Immunology 1997 **92** 180–187

Later development of Fas ligand-mediated cytotoxicity as compared with granule-mediated cytotoxicity during the maturation of natural killer cells

T. NAKAZAWA, K. AGEMATSU & A. YABUHARA *Department of Paediatrics, Shinshu University School of Medicine, Asahi, Matsumoto, Japan*

SUMMARY

We classified $CD56^+$ $CD3^-$ natural killer (NK) cells into $CD2^-$ $CD56^{dim}$ ($CD2^-$ NK), $CD2^+$ $CD56^{dim}$ ($CD2^+$ NK) and $CD2^+$ $CD56^{bright}$ populations, and investigated mainly functional differences between the former two populations. $CD2^-$ and $CD2^+$ NK cells were the same in their morphology and several surface molecules except for CD2. The percentages of $CD2^-$ NK cells in total NK cells were higher in the cord blood and bone marrow than in the peripheral blood of adults or children. Freshly isolated $CD2^-$ NK cells had CD2 in the cytoplasm, and gradually expressed it on the surface upon incubation with interleukin-2 (IL-2). These results demonstrated that CD2 is an antigen which appears on the surface during the maturation of NK cells. The granule-mediated cytotoxicities, which are mainly performed by the perforin molecule, of $CD2^+$ NK cells against K562 and Daudi cells were higher than those of $CD2^-$ NK cells, and they were inhibited to the levels of $CD2^-$ NK cells by the addition of a blocking anti-CD2 monoclonal antibody (mAb). Fas ligand (FasL) mRNA was expressed in freshly isolated $CD2^+$ NK cells but not in the $CD2^-$ NK cells. Neither freshly isolated NK populations showed FasL-mediated cytotoxicity, and only $CD2^+$ NK cells lysed Fas-transfected targets after the 24-hr incubation with IL-2. Based on these results, $CD2^-$ NK cells have already developed granule-mediated cytotoxicity equal to that of $CD2^+$ NK cells except for the CD2-associated activity, but they, unlike $CD2^+$ NK cells, totally lack FasL-mediated cytotoxicity. These findings suggest that FasL-mediated cytotoxicity may be acquired at more mature stages of NK-cell maturation than granule-mediated cytotoxicity.

INTRODUCTION

Natural killer (NK) cells are a population of lymphocytes that possess unique properties such as destruction of certain tumour cells and virus-infected cells without prior sensitization.[1] The NK-cell population, defined as the $CD3^-$ $CD56^+$ lymphocyte subset, comprises approximately 15% of the total lymphocytes in the human peripheral blood.[2,3] NK cells are characterized by cell surface expression of the CD56 and CD16 antigens.[2,3] It has been shown that approximately 80% of human NK cells express CD2 antigen, and a CD2-negative population resides in peripheral blood NK cells.[2–5] CD2 is a sheep erythrocyte receptor protein with 50 000 MW whose ligands are CD58 in human[6] and CD48 in rodents.[7] It is implicated in T-cell activation as an adhesion and co-stimulatory molecule.[8,9] CD2 is the earliest differentiation antigen to appear in human T-cell ontogeny and is expressed on virtually all thymocytes and mature T cells.[10] However, the details of NK-cell development and the contribution of CD2 antigen on NK cells remain to be elucidated.

NK cells are known to utilize mainly two different cytotoxic molecules; perforin and Fas ligand (FasL).[11–14] The perforin molecule, which is contained in the cytoplasmic granules, plays a major role in NK cell-mediated cytotoxicity.[11] Although differences in the granule-mediated cytotoxicity between $CD56^{dim}$ and $CD56^{bright}$ NK cells have been well investigated,[2,3] those between $CD2^-$ $CD56^{dim}$ and $CD2^+$ $CD56^{dim}$ NK cells have not been reported so far. FasL is a 40 000 MW type II transmembrane glycoprotein belonging to the tumour necrosis factor family, which induces apoptosis by binding to its receptor, Fas.[15] Arase *et al.*[12] have demonstrated that murine NK cells constitutively express FasL mRNA and kill Fas-expressing target cells. Similarly, human NK cells express FasL mRNA and show FasL-mediated cytotoxicity,[13,14] but little is known of whether this cytotoxicity is demonstrated in all NK-cell populations.

In the present studies, we have investigated the functional differences between $CD2^-$ $CD56^{dim}$ and $CD2^+$ $CD56^{dim}$ NK cells in the human peripheral blood with particular interest in

Received 8 May 1997; revised 4 July 1997; accepted 7 July 1997.

Abbreviations: FasL, Fas ligand; LAK, lymphokine-activated killer; mAb, monoclonal antibody; MNC, mononuclear cell; NK, natural killer.

Correspondence: Dr T. Nakazawa, Department of Paediatrics, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390, Japan.

the development of granule-mediated and FasL-mediated cytotoxicities.

MATERIALS AND METHODS

Monoclonal antibodies (mAb) and reagents

The following mAb were used in this study: anti-CD2 (T11, IgG1) and anti-CD56 (NKH1, IgG1) were purchased from Coulter Immunology (Hialeah, FL), anti-CD3 (Leu4, IgG1), anti-CD8 (Leu2a, IgG1), anti-CD16 (Leu11a, IgG1) and anti-CD57 (Leu7, IgM) from Becton-Dickinson (San Jose, CA), anti-CD11a (MHM24, IgG1), anti-CD11c (KB90, IgG1), anti-CD18 (MHM23, IgG1), anti-CD25 (ACT-1, IgG1) and anti-Bcl-2 (124, IgG1) from Dako (Carpinteria, CA) and anti-CD95 (UB2, IgG1) from MBL Co. (Nagoya, Japan). Anti-CD95 (7C11, IgM) was kindly provided by Dr J. Ritz (Dana-Farber Cancer Institute, Boston, MA). Anti-FasL (NOK2, IgG2a) was kindly provided by Dr K. Okumura (Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan). Interleukin-2 (IL-2) was purchased from Takeda Pharmaceutical Co. (Osaka, Japan) and interferon-γ (IFN-γ) from Shionogi Pharmaceutical Co. (Osaka, Japan). IL-12 was kindly provided by Dr M. Kobayashi (Genetics Institute Inc., Cambridge, MA). G-418 was purchased from Life Technologies (Grand Island, NY) and concanamycin A from Waco Pure Chemical Industries (Osaka, Japan).

Cell lines

We used the human erythroleukaemia cell line, K562, and human Burkitt's lymphoma cell line, Daudi, as target cells in the cytotoxicity assay. The mouse T-cell lymphoma cell line, WR19L and Fas transfected WR19L cell line (Fas/WR19L) were a generous gift from Dr K. Okumura. The K562, Daudi and WR19L cell lines were maintained in culture in RPMI-1640 medium (Flow Laboratories, Irvine, UK) containing 10% heat-inactivated fetal calf serum (FCS) (Upstate Biotechnology Inc., Lake Placid, NY), and the Fas/WR19L in RPMI-1640 medium containing G-418 (900 μg/ml) and 10% FCS in a humidified atmosphere of 5% CO_2 at 37°.

Isolation and purification of NK cells

Human peripheral blood mononuclear cells (MNC), cord blood MNC, and bone marrow MNC were obtained from normal volunteers after informed consent was given. Purification of NK cells was performed as described previously.[16] Briefly, MNC were isolated on a Ficoll–Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Adherent cells were depleted by incubation on plastic dishes for 1 hr at 37°. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD2 mAb, phycoerythrin (PE)-conjugated anti-CD56 mAb and biotinylated anti-CD3 mAb, followed by allophyocyanin (APC)-conjugated streptavidin (Becton-Dickinson). In some experiments, the cells were stained with FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD56 mAb and biotinylated anti-CD2 mAb, followed by streptavidin–APC. $CD2^-$ $CD56^{dim}$ $CD3^-$ and $CD2^+$ $CD56^{dim}$ $CD3^-$ NK cells were sorted with a fluorescence-activated cell sorter (FACStar plus) (Becton-Dickinson) under sterile conditions. Purity of the sorted NK populations was more than 98%.

Flow cytometric analysis

For analysis of cell surface antigen expression, aliquots of 1×10^6 MNC or purified NK cells were incubated with the indicated directly labelled mAb, properly diluted for 30 min at 4°. After washing twice with cold phosphate-buffered saline (PBS), the cells were resuspended in PBS. For analysis of cytoplasmic antigen expression, aliquots of 1×10^6 purified NK cells were fixed and permeabilized with Ortho Permeafix (Ortho Diagnostic System Inc., Raritan, NJ) for 40 min at room temperature. The cells were washed twice with PBS containing 2% FCS (PBS/FCS), and incubated with directly labelled mAb for 60 min at 4°. After washing again twice with PBS/FCS, the cells were resuspended in PBS/FCS. Single-, two-, or three-colour stained cells were analysed for relative fluorescence intensity on a FACScan cytofluorometer (Becton-Dickinson), and the percentage of positively stained cells was determined over 10 000 events. Fluorescence intensity is expressed in arbitrary units on a logarithmic scale. The isotype-matched direct-labelled controls were included in all experiments.

Cytotoxicity assays

Purified NK-cell cytotoxic activity was tested against various target cells in a standard 4-hr ^{51}Cr-release assay as previously described.[17] Briefly, freshly isolated NK cells and cultured NK cells in RPMI-1640 containing 10% FCS in the presence of IL-2 (200 U/ml), IL-12 (10 U/ml) or IFN-γ (1000 U/ml) at 37° for 24–72 hr as indicated were used as effector cells. The cells were washed once and assayed for cytotoxic activity against ^{51}Cr-labelled target cells (1×10^4/well) in a total volume of 200 μl in 96-well U-bottom microtitre plates (Nunc, Roskilde, Denmark). After 4 hr, 100 μl of supernatant was harvested and counted in a gamma-counter (Packard Instrument Co., Meriden, CT). Maximum and spontaneous releases were determined by incubating ^{51}Cr-labelled target cells with 1% Triton-X-100 (Sigma Chemical Co., St Louis, MO) or medium alone, respectively. All determinations were made in triplicate, and effector:target (E:T) ratios ranged from 10:1 to 0·3:1, as indicated. The percentage of specific lysis was determined as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The spontaneous release was routinely less than 10% of the maximum release.

Reverse transcription-polymerase chain reaction (RT-PCR) of FasL mRNA

Total RNA was extracted from sorted NK cells (1×10^6 cells) by the acid–guanidium thiocyanate–phenol–chloroform (AGPC) method using a RNAzol rapid RNA purification kit (Biotecx, Houston, TX). First-stranded cDNA copies were synthesized by using murine Moloney leukaemia virus (M-MLV) reverse transcriptase (Superscript II; GibcoBRL, Gaithersburg, MD) with oligo(dT) (GibcoBRL) as a primer in a total volume of 20 μl, and was used for PCR reaction. Primer sequences were as follows: FasL sense primer 5′-AAGGGTGGCCTTGTGATC-3′ and antisense primer 5′-GACCAGAGAGAGCTCAGA-3′,[15] β_2-microglobulin sense primer 5′-GCTATGTGTCTGGGTTTCAT-3′ and antisense primer 5′-CCCACTTAACTATCTTGGGC3′.[18] Two microlitres of cDNA were amplified in PCR under the following conditions using each primer and 5 U of Taq DNA

polymerase (GibcoBRL): 94° for 1 min, 55° for 2 min and 72° for 3 min for 30 cycles. After amplification, PCR products were separated by electrophoresis on 1·2% agarose gel containing ethidium bromide and visualized by ultraviolet light illumination.

RESULTS

Phenotypic characterization of three NK-cell populations

In the present study, we have identified three distinct populations of $CD3^-$ $CD56^+$ NK cells based on relative levels of CD2 and CD56 expressions: $CD2^-$ $CD56^{dim}$ ($CD2^-$) $CD2^+$ $CD56^{dim}$ ($CD2^+$) and $CD2^+$ $CD56^{bright}$ ($CD56^{bright}$) NK cells (Fig. 1a).

We first examined the percentages of these three NK populations in peripheral blood NK cells comparing those in cord blood and bone marrow NK cells. As shown in Table 1, there was no difference in the percentage of circulating $CD2^-$ population between adults and children. In contrast, the values of cord blood and bone marrow $CD2^-$ NK cells were higher than that of peripheral blood $CD2^-$ NK cells ($P<0{\cdot}001$). However, there was no difference in the percentage of $CD56^{bright}$ population among the peripheral blood, cord blood and bone marrow. The percentages of NK cells in total lymphocytes were almost the same among the peripheral blood, cord blood and bone marrow.

Morphology and antigen expression of $CD2^-$ and $CD2^+$ NK cells

As $CD56^{bright}$ cells are well investigated and known to have unique characteristics,[2,3,19] we here studied mainly characteristics of the $CD2^-$ and $CD2^+$ NK populations.

To delineate the functions and characteristics of these populations, we purified the two NK populations by sorting (Fig. 1b). May–Giemsa-staining showed that both of these populations were large granular lymphocytes, and had no differences in the number and size of granules (Fig. 1c,d). There were no differences in the surface expression of CD8, CD57, CD95 (Fas), adhesion molecules (CD11a, CD11c, CD18), activation antigen (CD25) and cytoplasmic expression of Bcl-2 between the two populations when analysed in freshly isolated populations (Fig. 2) and after IL-2 activation (data not shown). Both NK populations expressed substantial levels of CD16 molecule (FcδRIII) on the cell surface (Fig. 2). These results demonstrate that $CD2^-$ and $CD2^+$ NK cells have the same morphology and have several surface molecules in common except for CD2.

$CD2^-$ NK cells express cytoplasmic CD2 molecule and express surface CD2 after incubation with IL-2

We next investigated whether these cells reside in different subsets. We examined the cytoplasmic CD2 molecule of $CD2^-$

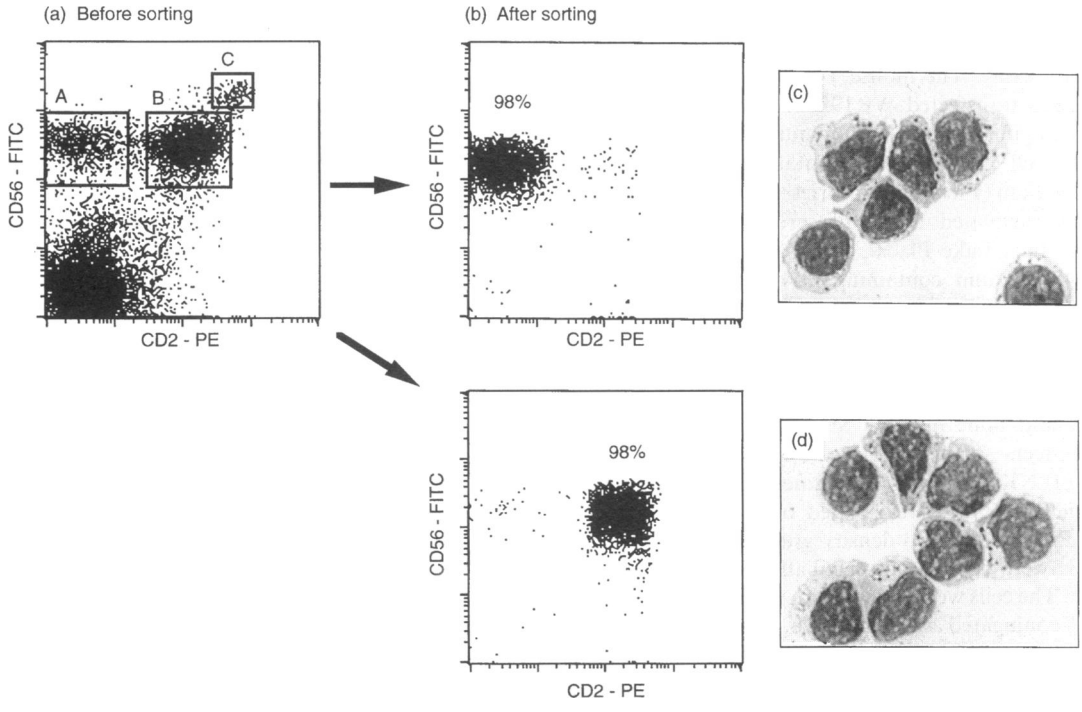

Figure 1. Phenotypic characterization and purification of NK cells, and morphology of these populations. MNC were stained with FITC-conjugated anti-CD2 mAb, PE-conjugated anti-CD56 mAb and biotinylated anti-CD3 mAb followed by streptavidin-APC, and were analysed and sorted using a FACStar plus. (a) Gated $CD3^-$ lymphocytes are shown. Three populations of $CD56^+$ NK cells are identified and indicated by the boxes labelled (A) to (C). In subsequent figures and tables, the $CD2^-$ $CD56^{dim}$ $CD3^-$ (A) cells are referred to as the $CD2^-$ NK population, and the $CD2^+$ $CD56^{dim}$ $CD3^-$ (B) cells are referred to as the $CD2^+$ NK population. (b) The purity of the sorted $CD2^-$ and $CD2^+$ NK cells was more than 98%. Purified $CD2^-$ (c) and $CD2^+$ (d) NK cells were cytospinned on the slide glass, and stained with May–Giemsa-staining. Magnification, ×1000. Each photograph is a representative from three independent experiments.

Table 1. Percentages of $CD56^{bright}$, $CD2^+$ and $CD2^-$ and NK-cell populations in total NK cells

	Peripheral blood			
	Adult*	Child*	Cord blood*	Bone marrow*
NK cells in total lymphocytes	15·1±6·8	13·3±6·0	11·8±5·8	18·2±6·9
$CD56^{bright}$ cells in total NK cells†	4·9±2·7	5·8±2·9	5·9±3·9	4·0±1·8
$CD2^+$ NK cells in total NK cells†	69·7±7·5	69·1±8·4	51·3±5·9‡	55·2±6·3‡
$CD2^-$ NK cells in total NK cells†	25·4±7·4	25·1±7·5	42·8±6·5‡	40·8±6·4‡

*The results represent mean percentages±SD collected from adults ($n=21$, age; 18–40 years old), children ($n=14$, age; 1–14 years old), cord blood samples ($n=13$, gestational age; 37–41 weeks), and bone marrow samples ($n=7$, age; 10–20 years old).

†NK cells were indicated as $CD56^+$ $CD3^-$ lymphocytes.

‡$P<0.001$ as compared to values of adults and children. Comparisons were done using ANOVA with Bonferroni method.

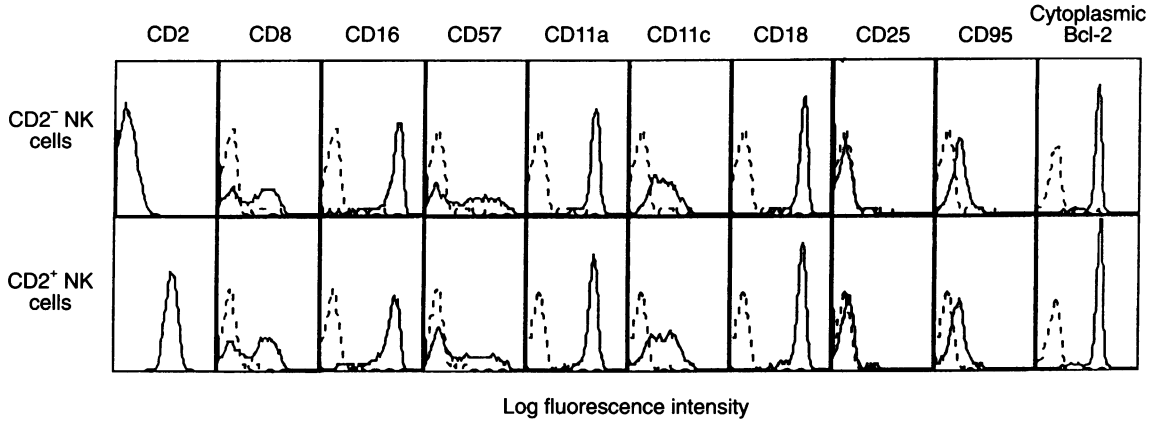

Figure 2. Phenotypic analysis of $CD2^-$ and $CD2^+$ NK populations by flow cytometry. MNC were stained with anti-CD3-FITC, anti-CD56-PE and anti-CD2-biotin plus streptavidin-APC, and sorted. Purified NK populations were stained with antibodies directly conjugated with FITC, as indicated (solid line). For Bcl-2 staining, these cells were permeabilized as described in the Materials and Methods. Isotype-matched FITC-labelled mAb was used as the negative control (dotted line). Similar results were obtained in three independent experiments.

NK cells by flow cytometry, and observed substantial levels of cytoplasmic CD2 expression (Fig. 3a). In addition, when we cultured $CD2^-$ NK cells with IL-2 for 4–14 days, we found the surface expression of CD2. The levels of CD2 expression were increased gradually (Fig. 3b), and a part of the cells expressed substantial levels of surface CD2. However, the other cells still expressed surface CD2 weakly. $CD2^-$ NK cells may require a longer time period and additional stimulation to shift totally to $CD2^+$ NK cells. These results suggest that $CD2^-$ NK cells have the ability to express CD2 on their surface, and may reside at earlier stages of maturation.

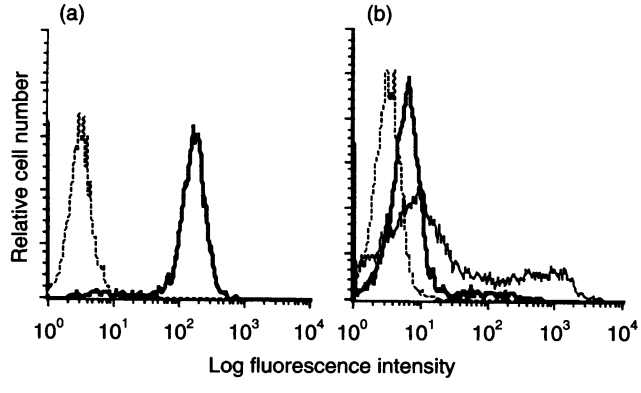

Figure 3. Flow cytometry analysis of cytoplasmic CD2 antigen expression in resting $CD2^-$ NK cells, and surface CD2 expression on cultured $CD2^-$ NK cells. (a) Freshly isolated $CD2^-$ NK cells were permeabilized and stained with FITC-conjugated anti-CD2 mAb and analysed by FACScan (solid line). (b) Sorted $CD2^-$ NK cells were incubated in the presence of IL-2 (200 U/ml) for 4 days (bold line) or 14 days (thin line), and stained with anti-CD2-FITC. Isotype-matched FITC-labelled mAb was used as the negative control (dotted line). Similar results were obtained in three independent experiments.

Granule-mediated cytotoxicity of the two NK populations

Since we demonstrated that $CD2^-$ and $CD2^+$ NK cells represent the stage of maturation of the NK cell, we investigated the functional differences of the two populations. Recent studies have demonstrated that perforin and FasL are major mechanisms of T and NK cell-mediated cytotoxicity.[11–14] To investigate granule-mediated cytotoxicity, which is mainly performed by perforin molecule, we used K562 (NK-sensitive tumour cell line) and Daudi (NK-resistant tumour cell line) cells as the targets. K562 and Daudi cells expressed CD58, the

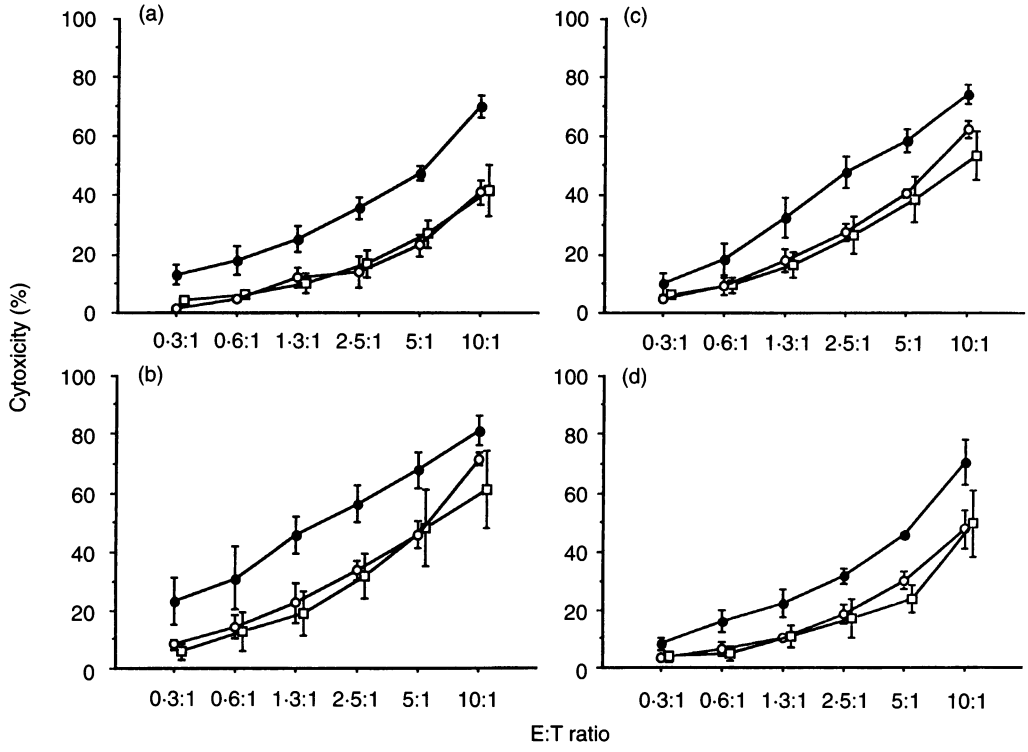

Figure 4. Cytotoxicity of CD2$^-$ and CD2$^+$ NK populations, and the blocking effect of anti-CD2 mAb. Freshly isolated (a) or cultured NK populations in the presence of IL-2 (200 U/ml) (b), IL-12 (10 U/ml) (c) and IFN-γ (1000 U/ml) (d) for 24 hr were used as effector cells. Freshly isolated or cultured CD2$^+$ NK cells (●), with anti-CD2 mAb (T11; 10 μg/ml) pretreatment (□) and CD2$^-$ NK cells (○) were tested for cytotoxicity against K562 target cells at various E:T ratios in a standard 4-hr ^{51}Cr-release assay. Results are expressed as the mean percentage of cytotoxicity ± SEM of four separate experiments.

ligand of CD2, on the surface, and these cells did not show apoptosis by the addition of stimulating anti-Fas mAb (7C11; 10 μg/ml) (data not shown). The cytotoxic activity of freshly isolated CD2$^+$ NK cells against K562 was higher than that of CD2$^-$ NK cells, which was inhibited to the levels of CD2$^-$ NK cells by the addition of blocking anti-CD2 mAb (Fig. 4a). After IL-2, IL-12 or IFN-γ stimulation for 24 hr, CD2$^+$ NK cells consistently exhibited higher cytolysis against K562 than did CD2$^-$ NK cells. The addition of anti-CD2 mAb showed a similar pattern in freshly isolated NK cells (Fig. 4b,c,d). Similarly, the cytotoxic activity of CD2$^+$ lymphokine-activated killer (LAK) cells against Daudi was higher than that of CD2$^-$ LAK cells, which was inhibited to the levels of CD2$^-$ LAK cells by the addition of anti-CD2 mAb (Fig. 5). These cytotoxic activities were completely blocked by the addition of concanamycin A (100 nM), an inhibitor of the perforin-associated system (data not shown), indicating that these cytotoxicities were mediated by the perforin molecule.[20] These results indicate that both CD2$^-$ and CD2$^+$ NK-cell populations have granule-mediated cytotoxicity, and the small difference in the cytotoxicity level between the two is due to the presence or absence of CD2 as an adhesion molecule.[5]

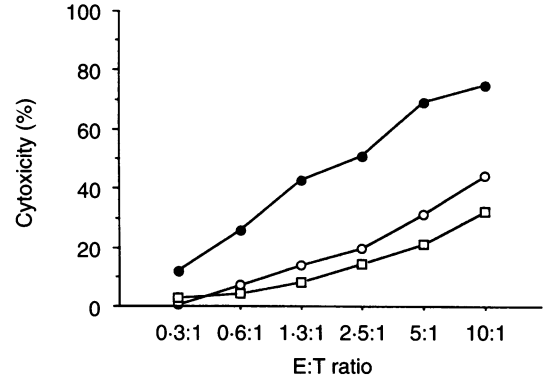

Figure 5. LAK activity of CD2$^-$ and CD2$^+$ NK populations, and the blocking effect of anti-CD2 mAb. Sorted NK populations were cultured in the presence of IL-2 (200 U/ml) for 4 days, and were used as LAK effector cells. CD2$^+$ LAK cells (●), with anti-CD2 mAb (T11; 10 μg/ml) pretreatment (□) and CD2$^-$ LAK cells (○) were tested for cytotoxicity against Daudi target cells at various E:T ratios in standard 4-hr ^{51}Cr-release assay. Representative data from three independent experiments are shown.

FasL-mediated cytotoxicity of the two NK populations

It has been reported that NK cells express FasL.[12–14] To investigate FasL-mediated cytotoxicity of CD2$^-$ and CD2$^+$ NK cells, we used the Fas-transfected WR19L cell line as the target. The WR19L and Fas/WR19L cells expressed CD48 on their surface, and only Fas/WR19L cells were induced to apoptosis by the addition of stimulating anti-Fas mAb (7C11; 10 μg/ml) within 4 hr (data not shown). Interestingly, the CD2$^+$ NK cells activated by IL-2 for 24 hr killed the Fas transfectant efficiently, but not non-transfected WR19L (Fig. 6b). This cytotoxicity was almost completely inhibited

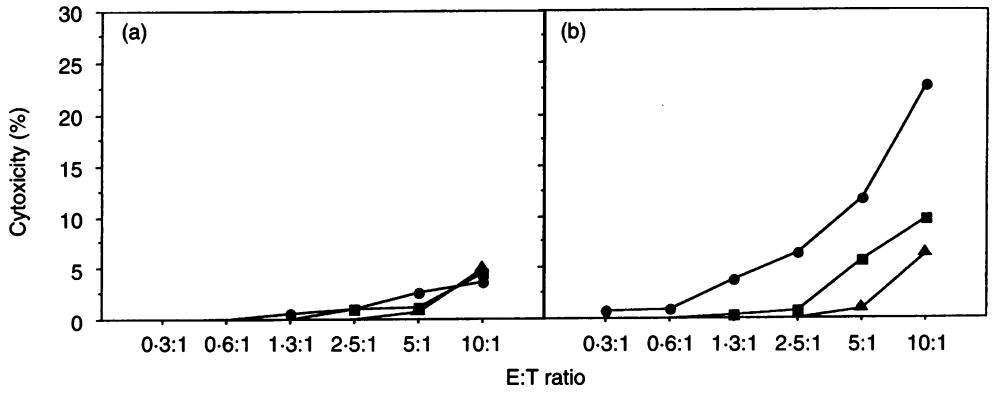

Figure 6. Cytotoxicity of NK populations against Fas-transfectants, and the effect of anti-FasL mAb on the cytotoxic activity. Sorted $CD2^-$ (a) and $CD2^+$ (b) NK cells were cultured in the presence of IL-2 (200 U/ml) for 24 hr, and used as effector cells. The cytotoxic activity was determined by the standard 4-hr ^{51}Cr-release assay using Fas/WR19L (●) or WR19L (▲) as the target cells. The blocking effect of anti-FasL mAb on the cytotoxic activity was determined using Fas/WR19L in the presence of anti-FasL mAb (NOK2; 10 μg/ml) (■). Representative data from four independent experiments are shown.

by the addition of anti-FasL mAb, whereas the concanamycin A (100 nM) pretreatment did not affect the cytotoxicity (data not shown). The addition of anti-CD2 mAb (T11; 10 μg/ml) showed approximately one-third inhibition of the cytotoxicity (data not shown). In contrast, the $CD2^-$ NK cells similarly treated with IL-2 did not lyse either Fas/WR19L or WR19L (Fig. 6a). Neither of the freshly isolated $CD2^-$ and $CD2^+$ NK populations exhibited FasL-mediated cytotoxicity in our system (data not shown). These results indicate that $CD2^+$ NK cells have the potential to exert FasL-mediated cytotoxicity only on short-term culture, but $CD2^-$ NK cells totally lack this ability.

Expression of FasL mRNA in freshly isolated and IL-2-activated NK populations

Since we observed the differences of FasL-mediated cytotoxicity between the two NK populations, we investigated the expression of FasL transcript in $CD2^-$ and $CD2^+$ NK cells by the RT-PCR method. Freshly isolated $CD2^+$ NK cells strongly expressed FasL mRNA, and the expression was up-regulated by IL-2 stimulation for 24 hr, whereas freshly isolated $CD2^-$ NK cells did not express FasL mRNA. After 24 hr of IL-2 activation, $CD2^-$ NK cells also expressed FasL mRNA, but the expression was very weak (Fig. 7). These findings support the view that IL-2-activated $CD2^+$ NK cells have the ability to lyse Fas-expressing targets by producing FasL.

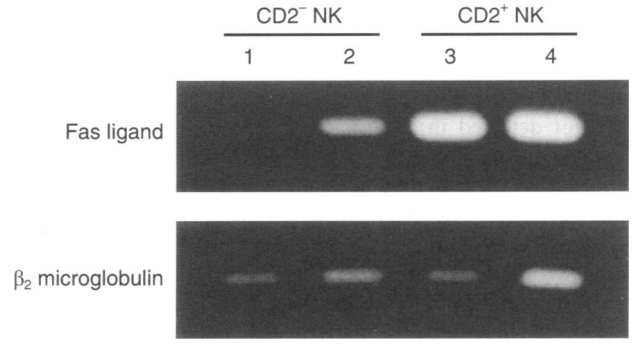

Figure 7. Expression of FasL mRNA on NK populations. The expression of FasL and β_2-microglobulin mRNA in freshly isolated $CD2^-$ NK cells (lane 1), $CD2^+$ NK cells (lane 3), 24 hr IL-2 (200 U/ml) activated $CD2^-$ NK cells (lane 2), $CD2^+$ NK cells (lane 4) were analysed by RT-PCR as described in the Materials and Methods. Representative data from three independent experiments are shown.

DISCUSSION

In the present study, we have identified three populations of NK cells based on relative levels of the CD2 and CD56 expression. The developmental relationship of these NK-cell populations remains controversial. It is known that $CD56^{bright}$ NK cells constitutively express high-affinity IL-2 receptors (α-, β- and γ-chain),[21] and possess substantially higher proliferative capacity[3,19] and IL-2-induced LAK activities[3] than $CD56^{dim}$ NK cells. Lanier *et al.*[2,3] suggested the hypothesis that the $CD56^{bright}$ NK cells represent an early NK progenitor cell subset that may differentiate into mature $CD56^{dim}$ NK cells. Studies from other laboratories demonstrated that human NK cells can differentiate from bone marrow[22,23] or cord blood[24] progenitor cells in long-term cultures, and these NK progenitor cells differentiate from $CD2^-$ to $CD2^+$ populations.[23,24] We have demonstrated here that $CD56^{bright}$ NK cells express high levels of CD2 antigen on the cell surface. The percentages of $CD2^-$ NK cells in cord blood and bone marrow NK cells were higher than the peripheral blood levels. Moreover, $CD2^-$ NK cells expressed substantial levels of CD2 molecule in the cytoplasm, and they gradually expressed CD2 on the cell surface upon incubation with IL-2. These findings support the notion that $CD2^-$ NK cells are at earlier stages of NK-cell maturation. Although Miller *et al.*[23] suggested that marrow stroma can stimulate CD2 expression on NK progenitors, we speculate that other stimulation in the peripheral blood or tissues can also induce the CD2 expression on the surface during the maturation.

In granule-mediated cytotoxicity, $CD2^+$ NK cells possessed higher NK and LAK activities than those of $CD2^-$ NK cells. However, these cytotoxic activities of $CD2^+$ NK cells were inhibited to the levels of $CD2^-$ NK cells by the addition of

blocking anti-CD2 mAb. It is known that NK cells can lyse a target cell through recognition, binding, killing and detaching, and they repeat this lytic sequence in a recycling capacity.[25] Our results suggest that the lower cytotoxic activities of the $CD2^-$ NK cells are due to the depressed binding through CD2 adhesion molecule.[5] Therefore, the killing step itself, which is mediated by the perforin molecule, appears not significantly to differ between the two NK populations. Perforin is contained in the cytoplasmic granules of NK cells.[26,27] In this respect, there was no difference in the number and size of the cytoplasmic granules between $CD2^-$ and $CD2^+$ NK population, which is consistent with the result that each of the two NK populations had substantial levels of granule-mediated cytotoxicity.

Fas/FasL-mediated cytotoxicity is one of the most important functions of NK cells, which induces apoptosis to the Fas-expressing target cells in a granule independent manner. The apoptosis shows characteristic morphological features, such as size reduction, chromatin condensation and nuclear fragmentation, and the dead cells are rapidly phagocytosed and digested by macrophages or by neighbouring cells.[28] Therefore, the Fas/FasL system produces no tissue damage in contrast to perforin-mediated immune reactions and their associated inflammatory responses.[28] It is reported that FasL transcript was not detected in freshly prepared T cells, whereas it was detected in freshly isolated NK cells under the same conditions.[12,14] We provided here interesting evidence that freshly isolated $CD2^+$ NK cells expressed FasL mRNA, but $CD2^-$ NK cells did not express it. Although both of the freshly isolated two NK populations could not lyse Fas-transfected target cells, $CD2^+$ NK cells showed FasL-mediated cytotoxicity upon short-term culture with IL-2. While $CD2^-$ NK cells expressed FasL mRNA after 24 hr IL-2 activation, the expression was weaker than that in $CD2^+$ NK cells, and they could not lyse the Fas transfectant. $CD2^-$ NK cells may require more maturation to develop detectable levels of FasL-mediated cytotoxicity. Anyway, these results demonstrate that there are significant differences in the FasL molecule and FasL-mediated cytotoxicity between the two NK populations.

In conclusion, CD2 is an antigen that is expressed on the surface during the maturation of NK cells. $CD2^-$ NK cells in the peripheral blood have already developed granule-mediated cytotoxicity equivalent to $CD2^+$ NK cells in the killing step itself, but do not have FasL-mediated cytotoxicity. FasL-mediated cytotoxicity may be acquired at more mature stages of NK-cell maturation than granule-mediated cytotoxicity.

ACKNOWLEDGMENTS

The authors thank Prof. A. Komiyama for advice and suggestions throughout this study and Dr S. Itoh for help with the sorting technique. We also thank Drs H. Yagita and K. Okumura for kindly providing anti-FasL mAb and cell lines.

REFERENCES

1. Robertson M.J. & Ritz J. (1990) Biology and clinical relevance of human natural killer cells. *Blood* **76,** 2421.
2. Lanier L.L., Le A.M., Civin C.I., Loken M.R. & Phillips J.H. (1986) The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* **136,** 4480.
3. Nagler A., Lanier L.L., Cwirla S. & Phillips J.H. (1989) Comparative studies of human FcRIII-positive and negative natural killer cells. *J Immunol* **143,** 3183.
4. Schmidt R.E., Michon J.M., Woronicz J., Schlossman S.F., Reinherz E.L. & Ritz J. (1987) Enhancement of natural killer function through activation of the T11 E rosette receptor. *J Clin Invest* **79,** 305.
5. Robertson M.J., Caligiuri M.A., Manley T.J., Levine H. & Ritz J. (1990) Human natural killer cell adhesion molecules: differential expression after activation and participation in cytolysis. *J Immunol* **145,** 3194.
6. Selvaraj P., Plunkett M.L., Dustin M., Sanders M.E., Shaw S. & Springer T.A. (1987) The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. *Nature* **326,** 400.
7. Arulanandam A.R.N., Moingeon P., Concino M.F. *et al.* (1993) A soluble multimeric recombinant CD2 protein identifies CD48 as a low affinity ligand for human CD2: divergence of CD2 ligands during the evolution of humans and mice. *J Exp Med* **177,** 1439.
8. Meuer S.C., Hussey R.E., Fabbi M. *et al.* (1984) An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. *Cell* **36,** 897.
9. Siliciano R.F., Pratt J.C., Schmidt R.E., Ritz J. & Reinherz E.L. (1985) Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature* **317,** 428.
10. Spits H., Lanier L.L. & Phillips J.H. (1995) Development of human T and natural killer cells. *Blood* **85,** 2654.
11. Kägi D., Ledermann B., Bürki K. *et al.* (1994) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369,** 31.
12. Arase H., Arase N. & Saito T. (1995) Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med* **181,** 1235.
13. Montel A.H., Bochan M.R., Hobbs J.A., Lynch D.H. & Brahmi Z. (1995) Fas involvement in cytotoxicity mediated by human NK cells. *Cell Immunol* **166,** 236.
14. Oshimi Y., Oda S., Honda Y., Nagata S. & Miyazaki S. (1996) Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. *J Immunol* **157,** 2909.
15. Takahashi T., Tanaka M., Inazawa J., Abe T., Suda T. & Nagata S. (1994) Human Fas ligand: gene structure, chromosomal location and species specificity. *Int Immunol* **6,** 1567.
16. Yang F.C., Agematsu K., Nakazawa T. *et al.* (1996) CD27/CD70 interaction directly induces natural killer cell killing activity. *Immunol* **88,** 289.
17. Yabuhara A., Kawai H. & Komiyama A. (1990) Development of natural killer cytotoxicity during childhood: marked increases in number of natural killer cells with adequate cytotoxic abilities during infancy to early childhood. *Pediatr Res* **28,** 316.
18. Güssow D., Rein R., Ginjaar I. *et al.* (1987) The human β_2-microglobulin gene: primary structure and definition of the transcriptional unit. *J Immunol* **139,** 3132.
19. Robertson M.J., Manley T.J., Donahue C., Levine H. & Ritz J. (1993) Costimulatory signals are required for optimal proliferation of human natural killer cells. *J Immunol* **150,** 1705.
20. Kataoka T., Shinohara N., Takayama H. *et al.* (1996) Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J Immunol* **156,** 3678.
21. Caligiuri M.A., Zmuidzinas A., Manley T.J., Levine H., Smith K.A. & Ritz J. (1990) Functional consequences of interleukin 2 receptor expression on resting human lymphocytes: identification of a novel natural killer cell subset with high affinity receptors. *J Exp Med* **171,** 1509.
22. Miller J.S., Verfaillie C. & McGlave P. (1992) The generation of human natural killer cells from $CD34^+/DR^-$ primitive progenitors in long-term bone marrow culture. *Blood* **80,** 2182.

23. MILLER J.S., ALLEY K.A. & MCGLAVE P. (1994) Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a $CD34^{+}7^{+}$ NK progenitor. *Blood* **83,** 2594.
24. CICUTTINI F.M., MARTIN M., PETRIE H.T. & BOYD A.W. (1993) A novel population of natural killer progenitor cells isolated from human umbilical cord blood. *J Immunol* **151,** 29.
25. ULLBERG M. & JONDAL M. (1981) Recycling and target binding capacity of human natural killer cells. *J Exp Med* **153,** 615.
26. PODACK E.R. & KONIGSBERG P.J. (1984) Cytolytic T cell granules: isolation, structural, biochemical, and functional characterization. *J Exp Med* **160,** 695.
27. YOUNG J.D., HENGARTNER H., PODACK E.R. & COHN Z.A. (1986) Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell* **44,** 849.
28. STELLER H. (1995) Mechanisms and genes of cellular suicide. *Science* **267,** 1445.