THE EFFECTS OF RECOMBINANT INTERLEUKIN 2-
ACTIVATED NATURAL KILLER CELLS ON AUTOLOGOUS
PERIPHERAL BLOOD HEMATOPOIETIC PROGENITORS

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Natural killer (NK) cells have been defined as non-MHC-restricted cytotoxic lymphocytes that do not rearrange TCR genes or express cell surface CD3/Ti complexes, but can be identified by the expression of the CD16 and/or Leu-19 antigens on the cell surface membranes (1). When activated with rIL-2, NK cells kill a broad range of fresh uncultured tumor cell targets, including autologous tumor cells (2–4). The ability of rIL-2-activated NK cells to lyse a variety of resistant tumor cell types in vitro has led to the clinical trials of lymphokine-activated killer (LAK) cell adoptive immunotherapy against neoplastic malignancies (5). The effect of rIL-2-activated NK cells on normal hematopoiesis, in particular hematopoietic progenitor (HP) cells, has been a controversial issue. Resting NK cells, although not inhibiting bone marrow–derived erythroid burst-forming unit (BFU-E), moderately inhibited bone marrow granulocyte/macrophage CFU (CFU-GM) (day 14) and granulocyte, erythroid, macrophage, megakaryocyte CFU (CFU-GEMM) (6–8). Activated NK cells have also been shown to inhibit bone marrow hematopoietic colony formation (6, 9, 10). The mechanisms by which NK cells regulate hematopoiesis remain largely unknown, however, soluble factors such as IF and TNF may play a major role in the phenomenon (9, 10). In contrast to prior results with bone marrow, in the present study, we have examined the effects of resting and rIL-2-activated NK cells on peripheral blood–derived HP cells in normal individuals and acute myelogenous leukemia (AML) patients in stable remission.

Materials and Methods

Lymphocytes. PBMC from normal donors (Stanford Blood Center, Stanford, CA) and AML patients induced into stable remission by chemotherapy (Stanford University Medical Center, Palo Alto, CA) were isolated by Ficoll/Hypaque centrifugation. After plastic adherence and passage through nylon wool to remove monocytes and B cells, the LBD cells were isolated by centrifugation through discontinuous gradients of Percoll (3). LBD cells were cultured

1 Abbreviations used in this paper: AML, acute myelogenous leukemia; BFU-E, erythroid burst-forming unit; CFU-GEMM, granulocyte, erythroid, macrophage, megakaryocyte CFU; CFU-GM, granulocyte/macrophage CFU; HBD, high buoyant density; HP, hematopoietic progenitor; LAK, lymphokine-activated killer; LBD, low buoyant density; LGL, large granular lymphocytes; PE, phycoerythrin.
in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) containing 1 mM glutamine, 100 µg/ml gentamicin (Gibco, Grand Island, NY), and FCS in the presence or absence of rIL-2 (Cetus Corp., Emeryville, CA).

**Immunofluorescence and Flow Cytometry.** Methods of immunofluorescence, flow cytometry, and data analysis have been described previously (3). Leu series antibodies were produced by the Becton Dickinson Monoclonal Center, Inc., Mountain View, CA.

**Cytotoxicity Assay.** Cytotoxic activity was measured using a 4-h ⁵¹Cr radioisotope release assay (3). Fresh uncultured AML tumor cells were obtained from AML patients during the acute phase of the disease and were frozen in liquid nitrogen to be used as fresh autologous leukemia cell targets.

**Hematopoietic Clonogenic Assay.** Cells were suspended in Iscove's modified Dulbecco's medium with 15% FCS, 9% BSA (Armour Pharmaceutical Co., Tarrytown, NY), 50 µM 2-ME, 1% penicillin/streptomycin, 1% glutamine, and 1.1% methycellulose. CFU-GM clonogenic assays were performed using 15% placental-conditioned media as an exogenous source of CSF. BFU-E and CFU-GEMM clonogenic assays contained 1% Mo T cell-conditioned media as a source of burst-promoting activity (kindly provided by Dr. David Golde, UCLA Medical Center, Los Angeles, CA) and 0.5 U/ml of purified human urinary erythropoietin. Cultures were incubated in humidified 5% CO₂ at 37°C and microscopically scored for colony growth on day 10 and 14, as previously described (11).

**Enrichment of Peripheral Blood HP Cells.** Peripheral blood HP cells (CD34⁺) were enriched from low buoyant density (LBD) cells using a magnetic bead depletion technique. LBD cells were stained with saturating amounts of anti-Leu-1 (CD5), anti-Leu-2 (CD8), anti-Leu-3 (CD4), anti-Leu-4 (CD3), anti-Leu-M3, anti-Leu-11a (CD16), anti-Leu-12 (CD19), and anti-Leu-16 (CD20) mAbs. The stained cells were washed, combined with goat anti-mouse Ig-conjugated magnetic beads (Advanced Magnetics Inc., Cambridge, MA), centrifuged at low speed, and incubated as a pellet for 40 min at 4°C. The pellet was then vortexed, underlayed with 10 ml of Ficoll/Hypaque, and centrifuged at 1,500 rpm for 20 min. The cells recovered at the interface (~5% of the total) were washed extensively and then applied to a plate magnet (Advanced Magnetics, Inc.) to remove any residual magnetic beads. By this procedure, CD34⁺ were enriched to 65-68% purity.

**Results and Discussion**

**Isolation and Characterization of Peripheral Blood HP Cells.** Human NK cells and HP cells are characterized as large LBD cells (12-14) and thus can be coenriched on discontinuous density gradients of Percoll. PBMC depleted of monocytes and B cells were separated into LBD and high buoyant density (HBD) cell populations on Percoll gradients (3). As previously reported (3), LBD cells from Percoll gradients of peripheral blood mononuclear cells are predominantly large granular lymphocytes (LGL) consisting of 45-60% NK cells (Leu-19⁺, CD16⁺, CD3⁻) and 35-50% T cells (CD3⁺). However, LBD cells from normal donors (n = 4) also contained 4-10% HP cells as identified by the mAb anti-HPCA-1, which recognizes the CD34 antigen. This represents a significant enrichment of HP cells, since HP cells usually make up <2% of PBMC. Peripheral blood CD34⁺ cells coexpressed HLA-DR antigen and transferrin receptors but did not coexpress CD2, CD3, CD4, CD8, CD5, CD16, or Leu-19 antigens. The phenotype of the peripheral blood HP cells is thus consistent with the phenotype of CD34⁺ HP cells of the bone marrow (15). To verify that the CD34⁺ cells generate in vitro hematopoietic colonies, peripheral blood LBD cells were stained with anti-CD34 mAb, separated into CD34⁺ and CD34⁻ populations using a FACS system, and cultured in methycellulose colony assays. CD34⁺ LBD cells were similar to bone marrow HP cells and demonstrated a broad spectrum of clonogenic capabilities (Table I). The CD34⁻ LBD cells showed essentially no hematopoietic colony formation.
TABLE I

Hematopoietic Colony Formation by Peripheral Blood CD34+ Cells

| Donor | FACS sorted | CFU-GM(10) | CFU-GM(14) | BFU-E | CFU-GEMM |
|-------|-------------|------------|------------|-------|----------|
|       | CD34*       | 600        | 260        | 2,400 | 18       |
|       | CD34−       | 1          | 1          | 4     | 0        |
| 2     | CD34*       | 532        | 248        | 2,148 | ND       |
|       | CD34−       | 1.2        | 1          | 4     | ND       |
| 3     | CD34*       | 436        | 296        | 1,828 | 84       |
|       | CD34−       | 0          | 0          | 0     | 0        |

LBD cells were isolated from the peripheral blood of three normal donors and stained with purified anti-HPCA 1 (CD34) mAb, followed by PE-conjugated rat anti-mouse IgG1 mAb. CD34+ and CD34− cells were isolated to >97% purity using a FACS and cultured in hematopoietic clonogenic assays. Results are presented as the mean colony number of duplicate platings per $1.5 \times 10^5$ cells plated.

Effects of rIL-2-activated NK Cells on Autologous Peripheral Blood HP Cells. To determine the feasibility of using rIL-2-activated NK cells to purge autologous HP cell preparation of residual leukemia cells, it was first essential to investigate the effects of NK cells (resting and rIL-2 activated) on the clonogenic colony formation capabilities of autologous HP cells. Peripheral blood NK cells (CD16+) were purified to homogeneity using a FACS and were incubated with and without rIL-2 for 24 h. Resting and rIL-2-activated NK cells were then combined with either autologous bone marrow cells or peripheral blood HP cell-enriched populations and cocultured in clonogenic methylcellulose colony formation assays. Consistent with prior reports (8, 9), resting NK cells did not effect autologous bone marrow BFU-E; however, they moderately inhibited the formation of CFU-GM (day 14) and strongly suppressed CFU-GEMM (Fig. 1 A). rIL-2-activated NK cells also inhibited CFU-GM...
The effects of coculture of NK cells and autologous HP cells. LBD cells obtained from normal donors were incubated at 37°C in the presence of 100 U/ml of rIL-2. At 0, 1, and 3 d, the LBD cells were plated in hematopoietic clonogenic assays (A): (I) BFU-E, (II) CFU-GM (day 10), (III) CFU-GM (day 14), (IV) BFU-E, (V) CFU-GEMM. Control cultures without rIL-2 remained unchanged throughout the duration of the experiment and contained 36 ± 6 CFU-GM (day 10), 48 ± 7 CFU-GM (day 14), 157 ± 12 BFU-E, and 8 ± 2 CFU-GEMM per 1.5 × 10⁵ plated cells. At each of these time points, the LBD cells were also assayed for cytolytic activity (B) against K562 (I), Daudi (II), and Colo 205 (III). Similar results were obtained in five additional experiments.

Figure 3. Characterization of LBD cells isolated from AML patients. Peripheral blood was obtained from AML patients induced into stable remission by inductive chemotherapy. (A) LBD cells were stained with PE-conjugated anti-Leu-19 and FITC-conjugated anti-Leu-4 (CD3). NK cells (I) were defined as Leu-19⁺ CD3⁻, while T cells (II) were defined as Leu-19⁻⁺ CD3⁺ (I). HP cells (III) were enumerated by staining with anti-HPCA 1 (CD34) followed by a PE-conjugated rat anti-mouse IgGl mAb. Results are presented as the percentage of positive staining cells for each of the four individuals studied. (B-E) LBD cells isolated from the four AML patient in remission were stained with PE-conjugated anti-Leu-19 and FITC-conjugated anti-Leu-4 (CD5). Anti-CD5 was used to identify and isolate T cells, since antibodies against CD5 have been shown to either inhibit or induce T cell-mediated cytotoxicity. NK cells (Leu-19⁺/Leu-1⁻) were isolated to >97% purity using a FACS, and were assayed for cytotoxic activity against Colo-205 (I) and two fresh uncultured allogeneic leukemias (ΔΔ), before culture (open symbols) and after overnight culture in 100 U/ml rIL-2 (solid symbols).
and monocytes. In contrast, the bone marrow preparations used in these studies were not enriched for HP cells and represented a heterogeneous cell population. Therefore, the NK cell-induced inhibition of bone marrow HP cells may be the result of indirect interactions of NK cells with non-HP cells of the bone marrow. Deglia-toni et al. (8, 9) have suggested that the NK cell–mediated inhibition of bone marrow HP cells is manifested by the secretion of NK cell–derived colony-inhibiting factors generated from the interaction of NK cells with HLA-DR + bone marrow cells. Since HP cells of the bone marrow express HLA-DR antigens, this study was unable to determine if NK cells were directly interacting with stem cells or other HLA-DR + bone marrow cells. Further experimentation on the interaction of autologous NK cells with various cellular elements of the bone marrow is indeed required to determine the mechanism of inhibition of bone marrow–derived HP cell colony formation by NK cells.

The Effects of Extended Coculture of NK Cells and HP Cells. To directly investigate the effects of activated NK cells on peripheral blood HP cells, LBD cells were incubated at 37°C in the presence of rIL-2. At 0, 1, and 3 d, the cells were plated for hematopoietic colony growth and assayed for cytotoxic activity against the NK-sensitive tumor line K562 (erythromyeloleukemia) and the NK-resistant cell lines Daudi (B lymphoblastoid) and Colo-205 (colon carcinoma). Culturing LBD cells for 3 d in the presence of rIL-2 had no inhibitory effects on the formation of hematopoietic colonies (Fig. 2A). Analysis of the cytotoxic activity of these cultures clearly showed that the NK cells were substantially activated 24 h after culture with rIL-2 and reached maximal levels of cytotoxic potential by 3 d (Fig. 2B). Peripheral blood HP cells cultured >3 d with or without NK cells showed decreases in hematopoietic colony formation and viability.

NK Cells and Peripheral Blood HP Cells from AML Patients. Studies were then performed using PBMC derived from patients with AML induced by chemotherapy into stable remission. LBD cells were isolated from AML remission patients and examined for percentages of NK cells, T cells, and CD34 + cells. These patients possessed normal percentages of NK cells and slightly elevated percentages of CD34 + HP cells (Fig. 3A). At the time of stable remission, the LBD cells from these patients contained no detectable leukemic blasts. The NK cells were functionally normal and when activated overnight with rIL-2, demonstrated potent activated cytolysis against fresh uncultured AML tumor cells as well as Colo-205 (Fig. 3 B-E). CD34 + HP cells from the LBD cells of AML remission patients demonstrated normal CFU-GM, BFU-E, and CFU-GEMM hematopoietic colonies in vitro (data not shown). rIL-2-activated NK cells from most AML patients were also capable of efficiently lysing autologous AML tumor cells (Table II). In these studies, PBMC from AML patients with varied percentages of tumor blasts were incubated in the presence of rIL-2 for 10–14 d. NK cells were then purified from these cultures using a FACS and were analyzed for cytolytic capabilities against a panel of tumor cells including autologous uncultured tumor and fresh uncultured allogeneic AML tumors (Table II). In three of the four patients studied, rIL-2-activated NK cells were very cytolytic against autologous AML tumor as well as allogeneic AML tumor, Colo-205, and K562. Patient AML-3 demonstrated strong levels of cytolytic activity against Colo-205, but showed only low levels of killing against autologous or allogeneic AML tumor cells.
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Table II
Cytolysis of Autologous Tumor by rIL-2-activated NK Cells from AML Patients

| Patient | E/T | K562 | Colo | AML-1 | AML-2 | AML-3 | AML-4 |
|---------|-----|------|------|-------|-------|-------|-------|
| AML-1   | 6   | 73   | 69   | 69    | 21    | -     | -     |
|         | 3   | 76   | 58   | 55    | 10    | -     | -     |
|         | 1.5 | 68   | 41   | 43    | 7     | -     | -     |
| AML-2   | 6   | 90   | 60   | 67    | 32    | -     | -     |
|         | 3   | 65   | 32   | 53    | 27    | -     | -     |
|         | 1.5 | 47   | 23   | 24    | 14    | -     | -     |
| AML-3   | 6   | 85   | 67   | -     | 22    | 11    | -     |
|         | 3   | 61   | 50   | -     | 14    | 5     | -     |
|         | 1.5 | 60   | 36   | -     | 10    | 1     | -     |
| AML-4   | 6   | 92   | 65   | -     | 52    | -     | 76    |
|         | 3   | 90   | 53   | -     | 40    | -     | 63    |
|         | 1.5 | 86   | 40   | -     | 25    | -     | 51    |

PBMC were obtained from AML patients in acute phase: AML-1, 80% blasts; AML-2, 85% blasts; AML-3, 72% blasts; AML-4, 50% blasts. LBD cells isolated from each of these patients were >90% leukemic blasts. A portion of these LBD cell preparations were frozen in liquid nitrogen to be used as fresh uncultured autologous leukemia cells, while the remaining LBD cells were cultured in 800 U/ml rIL-2 for 10-14 d. At this time, the cultures, which consisted exclusively of normal lymphocytes, were stained with PE-conjugated anti-Leu-19 and FITC-conjugated anti-Leu-4. NK cells (Leu-19+, CD8+) were isolated to >95% purity using a FACS and used as effector cells against various 51Cr labeled tumor cell targets, including fresh uncultured autologous and allogeneic AML leukemia cells.

Recently, several investigators have reported that leukemia patients in remission demonstrate elevated numbers of peripheral blood HP cells after induction chemotherapy (16-18). The use of autologous peripheral blood HP cells as an alternative to autologous bone marrow transplantation has gained a great deal of interest, particularly with the preliminary reports that blood-derived HP cells can successively reconstitute hematopoiesis after marrow-ablative radiotherapy (18-21). A major consideration in autologous HP cell reconstitution is the inability to efficiently remove residual tumor cells from the stem cell preparations. The use of peripheral blood-derived HP cells, however, may have several favorable advantages including the ability to obtain large numbers of HP cells through sequential leukapheresis that may contain less tumor cell contamination than the bone marrow. Our results demonstrate that the peripheral blood of normal donors, as well as AML patients in stable remission, have significant numbers of normal HP cells as well as NK cells that can be induced to kill autologous leukemia cells without inhibiting the in vitro clonogenic colony formation capabilities of the peripheral blood HP cells. Based on these results, we propose that rIL-2-activated NK cells may be successfully used to purge autologous peripheral blood HP cell preparations of residual leukemia cells in vitro before reconstitution of the recipient. The capability of rIL-2-activated NK cells to purge autologous peripheral blood HP cell preparations of residual leukemia cells is dependent upon the efficiency of the leukemic patients' activated NK cells to lyse autologous tumor cells. Although the majority of the AML patients in this study showed
potent activated NK cell lysis of autologous tumor, not all leukemia patients displayed the degree of autologous tumor killing required to achieve efficient tumor cell purging of peripheral blood HP cell preparations. This does not represent a major constraint, however, since leukemia cells can easily be frozen during the acute phase of the patient's disease and subsequently assessed for susceptibility to cytolysis by rIL-2-activated NK cells obtained from the peripheral blood at the time of remission. Moreover, this approach could potentially be combined with other recently developed methods of leukemia therapy to increase the efficiency of these treatment protocols. The results of our studies also suggest that it may be possible to effectively treat some leukemia patients by activating endogenous NK cells to lyse autologous tumor with in vivo rIL-2 therapy.

Summary
In the present study, we demonstrate that resting and rIL-2-activated NK cells had no inhibitory effects on peripheral blood-derived hematopoietic progenitor (HP) cells. Peripheral blood HP cells were similar to bone marrow progenitors in phenotype and clonogenic colony formation capabilities. Peripheral blood HP cells could be cocultured in vitro with rIL-2-activated autologous NK cells for 3 d without adverse effects on the HP cells. Acute myelogenous leukemia patients in stable remission were shown to have normal percentages of NK cells and elevated percentages of peripheral blood HP cells. NK cells from most of these patients could be activated with rIL-2 to lyse fresh uncultured tumor cells as well as autologous leukemia cells without effecting the peripheral blood HP cells. These results suggest that rIL-2-activated NK cells may be used to purge peripheral blood HP cell preparations of residual tumor cells before hematopoietic reconstitution.

Received for publication 12 February 1988 and in revised form 4 April 1988.

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