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Tumor-infiltrating lymphocytes from nonrenal urological malignancies

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Summary. Tumor-infiltrating lymphocytes (TIL) were isolated from 15 of 20 surgical specimens of transitional cell carcinoma of the urinary bladder, prostate cancer, testicular cancer, Wilms tumor and adrenal cancer. Expansion was carried out in four different culture conditions, each containing 1000 U/ml interleukin-2: RPMI medium with or without 20% (by volume) of lymphokine-activated killer cell (LAK) supernatant and AIM V medium with or without 20% LAK supernatant. The resultant cell populations were then assayed for cytotoxicity against a variety of autologous and allogeneic tumor targets and phenotypic analysis was performed with fluorescein-labeled monoclonal antibodies. TIL growth was unrelated to the initial percentage of lymphocytes or tumor cells present in the enzymatically dispersed specimens or whether fresh or cryopreserved tissue was utilized. Better growth was seen in AIM V than in RPMI medium ($P = 0.013$); the beneficial effect of the addition of LAK supernatant to RPMI was indicated ($P = 0.065$), and the addition of LAK supernatant to AIM V did not improve the ability to culture TIL ($P = 0.5$) from these cancers. TIL in long-term culture were predominantly $CD3^+$. The ratio of $CD4^+/CD8^+$ cells varied with time in culture and culture medium, but most cultures eventually became $CD4^+$. Cells bearing B cell, natural killer cell, and macrophage markers disappeared early in culture. Overall 14/15 TIL samples were lytic against one of the autologous and allogeneic targets tested, but specific lysis against the autologous tumor from which it was derived was seen in only one TIL culture originating from a bladder cancer. Our results suggest that TIL can be expanded to therapeutic levels from a variety of urological malignancies and that their potential role in future therapy should be further explored.

Introduction

Adoptive immunotherapy represents a novel form of cancer therapy in which lymphoid cells with antitumor activity are transferred to the tumor-bearing host. This approach is based on an attempt to amplify an immunological defense mechanism which may be present, but ineffective in the tumor-bearing host.

Tumor-infiltrating lymphocytes (TIL) comprise a lymphoid subset which is found within solid tumors and which can be selectively expanded from enzymatic digests of tumors cultured in the presence of interleukin-2 (IL-2). We have been able to grow TIL that could eliminate metastatic disease in murine colon adenocarcinoma and sarcoma models [15].

Utilizing techniques similar to those developed in the mouse we developed techniques for the generation of TIL from a variety of human tumors. TIL were successfully expanded from 24 of 25 consecutive human tumors, including 6 melanomas, 10 sarcomas, and 8 adenocarcinomas. Lymphocytes constituted from 3% to 74% of the single-cell tumor suspensions, and expanded from 2.9-fold to 9.1×10^8-fold over a culture period ranging from 14 to 100 days. Of 24 cultures, 9 lysed fresh autologous tumor targets in 4-h chromium-release assays; 2 cultures exhibited lysis restricted to the autologous target [18].

TIL were characterized from 37 consecutive fresh human renal cell cancer specimens and exhibited better growth in vitro than did peripheral blood lymphocytes obtained from the same patient and grown under identical conditions. Initially the majority of TIL were T cytotoxic/suppressor cells ($CD3^+8^+$), though with continued in vitro expansion (up to 50 days) there was a concomitant increase in the helper T ($CD3^+4^+$) cells and decrease in $CD8^+$ and HLA-DR^+ cell [1, 2].

Based on the therapeutic effects of TIL in murine tumor models and the technical feasibility of isolating and expanding potentially therapeutic TIL from various human cancers, clinical protocols utilizing TIL have been initiated [9, 16, 20]. Using therapy with TIL and IL-2, 11 objective responses were seen in 20 patients with metastatic melanoma, a type of cancer for which no effective chemotherapy exists [16]. This early success prompted us to evaluate TIL further and to study its potential applicability to the treatment of other malignancies.

The current study describes our efforts to grow and characterize TIL from common urological malignancies such as testicular, prostate, bladder and Wilms tumors.

Materials and methods

Tumor specimen. Tumor specimens were obtained directly from the operating room. The size of the tumor available for study varied according to the size of the surgical specimen and the amount of tissue required by the pathologist to make the histological diagnosis, but ranged from 2 g to 20 g. Using a sterile technique, the tumors were dissected

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into approximately 5-mm^3 fragments, which were immersed in medium consisting of RPMI 1640 (Whittaker Bioproducts, Walkerville, Md), hyaluronidase type V 0.01%, deoxyribonuclease type I 0.002%, collagenase type IV 0.1% (Sigma Chemical Company, St. Louis, Miss), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (NIH media unit), 50 μg/ml gentamycin (Gibco, Grand Island, NY) 20 μg/ml amphotericin (Flow Laboratories Inc., McLean, Va) 2 mM glutamine and 10 mM HEPES buffer (Biofluids, Rockville, Md). The mixture was stirred overnight at room temperature and filtered through a wire mesh to exclude undigested material. The cell suspension was washed three times in Hanks' balanced salt solution (HBSS) without calcium, magnesium or phenol red (Whittaker Bioproducts) and centrifuged each time at 400 *g* for 5 min. The suspension was layered over a density separation gradient (Lymphocyte Separation Medium, Bionetics, Kensington, Md) and centrifuged at 900 *g* for 15 min. The gradient interface containing predominantly viable lymphocytes, monocytes, and tumor cells was harvested and washed three more times in HBSS. The viable cell concentration was determined by trypan blue exclusion. The percentage of lymphocytes and tumor cells contained in the suspension was determined by cytological examination by a pathologist (D. S.).

Cytological analysis. Cell-suspension preparations of tumors were evaluated by routine cytological techniques to determine the percentages of tumor cells, lymphocytes, and monocytes present. In addition, immunocytochemistry was performed to identify specific lymphoid subsets in some tumor specimens.

Approximately 10^5 cells and 50 μl 22% bovine serum albumin (Ortho Diagnostic Systems, Raritan, NJ) were layered on glass slides using a Shandon cytospin (Shandon Inc., Pittsburgh, Pa) and air-dried. One slide was stained with Diff-Quik (American Scientific Products, McGaw Park, Ill) for routine cytological evaluation. Remaining slides were stored at 4° C until immunocytochemistry could be performed, usually within 24–48 h. Immunocytochemistry, using the avidin-biotin-peroxidase method was performed to determine lymphoid subsets present in the initial tumor preparation. A panel of monoclonal antibodies directed against a variety of leukocyte markers was employed: monoclonal antibodies included those directed against T-cell antigens CD3, CD4, and CD8; B-cell antigen CD22; macrophage marker CD14; HLA-DR (all the above, Becton-Dickinson, Mountain View, Calif); and leukocyte common antigen (Dako, Santa Barbara, Calif). An epithelial marker, anti-cytokeratin (Becton-Dickinson) was included in order to highlight the tumor cell population.

Culture of tumor-infiltrating lymphocytes. The single-cell solution was diluted to 5×10^5 viable cells/ml in four different culture conditions: (a) RPMI 1640 containing 10% heat-inactivated human serum, gentamycin, streptomycin, penicillin, amphotericin glutamine, HEPES buffer at the concentrations described above and 1000 U/ml recombinant interleukin-2 (rIL-2) (RPMI); (b) the above medium plus 20% by volume LAK cell culture supernatant (derived from culture of human peripheral blood lymphocytes PBL as previously described) (RPMI + LS); (c) AIM V solution (Gibco, Grand Island, NY) containing gentamycin, streptomycin, penicillin, amphotericin, glutamine, HEPES buffer, and 1000 U/ml rIL-2 (AIM); and (d) the above solution with 20% (by volume) LAK cell culture supernatant (AIM + LS). The cultures were maintained in 10-ml aliquots in 6-well culture plates (Costar, Cambridge, Mass) at 37° C in a humidified 5% CO_2 incubator. Cultures were harvested and resuspended to 2.5×10^5 TIL/ml in fresh medium with 1000 U/ml rIL-2 when the cells became confluent.

Cryopreservation of tumor cell suspension. Following preparation of the single-cell suspension, the cells were resuspended in 90% human AB serum (Advanced Biotechnologies, Silver Spring, Md) and 10% dimethylsulphoxide (Sigma) at 4° C. Using a programmed freezer (Virtis, Gardiner, NY) 1-ml aliquots were brought to a temperature of −40° C over 40 min and then stored in a liquid-nitrogen freezer at −180° C.

Phenotypic analysis. Lymphocytes were washed with cold staining buffer (HBSS without phenol red, with 5% heat-inactivated fetal calf serum and 0.02% sodium azide) and resuspended at 1×10^6 cells/ml in individual test-tubes. The tubes were centrifuged at 400 *g* for 5 min and all supernatant was removed. Samples of 0.015 ml fluorescein-conjugated monoclonal antibodies (Becton-Dickinson, Mountain View, Calif) were added separately to each tube. The antibodies used (and cell populations recognized) were: CD3 (T cell), CD8 (T cytotoxic/suppressor cell), CD4 (T helper/inducer cell), CD57 (NK and some T cells), CD56 (NK cells), CD20 (B cells), CD14 (macrophages) and HLA-DR (class II major histocompatibility complex MHC). After a 60-min staining period, the cells were washed twice with staining buffer and fixed with 1% paraformaldehyde. The cells were then stored at 4° C for 1–5 days until flow cytometric analysis was performed using a FACS 440 microfluorometer interfaced with a Consort 40 computer (Becton-Dickinson).

Chromium-release assay for cytotoxicity. The cytotoxic activity of TIL was tested in vitro in a standard 4-h ^{51}Cr-release assay against autologous, multiple allogeneic fresh tumor targets, and the cultured human tumor targets, Daudi (NK-resistant lymphoma cell line maintained in culture by serial passage) and K562 (NK-sensitive myeloid leukemia cell line maintained similarly). All steps of the assay were performed in RPMI 1640 with 2% human serum. The cryopreserved autologous and allogeneic targets were thawed and washed three times prior to labeling. Cultured tumor targets were washed similarly. After the last wash, 200 μCi $Na_2{}^{51}CrO_4$ (Amersham Corporation, Arlington Heights, Ill) in 0.2 ml was added to each pellet and the suspensions were incubated at 37° C for 60 min. Cells were then washed twice, reincubated for an additional 30 min, and after a third wash they were filtered through Nytex-110 (Lawshe Instrument Co., Rockville, Md) to remove clumps. A single-cell suspension was prepared and 5000 viable cells in 0.05 ml media were added to wells of 96-well round-bottom plates. TIL effectors were washed three times and used at an effector:target cell ratio of 10, 40, and 100. Each experiment included 3–4-day LAK effector cells (from normal human donors) as a control. Maximum target lysis was determined by incubation of cells with 2% sodium dodecyl sulphate detergent. All measurements

Table 1. Growth of tumor-infiltrating lymphocytes[a]

Histology	No. of specimens		(%)
	Total	Growth	
Testicular	4	3	(75)
Bladder	8	7	(88)
Prostate	4	4	(100)
Adrenal	2	0	(0)
Wilms	2	1	(50)
Total	20	15	(75)

[a] > 100-fold expansion in any medium

were performed in triplicate. The plates were centrifuged at 50 *g* for 5 min and incubated at 37° C for 4 h. Cultures were harvested with the Skatron-Titrek system (Skatron, Lier, Norway) and isotope release was measured in a LKB gamma counter.

Cytotoxicity as percentage of lysis was calculated as:

$$\text{cytotoxicity (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

where ^{51}Cr release is measured in cpm. Statistical significance was determined by the Fishers exact test. One lytic

Table 2. Source of tumor-infiltrating lymphocytes

Specimen no.	Patient Age/Sex	Tumor histology	Source of tissue	Prior therapy	Tumor (%)	Lymphocytes (%)	Tissue	Growth	Comments
1	20 M	Testicular embryonal	Pulmonary resection	Chemotherapy	N.A.[a]	N.A.	Cryopreserved	Yes	
2a	28 M	Testicular embryonal	Pulmonary resection	Chemotherapy	76	22	Cryopreserved	Yes	
2b			Resection of neck mass		78	20	Fresh	Yes	
3	29 M	Testicular seminoma	Orchiectomy	None	4	90	Fresh	No	Necrotic
4	58 M	Bladder	Transurethral resection	None	89	9	Fresh	No	
5	59 M	Prostate	Radical prostatectomy	None	86	10	Fresh	Yes	
6	72 M	Prostate	Radical prostatectomy	None	89	7	Fresh	Yes	
7	60 M	Bladder	Tranurethral resection	None	78	20	Fresh	Yes	
8	1 M	Neuroblastoma	Adrenalectomy	Chemotherapy	N.A.	N.A.	Fresh	No	
9	4 M	Wilms	Nephrectomy	None	N.A.	N.A.	Cryopreserved	No	
10	67 M	Prostate	Radical prostatectomy	None	89	9	Fresh	Yes	
11	47 F	Bladder	Cystectomy	Intravesical chemotherapy, phototherapy	35	60	Fresh	Yes	
12	60 M	Adrenal cortical	Adrenalectomy	None	N.A.	N.A.	Fresh	No	Necrotic, infected
13	68 M	Prostate	Radical prostatectomy	None	79	19	Fresh	Yes	
14	64 M	Bladder	Cystectomy	None	82	7	Cryopreserved	Yes	Fresh culture infected
15	68 M	Bladder	Transurethral resection	None	N.A.	N.A.	Fresh	Yes	
16	61 M	Bladder	Transurethral resection	None	N.A.	N.A.	Fresh	Yes	
17	83 M	Bladder	Transurethral resection	None	N.A.	N.A.	Fresh	Yes	
18	71 M	Bladder	Cystectomy	None	56	25	Fresh	Yes	
19	7 F	Wilms	Nephrectomy	None	97	2	Fresh	Yes	

[a] N.A., not available

Table 3. Expansion of tumor-infiltrating lymphocytes (TIL)

TIL	RPMI		RPMI + LAK supernatant		AIM V		AIM V + LAK supernatant	
	Time of growth[a, b] (days)	Expansion (-fold)[c]	Time of growth (days)	Expansion (-fold)	Time of growth (days)	Expansion (-fold)	Time of growth (days)	Expansion (-fold)
1	106	5.1×10^5	96	6.8×10^6	N.G.[d]		N.G.	
2 A	69	3.4×10^4	N.G.		78	2.8×10^6	26	5.5×10^3
2 B	N.G.		90	3.6×10^4	90	1.6×10^8	19	9.4×10^3
5	N.G.		N.G.		62	2.6×10^3	77	4.2×10^4
6	N.G.		N.G.		51	1.8×10^3	87	6.9×10^4
7	97	9.2×10^7	113	1.5×10^9	118.	5.3×10^{12}	118	1.4×10^{11}
10	N.G.		N.G.		64	4.8×10^4	64	1.4×10^4
11	27	7.3×10^2	43	1.7×10^3	N.G.		23	2.9×10^2
13	N.G.		71	1.4×10^4	66	3.2×10^3	66	2.0×10^3
14	N.G.		N.G.		24	4.3×10^2	54	1.7×10^4
15	N.G.		56	1.1×10^2	70	1.2×10^2	131	8.6×10^8
16	N.G.		85	$4 \times 5 \times 10^2$	107	1.1×10^7	107	1.2×10^7
17	N.G.		108	1.2×10^7	N.G.		N.G.	
18	77	3.0×10^4	100	4.3×10^4	114	3.3×10^5	N.G.	
19	N.G.		61	3.8×10^3	90	3.1×10^7	N.G.	
Total cultures with growth	5/15		10/15		12/15		11/15	

[a] Last day of expansion before culture became static or non-viable
[b] Growth = > 100-fold expansion
[c] Fold increase from original lymphocyte concentration
[d] N.G., No growth

unit was defined as the number of effector cells required to cause 20% lysis of 5×10^3 tumor target cells in a 4-h chromium-release assay.

Results

Expansion of TIL cultures. Tumor-infiltrating lymphocytes were successfully grown (more than 100-fold expansion of lymphocytes) from 15 of 20 (75%) cultures (Table 1) obtained from 19 different patients. At least two attempts were made to expand TIL from each tumor that failed to grow lymphocytes. The characteristics of the patient and tumor are presented in Table 2. With the exception of 1 patient with bladder cancer, 2 patients with primary testicular cancer, and the patient with neuroblastoma, no patient had received therapy other than surgery. Tissue was procured from metastatic deposits from 2 patients and from the primary tumor in 17. Sixteen cultures were plated fresh from the enzymatic digests, the others were started from cryopreserved tumor suspensions. TIL were successfully grown from 12/16 (75%) fresh and from 3/4 (75%) of cryopreserved tissues.

The lymphocytic infiltrate ranged from 2% to 60% of the tumor cell suspensions. One testicular and one adrenal tumor were mostly necrotic. In several tumors the total cell yield was insufficient to allow for both cytopathological analysis and the use of fresh tumors as targets in lysis assays. In general, $(1-2) \times 10^7$ fresh cells from the tumor suspension were adequate to initiate TIL cultures, but $(5-10) \times 10^7$ additional cells were required as target cells to carry out sequential cytotoxicity studies. The greatest difficulty in obtaining sufficient amounts of tissue for testing was encountered with prostate cancer and, in fact, several specimens offered for the study had to be rejected because of the small size of the tissue (< 2 g). The percentage of lymphocytes in the single-cell tumor suspension did not correlate with the ability to grow TIL or the amount of culture expansion. Culture growth was characterized by the gradual disappearance of tumor cells and a gradual increase in lymphocytes. There was usually no visually detectable tumor in growing cultures after 10–20 days. The first passage was usually performed in 5–7 days for fresh cultures and 10–14 days for cultures derived from cryopreserved specimens. Subsequent passages were usually performed weekly until the late states of the cultures, when the TIL growth rate decreased. After the initial lag phase, which was greater in cryopreserved specimens, there were no differences seen in the expansion patterns of TIL derived from fresh versus cryopreserved tumor suspensions. The rate of expansion was greatest in the early phases of the cultures. The cultures were expanded until growth ceased and viability decreased.

Effect of growth media

Table 3 lists all TIL cultures that expanded at least 100-fold from the initial number of lymphocytes under any of the culture conditions.

In RPMI-based medium without LAK supernatant, 5 of 15 (33%) TIL cultures grew. The mean culture duration to the last expansion was 75 days (range 27–106 days) and the range of expansion was 7.3×10^2–9.2×10^7-fold. The addition of 20% LAK supernatant to the basic medium resulted in growth of 10 of 15 (67%) ($P = 0.071$) cultures, the mean culture duration was 82 days (43–113 days) with 1.1×10^2–1.5×10^9-fold expansion. In AIM V, 12/15 (80%) TIL cultures grew for a mean of 78 days (24–118 days) and a 1.2×10^2–5.3×10^{12}-fold expansion. When 20% LAK

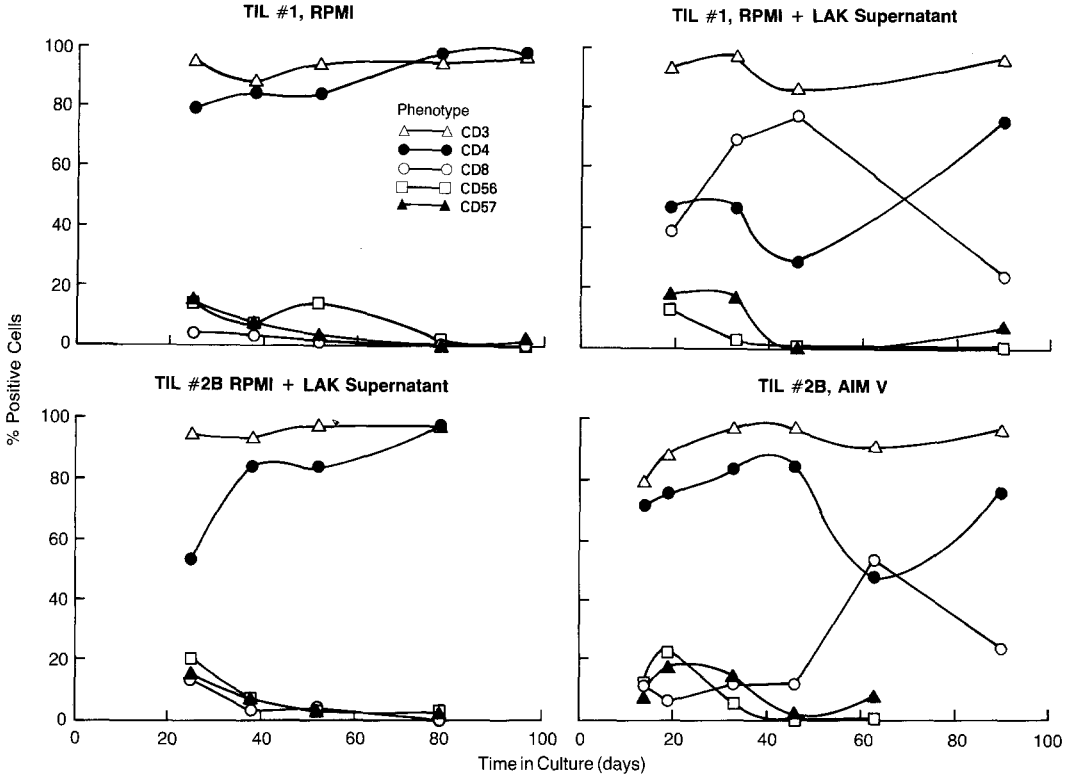

Fig. 1. Phenotypic variations of tumor-infiltrating lymphocytes. *Top left:* TIL culture 1 grown in RPMI are predominantly $CD3^+4^+$ during the entire course of growth. *Top right:* same TIL grown in RPMI and LAK supernatant also consist of mostly $CD3^+$ cells; however, the ratio of CD4/CD8 varies with time with a primarily $CD8^+$ cell population present at day 48. *Bottom left:* TIL culture 2b, grown in RPMI and LAK supernatant, are mostly $CD3^+4^+$ from day 40. *Bottom right:* TIL culture 2b, grown in AIM V, are initially $CD3^+4^+$; however, at day 62 a decline in this population is paralleled by the emergence of $CD3^+8^+$ cells. Eventually the culture reverts to $CD3^+4^+$. $CD56^+$ and $CD57^+$ cells are detected at very low levels only after the initial culture period

supernatant was added to AIM V, 11/15 (73%) TIL grew. The mean lifespan of cultures was 70 days (23–131 days) and a 2.9×10^2–1.4×10^{11}-fold expansion was observed. The use of AIM V medium provided better growth than RPMI in 12/15 (80%) cultures, while 2 cultures grew better in RPMI ($P = 0.013$). One culture failed to grow in either medium. The addition of LAK supernatant to RPMI was beneficial in 9/15 (60%) cultures, detrimental in 1/15 (7%) and did not result in altered expansion in 5 (33%) ($P = 0.065$). The addition of LAK supernatant to AIM V improved expansion of 5 of 15 cultures (33%), decreased it in 6/15 (40%) and resulted in no significant change in 4 (27%) ($P = 0.5$). Thus AIM V medium appears to be a better medium for TIL expansion than RPMI. The addition of LAK supernatant to RPMI appeared to improve TIL growth, although statistical significance was not achieved perhaps because of the small sample size. The addition of LAK supernatant to the AIM V medium offered no benefit.

Phenotypic analysis

The TIL phenotype varied with tumor histology, growth medium, and time in culture. All culture groups consisted mainly of $CD3^+$ cells by the 20th culture day. The majority of cells expressed the class II histocompatibility antigen HLA-DR. There was a concomitant decrease in cells bearing the NK cell or macrophage markers ($CD14^+$, $CD56^+$, or $CD57^+$) and no significant B cell population was observed. Figure 1 provides illustrative examples of the phenotypic variations with times. $CD4^+$ and $CD8^+$ cells varied inversely at all times. Most cultures demonstrated an early predominance of $CD4^+$ cells, and all aging cultures became almost exclusively $CD4^+$ by the time expansion ceased. However, there were two patterns seen during the middle of the expansion. Pattern 1, shown in Fig. 1 (upper left, lower left), demonstrates the constant dominance by $CD4^+$ cells with only minimal $CD8^+$ cells present. However, in some cultures, a transient increase in $CD8^+$ cells was observed (Fig. 1, upper right, lower right). This increase was parallelled by a decrease in $CD4^+$ cells. This pattern was unrelated to the histology of the original tumor or specific growth conditions, was most often seen between days 40 and 60, and did not directly correlate with results of cytotoxicity testing.

Cytotoxicity assays. Sequential assays of cytotoxic activity of each TIL culture under the various growth conditions were carried out in 4-h chromium-release assays, and the results are presented in Table 4. All tests were carried out within 24 h of performing an analysis of cell phenotype. All experiments included cryopreserved autologous targets, one or more allogeneic targets of the same or different histology, and the cultured targets Daudi and K562. Each experiment also included as a control, lysis by allogeneic 3–5-day LAK cells.

Table 4. Ability to lyse targets[a]

TIL	LAK lysis[b]	TIL lysis			
		Autologous target	Allogeneic target	Daudi target	K562 target
1	10	10	<2	3	20
2A	2	<2	<2	<2	2
2B	<2	<2	<2	2	9
5	<2	<2	<2	3	>20
6	<2	<2	<2	13	>20
7	8	7	3	10	10
10	2	<2	3	6	20
11	<2	<2	<2	4	8
13	10	3	4	13	>20
14	7	4	5	11	>20
15	10	<2	<2	3	13
16	3	8	4	>20	>20
17	4	<2	4	>20	>20
18	<2	<2	5	4	>20
19	<2	<2	<2	<2	<2

[a] Maximum cytotoxicity (LU/10^6 cells) in any experiment
[b] Allogeneic LAK cells from normal human donors tested against the tumor from which TIL were derived

LAK cells had an activity of 2 LU/10^6 cells or greater against 9/15 of the targets (Table 4); 14 of 15 TIL cultures were lytic against at least one of the autologous, allogeneic, Daudi, or K562, types of target cell. One prostate-derived TIL culture showed partial specificity for the autologous tumor, and a bladder-tumor-derived TIL sample was specific for its autologous tumor. TIL expanded from a Wilms tumor were non-lytic at all times tested. All TIL lost their lytic abilities against all targets in the final stages of the culture.

Specifically lytic TIL

TIL with specific cytotoxicity against the autologous tumor were derived from a bladder tumor (case 7). The large papillary tumor was transurethrally resected from a 60-year-old white man, who presented with hematuria. Enzymatic digestion yielded 10^8 cells, of which 2×10^7 cells were used to initiate TIL cultures in the four types of media. The pathological diagnosis was poorly differentiated transitional cell carcinoma, and cytopathological analysis indicated 78% tumor and 20% lymphocytes in the enzymatically digested single-cell suspension. Monoclonal antibody staining results showed 19% $CD3^+$, 10% $CD4^+$, 7% $CD8^+$, 1% B cells, 2% macrophages in the total suspensions (Fig. 2). TIL proliferated well under all four growth conditions. As an example, the phenotype and cytotoxicity assay results of the TIL cultured in AIM V are illustrated in Fig. 3. The proliferating culture reached a density of more than 10^6 cells/ml at 5–7-day intervals, and was resuspended to 2.5×10^5 cells/ml concentration. Cytotoxicity assays and phenotypic analysis were carried out on all groups every 15–20 days. $CD57^+$ cells decreased to minimal levels by day 40. Cells with $CD20^+$ or $CD15^+$ expression were minimal throughout the lifespans of the cultures. The cells were more than 78% $CD3^+$ at all times tested. A gradual increase of the $CD8^+$ cell population from 2% at day 20 to 42% at day 64 was noted in the AIM V group. A similar trend was noted in the other three growth conditions as well, although in the RPMI + LS group the $CD8^+$ cell population reached a maximum of only 5%. After reaching its peak, the $CD8^+$ population gradually declined to baseline levels. The number of $CD4^+$ cells was inversely proportional to the number of $CD8^+$ cells. Although the rise in the $CD8^+$ cell population took place over a slightly different time period in the various growth conditions, when the data were adjusted to the number of doubling times, the peak phenotypic changes coincided in all growth media, suggesting that the frequency of cell doubling rather than the medium or time in culture was the critical factor.

An example of the specific cytotoxicity of this culture for autologous tumor is shown in Fig. 4. Early in the culture period, the TIL lysed K562 targets. As cytotoxicity against Daudi and K562 cells declined after 40 days in culture, specific autologous lysis emerged. In cells cultured in RPMI and RPMI + LS, the emergence of specific lysis was delayed compared to what occurred in cells grown in AIM V and AIM V + LS, but if adjusted for the slight variation in doubling time, the occurrence of specificity coincided among the four groups. Specific lysis for autologous tumor corresponded to or shortly followed the emergence of the $CD8^+$ cell population. By day 80, specific lysis declined, as did the $CD8^+$ cell population, and the cultures grown in AIM V medium became completely non-lytic. Further studies to document the loss of specificity in the RPMI-cultured TIL could not be continued owing to the lack of availability of sufficient autologous tumor cells to serve as targets in lytic assays.

Discussion

It has been observed that animal and human malignancies frequently exhibit lymphocytic infiltrates, which may be evidence of a host immune response to the growing tumor [4, 5, 13]. Since 1980, tumor-infiltrating lymphocytes have been isolated from experimental animal tumors and successfully expanded in medium containing IL-2 [23]. When adoptively transferred to the tumor-bearing host in the presence of IL-2, TIL mediate tumor regression to a 100-fold greater extent than do LAK cells [15]. Encouraging results from animal studies led to the isolation and characterization of TIL derived from solid human tumors of several histologies, including melanoma [8, 18], head and neck [6, 10], lung [9], breast [22], ovarian [7] and renal cell cancer [1, 2]. Many of these studies suggested that tumor-infiltrating lymphocytes have a promising role in immunotherapy and therefore clinical trials have been instituted for patients with metastatic melanoma and a few other selected malignancies [9, 16].

The cytotoxic activity of lymphocytes isolated from the tumors of lung cancer patients against the autologous tumor target was significantly higher than the activity of lymphocytes isolated from peripheral blood [14].

TIL isolated from ovarian tumors grew well in long-term cultures in the presence of 1000 U/ml IL-2; however, specific lysis against the autologous tumor target could not be demonstrated [7]. TIL cultures were most active between days 20 and 30 and most cultures declined in activity by day 80.

The initial clinical success of TIL therapy, in combination with IL-2 and cytoxan in metastatic melanoma patients [16], has led us to investigate the characteristics of

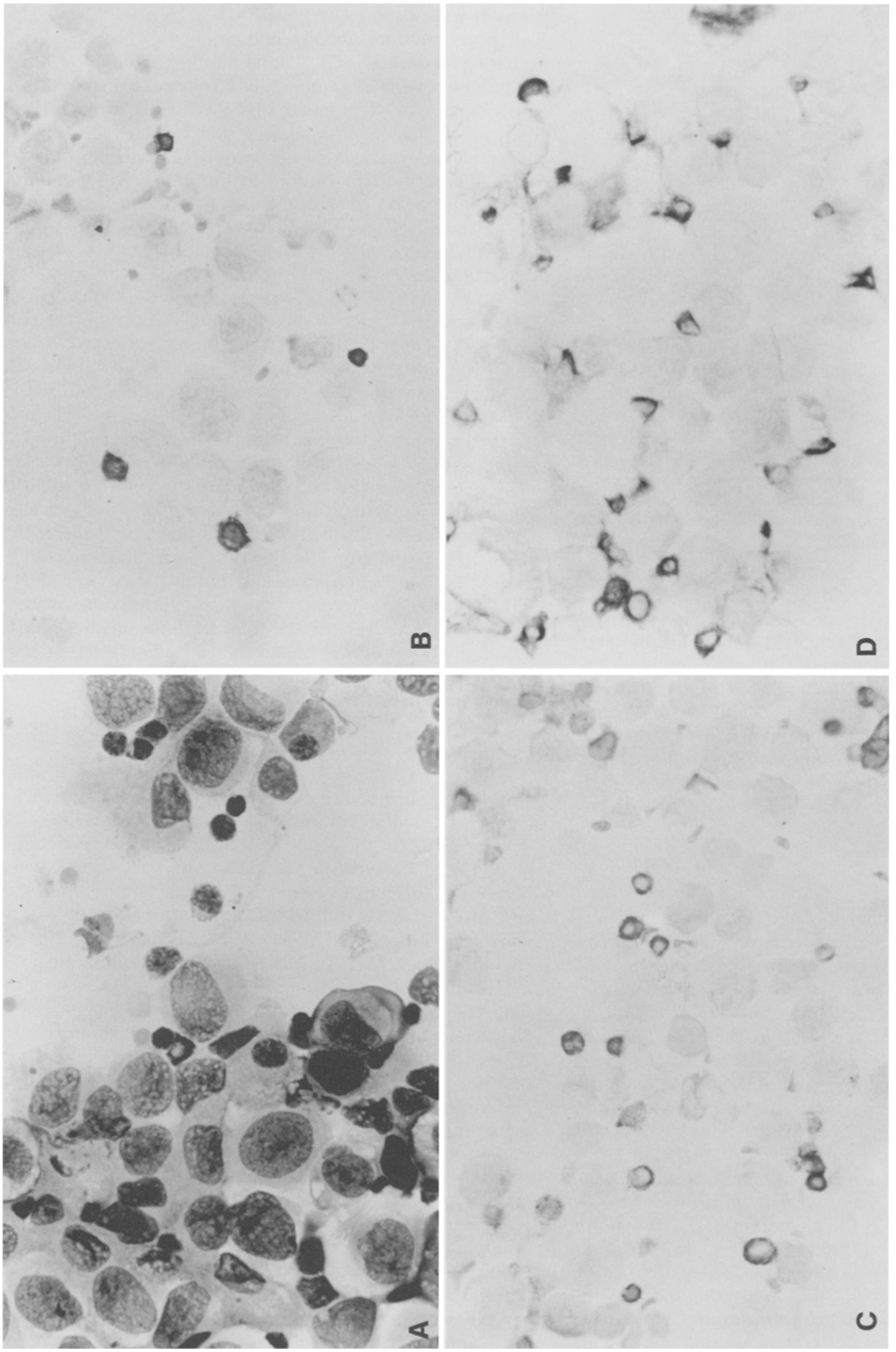

Fig. 2. **A** cell suspension of transitional cell carcinoma of bladder (Diff-Quik stain) shows population of large tumor cells and admixed small lymphocytes. **B–D** Immunocytochemical staining with monoclonal antibodies which highlight lymphoid subsets: **B** $CD8^+$, **C** $CD4^+$, and **D** $CD3^+$. Note that the tumor cells in the background are negative

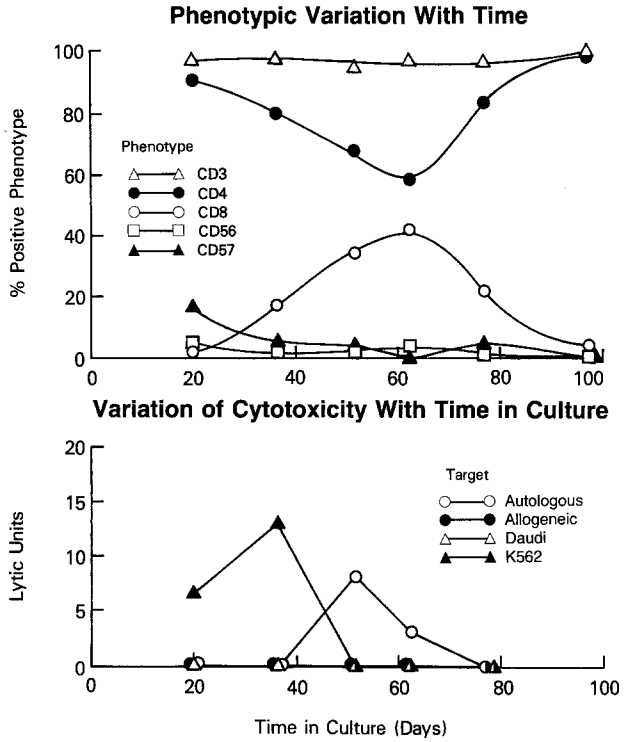

Fig. 3. Characterization of TIL culture 7 grown in AIM V medium. *Top:* phenotypic analysis shows the cell population is $CD3^+$ for the lifetime of the culture. $CD57^+$ cells decline from 17% at day 20 to less than 3% at later time points, and $CD56^+$ cells are minimal at all time points tested. The rise and fall of $CD8^+$ cells, with a peak at 62 days, is inversely proportional to the level of the $CD4^+$ population. *Bottom:* The population of TIL has less than 2 LU/10^6 cells of activity against several allogeneic targets. As activity against K562 declined with time, specific lytic activity against the autologous target was noted. Eventually TIL became inactive against all targets tested

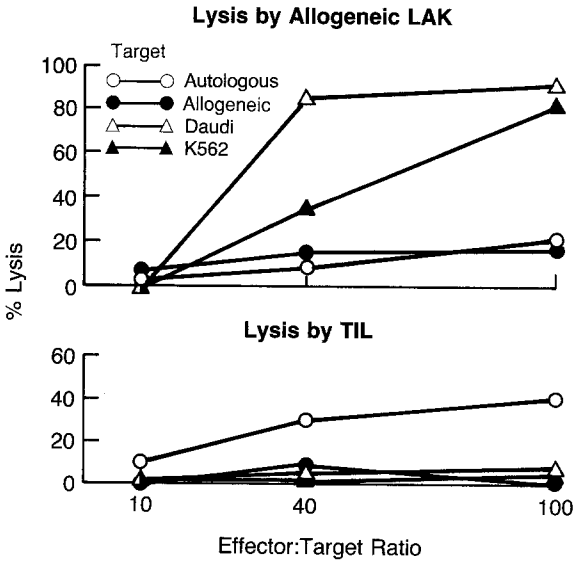

Fig. 4. Example of specific lysis of autologous target by TIL culture 7. *Top panel* demonstrates the ability of allogeneic LAK cells to lyse targets at various effector-to-target ratios. *Bottom panel* shows increasing lysis of the autologous target at increasing effector-to-target ratios, while lysis of the allogeneic cryopreserved, Daudi, and K562 targets was minimal

lymphocytes infiltrating urological malignancies. Belldegrun characterized tumor-infiltrating lymphocytes from renal cell cancer [1, 2] and Tsujihasi et al. investigated the lymphoid population present in bladder cancer initially, and after short-term culture in IL-2 [21]. The latter study noted low spontaneous NK cell activity present in the fresh TIL; however, marked augmentation was noted after short-term culture with IL-2. Cozzolino et al. studied tumor-involved lymph nodes from bladder cancer patients and found a tumor-specific lymphoproliferative response induced by the patient's malignant cells [3].

Our current study describes the isolation and expansion of TIL from common urological malignancies, including testicular, bladder, prostate, adrenal and Wilms tumors. A number of culture conditions were investigated in order to define the optimal conditions, and the groups were compared according to the ability to expand, growth characteristics, phenotype, and the ability to lyse autologous and allogeneic fresh or cultured tumor targets.

Our overall success rate in isolating and expanding TIL at least 100-fold was 75%. TIL were grown from all types of malignancies attempted except adrenal tumors. We were unable to isolate TIL from a neuroblastoma initially treated by multidrug chemotherapy and from an adrenal cortical carcinoma that was predominantly necrotic. A cryopreserved Wilms tumor failed to yield a TIL culture, and TIL cultured from a fresh Wilms tumor were non-lytic against several targets tested. TIL were grown from the majority of the other malignancies tested, namely testicular, bladder and prostate tumors. TIL from one particular bladder tumor (case 7) demonstrated specific lysis of the autologous tumor, suggestive of MHC-restricted lytic specificity against the tumor from which they were derived. This phenomenon was noted in approximately one-third of the melanomas tested, but rarely seen with other histologies [11, 18]. In one study T cells derived from patients with melanoma developed lytic specificity for autologous melanoma cells, and this specificity was enhanced by in vitro stimulation with autologous tumor [17]. Allogeneic LAK did not lyse six of our targets, therefore it is difficult to interpret the lack of autologous TIL lysis of these tumors.

We utilized only a portion of each specimen resected for our experimental protocol, the rest was submitted for pathological examinations in the original institutions. The ability to expand TIL to therapeutic levels is a factor of the original cell number and the ultimate expansion. On the basis of the usual yield of 10^8 total viable cells from each specimen, an expansion of 1000-fold would provide sufficient cells for therapy. Since in the clinical setting the majority of the specimens would be used for the generation of TIL, our results suggest that sufficient numbers of cells would be available from these urological tumors to perform immunotherapy.

The composition of the growth medium appears to be critical to the successful development of TIL therapy. The beneficial effects of LAK cell supernatants have been previously described [19], although the identities of particular components which promote TIL growth are not known. The improved ability of TIL to grow in RPMI medium containing LAK supernatant compared to growth in RPMI alone was suggested in our studies ($P = 0.065$). Compared to the RPMI-based medium, which contains 10% human serum and requires the presence of 20% LAK

supernatant, AIM V is a serum-free growth medium whose composition is well defined [12] and is free of the potential infectious complications associated with the large-scale usage of human serum. Our data indicate that the addition of LAK supernatant to AIM V does not improve the expansion capability of the TIL culture.

Utilization of different culture media for the outgrowth of TIL raises concerns about whether identical subpopulations are being selected. We observed phenotypic variations among some of our parallel cultures in different media; however, these trends were not consistent and they did not correspond to differences in lytic activity.

The relationship between cell phenotype and lytic activity needs further examination. We did not observe a consistent relationship between a particular phenotype and the pattern of cytotoxicity, although a correlation was noted in one culture between transient $CD8^+$ elevations and lytic specificity. This finding confirms the observations of Topalian and others, who found an association between cultures containing predominantly $CD8^+$ cells and high lytic activity [18]. As in the case of TIL derived from melanoma and renal cell cancer, lymphocytes cultured from urological tumors also became predominantly $CD4^+$ and non-lytic with extensive culture. In addition, we also observed an early decrease of $CD56^+$ and $CD57^+$ cell populations and a corresponding decrease in NK cell activity.

These results indicate that TIL from common urological malignancies may be isolated and expanded to levels required for therapy. The existence of MHC-restricted specific lysis in histologies other than melanoma is suggested. This study should lay the groundwork for additional characterization of TIL, further efforts to delineate autologous antitumor specificity, and the methods of expanding TIL for therapy in new clinical protocols directed against metastatic malignancies of the genitourinary tract.

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