LYMPHOKINE-ACTIVATED KILLER CELL PHENOMENON

II. Precursor Phenotype Is Serologically Distinct from Peripheral T Lymphocytes, Memory Cytotoxic Thymus-derived Lymphocytes, and Natural Killer Cells

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The lymphokine-activated killer cell (LAK)[1] system has been reported by us to represent a cytotoxic phenomenon distinct from either the classic cytotoxic thymus-derived lymphocyte (CTL) or natural killer (NK) cell system on the basis of development kinetics, stimulus requirement, cytotoxic specificity, precursor location, and serologic phenotype of the LAK effector cells (1). The LAK system is unique in its simplicity of activation, since culture of nonadherent peripheral blood lymphocytes (PBL) for a minimum of 3 d in lectin-free lymphokine preparations rich in interleukin-2 (IL-2) is the only requirement. As described earlier (1), the LAK phenomenon involves an activation mechanism that may be common to the many reports of nonclassic culture-induced lymphocyte-mediated cytotoxicities such as the mixed lymphocyte/leukocyte culture–induced anomalous killers (2–6), NK-like killers (7, 8), fetal calf serum–induced killers (9–11), and lectin-induced killer cells (12). Therefore, this system is a most likely candidate for unifying the growing body of literature concerning these cytotoxicities, and for approaching the study of their biological significance.

LAK can be generated from PBL of >90% of the cancer patients tested. One potential significance of the LAK cells is that they can efficiently lyse fresh single-cell suspensions of autologous fresh tumor cells, in addition to allogeneic fresh tumors and all cultured tumors tested, including those that are NK-resistant (1, 13), all of which LAK lyse optimally in a 4-h chromium-release assay. Since as yet no reproducible or consistent method is available for generation of human antitumor CTL, the potential for LAK as an important antitumor defense mechanism has encouraged further definition of the system. We have shown previously that the precursor of the LAK cell is nonadherent to plastic or nylon wool, and that its activation can be prevented by 2,000 rad of gamma irradiation. Therefore we studied in parallel the phenotype of the effectors and precursors of LAK cells, NK cells, and CTL and confirmed the distinct functional nature of each system by simultaneous activation cultures and

[1] *Abbreviations used in this paper:* C′, complement; CM, complete medium; C-IL-2, crude IL-2; CTL, cytotoxic thymus-derived lymphocyte; HBSS, Hanks' balanced salt solution; IL-2, interleukin 2; IVS, in vitro sensitization; LAK, lymphokine-activated killer cell; LGL, large granular lymphocyte; LSM, lymphocyte separation medium; NK, natural killer; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SRBC, sheep erythrocyte.

subsequent cytotoxicity assays. The results presented in this manuscript further confirm the uniqueness of the LAK system, since the precursor was found by both serologic and morphologic selection techniques to be different from peripheral blood T lymphocytes, NK cells, and memory CTL.

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 (6). 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 (KC Biologicals, Kansas City, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (National Institutes of Health [NIH] Median 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. Adherent cells were removed after suspending the PBL in CM at 3×10^7/ml, by placing into plastic tissue culture plates (Falcon 3023; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) for 1 h at 37°C. The nonadherent cells were gently washed from the plates and incubated in CM on scrubbed nylon wool columns for 60 min at 37°C.

Fresh Solid Tumor Cell Targets from Surgical Specimens. The preparation of single-cell suspensions from fresh tumor specimens was performed as described in detail previously (1). Briefly, fresh surgical specimens were collected and kept in HBSS at 4°C for processing. Necrotic tumor and connective tissues were dissected, and the remaining tumor tissue was minced in HBSS containing 5 U/ml hyaluronidase, 2 mg/ml collagenase, and 0.2–0.3 mg/ml deoxyribonuclease. The tumor fragments were further dissociated by trypsinization using 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, pelleted, and resuspended in 20–50 cm^3 of CM. An aliquot was sent for cytologic analysis, and the rest was used either immediately or cryopreserved in 90% human AB serum plus 10% dimethyl sulfoxide for future use. All samples were analyzed by the NIH Cytopathology Department using Papanicolaou staining to determine the percentage of tumor cells. All tumor preparations used in this study contained >80% malignant cells, and are identified further in the Results section as to the histologic type.

Cultured Tumor Targets. K562, the NK-sensitive myeloid leukemia line was cultured in CM, and passaged weekly.

Serologic Depletion Studies. PBL were washed twice in HBSS and resuspended at 1×10^7/ml at 4°C. The previously determined optimal dilution of monoclonal antibody was added directly to the cells (1:20 dilution for OKT-3, OKT-8, OKT-4, OKM-1, and Leu-7 and 1:50 for Leu-1 and OKT-11); the OKT reagents were purchased from Ortho Pharmaceutical Corp., Raritan, NJ, and the Leu reagents from Becton, Dickinson & Co., Sunnyvale, CA. Antibody incubation was performed for 1 h at 4°C with occasional mixing. The cells were pelleted, and complement (C′) was added in HBSS. Newborn rabbit serum was used as a source of C′ and was found to be optimal at a 1:6 dilution. C′ lysis was performed at 37°C for 1 h, after which the cells were washed once in HBSS, resuspended in CM, and counted for viability using trypan blue exclusion.

Percoll Gradient Separation of T Cells and Large Granular Lymphocytes (LGL). The nonadherent PBL cells were resuspended at 5×10^7/ml in CM supplemented with 10% AB serum, and separated by centrifugation on a discontinuous gradient of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) modified from the method described by Timonen et al. (14). Briefly, the 100% Percoll and CM were adjusted to 285 mosmol/kg H_2O with 10 × phosphate-buffered saline (PBS, pH 7.2) (Gibco Laboratories). Final concentrations of Percoll were prepared, at 40, 42, 45, 47, 50, 52, and 55%, or as described in the experiment. After carefully layering the fractions into 15-ml conical tubes (Falcon 2095), we layered 1 ml of nonadherent cells onto the top of the gradient, and centrifuged them at 550 *g* for 20 min at room temperature (or longer periods as described in the experiment). Cells from the individual layers were collected from

the top with a Pasteur pipette, and washed in CM. Cells from the NK-rich Percoll fraction (~45% Percoll) (containing LGL) were further depleted of low-density T lymphocytes via rosette formation with sheep erythrocytes (SRBC) for 2 h at 29°C (15). The non-rosette forming cells were >70% LGL. Preparations of unseparated nonadherent cells, LGL from the NK-rich Percoll fraction and 29°C SRBC rosetting, and T cells from the high-density (50–60%) Percoll fractions were then coded before further studies were performed.

Isolation by Panning. LAK precursors were separated from T cells found in fresh PBL by the panning procedure described in detail by Engleman et al. (16), except that human agamma serum was substituted for fetal calf serum. Briefly, nonadherent PBL were washed and resuspended in PBS at 10^7/ml. These cells were then incubated with various dilutions of either Leu-1 or OKT-3 for 20 min at room temperature. (We found the optimal quantities to be 0.01 ml of Leu-1 and 0.075 ml of OKT-3).

After two washes with PBS, the cells were resuspended in 4 ml of PBS containing 5% serum, added to plates previously coated with affinity-purified goat anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD), and incubated at 4°C for 2 h. Nonadherent cells were removed by gently swirling off the PBS, followed by one rinse with PBS. Adherent cells were removed by vigorous pipetting, and in some experiments by use of trypsin. All cells were then washed and resuspended in CM. The effectiveness of this panning procedure was confirmed by positive panning of allospecific CTL.

IL-2 Preparation and Partial Purification. Crude T cell growth factor was either purchased from Associated Biomedic Systems (Buffalo, NY) or prepared by us using a previously discussed modification (17) of our original method (18). 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 IL-2 (C-IL-2). Partial purification and removal of the PHA from these C-IL-2 preparations was performed as we described previously (19). In brief, the C-IL-2 was brought to 50% saturation with ammonium sulfate and the precipitate discarded. The supernatant was then raised to 75% saturation, the precipitate collected, resuspended in water to achieve an ~1/10 vol of C-IL-2 starting sample, dialyzed first vs. water, then vs. PBS, and passed over an affinity column (14 × 0.9 cm) of Sepharose 4B (Pharmacia) coupled to rabbit anti-PHA antibody. Tests for IL-2 activity were performed using IL-2-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 IL-2 titer.

Primary and Secondary In Vitro Sensitization for CTL Generation. Primary in vitro sensitization (IVS) was performed by co-culture of responder PBL with stimulator PBL incubated vertically in CM in Falcon 3013 flasks (20). Responder PBL were used at a final concentration of 1×10^6/ml in CM. Irradiation of PBL stimulator cells was performed using a cesium-137 source (Isomedex Inc., Parsippany, NJ) at 2,000 rad. Stimulator cells were washed once after irradiation and added at 1×10^6/ml. Primary CTL were tested on day 7 of culture.

Memory CTL were prepared by washing primary CTL with fresh CM, and reculturing for 7 d more at 1×10^6/ml. Serologic depletion studies were performed on day 14, after which time the memory cells were harvested (resulting in ≦25% of original cells) and again recultured at 1×10^6/ml in CM containing the optimal dilution of IL-2 previously titrated to activate memory cells into secondary CTL. The secondary CTL activity was then assayed on day 18.

Activation of LAK by Partially Purified IL-2. LAK were generated by culture of responder PBL at 1×10^6/ml in CM at the dilution of IL-2 determined to be optimal for growth of IL-2-dependent cells (usually 1:10 for our 10-fold-concentrated partially purified IL-2), and also titrated to be optimal for LAK activation. Cultures were for 6–7 d unless stated otherwise.

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, and labeled with 400 μCi of $Na^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) for 120 min in 0.5 cm^3 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 determined 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: ([experimental cpm − spontaneous cpm]/[maximal cpm − spontaneous cpm]) × 100%. All determinations were made in triplicate and data are reported as the mean ± SEM.

Lytic units were calculated by extrapolating the number of effector cells required to cause 33.3% lysis of 5×10^3 tumor or PBL target cells.

Preparation of Lymphoid Tissues. Human thymus (generously provided by Dr. Jack Roth, Surgery Branch, National Cancer Institute [NCI], NIH) spleen and lymph node, was processed immediately after surgical resection from cancer patients. The tissues were minced at room temperature in HBSS, and the dissociated cells were separated by filtration through nylon mesh. The erythrocytes were then removed by fractionation on LSM gradients as described for PBL. The mononuclear cell fraction was collected, washed, and cryopreserved until thawed for use in these experiments. Bone marrow cells were the generous gift of Dr. Thomas Sharp (Surgery Branch, NCI, NIH). The marrow cells were rinsed from the vertebrae body of a 23-yr-old cadaveric donor without cancer. They were further processed as for the PBL, and cryopreserved until used.

Results

LAK Effector Cells Express the Serologic Phenotype of Allospecific CTL. In our previous report (1) we showed that the autologous and allogeneic antitumor killer cells were generated by culture of PBL in partially purified preparations of IL-2; the resulting LAK effector cells are sensitive to C′ lysis by monoclonal antibodies known to identify allospecific CTL. These LAK effectors were extremely sensitive to Leu-1 (95% reduction) and partially sensitive to the OKT-3 and 4F2 (63% and 62%) T cell reagents and appeared insensitive to the monocyte–NK cell marker OKM-1 (1). Therefore, to determine the subclass of T cells mediating antitumor lysis and to directly compare their phenotype with that of CTL, we prepared LAK and CTL in parallel from the same responder PBL, and after activation tested their sensitivity to a variety of monoclonal anti–T cell subclass antibodies (Table I). Both LAK and CTL effector cells were found to be insensitive to the inducer-helper T cell antibody OKT-4 (21) (7% loss of activity), minimally sensitive to the monocyte/NK cell marker OKM-1 (22) (25% loss of activity), partially sensitive to the pan T cell reagent OKT-3 (23) (80% loss of activity), and extremely sensitive to the cytotoxic/suppressor T cell antibody OKT-8 (23) (90% and 71% loss of activity). The OKT-3 reagent has never been able to eliminate all LAK activity, even when the pan T Leu-1 reagent (24) did (1); therefore it was of interest to observe that OKT-3 plus C′ did not eliminate all CTL activity either. Thus LAK effector activity to autologous tumor and CTL activity to the specific sensitizing allogeneic cell were eliminated to relatively similar degrees by the same monoclonal antibodies.

LAK effectors appeared morphologically to be rather large cells when we attempted to separate the LAK from CTL by sedimentation on Percoll gradients. In data not shown, we found that the LAK and the CTL both sedimented in the 45% band. Since this area is identical to that for NK cell sedimentation, it is interesting to speculate that all cytolytic lymphocytes are larger cells of low density.

LAK Precursor Cells Are Distinct from Those Required for CTL Generation. Although LAK effector cells express the serologic phenotype of CTL, the LAK system differed from the primary CTL system in two major aspects: a more rapid kinetics of induction was seen for LAK (day 3 vs. days 6–7), and no known antigenic stimulus was required

TABLE I

Monoclonal Antibody Depletion of Autologous LAK vs. CTL Effector Function

Experiment	Treatment*	Post-IVS		Post-IL-2	
		Percent cells lysed‡	CTL activity	Percent cells lysed	LAK§ activity
			LU‖/10^6 cells		*LU/10^6 cells*
1	C′	0	20	0	50
	OKM-1 + C′	0	15	13	40
	OKT-3 + C′	27	4	13	25
	OKT-8 + C′	39	2	37	7
2	C′	0	14	0	4.5
	OKT-4 + C′	63	13	54	4.5
	OKT-8 + C′	29	4	36	0.5

* PBL from cancer patients were incubated for 7 d in a primary allogeneic IVS in one set of cultures, and with IL-2 in another. After treatment CTL lysis was tested toward fresh PBL targets autologous to the stimulator cell used in the IVS cultures, and LAK lysis tested to the autologous tumor target.

‡ The percent cells lysed was determined by trypan blue exclusion counts of viable cells.

§ The autologous tumor targets were uncultured single-cell preparations of melanoma in experiment 1, and sarcoma in experiment 2.

‖ Lytic units.

TABLE II

Sensitivity of Cells Required to Generate CTL vs. LAK to Monoclonal Antibodies plus C′

Treatment*	Percent cells lysed‡	Lytic units, 10^6 cells	
		CTL Allo-PBL targets post-IVS	LAK tumor targets§ post-IL-2 culture
None	0	10.5	11.0
C′ only	9	8.7	ND‖
OKT-3 + C′	56	<1	62.0
OKM-1 + C′	17	20.0	9.0
Leu-1 + C′	63	<1	66.0

* Nonadherent PBL were divided into five groups and treated as described. The nonlysed PBL from each treatment group were then divided equally, adjusted to the concentration of the untreated cells, and used as responders in an IVS or incubation with IL-2.

‡ The percent cells lysed was determined by trypan blue exclusion counts for viable cells.

§ The tumor targets used were from a single-cell suspension of fresh undifferentiated sarcoma.

‖ Not done.

for LAK (1). Therefore to further define the LAK phenomenon in relationship to the CTL system, we tested whether LAK precursor cells expressed any serologic similarities with CTL precursor cells. Table II shows the results from one of two experiments in which fresh PBL were treated with monoclonal antibodies plus C′ and then divided for use as responders in both LAK and allogeneic CTL activation cultures. As expected, the generation of allospecific CTL was totally eliminated by killing the precursors expressing the pan T antibodies OKT-3 or Leu-1 (24); however, the development of LAK activity from these same depleted populations was found to be

TABLE III

*Monoclonal Anti-T Cell Antibodies Eliminate Allospecific Memory CTL but not LAK Precursors**

Experiment	Treatment	Percent cells lysed‡	Lytic units, 10^6 cells		
			LAK	Memory CTL	
			Targets/ tumor§	Specific allo-PBL	Self-PBL
1	None	0	4.0	10.0	<1
	C′ only	24	5.6	16.0	<1
	OKT-3 + C′	94	>20.0	1.4	<1
	OKT-8 + C′	57	4.4	<1	<1
	Leu-1 + C′	95	>20.0	1.0	<1
2	C′ only	40	16.6	20.0	ND‖
	OKT-3 + C′	90	33.3	<1	ND
	OKM-1 + C′	86	10.0	15.0	ND
	Leu-1 + C′	95	50.0	<1	ND

* Memory CTL were prepared as described in Materials and Methods. On day 14, the memory cells were harvested and treated as shown, then recultured in IL-2 for 4 d more. The 2° CTL were then tested for lysis of the targets shown.

‡ The percent cells lysed was determined by trypan blue exclusion counts.

§ The tumor target cells used were a fresh malignant fibrous histiocytoma for experiment 1, and a malignant hemangiopericytoma for experiment 2.

‖ Not done.

dramatically augmented (five- to sixfold increase in lytic units). The anti-monocyte/NK cell antibody OKM-1 had little effect on either system. In experiments not shown, it was found that culture of cells treated with antibody alone (but not with C′) were not activated unless IL-2 was added, and that in the case of OKT-3, the presence of the antibody at the concentration used for lysis suppressed LAK development. Therefore, our results indicate that lysis of the T cells by specific antibody and C′ did not interrupt subsequent activation by IL-2 in the case of LAK but eliminated alloantigen reactivity in the case of CTL.

Panning techniques were also employed to positively select for T cells and to test the serologic phenotype of the LAK precursors. OKT-3 antibody was used in nine experiments with an average of 55.6 ± 5.9% of the cells adhering, and Leu-1 was used in four experiments yielding 50.5 ± 5.8% of cells adhering. Adherent, nonadherent, and unseparated PBL populations were then tested for ability to generate CTL and LAK. The nonadherent populations were found to be enriched in LAK precursors (2- to 17-fold increase of LAK generation). However, variable results were obtained with generation of CTL from the adherent cells. It was found that the presence of the antibodies significantly inhibited CTL generation, and even after trypsin treatment of the OKT-3 or Leu-1-positive panned cells, we were able to generate very little CTL activity. A recent report by Reinherz et al. (25) describing antigen nonresponsiveness after OKT-3 antibody modulation of the T3 molecule, provides the explanation for our lack of function by the adherent cells. Therefore, by the positive selection of T cells, we have found LAK precursors to be present in the OKT-3 and Leu-1 negative population; however, the activity of the purified T cell populations could not be confirmed in a direct manner.

LAK Precursors Are Distinct from Memory CTL. Secondary CTL are achieved from memory cell populations by culture with either of the SD or LD signals required for the generation of primary CTL (26), and the secondary CTL express lytic activity as early as 3 d after activation. These activation conditions are identical to those of LAK, since our human IL-2 preparations used for LAK activation are known to contain the second signal functional in generation of primary CTL (20). To test whether LAK precursors could be memory CTL cells, we performed several experiments in which we studied the serologic phenotype of memory CTL in parallel with that of LAK precursor cells. Our results in Table III demonstrated consistent elimination of secondary CTL production from memory populations by depletion with anti-T cell antibodies plus C′, whereas in the same cultures LAK generation was dramatically augmented. Therefore memory CTL cells do express the T cell markers OKT-3, OKT-8, and Leu-1, and cells required for LAK generation do not; again OKM-1 had no effect on the generation of LAK cells.

LAK Can Be Generated from NK-enriched LGL Populations. Sedimentation of nonadherent fresh PBL on Percoll gradients (14) was performed to further characterize the LAK precursors. In 10 out of 10 experiments we observed that the LAK precursors sedimented with the NK cells. Modification of the Percoll gradient procedure was unsuccessful in separating NK and LAK precursors, though all the LAK precursors could be localized in the higher density NK fractions expressing only 1/10th of the total NK activity when the time of centrifugation was increased. Though we have reported previously that LAK are generated from thoracic duct lymphocytes devoid of NK activity (1), it was uncertain whether NK cells in PBL could serve as LAK

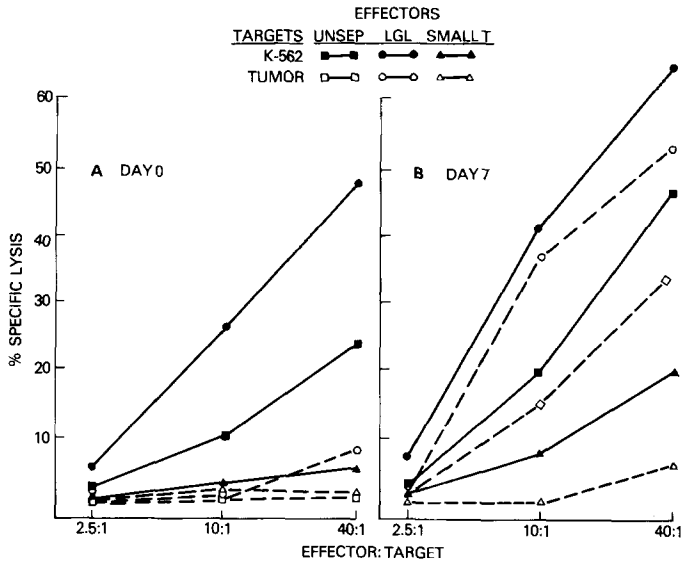

FIG. 1. Percoll gradient separation of LGL and small T cells. Nonadherent fresh PBL were separated into various densities on Percoll gradients (see Materials and Methods). The 42–45% fraction was further purified for NK cells by depletion of the 29°C SRBC rosetting cells; the resulting NK-enriched fraction, called LGL, were 5% of the starting cells. The small, more dense T cells from the bottom of the gradient were collected and tested in parallel; these represented 30% of the starting population. Unseparated cells, LGL, and small T cells were all tested in parallel on day 0 for NK activity, and for lysis of the NK resistant tumor (panel A). After 7 d of culture with IL-2 they were again tested for lytic activity (panel B).

precursors. Therefore we further purified the NK cells in the 42–45% Percoll fraction by SRBC rosetting at 29°C (Fig. 1). According to previously described results (14) these NK-enriched populations have been termed LGL. On day 0 after separation, the LGL were observed to display enriched NK activity while the T cells from the bottom of the gradient at 50 and 55% Percoll did not contain such activity toward the NK-sensitive target cell K562. None of the fractions of fresh PBL were observed to significantly lyse the LAK-sensitive sarcoma cell target. After the fractions were cultured for 7 d with IL-2 they were again tested for lysis of K562 and the NK-resistant sarcoma. The LAK activity was found to be enriched in the LGL fraction and reduced in the T cell fraction. In six experiments performed using the 29°C high affinity rosetting technique to separate NK cells from T cells, we consistently observed that the LAK precursors did not rosette with SRBC at 29°C.

LAK Precursors Are Serologically Distinct from NK Cells. Even the most highly purified LGL populations are reported to be quite heterogeneous (22) with the active NK cells representing an average of 50% of the total cells (27, 28). Because we were unable to distinguish NK cells from the cells required to generate LAK on the basis of Percoll sedimentation and high affinity SRBC rosetting, we further explored the serologic phenotype of LAK precursors using monoclonal antibodies that were reported to recognize NK cell antigens, either singly or in combination. Fig. 2 shows that treatment of nonadherent fresh PBL with either Leu-7 (also called HNK-1) (29) or OKM-1 plus complement significantly reduced or totally eliminated the NK activity, respectively. The combination of Leu-7 plus OKM-1 also totally abolished detectable NK activity. After culture of the remaining cells for 6 d in IL-2 to test for LAK generation it was found that the Leu-7-depleted population expressed an augmented level of LAK, while the OKM-1 and OKM-1 plus Leu-7 were no different from the control untreated cells. It is not known why the OKM-1-treated groups did not generate an augmented LAK response as would be expected if LAK precursors were enriched. We can only speculate that perhaps a minimal number of monocytes are required in activation of LAK cells.

In other experiments we tested whether prior treatment with OKT-11, which is reported to bind T cells as well as NK cells (30, 31) would eliminate LAK precursors

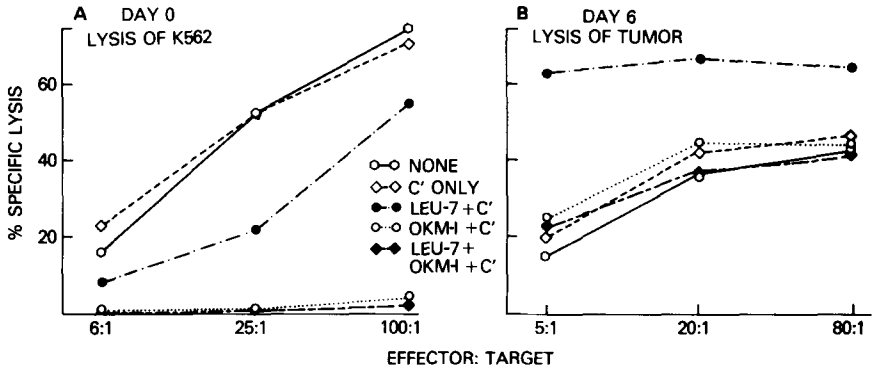

FIG. 2. LAK precursors are insensitive to anti-NK antibodies and C′. Fresh nonadherent PBL were treated with the antibodies and C′ as shown. The cell recoveries as determined by trypan blue exclusion counts immediately after treatment were: none (no treatment), 100%; C′ only, 95%; Leu-7 + C′, 95%; OKM-1 + C′, 75%; and Leu-7 + OKM-1 + C′, 70%. Panel A displays the day 0 NK activity of these groups, and panel B, the LAK activity generated after 6 d of culture in IL-2.

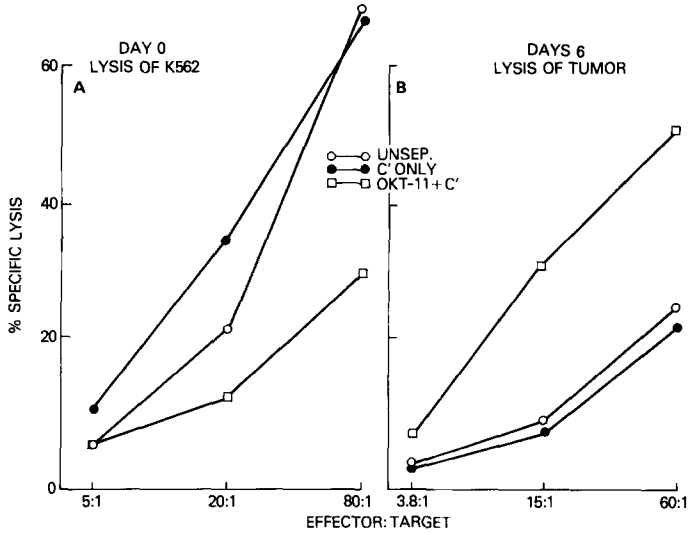

FIG. 3. LAK precursors are insensitive to OKT-11 + C′. Fresh nonadherent PBL were treated with the anti-OKT-11 and C′ as described in Materials and Methods. The cells treated with C′ only had a 70% recovery, and the OKT-11 + C′ treatment resulted in 3.3% recovery. Panel A displays the NK activity of these populations on day 0, and panel B displays the LAK activity generated after 6 d of culture in IL-2.

TABLE IV

*Distribution of LAK Precursors in Lymphoid Tissues**

Experiment		Lytic units/10^6 effector cells
1	PBL	50
	Thymus, I	33
	Thymus, II	57
2	Spleen	18
	Lymph node	80
	Bone marrow	22
3	Thoracic duct cells, I	16
	Thoracic duct cells, II	18
	Thoracic duct cells, III	67

* Single-cell suspensions were prepared from lymphoid tissues of either normal or cancer patients as described in Materials and Methods. The cells were then cultured in IL-2 and assayed for lysis of a sarcoma.

(Fig. 3). Our results confirm this, since 97% of the nonadherent PBL were lysed by OKT-11 and C′ (in other experiments at least 90%) and the NK activity was significantly depleted or abolished. After culture for 6 d in IL-2, however, the LAK activity of the OKT-11-depleted PBL was dramatically augmented, compared with the control cells. Thus the cells required to generate LAK appear to be distinct from NK cells, since the LAK cell precursors do not express OKM-1, Leu-7, or OKT-11, whereas NK cells do express these markers.

Distribution of LAK Precursors in Lymphoid Tissues. To determine whether the LAK precursor cells were unique to the PBL and thoracic duct lymphocytes (1) or could be found in other lymphoid tissues, we collected lymphocytes from a variety of human tissue, cultured them for 7 d in IL-2 and tested them for lysis of an NK-resistant

sarcoma (Table IV). We found that the LAK precursors do exist in all the tissues tested, including thymus thoracic duct, and in bone marrow. In the mouse it was reported that LAK could also be generated from tumor-infiltrating lymphocytes (32). Therefore, the LAK precursor appears to recirculate and distribute through all of the major lymphoid tissues.

Discussion

In previous studies (1, 13) we reported that partially purified IL-2 stimulated PBL from cancer patients and normals to become lytic for fresh autologous or allogeneic tumors, but not to lymphocytes or lymphoblasts. This system, which was named LAK, for lymphokine-activated killer cells, was found to be distinct from either the classic CTL or NK cell systems, based on a number of characteristics including kinetics of activation, stimulus requirement, target cell specificity, precursor cell location, and effector cell phenotype (1). Therefore this study was undertaken to further define the cell type involved in the LAK system. Our previous study had shown that monocytes and B lymphocytes found in PBLwere not the direct precursors or effectors of LAK cells, leaving mainly T cells, NK cells, and the ill-defined "null cells" as candidates. Our initial examination of the effector cell phenotype using monoclonal antibodies plus C′ showed that LAK were sensitive to the anti-T cell reagents OKT-3, Leu-1, 4F2, but not the monocyte reagent OKM-1 (1). This article continues the studies of LAK at both the precursor and effector cell stage, using the NK and CTL systems as the positive controls for our reagents.

We confirmed that LAK effector cells expressed the OKT-3 and OKT-8 phenotype of CTL when examined in parallel with allospecific CTL effector cells (Table I). Most surprisingly, the LAK precursors appeared totally negative for the same anti-T cell reagents that eliminated the development of CTL (Table II). Therefore not only were LAK precursors negative for expression of these T cell markers as determined by our C′ lysis technique, but it also suggested that T cells defined by the antibodies OKT-3, Leu-1 (Table II), and OKT-11 (Fig. 3) could not be involved in an accessory role.

LAK precursors were also found in memory CTL populations. Since LAK are generated under conditions known to activate secondary CTL from memory CTL, and since the phenotype of memory CTL is not known, we explored the sensitivity of memory CTL and LAK precursors to a variety of monoclonal antibodies. The pan anti-T cell antibodies OKT-3 and Leu-1 totally eliminated the 2° CTL precursors but resulted in an augmented expression of LAK. Since up to 95% of the memory CTL were lysed by the Leu-1, it appears that the LAK precursors must be <5% of the memory population. This finding reduces the possibility that LAK precursors in cancer patients represent memory CTL due to prior exposure to tumor. The LAK precursors also appeared to be contained in <5% of the fresh nonadherent PBL populations, as demonstrated by the killing of >90% of cells by OKT-11 plus C′ data with concomitant augmentation of LAK activity (Fig. 3). Therefore, if LAK is part of the T cell system, it is a subpopulation either resistant to C′ lysis or one not identified by the commercially available "pan" T reagent. We have shown (33) that the LAK precursors in the mouse are sensitive to Thy-1 plus C′ (the murine pan anti-T cell antibody), and suggesting that the human antibodies OKT-3, OKT-11, and Leu-1 may not recognize an antigen present on all T cells.

It has been reported that PBL populations enriched in NK activity grow for

extended periods in IL-2, and that the effector cells express the CTL phenotype of OKT-3 and OKT-8, but not OKM-1, which is expressed on fresh NK cells (34). It was not clear from those studies whether the actual NK cell became positive for the T cell markers, or whether they resulted merely from expansion of the LAK precursors and/or T cells contained in the population. Our previous results showed that TDL devoid of NK activity generated LAK (1), and therefore some LAK precursors were not NK cells. Since LAK precursors were found in the present study to be distinct from T cells, it became necessary to further examine their relationship to NK cells in a controlled manner. Percoll gradient sedimentation, followed by SRBC rosette depletion of T cells resulted in enriched NK fractions called LGL. Our results showed that the resultant NK-rich fraction, and not the small T cell fraction contained an enriched quantity of LAK precursors (Fig. 1). However, since these LGL were not pure NK cells (22), we again applied serologic techniques to examine the relationship between LAK and NK cells. Most LAK precursors were again negative for OKM-1, which eliminated NK activity (Fig. 2); LAK activity was augmented by pretreatment of PBL with Leu-7 (HNK-1) and also with OKT-11 (Fig. 3), which recognizes 95% of T cells plus a majority of NK cells. In all cases these antibodies significantly diminished or totally abolished the NK activity of the fresh PBL. We have never found LAK generation augmented after OKM-1 plus C′ treatment, suggesting that either some LAK precursors are OKM-1 positive, or that a small number of monocytes are required for LAK activation. Therefore LAK precursors do not express the NK cell markers of OKT-11 or Leu-7 and very little if any OKM-1. Since LAK precursors are found in all lymphoid tissues tested (Table IV) and NK cells are negative in most of these (34–36), we believe this data strongly suggests that the LAK did not originate from an active NK cell.

These results establish that the LAK precursor is distinct from the classic CTL or NK cell (though this interpretation must be qualified to state as determined by antibody and C′-mediated lysis using the pan T and NK reagents commercially available), and as yet we have no positive marker unique for the human LAK precursor. We are now employing fluorescent-activated cell sorting (FACS) analysis and a variety of subclass antibodies in an effort to identify the precursor based on techniques not dependent on C′-mediated lysis (E. Grimm, L. Neckers, and S. Rosenberg, manuscript submitted for publication). Our preliminary results from FACS analysis have confirmed that LAK precursors appear negative for the markers used in this study, plus they appear negative for a number of other T cell and NK cell markers; it was found that LAK precursors are positive for T-29/33 (37), directed toward the T200 antigen, which is broadly distributed within leukocytic system but not detectable on any other cell types. A clear description of the precursor cell phenotype is essential to understanding the nature of the LAK system, and its relationship to the other cytotoxic phenomena.

Further studies of both the signal(s) responsible for LAK activation and the specificity of LAK effectors are currently in progress. Highly purified human IL-2 has been found to be stimulatory, and, as yet, no other human lymphokines contained in our partially purified IL-2 appear responsible, including interferon and IL-1 (E. A. Grimm, J. A. Roth, R. Robb, L. Neckers, L. Lachman, D. Wilson, and S. A. Rosenberg, manuscript in preparation). The simplicity of LAK activation, its apparent unique antitumor reactivity, and the broad distribution of LAK precursors suggest

that the LAK system may be an expression of immune surveillance, and most intriguingly may provide a simple means for immunotherapy of cancers.

Summary

Culture of human peripheral blood lymphocytes (PBL) in partially purified and lectin-free interleukin 2 results in the generation of cytotoxic effector cells which have the unique property of lysing natural killer (NK)-resistant fresh human tumor cells. We have termed these effector cells "lymphokine-activated killer" cells (LAK). LAK are generated from both normal and cancer patients' PBL and are able to lyse both autologous and allogeneic tumor cells from all histologic tumor types tested. Our previous studies suggested that the LAK phenomenon was distinct from either the cytotoxic thymus-derived lymphocyte (CTL) or NK systems based on a variety of criteria. This study reports that the cell type involved is also distinct, as determined by phenotypic characteristics. The LAK effector cell phenotype was analyzed in parallel with alloimmune CTL, and LAK were found to be similarly susceptible to the monoclonal anti-T cell antibodies OKT-3 or OKT-8 plus complement. In contrast the LAK precursor was not susceptible to the OKT-3 or Leu-1 antibodies plus complement, while the ability to generate alloimmune CTL was totally obliterated when tested using the same PBL responder population; in fact, generation of LAK was found to be augmented five- to sixfold, clearly suggesting that LAK precursor cells are not T lymphocytes as defined by these antibodies. LAK precursors were found to be abundant in NK cell-enriched Percoll gradient fractions, which had been depleted of the 29°C E-rosetting "high affinity" T cells. However, LAK precursors were found to be distinct from the majority of NK cells since lysis of fresh PBL with the monoclonal antibodies OKM-1, Leu-7, or OKT-11 significantly depleted or totally eliminated NK activity, while subsequent activation of the remaining cells generated high levels of LAK and in some cases augmented levels of LAK. LAK precursors were found to be distributed in the thymus, bone marrow, spleen, lymph node, and thoracic duct in addition to the PBL. Therefore, while the cell(s) responsible for activation and expression of LAK activity have some common features with the classic T cell-mediated CTL and NK cell systems, the LAK precursor cells are clearly distinct as determined by phenotype analysis using monoclonal antibodies and complement, and at present must be classified as a "null" cell.

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