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Characterization of Transendothelial Migratory Lymphokine-activated Killer Cells

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We examined the killing activity of transmigrated lymphokine-activated killer (LAK) cells and their surface molecules associated with both transendothelial migration and cytotoxicity, using human umbilical vein-derived endothelial cell (HUVEC) monolayers on fibronectin with gelatin separating the upper chamber from the lower chamber. Migratory LAK cells were significantly more cytotoxic to Daudi target cells, expressed more LFA-1, and were more likely to be positive for CD2, compared to those LAK cells not adherent to the HUVEC monolayer. In contrast, in the absence of the HUVEC monolayer, there was no difference in LAK activity between migratory and non-adherent LAK cells. These results indicate that the interaction between LAK cells and the HUVEC monolayer allows selective migration of LAK cells with cytotoxic activity that is enhanced with respect to some surface molecules.

Key words: LAK — HUVEC — Adhesion molecules — Killing activity

Adoptive transfer of *in vitro*-expanded killer cells, such as LAK,[1,2] cytotoxic T lymphocytes, and TIL,[3,4] has been used to treat cancer patients. Pockaj *et al.*[5] recently demonstrated a positive association between the ability of ^{111}Indium (In)-labeled TILs to migrate to tumors and the clinical response. Further, it has been reported that regional injections of effector cells are more effective than systemic administration.[6] LAK cells accumulate briefly at tumor sites after regional intra-arterial perfusion[7] while intravenously infused LAK cells first accumulate in the lungs before migrating to the liver.[8,9] When ^{111}In-labeled LAK cells were infused into the maxillary artery (tumor feeding artery) of a patient with maxillary cancer, radioactivity persisted at the primary site at 48 h after the injection.[10] Thus, accumulation of the effector cells at tumor sites is an important step enabling these cells to kill tumor cells. To determine how killer cells migrate more effectively into tumor sites, it is important to explore the mechanism of the effector cell transendothelial migration. Since a model of transendothelial migration using a human umbilical vein-derived endothelial cells (HUVEC) monolayer has become available, many investigators have participated in studies of how adhesion molecules contribute to the migration of various types of effector cells.[11–14] A few studies have documented the influence of various cytokines on LAK cell migration by treating HUVEC with various cytokines[15] or by observing LAK cell chemotaxis.[16] However, they did not answer a critical question as to whether migrated LAK cells have the same or greater killing activity compared to non-separated LAK cells. The answer to this question will help determine if it is possible to produce LAK cells with greater killing activity that may migrate into tumor sites with more profound clinical effects. We employed an *in vitro* endothelial monolayer system in which a HUVEC monolayer is placed on gelatin and fibronectin to separate the upper chamber from the lower chamber, to examine the cytotoxic activity and surface molecules of transendothelially migrating LAK cells.

MATERIALS AND METHODS

Cell separation and induction of LAK cells Heparinized peripheral blood samples were collected from five normal healthy donors and peripheral blood mononuclear cells (PBMC) separated by Ficoll-Hypaque density gradient centrifugation. PBMC were cultured in culture medium (CM), composed of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), penicillin G (100 units/ml), streptomycin (100 μg/ml) and recombinant human interleukin (IL)-2 (800 IU; a generous gift from Takeda Chemical Industries Ltd., Osaka) for seven days at 37°C in a humidified 5% CO_2 atmosphere; one half of the medium was replaced every other day with fresh CM containing the same concentration of rIL-2.

Preparation of endothelial cells HUVEC were obtained and cultured as described by Jaffe *et al.*[17] with some modifications. Briefly, endothelial cells were isolated from umbilical cords after treatment with 0.25% trypsin (GIBCO-BRL, Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) and cultured on collagen-coated flasks (Corning Glass Works, Corning, NY) in Medium 199 containing 10% FCS, antibiotics, and 0.03 μg/ml endothelial cell growth factor (Becton Dickinson Two Oak

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Park, Bedford, MA). After confluence was reached, HUVEC were detached by treatment with 0.25% trypsin and 0.02% EDTA. Twenty thousand cells from the first passage were cultured on filters in Transwell culture inserts (6.5 mm diameter polycarbonate membrane with 5 μm pores; Transwell 3415, Costar, Cambridge, MA) for adhesion and migration assays. The filters were precoated with 2% gelatin, followed by additional coating with 5 μg/ml fibronectin (Becton Dickinson). The HUVEC formed a tight impermeable barrier in 5–7 days.

Cytotoxicity assay Ten million second-passage HUVEC or Daudi cells (LAK-sensitive lymphoma cell line) were suspended and incubated with 3.7 MBq of $Na^{51}CrO_4$ (Daiichi Radioisotope Laboratories, Tokyo) in 1 ml of CM without rIL-2 at 37°C in 5% CO_2. The ^{51}Cr-labeled target cells were then washed four times with RPMI-1640 and plated in triplicate at a final concentration of 1×10^4 cells/well in 96-well round-bottomed microtiter plates (Linbro, Flow Laboratories, McLean, VA). LAK cells were added to the target cells at an effector/target ratio of 1 or 10 in a final volume of 200 μl. The plate was centrifuged at 200g for 5 min and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. The cells were harvested with a Titertek collection system (Flow Laboratories) and the radioactivity present was determined using a gamma scintillation counter. Cytotoxicity (%) was calculated from the average of triplicate wells as $(E-S)/(M-S)\times100\%$, where E is experimental release, S is spontaneous release, and M is maximum release.

Blocking assays using monoclonal antibodies (mAb) LAK cells were treated with anti-human CD11a mAb (mouse IgG1; DAKO, Glostrup, Denmark) and/or anti-human CD2 mAb (mouse IgG1; Nichirei Ltd., Tokyo) for 30 min at 37°C, washed twice and then mixed with 1×10^4 ^{51}Cr-labeled Daudi cells. Cytotoxicity was measured by the same procedure as described for the cytotoxicity assay.

Transmigration assay LAK cells (1×10^5) were poured into the upper compartment of a Transwell culture system and incubated for 5 h. After 5-h incubation at 37°C in a 5% CO_2 atmosphere, the upper chambers were gently washed twice with 0.1 ml of RPMI-1640 to collect non-adherent LAK cells. The undersurface of the filter was then rinsed with 2 ml of cold PBS/0.2% EDTA solution to detach adherent transmigrated LAK cells and the detached LAK cells were combined with those in the lower chamber.

Fluorescence analysis Since the adherent LAK cell population always contained HUVEC after our separation procedure, we excluded this population from flow cytometry. Unseparated, non-adherent and migratory LAK cells were evaluated for their expression of CD2, CD11a, CD16, CD25 by flow cytometry. LAK cells in each fraction were incubated for 30 min on ice with mAb to CD11a (DAKO), CD16 (Nichirei Ltd.) or CD25 (Becton Dickinson), washed twice, and then incubated with fluorescence isothiocyanate (FITC)-conjugated rabbit antibodies to immunoglobulin G for another 30 min on ice. After two additional washes, the LAK cells were analyzed with a fluorescence-activated sorter (FACScan; Becton Dickinson). LAK cells were also incubated for 30 min on ice with mAb to CD2 (FITC conjugated, Nichirei Ltd.), washed twice, and analyzed with the FACScan. All incubations and washes were performed with PBS containing 0.1% NaN_3 and 2% FCS.

Statistical analysis The data are presented as means$\pm$SD. Significance was determined by using the unpaired two-tailed t test.

RESULTS

Appropriate LAK/HUVEC ratio for adhesion and migration assays We performed preliminary experiments to determine the non-toxic LAK/HUVEC ratio. In agreement with a previous report,[15] the cytotoxicity of LAK cells was negligible at an E/T ratio of 1.0 even after 12-h incubation (data not shown). Hence, we used a low LAK/HUVEC ratio (<1.0) in all subsequent adhesion and migration assays. Moreover, we stained the HUVEC monolayer with Diff-Quik (International Reagents Corp., Tokyo) and found neither morphologic change nor disruption of monolayer continuity under the light microscope. In addition, an incubation period of 5 h was selected for subsequent experiments on the basis of the kinetic study of adhesion and migration of LAK cells (data not shown).

Surface markers of non-adherent and migratory LAK cells Surface molecules of LAK cells are known to be involved in adhesion, migration and cytotoxicity. To characterize further the migratory LAK cells, we analyzed the surface markers of three populations (unseparated, non-adherent and migratory LAK cells) using immunofluorescence and flow cytometry. The major population of migratory LAK cells expressed more CD2 and CD11a compared to non-adherent LAK cells while over 99% of both LAK cells expressed CD11a (Fig. 1A). Sequential observation of the molecules of unseparated, non-adherent and migratory LAK cells demonstrated that non-adherent LAK cells consist mainly of a subpopulation which expressed relatively weakly both CD2 and CD11a in the unseparated LAK cells, while the population strongly expressing these molecule migrated preferentially through HUVEC (n=4).

As a control, without the HUVEC monolayer, non-adherent and migratory LAK cells were also examined for their expression of these surface markers (Fig. 1B). The migratory LAK cells expressed surface antigens

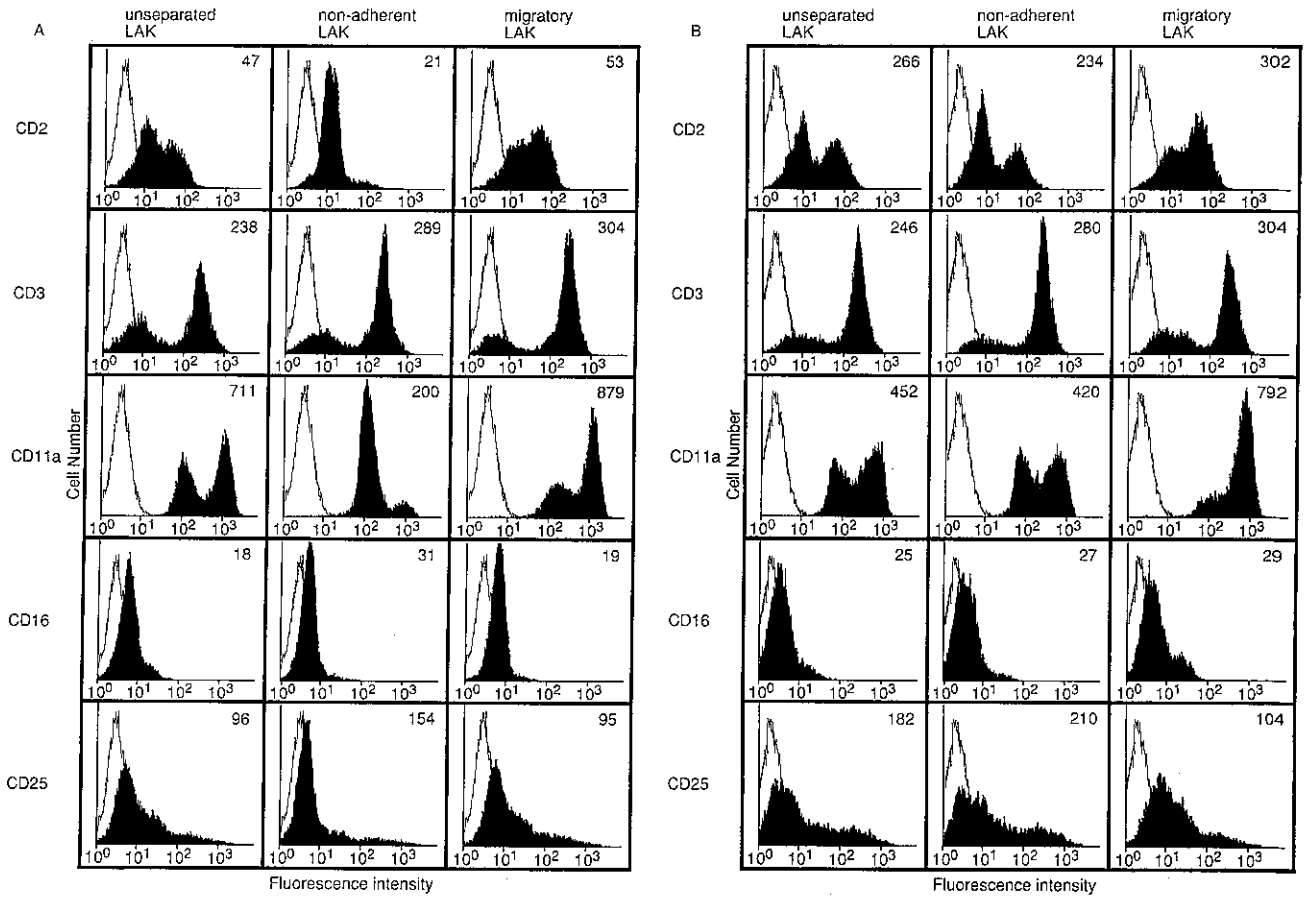

Fig. 1. CD2, CD3, CD11a, CD16 and CD25 expressions by unseparated, non-adherent, and migratory LAK cells, which were obtained as described under experimental procedures, stained with the indicated mAb, and enumerated in the FACS. There are marked differences in the expression of CD2 and CD11a among unseparated, non-adherent, and migratory LAK cells in the presence of HUVEC (panel A). Panel B, in the absence of HUVEC.

similar to those of CD2, CD3, CD11a and CD25-positive transendothelial migratory LAK cells. In contrast, non-adherent LAK cells in the absence of HUVEC expressed more intensely CD2 and CD11a than did those in the presence of HUVEC. The presence of HUVEC had no effect on migratory LAK cells with respect to their expression of CD3, CD16, or CD25 (Fig. 1A).

Cytotoxicity of non-adherent and migratory LAK cells Analysis of surface markers of unseparated, non-adherent, and migratory LAK cells showed that certain subpopulations migrate preferentially as a result of their interaction with HUVEC. To evaluate the killing activity of migratory LAK cells, we examined the cytotoxicity of non-adherent and migratory LAK cells with or without HUVEC against ^{51}Cr-labeled Daudi target cells. Without HUVEC, non-adherent LAK cells showed similar cytotoxicity to that of migratory LAK cells (% cytotoxicity at E/T ratio of 10: 49.7±4.4% vs. 53.7±5.4%, n=3) (Fig. 2A). In contrast, the cytotoxicity of LAK cells that migrated across HUVEC was significantly higher than that of non-adherent LAK cells at an E/T ratio of 10 (% cytotoxicity: 43.3±1.3% vs. 22.9±0.7%) (Fig. 2B). It was confirmed by experiments in four other cases (Fig. 3) that LAK cells migrating under HUVEC have a greater killing activity than do non-adherent LAK cells. These results indicate that LAK cells with killing activity migrate selectively and that their interaction with HUVEC plays a critical role in this selectivity.

Surface marker of LAK cells cytotoxic to Daudi cells We examined the inhibitory effect of anti-CD11a and CD2 mAbs on the cytotoxicity of LAK cells to Daudi cells. mAb to CD11a significantly suppressed the cytotoxicity. The combination of anti-CD2 and CD11a showed an additive inhibitory effect (Fig. 4), indicating that CD11a and CD2 contribute to the cytotoxicity of LAK cells against Daudi cells.

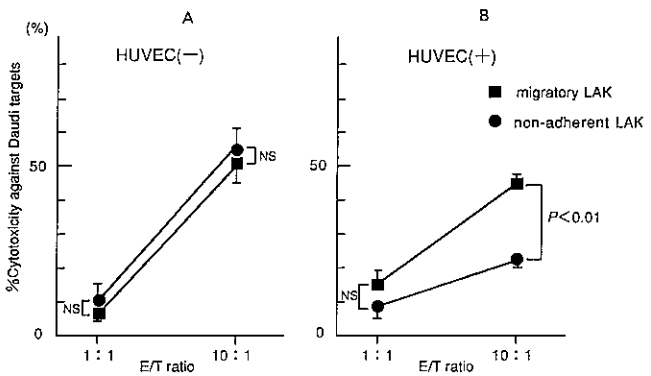

Fig. 2. Percent cytotoxicity of non-adherent and migratory LAK cells, examined in the presence or absence of HUVEC, against Daudi targets. In the absence of HUVEC (panel A), non-adherent and migratory LAK cells have the same level of cytotoxicity. In contrast, in the presence of HUVEC (panel B), migratory LAK cells are significantly more toxic than non-adherent LAK cells ($P<0.01$). Experiments shown in panels A and B were performed on different days. NS, not significant.

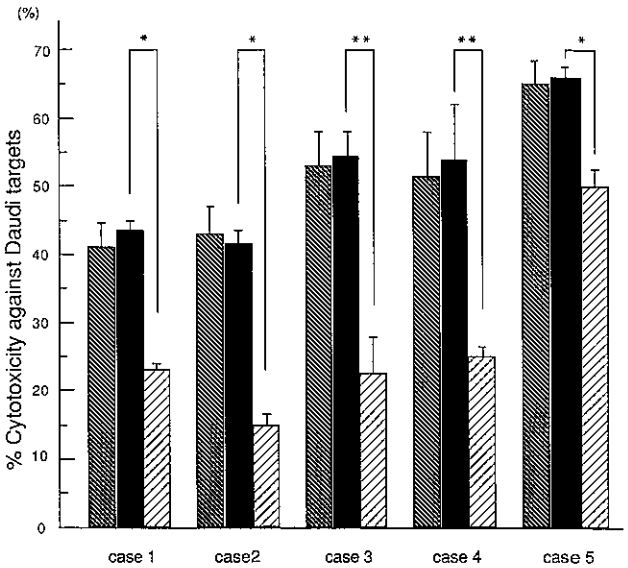

Fig. 3. Percent cytotoxicity of non-adherent or migratory LAK cells, in the presence of HUVEC, against Daudi target cells (10:1 E/T ratio). Migratory LAK cells are significantly more toxic than non-adherent LAK cells in all 5 cases tested (* $P<0.05$, ** $P<0.01$). Columns, mean of 5 experiments, each performed in triplicate; ▨ unseparated LAK; ■ migratory LAK; ▨ non-adherent LAK; bars, SE.

DISCUSSION

There is a consensus that LFA-1 plays a pivotal role in transendothelial migration of both NK and T cells.[18–20] LFA-1 is expressed on all T and NK cells. As expected, our fluorescence analysis showed that over 99% of LAK cells express LFA-1 (Fig. 1) and anti-LFA-1 (CD11a) mAb significantly inhibited the transendothelial migration of LAK (data not shown). Interestingly, most migrated LAK cells expressed a high intensity of LFA-1, in contrast to non-adherent LAK cells. These results may be explained in part by the observation of Shimizu *et al.*[21] that enhanced expression of LFA-1 is associated with increased capacity to bind to the relevant ligand (intracellular adhesion molecule-1).

The binding of LAK cells to target cells is an initial step in the cytolytic process. It is known that LFA-1 and CD2 molecules on LAK cells play a central role in establishing antigen-independent contact between LAK cells and target cells.[22, 23] LFA-1 and CD2 are also considered to be essential for the cytotoxicity of killer cells against certain types of tumor cells through signal transduction.[24, 25] We observed that mAb to LFA-1 significantly suppressed the cytotoxicity of LAK cells against Daudi target cells. In contrast to LFA-1, which is well known to have an important role in LAK cytotoxicity to tumor cells, it remains controversial whether CD2 contributes to such cytotoxicity. Nakamura *et al.*[26] proposed that the contribution of CD2 to the cytotoxicity exerted by LAK cells varies depending on the target cells. This hypothesis was based on the finding that anti-CD2 mAb

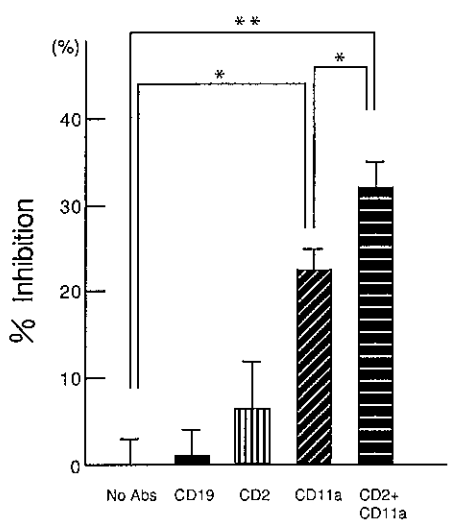

Fig. 4. Inhibition of cytotoxic activity against Daudi cells, expressed as percent ^{51}Cr release, by anti-CD2 and/or anti-CD11a mAbs (10:1 E/T ratio) (* $P<0.05$, ** $P<0.01$). Unseparated LAK cells were used to examine whether or not CD2 and/or CD11a molecules influence the cytotoxic activity of LAK cells against Daudi cells ($P<0.05$). Anti-CD11a mAb treatment significantly inhibited the cytotoxic activity, and combination treatment with anti-CD2 and anti-CD11a additively inhibited the cytotoxicity ($P<0.01$).

did not significantly inhibit the cytotoxicity against NK-sensitive target cells (in contrast to NK-resistant target cells, where the cytotoxicity was inhibited by anti-CD2 mAb). Since we used NK-resistant Daudi cells as the target for LAK activity, our study is concordant with their hypothesis in that it demonstrates that CD2 contributes to LAK killing activity towards resistant cells. CD2-positive cells comprised a larger population of migrated LAK cells than did non-adherent LAK cells (Fig. 1). Further, the intensity of CD2 expression by migratory LAK cells was strong. Accordingly, the cytotoxicity of the migratory LAK cells towards Daudi cells was significantly greater than that of non-adherent LAK cells (Fig. 2). Kato *et al.*[27] demonstrated in a murine model that LAK cells cultured with rIL-2 for 30 days expressed less CD2 compared to those cultured for 10 days and that the cytolytic activity of these LAK cells declined with their expression of CD2. It seems likely that the enhanced CD2 expression along with increased number of CD2-positive cells potentiates the killing activity of the migratory LAK cells.

It is noteworthy that in the absence of a HUVEC monolayer there was no difference in LAK activity between non-adherent LAK cells and migratory LAK cells. Interestingly, non-adherent LAK cells in the absence of a HUVEC monolayer consisted of LAK cells expressing more intensely both CD2 and CD11a compared to those in the presence of HUVEC (Figs. 1A and 1B). This may explain the lack of any difference in LAK activity between non-adherent LAK cells and migratory LAK cells in the absence of a HUVEC monolayer. These results also support the hypothesis that LAK cells which strongly express CD2 and CD11a are major contributors to LAK killing activity.

In conclusion, the interaction between LAK cells and the HUVEC monolayer allows the preferential migration of LAK cells which express more surface molecules and cytotoxic activity. These findings suggest that LAK cells that have killing activity migrate preferentially across capillary endothelial cells once they reach a tumor-feeding artery, resulting in effective tumor lysis. Reportedly, pretreatment of HUVEC with various cytokines such as IL-1, tumor necrosis factor-α, and interferon-γ facilitates the preferential migration of LAK cells.[15] Taking this result together with our findings, we suggest that the administration of these cytokines, followed by the infusion of effector cells into a tumor-feeding artery, may enhance the efficacy of adoptive immunotherapy.

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