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T Cell Receptor-independent Cell-mediated Cytotoxicity by Nude Mouse Lymphokine-activated Killer Cells

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Lymphokine-activated killer (LAK) cells, which can lyse a variety of tumor cells, can be induced from both normal and athymic nude mouse spleen cells by culture with high doses of recombinant interleukin 2 (rIL-2). LAK cells generated from nude mouse spleen cells (Nude-LAK cells) express just Thy 1.2 antigen, but not CD4 and CD8 antigens. Nude-LAK cells express neither T3 molecule, T cell receptor (TCR) $\alpha\beta$ nor TCR $\gamma\delta$ on their cell surface. The lack of TCR expression on Nude-LAK cells was confirmed by the results of northern blot analysis. LAK cells generated from normal mouse spleen cells (Nor-LAK) express TCR α, β transcripts, while Nude-LAK cells express only sterile TCR β transcript, but not TCR α transcript. TCR $\gamma\delta$ transcripts were scarcely detected in both Nor-LAK cells and Nude-LAK cells. Thus, it is strongly suggested that Nude-LAK cells can recognize and lyse tumor cells by TCR-independent mechanisms. Monoclonal antibody against lymphocyte function-associated antigen (LFA-1) molecule can block the cytotoxicity of Nude-LAK cells, indicating an important role of such accessory molecules in Nude-LAK cell-mediated cytotoxicity.

Key words: LAK cell — Nude mouse — T cell receptor — Lymphocyte function associated-antigen 1

It has been demonstrated that recombinant interleukin 2 (rIL-2)[7] induces the generation of lymphokine-activated killer (LAK) cells, which can lyse a variety of tumor cells, including natural killer (NK)-resistant fresh solid tumor cells.[1-4] LAK cells show a strong antitumor activity both *in vitro* and *in vivo* and adoptive transfer of LAK cells with rIL-2 was successful in experimental tumor therapy.[5-7] Moreover, an initial clinical trial has indicated that LAK cells may also be important in the treatment of human cancer.[8] Since LAK cells are functionally defined as IL-2-induced cytotoxic effector cells capable of lysing a variety of tumor cells,[1,2] the characteristics of LAK cells and their precursors have been a subject of controversy.[9-11] However, it has recently been accepted that LAK cells and their progenitors are heterogenous and LAK cells are inducible from both T lineage cells (T-LAK) and NK lineage cells (NK-LAK).[3,12,13)]

An important issue in connection with the LAK cell phenomenon is to understand how LAK cells recognize and kill a variety of tumor cells without presensitization. However, the mechanisms of LAK cell-mediated cytotoxicity remain unclear, although it is known that lymphocyte function-associated antigen-1 (LFA-1) is an important molecule for the binding between LAK and tumor cells.[13,14] Recent reports have demonstrated that some IL-2-dependent cytotoxic T lymphocytes (CTL) and NK-like cells expressing T cell receptor (TCR) $\gamma\delta$ are cytotoxic to a broad spectrum of tumor cells.[15-17] These results strongly suggested that TCR $\gamma\delta$ complex was a second receptor essential for the recognition of broad-reactive killer cells, such as LAK, NK and non-specific CTL. However, it is unclear whether TCR are involved in LAK cell-mediated cytotoxicity.

As reported previously,[18-28] we have demonstrated that LAK cells could be induced from immature thymocyte subpopulations ($CD4^-8^-$) and nude mouse spleen cells, which might express TCR $\gamma\delta$ complex.[21-23] Therefore, it was of interest to investigate the characteristics of LAK cells generated from immature lymphocytes. In this paper, we describe the characteristics of LAK cells generated from nude mouse spleen cells (Nude-LAK cells) and discuss whether TCR complexes are involved

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[7] Abbreviations: rIL-2, recombinant interleukin 2; LAK cells, lymphokine-activated killer cells; T-LAK cells, LAK cells generated from T cells; NK-LAK cells, LAK cells generated from NK cells; LFA-1, lymphocyte function-associated antigen-1; TCR, T cell receptor; Nude-LAK cells, LAK cells generated from nude mouse spleen cells; Nor-LAK cells, LAK cells generated from normal mouse spleen cells.

in target cell recognition and destruction by Thy 1^+ $CD4^-8^-$ Nude-LAK cells. Our results strongly suggest that Nude-LAK cells can recognize and lyse a wide spectrum of tumor cells by TCR-independent mechanisms.

MATERIALS AND METHODS

Animals and cell lines Normal or athymic nude BALB/c mice were purchased from SLC (Hamamatsu). Mouse lymphoma RDM-4 (H-2^k) and YAC-1 (H-2^a) cells were maintained in tissue culture using RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 m*M* glutamine and 10 m*M* HEPES.

Generation of LAK cells and cytotoxicity As reported previously,[14, 18-20] LAK cells were generated from normal or nude mouse spleen cells (5×10^6/ml) by culture with 2000 U/ml of recombinant human interleukin 2 (rIL-2; generous gift from Shionogi Pharmaceutical, Osaka) for 4 days at 37°C. After incubation, the LAK cells were harvested and their cytotoxicity against RDM-4 or YAC-1 cells was measured by ^{51}Cr release assay. Briefly, a 50 μl sample of ^{51}Cr-labeled target cells (5×10^4/ml) was mixed with 100 μl of effector cells at various effector-to-target ratios (E/T ratios). After centrifugation at 100*g*, the cells were incubated for 4 h at 37°C. The radioactivity of the culture supernatant (100 μl) was calculated as described previously.[24]

Blocking of LAK cell-mediated cytotoxicity by monoclonal anti LFA-1 antibody Two distinct mAbs (KBA mAb and M17/5.2), which recognize distinct epitopes of LFA-1 molecules, were used in this experiment. When the blocking effect of mAb on cytotoxicity was measured, 50 μl of mAb was added to the culture for cytotoxicity assay. Percentage inhibition of cytotoxicity was calculated by using the following equation: % inhibition of cytotoxicity = (1 − % cytotoxicity in the presence of mAb/% cytotoxicity in the absence of mAb) × 100.

Flow cytometric analysis Monoclonal antibodies, KBA, M17/5.2 (anti-LFA-1), 4A3 (anti-Thy1), GK 1.5 (anti-CD4), 53-6.72 (anti-CD8), H57-597 (anti-TCR $\alpha\beta$), 3A10 (anti-TCR $\gamma\delta$) and 2C11 (anti-CD3), were used in this experiment. In general, cell pellets (10^6) were incubated with mAb for 30 min on ice. The pellets were washed with PBS twice, then 5 μl of undiluted FITC-conjugated anti-rat Ig G antibody, anti-mouse Ig G (Cartago, San Francisco, CA) or anti-hamster Ig G (Cappel, West Chester, PA) was added and the mixture was incubated for 30 min on ice. After being washed twice with PBS, the cells were fixed with 1% paraformaldehyde and analyzed with FACScan. Fluorescence data were collected with logarithmic amplification. For each sample, data on 10,000 volume-gated viable cells were collected.

Probes cDNAs coding for murine TCRα chain (1280 bp *Eco*RI fragment from pT 816 donated by K. Imai), TCRβ chain (660 bp *Eco*RI fragment from 86T5 provided by M. Davis), TCR γ chain (1400 bp *Eco*RI fragment from 8/10-2 1.1 provided by Tak Mak), and TCRδ chain (430 bp *Eco*RI-*Xba*I fragment containing C region provided by Y. Yoshikai) were subcloned into the Bluescript SK plus vector (Stratagene) containing T3 and T7 promotors. ^{32}P-Labeled single-stranded (ss) RNA probes complementary to mRNA were prepared from the linearized plasmids by using T3 or T7 polymerase.

Northern blot analysis Preparation of cytoplasmic RNA, denaturation, electrophoresis in 1% agarose gel containing formaldehyde, and transfer to nitrocellulose filters were performed by standard methods.[25] Filters were hybridized overnight with ^{32}P-labeled ssRNA probes complementary to each mRNA in 50% formamide/6×SSPE (1×SSPE=180 m*M* NaCl/10 m*M* sodium phosphate, pH 7.7/1 m*M* EDTA)/2× Denhardt's solution/0.5% SDS/100 μg/ml salmon sperm DNA at 65°C. The filters were washed 4 times with 0.1×SSPE/0.1% SDS for 15 min each at 65°C before autoradiography.

RESULTS

Generation of LAK cells from nude mouse spleen cells As reported previously,[18] culture of nude mouse spleen cells with a high dose of rIL-2 caused the generation of killer cells, which could lyse a variety of tumor cells. As shown in Fig. 1, the kinetics of the generation of LAK cells from nude mouse spleen cells (Nude-LAK) was similar to that of LAK cells from normal mouse spleen cells (Nor-LAK cells). The Nude-LAK activity became obvious after 2 days of culture and reached the maximum at 4 days, as was also the case with Nor-LAK cells. It was also confirmed that Nude-LAK cells could lyse both NK-resistant RDM-4 lymphoma cells and NK-sensitive YAC-1 lymphoma cells, as did Nor-LAK cells. The induction of Nude-LAK cells was dependent on the concentration of added rIL-2. The Nude-LAK generation reached a plateau when 2000 U/ml of rIL-2 was added to the culture (Fig. 2).

Phenotypic characterization of Nude-LAK cells by flow cytometry The cell-surface phenotypes of Nude-LAK cells were investigated using mAbs against Thy 1, CD3, CD4, CD8, TCR$\alpha\beta$ and TCRγ. Nude mouse spleen cells did not express any T cell markers (Thy 1, CD4 or CD8) before culture (data not shown). However, culture of nude mouse spleen cells with 2000 U/ml of rIL 2 caused the induction of a high level of Thy 1 antigen on Nude-LAK cells (Fig. 3B). However, Nude-LAK cells did not express either CD4 or CD8 antigen (Fig. 3A). Thus, it

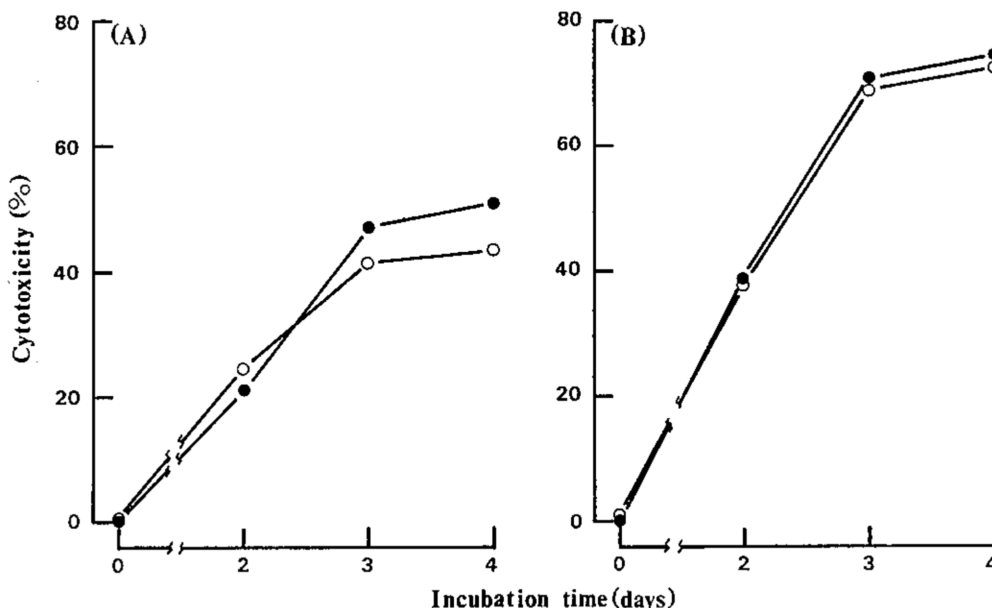

Fig. 1. The generation of LAK cells from normal and nude mouse spleen cells. Normal and nude mouse spleen cells were cultured with 2000 U/ml of r-IL 2 for various times and their cytotoxic activity against NK-resistant RDM-4 cells (A) and NK-sensitive YAC-1 cells (B) was measured by 4-h ^{51}Cr release assay. (○), Nor-LAK; (●), Nude-LAK.

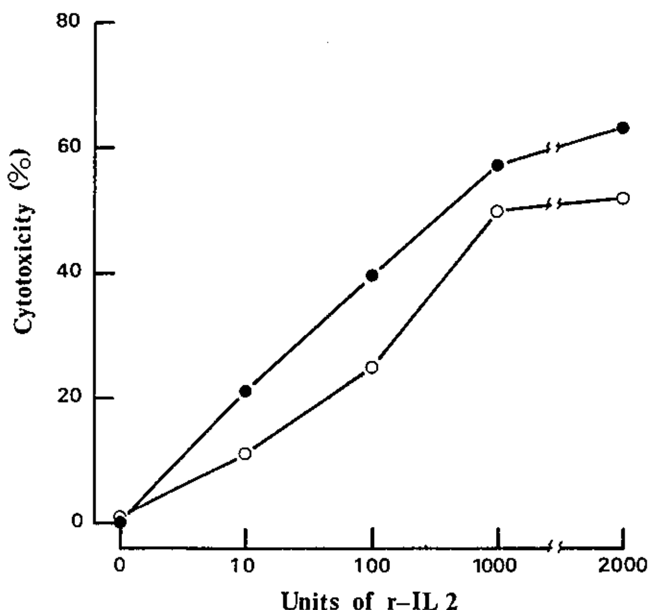

Fig. 2. Dose-dependent induction of Nude-LAK cells by r-IL 2. Nude mouse spleen cells were cultured with various doses of r-IL 2 for 4 days. After incubation, the cells were harvested and their cytotoxicity against RDM-4 was measured by ^{51}Cr release assay. (○), E/T ratio=5:1; (●), E/T ratio=10:1.

was demonstrated that Nude-LAK cells were Thy 1$^+$ CD4$^-$8$^-$, so-called double-negative cells. We further investigated the expression of TCR complexes on Nude-LAK cells. As illustrated in Fig. 3 (C-E), neither TCR$\alpha\beta$, TCR$\gamma\delta$ nor CD3 was expressed on Nude-LAK cells. These results strongly suggested that Thy 1$^+$ CD4$^-$CD8$^-$ Nude-LAK cells do not express functional TCR complexes on their cell surface.

Northern blot analysis of Nude-LAK cells To investigate TCR (α, β, γ, δ) messages in Nude-LAK cells, we extracted RNA from Nude-LAK cells. As a control, RNA was also obtained from Nor-LAK cells. The levels of these TCR genes were determined by using Northern blots. The results are shown in Fig. 4. Although Nor-LAK cells expressed TCRα, β transcripts, Nude-LAK cells showed only sterile TCR β transcript. Much lower levels of γ transcripts were found in both Nor-LAK cells and Nude-LAK cells after prolonged exposure. A TCR δ transcript of 2.0 kb was scarcely detected in both Nor-LAK and Nude-LAK cells. Thus, major populations of Nude-LAK cells appeared not to express any TCR gene.

Involvement of LFA-1 antigen in Nude-LAK cell-mediated cytotoxicity To evaluate the mechanism of Nude-LAK cell-mediated cytotoxicity, we next investigated the involvement of LFA-1 molecules, which

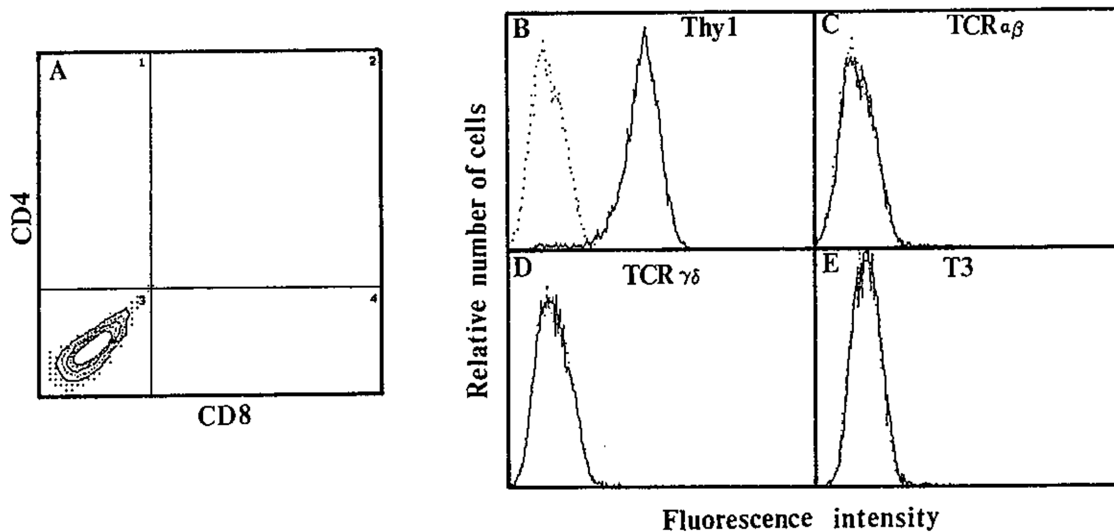

Fig. 3. Phenotypic characterization of Nude-LAK cells by flow cytometry. The expressions of CD4 and CD8 (A), Thy 1.2 (B), TCRαβ (C), TCRγδ (D), CD3 (E) on Nude-LAK cells were examined by FACScan as described in "Materials and Methods." Dotted lines show the unstained control curves. Solid lines show the stained cell curves.

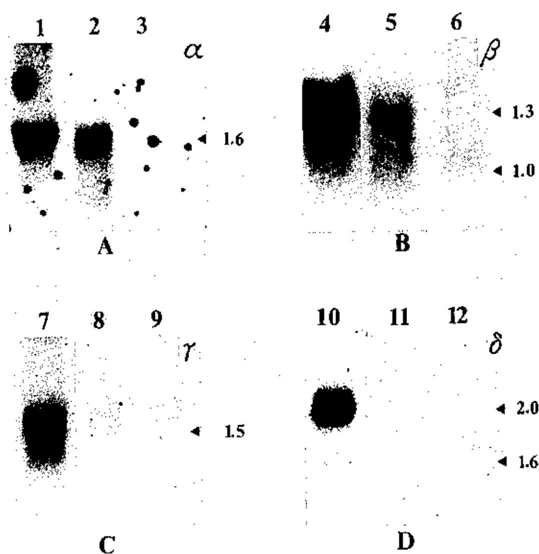

Fig. 4. Northern blot analysis of RNA isolated from Nor-LAK cells and Nude-LAK cells. The cellular RNA was prepared from Nor-LAK and Nude-LAK cells and TCR expression were determined by northern blot analysis as described in "Materials and Methods." (A) TCRα expression of CTLL-2 (lane 1), Nor-LAK cells (lane 2) and Nude-LAK cells (lane 3); (B) TCR β expression of CTLL-2 (lane 4), Nor-LAK cells (lane 5), and Nude-LAK cells (lane 6), (C) TCR γ expression of CTLL-2 (lane 7), Nor-LAK cells (lane 8), Nude-LAK cells (lane 9); (D) TCR δ expression of L8 lymphoma cells (lane 10), Nude-LAK cells (lane 11), and Nude-LAK cells (lane 12).

have been reported to be important in CTL-mediated cytotoxicity and LAK cell-mediated cytotoxicity.[14] As shown in Fig. 5, major populations of both nude spleen cells (Fig. 5A) and normal mouse spleen cells expressed lower levels of LFA-1 before culture. However, culture of these spleen cells with rIL-2 for 4 days resulted in the high-level expression of LFA-1 molecules. Therefore, we next examined whether the highly expressed LFA-1 molecule on Nude-LAK cells was essential for Nude-LAK cell-mediated cytotoxicity by using two distinct anti-LFA-1 mAbs. As shown in Fig. 6, both Nude-LAK cells and Nor-LAK cells showed remarkable cytotoxicity against both RDM-4 and YAC-1 cells. However, addition of mAb against LFA-1 (M17/5.2 and KBA) caused marked reduction of both Nude-LAK activity and Nor-LAK activity. These results strongly suggest an important role of the LFA-1 molecule in Nude-LAK cell-mediated cytotoxicity.

DISCUSSION

It has been demonstrated that CTL, NK cells and LAK cells are important antitumor effector cells in tumor-bearing hosts.[26-28] Although tumor recognition structures of specific CTL have been well defined,[29-31] little is known about the tumor recognition mechanisms of NK cells and LAK cells. Several investigators have demonstrated that cloned NK cells transcribe functional-length messages for γ and/or α and β.[32, 33] However,

recent results demonstrate that freshly isolated NK cells have no message for either TCR α, β, or γ.[34, 35] Thus, currently, it is accepted that freshly isolated NK cells recognize tumor cells by a mechanism distinct from that used by specific CTL.

LAK cells have been shown to be important for tumor rejection both *in vitro* and *in vivo*.[1-8] However, no evidence has been reported concerning the tumor recognition structure of LAK cells. LAK cells were initially defined as T cell-like cytotoxic effector cells induced by IL-2.[1] However, we demonstrated that mouse LAK cells were inducible from both T cells and NK cells.[3] These results were recently confirmed by Yang *et al.*[9] and Kalland *et al.*[12] In human systems, Itoh *et al.*[36] showed that the precursor cells for human LAK cells were Leu 11^+ NK cells. However, recently, Sawada *et al.*[37] demonstrated that human LAK cells could be generated from both NK cells and T cells if human PBL were cultured with rIL-2 in the presence of autologous serum and monocytes. Thus, both mouse and human cells appeared to be divided into T cell-LAK and NK-LAK. Moreover, we demonstrated that the precursor cells of LAK cells were heterogenous and LAK cells could be induced from immature lymphocytes such as $CD4^-8^-$

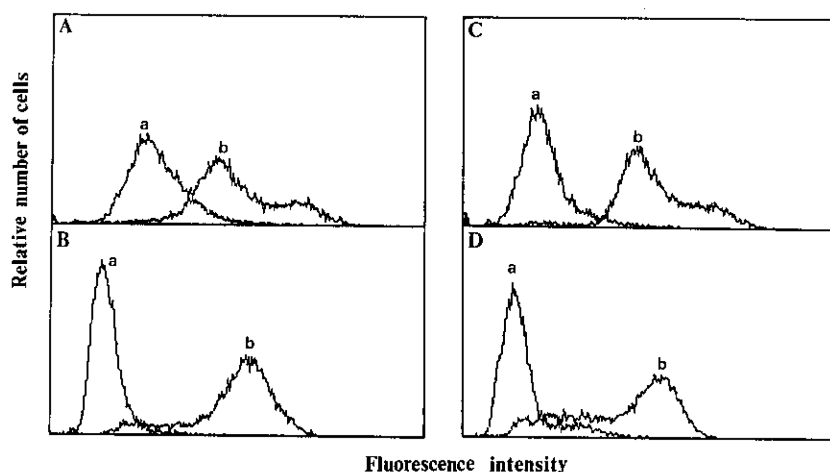

Fig. 5. Higher-level expression of LFA-1 antigen on LAK cells. The expression of LFA-1 antigen on unstimulated lymphocytes or r-IL 2-activated lymphocytes was measured by flow cytometry. (A), LFA-1 expression on unstimulated normal mouse spleen cells; (B), LFA-1 expression on Nor-LAK cells; (C), LFA-1 expression on unstimulated nude mouse spleen cells; (D) LFA-1 expression on Nude-LAK cells. Both control curves (a) and stained cell curves (b) are shown.

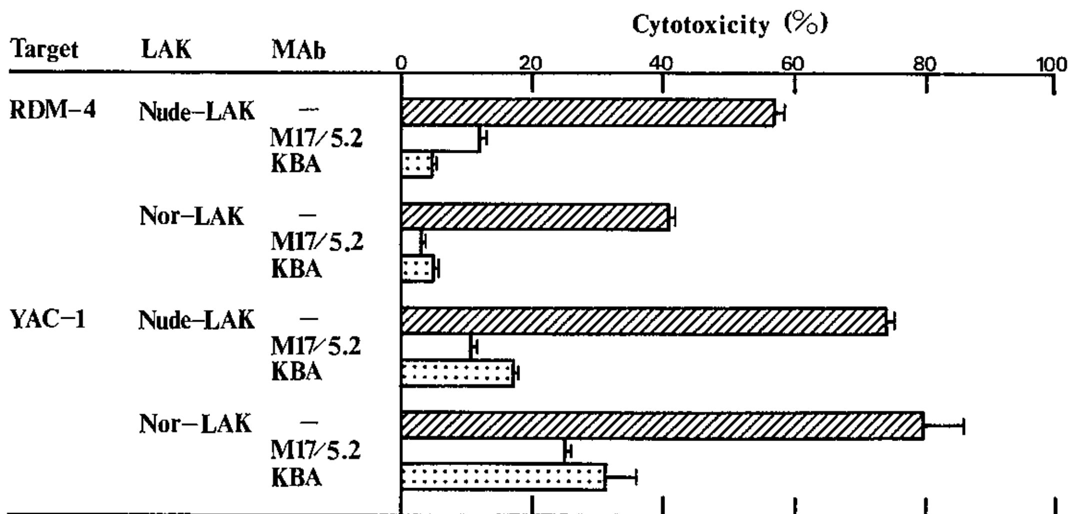

Fig. 6. The blocking of Nude-LAK cell-mediated cytotoxicity by anti-LFA-1 MAb. Both Nor-LAK cells and Nude-LAK cells were induced by culture with 2000 U/ml of r-IL 2 for 4 days. During cytotoxicity assay, 10 μg/ml of purified anti-LFA-1 MAb (M17/5.2 or KBA) was added to the culture and the blocking effect on the cytotoxic activity of LAK cells was measured. Both NK-resistant RDM-4 and NK-sensitive YAC-1 cells were used in this experiment. The E/T ratio was 20:1.

thymocytes and nude mouse spleen cells.[18-20] LAK cells induced from immature lymphocytes were considered to be suitable materials for the analysis of the mechanisms of LAK cell-mediated cytotoxicity, because we could easily eliminate contaminating LAK cells expressing mature TCR $\alpha\beta$ complex. If it became clear that LAK cells expressing no mature TCR $\alpha\beta$ complex could lyse a variety of tumor cells, one could postulate two possible tumor recognition mechanisms by LAK cells: one is that LAK cells can recognize tumor cells via TCR $\gamma\delta$ complex; the other is that LAK cells can recognize tumor cells via some unknown recognition molecules distinct from TCR complexes.

In this paper, we used LAK cells induced from nude mouse spleen cells. As described previously,[18] Nude-LAK cells were distinct from NK cells in the following respects. (1) Nude-LAK activity was not eliminated by the treatment with anti-asialo GM1 antibody plus complement, in contrast to resident NK activity. (2) The level of asialo GM1 expression on Nude-LAK cells was lower than that of resident NK cells. (3) Nude-LAK cells could be generated even if nude spleen cells were treated with anti asialo GM1 antibody plus complement, whereas the generation of activated NK cells was sensitive to anti-asialo GM1 antibody plus complement treatment.[12] In this paper, we extend our previous studies and demonstrate that Nude-LAK cells are Thy 1.2^+ $CD4^-8^-$ killer cells. Nude-LAK cells expressed neither CD3, TCR $\alpha\beta$ nor TCR $\gamma\delta$ on their cell surface as determined by flow cytometric analysis (Fig. 3). The absence of TCR complexes on Nude-LAK cells was confirmed by the results of northern blotting analysis (Fig. 4). Although Nor-LAK cells, used as control cells, have messages for TCR $\alpha\beta$, no detectable messages for TCR $\alpha\beta$ can be observed in Nude-LAK cells. The messages for TCR $\gamma\delta$ complex are scarcely detectable in both Nor-LAK and Nude-LAK cells. Recently, three groups reported that nonspecific killer cells with LAK activity expressed TCR $\gamma\delta$ complex on their cell surface, which strongly indicated the involvement of TCR $\gamma\delta$ complex in the action of broad-reactive killer cells such as NK cells and LAK cells.[15-17] However, the data presented in this paper demonstrate that this is not the case for Nude-LAK cells. We can not rule out the possibility that LAK cells derived from other precursor cells might use TCR $\gamma\delta$ complex, because LAK cells generated from $CD4^-8^-$ thymocytes express higher levels of CD3 and TCR $\gamma\delta$ molecules on their cell surface (data not shown). We are currently trying to evaluate the role of TCR $\gamma\delta$ complex in the tumor lysis mediated by LAK cells generated from $CD4^-8^-$ thymocyte subpopulations.

As reported previously,[14, 38, 39] we have demonstrated that LFA-1 molecules defined by KBA mAb produced by immunization with LAK cells are important in LAK cell-mediated cytotoxicity. In accordance with these results, we have now shown that Nude-LAK cells express higher levels of LFA-1 molecule on their cell surface and Nude-LAK activity is strongly blocked by the addition of anti-LFA-1 antibody (Figs. 5 and 6). The LFA-1 epitopes essential for Nude-LAK cell-mediated cytotoxicity are not unique, because both our antibody, KBA, and M17/5.2 produced by Springer *et al.* can block Nude-LAK activity. Recently, it was shown that the LFA-1 molecule is involved in signal transduction in T cell proliferation.[40] Therefore, LFA-1 itself might be involved in both binding and triggering of Nude-LAK cells. In addition to the LFA-1 molecule, LFA-2 (CD2) is well known as another important molecule for cell-mediated cytotoxicity.[41-43] Recently, one of our colleagues succeeded in cloning cDNA of mouse CD2[44] and we also examined whether Nude-LAK cells contained the message for CD2. Interestingly, Nude-LAK cells expressed high levels of message for CD2 (data not shown). Moreover, using mAb against mouse CD2 molecule,[45] it became clear that Nude-LAK cells express high levels of CD2 molecule on their cell surface (data not shown). However, the Nude-LAK activity was not blocked by mAb against CD2 (data not shown), indicating that CD2 is not involved in Nude-LAK cell-mediated cytotoxicity.

Thus, we conclude from our data described in this and previous reports[3, 4, 18-20] that (1) Nude-LAK cells are broad-reactive killer cells distinct from LAK cells generated from T cells (T-LAK) or NK cells (NK-LAK) in regard to their cell surface phenotypes and their sensitivity to antibody plus complement treatment; (2) Nude-LAK cells do not express TCR complexes on their cell surface and they can lyse a variety of tumor cells by TCR-independent mechanisms; (3) the LFA-1 molecule is essential at least for Nude-LAK cell-mediated killing.

Although the biological significance of LAK cells remains unclear, LAK cells are inducible *in vivo* [46] and may play an important role as antitumor effector cells at a local tumor rejection site. To develop a new strategy for adoptive tumor immunotherapy using LAK cells, it is essential to understand how LAK cells recognize and kill the tumor cells. We are convinced that Nude-LAK cells will be a valuable tool for investigating the mechanisms of LAK cell-mediated cytotoxicity.

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