Cancer Immunol Immunother (1989) 30: 247–253

Cancer Immunology Immunotherapy

Human lymphokine-activated killer (LAK) cells

I. Depletion of monocytes from peripheral blood mononuclear cells by L-Phenylalanine methyl ester: an optimization of LAK cell generation at high cell density

Kam H. Leung

E. I. du Pont de Nemours and Co., Medical Products Department, Business Planning & Development Division, Glenolden Laboratory, 500 S. Ridgeway Avenue, Glenolden, PA 19036, USA

Summary. Pretreatment of peripheral blood mononuclear cells (PBMC) with 5 mM L-phenylalanine methyl ester (PheOMe) provides an efficient means to deplete monocytes. PheOMe does not affect the number of large granular lymphocytes after the pretreatment, but does inhibit natural killer cell cytotoxicity temporarily after the pretreatment. However, depletion of monocytes by PheOMe allows lymphokine-activated killer (LAK) cell generation with recombinant interleukin-2 (rIL-2) at high cell density ($>5\times10^6$ cells/ml). The time of the PheOMe pretreatment is 40–60 min, though some effect could be observed within 15 min, and the pretreatment could be performed at room temperature. Pretreatment density of PBMC with 5 mM PheOMe could be achieved at cell density up to 3×10^7 cells/ml. PheOMe-pretreated cells could be activated by rIL-2 in serumless media at high cell density. Pretreatment of PBMC with 5 mM PheOMe provides an efficient means to deplete monocytes, as compared to plastic and nylon-wool adherence. LAK cell generation is similar in both methods of monocyte depletion; therefore, depletion of monocytes allows, LAK cell generation at high cell density. The PheOMe procedure provides an improved and convenient process for preparing LAK cells for adoptive immunotherapy.

Introduction

Human peripheral blood mononuclear cells (PBMC) incubated in the presence of interleukin-2 (IL-2) become cytotoxic to a broad spectrum of fresh tumor cell targets and cultured tumor cell line targets [2, 13]. These cytotoxic cells have been termed lymphokine-activated killer (LAK) cells. Natural killer (NK) cells and LAK cells have been implicated in immunosurveillance against tumor cells [1, 11]. It was disclosed by Rosenberg et al. [14] that the systemic administration of autologous LAK cells plus IL-2 to patients with advance cancer was beneficial. LAK cells were prepared at 1×10^6 cells/ml, but the process was tedious because a large number of cells was needed for each patient.

It has been shown that monocytes interfere with the activation of LAK activity by IL-2 [4, 5]. L-Leucine methyl ester (LeuOMe) and L-phenylalanine methyl ester (PheOMe) were shown to remove monocytes from human PBMC [4, 7]. However, LeuOMe also depleted NK cells [16]. We have reported that depletion of monocytes by PheOMe allows generation of LAK cells by rIL-2 at a cell density of 5×10^6 cells/ml or higher [7]. In the present studies, we have attempted to optimize the conditions for PheOMe pretreatment of PBMC for LAK activation at high cell density.

Offprint requests to: K. H. Leung

Materials and methods

Preparation of effector cells. Peripheral blood mononuclear cells were obtained from Biological Specialty (Lansdale, Pa) and separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient sedimentation [9].

Depletion of monocytes by PheOMe. PBMC (1×10^7 cells/ml) in RPMI-1640 medium after Ficoll separation were incubated with 5 mM freshly prepared PheOMe at room temperature for 40 min. PheOMe stock solution was adjusted to pH 7 and filtered through a 0.22-μm filter before the addition to PBMC. The cells were washed with RPMI-1640 medium.

Depletion of monocytes by plastic and nylon-wool adherence [9]. PBMC (3×10^6 cells/ml) in RPMI-1640 with 10% fetal calf serum were incubated plastic flasks for 1 h at 37° C. Nonadherent cells were decanted from the flasks into 50 ml conical tubes. The cell density was adjusted to 1×10^8 cells/ml and 1 ml cell suspension was layered on top of nylon-wool columns (0.5 g nylon wool in 5-ml syringes). The columns were incubated at 37° C for 40 min. The nonadherent cells were eluted from the column with 20 ml medium. The nonadherent cells were washed with RPMI-1640 medium.

LAK activation and cytotoxicity assay. The cells were cultured with RPMI-1640 medium supplemented with 10% fetal calf serum or various doses of rIL-2 (Du Pont) at various cell densities for 4 days at 37° C. In some experiments cells were cultured with RPMI-1640 supplemented with 4% pooled AB human serum or AIM-V serumless medium (Gibco, Grand Island, NY). After the culture period, the resulting cell were harvested for cytotoxicity assays [9] against Raji and K562 ^{51}Cr-labeled target cells. All assays were carried out in triplicate in round-bottom microtiter plates in a total volume of 0.2 ml. Labeled target cells in 0.1 ml (1×10^4 cells) were added to 0.1 ml effector cells at

various concentrations to obtain appropriate final effector cell: target cell ratios (E:T). The microtiter plates were centrifuged for 3 min at 80 g and then incubated for 2 h (NK assay) or 4 h (LAK assay). Three to four E:T ratios were assessed. The data shown are with an E:T ratio of 20:1 in 2-h assays for K562 and 4-h assays for Raji cells. To harvest for assay, plates were centrifuged for 5 min at 500 *g* and 0.1 ml each supernatant was removed for counting. The percentage specific lysis was calculated as follows:

$$\text{Specific } {}^{51}\text{Cr release (\%)} = \frac{\text{experimental release} - \text{background release}}{\text{maximal release} - \text{background release}}$$

The maximal release was obtained by adding 0.1 ml 1% Triton X-100 to 0.1 ml target cells. Background release is obtained as the radioactivity released from target cell incubated with medium alone; this value was usually 3%–5% of maximal release. All cytotoxicity data presented are from triplicate determinations with the SEM indicated. Each experiment was repeated at least three times with similar results.

Tumor target cells. Human B cell line, Raji, and erythroid-myeloid leukemia, K562, cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The cell lines were checked monthly for mycoplasma by the Hoechst dye method [3]. Fresh melanoma cells and lymphoma cells were gifts from Dr. J. Rinehart, Ohio State University, Columbus, Ohio. NCI fresh renal carcinoma tumor 466 was a gift from Dr. S. Rosenberg, NCI, Bethesda, Md.

[^{3}H]Thymidine uptake (proliferation). Microcultures were incubated in flat-bottom 96-well plates (Linbro) at 37° C and in 5% CO_2 in air, as previously described [10]. A 0.1-ml sample of culture: $(1-2) \times 10^6$ cells/ml, 0.05 ml culture medium and 0.05 ml (1 μCi) [*methyl*-^{3}H]thymidine (sp. act. 6.7 Ci/mmol) were added to the plates in quadruplicate. After 4-h of incubation the cultures were harvested on glass-fiber filter strips with an automatic cell harvester. The dried filters were counted in scintillation fluid in a Beckman scintillation counter.

Reagents. Recombinant IL-2 (1×10^6 U/mg protein) was from E. I. Du Pont de Nemours & Co. (Wilmington, Del). The unit of activity used throughout this report is equal to 33 BRMP units/ml activity. L-Phenylalanine methyl ester was purchased from Sigma (St. Louis, Miss) and Du Pont.

Flow cytometric analysis. For cell-surface marker analysis, 2×10^5 cells in 0.1 ml chilled staining buffer solution (phosphate-buffered saline, 15% BSA, and 0.1% sodium azide) were placed in 96-well round-bottom plate. Various antibodies tagged with a fluorescent marker were added to the cells for 30 min at 4° C. The cells were washed twice and resuspended in 1% paraformaldyhyde prior to analysis for fluoresence on a flow cytometer (Becton-Dickinson, Mountain View, Calif).

Results

Effect of rIL-2 on LAK activation.

We determined the effect of PheOMe pretreatment of PBMC on LAK generation by rIL-2. PBMC were isolated from the blood of healthy donors using Ficoll-Paque gra-

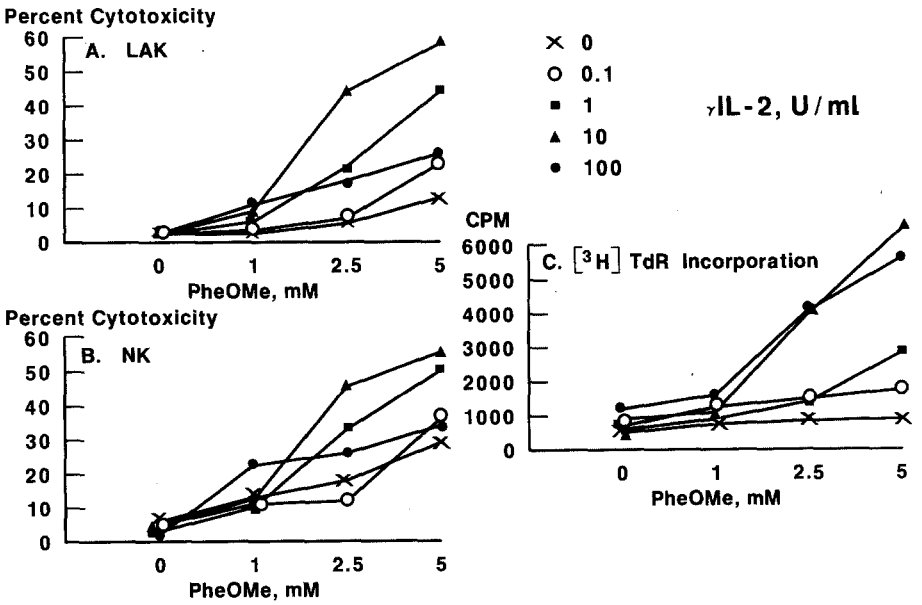

Fig. 1. Heparinized blood was separated by Ficoll-Paque density gradient sedimentation. The PBMC were suspended to 1×10^7 cells/ml in RPMI 1640 containing 10% fetal calf serum. PheOMe was dissolved in RPMI, the pH of the solution was adjusted to 7.0, and the solution was filtered through a 0.22-μm filter. The PBMC were incubated with 1–5 mM PheOMe in polypropylene 50-ml conical tubes for 40 min at room temperature. The resulting lymphocytes were washed twice with cold RPMI and resuspended in RPMI/10% fetal calf serum. The cells (1 ml) were cultured with medium or various doses of rIL-2 (Du Pont) at 5×10^6 cells/ml in polypropylene tubes for 4 days at 37° C. After the culture period, the resulting cells were harvested for cytotoxicity assays against Raji **(A)** and K562 **(B)** ^{51}Cr-labeled target cells. Three E:T ratios were assessed. The data shown are with an E:T ratio of 20:1 in 2-h assays for K562 and 4-h assays for Raji cells. Thymidine incorporation **(C)** of the cultures was assessed by incubation of 2×10^5 cells in 0.2 ml with 0.5 μCi [^{3}H]thymidine for 4 h

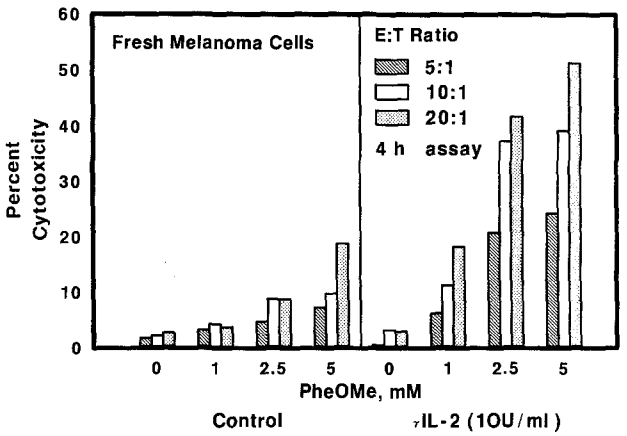

Fig. 2. Cells were prepared as described in the legend to Fig. 1 and were incubated with or without 10 U/ml rIL-2 at 5×10^6 cells/ml. Fresh melanoma cells (a gift from J. Rinehart, Ohio State University) were used as the labeled targets

dient centrifugation. PBMC were then incubated with 5 mM PheOMe at room temperature for 40 min. The washed cells (5×10^6/ml) were cultured with or without different doses of rIL-2 for 4 days. PheOMe treatment of PBMC enhanced some natural killer activity and LAK activity in the absence of rIL-2 (Fig. 1). For example, LAK cytotoxicity against Raji was 2% without PheOMe pretreatment and 12% with 5 mM PheOMe pretreatment. This enhancement of LAK activity was observed in over 20 different donors. The control cultures without PheOMe pretreatment were not responsive to rIL-2 activation at this cell density. However, PheOMe-treated cells were able to respond in a dose-dependent manner to rIL-2 between 0.1 U/ml and 10 U/ml. At 100 U/ml rIL-2 the activation was less than 1 U/ml. Therefore, PheOMe abrogates the unresponsiveness of cells cultured at high cell density to rIL-2. Similarly, [^{3}H]thymidine incorporation was augmented in PheOMe-treated cells in response to various doses of rIL-2. The enhancing effect of PheOMe treatment of PBMC on subsequent LAK generation could also be observed with fresh tumor cells (Fig. 2). Our data demonstrate that PheOMe-treated cells cultured for 4 days acquired some LAK activity against the fresh tumor cells and their activity was further augmented by rIL-2. The untreated cells did not response to rIL-2 at this cell density.

Table 1. Effect of PheOMe on natural killer cell lysis, monocytes and large granular lymphocytes (LGL) of peripheral blood mononuclear cells (PBMC)[a]

PheOMe (mM)	Monocytes (%)	LGL (%)	Cytotoxicity (%) + SEM: E:T ratio		
			20:1	10:1	5:1
0	25.5	16.0	13.8 ± 0.5	8.1 ± 1.4	4.5 ± 0.1
1	17.0	21.0	7.3 ± 2.7	2.7 ± 0.7	0.6 ± 0.3
2.5	12.0	22.0	1.3 ± 0.1	0.9 ± 0.5	0
5	5.0	16.0	0.7 ± 0.5	1.6 ± 0.6	0

[a] PBMC were treated with PheOMe for 40 min at room temperature. Natural killer cell lysis was measured by a 2-h ^{51}Cr-release assay against K562 cells. Percentages of monocytes and LGL were determined by Giemsa staining

Table 2. Phenotypic surface markers of PBMC pretreated with various doses of PheOMe[a]

PheOMe (mM)	Positive for surface marker (%)				
	CD4	CD8	CD3	Leu19	LeuM3
0	59	31	88	6	23
1	58	28	78	10	19
2.5	58	25	79	6	5
5	56	26	78	5	2

[a] PBMC from a different donor were treated with PheOMe for 40 min at room temperature. The cells were stained with the various monoclonal antibodies and analysed by flow cytometry. CD4 (Leu3), CD8 (Leu2) and CD3 (Leu4) are T cell markers. Leu19 (NKH-1) is a natural killer cell marker. LeuM3 (CD14) is monocyte/macrophage marker

Effect of PheOMe pretreatment on monocytes

PBMC were pretreated with PheOMe (1–5 mM) at room temperature for 40 min and washed. The cells were analyzed for NK activity against K562 target cells. As shown in Table 1, PheOMe temporarily abrogated NK activity and depleted monocytes from PBMC in a dose-dependent manner. PheOMe at 5 mM completely abrogated the NK activity. However, the treatment had little effect on the number of large granular lymphocytes isolated. The depletion of monocytes and the lack of effect on large granular lymphocytes with PheOMe were later confirmed in separate experiments by flow cytometric analyses (Table 2). Altogether the effect of PheOMe on PBMC was different from that of LeuOMe, which depleted monocytes, large granular lymphocytes and NK activity [16].

Comparison of PheOMe depletion of monocytes by physical treatments

PBMC were pretreated with 5 mM PheOMe at room temperature for 40 min and washed. At the same time, PBMC from the same donor were subjected to plastic and nylon-wool adherence. The resulting nonadherent cells were then pretreated with 5 mM PheOMe. As shown in Table 3, PheOMe at 5 mM depleted monocytes from 29.9% to 1.0% (eight donors). However, the treatment had little effect on the number of large granular lymphocytes isolated. The

Table 3. Effect of PheOMe and physical adherence treatment of peripheral blood mononuclear cells on monocytes and large granular lymphocytes (LGL)[a]

Cells	PheOMe (mM)	Monocytes (%)	LGL (%)
PBMC	0	29.9 ± 6.9	14.8 ± 4.7
	5	1.0 ± 1.0	20.0 ± 7.2
Nonadherent	0	3.9 ± 4.8	23.7 ± 6.4
	5	0.8 ± 0.8	27.2 ± 3.0

[a] Half of the PBMC were treated with medium alone or with medium containing 5 mM PheOMe for 40 min at room temperature. The other half of the PBMC (nonadherent) were depleted of monocytes by adherence to plastic flasks and nylon wool and treated as above (in medium with or without 5 mM PheOMe). Monocytes and LGL were determined by Giemsa staining. Data shown are the means ± SEM (eight experiments)

Table 4. Effect of PheOMe and physical adherence treatment of PBMC on recombinant human interleukin-2 activation[a]

Cytotoxicity against	Cells	PhOMe (mM)	E:T ratio		
			20:1	10:1	5:1
K562	PBMC	0	0.5	0	0.0±0.4
		5	50.7±0.8	29.1±1.0	14.8±0.9
	NA	0	45.5±0.2	27.2±1.4	13.6±1.3
		5	50.6±1.2	35.7±1.2	18.1±0.1
Raji	PBMC	0	2.5±0.5	0	0
		5	30.7±1.5	20.7±1.4	11.5±1.1
	NA	0	27.3±0.2	18.8±0.5	11.7±0.9
		5	37.0±1.3	25.3±0.2	12.4±0.6
Fresh lymphoma	PBMC	0	10.6±1.1	7.0±0.1	1.2±0.4
		5	23.5±0.9	18.6±0.1	11.2±1.6
	NA	0	24.7±1.4	19.3±2.3	12.3±1.2
		5	27.7±1.6	18.0±1.0	9.1±0.1
Fresh renal carcinoma	PBMC	0	2.0±0.5	1.0±0.5	0
		5	25.7±1.2	16.1±1.5	7.3±0.5
	NA	0	32.0±0.5	24.1±1.8	12.9±1.9
		5	29.6±0.2	21.4±1.0	13.6±1.4

[a] See Table 1 legend for details of cell treatments. Four-hour ^{51}Cr-release assays were performed as described in Materials and methods. NA, nonadherent

nonadherent cells from plastic and nylon-wool treatments contained 3.9% monocytes. PheOMe treatment of the nonadherent cells reduced the monocytes to 0.8%. PheOMe treatment of nonadherent cells had little effect on the percentage of large granular lymphocytes.

The four cell preparations were cultured at 5×10^6 cells/ml with 10 U/ml rIL-2 for 4 days and assayed for NK and LAK activity. As shown in Table 4, PBMC containing monocytes did not respond well to rIL-2 in lysing K562, Raji, and two fresh tumor targets. Depletion of monocytes from PBMC, either by PheOMe and physical adherence, enabled these cells to respond to rIL-2. Nonadherent cells were activated by rIL-2 to the same extent as PheOMe-treated cells. Treatment of nonadherent cells with PheOMe did not alter their response to rIL-2.

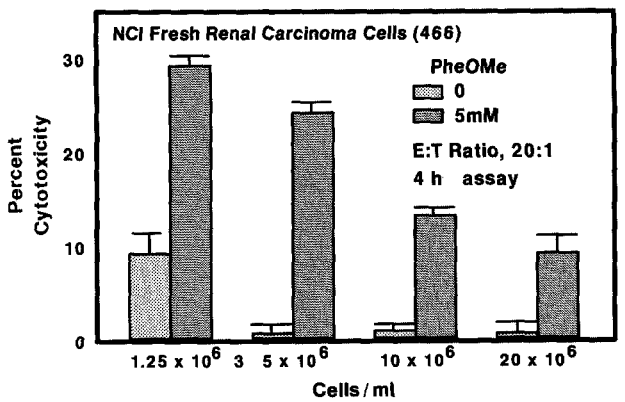

Fig. 3. PBMC were treated with or without 5 mM PheOMe. The resulting lymphocytes were cultured at $1.25 \times 10^6 - 20 \times 10^6$ cells/ml for 4 days. NCI fresh renal carcinoma tumor 466 (a gift from S. Rosenberg) was used as the source of labeled target cells

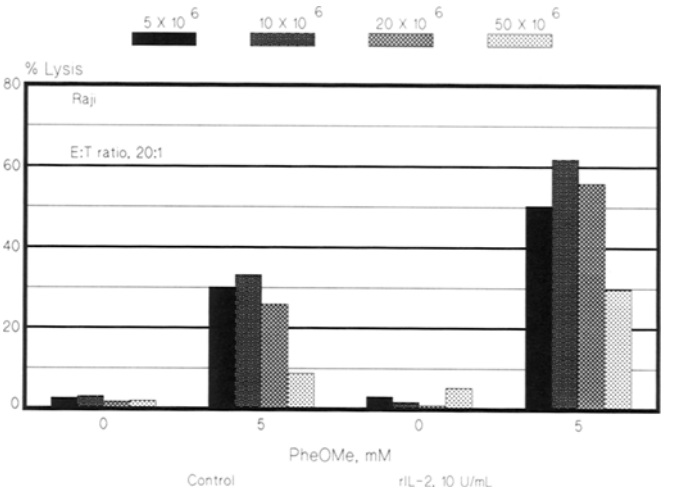

Fig. 4. Effect of cell density on PheOMe pretreatment. PBMC at various densities were incubated with 5 mM PheOMe for 40 min at room temperature. The washed cells were incubated with or without 10 U/ml rIL-2 for 3 days and assayed for cytotoxicity against Raji target cells

Effect of culture cell density on rIL-2 activation

Since most LAK generations from PBMC have been reported at $1-2 \times 10^6$ cells/ml we compared the effect of PheOMe in cultures with different cell density on LAK generation. Control PBMC- and PheOMe-treated cells were cultured at 1.25×10^6 5.0×10^6, 10.0×10^6 and 20.0×10^6 cells/ml with 10 U/ml rIL-2 for 4 days (Fig. 3). Control cells responded marginally to rIL-2 at a cell density of 1.25×10^6 cells/ml. As the cell density increased the control cells decreased their responsiveness to rIL-2. For example, rIL-2 could not generate LAK activity at 5×10^6 cells/ml. However, PheOMe-treated cells responded to rIL-2 better than the control cells cultured at the low cell density. Cells treated with PheOMe cultured at 1×10^7 or 2×10^7 cells/ml were still activated by rIL-2. Therefore, PheOMe treatment allows better LAK activation at the various cell densities.

Effect of cell density of PBMC on PheOMe pretreatment

PBMC were adjusted to different cell densities of 5×10^6, 10×10^6, 20×10^6, and 30×10^6 cells/ml for pretreatment with 5 mM PheOMe. The cells were cultured for 3 days with or without 10 U/ml rIL-2. As shown in Fig. 4, the lymphocytes treated with 5 mM PheOMe were about three times as potent as the control PBMC. There was little difference amongst the various cell densities for PheOMe treatment. Thus, PheOMe treatment of PBMC at cell densities of $5 \times 10^6 - 3 \times 10^7$ cells/ml results in cultures with LAK activity better than that of the untreated PBMC.

Next we compared higher cell densities of 5×10^7 and 10×10^7 with 1×10^7 cells/ml (Fig. 5) treated with PheOMe concentrations of 5 mM, 10 mM or 20 mM. Pretreatment of PBMC with 5 mM PheOMe was effective at 1×10^7 cells/ml and less effective at the higher cell densities of 5×10^7 and 10×10^7 cells/ml. At 10 mM PheOMe, it was less effective at 10×10^7 cells/ml than at the lower cell densities. At 20 mM PheOMe, little difference was observed amongst the three cell densities. However, they were not as effective as the cells treated with 5 mM or 10 mM PheOMe

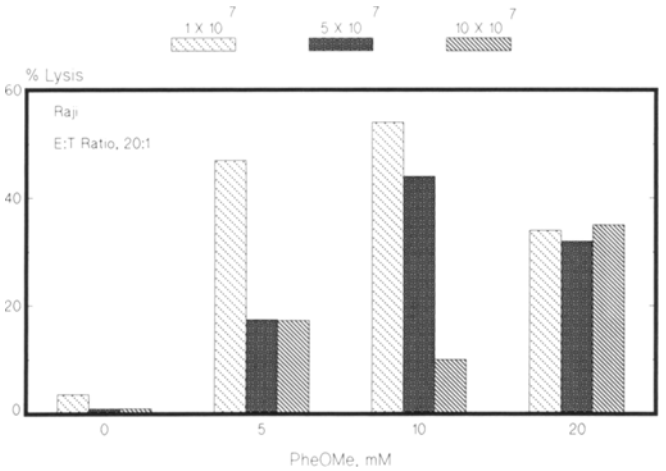

Fig. 5. Effect of cell density on PheOMe pretreatment. PBMC at 1×10^7 – 10×10^7 cells/ml were treated with 0, 5, 10 or 20 mM PheOMe for 40 min. The washed cells were then incubated with 10 U/ml rIL-2 for 3 days and assayed for cytotoxicity against Raji target cells

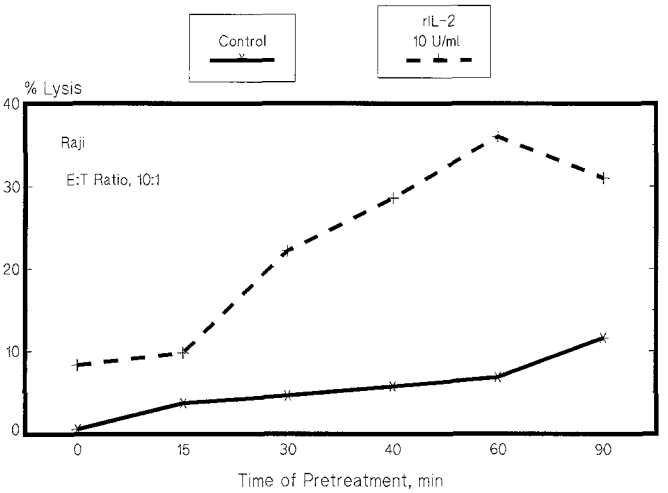

Fig. 6. Time of PheOMe pretreatment on LAK activation. PBMC (1×10^7 cells/ml) were treated with 5 mM PheOMe for 15–90 min and washed. The cells were then incubated with 10 U/ml rIL-2 for 3 days and assayed for cytotoxicity against Raji target cells

at 1×10^7 cells/ml. Hence, the PheOMe concentration range should be 5–10 mM and cell density should be less than 5×10^7 cells/ml during treatment.

Time of PheOMe pretreatment on LAK activation

PBMC were pretreated with 5 mM PheOMe for various time periods before rIL-2 activation (Fig. 6). It took about 30 min of pretreatment to observe enhanced LAK activation. The time of pretreatment was optimal at around 40–60 min.

Kinetics of LAK activation

PBMC were pretreated with 5 mM PheOMe and cultured at 5×10^6/ml with or without rIL-2 for 1, 2, 3, or 5 days. As shown in Fig. 7, control cells without rIL-2 had little LAK activity and some LAK activation was observed after 2 days in culture. PheOMe-pretreated cells generated some LAK activity by day 3. The LAK activity of PheOMe-

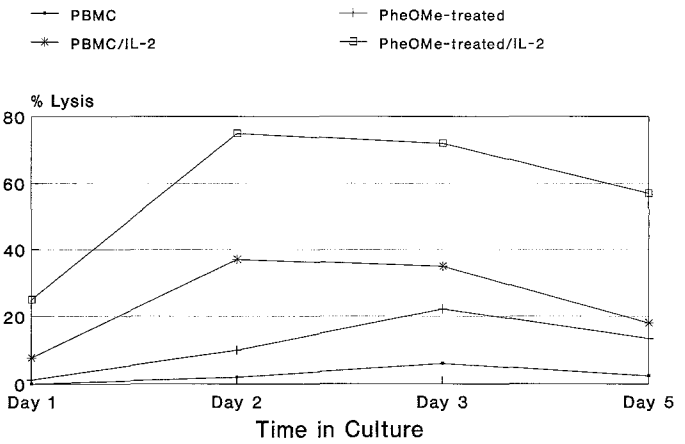

Fig. 7. Kinetics of LAK activation. PBMC were treated with medium or 5 mM PheOMe for 40 min. The washed cells were cultured with or without 10 U/ml rIL-2 for 1, 2, 3, or 5 days. LAK activity was assayed against Raji target cells

treated cells incubated with rIL-2 was observed after 1 day. The LAK activity remained high up to day 5 in culture. The kinetics of LAK activity are similar in the control and PheOMe-treated cells. However, the PheOMe-treated cells had higher cytotoxicity than the untreated cells.

Generation of LAK activity in various media

Since most of our LAK cell cultures were performed in RPMI-1640 supplemented with 10% fetal calf serum we have investigated other media. PBMC treated with 5 mM PheOMe were cultured at 5×10^6/ml in RPMI medium with 10% fetal calf serum, RPMI medium with 4% human serum or AIM-V serumless medium for 4 days in the presence of 10 U/ml rIL-2. Generation of LAK cytotoxicity ($LU_{30}/10^6$ cells) was better in all the three media with the PBMC that had been treated with PheOMe than with the untreated cells. However, LAK activity could be observed in AIM-V medium with the untreated PBMC. In summary, AIM-V medium and RPMI medium with 4% human serum gave similar LAK activation with PheOMe-treated PBMC.

Discussion

Our data demonstrate that PBMC containing monocytes did not respond well to rIL-2 at a cell density equal to or greater than 5×10^6/ml. Depletion of monocytes by PheOMe or physical adherence enables the cells to be responsive to rIL-2 activation. The cytotoxicity of these treated cells was increased against tumor cell lines that are either sensitive (K562) or resistant (Raji) to the killing effects of NK cells, as well as against fresh tumor target cells (Figs. 1–3). The proliferative response to rIL-2 was also augmented by monocyte depletion with PheOMe pretreatment (Fig. 1). Generation of LAK activity by rIL-2 from PBMC treated with PheOMe can be achieved in serumless medium, such as, AIM-V medium (Table 5).

Depletion of monocytes by PheOMe offers a one-step procedure: incubation with PBMC with 5 mM PheOMe at room temperature. Our study indicates that LAK cells could be generated from PBMC that had been treated with PheOMe. However, our procedure using PheOMe provides a few substantial differences, improvements and advantages over the initial procedures published. First, we

Table 5. Generation of LAK cells in various media[a]

Medium	Cytotoxicity ($LU_{30}/10^6$ cells)			
	Expt 1: PhOMe conc		Expt 2: PhOMe conc	
	0	5 mM	0	5 mM
RPMI/10% FCS	4.2	39.7	1.3	23.0
RPMI/4% HS	12.1	55.7	0.8	38.9
AIM-V	33.5	78.1	16.1	41.2

[a] PBMC were treated with or without 5 mM PheOMe as described in Materials and methods. Cells were incubated with 10 U/ml recombinant interleukin-2 at 5×10^6/ml (Expt. 1) or 1×10^7/ml (Expt. 2) for 3–4 days. LAK cytotoxicity was assessed against Raji target cells in 3-h assays. The experiments were done at separate times with a different donor. Data shown are representative of four different experiments. FCS, fetal calf serum; HS, human serum

use PheOMe as a chemical agent to deplete monocytes from PBMC. PheOMe is convenient to use and the degree of monocyte depletion by PheOMe can be monitored. Second, our cell culture densities are $(1-2) \times 10^7$ cells/ml as compared to 2×10^6 cells/ml. This will cut down the media used by five- to tenfold. We are also able to treat the PBMC before or after Ficoll sedimentation centrifugation and up to a density of 3×10^7 cells/ml. The depletion of monocytes by 5 mM PheOMe is efficient (95%–100%) and sufficient for LAK cell generation at high cell density-greater than 5×10^6 cells/ml. The procedure employing PheOMe-treated PBMC lends itself well to processing large volumes of blood and the cell numbers required for adoptive immunotherapy.

PheOMe is a lysosomotropic agent. Our data show that monocyte destruction began within 15 min after the incubation of PBMC with 5 mM PheOMe. However, it took about 40 min to observe the optimal depletion of monocytes from PBMC. As judged by Giesma stainings and cytometric analyses, only monocytes were depleted by PheOMe, and other lymphocytes, including NK cells, were mostly spared the depressive effect of PheOMe (Tables 1, 2). Though the NK activity of PheOMe-treated PBMC was initially inhibited, LAK activation by IL-2 was not affected. LeuOMe, on the other hand, is also a lysosomotropic agent to monocytes [16], but it is cytotoxic to NK cells. Therefore, little LAK cell activation by IL-2 could be observed from PBMC that had been treated with LeuOMe (data not shown).

PheOMe was able to deplete monocytes from PBMC at cell densities from 5×10^6 to 5×10^7 cells/ml. As the pretreatment cell density increased, the effective concentration of PheOMe also increased (Figs. 4, 5). Hence, PheOMe is a substrate being consumed by a population of cells in the PBMC – presumbly monocytes (Tables 1, 2). It was shown that monocytes could hydrolyse some L-amino acid methyl esters to free amino acids, which accumulate inside the lysosomes of monocytes [16]. The osmotic imbalance causes water to enter into the lysosomes, which release their enzymes to lyse the monocytes.

Depletion of monocytes by plastic and nylon-wool adherence demands more manipulations than PheOMe treatment. First the PBMC are incubated in plastic flasks at 3×10^6/ml. The nonadherent cells are then incubated in nylon-wool columns (1×10^8/column). The combined procedures give 90%–99% depletion of monocytes. The physical depletion of monocytes would require the use of more media, plastic ware, and time. Therefore, PheOMe treatment of PBMC provides an efficient and convenient method to deplete monocytes from PBMC. Our data show that there is little difference in LAK activation between the PheOMe and the physical depletion of monocytes (Table 3). This fact indicates that the monocytes are the prime target cells for destruction by PheOMe.

The role of monocytes in LAK cell generation has become a controversial issue. Monocytes have been shown both to augment and to suppress LAK generation [4, 5, 7, 12, 15]. A recent report shows that the enhancing effect of monocytes on lytic activity diminishes when they are used in high cell numbers [15]. Furthermore, their results show that T cells are the responder cells for the monocyte effect while purified NK cells are not affected by the monocytes. The cytolytic and proliferative responses to IL-2 of the T cells require monocytes. In our experiments, depletion of monocytes alone by PheOMe allowed LAK cell generation (cytolytic and proliferative responses) at high cell density (Fig. 1), and the degree of LAK activation was directly proportional to the degree of monocyte depletion by PheOMe (Fig. 1 and Tables 1, 2). Therefore, our studies suggest that monocytes interfere with LAK generation at high cell density.

The mechanisms by which monocytes inhibit LAK cell generation by IL-2 at high cell density are unclear and under investigation. Monocytes could compete with LAK precursor cells for IL-2. However, our data with high doses of IL-2 show that this may not be the case because 100 U/ml IL-2 did not generate better LAK activation than 1 U/ml or 10 U/ml IL-2 (Fig. 1). We have shown that prostaglandin E_2 inhibited LAK generation by low doses of IL-2 (Leung, unpublished observation) and monocytes were the source of prostaglandin E_2 [8]. However, indomethacin, an inhibitor of prostaglandin E_2 synthesis, did not prevent the monocytes from suppressing the LAK activation by IL-2 (data not shown). The likely explanation is that IL-2 may induce other cytokines and factors, such as IL-1, interferon, tumor growth factor β and tumor necrosis factor α and other metabolites from the monocytes, which may suppress the generation of LAK activity. Tumor growth factor β has been shown recently to inhibit LAK activation by IL-2 [6]. It is also possible that monocytes may suppress the generation of LAK activity by cell-to-cell interaction.

Taken together, PheOMe depletes monocytes from PBMC and allows LAK generation by rIL-2 at high cell density in 2–5 days. Our procedures are simple and convenient to be employed in the preparation of large numbers of LAK cells for adoptive immunotherapy.

Acknowledgements. The author thanks Ms. T. Maloney for her technical assistance, Mrs. AnnMarie O'Hara-Townsend for flow cytometry, Ms. E. Freas for her assistance in the preparation of the manuscript, Dr. J. Rinehart and Dr. S. Rosenberg for the fresh human tumor cells, and Dr. J. Linna for his helpful comments and discussions.

References

1. Barlozzari T, Reynolds CW, Herberman RB (1983) In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM_1-treated rats. J Immunol 131: 1024

2. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA (1982) 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
3. Helwig I, Groppe A (1972) Staining o constitutive heterochromatin in mammalian chromosomes with a new flurochrome. Exp Cell Res 75: 122
4. Hoyer M, Meineke T, Lewis W, Zwilling B, Rinehart J (1986) Characterization and modulation of human lymphokine (interleukin 2) activated killer cell induction. Cancer Res 46: 2834
5. Ibayashi Y, Hoon DSB, Golub SH (1987) The regulatory effect of adherent cells on lymphokine activated killer cells. Cell Immunol 110: 365
6. Kasid A, Bell GI, Director EP (1988) Effects of transforming growth factor-β on human lymphokine-activated killer cell precursors: autocrine inhibition of cellular proliferation and differentiation to immune killer cells. J Immunol 141: 690
7. Leung KH (1987) Improved process for preparing LAK cells by L-phenylalanine methyl ester pretreatment of peripheral blood mononuclear cells. Lymphokine Res 6, abstr 1817
8. Leung KH, Fischer DG, Koren HS (1983) Erythromyeloid tumor cells (K562) induce PGE synthesis in human peripheral blood monocytes. J Immunol 131: 445–449
9. Leung KH, Koren HS (1982) Regulation of human natural killing: II. Protective effect of interferon on NK cells from suppression by PGE_2. J Immunol 129: 1742
10. Leung KH, Mihich E (1980) Prostaglandin modulation of development of cell-mediated immunity in culture. Nature 288: 597
11. Mule JJ, Ettinghausen SE, Spiess PJ, Shu S, Rosenberg SA (1986) Antitumor efficacy of lymphokine-activated killer cells and recombinant interleukin-2 in vivo: survival benefit and mechanisms of tumor escape in mice undergoing immunotherapy. Cancer Res 46: 676
12. Nii A, Sone S, Utsugi T, Yangawa H, Ogura T (1988) Up- and down-regulation of human lymphokine (IL-2)-activated killer cell induction by monocytes, depending on their functional state. Int J Cancer 41: 33
13. Phillips JH, Lanier LL (1986) 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
14. Rosenberg SA, Lotze MT, Mul JJ (1988) New approaches to the immunotherapy of cancer using interleukin-2. Ann Intern Med 108: 853
15. Silvennoinen O, Vakkila J, Hurme M (1988) Accessory cells, dendritic cells, or monocytes, are required for the lymphokine-activated killer cell induction from resting T cell but not from natural killer cell precursors. J Immunol 141: 1404
16. Thiele DL, Lipsky PE (1985) Regulation of cellular function by products of lysosomal enzyme activity: elimination of human natural killer cells by a dipeptide ethyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes. Proc Natl Acad Sci USA 82: 2468

Received 28 February 1989/Accepted 19 June 1989