Two Populations of Mouse Lymphokine-activated Killer Cells Separated by Use of Soybean Agglutinin

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We separated lymphokine-activated killer (LAK) cells induced from spleen cells of BALB/c mice by culturing with recombinant interleukin-2 (rIL-2) into soybean agglutinin-positive (SBA⁺) and soybean agglutinin-negative (SBA⁻) fractions with a cell sorter at the time when LAK activity reached a maximum (day 3). We found that the cells with LAK activity were enriched in the SBA⁺ fraction. Analysis of cell surface phenotypes revealed that the SBA⁺ cells are of non-T cell origin, while the SBA⁻ fraction consists of T cells. We also found that the large granular lymphocyte (LGL) fraction of spleen cells obtained with a Percoll gradient became SBA⁺ after culture for 3 days with rIL-2, whereas cells of high density did not. The change in SBA binding sites was also examined on C57BL/6 mouse spleen cells and we found that NK1.1⁺ non-T cells selectively acquire SBA binding sites at an early stage of activation with rIL-2. On the other hand, sorted SBA⁻ cells gradually acquired SBA binding sites on extended culturing with rIL-2. These results suggest that the expression of SBA binding sites is related to the stage of cell activation with rIL-2, though cells of the NK-lineage became SBA⁺ at an earlier period of a culture with rIL-2 than cells of the T-lineage. Utilizing these findings, we separated NK-derived LAK cells by means of SBA-binding, and used the separated cells for adoptive immunotherapy for experimental pulmonary metastasis. We found that SBA⁺-NK cell-derived LAK cells showed stronger activity for the inhibition of experimental pulmonary metastasis than SBA⁻-T cell-derived LAK cells.

Key words: Lymphokine-activated killer cells — Interleukin-2 — Soybean agglutinin — Natural killer cells — T cells

Murine splenocytes or human peripheral blood lymphocytes incubated *in vitro* in the presence of recombinant interleukin-2 (rIL-2) acquire the ability to cause the cytolysis of a variety of tumor cells in short-term ⁵¹Cr release assays.[1, 2] Lymphokine-activated killer (LAK) cells have been considered to be potentially useful for adoptive immunotherapy for cancer patients.[3] However, the natures of the precursor and effector populations of LAK cells are controversial. LAK cells have been reported to be T cell-derived,[4, 5] natural killer (NK) cell-derived[6–8] or both.[9, 10] We previously reported that alloreactive cytotoxic T lymphocytes (CTLs)[11] or *in vitro*-activated killer T cells from tumor-bearing mice[12] have many sites for *Dolichos biflorus* agglutinin (DBA). We extended this work to determine whether or not rIL-2-generated LAK cells could be enriched by the use of lectins.

In this study, we found that the binding of soybean agglutinin (SBA) to mouse spleen cells increased in the course of the development of LAK cell activity. We separated SBA-positive and SBA-negative cells using a cell sorter when the total LAK activity reached a maxi-

mum. At this stage, we could separate NK-LAK cells and T-LAK cells on the basis of the binding to SBA.

MATERIALS AND METHODS

Mice Female BALB/c and C57BL/6 mice, 7 to 10 weeks old, were purchased from Charles River Japan Inc. (Kanagawa).

Tumor cells NL-17, a colon adenocarcinoma cell line with high lung metastatic activity after iv injection, was established from colon adenocarcinoma 26 cells by Tsuruo *et al.*, and kindly provided by Drs. T. Tsuruo and T. Yamori (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo).[13] NL-17, YAC-1 (A/Sn lymphoma), EL4 (C57BL/6 lymphoma) and B16 (C57BL/6 melanoma) cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, Md.), 20 mM N-2-hydroxy-ethylpiperazine-N-2-ethanesulfonic acid (HEPES; Wako Pure Chemicals, Co., Tokyo), penicillin (100 U/m; Sigma Chemicals, Co., St. Louis, Mo.), streptomycin (100 μg/ml; Sigma Chemicals) and 2 mM glutamine

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(Wako Pure Chemicals). P815 (DBA/2 mastocytoma), MM48 (C3H/He mammary carcinoma) and Meth A (BALB/c sarcoma cells) were passaged in the ascitic form in syngeneic mice.

rIL-2 Human IL-2, produced by means of recombinant DNA technology, with a specific activity of 35, 200 U/ mg protein was supplied by Takeda Chemical Industries (Osaka).

Lectins SBA, pokeweed mitogen (PWM) and bio-tinylated-SBA were purchased from Vector Laboratories (Burlingame, Calif.), and leucoagglutinating *P. vulgaris* agglutinin (PHA-L), concanavalin A (Con A) and *Lens culinaris* agglutinin (LCA) from Honen Oil Co. (Tokyo). Peanut agglutinin (PNA) was purified by the method reported by Lotan and Sharon.[14]

Antibodies Fluorescein isothiocyanate (FITC)-labeled monoclonal anti-mouse-Thy1.2 (clone TS) antibody was purchased from Bio-Yeda (Rehovot, Israel), FITC-anti-mouse-Lyt2 (clone 53-6.7) antibody and anti-mouse L3T4 (clone GK1.5) antibody from Beckton Dickinson (Mountain View, Calif.), and rabbit anti-asialo GM₁ antibody from Wako Pure Chemicals Co. The hamster mAb, 145-2C11, which recognizes the murine T3-ε chain, was the antibody reported by Leo *et al.*[15] Monoclonal anti-NK1.1 (PK136) antibody was a generous gift from Dr. G. C. Koo (Merck, Sharp & Dohme Research Laboratories, N.J.).[16] FITC-goat-anti-hamster IgG was obtained from E.-Y. Laboratories (San Mateo, Calif.), FITC-goat-anti-rat-IgG (Fc-specific), FITC-goat-anti-rabbit-Ig (IgA, IgG & IgM, heavy & light chain reactive) and FITC-anti-mouse-IgG [F(ab)$'_2$ fragment, Fc-specific] were from Cappel Laboratories (Cochranville, Pa.). FITC-avidin was from Vector Laboratories.

Generation of LAK cells Spleen cells from BALB/c mice were cultured with rIL-2 (2 U/ml) in complete medium for 3 days at 37°C in 5% CO_2. The complete medium comprised RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 4 mM glutamine, 1 mM sodium pyruvate (Wako Pure Chemicals), 20 mM HEPES (pH 7.2), 5×10^{-5} M 2-mercaptoethanol (Sigma), 1% nonessential amino acids (GIBCO), 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were seeded at 1.5×10^6/ ml in a 6-well tissue culture plate (6 ml/well) (Costar, Cambridge, Mass.).

Assay for cytolytic activity Target cells (2×10^6) were labeled with 100 μCi of Na^{51}CrO$_4$ (New England Nuclear, Boston, Mass.) in 0.5 ml of complete medium for 60 min at 37°C. After washing three times in complete medium, the labeled target cells (1×10^4 cells/well) were added to various numbers of effector lymphocytes in a 96-well round-bottomed plate (Sumitomo, Tokyo). After a 4-h incubation at 37°C under 5% CO_2, one half of each culture supernatant was harvested and counted with a gamma counter (Aloka, Tokyo). The maximum release

of radioactivity was determined with target cells dissolved in 0.5 M NaOH, and spontaneous release was determined by incubating target cells without effectors. Percent specific ^{51}Cr release was determined by applying the following formula.

$$\% \text{ specific release} = \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Fluorescent staining of cells For single color staining, 10^6 cells in 200 μl of 10 mM sodium phosphate buffer-0.15 M NaCl (PBS; pH 7.2) containing 0.1% bovine serum albumin (BSA) and 0.1% NaN₃ (PBS-BSA-NaN₃) were incubated on ice for 30 min in the presence of a saturating amount of an antibody or a subagglutinating amount of a lectin. The dilutions and concentrations of the antibodies and lectins, respectively, were as follows: FITC-anti-Thy1.2 (1:100), FITC-anti-Lyt2 (1:50), anti L3T4 (1:12.5), anti-asialo GM₁ (1:200), 145-2C11 (1:10), PK136 (1:10) and biotinyl-SBA (40 μg/ ml). For two-step staining, the cells were washed three times and then further incubated on ice for 30 min with a FITC-coupled second-step reagent. For staining with anti-L3T4, FITC-anti-rat-IgG (Fc-specific) was used at 1:50 in 200 μl of PBS-BSA-NaN₃; for anti-asialo GM₁, FITC-anti-rabbit-Ig (IgA, IgG & IgM, heavy & light chain reactive) at 1:200; for anti-145-2C11, FITC-anti hamster IgG at 1:100; for anti-PK136, FITC-anti-mouse IgG [F(ab)$'_2$ fragment, Fc-specific at 1:20; and for bio-tinyl-SBA, FITC-avidin at 1:100. The cells were washed again and then resuspended in PBS-BSA-NaN₃. Flow cytometric analysis was performed with an FCS-1 cell sorter (Japan Spectroscopic, Tokyo) as described previously.[17]

Preparation of LGL A suspension of BALB/c spleen cells was prepared in RPMI 1640 medium. Adherent cells were removed by incubation on a plastic tissue culture dish (Falcon #3003) for 1 h at 37°C, under 5% CO_2 ($2–3 \times 10^8$ cells/10 ml). Nonadherent splenocytes were resuspended in 2 ml of 7% FCS-Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Ltd., Tokyo) and then centrifuged through a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient prepared in a 15-ml plastic tube (Corning, #25311) by layering 2 ml aliquots of Percoll (57.5%, 55%, 52.5%, 50%, 47.5% and 45%, v/v, in 7% FCS-MEM). Nonadherent cells were layered on top of the Percoll gradient and then the tube was centrifuged at 1500 rpm for 30 min at room temperature. Each fraction was designated as follows; cells on 45% Percoll (Fr. 1), and at the 45%/47.5% interface (Fr. 2), 47.5%/50% interface (Fr. 3), 50%/52.5% interface (Fr. 4) and

52.5%/55% interface Fr. 5). LGL were concentrated at the 45%/47.5% Percoll interface.[18]

Experimental pulmonary metastasis NL-17 tumor cells (2×10^4) in 200 μl were injected iv into BALB/c mice to initiate pulmonary metastasis. On day 3, effector cells $(2 \times 10^6/\text{mouse})$ were injected iv in 200 μl of RPMI 1640 medium via the tail vein. On day 18 after tumor inoculation, mice were killed for enumeration of pulmonary tumor nodules.[19]

RESULTS

Lectin binding properties of LAK cells LAK cells were generated from spleen cells of BALB/c mice by culturing with recombinant IL-2 (rIL-2), and the lytic activities against NL-17, B16 and YAC-1 cells were found to reach a maximum on day 3 of the culture. We compared the lectin-binding properties of the spleen cells before and after culturing for 3 days with rIL-2. SBA, PHA-L, PWM and PNA were found to bind more strongly to 3-day LAK cells than to fresh spleen cells. Among these lectins, SBA showed the biggest difference in the binding to fresh spleen cells (Fig. 1A) and 3-day LAK cells (Fig. 1B). After binding of SBA to spleen cells, no significant incorporation of [^{3}H]thymidine was observed. The 3-day LAK cells consisted of two populations, SBA$^+$ and SBA$^-$ cells, as regards the binding sites for SBA, whereas SBA scarcely bound to fresh spleen cells. Some lectins, such as LCA and Con A, did not show any difference in binding to spleen cells before and after culture with rIL-

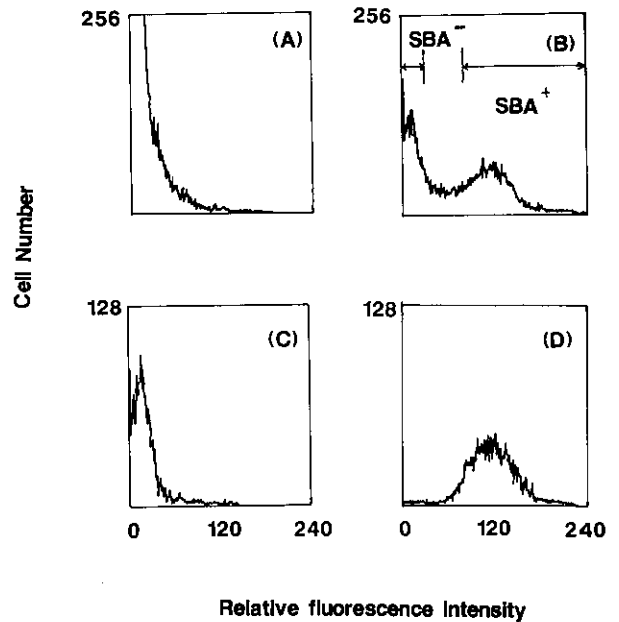Relative fluorescence intensity

Fig. 1. Cytofluorometric analysis of unsorted and sorted LAK cells with SBA. Fresh BALB/c spleen cells (A) and BALB/c spleen cells cultured in the presence of rIL-2 (2 U/ml) for 3 days (B) were stained with biotinyl SBA and FITC-avidin, and then separated with a cell sorter. After sorting with the cell sorter into SBA$^+$ (channels 87 to 255, in panel B) and SBA$^-$ (channels 0 to 28) fractions, each fraction was re-sorted with the cell sorter, respectively (C, D). The fluorescence intensity is plotted on a logarithmic scale.
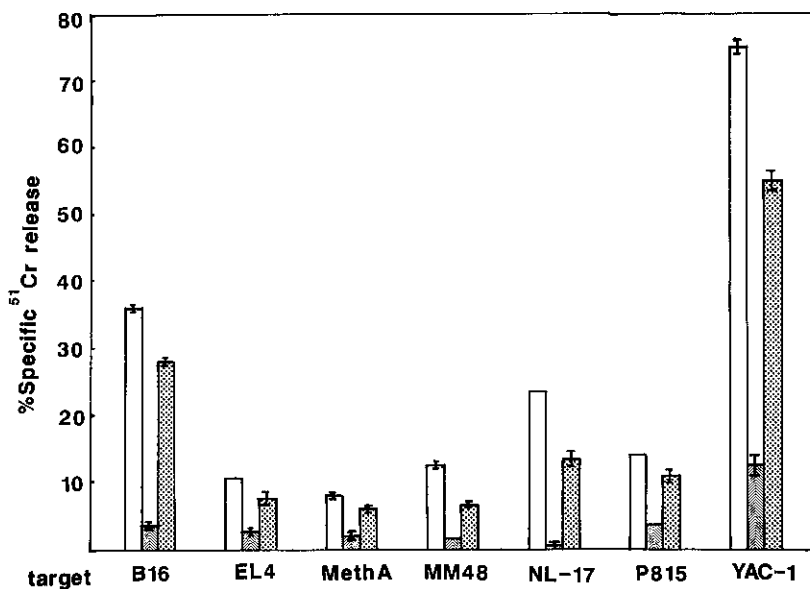

Fig. 2. Cytolytic activity of SBA$^+$ cells toward various targets. BALB/c spleen cells were cultured in the presence of rIL-2 (2 U/ml) for 3 days, and then separated with a cell sorter into SBA-positive and SBA-negative fractions. Each fraction was incubated at 37°C in the presence of rIL-2 until the 4-h ^{51}Cr release assay was performed on day 4 with various target cells. Cells in the SBA$^+$ fraction (☐) or the SBA$^-$ fraction (▨), or unsorted cells (▨) were used as effectors. The effector:target ratio was 5:1.

2. DBA, which has been shown to bind strongly to CTL[11] and to cultured spleen cells in the presence of Con A-activated spleen cell conditioned medium,[12] did not show strong binding to 3-day LAK cells (data not shown).

We then sorted the 3-day LAK cells into SBA$^+$ and SBA$^-$ fractions with a cell sorter to compare the cytolytic activities of these two fractions. Fig. 1 shows that SBA$^-$ (Fig. 1C) and SBA$^+$ (Fig. 1D) cells can be successfully separated with a cell sorter. The SBA$^+$ cells were 10–15% larger in diameter than the SBA$^-$ cells. The sorted cells were incubated in the presence of rIL-2 overnight before cytolytic activity was assessed against various target cells. As shown in Fig. 2, the cells with strong cytolytic activity were enriched in the SBA$^+$ fraction, and the SBA$^-$ cells showed only very weak activity, if any. The difference in cytolytic activity between the SBA$^+$ and SBA$^-$ fractions was seen irrespective of whether the target cells were NK-sensitive (YAC-1), NK-resistant syngeneic (NL-17, Meth A), NK-resistant H-2 compatible (P815) or NK-resistant H-2 incompatible (B16, EL4, MM48). When one unit of cytotoxicity was defined as the cell number required to kill 60% of 10^4 YAC-1 cells, 10^6 cells each of SBA$^+$, SBA$^-$ and unsorted fractions corresponded to 40, < 1 and 15 units, respectively. These results demonstrated that SBA could be used for the enrichment of LAK cells at least at the stage when the total LAK activity was maximum.

Cell surface markers of SBA$^+$- and SBA$^-$-LAK cells
The SBA$^+$- and SBA$^-$-LAK cells were compared as to their cell surface markers. Spleen cells of BALB/c mice were cultured with rIL-2 for 3 days, and then separated into SBA$^+$ and SBA$^-$ fractions with a cell sorter. These fractions were further incubated with rIL-2 overnight to internalize SBA and the fluorescent reagent. Cells were then stained with either anti-Lyt2 (CD8), anti-L3T4 (CD4), anti-T3-ε or anti-asialo GM$_1$, and then separated with a cell sorter (Fig. 3). Although most cells in both the SBA$^+$ and SBA$^-$ fractions were Thy1-positive (78% positive in the SBA$^+$ and 82% positive in the SBA$^-$ fraction), and the extents of the expression of Thy1 antigens were similar between SBA$^+$ and SBA$^-$ cells (Fig. 3E, J), SBA$^+$ cells did not express the T cell differentiation antigens, CD8 (Fig. 3A) and CD4 (Fig. 3B). Moreover, SBA$^+$ cells were negative as to T3-ε chain, which is a member of the T cell antigen receptor complex (Fig. 3C). On the other hand, 43% of SBA$^-$ cells were CD8-positive, 30% CD4-positive, and 60% T3-ε-positive. Furthermore, it was found that most SBA$^+$ cells were asialo GM$_1$-positive (83% positive), while only 45% of the SBA$^-$ cells were asialo GM$_1$-positive.
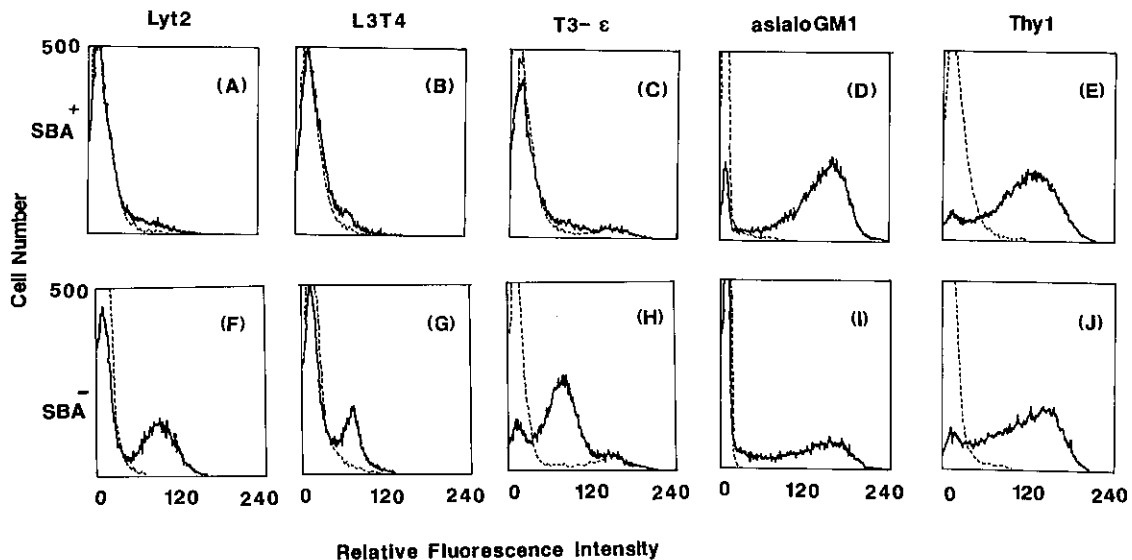

Fig. 3. Cells surface phenotypes of the SBA$^+$ and SBA$^-$ fractions. BALB/c spleen cells were cultured in the presence of rIL-2. On day 3, LAK cells were stained with SBA, and then sorted into SBA$^+$ and SBA$^-$ fractions (see Fig. 1). The sorted cells were incubated at 37°C for 18 h in the presence of rIL-2 to internalize the fluorescent SBA. SBA$^+$ cells (A–E) and SBA$^-$ cells (F–J) were stained with FITC-anti-Lyt2 (A, F), FITC-anti-Thy1 (E, J), anti-L3T4 plus FITC-goat anti-rat IgG (B, G), anti-mouse T3-ε (2C11) plus FITC-goat anti-hamster IgG (C, H) or anti-asialo GM$_1$ plus FITC-goat-anti-rabbit IgG (D, I), the fluorescence profiles being shown by solid lines on a logarithmic scale. In each panel the background level is shown by a broken line; second step reagent alone (B, C, D, F, G, H) or autofluorescence (A, E, F, J).

These results suggest that SBA binds selectively to non-T cell type LAK cells after 3 days of culture of BALB/c spleen cells. The results also indicate that Thy1 expression is not an unequivocal marker for T cell-derived-LAK cells, because SBA$^+$ cells express the Thy1 antigen, but do not express the T cell receptor complex or accessory molecules for T cell recognition, the CD4 and CD8 antigens, indicating that care is necessary in considering whether LAK cells are of T cell lineage based solely on the Thy1 expression.

Change in SBA binding sites on LGL during culture with rIL-2 To examine the possibility that SBA$^+$-LAK cells originated from NK cells, cells in the LGL fraction were cultured for 3 days in the presence of rIL-2, and then the lytic activity and the binding of SBA were examined. Since BALB/c spleen cells generally have very low NK activity, spleen cells were prepared from BALB/c mice that had been treated with polyI:C 24 h beforehand. The spleen cells were separated by Percoll density gradient centrifugation and the NK activity of cells in each fraction toward YAC-1 target cells was assessed (Fig. 4A). NK cells were found to be enriched in Fr. 2. In contrast, cells in Fr. 5 did not show NK activity (Fig. 4A). Fr. 2 contained a significant number of asialo GM$_1$-positive cells, while Fr. 5 did not (Fig. 5b, f). These results were consistent with the results of the NK activity assay. On the other hand, cells with a large number of SBA binding sites were almost completely absent in both Fr. 2 and Fr. 5, though Fr. 2 appeared to contain a small number of SBA-positive cells, whereas Fr. 5 almost completely lacked them (Fig. 5a, e). After cells in each fraction had been cultured with rIL-2 for 3 days, the lytic activity toward YAC-1 target cells and the SBA binding sites

were compared again between Fr. 2 and Fr. 5. After the culture with rIL-2, the Fr. 2 cells showed high lytic activity, but the Fr. 5 cells showed only very low lytic activity (Fig. 4B). SBA-positive cells appeared in Fr. 2
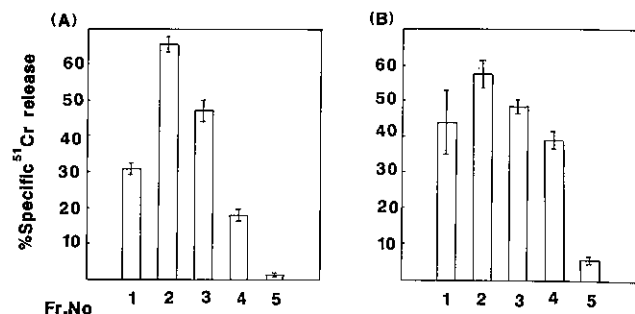

Fig. 4. Cytolytic activity of LGL toward YAC-1 target cells. LGL and high-density lymphocytes were separated from fresh spleen cells on a Percoll density gradient. BALB/c mice were treated with polyI:C (100 μg/mouse, ip) 24 h before the spleen cells were separated on the Percoll gradient. The fractions were as follows; cells on 45% Percoll (Fr. 1), and at the 45%/47.5% interface (Fr. 2), 47.5%/50% interface (Fr. 3), 50%/52.5% interface (Fr. 4) and 52.5%/55% interface (Fr. 5). (A) Cytolytic activity of fresh spleen cells in each fraction after separation on Percoll. Cells in each fraction were used as effectors in the 4-h ^{51}Cr release assay with YAC-1 cells, at the effector:target ratio of 20:1. (B) Cytolytic activity of cells in each fraction after culturing in the presence of rIL-2. Spleen cells were separated on Percoll and then the cells in each fraction were cultured in the presence of rIL-2 (2 U/ml) for 3 days. The 4-h ^{51}Cr release assay was carried out with YAC-1 cells, at the effector:target ratio of 5:1.
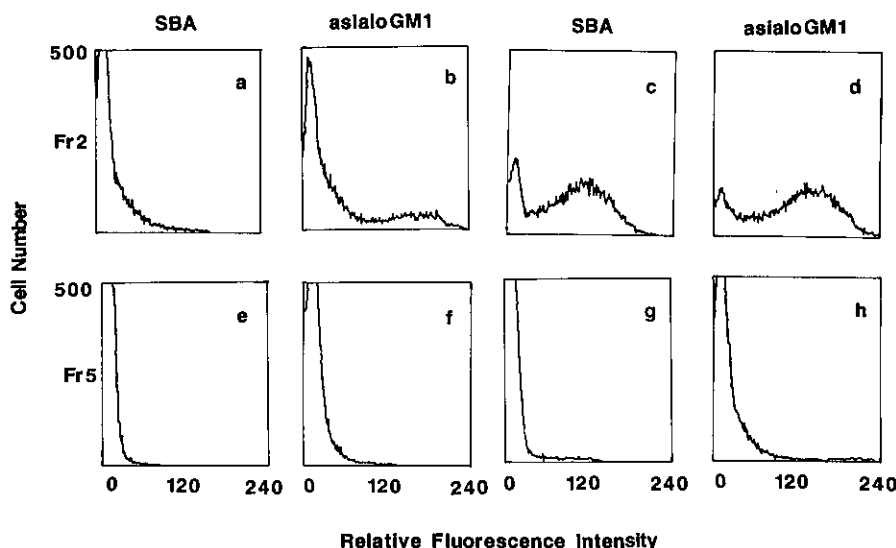

Relative Fluorescence Intensity

Fig. 5. Fluorescence profiles of Percoll-fractionated spleen cells and those after culture in the presence of rIL-2. Spleen cells from polyI:C treated BALB/c mice were fractionated on a Percoll gradient (see Fig. 4). Cells from Fr. 2 (a, b) and Fr. 5 (e, f) were stained with biotinyl-SBA plus FITC-avidin (a, e) or anti-asialo GM$_1$ plus FITC-anti-rabbit IgG (b, f), respectively. On the other hand, cells in each Percoll fraction were cultured in the presence of rIL-2 for 3 days. Cells derived from Fr. 2 (c, d) and Fr. 5 (g, h) were stained with biotinyl-SBA plus FITC-avidin (c, g) or anti-asialo GM$_1$ plus FITC-anti-rabbit IgG (d, h), respectively.

after 3 days of culture (Fig. 5c), whereas cells in Fr. 5 did not express a significant number of SBA binding sites even after culture with rIL-2 for 3 days (Fig. 5g). Although cells in Fr. 2 originally included a significant number of asialo GM_1-positive cells (Fig. 5b), the proportion of asialo GM_1-positive cells was significantly increased after 3 days of culture with rIL-2 (Fig. 5b, d). On the other hand, the appearance of asialo GM_1-positive cells in Fr. 5 was not observed even after 3 days of culture with rIL-2 (Fig. 5h). These results suggest that the SBA-positive cells which appeared during the 3-day culture with rIL-2 originated from low-density spleen cells, probably from LGL.

NK markers on SBA$^+$ cells To establish the origin of cells expressing the SBA binding sites during the culture with rIL-2, we investigated the expression of the NK1.1 alloantigen and the T3-ε chain on SBA$^+$ and SBA$^-$ cells simultaneously (Fig. 6). We used C57BL/6 mice, which are NK1.1-positive, whereas BALB/c mice are NK1.1-

negative. Spleen cells from C57BL/6 mice were cultured in the presence of rIL-2 for 2 days, and then sorted into SBA$^+$ and SBA$^-$ fractions. The cells in each fraction were cultured overnight with rIL-2, and then examined for the expression of NK1.1 and the T3-ε chain. Cells in the SBA$^+$ fraction expressed the NK1.1 (PK136) alloantigen, but did not express the T3-ε chain. On the contrary, cells in the SBA$^-$ fraction expressed the T3-ε chain, but did not express the NK1.1 alloantigen. The cytolytic activities of the SBA$^+$ and SBA$^-$ cells against B16 target cells amounted to 43.2% and 1.0%, respectively, at the E/T ratio of 4:1, and the cytolytic activities
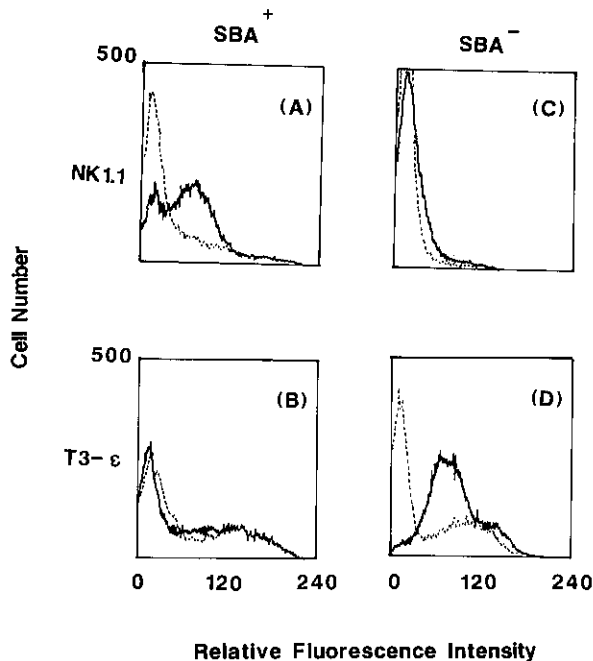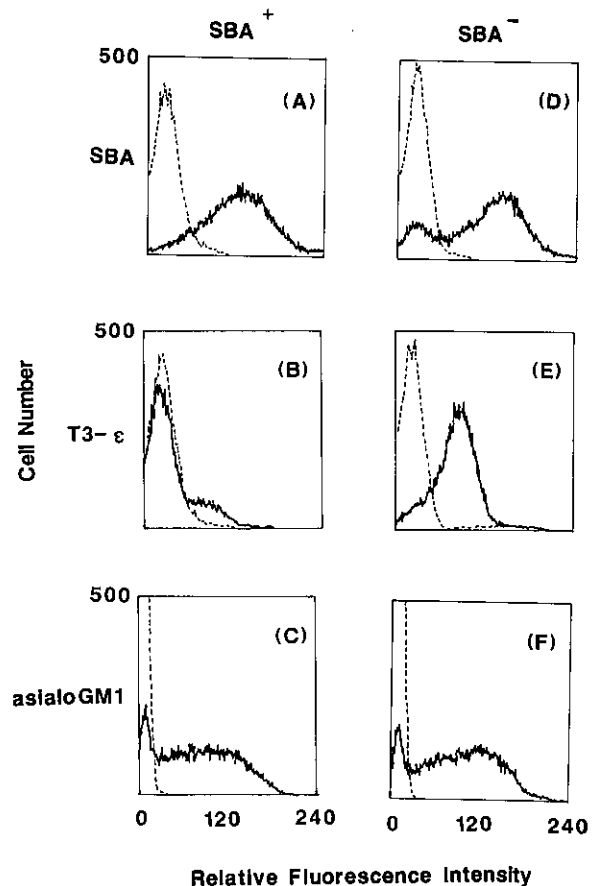

Fig. 6. NK surface marker expression of SBA$^+$ on C57BL/6 LAK cells. Spleen cells from C57BL/6 mice were cultured in the presence of rIL-2 for 2 days, and then the cells were stained with SBA and sorted into SBA$^+$ and SBA$^-$ fractions. The sorted cells were incubated with rIL-2 for 18 h at 37°C, and then stained with PK136 (NK1.1) plus FITC-anti-mouse IgG (A, C) or 145-2C11 (T3-ε) plus FITC-anti-hamster IgG (B, D). The fluorescence profiles of SBA$^+$ cells (A, B) and SBA$^-$ cells (C, D) are shown by solid lines. The background levels, FITC-anti-mouse IgG alone (A, C) or FITC-anti-hamster IgG alone (B, D), are shown by broken lines.

Fig. 7. Cell surface antigens on SBA$^+$- and SBA$^-$-LAK cells after extended culturing in the presence of rIL-2. BALB/c spleen cells were cultured in the presence of rIL-2 (2 U/ml) for 3 days, and then the cells were stained with SBA and sorted. The sorted cells were cultured in the presence of rIL-2 (2 U/ml) for an additional 3 days. On day 6, cells from the SBA$^+$ fraction (A–C) and from the SBA$^-$ fraction (D–F) were stained with biotinyl-SBA plus FITC-avidin (A, D), 145-2C11 plus FITC-anti-hamster IgG (B, E), or anti-asialo GM_1 plus FITC-anti-rabbit IgG (C, F). The broken lines indicate the profiles with the second step reagent alone.

of these fractions toward YAC-1 target cells were 71.9% and 4.6%, respectively, at the E/T ratio of 4:1. These results clearly demonstrated that the LAK cells originating from NK cells began to express SBA binding sites at an early stage during the culture with rIL-2.

Change in the expression of SBA binding sites during an extended culture with rIL-2 The proportion of SBA$^+$ cells was found to increase when cells were cultured for a longer period with rIL-2 (data not shown). We then examined whether or not SBA$^+$ cells with NK-like phenotypes would become the majority, and whether or not SBA$^-$ cells with T cell phenotypes would acquire binding sites for SBA. BALB/c spleen cells were cultured with rIL-2 for 3 days, and then sorted into SBA$^+$ and SBA$^-$ fractions. The cells in each fraction were recultured for another 3 days with rIL-2 and then the cell phenotypes

were examined. The binding sites for SBA appeared on the cells in the SBA$^-$ fraction after this reculture (Fig. 7A, D). Furthermore, we found that the cells in the SBA$^+$ fraction remained almost negative for T cell receptors (Fig. 7B), and that the cells in the SBA$^-$ fraction remained positive for T cell receptors even after the extended culture period (Fig. 7E). Moreover, the cells in the SBA$^+$ fraction were almost completely negative for CD4 (2% positive) and CD8 (6% positive), while a significant proportion of the cells in the SBA$^-$ fraction expressed these T cell differentiation antigens CD4, 26% positive, and CD8, 56% positive, after the extended culture. However, the cells in the SBA$^-$ fraction eventually expressed asialo GM$_1$ at a similar level to the cells in the SBA$^+$ fraction (Fig. 7C, F). These results suggest that the SBA$^-$ cells with T cell phenotypes obtained on 3-day culturing with rIL-2 express SBA binding sites after the extended culture with rIL-2.

This was confirmed in the case of spleen cells of C57BL/6 mice. Spleen cells of C57BL/6 mice were cultured with rIL-2 for 2 days, and then sorted into SBA$^+$ and SBA$^-$ fractions. Cells in each fraction were cultured for another 3 days with rIL-2 and then the cell phenotypes were examined. The expression of SBA binding sites was observed, after the extended culture period, on the cells that had been SBA-negative on day 2 of the culture (data not shown). Even after the extended culture period, cells in the SBA$^+$ fraction expressed the NK1.1 alloantigen and were almost completely negative for T cell receptors (Fig. 8A, B). On the other hand, cells
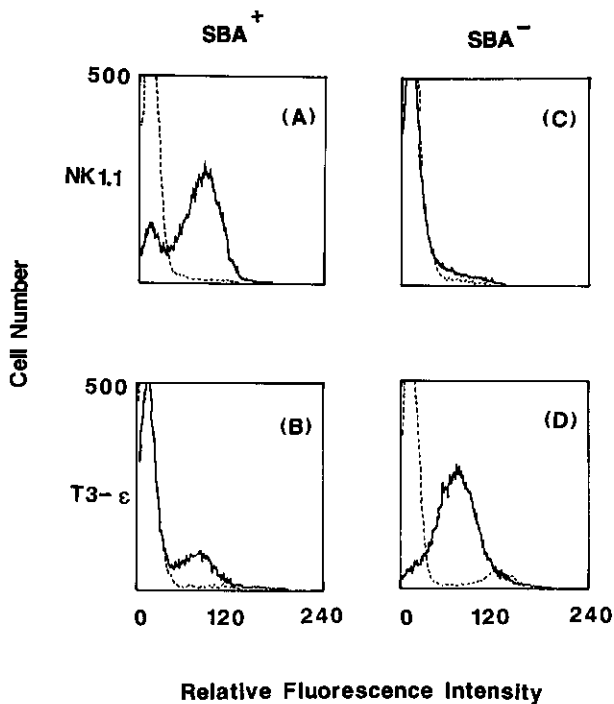

Fig. 8. SBA$^+$-LAK cells do, but SBA$^-$-LAK cells do not, express NK markers on their surface after extended culturing in the presence of rIL-2. C57BL/6 LAK cells, generated by culturing spleen cells in the presence of rIL-2 for 2 days, were stained with SBA, and then sorted into SBA-positive and SBA-negative fractions. The sorted cells were cultured for an additional 3 days. On day 5, the sorted cells were stained with PK136 plus FITC-anti-mouse IgG (A, C) or 145-2C11 plus FITC-anti-hamster IgG (B, D). The fluorescence profiles of the SBA$^+$ cells (A, B) and the SBA$^-$ cells (C, D) are shown by solid lines. The background levels, FITC-anti-mouse IgG alone (A, C) or FITC-anti-hamster IgG alone (B, D), are shown by broken lines.
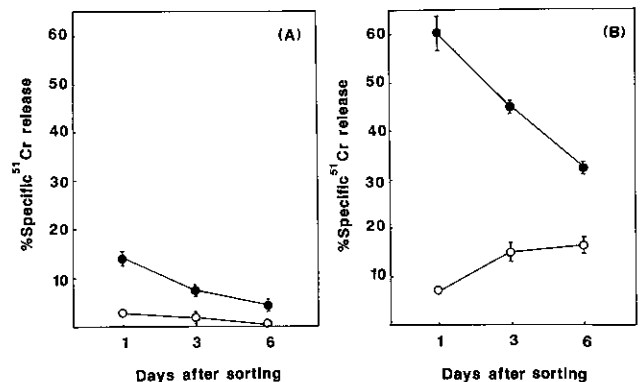

Fig. 9. Cytolytic activity of SBA$^+$ and SBA$^-$ cells after extended culturing. BALB/c spleen cells were cultured for 3 days in the presence of rIL-2, and then sorted into SBA$^+$ and SBA$^-$ fractions. The sorted cells were recultured in the presence of rIL-2. One, 3 and 6 days after sorting, the cytolytic activity of cells in the SBA$^+$ (●) or SBA$^-$ fraction (○) toward NL-17 (A) or YAC-1 (B) cells was determined by means of a 4-h ^{51}Cr release assay. The effector : traget ratio was 5:1.

in the SBA⁻ fraction expressed T cell receptors, but were negative for the NK1.1 alloantigen (Fig. 8C, D). These results are consistent with the results obtained for BALB/c mice.

We then examined the relationship between the expression of SBA binding sites and the cytolytic activity. BALB/c spleen cells were cultured for 3 days with rIL-2, sorted into SBA⁺ and SBA⁻ fractions, and then recultured for various periods with rIL-2 and examined for cytolytic activity. The longer the culture period after sorting, the weaker the lytic activity of the cells in the SBA⁺ fraction (Fig. 9). On the other hand, the cytolytic activity of the cells in the SBA⁻ fraction toward YAC-1 cells increased during the culture with rIL-2 (Fig. 9B). This increase paralleled the acquisition of SBA binding
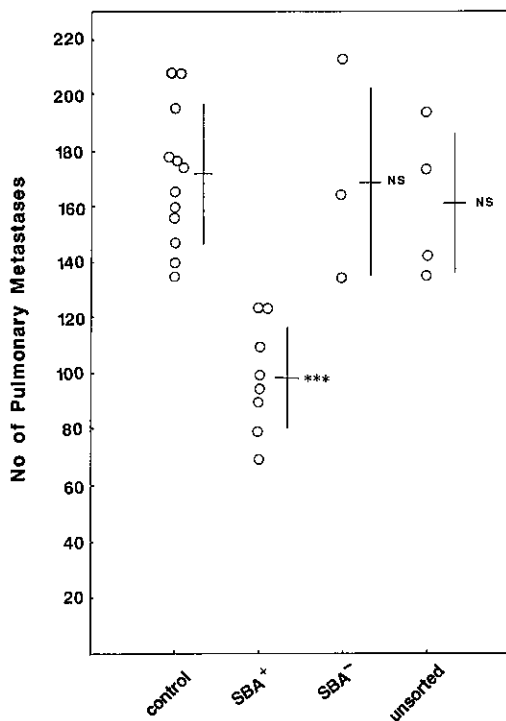
sites on these cells. However, the cells from the SBA⁻ fraction did not exhibit significant lytic activity toward NL-17 target cells, during the extended culture period of a total of 9 days. This may possibly be related to the fact that the selection of SBA⁻ cells on day 3 may deplete the accessory cells required for the generation of T-LAK activity.[20]

Effect of SBA⁺ cells on pulmonary metastasis We investigated the effect of SBA⁺ cells on experimental pulmonary metastasis. Injection of SBA⁺ (2×10^6/mouse) cells significantly reduced the number of metastases compared with that in control mice (Fig. 10). Since unsorted cells consist of 70–75% SBA⁻ cells and 25–30% SBA⁺ cells, injection of unsorted cells did not show significant inhibition of metastasis. It has been reported that rIL-2 given in combination with LAK cells increases the anti-tumor effectiveness of LAK cells.[21]

As the proliferation of SBA⁺ cells was faster than that of SBA⁻ cells in an extended culture (data not shown), the combined use of SBA⁺ cells with rIL-2 can be expected to have a greater effect.

DISCUSSION

Regarding the origin of LAK cells, conflicting results have been reported. The facts that LAK cells could be generated from thymocytes[4] or from Beige mouse spleen cells, which do not have NK activity,[5] constituted evidence for the existence of LAK cells of the T cell lineage. On the other hand, on positive selection with a FACS (fluorescence-activated cell sorter), Itoh found that LAK precursors in human peripheral blood mononuclear cells (PBL) were CD16⁺, and that strong LAK activity appeared in the NK-enriched low-density fraction obtained on a Percoll gradient.[6] In studies involving a limiting dilution technique, the cytotoxic precursor frequency in a culture containing IL-2 was shown to be much higher in the LGL fraction than in the small lymphocyte fraction prepared from human PBL.[7] On the other hand, the contribution of both T and NK cells to LAK activity has been reported. Ballas et al. reported that both NK cells and CTL precursors from mouse spleen could become LAK cells upon stimulation with IL-2.[9] Furthermore, Kalland et al. reported that 40% of the total LAK activity generated in mouse spleen was associated with NK-LAK and 60% with T-LAK.[10]

In this study, we found that the time course of the acquisition of SBA binding sites differed between NK cells and T cells upon activation with rIL-2. NK cells could acquire SBA binding sites earlier than T cells after activation with rIL-2. Utilizing these findings, we could compare the activities of NK-derived LAK cells and T cell-derived LAK cells in vitro and in vivo after separation on the basis of the expression of SBA binding sites. The

Fig. 10. Effect of SBA⁺ cells on experimental pulmonary metastasis. NL-17 tumor cells (2×10^4) were injected iv into BALB/c mice on day 0. On day 3, 2×10^6 effector cells were injected iv into each mouse. As effector cells, BALB/c spleen cells were incubated in the presence of rIL-2 (2 U/ml) for 3 days, and then the cells were stained with SBA and sorted into SBA⁺ and SBA⁻ fractions. After an 18-h incubation with rIL-2, the cells were used as effector cells. Lungs were harvested and metastatic nodules were counted after 18 days. Each dot represents the number of lung metastases in an individual mouse. Statistic significance of differences: control vs. SBA⁺, *** $P < 0.001$; control vs. SBA⁻, NS (not significant); control vs. unsorted, NS.

SBA$^+$-NK-derived LAK cells showed not only stronger LAK activity *in vitro* but also stronger inhibitory activity toward experimental pulmonary metastasis *in vivo* than SBA$^-$-T cell-derived LAK cells.

The time course of the acquisition of SBA binding sites was found to differ between mouse strains. BALB/c mouse T cells did not become SBA$^+$ until day 4, whereas C57BL/6 mouse T cells became SBA$^+$ on day 3 after culture initiation with rIL-2. For this reason, we separated LAK cells on day 3 in the case of BALB/c mice, but on day 2 in the case of C57BL/6 mice. However, since the cells of non-T cell origin acquired SBA binding sites earlier than T cells after culture initiation in both strains, the separation using SBA was effective if the timing of the separation was carefully selected.

A carbohydrate antigen, asialo GM$_1$, has been considered to be an NK marker. Negative selection with anti-asialo GM$_1$ and complement diminished the NK activity of fresh spleen cells,[22] and iv injection of anti-asialo GM$_1$ resulted in the reduction of NK activity in mouse spleen and also facilitated the growth of transplanted tumor cells.[23] However, the expression of the asialo GM$_1$ determinant was not restricted to NK cells, but its expression on alloreactive CTL[24] or activated macrophages has been reported.[25] In our study, the expression of asialo GM$_1$ determinants was related to the activation stage with rIL-2 despite that the expression on the LGL fraction of fresh spleen cells was confirmed. Thus, the extended culture with rIL-2 resulted in the expression of asialo GM$_1$ not only on NK-derived cells, but also on T cells.

In the extended culture with rIL-2, we found that a small portion of SBA$^+$ cells expressed T3-ε antigens (Figs. 7 and 8). Since SBA$^+$ cells separated on day 3 of the culture with rIL-2 did not express T3-ε antigens on day 4 (Fig. 3), immature T cells may possibly be included in the SBA$^+$ fraction, and these cells may acquire the ability of CD3 expression during the extended culture period. In fact, human CD2$^+$ CD3$^-$ cells with LAK activity have been reported.[26] However, the contribution of such putative immature T cells to LAK activity may be small, because the SBA$^+$-sorted fraction mainly consisted of NK-derived cells (Fig. 8), even after the extended culture period.

Taken together, the results show that the LAK cells with NK-phenotypes express binding sites for SBA at an early stage of the culture with rIL-2, and thus SBA could be efficiently used for the selection of LAK cells with NK-phenotypes at this stage. On the other hand, we observed that T cells expressed the binding sites for SBA at a later stage of the culture with rIL-2. Another carbohydrate determinant, asialo GM$_1$, was expressed not only on NK cells without *in vitro* activation with rIL-2, but also on the cells with NK phenotypes and T cell phenotypes after culturing with rIL-2. Unlike those for asialo GM$_1$, SBA binding sites were not significantly expressed on NK cells without *in vitro* activation with rIL-2. We also found SBA-selected NK-derived LAK cells were more effective as to *in vivo* inhibition of experimental pulmonary metastasis.

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