Depressed Natural Killer (NK) Function in Blood and Marrow is Related to the Decrease in CD 11+ Cells in Acute Leukemia

Keiko MORIKAWA, Akinobu NAKANO, Fumimaro OSEKO
and Shigeru MORIKAWA*

The natural killer (NK) cell activity of the blood and marrow was studied in patients with acute myelogenous leukemia (AML). NK activity of the cells from blood and marrow was significantly decreased in AML to three target cell lines. Both binding and killing capacities of the effector cells were deeply depressed in the blood as well as in the marrow at single cell assay. Surface phenotypic analysis showed a significant decrease in CD 11+ cell subsets, but not in CD 16+ or Leu-7+ cells, in both blood and marrow cells from AML. A significant decrease of large granular lymphocytes (LGL) was also displayed in these samples at morphological examination. The effector cells from AML patients poorly responded to interferon stimulation in NK cytolysis. Taken together, a decrease in CD 11+ cell population with LGL morphology appeared to be responsible for the impaired NK activity in patients with AML.

Key Words: NK activity, Acute myelogenous leukemia (AML), Bone marrow, Large granular lymphocytes (LGL), Interferon

Natural killer (NK) cells are a subpopulation of lymphocytes which have cytotoxic activity against a variety of tumor cells (1). Most NK cells are morphologically defined as large granular lymphocytes (LGL) (2). However, NK cells are heterogeneous cell populations in regard to expression of surface phenotypes (3). Although extensive efforts have been made to characterize them, the surface antigenic profiles and target cell specificity of heterogenous NK cells have not yet been fully clarified.

Reduced NK activity has been reported in patients with acute leukemia (4-8). The impaired NK activity in acute leukemia has been considered to be related to the dilution and replacement of normal lymphocytes with blast cells (7). However, recent investigation has indicated that patients with acute leukemia have primary defects in the cytotoxic capacity of their NK cells, therefore, NK cells could not attack the blasts as targets for NK (8). The observations that patients with pre-leukemic stages have shown reduced NK activity (9-11) and that NK cells restore their cytotoxic activity when cultured in the presence of interleukin-2 (IL-2) (8) are the evidences supporting the above possibility. However, it is still uncertain whether or not specific NK cell populations recognize leukemic blasts. The study in the cloning of LGL from human peripheral blood demonstrated that some of the NK cells showed a very limited pattern of specificity and others did multiple recognition patterns to a variety of target cell lines (12). In the above study, the possibility that recognition receptors on the surface of NK cells have been modulated during culture periods for several months could be considered.

In the present study, we examined NK activity of mononuclear cells from blood and marrow in patients with acute leukemia. Both blood and marrow mononuclear cells showed markedly de-
pressed cytotoxicity to three target cell lines. The impaired NK activity was observed in accordance with decreased numbers of CD11+ cells as well as LGL. The CD11+ cell population among heterogeneous NK cell populations appeared to be responsible for, at least, decreased NK activity in acute leukemia.

MATERIALS AND METHODS

Patients
Twenty-five patients with acute myelogenous leukemia (AML), 18 males (range: 17–80 years old) and 7 females (range: 32–72 years old) were examined in this study. Diagnosis was based on clinical, morphological, cytochemical and phenotypical findings in each patient. None had received chemotherapy or radiotherapy prior to the sample collection. As the haematological condition was various in AML, the patients were carefully selected on the bases of the percentages of blastic cells in this study. The morphological examination of mononuclear cells (MNC) obtained by F/H isolation from blood and marrow in these patients was in the following: MNC in blood, small lymphocytes 61.6±8.0%, monocytes 12.0±3.4%, blastic cells 12.2±5.3%; MNC in marrow, small lymphocytes 49.6±8.6%, monocytes, 13.6±4.4%, blastic cells 40.0±9.2%). The control group consisted of 24 normal subjects, 12 males of age range: 20–65 years and 12 females of age range: 28–70 years.

Preparation of effector cells
Bone marrow aspirates were obtained by sternal puncture in the usual manner. One to two ml aspirates were drawn into syringes containing heparin. Twenty ml of heparinized peripheral blood was obtained from the same donors at the time of bone marrow aspiration. Mononuclear cells of both the blood and the marrow were separated by using Ficoll-Hypeaque (F/H) density gradient centrifugation (Pharmacia Fine Chemicals, Uppsala, Sweden). Depletion of adherent cells was performed by incubating them on plastic dishes (Nunc, Roskild, Denmark) at 37°C in a 5% CO2 incubator for 45 minutes.

Target cells
Three NK-sensitive target cell lines, K-562, MOLT-4 and HSB-2, were used as target cells in this experiment. These cell lines were maintained in an RPMI-1640 medium supplemented with 10% fetal calf serum (FCS).

Cytotoxic assay
NK cell activity was determined according to a modification of 3H-uridine labelled methods described by Hashimoto and Sudo (13). Briefly, 1 × 10^6 target cells were incubated with 1 µCi/ml in final concentration of 3H-uridine (specific activity, 25–30 Ci/mmol, Amersham, England) at 37°C in a 5% CO2 incubator for 2 hours. Then, the cells were washed three times with Eagle’s minimum essential medium (MEM) and suspended in Click’s medium (14) plus 10% FCS. Cytotoxicity assay was performed with triplicate wells in microplate (Nunc) using a total volume of 200 µl in each well, 1 × 10^5 labelled target cells were added to 1 × 10^6 effector cells in each well with 10:1 effector to target ratio. The optimal ratio of effector to target cells was determined by previous experiments (15). The cytotoxicity assay was run for 6 hours at 37°C in the humidified atmosphere of 5% CO2 incubator. After that, cell mixtures were harvested with trichloroacetic acid in a multiple cell harvester (Labo Science, Tokyo), and the radioactivity of viable cells was counted in a liquid scintillation counter. Cytotoxicity was expressed in a percentage according to the formula shown in the following:

% lysis = [1−{(target + effector) cell cpm−background cpm}/(target cell cpm−background cpm)] ×100

Evaluation of cell morphology
Effector cell population was analyzed morphologically by a standard May-Grünwald-Giemsa staining. Differential counts were determined by oil immersion microscopy.

Cell surface immunofluorescence
1 × 10^5/0.2 ml cells were incubated at 0°C for 30 minutes with appropriated monoclonal antibodies, washed twice and incubated for additional 30 minutes with fluorescein-conjugated goat antimouse immunoglobulin (Cappel, West Chester, PA). After incubation, the cells were washed twice and prepared as wet mounts. The positive cells were counted using a fluorescent microscope.
Conjugate cytotoxicity assay

This assay was performed as described previously (16). In summary, effector to target conjugates are formed by incubating a 2:1 effector to target mixture of 0.5 ml effector (5 × 10⁵ cells) with 0.5 ml targets (2.5 × 10⁵ cells) in RPMI-1640 medium with 10% FCS in a 10 ml conical tube at 37°C for 15 minutes with subsequent centrifugation (5 minutes at 200 x g). After centrifugation, half of the supernatant was discarded and the pellet was resuspended by gently shaking. Two ml of melted agarose (39°C and not higher) is directly added to this resuspended pellet. The cell-agarose mixture was then quickly spread onto plastic 35-mm dishes (Nunc) which were precoated with poly-L-lysine. After the agarose had solidified, 1 ml of MEM was added to each dish. Dishes were subsequently incubated at 37°C under tissue culture conditions. Six hours later, the plates were removed from the incubator. The medium was removed and 1.5 ml of trypan blue was added for 5 minutes, then the dishes were washed with MEM. The lymphocyte to target binding was determined by counting the number of the lymphocyte-bound-target conjugates in 200 lymphocytes (free and bound) and was expressed as a percentage of lymphocyte to target binding. The lymphocyte killing was counted by the percentage of lymphocyte-bound targets killed in 200 lymphocytes (free and bound). The background death were checked by counting the percentage of dead cells in the absence of effector cells at the same time. The experiment was run in triplicate and expressed as mean ± SE.

Treatment of effector cells with interferon (IFN)

Effector cells were suspended at a concentration of 2 × 10⁶/ml in a culture medium containing human alpha-interferon (α-IFN, kindly provided by Dr. Hideo Yamaguchi, Osaka Red Cross Hospital) at a final concentration of 5,000 U/ml in a 5% CO₂ incubator at 37°C. After 2 hours of incubation, the cells were washed with a medium and immediately used for testing.

Monoclonal antibodies

Anti-human monoclonal antibodies, Leu-7, Leu-11 (CD 16) and Leu-M3 were purchased from Becton-Dickinson (Mountain View, CA), and OKM-1 (CD 11) were obtained from Ortho Diagnostic System (Raritan, NJ).

Statistical analysis

Statistical analysis of the data was performed by the Student's t-test.

RESULTS

Mononuclear cells from the blood as well as the marrow of AML showed markedly diminished NK activity

NK activity in the blood and marrow from patients with AML was examined against three NK-sensitive target cell lines, K-562, MOLT-4 and HSB-2 cells (Fig. 1). Blood NK activity of AML was at significantly low levels to three target cells (16.4 ± 3.6%* to K-562, 15.8 ± 2.9%* to MOLT-4, 15.7 ± 2.9%* to HSB-2: mean ± SEM; *p<0.005) (normal NK activity was mean ± SEM of 35.3 ± 3.5% to K-564, 28.1 ± 4.0% to MOLT-4, 29.5 ± 3.3% to HSB-2) (Fig. 1). The marrow cells from the patients with AML also showed very low levels of NK activity (4.1 ± 2.6% to K-562, 9.0 ± 3.9% to MOLT-4, 5.5 ± 3.5% to HSB-2) (Fig. 1). This data indicated that NK activity in patients with AML was remarkably depressed in the blood as well as in the marrow (marrow NK activity in the normal subjects: 15.2 ± 3.1% to K-562, 16.2 ± 2.8% to MOLT-4, 15.7 ± 2.2% to HSB-2).

Fig. 1. Cytotoxic activity of the mononuclear cells from blood and marrow in patients with AML (●) and normal subjects (○) against three target cell lines (K-562, MOLT-4, HSB-2). Each column represents mean ± SE (l |).
Analysis of surface phenotypes and LGL in patients with AML

Blood NK cells are morphologically defined as LGL, and phenotypically as CD11+, CD16+, and/or Leu-7+ cells (17-19). Next, the blood and marrow mononuclear cells of AML were examined for their morphology and phenotypes. The percentage of LGL in the blood was low in AML (6.8±2.5% in AML to 13.0±2.4% in control) (Fig. 2), but that in the marrow did not significantly decrease (4.4±2.4% in AML to 7.0±2.5% in control) (Fig. 3). LGL is not unique to NK cells as activated T cells are morphologically identical to LGL (3). Therefore, the decrease in LGL may reflect the reduced number of activated T cells. The blood and marrow mononuclear cells were then examined on the proportion of the cells expressing NK cell phenotypes using CD11, CD16 and Leu-7 monoclonal antibodies in addition to Leu-M3 monoclonal antibody (macrophage/monocyte specific). Blood mononuclear cells of AML patients showed an extreme decrease in CD11+ cells (15.0±2.9% in AML to 24.2±2.9% in control) (Fig. 2), while they did not in CD16+ or Leu-7+ cells (Fig. 2). The marrow cells from AML also showed markedly low levels of CD11+ cells and Leu-7+ cell to a lesser degree (17.3±3.2% in AML to 43.3±4.4% in control for CD11+ cells; 3.2±0.8% in AML to 7.0±2.4% in control for Leu-7+ cells) (Fig. 3). The above results demonstrated that a marked decrease in CD11+ cells and LGL existed in both blood and marrow cells from AML. This result suggested that a decrease in CD11+ cell subset with LGL morphology appeared to be responsible for impaired NK activity in AML, though there are no direct evidence that decreased CD11+ cells possessed LGL morphology.

Table 1. Target binding and killing activity of blood and marrow mononuclear cells

<table>
<thead>
<tr>
<th>Source</th>
<th>Binding (%)</th>
<th>Killing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood acute leukemia (4)</td>
<td>4.7±2.5</td>
<td>1.6±0.7*</td>
</tr>
<tr>
<td>normal subjects (6)</td>
<td>11.8±2.3</td>
<td>5.2±0.8</td>
</tr>
<tr>
<td>Marrow acute leukemia (3)</td>
<td>1.8±0.7*</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>normal subjects (6)</td>
<td>5.8±1.2</td>
<td>1.2±0.4</td>
</tr>
</tbody>
</table>

Blood and marrow mononuclear cells were tested target binding and killing activity against K-562 cell lines with 2:1 E:T ratio. Values represent mean ± SEM.
* Statistically significant low levels (*p<0.001, Student’s t-test)
Impairment of binding capacity in the interaction of NK cells and target cells

The mechanism of cytolysis in the NK system consists of two distinct events (20). Initially, NK cells bind to target cells, then, target cells stimulate intracellular lytic system of NK cells, finally resulting in target cell death. We next examined the target cell binding and killing activities of blood NK cells in AML. Binding activity of blood NK cells was approximately 40% of control (Table 2). Their killing activity was approximately 31% of control (Table 2). In marrow cells, the former was 31.0% of control binding activity and 41.6% of control killing activity, respectively (Table 2). Therefore, a remarkable depressed NK activity seemed to be the decrease in binding capacity in blood NK cells. While, in the marrow cells, a decrease in binding ability was observed in AML as well as in normal subjects (Table 2). The marrow cytotoxic cells seemed to have a primary weak killing and binding capacity to target cells.

IFN could not enhance NK cell activity in AML

NK activity in the blood has been shown to be augmented by pretreatment of effector cells with IFN (3, 21). When the blood MNC were pretreated with IFN, NK activity of the blood NK cells did not enhance in AML (17.1% to 19.4% against K-562, 15.6% to 15.8% against MOLT-4), though NK activity from normal subjects showed enhancement with IFN treatment (39.0% to 49.8% against K-562, 28.1% to 38.9% against MOLT-4) (Fig. 4). When the marrow MNC was treated with IFN, there was no increase in NK activity in AML as well as normal subjects (4.4% to 5.7% against K-562, 3.2% to 5.3% against MOLT-4 in AML; 13.4% to 16.3% against K-562, 13.9% to 9.7% against MOLT-4 in normal subjects) (Fig. 4). The NK-effector cells in the blood from AML appeared to be defective in responsiveness to IFN, though the effector cells in the marrow may be essentially insensitive to IFN boosting, because normal subjects did not respond to IFN, too.

DISCUSSION

In the present study, we have demonstrated that the surveillance system by NK cells was totally destroyed in AML. Not only blood cells but also marrow cells from patients with AML had an impairment in cytotoxic activity. Depressed NK cytotoxicity is likely to attribute to the decrease of CD 11+ cells with LGL morphology, because CD 11+ cells were at low levels in the blood as well as the marrow cells in AML. Whereas, other NK cell subsets, CD 16+ cells or Leu-7+ cells did not decrease in number. Not only NK cells but also monocytes and myeloid cells have CD 11 antigens on their cell surfaces. However, it is unlikely that the decrease in CD 11+ in AML is derived from the...
decrease in monocytes or myeloid cells in the blood, because the level of Leu-M 3+ (specific for monocytes) cells was not different from controls in AML (Figs. 2, 3) and any contamination of granulocytes was not detected in mononuclear cells of normal subjects as described in Materials and Methods. In marrow cells, however, the decrease in CD 11+ cells may be partly influenced in the decrease of mature myeloid series in AML. The blastic cells that possess CD 11 or CD 16 antigens on their surfaces in AML are very scarce as shown in the Leucocyte Typing Workshop (22). It is also conceivable that the low NK activity in AML may be caused by the decrease in CD 3+16+ T cells. Recent study have shown that cloned CD 3-4-8-16+ T cells exert nonspecific cytolysis (23). However, T cells expressing such phenotypes (CD 3-4-8- T cells) exist very small in peripheral blood lymphocytes (less than 2%) (24). Therefore, it is unlikely that such T cells are a major part in the decrease in NK activity in our study. The possibility that dilution of NK cells with blasts caused the decrease in CD 11+ cells and low NK cytotoxicity seemed to be unlikely. If the decrease in CD 11+ cells is the result of the dilution of effector cells with blasts, both Leu-7+ cells, CD 16+ cells or Leu-M 3+ cells are low in accordance with low CD 11+ cells. We carefully selected the AML patients not to contaminate a large number of blastic cells in the mononuclear cell fraction as described in Materials and Methods.

Our data also suggested that peripheral NK cells of AML may be functionally disturbed, as NK cells were insensitive to IFN boosting in AML.

Previous investigators reported the impairment of NK activity in patients with acute leukemia (4-8). Some investigators reported that depressed NK activity appeared to be based on the reduced number of effector cells (7) and others did that it was due to a defective killing activity in NK cells (5-8). The evidences that a low NK activity has been observed in remission stages of the patients with acute lymphoblastic leukemia (6) or preleukemic stages of leukemia patients (9) seem to be a clue to answer this question.

However, it is still unknown whether specific NK cells for blastic cell killing exist in acute leukemia. Although NK cells consist of heterogenous cell populations, the functional receptors of target cells on NK cells have not been clarified.

The known NK cell subpopulations at present are CD 3-/Leu-19+/CD 16+ or -, CD 3+/Leu 19+/CD 16+ or - cell subsets (3), Leu-7+/CD 16+ or - (17), Leu-7- or +/CD 11+ (18), CD 3-/CD 16+ or +/Leu-19+, CD 3+ or CD 16-/Leu 19+ cell subsets (19). Recent definition of NK cells (25) described that NK cells are necessary to be CD 3+. Marolda et al. (18) reported that among three subsets of Leu-7+/CD 11+, Leu-7-/CD 11+, and Leu-7-/CD 11- cell types, Leu-7-/CD 11+ phenotypic cells with morphologically LGL have an important role in NK cytolysis in some normal donors. The data that depressed NK function is possibly responsible for low levels of CD 11+ cells and LGL in AML are consistent with their results. It is difficult to estimate whether the unresponsiveness to IFN and depressed binding and killing capacities in AML were also attribute to a decreased number of CD 11+ cells alone or in concert with other NK cell subsets. CD 11+ cells with LGL may be powerful NK capacity among heterogenous NK cell populations.

Studies of cytotoxic activity in marrow cells have been very few. Lotzova (8) described that there was a scarcity of NK cells in bone marrow in patients with acute leukemia. Milleck et al. (4) reported that highly depressed NK activity of blood and marrow cells was observed in both the untreated and remission stages of patients with acute leukemia. As the true features of effector cells in charge of NK cytolysis has been uncertain in marrow elements, it is unknown whether low NK cytotoxicity and insensitivity to IFN is due to immaturity of the precursor NK cells, or whether distinct lineage of cell types attribute to this cytotoxic event.

In conclusion, this study suggested that CD 11+ cell with LGL subset among heterogenous NK cell populations plays an important role in NK cytolysis in AML.
REFERENCES


