Differential Activation of Lymphokine-activated Killer Cells with Different Surface Phenotypes by Cultivation with Recombinant Interleukin 2 or T-cell Growth Factor in Gastric Cancer Patients

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Fourteen days' culture of human peripheral blood lymphocytes (PBL) with recombinant interleukin 2 (rIL 2) or T cell growth factor (TCGF) results in the generation of lymphokine-activated killer (LAK) effector cells which have the unique property of lysing natural killer (NK)-resistant human tumor cells, Daudi, as well as NK-sensitive, K562 cells. LAK cells were generated from both normal and gastric cancer patients' PBL. However, LAK cell activities induced by rIL 2 or TCGF decreased with the progress of the tumor growth. In addition, TCGF-induced LAK cell activities were found to be lower than the rIL 2-induced LAK cell activities. Different mechanisms may be involved in the decreases of the rIL 2-induced and TCGF-induced LAK cell activities. This study further demonstrates that the cell types involved are also heterogeneous, as determined by phenotypic characteristics. The LAK-effector cell type was analyzed by two-color flow cytometry. RIL 2-induced LAK cells showed increased proportions of $CD4^+Leu\ 8^-$ and $Leu\ 7^+CD16^-$, and a decreased proportion of $CD8^+CD11^-$ cells, which are believed to be associated with killer T cell functions. In contrast, TCGF-induced LAK cells revealed significantly increased proportions of $CD8^+CD11^-$ and $CD4^+Leu\ 8^-$ cells, and a decreased proportion of $Leu\ 7^+CD16^-$ cells. Thus, LAK cells with different surface phenotypes were induced by the cultivations with rIL 2 and with TCGF.

Key words: Lymphokine-activated killer cells — Interleukin 2 — Gastric cancer

Incubation of human peripheral blood lymphocytes (PBL) with interleukin 2 (IL 2) induces the expression of potent cytotoxicity against both fresh and cultured tumor targets.[1] The effector cells have been named lymphokine-activated killer (LAK) cells. The LAK cells are functionally defined as any cell responding to IL 2 activation by the development of major histocompatibility complex (MHC)-unrestricted cytotoxicity against a natural killer (NK) cell-resistant tumor target without prior antigen priming.[2] Purified recombinant IL 2 (rIL 2) alone is sufficient for the induction of the tumor-selective oncolytic activity.[1-3] Although little is known concerning the regulation of LAK cells, Itoh et al.[4] reported that sera from cancer patients can suppress LAK development. It has also been demonstrated that LAK generation was suppressed by hydrocortisone[5] and by platelets,[5] some tumor cells[6] and/or their secretory factors, such as suppressor factor[7, 8] or transforming growth factor-beta (TGF-β).[9]

T-cell growth factor (TCGF) derived from supernatants of mitogen-stimulated lymphocytes have been shown to be indispensable to sustain the growth of T-cells with cytotoxic activity.[10, 11] However, we demonstrated that TCGF-dependent PBL or spleen cells from cancer patients or tumor-bearing animals could be serially

grown *in vitro* in large numbers with maintenance of cell-mediated immunosuppressive function.[12, 13] TCGF has been found to contain many kinds of cytokines, such as IL 1, IL 3, IL 4, IL 5, IL 6, interferon-gamma (IFNγ), and tumor necrosis factor (TNF), as well as IL 2. Therefore, the T-cell proliferative response and subsequent differentiation to effector cell function induced by TCGF may involve a complex series of interactions between many different cell types. The differences between rIL 2 and TCGF in the induction of LAK cells should be elucidated.

The nature of LAK-effector cells generated during culture with IL 2 has been extensively investigated.[6, 14-20] However, this is still a matter of debate, since LAK induction has been done by cultivation with different kinds of cytokines and by various methods. In an attempt to overcome the problems associated with distinguishing a diverse effector population with phenotypic markers, we tested whether two-color flow cytometric analysis could be used to identify the LAK-effector cells.

In the present study, we examined the effects of 14 days' cultivation with exogenous rIL 2 or TCGF on the induction of LAK cells with different phenotypes from PBL of gastric cancer patients, as well as those from healthy donors. It was found that rIL 2 was able to generate LAK cells with main surface phenotypes of $CD4^+Leu\ 8^-$ and $Leu\ 7^+CD16^-$. In contrast, TCGF in-

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fluenced mainly $CD8^+CD11^-$ and $CD4^+Leu\ 8^-$ LAK cells to proliferate. The nature of the different cell surface antigens involves in MHC-unrestricted killing is discussed.

MATERIALS AND METHODS

Patients Twenty patients with advanced gastric cancer were studied. Patients were divided into two groups: Group I consisted of ten patients who had resectable carcinoma with or without regional lymph node metastasis and were eligible for radical or nonradical resection; the ten patients in Group II had advanced nonresectable carcinoma with systemic metastasis. Blood samples were obtained from patients before surgery and/or chemotherapy (mitomycin C and/or tegafur). The patients ranged in age from 42 to 79 years (mean 60.5 years). The control group consisted of ten normal healthy persons (laboratory personnel, and hospital and technical staff), ranging in age from 40 to 72 years (mean 57.7 years).

Preparation of peripheral blood lymphocytes and TCGF-containing medium PBL were isolated from venous blood by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala) density gradient centrifugation.[21] As the TCGF source, we used culture supernatant from human spleen cells stimulated with 0.08% phytohemagglutinin (PHA-P, Difco, Detroit, MI). The details concerning TCGF preparation were presented in a previous paper.[12] The preparations were subsequently filtered through 0.45-μm filters (Millipore Corp., Bedford, MA), then stored at $-20°C$ until use.

Cell culture of rIL 2- or TCGF-activated PBL Approximately 2×10^6 PBL were placed in tissue culture flasks (Nunc, No. 163371, Roskilde, Denmark). RPMI-1640 medium supplemented with 10% FCS, 100 μg/ml kanamycin, and 50% TCGF preparations or 100 U/ml rIL 2 (Shionogi Co. Ltd., Osaka) was used to initiate the cultures. The IL 2 activity of 50% TCGF preparations used throughout the experiment was approximately equivalent to the activity of 100 U/ml rIL 2 assessed by the method of Gillis et al.[22] All cultures were maintained for 14 days in 5% CO_2 in air at 37°C and fed three times per week by changing the culture medium.

Monoclonal antibodies (MoAbs) and two-color flow cytometric analysis Fluorescein isothiocyanate (FITC)-conjugated anti Leu 2a (CD8), anti Leu 3a (CD4), anti HLA-DR and anti Leu 7, and phycoerythrin (PE)-conjugated anti Leu 15 (CD11), anti Leu 8, anti Leu 2a (CD8), anti Leu 11 (CD16) and anti IL 2R (CD25) were provided by Becton Dickinson (Mountain View, CA). Freshly isolated PBL or rIL 2- or TCGF-activated PBL were stained with fluorochrome-conjugated MoAb for 30 min at 4°C and were washed twice, as described previously.[12] The cells stained with FITC- and PE-

conjugated antibodies were analyzed for double labeling by flow cytometry (FACS-IV analyzer, Becton Dickinson Co., Mountain View, CA).

Assay for killer cell activities Killer cell activities of cell populations were tested against human NK-sensitive K562 cells[23] and NK-resistant Daudi cells.[24] The cell lines were kindly donated by Dr. H. Mizuno (Japanese Cancer Research Resources Bank, National Institute of Hygienic Science, Tokyo). Four million target tumor cells were incubated with 100 μCi of $Na^{51}CrO_4$ (Japan Atomic Energy Research Institute, Tokai, Ibaraki) for 90 min at 37°C with occasional gentle shaking. The cells were washed three times with Hanks' solution and finally resuspended in RPMI-1640 medium supplemented with 10% FCS and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Wako Chemical Co., Osaka). The rIL 2- or TCGF-induced cytolytic effect of cultured lymphoid cells on tumor targets was examined using a standard 4-h ^{51}Cr-release assay. The labeled target cells (1.5×10^4) and 3 effector cell concentrations which produced target cell: effector cell ratios (T/E ratios) of 1:10, 1:20 and 1:40, or 1:2.5, 1:5 and 1:10 were mixed and distributed to each well in a final volume of 200 μl on round-bottomed microtiter plates (Nunc, No. 163320) in quadruplicate. After incubation, the plates were centrifuged and cytolysis was evaluated by counting 0.1 ml of supernatant in a gamma-counter. Specific lysis was expressed according to the following formula:

$$\%\ \text{specific lysis} = \frac{\begin{array}{c}\text{experimental release}\\ -\ \text{spontaneous release}\end{array}}{\begin{array}{c}\text{maximum release}\\ -\ \text{spontaneous release}\end{array}}\times100.$$

The maximum releasable counts were determined by repeated freezing and thawing of the labeled cells, and amounted to 80–90% of the total radioactivity incorporated into the cells. Spontaneous release determined from target cells incubated in the culture medium was always 5% or less of the maximum release.

RESULTS

Table I shows that PBL obtained from normal healthy controls gave a substantial increase in cytolytic activities against NK-sensitive K562 cells at increased effector cell to target cell ratios. However, PBL from patients with resectable or nonresectable carcinoma showed significantly decreased killer cell activities as compared with those at the corresponding T/E ratios from normal individuals ($P<0.01$). Freshly prepared PBL from normal individuals and gastric cancer patients did not express significant levels of cytotoxicity against NK-resistant Daudi cells. Human PBL cultured with rIL 2 or TCGF for 14 days were further assayed for cytotoxicity against the K562 and Daudi cells. Table II summarizes

Table I. Killer Cell Activities of PBL

Subjects[a]	Target	Specific ^{51}Cr release (%)		
		1:10[b]	1:20[b]	1:40[b]
Normal	K562	22.6±1.5[c]	30.6±1.3	35.3±1.4
	Daudi	1.4±0.2	2.6±0.4	3.9±0.5
Resectable cancer	K562	10.3±1.1	17.0±1.7	24.6±2.5
	Daudi	0.8±0.1	2.0±0.2	3.6±0.4
Nonresectable cancer	K562	8.4±1.7	12.7±2.5	17.3±3.1
	Daudi	0.4±0.1	1.0±0.2	1.8±0.5

a) Each experimental group consisted of ten patients.
b) Target cell : effector cell ratio.
c) Mean±SE of ten cases.

Table II. Killer Cell Activities of Cells Cultured with rIL2

Subjects[a]	Target	Specific ^{51}Cr release (%)		
		1:2.5[b]	1:5[b]	1:10[b]
Normal	K562	30.2±2.6[c]	39.2±1.2	43.0±0.8
	Daudi	28.2±2.2	36.3±1.9	40.0±1.2
Resectable cancer	K562	28.1±2.5	33.4±3.5	39.1±1.6
	Daudi	24.0±2.9	31.1±2.3	36.7±1.9
Nonresectable cancer	K562	17.5±2.2	26.5±2.7	33.9±2.2
	Daudi	8.1±1.2	13.0±1.5	18.9±1.6

a) Each experimental group consisted of ten patients.
b) Target cell : effector cell ratio.
c) Mean±SE of ten cases.

Table III. Killer Cell Activities of Cells Cultured with TCGF

Subjects[a]	Target	Specific ^{51}Cr release (%)		
		1:10[b]	1:20[b]	1:40[b]
Normal	K562	24.6±1.8[c]	31.2±1.1	35.9±0.9
	Daudi	9.5±1.8	15.3±1.9	20.9±2.1
Resectable cancer	K562	20.1±2.6	26.6±2.5	30.4±2.3
	Daudi	9.4±2.2	14.1±2.6	19.1±2.9
Nonresectable cancer	K562	18.7±2.1	25.2±1.8	29.8±1.5
	Daudi	5.3±1.7	7.6±2.2	10.6±2.8

a) Each experimental group consisted of ten patients.
b) Target cell : effector cell ratio.
c) Mean±SE of ten cases.

the mean values of the killer cell activities of rIL 2-activated PBL in the three experimental groups. The rIL 2-activated PBL from normal controls and patients with resectable carcinoma showed significantly higher lytic activities against K562 cells than those at the corresponding T/E ratios in PBL described in Table I ($P<0.001$). On the other hand, killer (LAK) cell activities against Daudi cells were observed even at lower T/E ratios as compared with that of PBL in Table I. However, the

LAK cell activities against Daudi cells in rIL 2-activated PBL from patients with nonresectable carcinoma were significantly decreased as compared with those at the corresponding T/E ratios in patients with resectable carcinoma and normal healthy controls ($P<0.001$).

Table III summarizes the mean values of killer cell activities of TCGF-activated PBL from the three experimental groups. Killer cell activities against K562 cells were observed with cells cultivated with TCGF in each
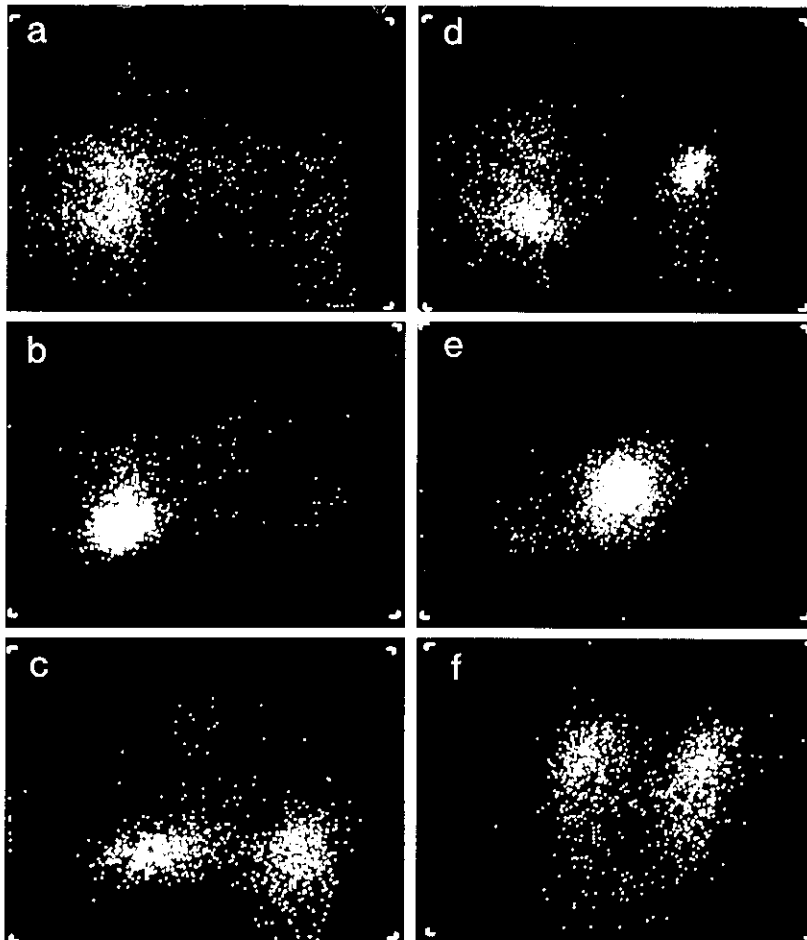
Fig. 1.   Representative FACS profiles of PBL (panels a and d), rIL-activated PBL (panels b and e) and TCGF-activated-PBL (panels c and f) from a patient with nonresectable gastric carcinoma analyzed by two-color flow cytometry. The cells were stained with FITC-conjugated CD8 and PE-conjugated CD11 (panels a, b and c), and with FITC-conjugated CD4 and PE-conjugated anti Leu 8 (panels d, e and f). Percent positive cells in each panel were as follows. Panel a: $CD8^+CD11^-$, 9.2%; $CD8^+CD11^+$, 9.8%; $CD8^-CD11^+$, 27.1%. Panel b: $CD8^+CD11^-$, 1.6%; $CD8^+CD11^+$, 1.3%; $CD8^-CD11^+$, 19.3%. Panel c: $CD8^+CD11^-$, 67.6%; $CD8^+CD11^+$, 3.0%; $CD8^-CD11^+$, 0%. Panel d: $CD4^+Leu 8^-$, 6.6%; $CD4^+Leu 8^+$, 25.6%; $CD4^-Leu 8^+$, 15.8%. Panel e: $CD4^+Leu 8^-$, 19.1%; $CD4^+Leu 8^+$, 74.7%; $CD4^-Leu 8^+$, 0.5%. Panel f: $CD4^+Leu 8^-$, 1.9%; $CD4^+Leu 8^+$, 48.4%; $CD4^-Leu 8^+$, 35.0%.

group. However, TCGF-activated PBL in all three groups showed a significant decrease in their ability to lyse the NK-resistant target, Daudi cells. The cells from patients with nonresectable carcinoma showed significantly lower killer cell activities against Daudi cells than those from normal individuals and patients with resectable carcinoma ($P<0.01$). In addition, the cell activities at the T/E ratio of 1:10 were significantly decreased as compared with these of rIL 2-activated PBL as shown in Table II ($P<0.001$).

To investigate the phenotypic characteristics of the killer cell population, the rIL 2- and TCGF-activated

PBL were analyzed by two-color immunofluorescence and flow cytometry. Figure 1 shows representative FACS profiles defined by CD8 and CD11, and CD4 and anti Leu 8 antibodies on PBL, and rIL 2- and TCGF-activated PBL from a patient with nonresectable carcinoma. The profile of rIL 2-activated PBL defined by CD8 and CD11 was indistinguishable from that of freshly isolated PBL from the patient, and differed markedly from that of TCGF-activated PBL (Fig. 1a, b and c). In contrast, the fluorescence profile of surface antigens of rIL 2-activated PBL defined by CD4 and anti Leu 8 was essentially different from those of PBL and TCGF-

Table IV. Two-color Flow Cytometric Analysis of PBL, rIL2-activated PBL and TCGF-activated PBL from Normal Healthy Controls and Gastric Cancer (Resectable and Nonresectable) Patients

	Normal[a]			Cancer (resectable)[a]			Cancer (nonresectable)[a]		
	PBL	rIL2	TCGF	PBL	rIL2	TCGF	PBL	rIL2	TCGF
CD8$^+$CD11$^-$	16.0±0.9[b]	17.9±2.5	58.6±4.8	16.4±1.6	15.5±3.2	48.0±6.5	19.4±1.9	10.2±2.5	51.6±4.3
CD8$^+$CD11$^+$	6.8±0.8	2.3±0.5	1.2±0.4	8.6±1.1	1.5±0.5	1.8±0.7	7.0±1.0	2.7±1.5	1.7±0.7
CD8$^-$CD11$^+$	12.3±1.2	8.0±1.9	0.1±0.1	12.3±1.2	6.3±2.3	0.3±0.2	12.9±1.6	10.9±2.5	0.4±0.3
CD4$^+$Leu 8$^-$	13.4±2.2	51.4±2.1	29.0±3.0	17.8±3.3	56.1±3.8	25.1±4.6	15.6±3.8	46.0±4.3	25.9±5.1
CD4$^+$Leu 8$^+$	31.1±2.5	13.9±1.0	11.6±2.3	21.7±3.5	18.9±1.3	25.0±2.2	25.7±4.6	23.9±2.4	22.2±3.8
CD4$^-$Leu 8$^+$	26.7±2.0	2.0±0.6	11.1±2.9	15.4±3.8	2.8±0.9	13.8±4.4	18.2±4.1	3.4±0.9	16.6±3.1
HLA-DR$^+$CD8$^-$	21.2±0.9	40.4±5.4	20.3±6.8	18.6±1.1	33.1±2.8	22.6±2.0	20.2±1.1	42.2±3.9	14.4±2.5
HLA-DR$^+$CD8$^+$	5.5±0.8	8.2±1.2	30.4±3.9	5.9±0.5	10.5±2.4	41.1±2.5	7.5±1.9	9.7±2.5	45.9±2.6
HLA-DR$^-$CD8$^+$	15.3±1.2	4.9±1.3	19.7±4.5	13.8±1.0	5.1±1.6	8.0±2.0	13.6±1.2	3.6±0.6	11.1±3.7
CD4$^+$HLA-DR$^-$	42.8±1.9	48.2±3.9	26.9±3.4	45.5±3.3	51.6±4.0	20.7±4.5	48.4±2.7	39.5±2.4	15.9±3.8
CD4$^+$HLA-DR$^+$	3.2±0.5	17.6±1.6	13.7±1.2	2.5±0.4	21.4±2.5	29.2±4.9	3.5±0.6	22.4±2.3	26.5±3.8
CD4$^-$HLA-DR$^+$	20.4±2.4	11.5±2.1	19.1±2.3	16.1±1.9	7.6±1.9	24.9±4.8	12.4±0.9	16.2±5.5	31.7±3.5
Leu 7$^+$CD16$^-$	7.7±1.2	17.5±2.3	2.3±0.8	6.6±1.2	11.5±2.2	2.8±0.8	7.1±2.4	10.3±2.3	3.5±1.1
Leu 7$^+$CD16$^+$	9.2±1.8	1.5±0.3	0.3±0.1	8.8±2.1	1.2±0.3	0.6±0.4	4.6±3.0	2.1±0.7	0.3±0.1
Leu 7$^-$CD16$^+$	3.7±1.1	1.6±0.6	0.3±0.2	3.5±0.5	1.7±0.9	0.1±0.1	2.5±1.0	1.7±0.7	0.5±0.3
HLA-DR$^+$CD25$^-$	25.4±1.3	26.0±2.3	34.9±4.8	24.9±1.3	24.2±3.9	29.2±6.1	25.4±1.7	18.7±3.3	29.6±4.8
HLA-DR$^+$CD25$^+$	1.0±0.2	25.3±3.8	18.0±5.6	0.8±0.1	21.9±3.1	36.0±7.1	0.7±0.2	35.5±4.9	39.0±6.4
HLA-DR$^-$CD25$^+$	1.5±0.3	5.7±0.8	4.5±1.5	0.9±0.2	12.2±2.4	5.6±1.4	0.9±0.2	14.6±3.1	6.0±2.1

a) Each experimental group consisted of ten patients.
b) Each value represents the mean percentage of positive cells ± standard error.

activated PBL (Fig. 1d, e and f). Table IV showed the percentages (mean±SE) of two-color fluorescent PBL, rIL 2- and TCGF-activated PBL reacting with MoAbs labeled with distinct fluorochromes in controls and patients with resectable and nonresectable carcinoma. The percentage of CD8$^+$CD11$^-$ cells was the same in PBL cultivated with rIL 2 from normal healthy controls and patients with resectable carcinoma. However, the percentage of CD8$^+$CD11$^-$ cells in rIL 2-activated PBL derived from patients with nonresectable carcinoma was significantly decreased as compared with that of PBL (19.4% ±1.9 vs. 10.2% ±2.5, $P<0.05$). The percentage of CD8$^+$CD11$^-$ cells was significantly increased by culture with TCGF in each experimental group. The percentage of CD8$^+$ cells also co-expressing the CD11$^+$ antigen was dramatically decreased by culture with rIL 2 or TCGF. More significant was the increase of the percentage of CD4$^+$Leu 8$^-$ cells in rIL 2- or TCGF-activated PBL. The percentage of CD4$^+$Leu 8$^+$ cells was significantly decreased in rIL 2- and TCGF-activated PBL from normal controls (31.1% ±2.5 vs. 13.9% ±2.0 and 11.6% ±2.3, $P<0.01$), but the percentages of CD4$^+$Leu 8$^+$ cells in rIL 2- and TCGF-activated PBL from gastric cancer patients were significantly increased

as compared with those of cells from normal healthy controls (18.9% ±1.3 and 23.9% ±2.4 vs. 13.9% ±1.0, $P<0.05$; 25.5% ±2.2 and 22.2% ±4.8 vs. 11.6% ±2.3, $P<0.01$). The results of FACS analysis of PBL, rIL 2- and TCGF-activated PBL from the three experimental groups using an anti HLA-DR and CD8 or DC4 or CD 25 are also shown in Table IV. The most prominent feature is the increased proportion of double-positive cells in rIL 2- and TCGF-activated PBL from cancer patients. The percentage of Leu 7$^+$CD16$^-$ cells in the three experimental groups was increased by cultivation with rIL 2. However, the proportion of Leu 7$^+$CD16$^-$ cells in rIL 2-activated PBL decreased with the progress of tumor growth. The percentage of Leu 7$^+$CD16$^-$ cells in TCGF-activated PBL was significantly decreased as compared with that of PBL ($P<0.01$).

DISCUSSION

In the present study, we compared the functional capability of LAK cells from PBL of patients with gastric carcinoma and normal individuals. The LAK cells were generated by 14 days' cultivation with rIL 2 or TCGF. Under the culture conditions used, the frequency

of T cells that underwent clonal expansion was comparable in gastric cancer and control PBL, and was as high as 82.8% to 100%. We demonstrated that rIL 2 or TCGF alone certainly caused differentiation into LAK cells. The growing LAK cells were cytotoxic to tumor cell lines insusceptible to NK lysis, as well as to NK target cells. However, these LAK cell activities were shown to decrease with the progress of tumor invasion. In addition, the LAK induction in these patients could not be restored to a normal level by culture with high concentrations of rIL 2 or TCGF (data not shown). We have attempted to determine whether decrease or loss of LAK cytotoxicity in the patients is a manifestation of an additional lymphokine requirement for signaling or an altered function of lymphoid cell growth. Shiiba et al.[8] stated that LAK induction by rIL-2 in cancer patients involved the production of human IFNγ. Indeed, we reported previously that exogenous rIFNγ could augment the lytic activity of rIL 2-activated killer cells to a normal level.[25, 26] The results suggested that lower cytotoxicity of rIL 2-activated PBL from these cancer patients might involve the dysfunction or the lack of IL 2 responder cells. However, the depressed TCGF-activated killer cell activities were not restored by additional culture with exogenous rIFNγ (data not shown). Previously, we have shown that suppressor cells are included among the TCGF-activated PBL from cancer patients.[12, 25, 27] The suppressor cells, which were named lymphokine-activated suppressor (LAS) cells,[25, 27] with a $CD8^+CD11^-$ surface phenotype inhibited the effector process of tumor cell lysis by LAK cells which had been activated in vitro by rIL 2. Though $CD8^+CD11^-$ cells are known to be killer T cells,[28] $CD8^+CD11^-$ LAS cells were found to contain a large cell population with $CD8^+CD28^-$ in our laboratory (data not shown). Thus, the depressed LAK activities in cultures with TCGF might be caused by induction of the suppressor cells. It has been reported that the generation of suppressor cells requires cofactors distinct from IL 2.[29] In fact, PBL from cancer patients did not develop the suppressor cell function when cultured with rIL 2 alone (data not shown). Thus, TCGF could be essentially inhibitory for LAK activation in cancer patients; the effect of TCGF on the induction of LAK cells also seems to be dependent upon host positive or negative immune response to the tumor. Therefore, it was suggested that the mechanisms of decrease of LAK cell activity of rIL 2- and TCGF-activated killer cells are very different.

More important, the increased LAK cell activities of rIL 2-activated PBL in gastric cancer patients and normal individuals were related to the unexpectedly elevated percentage of $CD4^+Leu\ 8^-$ cells, known to be helper T cells.[30] The findings of the analysis of the population level suggested that the majority of the rIL 2-

activated killer-effector cells in normal individuals and cancer patients expressed $CD4^+Leu\ 8^-$. Furthermore, the cells with Leu 7^+CD16^-, which is regarded as a marker of NK cells[31] were increased by cultivation with rIL 2 (Table IV). Leu 7^+CD16^- cells may develop LAK activity. We, therefore, assessed the contribution of Leu 7^+CD16^- cells to LAK effector function. Grimm et al.[14] showed that LAK effector cells were insensitive to CD4, minimally sensitive to CD11, partially sensitive to CD3, and extremely sensitive to CD8. Tilden et al.[17] reported that PBL cultured with rIL 2 for 3 days showed non-T cell markers, $CD5^-$, $CD16^+$, as the main antigenic phenotype, with LAK activity. These data are essentially different from our study. Short-term cultivation would not provide an effective approach for phenotypic analysis, since the cells might consist of several subsets of T lymphocytes and monocytes. Ferrini et al.[18] investigated the phenotypic and functional characteristics of human rIL 2-activated killer cells. They separated PBL into $CD3^+$ and $CD3^-$ cell fractions by the method of FACS sorting and cultured them for 14 to 21 days in 100 U/ml rIL 2. They reported that 11 clones with LAK activity maintained the original CD2/CD3 phenotype; three were $CD8^+$, $CD4^-$, and the others lacked both CD8 and CD4 antigen. The percentage of $CD8^+$ cells in our study presented here was not increased by 14 days' cultivation with rIL 2.

On the other hand, Rayner et al.[15] and Maggi et al.[16] reported functional and phenotypic analyses of LAK cells cultivated with supernatants from cultures of PHA-stimulated human PBL or spleen cells (referred to as TCGF in our study). Rayner et al.[15] stated that LAK cell clones obtained from PBL with high and intermediate cytolytic activity were of two types, expressing $CD8^+$ or $CD4^+$. In our study, TCGF resulted in preferential expansion of $CD8^+CD11^-$ and $CD4^+Leu\ 8^-$ cells and in unresponsiveness of $CD8^+CD11^+$ and $CD4^+Leu\ 8^+$ cells in PBL from normal healthy controls and gastric cancer (resectable and nonresectable) patients. Maggi et al.[16] reported that the majority of T cell clones isolated from spleen of untreated patients with Hodgkin's disease expressed $CD4^+Leu\ 8^-$ rather than $CD8^+CD11^-$. Thus, the different antigenic expressions between PBL and spleen cells might be related to the cultivation with TCGF.[32] The studies of Rayner et al.,[15] Maggi et al.[16] and other investigators[33, 34] have revealed that human killer T cells against tumors can express CD4 or CD8. Indeed, if rIL 2- or TCGF-activated PBL were treated with CD8 or CD4 and complement before the cell-mediated cytotoxicity test, cytotoxic activities were clearly noted (data not shown). Though it is usually difficult to discuss the cytotoxicity in relation to the percentages of the subsets of lymphocytes, it seems likely that rIL 2- or TCGF-activated PBL with $CD4^+Leu\ 8^-$ or $CD8^+CD11^-$ sur-

face phenotype exert LAK-effector activity. Our results are substantially in agreement with those of Rayner et al.[15] and Maggi et al.[16]

In addition, the proportions of HLA-DR$^+$CD8$^+$, CD4$^+$HLA-DR$^+$ and HLA-DR$^+$CD25$^+$ cells in rIL 2- and TCGF-activated PBL from cancer patients were increased as compared with those of normal healthy controls. Though the data do not establish whether HLA-DR antigen is necessary for cytotoxic effector activity or whether it is simply a marker of in vitro activation, the results suggest that immune response to tumor cells (antigens) might be enhanced in cancer patients.

In conclusion, cultivation of human PBL with rIL 2 or TCGF results in the generation of lymphoid cells with different surface phenotypes. It seems likely that CD4$^+$Leu 8$^-$, Leu 7$^+$CD16$^-$ and CD8$^+$CD11$^-$ cells from healthy donors and patients might be active as LAK-effector cells, though further examination by cell-sorting techniques remains necessary.

ACKNOWLEDGMENTS

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