JCI The Journal of Clinical Investigation

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J Clin Invest. 1996;97(2):515-521. https://doi.org/10.1172/JCI118443.

Research Article

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In Vivo Trafficking of Adoptively Transferred Interleukin-2 Expanded Tumor-infiltrating Lymphocytes and Peripheral Blood Lymphocytes

Results of a Double Gene Marking Trial

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Abstract

Adoptive immunotherapy with tumor-infiltrating lymphocytes (TIL) and IL-2 appears to produce dramatic regressions in patients with metastatic melanoma and renal cancer. However, the in vivo mechanism of TIL function is not known. We conducted an UCLA Human Subject Protection Committee, Recombinant DNA Advisory Committee, and FDA–approved clinical trial using genetically-marked TIL to test the hypothesis that these cells have unique, tumor-specific in vivo trafficking patterns. TIL and PBL (as a control effector cell population) were isolated and expanded in parallel in vitro in IL-2–containing medium for 4–6 wk. During the expansion, TIL and PBL were separately transduced with the amphotropic retroviral vectors LNL6 and G1Na. Transduced TIL and PBL were coinfused into patients and their respective numbers measured in tumor, peripheral blood, and normal tissues; integrated provirus could be quantitated and distinguished by DNA PCR.

Nine patients were treated (six melanoma, three renal) and received between 4.5×10^8 and 1.24×10^{10} total cells. Both "marked" TIL and PBL could be detected circulating in the peripheral blood, in some patients for up to 99 d after infusion. Marked TIL and/or PBL could be detected in tumor biopsies in six of nine patients as early as day 6 and as late as day 99 after infusion. No convincing pattern of preferential trafficking of TIL vs. PBL to tumor was noted. Moreover, concurrent biopsies of muscle, fat, and skin demonstrated the presence of TIL/PBL in comparable or greater numbers than in tumor in five patients.

The results of this double gene marking trial provide interesting insights into the life span and trafficking of adoptively transferred lymphocytes, but do not support the hypothesis that TIL specifically traffic to tumor deposits. (*J. Clin. Invest.* 1996. 97:515–521.) Key words: melanoma • renal carcinoma • cancer • retrovirus • neomycin resistance

This work was presented in part at the 48th Cancer Symposium of the Society of Surgical Oncology, Boston, MA on 23–26 March, 1995.

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Received for publication 8 June 1995 and accepted in revised form 11 October 1995.

J. Clin. Invest.

0021-9738/96/01/515/07 $2.00
Volume 97, Number 2, January 1996, 515–521

Introduction

Tumor-infiltrating lymphocytes (TIL)[1] are lymphocytes found within solid tumors. Although functionally suppressed in vivo, cultured populations often are enriched with activated T cells and demonstrate cytotoxicity for autologous tumor cells (1–12). Clinical trials involving the adoptive transfer of TIL supported with IL-2 have demonstrated regression of metastatic disease in a proportion of patients with renal cancer and melanoma (13–17).

The mechanism by which human TIL may contribute to an antitumor response has not been established. Most of the proposed mechanisms of TIL action, direct cytotoxicity or elaboration of inflammatory cytokines, would seem to require preferential enrichment at the tumor site. Several clinical trials have attempted to address the issue of human TIL trafficking in vivo. These trials have employed indium-111 labeling or genetic marking with retroviral vectors (18–22). While these clinical investigations provided very important insights into TIL lifespan and trafficking, their inherent design could not distinguish whether TIL detected in tumor arrived there randomly or preferentially after adoptive transfer. To address this issue, we conducted a TIL marking trial that allowed the direct in vivo comparison of renal or melanoma TIL with a putative "control" effector cell population, peripheral blood lymphocytes. We selected this control since it has been demonstrated that IL-2-expanded PBL (lymphokine-activated killer cells) do not contribute to IL-2–based cancer immunotherapy (23). If TIL do, in fact, specifically accumulate in tumors, one would expect a preferential enrichment of TIL versus PBL only at tumor sites, but not in normal tissues (muscle, fat, skin, blood). A number of investigators demonstrated the ability to genetically "mark" TIL and PBL with retroviral vectors containing the neomycin phosphotransferase (*neoR*) gene (21, 22, 24, 25). Two of these *neoR* vectors, LNL6 and G1Na, have been approved for clinical use and minor differences in their polylinkers allow the respective proviruses to be distinguished by DNA PCR (25–27). In a series of preclinical studies, we demonstrated the feasibility of transducing IL-2–expanded TIL and PBL with each of these vectors, to detect a single *neoR* gene copy in 10^5 cells by PCR, and the ability to quantitate provirus copy number in tissue samples (25). This preclinical data led to an UCLA Human Subject Protection Committee, Recombinant DNA Advisory Committee, and FDA–approved double-marking trial conducted at this institution. We report

1. *Abbreviations used in this paper: neoR*, neomycin phosphotransferase; NK, natural killer; TIL, tumor-infiltrating lymphocytes.

the results of this trial in which evidence for preferential TIL trafficking in vivo was not observed.

Methods

Clinical protocol. This double-gene marking TIL/PBL protocol was approved by the UCLA Human Subject Protection Committee (HSPC No. 91-10-442) which exerted continual oversight throughout the conduct of the trial. The protocol was also approved by the UCLA Recombinant DNA Committee, the National Institutes of Health (NIH) Human Gene Therapy Subcommittee, and the NIH Recombinant DNA Advisory Committee, and received an IND (Investigational New Drug) (#BB-IND-3286) from the Food and Drug Administration.

The trial was initiated in January 1993 and closed in April 1994. Nine patients (six melanoma, three renal carcinoma) were entered and underwent a thorough informed consent process. The complete protocol has been previously published (28). In brief, TIL and PBL from patients with metastatic, refractory melanoma or renal cancer were isolated and expanded in parallel in IL-2–containing medium. During the log phase of cell expansion, aliquots of cells were transduced with LNL6 or G1Na, Moloney murine leukemia virus–based retroviral vectors which contain the *neoR* gene (generously provided by Genetic Therapy, Inc., Gaithersburg, MD) (25–27). Transduced and untransduced TIL and PBL were pooled and infused into patients who were supported with IL-2 (melanoma) or IL-2/IFN-α2A (renal).

Transduced cells were shown to have integrated provirus and to be free of replication-competent retrovirus by the S+/L− method (conducted at Genetic Therapy, Inc.) (29). After adoptive transfer, serial blood, tumor, and normal tissue (muscle, skin, fat) biopsies were obtained and analyzed by quantitative DNA PCR for both LNL6 and G1Na provirus. These values were compared with the relative ratios of these proviruses in the pooled infusion cells (25). TIL/PBL phenotype, cytotoxicity profile, clinical toxicity, and clinical response were measured as described in the protocol (28).

Cell and tissue manipulations. TIL were isolated from the primary renal carcinoma obtained at the time of radical nephrectomy or from metastatic melanoma tumor biopsies, by overnight digestion in RPMI 1640 medium (Biofluids Inc., Rockville, MD) containing 0.1% collagenase type IV (Sigma Chemical Co., St. Louis, MO), 0.01% hyaluronidase type V (Sigma), 0.002% DNase type I (Sigma), and 50 μg/ml gentamicin (Biofluids), washed, and expanded in serum-free AIM-V medium (GIBCO BRL, Chagrin Falls, OH) supplemented with 400 IU/ml IL-2 (Hoffman-LaRoche, Nutley, NJ) at 37°C in 5% CO_2 as previously described (11, 25, 30). PBL were isolated from heparinized whole blood after centrifugation over Ficoll-Hypaque (LSM; Litten Bionetics, Charleston, SC) (31) and cultured in an identical manner as with TIL. PBL cultures were generally initiated 1 wk after TIL culture to optimize cell yield. During the log phase of cell expansion, generally 16 d after initiation of culture, aliquots of 5×10^7 to 10^8 cells were transduced with LNL6 or G1Na (titer range 4×10^5 to 1×10^7 cfu/ml) during a single 8-h supernatant infection at an moi of 2–6 in the presence of 10 μg/ml protamine sulfate (Elkins-Sinn, Inc., Cherry Hill, NJ). Cells washed multiple times were further expanded in AIM-V/IL-2.

A protocol requirement recommended by the Recombinant DNA Advisory Committee and FDA was that only a portion of TIL be retrovirally transduced so that a possible contamination would not prevent the patient from receiving (untransduced) TIL treatment. Transduction efficiencies ranged from < 1 to 7% determined by DNA PCR (described below). Expansion indices for untransduced TIL (range 12–74×, mean 37×) generally exceeded transduced TIL (range 1.44–9.5×, mean 3.4×) and transduced PBL (range 1.3–8.3×, mean 5.5×). Such differences in expansion indices between transduced and untransduced lymphocytes have been described (25). The overall viability of administered cells generally averaged > 70%: untransduced TIL (range 48–89%, mean 78%), transduced TIL (range 42–89%, mean 72%), and PBL (range 40–91%, mean 67%). Successful proviral integration, as determined by DNA PCR, was a requirement for the inclusion of transduced cells in the infusion pool. Patients received pooled cells (untransduced TIL, transduced TIL, transduced PBL); an aliquot of this infusion pool was used as a PCR standard to compare the infused LNL6/GINa ratio with those ratios found in tumor biopsies, blood, and normal tissue. The percentage of transduced TIL and PBL in this infusion pool are given in Table II. With the exceptions of patients 1.1 and 1.6, the remaining seven patients received transduced TIL and PBL, and LNL6/GINa ratios could be measured in all instances by DNA PCR. After the adoptive transfer of cells, PBL, tumor biopsies, and normal tissue samples (fat, skin, muscle) were obtained at intervals and subjected to DNA PCR analysis for LNL6 and GINa provirus. These samples were obtained by the principal investigator and processed with multiple safeguards to prevent cross-contamination. As a Recombinant DNA Advisory Committee and FDA requirement, these biopsies were obtained from superficial sites under local anesthesia through the same incision used for tumor biopsy.

Some postinfusion blood leukocyte samples and tumor biopsies (enzymatically digested into single cell suspensions) were cultured in AIM-V/IL-2 medium. Aliquots of these cultures were either enriched for CD8 cells using CELLector CD8 T150 flasks (Applied Immune Sciences, Menlo Park, CA) (32) or selected for transduced cells using the neomycin analogue, G418 (GIBCO), at concentrations of 100–400 μg/ml in AIM-V/IL-2 medium.

Patient treatment. Patients received an infusion of pooled transduced and untransduced TIL/PBL in the UCLA General Clinical Research Center. Recombinant IL-2 (Hoffman-LaRoche) was administered by continuous intravenous infusion at a dose of 2×10^6 IU/M^2 per d for 4 d/wk on weeks 2, 3, and 4. Renal cancer patients in addition received intravenous IFN-α2A (Hoffman-LaRoche) at a dose of 6×10^6 U/M^2 per d on days 1 and 4 on weeks 1, 2, 3, and 4. A single treatment cycle consisted of 4 consecutive wk of IL-2 therapy followed by 2 rest wk off all therapy. Patients were restaged after each cycle of therapy. Side effects of the cellular and biological therapy were managed as previously described (28).

DNA PCR. The DNA from blood, tumor, and normal tissue specimens was isolated by 16 h cell lysis at 55°C in DNA isolation buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% SDS, 25 mM EDTA, and 200 μg/ml proteinase K [Sigma]) followed by 25:24:1 phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation (33). A 0.5-μg aliquot of DNA was amplified by PCR in a total vol of 50 μl which contained 0.2 mM of each deoxynucleotide triphosphate, 1 μM of 5′- and 3′-oligonucleotide primers (DK250 and DK251 for LNL6; DK249 and DK248 for G1Na), 50 mM KCl, 5 mM Tris Cl, pH 8.3, 1.5 mM $MgCl_2$, and 2.5 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CN) on a DNA thermal cycler (Perkin-Elmer) (25). For quantitative "hot" PCR analysis, one of each pair of primers (DK251 for LNL6, DK249 for G1Na) was end labeled with [^{32}P]ATP (ICN Radiochemicals, Costa Mesa, CA) to a sp act of > 10^6 dpm/ng of DNA. The thermal cycle profile consisted of 1 cycle of 94°C for 5 min, 55°C for 2 min, and 72°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, and 1 cycle of 94°C for 1 min, 55°C for 2 min, and 72°C for 10 min. Oligonucleotide primer sequences for LNL6 were as follows: 5′-primer (DK250), GGT-GGA-GAG-GCT-ATT-CGG-CT, and 3′-primer (DK251), CAT-AGA-AGG-CGG-CGG-TGG-AA (fragment size 834 bp). The G1Na-specific PCR primers were as follows: 5′-primer (DK249), GAA-TTC-GCG-GCC-GCT-ACA-AT, and 3′-primer (DK248), GAT-AGA-AGG-CGA-TGC-GCT-GC (fragment size 829 bp). Both pairs of primers were designed at Genetic Therapy, Inc. using the Primer Designer software program. Serial dilutions of DNA from the PA317-LNL6 and PA317-G1Na amphotropic retrovirus packaging cells were used to generate PCR standard curves by which gene copy number of patient samples was determined. Total DNA per PCR was adjusted to 1 μg with λ phage DNA.

Amplified products were separated on 1% agarose gels with 0.5 μg/ml ethidium bromide or, for quantitative hot PCR, on 5% polyacrylamide gels which were dried and subjected to autoradiography on film (Fuji Photo Film Co., Tokyo, Japan) at −80°C for 2–16 h. The autoradiographs were then scanned (Color OneScanner; Apple Computers) using the Adobe Photoshop software program (Adobe Systems, Inc.), and densitometry performed using the NIH Image software program. Densities of patient sample PCR bands were then compared to standard curves generated by PCR of PA317-LNL6 or PA317-G1Na cell DNA to obtain gene copy numbers for test samples. Gene copy numbers for the standard curves were determined by converting the amount of PA317 DNA used for PCR using the factor ([1 gene copy/cell genome] × [1 cell genome/3 × 10^9 bp] × [1 bp/660 D] × [6.023 × 10^{23} D/g]). The gene copy numbers obtained by densitometry of the test samples were also compared to those obtained by cutting out the PCR product band from the dried polyacrylamide gel, counting radioactivity in a liquid scintillation counter (LS1800; Beckman Instruments Inc., Palo Alto, CA) in EconoSafe scintillation cocktail (Research Products, Inc., Mount Prospect, IL), and comparing to PA317-LNL6 or PA317-G1Na standard curves (34, 35).

Cytotoxicity assays. Cytotoxicity activity of TIL was tested in vitro in a standard 4-h ^{51}Cr release assay against fresh (cryopreserved) autologous tumor, one allogeneic tumor target (TU 59 for renal, M14 for melanoma), K562 (an natural killer (NK)-sensitive erythroleukemia cell line), and Daudi cells (an NK-resistant lymphoma) as previously described (12). Cytotoxicity is expressed as lytic units (LU) per 1 × 10^6 cells; 1 LU is defined as the number of effector cells needed to lyse 30% of 5 × 10^3 target cells. Effector to target ratios of 40:1, 20:1, 10:1, and 5:1 were used.

Phenotypic analysis. Phenotypic expression of TIL was determined by two-color fluorescence. Antibodies used were: anti–Leu-4 (CD3, pan-T cell)-FITC + anti–Leu-19 (CD56, NK cell, lymphokine-activated killer cells, T cell subsets)-PE; anti–T cell antigen receptor (T cell receptor–1 α/β)-FITC + anti–IL-2 receptor (CD25, activated T cells)-PE; anti-Leu-3a (CD4, T helper/inducer cells)-FITC + anti–Leu-2a (CD8, T cytotoxic/T suppressor cells)-PE; anti–Leu-28 (CD 28, cytotoxic T cells)-FITC + anti–Leu-15 (CD11b, NK cells, T suppressor cells); or + anti–Leu-3a and anti–Leu-2a, respectively. All antibodies were purchased from Becton Dickinson Inc. (San Jose, CA) except anti–Leu-15 (GenTrac, Plymouth Meeting, PA). FITC-IgG1 and PE-IgG2a (Simultest Control; Becton Dickinson) were used as negative controls.

Results

A catalogue of clinical characteristics (Table I) and TIL/PBL properties (Table II) may be summarized as follows: (*a*) patients received between 4.5 × 10^8 and 1.3 × 10^10 total cells; (*b*) transduced lymphocytes could be safely administered and treatment toxicity was not adversely affected in any identifiable manner by retroviral transduction; (*c*) all transduced cell populations were shown to be free of replication-competent retrovirus and to have integrated provirus; (*d*) seven patients received both TIL and PBL, in two patients only transduced TIL were administered (PBL either did not expand or transduce in two patients); (*e*) TIL/PBL phenotype and cytotoxicity profiles were similar to those from patients entered into other TIL trials and were not affected by transduction; and (*f*) the six melanoma patients exhibited progression of disease and all three renal patients clinically responded to treatment and classified as disease stabilization.

After TIL/PBL administration, patients were supported with infusional IL-2 (melanoma) or IL-2/α-IFN (renal). Serial blood leukocyte, tumor, and normal tissue (fat, skin, or muscle) biopsies were analyzed for LNL6 and GlNa provirus using quantitative DNA PCR. Fig. 1 provides a broad overview of this large DNA analysis. Several general observations can be made. Transduced TIL were detected in peripheral blood (9/9 patients), tumor biopsies (4/7 patients), and normal tissue biopsies (3/5 patients). Tumor and blood samples obtained as long as 99 and 80 d, respectively, after adoptive transfer con-

Table I. Clinical Characteristics of Enrolled Patients

Patient	Age/Gender	Disease	Sites of Disease	Clinical Response to TIL/PBL
1.1	50/M	melanoma	subcutaneous, liver, lung mediastinal LN	NR
1.2	44/F	melanoma	subcutaneous, lung, liver, R adrenal, pancreas, paraesophageal LN	NR
1.3	57/M	melanoma	subcutaneous, L axilla, liver, spleen, lung, mediastinal LN, paracaval LN	NR
1.4	60/M	renal carcinoma	renal, lungs, R scapula, subcutaneous, mediastinal LN	stable
1.5	47/M	melanoma	subcutaneous, lungs, spine, L humerus, liver, spleen, gallbladder, R inguinal LN	NR
1.6	45/M	melanoma	subcutaneous, liver, lung, bowel wall, mediastinal LN	NR
1.7	72/M	renal circinoma	subcutaneous, renal, retroperitoneal LN, frontal bone, sternum, L clavicle, mediastinal LN, lung, paraspinous, retrocrural LN	stable
1.8	55/M	renal carcinoma	subcutaneous, renal, liver, axillary LN, sternum, lungs, L supraclavicular, paraesophageal LN, periaortic LN	stable; regression of L supraclavicular lesion biopsied on day 76
1.9	62/M	melanoma	subcutaneous, L axilla, L inguinal LN, bilateral adrenal, internal mammary LN	NR

Table II. Properties of Administered TIL and PBL

Patient	Total cells infused*	Numbers of TIL/PBL exposed to retroviral vector‡	Percentage of transduced TIL/PBL in total pool of infused cells§		Surface phenotype					Cytotoxicity‖					
			TIL	PBL	CD 3	CD8	CD 4	CD 5 6	CD 2 5	auto	allo 1	allo 2	K562	Daudi	
1.1	1.06×10^{10}	TIL 8.7×10^{9}	0.227	0	78	90	4	3	0	1.31	−0.31	---	2.57	0.89	TIL
		PBL 0.0×10^{0}			---	---	---	---	---	---	---	---	---	---	PBL
1.2	1.05×10^{9}	TIL 8.4×10^{7}	0.15	0.077	69	89	1	5	1	−0.30	---	---	24.17	15.24	TIL
		PBL 4.0×10^{7}			67	81	4	6	1	---	---	---	---	---	PBL
1.3	4.82×10^{8}	TIL 2.6×10^{7}	0.185	0.055	50	63	4	22	2	0.69	−1.80	---	12.51	18.32	TIL
		PBL 1.4×10^{7}			38	51	20	27	1	0.07	−0.82	---	24.93	45.15	PBL
1.4	4.50×10^{8}	TIL 9.0×10^{7}	0.014	0.016	39	23	25	47	6	1.10	1.13	−1.11	−0.27	0.91	TIL
		PBL 8.0×10^{7}			65	30	42	23	1	1.88	2.28	−1.24	5.22	13.52	PBL
1.5	3.26×10^{9}	TIL 2.2×10^{8}	0.109	0.017	43	70	2	17	1	−0.11	−0.80	---	1.42	1.11	TIL
		PBL 1.2×10^{8}			14	32	6	74	1	0.16	−0.56	---	1.59	1.14	PBL
1.6	3.44×10^{9}	TIL 1.4×10^{8}	0.025	0	44	51	9	47	1	4.54	6.80	---	49.73	28.70	TIL
		PBL 0.0×10^{0}			---	---	---	---	---	---	---	---	---	---	PBL
1.7	1.04×10^{10}	TIL 1.8×10^{9}	ND	ND	92	83	9	1	0	−0.20	4.70	2.20	42.60	43.10	TIL
		PBL 4.0×10^{8}			50	24	33	38	2	---	---	---	---	---	PBL
1.8	1.09×10^{10}	TIL 8.9×10^{8}	0.015	0.087	26	22	17	42	11	1.30	0.60	0.60	28.30	28.00	TIL
		PBL 7.0×10^{8}			51	47	40	10	2	2.10	1.30	−0.06	8.80	8.10	PBL
1.9	1.24×10^{10}	TIL 1.1×10^{9}	ND	ND	44	68	4	3	1	−0.02	0.30	---	1.90	1.90	TIL
		PBL 1.7×10^{9}			39	45	19	29	3	−0.08	0.80	---	28.80	29.40	PBL

*This number represents the total number of TIL and PBL adoptively transferred to the patient. ‡These numbers of TIL and PBL were exposed to the LNL6 retroviral vectors. All PBL and an aliquot of TIL were transduced and expanded in parallel with untransduced TIL. After biosafety testing, all three populations of cells (transduced TIL, transduced PBL, untransduced TIL) were combined and infused into the patient. The total number of infused cells is given in the first column. The transduction efficiencies for these aliquots of TIL and PBL ranged from < 1 to 7%. §An aliquot of this infusion pool was retrieved and used as a TIL/PBL infusion ratio standard. The percentage of transduced TIL and PBL in this entire pool of infused cells was estimated by quantitative PCR. An ND entry means that the efficiencies could not be calculated for technical reasons. ‖Measured in lytic units.

tained *neoR*-transduced TIL. Transduced PBL were detected in peripheral blood (7/7 patients), tumor (3/7 patients), and normal tissue (1/5 patients). *NeoR*-transduced PBLs were detectable out to 99 d after infusion. In this figure, an estimate of the number of *neoR* gene copies/μg cellular DNA (10^5 nucleated cells) is given in parentheses near some data symbols.

The findings in several patients were instructive. Patient No. 1.2 (Fig. 2) received marked TIL and PBL. TIL(GlNa) could be detected in blood on days 1, 13, and 32 and in tumor biopsies on days 6, 13, and 32 at low copy number (< 3 copies/μg DNA). A day 13 muscle biopsy also had TIL at a comparable copy number (< 3 copies/μg DNA) in tumor. PBL(LNL6) were detected in blood on days 1, 6, and 13 and in tumor on days 6, 13, and 32. If anything, a greater number of marked PBL (14–40 copies/μg DNA) than TIL (< 3 copies/μg DNA) were present in these tumor biopsies. Patient No. 1.4 (Fig. 3) received renal TIL and PBL. PBL(LNL6) were detected in blood on days 48, 76, and 90. PBL(LNL6) retrieved from the tumor on day 76 could be cultured in AIM-V/IL-2 medium for up to 2 wk with persistence of the LNL6 provirus. Infused TIL(GlNa) could only be detected in a single peripheral blood sample on day 48 (Fig. 3, *arrow*). Patient No. 1.8 (Fig. 4) received renal TIL and PBL. PBL(GlNa) were detected in peripheral blood on days 8, 21, 42, and 63. Likewise TIL(LNL6) were detected on days 8, 14, 21, 42, and 63. PBL(GlNa) could also be detected in a day 21 tumor biopsy and successfully expanded in AIM-V/IL-2 for up to 42 d in culture. While these transduced cells could not be selected in G418 (100, 200, or 400 μg/ml), transduced cells with a CD8 phenotype could be positively selected on CELLector CD8 T150 flasks and further expanded in vitro. Negatively selected populations had 10% CD8 cells. PBL(GlNa) could likewise be retrieved from day 21 tumor biopsy in tissue culture. A very instructive observation was made when day 21 tumor, blood, skin, and muscle were analyzed for TIL and PBL provirus. An unequivocally and remarkably higher number of TIL(LNL6) gene copies could be detected in blood, muscle, and skin than in tumor (Fig. 4).

Discussion

Human tumor-infiltrating lymphocytes have generated significant research interest since their initial characterization (1, 2, 36–39). Although they are immunodepressed in this tumor microenvironment, when separated from tumor cells and expanded and activated in vitro, antitumor effectors can be readily generated (1–12). Investigators conducting clinical trials using adoptively transferred IL-2–expanded TIL in melanoma and renal cancer report clinical response rates that appear to be in excess of those achievable with biological therapy alone (13–17).

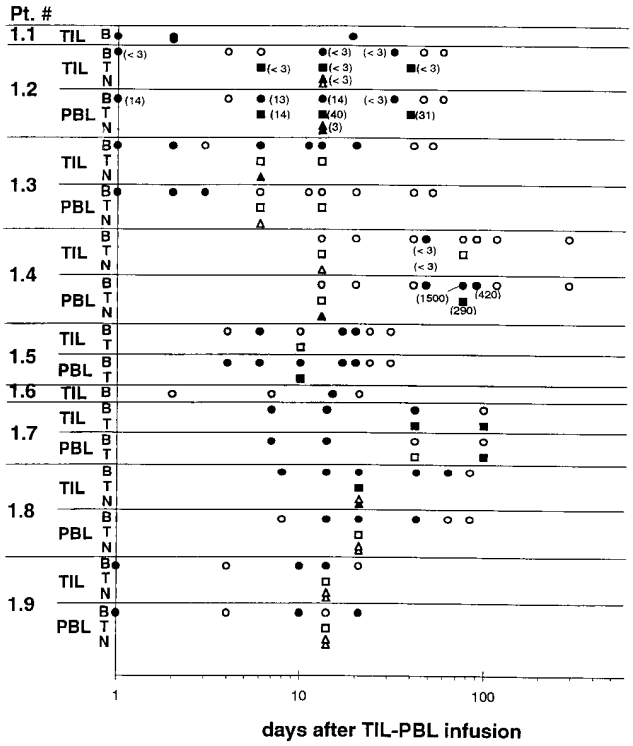

Figure 1. Overall summary of DNA PCR analysis of patient samples. Blood (*B*), tumor (*T*), and normal tissue (*N*) samples were obtained at various intervals after adoptive transfer and analyzed for TIL or PBL provirus. Filled symbols indicate a positive PCR product. Open symbols indicate a negative PCR product. Numbers in parentheses indicate "gene copy number/10^5 cells" determined by comparing the densities and/or radioactivity of the PCR product bands from patient's samples to those of PCR bands generated from serially diluted packaging cell line DNA (undiluted DNA = 1 gene copy per cell). *Pt.*, patient.

The UCLA experience in TIL adoptive immunotherapy of renal cancer has been recently reviewed (17). Of 48 patients treated with high dose (400 IU/ml) IL-2–expanded TIL and infusional IL-2, 17% achieved complete and 17% achieved partial responses. The complete responses were durable. However, the determinants and mechanism of response, and in particular the contribution of infused TIL, remains unknown. No clinical or laboratory marker has allowed differentiation between responding and nonresponding patients. A large multivariate analysis of responding versus nonresponding patients showed no correlation with TIL growth rate, culture duration, phenotype, cytotoxicity pattern, or cytokine profile (12). Moreover, in none of the patients treated were MHC-restricted tumor-specific cytotoxic T lymphocytes ever generated. Efforts at generating specific cytotoxic T lymphocytes using multiple culture manipulations (different cytokines, irradiated autologous tumor cells, low IL-2 concentrations) have been uniformly unsuccessful. These findings of impressive response rates with "nonspecific" cellular effectors are in contrast to melanoma TIL therapy in which cell numbers infused and tumor-specific cytotoxicity do correlate with response. It was against this backdrop of very different melanoma and renal cancer TIL biologies that a double gene marking trial was developed.

There remains the nagging question of whether TIL contribute anything to the biological therapy of melanoma and renal cancer, and, if so, how. The issue of clinical efficacy should be resolved in a well-designed, multiinstitution, randomized trial in kidney cancer that is currently ongoing. Patients are randomized to receive low dose, continuous infusion of IL-2

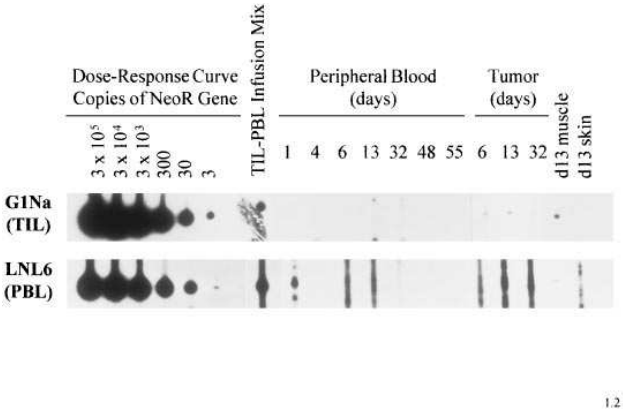

Figure 2. DNA PCR autoradiograph of samples from patient No. 1.2. Blood, tumor, and normal tissue samples obtained at different time points were analyzed for G1Na provirus (*TIL*) and LNL6 provirus (*PBL*). *NeoR* copy number dose-response curve is included. The estimated number of *neoR* copies per μg DNA is shown in parentheses.

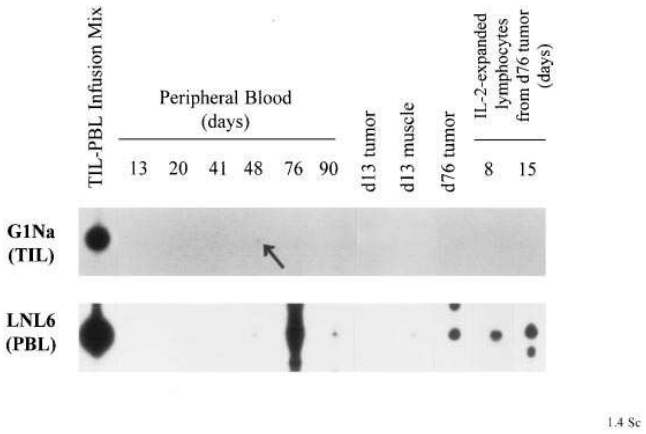

Figure 3. DNA PCR autoradiographs for patient No. 1.4. Arrow identifies faintly positive PCR product for TIL(G1Na) in day 48 blood sample. Day 76 PBL(LNL6) isolated from tumor biopsies could be cultured for 15 d.

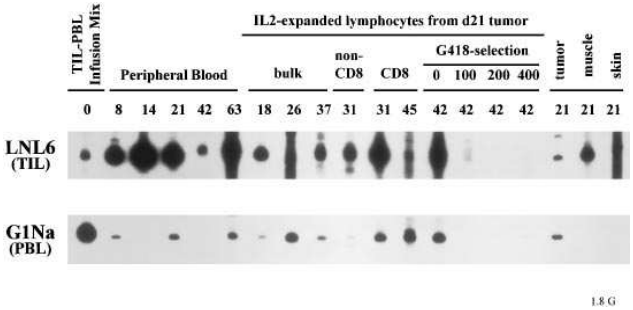

Figure 4. DNA PCR analysis of patient No. 1.8 samples. Lymphocytes from day 21 tumor biopsy could be grown in tissue culture but could not be selected in G418. The CD8+ subpopulation could be isolated and cultured.

alone or IL-2 plus $CD8^+$ TIL after radical nephrectomy (Belldegrun, A. S., personal communication).

Some insight into TIL immunotherapy might be gained if it was known where TIL went and what they did. Murine TIL–tumor models have allowed several important immunological hypotheses to be tested. While murine TIL have unequivocal and potent antitumor activity, the most important reservation is that only lung metastases, and not subcutaneous tumors, are susceptible (40, 41). This finding calls into question a hypothetical requirement for TIL antitumor activity, namely, their ability to traffic to all tumor sites.

Based on our current understanding of tumor immunobiology, the most useful place for adoptively transferred TIL to go would be the tumor, to mediate either direct or indirect effects. Thus, the notion that TIL "homed" to tumors represented a useful hypothesis to test experimentally and clinically. Remarkably, even in murine TIL–tumor models in which the antitumor effect of TIL is undisputed, there is no evidence that TIL specifically localize to or are retained in metastatic lung nodules (42). Clinical investigations using indium-111–labeled TIL improved our knowledge of the biodistribution of infused TIL but could not definitively address the issue of specific (versus random) lodging of cells in tumor (19, 20). Likewise, the landmark TIL-retroviral marking trial helped to usher in the field of human gene therapy and provided information about TIL life span only (21).

This clinical investigation built upon these important contributions by adding an additional refinement, namely the use of a control cell population. By coinfusing both TIL and PBL (control), activated and expanded in parallel, but genetically "marked" with different retroviral vectors, a quantitative comparison could be made. This study design allowed the hypothesis of preferential TIL localization to tumor to be directly tested. One would have to observe preferential enrichment of TIL versus PBL at tumor sites, and only at tumor sites, for this hypothesis to be supportable.

In this clinical investigation of nine patients, no evidence of preferential TIL trafficking to tumor was found. We confirmed that TIL (and PBL) could be detected in the peripheral blood months after adoptive transfer. We could detect TIL (but also PBL) in tumor and normal tissue biopsies, but there was no evidence of preferential enrichment of TIL in tumor. We were able to retrieve marked TIL and PBL from blood and tumor samples and could manipulate and study them (at least as part of a bulk population) in vitro for several weeks. This finding underscored the longevity and hardiness of adoptively transferred lymphocytes. Although they constituted "secondary" TIL, their presence in tumor biopsies in no way supported a homing theory. A legitimate counter argument to these conclusions is that, in this particular group of patients, no patient enjoyed a dramatic clinical response and that TIL "homing" would be a necessary prerequisite to a good antitumor effect. The three renal patients (patients 1.4, 1.7, 1.8) showed a clear response to therapy scored as stabilization of disease. Biopsies were retrieved in one patient (patient 1.4) when one metastatic lesion was getting smaller and softer. This lesion (patient 1.4, day 76) showed no evidence of TIL localization. The study design of this clinical trial also could not exclude the presence of a subpopulation of TIL with specific in vivo behavior, certainly a possibility. Genetically marked cells represented only a fraction of the bulk population, a bulk population generally oligoclonal with respect to T cell receptor specificity. Even our ability to retrieve "secondary" TIL, which we felt might allow us to study lymphocytes that found their way into tumors a second time, were limited by our inability to purify them under the selective pressure of G418.

This clinical investigation clearly demonstrated that: (*a*) retrovirally transduced human lymphocytes could be safely administered to patients and that fairly complicated series of manipulations of TIL could be performed yielding quantitative data; (*b*) human lymphocytes persist in peripheral blood and tissues months after infusion, and the possibility of an indirect antitumor activity via systemic production of cytokines by these activated lymphoid cell populations might explain the dramatic antitumor responses in some of these patients; and (*c*) in nine consecutive patients, no clear evidence of preferential TIL trafficking to tumor was found.

Acknowledgments

We are grateful to Drs. E. Carmack Holmes, Sidney Golub, Isidro Salusky, and Dennis Slamon for their continued support.

This work was also supported by National Institutes of Health grants #P01CA59326 (J. S. Economou), R29CA50780 (J. S. Economou), and P30CA16042 (J. S. Economou), a National Cancer Institute Institutional Training Grant in Surgical Oncology, T32CA09010, and the Monkarsh, Kesselman, and Wolfberg Funds. This study was also supported by Clinical Research Center NIH grant MO1-RR00865. We are grateful to Genetic Therapy, Inc. (Gaithersburg, MD) for generously providing LNL6 and G1Na retrovirus vectors and for conducting S+/L− assays. We are also grateful to Hoffman-LaRoche (Nutley, N.J.) for providing recombinant IL-2 and IFN-α2A and to Applied Immune Sciences (Menlo Park, CA) for providing CELLector CD8 flasks.

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