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IN VIVO EFFECTS OF RECOMBINANT IL-2

I. Isolation of Circulating Leu-19$^+$ Lymphokine-Activated Killer Effector Cells from Cancer Patients Receiving Recombinant IL-2

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This study was designed to isolate and phenotypically characterize lymphokine-activated killer (LAK) cells generated in vivo during administration of high dose rIL-2 to cancer patients. The development of circulating LAK effector cells in these patients was demonstrated by the ability of fresh PBL to exhibit lytic activity against the NK-resistant Daudi cell line and fresh tumor cells without prior in vitro culture with rIL-2. Kinetic studies demonstrated that circulating LAK effector cells are detectable 4 to 6 wk after the initiation of rIL-2 therapy. Cells isolated by FACS revealed that circulating LAK cells are Leu-19$^+$, Leu-17$^+$ but CD5$^-$. We have previously reported that circulating Leu-19$^+$ cells are heterogeneous with regard to the expression of CD16 and CD8. Since sorting of cells expressing Leu-19 and either low quantities of CD8 or CD16 resulted in cytolytic activity in both the positive and negative fractions, these latter two markers do not identify subpopulations of Leu-19$^+$ cells with or without LAK cytolytic activity. Although all LAK cells generated in vivo were Leu-19$^+$, we generated LAK cells from the Leu-19$^-$ subpopulation after in vitro culture with rIL-2, suggesting that at least some of in vitro generated LAK cells are derived from Leu-19$^-$ precursor cells. These LAK cells did not, however, express the Leu-19 surface marker. Based on the functional data reported in this paper, we conclude that circulating LAK effector cells are a phenotypically heterogeneous population that express surface Ag in association with NK cells and not T lymphocytes.

LAK[2] cells are generated after culture of PBL with IL-2 (1–4). LAK cells are distinguished from NK cells and cytolytic T cells by their ability to lyse NK-resistant cell lines and fresh tumor cells in a non-MHC restricted fashion (2). Because of their ability to lyse fresh tumor cells, LAK cells may play an important role in immune surveillance against malignant cells. In fact, Rosenberg and others recently reported that infusion of LAK cells in vivo along with rIL-2 resulted in a greater than 50% reduction in tumor size of patients with a variety of otherwise refractory malignancies (5–9).

Historically, LAK cells have been defined functionally by their ability to lyse fresh tumor and NK-resistant cell lines. Previous experiments attempting to phenotypically characterize these cells have been controversial. These studies have suggested a role for both T lymphocytes and/or NK cells at both the precursor and LAK effector cell stage of differentiation (2, 3, 10–16). Many of these studies have utilized negative selection of a population of cells (antibody and C pretreatment or panning) to characterize the phenotype of LAK cells (2, 3, 13, 16). At the precursor level, these experiments have been difficult to interpret since a failure to generate LAK cells could be due to removal of a cell indirectly involved in differentiation rather than the depletion of the LAK precursor cell. At the effector cell level, data have suggested that LAK effector cells are a heterogeneous population (16). Therefore, the removal of one effector cell population by negative selection may not eliminate all cytolytic activity.

In our previous studies we demonstrated that cancer patients treated with high dose rIL-2 have significant alterations in the phenotypic composition of circulating PBL (17) (T. M. Ellis, S. P. Creekmore, D. P. Braun, J. E. Harris, and R. I. Fisher, manuscript in preparation). These changes include: 1) increased percentages of Leu-19$^+$ and CD16$^+$ cells, 2) variable decreases in percentages of CD3$^+$ cells, 3) increased percentages of Leu-17$^+$ cells, and 4) decreased percentages of CD8$^+$ (bright) cells with concomitant increase in percentages of CD8$^+$ dull (CD8d$^+$) cells.

In this report, we present data on the phenotypic identification of LAK effector cells generated in vivo. After approximately 4 to 6 wk of high dose rIL-2 therapy, fresh PBL are able to lyse the NK-resistant Daudi cell line and fresh tumor cells in a 4-h ^{51}Cr-release assay. Our results demonstrate that LAK effector cells isolated from fresh PBL by FACS are Leu-19$^+$ and Leu-17$^+$ but CD5$^-$. Furthermore, Leu-19$^+$ LAK effector cells activated in vivo are a phenotypically heterogeneous population with regard to the expression of the CD8 and CD16 antigens.

MATERIALS AND METHODS

Reagents and cell lines. The MAb anti-Leu-1 (CD5), anti-Leu-2 (CD8), anti-Leu-11c (CD16), anti-Leu-17, and anti-Leu-19 were purchased from

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[2] Abbreviations used in this paper: LAK cells, lymphokine-activated killer cells; CD8d, CD8$^+$ dull cells; LGL, large granular lymphocytes; LU, lytic units; PE, phycoerythrin.

Becton Dickinson (Mountain View, CA). These antibodies were directly conjugated with either FITC (Leu-1 and Leu-2) or PE (Leu-11c, Leu-17, and Leu-19). The NK-sensitive K562 cell line and the NK-resistant Daudi cell line were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 with 10% FCS (K562) or 20% FCS (Daudi) plus L-glutamine.

Patient population. In the first protocol (A), five patients with incurable malignant melanoma or colon carcinoma were selected for study and had received no prior chemotherapy or radiotherapy. Patients received 3×10^7 U/m^2 rIL-2 via 24-h i.v. infusion, once weekly the first 4 wk and then twice weekly the next 4 wk. Some patients received a maintenance course of rIL-2 (3×10 U/m^2) for an additional 8 wk. Surgically resected colon carcinoma patients with poor prognosis stage C disease were entered onto a second protocol (B). These patients received 1×10^6 U/m^2 rIL-2 twice weekly delivered i.v. via an indwelling infusion pump. The patients on the second protocol received multiple rIL-2 infusions for up to 7 mo.

Flow cytometry and cell sorting. Fresh PBL were obtained from patients at various times during therapy but always before the infusion of rIL-2. Blood was diluted 1/2 with HBSS and then 40 ml were overlayed onto 10 ml of lymphocyte separation medium (Organon Teknika Corporation, Durham, NC) in 50-ml Falcon tubes (Falcon Labware, Oxnard, CA). After centrifugation ($500 \times g$) for 20 min, the lymphocyte-enriched interface was collected and washed extensively in HBSS with 5% FCS. PBL (2.5 to 3.0×10^7) were stained with saturating amounts of mAb for 30 min at 4°C. Cells were then washed three times with HBSS/5% FCS before analysis. Cell populations were purified by cell sorting with a FACS 420 (Becton Dickinson). Cells were sorted at a rate of 1000 cells/s with the abort circuitry activated. Purity was monitored after each sort by flow cytometry. Positive fractions were routinely greater than 90% pure and negative fractions were greater than 95% pure.

In vitro generation of LAK cells. Unseparated PBL or sorted cell populations were cultured at 5.0×10^5 cells/ml in 24-well flat-bottomed plates (Costar Laboratories, Cambridge, MA) containing RPMI 1640 supplemented with 2% human AB serum, 2 mM L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, 50 μg/ml gentamycin sulfate, and 1500 U/ml rIL-2 (Cetus Corp., Emeryville, CA). The plates were incubated at 37°C for 4 days at which time cells were harvested and assayed for cytolytic activity in a ^{51}Cr-release assay or stained with mAb and analyzed for cell surface determinants.

Preparation of fresh tumor cell targets. Fresh tumor cells were obtained by slight modification of the methods of Grimm et al (2). After removal of necrotic and fatty tissue, tumor was cut into 1 cm^3 fragments and incubated for 30 min in RPMI 1640 containing 0.01% hyaluronidase, 0.1 collagenase, and 0.002% DNase with constant stirring. Supernates were removed and the remaining tumor fragments were incubated with the enzymes an additional two times. The supernates from each enzyme treatment were pooled and the cells were washed three times with HBSS. If necessary, cells were centrifuged over a Ficoll-Hypaque gradient to remove dead cells and RBC. The tumor cells were resuspended in 90% human AB$^+$ serum/10% DMSO and frozen in liquid nitrogen. On the day of assay, cells were thawed, washed three times, and labeled with 200 μCi sodium chromate.

Assessment of LAK effector cell activity. K562 cells, Daudi cells, or fresh tumor cells were labeled with 200 μCi ^{51}Cr (New England Nuclear, Boston, MA) for 1 h at 37°C and then washed three times in RPMI 1640 containing 2% human AB serum. Target cells were subsequently incubated for 30 min at 37°C and washed once before resuspension in complete medium and were plated at 5000 cells/well in a 96-well round-bottom plate (Falcon Labware). Effector cells were added at various numbers to achieve a range of E/T ratios. The plates were centrifuged ($40 \times g$, 5 min) and then incubated at 37°C in a 5% CO_2 incubator. After 4 h, supernates were collected with the use of the Skatron Supernatant Collection System (Skatron Inc., Sterling, VA) and assayed for radioactivity by gamma scintillation counting. Data are presented as percent specific ^{51}Cr-release or as LU. Percent specific ^{51}Cr-release was determined as follows:

$$\text{Percent specific } {}^{51}\text{Cr-release} = \left(\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}\right) \times 100.$$

Spontaneous cpm were determined by measuring the amount of ^{51}Cr released from 5000 targets (six wells) incubated in complete medium for 4 h. Total cpm were determined by measuring the amount of ^{51}Cr released from 5000 target cells (six wells) incubated with 0.5% Triton-X detergent. One LU is defined as the number of effector cells required to lyse 30% of the target cells.

RESULTS

LAK cell generation in vivo. PBL from patients receiving doses of rIL-2 greater than or equal to 1×10^6 U/m^2 were assayed at various times during the course of therapy for NK and/or LAK effector cell activity. LAK activity was measured by the ability of fresh PBL without further in vitro stimulation with rIL-2 to lyse the NK-resistant Daudi cell line or fresh tumor cells in a 4-h ^{51}Cr-release assay. Seven patients undergoing rIL-2 therapy were examined for circulating LAK effector cells. PBL from these patients were incapable of lysing Daudi cell line before therapy (Table I). LAK cytolytic activity was observed, however, in all patients after multiple infusions of rIL-2. The cytolytic capacity of PBL to lyse the NK-sensitive cell line, K562, was also augmented after rIL-2 therapy. The augmentation in NK activity ranged between 2.9- and 43.6-fold.

Representative cytolytic data on one patient are presented in Figure 1. Although the patient was incapable of lysing Daudi cells before therapy, after 5 wk of rIL-2 infusion (six treatment courses) fresh PBL lysed Daudi cells and fresh tumor cells to a similar extent.

Three patients were studied to determine the time course of LAK effector cell development in peripheral blood. The results of these kinetic studies are presented in Figure 2. Although PBL were incapable of lysing the Daudi cell line at wk 1 to 3, one patient had significant cytolytic activity at wk 4 (Fig. 2C). By wk 6, PBL from all three patients demonstrated LAK activity in a cytotoxicity assay. We conclude that circulating LAK cells were detectable in patients receiving 3×10^7 U/m^2 rIL-2 per infusion 4 to 6 wk after the initiation of therapy.

LAK effector cells generated in vivo are Leu-19$^+$. We have observed that patients receiving rIL-2 have significant increases in circulating Leu-19$^+$ cells (17). Recently, it was reported that LAK effector cells generated in vitro express the cell surface Ag Leu-19 (12, 13, 18). We determined, therefore, if LAK effector cells developing in vivo express the Leu-19 Ag.

Leu-19 cell sorts have been performed on a total of four patients receiving rIL-2 therapy. In all cases, LAK effector cells isolated from peripheral blood were contained within the Leu-19$^+$ cell population (Table II). Although Leu-19$^-$ cells were capable of lysing the K562 cell line in some experiments (data not shown), these cells have not lysed the NK-resistant Daudi cell line. In other experiments, the Leu-19$^-$ cells failed to demonstrate significant anti-Daudi effector activity even after 18 h of incubation with ^{51}Cr-labeled target cells (data not shown).

Leu-19$^+$ and Leu-19$^-$ cells were isolated from a normal donor and then assayed for cytolytic activity against the NK-sensitive K562 cell line and NK-resistant Daudi cell line. As reported by others (18, 19), the Leu-19$^+$ cell population was capable of lysing the K562 cell line. Neither the unsorted, Leu-19$^+$, nor Leu-19$^-$ cells were capable of lysing ^{51}Cr-labeled Daudi cells (Table II). Therefore, isolation of Leu-19$^+$ cells by cell sorting is not inducing LAK activity but rather rIL-2 administration in vivo results in the generation of Leu-19$^+$ cells with LAK cytolytic activity.

Data from a representative Leu-19 sorting experiment on one patient are presented in Figure 3. Fresh PBL were stained with PE-conjugated anti-Leu-19 and separated into positive and negative populations by cell sorting. These cell populations were then assayed for cytolytic activity in a 4-h ^{51}Cr-

TABLE I

Summary of in vivo generated LAK cells

Patient	Protocol	LU/10^6 Cells			
		Anti-Daudi activity		Anti-K562 activity	
		Pre	Post	Pre	Post
1	A[a,b]	0	25.6	13.8	80.0
2	A	0	5.9	2.7	117.6
3	A	0	5.1	4.1	>80.0
4	A	0	13.1	7.1	20.8
5	A	0	3.6	5.4	>80.0
6	B[c]	0	8.0[d]	10.0	>80.0
7	B	0	14.5[e]	2.4	29.1

[a] rIL-2 (3×10^7) U/m^2: 1×/wk (wk 1 to 4), 2×/wk (wk 5 to 8).
[b] Cytolytic activity of fresh PBL was measured at wk 0 and wk 8.
[c] rIL-2 (1×10^6) U/m^2: 2×/wk.
[d] Cytolytic activity of fresh PBL was measured at 6 mo.
[e] Cytolytic activity of fresh PBL was measured at 3 mo.

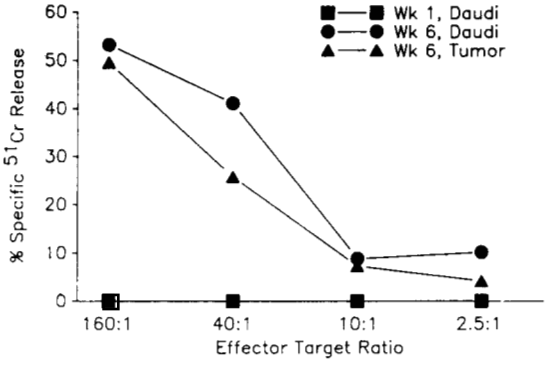

Figure 1. Effect of rIL-2 infusion on circulating LAK effector cell cytotoxicity. PBL from patient 3 on protocol A were obtained before therapy and assayed for cytolytic activity against Daudi cells (■) or after wk 5 of therapy and assayed for cytolytic activity against Daudi cells (●) or fresh tumor cells (▲) in a 4-h ^{51}Cr-release assay.

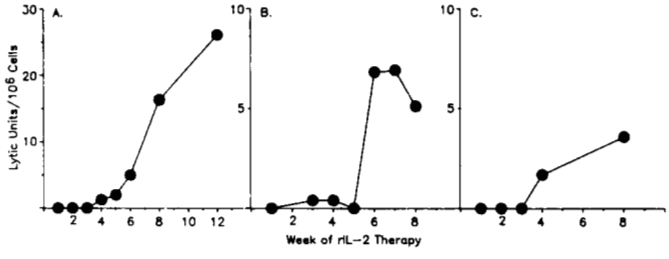

Figure 2. Kinetics of development of circulating LAK effector cells. PBL from patient 4 (*A*), patient 3 (*B*), and patient 5 (*C*) were obtained at weekly intervals and assayed for LAK activity against Daudi cells in a 4-h ^{51}Cr-release assay.

release assay. LAK effector cells defined by their ability to lyse the NK-resistant Daudi cell line were contained exclusively within the Leu-19$^+$ population (Fig. 3). Leu-19$^-$ cells were incapable of lysing the Daudi cell line even when assayed at fourfold higher concentrations than the Leu-19$^+$ cells (80:1 E/T ratio for Leu-19$^-$ cells vs 20:1 for Leu-19$^+$ cells). Comparison of LAK activity in terms of LU demonstrated that the isolation of Leu-19$^+$ cells resulted in a ninefold enrichment in cytolytic activity (5.8 LU for unsorted cells vs 51.3 LU for Leu-19$^+$ cells).

Phenotypic studies performed in our laboratory also demonstrated an increase in the percentage of Leu-17$^+$ PBL in patients undergoing rIL-2 therapy (17). Since a majority of Leu-19$^+$ PBL were also Leu-17$^+$, experiments were performed to determine if circulating LAK effector cells were restricted to the Leu-17$^+$ subpopulation. Leu-17 cell sorts were performed on three different patients receiving rIL-2 therapy. A summary of the cytolytic data of two of these patients is presented in Table II. LAK effector cell activity was associated entirely with the Leu-17$^+$ cell population with no detectable activity in the Leu-17$^-$ cell population. The Leu-17 sort on the third patient is presented in Figure 4. LAK activity was not evident in PBL of this patient yet was detectable in the Leu-17$^+$ sorted population. This is probably due to the fact that this patient had not received rIL-2 for 2 wk and circulating LAK activity had diminished. There was no cytolytic activity against Daudi cells in the Leu-17$^-$ enriched cell population. Cell populations from this patient were also assayed for cytolytic activity against fresh tumor cells. Although the Leu-17$^+$ cells were able to lyse fresh sarcoma cells, the Leu-17$^-$ cells were incapable of lysing these tumor cells (Fig. 4*B*).

Leu-19$^-$ cells contain LAK precursors. Previous reports demonstrated that LAK precursors were contained within the Leu-19$^+$ population of Percoll-enriched LGL (12). To evaluate the distribution of LAK precursor cells in patients receiving rIL-2, unsorted, Leu-19$^+$ and Leu-19$^-$ cell populations were cultured 4 days with rIL-2 and then assayed for cytolytic activity. Although the Leu-19$^-$ cells did not

TABLE II

Summary of Leu-19 and Leu-17 cell sorts

Patient	Cells Sorted	Percent Positive[a]	Target	LU/10^6 Cells		
				Unseparated	Positive	Negative
4	Leu-19	31	Daudi	13.7	40.0	0
5	Leu-19	25	Daudi	8.3	4.9	0
6	Leu-19	23	Daudi	7.1	29.0	0
7	Leu-19	7	Daudi	14.3	39.2	0
Normal	Leu-19	15	K562	8.0	13.3	0
			Daudi	0	0	0
6	Leu-17	22	Daudi	>40.0	8.1	0
7	Leu-17	28	Daudi	9.1	12.5	0

[a] Percent of total PBL which stained with antibody.

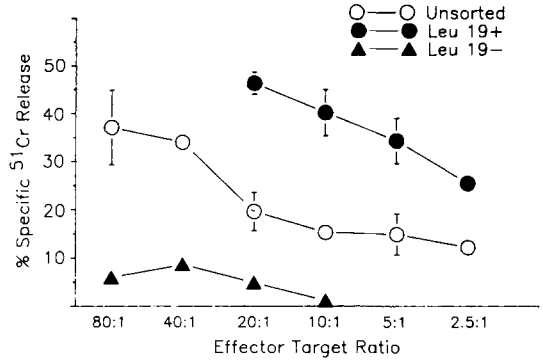

Figure 3.. Role of Leu-19+ PBL in circulating LAK effector cell activity. PBL were isolated from patient 6 on protocol B after 6 mo of therapy. The cells were stained with Leu-19 and sorted into positive and negative fractions. The Leu-19+ population represented 17% of the total PBL. Either unsorted (○), Leu 19+ (●) or Leu-19− (▲) cells were assayed for LAK cytolytic activity in a 4-h ^{51}Cr-release assay.

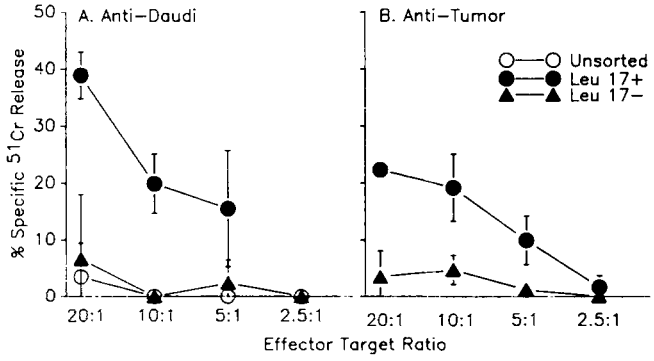

Figure 4. Contribution of Leu-17+ cells to circulating LAK effector cell activity. PBL from patient 4 (wk 14) were isolated, stained with Leu-17, and then sorted into Leu-17+ and Leu-17− fractions. Forty-eight percent of the PBL were Leu-17+. Unsorted (○), Leu-17+ (●), or Leu-17− (▲) cells were assayed for LAK activity against Daudi cells (*A*) or fresh tumor cells (*B*) in a 4-h ^{51}Cr-release assay.

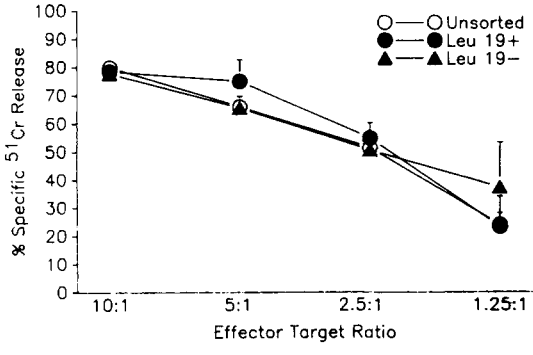

Figure 5. LAK activity after in vitro culturing of Leu-19+ and Leu-19− cells with rIL-2. PBL from patient 4 on protocol A (wk 8) were sorted into Leu-19+ and Leu-19− cell populations and then cultured with rIL-2. After 4 days, the unsorted (○), Leu-19+ (●), or Leu-19− (▲) cells were assayed for LAK activity in a 4-h ^{51}Cr-release assay.

lyse the NK-resistant Daudi cell line on day 0 (data not shown), these cells had cytolytic activity comparable to either the Leu-19+ or unseparated cell populations after a 4-day incubation with rIL-2 (Fig. 5).

Leu-19− cells that were in culture for 4 days with rIL-2 were also evaluated for the expression of Leu-19 on their cell surface and were shown to maintain Leu-19− phenotype after the culture period. These experiments were performed on three separate occasions with similar results. Therefore, there exist at least two distinct LAK effector cell populations. LAK cells generated in vivo all express the Leu-19 cell surface determinant. A second LAK effector cell which is Leu-19− can be generated in vitro from PBL of patients who have received rIL-2.

Circulating LAK effector cells are CD5−. Patients on long term rIL-2 therapy have moderate increases in the absolute number of CD3+ cells (17). Previous reports indicated that LAK effector cells generated in vitro can express the T cell-associated surface markers CD3 and CD8 (2, 3, 11, 16). We therefore examined the contribution of T lymphocytes to circulating LAK effector cell activity. Inasmuch as anti-CD3 antibodies have been shown to be inhibitory in cytolytic assays (20), we isolated T lymphocytes by using an anti-CD5 (FITC-Leu-1) antibody. Stained and unstained PBL were assayed for cytotoxicity against Daudi cells to determine whether anti-CD5 had any inhibitory effects on functional activity. The cytolytic capacity of these two groups were superimposable. Therefore, the control cells in Figure 6 represent PBL that have been stained with CD5 but not sorted. Purified CD5+ cells were not able to lyse the Daudi cell line. LAK effector cells were detectable, however, in the CD5− cell population (Fig. 6*B*). Taken together with the Leu-19 cell sorts, these data demonstrate that LAK effector cells generated in vivo express surface markers associated with NK cells, with no detectable activity in the T cell population.

LAK effector cells are phenotypically heterogeneous. Our laboratory has previously reported that the Leu-19+ cells developing in rIL-2-treated patients were heterogeneous with respect to other surface markers including CD8 and CD16 (17). Experiments were therefore performed to determine if circulating LAK effector cell activity were restricted to or enriched in either of these Leu-19+ subpopulations.

In the first experiment, PBL were isolated, stained with anti-CD16 (PE anti-Leu-11c) and then sorted into CD16 positive and negative fractions. Phenotypic analysis on cells before sorting revealed that all CD16+ cells were Leu-19+, whereas the CD16− population was composed of both Leu-19+ and Leu-19− cells. Cytolytic activity against the Daudi cell line was present in both CD16+ and CD16− fractions (Table III, Expt. 1). Thus, further purification of Leu-19+ cells based on co-expression of CD16 antigen did not result in the isolation of LAK effector cells.

Phenotypic studies of PBL from patients receiving rIL-2 therapy have demonstrated that with time there occurs a marked decreased in the relative number of CD8+ bright cells with an accompanying increase in CD8 dull (CD8d+) cells (17). All CD8d+ cells also expressed the Leu-19 marker.

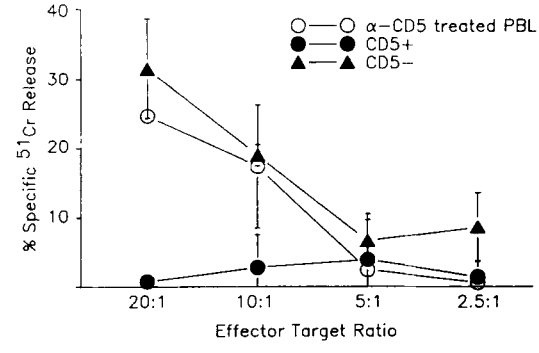

Figure 6. Contribution of CD5+ cells to circulating LAK effector cell activity. PBLs from patient 4 (wk 16) were sorted into CD5+ (Leu-1) or CD5− cell population. The CD5+ population represents 55% of the total population. CD5 stained but unsorted (○), CD5+ (●) or CD5− (▲) cells were assayed for LAK activity in a 4-h ^{51}Cr-release assay.

TABLE III
Phenotypic heterogeneity of LAK cells

Expt.	Effectors	Targets	E/T Ratio					
			40:1	20:1	10:1	5:1	2.5:1	1.25:1
1[a]	Unseparated	Daudi	ND	ND	30.5	8.3	4.3	1.5
	CD16$^+$	Daudi	ND	ND	38.9	6.8	8.1	0
	CD16$^-$	Daudi	ND	ND	41.3	12.0	0	3.5
2[b]	Unseparated	Daudi	20.6	10.4	1.1	0	ND	ND
	CD8d$^+$Leu-19$^+$	Daudi	22.7	17.5	6.5	4.5	ND	ND
	CD8d$^-$Leu-19$^+$	Daudi	25.6	27.3	14.4	5.9	ND	ND

[a] Patient on protocol A (wk 8).
[b] Patient on protocol A (wk 11).

However, a significant number of Leu-19$^+$ cells do not express the CD8 antigen. We determined if LAK effector cells co-express both Leu-19 and CD8d. Fresh PBL were sorted into the CD8d$^+$Leu-19$^+$ and CD8d$^-$Leu-19$^+$ populations. When assayed in a ^{51}Cr-release assay, both CD8d$^+$Leu-19$^+$ and CD8d$^-$Leu-19$^+$ cell populations had comparable cytolytic activity (Table III, Expt. 2).

DISCUSSION

In 1982, Grimm et al. (2) reported that culturing PBL with lectin-free, IL-2-containing supernates resulted in the generation of LAK cells capable of lysing fresh solid tumor cells (2). Since then, there have been numerous attempts to phenotypically define the LAK effector cells. Conflicting data have suggested that LAK effector cells develop from either T lymphocyte or NK cell populations. Initial studies with antibody and C pretreatment indicated that LAK effector cells in both the murine and human system express T lymphocyte cell surface determinants (2, 21–23). However, more recent reports have demonstrated that the bulk of LAK activity is mediated by cells expressing NK-associated cell markers (12–15, 17, 24–27).

We have evaluated the phenotype of LAK cells developing in malignant melanoma and colon carcinoma patients who received high dose rIL-2 therapy. In all patients studied to date, LAK effector cells have been detected in PBL. Low levels of activity were detectable between 4 and 6 wk after initiation of therapy. At 6 wk there was a significant increase in circulating LAK effector cells. This change reflects a true increase in circulating cells and not due to a change in time points at which activity is measured (3 days post-IL-2 infusion (wk 6 to 9) vs 7 days post-IL-2 (wk 2 to 5 and 12)) since the greatest activity was observed at wk 12 after rIL-2 infusion returned to once a week. This suggests that detection of circulating LAK effector cells is primarily dependent on the quantity of rIL-2 infused or the duration of rIL-2 treatment.

The development of circulating LAK effector cells presented us with an opportunity to phenotypically identify those cells generated in vivo that have functional LAK activity. There are numerous reasons why the isolation and phenotypic characterization of in vivo generated LAK cells would be advantageous to previous studies on in vitro generated LAK cells. First, our previous data demonstrating the increase in Leu-19$^+$ cells in rIL-2 treated patients and recent reports (12, 13, 18) that LAK cells generated in vitro are Leu-19$^+$ suggested that a large number of PBL in these patients had LAK effector cell activity. Therefore, these cells could easily be isolated by positive selection techniques. Second, if circulating LAK effector cells were Leu-19$^+$, then the phenotypic heterogeneity of Leu-19$^+$ cells also suggested that these cells were arising from multiple precursor pools. Hence, the relative contributions of subpopulations to total LAK activity could be analyzed by selecting for each subpopulation. Finally, the functional cells isolated from peripheral blood represent the cells responding to rIL-2 in vivo, thereby eliminating in vitro induced artifacts.

The Leu-19$^+$ cell population in normal individuals represents approximately 15% of PBL and is comprised predominantly of LGL that possess NK activity (19). Patients on high dose rIL-2 therapy exhibit significant relative and absolute increases in circulating Leu-19$^+$ PBL (17). The experiments reported in this paper demonstrate that circulating LAK effector cells obtained from these patients are all Leu-19$^+$ cells. In fact, in four out of five Leu-19 sorting experiments, the isolation of Leu-19$^+$ cells resulted in an enrichment in cytolytic activity. The increase in cytolytic activity is due to enrichment of functional cells rather than an antibody (Leu-19)-mediated enhancing effect inasmuch as the anti-Daudi lytic activity of Leu-19-treated but unsorted PBL is comparable with untreated cells. Also, the addition the Leu-19 antibody to the 4-h ^{51}Cr-release assay had no effect on LAK activity of PBL (data not shown). These data are in agreement with recent reports that LAK cells generated in vitro express the Leu-19 Ag (12, 13, 18). We have also fractionated fresh PBL from a normal donor into Leu-19$^+$ and Leu-19$^-$ cells. Although Leu-19$^+$ cells were able to lyse the K562 cell line, they did not lyse Daudi cells. Therefore, the cytolytic activity mediated by Leu-19$^+$ cells from patients is a direct result of rIL-2 infusion.

Leu-19$^+$ cells developing in patients receiving rIL-2 have been shown to be comprised of two subpopulations (Leu-19$^+$ and "bright" Leu-19$^+$) based on the intensity of Leu-19 staining (17). It should be noted that in the present studies Leu-19$^+$ cells were isolated without regard for the intensity of staining. Therefore, the Leu-19$^+$ cell populations assayed for LAK activity consisted of both Leu-19$^+$ and "bright" Leu-19$^+$ cells. Experiments are presently being performed to determine which of these Leu-19$^+$ cell populations is responsible for LAK cytolytic activity.

In the phenotypic studies on these patients, we demonstrated that the Leu-19$^+$ PBL are heterogeneous with respect to other cell surface determinants. A majority of Leu-19$^+$ cells also express Leu-17$^+$. Subpopulations of Leu-19$^+$ cells express the CD8d and CD16 cell surface markers. Although the CD8 marker is primarily expressed on cytotoxic and suppressor T lymphocytes (28), NK cells have been shown to express low levels of CD8 (29). The cell sorting data demonstrate that all circulating LAK effector cells are Leu-17$^+$. Similar to the Leu-19 experiments, the increase in cytotoxicity was due to enrichment of the Leu-17$^+$ population rather than an antibody-mediated effect inasmuch as the lytic activity of Leu-17-stained PBL was comparable to unstained cells. However, when fresh PBL were sorted

for CD8d or CD16 expression, LAK effector cells were present in both the positive and negative populations. Based on cytolytic units, the cytolytic activity is comparable in the positive and negative fractions of both the CD8d and CD16 cell sorts. Hence, cell sorting based on co-expression of Leu-19 and either CD8d or CD16 did not further enrich for LAK effector cells. Circulating LAK effector cells, therefore, are homogeneous with respect to Leu-19 and Leu-17 expression but heterogeneous when further separated by co-expression of CD8 or CD16.

There is a small population of cells in normal individuals that expresses both the Leu-19 and CD3 Ag. Our phenotypic studies demonstrate that this population is not expanded significantly during rIL-2 infusion (17). This conclusion was extended in this paper by demonstrating that CD5$^+$ cells, which include all CD3 cells, do not contribute to circulating LAK effector cell activity.

We were able to generate LAK cells from the Leu-19$^-$ cell population after 4 days in culture with rIL-2. These LAK effector cells, however, did not express the Leu-19 surface Ag after culture. Preliminary data suggest these LAK effector cells may be CD3$^+$. It is possible that this cytolytic cell may be the CD3$^+$CD16$^-$ effector cells described by others (2, 10, 11, 13–16). These data would support the work of Ortaldo and co-workers (13) who proposed that there were at least two populations of LAK effector cells: a CD3$^-$CDw16$^+$ population and a CD3$^+$CDw16$^-$ population. However, the generation of LAK cells from the Leu-19- population is in contrast with the work of Phillips and Lanier (12) who postulated that all LAK precursors are contained within the Leu-19$^+$ subpopulation. In the present studies, PBL from either rIL-2-treated patients or untreated normal volunteers were used for LAK generation, whereas their studies enriched for LGL before culturing. It is possible that the Leu-19$^-$ precursor is contained within the lymphocyte population and not the LGL fraction.

We conclude that, although both Leu-19$^+$ and Leu-19$^-$ cells can mediate LAK effector cell cytotoxicity after in vitro generation, circulating LAK effector cells generated in vivo are all Leu-19$^+$. Also, the data demonstrate that circulating LAK effector cells express cell surface markers in common with NK cells but not T lymphocytes. LAK effector cells are Leu-19$^+$, Leu-17$^+$ but CD5$^-$. Some LAK effector cells are CD8d$^+$ and CD16$^+$. Whether these differences in CD8 and CD16 expression represent multiple stages in LAK effector cell differentiation or rather separate effector cells arising from distinct precursors is presently being examined.

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