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Expression of Membrane-associated Lymphotoxin/Tumor Necrosis Factor-β on Human Lymphokine-activated Killer Cells

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A membrane-associated lymphotoxin (LT)-related molecule was detected on human lymphokine-activated killer (LAK) cells by flow cytometric analysis. Kinetic analysis revealed that the LT antigenicity on LAK cells appeared at 9 h after the beginning of culture and was maintained thereafter. By autoradiography, the molecular weight of membrane LT was estimated to be 31 kD and/or 62 kD.

Key words: Lymphokine-activated killer — Lymphotoxin — Membrane-associated form

Lymphokine-activated killer (LAK) activity is induced when human peripheral blood mononuclear cells (PBMC) are cultured with interleukin 2 (IL-2).[1,2] Although the tumor cell killing mechanism by LAK cells remains to be clarified, induction of certain effector molecules on the plasma membrane of LAK cells is expected since cell-cell contact between effector lymphocytes and target tumor cells is required for killing.[3] Several cytokines and cytokine-related molecules have been found to be expressed on macrophage or lymphocyte membranes, and their significance in cell-cell interactions and cytolytic reactions has been widely debated. Tumor necrosis factor (TNF-α) on activated macrophage is known to have activity to kill TNF-sensitive tumor cell lines.[4–6] A TNF-α-like molecule was also found on a murine cytotoxic T cell line.[7] Membrane-associated interleukin 1α (IL-1α) activity of macrophages has been reported and may have roles in macrophage-T cell collaboration and tumor cell killing.[8–10] In this report, we describe the expression of a lymphotoxin(LT)/TNF-β-related molecule on LAK cells and the results of a partial biochemical characterization.

PBMC from a healthy donor were separated by density gradient sedimentation with Ficoll-Paque (Pharmacia) and cultured in 24-well microplates (Nunclon) at a cell concentration of 1×10^6/ml in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 15% of AB-type human fresh frozen plasma (FFP) and 5 U/ml human recombinant IL-2 (TGP-3; Takeda Pharmaceutical Co., Osaka), at 37°C and 5% CO_2. After incubation for 9 h, the cells were washed three times with Dulbecco's phosphate-buffered saline and then reacted with $F(ab')_2$ fragments of affinity-purified rabbit anti-LT, rabbit anti-IL-1α or mouse monoclonal anti-TNF-α antibody (UTA-1). All the anti-cytokine antibodies were prepared in our laboratory with recombinant human LT (Kanegafuchi Chem. Co., Tokyo), recombinant human IL-1α (Dainippon Pharmaceutical Co., Tokyo) or recombinant human TNF-α (Dainippon Pharmaceutical Co.) as the antigens. Their specificities for each antigen were assured by enzyme-linked immunosorbent assay (ELISA).[11] The cells that reacted with anti-cytokine antibodies were developed with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG (Tago Inc., USA) or goat anti-mouse IgG (Cappel, USA) antibody and then subjected to flow cytometric analysis on an Epics Profile (Coulter, USA). LAK cells were stained with anti-LT but not with anti-TNF-α or anti-IL-1α (Fig. 1). The monophasic staining profile with anti-LT suggested that almost all the cells cultured with IL-2 expressed membrane LT. This is somewhat surprising because the lymphocyte subset which produces LT predominantly is known to be type 1 helper T cells, although there is evidence that B lymphoblastoid cell lines could also secrete LT.[12] LAK cells are known to consist of heterogenous cell populations of NK and T cell type.[13] Preliminary results obtained from a two-color flow cytometric analysis show that most of the LAK cells positively stained with anti-LT are CD2-positive, and that they include both CD3-positive and CD3-negative populations (data not shown). Further studies are under way to verify the type of LAK cells which bear the membrane-associated LT.

For long-term culture, PBMC were cultured with IL-2 in a gas-permeable bag (Karmicell; Kawasumi Lab., Tokyo) at rest for 5 days, and then transferred into the inner compartment of a "concentrate rotary tissue culture bag" (Kawasumi) with FFP-free medium in the outer compartment and cultured for a further 11 days with exchange of the medium every 2 or 3 days. At the

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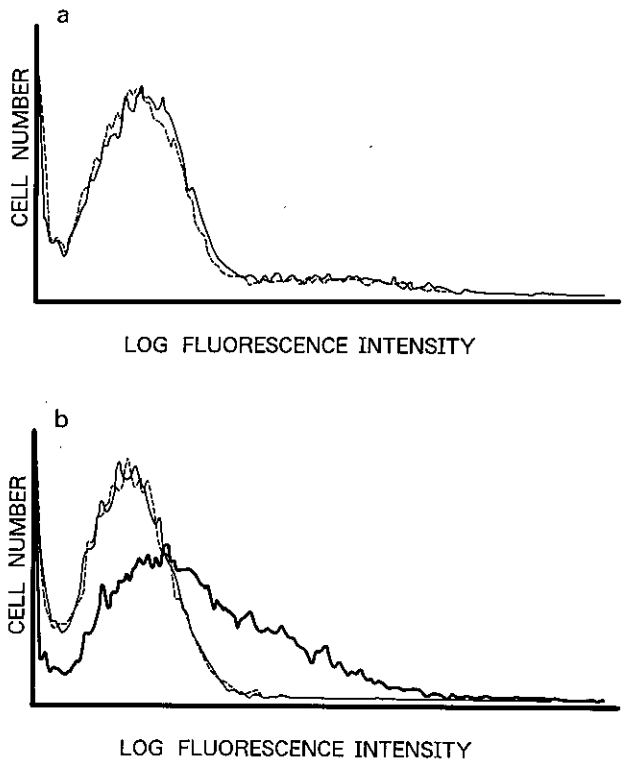

Fig. 1. Flow cytometric analysis of LAK cells with anti-cytokine antibodies. LAK cells incubated for 9 h were stained with (a) anti-TNF-α MAb IgG (UTA-1), (b) F(ab′)$_2$ fragments of rabbit anti-IL-1α(—) or anti-LT (—). Controls, reacted with normal mouse IgG or normal rabbit F(ab′)$_2$ as the first antibodies, are indicated with broken lines.

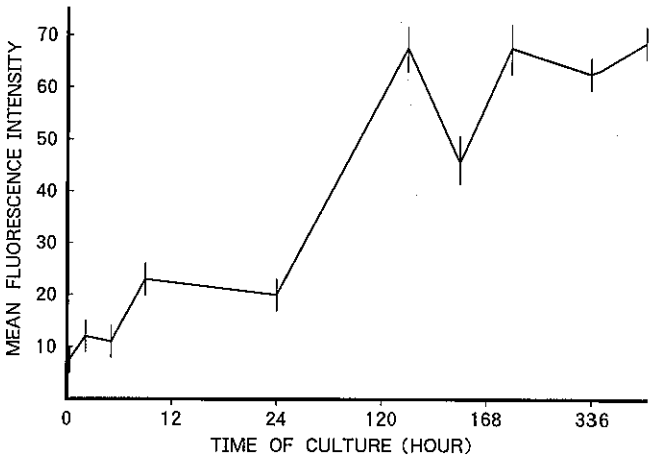

Fig. 2. Kinetics of membrane-associated LT expression. LAK cells incubated for various lengths of time were reacted with anti-LT antibody and flow cytometric analyses were performed. Each point indicates the mean of fluorescence intensity.

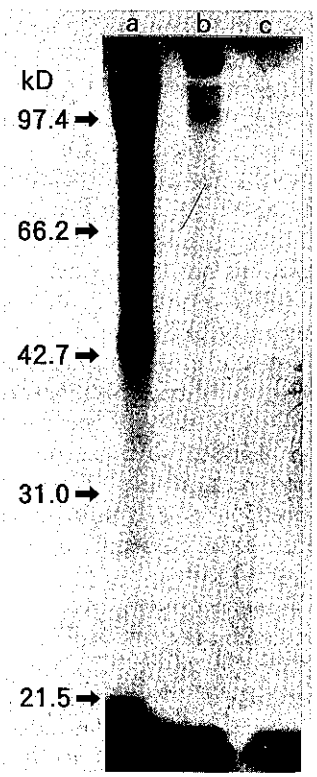

Fig. 3. Autoradiography of membrane LT. LAK cells incubated for 5 days were surface-radioiodinated by lactoperoxidase and solubilized with 0.5% NP-40. Cell lysates were immunoprecipitated with anti-LT antibody or normal rabbit IgG and *Staphylococcus aureus* COWAN 1. Then, the samples were subjected to SDS-PAGE and autoradiography. Lane a: crude radioiodinated cell lysate. Lane b: immunoprecipitates with anti-LT antibody. Lane c: immunoprecipitates with normal rabbit IgG.

indicated time (Fig. 2), aliquots were removed for measurement of the membrane LT expression on the cells. Figure 2 shows that membrane-associated LT was detected after 9 h from the beginning of culture and its expression reached the maximum by 5 days, thereafter being sustained for up to 16 days.

To estimate the molecular weight of membrane LT, PBMC cultured with IL-2 for 5 days were surface-radioiodinated with lactoperoxidase and lysed with 20 m*M* Tris-HCl-buffered saline, pH 7.3, containing 1 m*M* PMSF and 0.5% NP-40. Aliquots of cell lysates were reacted with either rabbit anti-LT or normal rabbit IgG and then *Staphylococcus aureus* COWAN 1 (SAC) was added. The precipitated SAC were washed and sample buffers for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were added. The SAC were then electrophoresed according to the method of Laemmli.[14] By autoradiography performed with XAR film (Kodak, USA) and a Cronex intensifying screen (Du Pont, USA),

a major band with a molecular weight of 31 kD and two minor bands with molecular weights of 62 kD and 25 kD were detected in the sample immunoprecipitated with anti-LT (Fig. 3). A 98 kD band was probably non-specific because the same band appeared in the control lane.

This is the first report to present evidence for a membrane-associated form of LT. Although the 31 kD and/or 62 kD molecule(s) (Fig. 3) seem to be membrane LT, it is possible that some LT molecules released from LAK cells were passively attached to the plasma membrane non-specifically or through their receptor; this could account for the molecules at 25 kD (Fig. 3), because native secreted LT was shown to have a molecular weight of 25 kD.[15] This possibility is supported by the evidence that the amount of anti-LT antibody bound to LAK cells was decreased by about 20% by treatment of the cells with high salt (0.3 *M* NaCl) and low pH (pH= 3.0) before staining (data not shown). At present, it is not known whether the membrane LT is a precursor form of the secreted LT, as is the case with TNF-α[6, 16] and IL-1α,[17, 18] or whether the membrane LT is a distinct molecule from the secreted LT. However, the molecular size (31 kD) of membrane LT estimated by SDS-PAGE could be explained if one postulates that the putative signal peptide which contains 34 amino acid residues is not removed from the secreted LT of 25 kD. Nedwin *et al.*[19] showed that both TNF-α and LT were secreted from PBMC cultured with IL-2. Thus, it is noteworthy that only membrane LT was expressed on the cells. As the membrane TNF-α is known to be expressed on activated macrophages, these two kinds of membrane-associated cytokine may have disparate functions.

The kinetics of the membrane LT expression on LAK cells were almost parallel with the kinetics of tumor cell killing activity of LAK cells (data not shown). Thus, it is possible that membrane LT could deliver some signal to the target tumor cells. In fact, paraformaldehyde-fixed LAK cells could kill actinomycin D-treated L929 cells and this killing activity was suppressed by anti-LT antibody (data not shown). It was, however, reported that Daudi cells and Raji cells, which have been used as target cells of LAK activity, lack TNF-receptor or mRNA of TNF-receptor, respectively.[20, 21] Because both TNF-α and LT are known to bind to TNF-receptor equally, membrane LT might not, therefore, play an essential role in the killing mechanism of LAK cells, at least against Daudi or Raji cells.

Since LT is known to possess various biological activities on a wide variety of cells and tissues,[22–26] it is anticipated that the membrane LT play some roles in cell-cell interactions other than tumor cell killing. Further investigations are required to clarify the significance of membrane LT.

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