6/9</td>
<td>2/9</td>
<td></td>
</tr>
<tr>
<td>J1S6C1</td>
<td>SLSSVPNLQPOH 1/8</td>
<td>1/12</td>
<td>1/2</td>
<td>1/9</td>
<td></td>
</tr>
<tr>
<td>J2S1C2</td>
<td>SFGLNLEQEF 1/12</td>
<td>1/12</td>
<td>1/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequences occurring no more than twice in single postvaccine metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1S1C1</td>
<td>SLGGSTEAF 1/12</td>
<td>1/12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1S2C1</td>
<td>SLLGADTEAF 1/8</td>
<td>2/12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1S5C1</td>
<td>SLTQOPOH 1/9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1S6C1</td>
<td>SNTISPLH 1/12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J2S1C2</td>
<td>SYSGTYNQEF 1/12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J2S7C2</td>
<td>SWGRGEOY 1/12</td>
<td></td>
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</tbody>
</table>

* Amino acid sequences in the single letter code.

S-3, S-4) of patient JB. TIL were not available from the others. Expression of BV14 could be assessed by immunofluorescence in S-3 where it accounted for 21% of T cells, confirming that this subpopulation had expanded compared with both pre- (11%) and matched postvaccine (12%) PBL. All T lymphocyte cultures were initiated at the same time and lymphocytes were grown in vitro in the presence of anti-BV14 and antihuman T lymphocyte antibodies (anti–HLA-A2/A28 mAb CR11.351). Sequences occurring identically in multiple postvaccine metastases (Table IV) that were tested for cytotoxicity on the autologous target. After 4 wk of in vitro restimulation, BV14-enriched T cell lines derived from the two inflamed metastases (S-2, S-3) but not from the minimally inflamed metastasis (S-4) lysed the autologous tumor line derived from S-3 (Table IV). Inhibition of lysis by mAbs W6/32 (anti–MHC class I antigen) and CR11.351, which recognizes HLA-A28 in addition to HLA-A2 (24), indicated recognition of antigen(s) in association with this restriction element (Table IV). Similar results were obtained by testing VB14-enriched T cell lines after 5 wk of in vitro restimulation.

Discussion

Immunization of melanoma patients with autologous tumor cells modified with the hapten DNP frequently induces an inflammatory response in metastatic tumors (1). Immunohistochemical and flow cytometric studies have shown that such tumors are infiltrated with T lymphocytes, most of which are CD8+ (2). If T cells are triggered by the tumor vaccine and their presence reflects an in vivo cellular immune response to tumor cells, one would expect to find selective clonal expansions within these infiltrating T cells. TCRBV repertoire analysis in metastases that developed after vaccine treatment showed major expansions of selected T cell subpopulations in 5/6 patients. In three patients, expansion of T cells expressing BV14 could be observed and, in 2/3 whose material was enough for further analysis, we could demonstrate expansion of distinct T cell clones. Although normal skin from these patients was not available, we have previously shown that BV14 overexpression in melanomas did not reflect an intrinsic property of the skin (11). Moreover, a recent analysis of BV transcript composition expressed in normal skin of three melanoma patients indicated that it was different from that in the autologous matched tumor (Parmiani, G., and M.L. Sensi, unpublished data).

A striking finding of this study is the identification of the same dominant clone in different metastases sampled several months apart in patient JB. Two other clones were found identical in 3/4 and 2/4 of the metastases. In vivo persistence in PBL of cytotoxic T lymphocytes (CTL) clones with identical TCR clonotype composition and defined anti–tumor-peptide specificity has been recently described for the anti–MAGE-1/A1, MAGE-1/Cw*1601, and BAGE/Cw*1601 (25, 26). Two of the three recurring clones described in this study showed a bias in the use of J1S5 and displayed a basic, non–germline-encoded arginine residue at the same CDR3 position. The recurrence and structural constraints of these clonal T cells strongly suggests a role for melanoma-associated antigen(s) in the generation of the expansion.

This explanation is strengthened by the presence of CTL displaying an HLA-A28–restricted lysis of autologous melanoma cells within the BV14+ subset obtained from two out of three postvaccine tumors. Our demonstration that the overexpressed TCRBV had functional significance is unique: none of the previous studies reporting overrepresentation of BV gene families in lesions of immunotherapy-treated patients provided evidence for their role in the immune response to their autologous tumor. Immunization with autologous DNP-modified melanoma cells induces a CTL response to those hapten-
modified cells (27). Based on the work of Martin and Wetzlitz (28), we hypothesize that a subpopulation of T cells elicited by DNP-modified melanoma antigens recognized unmodified melanoma antigens as well.

It is of interest that patient JB was treated with multiple courses of DNP-modified vaccine over a period of 2 yr, and developed clinically and histologically evident inflammatory responses in multiple metastases. Moreover, he had a striking antitumor response with regression of multiple pulmonary metastases. The final metastatic lesion (S-4) was obtained from this patient when he had developed rapidly progressing metastases. This tumor contained mRNA for IL-10 but, in contrast with the other lesions, no IFN-γ mRNA could be detected (29). BV14 was no longer overexpressed in this metastasis, and BV14+ T cell lines derived from it were not cytotoxic for the autologous tumor. However, the presence of the two dominant BV14J1S5 transcripts in tumor S4 suggests that an immune response persisted. Lack of cytotoxicity of the BV14+ T cells recovered from this metastasis may reflect a state of anergy in vivo of melanoma-specific T cells that prevented their in vitro expansion. Indeed, it has been shown that melanoma cells may render T cells anergic through autocrine IL-10 production (30) and that IL-10 may induce a long-lasting unresponsiveness in CD4+ human T cell clones as shown by a block in proliferation and cytokine production, including IFN-γ (31). Alternatively, emergence of antigen-loss variants would be compatible with these results. Identification of the TCR clonotypes that mediate cytotoxicity in BV14+ T cells obtained from tumors S-2 and S-3 and of the antigen that they recognize will help in resolving this question.

It should be noted that some of the BV overexpressed in postvaccine tumors were already expanded, compared with PBL, in prevaccine metastases. This observation supports the hypothesis of immunological priming by tumor-associated antigens in the course of tumor growth and is corroborated by similar findings made in untreated primary melanomas (11, 32, 33) and metastatic lesions of patients who had not received any immunological manipulation (34). It is also supported by the presence of a high frequency of antimelanoma CTL precursors in PBL of some metastatic patients (35, 36).

BV14 has been shown to be involved in the response to the melanocytic differentiation antigen Melan-A/MART-1 by HLA-A2–restricted clones (37) and, in a previous study, was found to be overexpressed in primary melanomas of untreated HLA-A2+ patients (11). All the tumors used in the present study expressed mRNA for Melan-A/MART-1 and gp100 (data not shown). However, of the three patients whose postvaccine tumors overexpressed TCRBV14, only one (RS) was positive for HLA-A2. A second patient (JB) expressed HLA-A1/A28, and the third patient (FC) expressed HLA-A1. Thus it seems likely that TIL from these patients recognize novel melanoma antigens rather than the HLA-A2–binding melanoma differentiation antigens that have already been characterized.

These findings constitute the first demonstration of clonal T cell expansion elicited at the tumor site by a human tumor vaccine. Thus they provide immunological validation for the therapeutic approach with autologous DNP-modified tumor cells. In addition, this study shows that analysis of TCR expression at the tumor site is a powerful tool for tracing the evolution of the immune response to autologous tumor that is induced by immunotherapy.

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References

20. Anichini, A., A. Mazzocchi, G. Fossati, and G. Parmiani. 1989. Cytotoxic T lymphocyte clones from peripheral blood and from tumor site detect in...
tratumor heterogeneity of melanoma cells. Analysis of specificity and mecha-
clonal antibody (W6/32) in structural studies of HLA-A,B,C, antigens. *J. Im-
umol.* 123:342–349.
22. Russo, C., A.K. Ng, M.A. Pellegrino, and S. Ferrone. 1983. The mono-
clonal antibody CR11-351 discriminates HLA-A2 variants identified by T cells. *Immunogenetics.* 18:23–35.
Multiple genetic mechanisms have contributed to the generation of the HLA-
27. Sato, T., H.C. Maguire, Jr., M.J. Mastrangelo, and D. Berd. 1995. Hu-
31. Groux, H., M. Bigler, J.E. De Vries, and M.G. Roncarolo. 1996. Inter-
33. thor Straten, P., J.C. Becker, T. Seremet, E.B. Brocker, and J. Zeuthen. 1996. Clonal T cell responses in tumor infiltrating lymphocytes from both re-
acting with autologous tumor via the T-cell receptor: limiting dilution analysis of specific CTLp in peripheral blood and tumor-invaded lymph nodes of mela-
36. Anichini, A., R. Mortarini, C. Maccalli, P. Squareina, K. Fleischhauer, L. Mascheroni, and G. Parmiani. 1996. Cytotoxic T cells directed to tumor anti-
gens not expressed on normal melanocytes dominate HLA-A2.1–restricted im-
37. Sensi, M., C. Traversari, M. Radrizzani, S. Salvi, C. Maccalli, R. Mor-