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In vivo accumulation of the same anti-melanoma T cell clone in two different metastatic sites

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ABSTRACT **In a patient with progressing metastatic melanoma, we showed that the same autologous tumor-cytolytic CD8$^+$ tumor infiltrating lymphocyte (TIL) clone accumulated in two separate metastatic sites. This clone, which represented three of eight independently derived clones from a tumor deposit on the skin of the abdomen, also represented two of eight clones derived from a skin lesion on the shoulder. This clone could be identified by its use of a unique TCRBV2-nD1n-J1S6 sequence, and could also be detected by single-stranded conformational polymorphism (SSCP) as the dominant TCRBV2-expressing clone among CD8$^+$ TILs propagated from both shoulder and abdominal lesions. Using SSCP analysis, we also demonstrated that this clone was dominant in the fresh tumor tissue and in all TILs in which CD8$^+$ were strongly represented, including several separate but parallel cultures. The SSCP pattern for this clone was not apparent among CD4$^+$ TILs or CD8$^+$ peripheral blood mononuclear cells. The SSCP analysis of the tumor tissue prior to *in vitro* culture is an indication that the selection for this anti-tumor cytotoxic T cell clone was a reflection of its *in vivo* accumulation. Thus, we provide evidence that melanomas are immunogenic and able to select for cytotoxic anti-tumor-specific TIL clones that are expanded *in vivo* and can circulate to accumulate in different tumor sites. However, because these clones were isolated from progressing tumor metastases, the accumulation of these specific cytotoxic T cells was not sufficient to contain tumor growth.**

It has been shown that the immune response to human melanoma includes cytotoxic T lymphocytes capable of lysing autologous tumor cells *in vitro* (1–15). Some of the melanoma tumor antigens recognized have been identified and cloned (16–22). Despite the presence of these cytotoxic lymphocytes, it is evident that the immune response fails to eradicate clinically apparent tumors. Several mechanisms have been proposed to explain tumor escape from immune surveillance, including the loss of HLA alleles needed for T cell recognition (23, 24), as well as other cell surface molecules necessary for induction of T cell activation and proliferation (25).

Several laboratories have shown that the tumor infiltrating lymphocytes (TILs) propagated from human melanoma often show restricted T cell antigen receptor (TCR) usage (1, 4, 12, 26–34). In some cases it has also been possible to demonstrate that the dominant T cells among the TILs are able to lyse autologous tumors (3, 12, 35). Still, an unresolved concern has been the lack of unequivocal evidence that the dominant clones isolated *in vitro* were representative of the *in vivo* accumulation of these cells within the tumor, rather than a reflection of an *in vitro* selection. By comparing the TCR usage among the freshly isolated TILs with the TCR which could be sequenced from anti-tumor cytotoxic clones, we addressed the question of whether significant accumulations of melanoma-specific cytotoxic T cells occurred in tumors *in vivo*.

Combining the propagation and cloning of tumor-specific TILs with analysis of the TCR usage within the fresh tumor, we provide evidence that the *in vitro* "dominance" of an anti-melanoma cytotoxic TIL clone is a true reflection of the *in vivo* accumulation of this clone. In addition, our data show that identical anti-tumor cytotoxic TIL clones could be derived from two different cutaneous melanoma tumor deposits within the same patient.

MATERIALS AND METHODS

Isolation of TILs and Tumors. Tumor specimens were isolated from two skin biopsies obtained simultaneously from a 71-year-old male patient undergoing surgical removal of cutaneous nodules (on the shoulder and abdomen) from a metastatic malignant melanoma, according to approved institutional guidelines at the Massachusetts General Hospital. The patient was first diagnosed with malignant melanoma in a lesion from the skin of the back 15 years previously. One month before the removal of these cutaneous nodules, he developed a hemorrhagic left pectoral mass that was diagnosed as malignant melanoma. Two nodules were also detected in the lungs on a chest x-ray. In the intervening month he developed multiple skin nodules, two of which were removed for possible enrollment in an immunotherapy trial. However, discovery of a right pareto-occipital mass made the patient ineligible for cytokine therapy. He was treated with radiotherapy to the head, with ensuing shrinkage of the intracranial mass. In the year following the skin tumor excision, the patient's tumor progressed to include multiple visceral metastases.

The tissue fragment from the skin of the shoulder was divided for *in vitro* culture and for fresh extraction of RNA. The smaller of the two tumor biopsies (from the skin of the abdomen) was processed only for tissue culture.

For functional analyses and cloning, TILs were propagated in recombinant interleukin 2 (IL-2) as described to foster the outgrowth of activated T lymphocytes (5, 36). In brief, tumor tissue was cut into 1–2 mm tissue fragments with a scalpel blade, and different fragments were placed into 24-well tissue culture plates containing RPMI 1640 medium supplemented with 5% normal human serum in the following four parallel and independent conditions: (*i*) 100 units/ml IL-2; (*ii*) IL-2 and anti-CD3 mAb [obtained from clone 12F6 (15); final concentration, 0.1 μg/ml]; (*iii*) IL-2 and anti-CD3,4 bispecific mAb (final concen-

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Abbreviations: TIL, tumor infiltrating lymphocyte; SSCP, single-stranded conformational polymorphism; TCR, T cell antigen receptor; IL-2, interleukin 2; AB, abdominal; SH, shoulder; PBMC, peripheral blood mononuclear cell.

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tration, 3 μg/ml); and (*iv*) IL-2 and CD3,8 bispecific mAb (final concentration, 2.5 μg/ml). These bispecific monoclonal antibodies allow the selective outgrowth of highly enriched $CD8^+$ or $CD4^+$ lymphocytes, respectively (15). Four cultures were also derived with the same protocol from peripheral blood mononuclear cells (PBMCs) isolated through a Ficoll/Isopaque gradient from the patient. Cytofluorimetric staining of the cultures was performed with standard techniques (37), including analysis of TCR Vβ2 using clone E22E7.2 (Immunotech, Westbrook, ME).

As growing lymphocytes filled the initial wells, they were passaged into flasks, and IL-2 responsiveness was maintained by periodic restimulation with irradiated feeder cells and phytohemagglutinin (PHA) (1 μg/ml for polyclonal activation) as reported (5, 38).

T Cell Cloning. TILs were cloned from the CD8-enriched cultures obtained with the bispecific mAb CD3,4 by limiting dilution using irradiated mononuclear cells as feeder cells together with PHA as a polyclonal stimulus, as reported (12, 39, 40). Clones were expanded in IL-2 and grown to 2–5 $\times$ 10^7 for functional assays and to extract RNA. The HLA type of the patient's T cell line (kindly performed by S. Saidman, Massachusetts General Hospital Tissue-Typing Laboratory), by standard serological methods for class I and DNA typing for class II, was: HLA-A2, 24, B35, 61, Bw6, DR3(17)(DRB1*0301), DR7(DRB1*0701, DRB3*, DRB4*, DR52, DR53).

Melanoma Tumor Cell Lines. An autologous tumor cell line was established from tissue fragments cultured in RPMI 1640 medium supplemented with 5% human serum, in the absence of IL-2. The autologous melanoma cell line grew slowly for several weeks allowing us to perform the initial cytotoxicity assay experiments. However, this line could not be maintained beyond 3 months and is no longer available.

Six additional melanoma cell lines, derived from six different patients, were also used in the functional experiments. Cell lines M1, M4, M5, and M7 have been previously reported (37). The following is the HLA typing of these cell lines. M1: HLA A1,2; B7,8; Cw7;DR2, DQw1. M4: A1,3; B8,8; Cw7; DR3; DRw52. M5: A2,3; B14,39 (Bw6). M7: A10,11; B7,35 (Bw6), Cw4,w7; DR5; DRw52, DQw1,w3. M8 is a newly established cell line from a patient with metastatic melanoma with A2,24; B56,62; Bw6; DR1, 13, DR52. SK-Mel is an HLA-$A2^+$ cell line (19) kindly provided by J. Walter (Massachusetts Institute of Technology, Cambridge, MA).

Functional Analyses. Screening of the cytotoxic activity of TIL bulk and cloned progeny was determined against autologous tumors, allogeneic melanomas, as well as NK (K562) and LAK (Daudi) targets, using a ^{51}Cr-release assay, as described (5) at 50:1 target-to-effector cell ratios. In addition, the HLA-defective tumor line, CEM-T2, was used to present HLA-A2-restricted tyrosinase peptides to determine whether the peptides could be recognized by the bulk TILs or TIL clones. CEM-T2 was pulsed with 5 μg/ml of tyrosinase peptide YMNGTMSQV or 5 μg/ml of N-terminated tyrosinase peptide MLLAVLTCL (19). Unpulsed and peptide-pulsed CEM-T2 lines were labeled with ^{51}Cr and used as targets for melanoma-derived TILs.

Analysis of TCR Usage. TCR usage was assessed using a PCR-amplification system for TCRAV and TCRBV genes as described (41). For isolation of RNA from the freshly isolated tumor, the tissue was cut into 1–2 mm tissue fragments, and fragments were snap frozen in liquid nitrogen. Frozen tissue (50–100 mg) was stored at -80°C in 1 ml of Trizol (GIBCO/BRL; Life Technologies, Gaithersburg, MD) until it was subjected to RNA extraction. Total RNA was isolated by emersing the frozen tissue and homogenizing in the presence of Trizol by multiple pipetting of the small tissue fragments in a 2.2-ml microcentrifuge test tube. Chloroform (0.2 ml) was added to the tissue-Trizol slurry, and the RNA was extracted following separation by microfuge centrifugation for 15 min at 12,000 $\times$ *g*. The RNA was precipitated from the aqueous phase by the addition of 0.5 ml isopropyl alcohol and centrifugation at 4°C. The RNA pellet was washed with 75% ethanol and dissolved in 25 μl RNase-free H_2O. For extraction of RNA from cultured TILs, total RNA was isolated from 10^7 actively growing cells with Trizol, as above.

Total RNA (1 μg) was converted into first-strand cDNA using an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase according to the manufacturer's specifications (Promega). PCR amplification (25 or 30 cycles) was carried out using V region sequence-specific primers for 29 TCRAV families and 24 TCRBV families. Primer sequences and thermal cycling conditions were as described (41), with the following exceptions. A new TCRAV4 primer (5′-TGATGCTAAGACCACACAGCC-3′) was used because of inappropriate annealing of the previously described primer. Southern blotting and hybridization was carried out with ^{32}P-labeled constant-region probes. Identification of TCRAV and TCRBV usage was performed on individual clones, followed by gene sequencing of the clones that expressed the TCRBV2 gene to determine their identity, as described below.

To study the TCRAV gene usage by two shoulder-derived clones that had TCRBV gene sequence levels identical to the three abdomen-derived clones, we designed a primer to amplify, by PCR, the unique TCRAV-n-J joining gene region. The primer, designed on the basis of the TCRAV gene sequence of clone AB-1, was 5′-GTGAAGATCCCAAAGCCAAGGAAA-3′. It was used in PCR amplification together with a primer annealing to the TCRAC gene. The TCRAC primer used was 5′-CTTACTTTGTGACACATTTG-3′.

TCR Gene Sequencing. PCR products from V-region transcripts of TIL clones were purified from low-melting temperature agarose gels with the Wizard Prep PCR clean-up kit (Promega). Sequencing reactions were performed by the Sanger dideoxy method (42) using the Sequenase version-2 kit from United States Biochemical, as described by the manufacturer, except that the PCR products were denatured and quick-chilled in a dry ice ethanol bath and briefly stored on ice before commencing the sequencing reaction (43). Sequencing primers (final concentration of 1.2 μM) were designed to obtain sequence information through the TCRBV-n-D-n-J joining region. The TCRBC primer sequence was 5′-TCTGCTTCTGATGGCTCAAACACA-3′ and the TCRBV2 primer was 5′-CTGACCTTGTCCACTCTGACAGT-3′.

Single-Stranded Conformational Polymorphism (SSCP) Analysis. We have adapted SSCP (44, 45) for analysis of the TCR. After the variable region gene usage was identified for each of the

FIG. 1. TCRβ chain gene sequence of clones AB-1, -2, and -3. This sequence is available through the GenBank database (accession number U40815).

clones, it was noted that three of the clones contained TCRBV2-family transcripts. A nested 5′ TCRBV2-specific primer and a nested 3′ TCRBC primer were designed to generate a PCR product of ≈200 bp that spanned the V-n-D-n-J region. The nested primers employed for TCRBV2 analysis were the same as described in the previous section. For the SSCP analysis of TCRBV20, the following primer pair was utilized: TCRBV20 (nested) 5′-TGGCCAGATCAGCTCTGAGGTG-3′ together with a nested TCRBC primer, 5′-GGCCTTTTGGGTGTGG-GAGATCT-3′.

The PCR product (80 μl) was purified with the Wizard Prep PCR clean-up kit. Purified, heat-denatured PCR products were labeled with ATP[γ-^{32}P] by T4 polynucleotide kinase (New England Biolabs), and purified over a Sephadex G-25 column equilibrated in 1 mM Tris·HCl (pH 9.0), 5 mM KCl, and 0.1 mM EDTA. The ^{32}P-labeled PCR product was diluted 1:50 with formamide (20 mM EDTA) to a final formamide concentration of 94%. This mixture was heated for 8 min at 95°C, quick-chilled in a dry-ice ethanol bath, and 5 μl was loaded on a 0.4-mm thick sequencing gel. Electrophoresis was performed through 8% polyacrylamide (19:1, acrylamide:bisacrylamide) in 0.5× TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) containing 10% glycerol. After running for 15 hr at 15 W at 4°C, the gel was fixed with 10% methanol and 10% acetic acid solution for 30 min, dried to paper, and exposed to Kodak X-Omat film overnight with an intensifying screen.

To determine the sequence of the products visualized in the SSCP analysis, discrete bands were cut out of the gel after alignment with the corresponding autoradiogram. Strips of gel were boiled for 1 hr in 100 μl of PCR buffer (10 mM Tris·HCl/50 mM KCl), followed by reamplification with the nested PCR primer pair to generate a PCR product for direct DNA sequencing.

RESULTS

Phenotype and Cytotoxic Activity of the TIL Bulk Cultures and Clones. TILs derived from abdomen and shoulder in IL-2 alone and in IL-2 plus anti-CD3 resulted in predominantly CD8$^+$ populations (CD8$^+$ cells were >80% in all of these cultures, data not shown). In parallel wells, to enrich the CD8$^+$ population further, we grew the TILs in the presence of anti-CD3,4 bispecific mAbs, and this resulted in a population containing >99% CD8$^+$ cells from both abdomen and shoulder. A CD8$^+$ T cell culture was also obtained in a similar fashion from PBMCs and contained 97.8% CD8$^+$ cells.

Both CD8$^+$-enriched TIL bulk cultures, established from the abdominal metastatic lesion (AB-TIL) and from the shoulder metastatic lesion (SH-TIL), had strong cytotoxic activity against autologous melanoma target cells (Table 1). AB-TIL were also tested against six allogeneic melanoma cell lines and showed <5% cytotoxicity against all of these tumor lines. SH-TIL also failed to kill allogeneic melanoma (data not shown). These lines also failed to kill CEM-T2 alone or when this cell line was pulsed with the two tyrosinase peptides. Although we did not assess direct involvement of the TCR in tumor recognition, there was less than 10% lysis of NK (K562) or LAK (Daudi) target cells, indicating that the anti-autologous tumor cytotoxicity was not due to nonspecific activated killer activity.

Clones were derived from AB-TIL and, later, from SH-TIL. The eight AB-TIL clones were tested for their cytotoxic activity against autologous tumors (Table 1). Seven of the eight clones demonstrated cytotoxic activity against autologous melanoma cells but lacked significant cytotoxicity against the K562 and Daudi targets. Clones AB-1, AB-2, and AB-3 showed the strongest anti-autologous cytotoxic activity. It was determined that clones AB-1, -2, and -3, although isolated independently, shared identical TCR gene expression (see below). Similarly, clones AB-4, -5, and -6 also proved to be three independent isolates of another anti-autologous melanoma cytotoxic T cell clone.

Having found multiple copies of anti-melanoma clones within the abdominal tumor cultures, we went back to the CD8$^+$-enriched SH-TIL and performed a limiting dilution cloning on this culture to determine whether the clones found in the AB-TIL were also represented among the SH-TIL. As detailed below, we detected two SH clones with TCR identity to AB-1, -2, and -3.

TCRAV and TCRBV Gene Usage by TIL Clones. TCRAV and TCRBV usage by the eight AB-TIL clones, as determined by PCR, is shown in Table 1. In all clones, a single TCRBV band was visualized in the ethidium bromide-stained gel. Four of the clones expressed only one TCRAV; in the other four clones, two TCRAV were detected.

Three clones (AB-1, -2, and -3), which had strong autologous tumor-specific cytotoxicity, utilized TCRAV1 and TCRBV2 genes. Three other cytotoxic clones (AB-4, -5, and -6) expressed TCRAV8 together with TCRAV16 and TCRBV20. By direct sequencing, we detected the same TCRBV2-nD1n-J1S6 sequence (Fig. 2) on AB-1, -2, and -3 clones, thus confirming that these were three independently isolated, identical clones.

Later, a cloning was obtained from the CD8$^+$ SH-TIL, yielding eight clones (clones SH-1 to SH-8). These clones could not be tested for their autologous tumor-cytotoxic activity because the autologous melanoma cell line was no longer available, but they

Table 1. Cytotoxic functions of CD8$^+$ bulk TIL cultures derived from two metastatic lesions, abdomen (AB) and shoulder (SH), and of eight clones derived from AB-TIL

Effector cells	% lysis of autologous melanoma*	% lysis of K562	% lysis of Daudi	TCRAV usage	TCRBV usage
CD8$^+$ AB-TIL	100	75	45	—	—
CD8$^+$ SH-TIL	82	87	38	—	—
Clone AB-1	100	2	0	TCRAV1	TCRBV2
AB-2	81	1	2	TCRAV1	TCRBV2
AB-3	54	1	0	TCRAV1	TCRBV2
AB-4	31	0	0	TCRAV8 TCRAV16	TCRBV20
AB-5	27	0	2	TCRAV8 TCRAV16	TCRBV20
AB-6	22	1	2	TCRAV8 TCRAV16	TCRBV20
AB-7	29	3	0	TCRAV11 TCRAV16	TCRBV11
AB-8	3	4	9	TCRAV3	TCRBV7

The table shows the TCRAV and TCRBV gene usage by these clones.
*Percent lysis at 50:1 (effector-to-target) ratio.

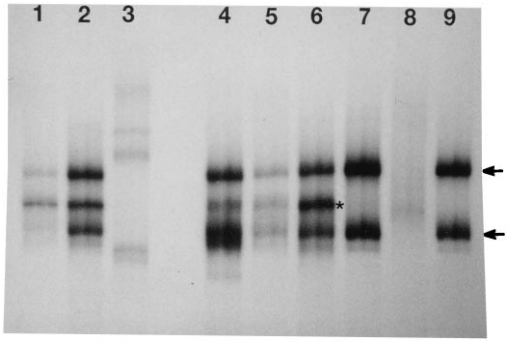

FIG. 2. PCR-amplified TCRBV2 transcripts from different sources were analyzed by SSCP. Lanes: 1, AB-TIL grown with IL-2 alone; 2, AB-TIL separately derived from a tissue fragment and grown with IL-2 and anti-CD3; 3, AB-TIL separately derived from a tissue fragment and grown with anti-CD3,8 bispecific mAbs and enriched for $CD4^+$ cells; 4, freshly frozen tumor tissue from the shoulder metastatic lesion; 5, same as in lane 4 (duplicate conditions from a separate tumor tissue fragment); 6, AB-TIL separately derived from a tissue fragment and grown with anti-CD3,4 bispecific mAbs and highly enriched for $CD8^+$ cells; 7, SH-TIL grown with anti-CD3,4 bispecific mAbs and highly enriched for $CD8^+$ cells; 8, PBLs grown with anti-CD3,4 bispecific mAbs and highly enriched for $CD8^+$ cells; 9, cytotoxic clone AB-1.

were analyzed for their TCR gene usage. Two of these clones (SH-2 and -3) showed the same TCRAV1 and TCRBV2 gene usage, as did autologous cytotoxic clones derived from abdomen (clones AB-1, -2, and -3). The TCRβ chains of clones SH-2 and -3 were sequenced and found to be identical to the TCRβ gene used by AB-1, -2, and -3 clones (Fig. 1).

The TCRα chain of SH-2 and -3 was also shared with the AB-1, -2, and -3 clones because the TCRα of these clones could be amplified with a primer that annealed to the unique TCRAV-n-J joining sequence of these clones (data not shown). None of the eight clones isolated from the SH-TIL corresponded to the TCRAV8, 16 and TCRBV20 noted among the abdominal tumor-derived clones AB-4, -5, and -6.

TCRBV2 Usage by TIL Cultures Derived from PBMCs and Two Different Metastatic Lesions. To determine whether the multiple copies of a clone expressing TCRBV2 was a reflection of an increased frequency of TCRBV2-expressing cells in the TIL cultures and in the PBMCs, we utilized a mAb specific for Vβ2 protein to stain these cultures. We determined that the PBMCs contained 5.4% Vβ2, AB-TIL contained 14.5%, and SH-TIL contained 18.4% Vβ2.

In addition, we compared the TCRBV gene usage repertoire by PCR of PBMCs, AB-TIL, and SH-TIL, which was directed at most of the currently known individual TCRBV gene families. We found that the TIL cultures were somewhat more restricted in their TCRBV repertoire than were PBMCs. Although most TCRBV gene families that could be readily detected in the TILs were also those detected in PBMCs, some TCRBV were reduced in TILs, including TCRBV11, 12, 16, and 23, in both SH- and AB-TIL, and TCRBV 18, 21, 22, and 24, which were also reduced in SH-TIL compared with the $CD8^+$ peripheral blood lymphocyte (PBL). TCRBV2 and TCRBV4 were increased more in SH- and AB-TIL than in the PBL, particularly because TCRBV2 was virtually undetectable among the $CD8^+$ PBL. This confirmed the selective accumulation of Vβ2-expressing cells in the TILs, as shown with the antibody studies. TCRBV2 expression was comparable in both AB-TIL and SH-TIL (data not shown). We analyzed the TCRBV2 gene transcripts from these cultures and the fresh tumor tissue to determine whether the increased TCRBV2 expression was related to a selective accumulation of unique anti-melanoma clones.

PCR-SSCP Analysis of TCRBV Usage in Cytotoxic Clones, Bulk TIL Cultures, and Fresh Tumor Tissue. To assess whether the anti-tumor cytotoxic clones represented a major component of the TCRBV2 usage among TILs, we utilized PCR-SSCP analysis to "fingerprint" the tumor-cytotoxic clones and to evaluate the presence of these clones among the various TIL cultures. Fig. 2 shows the TCRBV2 fingerprint of melanoma TILs, comparing the results from one cytotoxic T cell clone (clone AB-1) with RNA extracted from fresh frozen tumor tissue, vs. various parallel TIL cultures. The cultured TILs included $CD8^+$ AB-TIL and SH-TIL, as well as $CD8^+$ PBMCs of the patient. Parallel but separate cultures of AB-TIL were stimulated with IL-2 alone or with IL-2 and anti-CD3 antibody. Both gave rise to predominantly $CD8^+$ cultures and were also evaluated in PCR-SSCP. $CD4^+$ AB-TIL (enriched to 78% $CD4^+$), which were propagated using bispecific mAb (CD3, 8), were also assessed.

The results of the SSCP showed that the same two bands that corresponded to the TCRβ transcripts from the tumor-specific clone (AB-1, lane 9) could be demonstrated in the fresh tissue extracted from the shoulder tumor (lanes 4 and 5), as well as $CD8^+$ AB-TIL and SH-TIL cultured from the two different biopsy sites. To confirm that the banding pattern in the fresh tumor, bulk TIL cultures, and cytotoxic clones were truly reflective of the same TCR sequence, the corresponding bands from the SSCP were cut out, and the DNA was eluted, reamplified, and sequenced. The sequence from the upper dominant band (upper arrow in Fig. 2) from the SSCP gel matched perfectly with the sequence obtained from the TCRβ transcripts of the identical cytotoxic clones AB-1, -2, and -3, shown in Fig. 1.

In addition to appearing in the two different tumor sites, the same TIL clones were also isolated from multiple culture wells that contained different fragments of the tumor biopsy (parallel cultures with IL-2 only, anti-CD3, and bispecific antibodies), indicating that these clones were probably disseminated within each tumor biopsy, with the caveat that manipulation of the tissue during processing could have seeded the same IL-2-responsive clone into different wells. A third strong band could be visualized in lanes 1, 2, 4, 5, and 6 between the two arrowed bands. This band was also cut out from lane 6, the DNA was eluted and reamplified, and the resulting DNA sequence was identical to the clonal sequence shown in Fig. 1, showing that this extra dominant band reflected double-stranded DNA due to incomplete denaturation or reannealing of the two strands in the SSCP gel.

In contrast, analysis of the PBMCs from the same patient showed predominantly a smear pattern (lane 8), consistent with this patient's peripheral blood $CD8^+$ T cells having highly diverse CDR3s among the TCRBV2 transcripts. The $CD4^+$ AB-TIL culture (lane 3) showed a different banding pattern from the cytotoxic clone or the $CD8^+$ AB-TIL and SH-TIL.

The SSCP pattern of the TCRBV20 (AB-4, -5, and -6 clones) could also be demonstrated as the dominant pattern among the TCRBV20-using lymphocytes from AB-TIL (data not shown). The banding pattern of these three clones was also detected in one of the two pieces of fresh tumor tissue from the shoulder. Both the fresh tissue and the SH-TIL showed additional bands in the TCRBV20 SSCP analysis. Although the SSCP data indicated that these clones were also present within the SH-TIL, none of the eight SH-derived clones shared the TCRBV20, TCRAV8, and TCRAV16, characteristic of clones AB-4, -5, and -6.

DISCUSSION

In the analysis of anti-tumor cytotoxic T cell clones from an individual patient with multiple cutaneous deposits of malignant melanoma, we have demonstrated the predominance of the same tumor-cytotoxic T cell clones in two different anatomical sites. Combining SSCP analyses of DNA from fresh tumor tissues and *in vitro*-propagated TILs, it is apparent that the accumulation of anti-tumor cytotoxic T cell clones within these tumor lesions was a reflection of the *in vivo* selective process. This $CD8^+$ TIL clone, which represented three of eight independently derived clones from a tumor deposit on the skin of the abdomen, also repre-

sented two of eight clones derived from a tumor lesion on the skin of the shoulder. SSCP and direct DNA sequencing demonstrated this clone in the fresh tumor tissue by virtue of its use of a unique TCRBV2-nD1n-J1S6 sequence. This was the dominant clone among the TCRBV2 gene-expressing cells in the $CD8^+$ TILs propagated from both shoulder and abdominal tumor deposits. mAb studies also demonstrated the concentration of T cells expressing surface TCR-Vβ2, providing further quantitative proof of the selection for this phenotype TIL.

The SSCP analysis provides molecular evidence that reflects the presence and relative abundance of these anti-tumor TIL clones in both the abdominal and shoulder tumor nodules, as well as in the bulk TIL cultures from which they were derived. The ability to obtain readable DNA sequence information in the highly variable CDR3 region is a reflection of the homogeneity of that PCR signal. Because we have been able to perform direct sequencing of the TCRβ chain PCR products after elution from the SSCP gel, this provides further evidence that this unique TCRβ chain rearrangement is predominant among the TCRBV2-expressing lymphocytes. By this approach, we have established a direct molecular link between the tumor tissue, *in vitro* propagated bulk TIL cultures, and anti-tumor TIL clones. Another study using SSCP indicated the predominant usage of individual TCRs repopulating a patient who had received a bone marrow transplant (46), but our study extends this finding to demonstrate the functional relationship of a dominant clone to its *in vivo* accumulation. Although obtained in a single patient, these data support the proposition that a tumor-cytotoxic T cell clone could propagate, circulate, and accumulate within the tumor deposit *in vivo*, where it might be expected to inhibit tumor growth.

Unfortunately, we have not been able to establish the antigen specificity of the anti-melanoma response in this patient. The failure of the TIL cultures to lyse a variety of melanoma targets that share HLA-HLA-A2 with the tumor patient suggests that the TILs are unlikely to recognize MART-1/Melan-A, which is said to have a wide distribution among melanomas (18). It is not surprising that the TILs fail to recognize the less widely recognized tyrosinase peptides (19). As several "private" tumor antigens have been demonstrated, it is likely that the tumor-reactive clones we have isolated react with a restricted tumor-associated antigen. The scope of this investigation has not allowed us to complete the search for the fine specificity of the TIL clones, but their accumulation *in vivo* is an indication of the biological significance of these cells in this patient. Because we and others (3, 4, 10) have noted clonal dominance of anti-melanoma cytotoxic TILs in other patients, the phenomenon noted in this patient is likely to be reflective of the status of additional tumor hosts.

The demonstration of the same tumor-specific cytotoxic T cell clone in two different tumor deposits helps to address the lingering concern from several TIL studies that the clonal dominance seen among propagated TILs was the result of culture artifact, rather than a reflection of the true anti-tumor response. This study argues cogently that the dominance of this same clone in the freshly isolated tissue is due to an *in vivo* accumulation of anti-tumor-specific T cells within the tumor. The current study reiterates the points raised by Mackensen *et al.* (10), that tumor-specific cytotoxic T cells can concentrate within melanomas. In addition, the results from our study further emphasize that the same dominant clones can concentrate in two different metastatic deposits, but that this anti-tumor response is not necessarily associated with tumor regression, as we have observed them in progressing tumors. We have also noted selection for tumor-specific clones in a second patient where the anti-tumor response was characterized by accumulation of predominant autologous tumorlytic TILs specific for Melan-A/MART-1 (unpublished data).

The fact that the same anti-tumor-specific clone can accumulate in two different tumor lesions emphasizes additional aspects of the anti-tumor response. First, the T cells we have detected (via a clonal T cell receptor) have been propagated *in vitro* because of their IL-2 responsiveness. This indicates that they expressed IL-2 receptors *in vivo* as an indication of their state of activation. Second, the fact that they have accumulated and even predominate in two different sites indicates that they must have been able to proliferate *in vivo* as well as *in vitro*. Thus, the necessary activation, proliferation, and maturation signals that drive the cytotoxic T cell response against the tumor are present *in vivo*. Although it is not known whether the initial T cell proliferation occurs within the tumor nodules or elsewhere, such as lymphoid tissues, it is clear that a single clone must have been rendered competent to circulate and react to the tumor antigens *in vivo*. The cytotoxic T cell response appears to play a vital role in the rejection of allografts (47) and may even signal impending rejection (48). However, if the "desired" tumor-specific cytotoxic TILs can accumulate within tumors without effecting tumor rejection, one must again emphasize the need for additional factors to achieve tumor rejection.

The failure of the immune response to control the growth of malignancy has been ascribed to a variety of immunological escape mechanisms for the tumor, including lack of immunogenicity due to loss of major histocompatibility complex (MHC) molecules needed for antigen presentation (23, 24), lack of adequate accessory cell function (such as that mediated via B7-costimulatory function; ref. 25), loss or absence of tumor-specific antigens (49–51), or failure to induce appropriate cytokines (52, 53). Tumor models have shown that cytotoxic T cell clones can destroy tumor deposits in the presence of adequate amounts of IL-2 (54). These findings have led to the utilization of both cytokines alone and *in vitro*-propagated TILs and cytokines to treat advanced human malignancy, but these studies showed tumor regression in a small minority of patients (14, 55). If either the activation of TILs by cytokine or the induction of proliferation were the missing elements in the anti-tumor response, these immunological interventions might have been expected to be more successful. The lack of efficacy *in vivo* may also be due to an anergic state that results in decreased TCR-mediated signaling, which can be reversed by *in vitro* culture (56). Thus, the *in vitro* efficacy of the clones we derived might not show a parallel *in vivo* cytotoxicity, even if therapeutic cytokines were present.

Ironically, the clonal nature of the anti-tumor response may facilitate the tumor's escape from immunological destruction as the loss of a single HLA allele, or loss of the recognized peptide could render such clones ineffective. Although it is clear that anti-tumor T cells can be effective in both animal models (54) and humans (10), it is likely that tumor escape includes loss of HLA (23, 24) or tumor gene products (17) as well as ineffective immune recruitment *in vivo* (25, 56). More perplexing is the prolonged latency seen in some melanoma patients, including the 15-year apparent disease-free interval experienced by this patient, in which the host–tumor relationship remains enigmatic. Outgrowth of a "loss mutant" tumor cell that escapes immune recognition could explain part of the rapid progression of tumors once they reappear.

Of course, other cell types, including $CD4^+$ T lymphocytes, can act directly, or via soluble mediators, including both Th1- and Th2-type cytokines to regulate the anti-tumor response (52, 57, 58). A role for $CD4^+$ T cells has been suggested in regressing tumors that contain significant numbers of these cells in concert with up-regulated class II MHC expression on the tumor cells (59). Furthermore, in early melanoma lesions (<0.75 mm), the vast majority of the TILs are $CD4^+$, whereas in more advanced tumors, $CD8^+$ cells often predominate (60), suggesting that $CD8^+$ cells are not sufficient to prevent tumor growth. Thus, loss of effective T cell help could prevent adequate expansion of cytotoxic T cells, allowing tumor progression even in the presence of a specific cellular immune response. Unfortunately, even though these anti-tumor-specific TIL clones may be predominant among the lymphocytic infiltrate, it cannot be concluded that their *in vitro* ability to destroy tumor cells in short-term lysis assays

parallels their *in vivo* efficacy, where some immunoregulatory element may be limiting. Further studies are required to distinguish among the many mechanisms of tumor escape.

Combining SSCP analysis of TCR gene expression with functional analysis of TIL clones has allowed us to demonstrate that the immune response to tumors includes activation, proliferation, and accumulation of anti-tumor-specific cytotoxic T cells. The SSCP analysis of both the fresh tissue and propagated TILs indicates the *in vivo* selection for tumor-reactive T lymphocytes. The ability to fingerprint an individual T cell receptor gene sequence in a heterogeneous lymphocyte population has implications for tracking of individual clonal T cell infiltrates in different tumor deposits and other inflamed tissues, as well as detection of T cell malignancies.

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