Jpn. J. Cancer Res. **80**, 662–669, July 1989

Killing of Alveolar Macrophages and of Monocytes that Have Responded to Granulocyte-Macrophage Colony-stimulating Factor by Human Lymphokine-activated Killer Cells

Saburo Sone,[1] Noriaki Inamura, Sukh Mahendra Singh, Akio Okubo, Hiroaki Yanagawa, Mie Nakanishi and Takeshi Ogura

Third Department of Internal Medicine, The University of Tokushima School of Medicine, Kuramoto-cho 3, Tokushima 770

The susceptibilities of human blood monocytes and alveolar macrophages (AM) to cytotoxicity mediated by lymphokine (IL-2)-activated killer (LAK) cells were examined. Monocytes and AM of healthy donors were obtained by counter-flow centrifugal elutriation (CCE) and bronchoalveolar lavage, respectively. The LAK activity induced by incubation of blood mononuclear cells (MNC) for 4 days with recombinant interleukin 2 (IL-2) was measured by a 4-h ^{51}Cr release assay. The LAK cells were not cytotoxic to freshly isolated monocytes, but were cytotoxic to autologous fresh AM and monocytes that had been incubated for more than 4 days in medium alone. Blood monocytes that had been incubated for 4 days in medium with granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) or interleukin 3 (IL-3) were much more susceptible than untreated monocytes to the cytotoxicity of LAK cells. When blood monocytes were separated by CCE into subpopulations of three sizes (small, medium and large), the medium- and large-sized monocytes showed greater responses to GM-CSF in terms of DNA synthesis and colony formation than the small-sized cells. After treatment with GM-CSF for 4 days, these medium and large monocytes were more susceptible than the small monocytes to the cytotoxic action of LAK cells. These results suggest that LAK cells may be important *in situ* in down-regulating the functions of mature macrophages and blood monocytes that have responded to GM-CSF.

Key words: Macrophages — Interleukin 2 — Colony-stimulating factor — Lymphokine-activated killer cell

Monocyte-macrophages are important regulatory and effector cells in host defense.[1-3] For potentiating non-specific host defense, much attention has been paid to colony stimulating factors (CSFs)[2] such as granulocyte-macrophage CSF (GM-CSF), macrophage CSF (M-CSF) and multi-CSF (IL-3), because these CSFs are known to support the growth and proliferation of mononuclear phagocyte progenitor cells.[4-6] In addition to regulating the proliferation of progenitor cells, these CSFs are known to influence various functions of monocytes and macrophages, such as their production of $H_2O_2^-$,[7,8] Ia antigen expression,[8,9] tumoricidal activity,[10] induction of prostaglandin[11] and production of monokines.[12,13]

Cells with cytotoxicity towards a wide spectrum of tumors can be induced without antigenic stimulation by *in vitro* culture of unprimed lymphocytes for 4 to 6 days with lymphokines including IL-2.[14,15] These cells, referred to as lymphokine-activated killer (LAK) cells, destroy various fresh autologous and allogeneic tumor cells.[14,16,17] Recently, we demonstrated that human blood monocytes and AM up- or down-regulate the induction of LAK activity depending on their functional state.[18-20] On the other hand, there is accumulating evidence that LAK cells are cytotoxic to hematopoietic progenitor cells in the bone marrow[21] and peripheral blood.[22] Moreover, Djeu and Blanchard[23,24] recently reported that blood monocytes, isolated by adherence and detachment, were susceptible to the cytotoxicity of LAK cells. Previously, we found that detachment of monocytes adhering to plastic resulted in their stimulation and/or activation.[25] These findings raise the question of whether the susceptibilities to LAK-mediated cytotoxicity of blood monocytes and mature macrophages located in tissues or organs depend on their activation and maturation. Moreover, it is also important to examine whether various cytokines may influence the susceptibilities of monocytes and macrophages to LAK

---

[1] To whom correspondence should be addressed.

[2] Abbreviations used: AM, alveolar macrophages; CCE, counter-flow centrifugal elutriation; CRPMI 1640 medium, complete RPMI 1640 medium supplemented with 5% FBS and gentamicin; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-2, interleukin 2; LAK, lymphokine-activated killer; LU, lytic unit; MNC, mononuclear cells; M-CSF, macrophage colony-stimulating factor; TNF, tumor necrosis factor.

activity. In this study, we found that LAK cells were not cytotoxic to freshly isolated monocytes, but killed alveolar macrophages and mature blood monocytes that had responded to GM-CSF.

MATERIALS AND METHODS

Cell cultures A human Burkitt lymphoma cell (Daudi) line was purchased from the American Type Culture Collection (ATCC), Rockville, MD. Daudi cells were maintained as stationary suspension cultures in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin, designated as CRPMI 1640, at 37°C in a 5% CO_2 humidified atmosphere.

Reagents Fetal bovine serum (FBS) was purchased from M.A. Bioproducts, Walkersville, MD. Human recombinant interleukin 2 (IL-2) was prepared at Takeda Pharmaceutical Co., (Osaka), and had a specific activity of 3.5×10^4 U/mg as assayed on IL-2-dependent murine NKC3 cells.[18] Recombinant human interferon γ (IFN-γ; specific activity, 5.36×10^4 U/mg protein) and human recombinant IFN-α subtype A (IFN-αA; specific activity, 1.975×10^8 U/mg protein) were kindly supplied by Nippon Roche (Tokyo). Recombinant human IL-1α and IL-1β were kindly supplied through Dr. Y. Hirai (Otsuka Pharmaceutical Co., Tokushima).[26] Recombinant macrophage colony-stimulating factor (M-CSF; specific activity, 0.8×10^6 U/mg protein), recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (specific activity, 1.7×10^7 U/mg protein), and recombinant human IL-3 (specific activity, $2\text{–}4 \times 10^6$ U/mg protein) were supplied by the Genetics Inst., Cambridge, MA. Recombinant human IL-6 (specific activity, 4.0×10^3 U/mg protein) was supplied by Ajinomoto Co., Tokyo. Natural tumor necrosis factor (TNF; specific activity, 4.0×10^5 JRU/mg protein) was a gift from the Hayashibara Institute (Okayama). These agents did not contain any endotoxins detected by *Limulus* amebocyte lysate assay (sensitivity limit, 0.1 ng/ml; Seikagaku Kogyo Co., Tokyo). Lipopolysaccharide (LPS; *E. coli* 055: B5) was obtained from Difco Laboratories, Detroit, MI.

Isolation and culture of human peripheral blood monocytes Leukocyte concentrates were collected from peripheral blood (200 ml) of healthy donors in a Kubota KR-400 centrifuge with an RS-6600 rotor, and mononuclear cells were separated from the leukocyte concentrates in lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD). Then, lymphocytes and monocytes were separated from the mononuclear cell samples by centrifugal elutriation in a Hitachi SRR6Y elutriation rotor.[18, 19] Fractions containing lymphocyte-rich cells and monocyte-rich cells were obtained at 2,000 rpm and flow rates of 15 ml/min and 20 ml/min, respectively. More than 99% of the cells in the lymphocyte-rich fraction were lymphocytes and more than 90% of those in the monocyte-rich fraction were monocytes as determined by nonspecific esterase staining and morphological examination. More than 97% of the cells in both fractions were viable, as judged by the trypan blue dye exclusion test.

For separation of the monocyte fractions by CCE, monocyte-rich fractions were obtained at a speed of 2000 rpm with an increase in flow rate every 50 ml starting from 20 ml/min. The percentage of monocytes in each fraction was determined by nonspecific esterase staining and morphological examination. These fractions were washed twice with balanced salt solution, and resuspended in CRPMI-1640 at appropriate concentrations. They were used as targets of LAK cells immediately or after incubation for various periods in CRPMI 1640 medium with test cytokines.

Harvesting of human AM Bronchoalveolar lavage was performed as described in detail elsewhere.[18, 27] Briefly, the oral cavity and the upper airway were anesthesized with lidocaine spray, and the tip of an Olympus fiberoptic bronchoscope (Model BF-1T; Olympus Co., Tokyo) was wedged into one of the segments of the right or left lobe. Then 50 ml of sterilized saline (0.9% NaCl) at 37°C was instilled into the lung and the fluid was gently sucked out with a 50-ml syringe. This process was repeated three times. Of the total of 150 ml of saline instilled, about 65% was recovered. About 1.8×10^7 viable AM per wedge segment (>93% viable as determined by trypan blue dye exclusion) were obtained from normal volunteers. Differetial counts of cells stained for nonspecific esterase established that 90% of these cells were AM. These AM were used as targets of LAK activity immediately or after cultivation for 4 days in medium with GM-CSF. In one preliminary experiment, cells in the lavage were plated for 1 h on a plastic surface, and then nonadherent cells were harvested by washing with RPMI 1640 prewarmed at 37°C, and used as targets.

Assay of LAK activity LAK activity was assayed by measuring ^{51}Cr release as described in detail previously.[18-20] Briefly, human blood effector lymphocytes (10^6 cells/ml) were incubated in the presence or absence of an optimal concentration of IL-2 (1 U/ml) at 37°C under 5% CO_2 in air for 4 days. The cultures were then thoroughly washed and their cytotoxicity against ^{51}Cr-labeled 10^4 target cells was measured at various effector/target (E/T) cell ratios. Incubations were terminated after 4 h, the supernatants (0.1 ml per well) were harvested by brief centrifugation at 1,500 rpm, and their radioactivities were determined in a gamma counter. Percentage cytotoxicity was calculatd as follows:

% Cytotoxicity

$$=100\times\frac{\text{experimental cpm}-\text{spontaneous cpm}}{\text{total cpm}-\text{spontaneous cpm}}$$

The spontaneous release observed with different target cells ranged from 5 to 15% (total lysis). One lytic unit (LU) was defined as the number of effector cells required to cause 20% lysis of target cells.

Assay of proliferative response of monocytes to GM-CSF The 3 fractions of blood monocytes separated by CCE were each incubated in suspension with GM-CSF (100 U/ml) for 5 days. The cultures were labeled for the final 18 h with ^{3}H-TdR (6.8 Ci/mmol; Amersham, Arlington Heights, IL) at 0.5 μCi/well. At the end of the incubation period, the cells were harvested on a glass-fiber filter in a cell harvester, MASH II, and cellular ^{3}H-TdR incorporation was assessed by scintillation counting.

Statistical analysis The statistical significance of differences between test groups was analyzed by means of Student's t test (two-tailed).

RESULTS

Killing of monocytes and AM by LAK cells In preliminary experiments, we found that blood lymphocytes separated by CCE and nonadherent cells, obtained by washing after plating bronchoalveolar lavage cells, were not susceptible to the cytotoxicity of LAK cells (data not shown), thus ruling out the possibility that small populations of lymphocytes or nonadherent cells contaminating the monocyte- or AM-rich populations used as targets could account for some, if not all, the monocyte or macrophage killing mediated by LAK cells. Based on these observations, we examined whether blood monocytes isolated by CCE and AM freshly obtained by bronchoalveolar lavage from healthy donors were sus-

Table I. Susceptibilities of Freshly Harvested Blood Monocytes and AM Freshly Harvested from Healthy Donors to Cytotoxicity Mediated by Autologous LAK Cells

Experimental no.	Cytotoxicity (LU per 10^6 cells) against: Fresh monocytes	Fresh AM	Daudi cells
1	0	30.7	123.4
2	2.7	64.6	274.1
3	1.9	61.1	384.2

Blood MNC were incubated for 4 days in medium with IL-2 (1 U/ml), and then their LAK activities on Daudi cells and freshly isolated monocytes and AM obtained from healthy donors were examined as described in "Materials and Methods."

Table II. Effects of Pretreatment of Monocytes with Various Cytokines on Killing of the Cells by LAK Cells

Cytokine	(concentration, U/ml)	% Cytotoxicity against monocytes, E/T ratio 40:1	20:1	10:1	UL/10^6 cells
Exp. 1					
Medium		5.9±1.4[a]	3.6±0.6	2.3±0.6	1.5
IFN-αA	1000	2.3±0.4	2.3±0.2	1.0±0.4	0
IFN-γ	100	6.6±0.2	5.1±0.6	3.8±1.2	1.4
TNF	100	13.4±1.7[b]	11.7±0.1[b]	7.2±2.3[b]	9.8
IL-3	10	23.9±0.9[b]	15.7±1.5[b]	14.6±4.2[b]	32.6
M-CSF	1000	19.1±2.5[b]	13.9±2.2[b]	7.3±2.5[b]	20.4
GM-CSF	100	26.5±2.6[b]	20.0±0.4[b]	14.3±0.9[b]	52.4
Exp. 2					
Medium		13.7±0.4	9.3±1.9	6.8±0.7	7.0
IL-1β	100	31.1±1.6[b]	24.2±2.3[b]	16.0±2.6[b]	84.0
IL-2	1	19.6±3.4	11.7±0.4	7.0±1.2	15.3
IL-3	10	32.5±5.5[b]	25.7±1.1[b]	19.6±4.6[b]	100.2
IL-6	10	20.7±4.0	14.3±1.7	9.1±5.4	19.4
GM-CSF	100	57.1±1.2[b]	46.0±0.6[b]	32.7±0.7[b]	307.4

Blood MNC were incubated for 5 days with IL-2 (1 U/ml) and then their LAK activities on monocytes that had been incubated for 5 days in medium with the indicated cytokines were examined as described in "Materials and Methods."

a) Mean±SD for triplicate cultures. Data were obtained in two separate experiments.

b) $P<0.05$ vs. value at the same E/T ratio for monocytes incubated in medium alone.

ceptible to the cytotoxicity of LAK cells. LAK cells were induced by culture of blood MNC of healthy donors for 4 days in medium with 1 U/ml of IL-2 before measurement of LAK activity against fresh autologous blood monocytes and AM at various E/T ratios. The results are shown in Table I. LAK cells were highly cytotoxic to NK-resistant Daudi cells. Under these experimental conditions, LAK cells did not affect freshly isolated blood monocytes, but destroyed significant numbers of freshly isolated autologous AM.

Effect of pretreatment of monocytes with various cytokines on their killing by LAK cells We examined the effect of pretreatment of blood monocytes with various cytokines on their susceptibility to LAK-mediated cytotoxicity. Blood monocytes were incubated for 4 days in medium with the indicated cytokines at their optimal concentrations, and then used as target cells of autologous LAK cells obtained by incubation of blood MNC with IL-2 (1 U/ml). LAK activity was assessed by 4-h ^{51}Cr release assay. The results are given in Table II, IFN-αA and IFN-γ did not affect the killing of monocytes by LAK cells. But pretreatment of monocytes with TNFα, IL-1β, IL-3, IL-6, M-CSF or GM-CSF significantly increased the susceptibility of the cells to the cytotoxicity of LAK cells. Of these cytokines, GM-CSF caused the greatest increase in susceptibility to LAK cells.

Enhancement of LAK-mediated monocyte killing by GM-CSF We examined the effect of pretreatment of monocytes and AM with GM-CSF on their susceptibilities to LAK-mediated cytotoxicity. Blood monocytes and AM were incubated for 4 days in medium containing 100 U/ml of GM-CSF, and then used as targets of LAK cells induced by culturing blood MNC with an optimal concentration of IL-2 (1 U/ml). As shown in Table III, LAK cells were highly cytotoxic to allogeneic Daudi cells, and monocytes treated with GM-CSF were

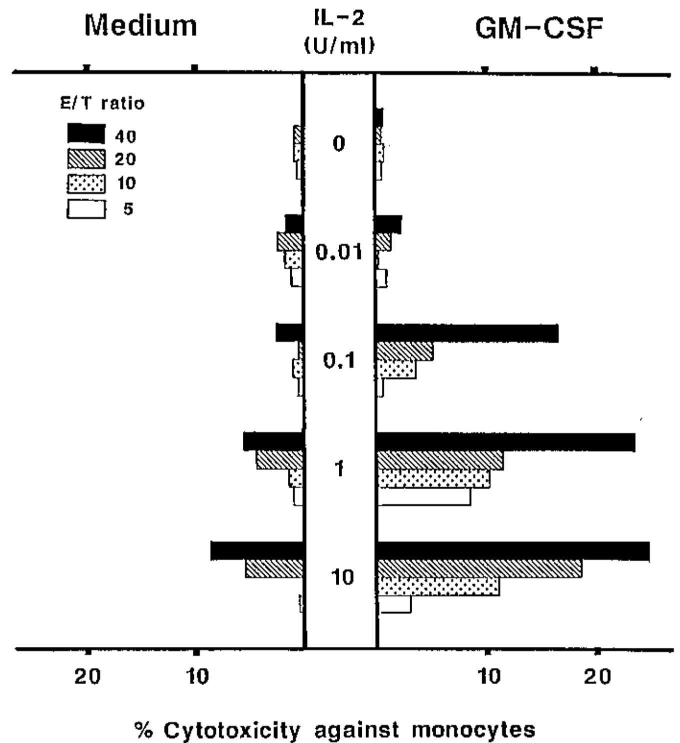

Fig. 1. Effect of IL-2 concentration on induction of LAK activity against monocytes. Blood monocytes were incubated in medium with or without GM-CSF (100 U/ml) for 4 days, and then used as target cells. Blood MNC were incubated for 4 days in medium with various concentrations of IL-2 before assay of LAK activity against these monocytes (1×10^4 cells) or Daudi cells (1×10^4 cells). Percentage LAK activity was determined as described in "Materials and Methods." Values are means for triplicate cultures. Data are representative of two separate experiments. The SD was consistently $<10\%$ of the mean.

Table III. Effect of Treatment of Monocytes or AM with GM-CSF on Their Susceptibility to LAK-mediated Cytotoxicity

No. of exp.	Cytotoxicity (LU/10^6 cells) against: Monocytes		AM		Daudi cells
	Medium	GM-CSF	Medium	GM-CSF	
1	0.3	51.7	25.1	27.4	444.7
2	0	31.4	15.0	11.0	79.9
3	0	34.0	4.1	4.0	916.0

Blood lymphocytes were incubated for 4 days in medium with 1 U/ml of IL-2, and then their LAK activities were tested on Daudi cells, and monocytes or AM that had been incubated for 4 days in medium with or without 100 U/ml of GM-CSF.

Table IV. Susceptibility of Blood Monocytes Treated with GM-CSF to Cytotoxicity of LAK Cells

Incubation time (h)	Cytotoxicity (LU/10^6 cells) Untreated monocytes	GM-CSF-treated monocytes	Daudi cells
5	0.1	0	862.7
24	0.5	0.4	1107.3
96	4.2	60.9	1102.6
192	10.9	104.6	989.1

Monocytes were incubated in medium with or without GM-CSF (100 U/ml) for the indicated periods, and then washed and used as target cells. Autologous blood MNC were separately incubated for 4 days in medium containing IL-2 (1 U/ml), and then their LAK activities on the monocytes and on Daudi cells were tested at E/T ratios of 40:1, 20:1 and 10:1. Data are representative of two separate experiments which gave similar results.

Table V. Proliferative Response of Blood Monocyte Subpopulations to GM-CSF

Monocyte fraction	Cell size (μm)	Population (%)		Proliferative response (^{3}H-TdR uptake)			
				Exp. 1		Exp. 2	
		Monocytes	Lymphocytes	Medium	GM-CSF	Medium	GM-CSF
I	8.99	77.1	22.9	86±63	1313±503	259±10	433±238
II	9.71	91.6	5.3	80±55	5092±286	109±70	2626±25
III	11.43	90.9	2.7	28±4	4534±715	31±6	2009±222

Monocyte fractions separated by CCE were cultured in medium with or without 100 U/ml of GM-CSF. ^{3}H-TdR incorporation was determined after incubation for 5 days.

Table VI. LAK Activity against GM-CSF-treated Monocytes

No. of exp.	Cytotoxicity (LU/10^6 cells) against:			
	Monocyte fraction			Daudi cells
	I	II	III	
1	0.1	61.3	93.2	2709.2
2	0.1	56.7	52.4	491.5
3	41.4	141.9	197.9	2206.8

Monocyte fractions separated by CCE were incubated for 4 days in medium with 100 U/ml of GM-CSF and then used as targets of LAK cells induced by incubation of blood MNC with IL-2 (1 U/ml) for 4 days.

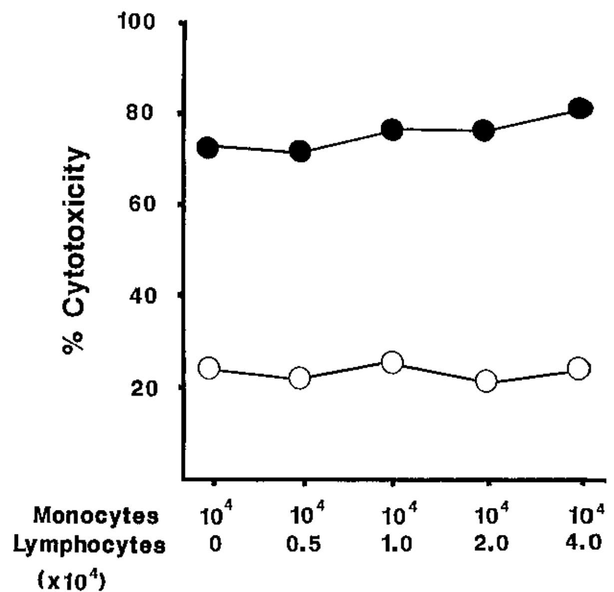

Fig. 2. Absence of effect of coexistence of lymphocytes on monocyte killing by LAK cells. Blood MNC were incubated for 5 days in medium with 1 U/ml of IL-2 before assay of LAK activity at an E/T ratio of 20:1 against 1×10^4 Daudi cells (●) or 1×10^4 monocytes (○) with the indicated numbers of lymphocytes that had been incubated for 5 days in medium with 100 U/ml of GM-CSF. Percentage LAK activity was determined as described in "Materials and Methods." Values are means for triplicate cultures. Data are representative of two separate experiments with similar results. The SD was consistently $<10\%$ of the mean.

significantly more susceptible to the cytotoxicity of autologous LAK cells than untreated monocytes. AM were slightly susceptible to LAK cells, but pretreatment with GM-CSF did not affect their susceptibility. Again, LAK cells were not significantly cytotoxic to lymphocytes that had been incubated for 4 days in medium with or without 100 U/ml of GM-CSF (data not shown).

Next, we examined the dose-dependence of the effect of IL-2 on the killing of monocytes by LAK cells. Blood MNC were incubated for 4 days in medium with various concentrations of IL-2, and then tested for LAK activity against monocytes that had been incubated for 4 days with or without 100 U/ml of GM-CSF. As shown in Fig. 1, blood MNC treated with IL-2 at concentrations of more than 0.1 U/ml were slightly cytotoxic to monocytes that had been incubated in medium alone, but highly cytotoxic to monocytes that had been incubated with GM-CSF.

The effect of the period of monocyte treatment with GM-CSF on their susceptibility to LAK cells was also examined. Blood monocytes were first incubated in suspension for up to 192 h in medium with or without GM-CSF (100 U/ml), and then their viable cell numbers were determined. The recoveries of viable monocytes cultured in medium without and with GM-CSF was similar after 24 h ($>90\%$), but were 42% and 70%, respectively, after 96 h. These remaining monocytes were then used as target cells for autologous LAK cells induced by cultivation of blood MNC for 4–5 days in medium with IL-2. LAK activities were measured at various E/T ratios, and the results are given in Table IV. Treatment of blood monocytes with GM-CSF for 96 h or more resulted in significantly greater susceptibility to LAK cell-mediated cytotoxicity than that of untreated monocytes.

Killing by LAK cells of monocytes that have responded to GM-CSF We recently found that blood monocytes were heterogenous with respect to their response to GM-CSF (submitted for publication). Therefore, we examined whether LAK cells killed blood monocytes capable of responding to GM-CSF. Blood monocytes were separated into subpopulations of three sizes by CCE and the abilities of these subpopulations to proliferate and form colonies in response to GM-CSF were measured. As shown in Table V, monocytes in Fr. II (intermediate size) and Fr. III (large size) responded to GM-CSF more than those in Fr. I (small size). We examined the susceptibilities of these monocyte subpopulations treated with GM-CSF to LAK cells. Monocytes in Frs. II and III were more susceptible than those in Fr. I (Table VI). In a parallel experiment, we examined whether the coexistence of lymphocytes with monocytes influenced the killing of monocytes by LAK cells. Monocytes in Fr. III were incubated for 5 days in medium with 100 U/ml of GM-CSF, and then mixed at various ratios with lymphocytes that had been separately incubated in medium with 100 U/ml of GM-CSF. These monocytes were used as targets of LAK cells that had been induced by 5-day incubation with IL-2 (1 U/ml). As shown in Fig. 2, killing of GM-CSF-treated monocytes by LAK cells was not affected by the presence of lymphocytes.

DISCUSSION

The present study demonstrated that LAK cells were cytotoxic to fresh autologous AM but not monocytes obtained from healthy donors, and that the susceptibility of blood monocytes, but not the AM, to LAK cells was induced by treatment with GM-CSF. We also showed that the cytokines IL-1, IL-3, TNFα and M-CSF had various augmenting effects on killing of monocytes by LAK cells.

Recently, blood monocytes separated as adherent cells from fresh blood were found to be susceptible to LAK cells.[23, 24] It is of interest to know whether circulating blood monocytes are susceptible to LAK-mediated killing, because monocyte-macrophages and monokines are important in the production of IL-2 and IL-2 receptors on T cells and NK cells[28, 29] and in up-regulation of expression of IL-2-induced LAK activity.[18, 19] In the present study we showed that this was not the case because human blood monocytes from healthy donors freshly isolated by CCE were not killed by autologous LAK cells. There are several possible explanations for this finding: many factors, such as variabilities of blood donors and serum, the use of medium contaminated by small amounts of endotoxin and the method of assay, influence the functional properties of isolated monocytes, but the method of collecting blood monocytes seems to have the greatest influence on their functional state. For example, adherence to plastic surfaces or mechanical scraping of adherent monocytes from a surface might damage them, and so make them susceptible to LAK-mediated cytotoxicity. Moreover, activated monocytes seem to be more susceptible than unstimulated monocytes to the cytotoxicity of LAK cells for the following reasons: 1) adherence is known to trigger activation of monocytes.[30] 2) after treatment with LPS for 16 h, monocytes were significantly susceptible to LAK cells (data not shown). 3) Human AM in the so called "stimulated and/or activated state,"[27, 31] showed variable susceptibility to LAK cells (Table I). In contrast, we[32] and others[33] found that fresh human monocytes isolated by CCE are not functionally activated. The present results again clearly showed that functionally intact, freshly isolated blood monocytes were not affected by LAK cells.

In the present study we found that of various cytokines tested, monokines (IL-1 and TNFα) and CSFs (IL-3, M-CSF and GM-CSF) increased the susceptibility of blood monocytes to LAK cells (Table II). The mechanism of this effect of these cytokines is unknown. One possibility is that the increase in susceptibility of monocytes is due to their activation by these cytokines. Indeed, monokines (IL-1 and TNFα) and CSFs are known to activate macrophages.[4, 5, 10, 34, 35] Another possibility is that the increased susceptibility of IL-1β- or TNFα-treated monocytes is due to their production of CSF, because these monokines are known to stimulate monocyte-macrophages to produce CSFs.[5] Of the CSFs tested, GM-CSF caused the greatest increase in susceptibility of monocytes to LAK-mediated killing. Interestingly, it did not affect the susceptibility of AM, suggesting a difference in the responses of monocytes and AM to GM-CSF.

In the present study, we examined whether all or only some of the blood monocytes became susceptible to LAK cells on treatment with GM-CSF, because blood monocytes are known to show heterogeneous metabolic, biochemical and physiological properties.[36-38] Using CCE, we separated blood monocytes by size into subfractions of large, medium and small size and found that the proliferation of the two fractions of larger cells in response to GM-CSF was greater than that of the smallest monocytes. Under these experimental conditions, there was a close correlation between the proliferative responses of these monocyte fractions to GM-CSF and their susceptibilities to the cytotoxicity of LAK cells, suggesting that monocytes that have responded to GM-CSF may be the cells that are susceptible to LAK-mediated killing.

It is interesting to consider the relationship between induction and maintenance of LAK activity and mono-

cyte-macrophage killing by LAK cells. LAK cells were found to be highly cytotoxic to blood monocytes cultured for 4 days with GM-CSF, but not to be cytotoxic to monocytes that had been cultured for less than 24 h in medium with or without GM-CSF (Table IV). We found previously that blood monocytes up-regulated induction of LAK activity by IL-2 from T cells and a subpopulation of NK cells,[18] and that on incubation of lymphocytes with IL-2 the monocyte dependency of LAK induction was maximal within 24 h.[18, 19] Lymphocytes incubated with IL-2 were also found to show maximal LAK activity on days 4–6, and the activity subsequently declined.[11] CSF-like mediators were previously found to be produced by activated T lymphocytes.[4] These findings suggest that generation of LAK activity during immune responses may explain the suppression of monocyte-mediated up-regulation of LAK induction: first enhancement of expression of LAK activity by monocytes and then its decrease resulting from killing of monocytes treated with cytokines such as CSF.

There is encouraging evidence that local and/or systemic administration of LAK cells and IL-2 results in regression of cancer in patients in the terminal state.[39, 40] GM-CSF is expected to increase the number and function of granulocytes and macrophages, but the present and previous[18, 19] findings indicate that caution is required when GM-CSF is used in combination with IL-2 for treatment of malignant diseases.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and a grant from the Foundation for Promotion of Cancer Research of Japan.

(Received April 12, 1989/Accepted May 26, 1989)

REFERENCES

1) Unanue, E. R. The regulatory role of macrophages in antigenic stimulation. Part two. Symbiotic relationship between lymphocytes and macrophages. *Adv. Immunol.*, **31**, 1–15 (1981).
2) Fidler, I. J. Macrophages and metastasis: a biological approach to cancer therapy. Presidential address. *Cancer Res.*, **45**, 4714–4728 (1985).
3) Sone, S. Role of alveolar macrophages in pulmonary neoplasias. *Biochim. Biophys. Acta*, **823**, 227–245 (1986).
4) Metcalf, D. The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. *Blood*, **67**, 257–267 (1986).
5) Sieff, C. A. Hematopoietic growth factors. *J. Clin. Invest.*, **79**, 1549–1557 (1987).
6) Chen, B. D-M., Clark, C. R. and Chou, T. Granulocyte/macrophage colony-stimulating factor stimulates monocyte and tissue macrophage proliferation and enhances their responsiveness to macrophage colony-stimulating factor. *Blood*, **71**, 997–1002 (1988).
7) Reed, S. G., Nathan, C. F., Phil, D. A., Rodricks, P., Shanebeck, K., Conlon, P. J. and Grabstein, K. H. Recombinant granulocyte/macrophage colony stimulating factor activates the macrophages to inhibit *Trypanosoma cruzi* and release hydrogen peroxide. Comparison with interferon gamma. *J. Exp. Med.*, **166**, 1734–1746 (1987).
8) Coleman, D. L., Chodakewitz, J. A., Bartiss, A. H. and Mellors, J. W. Granulocyte-macrophage colony-stimulating factor enhances selective effector functions of tissue derived macrophages. *Blood*, **72**, 573–578 (1988).
9) Falk, L. A., Wahl, L. M. and Vogel, S. N. Analysis of Ia antigen expression in macrophages derived from bone marrow cells cultured with granulocyte-macrophage colony stimulating factor. *J. Immunol.*, **140**, 2652–2660 (1988).
10) Grabstein, K. H., Urdal, D. L., Tushinski, R. J., Mochizuki, D. Y., Prince, V. L., Cantrell, M. A., Gillis, S. and Conlon, P. J. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. *Science*, **232**, 506–508 (1986).
11) Kurland, J. I., Pelus, L. M., Ralph, R., Bockman, R. S. and Moore, M. A. Induction of prostaglandin E synthesis in normal and neoplastic macrophages: role of colony-stimulating factor(s) distinct from effects on myeloid progenitor cell proliferation. *Proc. Natl. Acad. Sci. USA*, **76**, 2326–2330 (1979).
12) Cannistra, S. A., Vallenga, E., Groshek, P., Rambaldi, A. and Griffin, J. D. Human granulocyte-monocyte colony stimulating factor and interleukin 3 stimulate monocyte cytotoxicity through a tumor necrosis factor-dependent mechanism. *Blood*, **71**, 672–676 (1988).
13) Warren, M. K. and Ralph, P. Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity. *J. Immunol.*, **137**, 2281–2285 (1986).
14) 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).
15) Lotze, M. T., Grimm, E. A., Mazumder, A., Strausser, J. L. and Rosenberg, S. A. Lysis of fresh and cultured autologous tumor by lymphocytes cultured in T-cell growth factor. *Cancer Res.*, **41**, 4420–4425 (1981).

16) Hersey, S., Bindon, D., Edwards, A., Murray, E., Phillips, G. and McCarthy, W. H. Induction of cytotoxic activity in human lymphocytes against autologous and allogeneic melanoma cells *in vitro* by cultures with interleukin-2. *Int. J. Cancer,* **28,** 695–703 (1981).
17) Itoh, K., Tilden, A. B. and Balch, C. M. Role of interleukin 2 and a serum suppressive factor on the induction of activated killer cell cytotoxic for autologous human melanoma cells. *Cancer Res.,* **45,** 3173–3178 (1985).
18) Sone, S., Utsugi, T., Nii, A. and Ogura, T. Effects of human alveolar macrophages on the induction of lymphokine (IL-2)-activated killer cells. *J. Immunol.,* **139,** 29–34 (1987).
19) Nii, A., Sone, S., Utsugi, T. and Ogura, T. Up- and down-regulation of human lymphokine (IL-2)-activated killer cell induction by monocytes depending on their functional state. *Int. J. Cancer,* **41,** 33–40 (1988).
20) Sone, S., Inamura, N., Nii, A. and Ogura, T. Heterogeneity of human lymphokine (IL-2)-activated killer cell precursors and regulation of their LAK induction by blood monocytes. *Int. J. Cancer,* **42,** 428–434 (1988).
21) Fujimori, Y., Hara, H. and Nagai, K. Effect of lymphokine-activated killer cell fraction on the development of human hematopoietic progenitor cells. *Cancer Res.,* **48,** 534–538 (1987).
22) Nagler, A., Greenberg, P. L., Lanier, L. L. and Phillips, J. H. The effects of recombinant interleukin 2-activated natural killer cells on autologous peripheral blood hematopoietic progenitors. *J. Exp. Med.,* **168,** 47–54 (1988).
23) Djeu, J. Y. and Blanchard, D. K. Lysis of human monocytes by lymphokine-activated killer cells. *Cell. Immunol.,* **111,** 55–65 (1988).
24) Djeu, J. Y. and Blanchard, D. K. Interferon-gamma-induced alterations of monocyte susceptibility to lysis by autologous lymphokine-activated killer (LAK) cells. *Int. J. Cancer,* **42,** 449–454 (1988).
25) Sone, S., Mutsuura, S., Ogawara, M. and Tsubura, E. Potentiating effect of muramyl dipeptide and its lipophilic analog encapsulated in liposomes on tumor cell killing by human monocytes. *J. Immunol.,* **132,** 2105–2110 (1984).
26) Nishida, T., Nishino, N., Takano, M., Kawai, K., Bando, K., Masui, Y., Nakai, S. and Hirai, Y. cDNA cloning of IL-1α and IL-1β from mRNA of U937 cell line. *Biochem. Biophys. Res. Commun.,* **143,** 345–352 (1987).
27) Sone, S. and Tsubura, E. Human alveolar macrophages: potentiation of their tumoricidal activity by liposome-encapsulated muramyl dipeptide. *J. Immunol.,* **129,** 1313–1317 (1982).
28) Wakasugi, H., Bertoglio, J., Tursz, T. and Fradelizi, D. IL-2 receptor induction on human T lymphocytes: role for IL-2 and monocytes. *J. Immunol.,* **135,** 321–327 (1985).
29) Shirakawa, F., Tanaka, Y., Eto, S., Suzuki, H., Yodoi, J. and Yamashita, Y. Effect of interleukin 1 on the expression of interleukin 2 receptor (Tac antigen) on human natural killer cells and natural killer-like cells (YT cells). *J. Immunol.,* **137,** 551–556 (1986).
30) Haskill, S., Johnson, C., Eierman, D., Becker, S. and Warren, K. Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. *J. Immunol.,* **140,** 1690–1694 (1988).
31) Sone, S., Moriguchi, S., Shimizu, E., Ogushi, F. and Tsubura, E. *In vitro* generation of tumoricidal properties in human alveolar macrophages following interaction with endotoxin. *Cancer Res.,* **42,** 2227–2231 (1982).
32) Utsugi, T. and Sone, S. Comparative analysis of the priming effects of human interferon-γ, α and β on synergism with muramyl dipeptide analog for anti-tumor expression of human blood monocytes. *J. Immunol.,* **136,** 1117–1122 (1986).
33) Kleinerman, E. S. and Herberman, R. B. Tumoricidal activity of human monocytes: evidence for cytolytic function distinct from that of NK cells. *J. Immunol.,* **133,** 4–8 (1984).
34) Onozaki, K., Matsushima, K., Kleinerman, E. S., Saito, T. and Oppenheim, J. J. Role of interleukin 1 in promoting human monocyte-mediated tumor cytotoxicity. *J. Immunol.,* **135,** 314–320 (1985).
35) Mace, K. F., Ehrke, M. J., Hori, K., Maccubbin, D. L. and Mihich, E. Role of tumor necrosis factor in macrophage activation and tumoricidal activity. *Cancer Res.,* **48,** 5427–5432 (1988).
36) Esa, A. H., Donnenberg, A. D. and Hess, A. D. Immunological heterogeneity of human monocyte subsets prepared by counterflow centrifugal elutriation. *Immunology,* **59,** 95–99 (1986).
37) MaCarley, D. L., Shah, V. O. and Weiner, R. S. Purified human monocyte subsets as effector cells in antibody dependent cellular cytotoxicity (ADCC). *J. Immunol.,* **131,** 1780–1783 (1983).
38) Khansari, N., Chou, Y. and Fudenberg, H. Human monocyte heterogeneity: interleukin-1 and prostaglandin E_2 production by separate subsets. *Eur. J. Immunol.,* **15,** 48–51 (1985).
39) Rosenberg, S. A., Lotze, M. T., Muul, L. M., Chang, A. E., Avis, F. P., Leitman, S., Linehan, W. M., Robertson, C. N., Lee, R. E., Rubin, J. T., Seipp, C. A., Simpson, C. G. and White, D. E. A progress report of the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose of interleukin 2 alone. *N. Engl. J. Med.,* **316,** 889–897 (1987).
40) West, W. H., Tauer, K. W., Yannelli, J. R., Marshall, G. D., Orr, D. W., Thurman, G. B. and Oldham, R. K. Constant infusion of recombinant interleukin-2 in adoptive immunotherapy of advanced cancer. *N. Engl. J. Med.,* **316,** 898–905 (1987).