LYMPHOKINE-ACTIVATED KILLER CELLS IN RATS
III. A Simple Method for the Purification of Large Granular Lymphocytes and Their Rapid Expansion and Conversion into Lymphokine-activated Killer Cells

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Recent advances in adoptive immunotherapy have used cytotoxic lymphocytes with broad antitumor cytotoxicity. Studies have shown that the coculture of lymphocytes with rIL-2 induces the generation of lymphokine-activated killer (LAK)[1] cell activity and this activity has proven useful in the control of metastatic tumors in animal models and in some cancer patients (1–3).

Adoptive immunotherapy using cells with LAK activity first requires the removal of large numbers of lymphocytes from the patient followed by a period of in vitro culture in IL-2 (1, 4). In general, an overall expansion of lymphocytes in bulk cultures is seldom achieved (4) and it is difficult to accurately assess the number of cells with LAK activity reinfused into the patient. The development of methods that would enable the rapid expansion of purified populations of cells with LAK activity would provide an acceptable and presumably preferable alternative to conventional culture methods.

We have recently reported on the generation of cells with LAK activity in rats (5) and have demonstrated that the majority of LAK progenitors are contained within the large granular lymphocyte (LGL) population, expressing surface markers characteristic of rat NK cells (5–7). In the present studies, we demonstrate that one of the first responses by LGL to rIL-2 is their attachment to plastic surfaces. Exploiting this observation, we now show that rIL-2–activated plastic adherent LGL can expand between 30- and 100-fold in 3–4 d of culture to generate exceedingly high levels of broad antitumor cytotoxicity.

Materials and Methods

Animals. Male Fischer 344 rats (75–100 g) were purchased from Taconic Farms, Inc. (Germantown, NY) and were housed in a specific pathogen-free animal facility at the Pittsburgh Cancer Institute.

Tumor Cells. Routinely, the lysis of the NK-resistant mastocytoma, P815, was used as

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[1] *Abbreviations used in this paper:* LAK, lymphokine-activated killer; LGL, large granular lymphocyte.

an indicator of LAK activity. Other targets included two NK-resistant syngeneic rat tumor cells: MADB106 (F344 mammary adenocarcinoma) (8) and CRNK-16 (F344 LGL leukemia) (9). All of these lines were grown in RPMI 1640 medium with 10% FCS and antibiotics. In several cases, fresh tumor explants were used as targets including fresh ascites tumor of CRNK-16 leukemia and fresh solid tumor explants of the MADB106 adenocarcinoma. The NK-sensitive Moloney virus–induced YAC-1 lymphoma was used as the indicator of NK activity (10).

Interleukin 2. Human rIL-2 was kindly provided by the Cetus Corp. (Emeryville, CA) and contained 1.25×10^6 U of IL-2/mg of protein (as defined by [^{3}H]thymidine incorporation into CTLL-2 cells).

Preparation of Lymphoid Cells. Spleens were aseptically removed and single-cell suspensions prepared in RPMI 1640 with 10% FCS. Splenic mononuclear cells were obtained after centrifugation on Ficoll/Hypaque gradients (density = 1.077) at 300 *g* for 20 min. Peripheral blood was obtained by cardiac puncture into heparinized syringes. Mononuclear cells were then obtained after centrifugation on Ficoll/Hypaque gradients (density = 1.077) at 300 *g* for 30 min. Spleen or peripheral blood mononuclear leukocytes were routinely passed over nylon-wool columns to remove monocytes/macrophages and B cells (11). Thus, 10^8 spleen cells in 2 ml of RPMI 1640, 10% FCS, were added to a 10-cc syringe containing 6 g of sterile nylon wool (Cellular Products, Buffalo, NY). The cells were incubated for 1 h at 37°C and the nylon wool was gently washed (without squeezing) with 20 ml of 37°C RPMI 1640, 10% FCS. The nonadherent cells were collected, washed, and used. By this procedure, we consistently reduced the percentage of B cells in the spleen preparations to <2% (by flow cytometric analysis using anti-Ig antibodies) and the percentage of monocyte/macrophages to <0.3% (by morphologic analysis of Giemsa-stained cytocentrifuge preparations). In some experiments LGL were purified from the nylon-wool nonadherent blood or spleen cells by Percoll density centrifugation, as described by Reynolds et al. (12). Briefly, nylon-wool nonadherent leukocytes were placed on four-step Percoll gradients having densities of 48, 52, 56, and 60% Percoll. The gradients were centrifuged at 400 *g* for 30 min and the LGLs were obtained from the 48/52% interface.

Generation of Cells with LAK Activity from Standard Cultures. Cells with LAK activity were produced by culture in human rIL-2. Medium conditions for the generation of LAK activity were determined in preliminary experiments and included the following: RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Gibco Laboratories), 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics (streptomycin/penicillin) (hereafter referred to as LAK medium) containing 10^3 U/ml rIL-2. Hepes buffer was routinely omitted from the medium. The lymphoid cells were cultured at an optimal density of 2×10^6 viable cells/ml in LAK medium in 5% CO_2/95% air at 37°C.

Generation of Cells with LAK Activity from Adherence Cultures. 50×10^6 nylon-wool nonadherent mononuclear leukocytes were cultured in 25 ml of LAK medium (containing 1,000 U/ml rIL-2) in T-75 flasks (Corning Glass Works, Corning, NY). The cells were cultured for various periods of time (from 2 to 72 h) at 37°C, after which the nonadherent cells were decanted and the adherent cells washed three times with 20 ml of warm (37°C) RPMI 1640 containing 2% FCS. The adherent cells then received 25 ml of either fresh LAK medium containing fresh rIL-2 or the conditioned medium from which they were initially cultured. This conditioned medium was prepared by removing the nonadherent cells by centrifugation and passing the medium through a 0.45-μm millipore filter. Conditioned medium was routinely used fresh at a 100% concentration, but could also be used when diluted 1:1 with fresh medium. This medium could also be stored at −20°C without loss of growth-promoting activity. The cultures were then continued at 37°C for a total of 5 to 7 d. To remove the adherent LGLs, the medium was decanted and 5 ml of 5 mM EDTA in PBS was added, and the flask was scraped with a rubber policeman.

Antibodies Used. A panel of antibodies was used in these studies. These included the mouse mAbs OX8 (CD8, γ1), OX19 (CD5, γ1), OX6 (Ia, γ1), OX39 (CD25, [IL-2 receptor, γ1]); all purchased from Accurate Chemical and Scientific Corp. (Westbury,

NY). Each of these antibodies was used at 1:100 dilution based on preliminary dose-response titrations. Monoclonal R1-3B3 (γ2b) (CD5) was obtained from Dr. Craig Reynolds (Biological Response Modifier's Program, Frederick, MD) and used at 1:1,000 dilution. Anti-asialo GM_1 was purchased from Wako Chemical Co. (Dallas, TX) and used at a 1:200 dilution. Rabbit antilaminin antiserum (R601) was provided by Dr. James Varani (Department of Pathology, University of Michigan, Ann Arbor, MI) and its reactivity with NK cells has been previously described (13, 14). Monoclonal antilaminin B_2 chain (Lam-1) (γ2b) was provided by Dr. Albert Chung (Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA) (15). All second-step reagents were FITC-labeled $F(ab')_2$ fragments of goat antibody against the primary antibody and were purchased from Cappel Laboratories (Malvern, PA).

Flow Cytometry. For surface marker analysis, 2×10^5 lymphocytes were placed in 12 × 75-mm glass tubes in 0.1 ml of staining buffer (PBS, pH 7.3, 0.1% sodium azide, 2% FCS). Various antisera or normal sera were added (1:20 to 1:100 final dilution) for 30 min at 4°C. The cells were washed twice and resuspended in FITC-labeled $F(ab')_2$ fragments of anti-IgG of the primary antibody (Cappel Laboratories). After 30 min at 4°C, the cells were washed twice, resuspended on 1% paraformaldehyde, and analyzed for fluorescence on a FACStar flow cytometer (Becton Dickinson and Co., Mountain View, CA).

Cytotoxicity Assay. Cytotoxicity was measured in a standard 4-h ^{51}Cr-release microcytotoxicity assay using 96-well, round-bottomed microplates (Costar, Cambridge, MA). The target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ per 2×10^6 cells, washed, and seeded into 96-well cultures dishes at 5×10^3 cells/well. Suspensions of effector cells were then added to triplicate wells to give various E/T ratios in a final volume of 200 μl. After an additional incubation at 37°C for 4 h, 100 μl of supernatant was removed from each well and was counted in a γ counter to determine experimental release. Spontaneous release was obtained from wells receiving target cells and medium only, and total release was obtained from wells receiving 1% Triton X-100. The spontaneous release never exceeded 20% of the total release, and in most experiments it ranged between 5 and 15% of the total release. The percent cytotoxicity was calculated by the following formula: Percent cytotoxicity = 100 × [(experimental release − spontaneous release)/(total release − spontaneous release)].

Lytic units of cytotoxic activity were determined from linear regression curves plotted from various E/T ratios. In all cases, 1 LU was defined as the number of effector cells required to cause 20% specific ^{51}Cr release from 5×10^3 target cells. Total lytic units per culture were calculated by multiplying the LU_{20} value by the total number of cells in the culture.

Proliferation Assays. Cell proliferation was measured by the incorporation of [^{3}H]TdR into DNA. Cells were plated at 5×10^4 cells in 0.2 ml of LAK medium in 96-well flat-bottomed microplates. At the time of testing for proliferation the cells were pulsed with 1 μCi of [^{3}H]TdR (sp act, 2 Ci/mmol; New England Nuclear, Boston, MA) for 4 h. [^{3}H]-TdR incorporated into DNA was determined after harvesting using a MASH II harvester and was counted in a scintillation counter.

Autoradiography. 2×10^5 cells were incubated with 2 μCi [^{3}H]TdR in 200 μl of LAK medium for 2 h at 37°C. The cells were then washed, cytospin preparations prepared, and the cells fixed with absolute methanol for 30 min. The cells were covered with NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), developed after a 2-d exposure, and counterstained with Giemsa solution.

Statistics. Statistical analysis was performed using the Student's *t* test.

Results

Generation of LAK Activity from Plastic Adherent and Nonadherent Lymphocytes. Nylon-wool nonadherent F344 spleen cells were cultured at 2×10^6/ml in LAK medium containing 1,000 U/ml rIL-2. After 2, 24, 48, or 72 h, the plastic adherent or nonadherent lymphocytes were collected, counted, and

TABLE I

Generation of LAK Activity from Purified LGL Selected by Their Adherence to Plastic Surfaces

Group	Incubation	Cells	Cells/ml	LGL	Proliferation	Cytolytic activity	
						YAC-1	P815
	h		$\times 10^{-6}$	%	*dpm* $\times 10^{-3}$	$LU_{20}/10^7$ *cells*	
1	0	Spleen	2.00	3	NT	40	0
	2	NA	2.00	3	2.9	102	0
		ADH	0.02	94	5.7	1,008	0
	24	NA	2.00	3	32.1	110	20
		ADH	0.03	96	144.3	5,458	1,105
	48	NA	2.00	7	32.3	195	110
		ADH	0.09	97	324.3	7,266	1,581
	72	NA	2.00	14	118.2	841	145
		ADH	0.13	97	397.4	5,221	948
2	48	NA	2.00	9	NT	123	11
		ADH	0.02	98	NT	2,011	221

Nylon-wool nonadherent F344 spleen (group 1) or peripheral blood (group 2) mononuclear cells were cultured at 2×10^6/ml in 1,000 U/ml rIL-2 for 2, 24, 48, or 72 h. At each time point, the adherent (ADH) and nonadherent (NA) cells were harvested, counted, and assayed for proliferation ([^{3}H]TdR incorporation into DNA in 4 h), cytotoxicity against YAC-1 and P815 target cells, and the percentage of LGL. The time 0 point represents fresh nylon-wool nonadherent spleen cells.

analyzed for morphology, surface markers, proliferation, and cytotoxic activity against YAC-1 and P815 targets. The representative data in Table I show that within 2 h of culture in rIL-2, a population of lymphocytes expressing high levels of YAC-1 cytolytic activity (but not P815 cytolytic activity) became attached to the plastic surface. Giemsa-stained cytospin preparations revealed that these adherent cells were 94–98% LGL and <1% monocytes/macrophages by morphological criteria (Fig. 1, *A* and *C*). Using spleen cells, the percentage of cells adhering to the plastic was low (~1–2%) at 2 h but increased to ~4.5% of the input cells by 48 h (Table I).

Cytolytic activity against the NK-sensitive YAC-1 target was high when 2-h adherent cells were tested and continued to increase when the adherent cells were collected and tested at 24 or 48 h. Cytolytic activity against YAC-1 from 48-h adherent cells was ~40 times higher than that seen with the nonadherent population and ~180 times higher than the cytotoxic activity in fresh unactivated, nylon-wool nonadherent spleen cells. Cytolytic activity against the P815 target was undetectable with 2-h adherent cells but was high in 24-h adherent cells and peaked with 48-h adherent cells. The data in Table I also show that the proliferative activity of adherent LGLs was low with 2-h adherent cells but became marked in 24, 48, and 72-h adherent cells.

Surface Marker Phenotype of Plastic Adherent LGL. Surface marker analysis was performed to determine the relative contribution of lymphocyte subsets to the population of plastic adherent LGLs. The data shown in Table II indicate that rIL-2–induced plastic adherent splenic LGL (24-h adherent cells, 96% LGL) expressed surface markers characteristic of rat NK cells. These cells were OX8(CD8)$^+$, asialo GM_1^+, and laminin-positive, but OX19(CD5)$^-$, R1-3B3(CD5)$^-$, W3/25(CD4)$^-$, OX39(CD25)$^-$, Ia$^-$, and Ig$^-$. A similar marker profile

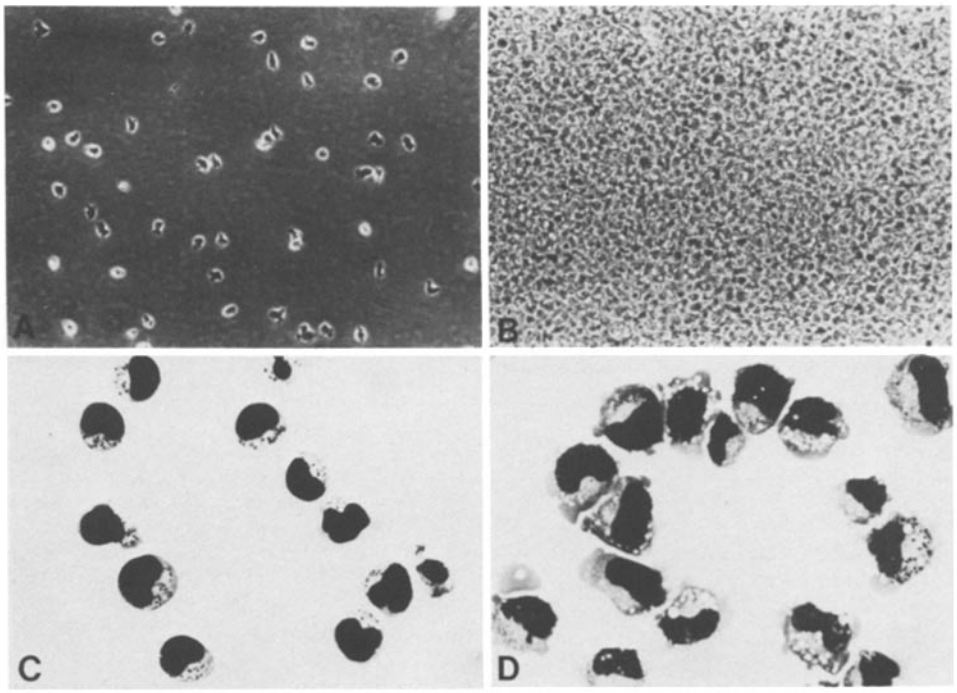

FIGURE 1. Photomicrographs of plastic adherent LGL in response to rIL-2. Nylon-wool nonadherent F344 spleen cells were cultured in LAK medium for 24 h and the plastic adherent cells were refed with the 24-h conditioned medium and cultured for an additional 4 d. (*A*) Phase-contrast photomicrograph of 24-h adherent LGLs; (*B*) the same cells as in *A* after an additional 4 d of culture; (*C*) Giemsa-stained cytospin preparation of adherent LGL from *A*; (*D*) Giemsa-stained cytospin preparation of cultured cells from *B*.

TABLE II

Surface Marker Phenotype of rIL-2–induced Adherent LGLs vs. LGLs Obtained from Percoll Gradients

Cell population	LGL	Positive cells								
		NK markers			T cell subset markers				Others	
		OX8	Asialo GM_1	Laminin	OX19	R1-3B3	W3/25	OX39	Ia	Ig
	%		%				%		%	
Adherent (24 h, spleen)	96	94	91	74	3	2	2	4	13	2
Adherent (48 h, spleen)	98	95	97	95	5	4	1	3	46	3
Percoll (PBL)	92	96	86	62	4	4	2	2	1	4
Percoll (spleen)	55	50	84	NT	9	11	9	3	NT	4

Nylon-wool nonadherent F344 spleen or PBLs were obtained and the LGLs were purified by plastic adherence (24 or 48 h) or Percoll gradient centrifugation. These cells were analyzed for their expression of various surface markers by flow cytometry and LGLs were quantitated by Giemsa-stained cytospin preparations (1,000 cells counted).

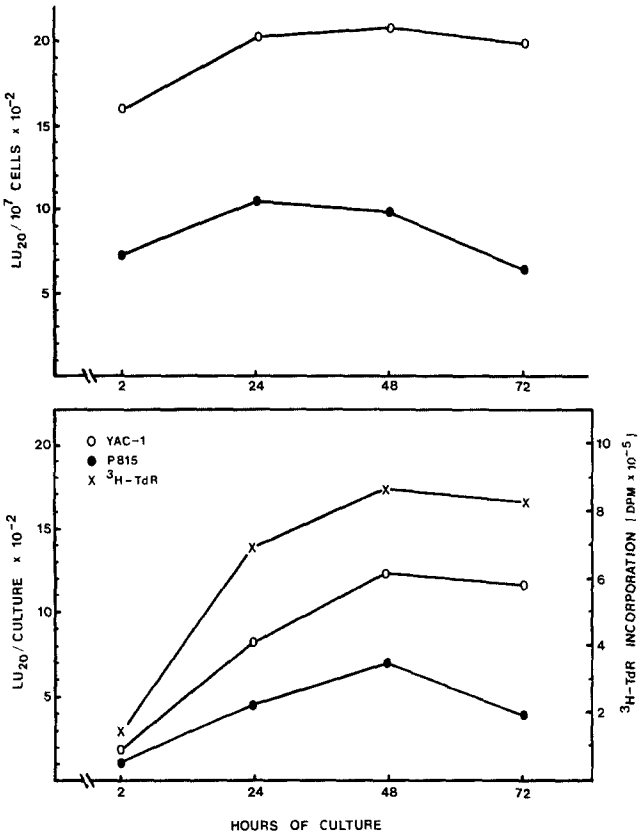

FIGURE 2. Generation of LAK activity from adherent LGLs collected at different times after addition of rIL-2. Nylon-wool nonadherent F344 spleen cells were cultured in LAK medium for 2, 24, 48, or 72 h. At each time point, the nonadherent cells were removed and the adherent cells refed with the conditioned medium. All cultures were then continued for a total of 5 d. Cytolytic activity on YAC-1 and P815 targets, as well as [^{3}H]TdR incorporation, were determined on day 5. (*Top*) Lytic U/10^7 cells. (*Bottom*) Total lytic U/culture and [^{3}H]TdR incorporation into DNA.

was observed on 48-h adherent splenic LGL (Table II) as well as adherent cells isolated from peripheral blood (data not shown). Although the OX6 (Ia) marker was present on a low percentage of 24-h adherent cells, nearly 50% of these cells expressed this marker at 48 h. It should be noted that the increased expression of the OX6 (Ia) antigen on 24- and 48-h adherent LGL indicates that these cells have undergone activation and does not represent monocyte/macrophage contamination. These adherent cells were not phagocytic (for latex beads) and macrophages were not recognized morphologically in Giemsa-stained cytospin preparations (data not shown).

The data in Table II indicate that the phenotype of plastic adherent splenic LGLs was essentially identical to that of blood LGLs purified by Percoll gradient centrifugation. The advantage of using the plastic adherence method becomes obvious when the phenotypes of adherent splenic LGL are compared with Percoll-purified splenic LGL. In this case, only 55% of the Percoll-purified spleen cells were LGL, with a substantial level of T cell contamination (~10%).

Generation of LAK Activity from Adherent LGLs Collected at Different Times after Addition of rIL-2. To determine the optimal time for selecting the adherent LGL for expansion and generation of LAK activity, kinetic experiments were performed. These data are shown in Fig. 2. Adherent splenic LGLs were obtained at 2, 24, 48, or 72 h and cultured in their own conditioned medium

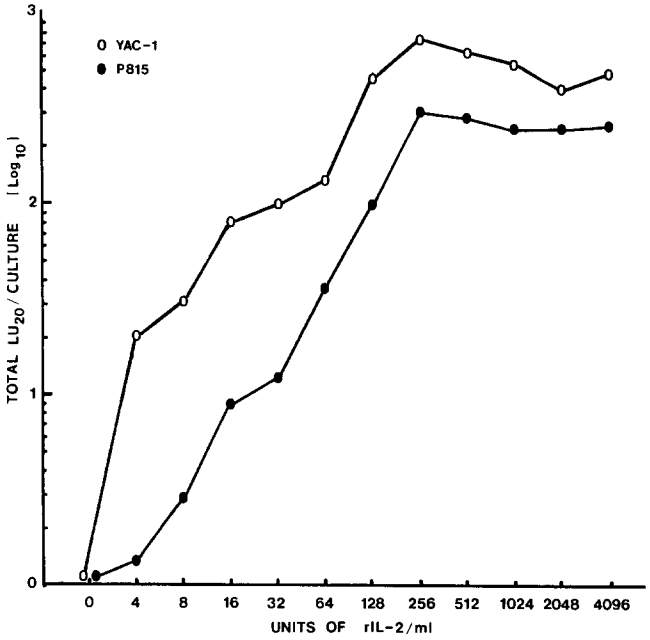

FIGURE 3. IL-2 dose–response relationship for the generation of LAK activity from plastic adherent F344 spleen cells. 20 × 10^6 nylon-wool nonadherent spleen cells were cultured in T-25 flasks in LAK medium containing various amounts of rIL-2. After 48 h the nonadherent cells were removed and the adherent cells were refed with the conditioned medium and continued in culture for an additional 3 d. Total cytolytic U/culture were then determined for YAC-1 and P815 target cells.

for a total of 5 d. Although similar levels of cytotoxicity were generated on a per cell basis ($LU_{20}/10^7$ cells) regardless of when the adherent cells were collected, total lytic units per culture were clearly highest from adherent cells collected at 48 h. The low levels of total lytic units per culture obtained with 2-h adherent cells is indicative of the low number of adherent cells obtained at 2 h as well as their low level of expansion over the next 5 d. Because of results such as those shown in Fig. 2, we chose 48-h cultures as the optimal time for selecting adherent cells for expansion in all subsequent experiments.

IL-2 Dose–Response Relationship for Optimal Growth of Plastic Adherent LGL. Dose–response experiments were conducted to determine the optimal level of rIL-2 required to generate and expand adherent spleen cells. Thus, 20 × 10^6 nylon-wool nonadherent spleen cells were placed in different T-25 flasks and cultured in different levels of rIL-2. After 48 h the adherent cells were collected, refed with their conditioned medium, and allowed to grow for an additional 3 d (5 d total). The results of these experiments are shown in Fig. 3. While adherent cells could be generated in as little as 4 U/ml rIL-2, optimal levels of expansion and generation of total cytolytic activity per culture were obtained in cultures containing at least 100 U/ml rIL-2. For rats, this is also the optimal rIL-2 dose range for generating LAK activity in standard bulk cultures.

Phenotype of Adherent LAK Cells. The phenotype of the LAK effector cells generated from plastic adherent LGLs was also determined. These data are presented in Table III. 48-h adherent spleen cells were cultured for an additional 3 d to expand and generate LAK effector cells. Morphologically, these cells were ~98% LGL with vacuolated cytoplasm and undulating surfaces (Fig. 1 *D*). Surface marker analysis revealed these cells expressed high levels of OX8, asialo GM_1, laminin, and Ia surface markers. Few cells (0–5%) expressed pan-T cell markers

TABLE III

Phenotype of 5-d Expanded Cells Generated from Plastic Adherent LGLs, Percoll-purified LGLs, or in Standard Bulk Cultures

LAK cells derived from:	LGL‡	Positive cells*								
		NK markers			T cell markers				Others	
		OX8	Asialo GM1	Laminin	OX19	R1-3B3	W3/25	OX39	Ia	Ig
	%		%			%			%	
Adherent LGLs§	98	80	95	94	4	1	2	9	63	2
Percoll-purified LGLs‖	95	84	78	68	6	5	6	NT	60	4
Standard LAK¶	42	85	92	54	41	32	4	27	56	12

* Flow cytometric determination.
‡ Giemsa-stained cytospin preparation (1,000 cells counted).
§ 48-h adherent LGLs cultured from nylon wool–passed spleen cells. The percent LGL in the 48-h adherent population was 98%. The adherent cells were cultured for an additional 3 d.
‖ Percoll-purified peripheral blood LGLs. The percent LGL in the purified population was 94% at the initiation of culture. Cells were cultured for 5 d.
¶ Nylon-wool nonadherent F344 spleen cells cultured for 5 d.

(OX19, R1-383), helper T cell markers (W3/25), or B cell markers (Ig). In addition, the OX39 marker (IL-2-R) was expressed on only 9% of the responding adherent LGLs, and in relative low intensity. This phenotype was similar to that obtained from Percoll-purified peripheral blood LGLs after growth in rIL-2 for 5 d. The phenotype of cells present in standard bulk cultures is also shown for comparison.

IL-2-activated Adherent LGLs Expand Rapidly in Culture. The in vitro expansion of rIL-2–activated plastic adherent LGL was investigated. The data shown in Fig. 4 indicate that when 48-h adherent cells were allowed to expand in rIL-2 for an additional 3–4 d (i.e., 5–6 d of total culture), the expansion indices often reached as much as 90-fold. To determine this, 48-h adherent cells (97% LGL) were collected, then replated at a density of 5×10^4 cells/ml, and allowed to grow for an additional 3–4 d. These cells reached densities between 1.8 and 3.0×10^6 cells/ml over the next 3–4 d (Fig. 1, *B* and *D*; and Fig. 4, *A* and *B*). In some experiments (2 of 6), densities reached as high as 4.5×10^6 cells/ml (or an expansion of 90-fold). Furthermore, we noted that when the adherent cells were cultured with the conditioned medium from which they were originally growing, the expansion indices were two- to threefold higher than when adherent cells were cultured with fresh LAK medium containing fresh rIL-2 (Fig. 4 *A*). The rapid expansion of LGLs was also accompanied by the accumulation of high levels of total cytolytic activity per culture (Fig. 4 *B*).

Although we noted high levels of cellular expansion as well as [^{3}H]TdR incorporation, experiments were designed to determine the percentage of cells actively synthesizing DNA. To determine this, we used [^{3}H]TdR pulsing and autoradiography. Typical autoradiographs of 2-h and 48-h adherent splenic LGLs are shown in Fig. 5. The cumulative data shown in Fig. 6 indicate that by 48 h in culture, 85% of the adherent cells were synthesizing DNA as detected in

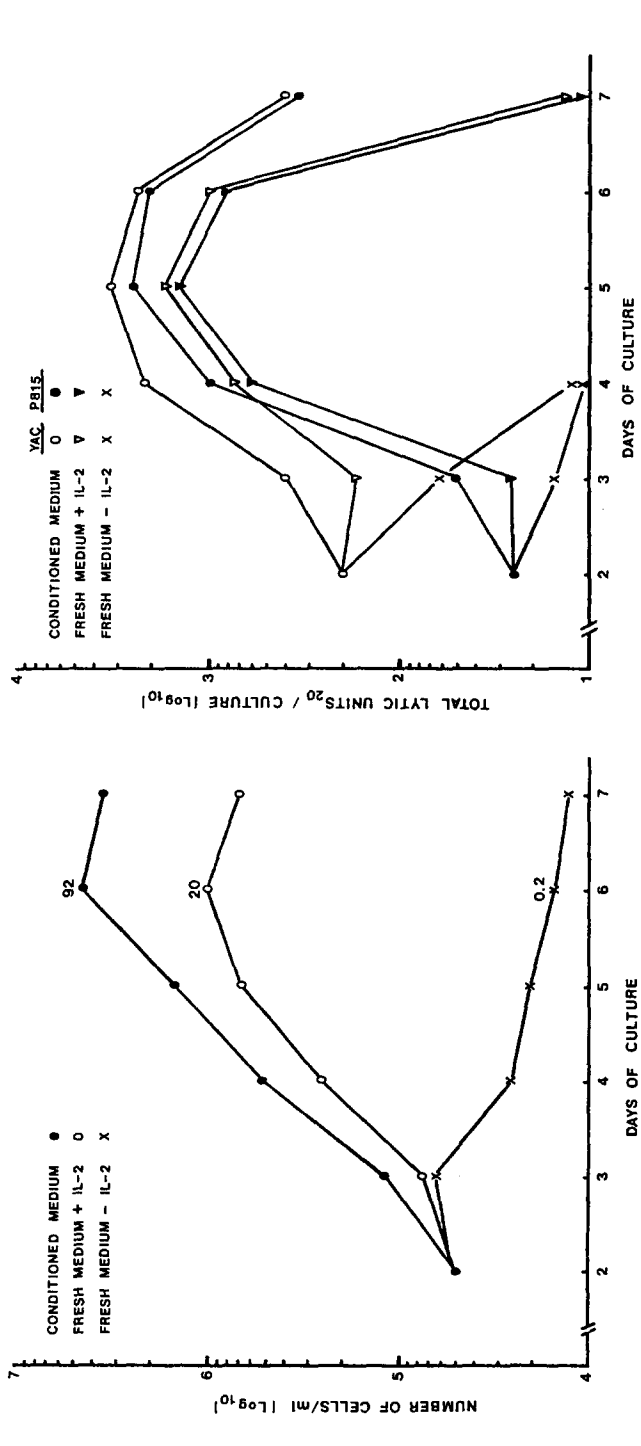

FIGURE 4. Expansion of plastic-adherent LGLs over a 5-d period. Nylon-wool nonadherent F344 spleen cells were cultured for 48 h in LAK medium. The plastic adherent cells were collected, and plated at 5×10^4 cells/ml in 48-h conditioned medium or fresh medium with or without rIL-2. The growth of the cells was then monitored over the next 5 d (*left*). The numbers above each curve indicate the fold expansion of the LGL at the peak of expansion without refeeding. (*Right*) The expansion of cytotoxic activity against YAC-1 and P815 targets, expressed as total lytic U/culture over the same period.

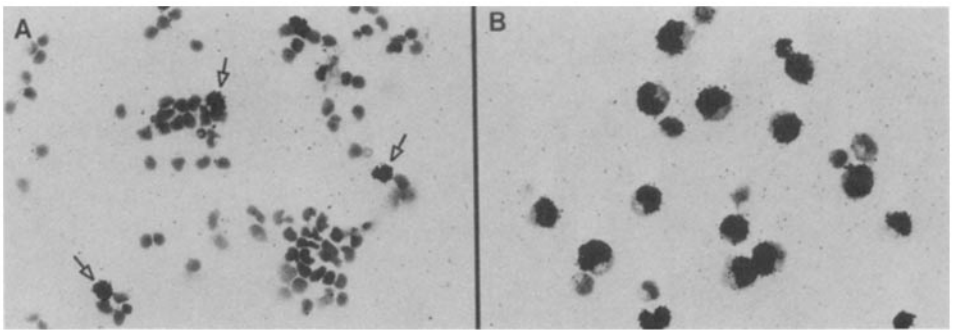

FIGURE 5. DNA synthesis by plastic adherent LGL in response to rIL-2. Nylon-wool nonadherent F344 spleen cells were cultured for 2 or 48 h in LAK medium. At each time point, the plastic adherent LGLs were collected, incubated with [^{3}H]TdR (1 μCi) for 2 h, washed, and a cytospin was prepared. Autoradiography reveals intense grain counts over positive (DNA-synthesizing) nuclei in both 2-h (*A*) or 48-h (*B*) [^{3}H]TdR-pulsed adherent LGLs. Arrows indicate positive cells in *A*.

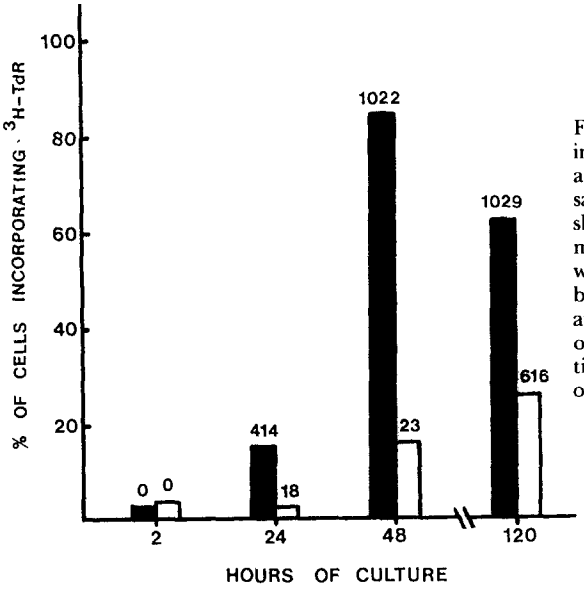

FIGURE 6. Kinetics of DNA synthesis in adherent vs. nonadherent spleen cells at different times in culture. Using the same protocol as described in Fig. 5, cells showing intense nuclear grain development were scored as positive. 500 cells were counted for each data point. Solid bars represent adherent cells; open bars are the nonadherent cells. The numbers over the bars indicate the cytotoxic activity $LU_{20}/10^7$ cells of each population on P815 targets.

a 2-h pulse of [^{3}H]TdR. This was in sharp contrast to the nonadherent cells in which only 18% of the cells were synthesizing DNA.

Expansion of Plastic Adherent LGLs Generates More Efficient Broadly Cytotoxic (LAK) Killer Cells. Nylon-wool nonadherent F344 spleen cells were cultured for 48 h and the plastic adherent LGLs were collected and cultured for an additional 3 d in conditioned medium. These cells were then tested for cytolytic activity against several tumor cells including fresh explants of two different syngeneic NK-resistant targets (MADB106 and CRNK-16). The cytolytic activity of the expanded adherent LGL was compared with bulk cultures of LAK cells generated under standard conditions and to Percoll-purified splenic LGLs (which contained 55% LGL and at least 10% mature T cells at the initiation of the

TABLE IV
LAK Activity Generated from 48-h Adherent LGLs, Percoll-purified LGLs, and in Standard Bulk Cultures

Effector cells	Cells/ml*	Cytotoxic activity			
		YAC-1	P815	MADB106	CRNK-16
		$LU_{20}/10^7$ cells			
Adherent LGL	1.6×10^6	3,564	1,269	1,649	207
Percoll-purified splenic LGL	1.8×10^6	1,270	291	1,041	120
Standard bulk culture	2.1×10^6	893	273	427	67

Nylon-wool nonadherent spleen cells were obtained from F344 rats. LGLs were purified by Percoll gradient centrifugation (55% LGL) or by rIL-2–induced plastic adherence (48 h) (93% LGL). The Percoll-purified LGLs were cultured in LAK medium for 5 d. The 48-h adherent LGLs were cultured in their conditioned medium for an additional 3 d (for a total of 5 d). Cytotoxicity was tested on YAC-1, P815, and fresh syngeneic MADB106 tumor explants or fresh ascites tumor of CRNK-16 leukemia.

* Cells were measured on day 5.

culture). The data in Table IV indicate that substantially higher levels of cytolytic (LAK) activity were generated in cultured, purified, adherent LGLs compared with either standard LAK cultures or partially purified splenic LGLs.

Discussion

These studies provide a novel method for the purification and rapid expansion of large numbers of highly purified LGL expressing broad antitumor (LAK) cytotoxicity. A major limitation to the widespread use of LAK therapy is the difficulty in generating sufficient quantities of effector cells with efficient broad antitumor reactivity. Cell numbers in standard LAK cultures rarely expanded over 3–5 d in culture, and more often decreased in cell yield by as much as 50% (4). Furthermore, when bulk cultures are generated, it is unknown what the actual frequency of LAK effector cells is within the culture. This could, in fact, vary as much as 10-fold from culture to culture. In this study, we provide a method in which it is possible to consistently achieve high levels of expansion (up to 100-fold) of highly purified LAK precursor cells, thus achieving cultures of pure LAK effector cells in sufficient numbers to be used for therapy or for in vitro experimentation.

These studies indicate that upon activation by rIL-2, LGLs rapidly undergo surface changes that enable their adherence to plastic. Evidence that the responding cells were LGL/NK cells included the following: (*a*) the cells were LGLs by morphological criteria; (*b*) the cells expressed surface markers characteristic of rat NK cells; (*c*) the cells initially contained high levels of YAC-1 (but not P815) cytolytic activity; and (*d*) the cells developed LAK cytolytic activity in response to rIL-2. This observation is consistent with previous results in rats, humans, and mice that have indicated that the majority of LAK precursor activity is contained within the LGL/NK subset of lymphocytes (5–7, 16–22).

The observation of the acquisition of adherent properties by NK cells upon activation with rIL-2 is reminiscent of previous observations of some adherence of in vitro–activated NK cells to nylon-wool columns or plastic (23, 24). However,

we found that not all LGLs respond to rIL-2 in unison, since not all LGLs became adherent by 2–4 h. In fact, LAK activity could also be generated from the nonadherent population regardless of when the nonadherent LGLs were harvested. However, we noted that new generations of adherent LGLs developed from the nonadherent populations and these adherent cells also developed LAK activity.

Kinetic experiments demonstrated that the optimal time for harvesting adherent cells to achieve optimal expansion and generation of optimal LAK activity was 48 h. Dose–response studies indicated that 48-h adherent cells responded best to LAK medium containing between 100 and 1,000 U/ml of rIL-2. Furthermore, it was clear that refeeding the adherent LGLs with the 48-h conditioned medium was necessary for achieving optimal expansion of these cells. The data suggest that factors in addition to IL-2 present in the conditioned medium may be required for the optimal expansion of LGL. This could possibly explain why 2-h adherent LGL could not expand as well as 24- or 48-h adherent LGL, since such factors would not have been produced to significant levels.

Our findings confirm previous findings with human cells that LGLs can synthesize DNA and proliferate very rapidly in response to rIL-2 (25, 26). Proliferation of adherent LGLs was noted as early as 24 h after culture and reached plateau levels by 48 to 72 h. Autoradiography of [^{3}H]TdR-labeled 48-h adherent cells revealed that up to 85% of these LGLs were synthesizing DNA. This was in contrast to the nonadherent population in which only 10–20% of the cells were synthesizing DNA. Based upon this level of proliferation and the quantitation of cell numbers at the peak of cell expansion (days 3 and 4), we estimated that the doubling time for LGLs was ~10 h. However, we have also noted that such high levels of proliferation cannot be maintained for extended periods (>10 d), and the rapid expansion of the LGLs ultimately declines. This occurs even after refeeding with conditioned medium or with fresh rIL-2. We do not know what limiting signals prevent the continuous, rapid growth of the LGL/LAK cells, although cultures tend to become inhibited when they reach densities above 10^6 cells/ml.

The expansion of LGL/NK cells using this methodology appears superior to standard LAK cultures methodology. These cultures generated significantly higher levels of total cytolytic activity per culture and higher levels of LAK activity against several NK-resistant fresh tumor targets. Since adherent LGL represent only a small percentage of the cells in a bulk culture (~1–3%), and since these cells are capable of rapid expansion to generate levels of cytolytic activity substantially higher than that seen in bulk cultures, it appears that suppressor activity may be manifest within bulk cultures. Such activity is presumably purified away from the adherent cells, thus allowing expression of their full expansion capabilities. Indeed, we now have evidence that both suppressor T cells as well as monocytes may contribute to inhibition of the generation of the full potential of LAK activity within bulk cultures.

In addition to the rapid expansion of cells with broad antitumor reactivity, these studies provide a simple and reproducible method for generating highly purified LGL for in vitro studies of NK function. We were able to generate LGL to >95% purity from rat spleen or peripheral blood. Even under the best

conditions, the purity of LGL isolated from rat spleen cells using Percoll gradients never exceeds 50–70%. This limitation is due to the high level of agranular lymphocytes in the spleen cell preparations that copurify with the LGL. Furthermore, we and our colleagues (Melder, R. J., T. L. Whiteside, N. L. Vujanovic, J. C. Hiserodt, and R. B. Herkerman, manuscript in preparation) have performed similar studies in humans and mice and have observed very similar findings. LGLs from both human PBL and mouse spleen cells adhere to plastic during the first 24 h of culture in rIL-2 yielding highly purified (>90%) LGL. These cells also rapidly expand in rIL-2 to generate high densities of LGL cells with very high levels of broad antitumor (LAK) cytotoxicity. Thus, it appears the methods reported in this study represent a general biological phenomenon and could be applied to LGL/LAK cells from a variety of species.

Summary

A simple method for the purification and rapid expansion of large granular lymphocytes into cells with efficient broad antitumor cytotoxicity after stimulation by human rIL-2 is described. Nylon-wool nonadherent splenic mononuclear leukocytes from Fischer 344 rats were cultured in medium containing 1,000 U/ml rIL-2. The initial response of a small subpopulation of cells (<2%) to rIL-2 was their adherence to the plastic surface. This response was noted as soon as 2 h after addition of rIL-2. 2-h rIL-2-activated plastic adherent lymphocytes were 90–98% LGL, expressed surface markers characteristic of rat NK cells (OX8 $[CD8]^+$, asialo GM_1, laminin$^+$, OX19 $[CD5]^-$, R1-3B3 $[CD5]^-$, W3/25 $[CD4]^-$, OX39 $[CD25]^-$, Ia^-, and Ig^-), and expressed very high levels of cytotoxicity against YAC-1 target cells. In addition to the above markers, plastic-adherent LGLs obtained at 24, 48, or 72 h progressively expressed Ia surface antigens, but were not phagocytic and contained <1% monocytes/macrophages by morphology. When 24- or 48-h plastic-adherent LGL/NK cells were cultured over 3–4 d in rIL-2, the cells expanded between 30- and 100-fold, reaching densities between $2–3 \times 10^6$ cells/ml. These rapidly expanding LGL/NK cells also generated very high levels of LAK activity (including lysis of fresh NK-resistant solid tumor cells), expressed a phenotype characteristic of activated rat NK/LAK cells, and incorporated $[^3H]$TdR into DNA. This technique not only provides a novel method for the purification of LGL/NK cells for in vitro studies but also provides a means for the rapid expansion of highly purified cells with high levels of broad antitumor (LAK) cytotoxicity.

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