Establishment of Mouse Lymphokine-activated Killer Cell Clones and Their Properties

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To assess the properties of lymphokine-activated killer (LAK) cells, we established mouse LAK cell clones from LAK cell lines induced from C57BL/6 mouse spleen cells. Although these clones expressed similar phenotypes to the parent LAK cells, Lyt-2 was expressed in a restricted portion of the clones. All clones were found to express T3 CD_3 and T cell receptor (TcR) $\alpha\beta$ on their cell surface. Rearrangement patterns of TcR β were the same among the clones derived from the same parent cell line but differed in those from different cell lines as determined by using $C\beta_1$ and $J\beta_2$ probes. The molecules responsible for LAK-target cell binding were examined by using a monoclonal antibody (mAb) against lymphocyte function associated antigen 1 (LFA-1). This mAb (termed KBA) showed inhibitory effects on both LAK-target cell binding and cytolytic activity of LAK cell clones, indicating a principal role of LFA-1 in LAK cell clones. The magnitude of perforin mRNA expression in LAK cell clones was unrelated to their cytolytic activities.

Key words: Lymphokine-activated killer cell clone — T cell receptor $\alpha\beta$ — Lymphocyte function-associated antigen 1 — Perforin

Lymphokine-activated killer (LAK[4]) cells are inducible from lymphoid cells of experimental animals and humans by stimulating them with interleukin 2 (IL2). They show strong cytotoxicity to a variety of tumor cells of the same and different species of animals. The identified phenotypes of LAK cells are not always consistent in various papers[1-6] which stated that LAK cells mainly consist of either T cell type or natural killer (NK) cell type of lymphocytes. This could be ascribed to the differences in their cell source and culture conditions for the derivation. Since LAK cells are polyclonal cell populations consisting of multiple killer cells of different lymphoid cell lineages, it is necessary to determine the properties of the cell population at a clonal level. Moreover, to understand precisely the *in vivo* function of LAK cells, it is desirable to establish LAK cell clones from experimental animals.

In an attempt to better define the characteristics of cells expressing LAK activity, we have derived several LAK cell clones from mouse spleen cells and thymocytes and analyzed their LAK activities, surface phenotypes, and molecules responsible for target cell binding and killing.

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[4] Abbreviations: LAK, lymphokine-activated killer; IL2, interleukin 2; NK, natural killer; LFA-1, lymphocyte function-associated antigen 1; TcR, T cell receptor; CFC, conjugate-forming cells; LGL, large granular lymphocyte; CTL, cytotoxic T lymphocyte; r-ADCC, reverse-type antibody-dependent cellular cytotoxicity.

MATERIALS AND METHODS

Animals Male C57BL/6 mice were obtained from Shizuoka Agriculture Cooperative Association for Laboratory Animals, Hamamatsu, and used at ages of 7–8 weeks.

Interleukin 2 Recombinant human IL2 (rh-IL2) 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.

Culture medium In all cell cultures, we used RPMI 1640 medium containing penicillin G at 100 unit/ml, kanamycin sulfate at $60\,\mu$g/ml, 1 mM sodium pyruvate, 2 mM 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).

Derivation of LAK cells, LAK cell lines and the clones Aliquots (1 ml) of cell suspensions containing fresh splenocytes or thymocytes at 2.5×10^6 cells/ml were placed into wells of a Costar 12-well plastic plate and were incubated in a CO_2 incubator for 4 to 5 days at 37°C in the presence of rh-IL2 at 500 JU/ml. The cells collected from the plate were washed with medium and used at LAK cells. After confirmation of their LAK activity, the cells were subcultured in the presence of rh-IL2. LAK cell lines were established by subcultures of LAK cells at 3-day intervals by keeping the maximum cell density below 1×10^6 cells/ml and then cloned by a limiting dilution method with or without the help of a feeder layer of mitomycin C-treated C57BL/6 macrophages.

Tumor cells Mouse cell lines, FBL-3 (H-2^b), MBL-2 (H-2^b), YAC-1 (H-2^a) lymphomas, Meth A fibrosarcoma (H-2^d), BW5147 thymoma (H-2^k), P815 mastocytoma (H-2^d) and MOLT-4 human T lymphoma were used. These tumor cell lines were maintained by *in vitro* culture.

Antibodies The KBA mAb reactive with the LFA-1 molecule was prepared from hybridoma cells wich had been established in our laboratory as described previously.[7] Anti-LFA-2 (CD2) mAb, RM2-2, anti-T3(CD3)ε mAb, 145-2C11, and anti-IL2 receptor (CD25) mAb, 3C7, were kindly donated by Dr. K. Okumura, Medical School, Juntendo University, Tokyo. Anti-L3T4 (CD4) mAb, GK1.5, anti-MHC class II (Ia) mAb, FITC-conjugated anti-Lyt-2 (CD8) and Thy1.2 mAbs were purhased from Becton Dickinson (Mountain View, CA).

Gene probes cDNAs used were as follows: cDNA coding for murine TcR α chain, 1280 bp EcoRI fragment from pTα816,[8] (provided by K. Imai); for TcR β chain, 660 bp EcoRI fragment from 86T5[9] (provided by M. Davis); for TcR γ chain, 1400 bp EcoRI fragment from 8/10-2γ1.1,[10] (provided by T. Mak); for TcR δ chain, 430 bp EcoRI-XbaI fragment containing Cδ region,[11] (provided by Y. Yoshikai); and for murine perforin (1630 bp EcoRI-EcoRV fragment) from λPFP-7.[12] They were subcloned into the Bluescript SK plus vector (Stratagene) containing T3 and T7 promoters. ^{32}P-Labeled single-stranded (ss) RNA probes complementary to mRNA were prepared from the linearized plasmids by using T3 or T7 polymerase. Murine TcR Jβ2 genomic DNA probe, 2.3 kbp EcoRI fragment containing Jβ2 region[13] and murine TcR Cβ1 cDNA probe, 660 bp EcoRI fragment from 86T5, were ^{32}P-labeled by random priming as described.[14]

Assay for cell surface phenotypes A cell pellet of 10^6 cells was incubated with mAbs for 1 h at 4°C followed by FITC-conjugated anti-rat IgG antibody (Tago Inc., Burlingame, CA) or anti-hamster Ig antibody (Cappel Co., West Chester, PA). After 3 washings, the cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) and data were processed by using the Consort 30 program.

Cytotoxicity assay Destruction of target cells was assessed by the ^{3}H-uridine method as reported previously.[15] Briefly, target cells were incubated overnight at 37°C in RPMI 1640 medium containing 10% FCS with 2 μCi/ml of ^{3}H-uridine (^{3}H-UdR; specific activity 28 Ci/mmol, Amersham Japan, Tokyo), washed 3 times with PBS and adjusted to 5×10^4 cells/ml in culture medium. Various numbers of effector cells were mixed with 5×10^3 target cells (a final volume of 0.2 ml) in a Costar No. 3799 96-well U-bottomed plastic plate, and the cell mixture was cutured for 18 h at 37°C in the presence or absence of mAb. After incubation, cells were collected by use of a Skatron cell harvester, and their radioactivity

was measured by a standard liquid scintillation technique. The percentage of target cell lysis was calculated from the following equation:

$$\text{Cytotoxicity } (\%) = \{1 - (\text{cpm of target cells cultured with effector cells/cpm of target cells cultured alone})\} \times 100.$$

Assay for conjugate-forming cells Target cells (1×10^7) were labeled with 10 ng/ml of hydroethidine (HE; Polysciences Inc., Warrington, PA) in culture medium for 15 min at 37°C. Effector cells (5×10^6) were labeled with 20 μg/ml of azidofluorescein diacetate (azido-FDA) (Wako Pure Chemical Industries, Osaka) in RPMI 1640 medium without FCS 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 culture medium and centrifuged at 80g. After incubation for 15 min at 37°C, the cells were gently resuspended and analyzed on a FACScan. The percentage of conjugate-forming cells (CFC) was calculated by using the following formula:

$$\text{CFC } (\%) = (\text{number of fluorescent cells bound with target cells/number of all fluorescent cells}) \times 100.$$

Northern and Southern blot analyses Northern and Southern blots were performed as described previously.[14] For Nothern blotting, RNA transferred onto filters was hybridized overnight with ^{32}P-labeled single-stranded RNA probes complementary to each mRNA at 65°C. The filters were washed 4 times at 65°C and then subjected to autoradiography.

For Southern blotting, 10 μg of genomic DNA digested with HindIII was subjected to electrophoresis in 0.8% agarose. After transfer of DNA in a gel sheet to a nylon membrane filter, DNA was hybridized overnight with murine TcR Cβ1 cDNA probe or with murine TcR Jβ2 genomic DNA probe, and then ^{32}P-labeled by random priming at 65°C. The filter was washed twice at 65°C and then subjected to autoradiography.

RESULTS AND DISCUSSION

Establishment of LAK cell clones Although human LAK cell clones have been established by several laboratories,[16-18] attempts to prepare LAK cell lines and clones from murine lymphoid cells have been unsuccessful. However, by devising suitable culture conditions, we were able to establish LAK cell lines and clones from mouse lymphocytes. LAK cells were induced from spleen cells or thymocytes of C57BL/6 mice in the presence of IL2. After confirmation of their LAK activity, the cells were subcultured in the presence of IL2. The interval of subcultue or cell density in culture was critical for estab-

lishment or LAK cell lines. Subculture with an interval of more than 5 days did not lead to LAK cells capable of further passages. By shortening the subculture interval to 3 days and keeping the maximum cell density below 1×10^6 cells/ml, we obtained subcultured LAK cells which sustained the growth activity in the presence of IL2. LAK cell clones were prepared from the established LAK cell lines by a limiting dilution method. The cell lines termed 4SL and MSL626 were cloned at 102 and 90
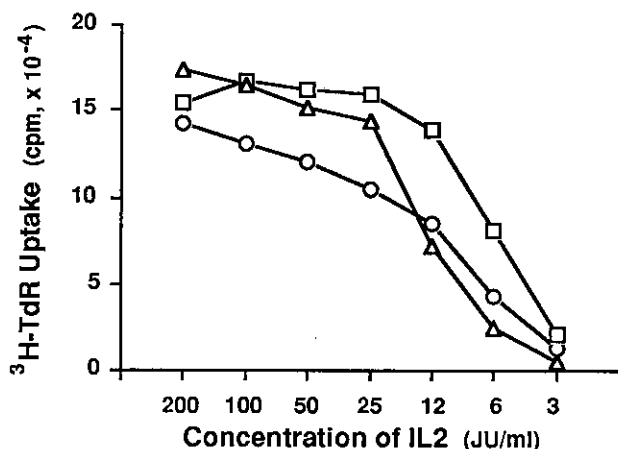

Fig. 1. IL2-dependent cell growth of mouse LAK cell clones. LAK cell clones, ESK1.3-1 (□), ESK2.2 (○), ESK2.5 (△), were cultured with serially diluted recombinant IL2 for 44 h and then pulsed with ³H-thymidine for 4 h.

days after starting the LAK cell cultures, respectively. We obtained 4 clones, termed ESK1.1 to ESK1.4, from the 4SL line by culture using mitomycin C-treated syngeneic macrophages as a feeder layer, and 2 of them were recloned by day 221. Cloning of MSL626 was successful without a feeder layer and we obtained 7 clones (ESK2.1 to ESK2.7) from the cell line. From thymocytes, we obtained one clone (ETK1.1). All LAK cell lines and clones were morphologically large granular lymphocytes (LGL) containing azure granules. LAK clones required IL2 for cell growth and maintenance (Fig. 1).

Phenotypes of LAK cell clones The phenotypes of LAK cell lines and clones were determined by flow cytometry. The results are summarized in Table I. All cell clones as well as the parent cell lines were found to express Thy 1.2, T3 (CD3), and IL2 receptor (CD25) as common phenotypes, but not L3T4 (CD4). As to other lymphocyte markers, Lyt-2 (CD8) was expressed only in ESK1.3, ESK2.2 and ESK2.6 clones, major histocompatibility complex (MHC) class II (Ia) antigen in ESK2.5, ESK2.6, and ESK1.3 (week), and a low level of asialo-GM1 in 8 cell clones other than ESK1.2 and ESK2.1 clones. As to cell surface adhesion molecules, LFA-1 was strongly expressed on all the LAK cell clones, but LFA-2 (CD2) was undetectable. These findings indicated that all established murine LAK cell clones are of T cell type but differ in the expression of CD8 and MHC class II antigen.

Cytolytic activities of LAK cell clones against various tumor target cell lines Eleven LAK cell clones derived from mouse splenocytes or thymocytes were analyzed for

Table I. Surface Marker Analysis of LAK Cell Clones

LAK clones	Surface antigen[a]						
	T3	Thy1.2	L3T4	Lyt-2	LFA-1	IL2R	Ia
ESK1.3-1[b]	+	+	−	+	+	+	+
ESK1.3-2	+	+	−	+	+	+	+
ESK1.4-1	+	+	−	−	+	+	−
ESK2.2	+	+	−	+[c]	+	+	−
ESK2.6	+	+	−	+[c]	+	+	+
ESK2.1	+	+	−	−	+	+	−
ESK2.3	+	+	−	−	+	+	−
ESK2.4	+	+	−	−	+	+	−
ESK2.5	+	+	−	−	+	+	+
ESK2.7	+	+	−	−	+	+	−
ETK1.1[d]	+	+	−	−	+	+	−

a) Surface antigen expression was analyzed by using a FACS analyzer.
b) ESK clones were derived from LAK cells generated from C57BL/6 spleen cells.
c) Not all the cells expessed Lyt-2 antigens.
d) ETK clone was derived from LAK cells generated from C57BL/6 thymus cells.

Table II. Cytotoxicity of LAK Cell Clones against Various Target Cells

LAK cell clones	% Cytotoxicity[a]					
	FBL-3	MBL-2	Meth A	YAC-1	BW5147	MOLT-4
ESK1.1	22	nt[b]	23	14	2	nt
ESK1.2	0	nt	24	14	20	nt
ESK1.3	1	nt	28	20	39	nt
ESK1.4	12	nt	28	7	1	nt
ESK2.1	51	60	18	38	31	nt
ESK2.2	38	57	5	39	65	23
ESK2.3	34	67	nt	19	38	35
ESK2.4	61	60	nt	34	49	26
ESK2.5	78	56	30	58	77	24
ESK2.6	80	68	5	62	78	22
ESK2.7	65	27	nt	23	38	nt

a) Effector-to-target ratio was 10:1.
b) Not tested.
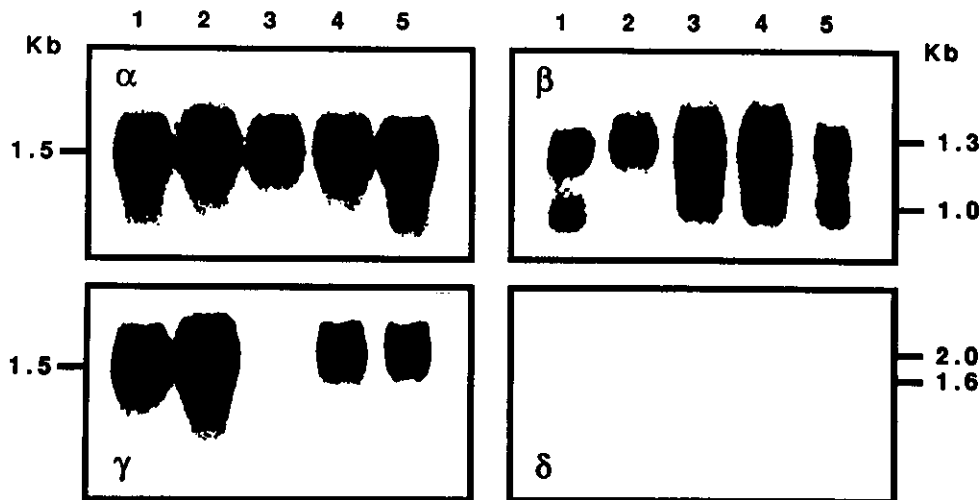
Fig. 2. Northern blot analysis for TcR transcripts in mouse LAK cell clones. Ten μg each of cytoplasmic RNA prepared from the murine LAK cell clones: ESK1.3-1 (lane 1), ESK1.3-2 (lane 2), ESK2.2 (lane 3), ESK2.5 (lane 4) and ESK2.6 (lane 5), were loaded. α, TcR α; β, TcR β; γ, TcR γ; δ, TcR δ. Sizes of the transcripts are indicated in kb at left or right.

their cytolytic activity aginst a panel of mouse or human tumor target cells. The results are shown in Table II. Individual LAK cell clones lysed multiple target tumor cells of mouse and human origins including NK-resistant cell lines, though the activities toward target tumor cell lines were sligtly different among each LAK cell clone.

Expression of TcR $\alpha\beta$ in murine LAK cell clones and TcR β gene rearrangement Recently, several human T cell clones and lines bearing TR $\gamma\delta$ have been demon-

strated to exhibit an anomalous killer activity,[19-25] and Yagita et al. also observed that the majority of human LAK cells cultured for long periods bore TcR $\gamma\delta$ (unpublished data). Since all our murine LAK cell clones expressed T3 (CD3), it is likely that they expressed TcR on their cell surface. Therefore, we examined the type of TcR expressed in our murine LAK clones by Northern blot analysis for TcR α, β, γ and δ transcripts (Fig. 2). The TcR α transcript of 1.5 kb and the TcR β transcript

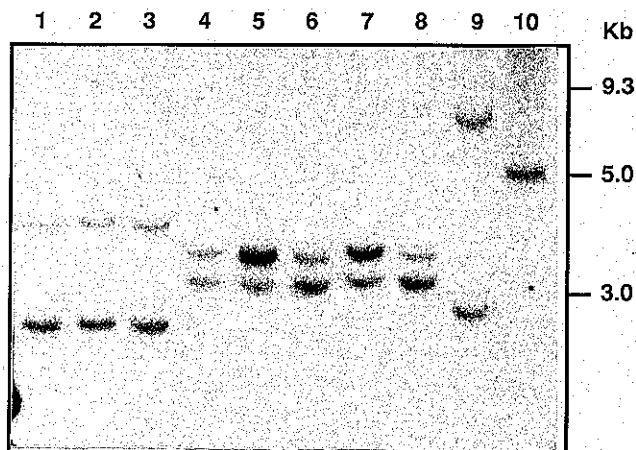Fig. 3. Southern blot analysis of DNA from LAK cell clones using Jβ2 probe of TcR. DNA from LAK cell clones, ESK1.3-1 (lane 1), ESK1.3-2 (lane 2), ESK1.4-1 (lane 3), ESK2.2 (lane 4), ESK2.5 (lane 5), ESK2.6 (lane 6), ESK2.2-1 (lane 7), ESK2.6-1 (lane 8), ETK1.1 (lane 9) and liver (lane 10), were digested with HindIII, electrophoresed, transferred to a nylon filter, and hybridized with a ^{32}P-labeled Jβ2 probe.
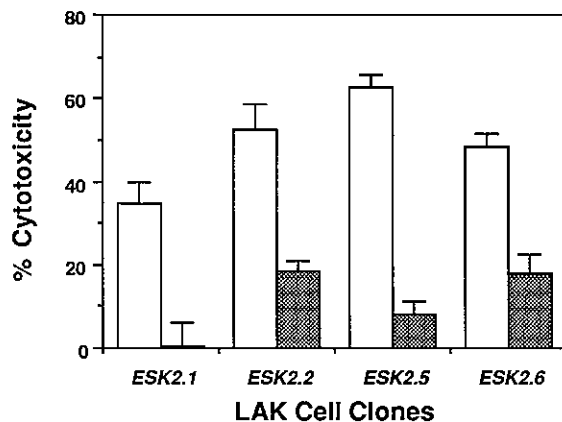

Fig. 5. Inhibitory effect of anti-LFA-1 mAb (KBA) on cytolytic activities of various LAK cell clones. LAK cell clones were cocultured with ^{3}H-uridine-labeled MBL-2 for 18 h in the presence (▨) or absence (□) of KBA mAb and their cytotoxicity was measured. Assays were performed with triplicate samples and the standard deviations are shown as bars. The effector-to-target ratio was 10.
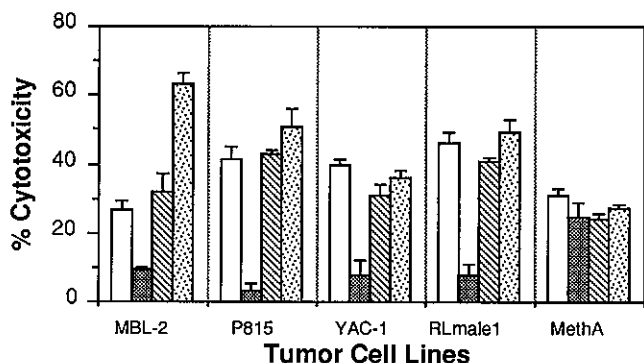

Fig. 4. Effect of monoclonal antibodies on the cytolytic activity of mouse LAK cell clone against various murine tumor cells. LAK cell clone, ESK2.2, was assayed using ^{3}H-uridine-labeled target cells in triplicate. MAb to LFA-1 (KBA, ▨), Thy1.2 (4A3, ▧) and T3 (145-2C11, ▥) were added at the initiation of the assay. □, No antibody. The effector-to-target ratio was 10.

of 1.3 kb were abundant in all the cell clones examined. TcR γ transcript, but not TcR δ transcript, was detected from 4 out of 5 cell clones. These results clearly indicated that the murine LAK clones express TcR αβ, but not TcR γδ, on their cell surface.

The DNA extracted from LAK cell clones was treated with HindIII and then subjected to Southern blotting using Cβ1 and Jβ2 cDNAs as probes. With both probes, all LAK cell clones showed the rearranged TcR β gene as

compared to the liver DNA (Fig. 3). The rearrangement patterns were the same among the cell clones derived from the same LAK cell line but different among the clones from different LAK cell lines. This finding suggests two possibilities; 1) LAK cell clones derived from a LAK cell line might have originated from one precursor clone or 2) these LAK cell clones might have originated from different precursor clones haveing TcR gene with the same β gene rearrangement pattern. Although the second possibility is unlikely, it will be necessary to establish LAK cell clones from several different LAK cell lines induced from the same individual and to compare their rearrangement patterns of the TcR gene, in order to check it.

Role of LFA-1 molecule in LAK cell-mediated tumor cell killing Because it is well known that TcR/CD3 complex, CD2, CD8 and LFA-1 molecules are involved in the process of target cell lysis by alloreactive cytotoxic T lymphocytes (CTL),[26-29] we decided to investigate their possible involvement in the target cell lysis by LAK cell clones by using mAbs against these cell surface molecules.

As shown in Fig. 4, anti-T3ε mAb (145-2C11) and anti-Thy1.2 mAb (4A3) did not inhibit cytolysis of YAC-1 and Meth A tumor cells by LAK cell clones, although 145-2C11 mAb could induce reverse-type antibody-dependent cellular cytotoxicity (r-ADCC) toward Fc receptor-bearing target cells such as MBL-2 and P815.[30] In contrast, treatment of ESK2.2 with anti-LFA-1 mAb (KBA) efficiently inhibited the lysis of various target tumor cells (64 to 92% inhibition). How-
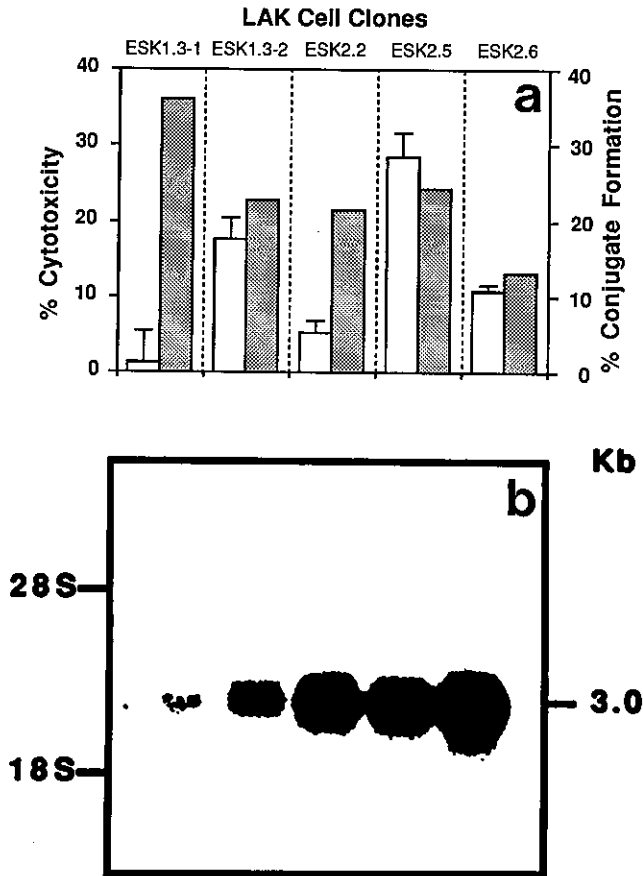
LAK Cell Clones

Relationship between cytotoxic activity, target cell-binding capacity, and expression of a cytotoxic factor, perforin, in LAK cell clones We examined the relationship between cytotoxic activity, target cell-binding capacity, and expression of perforin message of LAK cell clones. The results are illustrated in Fig. 6. In general, the cytotoxic activity of LAK cell clones tended to decrease during long-term culture without any decrease of their binding capacity to target cells (data not shown). Furthermore, although the target cell binding capacity was not much different among the established LAK cell clone, their cytotoxic activities differed significantly (Fig. 6a). Therefore, it was indicated that the difference in target cell binding capacity of the LAK cell clones is not directly related to the cytotoxic activity.

As for the effector molecules responsible for the target cell killing, perforin has been found to be a principal cytotoxic factor of both CTL and NK cells.[31-34] Recently, Shinkai et al. cloned the cDNA encoding murine perforin.[12] With the use of this cDNA, we examined the expression of the perforin mRNA in our LAK cell clones. The blotting patterns revealed that the expression of perforin mRNA was unrelated to either binding ability or cytolytic activity of LAK cell clones (Fig. 6b). Recently, it has been reported that interaction of LAK cells with target cells does not induce the exocytosis of cytolytic granules containing perforin and serine esterase.[35] Therefore, it is suggested that perforin is not a principal cytolytic factor of the LAK cell clones in their direct target cell killing and that probably another unknown cytolytic molecule might be involved in the LAK cell-mediated target cell destruction.

ever, the inhibition was less evident when Meth A was used as target cells (20% inhibition). Similar results were also obtained when other LAK cell clones were used as effector cells (Fig. 5). Therefore, the ability of KBA to inhibit cytolysis seems to be dependent upon the type of target cells rather than the type of effector cells. These findings indicate that LFA-1 plays a leading role in the LAK cell-mediated cytotoxicity, rather than in antigen-specific CTL cytotoxicity.

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