Jpn. J. Cancer Res. 80, 59–64, January 1989

Lysis of Human Leukemic Cells by Monocyte-derived Macrophages Activated with Interferon-γ and Interleukin-2

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Cytolysis of leukemic cells by peripheral blood-derived macrophages was examined by means of an *in vitro* ^{111}In release assay. Monocytes prepared on culture dishes lyse YK-M2. However, when monocytes were cultured *in vitro* and transformed into macrophages, they lost most of their lytic activity. The addition of human recombinant interleukin-2 (rIL-2) on day 5 in culture enhanced the lytic activity significantly. Similarly, treatment of macrophages with human recombinant interferon gamma (rIFN-γ) promoted the lysis of YK-M2 and K-562, although the extent of lysis was smaller than that by rIL-2. Macrophages activated with rIL-2 and rIFN-γ also lysed human leukemic cells. Activated macrophages lysed leukemic cells of acute myelocytic leukemia more than acute lymphocytic leukemia cells. Macrophages derived from the peripheral blood of patients with leukemia were examined for their lytic activity against YK-M2. The patient's macrophages lysed more YK-M2 than did control macrophages when they were activated with rIL-2 and rIFN-γ. The macrophages of two patients also demonstrated autologous leukemic cell lysis.

Key words: Macrophage — Recombinant interleukin-2 — Recombinant interferon-γ — Leukemic cell — Leukemia

Macrophages play an important role in host defense against microbes.[1-3] They have also been demonstrated to exhibit tumoricidal activity *in vitro*[4-6] and antitumor activity *in vivo*.[7,8]

Macrophages must be activated to acquire the capacity to destroy tumor cells. Macrophages acquire competence for antitumor function on treatment with activating agents, such as bacterial lipopolysaccharide (LPS),[9] muramyl dipeptide[10] and interferon gamma (IFN-γ).[6,11]

Recently, interleukin-2 (IL-2) was reported to augment the cytotoxicity of human monocytes against some human tumor cell lines.[12,13] We report here that monocyte-derived human macrophages activated with recombinant interferon-γ (rIFN-γ) or recombinant interleukin-2 (rIL-2) exhibit lytic activity against human leukemic cells, and that macrophages from leukemia patients also lyse human leukemic cells.

MATERIALS AND METHODS

Preparation of macrophages Heparinized venous blood obtained from healthy donors was mixed with an equal volume of 3% dextran in 0.9% sodium chloride and kept for 30 min at room temperature to allow sedimentation of erythrocytes. Leukocyte-rich plasma was collected and centrifuged at 150g for 10 min. Pellets were washed with phosphate-buffered saline and suspended in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo) supplemented with 2 mM glutamine (Nissui) at a concentration of 10^7 cells/ml. The suspension was layered over Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged first at 70g for 10 min and then at 700g for 15 min at 25°C. The mononuclear cell fraction was collected and washed with RPMI 1640, and the differential count was obtained by microscopical observation. The cells were resuspended in RPMI 1640 containing 10% heat-inactivated human AB serum at 2×10^6 monocytes/ml. Unless otherwise noted, 2×10^5 monocytes in 0.1 ml were plated on 96-well flat-bottomed microplates (well diameter 6.4 mm, Corning Glass Works, New York). After incubation for 2 h at 37°C in a 5% CO_2 incubator, plated cells were washed vigorously and 0.1 ml of complete medium was added to the well. Adherent cells were incubated for 24 h in a 5% CO_2 incubator, and washed again vigorously. Then 0.1 ml of complete medium was added. Cells adherent after overnight culture were monocytes as estimated by differential counts of stained cells and by phagocytic capacity.[14] These monocytes changed in appearance into macrophages during the cultivation[14]; more than 99% of adherent cells after day 5 showed vigorous ingestion of candida and strong positivity in non-specific esterase staining. They are referred to as macrophages and were used as effector cells in the cytotoxicity assay. The number of macrophages adherent on day 7 was about 1×10^5 cells per well (counted with a hemocytometer after collection with a rubber police-

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man). Macrophages from patients with leukemia were prepared similarly.

Target cells Leukemic cell lines, YK-M2 established from the peripheral blood of a patient with acute monoblastic leukemia[15] and K-562,[16] were used as target cells. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, 2 m*M* glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Viability of YK-M2 and K-562 was >95% by the dye exclusion method with trypan blue. We used human serum instead of fetal calf serum since we found that the fetal calf serum we used contained a significant amount of LPS when assayed by the limulus colorimetric test (>300 pg/ml).[17] All materials we used in this study contained less than 20 pg/ml LPS. Human leukemic cells were prepared from peripheral blood obtained from patients with leukemia by dextran sedimentation and the Ficoll-Paque technique, and they were cryopreserved until use. All preparations contained >80% leukemic cells. The cryopreserved leukemic cells were recovered before use by thawing rapidly in a water-bath at 37°C. They were used in cytotoxicity assay only when the viability of leukemic cells was >80% (determined by the dye exclusion method).

Human recombinant interferon gamma (rIFN-γ) Originally rIFN-γ was cloned by Genentech, Inc. (South San Francisco) and kindly supplied by Toray Industries, Inc. and Daiichi Seiyaku Co., Ltd. (Tokyo). The specific activity of rIFN-γ was approximately 4×10^7 U/mg protein based on antiviral activity assessed by measuring inhibition of sindbis virus replication in FL cells (human amnion cell line), corrected to the reference IFN-γ standard Gg 23-901-530 of the National Institute of Health (NIH; Bethesda, MD).[18]

Human recombinant interleukin 2 (rIL-2) Purified rIL-2 was produced by the recombinant DNA technology and kindly supplied by Takeda Chemical Industries (Osaka).[19] The *in vitro* biological activity of rIL-2, as measured in terms of the ability to maintain NKC3 cells, was $3.5\pm0.5\times10^4$ U/mg of protein. It corresponded to 1.2×10^7 U/mg, calculated with reference to the Biological Response Modifiers Program reagent human IL-2 (Jurkat).[20]

Treatments of macrophages Adherent monocytes were cultured in a 5% CO_2 incubator at 37°C and rIFN-γ or rIL-2 was added on day 5. After 48 h of co-culture at 37°C in a 5% CO_2 incubator, monocyte-derived macrophages were used as effector cells.

Cytotoxicity assay Cytotoxicity of macrophages was evaluated by an ^{111}In oxine release assay as described previously by Wiltrout *et al.*[21] [^{111}In]chloride was obtained from Nihon Medi-Physics, Inc. (Tokyo) and 8-hydroxyquinoline (oxine) was obtained from Sigma Chemical Co. (St. Louis, MO).

Labeling of 1×10^7 tumor cells was performed by incubation for 15 min with 50 μCi of ^{111}In oxine at room temperature in 0.5 ml of RPMI 1640 with 10% human AB serum. The cells were then washed 3 times in 15 ml of medium. Labeled targets (5×10^3) were added to wells in which 2×10^5 monocytes had been plated. The total reaction volume was 0.2 ml and the plates were incubated for 24 h with YK-M2 or K-562 and for 12 h with human leukemic cells. After incubation, the microplates were centrifuged for 5 min at 250*g* and a 0.05 ml aliquot of supernatant was removed from each well for counting of radioactivity by an automatic gamma spectrometer (ANSR, Abbott Laboratories). The percent specific lysis for each target was calculated as follows:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}\times100\%$$

where the maximum release was that obtained from target cells exposed to 1% sodium dodecyl sulfate (Sigma). With all the targets tested, the spontaneous release of YK-M2 and K-562 was less than 10% of the maximum release and that of human leukemic cells were less than 25% of the maximum release. As a reference, the spontaneous release of YK-M2 and K-562 labeled with ^{51}Cr was 15–20%.

RESULTS

Lysis of human leukemic cell lines by normal macrophages Lytic activity of monocyte-derived macrophages against human leukemic cell line, YK-M2, was measured

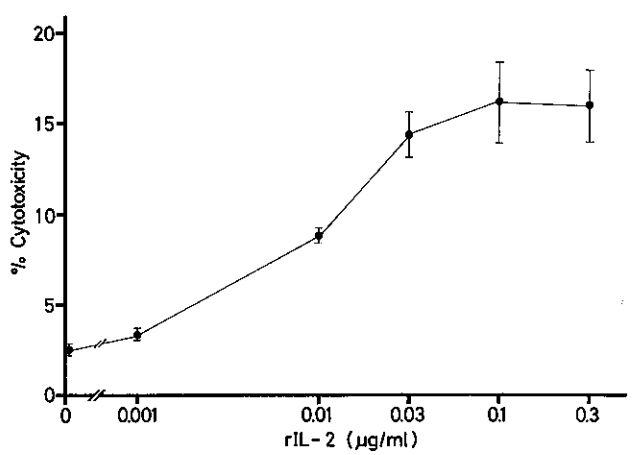

Fig. 1. Effect of rIL-2 on macrophage cytotoxicity against YK-M2 cells. Macrophages were cultured in the presence of rIL-2 at different concentrations for 48 h. Then YK-M2 targets were added. The cytotoxic activity of macrophages was determined at E:T=20:1. The results represent the means±SEM for three separate experiments.

during culture of macrophages *in vitro*. Adherent monocytes on day 0 exhibited significant lysis of YK-M2 (11.7 ±3.4%, n=7). After day 3 their lytic activity decreased markedly, and the extents of lysis were 2.3% and 2.6± 0.5% on day 3 and day 7, respectively. Addition of rIL-2 to the culture on day 5 significantly increased the lytic activity of macrophages on day 7. Figure 1 shows the effect of rIL-2 at different concentrations on macrophage lytic activity against YK-M2. At concentrations higher than 0.001 μg/ml, rIL-2 enhanced the lytic activity in macrophages, and the maximum effect of rIL-2 was obtained at 0.1 μg/ml. Figure 2 shows the influence of different numbers of macrophages as effector cells on the lysis of 5×10^3 YK-M2 cells. The maximum activity was obtained at effector-to-target cell ratios higher than 20:1. The effect of rIFN-γ was also examined in a similar way, and the addition of rIFN-γ on day 5 enhanced the activity of macrophages to lyse YK-M2 cells although the extent of augmentation was smaller than that by rIL-2 (Table I). Similar results were obtained with K-562 as the target except that K-562 was more sensitive to the lysis by macrophages (Table I).

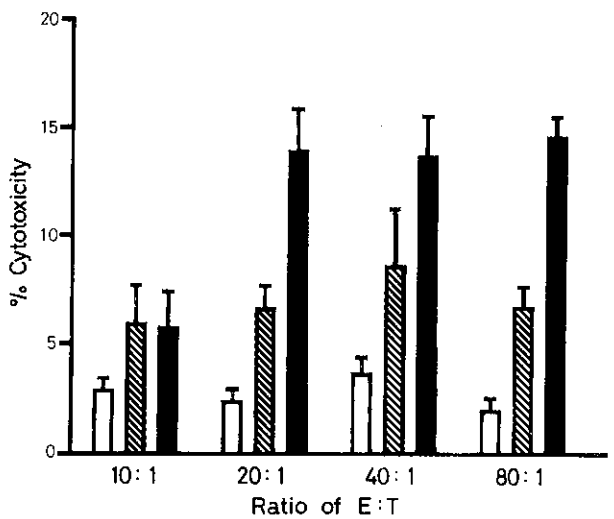

Fig. 2. Effect of macrophage number on the cytolysis of 5×10^3 YK-M2. Macrophage cytotoxicity was determined at different E:T ratios in the presence of 100 U/ml rIFN-γ or 0.1 μg/ml rIL-2. The results represent the means±SEM for four separate experiments. □, Medium; ▨, rIFN-γ; ■, rIL-2.

Table I. Lysis of Leukemic Cell Lines by Macrophages Prepared from Healthy Donors

Target cells	Treatment of macrophages[a]		
	Medium	rIFN-γ	rIL-2
	% Cytotoxicity[b]		
K-562	3.4±1.4 (8)	8.9±2.0 (8)*	28.1±6.5 (8)*
YK-M2	2.6±0.5 (19)	6.5±1.2 (19)*	16.8±2.2 (19)*

a) Macrophages were cultured in the absence or presence of rIFN-γ (100 U/ml) or rIL-2 (0.1 μg/ml) for 2 days.
b) The cytotoxic activity of the macrophages was determined at E:T=20:1. Results are expressed as the mean±SEM. The number of experiments is given in parentheses.
* The difference in the treatment of macrophages (medium only vs. rIFN-γ or rIL-2) was significant ($P<0.01$).

Table II. Lysis of Human Leukemic Cells by Macrophages Prepared from Healthy Donors

Source of leukemic cells		Treatment of macrophages[a]		
		Medium	rIFN-γ	rIL-2
		% Cytotoxicity[b]		
HT (52F)	AML	−0.1	0.8	4.0
HK (73F)	AML	1.5	9.1	11.6
KI (48F)	AML	1.7±0.2(6)[c]	4.7±0.8(6)	14.0±2.5(6)
KM (62M)	AML	1.0	4.7	7.1
TF (34M)	AML	3.5	2.7	6.7
TN (18F)	AML	1.6	4.7	3.9
SK (27M)	ALL	2.1±0.6(4)	1.1±0.4(4)	4.9±1.1(4)
KK (53M)	ALL	0.2	−0.6	−0.1
YO (38F)	ALL	0.2	0.8	2.9
MS (63M)	ALL	−2.1	−2.6	5.6
SG (39M)	ALL	0.5	−2.0	0.6
EK (44F)	ALL	2.9	−0.9	5.5

a) Macrophages were cultured in the absence or presence of rIFN-γ (100 U/ml) or rIL-2 (0.1 μg/ml) for 2 days.
b) The cytotoxic activity of macrophages was determined at E:T=20:1.
c) Mean±SEM (n).

Table III. Lysis of YK-M2 by Macrophages Prepared from Patients with Leukemia

Source of macrophages			Treatment of macrophages[a]		
			Medium	rIFN-γ	rIL-2
			% Cytotoxicity[b]		
1	KK (64M)	AML	1.4 (1.0)[c]	4.3 (3.9)	29.2 (13.2)
1′	KK (64M)	AML	0.0 (0.6)	2.0 (0.1)	26.4 (9.0)
2	TY (18M)	APL	0.9 (1.0)	3.3 (1.1)	28.2 (4.4)
3	SK (27M)	ALL	5.6 (5.4)	11.5 (7.7)	10.4 (26.7)
3′	SK (27M)	ALL	3.5 (0.0)	5.5 (6.5)	14.7 (15.8)
4	EH (46F)	AML	0.3 (0.3)	4.9 (4.4)	29.1 (5.2)
5	YY (39F)	ALL	4.1 (3.5)	6.4 (7.3)	29.2 (10.8)
6	KI (48F)	AML	2.2 (2.0)	7.2 (5.6)	20.2 (14.1)
6′	KI (48F)	AML	9.2 (0.0)	18.0 (0.7)	32.3 (10.1)
7	SU (66F)	AML	0.4 (0.0)	4.0 (6.5)	6.6 (15.8)

a) Macrophages were prepared from 7 patients with leukemia in complete hematological remission and from normal controls. Macrophages were cultured in the absence or presence of rIFN-γ (100 U/ml) or rIL-2 (0.1 μg/ml) for 2 days.
b) The cytotoxic activity of macrophages was determined at E:T=20:1.
c) Values in parenthesis indicate the lysis of YK-M2 by macrophages from healthy donors assayed at the same time.

Table IV. Lysis of Autologous Leukemic Cells by Macrophages

Source of macrophages and leukemic cells			Treatment of macrophages[a]		
			Medium	rIFN-γ	rIL-2
				% Cytotoxicity[b]	
SK (27M)	ALL		0.1	0.5	13.9
KI (48F)	AML		2.4±0.7 (4)[c]	5.4±1.0 (4)	12.6±1.4 (4)

a) Leukemic cells were obtained from two patients before they received chemotherapy, and were stored in liquid nitrogen. Macrophages were prepared from these patients after they achieved complete hematological remission. Macrophages were cultured in the absence or presence of rIFN-γ (100 U/ml) or rIL-2 (0.1 μg/ml) for 2 days.
b) The cytotoxic activity of macrophages was determined at E:T=20:1.
c) Mean±SEM(n).

Susceptibility of leukemic cells obtained from leukemia patients to normal macrophages As shown in Table II, macrophages incubated with medium only showed minimum lytic activity. Macrophages activated with rIL-2 exhibited enhanced cytolysis against human leukemic cells, and the augmentation by rIL-2 was greater than that by rIFN-γ against leukemic cells. Although the lysis of leukemic cells by rIL-2-activated macrophages was significant, the extent was far smaller than that of macrophage cytolysis of YK-M2 and K-562 (Tables I and II).

The susceptibility of leukemic cells to macrophage cytotoxicity seemed to be different depending on the type of leukemic cells. Recombinant IFN-γ-activated macrophages lysed acute lymphocytic leukemia (ALL) cells at $-0.7\pm0.6\%$ and acute myelocytic leukemia (AML) cells at $4.4\pm1.1\%$ (Table II). The lytic activity of rIL-2-activated macrophages against AML cells was stronger ($3.2\pm1.0\%$ of ALL cells and $7.9\pm1.7\%$ AML cells).

Lysis of YK-M2 by patient's macrophages In leukemia patients treated successfully and in complete remission, monocytosis is usually observed at the recovery phase after intensive chemotherapy.[22] We obtained monocytes from leukemia patients at this phase, and examined their cytolytic activity against YK-M2. In all cases, macrophages prepared from leukemia patients showed lytic activity against YK-M2 when macrophages were activated with rIFN-γ or rIL-2. As indicated in Table III, rIL-2-activated macrophages lysed three to four times more YK-M2 than did rIFN-γ-treated macrophages. The mean lysis of YK-M2 by patient's macrophages activated with rIL-2 was $22.6\pm2.9\%$, which was about 1.8 times that by normal macrophages activated with rIL-2 assayed at the same time ($P<0.05$).

Autologous leukemic cell lysis Autologous leukemic cell lysis by macrophages was studied in two cases. Leukemic cells had been obtained at the onset of the disease and cryopreserved until the assay. Macrophages were prepared from monocytes obtained during complete remission. As shown in Table IV, both cases showed significant lysis when macrophages were activated with rIL-2.

DISCUSSION

Monocyte-derived macrophages activated with rIL-2 lysed human leukemic cells, but the monocytes cultured *in vitro* had no ability to lyse leukemic cells. We previously reported that cultured monocytes did not kill candida at all after 3 days of culture even though they retained the capacity to ingest the microorganism.[14] The addition of rIL-2 on day 5 in monocyte culture induced significant lysis of human leukemic cells, although the extent was not large. The indium-releasing method used in this study was suitable to quantitate such a small extent of lytic activity of human macrophages against human leukemic cells since spontaneous release was smaller than that by other methods such as ^{51}Cr release assay.[21]

Recently, Malkovsky *et al.*[12] reported that human monocytes incubated with rIL-2 for 24 h exhibited lytic activity against a cell line, T24 targets,[23] and Philip[13] also demonstrated significant lysis of several cell lines by monocytes activated with rIL-2. Our results coincided with their findings in that rIL-2 augmented the lytic activity of tumor cells by monocytes. Interestingly, the lytic activity of rIL-2-activated macrophages differed depending on the target leukemic cells, and the macrophages killed AML cells more effectively than ALL cells. The reason for this difference is unknown.

Several agents are well characterized to be potent in macrophage activation.[24] One of them, IFN-γ, has been reported to enhance tumor cell lysis of monocytes and macrophages.[6, 11] Our findings indicated that rIFN-γ-activated macrophages significantly to lyse leukemic cell lines, YK-M2 and K-562, but the extent of lysis was smaller than that by rIL-2-activated macrophages. As Adams reported,[24] an additional agent such as LPS may be necessary for IFN-γ-activated macrophages to achieve optimal activity to lyse tumor cells.

Macrophages prepared from patients with leukemia also showed significant lysis of leukemic cells. We compared their lytic activity against YK-M2 with that of normal macrophages obtained from healthy donors. When macrophages were activated with rIL-2, their lytic activity was significantly higher than that of normal macrophages activated with rIL-2. Such enhancement may be explained by the fact that the monocytes were obtained from patients at the recovery phase of hematopoiesis after intensive chemotherapy. The mechanism(s) of such functional enhancement of macrophages remains to be identified.

Owing to recent advances in chemotherapy, leukemia can now be brought into remission.[25] However, recurrence is frequently observed in patients in complete remission, which indicates that a small number of leukemic cells remains in the body.[26] Therefore, a new strategy is necessary to expel the remaining leukemic cells and achieve a cure. The use of macrophages that are activated with rIL-2 to exhibit cytotoxicity against human leukemic cells might be one way to destroy the remaining leukemic cells in the patients.

ACKNOWLEDGMENTS

We thank Ms. E. Shibata and Ms. J. Hirai for their technical assistance.

(Received September 17, 1988/Accepted November 24, 1988)

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