Impaired Natural Killer Cell Recycling in Childhood Chronic Neutropenia With Morphological Abnormalities and Defective Chemotaxis of Neutrophils

By Atsushi Komiyama, Hiroshi Kawai, Sachihiro Yamada, Kohki Aoyama, Munehiro Yamazaki, Hiroharu Saitoh, Yukiai Miyagawa, Taro Akabane, and Yoshio Uehara

Natural killer (NK) cell activity was measured by a \(^{51}\)Cr-release assay using K562 target cells in 12 neutropenic children. NK cell activity was depressed in four patients who had childhood chronic neutropenia with abnormal neutrophil morphology and chemotaxis. The percentage of lysis at a 40:1 effector-target ratio was 28.4% to 42.1% (P < .001) of the normal lymphocyte value during the study period (32 to 40 months). NK cell activity was normal in the other eight children with chronic neutropenia without any of these neutrophil abnormalities: lazy leukocyte syndrome, Shwachman syndrome, or dysgammaglobulinemia type I with neutrophil defects. NK cell activity of the four patients was depressed at 5:1 to 40:1 effector-target ratios. The NK cells responded to in vitro interferon (IFN)-\(\alpha\) and interleukin 2, as did normal lymphocytes, but the activated levels were still lower than those of normal lymphocytes (P < .01). Because NK cells kill a target through recognition, binding, killing, and detaching, and they repeat this lytic sequence (ie, recycling), the localization of the NK cell defect was further analyzed in the four patients using both \(^{51}\)Cr-release and single-cell-in-agarose assays. The patients' NK cells were normal in recognizing, binding, and killing a target but were defective in recycling; the estimated maximum recycling capacity (MRC) values in a four-hour assay were 1.8 to 2.4 (P < .01), as compared with the normal lymphocyte value of 5.5 ± 0.6 (mean ± SD). The stimulation of the effector cells with 1,000 U/mL IFN-\(\alpha\) did not significantly increase the estimated MRC. These results demonstrate that NK cells are defective in recycling in some type of childhood chronic neutropenia with abnormal neutrophil morphology and chemotaxis. The NK cell deficiency is of clinical interest in terms of its relationship to the recurrent infections, development of malignancy, and dysgranulopoiesis in the disorder.

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In recent years, much interest has been focused on natural killer (NK) cells that can provide protection against infections\(^{1,2}\) and tumors.\(^{3,4}\) There is also evidence that NK cells may play an important physiologic or pathologic role in the regulation of granulopoiesis.\(^{5}\) When NK cells work as an effector for cytolysis, they first recognize and bind to a target cell, next kill the bound target, then detach from the dead target and repeat this sequence by their recycling capacity.\(^{1,9}\)

Childhood chronic neutropenia comprises a heterogeneous group of neutropenias, usually developing early in life,\(^{10,11}\) that have been described with diagnoses such as congenital neutropenia,\(^{12,13}\) congenital agranulocytosis,\(^{14,15}\) and congenital dysgranulopoietic neutropenia.\(^{16}\) Some children with such neutropenia suffer from frequent infections. Several reports describe the occurrence of a malignancy of acute leukemia in long-term survivors of the patients with some type of childhood chronic neutropenia.\(^{14,15}\) It thus appears of interest to examine whether there are abnormalities of NK cells in childhood chronic neutropenia. Because NK cells are considered to be a possible regulator of granulopoiesis in vivo\(^6\) and there is dysgranulopoiesis in childhood chronic neutropenia,\(^{16}\) we further expected that the NK cell assays might provide some information about the pathogenesis of this disorder. In the present report, we demonstrate depressed activity of NK cells due to their impaired recycling in four children who have chronic neutropenia with morphological abnormalities and defective chemotaxis of neutrophils.

**MATERIALS AND METHODS**

**Patient data and routine laboratory studies.** Twelve neutropenic children were studied. Of the patients, nine had childhood chronic neutropenia; one, lazy leukocyte syndrome; one, Shwachman syndrome; and one, dysgammaglobulinemia type I with neutrophil defects. The major clinical and laboratory findings for these children are presented in Table 1. Patients No. 1 through 4 and 10 through 12 have experienced frequent infections, such as upper and lower respiratory tract infections, superficial abscess, and lymphadenitis, sometimes requiring hospitalization, since early infancy. In Patients No. 1 and 4, varicella infection was somewhat serious; varicella eruptions were larger, resulting in the persistence of many scars on the skin. Patient No. 2 had a severe episode of mycoplasmal pneumonia. In Patients No. 5 through 9, infections were not frequent and were well controlled by treatment with appropriate antibiotics. All the patients had persistent or intermittent neutropenia. Patients No. 1 through 4 were confirmed as neutropenic when they first visited our clinic during infancy to early childhood; we have followed them for six to 15 years without observing resolution from severe neutropenia. Patients No. 5 through 9 recovered spontaneously from neutropenia at 11 to 26 months of age. Patient No. 10 has been neutropenic during the observation period for 28 months, and Patients No. 11 and 12 have had intermittent neutropenia for longer than six years. Serum IgG, IgA, and IgM levels were normal to elevated in all the patients except Patient No. 12, in whom IgM levels were high (930 to 1,020 mg/dL) and IgG and IgA levels were low (<20 mg/dL).

**Special hematologic studies.** For morphological examinations, blood smears were stained with May-Giemsa stain. In addition, peripheral blood samples were routinely processed for electron microscopy as previously described\(^{13,17}\). Briefly, cells were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated, embedded, sectioned, and examined with a Hitachi HS-8 electron microscope (Hitachi Ltd, Tokyo).

Neutrophil chemotaxis was examined by a modification of the Boyden's method using a Millipore filter (3 \(\mu\)m pore size; Millipore Co, Bedford, Mass) and a culture filtrate of *Escherichia coli* as a chemotactic factor. In the present experiments, neutrophil suspension was prepared at a concentration of 5 \(\times\) 10\(^3\) cells per
Table 1. Major Clinical and Laboratory Features of Neutropenic Patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patient No.</th>
<th>Age (yr)†</th>
<th>Sex</th>
<th>Infections Requiring Hospitalization</th>
<th>Neutrophil Count (10³/mm³)</th>
<th>Mono-cytosis</th>
<th>Bone Marrow Morphology</th>
<th>Special Studies on Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Childhood chronic neutropenia</td>
<td>1</td>
<td>5/F</td>
<td></td>
<td>(+)</td>
<td>0–100</td>
<td>(+ +)</td>
<td>Decrease in primary and secondary granules, myelin figure</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6/F</td>
<td></td>
<td>(+)</td>
<td>0–420</td>
<td>(+)</td>
<td>Decrease in primary granules</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12/F</td>
<td></td>
<td>(+)</td>
<td>0–240</td>
<td>(+)</td>
<td>Decrease in primary granules, myelin figure</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>14/M</td>
<td></td>
<td>(+)</td>
<td>0–80</td>
<td>(+ +)</td>
<td>Decrease in granuloid cells, maturation arrest at promyelocyte stage</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2/M</td>
<td></td>
<td>(−)</td>
<td>600–1,200</td>
<td>(−)</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3/F</td>
<td></td>
<td>(−)</td>
<td>820–1,400</td>
<td>(−)</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1/F</td>
<td></td>
<td>(−)</td>
<td>460–880</td>
<td>(−)</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3/M</td>
<td></td>
<td>(−)</td>
<td>800–1,600</td>
<td>(−)</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>6/M</td>
<td></td>
<td>(−)</td>
<td>400–1,460</td>
<td>(−)</td>
<td>Decrease in mature neutrophils</td>
<td>Normal</td>
</tr>
<tr>
<td>Lazy leukocyte syndrome</td>
<td>10</td>
<td>1/F</td>
<td></td>
<td>(+)</td>
<td>0–400</td>
<td>(−)</td>
<td>Normal</td>
<td>Decreased</td>
</tr>
<tr>
<td>Shwachman syndrome</td>
<td>11</td>
<td>8/M</td>
<td></td>
<td>(+)</td>
<td>620–3,600</td>
<td>(−)</td>
<td>Normal</td>
<td>Normal to decreased</td>
</tr>
<tr>
<td>Dysgammaglobulinemia type I</td>
<td>12</td>
<td>7/M</td>
<td></td>
<td>(+)</td>
<td>880–8,600</td>
<td>(−)</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*The patient’s age at the first NK cell assay is given.
†Neutrophil morphology was examined on May-Giemsa–stained smears and by electron microscopy.
‡Neutrophil chemotaxis was studied by the filter method using a culture filtrate of E coli as a chemotactic factor.

milliliter because of the paucity of neutrophils according to our method.1 A chemotaxis index was calculated by dividing the number of neutrophils in ten fields of the finishing side by that of the starting side of the filter (×1000).

Assay for CFU-GM was done as previously reported.14,17 Briefly, 2 × 10⁵ nucleated marrow cells were immobilized in 0.3% agar with McCoy’s 5A medium and plated on the top of a feeder layer containing 1 × 10⁶ normal peripheral leukocytes immobilized in 0.5% agar. The cells were cultured for 14 days, and colony numbers were evaluated.

Sample preparations for NK cell assays. Heparinized peripheral blood samples were obtained from the patients when they were free of overt infection and were not undergoing any treatment. Mononuclear cells (MNCs) were separated by Ficoll-Hyphaque centrifugation from the blood samples and washed three times with Hank’s balanced salt solution (HBSS). MNCs were then depleted of monocytes by adherence to plastic surfaces14 and used as the patients’ lymphocytes. Normal lymphocytes were also obtained in an identical manner from healthy volunteers. As another control, lymphocytes were obtained from age-matched children without any hematologic or immunologic disorder.

Treatment of effector cells with interferon or interleukin 2. In some experiments, lymphocytes at concentrations of 2 to 3 × 10⁶/mL were incubated with 500 to 5,000 U/mL human interferon (IFN)-α (Kyoto Red Cross Hospital, Kyoto, Japan) or 20% interleukin 2 (IL 2) preparation (Ohtsuka Assay Lab, Tokushima, Japan) at 37 °C for 20 hours in 5% CO₂ atmosphere. They were then washed three times with HBSS and used as effector cells.

§Cr-release assay. NK cell activity was assayed on §Cr-labeled K562 target cells as previously described.14 Effector cells included the patients’ and control lymphocytes. For labeling, 2 × 10⁶ K562 cells in 2 mL RPMI 1640 containing 10% fetal calf serum (FCS; Flow Laboratories, Rockville, Md) were incubated with 100 μCi §Cr (Amersham, Arlington Heights, III) at 37 °C for 60 minutes and washed four times with the culture medium. Cell mixtures, prepared at various effector:target ratios, were brougel to a 1-mL final volume and incubated at 37 °C for four hours in 5% CO₂ in air. In some experiments, effector and target cells were mixed at a ratio of 5:1 (5 × 10⁶ effector cells and 10⁶ target cells). The following equation was used to express cytotoxicity: percent lysis = [(cpm experimental release – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release)] × 100. In all the experiments, spontaneous release was <20%. All assays were conducted in triplicate.

Single cell-in-agarose assay. This assay was done according to the method of Grimm and Bonavida1 with some modifications.19 Briefly, 2 × 10⁴ of the patients’ or normal lymphocytes (effector cells) and an equal number of unlabeled K562 cells (target cells) were mixed in a total volume of 2 mL RPMI 1640 containing 10% FCS in a round-bottom tube. The tubes were centrifuged at 500 g for
two minutes and incubated at 37 °C for ten minutes. Control tubes of
target cell alone were prepared in an identical manner. Subsequent-
ly, most of the supernatant was aspirated, leaving a small volume
of the medium, in which the pellet was resuspended. The cell mixture
was then added to 0.5 mL of 0.5% agarose in RPMI 1640 with 10
mmol/L HEPES, which had been melted in boiling water and
maintained in liquid form at 40 °C in a water bath. The cells and
agarose were mixed, and 0.1 mL to 0.2 mL of the mixture was
quickly poured onto tissue culture plates precoated with 0.5% agarose.
After the cell mixture in agarose had solidified, RPMI 1640
with 10% FCS was layered on top of the plates to prevent dehydration
and the plates were incubated at 37 °C for four hours in a 5%
CO2 humidified incubator. In some experiments, the plates were
incubated for ½, 1, 2, and 3 hours. After the incubation, the medium
was removed and 0.1% trypan blue was added for ten minutes.
The plates were washed with cold phosphate-buffered saline, fixed with
1% formaldehyde, and examined with a microscope.

The percentage of target-binding cells (TBCs) was determined by
counting the number of lymphocytes binding to K562 target cells in
400 to 500 counted lymphocytes. There was no difficulty distinguish-
ing between lymphocytes and K562 cells on the basis of their cell
size: the latter cells were much larger than the former. The percent-
age of TBCs with dead targets was determined by counting the
number of dead K562 cells in 100 effector-target conjugates.
Spontaneous target cell death was determined by calculating the
percentage of dead K562 cells in the control cultures without
effector lymphocytes. Corrections allowing for spontaneous target
death were made using the following formula to calculate the
percentage of TBCs with dead targets: (percentage of TBCs with
dead targets) – (percentage of spontaneously dead targets) ×
(percentage of TBCs with dead targets).

Analysis of data. Data from the 51Cr-release assay and from the
single cell-in-agarose assay were combined, and maximum NK
capacity (Vmax) was determined as done by Ullberg and Jondal.9
They demonstrated that it is possible to get rough estimation of
Vmax for 107 effector cells using a 5:1 (5 × 106 effector lymphocytes
and 104 target cells) 51Cr-release assay and then using the following
formula: Vmax = 1.4 × 107 + 4.2 × 102 × (percentage 51Cr release
at a 5:1 effector-target ratio). We thus calculated Vmax in such a
manner. The percentage of active NK cells (lymphocytes with bound
and dead targets) was estimated from the single cell-in-agarose
assay by multiplying the percentage of TBCs by that of TBCs with
dead targets. The maximum capacity (MRC) was then
calculated by dividing Vmax by the absolute number of active NK
cells in the Vmax (106 cells). MRC estimates the average number
of target cells killed by an active NK cell in the four-hour assay.

RESULTS

Further characterization of neutropenia. The neutrope-
nia was further characterized by microscopic morphology,
neutrophil chemotaxis, and CFU-GM assays.

Light and electron microscopy demonstrated some morpho-
logical abnormalities of neutrophils in patients No. 1
through 4 and 11 (Table 1). Neutrophils from patients No. 1
through 4 had decreased numbers of primary granules
frequently showing myeloid figures, and those from patient
No. 1 had decreased numbers of secondary granules as well
at the electron microscopic level; an electron micrograph of a
representative neutrophil from patient No. 1 is presented
elsewhere.11 At the light microscopic level, the neutrophils of
patient No. 11 had kidney-shaped or bilobed nuclei, resembling
Pelger-Huet anomaly, on several occasions during the
five-year observation period.

Neutrophils from patients No. 1 through 4 were defective
in chemotaxis: their chemotaxis index ranged from 97.6 to
122.6, with a mean ± SD of 112.3 ± 11.1 (P < .001), as
compared with the value of normal neutrophils from healthy
volunteers of 396.3 ± 62.5 (mean ± SD, N = 20). In patient
No. 10, neutrophil random mobility (41.0% of the control
value) and chemotaxis (44.7% of the control value) were
decreased. The neutrophils of patient No. 11 were impaired
in chemotaxis (50.6% of the control value) when they had an
abnormal nuclear shape. The neutrophils of patient No. 12
were defective in chemotaxis (16% to 36% of the control
value). Sera from these patients did not inhibit chemotaxis of
normal neutrophils.

The number of CFU-GM was assayed in patients No. 1
through 6 and 9 through 12. The bone marrow cells from all
patients except patient No. 2 grew normal numbers of
CFU-GM when normal leukocytes were used as a feeder
layer: the numbers ranged from 142 to 258 per 2 × 105 cells
as compared with the control value of 189 ± 50
(mean ± SD). In patient No. 2, at age 5 years, the number
of CFU-GM was decreased (19/2 × 105 cells).

NK cell assays. NK cell activity was measured against
K562 by the standard 51Cr-release assay at a 40:1 effector-
target ratio on two to six separate occasions in each of the 12
neutropenic children (Table 2). In every experiment, the
results were presented as the percentage of the control value
obtained using normal lymphocytes in the parallel experi-
ment; percentage of lysis of normal lymphocytes from 20
healthy individuals was 47.2% ± 5.7% (mean ± SD) in the
present studies. NK cell activity was significantly depressed

Table 2. NK Cell Activity

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number of Assays</th>
<th>Intervals Between First and Last NK Cell Assay (mol)</th>
<th>NK Cell Activity as Percent Control* (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>34</td>
<td>42.1 ± 24.4†</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>40</td>
<td>28.4 ± 20.1†</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>35</td>
<td>34.5 ± 13.8†</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>32</td>
<td>35.3 ± 20.7†</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>12</td>
<td>95.9 ± 19.3</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>8</td>
<td>176.6 ± 74.1</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>10</td>
<td>93.4 ± 9.3</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>6</td>
<td>109.4 ± 3.7</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>14</td>
<td>143.6 ± 33.7</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>12</td>
<td>106.5 ± 9.2</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>20</td>
<td>151.8 ± 48.4</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>9</td>
<td>130.1 ± 15.8</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control children</td>
<td>5–10 yr (N = 7)</td>
<td>96.2 ± 12.5</td>
<td></td>
</tr>
<tr>
<td>11–15 yr (N = 8)</td>
<td></td>
<td>98.6 ± 15.8</td>
<td></td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>N = 20</td>
<td>100.0 ± 12.1</td>
<td></td>
</tr>
</tbody>
</table>

*NK cell activity was measured by a 51Cr-release assay at a 40:1
effector-target ratio. Results were expressed as percentage of the
control value obtained in the parallel experiment using normal lymphocytes from
healthy individuals. NK cell activity of 20 healthy individuals was 47.2% ±
5.7% (mean ± SD) as a percentage of lysis in the present studies.
†P < .001.
in four patients (patients No. 1 through 4) who had childhood chronic neutropenia with morphological abnormalities and defective neutrophil chemotaxis (see Table 1): the percentage of lysis was 28.4% to 42.1% (P < .001) of the control value of normal lymphocytes. The patients' values were also depressed as compared with those of the age-matched controls (P < .001). In the remaining five patients with childhood chronic neutropenia without any of these neutrophil abnormalities, NK cell activity was normal. NK cell activity was also normal in the patients with lazy leukocyte syndrome, Shwachman syndrome, or dysgamma-globulinemia type I with neutrophil defects.

In the next experiments, NK cell activity of the four patients (patients No. 1 through 4) was assayed at various effector-target ratios. As shown in Fig 1, NK cell activity was depressed in all the four patients (P < .001 to P < .02) at 5:1 to 40:1 effector-target ratios and was depressed in patients No. 2 through 4 (P < .01), but normal in patient No. 1 at a higher effector-target ratio of 80:1.

Experiments were then performed to examine whether NK cells from the four patients could respond to in vitro IFN, and the results are shown in Fig 2. The patients' NK cells responded to 500 to 5,000 U/mL IFN-α, as did normal lymphocytes, but their IFN-activated cytotoxicity levels remained lower than the activated normal lymphocyte level (P < .01). Namely, the absolute IFN-induced increase of the percentage of lysis of the patients' NK cells was the same as that observed with normal lymphocytes, but their NK cell deficiency could not be totally corrected by IFN.

Because IL 2 is known to augment NK cell activity, the patients' lymphocytes were treated with IL 2 preparation and assayed for their NK cell activity in another stimulation study. As observed with IFN, the patients' NK cells responded to the IL 2 stimulation, as did normal lymphocytes, but their activated cytotoxicity levels still remained below the activated normal lymphocyte level (P < .01): NK cell activity levels as percentage of lysis (mean ± SD) were increased from 25.0% ± 1.0% to 42.9% ± 1.2%, 13.8% ± 0.8% to 40.0% ± 6.0%, 16.7% ± 1.4% to 39.3% ± 4.2%, 10.8% ± 1.0% to 37.5% ± 1.8% in patients No. 1, 2, 3, and 4, respectively, when the normal lymphocyte level (N - 7) was increased from 43.8% ± 4.2% to 78.2% ± 7.2%.

There are several steps in the cytolytic process of NK cells. We therefore examined which step(s) was defective in the four patients. For this purpose, we determined the Vmax, percentage of TBCs, percentage of TBCs with dead targets, percentage of active NK cells, and estimated MRC using the 5:1 (5 × 10⁶ lymphocytes and 10⁶ target cells) ⁵¹Cr-release assay and the single cell-in-agarose assay simultaneously; the results are summarized in Table 3. In all the four patients, the percentage of TBCs, percentage of TBCs with dead targets, and percentage of active NK cells were normal, and the latter two were increased in a normal manner by in vitro IFN stimulation, indicating normal capacity of their NK cells in recognizing, binding, and killing the target. The NK cells were defective, however, in recycling capacity: the estimated MRC values were 1.8 to 2.4
(P < .01) as compared with the normal lymphocyte value of 5.5 ± 0.6 (mean ± SD). The effector cell stimulation with 1,000 U/mL IFN-α did not significantly increase the estimated MRC of both the patients' and normal cells.

The recycling may reflect not only speed of killing, but also the "chemotaxis" of NK cells to find and bind to additional target cells. To analyze further the defective lytic step(s) of the patients' NK cells, we examined their killing at earlier timepoints by the single cell-in-agarose method. As shown in Fig 3, the killing of the patients' NK cells was normal at 1/2, 1, 2 and 3 hours, indicating normal speed of cell killing.

**DISCUSSION**

Childhood chronic neutropenia is a heterogeneous set of disorders with persistent neutropenia usually developing early in life. We and other investigators have recently demonstrated several patients having childhood chronic neutropenia with morphological abnormalities of neutrophils. These abnormalities include a decrease of cytoplasmic granules and the appearance of myelin figures, being identical to those of our four patients (patients No. 1 through 4) cells. Our previous and present studies have further demonstrated defective neutrophil chemotaxis in such neutropenia with dysmorphic neutrophils, as in patients No. 1 through 4. The history of recurrent infections since early infancy and the development of persistent neutropenia early in childhood in the four patients indicate that their neutropenia is probably congenital.

It is now evident from the present NK cell assays that NK cells are deficient in some type of childhood chronic neutropenia with abnormal neutrophils with respect to morphology and chemotaxis. In fact, NK cell activity was normal in childhood chronic neutropenia without any of these neutrophil abnormalities and in the other neutropenic disorders, such as lazy leukocyte syndrome, Shwachman syndrome, and dysgammaglobulinemia type I with neutrophil defects. Because NK cells are involved in the defense against infections, there may be a possibility that NK cells of our four patients could be too exhausted by the frequent episodes of infection to operate in a normal manner. This possibility is unlikely, however, because NK cells were normal in the other neutropenic patients in whom infections were also frequent. Therefore, it would not seem that the chemotherapy is a likely explanation for the NK cell deficiency. Actually, the patients were not undergoing any treatment when NK cell assays were done. It thus appears that the impairment of their NK cell activity is a primary defect.

NK cell assays with various effector-target ratios and the effector cell stimulation can elucidate the characteristics of

![Fig 3. Killing of K562 target cells by NK cells at early timepoints.](https://example.com/fig3.png)
NK cells in diseases. In fact, NK cell activity was depressed at 5:1 to 40:1 effector-target ratios in all the four patients but normal at a higher effector-target ratio of 80:1 in one of them. Additionally, the patients' effector cells responded to various concentrations of IFN-α as did normal lymphocytes, but the activated NK cell activity levels still remained depressed as compared with those observed with normal lymphocytes. Similar results were obtained with IL 2 stimulation as well. Such normal NK cell activity with sufficient numbers of effector cells and failure of an increase of the activity up to the normal lymphocyte level by IFN and IL 2 prompted us to analyze further the NK cell defect using both 51Cr-release and single cell-in-agarose assays.

In the cytolytic event of NK cells, they first recognize and bind to a target cell, next kill the bound target cell, then detach from the dead target and repeat this process through their recycling capacity. It is of interest, therefore, to examine which step(s) of the lytic process is defective in our patients. In the patients, the percentage of TBCs was normal, indicating normal numbers of NK cells capable of recognizing and binding the target. In addition, normal percentages of TBCs with dead targets and active NK cells indicate that their NK cells are also normal in killing the bound targets. In vitro IFN can augment cytolytic function of NK cells by recruiting nonlytic pre-NK cells to active NK cells as well as by enhancing the kinetics of lysis at the single effector-target cell level; this was indeed the case in our patients, as demonstrated by the increase of active NK cells by IFN.

The MRC estimates the number of target cells killed by a given NK cell in a four-hour assay. The decrease of MRC in our patients indicates that their NK cells are defective in their recycling capacity. Normal killing at early timepoints by the patients' NK cells certainly precludes the possibility of decreased speed of killing as a cause of their abnormal recycling capacity. It is noteworthy that the IFN stimulation of effector cells did not have significant effects on their MRC, not only in the patients, but also in healthy individuals. The failure of improvement of MRC by IFN appears to be responsible for the depressed IFN-activated NK cell activity, despite the normal increase of active NK cells in the patients.

Because cell mobility is largely involved in the recycling of NK cells, it is noteworthy that the patients' neutrophils were defective in chemotaxis. In Chédiak-Higashi syndrome, NK cells and neutrophils have similar morphological and functional abnormalities, such as giant cytoplasmic granules and delayed killing ability. Davies et al have recently reported two infants with a combined abnormality of NK cells and neutrophil chemotaxis, although the data of NK cell MRC are not presented. Our results, coupled with these findings, suggest the preferential coexistence of similar abnormalities of NK cells and neutrophils in some disorders.

The clinical significance of the NK cell deficiency in childhood chronic neutropenia deserves discussion, as NK cells are operative in the protection against infections and tumors and in the regulation of granulopoiesis.

There are several lines of evidence for a role of NK cells in resistance against some microbial infections. It is likely that NK cells play an important role in the defense against viral infections. A possible role for NK cells has also been found for natural resistance of mice to infections by a certain type of fungus. Our patients have experienced somewhat serious varicella and mycoplasmal infections in addition to a variety of bacterial infections. These clinical features suggest the possibility that the patients' NK cell deficiency was associated with the recurrence or severity of some infections.

Several patients have been reported with some type of childhood chronic neutropenia terminating in acute leukemia. In 1979, Rosen and Kang demonstrated that acute leukemia had occurred in two (3.3% to 6.7%) of the 30 to 60 published examples of childhood chronic neutropenia and added another case of the disorder terminating in acute myelomonocytic leukemia. Considering that most of the patients might die early in childhood from infections, these findings indicate a somewhat high incidence of malignancy in long-term survivors with childhood chronic neutropenia. Although it remains undetermined whether the NK cell deficiency in neutropenic disorders would account for the development of malignancy, it should be borne in mind that NK cells are defective in some type of childhood chronic neutropenia with abnormal neutrophils with respect to morphology and chemotaxis.

Hansson et al have recently shown that NK cells have the ability to inhibit CFU-GM proliferation in vitro. If so, a decrease of NK cell activity might be rather convenient for CFU-GM growth. In fact, numbers of CFU-GM were normal in three of our four patients. Further studies are now in progress in our laboratory to determine whether the NK cell deficiency in our patients is operative in the upregulation of their CFU-GM proliferation or, adversely, in the pathogenesis of the neutropenia, or whether it is not related to granulopoiesis.

REFERENCES


