High Cytotoxic Cell Activity in the Marrow from Patients with Aplastic Anemia

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The mechanism which produces marrow failure in idiopathic aplastic anemia is still unknown. Recent investigations have suggested the crucial role of NK cells in the regulation of normal hematopoiesis. In this study, the cytotoxic activity of mononuclear cells from human bone marrow and peripheral blood was examined against three NK-sensitive target cell lines in 15 patients with aplastic anemia as well as 21 normal subjects. Marrow mononuclear cells from aplastic anemia demonstrated a high cytotoxicity comparable to peripheral blood NK cells to these target cells. Neither large granular lymphocytes nor the cells expressing known NK cell surface phenotypes increased in aplastic marrow cell elements. The aplastic marrow cells showed strong killing activity rather than binding at single cell assay. They consisted of non-adherent and adherent cell population in plastic adherence and were unresponsive to IFN treatment. The existence of cytotoxic cells with high NK-like activity may be responsible for the mechanism of marrow failure in aplastic anemia.

Key words: Aplastic anemia, Bone marrow, NK activity, Large granular lymphocyte (LGL)

The pathophysiologic mechanisms producing marrow failure in idiopathic aplastic anemia have not yet been clarified. Several evidences suggest that an immune mechanism may destroy or suppress hematopoietic progenitor cells in aplastic anemia. Removal of T lymphocytes from the blood (1) or phagocytic cells in the marrow (2) enhanced hematopoietic colony formation in aplastic anemia. The effectiveness of anti-thoracic duct globulin (3) and of antithymocyte globulin (4) in the treatment of aplastic anemia has been reported. Lymphocytes acting in an inhibitory manner on an in vitro hematopoietic colony formation have been found in aplastic anemia (5, 6). However, other features of aplastic anemia do not support an immune-mediated mechanism in aplastic anemia. Patients with aplastic anemia do not respond to conventional immunosuppressive therapy (7). Antilymphocyte globulin did not enhance colony formation of aplastic marrow (8).

Natural killer (NK) cells have an important role in immunosurveillance mechanism against tumor and in resistance to infections (9). NK cells also play a role in the regulation of hematopoiesis (10). NK cells are involved in the mechanism of rejection of allogeneic and parenteral bone marrow transplants (11, 12). NK cells suppress erythropoiesis and granulopoiesis in vitro (13, 14). These evidences suggest that peripheral NK cells may influence on hematopoiesis in vivo. Then, the question is whether NK-like cells in the marrow play a role in pathological condition. In the present study, we examined the natural cytotoxic activity of the marrow cells from patients with aplastic anemia to examine the above possibility.

MATERIALS AND METHODS

Patients

The patient group consisted of seven males (range, 28–70 years old) and 8 females (range, 34–72
years old) with a chronic form of idiopathic aplastic anemia. Approximately one third of the patients had a history of receiving some blood transfusion in the past. However, none of them have a necessity of it in these several years. They have been given androgen treatment for years, which acted effectively in improving the severity of clinical condition. Any of them had not suffered from viral infection at the time of the examination. The control group consisted of 24 volunteers, 12 males (range, 20–65 years old) and 12 females (range, 28–70 years old).

Collection of mononuclear cells from bone marrow and peripheral blood

Bone marrow aspirates were obtained from patients and adult healthy volunteers by sternal puncture in the usual manner. One to two-milliliter aspirates were drawn into syringes containing heparin. Twenty milliliters of heparinized peripheral blood were obtained from the same subjects at the time of bone marrow puncture.

Preparation of effector cells

Mononuclear cells from the blood and marrow were separated by Ficoll/Hypaque (F/H) density gradient centrifugation (Pharmacia, Uppsala, Sweden). Depletion of adherent cells was performed by incubating the cells in plastic dishes (Nunc Roskild, Denmark) at 37°C in a 5% CO₂ atmosphere for 45 minutes.

Target cells

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

Cytotoxic assay

The cytotoxic activity was determined according to a modification of ³H-uridine labeling methods described (15). Briefly, 10⁶ target cells were incubated with ³H-uridine (Specific activity, 25–30 Ci/mmol, Amersham, England) at a final concentration of 1 μCi/ml at 37°C in 5% CO₂ incubator for 2 hours. The cells were washed three times with MEM and suspended in Click’s medium plus 10% FCS. The cytotoxic assay was performed in triplicate in 96-well microplates (Nunc) with a total volume of 200 μl in each well in an effector-to-target cell ratio of 10:1. The cytotoxicity was run for 6 hours at 37°C in a humidified atmosphere of 5% CO₂. The cell mixtures were harvested with trichloroacetic acid in a multiple cell harvester (Labo Science, Tokyo) and the radioactivity of viable target cells was expressed as a percentage according to the following formula:

\[
\% \text{ lysis} = \left[1 - \frac{\text{target+effector cell cpm} - \text{background}}{\text{target cell cpm} - \text{background}}\right] \times 100
\]

Cytotoxicity assay at single cell level

This assay was performed as described by Grimm and Bonavida (16). In summary, an effector-to-target mixture of 0.5 ml effector (5 × 10⁵) cells with 0.5 ml target (2.5 × 10⁵) cells was incubated in RPMI-1640 medium with 10% FCS in a 10 ml conical tube at 37°C for 15 minutes with subsequent centrifugation (5 min at 200 × g). One-half of the supernatant was discarded and the pellet was resuspended by gentle shaking. Two ml of melted agarose (39°C and no higher) were directly added to this re-suspended pellet. The cell-agarose mixture was then quickly spread onto plastic 35 mm petri dishes (Nunc) pre-coated 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. After 6 hours the medium was removed and 1.5 ml of trypan blue was added for 5 minutes. The dishes were washed with MEM. The percentages of lymphocytes which bind with target cells was determined as target binding activity by counting the number of lymphocytes-to-target conjugates per 200 lymphocytes in the petri dishes. The frequencies of target killing activity of the lymphocytes was expressed as the percentage of dead conjugates relative to the total number of conjugates. The background percentage of cell death was determined by calculating the percentages of dead cells in the control dishes. The experiment was run in duplicate.

Cell surface immunofluorescence

The cells (10⁵/0.2 ml) were incubated on ice for 30 minutes with the appropriate monoclonal antibodies, washed twice and incubated for a further 30 minutes with fluorescein-conjugated goat anti-mouse immunoglobulin. After incubation, the staining cells...
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were counted with fluorescence microscopy.

**Monoclonal antibodies**

OKM-1 (CD 11b) was purchased from Ortho Pharmaceutical Corporation (Raritan, NY). Leu 7, Leu 11 (CD 16) and Leu M3 were obtained from the Becton Dickinson Monoclonal Center (Mountain View, CA).

**Interferon boosting**

Effector cells \((1 \times 10^6)\) were pretreated with 5000 units of purified human alpha interferon (provided by Dr. H. Yamaguchi, Osaka Red Cross Hospital) for 2 hours at 37°C.

**Evaluation of cell morphology**

Effector cell populations were analyzed morphologically by standard May-Grunwald-Giemsa staining procedure. Differential counts were determined by oil immersion microscopy.

**Statistical analysis**

Analysis of the data was performed by Student’s T-test.

## RESULTS

**Cytotoxic activity of the marrow and blood mononuclear cells from patients with aplastic anemia and normal subjects**

The mononuclear cells of the marrow from patients with aplastic anemia demonstrated strong cytolysis to three NK-sensitive target cell lines, which was at considerable levels when compared to that of the blood mononuclear cells with a mean percentage of cytotoxicity, \(26.2 \pm 3.7\%^*\) to K-562, \(27.7 \pm 3.0\%^*\) to Molt-4, and \(22.7 \pm 3.7\%\) to HSB-2 (mean ± SEM; *significant, \(p<0.05\)) (Fig. 1). In contrast, the mononuclear cells from normal marrow showed a much lower cytotoxicity to the target cells than the blood mononuclear cells (\(13.8 \pm 3.3\%\) to K-562, \(12.0 \pm 2.2\%\) to MOLT-4, and \(13.1 \pm 1.9\%\) to HSB-2). It is possible that the contamination of blood NK cells may affect the cytotoxic activity in the marrow of aplastic anemia. To check this possibility, the following experiments were undertaken.

**Membrane characterization of the marrow and blood mononuclear cells in aplastic anemia and normal subjects**

The surface phenotypes of the blood NK cells have been identified as CD 11b\(^+\) (17), CD 16\(^+\) (18, 19) and Leu 7\(^+\) (20). We examined the phenotypes of the mononuclear cells of the marrow and blood from aplastic anemia and from normal subjects using CD 11b, CD 16, and Leu 7 monoclonal
antibodies. We also examined the frequencies of monocytes/macrophages using Leu-M 3 monoclonal antibodies (monocyte/macrophage specific), because monocytes/macrophages demonstrate cytotoxic activity to NK-target cells (21–25). The cell expressing NK-specific phenotypes (Leu7+, CD16+, CD11b+) did not significantly increase in aplastic marrow cells nor in aplastic blood cells compared with those from controls (Table 1). Leu-M 3 + cells did not increase either in the blood and marrow cells of aplastic anemia. This result indicated that the high NK activity of aplastic marrow cells did not derive from the increase in number of NK cells or monocytes which might be contaminated by peripheral blood.

**Morphological characterization of marrow and blood mononuclear cells**

NK cells are morphologically characterized as large granular lymphocytes (LGL), large lymphocytes containing granules in their cytoplasm (26). The leucocyte differentials of mononuclear cells collected by F/H isolation in this experiments was examined. Frequency of LGL from aplastic marrow did not significantly increase in number (6.5 ± 3.4%) when compared with normal marrow cells (7.9 ± 2.5%) (Table 2). The frequencies of LGL in blood cells from aplastic anemia (14.5 ± 5.0%) also did not (controls: 11.8 ± 2.4%) (Table 2).

**Separation of the effector cells by plastic adherence**

Though large population of NK cells have been defined as nonadherent to plastic dishes (19), other studies showed that NK cells present in adherent cell

### Table 1. Membrane phenotypes of blood and marrow mononuclear cells from patients with aplastic anemia and normal subjects.

<table>
<thead>
<tr>
<th>monoclonal antibody</th>
<th>Leu-7</th>
<th>CD 16</th>
<th>CD 11b</th>
<th>Leu-M3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aplastic anemia</td>
<td>(10)</td>
<td>14.3±2.8</td>
<td>13.6±4.6</td>
<td>18.1±1.7</td>
</tr>
<tr>
<td>normal subjects</td>
<td>(21)</td>
<td>14.8±1.5</td>
<td>14.5±1.5</td>
<td>28.2±2.8</td>
</tr>
<tr>
<td><strong>marrow</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aplastic anemia</td>
<td>(8)</td>
<td>8.2±2.6</td>
<td>7.0±1.3</td>
<td>29.4±3.1</td>
</tr>
<tr>
<td>normal subjects</td>
<td>(8)</td>
<td>7.0±2.4</td>
<td>8.4±2.7</td>
<td>43.3±4.4</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM of % cells labeled with each monoclonal antibody. Number in parenthesis represents number of subjects.

### Table 2. Morphological characterization of blood and marrow mononuclear cells from patients with aplastic anemia and normal subjects.

<table>
<thead>
<tr>
<th></th>
<th>LGL</th>
<th>lymphocytes</th>
<th>monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>%</strong></td>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>aplastic anemia (8) blood</td>
<td>14.5±5.0</td>
<td>68.5±8.3</td>
<td>14.5±3.8</td>
</tr>
<tr>
<td>aplastic anemia (8) marrow</td>
<td>6.5±3.4</td>
<td>31.1±10.5</td>
<td>11.4±2.0</td>
</tr>
<tr>
<td>normal subjects (15) blood</td>
<td>11.8±2.4</td>
<td>74.3±4.9</td>
<td>11.1±3.5</td>
</tr>
<tr>
<td>normal subjects (15) marrow</td>
<td>7.9±2.5</td>
<td>38.7±4.1</td>
<td>13.0±2.4</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM. Number in parenthesis represents number of subjects. LGL: large granular lymphocytes.
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populations (24, 25). Therefore, NK cells appeared to be heterogeneous in relation to plastic adherence. The investigation of Freundlich et al (24) demonstrated that an adherent NK cell population showed higher cytotoxicity to target cells than nonadherent cell population in the peripheral blood. When the marrow mononuclear cells from aplastic and normal subjects were divided into adherent and nonadherent cell populations by plastic dishes, adherent cell populations showed a slightly higher cytotoxicity than non-adherent cell population in both groups, but this was not statistically significant (Table 3). This results suggested that approximately half of the cytotoxic cells in the marrow have a character to adherent to the plastic dishes.

**Sensitivity to interferon treatment**

Interferon (IFN) is known to have a profound boosting effect on human NK cell activity (19). When the blood mononuclear cells were pretreated with IFN, cytotoxic cell activities to two target cell lines, MOLT-4 and HSB-2, were increased in aplastic anemia (25.9 to 33.1%, 23.5 to 32.5%) and in controls (29.9 to 38.5%, 29.6 to 38.9%) (Fig. 2), however, cytotoxic cell activity in the marrow mononuclear cells did not increase in the aplastic anemia (20.2 to 19.2%, 20.0 to 20.7%) as well as it did in normals (12.4 to 14.2%, 10.1 to 11.8%) when they were pretreated with IFN for a short time (Fig. 2). Cytotoxic cells in the marrow appeared to be insensitive to IFN boosting in our results.

**Target cell binding and killing activity at single cell level**

In this experiment, the cytotoxic activity of the mononuclear cells from marrow and blood was examined at single cell level as described by Grimm et al (16). Target cell binding and killing activity

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**Table 3. Comparison of NK activity of adherent and nonadherent bone marrow cells from patients with aplastic anemia and normal subjects.**

<table>
<thead>
<tr>
<th></th>
<th>cytotoxicity %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aplastic anemia</strong> (6)</td>
<td></td>
</tr>
<tr>
<td>NA-BMMC</td>
<td>17.6±2.8</td>
</tr>
<tr>
<td>Ad-BMMC</td>
<td>21.8±2.4</td>
</tr>
<tr>
<td><strong>normal subjects</strong> (6)</td>
<td></td>
</tr>
<tr>
<td>NA-BMMC</td>
<td>8.9±2.9</td>
</tr>
<tr>
<td>Ad-BMMC</td>
<td>13.5±3.0</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.

Bone marrow mononuclear cells were treated by plastic dish for 40 min at 37°C and were separated into nonadherent (NA-BMMC) and adherent (Ad-BMMC) cells. These cells were tested in NK activity against K-562 target cells with 10:1 E:T ratio.

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![Graph showing cytotoxicity (% of target cells) against IFN concentration (in a-log scale) for Blood and Marrow samples from Aplastic Anemia and Normal subjects.](image-url)
Table 4. Target binding and killing activity of marrow and blood mononuclear cells.

<table>
<thead>
<tr>
<th>source</th>
<th>binding (%)</th>
<th>killing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aplastic anemia</td>
<td>11.2± 1.2</td>
<td>4.9± 0.5</td>
</tr>
<tr>
<td>normal subjects</td>
<td>10.7± 2.3</td>
<td>5.8± 1.2</td>
</tr>
<tr>
<td>Marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aplastic anemia</td>
<td>7.5± 3.5</td>
<td>3.8± 1.8</td>
</tr>
<tr>
<td>normal subjects</td>
<td>6.3± 1.0</td>
<td>1.2± 0.2</td>
</tr>
</tbody>
</table>

Blood and marrow cells were tested target binding and killing activity to K-562 cells at an E:T ratio of 2:1. (see materials and methods). Values represent mean ± SEM. Number in parenthesis represents number of subjects.

Blood NK cells were very similar between aplastic anemia and normals. Though the aplastic marrow cells did not show significant increase than the normal marrow cells in binding activity to target cells, a pronounced killing activity to target cells was observed in marrow cells from aplastic anemia (3.8 ± 1.8% to 1.2 ± 0.2%) (Table 4). This result indicated that the marrow cytotoxic cells were functionally active in aplastic anemia.

**DISCUSSION**

In the present study, we demonstrated that mononuclear cells from bone marrow of patients with aplastic anemia have a pronounced cytotoxic activity against three NK-sensitive target cell lines. Though normal marrow cells showed weak cytotoxicity to these target cells compared with blood NK cells, the aplastic marrow cells displayed comparable levels of cytotoxicity with the blood NK cells from aplastic patients as well as that from normal subjects. The question is whether conventional NK cells from blood may be contaminated in the marrow elements at the time of marrow aspiration, even though the amounts of marrow aspirated were very small. However, neither LGL nor CD 16+, CD 11+, CD 13+ and/or Leu 7+ cells increased in aplastic marrow cells when compared with normal blood and marrow cells. These results suggested that cytotoxic cells in the marrow from aplastic anemia may not have the known NK cell phenotypes in the peripheral blood. Other data regarding plastic adherence and responsiveness to IFN also showed that the marrow cytotoxic cells possess different characteristics to blood NK cells. The results of cytotoxicity at single cell levels indicated that the cytotoxic cells in the aplastic marrow seem to be more active in target cell killing function than that in normal marrow.

There are growing evidences suggesting the presence of cytotoxic cells in normal marrow (12, 20, 27–34). Cytotoxic activity are in low levels in normal marrow (30, 33, 35). Induction of marrow cytotoxic cells are experimentally modulated by pokeweed mitogens, B cell lines or FCS (30). Recent investigation (36) demonstrated that bone marrow-derived killer cells were possible to be activated by interleukin-2. Another evidences suggested that the cells work suppressively to natural killing, suppressor NK cells, may exist in bone marrow as well as blood (37). Taken together, it is possible that the cytotoxic cells in the marrow may injure the hematopoiesis in some way in vivo.

Although the true feature of marrow cytotoxic cells are unknown, Lohman-Matthes et al (38) have reported that a highly enriched preparation of murine promonocytes showed NK-like cytotoxicity. These effector cells resemble medium-sized lymphocytes, non-adherent, nonphagocytic and negative in esterase activity. Cytotoxic activity was enhanced in the presence of IFN in the culture. Claesson et al (32) reported another observation. They examined the cytotoxic activity of murine marrow cells after 6 to 14 day culture. Diffuse colony-forming cells in the group of colonies showed high killer cell activity. These cells were small, non-adherent, and possibly more immature myeloid lineage cells than promonocytes. The effector cells with cytotoxic activity observed in our study may belong to these immature cytotoxic cell populations in human. Both of the cultured cytotoxic cell clones derived from murine
were non-adherent, but our freshly prepared marrow cells from human are heterogeneous, adherent and non-adherent cell populations. Though the difference between the cytotoxic cells in human and murine is uncertain, the cultured cytotoxic cell clones may represent an expansion of the non-adherent cell population.

Uchida et al (39) reported high NK activity in the bone marrow of myeloma patients. In their study, cytotoxic activity was reduced after treatment of marrow cells with OKM-1 or Leu-7 monoclonal antibody and complement. Therefore, they concluded that NK precursor cells differentiate to mature NK cells in the bone marrow of myeloma patients. As there was no increase in conventional NK cells in the marrow elements of aplastic anemia, a different mechanism may work as a cause of the high killing activity in aplastic anemia and myeloma.

Blood mononuclear cells in patients with aplastic anemia showed diminished NK activity before and after bone marrow transplantation (40) and before receiving a treatment with antithymocyte globulin (41). In both cases, the patients had a severe aplastic anemia. In the former cases, they have received extensive immunosuppressive therapy before and after marrow transplantation. The patients in the latter report had antithymocyte globulin treatment. The patients in our study have a chronic form and had not received any immunosuppressive drugs, so these patients appeared to be less severe in clinical condition than the patients reported in the literatures (40, 41). The different observations between theirs and ours may suggest the differences of the pathogenesis in acute and chronic form of aplastic anemia.

On the other hand, it has been reported that macrophages as well as NK cells have cytotoxic activity against various target cell lines (21–25). It is unlikely that monocytes play a main role in cytotoxicity in the aplastic marrows examined in our study, because neither morphological monocytes nor Leu-M3 (monocyte specific antibody) positive cells changed in number in either aplastic marrow or in normal marrow (Table 1, 2).

The use of 3H-Uridine release assay (15) proved to be no problem for the assessment of NK cell activity to target cells (42). Large amounts of unlabeled uridine in Click’s medium prevent the re-utilization of labeled uridine which is released in very small amounts from killed targets in 6 hours of culture. The effector to target ratio of 10:1 was optimal in our experimental method (42).

In conclusion, the presence of cytotoxic cells with high NK-like activity in aplastic marrow is meaningful in view of the pathophysiological role of the cytotoxic cells in marrow.

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REFERENCES


