Jpn. J. Cancer Res. 82, 1139–1144, October 1991

Adhesion Molecules on Murine Lymphokine-activated Killer Cells Responsible for Target Cell Killing: A Role of CD2

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Lymphokine-activated killer (LAK) cells were induced from C57BL/6 mouse spleen cells and the effects of culture time on the expression of cell surface phenotypes and cytotoxic activity of LAK cells were determined. The expression of CD2 remarkably decreased after culture of LAK cells for 30 days, while LFA-1, a principal adhesion molecule in LAK cells, and CD3 were not changed by the culture. LAK cells cultured for 90 days completely lost CD2. In accordance with the decrease of CD2, the cytotoxic activity of LAK cells declined but a certain leven was retained even after the complete loss of CD2. The established LAK cell clones were also strongly positive for the expression of LFA-1 but negative for CD2. When the LAK cell clones were transfected with the CD2 cDNA, they started to express CD2 on their cell surface and to show greater binding ability and stronger cytotoxicity to target tumor cells. These results indicated that CD2 plays a role as an adhesion molecule responsible for target cell killing in murine LAK cells.

Key words: Lymphokine-activated killer cell — CD2 — LFA-1 — Target cell lysis

Culture of lymphoid cells in the presence of IL-2 generates cytolytic cells, termed lymphokine-activated killer (LAK[4]) cells, which possess cytolytic activity against a broad range of NK-sensitive and NK-resistant tumor target cells without MHC restriction.[1,2] Since the first obligatory step of the cytotoxic reaction is the binding between killer and target cells, it is crucial to identify the adhesion molecules in these cells.

Many types of killer cells such as killer T, NK and LAK cells express LFA-1 as an adhesion molecule.[3,4] CD2 is also expressed in these cells,[5,6] but its role in murine LAK cells has not been established.

We report herein that murine LAK cells strongly express CD2 in the early stage of their induction, but the expression declines upon long-term culture, and it appears to be correlated with the binding and cytolytic activities of LAK toward target tumor cells.

MATERIALS AND METHODS

Animals Male C57BL/6 mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, and used at ages of 5–8 weeks.

Tumor cells Murine cell lines, EL-4 (H-2^b), FBL-3 (H-2^b), BALB/c RL♂1 (H-2^d) and YAC-1 (H-2^a) lymphomas, P-815 mastocytoma (H-2^d) and Meth A fibrosarcoma (H-2^d) were used. These tumor cell lines were maintained by *in vitro* culture in RPMI 1640 medium containing penicillin G at 100 unit/ml, kanamycin sulfate at 60 μg/ml, 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, HEPES at 2.3 mg/ml, $NaHCO_3$ at 2.0 mg/ml and 10% heat-inactivated fetal calf serum (FCS) (Flow Laboratories, North Ryde, Australia). Meth A cells were dispersed by incubation in phosphate-buffered saline (PBS) containing 0.1% ethylenediaminetetraacetic acid for a few minutes at 37°C.

Interleukin-2 Recombinant human interleukin-2 (rh-IL-2) having a specific activity of 1.4×10^7 JU (Jurkat unit)/ml was kindly donated by Shionogi Pharmaceutical Co., Osaka, and used at appropriate dilutions.

Derivation of murine LAK cells, LAK cell lines and LAK cell clones Fresh splenocytes (2.5×10^6/ml) from mice suspended in RPMI1640 medium containing 10% FCS were distributed into a Costar 12-well plastic plate and were incubated at 37°C in a CO_2-incubator for 4 to 5 days in the presence of 500 JU/ml of rh-IL-2. The cells were collected from the plate, washed with medium and used as LAK cells. After confirmation of their LAK activity, the cells were subcultured in the presence of rh-IL-2. LAK cell lines were established by subculturing LAK cells at 3-day intervals, keeping the maximum cell density

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[4] Abbreviations used in this paper: LAK, lymphokine-activated killer; IL-2, interleukin 2; LFA, lymphocyte function-associated antigen; asGM1, asialoGM1; TcR, T cell receptor; CTL, cytotoxic T lymphocyte; ICAM, intercellular adhesion molecule; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; FDA, fluorescein diacetate; HE, hydroethidine; CFC, conjugate-forming cells.

below 1×10^6/ml, and then cloned by a limiting dilution method with the aid of mitomycin C-treated macrophages as a feeder layer.

Antibodies The KBA monoclonal antibody (mAb) reactive with the LFA-1 molecule was prepared from hybridoma cells which had been established in our laboratory as described previously.[7] Anti-LFA-2 (CD2) mAb, RM2-2, anti-T3ε (CD3) mAb, 145-2C11, and anti-T200 (CD45) mAb, RA3-6B2, were kindly donated by Dr. K. Okumura, Medical School, Juntendo University, Tokyo. FITC-conjugated anti-mouse Lyt 2 (CD8) and Thy 1.2 mAbs were purchased from Becton Dickinson (Mountain View, CA).

Assays for cell surface phenotypes and cytotoxicity A cell pellet of 10^6 cells was incubated with FITC-conjugated mAbs for 1 h at 4°C. After incubation, the cells were washed 3 times with PBS and then analyzed on a FACScan (Becton Dickinson).

Target cell lysis was assessed by the ^{3}H-uridine method as reported previously.[8] Briefly, target cells were incubated overnight at 37°C in RPMI 1640 medium containing 10% FCS fortified with 2 μCi/ml of ^{3}H-uridine (specific activity 28 Ci/mmol, Amersham Japan, Tokyo). After 3 washings with PBS, the cells were suspended in medium at 5×10^4 cells/ml. Various numbers of LAK cells were mixed with 5×10^3 target cells in a final volume of 0.2 ml and distributed to wells of a Costar No. 3799 96-well U-bottomed plastic plate, and the cell mixtures were cultured in the presence or absence of mAb for 18 h at 37°C. After incubation, cells were collected by the use of a Skatron cell harvester, and the radioactivity that remained in the cells was measured by a standard liquid scintillation technique. The percentage of target cell lysis was calculated from the following equation:

%Cytotoxicity$=100-100\times$cpm of target cells cultured with effector cells/cpm of target cells cultured alone.

Assay for conjugate-forming cells Target cells (1×10^7) were labeled with 10 ng/ml of hydroethidine (HE) (Polysciences Inc., Warrington, PA) in RPMI 1640 medium containing 10% FCS for 15 min at 37°C. Effector cells (5×10^6) were treated with 20 μg/ml of azidofluorescein diacetate (azido-FDA; Wako Pure Chemical Industries, Osaka) in FCS-free RPMI 1640 medium for 10 min at 37°C. After 3 washings with PBS, the fluorescently labeled effector cells (5×10^5) were mixed with target cells (2.5×10^6) in 0.5 ml of RPMI 1640 medium containing 10% FCS and centrifuged at 80g. After incubation for 15 min at 37°C, the cells were gently resuspended and analyzed on a FACScan. The value of percent conjugate-forming cells (CFC) was calculated from the following formula:

%CFC$=100\times$number of fluorescent cells bound to target cells/number of all fluorescent cells

RESULTS

Effect of culture time on the expression of CD2 in LAK cells LAK cells were induced from splenocytes and the expression of cell surface molecules on LAK cells was analyzed by flow cytometry. The results are shown in Fig. 1. When splenocytes were cultured for 10 days in the presence of IL-2, the induced LAK cells expressed a greater amount of CD2 as compared with the original spleen cells. However, if the LAK cells were further cultured in the presence of IL-2, the cell surface CD2 tended to decrease and at 90 days after culture (90-day LAK), CD2 was no longer detected. The LAK cell lines established from the 90-day LAK also lacked CD2.

In addition to CD2, LAK cells expressed Thy 1, CD3, CD8 and LFA-1 but these phenotypes were not significantly altered by culture as manifested by the FACS profiles of LAK cells cultured for 10 or 30 days (Fig. 2).

Decrease in cytolytic activity of LAK cells upon long-term culture To determine whether the change of CD2 expression affects the ability of LAK cells to bind and exhibit cytotoxicity to target cells, we examined the cytolytic activities of the 10-day and 30-day LAK cells against FBL-3, RL♂1, P815, YAC-1 and Meth A by the ^{3}H-uridine method. Although the activity of LAK cells significantly declined as the culture time was prolonged, the change of the activity was different among the target cells used (Table I). The 90-day LAK cells had almost lost cytotoxic activity against MBL-2, FBL-3 and Meth

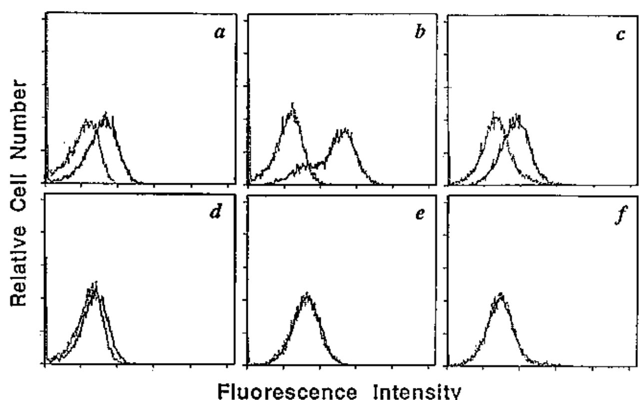

Fig. 1. Expression of CD2 on murine splenocytes and LAK cells at various culture periods. Murine splenocytes (a) and LAK cells, cultured with IL-2 for 10 days (b), 30 days (c), 60 days (d), 90 days (e) and 1 year (f), were surface-labeled with anti-CD2 mAb (RM2-2, 1 μg) followed by fluorescein isothiocyanate (FITC)-labeled anti-rat immunoglobulins. Samples were analyzed on a FACScan. Fluorescence profiles shown in solid lines represent the cells treated with RM2-2. Control cells were treated with the second antibody only (dotted lines).

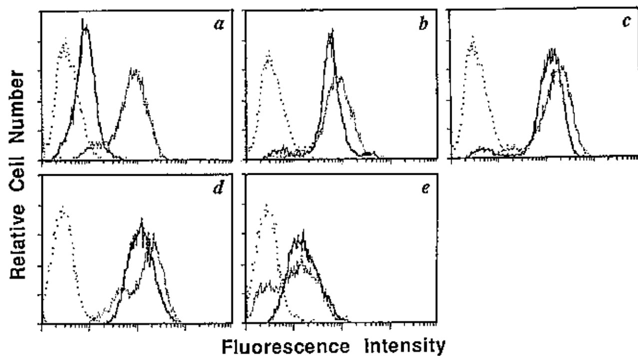

Fig. 2. Expression of various surface molecules on LAK cells. LAK cells, cultured for 10 days (thin lines) and 30 days (thick lines), were stained with FITC-conjugated anti-CD2 mAb (a), anti-LFA-1 mAb (b), anti-CD3ε mAb (c), anti-Thy-1.2 mAb (d) and anti-CD8 mAb (e). The dotted lines represent autofluorescence in the absence of mAb.

Table I. Effect of Culture Period on Cytolytic Activity of LAK Cells

Target cells	% Cytotoxicity[a] (E/T=10)		
	10-day LAK[b]	30-day LAK	90-day LAK
FBL-3	79.0±2.8[c]	26.4±5.7	6.3±3.3
RL♂1	68.3±5.1	46.2±3.3	28.0±6.9
P815	72.4±1.0	40.8±5.1	21.5±5.4
YAC-1	66.7±2.0	23.8±5.8	ND[d]
Meth A	53.7±1.2	7.0±4.7	4.4±3.6

a) Cytotoxicity assay was performed by the ^{3}H-uridine method for 16 h as described in "Materials and Methods."
b) LAK cells were induced from murine splenocytes with IL-2 and cultured for 10 days, 30 days and 90 days.
c) Mean±SD of triplicates, expressed as percent cytotoxicity.
d) Not done.

A cells, but retained the activity towards RL♂1 and P815 cells. Similar results were also obtained in a 4-h ^{51}Cr-release assay (data not shown).

Decrease in target-binding ability of LAK cells upon long-term culture To examine the correlation between cytotoxic activity of LAK cells and expression of CD2 on LAK cells, we investigated the binding ability of LAK cells expressing a relatively large amount (10-day LAK) or an undetectable amount of CD2 (60-day LAK). The results are depicted in Fig. 3. Binding capacity (conjugate-forming cells) of 10-day LAK cells was larger than that of 60-day LAK cells for all target tumor cells tested, especially for FBL-3, P815 and Meth A. Similar results were obtained when conjugate-forming cell assay was performed at 4°C (data not shown).

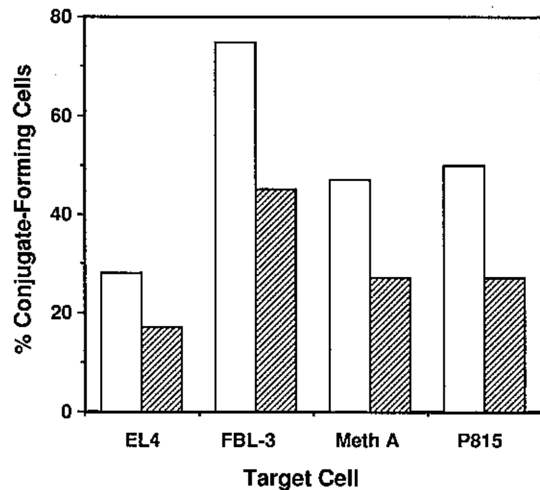

Fig. 3. Conjugate formation of LAK cells with various murine tumor cell lines. Azido-fluorescein diacetate (azido-FDA)-labeled LAK cells (10d LAK, open bars; 60d LAK, shaded bars) were incubated with hydroethidine (HE)-labeled tumor cell lines for 15 min at a target:effector ratio of 5:1 as described in "Materials and Methods." LAK cells conjugated with target cells were analyzed on a FACScan and calculated by using the Consort 30 program.

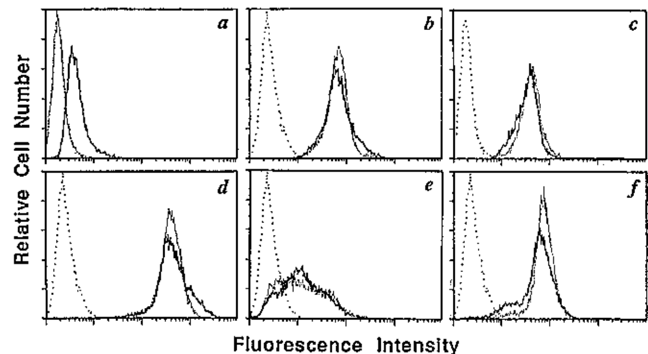

Fig. 4. Expression of various surface molecules on LAK cell clone ESK1.3-1 (thin lines) and CD2-transfected LAK cell clone CD2.7 (think lines). Cells were stained with FITC-conjugated anti-CD 2 mAb (a), anti-LFA- 1 mAb (b), anti-CD3ε mAb (c), anti-Thy1.2 mAb (d), anti-CD8 mAb (e) and anti-T200 mAb (f). The dotted lines represent autofluorescence in the absence of mAb.

Target cell binding and cytotoxicity of CD2 gene-transfected LAK cell clones The cDNA coding for murine CD2 was introduced by electroporation into the ESK1.3-1 LAK cell clone which practically lacked CD2 expression.[9] Transfected cells were selected for their resistance to G418 and analyzed for the surface expres-

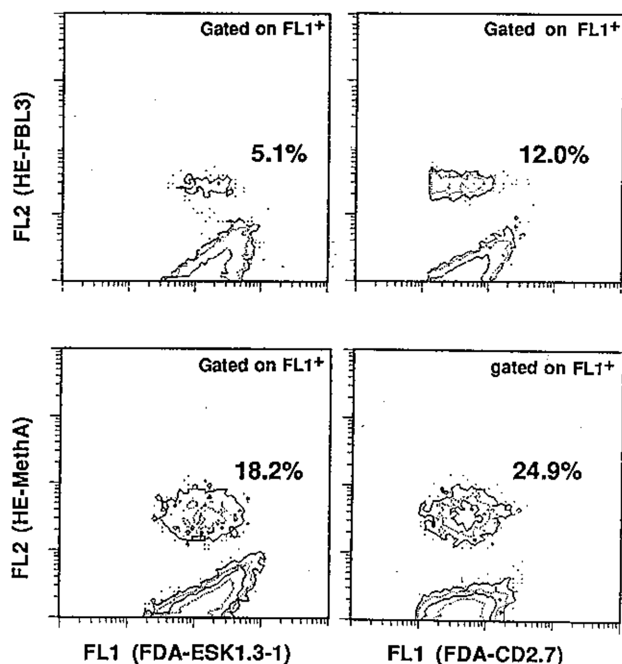

Fig. 5. Conjugate formation of CD2-negative or positive LAK cell clones against tumor cell lines. FDA-labeled ESK-1.3-1 cells (left) or CD2.7 cells (right) were incubated with HE-labeled FBL-3 (upper) or Meth A (lower) at a cell ratio of 5:1 as described in "Materials and Methods" and analyzed on a FACSan. The percentages of LAK cells conjugated with target cells (positive in both fluorescence intensities; $FL1^+$ and $FL2^+$) represented in the figures were calculated by using the Consort 30 program and are indicated in the figures.

sion of CD2 by an indirect immunofluorescence assay using anti-CD2 mAb, RM2-2. One *CD2*-transfected cell line was cloned by limiting dilution and two CD2-positive clones were obtained. These clones, termed CD2.3 and CD2.7, grew in medium containing IL-2. Except for CD2, the phenotypes of CD2.7 cells and the parent ESK1.3-1 cells were similar (Fig. 4).

A *CD2*-transfected LAK cell clone, CD2.7 cells and the parental CD2-negative ESK1.3-1 cells were assayed for their binding ability with FBL-3 and Meth A target tumor cells. The results are shown in Fig. 5. When FBL-3 cells were used as a target, the percentage of conjugate formation of CD2.7 cells (12.0%) was greater than that of ESK1.3-1 cells (5.1%). A similar result was obtained using Meth A as a target. The treatment of these target tumor cells with RM2-2 mAb inhibited the binding capacity of CD2.7 cells. These results indicated that increase of the binding capacity in CD2.7 cells is attributable to the transfected *CD2* gene coding for the cell surface CD2 molecule.

Table II. Effect of *CD2* Gene Transfection on Cytolytic Activity of LAK Cell Clones

Target cells		% Cytotoxicity[a] (E/T=10)	
		ESK1.3-1	CD2.7
Exp.1	YAC-1	6.9±0.1[b]	13.2±2.3
	Meth A	22.2±2.1	40.2±5.1[c]**
Exp.2	FBL-3	15.9±3.0	22.0±1.9*
	Meth A	14.4±2.1	23.2±2.3**

a) Cytotoxicity assay was performed by the ^{3}H-uridine method for 16 h.
b) Mean±SD of triplicates.
c) Significant differences between ESK 1.3-1 and CD 2.7 (* $P<0.05$, ** $P<0.01$ by Student's *t* test).

We next examined the cytolytic activity of CD2.7 and ESK1.3-1 cells. The results are summarized in Table II. When YAC-1, FBL-3 or Meth A cells were used as targets, CD2.7 cells showed stronger cytotoxicity than ESK1.3-1 cells, especially for Meth A cells, indicating that increase in the CD2 expression on LAK cells augmented the activity of LAK cells in terms of both target cell binding and killing.

DISCUSSION

The principal adhesion molecule on LAK cells responsible for target cell killing is indicated to be LFA-1, which is commonly expressed on the surface of LAK cells, NK cells and killer T cells.[10-12] In addition to LFA-1, CD2 has been shown to mediate T cell adhesion.[10, 11, 13-15] CD2 is one of the markers of intrathymic maturation,[16] and is present on T cells, NK cells and murine B cells.[17, 18] Anti-CD2 mAb is able to inhibit the conjugate formation between CTL or NK cells and target cells,[19, 20] indicating the involvement of CD2-mediated adhesion in effector-target cell interaction. Moreover, it is known that CD2 plays a role in T cell activation, and stimulation of T cells with anti-CD2 mAb causes the translocation of protein kinase C from cytosol to cell membrane,[21] the increase of intracellular calcium level,[22] the regulation of T cell proliferation and the induction of nonspecific cytolysis by CTL and NK cells.[19, 23-25] These observations are, however, restricted to human and rat systems because of the lack of appropriate mAbs against murine CD2, with the exception of RM2-2 mAb prepared by Yagita *et al.*[26] RM2-2 is reactive with CD2 but does not inhibit the adhesion of $CD2^+$ lymphocytes to target cells.

To determine the role of murine CD2 in LAK cell function, we analyzed the relationship between expression of CD2 and cytolytic activity of LAK cells and

found that both binding ability and cytolytic activity of murine LAK cells decrease during long-term culture. As to the changes of adhesion molecules and cytotoxic molecules in LAK cells during long-term culture, only the expression of the CD2 molecule decreased, while neither LFA-1 nor cytolytic molecules such as serine esterase[27] and perfolin[28] decreased. Like culture lines, established LAK cell clones were completely negative in the expression of cell surface CD2, but retained other molecules at the level of fresh LAK cells.

Since $CD2^-$ murine LAK cell clones retained the LAK activity, CD2 seemed not to be a major cell surface molecule responsible for target cell killing by LAK cells. However, the ability to bind target cells and the cytolytic activity of LAK cells apparently decreased in parallel with the decrease of CD2 expression. Therefore, CD2 appeared to be a second-order adhesion molecule in the murine LAK cells, if LFA-1 is classified as a first-order adhesion molecule. This was confirmed by the introduction of CD2 gene into $CD2^-$ LAK cell clones. As expected, introduction of CD2 gene resulted in emergence of CD2 molecules and augmented the cytotoxic activity of $CD2^-$ LAK cells. This observation also strongly supported the hypothesis that CD2 is not essential for murine LAK cells, but enhances the LFA-1-mediated target cell binding and killing. It seems important to investigate whether or not the amount of LFA-3, a target molecule of CD2, on target cells also controls the binding and killing activity of LAK cells. However, we could not perform such an investigation, because a mAb that recognizes murine LFA-3 is not yet available.

ACKNOWLEDGMENTS

We thank Dr. K. Okumura, Medical School, Juntendo University, for providing several kinds of mAbs used. This work was supported in part by a Grant-in-Aid for Special Project Research in Cancer-Bioscience (No. 62614502) from the Ministry of Education, Science and Culture, Japan.

(Received June 21, 1991/Accepted July 22, 1991)

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