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Analysis of T cell receptor β and γ genes from peripheral blood, regional lymph node and tumor-infiltrating lymphocyte clones from melanoma patients*

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Summary. A total of 199 T cell clones from two melanoma patients were derived from progenitor T cells from recurrent melanoma, regional lymph nodes (either involved or uninvolved with malignancy) and peripheral blood by inoculating single cells directly into the wells of microtiter plates before in vitro expansion. The surface marker phenotype of most clones was $CD4^+CD8^-$, although some were $CD4^-CD8^+$. Genomic DNA prepared from all clones was analyzed by Southern blot hybridization using T cell receptor (TCR) β and γ gene probes, seeking clones with identical TCR gene rearrangement patterns as direct evidence for in vivo progenitor T cell clonal amplification. Probing *Hin*dIII-digested DNA with TCRβ and TCRγ probes revealed several clones with identical TCR gene rearrangement patterns. These clones had subsequent probing of *Bam*HI-digested DNA with TCRβ and TCRγ probes, which showed all but 2 clones to have distinct rearrangement patterns. These analyses provide clear molecular evidence for in vivo polyclonal $CD4^+$ T cell populations in each of several separate immune compartments in these patients.

Introduction

Many human tumors have been known for some time to have an associated cellular T lymphocyte infiltration [9]. Functional assays of lymphocytes infiltrating these tumors have not been able consistently to identify populations of T cells with increased tumor reactivitiy [8, 13]. Tumor-reactive lymphocytes, if present, may mediate functions such as cytotoxicity, cytokine release, "helper" activity, "suppressor" activity, or a potential "network" of effects. Determining whether lymphocytes infiltrating tumors and regional lymph nodes represent oligoclonal or polyclonal populations of cells may assist understanding the means by which these cells may recognize tumor.

Molecular mechanisms for T cells recognition and activation by antigen have received intense investigation [3, 10, 11, 15, 18, 19, 21, 29–31, 33, 36]. Specific recognition by T lymphocytes of autologous tumor-associated antigens may be via the classical T cell receptor (TCR) complex (a disulfide-linked α/β heterodimer) and/or by the more recently described γ/δ receptor. Once activated, a T cell will undergo proliferation and clonal expansion to allow production of a large number of T cells with a similar pattern of TCR gene rearrangement. While a population of lymphocytes specifically reactive to a given antigenic stimulus could reflect a single expanded clone, it could also be comprised of multiple separate clones, that share variable-region genes or separate clones that have a similar recognition function mediated by distinct receptors. The relative importance of a clonally amplified T cell population, versus multiple separate tumor-specific or non-specific T-cell clones, has not been critically evaluated for lymphocytes infiltrating tumors and regional lymph nodes of patients with cancer. Similar types of analyses have suggested limited T cell heterogeneity in the immune compartments of nonmalignant diseases including the cerebrospinal fluid of patients with multiple sclerosis (and mice with autoimmune encephalomyelitis), the synovial fluid of patients with arthritis and immune compartments from patients with other diseases with a postulated autoimmune lymphocytic infiltration [1, 6, 7, 16, 22, 28, 35]. This report describes the molecular analysis of 199 T-cell clones expanded from two patients with malignant melanoma. T cell receptor gene rearrangement patterns of these clones demonstrate the heterogeneity of polyclonal $CD4^+$ T cells that are found in separate immune compartments in these patients.

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Materials and methods

Case history 1. A 63-year-old man presented 3 months after excision of a malignant melanoma from the right distal forearm with a recurrent lesion in the area of the primary excision. A wide surgical excision revealed nodular malignant melanoma invasive to Clark's level V with a depth of 11 mm and surgical margins free of malignancy. A right axillary lymph node dissection subsequently revealed 2 of 18 lymph nodes to be positive for malignant melanoma.

Case history 2. A 25-year-old man presented with a left axillary mass 5 years after excision of a Clark's level IV melanoma (1.62 mm) from the left chest wall. A left axillary lymph node dissection revealed 1 (3.5 cm in size) of 23 axillary lymph nodes to be involved with metastatic melanoma.

Cells and culture conditions. Peripheral blood lymphocytes (PBL) were obtained from both patients (PBL1; PBL2) by centrifugation of human peripheral blood samples collected with heparin (beef lung heparin, 10 units/ml whole blood) on Ficoll/Hypaque density gradients at approximately 300 *g* for 30 min. The mononuclear cell fraction was washed with phosphate-buffered saline (PBS) twice and resuspended in modified RPMI-1640 medium (containing 25 mM HEPES, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) with 10% pooled human serum. PBL were then cryopreserved in modified RPMI-1640 medium containing 20% pooled human serum and 8% dimethylsulfoxide for subsequent analysis or incubated for 40 h in modified RPMI-1640 medium with 10% pooled human serum with 0.5 μg/ml phytohemagglutinin (HA-17, Wellcome Diagnostics; Research Triangle Park, N. C.) for mitogenic stimulation. The PBL were then centrifuged, washed and the cell number determined. PBL were obtained from the patient at the time of surgical excision of the recurrent melanoma.

Surgical specimens were obtained from the first patient of the recurrent melanoma tumor (T1), a regional lymph node that was histologically positive for malignancy ($LN1^+$) and a regional lymph node that was histologically negative for malignancy ($LN1^-$). The tumor and lymph nodes were initially rinsed in modified RPMI-1640 medium containing 10% human serum and were then finely minced into less than 1-mm × 1-mm pieces with surgical iris scissors. The mechanically disaggregated specimens were incubated in vitro for 40 h in modified RPMI-1640 medium containing 10% pooled human serum and 20% nutrient medium HL-1 (Ventrex Laboratories, Portland, Me.) with intermittent agitation of the suspension during the incubation. One half of the tumor sample was also incubated with 100 units/ml recombinant interleukin-2 (Hoffmann-LaRoche, Nutley, N. J.). Each specimen was then filtered with a 53-μm filter to separate mononuclear cells from surrounding tissue fragments. The mononuclear cells were then centrifuged and the cell number determined. A portion of each sample was cryopreserved as above for subsequent analyses. Lymphocyte cloning plates were established both from fresh lymphocyte populations and from previously cryopreserved samples after washing cells twice in modified RPMI-1640 medium and resting them in this medium overnight before plating. A surgical specimen from the second patient of a lymph node involved with melanoma ($LN2^+$) was handled with the same procedure.

Lymphokine-activated killer (LAK) cell supernatant production. LAK cell supernatant was prepared during the in vitro generation of LAK cells. Briefly, PBL were cultured at 3×10^6 cells/ml in modified RPMI-1640 medium containing 2.5% human serum and 1000 units/ml recombinant interleukin-2 (Hoffmann-La Roche) for 48–72 h. The lymphocytes were then separated from the LAK cell supernatant utilizing a Fenwall CS 3000 Apheresis centrifuge. The LAK cell supernatant was frozen and stored for subsequent use. The LAK cell supernatant was filtered with a 0.2-μm Nalgene filter prior to use for lymphocyte culture.

Lymphocyte cloning conditions. The method described here is based on a few modifications from the cloning procedure described in a previous report [27]. Lymphocytes obtained from PBL1, T1, $LN1^+$, $LN1^-$, PBL2 and $LN2^+$ were handled in an identical manner. Lymphocytes were plated by limiting dilution into 96-well microtiter plates to establish cloning plates using up to four lymphocytes per well. Growth medium consisted of modified RPMI-1640 medium containing 25% LAK cell supernatant, 20% nutrient medium HL-1, 10% pooled human serum and 0.25 μg/ml phytohemagglutinin. Accessory feeder cells used are a derivative of WI-L2 lymphoblastoid cells designated TK6, obtained initially from W. G. Thilly (Massachusetts Institute of Technology, Cambridge, Mass.) and grown in modified RPMI-1640 medium containing 10% pooled human serum. The irradiated TK6 cells (10×10^3 cGy) were plated at an initial cell density of 5×10^3 cells/microtiter well. An inverted phase-contrast microscope was used on day 14 of incubation to score wells for visible colony growth. The lymphocyte clones with visible growth were sequentially fed with growth medium containing 2.5×10^5 irradiated TK6 cells/ml, split and advanced into larger wells every 3–4 days and maintained in culture until a final lymphocyte count of $(5-30) \times 10^6$ cells/clone was obtained. Lymphocytes were then cryopreserved to allow for both molecular and phenotypic analysis of each expanded clone.

Molecular analysis. Molecular analysis consisted of initial Southern blot analysis for the TCRβ and TCRγ gene rearrangements after *Hin*dIII restriction endonuclease digestion, essentially as described in a prior report [24]. Clones that had identical gene rearrangements after this analysis had their DNA digested with an additional restriction endonuclease (*Bam*HI) to allow for additional Southern blot analysis for the TCRβ and TCRγ gene rearrangement patterns. Briefly, $(3-15) \times 10^6$ frozen cells for each clone were thawed, washed with PBS and resuspended in 1 ml TRIS/EDTA (10 mM TRIS, 1 mM EDTA, ph 8.0). Then 2.5 ml TENS [25 mM TRIS/HC1 pH 8, 100 mM NaCl, 10 mM EDTA, 0.6% sodium dodecyl sulfate (SDS)] was added and the solution was heated to 65° C for 15 min and then cooled. Proteinase K (1.5 mg) in 100 μl TENS was added and the mixture was incubated at 37° C overnight. An additional 1.0 mg proteinase K in 50 μl TENS was then added and the digestion continued for 2 h. The resulting solution was phenol-extracted twice; this was followed by three chloroform/isoamyl alcohol (24 : 1) extractions and the solution was precipitated in ethanol and resuspended in TRIS/EDTA. For the Southern blots; approximately 7.5 μg/lane genomic DNA was digested with restriction enzymes (initially *Hin*dIII for all clones and then with *Bam*HI for some clones), fractionated on a 0.7% agarose gel in 40 mM TRIS/acetate, 2 mM EDTA buffer and transferred to nitrocellulose filters (Schleicher and Schuell, Keene, N. H.). Prehybridization was for 4–6 h at 42° C in 40 ml 50% formamide, fivefold concentrated sodium chloride/sodium citrate (SSC), fivefold concentrated Denhardt's solution and 50 mM TRIS pH 7.5, with 250 μg/ml sheared and denatured salmon sperm DNA. Hybridization was overnight at 42° C in 20 ml 50% formamide, fivefold concentrated SSC, single-strength Denhardt's, 20 mM TRIS pH 7.5 and 10% dextran sulfate, with 250 mg/ml sheared and denatured salmon sperm DNA and $(1.0-1.5) \times 10^6$ cpm/ml oligonucleotide-labeled probe.

The TCRβ probe used was the cDNA insert from Jurkat 2 obtained from Dr. T. W. Mak. It contains nucleotides 100–870 cloned into the *Pst*I site of pBR322 [37]. The TCRγ probe used was obtained from Dr. T. H. Rabbits and is the 700-bp *Hin*dIII-*Eco*RI insert of pH60, a genomic clone containing Jγ1 [17].

After hybridization, washing of the TCRβ and TCRγ blots consisted of three 5-min washes in twofold concentrated SSC, 0.2% SDS, and four 30-min washes in twofold concentrated SSC, 0.2% SDS at 65° C. Autoradiography was performed at –80° C with Kodak XAR-5 film for 3–5 days. For re-use of filters, the probe was removed from the nitrocellulose by two 15-min washes in distilled water at 65° C. The filter was then placed in 40 ml prehybridization solution and left in the refrigerator until ready for additional hybridization.

Phenotypic analysis. Phenotypic analysis consisted of flow cytometry evaluation for surface marker expression of CD3 (T cell), CD4 (helper T cell), CD8 (cytotoxic T cell), CD19 (B cell), CD56 (NK cell) and the T cell α/β receptor (WT31), all using monoclonal antibodies obtained from Becton Dickinson, Mountain View, Calif. Briefly, cryopreserved cells were initially washed twice in PBS. Cells were then resuspended in PBS at 5×10^6 cells/ml, and 100 μl was utilized for each marker. A 20-μl sample of fluorescin-isothioyanate-conjugated antibody was added, vortexed and incubated on ice for 30 min. The sample was then washed twice in cold PBS and resuspended in 0.5 ml cold PBS for subsequent fluorescent-activated cell sorter (FACS) analysis. For the unconjugated antibody, 20 μl antibody was added to 100 μl cells, vortexed and incubat-

Table 1. Phenotypic characterization of bulk lymphocyte populations[a]

Patient	Lymphocyte origin	Cells expressing surface markers (%)				
		CD3	CD4	CD8	CD56	CD19
1	PBL	75	35	50	11	NT
	LN^+	70	40	30	2	NT
	LN^-	61	30	22	2	NT
	Tumor	85	27	76	6	NT
2	PBL	72	45	17	12	6
	LN^+	66	50	15	8	27

[a] Flow cytometry evaluation for surface marker expression of CD3, CD4, CD8, CD56, and CD19 was performed after separation of lymphocytes from the peripheral blood (PBL), from lymph nodes either involved (LN^+) or uninvolved (LN^-) with malignancy and from the tumor infiltrate (Tumor) of melanoma patients. The bulk lymphocyte populations from each source were analyzed prior to in vitro plating of lymphocytes to generate T cell clones. NT, not tested

ed on ice for 30 min. The sample was then washed twice in cold PBS. A sample of 20 µl goat anti-(mouse Ig) (fluorescein conjugate) was then added, vortexed and incubated on ice for 30 min. The sample was then washed twice in cold PBS and resuspended in 0.5 ml cold PBS for subsequent FACS analysis.

Results

Phenotype of bulk lymphocyte populations

Phenotypic markers of bulk lymphocyte populations were obtained before plating of lymphocytes. Table 1 compares the bulk lymphocyte populations that were derived from different sources in the patients with malignant melanoma. Lymphocytes from all sources were predominantly $CD3^+$. Lymphocytes from the tumor were predominantly $CD8^+$, while lymphocytes obtained from the lymph nodes showed more $CD4^+$ than $CD8^+$ cells. The PBL from patient 1 exhibited a surprisingly high proportion of $CD8^+$ cells. This surface marker analysis was repeated with reproducible results (i.e. CD3 = 90%, CD4 = 37%, CD8 = 46%).

Direct cloning of lymphocytes

From the first patient 98 single-cell-derived T cell clones were expanded. Sources for these clones were lymphocytes within the recurrent melanoma tumor (43 clones), lymphocytes in a regional lymph node histologically positive for malignancy (24 clones), lymphocytes in a regional lymph node histologically negative for malignancy (22 clones) and peripheral blood lymphocytes (9 clones). Cloning efficiencies at 1 cell/well from each source were 7.0% (±3.0%). In addition, 6 "clones" were expanded that probably were expanded TK6 feeder cells as they were $CD3^-$ $CD4^-$ $CD8^-$ $CD56^-$ and lacked a polymorphic fragment of DNA after *Bam*HI digestion characteristic of all other examined T cell clones from this patient. These TK6 "clones" are therefore not included in our analysis of T cell clones from this patient.

From the second melanoma patient 101 single-cell-derived T cell clones were expanded from an axillary lymph node involved with melanoma (55 clones) and from peripheral blood lymphocytes (46 clones). Cloning efficiency at 1 cell/well was 37.6% for the lymph node lymphocytes and 41.3% for the peripheral blood lymphocytes. The actual cloning efficiency of T cells was even higher than the above values because of B cells (CD19 monoclonal antibody, Becton Dickinson, Mountain View, Calif.) present in both the lymph node lymphocytes (27.1%) and peripheral blood lymphocytes (5.6%) prior to generating T cell clones.

Table 2. Phenotype of expanded T lymphocyte clones[a]

Origin of clones	T lymphocyte clones evaluated	$CD4^+$ $CD8^-$ $CD56^-$	$CD4^-$ $CD8^+$ $CD56^-$	$CD4^+$ $CD8^+$ $CD56^-$	$CD4^-$ $CD8^+$ $CD56^+$
PBL	9	5	0	1	3
LN^+	17	15	1	1	0
LN^-	13	13	0	0	0
Tumor	23	17	4	1	1
Total clones	62	50	5	3	4

[a] Flow cytometry evaluation for surface marker expression of CD4, CD8 and CD56 was performed on 62 of the expanded T cell clones of melanoma patient 1. Progenitor T cells for these single-cell-derived T cell clones included lymphocytes from the peripheral blood (PBL), positive lymph node (LN^+), negative lymph node (LN^-) and melanoma tumor infiltrate (Tumor). Lymphocyte cloning was performed after separation of lymphocytes from each source and prior to in vitro expansion of the bulk lymphocyte populations. Of these 62 clones, 44 were also evaluated for expression of CD3 and all 44 were positive. All 18 clones not evaluated for CD3 expression had molecular evidence for rearranged patterns for both β and γ T cell receptor genes thus implying CD3 expression to be likely

Table 3. Expression of α/β molecules of the T cell receptor on the surface of expanded T lymphocyte clones[a]

Origin of clones	T lymphocyte clones evaluated	α/β expression
PBL	8	7[b]
LN^+	6	6
LN^-	10	9[c]
Tumor	9	9
Total clones	33	31

[a] Flow cytometry evaluation for surface marker expression of the α/β T cell receptor was performed for 33 of the expanded T cell clones from the peripheral blood (PBL), from lymph nodes either involved (LN^+) or uninvolved (LN^-) with malignancy and from the tumor infiltrate (Tumor) of melanoma patient 1. Additional evaluation of α/β-receptor-negative clones with a γ/δ monoclonal antibody would be of interest in subsequent patients, but was not performed in this patient

[b] The negative clone is $CD3^+$ $CD4^-$ $CD8^+$ $CD56^+$

[c] The negative clone is $CD3^+$ $CD4^+$ $CD8^-$ $CD56^-$

Phenotypic analysis of expanded lymphocyte clones

Table 2 characterizes the phenotype of 62 of the expanded T cell clones from patient 1, which had phenotypic analysis for CD3, CD4, CD8 and CD56 surface marker expression. Preferential expansion of $CD4^+$ clones can be seen, introducing some selection bias for $CD4^+$ clones. This is similar to our prior experience utilizing this cloning technique [2]. $CD8^+$ clones were infrequently expanded from the lymph nodes. No $CD4^+$ clone was seen to co-express the CD56 surface marker, while 4 $CD8^+$ clones also co-ex-

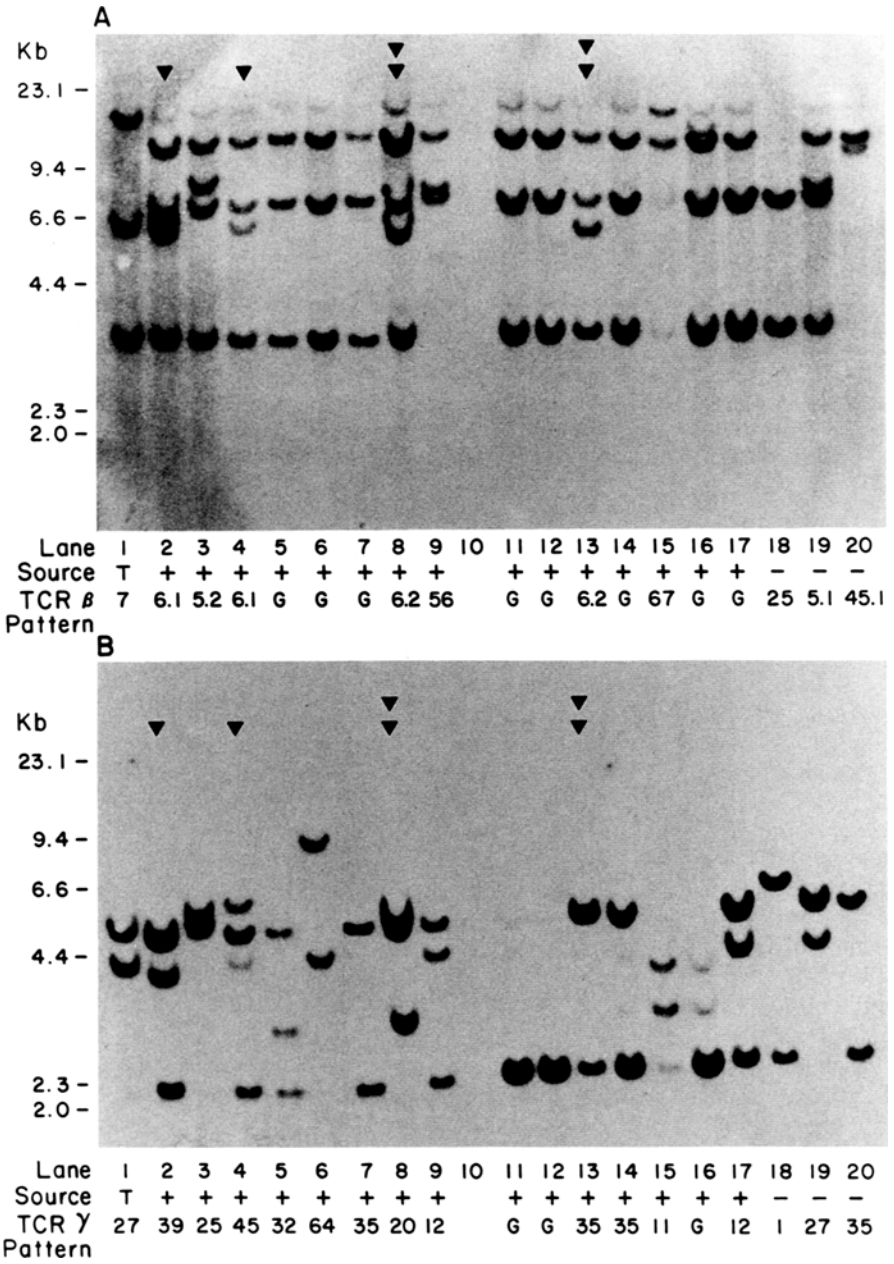

Fig. 1 A, B. Southern blot analysis for the TCRβ and TCRγ gene rearrangements after *Hin*dIII restriction endonuclease digestion. Shown are representative clones from the melanoma infiltrate (*T*), positive lymph node (+) and negative lymph node (–) of melanoma patient 1. Each clone was analyzed for both β and γ TCR patterns; clones with germ line patterns are indicated (*G*), and each rearranged pattern is given a distinct number (i.e., 7 in lane 1 for TCRβ) and compared to the TCR pattern obtained from all of the clones from the same patient to determine uniqueness. **A** TCRβ patterns for 19 of the clones from patient 1 are shown(*lanes 1–9 and 11–20*). Similar *arrows* identify clones with identical rearranged TCR-β patterns (i.e., *single arrow* for pattern 6.1, and *double arrow* for pattern (6.2). **B** TCRγ patterns for the same 19 clones, in the same order, as in panel **A**. *Arrows* designate the different TCR-γ patterns that correspond to clones identified as sharing a rearranged TCRβ pattern in **A**

pressed the CD56 surface marker. Table 3 shows that 31 of 33 clones evaluated were positive for expression of the α/β molecule of the T cell receptor (Becton Dickinson WT31 monoclonal antibody). Additional evaluation of the WT31-negative clones with a γ/δ monoclonal antibody was not performed but will be of interest in subsequent patients.

Southern blot analysis of expanded lymphocyte clones

Southern blot analysis of *Hin*dIII-digested DNA from each T cell clone revealed that most clones showed rearranged fragments with both TCRβ and TCRγ probes (Fig. 1). Similar arrows designate clones that all exhibited identical TCRβ rearrangement patterns; the corresponding TCRγ patterns were, however, different for these clones. Altogether 21 clones from the first patient (6 "pairs", 1 "triplet"; 1 "sextuplet") and 24 clones from the second patient (6 "pairs"; 1 "triplet"; 1 "quadruplet"; 1 "quintuplet") had evidence for sharing TCR gene rearrangement patterns within an individual after *Hin*dIII digestion and were further analyzed after *Bam*HI digestion. Table 4 demonstrates that all but two of the clones sharing TCR gene rearrangement patterns in the initial analyses after *Hin*dIII digestion were distinguished from one another and shown to contain distinct rearrangement patterns for their TCRβ and γ genes. One clone from the first patient and two clones from the second patient had no DNA available for *Bam*HI digestion. The one identical pair appeared to have germline TCR patterns on all blots except for a faint new fragment on a *Bam*HI TCRβ blot. Thus, 95 of the 97 independent clones evaluated from patient 1 and 99 of 99 independent clones from patient 2 showed distinct TCR gene rearrangement patterns after two different restriction endonuclease digestions and analyses with both TCRβ and TCRγ probes.

Table 4. Frequency of β and γ gene patterns of the T cell receptor after digestion with *Hin*dIII and *Bam*HI[a]

Patient	Origin of clones	Total clones	Pattern of β and γ gene rearrangements	
			Clones sharing a particular pattern	Clones expressing a unique pattern
1	PBL	9	0	9
	LN^+	24	2	21(1)[b]
	LN^-	22	0	22
	Tumor	43	0	43
2	PBL	46	0	45(1)[b]
	LN^+	55	0	54(1)[b]

[a] Southern blot analysis for the TCRβ and TCRγ gene rearrangements after both *Hin*dIII and *Bam*-HI restriction endonuclease digestion was performed on DNA from expanded single-cell-derived T cell clones. Progenitor T cells for these clones were obtained from the peripheral blood (PBL), a positive lymph node (LN^+), a negative lymph node (LN^-) and the melanoma tumor infiltrate (Tumor) of two patients with recurrent melanoma. T cell clones were evaluated for possible sharing of a particular pattern of β and γ T cell receptor gene rearrangement with any other clone from any of the groups of clones from the same patient

[b] Three clones, which each shared a pattern with one additional clone after *Hin*dIII digestion, were unable to have a second restriction digestion with *Bam*HI because of insufficient remaining DNA, thus a complete analysis of these three clones was not possible. All but two of the remaining clones that initially shared a particular pattern for β and γ gene rearrangement after *Hin*dIII digestion were shown to be different and to consist of clones with distinct rearrangement patterns for their T cell receptor after additional digestion with *Bam*HI

Discussion

T-lymphocyte clones can be reliably established and successfully expanded from several tissue sources including tumor, lymph nodes and peripheral blood. However, current culture conditions preferentially expand CD4-positive lymphocytes, as has been noted for clonal expansion of T lymphocytes infiltrating other human solid tumors [20]. The reason for the surprisingly high proportion of $CD8^+$ cells in the PBL from patient 1 is not clear. Surface marker analysis of PBL from patient 2 reported in this manuscript exhibited the more typical pattern with a higher percentage of $CD4^+$ cells than $CD8^+$ cells. The T cell clones described here were generated from several different immune compartments with different proportions of $CD4^+$ cells and $CD8^+$ cells.

While recent studies have documented monoclonal or oligoclonal in vivo T lymphocyte responses for some human diseases [1, 6, 7, 16, 22, 28, 35], this has not been reported for tumor-infiltrating lymphocytes. Some studies have presented functional data suggesting that a tumor-specific T lymphocyte response for some patients with melanoma can be generated in vitro [12, 13, 23]. Recent molecular data have shown oligoclonal T cell responses by bulk populations of tumor-infiltrating lymphocytes from human renal cell carcinoma expanded with IL-2 in vitro [5]. It remains uncertain whether an antigen-specific T cell response occurs in vivo for melanoma or whether the documented tumor specificity seen in vitro may reflect stimulation seen only during in vitro lymphocyte cultivation with tumor cells. Direct in vitro cloning of T lymphocytes following in vivo harvesting, with subsequent analysis of the patterns of TCRβ and TCRγ gene rearrangements, can serve as a useful means to identify particular T cell clones that were expanded in vivo.

Analysis of 199 T cell clones from separate immune compartments in these two patients revealed that all but 2 clones that were analyzed with two restriction enzymes and two TCR probes had originated from unique precursors. This is similar to our recent analysis of 94 T cell clones generated directly from the peripheral blood of three normal individuals, in which we found all clones evaluated within an individual to have a distinct pattern of β and γ gene rearrangement after analysis with two restriction enzymes and two TCR probes [25].

This analysis demonstrates a heterogeneous $CD4^+$ T lymphocyte population in the tumor and regional lymph nodes of these melanoma patients. The heterogeneity of these T cell clones could reflect at least four spearate possibilities. First, this could reflect an immune infiltrate of lymphocytes within the tumor and regional lymph nodes that do not recognize a specific antigen on the tumor and represent a non-specific collection of cells able to recognize a variety of antigens. Second, some distinct T cell clones might recognize a variety of tumor-specific antigens [4, 14], or antigens restricted by a variety of major histocompatibility complex restricting element epitopes. Third, independently derived T cell clones may share variable-region genes, which would have not been apparent with this current level of molecular analysis. While the analysis presented here could have detected in vivo amplification of an individual T cell clone, it would have missed a possible amplification of several different T cell clones that share relevant variable-region genes. Finally, homogeneous clones might have been present in the tumor or nodes, but not analyzed because they did not grow out in the cloning process. The 7% cloning efficiency for patient 1 and the preponderance of $CD4^+$ clones obtained would be consistent with this last possibility. However, the much higher cloning efficiencies obtained with the second patient, who also had a heterogeneous T lymphocyte response, makes this possibility less likely. It is also possible that relevant antitumor specificity resided in the $CD8^+$ T cell population, which is not expanded by this cloning technique.

We are continuing these studies to determine whether additional patients with melanoma have similar T cell heterogeneity in their tumor and regional lymph node populations. It will be important to examine whether distinct patterns of T cell receptor variable-region gene usage can be identified in melanoma-infiltrating T-cell clones [26]. Shared patterns of T cell receptor variable-region genes, if present, would also provide indirect evidence for possible "melanoma-specific" antigens. Functional analysis for specific immune reactivity by these T cell clones, utilizing fresh autologous tumor cells or an autologous tumor cell line, will be of great interest.

The molecular data from these 199 T-cell clones provide a clear demonstration that, for at least some patients with melanoma, the in vivo distribution of T cells in the tumor and related lymph nodes reflects a polyclonal population of $CD4^+$ T cells. This type of analysis can be applied to other patients with melanoma, as well as patients with other malignancies, to determine if a polyclonal in vivo T lymphocyte response occurs in most human malignan-

cies. Functional analyses of these clones will help determine the relative importance of antigen-specific versus nonspecific T cell immune recognition in the immune response to cancer. While antitumor responses are seen in some patients following therapy with bulk populations of tumor-infiltrating lymphocytes together with interleukin-2 infusions, further improvements are needed in order to prolong survival substantially for the majority of patients with metastatic malignant melanoma [32, 34]. It is hoped that further analyses of the in vivo immune response to autologous malignant melanoma will assist in the planning of new therapeutic strategies for these patients.

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