CD56^{bright} and CD56^{dim} Natural Killer Cells in Patients With Chronic Myelogenous Leukemia Progressively Decrease in Number, Respond Less to Stimuli That Recruit Clonogenic Natural Killer Cells, and Exhibit Decreased Proliferation on a Per Cell Basis

By Bryce A. Pierson and Jeffrey S. Miller

Human natural killer cells (NK) require accessory cell-derived contact and soluble factors for maximal expansion. However, it is unclear whether increased recruitment of clonogenic NK, increased proliferation on a per cell basis, or a combination of both is responsible for the increased expansion. We show that expansion of both CD56^{bright} and CD56^{dim} NK from normal donors is increased in the presence of M2-10B4 accessory cell-soluble factors. In contrast, the addition of M2-10B4 stromal ligands further augments contact and soluble factors for maximal expansion. How additional differences are based on relative CD56 fluorescence, though CD2 and CD8 have been shown to subdivide the population, the most informative functional distinction may be decreased in patients with CML. The vast majority of circulating NK pool, is significantly reduced in all patients with CML (normal, 5.7% ± 0.8% v CML [all stages combined], 2.5% ± 0.5%, *p* = .001). After purification of NK to correct for differences in circulating NK number, resting NK cytotoxicity against K562 tumor targets is significantly reduced in patients with CML on or recently on hydroxyurea therapy. However, this reduced cytotoxicity can be corrected by 18 hours of incubation with 1,000 U/mL recombinant IL-2. When plated in limiting dilution on viable M2-10B4, which maximally stimulates NK from normal donors, we show that both NK clonogenic frequency and proliferative capacity are significantly reduced as CML progresses, demonstrating an inherent defect in their ability to respond to normal NK stimuli. Although NK cloning efficiency between normal donors and CML patients was the same, significant differences were observed in (1) the absolute number of circulating CD56^{bright}/CD3^- NK, (2) the absolute number of circulating CD56^{bright} NK, and (3) proliferation on a per cell basis. Unlike resting NK function, prior cytotoxic therapy alone did not account for these observed abnormalities. These data suggest that, although NK are not derived from the malignant clone, they are inherently affected by their malignant microenvironment.

© 1996 by The American Society of Hematology.
We have previously shown that, in normal donors, maximal NK expansion is mediated by contact and soluble factors from accessory cells.\textsuperscript{5,14,15,20} We have further defined this observation here by showing that the CD56\textsuperscript{+}CD3\textsuperscript{−} and CD56\textsuperscript{−}CD3\textsuperscript{+} NK subsets have different proliferative potentials and clonogenic frequencies and respond differently to contact and soluble factors from accessory cells. In addition, using single-cell sorting, the increased NK expansion observed in the presence of M2-10B4 contact and soluble factors was found to be a combination of increased recruitment of clonogenic NK and increased proliferation on a per cell basis. We hypothesize that NK from patients with CML may exhibit reduced cytotoxicity, proliferative capacity, and clonogenic frequency and that these defects may play a role in disease progression. This may contribute to the rapid transformation observed in accelerated phase and blast crisis CML. The M210-B4 accessory cell system was used to differentiate whether CML NK abnormalities are mediated by defective accessory cells or by the CML NK themselves.

**MATERIALS AND METHODS**

**Patient population.** Heparinized peripheral blood (PB) samples (35 to 50 mL) were obtained with informed consent from 21 patients with CML. We investigated NK proliferation and function from 7 patients with CML in early chronic phase (ECF; <12 months from diagnosis), 10 patients with CML in late chronic phase (>12 months from diagnosis) or accelerated phase (LCAP), and 4 patients with CML in blast crisis (BC). In addition, 15 healthy normal adult volunteers 21 to 45 years of age were studied as controls. The guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota were followed in obtaining all samples.

**Purification of PB NK.** PB mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (specific gravity, 1.077; Sigma Diagnostics, St Louis, MO) density centrifugation (30 minutes at 37°C and 400g). Normal PBMC were depleted of T cells by anti-CD3 and anti-CD5 mouse mononuclear antibodies (MoAbs; Becton Dickinson, Mountain View, CA) and goat antitoxin immunomagnetic beads (15 beads/cell; Advanced Magnetics Inc, Cambridge, MA).\textsuperscript{14,15,21} For samples from patients with CML, anti-CD19 (Becton Dickinson), M1Y-8 ( Coulter Cytometry, Hialeah, FL), and anti-glycoporphin A (AMAC, Westbrook, MD) MoAbs were also added to the immunomagnetic bead depletion cocktail. The depleted PBMC from normal donors were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 or anti-CD2 MoAb and phycoerythrin (PE)-conjugated anti-CD56 MoAb (250 ng/10\textsuperscript{6} cells; Becton Dickinson) and sorted on a FACS Star Plus flow cytometer (Becton Dickinson) equipped with a Consort 32 computer (Hewlitt Packard 340; Hewlitt Packard, Palo Alto, CA) into CD56\textsuperscript{−}CD3\textsuperscript{−}, as previously described.\textsuperscript{14,15,21} or the CD56\textsuperscript{+}CD3\textsuperscript{−}CD2\textsuperscript{+}, CD56\textsuperscript{−}CD3\textsuperscript{−}CD2\textsuperscript{+}, and CD56\textsuperscript{−}CD3\textsuperscript{−}CD2\textsuperscript{+} NK subpopulations using established criteria for CD56\textsuperscript{+}NK.\textsuperscript{16,17,22} The depleted PBMC from patients with CML were labeled with FITC-conjugated anti-CD2 MoAb, PE-conjugated anti-CD56 MoAb, and streptavidin (SA670)-conjugated anti-CD3 MoAb (250 ng/10\textsuperscript{6} cells; Becton Dickinson) and CD5\textsuperscript{−} cells were sorted into CD56\textsuperscript{−}CD3\textsuperscript{−}CD2\textsuperscript{−}, CD56\textsuperscript{−}CD3\textsuperscript{−}CD2\textsuperscript{−} NK populations using established criteria for CD56\textsuperscript{+}NK.\textsuperscript{16,17,22}

**NK cultures.** NK were cultured in a 2:1 (vol/vol) Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12-based NK medium, which significantly increases NK expansion compared with standard RPMI 1640 based medium.\textsuperscript{27} The DMEM/F12-based NK medium (DMEM and Ham’s F12 were obtained from Gibco Laboratories, Grand Island, NY) was supplemented with 24 μM/L 2-mercaptoethanol, 50 μM/L ethanolamine, 20 mg/L L-ascorbic acid, 5 μg/L sodium selenite (NaSeO\textsubscript{4}), 1,000 U/mL recombinant IL-2 (rIL-2; a generous gift from Amgen, Thousand Oaks, CA), and 10% heat-inactivated human AB serum (North American Biologicals, Miami, FL), 100 μM/L penicillin, and 100 μM/L streptomycin (GIBCO). Fluorescence-activated cell sorting (FACS)-purified CD56\textsuperscript{+}CD3\textsuperscript{−} NK (1 × 10\textsuperscript{6}), CD56\textsuperscript{−}CD3\textsuperscript{+}CD2\textsuperscript{+} (2 × 10\textsuperscript{6}), CD56\textsuperscript{−}CD3\textsuperscript{−}CD2\textsuperscript{−} (1 × 10\textsuperscript{6}), or CD56\textsuperscript{+}CD3\textsuperscript{−}CD2\textsuperscript{−} NK were cocultured in 1 mL medium in 24-well plates in direct contact with irradiated M2-10B4 (a murine fibroblast stromal cell line provided by Dr Connie Eaves, Vancouver, British Columbia, Canada), as previously described.\textsuperscript{29} At day 7, culture volumes were doubled to 2.0 mL and subsequently half medium changes were performed every 3 to 4 days. Contents of culture wells were split 1:2 as needed to maintain cell concentrations less than 2 to 3 × 10\textsuperscript{5} cells/mL. Cultures were terminated on day 20 or 28, as indicated, and cells were enumerated with a hemocytometer, phenotyped, and analyzed for cytotoxicity. NK fold expansion was determined as the total number of cultured cells at day of harvest divided by the initial number of NK plated at day 0.

For single-cell experiments with CD56\textsuperscript{−}CD3\textsuperscript{−}CD2\textsuperscript{−} NK from normal donors, individual CD56\textsuperscript{−}CD3\textsuperscript{−}CD2\textsuperscript{−} NK were deposited in 96-well plates containing 100 μL of fresh NK medium, M2-10B4 conditioned medium, or fresh NK medium with viable irradiated M2-10B4 monolayers using the ACDU single cell deposition software on a modified FACS Star\textsuperscript{™} (Becton Dickinson). At day 7, the culture volume was doubled to 200 μL with fresh NK medium or a 50/50 mixture of fresh medium with M2-10B4 conditioned medium, as indicated. Beginning on day 10, half medium changes were performed every 3 to 4 days. Cultures were terminated after 40 days of culture. Clonogenic frequency was determined as the percentage of wells positive for NK proliferation. Wells were determined to be positive for NK proliferation if the specific 51Cr release was two standard deviations greater than the mean spontaneous release observed after the addition of 5,000 51Cr-labeled cells to each well in a standard 4-hour chromium release assay. Specific lysis for each positive well was determined as previously described.\textsuperscript{14}

**Cytotoxicity.** Cultured activated NK (ANK) populations were tested for cytotoxicity against the NK-sensitive cell line K562 and the NK-resistant cell line Raji in a 4-hour Cr release assay. Effector to target ratios ranged from 20:1 to 0.082:1. Target cells were labeled with 200 μCi sodium chromate-trCr (DuPont, Wilmington, DE) for 60 to 90 minutes. All determinations were performed in triplicate, and the percentage of lysis was determined.

**Limiting dilution assays.** Irradiated M2-10B4 in 96-well plates was used as a feeder layer. NK populations were plated at 1 to 800 cells/well with 24 replicates per concentration in 100 μL supplemented DMEM/F12 medium. The well volume was doubled at day 7 and half medium changes were performed with fresh medium on days 10, 14, and 17. At day 20, wells were determined to be positive for NK proliferation if the specific 51Cr release was three standard deviations greater than the mean spontaneous release observed after the addition of 5,000 51Cr-labeled K562 targets in a 4-hour chromium release assay, as described above. Forty-eight wells were used to determine each total lysis and spontaneous release (spontaneous release was less than 15% of total). The frequency of NK responding in each population was calculated as the reciprocal of the concentration of cells that resulted in 37% negative wells using Poisson statistics and the weighted mean method.\textsuperscript{23,24}

**Statistics.** Results of experimental points obtained from multiple experiments are reported as mean ± standard error of the mean (SEM). Significance levels were determined by two-sided Student’s t-test analysis.
RESULTS

Bulk CD56+/CD3- NK from normal donors were divided into three subsets on the basis of CD56 and CD2 expression. The CD56+bright/CD2+, CD56+dim/CD2+, and CD56+dim/CD2- NK subsets account for 5.7% ± 0.8%, 61.2% ± 2.4%, and 22.0% ± 2.7%, respectively, of the total resting NK pool. We hypothesized that these subsets may respond differently to accessory cell-produced contact and soluble factors, as previously described.\(^1\) Normal NK were sorted into the three subsets and plated in medium alone, in a Transwell insert over an irradiated M2-10B4 feeder layer (soluble factors alone), or in direct contact with the M2-10B4 feeder layer (soluble + contact factors). NK fold expansion was measured after 20 days of culture by hemocytometer enumeration. The CD56+bright/CD2+ NK subset showed the highest proliferative capacity, whereas the CD56+dim NK subsets expanded less (Table 1). M2-10B4 soluble factors significantly increased expansion of all NK subsets. However, in the additional presence of stromal ligands (soluble + contact factors), the CD56+bright/CD2+ NK subset was solely responsible for the contact-mediated expansion observed in the bulk CD56+/CD3- NK pool.

Although accessory cell-soluble and contact factors increase CD56+high NK expansion, it is unclear whether this occurs due to increased proliferation per NK cell, to the recruitment of additional clonogenic NK, or to a combination of both. To answer this question, single CD56+bright/CD2+ NK cells (3 normal donors) were sorted into 96-well plates containing medium alone, M2-10B4 conditioned medium as a source of soluble factors,\(^2\) or viable M2-10B4 monolayers. After 40 days of culture, clonogenic frequency and NK expansion were determined. The clonogenic frequency of single sorted CD56+high/CD2+ NK was significantly increased in the presence of soluble factors alone (38.6%) and maximal with soluble + contact factors (56.5%) compared with medium alone (16.7%; Table 2). NK fold expansion from single cells was measured in two ways. First, NK were enumerated using a hemocytometer. NK expansion from single cells was significantly increased by culture in direct contact with viable M2-10B4 compared with medium alone (M2-10B4, 11,600 ± 1600 NK from a single cell, n = 100 wells; medium alone, 720 ± 95 NK from a single cell, n = 68 wells, P < .001). Second, because low cell counts are inaccurate, the percentage of specific lysis for all of the positive wells that exhibited clonogenic growth was calculated as a more sensitive measure of single-cell NK proliferation. Because the percentage of specific lysis is dependent on the effector to target ratio and a constant number of labeled K562 targets were added to each well, the differences in specific lysis are representative of effector cell number. NK cultured for 35 days in fresh NK medium alone, in M210-B4 conditioned medium, or in direct contact with viable M210-B4 exhibited no differences in killing of K562 tumor targets, showing that the addition of stromal factors to culture in the presence of maximal (1,000 U/mL) IL-2 does not independently affect NK killing (Fig 1). These cytotoxicity curves, which were assayed at known effector to target ratios, exhibited a linear correlation between effector number and specific lysis for effector to target ratios of 2.2 to 0.24. Therefore, the average percentage of specific lysis of positive wells can be used as a comparative measure of single-cell NK proliferation. Importantly, there was no difference in average percentage of specific lysis between the spontaneous release control wells, the 634 negative wells in medium alone (−2.7% ± 0.1%), the 494 negative wells grown with soluble factors (−1.9% ± 0.1%), and the 371 negative wells grown with contact and soluble factors (−1.1% ± 0.2%), showing that this assay clearly distinguishes between positive and negative wells. In contrast, the 127 wells positive for NK clonogenic growth in medium alone exhibit 15.7% ± 1.2% average specific lysis, which increased to 19.5% ± 0.1% (n = 311) by the addition of M2-10B4-conditioned medium. M2-10B4 contact factors further augmented the average percentage of specific lysis to 27.3% ± 0.1% (n = 481). Taken together, these data show that M2-10B4 contact and soluble factors increase both the clonogenic recruitment and the absolute proliferation on a per cell basis.

As a final comparative measure of the effects of soluble and contact factors in culture, the total killing capacity, which is representative of total cell proliferation, was calculated. This measure takes into account the total number of positive wells and the average specific lysis as a measure of proliferation in each culture condition. Single sorted CD56+bright/CD2+ NK cells cultured on viable M210-B4 exhibited twofold and sixfold increases in total killing capacity compared with M2-10B4 conditioned medium or medium alone, respectively, which was similar to the actual cell

Table 1. NK Subsets Respond Differently to M2-10B4 Contact and Soluble Factors

<table>
<thead>
<tr>
<th>Population</th>
<th>Medium Alone*</th>
<th>Soluble Factors Alone†</th>
<th>P Value†</th>
<th>Soluble + Contact Factors‡</th>
<th>P Value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56+/CD3-</td>
<td>6</td>
<td>16.3 ± 2.5</td>
<td>27.4 ± 2.9</td>
<td>.015</td>
<td>44.3 ± 3.8</td>
</tr>
<tr>
<td>CD56+bright/CD2+</td>
<td>5</td>
<td>105.1 ± 10.9</td>
<td>486.0 ± 105.3</td>
<td>.042</td>
<td>1443.7 ± 398.1</td>
</tr>
<tr>
<td>CD56+dim/CD2-</td>
<td>3</td>
<td>3.1 ± 0.6</td>
<td>26.5 ± 3.9</td>
<td>.001</td>
<td>29.5 ± 11.3</td>
</tr>
<tr>
<td>CD56+low/CD2-</td>
<td>3</td>
<td>0.8 ± 0.2</td>
<td>13.5 ± 7.7</td>
<td>.002</td>
<td>11.3 ± 5.9</td>
</tr>
</tbody>
</table>

Values reported are NK fold expansion.

* Fresh unconditioned complete IL-2-containing NK medium.
†NK cocultured in a transwell above viable M2-10B4 monolayers.
‡NK cocultured in direct contact with viable M2-10B4.
counts at day 40 (Table 2). Because NK cultured in direct contact with viable M2-1OB4 feeder layers provided maximal NK recruitment and proliferation on a single cell basis, viable M2-1OB4 was used in all further experiments as a constant accessory cell source to assess NK function in patients with CML.

Although diminished NK outgrowth and bulk function have been correlated with CML disease progression using the adherent lymphokine-activated killer cell methods, we investigated NK proliferation and function from 7 patients with CML in ECP, 10 patients with CML in LCP/AP, and 4 patients with CML in BC (Table 3). The number of circulating NK per milliliter of PB was calculated using NK-enriched populations obtained after immunomagnetic bead depletion. The absolute number of circulating NK in normal individuals was 63,700 ± 6,400 NK/mL PB (n = 14). In contrast, the absolute number of NK per milliliter was significantly and progressively decreased in patients with CML in ECP (P = .037), in LCP/AP (P = .005), and in BC (P = .003; Table 4). In addition to a decrease in the total NK pool, the number of CD56+bright/CD2+ NK was even further decreased in patients at all stages of CML (2.5% ± 0.5%, n = 18) compared with the normal donor controls (5.7% ± 0.8%, n = 15; P = .001; Table 4). However, an alternative explanation for the decrease in NK number may be the effect of cytotoxic therapy. To address this issue, we divided patients with CML into three distinct therapy groups (Table 3): group 1, 6 patients with ≤4.5 cumulative months of hydroxyurea therapy, off therapy for ≥7 months before study (off hydroxyurea); group 2, 6 patients with ≤6 cumulative months of hydroxyurea therapy, on therapy up to 0 to 3 weeks before study (on hydroxyurea); and group 3, 9 patients having received extensive prior hydroxyurea and/or other chemotherapy (extensive therapy). The off hydroxyurea group received more interferon-α (INF-α) therapy (mean, 11.2 months/patient) and was therefore further from diagnosis (median, 14.5 months); 5 of 6 patients were classified as LCP/AP CML. There was no significant difference in bulk circulating CD56+CD3- NK number (42,600 ± 7500 v 37,700 ± 3,700 NK/mL PB; P = not significant [NS]) or in circulating CD56+bright/CD2+ NK number (1,165 ± 410 v 790 ± 320 NK/mL PB; P = NS) for CML patients off hydroxyurea and on hydroxyurea, respectively. White blood cell (WBC) counts were slightly higher in patients off hydroxyurea compared with those on hydroxyurea (8.3 ± 2.2 v 14.0 ± 3.3; P = NS) and there was no correlation between WBC counts and circulating NK numbers.

NK from patients with CML were next evaluated for their clonogenic capacity after subsets were purified by FACS. NK clonogenic frequency was quantified by limiting dilution analysis (LDA) after 20 days of culture in direct contact with irradiated M2-1OB4 for CML CD56+bright/CD2+, CD56+dim/CD2+, and CD56+dim/CD2- NK subsets. There was no significant difference in clonogenic frequency for any of the NK subsets in ECP CML patients compared with normal individuals (Table 4). However, in the later stages of CML (LCP/AP and BC), the clonogenic frequency of all three NK subsets was significantly reduced. When NK expansion was measured for bulk CD56+CD3- and CD56+bright/CD2+ NK
cultured in direct contact with M2-10B4 for 28 days, the NK proliferative capacity was significantly decreased for CML patients in all stages of CML compared with normal controls, even for CML patients in ECP (Table 4). NK expansion correlates well with the time from diagnosis for both the bulk CD56+/CD3- NK pool (R = .806; data not shown) and the CD56^bright/CD2+ NK subset (R = .739; Fig 2).

Although several laboratories have reported decreased NK function in patients with CML, the potential for dilution of NK activity by the vast myeloid compartment has confounded the results and it is unclear whether NK are inherently less active or just diminished in number. We used freshly purified CD56+/CD3- NK in standard chromium release assays against K562 tumor targets to address this question. Even purified resting NK, used to correct for differences

### Table 3. Patient Characteristics and Prior Therapy History

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Hydroxyurea Therapy</th>
<th>IFNα Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>Age/TF</td>
<td>WBC Count</td>
</tr>
<tr>
<td>ECP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46/M</td>
<td>3</td>
<td>17.2</td>
</tr>
<tr>
<td>57/F</td>
<td>3</td>
<td>11.8</td>
</tr>
<tr>
<td>22/M</td>
<td>5</td>
<td>6.7</td>
</tr>
<tr>
<td>17/M</td>
<td>5</td>
<td>5.6</td>
</tr>
<tr>
<td>54/F</td>
<td>6</td>
<td>6.3</td>
</tr>
<tr>
<td>46/F</td>
<td>10</td>
<td>2.9</td>
</tr>
<tr>
<td>45/F</td>
<td>11</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* Comparison between LCPIAP and ECP.

### Table 4. Circulating NK Number, Clonogenic Frequency, and Expansion Progressively Decrease as CML Progresses

<table>
<thead>
<tr>
<th>Stage</th>
<th>Circulating NK Number/mL PB (×10^5)</th>
<th>Clonogenic Frequency (%)</th>
<th>NK Expansion* (d 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bulk CD56+/CD3 NK</td>
<td>CD56^bright/CD2+ NK</td>
<td>CD56^bright+CD2+ NK</td>
</tr>
<tr>
<td></td>
<td>(NK)</td>
<td>(NK)</td>
<td>(NK)</td>
</tr>
<tr>
<td>Normal</td>
<td>6.37 ± 6.4 (14)</td>
<td>3.1 ± 0.7 (12)</td>
<td>4.0 ± 0.1 (5)</td>
</tr>
<tr>
<td></td>
<td>4.07 ± 6.7 (7)</td>
<td>1.1 ± 0.4 (7)</td>
<td>3.79 ± 0.6 (5)</td>
</tr>
<tr>
<td></td>
<td>.027</td>
<td>.026</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>.03</td>
<td>.038</td>
<td>NS</td>
</tr>
<tr>
<td>ECP</td>
<td>31.9 ± 6.0 (9)</td>
<td>0.69 ± 0.22 (8)</td>
<td>8.2 ± 3.9 (9)</td>
</tr>
<tr>
<td></td>
<td>31.9 ± 6.0 (9)</td>
<td>0.69 ± 0.22 (8)</td>
<td>8.2 ± 3.9 (9)</td>
</tr>
<tr>
<td></td>
<td>31.9 ± 6.0 (9)</td>
<td>0.69 ± 0.22 (8)</td>
<td>8.2 ± 3.9 (9)</td>
</tr>
<tr>
<td></td>
<td>.003</td>
<td>.003</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>.004</td>
<td>.004</td>
<td>NS</td>
</tr>
<tr>
<td>ECP</td>
<td>10.7 ± 5.2 (4)</td>
<td>0.15 ± 0.08</td>
<td>2.7 ± 0.9 (4)</td>
</tr>
<tr>
<td></td>
<td>10.7 ± 5.2 (4)</td>
<td>0.15 ± 0.08</td>
<td>2.7 ± 0.9 (4)</td>
</tr>
<tr>
<td></td>
<td>.004</td>
<td>.004</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>.004</td>
<td>.004</td>
<td>NS</td>
</tr>
</tbody>
</table>

The number of experiments for each point is in parentheses (n).

* Fold expansion of NK cultured for 28 days in direct contact with viable M2-10B4.

† Comparison between normal and ECP.

‡ Comparison between LCPIAP and ECP.
in circulating NK number, show a significant decrease in cytolytic activity in ECP and LCP/AP patients and nearly a complete loss of cytolytic function in BC patients (Fig 3A). However, analysis of resting NK function by prior cytotoxic therapy showed that NK from patients off hydroxyurea were not different from normal controls. In contrast, patients on hydroxyurea or after extensive therapy exhibited significantly reduced function (Fig 3B). Short-term (18 hours) incubation with high-dose IL-2 restores cytotoxic activity to normal levels in both the on hydroxyurea and extensive therapy groups, showing a reversible defect in NK function (Fig 3C).

To test whether the effects of the short-term IL-2 incubation could be maintained during long-term culture with IL-2, NK from patients with CML were initially plated in direct contact with M2-10B4 and tested for cytotoxic function after 28 days of culture. We observed no significant differences in cytotoxic function against either K562 or NK-resistant Raji tumor targets compared with normal NK at any effector to target ratio (data not shown).

In the experiments with single sorted normal NK, we established that the average percentage of specific lysis can be used as a comparative measure of proliferation of single cells. Because CML NK cultured in direct contact with viable M210-B4 kill K562 tumor targets similar to normal NK (as seen for K562 targets in Fig 1), we compared the average percentage of specific lysis from positive wells of CML NK plated in limiting dilution to determine if proliferation per cell was affected in CML. There is a significant decrease in NK proliferation (NK specific lysis) as CML progresses through ECP to LCP/AP and finally BC (Table 5). The five LCP/AP patients off hydroxyurea still exhibited significantly reduced NK proliferation ($P < .001$) compared with ECP and normal controls, suggesting that therapy is not solely responsible for these observed decreases. This was consistent

---

**Fig 2.** Correlation between NK fold expansion and time from diagnosis. Log of NK fold expansion at day 28 from CD56$^{+}$/CD2$^{-}$/CD3$^{-}$/CD56$^{+$} NK cultured in direct contact with M2-10B4 is plotted versus the log of time from diagnosis in months. A linear relationship is observed with a correlation coefficient of $R = .740$. A similar correlation was observed for the bulk CD56$^{+}$/CD3$^{-}$/NK, with a correlation coefficient of $R = .802$.

---

**Fig 3.** Defective resting cytotoxicity of CML NK can be attributed to prior hydroxyurea therapy. (A) CD56$^{+}$/CD3$^{-}$/NK from 6 normal donors (○), 6 patients with ECP CML (▲), 5 patients with LCP/AP CML (●), and 3 patients with BC CML (△) were tested for cytotoxicity against K562 tumor targets after FACS purification. Resting cytotoxicity was significantly reduced in ECP CML and LCP/AP CML compared with normal controls and was nearly absent in NK from patients with BC CML. (B) NK from patients with CML were grouped according to prior cytotoxic therapy into three groups, as described in the text: group 1 (off hydroxyurea; ▲); group 2 (on hydroxyurea; ●); and group 3 (extensive therapy; ○). There is no significant difference in resting NK function between normal donors and off hydroxyurea patients, whereas on hydroxyurea and extensive therapy patients showed significantly reduced resting cytotoxic function. (C) After 18 hours of incubation with 1,000 U/mL IL-2, no significant differences in cytotoxicity were observed for any of the conditions tested ($^{*}P < .05$, $^{*}P = .02$, $^{*}P = .01$).
with bulk proliferation at day 28 from both CD56^bright/CD3^- NK (26.2-fold \pm 1.2-fold v 67.3-fold \pm 28.8-fold expansion, P = .049) and CD56^bright/CD2^+ NK (194-fold \pm 67-fold v 1.624-fold \pm 676-fold expansion, P = .048) from patients with CML analyzed by therapy: off hydroxyurea versus on hydroxyurea, respectively. The decrease in average percentage of specific lysis was observed for all three NK subsets at each initial concentration of NK plated, showing less proliferation per NK cell in all stages of CML compared with NK proliferation from normal donors. Decreased NK proliferation per cell was observed even with NK from patients with CML in ECP in which clonogenic capacity was unaffected, identifying a subtle functional abnormality of these ECP CML NK cells.

**DISCUSSION**

We have previously shown that bulk CD56^bright/CD3^- NK expansion is mediated by two independent stimuli from accessory cells: (1) direct contact with ligand(s) present on accessory cells and (2) soluble factors produced by these cells. These contact and soluble factors are synergistic late in culture and together stimulate maximal NK expansion. In this report, we extend these findings by showing that the CD56^bright and CD56^dim NK subsets also respond independently to the accessory cell-derived contact and soluble factors. However, the CD56^bright NK are the only NK to respond to contact factors and are solely responsible for the contact-mediated increased expansion observed in the bulk CD56^bright/CD3^- NK population reported previously. By sorting single CD56^bright NK, the increase in bulk expansion is the result of both an increase in recruitment of clonogenic NK and an increase in proliferation on a per cell basis.

The CD56^bright NK subset is both phenotypically and functionally distinct from the larger CD56^dim NK subset, suggesting that CD56^bright NK may represent a more immature NK population. Phenotypically, in addition to differences in CD56 expression, the CD56^bright NK constitutively express the high-affinity IL-2 receptor and have recently been reported to express the c-kit receptor, which further distinguishes them from CD56^dim NK. Functionally, unstimulated CD56^bright NK exhibit lower cytotoxicity against K562 compared with CD56^dim NK. However, in the presence of IL-2, CD56^bright NK acquire potent lytic activity similar to that of CD56^dim NK. We and others have shown that primitive CD34^- marrow progenitors can develop into phenotypic and functional NK. Direct contact with stromal ligands potentiates NK expansion but may not be an absolute requirement for maturation, especially with marrow progenitors already showing signs of lymphoid commitment. Our observation that CD56^bright NK alone respond to contact with accessory cell ligands further supports the hypothesis of CD56^bright NK as a more immature population than CD56^dim NK.

CML is usually diagnosed in chronic phase with eventual progression to acceleration and then to blast crisis. The mechanism of this progression is unknown, but correlation with immune function may be involved. Different methods of obtaining NK-enriched populations have been used. One study reported the use of sheep red blood cell (sRBC) rosetting to remove myeloid contamination before FACS. However, we have shown that, under some conditions, sRBC rosetting with AET-treated sRBC also removes CD2^+ NK cells (including all CD56^bright NK); thus, the results may not be representative of the total CD56^bright NK pool. Using the adherent lymphokine-activated killer cell (A-LAK) system to obtain NK-enriched populations,
our laboratory previously showed decreased cytotoxicity and expansion in 14-day cultured A-LAK populations from patients with CML as CML progressed from chronic phase to advanced disease.13 However, because A-LAK cells are generated by an adherence step, the starting population could contain malignant monocytes that may affect both NK activity and expansion differently than do normal monocytes.14 Therefore, definitive conclusions cannot be made about NK activity and proliferative capacity in the absence of the malignant microenvironment.

To avoid these confounding variables, FACS-purified CD56+1/CD3− NK with a well-defined nonmalignant accessory cell source (M2-10B4) were used to further investigate NK in patients with CML. Purified NK from patients with CML exhibit significantly reduced resting cytotoxicity against K562 tumor targets when analyzed by disease stage. However, an alternative explanation for the defective resting NK function might be prior cytotoxic therapy, which may have profound effects on hematopoietic cells. We show that CML patients on hydroxyurea (within 0 to 3 weeks of study) exhibit loss of resting NK function compared with patients off hydroxyurea (for at least 7 months before study). Although both patients on hydroxyurea and off hydroxyurea were off INF-α for 1 to 2 months before study, LCP/AP off hydroxyurea patients did receive more cumulative months of INF-α therapy than the ECP on hydroxyurea patients (mean, 11.2 vs 2.8 months/patient). Therefore, we cannot exclude that the long-acting biologic effects of INF-α played a role in preserving resting NK function. NK functional defects were reversible because 18 hours of incubation with 1,000 U/mL rIL-2 restored CML NK function to normal levels in all patients tested independent of CML stage or prior therapy. Long-term NK culture (28 days) from patients in all stages of CML produced NK that were not different in cytotoxic function from normal individuals, suggesting that, although proliferative differences and resting functional differences exist in CML, once activated and expanded, the acquisition of cytolytic machinery is intact. Correction of the defective NK cytotoxicity with IL-2 in patients with CML may prove to be a useful therapeutic strategy. Because NK themselves are not malignant7 and CML ANK lyse primarily of both circulating bulk CD56+brgh+ NK and the highly proliferative CD56+brght NK subset compared with normal controls, which was independent of prior cytotoxic therapy. CML NK show specific abnormalities in NK responses to cytokine-activated killer cell phenomenon. Lysis of natural killer-resistant and CD56+brght NK remain unchanged from normal values in ECP CML, four significant therapy-independent defects can be observed in NK from patients with ECP CML compared with normal donors: (1) the absolute number of circulating NK is reduced, (2) the absolute number of CD56+brght NK is reduced, (3) bulk NK expansion is reduced, and (4) proliferation per NK cell in all NK subsets is reduced. Taken together, these data suggest that modulation of the NK compartment begins early in CML and becomes more pronounced as disease progresses. Further investigation of these defects may illuminate the mechanism by which CML gradually escapes from immune control. Using the system of purified NK with defined accessory cells, it will be possible to systematically explore the role of CML malignant microenvironment components in the modulation of NK activity and proliferation.

ACKNOWLEDGMENT

The authors thank Drs P.B. McGlave and C. Verfaillie for their ongoing support of this work and B. Anderson for his excellent technical help with FACS.

REFERENCES

CD56+bright and CD56+dim natural killer cells in patients with chronic myelogenous leukemia progressively decrease in number, respond less to stimuli that recruit clonogenic natural killer cells, and exhibit decreased proliferation on a per cell basis

BA Pierson and JS Miller