Natural Killer Cells Suppress Human Erythroid Stem Cell Proliferation In Vitro

By Kenneth F. Mangan, Mary E. Hartnett, Sherri A. Matis, Alan Winkelstein, and Toru Abo

To determine the role of natural killer (NK) cells in the regulation of human erythropoiesis, we studied the effects of NK-enriched cell populations on the in vitro proliferation of erythroid stem cells at three different levels of maturation (day 14 blood BFU-E, day 5–6 marrow CFU-E, and day 10–12 marrow BFU-E). NK cells were enriched from blood by Percoll density gradient centrifugation and by fluorescence-activated cell sorting (FACS), using the human natural killer cell monoclonal antibody, HNK-1. The isolated enriched fractions were cocultured with autologous nonadherent marrow cells or blood null cells and erythropoietin in a methylcellulose erythroid culture system. Cells from low-density Percoll fractions (NK-enriched cells) were predominantly large granular lymphocytes with cytotoxic activity against K562 targets 6–10-fold greater than cells obtained from high-density Percoll fractions (NK-depleted cells). In coculture with marrow nonadherent cells (NA) at NK:NA ratios of 2:1, NK-enriched cells suppressed day 5–6 CFU-E to 62% (p < 0.025) of controls, whereas NK-depleted cells slightly augmented CFU-E to 130% of controls (p > 0.05). In contrast, no suppression of day 10–12 marrow BFU-E was observed employing NK-enriched cells. The NK CFU-E suppressor effects were abolished by complement-mediated lysis of NK-enriched cells with the natural killer cell antibody, HNK-1. Highly purified HNK-1+ cells separated by FACS suppressed marrow CFU-E to 34% (p < 0.025) and marrow BFU-E to 41% (p < 0.025) of controls. HNK- cells had no significant effect on either BFU-E or CFU-E growth. NK-enriched cells were poor stimulators of day 14 blood BFU-E in comparison to equal numbers of NK-depleted cells or T cells isolated by E-rosetting (p < 0.01). Interferon boosting of NK-enriched cells abolished their suboptimal burst-promoting effects and augmented their CFU-E suppressor effects. These studies provide evidence for a potential regulatory role of NK cells in erythropoiesis. The NK suppressor effect is maximal at the level of the mature erythroid stem cell CFU-E. These findings may explain some hypoproliferative anemias that develop in certain NK cell-activated states.

NK cells as effectors of genetically determined resistance to bone marrow transplants. Hansson and coworkers have recently shown that NK cells inhibit human granulocyte progenitor cell (CFU-C) proliferation in vitro. Collectively, these observations suggest that NK cells may also play an important physiologic or pathologic role in the regulation of hematopoiesis.

To date, there is no direct evidence implicating NK cells in the regulation of erythropoiesis. However, several indirect lines of evidence suggest that NK cells might also modulate erythroid stem cell proliferation. First, a leukemic cell line, K562, displaying prominent erythroid features, is highly reactive to natural killer cells. Second, interferon, a potent inducer of NK cell activity, also inhibits erythropoietic stem cell proliferation in vitro. Third, as we had previously noted, Tgamma cells, which contain substantial NK cell activity, can both retard BFU-E proliferation and suppress CFU-E proliferation.

To directly determine the effects of NK cells on erythroid stem cell proliferation, we concentrated blood NK cells by discontinuous density gradient centrifugation on Percoll or by fluorescence-activated cell sorting, employing the human natural killer cell monoclonal antibody, HNK1. The isolated NK-enriched fractions were then cocultured with autologous blood null cells or nonadherent marrow cells in the presence of erythropoietin. The results indicate that normal NK cells have little or no stimulatory activity on blood or marrow BFU-E and are capable of suppressing proliferation of both marrow BFU-E and

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CFU-E. The suppressive influence of NK cells appears to be maximal at the level of the more mature erythroid stem cell (CFU-E) and can be augmented by NK cell boosting with interferon.

**MATERIALS AND METHODS**

*Isolation of Blood Null Cells and Marrow Nonadherent (NA) Target Cells*

All blood and marrow samples were collected into heparinized saline (50–100 U/ml) syringes under aseptic conditions from healthy adult volunteers. Volunteers had given written consent according to the Human Studies Committee at Montefiore Hospital. Blood null cells were employed as targets in cocultures with NK cells for study of primitive day 14 BFU-E (erythroid burst-forming units). The methods for the isolation of blood null cells have been described extensively.\(^1\) In brief, blood mononuclear cells obtained at the interface of a standard Ficoll-Hypaque (FH) (Pharmacia Fine Chemicals, Piscataway, NJ, density 1.077 g/cm\(^3\)) gradient centrifugation\(^2\) were first depleted of adherent cells by incubation on plastic petri dishes coated with fetal calf serum (25–50 × 10\(^6\) cells/78 sq cm). B cells were then removed by adherence to antimunoglobulin-coated petri dishes.\(^1\) T cells were separated from monocyte and B-depleted fractions by rosetting with 2-aminoethylisothiouronium-bromide-treated sheep erythrocytes.\(^3\) In some experiments, T cells were retrieved for cocultures by lysing sheep erythrocytes with Tris-buffered ammonium chloride. The BFU-E enriched null cells remaining at the interface of subsequent FH density gradient centrifugations were routinely found to contain ≤5% B or T cells and less than 2% monocytes, as judged by cell surface marker analyses and histochemical stains.\(^4\)

Marrow nonadherent (NA) mononuclear cells were employed as target cells in studies of day 5–6 CFU-E (erythroid colony-forming units) or day 10–12 intermediate BFU-E. To obtain NA cells, aspirated marrow was strained through 20–25-gauge needles, and whole mononuclear marrow cells were obtained after a standard Ficoll-Hypaque density gradient centrifugation. The mononuclear cells were then depleted of adherent cells by incubation on plastic petri dishes coated with fetal calf serum, as described above. Prior to erythroid colony assays, blood null or marrow NA target cells were washed 3 times in alphaminimal essential medium (αMEM) containing 10% fetal calf serum (FCS), (GIBCO, Grand Island, NY).

*Isolation of NK Cells on Percoll Gradients*

For some experiments, NK cells were enriched on discontinuous Percoll (polyvinylpyrrolidone-coated colloidal silica particles; Pharmacia) gradients, as previously described.\(^5\) In brief, a stock solution of nonisoosmotic Percoll was diluted with αMEM containing 10% FCS to final concentrations of 37.5%, 40%, 42.5%, 45%, 47.5%, and 50% Percoll. These corresponded to fractions (F) F-0 through F-5, respectively. Six-milliliter aliquots of each concentration of diluted Percoll were carefully layered on 50-ml conical centrifuge tubes. Blood NA cells, obtained as described above, were suspended in αMEM with 10% FCS, layered on fraction 0 (density ≤ 1.048 g/cm\(^3\)), and centrifuged (300 g) for 45 min at 20°C. NK-enriched cells from F-0 and F-1 (density 1.062 g/cm\(^3\)) were pooled (low-density cells) and washed 3 times in αMEM with 10% FCS prior to cocultures. Cells from F-3 and F-5 (density 1.075 g/cm\(^3\)) were also pooled (high-density cells) and washed 3 times and used in some experiments as NK-depleted T-enriched cells (see below, Table 1).

For the NK dose-response experiments, NK cells were further enriched, employing isoosmotic Percoll as described by Timonen et al.\(^6\) In brief, cells retrieved from low density (1.062 g/cm\(^3\)) fractions (F2 and F3) were depleted of high-affinity E-rosette cells by mixing F2 and F3 cells with sheep erythrocytes for 1 hr at 29°C, followed by removal of E rosettes by a FH density gradient centrifugation. Cells remaining at the interface were highly enriched for NK cells (post-rosetted, low-density cells) (Table I). High-density fractions (>1.075 g/cm\(^3\)) (F5–F7) were employed as NK-depleted cells (Table I). Densities of gradients were routinely checked, using size marker beads (Pharmacia). Cell recoveries were always greater than 70%, and viabilities were always greater than 95%, as determined by trypan blue staining.

*Cell Marker Characterization of NK-Enriched and NK-Depleted Fractions*

Pooled cells from low- and high-density Percoll fractions were further characterized by their reaction with monoclonal antibodies and histochemical stains. The OKT3 antibody (Ortho Pharmaceutical, Raritan, NJ), which defines a pan-T-cell antigen, and the OKM1 antibody (Ortho Pharmaceutical), which is found on some

<table>
<thead>
<tr>
<th>Table 1. Characteristics of NK Cell-Enriched and -Depleted Fractions Isolated by Percoll or FACS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test</strong></td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>LGL(\dagger)</td>
</tr>
<tr>
<td>Small and medium lymphocytes</td>
</tr>
<tr>
<td>Monocytes(\dagger)</td>
</tr>
<tr>
<td>OKM-1 cells(\dagger)</td>
</tr>
<tr>
<td>HKN+ cells(\dagger)</td>
</tr>
<tr>
<td>OKT3+ cells(\dagger)</td>
</tr>
<tr>
<td>Cytotoxicity</td>
</tr>
</tbody>
</table>

\*Values indicate percent positive, reactive or cytotoxicity; mean ± 1 SD given for (n) observations in parentheses.
\(\dagger\)Low-density = 1.062 g/cm\(^3\); high-density = 1.075 g/cm\(^3\).
\(\ddagger\)LGL, large granular lymphocytes (Wright-Giemsa cytopsin smears.
\$As determined by reaction with α-naphthyl acetate esterase.
\[\]Reactivity with monoclonal antibodies determined by FACS analysis, as described in Materials and Methods.
\[\]Against K562 target cells at E:T ratio of 25:1, as described in text.
\**\*Determinations before removal of high avidity E-rosetted cells.
ND, not done.
monocytes, T cells, null cells, and NK cells, were employed in a manual indirect immunofluorescence assay with a fluorescein isothiocyanate (FITC)-labeled IgG fraction of goat anti-mouse IgG (Meloy Labs, Springfield, VA), as previously described. The HK-1 antibody, which defines both natural killer cells and K cells, was employed with a FITC-labeled goat anti-mouse IgM (Cappel Labs, Cochranville, PA) in an indirect immunofluorescence assay, as described. In some studies, the FITC-labeled HK-1 antibody (Leu-7, Becton-Dickinson, Mountain View, CA) was employed in a direct immunofluorescence assay, and cells were analyzed using the fluorescence-activated cell sorter (FACS). Cells from Percoll- or FACS-separated fractions were prepared by cytocentrifugation (Shandon, Southern Instrument, Inc., Sewickley, PA) and were analyzed for morphology or reaction with Wright-Giemsa or alpha-naphthyl acetate esterase stains.

**NK Cytotoxicity Assay**

K562 cells obtained from a patient with chronic myelogenous leukemia in blast crisis were employed as targets. Percoll low-density or high-density cells, T cells, or null cells were employed as effector cells. A quantity of $5 \times 10^5$ K562 cells was labeled with 100 µCi $^5$Cr (Amersham, Arlington Heights, IL) in 1.0 ml of Tris-phosphate buffer, pH 7.4, for 30 min at 37°C, with gentle shaking every 10 min, then washed 4 times in RPMI media containing 10% FCS (growth media). Cytotoxicity assays were carried out in 0.25 ml plastic wells (Linbro Scientific, Hamden, CT) at effector: target (E:T) cell ratios of 25:1. Seventy-five microliters of effector cells in growth media was added to $10^5$ labeled K562 target cells in 50 µl of growth media (total volume 125 µl). The cells were centrifuged for 3 min at 37°C in a 5% CO2 humidified atmosphere, then centrifuged at 250 g for 10 min. The radioactive supernatants (X) were then retrieved employing the Titertek Collection System (Flow Labs, Inc., McLean, VA). Radioactivity was measured using the auto-gamma scintillation spectrometer (Packard 5130). Each sample was counted for 3 min. Spontaneous (Sp) target cell lysis was determined from 6 wells containing $10^5$ labeled cells with growth media only. Maximal release (Max) of labels was determined by addition of 75 µl of 10% aqueous sodium lauryl sulfate (SLS), Fisher Scientific, to $10^5$ labeled target cells.

The percentage cytotoxicity (Cx) was calculated as follows:

$$\text{%Cx} = \frac{X - \text{Sp}}{\text{Max} - \text{Sp}} \times 100$$

where X is radioactivity released by effector cells, Sp is radioactivity released by growth media only, and Max is radioactivity released by 10% SLS.

**Isolation of HNK-1+ and HNK-1− Cells by FACS**

The human natural killer cell antibody (HNK-1), a monoclonal IgM antibody developed by Abo and Balch, was employed to isolate highly purified populations of NK cells from blood NA mononuclear cells. HNK-1 antibody (25 µg/ml) was added to aliquots of $3 \times 10^6$ pelleted NA mononuclear cells for 30 min at 4°C, with gentle mixing every 10 min. The HNK-1 antibody labeled NA cells were then washed 3 times in RPMI with 10% FCS, pelleted, and exposed to a fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgM antibody (1:20 or 1:40 dilution, 100 µl/106 cells). The labeled cells were washed 3 times in RPMI with 10% FCS and sorted aseptically using FACS (Becton-Dickinson). For each experiment, 2–3 x $10^5$ cells were stained and sorted. In three experiments, FACS analysis indicated that NA mononuclear cells were 15% ± 4.9% HNK+ (mean ± 1 SD). Both HNK+ and HNK− cells were collected and washed 3 times in RPMI with 10% FCS prior to cocultures. Sorted HNK+ cells were 92% ± 4% HNK+ on reanalysis by FACS. Label controls were 2.4% ± 1.3%. Viabilities of HNK+ or HNK− cells were always >92%.

**Augmentation of NK Activity**

In some experiments, NK activity was boosted by preincubating $4 \times 10^5$ low-density cells in αMEM with 20% FCS, containing $2.4 \times 10^6$ IU human lymphoblastoid interferon (specific activity, $1.0 \times 10^5$ U/mg protein Hu-IFN-α, Electro-Nucleonics Inc., Bethesda, MD) or $2 \times 10^5$ IU ultrapurified human leukocyte interferon (Science Inc., New Brunswick, NJ), for 18 hr at 37°C in a 5% CO2 atmosphere. Prior to cocultures, the cells were washed 3 times in αMEM containing 5% FCS.

**Complement-Mediated Lysis of NK Cells**

A quantity of $5 \times 10^5$ post-E rosetted low-density Percoll cells was exposed to 100 µg/ml HNK-1 antibody at 4°C, washed in αMEM, treated with a 1:40 dilution of rabbit complement (C′) (Low Tox, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) for 1 hr at 37°C, then rewashed 3 times in αMEM before plating. The $2 \times 10^5$ viable cells remaining after complement-mediated lysis was cocultured with $2 \times 10^5$ nonadherent marrow target cells. Controls were $2 \times 10^5$ untreated or C′-treated post-rosetted low-density Percoll cells.

**Erythroid Colony Assays**

A quantity of $2 \times 10^6$ blood null cells or $2 \times 10^5$ marrow NA target cells were cocultured with $10^4$–$10^5$ autologous NK-enriched cells (low-density Percoll or FACS, HNK+ cells) or NK-depleted cells (high-density Percoll, E rosette+, or FACS HNK− cells) in a 0.8% methylcellulose erythroid culture system containing 10 µg mercaptoethanol, 1% bovine serum albumin, and 30% fetal calf serum, as previously described. One International Unit for CFU-E or 2.0 IU for BFU-E of erythropoietin per 1.3-ml plate was added. The human urinary erythropoietin (fraction PS-1031-SL or CAT-I), kindly provided by the Division of Blood Diseases and Resources, National Heart, Lung and Blood Institute, was used for these studies. Each culture was performed in triplicate or quadruplicate (FACS experiment). Cultures were incubated for 5–14 days in a 5% CO2 atmosphere under high humidity, then stained in situ with benzidine. Day 5–6 benzidine-positive colonies containing at least 8 cells/aggregate were scored as 1 BFU-E. Blood BFU-E were scored on day 14. Marrow BFU-E were scored on days 10–12. Results are expressed as the number of CFU-E or BFU-E per $2 \times 10^4$ nonadherent or null cells plated.

**Statistics**

Comparisons between cohorts were made using Student’s t test.

**RESULTS**

**Characterization of Cell Fractions**

Morphological, histochemical, antigenic, and functional characteristics of the high- and low-density Percoll and HNK+ and HNK− cells used in the coculture experiments are summarized in Table 1. Cells from low-density (1.062 g/cu cm) Percoll were
predominantly OKM1+, HNK1+, OKT3−, large granular lymphocytes (LGL). In contrast, cells from high density (≥1.075 g/cu cm) Percoll were predominantly OKM1−, HNK−, OKT3+ small or medium round lymphocytes. The cytotoxic activity of low-density cells against K562 targets at E:T ratios of 25:1 was 6–10-fold that of high-density cells. Based on esterase stains, monocytes were nearly absent from these fractions. FACS-separated HNK+ cells were predominantly LGL (Fig. IA), whereas FACS-separated HNK− cells were predominantly small round lymphocytes (Fig. IB). Cytotoxic activity against K562 targets was concentrated in the HNK+ cells; however, FACS-separated HNK− cells (i.e., 3% ± 2% HNK+) still showed some cytotoxic activity against K562 targets.

**Effect of NK Cells on Marrow CFU-E Proliferation**

The effects of low-density (F0–F1) or high-density (F4–F5) cells, isolated from Percoll gradients on day 5–6, CFU-E proliferation from marrow nonadherent cells are shown in Table 2. In 6 separate experiments, CFU-E proliferation in the presence of $2.0 \times 10^5$ F0–F1 cells were reduced to 57% of controls without NK cells (151 ± 36 versus 86 ± 4/2 ± 10^4 NA cells, $p < 0.05$). In 4 of 6 individual experiments, the suppression of CFU-E proliferation was significant ($p < 0.05$). In contrast, high-density F4–F5 cells, under similar conditions, either stimulated or caused no significant change in CFU-E proliferation in vitro ($p > 0.05$). The differences between the effects of F0–F1 cells and F4–F5 cells (columns 3 and 4, Table

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**Fig. 1.** Wright-Giemsa cytopsin preparation of cells obtained by FACS, employing the HNK-1 monoclonal antibody. HNK-1+ cells (A) were predominantly large granular lymphocytes. HNK-1− cells (B) were predominantly small or medium-sized lymphocytes without cytoplasmic granules.
Table 2. Effect of NK Cell or T Cell Enriched Fractions From Percoll Gradients on CFU-E Proliferation From Marrow Nonadherent Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nonadherent</th>
<th>Nonadherent + NK</th>
<th>Nonadherent + T</th>
<th>p Value§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>166 ± 31†</td>
<td>130 ± 31</td>
<td>250 ± 46</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2</td>
<td>98 ± 34</td>
<td>35 ± 14</td>
<td>113 ± 27</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>3</td>
<td>162 ± 32</td>
<td>93 ± 13</td>
<td>178 ± 44</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>168 ± 6</td>
<td>131 ± 29</td>
<td>182 ± 6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>5</td>
<td>195 ± 12</td>
<td>46 ± 17</td>
<td>161 ± 29</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>6</td>
<td>116 ± 22</td>
<td>81 ± 9</td>
<td>117 ± 2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>n = 6</td>
<td>151 ± 36</td>
<td>86 ± 41</td>
<td>167 ± 50</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

*2.0 × 10^5 nonadherent marrow cells were cocultured with 2.0 × 10^6 NK cells obtained from Percoll fractions 0 and 1 or T cells obtained from Percoll fractions 4 and 5 in the presence of 1.0 IU erythropoietin/1.3-ml plate.
†Experiments 1 and 5 were allogeneic cocultures; all others were autologous.
‡Values indicate mean ± 1 SD CFU-E/2 × 10^5 nonadherent cells for triplicate plates.
§Comparison between nonadherent + NK versus nonadherent + T for individual experiments.

2) on CFU-E proliferation were significant in every instance (p < 0.025, column 5).

To test the possibility of an NK suppressor effect on CFU-E further, 10^4–4 × 10^5 NK-enriched or NK-depleted cells, obtained from low- or high-density Percoll fractions, respectively, were cocultured in a dose–response fashion with marrow nonadherent cells and scored for CFU-E. The combined results of 6 separate experiments are shown in Fig. 2. Some 145 ± 40 CFU-E proliferated in control NA marrow cells plated alone. Relative to control cultures, a progressive decrease in CFU-E numbers occurred upon addition of increasing numbers of NK-enriched, low-density cells (Fig. 2). At NK:NA marrow cell ratios of 2:1, CFU-E numbers were reduced, on the average, to 62% of controls (p < 0.025). In contrast, equal numbers of NK-depleted, high-density cells caused a slight augmentation in CFU-E numbers to 130% of controls (p > 0.05). The differences between the effects of low-density versus high-density cells at NK:NA ratios of 2:1 were significant (p < 0.005).

To confirm the NK cell suppressor effects on CFU-E, NK cells from low-density post-rosetted Percoll fractions were treated with the HNK-1 monoclonal antibody in the presence of complement before coculture with marrow NA cells. Low-density NK-enriched cells, either untreated or treated with complement only, suppressed CFU-E compared to controls without NK-enriched cells (Fig. 3). In contrast, NK-enriched cells treated with HNK-1 antibody and complement no longer suppressed CFU-E growth in three experiments.

![Fig. 2. Dose–response effects of NK-enriched low-density cells (broken line) or NK-depleted high-density cells (solid line) on day 5–6 CFU-E proliferating from 2.0 × 10^6 autologous marrow nonadherent (NA) cells. Values represent mean ± 1 SD for 6 separate experiments. A quantity of 145 ± 40 CFU-E (mean ± 1 SD) proliferated in marrow NA cell controls.](image-url)
Effects of NK Cells on Marrow BFU-E Proliferation

To determine the effects of NK cells on day 10–12 marrow BFU-E proliferation, similar dose–response experiments were performed, employing low- and high-density Percoll cells and marrow nonadherent cells. The combined results of 5 separate experiments are shown in Fig. 4. A quantity of $34 \pm 16$ BFU-E proliferated in marrow nonadherent cells plated alone. At low concentrations ($10^3$–$10^4$ cells), NK-enriched or NK-depleted cells caused no significant burst-promoting effects. At greater concentrations ($2.0 \times 10^5$ cells), high-density cells augmented BFU-E numbers twofold over controls ($p < 0.05$). The burst-promoting effects of low-density NK-enriched cells was slightly less ($p > 0.05$). Marrow BFU-E proliferation was not suppressed by either low- or high-density Percoll cells within the range of cells tested.

Effects of FACS-Separated HNK+ and HNK- Cells on Marrow CFU-E and BFU-E Proliferation

Although low-density cells obtained from Percoll gradients are NK cell-enriched, it was important to exclude the possibility that other cells (possibly T cells) might be responsible for the suppression of erythropoiesis observed when employing the Percoll fractions. Therefore, highly purified (>90%) NK cells were isolated by FACS, employing the HNK-1 monoclonal antibody that identifies LGL having NK activity. As shown in Fig. 5, in 3 separate experiments, addition of HNK-1+ cells suppressed BFU-E proliferation on the average to 34% of controls without NK cells ($118 \pm 29$ versus 40 ± 22) and to 31% of CFU-E numbers proliferating in the presence of FACS-separated HNK-1- cells ($127 \pm 21$ versus 40 ± 22, $p < 0.025$). As shown in Fig. 6, HNK+ cells also suppressed intermediate day 10–12 BFU-E, on the average, to 41% of BFU-E proliferating in marrow nonadherent...
cells (92 ± 39 versus 38 ± 19) and to 32% of BFU-E proliferating in the presence of HNK− cells (119 ± 43 versus 38 ± 19, p < 0.025). HNK-1− cells isolated by FACS had no significant effects on either CFU-E or BFU-E proliferation under similar conditions.

Effects of NK Cells on Blood BFU-E Proliferation

The burst-promoting effect of 2.0 × 10⁴ NK cells was compared to the activity of equal numbers of T cells in cocultures using autologous blood null cells in 10 separate experiments (Table 3). For these experiments, NK cells were obtained by separation on low-density Percoll gradients, F0–F1. T cells were obtained either by E rosetting technique (experiments 1–5, Table 1) or from the high-density Percoll fractions (F4–F5) (experiments 6–10, Table 1).

Addition of 2 × 10⁴ NK cells (Table 3, column 3) increased BFU-E proliferation approximately fourfold (32 ± 16 versus 8 ± 3 BFU-E/2 × 10⁴ null cells, p < 0.01, n = 10). However, compared to T cells, isolated by either E rosetting or F4–F5 Percoll gradients, the burst-promoting stimulus was significantly less under identical culture conditions (72 ± 35 versus 32 ± 16, p < 0.01; Table 3, columns 3 plus 4). These differences could not be explained by enrichment of BFU-E in either F4–F5 cells or ER+ T cells, because only 2 ± 4 BFU-E proliferated in T cells plated separately (column 6) as compared to 6 ± 6 BFU-E in F0–F1 cells (column 5). The majority of blood BFU-E were found in F2–F3 of Percoll gradients (data not shown). T cells isolated by E rosetting (experiments 1–5) provided a significantly greater burst-promoting stimulus than T cells enriched on F4–F5 Percoll gradients (experiments 6–10, Table 1; 94 ± 37 versus 51 ± 16 BFU-E/2 × 10⁴ null cells, p < 0.025).

Effect of NK Cell Boosting With Interferon on BFU-E and CFU-E Proliferation

Because interferon is a potent inducer of NK cell activity, the NK cell suppressor effects on erythropoietic stem cell proliferation were tested after exposure of NK cells overnight to type I interferon. The results of separate coculture experiments for blood BFU-E (experiments 1–4) and marrow CFU-E (experiments 5–8) are given in Table 4. In every instance, NK-enriched cells obtained from low-density Percoll were more potent suppressors of both blood BFU-E and marrow CFU-E after preincubation with interferon.

**DISCUSSION**

The results of experiments employing blood lymphocytes obtained by Percoll density gradient centrifugation provide new evidence for a potential regulatory role of NK cells in human erythroid stem cell proliferation. Cells retrieved from low-density Percoll fractions were predominantly OKM1 +, HNK-1 +, OKT3− large granular lymphocytes (LGL), whereas cells from high-density fractions were mostly OKM1−, HNK-1−, OKT3 + small round lymphocytes, as previously reported.2,3 Spontaneous cytolytic activity against K562 targets by low-density cells was 6–10-fold greater than high-density cells. When NK-enriched low-density cells were cocultured with marrow nonadherent (NA) target cells at various cell ratios, a consistent inhibition of day 5–6 CFU-E growth was observed. In contrast, the NK-depleted high-density cells augmented CFU-E proliferation in a dose-response fashion. The NK suppressor effect on CFU-E was confirmed by the additional observations that: (1)

### Table 3. Comparison of BFU-E Promoting Effects of NK Cells Versus T Cells in Cultures With Blood Null Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Null</th>
<th>Null + NK</th>
<th>Null + T</th>
<th>NK</th>
<th>T</th>
<th>p Value</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9 ± 2</td>
<td>45 ± 5</td>
<td>95 ± 10</td>
<td>11 ± 8</td>
<td>0 ± 0</td>
<td>&lt;0.01</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>2</td>
<td>7 ± 2</td>
<td>35 ± 3</td>
<td>110 ± 18</td>
<td>13 ± 5</td>
<td>0 ± 0</td>
<td>&lt;0.01</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>3</td>
<td>10 ± 5</td>
<td>12 ± 4</td>
<td>65 ± 9</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>12 ± 4</td>
<td>33 ± 4</td>
<td>146 ± 14</td>
<td>18 ± 10</td>
<td>5 ± 5</td>
<td>&lt;0.005</td>
<td>&lt;0.01</td>
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<td>22 ± 8</td>
<td>53 ± 11</td>
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<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>6</td>
<td>6 ± 3</td>
<td>21 ± 8</td>
<td>33 ± 6</td>
<td>8 ± 4</td>
<td>2 ± 2</td>
<td>&lt;0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>7</td>
<td>9 ± 3</td>
<td>18 ± 8</td>
<td>50 ± 8</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>&lt;0.025</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>8</td>
<td>12 ± 4</td>
<td>58 ± 6</td>
<td>72 ± 9</td>
<td>10 ± 4</td>
<td>11 ± 3</td>
<td>&lt;0.01</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>9</td>
<td>2 ± 1</td>
<td>23 ± 12</td>
<td>39 ± 3</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>&lt;0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>10</td>
<td>7 ± 6</td>
<td>52 ± 6</td>
<td>60 ± 9</td>
<td>1 ± 1</td>
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<td>&lt;0.01</td>
</tr>
<tr>
<td>n = 10</td>
<td>8 ± 3</td>
<td>32 ± 16</td>
<td>72 ± 35</td>
<td>6 ± 6</td>
<td>2 ± 4</td>
<td>&lt;0.01</td>
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</table>

*2.0 × 10⁴ blood null cells were cocultured with 2.0 × 10⁴ autologous NK or T cells in the presence of 2.0 IU of erythropoietin/1.3-ml plate. NK cells: cells from Percoll fractions 0 and 1. T cells were ER+ cells (experiments 1–5) or from Percoll fractions 4 and 5 (experiments 6–10). Cytotoxicity or blood null cells against K562 targets was 40% ± 7% (N = 3). Percent cytotoxicity of E-rosetted T cells was <2%.

†Values indicate mean ± 1 SD. BFU-E/2 × 10⁴ null cells for triplicate plates.

‡Comparisons between null + NK versus null + T cohorts for individual experiments.

§Comparisons between null versus null + NK cohorts for individual experiments.

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direct addition of FACS-separated highly purified HNK-1+ cells, but not HNK-1−, cells suppressed CFU-E growth, and (2) removal of HNK + cells from NK-enriched low-density Percoll fractions abrogated the suppressor effects of these cells.

Recent evidence has suggested that T-cell-mediated suppression of erythroid stem cell growth may be restricted by HLA-DR. The NK suppressor effects on CFU-E did not require a preincubation period with marrow NA cells, could be boosted with interferon, and were also observed in allogeneic cultures (Table 3). As residual T cells or monocytes in low-density Percoll fractions may contribute to their BFU-E-enhancing effect, it appears that NK cells themselves have little or no burst-promoting effects.

Interferon boosting of NK cells augmented their suppressor effects on CFU-E and BFU-E proliferation. A direct effect of exogenous interferon on erythroid stem cell proliferation is unlikely, as any residual interferon would be unstable in the mercaptoethanol-containing erythroid cultures. However, it is possible that endogenous interferon, produced by activated NK cells, may be transmitted through direct cell contacts to suppress erythroid stem cell proliferation. Further studies are in progress to clarify this possibility. However, these studies suggest that the previously observed suppression of erythroid stem cell proliferation by interferon could be mediated in part through activation of NK cells in culture.

NK cells are normally found in only small numbers in the bone marrow. Therefore, the physiologic significance of our in vitro finding is not clear. However, our observations that the suppressor effects of NK cells on
erythropoiesis appear maximal at the level of the more mature erythroid stem cells raises the possibility that NK cells may be important in regulating the size of the rapidly cycling and proliferating mature erythroid stem cell compartment. In pathologic marrow states, such as aplastic anemia or pure red cell aplasia, recent attention has focused on the pathogenic roles of suppressor/cytotoxic T cells.\(^{16,30}\) However, the present studies suggest that the mediators of these disorders might be NK cells. Three additional recent observations support this notion: (1) OKT8+ (cytotoxic/suppressor cells) predominate in the bone marrow;\(^{31}\) (2) a proportion of OKT8+ cells in this marrow are HNK-1+;\(^ {32}\) and (3) Tγ cells, previously implicated in the suppression of hematopoiesis in aplastic anemia\(^ {10}\) and CLL,\(^ {16}\) are largely HNK+, OKT8+ LGL.\(^ {3,32}\)

Finally, these findings may have relevance to the suppression of erythropoiesis observed in some postviral states, where interferon levels are high; in the development of marrow suppression after interferon therapy\(^ {15}\) or posttransplant;\(^ {10}\) or in patients with a wide variety of tumors, where NK cells may be endogenously activated. Further attention to the pathologic roles of NK cells in suppressed states of erythropoiesis is likely to provide potentially important pathogenic information.

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REFERENCES


Natural killer cells suppress human erythroid stem cell proliferation in vitro

KF Mangan, ME Hartnett, SA Matis, A Winkelstein and T Abo

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