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Membrane-associated Lymphotoxin Expression and Functional Analysis of Lymphokine-activated Killer Cells Derived from Tumor-infiltrating Lymphocytes

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The expression of a membrane-associated lymphotoxin molecule (mLT) on lymphokine-activated killer (LAK) cells obtained from 18 patients with malignant tumors and its role in the tumor cell killing mechanisms were investigated. LAK cells from tumor-infiltrating lymphocytes (TIL-LAK cells) were mainly composed of CD3-positive cells, whereas LAK cells from peripheral blood lymphocytes (PBL-LAK cells) were mainly composed of CD16- and CD56-positive cells. However, mLT was found to be expressed on TIL-LAK cells as well as PBL-LAK cells. The degree of mLT expression correlated with the killing activity of LAK cells towards L929 cells ($r=0.806$, $P<0.01$, $n=15$), but not with that towards Daudi or K562 cells. Although the degree of mLT expression correlated with the amount of secreted lymphotoxin (LT) in the supernatant of LAK cell culture, the secreted LT itself could not account for the tumor cell killing activity of LAK cells. Polyclonal rabbit anti-LT antibody partially inhibited the killing activities of LAK cells towards L929 cells and this inhibition was found in the combination of autologous tumor cells and PBL-LAK cells. These findings suggest the possibility that the mLT-related cytotoxicity is involved in the tumor cell killing mechanisms of TIL-LAK cells as well as PBL-LAK cells.

Key words: LAK — TIL — Lymphotoxin — Membrane-associated cytokine

Lymphokine-activated killer (LAK) cells derived from peripheral lymphocytes (PBL-LAK) and tumor-infiltrating lymphocytes (TIL-LAK) were introduced into clinical use by Rosenberg *et al.*[1] Many trials of adoptive immunotherapy using PBL-LAK or TIL-LAK cells have been performed and several clinical cases in which tumors were significantly regressed have been reported.[2–4] However, the clinical results were not necessarily satisfactory.[5–7] Along with clinical trials of LAK cells, studies to clarify the mechanisms of killing and recognition of tumor cells are required. To date, various effector molecules, including perforin, cytokines and serine esterase, have been suggested to be involved in tumor cell killing by LAK cells.[8–10] Among them, cytokines released from LAK cells may play an important role in cytolysis of tumor target cells.[11–13]

It has recently been reported that a membrane-associated lymphotoxin (LT) molecule (mLT) is expressed on human PBL-LAK cells.[14, 15] This molecule is expressed as early as 9 h after culturing human PBL with interleukin-2 (IL-2), and expression reaches a maximum at 5 days. It was found that a murine fibroblast cell line, L929 cell, was killed by mLT-expressing PBL-LAK cells but not by mLT-non-expressing PBL-LAK cells which were obtained by culturing PBL-LAK without IL-2 for 24 h.[16] A similar correlation between the mLT expression and the tumor cell killing activity of LAK cells was also observed when human tumor cell lines were used as target cells. Further, the tumor cell killing activity of PBL-LAK cells was partially inhibited in the presence of anti-LT antibody.[16] These findings prompted us to investigate the relationship between the mLT expression and the tumor cell killing activity of TIL-LAK cells. In the present study, we conducted phenotypic and functional analyses of human TIL-LAK cells along with PBL-LAK cells from the viewpoint of mLT expression.

MATERIALS AND METHODS

Reagents Recombinant human IL-2 (rIL-2; TGP-3) was a kind gift from Takeda Pharmaceutical Co. (Osaka). Its specific activity was 4.2×10^3 U/mg protein. The biological titer of rIL-2 was designated on the basis of Takeda's own standard, and one unit is equivalent to 364 U of the international IL-2 standard. The recombinant human lymphotoxin (rLT) was kindly provided by Kanegafuchi Chemical Co. (Takasago). The recombinant human tumor necrosis factor-α (rTNF-α) was kindly provided by Dainippon Pharmaceutical Co. (Osaka). Polyclonal rabbit anti-LT and anti-TNF-α were prepared by immunizing rabbits with rLT or rTNF-α emulsified in complete Freund's adjuvant. IgG fractions of each antiserum were obtained by protein A-conjugated Sepharose column chromatography (Pharmacia, Uppsala), then affinity purifications were performed with each cytokine-conjugated Formyl-Cellulofine (Seikagaku Kogyo Co.,

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Table I. Clinical Information on Patients

Patients No.	Age	Sex	Site	Histology
1	65	female	lung	squamous cell carcinoma
2	55	male	lung	small cell carcinoma
3	75	male	lung	adenocarcinoma
4	57	male	lung	adenocarcinoma
5	44	female	lung	adenocarcinoma
6	69	female	lung	adenocarcinoma
7	42	female	breast	adenocarcinoma
8	24	male	mediastinum	adenocarcinoma
9	66	male	stomach	adenocarcinoma
10	76	male	colon	adenocarcinoma
11	76	female	colon	adenocarcinoma
12	22	male	liver	hepatocellular carcinoma
13	24	male	liver	hepatocellular carcinoma
14	62	female	liver	adenocarcinoma
15	66	male	pancreas	adenocarcinoma
16	69	female	pancreas	adenocarcinoma
17	65	male	gallbladder	adenocarcinoma
18	1	male	kidney	Wilms' tumor

Tokyo). $F(ab')_2$ fragments of each antibody were prepared by digestion of the purified IgG with pepsin (Difco Lab., Detroit).

Induction of TIL-LAK cells Clinical information on patients is summarized in Table I. These patients had received no anti-tumor therapy at the time of operation. Fresh tumor tissues were resected from surgical specimens under sterile conditions. Necrotic and fatty tissues associated with the tumor mass were removed. These tissues were minced with scissors to give pieces 4–6 mm in diameter in RPMI 1640 medium (Flow Lab., Irvine) containing 100 U/ml penicillin and 100 μg/ml streptomycin (washing medium) and washed three times with washing medium. These minced tissues were suspended in RPMI 1640 medium containing 15% AB-type human fresh frozen plasma (FFP) and 5 U/ml rIL-2 and then cultured in a 24-well microplate (Corning, New York) at 37°C under 5% CO_2 in a humidified incubator for one or two weeks. Medium was exchanged every 3 or 4 days. After cultivation, the tissue suspension was filtered through a steel mesh to obtain a single cell suspension followed by density gradient sedimentation with Ficoll-Paque (Pharmacia) and then the TIL-LAK-enriched cells were cultured in a 24-well microplate at a cell concentration of 1×10^6/ml for a further 2 or 3 weeks.

Induction of PBL-LAK cells Peripheral blood from patients before operation was obtained by venepuncture with 10 U/ml of heparin, and PBL were isolated by centrifugation using Ficoll-Paque and washed 3 times with washing medium. PBL were suspended in RPMI 1640 medium containing 15% AB-type human fresh frozen plasma (FFP) and 5 U/ml rIL-2 at a cell concentration of 1×10^6/ml and cultured in a 24-well microplate at 37°C under 5% CO_2 for 4 or 5 weeks. Medium was exchanged every 3 or 4 days.

Preparation of fresh tumor cells Fresh minced tumor tissue was digested in RPMI 1640 medium containing 0.01% hyaluronidase type V (Sigma, St. Louis), 0.002% DNAse type I (Sigma), 0.1% collagenase type IV (Sigma) for 6–24 h at room temperature, followed by filtration through a steel mesh to exclude undigested tissue fragments. The tumor cell-enriched fraction separated by centrifugation on Ficoll-Hypaque gradients was stored in the medium supplemented with 40% FFP and 10% dimethyl sulfoxide at −80°C until used as autologous tumor cells.

Flow cytometric analysis TIL-LAK and PBL-LAK cells were washed 3 times with Dulbecco's phosphate-buffered saline (PBS) containing 2% fetal calf serum (Filtron, Brooklyn) and 0.02% NaN_3 and then reacted at 4°C for 1 h with both $F(ab')_2$ of affinity-purified rabbit anti-LT IgG and one of the following monoclonal antibodies: anti-CD2, anti-CD3, anti-CD16, anti-CD56, anti-HLA-DR (Becton Dickinson Immunocytometry, San Jose), anti-CD4, and anti-CD8 (Coulter Immunology, Hialeah). As a negative control, $F(ab')_2$ of normal rabbit IgG and normal mouse IgG were used. After the reaction, these cells were washed 3 times with PBS and then allowed to react at 4°C for 30 min with both FITC conjugated-$F(ab')_2$ of goat anti-rabbit IgG and phycoerythrin-conjugated $F(ab')_2$ of goat anti-mouse IgG (Tago Inc., Burlingame). These stained cells were then analyzed on an EPICS Profile flow cytometer (Coulter). Expressions of mLT and other surface marker

were visualized by the shift of the mean channel of the fluorescence intensity.

Enzyme-linked immunosorbent assay (ELISA) ELISA methods for TNF-α and LT by the sandwich method using polyclonal rabbit anti-TNF-α and LT antibodies were established in our laboratory.[17] $F(ab')_2$ fragments were used for the solid phase and IgG conjugated with horseradish peroxidase for the liquid phase. Culture supernatants were collected after 4–5 weeks of culture. The supernatants were separated by centrifugation, and stored at −80°C until measurement.

Tumor cell killing assay Daudi, K562 and autologous tumor cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Buckinghamshire) for 1 h at 37°C and washed extensively before use. The radiolabeled cells were plated at 1×10^4 cells/well in RPMI 1640 medium supplemented with 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum (Filtron) and incubated with $1–5\times10^5$ cells/well of TIL-LAK or PBL-LAK at 37°C under 5% CO_2 for 4 h. After incubation, the amount of ^{51}Cr released in supernatants was measured by using a gamma scintillation counter (Aloka, Tokyo). Maximum ^{51}Cr release was obtained by addition of 10% sodium dodecyl sulfate (SDS). Triplicate measurements were taken for each sample and the percentage of specific target cell lysis was calculated as:

$$\frac{(\text{experimental } {}^{51}\text{Cr release} - \text{spontaneous } {}^{51}\text{Cr release})\times100}{\text{maximum } {}^{51}\text{Cr release} - \text{spontaneous } {}^{51}\text{Cr release}}$$

Standard deviations were within 15%. Student's *t* test was used for statistical analysis.

L929 cytotoxicity assay L929 cell cytotoxicity was measured by crystal violet assay. L929 cells were seeded into a 96-well microplate (Corning) at a density of 1.25×10^4 cells/well and incubated overnight. Following incubation, TIL-LAK or PBL-LAK were added at a density of 12.5×10^4 cells/well. For measurement of L929 cell cytotoxic activity of rLT or culture supernatants, diluted sample solutions were added in the presence or absence of actinomycin D. After 16 h incubation with TIL or PBL, each well was washed 3 times and then stained with 2% crystal violet solution. Our hundred percent killing was obtained by the addition of 5% SDS. Absorbance at 540 nm was measured with a Titertek Multiskan (Flow, Rockville) and the percent specific cytotoxicity was calculated as:

$$\frac{(\text{A540 in control well} - \text{A540 in experimental well})\times100}{\text{A540 in control well} - \text{A540 in 100\% killing well}}$$

RESULTS

Kinetics of mLT expression A typical time course of mLT expression on both TIL-LAK and PBL-LAK cells derived from a patient with hepatocellular carcinoma is shown in Fig. 1. No mLT-expressing cells were found in TIL or PBL at the beginning of culture. The mLT expression on PBL-LAK reached a maximum at 5–8 days of culture and then decreased gradually. TIL-LAK expressed mLT maximally at 10–14 days of culture and the expression decreased thereafter.

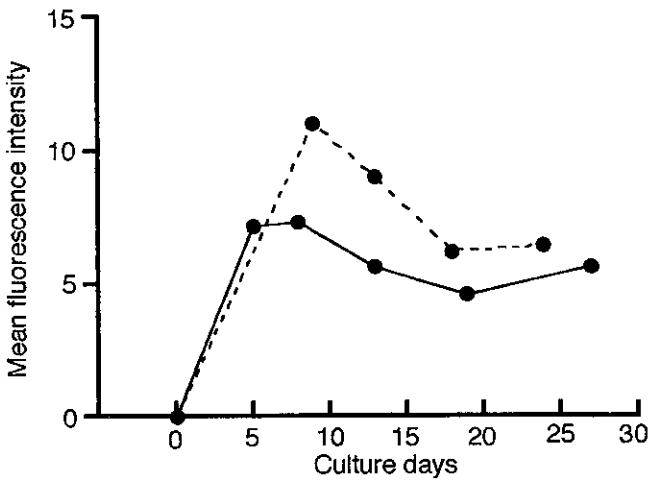

Fig. 1. Time courses of the mLT expression on TIL-LAK (○) and PBL-LAK (●) cells derived from a patient with hepatocellular carcinoma (patient No. 12). The mean channel of fluorescence intensity is indicated.

Expression of mLT and other surface markers on TIL-LAK and PBL-LAK The expressions of mLT and other surface markers on 4-week-cultured LAK cells derived from either TIL or autologous PBL were measured by flow cytometric analysis (Fig. 2). The mean fluorescence values of mLT expression were 0.3–6.7. In all except for two cases, the mLT expressions on TIL-LAK were more than those on the corresponding individual PBL-LAK. There was, however, no significant difference in mLT expression between TIL-LAK and PBL-LAK cells. The expressions of both CD3 and DR on TIL-LAK were greater than those on PBL-LAK ($P<0.005$, $P<0.01$) whereas the expressions of both CD16 and CD56, surface markers of NK-cells, on TIL-LAK cells were significantly less than those on PBL-LAK ($P<0.005$). There were no significant differences in CD2, CD4 and CD8 expressions between TIL-LAK and PBL-LAK cells.

Secretion of LT and TNF-α in culture supernatant LT and TNF-α in the culture supernatants of 4-week-cultured TIL-LAK and PBL-LAK cells were measured by ELISA. All the supernatants used were taken from the 3-day culture of LAK cells from the last medium exchange so that LT and TNF-α released were accumulated (Fig. 3). The concentration of LT in the superna-

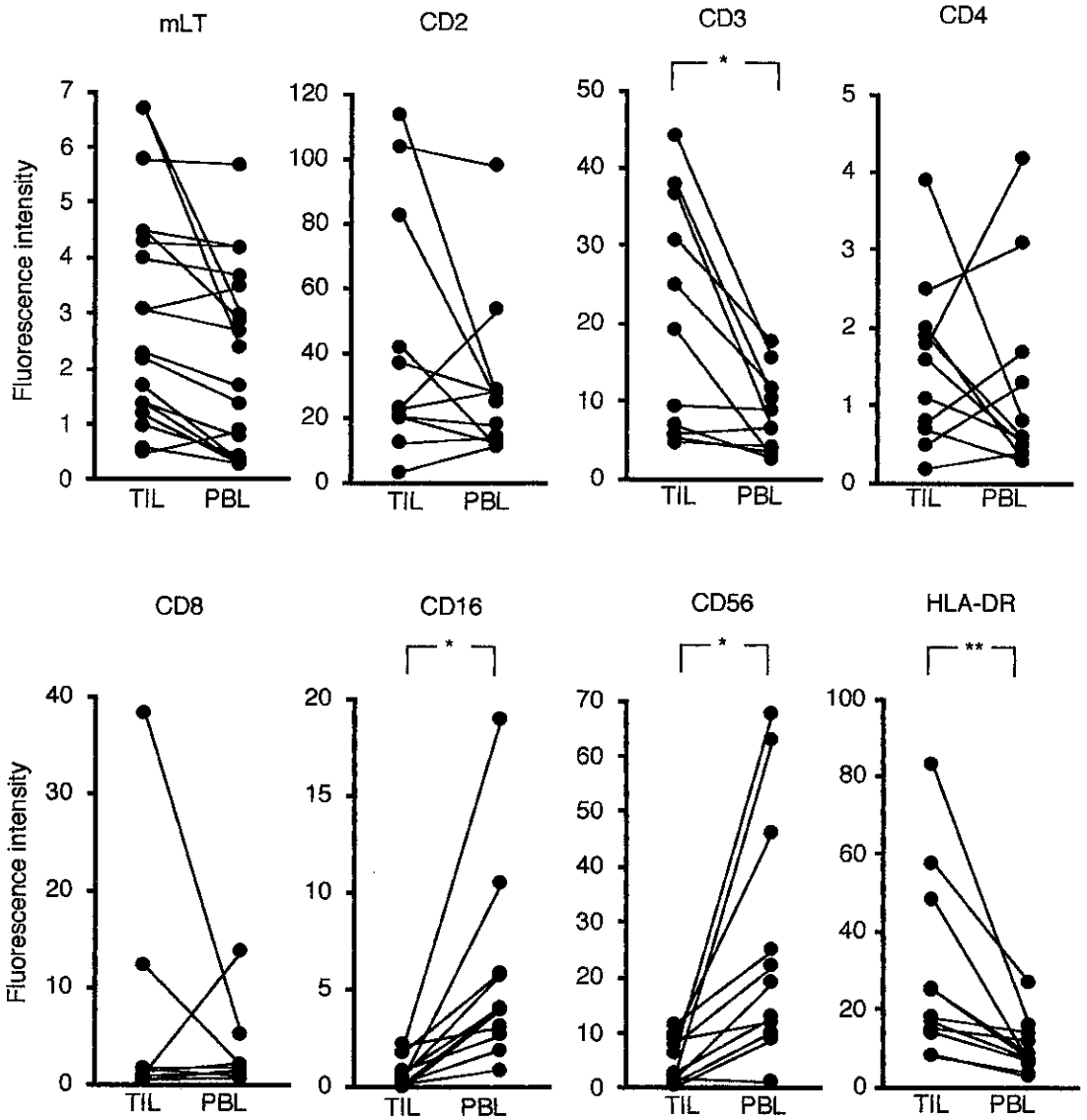

Fig. 2. Expressions of mLT and other surface markers on TIL-LAK and PBL-LAK cells. Each surface antigen expression is shown as the mean channel of fluorescence intensity. Samples from 18 patients were used for measurements of mLT expression and samples from 11 patients for those of the other surface markers. *, $P<0.005$; **, $P<0.01$.

tants ranged from 0 to 2300 pg/ml. There was a significant positive correlation between secreted LT and mLT expression ($r=0.850$, $P<0.01$, 9 cases). In contrast, there was no secretion of TNF-α except for one case.

Tumor cell killing activity of TIL-LAK and PBL-LAK

The mLT expression and the killing activities of 4-week-cultured TIL-LAK and PBL-LAK towards Daudi, K562 and L929 cell lines were investigated. The killing activities of LAK cells towards both Daudi and K562 cells were measured by using the 4-h ^{51}Cr release assay. The killing activities towards L929 cells were measured by means of the 16-h crystal violet assay. There was no correlation between the mLT expression and the killing activities towards Daudi and K562 cells (Fig. 4). In contrast, the killing activities towards L929 cells significantly correlated to the mLT expression on both TIL-LAK and PBL-LAK cells ($r=0.806$, $P<0.01$, 15 cases). When the ^{51}Cr release assay was used for the measurement of killing activities of LAK cells towards L929 cells, the results were similar to those obtained by the

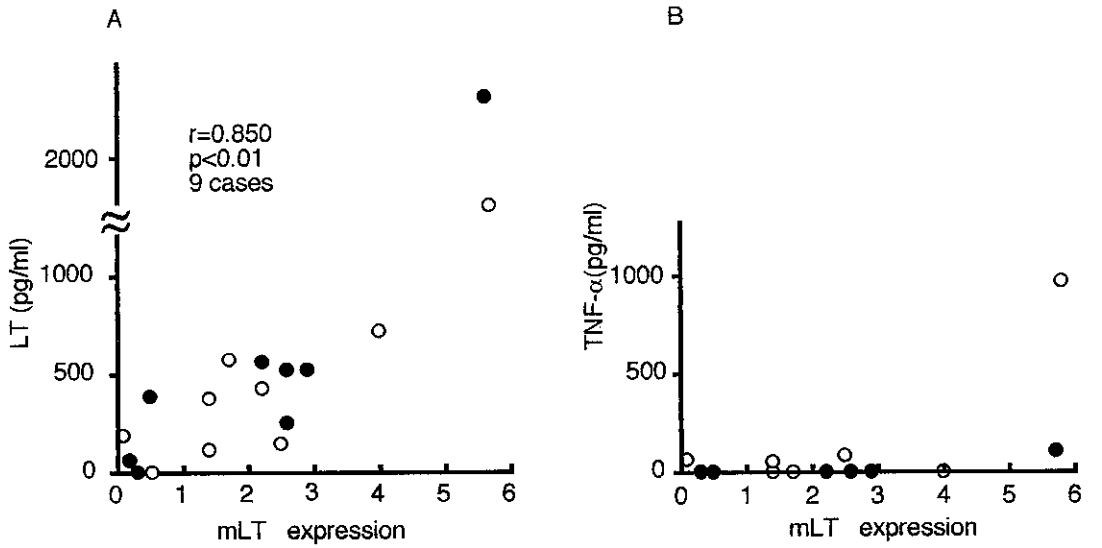

Fig. 3. Correlation between mLT expression and secretion of LT (A) and TNF-α (B) by TIL-LAK and PBL-LAK cells. LT and TNF-α in the culture supernatants of 4-week-cultured TIL-LAK (○) and PBL-LAK (●) cells were measured by ELISA.

crystal violet assay (data not shown). Furthermore, when the 4-h assay instead of 16-h assay was employed, there was also a significant correlation between the mLT expression and the killing activity towards L929 cells (data not shown).

To demonstrate that the killing activity of LAK cells towards L929 cells was due not to the LT secreted from LAK cells but to mLT expressed on LAK cells, L929 cell cytotoxicities of the supernatants of PBL-LAK cells cultured for 16 h and those of rLT were measured (Table II). Several hundred pg/ml of rLT was enough to kill the L929 cells in the presence of actinomycin D, but was ineffective in the absence of actinomycin D. One hundred ng/ml of rLT was required for 40.9% specific cytotoxicity towards L929 cells in the absence of actinomycin D. The LT secreted by LAK cells, therefore, cannot account for the L929 cell killing activity of LAK cells in the absence of actinomycin D because the concentration of secreted LT was less than 1000 pg/ml (data not shown, refer to Fig. 3).

Effects of antibodies on the killing activities of LAK cells towards L929 cells and autologous tumor cells An involvement of mLT in L929 cell killing by LAK cells was suggested by the result in Fig. 4. This was further supported by the evidence that polyclonal anti-LT antibody could partially suppress the killing activity of LAK cells towards L929 cells (Table III). These suppressions were observed in killings both by TIL-LAK cells and by PBL-LAK cells. The degree of suppression ranged from 12.9% to 71.8%. There was a dose-response relationship between the degree of suppression and the concentration of anti-LT antibody (data not shown). The killing activities of both TIL-LAK and PBL-LAK cells towards corresponding autologous tumor cells were measured (Table IV). Although the cytotoxicities were relatively low (8.4–31.6%), partial inhibition of the cytotoxicity of PBL-LAK cells by anti-LT antibody was found, whereas only marginal inhibition was found in the case of TIL-LAK cells.

DISCUSSION

In the present study, we showed that mLT is expressed on TIL-LAK cells as well as PBL-LAK cells,[14–16] and there is a positive correlation between the mLT expression and the killing activity of LAK cells towards L929 cells. Although the biological significance of mLT has not been fully established, an involvement in the tumor cell killing mechanisms of LAK cells is strongly suggested. In fact, surface LT on phytohemagglutin- or concanavalin A-stimulated lymphocytes[18] and mLT on $CD4^+$ CTL clones[19] have been reported to play a role in target cell killing.

The time course of the mLT expression on TIL-LAK cells, which depended on the presence of IL-2, was similar to that on PBL-LAK cells.[15] Whether the TIL *in situ* expresses mLT is an important question, because such lymphocytes invading a tumor mass are expected to be activated. In spite of many examinations, no mLT expression on TIL was found at the beginning of culture.

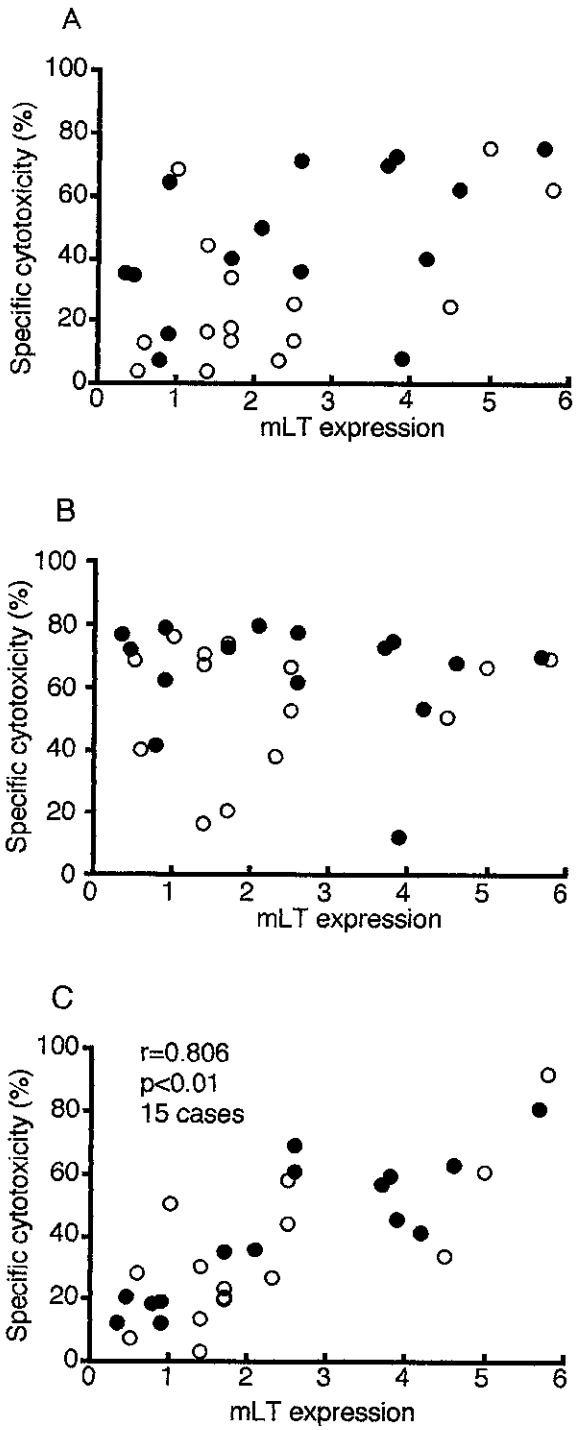

Fig. 4. Correlation between mLT expression and killing activity of 4-week-cultured TIL-LAK and PBL-LAK cells towards tumor cell lines. Killing activities of LAK cells towards Daudi (A) and K562 (B) cells were measured by 4-h ^{51}Cr release assay and killing activity towards L929 cells (C) was measured by 16-h crystal violet assay. Correlations between mLT expression and killing activities of TIL-LAK (○) and PBL-LAK (●) are shown.

Table II. L929 Cell Cytotoxicity of rLT and Supernatant in the Presence or Absence of Actinomycin D[a)]

	% Specific cytotoxicity	
	AcD(+)	AcD(−)
LT(pg/ml)		
100000	NT[b)]	40.9
10000	NT	7.9
1000	97.8	0
500	95.4	0
250	83.8	0
125	63.4	0
62.5	39.6	0
31.5	31.4	0
15.6	27.6	0
Supernatant (% volume)		
50	91	0
25	65.4	0
12.5	36.7	0

a) Cytotoxicity was measured by 16 h crystal violet assay as described in "Materials and Methods." Supernatant was taken from the co-culture of PBL-LAK cells plus L929 cells for 16 h.
b) NT, not tested.

Table III. Effects of Anti-LT Antibody on the Killing Activity of LAK Cells towards L929 Cells[a)]

Patient No.	Effector	NR-IgG	Anti-LT
8	TIL	54.3±4.98	15.3±1.55 (71.8)[b)]
	PBL	46.6±2.85	18.9±4.20 (59.4)
9	TIL	58.2±4.45	50.7±0.75 (12.8)
	PBL	58.7±1.51	45±2.45 (23.3)
15	TIL	58.4±3.51	43.8±4.01 (25)
	PBL	51.9±5.97	38.9±2.73 (25)
17	PBL	30.8±0.38	16.4±3.91 (41.2)

a) Percent specific cytotoxicity of either TIL-LAK or PBL-LAK cells cultured for 3–4 weeks towards L929 cells was measured by 16-h crystal violet assay. Effector-to-target ratio was 10:1. Rabbit IgG of anti-LT antibody or normal rabbit IgG (NR-IgG) was added at a concentration of 50 μg/ml. Values are mean±SE.
b) Percent suppression.

However, the possibilities still remain that the actual cell number of TIL is so small that we cannot detect them and/or that activated TIL which have invaded deeply into the tumor mass are hardly detached from it during the early time of culture.

The greater expression of CD3 on TIL-LAK cells than that on PBL-LAK cells, which is consistent with the results of others,[1,20–23] indicates that TIL-LAK cells are of T-cell lineage. In contrast, the expressions of both CD16 and CD56 on PBL-LAK cells are more marked, which supports the notion that PBL-LAK cells are

Table IV. Effects of Anti-LT Antibody on the Killing Activity of LAK Cells towards Autologous Tumor Cells[a)]

Patient No.	Effector	NR-IgG	Anti-LT
6	TIL	8.4±0.32	5.3±0.33 (36.9)[b)]
	PBL	12.4±2.31	6.7±2.21 (45.9)
18	TIL	23.4±1.96	21.0±2.33 (10.2)
	PBL	31.6±4.41	10.9±0.21 (65.5)

a) Percent specific cytotoxicity of TIL-LAK cells and PBL-LAK cells towards autologous tumor cells cultured for 3–4 weeks was measured by 4-h ^{51}Cr release assay. Effector-to-target ratio was 50:1. Rabbit IgG of anti-LT antibody or normal rabbit IgG (NR-IgG) was added at a concentration of 50 μg/ml. Values are mean±SE.
b) Percent suppression.

mainly derived from NK cells. It should be pointed out that mLT is equally expressed on both kinds of LAK cell populations regardless of the cell lineage.

It is an important problem how the mLT is related to the secreted LT. As an analogous case, the existence of two forms of membrane-associated TNF-α should be noted.[24)] One of them is the TNF-α molecule (17 kDa) attached to a receptor[25)] and the other is an integral membrane protein (26 kDa) which is a precursor to the secreted TNF-α.[26)] Thus, all the secreted TNF-α is produced by cleavage of the membrane-associated TNF-α. In the case of LT, Androlewicz *et al.*[27)] showed that an activated human T cell hybridoma expressed a heteromeric complex composed of LT and a distinct 33 kDa glycoprotein. They proposed a novel model in which biosynthesized LT underwent two processes, i.e., trimer formation of LT followed by secretion, and attachment to the 33 kDa molecule followed by expression on the plasma membrane. Although it is not clear at present whether this is the case in mLT on LAK cells, similar mechanisms may be suggested by the finding that the amount of secreted LT in the supernatant correlated with the mLT expression of LAK cells (Fig. 3). The significance of secreted cytokines in the tumor cell killing activity of LAK cells is still controversial.[12,28–35)] It has been reported recently that membrane-associated TNF-α (mTNF-α) on murine macrophages had a TNF-α-sensitive tumor cell killing activity.[36)] Moreover, mTNF-α on the $CD8^+$ cytotoxic T lymphocytes has been found to be involved in the slow lysis of TNF-α-sensitive cells.[37)] Therefore, these findings on mTNFα raise the possibility that membrane-form but not secreted cytokines play an important role in the tumor cell killing.

We propose here that the mLT on LAK cells plays at least a partial role in tumor cell killing. This notion is supported by the findings that the mLT expression correlated to the killing activity of LAK cells towards L929 cells and that such killing activity was inhibited by the presence of anti-LT antibody. Moreover, it is further supported by the evidence that the mLT-non-expressing LAK cells which could be obtained by culturing LAK cells without exogenous IL-2 for 24 h had weaker activity to kill L929 cells than mLT-expressing LAK cells.[16)] Secreted LT cannot account for the L929 cell killing ability because the amount of LT secreted from LAK cells was far less than that required for L929 cell killing in the absence of actinomycin D (Table II). On the other hand, mLT does not appear essential to the killing of Daudi and K562 cells by LAK cells because the killing activity of LAK cells towards these cells did not correlate to mLT expression (Fig. 4). LAK cells are known to have several killing mechanisms including those involving pore-forming protein, TNF-related molecules and serine esterase.[8–10)] Such mechanisms may be involved in the killing of Daudi and K562 cells by LAK cells. However, these two cell lines appear exceptional, because seven out of nine human tumor cell lines were susceptible to mLT-mediated killing by LAK cells.[38)] This notion is supported by the evidence of the partial inhibition of killing activity of LAK cells by anti-LT antibody towards autologous tumor cells (Table IV).

In conclusion, our data have revealed a new aspect of TIL-LAK cells, namely expression of mLT. Although the involvement of mLT in the effector system of LAK cells against tumor cells is indicated by the present results, elucidation of the precise mechanisms and biological significance must await further investigations.

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