Jpn. J. Cancer Res. 81, 927–935, September 1990

The Heterogeneity of Target Recognition by Lymphokine-activated Killer Precursor Cells

Yukihiro Ibayashi,[1,3] J. Dixon Gray,[2] Sidney H. Golub,[2] Masahiko Daibo,[1] Toshiaki Yamaki,[1] Takahisa Kawahara,[1] Tsukasa Kubota[1] and Kazuo Hashi[1]

[1]*Department of Neurosurgery, Sapporo Medical College, S.1, W.16, Chuo-ku, Sapporo 060, Japan and*
[2]*Division of Oncology, Department of Surgery, UCLA School of Medicine, Los Angeles, CA 90024, USA*

Lymphokine-activated killer (LAK) cells were generated from peripheral blood lymphocytes (PBL) that were depleted of mature cytotoxic natural killer (NK) cells. PBL NK activity was abolished by pretreatment of effector cells with the toxic lysosomotropic agent L-leucine methyl ester (LME) or by depletion of effector cells by K562 monolayer absorption (MA). Both treatments markedly reduced the proportion of cells expressing NK-associated markers such as CD 16 (Leu 11b, B73.1), Leu 7, and NKH-1 (Leu 19), whereas these treatments had minimal effects on cells expressing T cell markers (CD 3, CD 4, and CD 8). LME and MA also drastically decreased the proportion of K562 target-binding lymphocytes. LAK activity against NK-sensitive and NK-resistant targets can be generated from the NK cell-depleted PBL by incubation with interleukin-2. Peak LAK activity generated from MA-treated PBL was later than the peak of LAK activity generated from either untreated or LME-treated PBL. Although MA of PBL on NK-resistant S4 sarcoma targets had little effect on NK activity, LAK activity against both K562 and S4 targets was reduced. These results suggest that there are at least three LAK precursor subpopulations in PBL: mature NK cells that can bind and kill K562 targets (LME-sensitive and MA-sensitive); "pre-NK" cells that can bind but cannot kill (LME-resistant and MA-sensitive); and non-NK cells that cannot bind and cannot kill K562 targets (MA-resistant).

Key words: Lymphokine-activated killer (LAK) cell — LAK precursor cell — Leucine methyl ester — Tumor monolayer absorption — Natural killer cell

The culture of human peripheral blood lymphocytes (PBL)[4] with interleukin-2 (IL-2) in the absence of any other stimulus results in the generation of cells that are cytotoxic against a wide variety of target cells including natural killer (NK)-sensitive and NK-resistant target cells.[1,2] These "lymphokine-activated killer" (LAK) cells have a number of similarities to NK cells.[3–5] However, there are many systems, such as thymocytes, where LAK cells can be derived from precursors that are clearly not mature NK cells.[6–8] The purpose of this study was to determine if NK or non-NK LAK precursors generate LAK effector cells of different cytotoxic specificities.

There are many techniques which can distinguish non-NK cells from NK cells. The identification of NK cells has been based on the separation of cells on Percoll discontinuous gradients or on their reactivity with monoclonal antibodies (MoAb) which are selective for NK cells. However, such techniques still yield rather heterogeneous cell populations as judged by immunologic function, lymphokine production, or expression of other surface markers.[9,10] In this study, we used two techniques which can eliminate NK cells from PBL based on NK function. We previously demonstrated that NK activity in PBL is abolished by pretreatment of effector cells with the toxic lysosomotropic agent L-leucine methyl ester (LME).[11] We and other investigators[12] also found that absorption of PBL on monolayers of NK-sensitive K562 targets totally inhibited NK activity. We sought to determine if cells that can kill NK-sensitive targets (LME-sensitive cells), cells that can bind targets (target monolayer adherent cells), or cells that can do neither differ in their ability to generate LAK effector cells. This information would help to define the relationship between LAK cells and NK cells.

MATERIALS AND METHODS

Lymphocyte preparation Human PBL obtained from healthy volunteers were prepared by centrifugation on Ficoll-Hypaque (Ficoll-Paque, Pharmacia, Piscataway, NJ) density gradients and the adherent cells were depleted by adherence to plastic tissue culture dishes (Falcon 1029; Falcon Labware, Oxnard, CA) or to a scrubbed nylon wool column for 1 h.[13] The mononuclear cells were washed three times and resuspended in com-

[3] To whom correspondence should be addressed.

[4] Abbreviations: IFN, interferon; IL-2, interleukin-2; LAK, lymphokine-activated killer; LME, leucine methyl ester; MA, monolayer absorption; MoAb, monoclonal antibody; NK, natural killer; PBL, peripheral blood lymphocytes.

plete medium (CM) consisting of RPMI 1640 medium (Flow Laboratory, McLean, VA) containing antibiotics (Gibco Laboratory, Grand Island, NY) and HEPES buffer supplemented with 10% human AB serum (Biocell Laboratory, Carson, CA).

LME treatment LME (Sigma Chemical Co., St. Louis, MO) was prepared fresh for each experiment in RPMI 1640 medium and the pH was adjusted to 7.4 with 1 *N* NaOH. PBL at 8×10^6 cells/ml in RPMI 1640 supplemented with 5% AB serum were treated with 40 m*M* LME at 37°C for 30 min and were then washed three times before use.[11]

Monolayer absorption (MA) treatment Tumor cells in RPMI 1640 medium were added to 60×15 plastic culture dishes (Falcon) which were coated with 50 μg/ml of poly-L-lysine (Sigma Chemical Co.). After 1 h at 37°C, the plates were washed three times to remove non-adherent cells then incubated with CM for 10 min to block residual poly-L-lysine. Lymphocytes in RPMI 1640 medium were added to the tumor cell monolayers. After 1 h at 37°C, non-adherent lymphocytes were removed and used as effector cells in cytotoxicity assays or cultured with IL-2 for 4 days. For some experiments, tumor cells were treated with 100 μg/ml of mitomycin C (ICN Nutritional Biochemicals, Cleveland, OH) for 30 min at 37°C to prevent contaminating tumor cell growth in the LAK culture.

MoAb and surface marker analysis Leu series antibodies were obtained from Becton, Dickinson and Co. (Mountain View, CA). B73.1 MoAb was a generous gift from Dr. G. Trinchieri (Wistar Institute of Anatomy and Biology).[14] Lymphocyte surface markers were detected by incubating cells with mouse MoAb against subsets of human lymphocytes followed by rosetting with a universal rosetting reagent.[15] Briefly, 100 μl of lymphocytes at a concentration of 5×10^6/ml were mixed with MoAb and then incubated on ice for 30 min. After the cells were washed and resuspended, 100 μl of anti-mouse Ig rosetting reagent was added, and the mixture was centrifuged at 50*g* for 4 min. The rosettes were read after incubation for 1 h at 4°C. The percentage of positive cells was determined by counting at least 600 cells.

Single cell conjugate assays The assay employed is a modification of the procedure described by D'Amore and Golub.[16] Equal numbers of effector cells and target cells (2.5×10^5 each) were mixed in 100 μl of RPMI 1640 with 10% AB serum, and were incubated at 37°C for 10 min. The cells were centrifuged at 200*g* for 5 min and were then mixed with 100 μl of 1% molten (39°C) agarose. The mixture was quickly spread on a glass slide precoated with 2% agar, and the numbers of lymphocytes conjugated with tumor cells were counted. The percentage of effector cells bound to tumor cells was assessed by counting at least 600 lymphocytes.

LAK cell culture Purified human recombinant IL-2 (alanine-125 analog) was generously provided by Amgen, Inc. (Thousand Oaks, CA). Ten U/ml of IL-2 was used in lymphocyte culture for LAK induction. This dose had previously been shown to induce strong cytotoxic activity.[17] LAK cells were induced by *in vitro* incubation for 4 days with IL-2. PBL were cultured in 25 cm^2 flasks (3050 Costar, Cambridge, MA) in 5 ml of CM at a concentration of 2×10^6 cells/ml.

Target cells and cytotoxicity assays The erythroleukemia cell line K562, the sarcoma cell line UCLA-SO-S4 (S4), and the melanoma cell line UCLA-SO-M14 (M14) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Flow Laboratory). To obtain a single cell suspension of S4 or M14 cells, the cultures were treated briefly with a 0.25% trypsin solution. The S4 or M14 cells were used as "LAK" targets and K562 as the NK targets. Standard cytotoxicity assays were performed in RPMI 1640 medium supplemented with 10% AB serum.[18] Target cells were labeled with 100 μCi of sodium ^{51}Cr chromate (Amersham International, Arlington Heights, IL) for 60 min at 37°C and washed by centrifugation. Cytotoxicity was tested in V-shaped microwells with 200 μl of assay medium containing 5×10^3 target cells and various numbers of effector cells. For some experiments, the same volumes of various tumor cells (inhibitors) were added at 2:1 to 32:1 competitor: target ratios (cold target competition assays). The assays were initiated by low-speed centrifugation (50*g* for 4 min) followed by incubation for 4 h at 37°C. At the end of the incubation, 100 μl of supernatant was collected from each well for counting of the amount of chromium released. The amount of cytotoxic activity was expressed by the following formula:

$$\%\ \text{cytotoxicity}=\frac{\text{experimental release}-\text{spontaneous release}}{\text{total release}-\text{spontaneous release}}\times100.$$

Spontaneous release was determined from wells containing no effector cells and total release was obtained from wells containing target cells lysed by 5% NP40 detergent. The results were also expressed as lytic units/10^6 cells, with 1 lytic unit being the number of effector cells required to cause 30% lysis of target cells.[19]

RESULTS

Effect of LME or MA on NK cytotoxicity PBL were treated with LME or K562 monolayer absorption (K562 MA) and were tested for cytotoxicity against K562 targets. The percent tumor cell contamination following K562 MA was 1.3–2.8%, which was shown not to affect the cytotoxicity assays in which it was employed. The percent cell recovery of PBL after K562 MA was 74.8±

2.8% (five experiments). Treatment of PBL with LME or K562 MA resulted in an inhibition of NK activity at all effector: target ratios tested (Fig. 1A). NK cells have been reported to be the major cell population in PBL able to bind K562 target cells, and binding of NK cells to target cells is an early and obligatory step in NK cytolysis.[20] Therefore, the ability of cells to mediate K562 target binding after LME or K562 MA treatment was examined (Table I). Both treatments significantly decreased PBL K562-binding cells ($P < 0.001$ by Student's t test). The effects of LME and K562 MA on the subpopulations of PBL were determined by comparing the expression of surface markers among control PBL, LME-treated PBL, and K562 MA-treated PBL (Table II). Cells expressing NK-associated markers such as CD 16 (Leu 11b, B73.1), Leu 7, or Leu 19 were significantly decreased ($P<0.005$), whereas the proportion of cells expressing T cell surface markers such as CD 3 (OKT 3, Leu 4), CD 4 (Leu 3a), or CD 8 (Leu 2a) were not affected by the same treatments. Thus, treatment of PBL with LME or K562 MA resulted in an elimination of NK cell activity by cytotoxicity, binding, and cell surface marker analysis. We also examined the effects of LME or K562 MA on NK cytotoxicity against NK-resistant S4 target cells. Like untreated PBL, LME- or K562 MA-treated PBL had no cytotoxicity against S4 target cells. These treatments decreased further the already very low level of cells that can bind S4 target cells (from 3.7% to 3.0% by LME and to 2.2% by MA).

To determine if the effects of K562 MA were specific for NK cells, we also examined the effects of NK-resistant tumor MA treatment on PBL. PBL were depleted on NK-resistant S4 cells (S4 MA) and com-

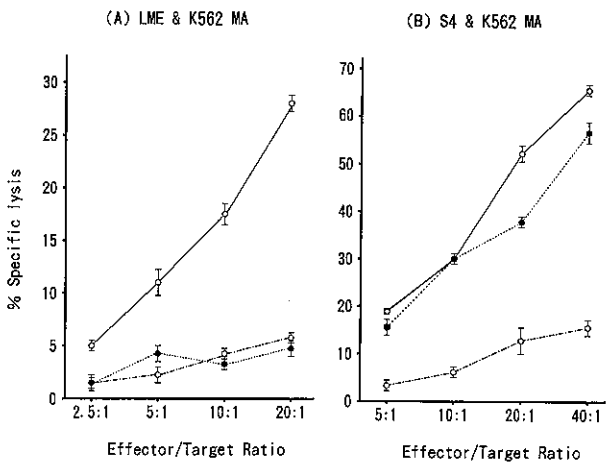

Fig. 1. Effect of LME and target cell MA treatment on NK activity. PBL were treated with medium alone (○——○), 40 mM LME (●----●), or K562 MA (○—•—○) (Panel A), or with K562 MA (○—•—○) or S4 MA (●----●) (Panel B), then washed and assayed for NK activity. LME or K562 MA treatments markedly decreased NK activity. S4 MA resulted in little or no decrease of NK activity. Vertical bars indicate SD of the mean for % specific lysis.

Table I. Effect of LME and Target Cell MA Treatment on K562 Target-binding Cells

Treatment	% binding cells
Medium	11.7±0.4[a]
LME	4.0±0.3[b]
K562 MA	2.0±0.2[b]
Medium	14.8±1.0
S4 MA	8.7±0.6[b]
K562 MA	4.3±0.4[b]

a) Values are expressed as mean±SEM of five separate experiments.
b) $P<0.001$ by Student's t test.

Table II. Effects of LME and MA on Lymphocytes Expressing NK Markers

Expt.	Treatment[a]	% of cells reactive with MoAb							
		Leu 11b	Leu 19	Leu 7	B73.1	OKT 3	Leu 4	Leu 2a	Leu 3a
1	Medium	12.0±0.4[b]	9.8±0.5	10.8±0.8	10.3±0.5	89.5±1.3	83.5±1.2	33.0±1.4	63.0±2.1
	LME	1.2±0.3[c]	1.3±0.3[c]	1.2±0.2[d]	2.2±0.5[c]	83.3±2.5	80.5±1.1	31.0±1.2	65.0±1.3
	MA	2.2±0.5[c]	1.8±0.4[d]	1.8±0.5[d]	2.8±0.6[c]	86.8±1.9	79.5±0.9	32.8±1.4	65.8±0.9
2	Medium	8.3±0.5	4.8±0.3	—[e]	—	—	74.8±1.7	36.8±2.0	53.5±0.5
	LME	1.0±0.4[d]	0.3±0.1[d]	—	—	—	78.5±1.4	42.0±1.9	62.3±1.0
	MA	2.0±0.4[d]	0.3±0.2[d]	—	—	—	82.5±2.5	36.8±1.9	48.8±1.3

a) PBL were treated with medium alone, 40 mM LME for 30 min, or MA for 1 h.
b) Values are expressed as mean±SEM.
c) $P<0.001$ *d*) $P<0.005$ by Student's t test.
e) Not tested.

Table III. Effect of S4 MA on Lymphocytes Expressing NK Markers

Expt.	Treatment[a]	% of cells reactive with MoAb							
		Leu 11b	Leu 19	Leu 7	B73.1	OKT 3	Leu 4	Leu 2a	Leu 3a
1	Medium	12.0±0.4[b]	9.8±0.5	10.8±0.8	10.3±0.5	89.5±1.3	83.5±1.2	33.0±1.4	63.0±2.1
	S4 MA	10.8±0.8	8.3±0.5	9.5±0.6	10.8±0.5	84.3±0.9	82.3±1.4	29.8±0.8	65.3±1.1
2	Medium	7.6±0.5	5.8±0.4	9.2±0.6	9.4±0.2	—[c]	71.3±3.4	30.0±1.3	51.3±2.2
	S4 MA	7.2±0.4	4.8±0.4	8.4±0.5	9.6±0.7	—	72.5±1.4	29.5±2.0	56.8±2.6

a) PBL were treated with medium alone or S4 MA for 1 h.
b) Values are expressed as mean±SEM.
c) Not tested.

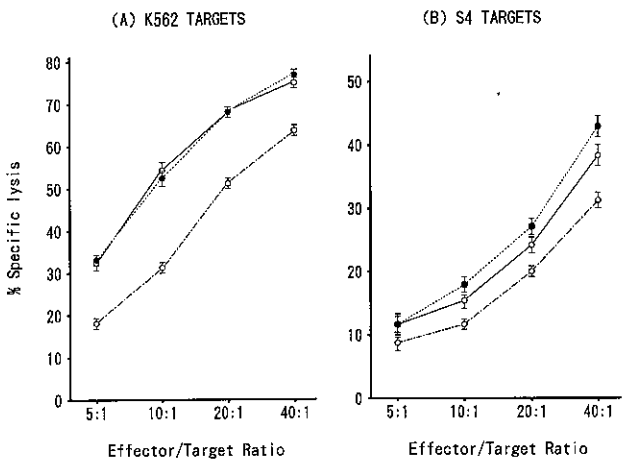

Fig. 2. Regeneration of cytotoxic activity against S4 and K562 targets in LME- or K562 MA-treated PBL. PBL were treated with medium alone (○——○), LME (●----●), or K562 MA (○—•—○), then washed and prepared for cytotoxicity assays against K562 (Panel A) and S4 target cells (Panel B). Vertical bars indicate SD of the mean for % specific lysis.

Table IV. Regeneration of Target-binding Cells in LME- or K562 MA-treated PBL

Target	Treatment	% binding cells
S4	Medium	8.7±0.4[a]
	LME	6.7±0.3[b]
	K562 MA	5.0±0.4[b]
K562	Medium	20.3±0.8
	LME	14.2±0.4[c]
	K562 MA	11.8±0.3[d]

a) Values are expressed as mean±SEM of five separate experiments.
b) $P<0.01$ *c*) $P<0.005$ *d*) $P<0.001$ by Student's t test.

pared to K562 monolayers (K562 MA) before assay of NK activity. The percent tumor cell contamination of S4 MA was 0.1–1.3%, which was shown not to affect the cytotoxicity assays and the percent cell recovery of PBL after S4 MA was 85.3±2.2% (five experiments). Treatment of PBL with K562 MA resulted in a marked decrease in NK activity, while in contrast S4 MA resulted in little or no decrease of NK activity (Fig. 1B). Single cell binding assays revealed that treatment of PBL with S4 MA reduced the number of cells that can bind to K562 target cells, but to a lesser extent than K562 MA (Table I). Cell surface marker analysis also revealed that cells expressing NK-associated markers such as CD 16, Leu 7, or Leu 19 were only slightly reduced after treatment of PBL with S4 MA (Table III). The proportion of cells expressing T cell surface markers such as CD 3, CD 4, or CD 8 was not affected. These results indicate that S4 MA resulted in a partial reduction of mature NK cells and in a substantial reduction of cells that can bind S4 target cells but cannot kill K562 targets. These results verify that K562 cytotoxic NK cells are granular (and therefore sensitive to LME) and bind to K562 (can be removed on K562 monolayers). In contrast, the relatively few S4 binding cells may represent low-efficiency NK cells, as their removal did significantly decrease binding cells to K562 target but had little effect on NK cytotoxicity.

Regeneration of cytotoxic activity in LME- or MA-treated PBL We previously demonstrated the regeneration of cytotoxic activity in LME-treated PBL with IL-2, indicating that cells besides mature cytotoxic NK cells can generate LAK cells.[11,21] Experiments were designed to determine if IL-2 can also regenerate cytotoxic cell populations for MA-treated PBL. These experiments would test the hypothesis that "pre-NK" cells can function as LAK precursor cells. LME- or K562 MA-treated lymphocytes were incubated with 10 U/ml of IL-2 for 4 days. The IL-2 concentration was selected on the basis of optimal induction of LAK activity.[22] Incubation of PBL with IL-2 resulted in a strong cytotoxic activity against K562 targets (Fig. 2A). Cytotoxicity generated from

LME-treated PBL was equal to cytotoxicity generated from untreated PBL. In contrast, cytotoxicity generated from K562 MA-treated PBL was markedly lower than cytotoxicity generated from untreated PBL. The proportion of K562-binding cells generated from LME- or K562 MA-treated PBL was comparable to the levels found in fresh PBL (Table IV). Furthermore, the cultured LME- or MA-depleted PBL were cytotoxic against S4 (Fig. 2B). In single cell binding assays, the number of S4 binding cells generated from LME- or K562 MA-treated PBL was increased when compared to fresh PBL (Table IV). Thus, there are LME- or K562 MA-resistant precursor cells which can generate cytotoxic cells against NK-sensitive and NK-resistant targets.

Depletion of PBL by K562 MA resulted in a reduction of IL-2-induced cytotoxicity against both NK-sensitive (Fig. 3A) and NK-resistant (Fig. 3B) targets. Although S4 MA had only a slight inhibitory effect on NK cytotoxicity (Fig. 1B), the cytotoxicity generated from S4 MA-treated PBL was decreased against both NK-sensitive and NK-resistant target cells (Fig. 3). We examined the time course of regeneration of cytotoxic cells by incubating depleted cell populations with 10 U/ml of IL-2. Peak cytotoxicity against K562 targets generated from K562 MA-depleted PBL was later than the peak of cytotoxicity generated from untreated or LME-treated PBL (Fig. 4).

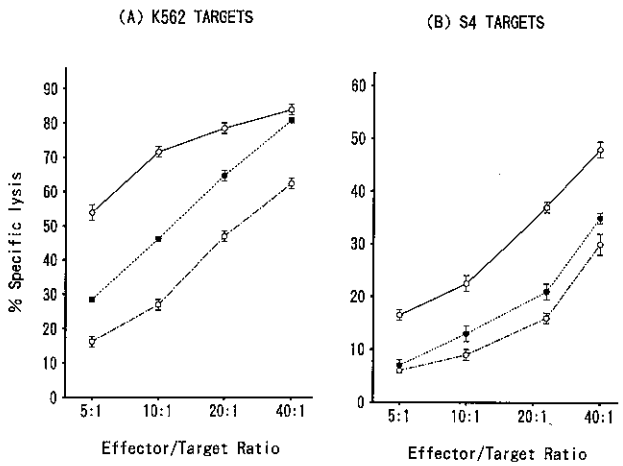

Fig. 3. Regeneration of cytotoxic activity against S4 and K562 targets in S4 MA- or K562 MA-treated PBL. PBL were treated with medium alone (○——○), S4 MA (●----●), or K562 MA (○—•—○), then cultured with 10 U/ml of IL-2 for 4 days. Cytotoxicity was assayed on K562 (Panel A) and S4 target cells (Panel B). Vertical bars indicate SD of the mean for % specific lysis.

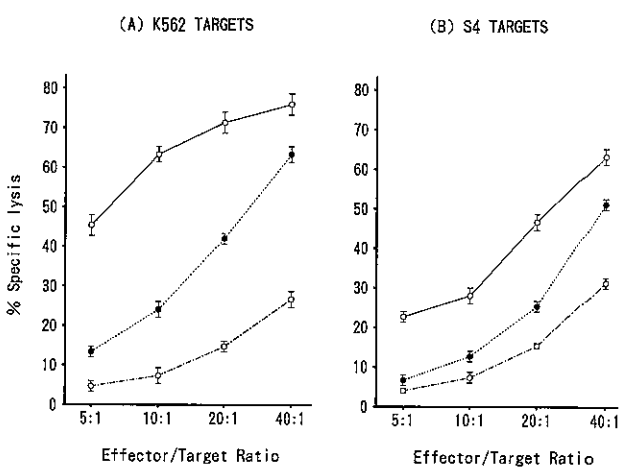

Fig. 5. Effect of S4 MA or K562 MA on LAK effector cells. PBL were cultured with 10 U/ml of IL-2 for 4 days and then treated with medium alone (○——○), S4 MA (●----●), or K562 MA (○—•—○). Cytotoxicity was assayed on K562 (Panel A) and S4 target cells (Panel B). Vertical bars indicate SD of the mean for % specific lysis.

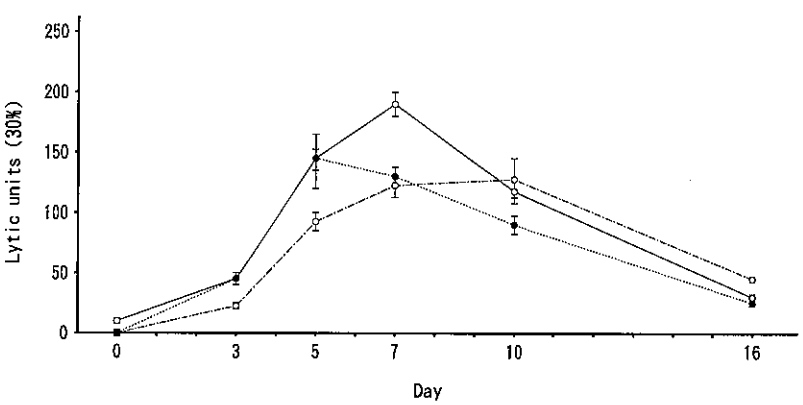

Fig. 4. Kinetics of cytotoxicity against K562 target cells generated from untreated (○——○), LME-treated (●----●), and K562 MA-treated PBL (○—•—○). All results are the average lytic units of 3 samples. Vertical bars indicate SD of the mean for lytic units.

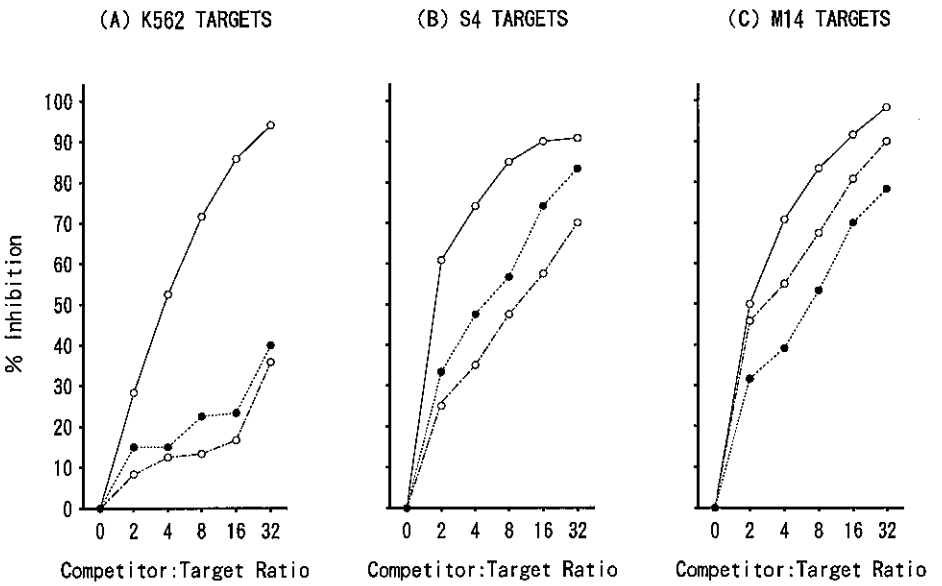

Fig. 6. Specificity of LAK effector cells by cold target competition analysis. PBL were cultured with 10 U/ml of IL-2 for 4 days and tested at a 20:1 effector:target ratio against K562 (Panel A), S4 (Panel B), and M14 targets (Panel C). Inhibitors, K562 (○——○), S4 (●----●), or M14 (○—•—○) were added at 2:1 to 32:1 competitor:target ratios.

Effect of MA on LAK effector cells Experiments were designed to determine if heterogeneity exists in LAK effector cells. We examined the effects of MA on the LAK effector phase. PBL were cultured with 10 U/ml of IL-2 for 4 days then depleted on S4 or K562 monolayers before cytotoxicity assays against S4 and K562 targets. The percent tumor cell contamination, which was shown not to affect the cytotoxicity assays, was 1.3–2.8% of K562 cells in K562 MA and 0.1–1.3% of S4 cells in S4 MA. Figure 5 shows that treatment of LAK effector cells with K562 MA resulted in a marked reduction of LAK activity against both S4 and K562 targets. Treatment of LAK effector cells with S4 MA also resulted in a reduction of LAK activity against both S4 and K562 targets, but the effect of S4 MA was weaker than that of K562 MA.

Another means of assessing LAK effector cell specificity is cold target competition analysis (Fig. 6). PBL were cultured with 10 U/ml of IL-2 for 4 days and then LAK cells were tested at a 20:1 effector: target ratio. With labeled S4 or labeled M14 target cells, K562, S4, and M14 were equally competitive and resulted in a reduction of cytotoxicity. However, with labeled K562 target cells, S4 and M14 competed very poorly whereas K562 was an effective competitor. Thus, heterogeneity of LAK precursor cells was also seen in the LAK effector phase.

DISCUSSION

Adoptive immunotherapy with LAK and/or IL-2 can be an effective treatment for established pulmonary metastases in animal models.[23] There are also reports of successful immunotherapy with LAK cells and IL-2 in cancer patients.[24] However, the precise nature of LAK cells and their precursors remains to be determined. Although LAK precursor cells have been described as a part of the Leu 7^- and CD 16^+ large granular lymphocytes that include NK cells,[4] there are number of systems, such as thymocytes, whereby LAK cells can be derived from precursors that are clearly distinct from mature NK cells.[6–8] However, the populations obtained on the basis of either buoyant density or cell surface markers are still heterogeneous as judged by function and other surface markers.[9, 10] Therefore, to analyze the relationship between NK and LAK cells, we analyzed LAK precursor cells by depleting NK cells from PBL using two techniques which can eliminate NK cells based on NK functions.

We previously demonstrated that PBL NK activity is abolished by treatment with the toxic lysosomotropic agent LME.[11] The results of the present study indicate that MA on NK-sensitive targets also eliminates NK activity (Fig. 1). The absence of NK activity following

LME or MA treatment was verified by NK cytotoxicity assay and by cell surface marker analysis (Fig. 1 and Tables I and II). However, there was a subpopulation in the remaining cells that could bind to K562 target cells but could not lyse those targets. NK cytotoxicity results from IFN- or IL-2-treated cancer patients suggested an *in vivo* increase in the development of NK cells generated from noncytotoxic NK precursors.[25,26] Similarly, these results suggest the existence of non-cytotoxic precursors. The K562 MA resistant pre-cytotoxic cells appear to be less mature NK precursor cells than LME-resistant precursor cells as they require a longer time to generate mature cytotoxic cells (Fig. 4). Thus, these results suggest that there are at least three types of LAK precursor cells, one that can bind and kill K562 target cells (mature NK cells that are LME-sensitive and K562 MA-sensitive), one population that can bind but cannot kill ("pre-NK" pre-cytotoxic cells that are LME-resistant), and one that cannot bind and cannot kill K562 target cells (probably non-NK cells that are K562 MA-resistant). It is not clear whether precursors are phenotypically of the same lineage as the mature NK cells. However, we reported previously that cells with NK cell marker HNK-1 were highly enriched in low Percoll density fractions, whereas high-density PBL were depleted of HNK-1^+ cells.[11] LME treatment eliminated the HNK-1^+ cells in low-density PBL populations and IL-2 could regenerate LAK activity after LME treatment only in the low-density fractions. Therefore, these results would indicate that there are some LAK precursors of NK lineage in the low-density fractions.

There are a number of reports of cell populations which can bind but cannot kill target cells. Velardi *et al.*[27] reported that 21% of Leu3/T4^+ cells had granular lymphocyte morphology and were able to bind to NK-sensitive target cells but failed to lyse those targets. Wright and Bonavida[28] have shown that NK cells could also bind to NK-resistant target cells. In their study, NK cells appear to be non-lytic binding cells to NK-resistant target cells. Therefore, it is likely that there are nonlytic binding cells of NK lineage and LAK cell development may involve the differentiation of these nonbinding and nonlytic precursors into binding, nonlytic cells followed by further maturation into binding and lytic effector cells.

The discrimination of LAK cells from NK cells is primarily based on target specificity. K562 MA treatment totally eliminated NK activity (Fig. 1A) but only partially decreased LAK activity and K562 binding cells (Fig. 2, Table IV). On the other hand, S4 MA treatment did not affect NK activity (Fig. 1B) or reactivity with monoclonal antibodies to NK-associated markers (Table III) but still partially decreased LAK activity against both K562 and S4 targets (Fig. 3). Therefore, some precursors for NK-sensitive targets can also recognize NK-resistant targets and these precursors probably include the NK cells with the highest binding affinity for NK targets. The heterogeneity of specificity or maturational state of LAK precursor cells is shown by the pronounced depletion of LAK precursor cells on K562 MA and the lesser depletion by S4 MA or LME treatment.

In the LAK effector phase, similar results were observed. Treatment of LAK effector cells with S4 or K562 MA resulted in a reduction of LAK activity against both S4 and K562 target cells, whereas S4 MA was less effective than K562 MA (Fig. 5). In cold target competition analysis, K562 competed effectively against NK-sensitive and NK-resistant targets (Fig. 6). However, S4 or M14 competed very poorly against K562 targets. These results again indicate at least two possible models of target cell recognition by LAK effector cells (i.e. heterogeneity of target structures or quantitative differences in target structure expression). One possibility is that K562 target cells have several types of target recognition receptors, while NK-resistant targets share at least one such structure but lack others. A second possibility is that K562 and S4 (or M14) share the same target structure, but it is quantitatively better expressed on K562 targets. In either case, one would predict that all LAK cells and LAK precursors would recognize K562, and some but not all effectors would recognize NK-resistant targets such as S4 or M14.

It is not clear what kind of target recognition molecules are recognized by LAK precursor and effector cells. However, it is well known that target cell susceptibility to NK-mediated cytolysis may in part be dependent on the stage of differentiation of the tumor cell target structure.[29] Ando *et al.*[30] demonstrated that a significant correlation is observed between the quantity of the differentiation antigen ganglioside GM 2 on the target cells and their sensitivity to NK lysis. This raised the possibility that GM 2 is an NK target structure. Werkmeister *et al.*[31] demonstrated that NK-resistant butyrate-induced K562 target cells have significantly elevated levels of sialic acid within sialyl glycoproteins and particularly ganglioside extracts. Treatment of these cells with neuraminidase, which releases cell surface sialyl glycoproteins, was associated with an increase in NK susceptibility. They suggested the importance of sialic acid in masking putative NK target recognition structures. In cold target competition assays, treatment of S4 target cells with neuraminidase resulted in more effective competition (data not shown). Therefore, it seems more likely that differences between LAK precursors for NK-sensitive and NK-resistant target cells may reflect differences in the ability to recognize low versus high expression of one single target structure. Our results indicate that NK-resistant targets would be lysed only by high-

efficiency (LAK) effectors and that NK-sensitive targets such as K562 could be lysed by low-affinity/efficiency effector cells (NK cells) because of their high expression of target structures. In summary, there is considerable heterogeneity of target recognition by LAK precursor and effector cells. This heterogeneity might be derived from differences in target structure expression and in the affinity of precursor or effector cells for these targets.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the provision of recombinant IL-2 by Amgen, Inc., the generous gift of MoAb B73.1 by Dr. G. Trinchieri, and valuable comments on the manuscript by Dr. H. Shau. This study was supported in part by USPHS grant CA 34442.

(Received February 9, 1990/Accepted June 2, 1990)

REFERENCES

1) Grimm, E. A., Mazumder, A., Zhang, H. Z. and Rosenberg, S. A. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.*, **155**, 1823–1841 (1982).
2) Rosenberg, S. A., Grimm, E. A., McGrogan, M., Doyle, M., Kawasaki, E., Koths, K. and Mark, D. F. Biological activity of recombinant human interleukin 2 produced in *Escherichia coli*. *Science*, **223**, 1412–1415 (1984).
3) Ortaldo, J. R., Mason, A. T., Gerald, J. P., Henderson, L. E., Farrar, W., Hopkins, R. F., III, Herberman, R. B. and Rabin, H. Effects of natural and recombinant IL 2 on regulation of IFN production and natural killer activity: lack of involvement of the Tac antigen for these immunoregulatory effects. *J. Immunol.*, **133**, 779–783 (1984).
4) Itoh, K., Tilden, A. B., Kumagai, K. and Balch, C. M. Leu-11$^+$ lymphocytes with natural killer (NK) activity are precursors of recombinant interleukin 2 (rIL 2)-induced activated killer (AK) cells. *J. Immunol.*, **134**, 802–807 (1985).
5) Phillips, J. H. and Lanier, L. L. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. *J. Exp. Med.*, **164**, 814–825 (1986).
6) Toribio, M. L., DeLandazuri, M. O. and Lopez-Botet, M. Induction of natural killer-like cytotoxicity in cultured human thymocytes. *Eur. J. Immunol.*, **13**, 964–969 (1983).
7) Gray, J. D., Torten, M. and Golub, S. H. Generation of NK-like cytotoxicity from human thymocyte with IL-2. *Natl. Immun. Cell Growth Regul.*, **3**, 124–133 (1984).
8) Ramsdell, F. J. and Golub, S. H. Generation of lymphokine-activated killer cell activity from human thymocytes. *J. Immunol.*, **139**, 1446–1453 (1987).
9) Abo, T., Cooper, M. D. and Balch, C. M. Characterization of HNK-1$^+$ (Leu-7) human lymphocytes. I. Two distinct phenotypes of human NK cells with different cytotoxic capability. *J. Immunol.*, **129**, 1752–1757 (1982).
10) Scala, G., Allavena, P., Djeu, J. Y., Kasahara, T., Ortaldo, J. R., Herberman, R. B. and Oppenheim, J. J. Human large granular lymphocytes (LGL) are potent producers of interleukin 1. *Nature*, **309**, 56–59 (1984).
11) Shau, H. and Golub, S. H. Depletion of NK cells with the lysosomotropic agent L-leucine methyl ester and the *in vitro* generation of NK activity from NK precursor cells. *J. Immunol.*, **134**, 1136–1141 (1985).
12) Jensen, P. J. and Koren, H. S. Heterogeneity within the population of NK and K cells. *J. Immunol.*, **124**, 395–398 (1980).
13) Uede, T., Kohda, H., Ibayashi, Y., Osawa, H., Diamantstein, T. and Kikuchi, K. Functional analysis of mononuclear cells infiltrating into tumors. II. Differential ability of mononuclear cells obtained from various tissues to produce helper factors that are involved in the generation of cytotoxic cells. *J. Immunol.*, **135**, 3243–3251 (1985).
14) Perussia, B., Starr, S., Abraham, S., Fanning, V. and Trinchieri, B. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor function. I. Characterization of the lymphocyte subset reactive with B73.1. *J. Immunol.*, **130**, 2133–2141 (1983).
15) Karavodin, L. M. and Golub, S. H. Universal rosetting reagent for the detection of human cell surface markers. *J. Immunol. Methods*, **61**, 293–300 (1983).
16) D'Amore, P. J. and Golub, S. H. Suppression of human NK cell cytotoxicity by an MLC-generated cell population. *J. Immunol.*, **134**, 272–279 (1985).
17) Ibayashi, Y. and Golub, S. H. Functional studies on lymphokine activated killer (LAK) cells against NK-sensitive and NK-resistant targets. *Proc. Am. Assoc. Cancer Res.*, **27**, 352 (1986).
18) Ibayashi, Y., Uede, T., Uede, T. and Kikuchi, K. Functional analysis of mononuclear cells infiltrating into tumors: differential cytotoxicity of mononuclear cells from tumors of immune and nonimmune rats. *J. Immunol.*, **134**, 648–653 (1985).
19) Pross, H. F., Baines, M. G., Rubin, P., Shragge, P. and Patterson, M. S. Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. *J. Clin. Immunol.*, **1**, 51–63 (1981).
20) Timonen, T., Ortaldo, J. R. and Herberman, R. B. Analysis by a single cell cytotoxicity assay of natural killer (NK) cell frequencies among human large granular lym-

phocytes and of the effect of interferon on their activity. *J. Immunol.*, **128,** 2514–2521 (1982).
21) Gray, J. D., Shau, H. and Golub, S. H. Functional studies on the precursors of human lymphokine-activated killer cells. *Cell. Immunol.*, **96,** 338–350 (1985).
22) Ibayashi, Y., Hoon, D. S. B. and Golub, S. H. The regulatory effect of adherent cells on lymphokine activated killer (LAK) cells. *Cell. Immunol.*, **110,** 365–378 (1987).
23) Mazumder, A. and Rosenberg, S. A. Successful immunotherapy of natural killer-resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated *in vitro* by interleukin 2. *J. Exp. Med.*, **159,** 495–507 (1984).
24) Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E., Ettinghausen, S. E., Matory, Y. L. Skibber, J. M., Shiloni, E., Vetto, J. T., Seipp, C. A., Simpson, C. and Reichert, C. M. Observations on the systemic administration of autologous lymphokine-activated killer cell and recombinant interleukin-2 to patients with metastatic cancer. *N. Engl. J. Med.*, **313,** 1485–1492 (1985).
25) Golub, S. H., Dorey, F., Hara, D., Morton, D. L. and Burk, M. W. Systemic administration of human leukocyte interferon to melanoma patients. I. Effects on natural killer function and cell populations. *J. Natl. Cancer Inst.*, **68,** 703–710 (1982).
26) Ibayashi, Y., Tokuda, Y., Saks, E. R., Sarna, G. P. and Golub, S. H. *In vivo* and *in vitro* activation on NK cytotoxicity with IL-2. *In* "Cellular Immunotherapy of Cancer," ed. R. Truitt, M. M. Bortin and R. P. Gale, pp. 275–285 (1987). Alan R. Liss Inc., New York.
27) Velardi, A., Grossi, C. E. and Cooper, M. D. A large subpopulation of lymphocytes with T helper phenotype (Leu-3/T4$^+$) exhibits the property of binding to NK cell targets and granular lymphocyte morphology. *J. Immunol.*, **134,** 58–64 (1985).
28) Wright, S. C. and Bonavida, B. Studies on the mechanism of natural killer cell-mediated cytotoxicity. V. Lack of NK specificity at the level of induction of natural killer cytotoxic factors in culture of human, murine, or rat effector cells stimulated with Mycoplasma-free cell lines. *J. Immunol.*, **133,** 3415–3423 (1984).
29) Gidlund, M., Orn, A., Pattengale, P. K., Janson, M., Wigzell, H. and Nilsson, K. Natural killer cells will only kill tumor cells at a given stage of differentiation. *Nature,* **292,** 848–850 (1981).
30) Ando, I., Hoon, D. B., Suzuki, Y., Saxton, R. E., Golub, S. H. and Irie, F. R. Ganglioside GM 2 on the K562 cell line is recognized as a target structure by human natural killer cells. *Int. J. Cancer,* **40,** 12–17 (1987).
31) Werkmeister, J. A., Pross, H. F. and Roder. J. C. Modulation of K562 cells with sodium butyrate. Association of impaired NK susceptibility with sialic acid and analysis of other parameters. *Int. J. Cancer,* **32,** 71–78 (1983).