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Cytolytic antitumor effector cells in long-term cultures of human tumor-infiltrating lymphocytes in recombinant interleukin 2

Theresa L. Whiteside[1, 5], D. S. Heo[5], S. Takagi[5], J. T. Johnson[2, 5], S. Iwatsuki[3, 5], and R. B. Herberman[1, 4, 5]

Division of Clinical Immunopathology, Departments of Pathology[1], Otolaryngology[2], Surgery[3], and Medicine[4], University of Pittsburgh School of Medicine and Pittsburgh Cancer Institute[5], Pittsburgh, PA 15261, USA

Summary. Lymphocytes infiltrating human solid tumors (TIL) and autologous peripheral blood lymphocytes (A-PBL) were cultured with 1000 units/ml of recombinant interleukin 2 (rIL2) in long-term cultures. TIL isolated from 26 primary squamous cell carcinomas of the head and neck expanded better ($P < 0.01$) and achieved higher total lytic units of activity against fresh tumor cell targets ($P < 0.05$) than A-PBL. TIL obtained from primary hepatocellular carcinomas ($n = 7$) showed a higher degree of expansion than those from metastatic liver tumors ($n = 7$). Further, TIL from metastatic tumors of the head and neck, liver, and ovary were delayed up to 50 days in their proliferative response to rIL2. Long-term mass cultures in rIL2 of TIL, A-PBL, or normal PBL were serially monitored for cytotoxicity with different cultured and fresh tumor cell targets and for phenotypic markers of the predominating cell populations. Antitumor cytotoxicity was found in cultures enriched in CD3+Leu19+ and/or CD3-Leu19+ cells. Two-color sorting of such cultures followed by cytotoxicity assays confirmed that the human antitumor effectors expressed either the CD3+Leu19+ or CD3-Leu19+ phenotype. CD3+Leu19- cells had little or no antitumor cytotoxicity. The two types of Leu19+ effector cells were present in low numbers in fresh TIL, A-PBL, or normal PBL; in contrast, in some rIL2-expanded long-term cultures, they represented a majority of proliferating cells. This study identifies for the first time two types of antitumor effector cells in rIL2 cultures of human TIL, one of which may represent activated natural killer cells on the basis of the absence of the CD3 and expression of the Leu19 antigen. These antitumor effector cells mediate non-MHC-restricted cytotoxicity of fresh or cultured tumor cell targets of different histologic types.

Introduction

Tumor-infiltrating lymphocytes (TIL) isolated from murine tumors were recently reported to be 50–100 times more potent than lymphokine-activated killer (LAK) cells in eliminating established tumors, expand more rapidly in culture, exhibit specificity for autologous tumors, and mediate antitumor reactivity in vivo in the absence of recombinant interleukin 2 (rIL2) [16]. Furthermore, these murine TIL were effective in eliminating large metastatic tumors in mice pretreated with cyclophosphamide [16]. Little is known about functional properties of TIL obtained from human solid tumors and expanded in high concentrations of rIL2. Muul and colleagues found that TIL isolated from human melanomas proliferated briskly in rIL2 and, in some cases, exhibited cytotoxicity that appeared to be specific for the autologous tumor [13]. Also, Kurnick and his collaborators were successful in culturing TIL from primary lung tumors in rIL2, and these TIL showed antitumor reactivity, including cytotoxicity against autologous lung tumors [5].

In this study our objectives were to: (a) demonstrate that TIL from various human solid tumors could be reliably and consistently grown in long-term cultures in the presence of rIL2; (b) compare antitumor cytotoxic activities of TIL with those of autologous LAK cells from the peripheral blood of patients with cancer; and (c) define the nature and cytotoxic profile of antitumor effector cells in long-term TIL cultures. It appeared to us essential to characterize the effector cells responsible for autologous antitumor reactivity, if cultured human TIL are to be considered as a therapeutic modality for the immunotherapy of cancer.

Materials and methods

Patients. Fresh tumor tissues were obtained from 29 patients with squamous cell carcinoma of the head and neck (SCCH&N) and 4 patients with non-squamous cell cancer of the head and neck all of whom underwent surgery at the Eye and Ear Hospital of Pittsburgh. The patients included females (ages 51–74) and males (ages 25–76). Among patients with SCCH&N, 26 had primary and 3 had metastatic tumors (see Table 1), and 7 of the primary SCCH&N had received previous radiation therapy. Tumor tissues were also obtained from 14 patients with liver cancers; 7 of these had primary hepatocellular carcinoma, and the other 7 had colon adenocarcinoma which metastasized to the liver. All the patients had liver resections at Presbyterian University Hospital. In addition, tumor specimens were available from 5 patients with ovarian adenocarcinomas, 2 of which were primary and three metastatic (Table 1). The ovarian tumor specimens were obtained from Magee Women's Hospital.

Offprint requests to: Theresa L. Whiteside, Room 5725, One Children's Place, 3705 Fifth Avenue at DeSato Street, Pittsburgh, Pa 15213-3417, USA

Tumor tissues. Freshly excised tumor tissues (0.1 g to 20 g wet weight) were placed in sterile tissue culture medium (TCM) containing antibiotics immediately after surgery, and hand-delivered to the laboratory. Tumor tissues were processed as described elsewhere [23]. Briefly, the tissue was minced with scalpels into pieces smaller than 1 mm^3. The pieces were subjected to enzymatic digestion with a mixture of collagenase (2 mg/ml, Cooper Biochemicals, Malvern, Pa.) and DNase (100 µg/ml, Sigma Chemical Co., St. Louis, Mo.) in TCM containing antibiotics and 5% human AB serum for 1–2 h at 37° C. Following enzymatic digestion, suspensions containing tumor cells, tissue cells, and TIL were placed on differential Ficoll-Hypaque gradients, centrifuged, washed twice in TCM and checked for viability using trypan blue. The numbers of TIL and tumor cells present in these suspensions were determined by counting in a hemocytometer, and the suspensions were plated in 16-mm wells of Costar plates (≠3424, Cambridge, Mass).

Autologous peripheral blood lymphocytes. Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood collected from the patients with head and neck tumors prior to surgery and centrifuged over Ficoll-Hypaque gradients. Isolated mononuclear cells were washed 3X in Hanks' balanced salt solution and counted in a hemocytometer in trypan blue.

Cultures of TIL and A-PBL. TIL suspensions and autologous (A)-PBL were cultured under identical conditions, initially in 16-mm wells (Costar ≠3424) in 1 ml of TCM which consisted of RPMI 1640 supplemented with 2 m*M* L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin (Gibco, Grand Island, NY), and with 5% pooled, prescreened AB human serum. The rIL2 from *Eschericia coli*, donated by Cetus Corp., Emeryville, Calif., was used at 1,000 units/ml. Cultures were started at a cell density of 1.0×10^6 TIL or A-PBL per ml and incubated at 37° C in a humidified atmosphere of 5% CO_2. Cell densities were determined by counting cells every 48 h. Expanding cultures were transferred to 75-cm^2 flasks (Costar, ≠3075) and maintained at 1×10^6 cells/ml by adding fresh TCM plus rIL2 or subculturing as needed. Expansion was calculated for every culture on the basis of cell counts in trypan blue. As cells were removed at regular intervals for assays, the total cell numbers in culture were estimated to give the number that had been present had all the cells been expanding together.

Fresh allogeneic and autologous tumor cell targets. Human solid tumors, ovarian and endometrial carcinomas, hepatic carcinomas, and renal cell carcinomas obtained freshly from surgery were dissociated by enzymatic treatment and the resultant suspensions centrifuged on differential Ficoll-Hypaque gradients as described elsewhere [23]. The tumor cells were collected from the upper interface, washed, checked for viability in trypan blue, and counted. Tumor cells were either used fresh or cryopreserved in 90% human serum and 10% dimethyl sulfoxide by controlled-rate freezing (Cryo-Med, Mt. Clemens, Mich.). Immediately prior to cytotoxicity assays, tumor cells were thawed, washed in TCM, checked for viability and labeled with radioactive chromium to serve as targets. Tumor cells were tested for natural killer (NK) sensitivity with fresh normal PBL and were shown to be NK-insensitive prior to use in assays with TIL.

Tumor lines. Tumor lines K562, Daudi and Raji were maintained in culture in RPMI 1640 with 10% fetal bovine serum. Cells were subcultured as needed, and the cells in log phase were used for cytotoxicity assays. In addition, a melanoma cell line IGR3 obtained from S. Carrel, Ludwig Institute, Switzerland, was maintained in the same medium, passaged by trypsinization, and used as a target.

Cytotoxicity assays. Fresh or cultured tumor cell targets were labeled with 100–250 µCi of sodium chromate (5 mCi/ml, New England Nuclear, Mass.) for 1–2 h at 37° C. Cells were then washed 4X in TCM, resuspended in fresh medium, counted, and aliquoted at 5×10^3 targets/well in a 96-well U-bottomed plate (Costar) into which the effector cells had been previously aliquoted at predetermined concentrations. The effector to target cell ratios (E:T) ranged from 50:1 to 6:1. In some experiments with rIL2-expanded cells, E:T ratios were from 12:1 to 0.4:1. Plates were centrifuged at 65 *g* for 5 min and incubated in 5% CO_2 in air at 37° C for 4 h, after which medium was harvested from each well using a Skatron supernatant harvesting apparatus (Skatron, Sterling, Va.). All determinations were done in triplicate. Radioactivity was counted in a gamma counter and percent specific lysis was determined according to the formula:

$$\frac{\text{experimental mean cpm} - \text{spont. release mean cpm}}{\text{maximal mean cpm} - \text{spont. release mean cpm}} \times 100$$

Lytic units (LU) were calculated according to the formula of Pross et al. [15]. One LU was defined as the number of cells needed to lyse 20% of the targets and calculated per 10^7 effector cells. Since TIL and A-PBL cultures were subcultured repeatedly and used for assays at different times during culture, LU per culture were calculated using the initial number of cells in a culture which was multiplied by the degree of expansion, to reflect the total number of cells that would have been present had all the cells been recovered at the time of subculture. Total lytic units (TLU) per culture were calculated by multiplying LU by the magnitude of expansion.

Flow cytometry and sorting. Cells were adjusted to the concentration of 2×10^5/ml and stained with fluorescein- or phycoerythrin-labeled monoclonal antibodies (10 µl) for 30 min at 4° C for two-color flow cytometry analyses. Antibodies were purchased from Becton Dickinson, Mountain View, Calif. and included: Leu 4, Leu 3a, Leu 2a, Leu 5b, Leu 19, Leu 11a, anti-IL2R, and anti-HLA-DR. Cells were washed 2X with the PBS-0.1% sodium azide buffer and resuspended in the same buffer for flow cytometry. A FACStar instrument was used for the two-color analysis and for cell sorting.

Statistical analysis. The paired data (A-PBL and TIL) were analyzed by signed ranks test, and Wilcoxon's statistic was used to calculate the significance of differences between the two experimental groups. In addition, Student's *t*-test was used to determine significance of differences between cytotoxicity of rIL2-expanded cultures of TIL grouped on the basis of a predominant cellular phenotype.

Table 1. Patients with primary and metastatic cancer whose tumor biopsies were studied

I. *Squamous cell carcinomas of the head and neck* (SCCH&N)	
n = 29 (paired TIL and A-PBL)	
Primary	26
No XRT[a]	19
Previous XRT	7
Metastatic	3
II. *Non-squamous cell cancers of the head and neck* (NSC)	
n = 4 (paired TIL and A-PBL)	
Cystic adenocarcinoma (parotid), no XRT	
Clear cell carcinoma (parotid), previous XRT	
Undifferentiated carcinoma (parotid), 2 specimens, primary and metastatic, 2 months apart	
III. *Hepatic carcinoma*	
n = 14 (TIL only)	
Primary (hepatocellular)	7
Metastatic (adenocarcinoma, colon)	7
IV. *Ovarian adenocarcinoma*	
n = 5 (TIL only)	
Primary	2
Metastatic	3

[a] XRT = X-ray therapy

Results

Expansion of TIL and A-PBL in rIL2

In order to determine the ability of TIL to grow in rIL2 and to compare their growth with that of A-PBL, we established paired cultures of TIL and A-PBL obtained from 32 patients (33 specimens) with head and neck cancer (Table 1). Only 6 out of 64 cultures (9%) failed to expand under the growth conditions used. Two of the TIL preparations began to grow but were contaminated by fungi, and the cultures had to be terminated. Most of the cultures (n = 43 or 67%) reached between 2- and 100-fold expansion in 30–50 day cultures. A third (n = 21 or 33%) of the cultures achieved expansion equal to or greater than 100-fold, and in some cases greater than 1,000-fold (Fig. 1). The median expansion fold achieved by paired TIL and A-PBL cultures was 100 for TIL and 27 for A-PBL from patients with the head and neck cancer (Fig. 1), a difference significant at $P < 0.01$. Figure 2 shows the expansion achieved by 10 selected pairs of TIL and A-PBL from head and neck cancers. Clearly, the TIL obtained from these tumors and growing in rIL2 reached higher total cell numbers per culture than A-PBL in most cases (25 of 31 pairs studied), but clearly not in every case (see Fig. 2). The TIL from metastatic head and neck cancers either failed to expand in rIL2 or demonstrated delayed proliferative responses.

To further determine whether TIL from metastatic and primary tumors expanded comparably in rIL2, TIL were obtained from 14 hepatic tumors (7 primary and 7 metastatic, see Table 1) and cultured in rIL2. As shown in Fig. 3, TIL from primary hepatic tumors expanded better than those from metastatic tumors. The TIL from 2 primary and 3 metastatic liver tumors failed to grow altogether. However, TIL from 1 metastatic tumor (≠4 in Fig. 3) achieved a remarkably high number of cells in a long-term culture. Significantly, these expanded cells had no antitumor cytotoxicity.

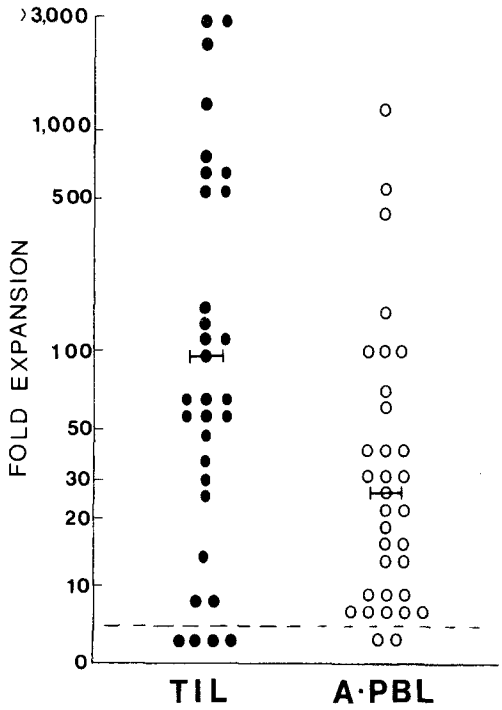

Fig. 1. Expansion achieved in long-term cultures with recombinant interleukin 2 (rIL2) by all tumor-infiltrating lymphocytes (TIL) and autologous peripheral blood lymphocytes (A-PBL) obtained from patients with head and neck tumors. The median expansion for each group is designated by the *bar*. The *interrupted line* separates those cultures that did not expand under the experimental conditions used.

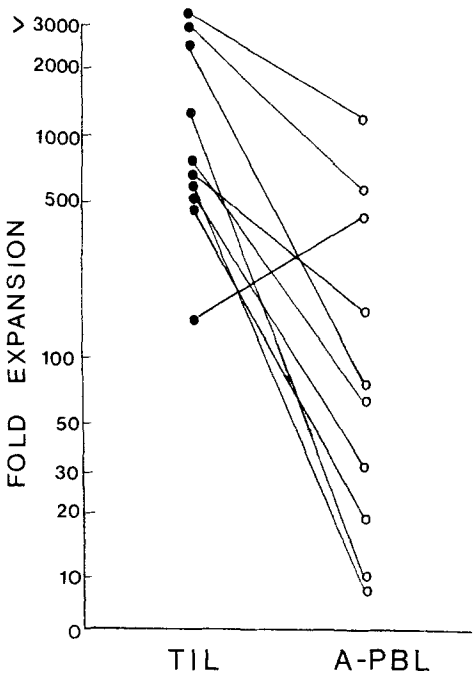

Fig. 2. Ten pairs of TIL and A-PBL from patients with head and neck cancer were selected to compare the fold expansion obtained in long-term cultures with rIL2. The TIL expanded better than A-PBL in 9 out of 10 cases

The kinetics of growth of TIL and A-PBL in long-term rIL2 cultures were studied in all cases of head and neck cancers. In Fig. 4, the representative growth curves for 2 TIL and 2 A-PBL obtained from patients with primary

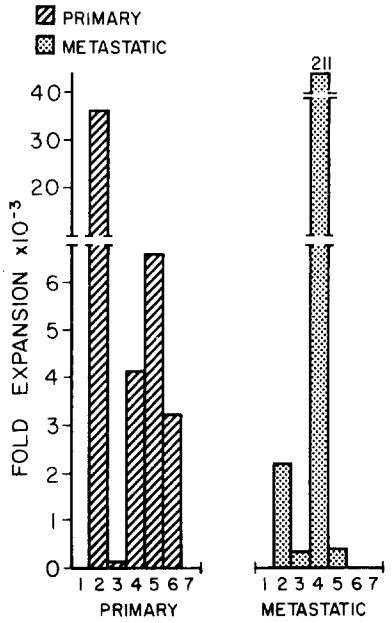

Fig. 3. TIL from seven primary hepatocellular carcinomas and seven metastatic liver tumors (all adenocarcinomas of the colon) are compared in terms of the fold expansion in long-term cultures in rIL2. Some TIL achieved remarkable expansions in rIL2 regardless of whether they originated from primary or metastatic tumors (e.g., #2 in the primary group versus #4 among metastatic tumors). TIL from primary tumors #1 and 7 and those from metastatic tumors #1, 6 and 7 failed to grow in rIL2

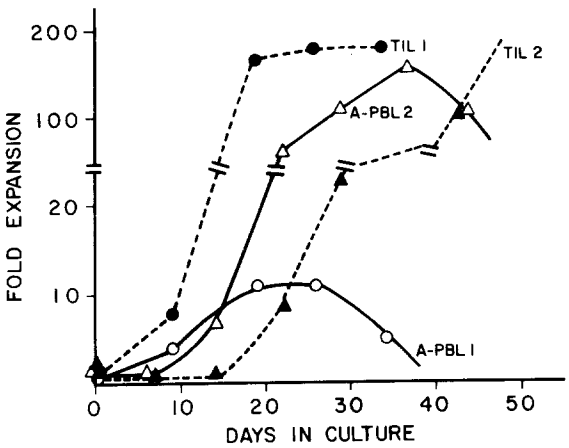

Fig. 4. Representative growth curves of two paired TIL and A-PBL cultures established with cells obtained from patients with primary squamous cell carcinoma of the head and neck. Cells were obtained from a patient treated with XRT (case #1; circles) and from an untreated patient with SCCH&N (case #2; triangles)

SCCH&N are shown. Two different patterns of growth were observed: (a) TIL expanded within the first few days of culture, and A-PBL lagged behind and never achieved cell numbers comparable to those of TIL (e.g., case #1 in Fig. 4). The TIL and PBL obtained from primary SCCH&N previously treated with X-ray therapy had this pattern of growth kinetics; (b) TIL showed a lag period lasting for up to 20 days and then expanded rapidly to reach very high cell numbers by day 50. The A-PBL began proliferating around day 7, reached peak cell numbers by day 30–40 and then declined in growth (e.g., case #2 in

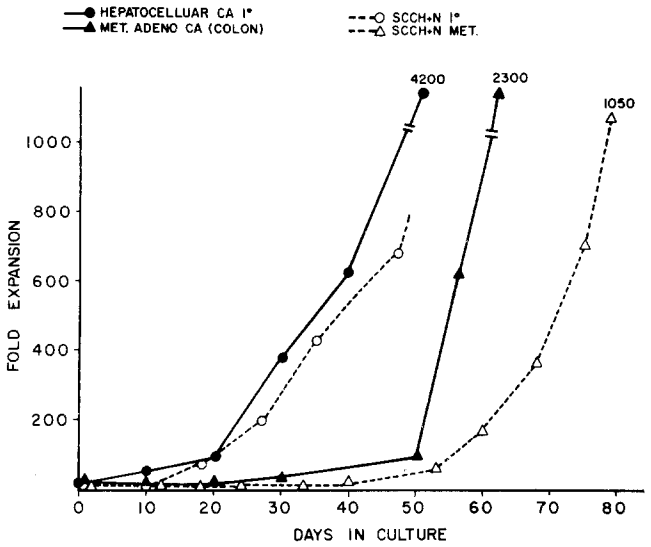

Fig. 5. Representative growth curves for TIL obtained from two primary tumors: hepatocellular carcinoma (●) and squamous cell carcinoma of the head and neck (○) and from two metastatic tumors: colon adenocarcinoma metastatic into liver (▲) and squamous cell carcinoma of the head and neck (△)

Fig. 4). The TIL and PBL obtained from primary untreated SCCH&N showed this growth pattern.

TIL outgrowing from metastatic tumors exhibited an especially prolonged lag period, which lasted up to 40 and 50 days in culture. The growth kinetics of TIL from metastatic tumors contrasted with those of TIL from primary tumors as illustrated in Fig. 5. TIL from metastatic tumors of different histologic types were delayed in their expansion in rIL2 (Fig. 5). After the long lag period, these cells started growing rapidly and often, but not always, reached greater than 1,000-fold expansion.

Cytotoxicity of TIL grown in long-term cultures with rIL2

TIL and A-PBL cultures were serially tested for antitumor cytotoxicity against fresh (allogeneic and autologous when available) and cultured (K562 and Raji) tumor cell targets. The cultures were assayed at weekly intervals. Table 2 summarizes the results of antitumor cytotoxicity measured against an allogeneic fresh tumor cell target (ovarian carcinoma) of paired TIL and A-PBL cultures established from 25 patients with head and neck tumors. The highest total cytotoxic activity (TLU) achieved by each TIL and A-PBL culture, and the day in culture on which this level was achieved are presented. The TIL cultures attained higher TLU of activity ($P < 0.05$) and generally did so later in culture than A-PBL. The same pattern of results was obtained when cytotoxicity was measured against K562, Raji, and a cultured tumor cell target (IGR3 melanoma line; data not shown). TIL isolated from metastatic tumors generally had lower cytotoxicity than A-PBL. In patients with metastatic liver tumors, TIL had low or no antitumor effector function (manuscript in preparation).

Phenotypic characteristics of TIL cultures with antitumor reactivity

Flow cytometry studies were performed in all expanding TIL cultures at weekly intervals. Staining with monoclonal antibodies and two-color analyses revealed that most

Table 2. The highest antitumor cytotoxicity achieved in long-term IL2 cultures of TIL and A-PBL obtained from patients with head and neck tumors[a]

Patient no.	TIL		A-PBL	
	Day	TLU[c]	Day	TLU
	TLU culture			
2		ND	53	6,800
7	42	700	42	67
8	36	380	36	1,100
9		ND	31	460
10[b]	68	6,400	24	700
11[b]	14	110	14	490
12	38	28,000	23	15,000
13	44	1,500	22	4,300
14	30	75,000	30	2,500
15		ND	1	640
16	40	990	8	410
17	29	450	29	4,400
18[b]	24	900	38	4,700
19	26	8,900	12	1,500
20	19	5	19	0
21		ND	16	744
22	16	980	8	72
23	10	8	10	172
24	17	7,000	17	2,550
25	19	3,190	9	380
27	8	460	8	150
28[b]	20	24	20	4
32	43	850	12	1
33	37	428	37	0
34	30	1,162	30	332
Median values	26	850	19	460

[a] Fresh cryopreserved ovarian carcinoma cells were used as targets in the cytotoxicity assays

[b] TIL/A-PBL cultures established from metastatic head and neck cancers

[c] TLU, total lytic units

of these cultures contained three predominant cell populations in various proportions: CD3+Leu19−, CD3+Leu19+, and CD3−Leu19+. For example, Table 3 summarizes the antitumor reactivity and phenotypic characteristics of those TIL cultures obtained from SCCH&N, liver and ovarian tumors that were studied by two-color flow cytometry for the expression of the CD3/Leu19 phenotype. The cultures were divided into three groups on the basis of a predominant cellular phenotype detected in these cultures. The TIL cultures in which CD3+Leu19− cells predominated (Group-I) had only moderate antitumor cytotoxicity. In contrast, TIL cultures in which substantial numbers of CD3−Leu19+ were present (Group-III) had comparatively high cytotoxicity. The differences in cytotoxicity between Groups I and III were statistically significant as shown in Table 3. The TIL cultures containing both CD3+Leu19+ and CD3+Leu19− populations (Group-II) showed a kinetically bimodal cytotoxicity profile, with high cytotoxicity early in culture and low or no cytotoxicity after 50 days in culture (Table 3). These observations indicated that the populations of TIL expressing the Leu19 antigen might be responsible for lysis of tumor cells. Furthermore, when the long-term IL2 cultures of patients' PBL were analyzed for antitumor effector function, it appeared that the highest cytolytic activities were found in cultures containing substantial levels of all three (CD3+Leu19−, CD3+Leu19+, and CD3−Leu19+) populations (Group-II in Table 4). Cultures of patients' PBL in which CD3−Leu19+ cells were in low proportions (0%–11% in Group-I, Table 4) had comparatively low antitumor cytotoxicity, as measured with four different tumor cell targets.

The Leu19+ cell populations were also detected by two-color flow cytometry in long-term rIL2 cultures of normal PBL (Table 5). The CD3+Leu19+ and CD3−Leu19+ cells were present in relatively high proportions in these cultures, and this was accompanied by high levels of antitumor cytotoxicity (Table 5). In culture #4, where only 1%–11% of CD3+Leu19− cells but 79%–84% CD3−Leu19+ cells were present, cytotoxicity was high, a finding suggestive of a possibility that the latter cell population mediated this cytotoxicity. The overall impression was that normal PBL cultured in rIL2 had higher antitumor effector function than that of cultures of patients lymphocytes, and they also contained higher proportions of CD3−Leu19+ cells.

Phenotypic analysis of fresh TIL

In view of the possible association between the CD3−Leu19+ and CD3+Leu19+ populations and antitumor cytotoxicity in long-term IL2 cultures, it became important to determine to what extent these two populations were represented in the freshly isolated TIL populations. As shown in Table 6, CD3+Leu19− cells consistently represented between 60% to 90% of cells in the TIL suspensions obtained from human solid tumors of different histologic types. Leu19+ and CD16+ cells were <7% in SCCH&N, but were more frequent in A-PBL of patients with SCCH&N and in some TIL suspensions isolated from liver and ovarian tumors. The CD3+Leu19+ cells consistently accounted for 0%–8% in fresh TIL suspensions or A-PBL in SCCH&N patients. In normal fresh PBL, both the CD3−Leu19+ and CD3+Leu19+ populations were a relatively minor cellular component (mean 9%±5 (SD); $n = 50$ and 2%±2; $n = 15$, respectively). It should be noted that all TIL suspensions from SCCH&N tumors were enriched in activated (CD2+HLA-DR+) T lymphocytes (Table 6).

Sorting experiments

In order to confirm the nature of antitumor effector cells in cultures of TIL expanding in rIL2, we selected cultures containing appreciable levels of Leu19+ cells, labeled the cultured cells with monoclonal antibodies Leu4 and Leu19, and performed two-color sorting. Following the sort, the separated populations were assayed for antitumor cytotoxicity. Table 7 shows results of a representative experiment performed with TIL obtained from a patient with SCCH&N. The unsorted culture contained considerable proportions of all three (CD3+Leu19−, CD3+Leu19+, and CD3−Leu19+) populations (Table 7). The results with sorted cells indicated that CD3+Leu19+ as well as CD3−Leu19+ cells were responsible for lysis of fresh allogeneic tumor cell targets in this TIL culture (Fig. 6, Table 7). The CD3+Leu19− cells were poorly cytotoxic against fresh carcinoma cells and not cytotoxic against the fresh sarcoma targets. It is of interest that the two effector

Table 3. Cytotoxicity and phenotypic profiles of TIL populations obtained from human solid tumors and expanded in rIL2[a]

Tumor type	Specimen no.	Culture day	Cytotoxicity (LU/10^7 cells)				Phenotype (% positive)		
			K562	RAJI	TU target[b]	TU target[c]	CD3+ Leu19−	CD3+ Leu19+	CD3− Leu19+
Group – I.									
SCCH&N	(23)	10	104	36	ND	5	81	2	5
SCCH&N	(12)	12	160	42	36	26	59	2	3
Ovarian carcinoma	(1)	16	192	62	70	25	92	4	3
SCCH&N	(22)	16	741	105	ND	49	84	2	6
SCCH&N	(19)	19	167	20	26	21	93	3	1
SCCH&N	(12)	20	235	115	40	36	86	0	11
Hepatocellular carcinoma (HCC) (R)		30	377	287	33	63	89	4	6
SCCH&N	(12)	39	134	196	33	21	98	4	1
NSCH&N	(18)	43	4	0	56	ND	88	2	0
Group – II.									
SCCH&N	(25)	9	530	100	ND	29	59	23	0
SCCH&N	(24)	10	875	95	ND	110	68	17	7
SCCH&N	(24)	17	1000	200	ND	87	68	20	9
HCC (W)		30	1725	14	73	7	6	88	1
SCCH&N	(19)	32	115	5	78	2	83	16	1
NSCH&N	(16)	50	30	0	ND	10	43	53	3
SCCH&N	(14)	51	0	0	0	0	65	34	0
Ovarian carcinoma	(2)	51	8	5	31	ND	53	14	8
Ovarian carcinoma	(1)	51	48	39	30	ND	50	42	8
SCCH&N	(12)	72	44	89	0	4	70	10	10
Group – III.									
SCCH&N	(28)	6	940	ND	515	ND	61	6	26
SCCH&N	(27)	8	752	181	ND	184	64	4	20
HCC (V)		14	1530	498	141	203	7	4	85
Ovarian carcinoma	(3)	16	1232	218	ND	118	31	22	30
Ovarian carcinoma	(2)	19	860	210	138	33	51	3	42
NSCH&N	(18)	24	416	86	84	30	79	1	17
SCCH&N	(17)	43	410	400	134	45	3	5	91
		[d]	$P < 0.005$	$P < 0.025$	$P < 0.05$	$P < 0.05$			

[a] The TIL cultures listed were monitored during their expansion in long-term cultures in rIL2 for antitumor cytotoxicity and for the percentages of cells expressing the Leu19 and/or CD3 antigens

[b] Fresh tumor cell targets were: sarcoma cells for TIL from SCCH&N and ovarian carcinoma, allogeneic hepatocellular carcinoma for TIL from liver tumor

[c] Fresh tumor cell targets were: renal cell carcinoma cells for TIL from SCCH&N and ovarian carcinoma allogeneic hepatocellular carcinoma for TIL from liver tumors

[d] Differences in cytotoxicity between Group-III and Group-I cultures were calculated for each target using Student's *t*-test

cell populations expressing the Leu19 antigen exhibited very high NK cell activity as well as LAK activity. The CD3+Leu19− cells lysed less efficiently. For example, as shown in Table 8, TIL obtained from an ovarian carcinoma and expanded in rIL2 for 10 days showed very high cytotoxicity on K562 targets (16,905 LU). After sorting, it became apparent that the two Leu19+ cells mediated this activity, whereas CD3+Leu19− cells were relatively poorly effective. The same pattern was seen with Raji and fresh allogeneic/autologous tumor cells (Fig. 6). Also, it should be noted that the cytotoxicity levels of the unsorted cultures were generally lower than those measured with purified effector cells (Tables 7, 8 and Fig. 6).

Discussion

Tumor infiltrating lymphocytes from human solid tumors have been of considerable interest for the last two decades, largely because of their presumed importance in tumor growth. Experimental murine models of tumor growth and metastasis supported the notion that TIL represented an immune response of the host to the tumor [1, 12]. Until recently, functional studies of human TIL were not feasible due to the paucity of these cells in tumor cell suspensions [22]. In a few cases where it was possible to examine proliferative and cytotoxic activities of human TIL, they were found to be depressed [4, 9, 11]. More recent availability of a T cell growth factor (TCGF) facilitated studies of human TIL and their in vitro culture [21]. The TCGF-expanded populations as well as microcultures of TIL obtained by limiting dilution exhibited a broad antitumor cytotoxicity profile [20, 24], and autologous tumor reactivity, when assessed, was rarely demonstrable [5, 13]. The poor in vitro proliferation of TIL in response to mitogens and alloantigens was shown to be an intrinsic property of TIL, possibly a result of tumor-induced inhibition [9, 10, 17].

Table 4. Cytotoxicity and phenotypic profiles of mononuclear cells (MNC) obtained from the peripheral blood of patients with cancer and expanded in rIL2[a]

MNC	Specimen no.	Culture day	Cytotoxicity (LU/10^7 cells)				Phenotype (% positive)		
			K562	RAJI	Cultured TU target[b]	Fresh TU target[c]	CD3+ Leu19−	CD3+ Leu19+	CD3− Leu19+
Group − I.									
SCCH&N	(27)	8	1017	52	ND	124	40	47	11
SCCH&N	(25)	9	1270	225	ND	95	52	21	6
SCCH&N	(23)	10	332	23	ND	39	65	23	4
NSCH&N	(16)	18	476	205	550	82	76	9	11
SCCH&N	(14)	23	268	173	7	11	84	9	3
NSCH&N	(18)	24	103	65	190	92	78	17	3
NSCH&N	(18)	45	15	12	85	0	50	49	1
NSCH&N	(18)	56	4	ND	0	ND	53	47	0
Group-II.									
SCCH&N	(28)	6	2255	ND	1025	ND	55	8	23
SCCH&N	(26)	8	1241	326	ND	263	30	18	35
SCCH&N	(21)	8	1080	240	ND	53	51	12	35
SCCH&N	(19)	8	1800	630	940	170	22	10	62
SCCH&N	(24)	10	1490	270	ND	313	56	13	17
SCCH&N	(21)	16	570	52	ND	31	48	14	24
SCCH&N	(24)	17	1380	1003	ND	170	66	12	20
SCCH&N	(19)	33	625	224	220	3	22	48	30
SCCH&N	(17)	43	1440	105	170	48	3	5	91

[a] See the footnote to Table 3
[b] Cultured tumor cell targets were IGR3 melanoma cells
[c] Fresh tumor cell targets were sarcoma cells

Table 5. Cytotoxicity and phenotype profiles of MNC obtained from the peripheral blood of normal volunteers and expanded in rIL2[a]

MNC	Specimen no.	Culture day	Cytotoxicity (LU/10^7 cells)				Phenotype (% positive)		
			K562	RAJI	Cultured TU target[b]	Fresh TU target[c]	CD+ Leu19−	CD3+ Leu19+	CD3− Leu19+
	(1)	19	2090	390	560	130	82	7	10
	(1)	44	1380	211	384	ND	31	60	9
	(2)	19	1000	143	164	41	59	25	15
	(2)	26	1770	630	1310	170	30	47	23
	(3)	19	2570	670	1150	120	46	8	37
	(4)	19	2160	1010	164	90	11	13	74
	(4)	26	1920	790	1144	290	4	11	84
	(4)	51	3712	1096	1033	119	1	15	83
	(4)	58	2093	151	ND	18	<1	16	84

[a] See the footnote to Table 3
[b] Cultured tumor cell targets were IGR3 melanoma cells
[c] Fresh tumor cell targets were sarcoma cells

Recent development of methods for culturing of human lymphocytes in long-term cultures with rIL2 [12] has revived interest in reexamining the functional repertoire of TIL from human solid tumors. These methods allowed Rosenberg and his colleagues [16] to expand murine TIL in rIL2 and demonstrate that they were 50 to 100 times more effective than rIL2-cultured spleen cells in reducing established pulmonary metastases. On the basis of such results, these authors suggested that TIL from human tumors may achieve a comparable degree of expansion and antitumor effector function [16]. We have recently compared expansion and cytotoxic reactivity of rIL2-expanded TIL and A-PBL from a small series of human SCCH&N and showed that both these cell populations could give rise to antitumor effector cells in long-term cultures in high concentrations of rIL2 [13]. Furthermore, TIL appeared to expand better and retain antitumor cytotoxicity longer in these cultures than A-PBL [3, 25]. These preliminary observations are now extended to a larger series of paired TIL and A-PBL from SCCH&N ($n = 29$) and from non-squamous cell cancer of the head and neck ($n = 4$). In addition, we studied TIL from liver and ovarian tumors to determine if TIL from tumors of different histologic types can be established in culture with rIL2 and retain antitumor cytotoxicity. The experiments reported here confirm the feasibility of establishing long-term cultures in rIL2 of TIL from different human solid tumors.

The TIL obtained from metastatic tumors were less responsive to rIL2 than TIL from primary cancer. They only proliferated after a lag period of 30–40 days or not at all,

Table 6. Phenotypic markers of fresh TIL from human solid tumors and A-PBL prior to expansion in IL2[a]

% Positive cells SCCH&N paired TIL/A-PBL ($n = 9$)		
Phenotype	TIL	A-PBL
CD3+	74 (70–94)	66 (52–81)
CD2+	83 (65–94)	85 (56–91)
CD4+	30 (13–60)	42 (13–64)
CD8+	40 (9–60)	31 (14–53)
Leu19+	3 (1–7)	8 (1–25)*
CD16+	4 (0–60)	19 (0–74)*
CD3+Leu19+	1 (0–5)	2 (0–7)
CD3+IL2R+	6 (1–37)	5 (2–13)
CD2+HLA−DR+	32 (3–59)	4 (1–9)*

TIL only		
Phenotype	Hepatic tumors $n = 7$	Ovarian carcinoma $n = 5$
CD3+	75 (60–90)	72 (68–94)
CD2+	78 (69–91)	87 (69–95)
CD4+	30 (20–55)	42 (9–70)
CD8+	23 (22–57)	32 (25–40)
Leu19+	5 (1–20)	9 (7–11)
CD16+	3 (2–21)	13 (10–63)
CD3+Leu19+	2 (0–7)	3 (2–8)
CD3+IL2R+	7 (0–27)	5 (3–49)
CD3+HLA−DR+	20 (7–30)	5 (2–87)

[a] TIL were isolated from fresh tumor biopsies as described in *Materials and methods*, stained with monoclonal antibodies and analyzed by two-color flow cytometry. Asterisks indicate significant ($P < 0.05$) differences between TIL and A-PBL. Data are presented as median percentages with ranges given in parenthesis

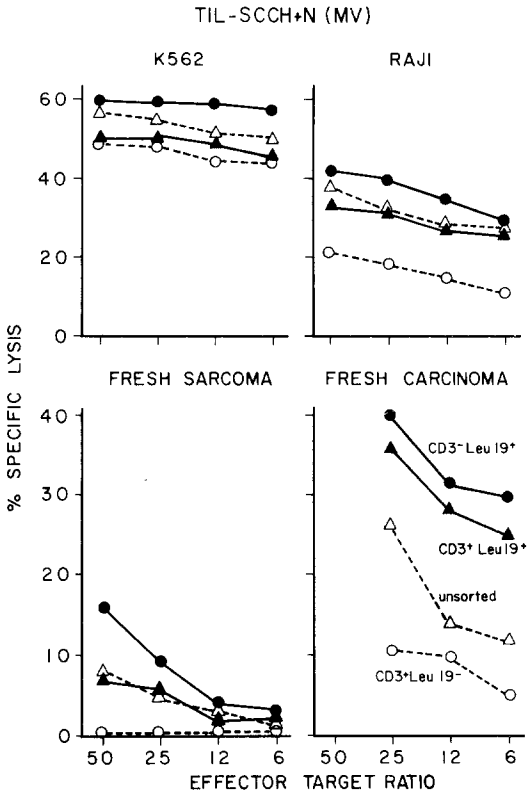

Fig. 6. Cytotoxicity assays performed with the effector cells obtained by sorting of a representative long-term TIL culture established from a patient with squamous cell carcinoma of the head and neck. Both cultured and fresh tumor cells were used as targets

and this in vitro behavior was seen with TIL from the head and neck, liver, and ovarian tumors. Furthermore, rIL2-expanded TIL from metastatic tumors had low antitumor cytotoxicity (manuscript in preparation) or, even when they vigorously expanded (as in case #4, Fig. 3), had little or no cytotoxic activity against tumor cell targets. At this time, the reasons for decreased responsiveness to rIL2 of TIL from metastatic cancers is unknown and may indeed be related to inhibitory influences at the tumor site [17, 19, 24]. It could be argued that metastatic tumors may be less infiltrated with mononuclear cells or contain fewer activated lymphocytes than primary cancers. Although the former may be true for some tumors, as estimated by immunoperoxidase in situ [3], all TIL cell suspensions prepared for culture with rIL2 were adjusted to the same cell concentration of 1×10^6 TIL/ml. Activated (IL2R+ or HLA-DR+) lymphocytes in metastatic head and neck, liver, and ovarian tumors were enumerated in situ and, whenever possible, in suspensions of TIL and found to be present in a variable proportion (e.g., 8%–20% IL2R+ and 7%–27% HLA-DR+ for liver tumors). In our earlier studies of TIL from primary and metastatic breast carcinoma and other solid tumors including melanoma, glioblastoma, and colon adenocarcinoma, we recovered few HLA-DR+ and IL2R+ T lymphocytes [23, 24]. In view of these discrepancies, which may be attributable to biologic variability between different tumor types, the issue of TIL activation in situ and its relationship to in vitro responsiveness to rIL2 in metastatic versus primary tumors, merits further investigation. Since adoptive immunotherapy with TIL would be particularly attractive in established, metastatic tumors unresponsive to other forms of conventional therapy, it is especially important to assess both in vitro proliferative responses to rIL2 and antitumor cytotoxicity of TIL obtained from human metastatic cancers.

The identity of the antitumor effector cell or cells in rIL2-expanded TIL cultures is of basic and practical importance, but has not been addressed in previous studies with human TIL. In bulk rIL2 cultures, cell populations undoubtedly interact with each other, and the net expansion and cytotoxicity are the sum of cellular interactions. Even in bulk rIL2 cultures, however, the highest antitumor cytotoxicity was found to be associated with the Leu19+ cell populations (Tables 3–5). The Leu 19 antigen, a 200,000 dalton surface glycoprotein is expressed on about 15% of normal circulating lymphocytes in humans [6], and this subset of lymphocytes has been shown to contain both NK cells and T lymphocytes that mediate MHC-unrestricted cytotoxicity [6, 18]. In fresh TIL, Leu19+ cells ranged from 1% to 7% in SCCH&N, 1% to 20% in liver tumors, and 7% to 11% in ovarian carcinomas. Two-color phenotyping disclosed the presence of two Leu19+ cell populations: CD3+Leu19+ and CD3−Leu19+. The former cell type was rare in normal PBL (mean 2%±2 [SD]; $n = 15$), and in fresh TIL suspensions it ranged from 0% to 7% (mean 3%±2 [SD]; $n = 21$). The CD3−Leu19+ cells represented 9%±5 (mean ± SD; $n = 50$) in normal PBL and 1%–20% (mean 7% ± 5 [SD]; $n = 20$) of fresh TIL. In long-term cultures (in 1000 units/ml of rIL2) of TIL, A-PBL, or normal PBL, the percentages of the

Table 7. Analysis of cytotoxicity and cellular phenotypes following sorting of TIL obtained from a patient with SCCH&N and cultured in rIL2[a]

	Cytotoxicity (LU/10^7 cells)		Phenotype (% positive)		
	Fresh sarcoma	Fresh carcinoma	CD3+Leu19−	CD3+Leu19+	CD3−Leu19+
Unsorted	14	29	60	20	10
CD3+Leu19−	0	10	100	0	0
CD3+Leu19+	14	70	9	88	3
CD3−Leu19+	30	129	0	2	98

[a] TIL were isolated from the tumor and expanded in culture with rIL2 as described in *Materials and methods*. The cultured cells were stained with Leu4-phycoerythrin and Leu19-fluorescein isothiocyanate and two-color sorting was performed on FACStar. The sorted cells were incubated for 12 h and then used as effectors in cytotoxicity assays

Table 8. Cytotoxic activity on K562 targets of unsorted and sorted TIL obtained from an ovarian carcinoma and cultured in rIL2[a]

	E/T ratio						
	12:1	6:1	3:1	1.5:1	0.8:1	0.4:1	LU
Unsorted	77.4	74.2	78.3	80.5	65.5	52.0	16905
CD3−Leu19+	81.2	81.4	77.4	76.1	71.6	63.1	23910
CD3+Leu19+	69.1	69.0	66.3	67.4	57.5	45.3	11530
CD3+Leu19−	36.1	29.9	18.8	12.2	8.0	3.0	445

[a] TIL were isolated from an ovarian carcinoma, cultured in rIL2 for 10 days, and sorted. Cytotoxicity of the sorted cells was determined at different E : T ratios against K562 targets. Data represent % specific lysis

CD3−Leu19+ cells varied considerably between patients (9%–91%; see Tables 3, 4, 5) and were often lower than those of CD3+Leu19− cells. While the latter cells were the major component of most of our cultures, they exhibited little or no cytotoxicity, as shown in cell sorting experiments. The antitumor activity against fresh tumor targets of different histologic types was associated with CD3+Leu19+ and CD3−Leu19+ cells (Fig. 6) in TIL and A-PBL as well as in normal PBL cultures [3, 26] (Tables 3, 4, 5).

The cells expressing the Leu19 antigen were thus responsible for the cytotoxicity of lymphocytes maintained in long-term cultures with rIL2, while the classic T lymphocytes CD3+Leu19− were not. Preliminary results indicate that the CD3+Leu19+ cells also are CD8-positive, and experiments are underway to further characterize the Leu19+ effectors phenotypically as well as functionally.

Our experiments indicated that antitumor cytotoxicity mediated by the CD3+Leu19+ and CD3−Leu19+ effectors and demonstrable in rIL2 cultures of TIL and PBL is neither MHC-restricted [3, 26] nor autologous tumor-restricted (unpublished data, manuscript in preparation). The presence of Leu19+ antigen on effectors of cytotoxicity against human solid tumor targets suggests that rIL2-activated NK cells (CD3−Leu19+) as well as T cells (CD3+Leu19+) are the major effectors of non-MHC-restricted cytotoxicity. A similar conclusion was reached by Phillips, Lanier and their collaborators on the basis of both in vitro and clinical experiments in humans [6, 7, 14]. On the other hand, the recent finding by the same group of the Leu19 antigen on cultured cell lines may be indicative of a broader expression, not restricted to cytotoxic cells, of this cell surface antigen [8]. Indeed, we have preliminary evidence that in long-term TIL and PBL cultures in rIL2, the Leu19 antigen may be also expressed on noncytotoxic CD3+ cells. Further studies are necessary to delineate the relationship between antitumor cytotoxicity and the expression of the Leu19 antigen on in vivo and in vitro activated effector cells.

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