

LYMPHOKINE-ACTIVATED KILLER CELL PHENOMENON

Lysis of Natural Killer-resistant Fresh Solid Tumor Cells

by Interleukin 2-activated Autologous

Human Peripheral Blood Lymphocytes

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Although cultured human tumor cell lines have been shown to be susceptible to lysis by a variety of immunologic effector mechanisms including natural killing (NK)¹ and spontaneous cell-mediated cytotoxicity (SCMC) (1–7), few instances of lysis of fresh uncultured human tumor cells have been reported. When lysis of fresh human tumor cells was observed, it was present in only a small percentage of the samples tested (8–11). Recently (12) we reported that peripheral blood leukocytes (PBL) from cancer patients could be activated in culture in preparations of interleukin-2 (IL-2), resulting in the development of effector cells cytotoxic for autologous fresh solid tumor cells in short-term ⁵¹chromium release assays in >90% of cancer patients tested. This paper further characterizes the IL-2-induced antitumor cytotoxicity phenomenon and provides evidence that it represents a previously unappreciated effector system distinct from the NK and the cytotoxic T lymphocyte (CTL) systems.

The IL-2-mediated activation of PBL to become cytotoxic to fresh autologous solid tumor cells probably reflects a common activation mechanism that can unify the growing body of literature describing nonclassical cytotoxicities. Such reports include anomalous cytotoxicities, distinct from CTL during alloactivation (13–17), lectin-activated killing, distinct from lectin-dependent killing (18), fetal calf serum culture-induced killers (16, 19–21), and mixed-lymphocyte tumor interaction (MLTI)-induced killers, found only when proliferation was stimulated (8–11, 22). Though fresh tumor targets were rarely used in those studies, our previous reports of lysis of autologous fresh solid tumor cells after allosensitization (23) or lectin activation (24), and those of others after MLTI (8–11), support this hypothesis and suggest that lymphokine activated killers (LAK) represent a unique and fundamental cytotoxic effector system that may play a role in immune surveillance against NK resistant solid tumor cells, and may have a possible role in the adoptive immunotherapy of tumors.

¹ *Abbreviations used in this paper:* CTL, cytotoxic thymus-derived lymphocytes; CM, complete medium; DMSO, dimethyl sulfoxide; HBSS, Hanks' balanced salt solution; IF, immune interferon; IL-2, interleukin 2; LAK, lymphokine-activated killer cells; LBC, lymphoblastoid cells; MLTI, mixed lymphocyte tumor interactions; NK, natural killer; PBL, peripheral blood mononuclear leukocytes; PHA-P, phytohemagglutinin; SCMC, spontaneous cell-mediated cytotoxicity; TCGF, T cell growth factor, C-TCGF, crude TCGF, PP-TCGF, partially purified TCGF; TDL, thoracic duct lymphocytes.

Materials and Methods

PBL. PBL were obtained from normal volunteers and cancer patients by fractionation of peripheral blood cells on LSM gradients (Litton Bionetics, Kensington, MD) as previously described (23). The cells were washed twice with Hanks' balanced salt solution (HBSS) and resuspended in complete medium (CM) consisting of RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% heat inactivated human AB serum (K. C. Biologicals, Kansas City, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin (National Institutes of Health [NIH] Media Unit). PBL were used fresh or cryopreserved and subsequently thawed when needed. Blood from cancer patients was drawn before surgery or at least 2 wk after surgery.

Fresh Tumor Targets. Single-cell suspensions of fresh autologous tumor were prepared in one of two ways:

FROM MALIGNANT EFFUSIONS. Fresh tumor cells were harvested in heparin (100 U/ml) from the ascites of a patient with breast cancer. The cell suspension was washed into HBSS three times at 100 g for 5 min, and the final cell pellet resuspended in CM.

FROM SOLID SURGICAL SPECIMENS. Fresh-resected tumor was collected from surgery and transported in HBSS at 4°C until processing. Necrotic tumor and connective tissue were removed, and the remaining specimen was minced, using scissors, in HBSS containing 5 U/ml hyaluronidase, 2 mg/ml collagenase, and 0.2–0.3 mg/ml deoxyribonuclease. The fragments were further dissociated by trypsinization by mechanical stirring in flasks with 10–20 ml of 0.25% trypsin in Dulbecco's phosphate-buffered saline with EDTA without calcium or magnesium (NIH Media Unit) for 10 min. The resulting cell suspension was decanted into a tube containing heat-inactivated human AB serum, and the trypsinization repeated with the remaining fragments in the flask, adding more deoxyribonuclease if necessary. The cells in the total suspension were then pelleted and resuspended in 20–50 cc of CM. Some specimens were processed further to eliminate contaminating lymphocytes according to the method of Kedar.² A discontinuous gradient of 10 cc each of 25%, 15%, and 10% Percoll in CM was layered, and 10 cc of the tumor cell suspension was layered on top. The gradient was centrifuged at room temperature for 7 min at 25 g. The layers, bottom (tumor) and top (debris and unwanted cells), were collected separately, washed twice with HBSS, resuspended in CM, and counted for viability in trypan blue. An aliquot was sent for cytologic analysis, and the rest used either immediately or cryopreserved in 90% human AB serum plus 10% dimethyl sulfoxide (DMSO) for future use. All samples were analyzed by the NIH Cytopathology Department using Papanicolaou staining (25) to determine the percentage of tumor cells. The tumor preparations used in the experiments reported in this paper are summarized in Table I.

Removal of Adherent Cells. PBL were incubated at 2×10^6 /ml in CM in petri dishes for 1 h at 37°C. Nonadherent cells were recovered in the media after gentle swirling of the dish. Each dish received one rinse with fresh CM, followed by swirling and aspiration of the nonadherent cells. Nylon-wool adsorption was performed for 1 h at 37°C in CM using scrubbed nylon wool (27).

Serologic Depletion Studies. Cells were washed twice in HBSS and resuspended at 4×10^6 /ml in HBSS at room temperature. The previously determined optimal dilution of monoclonal antibody was added directly to the cells (1:20 dilution for OKT.3 and OKT.8 from Ortho Pharmaceutical Corp., Raritan, NJ; OKM1, 1:20 for 4F2 from Dr. A. Fauci, NIH; and 1:50 for Leu-1 from Becton, Dickinson & Co., Sunnyvale, CA). Incubation was performed for 1 h at 4°C with occasional mixing. The cells were then pelleted by centrifugation, and the second antibody (polyspecific rabbit anti-mouse immunoglobulin, Cedarlane Laboratories, Ontario, Canada) was added at a 1:10 dilution in HBSS. Incubation was continued for 30 min at 4°C. The cells were pelleted, and complement added in HBSS. Newborn rabbit serum was used as a source of complement and was found to be optimal at a 1:6 dilution. Complement lysis was performed at 37°C for 1 h, after which the cells were washed twice in HBSS, resuspended in CM, and counted for viability using trypan blue.

T Cell Growth Factor (TCGF) Preparation and Partial Purification. Crude TCGF was either purchased from Associated Biomedic Systems (Buffalo, NY) or prepared by us using a previously

² Kedar, E. A rapid technique for isolation of viable tumor cells from solid tumors. Use of tumor cells for induction and measure of cell-mediated cytotoxic responses. Manuscript submitted for publication.

TABLE I
Patient-Tumor Systems Used

Patient	Patient		Tumor type	Processing method*	Percent malignant cells
	Age	Sex			
6	52	F	Ovarian cancer	C	NT‡
7	49	F	Ascites, breast cancer	A	82
12	36	F	Osteosarcoma	C	50
13	31	F	Stromal cell sarcoma	B	95
14	69	M	Melanoma	B	94
15	19	F	Osteosarcoma, metastases	B	40
19	61	M	Melanoma	B	90
23	20	M	Synovial cell sarcoma	B	67
25	42	M	Synovial cell sarcoma	B	90
30b	47	M	Melanoma	C	91
30c	47	M	Melanoma	D	78
36	42	M	Undifferentiated sarcoma	C	60
37	42	F	Leiomyosarcoma	B	76
41	40	F	Melanoma	C	72
44	47	M	Melanoma	C	85
45	24	M	Synovial cell sarcoma	C	90
48	23	F	Synovial cell sarcoma	C	72
51	20	F	Synovial cell sarcoma	C	65
52	74	M	Pleomorphic liposarcoma	C	87
53	39	M	Liposarcoma	C	50
54	38	M	Synovial cell sarcoma	C	73
56	58	M	Rhabdomyosarcoma	C	80
57	38	F	Malignant fibrous histiocytoma	C	68
58	17	F	Sarcoma	C	44
59	78	M	Malignant fibrous histiocytoma	C	51
63	56	F	Sarcoma	C	72
64	24	M	Osteogenic sarcoma	C	29
67	19	M	Sarcoma	C	68

* Tumor processing methods used: A, wash only; B, collagenase, hyaluronidase, deoxyribonuclease, and trypsin digestion, followed by percoll gradient; C, cell enzymes as in (B), but no percoll gradient; D, collagenase, hyaluronidase, and deoxyribonuclease only. All tumors were cryopreserved in human AB serum containing 10% DMSO and stored in liquid nitrogen.

‡ Not tested.

discussed modification (27) of our original method (28). In brief, PBL at 5×10^6 /ml were incubated in RPMI 1640 containing 2% human AB serum and 0.2% phytohemagglutinin (PHA-P) (Difco Laboratories, Detroit, MI). The 2-d culture supernatant was collected by centrifugation and used as crude TCGF (C-TCGF). Partial purification and removal of the PHA from these C-TCGF preparations was performed as we described previously (29). In brief, the C-TCGF was brought to 50% saturation with ammonium sulfate and the precipitate discarded. The supernatant was then brought to 75% ammonium sulfate saturation, the precipitate resuspended in water to achieve an $\sim 1/10$ vol of C-TCGF starting sample, dialysed, first vs. water then vs. phosphate-buffered saline, and passed over an affinity column (14×0.9 cm) of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled to rabbit anti-PHA antibody. Tests for TCGF activity were performed using TCGF-dependent human PBL after at least 14 d in culture. Both short-term [3 H]thymidine uptake assays and 7-d growth assays were used to determine optimal TCGF titer (27-29).

Lymphoblast (LBC) Targets. Lymphoblast cells were prepared by incubating fresh PBL at 1×10^6 /ml in CM with 10 μ g/ml Con A for 2 d.

Cultured Tumor Targets. K562, the NK-sensitive myeloid leukemia cell line, and Daudi, the

NK-resistant lymphoma line were cultured in CM (containing human AB serum). These lines were passaged at 1:40 cell dilution every week. Repeated testing for mycoplasma contamination showed that both lines were negative.

In Vitro Sensitization and Partially Purified TCGF (PP-TCGF) Activation of LAK. Primary in vitro sensitization was performed by co-culture of responder PBL with stimulator PBL in Falcon 3013 flasks (Falcon Labware, Oxnard, CA) incubated vertically in CM. Responder PBL were used at a final concentration of 1×10^6 /ml in CM. Irradiation of PBL stimulator cells was performed using a 137 cesium Source. (Isomedex Inc., Parsippany, NJ) at 2,000 rad. Stimulator cells were washed once after irradiation and added at 1×10^6 /ml. For generation of LAK, PP-TCGF was added to 1×10^6 /ml cultures of responder PBL at the dilution determined to be optimal for growth of TCGF-dependent cells (usually a 1:10 for the 10-fold-concentrated PP-TCGF).

Measurement of Cytotoxicity. A 4-h ^{51}Cr release assay was used to measure cytotoxicity of fresh tumor or PBL target cells. Target cells were thawed the morning of the assay, labeled with 400 μCi of $\text{Na}^{51}\text{CrO}_4$ (Amersham Corp., Arlington Heights, IL) for 120 min in 0.5 cc of CM. The cells were then washed four times with CM and added at 5×10^3 cells/well to various numbers of the effector lymphocytes in round-bottomed microtiter plates (Linbro Chemical Co., Hamden, CT). The plates were centrifuged at 500 rpm for 3 min and incubated for 4 h at 37°C, 5% CO_2 . The culture supernatants were harvested with the Skatron-Titertek System (Skatron A.S., Lierbyen, Norway) and counted in a gamma counter. Maximum isotope release was produced by incubation of the targets with 0.1 N HCl. Spontaneous release was produced by incubation of targets with CM alone. Fresh tumor targets were found to express between 10 and 30% spontaneous release during the 4-h assay. The percentage of specific lysis was calculated by the formula: $([\text{experimental cpm} - \text{spontaneous cpm}]/[\text{maximal cpm} - \text{spontaneous cpm}]) \times 100\%$. All determinations were made in triplicate and data are reported as the mean \pm SEM.

Results

Fresh Solid Tumor Cells Are Resistant to Lysis by Autologous PBL Containing NK Cells, But Are Lysed by Autologous LAK Cells. PBL from 10 cancer patients were tested for lysis of the known NK sensitive target cell, K562, and the patients' fresh autologous tumor target cells (Table II). In the same experiment, the PBL were also activated with PP-TCGF and tested for lysis against the fresh autologous tumor cells. In all 10 patients, fresh autologous tumor cells were lysed by the LAK cells. Also, fresh PBL from all 10 patients demonstrated NK mediated lysis of K562, however, no significant lysis of the autologous tumor target cells was found by these same fresh PBL. These experiments demonstrated that fresh PBL from cancer patients contain NK cells as defined by killing of K562 targets, but that these cells are incapable of causing detectable lysis of fresh tumor cells. All 10 fresh tumor samples were lysed by autologous LAK during the same 4-h ^{51}Cr release assay.

In other experiments we found that LAK were generated in 8 of 11 autologous combinations (patients 6, 7, 12-15, 19, 25, 30, 36, and 37, data not shown). In these experiments, lysis of autologous fresh PBL was also tested. Fresh PBL were lysed in four of eight combinations, but at a much lower level than lysis of autologous tumor. The highest lysis of autologous PBL observed was half of the percent lysis of tumor. The 3 of 11 LAK-negative combinations were found to be caused by effector immune incompetence in 2 cases (patients' PBL were unable to respond in an in vitro allosensitization, and patients died within 2 weeks), and in one case the tumor was not lysable by any LAK (neither autologous or allogeneic).

In total, we have found that 18 of 21 (85%) autologous patient-tumor combinations are positive for LAK-mediated lysis of fresh tumor cells, and that 20 of 21 (95%) fresh

TABLE II
Lack of Autologous Fresh Tumor Lysis by Fresh PBL

Patient	Percent specific lysis (\pm SEM)		
	Fresh PBL (effector)/K562 (target)	Autologous tumor	
		Fresh BPL	LAK \ddagger
30b	64.2 \pm 0.5	-3.7 \pm 2.4	89.8 \pm 1.6
	46.3 \pm 0.5	-1.0 \pm 3.6	63.4 \pm 4.8
	19.0 \pm 0.9	-1.6 \pm 7.6	37.3 \pm 4.6
37	44.0 \pm 0.4	-0.9 \pm 0.2	44.1 \pm 2.3
	26.3 \pm 1.3	-0.8 \pm 0.2	34.1 \pm 1.8
	NT \S	NT	22.3 \pm 1.4
48	22.2 \pm 0.2	2.0 \pm 1.6	51.4 \pm 2.8
	8.6 \pm 0.2	0.0 \pm 1.1	40.4 \pm 2.0
	1.6 \pm 0.2	3.4 \pm 0.6	16.3 \pm 0.8
52	14.0 \pm 0.8	-3.6 \pm 0.4	30.0 \pm 0
	4.0 \pm 0.7	-1.7 \pm 0.6	21.0 \pm 1
	1.0 \pm 0.4	1.6 \pm 1.1	13.0 \pm 1.0
54	24.1 \pm 0.0	2.4 \pm 1.8	24.6 \pm 1.9
	14.9 \pm 0.8	3.7 \pm 2.5	9.3 \pm 0.4
	7.3 \pm 0.8	1.8 \pm 0.2	4.8 \pm 0.1
56	24.6 \pm 2.2	-3.2 \pm 3.2	38.6 \pm 8.9
	7.5 \pm 0.0	3.4 \pm 4.0	19.2 \pm 7.2
	1.7 \pm 0.3	3.9 \pm 0.9	6.1 \pm 8.3
57	27.8 \pm 1.2	-7.9 \pm 0.4	50.2 \pm 0.7
	11.4 \pm 1.1	-3.4 \pm 1.0	14.6 \pm 1.2
	3.3 \pm 0.4	1.8 \pm 3.7	5.1 \pm 1.1
58	59.9 \pm 3.3	-5.4 \pm 0.6	27.5 \pm 4.7
	27.3 \pm 0.5	0.4 \pm 5.6	18.4 \pm 0.7
	8.8 \pm 0.4	-4.4 \pm 2.7	3.5 \pm 1.6
63	39.3 \pm 0.3	-0.6 \pm 0.8	43.0 \pm 1.4
	14.4 \pm 0.2	5.6 \pm 4.3	26.2 \pm 3.4
	3.8 \pm 0.2	1.9 \pm 0.8	7.6 \pm 0.4
67	34.9 \pm 0.9	1.0 \pm 1.0	16.1 \pm 1.1
	14.5 \pm 0.7	-3.6 \pm 1.1	11.5 \pm 1.9
	4.7 \pm 0.1	2.8 \pm 0.9	7.2 \pm 0.9

* Fresh PBL were used at effector/target ratios of 100:1, 25:1, and 6:1.

\ddagger PBL were cultured in CM with 10% PP-TCGF to produce LAK, which were tested for lysis at effector/target ratios of 40:1, 10:1, and 2.5:1.

\S Not tested.

tumors are lysable by LAK (either autologous or allogeneic, see Figs. 1 and 2), but that no (0 of 10) fresh autologous tumor cells are lysed by autologous NK.

Fresh Solid Tumor Cells Are Resistant to Lysis by Fresh NK-containing PBL of Normal Individuals. The data in Table II show that PBL from cancer patients expressed NK

	K562	Tumor												
		7	30b	41	44	45	48	14	19	30c	51	53	54	57
Activated PBL														
Normal Fresh PBL 1														
Normal Fresh PBL 2														
Normal Fresh PBL 3														
Normal Fresh PBL 4														
Normal Fresh PBL 5														
Normal Fresh PBL 6														
Normal Fresh PBL 7														
Normal Fresh PBL 8														
Normal Fresh PBL 9														
Normal Fresh PBL 10														
Normal Fresh PBL 11														
Normal Fresh PBL 12														
Normal Fresh PBL 13														
Normal Fresh PBL 14														
Normal Fresh PBL 15														
Normal Fresh PBL 16														
Normal Fresh PBL 17														

FIG. 1. Fresh solid tumor cells are NK resistant and LAK sensitive. The combined data from two 4-h ^{51}Cr release assays is depicted. 13 uncultured solid tumor samples were tested as targets for their susceptibility to lysis by NK-containing PBL of 17 normal individuals, at an effector/target ratio of 150:1. The normal individuals consisted of eight females and nine males, and ranged in age from 19 to 41. PBL from the first eight individuals were tested in both experiments. Lysis was considered positive when the percent specific lysis was statistically significant at the $P \leq 0.01$ level using the Student's t test. Activated PBL are normal PBL from patients 7 and 12 cultured in PP-TCGF for 5 d or allo sensitized. ■, positive lysis; □, negative lysis; □, not tested.

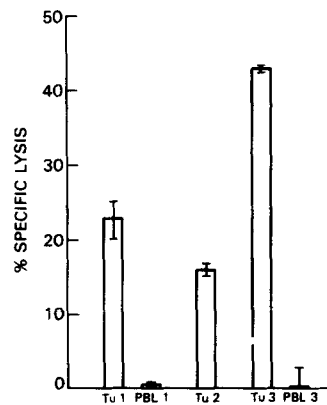


FIG. 2. LAK lyse allogeneic fresh tumor cells. PBL from melanoma patient 14 were activated by culture in PP-TCGF for 7 d and tested for LAK-mediated lysis of fresh autologous tumor and autologous PBL as well as allogeneic PBL and allogeneic tumor 15. Data are shown for the effector/target ratio of 20:1. Tu, tumor; 1, autologous melanoma; 2, allogeneic melanoma; 3, allogeneic osteosarcoma.

activity toward K562 but did not express detectable NK activity toward their fresh solid tumor cells. However, it remained possible that fresh tumor cells were sensitive to lysis by subpopulations of NK cells deficient in cancer patients. Therefore, to determine whether fresh tumor cells were sensitive to any NK-mediated cytotoxicity, we tested PBL from 17 normal individuals for lysis of K562 and for lysis of fresh allogeneic solid tumor cells (Fig. 1).

All tumors were sensitive to lysis by the LAK (26 of 26 tests) and all normal PBL contained high levels of NK activity in 25 of 25 tests directed against K562 targets. However, only 6% of the normal PBL-fresh tumor combinations (15 of 239) displayed significant lysis of the solid tumors, and of those tested a second time, only 2 of 6 were lytic. Of the 13 solid tumor preparations tested, 8 were lysed by PBL from any normal individual, and 3 were lysed by PBL from only a single donor. Two tumor preparations (numbers 41 and 45) were lysed by the PBL from 5 of the 17 donors. 15 of the 17 normal PBL lysed none or only 1 of tumor specimens; 1 donor (patient 15) lysed 2 of the 10 tumor targets and 1 donor (patient 5) lysed 4 of the 13 targets tested.

A Mantel-Haenszel stratified analysis (30) revealed that the lysis of fresh tumor by fresh PBL was significantly less ($P < 0.0001$) than their lysis by the LAK. A chi-square analysis for multinomial observations (31) showed that the susceptibility of fresh tumors to lysis by fresh PBL was not normally distributed, with some tumors more susceptible than expected ($P = 0.0025$). Using the same statistical test, it was also determined that the normal PBL population appeared skewed, with PBL from one normal individual (patient 5) expressing a level of killing not expected to be observed by chance alone ($P = 0.0009$); therefore, it is possible that some normals (~5% based on our 1 of 17) do contain killer cells circulating in their PBL capable of killing fresh tumor. Though these chi-square analyses suggest that fresh PBL from some normals are capable of killing fresh tumor, and that some tumor cells are more susceptible to lysis than others, the main conclusion is that LAK-sensitive fresh solid tumor cells are in general insensitive to lysis mediated by NK cells.

The resistance of uncultured human solid tumor cells to NK and their susceptibility to LAK is not due to any processing artifact of the tumor cells. Most of the target cells used for the experiments in Table II and Fig. 1 were prepared by enzymatic digestion from the connective tissue and ground substance, using a cocktail of enzymes (see Materials and Methods) including trypsin. Our results show that this enzymatic treatment neither creates nor destroys the antigenic determinant(s) recognized by the LAK, because a tumor from patient 7, malignant ascites in a patient with breast carcinoma, received no enzymatic processing and was easily killed by LAK, and not by NK. Melanoma tumor 30b was prepared by using hyaluronidase, collagenase, and deoxyribonuclease, but not by trypsin, and was also not lysed by the NK cells, but was lysed by the LAK. Trypsinization of K562 cells does not affect their lysis by NK cells (32).

These experiments demonstrated that PBL from normal individuals contain NK cells as evidenced by lysis of K562 target cells, but that these whole PBL populations are incapable of lysing the great majority of fresh allogeneic tumor cells. Thus, LAK lysis of uncultured tumor cells is not unique to cancer patients, and therefore cannot be considered as a specific anamnestic response, because PBL from normal, tumor-free individuals can be induced to express this activity.

Fresh Tumor Cells Are Lysed by Allogeneic LAK from Cancer Patients and Normal Individu-

als. As shown in Fig. 1, allogeneic normal LAK cells were capable of lysing fresh tumor from all fresh tumor specimens tested. To further study this phenomenon, PBL from melanoma patient 14 were cultured for 7 d in PP-TCGF and then tested for the expression of LAK activity toward the autologous tumor and PBL and to an allogeneic melanoma and an allogeneic osteosarcoma and PBL (Fig. 2). It was found that allogeneic fresh tumor, but not allogeneic PBL, was lysed. Therefore, the PP-TCGF-induced lysis of allogeneic tumors represents LAK that preferentially kills tumor cells, and not usually the corresponding PBL, suggesting that LAK lysis of allogeneic tumors is not merely an allogeneic response. Lotze et al. (12) reported that allogeneic cultured tumors and fibroblasts were also lysed by TCGF activated PBL. Therefore, LAK killing is not restricted to autologous tumor cells or to single histologic tumor types, but is expressed to allogeneic fresh and cultured lines, implying that the target specificity is broad.

Autologous PP-TCGF Stimulates LAK Lytic for Autologous Tumor. To test whether allogeneic PP-TCGF was required for LAK generation (allogeneic PP-TCGF was used in all other experiments in this paper) and whether PBL from cancer patients were competent to generate TCGF suitable for LAK activation, we prepared PP-TCGF by PHA stimulation of PBL from melanoma patient 14. Culture of fresh PBL from patient 14 with his autologous PP-TCGF did generate LAK (Table III). Patient 14 was known to have a moderate tumor burden at the time of his leukapheresis, and died ~6 mo later. Thus, PBL from cancer patients are competent to produce TCGF. Autologous TCGF stimulates LAK, thereby ruling out the possibility that LAK stimulation depends on an allogeneic effect.

Immune Interferon (IF) Is Not the Primary Stimulus in PP-TCGF. Interferon has been shown to augment NK activity and to stimulate lysis of allogeneic but not of autologous fresh tumors (33, 34). Because IF is known to be present in PP-TCGF, we tested whether IF and not IL-2, as presumed, was the primary stimulus for LAK. To examine this, we tested for LAK development in PP-TCGF depleted of IF by exposure to acid pH. Table IV shows that the IF-depleted PP-TCGF was fully capable of

TABLE III
Autologous PP-TCGF Generates LAK

Dilution PP-TCGF*	Effector/ Target ratio	Percent specific lysis of autologous	
		Tumor	PBL
1:4	40:1	27.7 ± 1	15.7 ± 2.8
	10:1	16.6 ± 0.4	8.5 ± 1.1
	2.5:1	5.2 ± 0.7	-3.0 ± 0.2
1:8	40:1	28.0 ± 2.0	-4 ± 1
	10:1	14.0 ± 0.4	-2 ± 1
	2.5:1	4.8 ± 0.1	—
No PP-TCGF	40:1	0.2 ± 0.7	-3 ± 2.7
	10:1	-9 ± 0.2	-8 ± 1.0

* PBL from melanoma patient 14 were collected by leukapheresis, and PP-TCGF prepared and tested exactly as from normal PBL. A fresh sample of PBL from patient 14 was then collected and cultured in his autologous PP-TCGF for 7 d. The cultures were then tested for LAK activity toward autologous tumor and PBL.

TABLE IV
Interferon-depleted PP-TCGF Maintains Stimulatory Activity

	Percent specific lysis of autologous*	
	Tumor 37	PBL 37
PP-TCGF, 630 U/ml‡	28.9 ± 2.3	3.5 ± 2.4
	10.8 ± 1.8	2.3 ± 2.8
	3.8 ± 0.9	-2.5 ± 0.8
PP-TCGF, <5 U/ml‡§	26.8 ± 4.3	1.2 ± 0.3
	18.0 ± 0.0	0.2 ± 0.2
	13.2 ± 4.8	0.6 ± 1.9

* Effector/target ratios used were 40:1, 5:1, and 1.25:1.

‡ Determination of leukocyte interferon units was performed by Biofluids, Rockville, MD, courtesy of Dr. John Ortaldo, National Cancer Institute.

§ Interferon was denatured by an 8-h dialysis vs. glycine-HCl at pH 2.0, followed by dialysis vs. PBS to restore PP-TCGF to neutral pH.

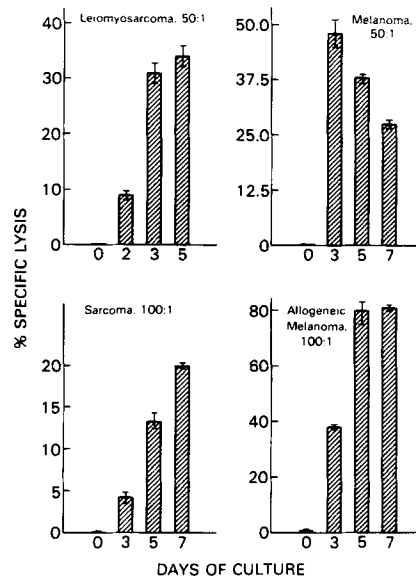


FIG. 3. Kinetics of LAK development. PBL from leiomyosarcoma patient 37, melanoma patient 14, and sarcoma patient 52 were cultured in PP-TCGF and tested for lysis of autologous tumor on the days shown. Patient 52 LAK were also tested for lysis of an allogeneic melanoma tumor 30 b. All assays were for 4 h at effector/target ratios shown.

generating LAK. Therefore, a factor(s) in PP-TCGF other than IF is the primary stimulus for LAK.

Kinetics of LAK Development. Because fresh PBL from cancer patients or normal individuals did not lyse fresh autologous or allogeneic tumor cells (Table I and Fig. 1), but did exhibit this lysis after 7 d in culture in PP-TCGF, we investigated the kinetics of the development of LAK lytic activity. PBL from three cancer patients were cultured in PP-TCGF and assayed for lysis of autologous tumor target cells at various times (Fig. 3). PBL from one patient was also tested for lysis of an allogeneic tumor. In all tests, lytic activity was evident by day 3, and in the one combination

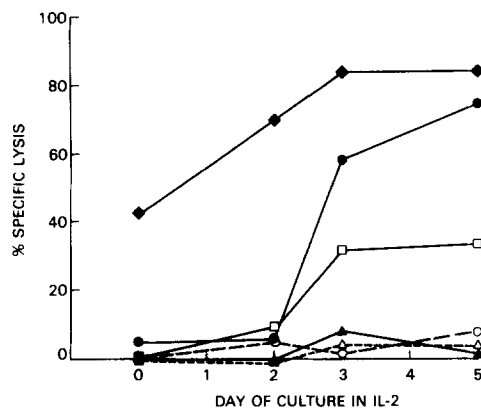


FIG. 4. Kinetics of development and specificity of LAK lysis. PBL from patient 37 were tested for lysis of autologous tumor (also shown in Fig. 3), and for the development of lysis to other target cells. All tests were performed in parallel in a 4-h assay, and the data shown are for an effector/target ratio of 50:1. ▲, autologous PBL; ○, autologous Con A lymphoblasts; △, allogeneic PBL; □, autologous tumor; ◆, K562; ●, Daudi.

tested, a low level of lysis was evident on day 2. In data not shown, no lysis was ever detected on day 1. LAK lysis of tumor cells continued to increase to day 5 in three of four cases. Therefore, it can be concluded that LAK are evident by day 2 or 3 of culture in PP-TCGF and persist at least until day 7. Our previously published data (12) showed that lysis of autologous tumor was expressed as long as 14 d after culture in PP-TCGF, and preliminary cloning experiments revealed that LAK activity persisted even longer, but that not all TCGF stimulated clones expressed LAK activity (data not shown).

In two of the patients tested for kinetics of development of LAK cells, other cell types were also used as targets. The results from patient 37 are shown in Fig. 4. At no time was cytotoxicity expressed toward the autologous PBL, Con A lymphoblasts, or to allogeneic PBL; however, cytotoxicity to the autologous tumor and to the NK-resistant, cultured lymphoma line, Daudi, developed in parallel. NK activity to K562 was clearly positive in the fresh PBL, but did increase in parallel with the development of LAK. It is reported (35) that highly enriched NK populations grow in TCGF, and their persistence in culture along with the LAK-mediated lysis toward K562 after days 2 and 3 can be responsible for the cumulative cytotoxicity expressed toward K562.

Proliferation of PBL Is Required for LAK Expression. Because LAK cytotoxicity was evident early (days 2 and 3), we questioned whether the initial expression of LAK required proliferation of the PBL. PBL from three patients, irradiated at 2,000 rad, were therefore tested for PP-TCGF-induced LAK development (Table V). Whereas the nonirradiated PBL consistently developed LAK, the irradiated PBL did not, even though viable cells were recovered from the cultures and the cultures were tested on days 3 and 4. Therefore, it is concluded that an irradiation-sensitive, differentiation phase is required for LAK expression. Although LAK populations appear heterogeneous at the light-microscope level, they do contain blastic appearing lymphoid cells, showing evidence of activation.

Precursors of LAK Are Not Cells and Are Nonadherent. To determine if LAK could

TABLE V
Induction of LAK Is Radiation Sensitive

Autologous PBL	Percent cytotoxicity of autologous tumor					
		44		57		4.6
	E/T‡		E/T		E/T	
Irradiated	40:1	9.3 ± 4.0	40:1	7.8 ± 4.4	50:1	-4.5 ± 2.5
	10:1	2.8 ± 1.3	10:1	-0.8 ± 0.9	12.5:1	-2.4 ± 5.5
	2.5:1	-5.2 ± 3.1	2.5:1	-3.7 ± 0.8	3.1:1	7.8 ± 8.2
Nonirradiated	40:1	89.8 ± 1.6	40:1	50.2 ± 0.7	50:1	23.0 ± 0
	10:1	63.4 ± 4.8	10:1	14.6 ± 1.2	12.5:1	8.1 ± 1.2
	2.5:1	37.3 ± 4.6	2.5:1	5.1 ± 1.1	3.1:1	-0.4 ± 2.8

* Fresh PBL from each patient were either irradiated (2,000 rad), or not irradiated, followed by culture in PP-TCGF. Lysis of the autologous tumor was tested in a 4-h ⁵¹Cr release assay. Culture was 3 d for patient 63, and 4 d for patients 44 and 57.

‡ Effector/target ratio.

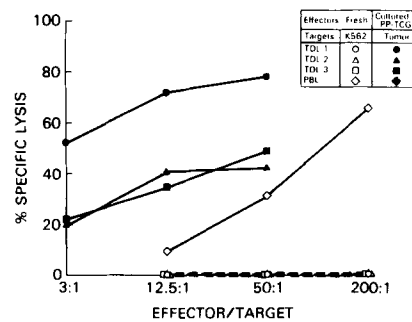


FIG. 5. TDL void of NK activity are stimulated to express LAK activity. Early TDL drainage from three noncancer patients being prepared for kidney transplantation were washed and purified as described for PBL. To stimulate LAK activity, the TDL were cultured for 7 d in CM containing PP-TCGF, and then tested for lysis of melanoma tumor 44.

represent a form of “activated NK” cell, we examined whether fresh thoracic duct lymphocytes (TDL), known to be void of NK cells (36), would respond to PP-TCGF and develop antitumor lytic activity. Fig. 5 confirms that fresh TDL from three different patients are devoid of anti-K562 NK activity, but that LAK did develop from these TDL. In the same assay, the K562 target cells were lysed by fresh PBL, as expected. Therefore, LAK precursors do not express NK activity and are most likely not classical NK cells.

Depletion of plastic- and nylon wool-adherent cells from PBL had no effect on the generation of LAK (Table VI). Depletion of adherent cells from PBL was performed in 12 experiments with a cell recovery from 33 to 77%, with a mean of 56.2%. In all experiments, no decrease, and an occasional augmentation in the generation of LAK, were found. Wright-Giemsa staining of the plastic culture plates after PBL adherence confirmed that monocytes did adhere, and after the nylon-wool incubation, the resulting cells were >79% lymphocytes. Because no decrease in the generation of LAK was evident, it appears that LAK precursors are neither monocytes nor B lymphocytes. Our data strongly suggest that adherent cells are unnecessary in LAK activation,

TABLE VI
Precursors of LAK Are Nonadherent PBL

Starting population	Percent specific lysis*	
	Tumor	PBL
Whole PBL	50.4 ± 2.9	14.7 ± 1.5
	28.5 ± 2.8	4.5 ± 1.6
	12.5 ± 2.6	0.1 ± 0.1
Nonadherent PBL‡	53.4 ± 1.7	22.9 ± 3.1
	39.4 ± 1.8	9.4 ± 1.8
	13.9 ± 2.2	-2.2 ± 1.1

* Lysis of autologous tumor and PBL was performed in a 4-h ⁵¹Cr release assay at effector/target ratios of 20:1, 5:1, and 1.25:1.

‡ Nonadherent cells were recovered after 1 h of plastic-plate adsorption, followed by 1 h of nylon-wool adsorption at 37° as described in Materials and Methods. In this experiment, 70% of the PBL were recovered from the procedure and then cultured in PP-TCGF for 7 d.

however, they do not rule out a role for a minimal number of adherent cells in an accessory function.

LAK Effectors Are Nonadherent and Express Serologically Detectable T Cell Markers. Because LAK effectors are recovered from plastic tissue culture flasks, it was likely that they were nonadherent to the plastic of the flask. However, to establish the nonadherent nature of the LAK, effector populations were incubated on scrubbed nylon wool columns for 1 h at 37°C immediately before the ⁵¹Cr release assay. Neither autologous nor allogeneic LAK activity were eliminated by the nylon wool adherence, although significant numbers of cells were retained by the columns (data not shown).

A variety of monoclonal antibodies were used for detection of serologically defined markers on LAK effector cells using complement-mediated lysis (Table VII). LAK activity was found to be significantly diminished by antibodies defining T cell markers OKT.3 (37), Leu-1 (38), and 4F2 (39), but not the monocyte and NK marker, OKM-1 (40, 42). However, the depletion was not total. Attempts using various dilutions of antibodies or complement, both with and without a second antibody, have failed to achieve a total depletion of lytic activity. Therefore we repeated the depletion of LAK effectors by the T-specific OKT.3 and OKT.8 (cytotoxic T cell marker) (42) antibodies in parallel with depletion of CTL tested against PBL expressing the specific alloantigen as a positive control for depletion of a known effector function. The OKT.3 and OKT.8 complement-mediated treatment diminished, but did not eliminate CTL activity (data not shown). Again these antibodies diminished but did not totally eliminate LAK activity. Therefore, the LAK effectors are as sensitive to OKT.3 and OKT.8 as are CTL using our complement-mediated lysis technique. We believe that our technique was optimal, but that the reagents were not as sensitive for the complement-mediated detection of antigens as has been reported using fluorescence techniques (37-42) and that LAK do express markers that are in common with CTL and activated T cells.

Discussion

This paper describes the human LAK phenomenon as distinct from the NK and CTL systems (summarized in Table VIII). Major features of the LAK system include:

TABLE VII
Serologic Profile of LAK Effector Cells

Treatment of LAK effector cells*			Percent specific lysis of tumor 63‡	Lytic units per 10 ⁶ cells	Total lytic units recovered§
Antibody	Comple-ment	Percent cells re-covered¶			
—	—	100	78.6 ± 2.1	21.3	21.3
			48.4 ± 1.8		
			15.9 ± 0.8		
—	+	100	85.2 ± 6.3	30.0	30.0
			54.7 ± 5.4		
			24.4 ± 2.2		
OKM.1	+	100	79.3 ± 2.2	22.0	22.0
			43.1 ± 1.3		
			13.4 ± 0.2		
OKT.3	+	71	56.7 ± 3.8	11.1	7.9
			24.5 ± 0.5		
			9.1 ± 1.1		
Leu-1	+	11	54.4 ± 1.1	10.0	1.1
			24.0 ± 2.6		
			6.0 ± 1.8		
4F2	+	100	43.3 ± 1.2	7.7	7.7
			22.3 ± 0.8		
			10.7 ± 2.1		

* LAK were prepared from PBL by culture for 3 d in PP-TCGF.

‡ Lysis was tested at effector/target ratios of 50:1, 12.5:1, and 3:1.

§ Lytic units per 10⁶ cells (10⁶ divided by the number of cells required to cause 33.3% lysis of 5 × 10⁴ tumor cells), corrected for the number of cells recovered after treatment.

¶ Percentage of cells recovered determined by trypan blue exclusion counts for viable cells.

(a) LAK lyse NK-resistant fresh solid tumor cells in a 4-h ⁵¹Cr release assay; (b) LAK can be generated by a 2–3-d culture in IL-2 containing supernatants, from either allogeneic or autologous sources; (c) IF is not the primary stimulus responsible for LAK; (d) neither precursors nor LAK effectors are adherent cells; (e) LAK precursors are found in NK-void TDL populations, as well as in normal and cancer patients' PBL; (f) LAK development is radiation sensitive; and (g) LAK effectors express the serologically defined phenotype of T cells. The kinetics of development shows that LAK cells are generated after several days of in vitro culture with PP-TCGF. The kinetics of development and the stimulus required serve to distinguish LAK from the CTL system. The development of LAK expression is much faster than that of primary CTL, but is not different from that of secondary CTL. PBL from normal individuals, however, can be induced to kill tumors by exposure to IL-2, indicating that LAK is probably not an expression of classical secondary CTL from memory populations. A further indication distinguishing the LAK system from CR is the observation that depletion of OKT.3-positive cells from fresh PBL, followed by activation with PP-

TABLE VIII
LAK Mediated Antisolid Tumor Killing Represents a Previously Undefined Lytic System

Characteristic	Lytic Activity		
	NK	LAK	CTL
Development kinetics	Fresh PBL	Day 2, 3	Day 5, 6
Stimulus	None	IL-2 (or cellular interactions generating IL-2)	Specific antigen
Specificity of cytotoxicity	Bone marrow; cultured cells; leukemia	Fresh solid tumors (plus all targets killed by NK)	Specific antigen
Precursor location	TDL- PBL+	TDL+ PBL+	TDL Unknown PBL+
Serologic Phenotype of effector	OKM.1+ OKT.3-	OKM.1- OKT.3+	OKM.1- OKT.3+

TCGF, greatly augmented LAK production (E. A. Grimm, A. Mazumder, and S. A. Rosenberg, manuscript in preparation), indicating that the LAK precursor is not a mature T cell.

The kinetics of LAK generation is reminiscent of the kinetics of development of the anomalous killing of NK-resistant cultured tumors described by Seeley and Golub (13), because lytic activity toward NK-resistant lines was evident 2-3 d earlier than CTL during an allosensitization. The LAK killing system is probably the final common pathway that explains many of the nonclassical cellular cytotoxicities reported previously, though fresh tumor cells were usually not used as target cells in such studies. These reports include activated cell killing (16), fetal calf serum-induced killing (19-21), and lectin-stimulated killing (18). Previous reports from our laboratory support the hypothesis that LAK may be generated from primary stimuli other than IL-2, but in which IL-2 is also generated, such as during allosensitization (23) or during lectin activation with T cell mitogens (24), because in our reports, effectors were generated that did kill fresh autologous solid tumor cells. Therefore, the stimulus for production of LAK cells may be found during a variety of culture conditions, all of which are known to involve IL-2 production (27).

The stimulus required for LAK generation further serves to distinguish the LAK from the NK and CTL systems. No stimulus is known to be required for NK generation, though presumably a signal exists for NK differentiation *in vivo*. Leukocyte interferon is considered as a potent stimulus for NK augmentation, but was found not to be the primary stimulus in PP-TCGF for LAK development. NK cells are apparent in fresh PBL without exogenous stimulation, whereas LAK cells do require exogenous stimulation and proliferation to be expressed. CTL require two signals: a proliferative stimulus, and a specific antigenic stimulus. We have previously shown that our PP-TCGF contains the factor(s) that synergize with nonimmunogenic alloantigen to induce a competent *in vitro* sensitization toward alloantigen, but that the PP-TCGF culture of responder PBL alone in PP-TCGF will not generate any

allospecific lysis.³ Therefore, LAK require activation with a proliferative stimulus, but they do not appear to require antigenic stimulation as do CTL.

The final criteria for distinguishing LAK from NK or CTL are those of target cell specificity and effector cell phenotype. Although we do not know if absolutely pure populations of NK cells are lytic for solid tumors, we have shown that populations of fresh PBL that demonstrate NK activity toward K562 do not demonstrate lysis of solid tumors, suggesting that the antigen recognized on fresh tumor cells is not the same determinant responsible for NK-mediated lysis of K562 cells. The NK and LAK systems are also distinguishable based on the serologic phenotype of the effector cells. NK populations are sensitive to certain monocyte markers such as OKM-1, and resistant to T cell markers such as OKT.3 (41, 42). We found that LAK do not express OKM-1 and do express OKT.3. The serologic profile of LAK is identical to that of CTL, but the specificity of lysis is clearly different in that CTL lyse target cells expressing the stimulating cell alloantigen. LAK are observed in populations of CTL after allosensitization (23), but it is not yet known whether these killing functions are performed by the same or different cells. Neither the precursors nor the LAK effectors are adherent to plastic or nylon, further ruling out monocytes or B lymphocytes as playing a role in the LAK system. LAK precursors are evident in TDL, which are devoid of NK cells, implying that the precursors are not NK cells.

It is unclear from our data what antigen(s) is being recognized on fresh solid tumor cells. The lysis of allogeneic tumors of varying histologies, as well as autologous tumors, indicates that a "common" antigen is responsible. The determinant recognized is neither created nor destroyed by trypsin, and tumors that have not been exposed to enzymes are lysed as easily as the treated ones. In results not shown, we also found no difference in the lysis of tumors that were cultured overnight before use as target cells, indicating that there was no requirement for a "regrowth" of antigen, as suggested by others (9). The broad specificity of LAK lysis of all allogeneic tumor cells, whether cultured or fresh, may indicate that antigen recognition per se is not involved, but rather some form of nonimmunogenic lectin-like recognition. A variety of tumor cells have recently been described to express unique lectin-like molecules on their surface (43). A final possibility for the broad specificity of lysis of LAK is that the phenomenon may represent an antibody-mediated cellular cytotoxicity ADCC effect, from naturally occurring antibodies to tumor cells found in normal human serum. This possibility is highly unlikely, as ADCC effectors may be identical to NK effectors, and are found in fresh PBL (for review see 44).

Further studies of the specificity of LAK lysis are currently in progress (E. A. Grimm and S. A. Rosenberg, manuscript in preparation). It should be noted that autologous normal PBL are occasionally lysed by LAK cells, but to a much lower degree than that of tumor, even though both PBL and tumor are equally lysable by allo-CTL (12).

In conclusion, we have described a unique cytolytic effector system that is distinct from the NK and CTL systems, and that efficiently kills at least fresh human tumor cells from 95% of patients tested. The LAK cells are generated from PBL of normal individuals as well as cancer patients and also from thoracic duct lymphocytes devoid

³ Grimm, E. A., A. Mazumder, and S. A. Rosenberg. In vitro growth of cytotoxic human lymphocytes. V. Generation of allospecific cytotoxic lymphocytes to nonimmunogenic antigen by supplementation of in vitro sensitization with partially purified T cell growth factor. Manuscript submitted for publication.

of NK cells. The abundant expression of antitumor cytotoxicity and broad distribution of LAK precursor cells makes it a likely candidate for use in adoptive immunotherapy and for consideration as an effector mechanism responsible for immune surveillance.

Summary

Activation in lectin-free interleukin 2 (IL-2) containing supernatants of peripheral blood mononuclear leukocytes (PBL) from cancer patients or normal individuals resulted in expression of cytotoxicity toward 20 of 21 natural killer (NK)-resistant fresh solid tumor cells tested. Fresh solid tumor cells were resistant to NK-mediated lysis in 10 autologous patients' PBL-tumor interactions, and from 17 normal individuals tested against 13 allogeneic fresh tumors. Culture of PBL in IL-2 for 2-3 d was required for the lymphokine activated killers (LAK) to be expressed, and lytic activity toward a variety of NK-resistant fresh and cultured tumor targets developed in parallel. Autologous IL-2 was functional in LAK activation, as well as interferon-depleted IL-2 preparations. Irradiation of responder PBL before culture in IL-2 prevented LAK development. Precursors of LAK were present in PBL depleted of adherent cells and in NK-void thoracic duct lymphocytes, suggesting that the precursor is neither a monocyte nor an NK cell. LAK effectors expressed the serologically defined T cell markers of OKT'3, Leu-1, and 4F2, but did not express the monocyte/NK marker OKM-1. Lysis of autologous fresh solid tumors by LAK from cancer patients' PBL was demonstrated in 85% of the patient-fresh tumor combinations.

Our data present evidence that the LAK system is a phenomenon distinct from either NK or CTL systems that probably accounts for a large number of reported nonclassical cytotoxicities. The biological role of LAK cells is not yet known, although it is suggested that these cells may be functional in immune surveillance against human solid tumors.

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References

1. Hersey, P., A. Edwards, J. Edwards, E. Adams, G. W. Milton, and D. S. Nelson. 1975. Specificity of cell-mediated cytotoxicity against human melanoma lines: evidence for "nonspecific" killing by activated T cells. *Int. J. Cancer*. **16**:173.
2. Jondal, M. and H. Pross. 1975. Surface markers on human B and T lymphocytes. VI. Cytotoxicity against cell lines as a functional marker for lymphocyte subpopulations. *Int. J. Cancer*. **15**:596.
3. Peter, H. H., J. Pavie-Fischer, W. H. Fridman, C. Aubert, J. P. Cesarini, R. Roubin, and F. M. Kunlsky. 1975. Cell-mediated cytotoxicity in vitro of human lymphocytes against a tissue cultured melanoma line. *J. Immunol.* **115**:539.

4. Takasugi, M., and M. R. Mickey. 1976. Interaction analysis of selective and nonselective cell-mediated cytotoxicity. *J. Natl. Cancer Inst.* **57**:255.
5. Lotzova, E., and K. B. McCredie. 1978. Natural killer cells in mice and man and their possible biological significance. *Cancer Immunol. Immunother.* **4**:215.
6. Menon, M., and S. S. Stefani. 1978. Lymphocyte mediated natural cytotoxicity in Neoplasia. *Oncology.* **35**:63.
7. Herberman, R. B., J. Y. Djeu, H. D. Kay, J. R. Ortaldo, C. Riccardi, G. D. Bonnard, H. T. Holden, R. Fagnani, A. Santoni, and P. Puccetti. 1979. Natural killer cells: Characteristics and regulation of activity. *Immunol. Rev.* **44**:43.
8. Vose, B. M., F. Vanky, M. Fopp, and E. Klein. 1978. In vitro generation of cytotoxicity against autologous human tumor biopsy cells. *Int. J. Cancer.* **21**:588.
9. Vanky, F. T., B. M. Vose, M. Fopp, and E. Klein. 1979. Human tumor-lymphocyte interaction in vitro. VI. Specificity of primary and secondary autologous lymphocyte-mediated cytotoxicity. *J. Natl. Cancer Inst.* **62**:1407.
10. Vose, B. M. 1980. Specific T-cell-mediated killing of autologous lung tumor cells. *Cell. Immunol.* **55**:12.
11. Klein, E., and F. Vanky. 1981. A. Review. Natural and activated cytotoxic lymphocytes which act on autologous and allogeneic tumor cells. *Cancer Immunol. Immunother.* **11**:183.
12. Lotze, M. T., E. A. Grimm, A. Mazumder, J. L. Strausser, and S. A. Rosenberg. In vitro growth of cytotoxic human lymphocytes. IV. Lysis of fresh and cultured autologous tumor by lymphocytes cultured in T cell growth factor. *Cancer Res.* **41**:4420.
13. Seeley, J. K., and S. H. Golub. 1978. Studies on cytotoxicity generated in human mixed lymphocyte cultures. I. Time course and target spectrum of several distinct concomitant cytotoxic activities. *J. Immunol.* **120**:1415.
14. Zarlring, J. M., H. I. Robins, P. C. Raich, F. H. Bach, and M. L. Bach. 1978. Generation of cytotoxic T lymphocytes to autologous human leukemia cells by sensitization to pooled allogeneic normal cells. *Nature (Lond.).* **274**:269.
15. Seeley, J. K., G. Mascucci, A. Poros, E. Klein, and S. H. Golub. 1979. Studies on cytotoxicity generated in human mixed lymphocyte cultures II. Anti-K562 effectors are distinct from allospecific CTL and can be generated from NK-depleted T-Cells. *J. Immunol.* **123**:1303.
16. Masucci, M. G., E. Klein, and S. Argov. 1980. Disappearance of the NK effect after explanation of lymphocytes and generation of similar nonspecific cytotoxicity correlated to the level of blastogenesis in activated cultures. *J. Immunol.* **124**:2458.
17. Bolhuis, R. L. H., and H. Schellekens. 1981. Induction of natural killer cell activity and allocytotoxicity in human peripheral blood lymphocytes after mixed lymphocyte culture. *Scand. J. Immunol.* **13**:401.
18. Stejskal, V., G. Holm, and P. Perlman. 1973. Differentiated cytotoxicity of activated lymphocytes on allogeneic and xenogeneic target cells. I. Activation by tuberculin and by staphylococcus filtrate. *Cell. Immunol.* **8**:71.
19. Zielske, J. V. and S. H. Golub. 1976. Fetal calf serum-induced blastogenic and cytotoxic responses of human lymphocytes. *Cancer Res.* **36**:3842.
20. Ortaldo, J. R., G. D. Bonnard, and R. B. Herberman. 1977. Cytotoxic reactivity of human lymphocytes cultured in vitro. *J. Immunol.* **119**:1351.
21. Golub, S. H., M. G. Golightly, and J. V. Zielske. 1979. "NK-like" cytotoxicity of human lymphocytes cultured in media containing fetal bovine serum. *Int. J. Cancer.* **24**:273.
22. Martin-Chandon, M. R., F. Vanky, C. Carnaud, and E. Klein. 1975. In vitro education on autologous human sarcoma generates non-specific killer cells. *Int. J. Cancer.* **15**:342.
23. Strausser, J. L., A. Mazumder, E. A. Grimm, M. T. Lotze, and S. A. Rosenberg. 1981.

- Lysis of human solid tumors by autologous cells sensitized in vitro to alloantigens. *J. Immunol.* **127**:266.
24. Mazumder, A., E. A. Grimm, H. Z. Zhang, and S. A. Rosenberg. 1982. Lysis of fresh human solid tumors by autologous lymphocytes activated in vitro with lectins. *Cancer Res.* **42**:913.
 25. Cytopathology Section, National Cancer Institute, National Institutes of Health. 1974. *Cytopathology Handbook*, 7th Revision. 42.
 26. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**:645.
 27. Grimm, E. A., and S. A. Rosenberg. 1982. Production and properties of human IL-2. In *Characterization and Utilization of T Lymphocyte Clones*. G. Fathman and F. Fitch, editors. Academic Press, Inc., New York. In press.
 28. Strausser, J. L., and S. A. Rosenberg. 1978. In vitro growth of cytotoxic human lymphocytes. I. Growth of cells sensitized in vitro to alloantigens. *J. Immunol.* **121**:1491.
 29. Lotze, M. T., and S. A. Rosenberg. 1981. In vitro growth of cytotoxic human lymphocytes. III. The preparation of lectin-free T cell growth factor (TCGF) and an analysis of its activity. *J. Immunol.* **126**:2215.
 30. Mantel, N., and W. Haenszel. 1959. Statistical aspects of the analysis of data from retrospective studies of disease. *J. Natl. Cancer Inst.* **22**:719.
 31. Brown, W., and M. Hollander. 1977. *Statistics: A Biomedical Introduction*. John Wiley & Sons, New York. 201-208.
 32. Vose, B. M., and M. Moore. 1980. Natural cytotoxicity in humans. Susceptibility of freshly isolated tumor cells to lysis. *J. Natl. Cancer Inst.* **65**:257.
 33. Vanky, F., and S. Argov. 1979. Human tumor lymphocyte interactions *in vitro*. VII. Blastogenesis and generation of cytotoxicity against autologous tumor biopsy cells are inhibited by interferon. *Int. J. Cancer.* **26**:405.
 34. Vanky, F. T., S. A. Argov, S. A. Einhorn, and E. Klein. 1980. Role of alloantigens in natural killing. Allogeneic but not autologous tumor biopsy cells are sensitive for interferon-induced cytotoxicity of human blood lymphocytes. *J. Exp. Med.* **150**:1151.
 35. Timonen, T., J. R. Otaldo, and R. B. Herberman. 1982. Cultures of purified human NK cells. In *NK Cells and Other Natural Effector Cells*. R. B. Herberman, editor. Academic Press, Inc., New York. In press.
 36. Forbes, J. T., G. D. Niblack, R. Fuchs, R. E. Richie, H. K. Johnson, and R. K. Oldham. 1981. Human natural cell-mediated cytotoxicity. I. Levels in peripheral blood, cord blood, and thoracic duct lymphocytes. *Cancer Immunol. Immunother.* **11**:139.
 37. Kung, P. C., G. Goldstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T-cell surface antigens. *Science (Wash. D.C.)*. **206**:349.
 38. Engleman, E. G., R. Warnke, R. I. Fox, and R. Levy. 1981. Studies of a Human T Lymphocyte Antigen recognized by a monoclonal antibody. *Proc. Natl. Acad. Sci. U. S. A.* **58**:1791.
 39. Moretta, A., M. C. Mingari, B. F. Haynes, R. P. Sekaly, L. Moretta, and A. S. Fauci. 1981. Phenotypic characterization of human cytotoxic T lymphocytes in mixed lymphocyte culture. *J. Exp. Med.* **153**:213.
 40. Zarling, J. M., and P. C. Kung. 1980. Monoclonal antibodies which distinguish between human NK cells and cytotoxic T lymphocytes. *Nature (Lond.)*. **288**:394.
 41. Ortaldo, J. R., S. O. Sharrow, T. Timonen, and R. B. Herberman. 1981. Determination of surface antigens on highly purified human NK cells by flow cytometry with monoclonal antibodies. *J. Immunol.* **127**:2401.
 42. Reinherz, E. L., P. C. Kung, G. Goldstein, and S. F. Schlossman. 1980. A monoclonal antibody reactive with the human cytotoxic suppressor T cells subset previously defined by a heteroantiserum termed TH₂. *J. Immunol.* **124**:1301.

43. Raz, A., and R. Lotan. 1981. Lectin-like activities associated with human and murine neoplastic cells. *Cancer Res.* **41**:3642.
44. Bonnard, G. D., H. D. Kay, R. P. Herberman, J. R. Ortaldo, J. Djeu, K. J. Pfiffner, and J. R. Oehler. 1979. Models for mechanisms of natural cytotoxicity. *In* Natural and Induced Cell-Mediated Cytotoxicity. Academic Press, Inc., New York. 63.