RAPID COMMUNICATION

The Generation of Human Natural Killer Cells From CD34+/DR- Primitive Progenitors in Long-Term Bone Marrow Culture

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We have adapted the stroma-dependent long-term bone marrow culture (LTBMC) system to study the development of human natural killer cells (NK) from the CD34+/HLA-DR- (CD34+/DR-) BM mononuclear cell (BMMNC) population. The CD34+/DR- population does not express any known antigens associated with myeloid or lymphoid lineage and has been shown by us and others to contain primitive hematopoietic progenitors capable of both self-renewal and differentiation to myeloid lineage. CD34+/DR- cells obtained from normal human BM by fluorescence-activated cell sorting were plated on allogeneic, irradiated BM stromal layers. After 5 weeks of culture in the presence of media containing recombinant interleukin-2 and human serum, 147- to 21-fold expansion of cells with the morphologic appearance of large granular lymphocytes was observed. Cultured cells (84.8% ± 1.5%) expressed the characteristic CD56+/CD3- phenotype of NK. A proportion of CD56+/CD3- cells expressed other markers of lymphoid lineage that have been associated with mature NK, including CD2 (7.8% ± 1.2%), CD7 (19.5% ± 2.8), CD8 (3.1% ± 1.0%), and CD16 (4.5% ± 1.3%). The cultured cells did not express other antigens associated with T-lymphocyte (CD3, CD5, T-cell receptor [TCR] α/β and TCR γ/δ), B-lymphocyte (CD19), myeloid (MY8, CD33, and CD71), or monocytoid (CD14 and CD15) lineage and did not express the CD34 antigen associated with hematopoietic progenitors present on the starting population. This NK population was cytotoxic against both K562 (E:T 20:1; 79% ± 1.9%) and Raji (E:T 20:1; 38% ± 5.7%) target cell lines. The NK progenitor frequency in the CD34+/DR- cell population determined by limiting dilution of CD34+/DR- on stromal layers followed by a functional chromium release assay against K562 targets was 1:169 ± 50 CD34+/DR- cells. The data suggest that human LTBMC developed to study myeloid differentiation can be modified to study the origin and development of the NK and possibly other lymphoid lineages. Modified cultures show that cells with morphologic, phenotypic, and functional characteristics of NK can be derived from a population of BMMNC with the phenotype of primitive hematopoietic progenitors and without phenotypic evidence of lymphoid- or myeloid-lineage commitment. Further studies will address the cell of origin and the ontogeny of human NK and other lymphoid lineages.

HUMAN NATURAL KILLER (NK) cells are a subset of mononuclear cells (MNC) that express the CD56+/CD3- phenotype and have the appearance of large granular lymphocytes. These cells have a highly specialized function associated with the recognition and destruction of infected or transformed cells. Although attributes of both lymphoid and myeloid cells have been ascribed to the NK population, the NK lineage is not known and an early NK progenitor has not been described. It has been hypothesized that primitive progenitors of both myeloid and lymphoid lineage can be found in the population of bone marrow MNC (BMMNC) expressing CD34, but not other antigens associated with myeloid- or lymphoid-lineage commitment. Recently, cultivation of the CD34+/HLA-DR- (CD34+/DR-) population in stromal-dependent long-term BM culture (LTBMC) showed that primitive progenitors can be maintained and differentiate into committed myeloid precursors. The culture system requires the presence of human BM stroma to initiate, sustain, and regulate myeloid population growth. Recently, it has been suggested that cultivation of subsets of the human CD34+ population in vitro or transplantaion into SCID mice can result in the production of human T and B lymphocytes. Several investigators have cultured BM populations with or without the depletion of mature NK to obtain various activated NK populations; however, propagation of NK cells from normal human BM populations bearing the CD34 marker associated with primitive progenitors has not been reported. We show here the generation of human NK from CD34+/DR- cells after modification of LTBMC and begin to use this system to explore human lymphoid ontogeny.

MATERIALS AND METHODS

Patient population. Twenty healthy adult volunteers aged 21 to 45 years were studied. Peripheral blood (PB) or BM obtained from the posterior iliac crest was collected in syringes containing preservative-free heparin. All samples were obtained using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. BMMNC or PBMC were obtained by Ficoll-Hypaque (specific gravity, 1.077; Sigma Diagnostics, St Louis, MO) density gradient centrifugation (30 minutes at 37°C and 400g).

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**Purification of BM primitive progenitors.** BMMNC were initially purified by a counterflow elutriation step. BMMNC were resuspended in phosphate-buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA) and 0.01% EDTA. The cells were injected into an elutriation system with a standard separation chamber (Beckman Instruments, Palo Alto, CA) primed with PBS-BSA-EDTA. Rotor speed and temperature were maintained at 1,950 rpm and 10°C. After loading, 200 mL of effluent was collected at a flow rate of 14 mL/min in fraction 14 (F14). The rotor was then stopped and the remaining BMMNC were flushed from the chamber and discarded. F14-BMMNC were further purified by depletion of T lymphocytes with 2-amino ethylisothiouronium bromide (AET)-treated sheep red blood cells (Kroy Medical Inc, Stillwater, MN) as previously described.1 This enriched progenitor population was labeled (250 ng/10^6 cells) with phycoerythrin (PE)-conjugated anti-CD34 antibody (HPICA-2; Becton Dickinson, Mountain View, CA) and fluorescein (FITC)-conjugated HLA-DR (250 ng/10^6 cells; Becton Dickinson). Cells were sorted on a FACS-Star laser flow cytometry system (Becton Dickinson) equipped with a consort 40 computer. Cells were first selected for low vertical and very low horizontal light scatter properties and then sorted for high-density expression of the CD34 antigen but absence of the HLA-DR antigen as previously described.11

**Purification of PB NK cell progenitors.** NK cells were enriched by an initial negative depletion followed by fluorescence-activated cell sorting (FACS). Briefly, PBMC were labeled with anti-CD3 (15 ng/10^6 PBMC; Becton Dickinson) for 30 minutes at 4°C, washed, and then incubated with goat antimouse IgG-coated immunomagnetic beads (30 beads/cell; Advanced Magnetics Inc, Cambridge, MA). The depleted population was obtained by removing rosoted cells after exposure to a strong magnetic field. In addition to T-lymphocyte depletion, monocytes were also depleted during this step by phagocytosis of the magnetic beads. This NK-enriched population was labeled with 250 ng of FITC-conjugated anti-CD3 and PE-conjugated anti-CD56 (Leu-19) per 10^6 cells. NK cells were sorted to exclude all T lymphocytes and to select cells bearing CD56 (CD56+CD3-).

**Culture of NK cells.** Fifteen thousand BM-derived CD34+/DR- or PB-derived CD56+/CD3- were plated on allogeneic irradiated stroma, as previously described,11 with modifications. Stromal layers were generated by plating BMMNC in LTBMC media consisting of Iscove's modified Dulbecco's medium (IMDM) supplemented with 12.5% fetal calf serum, 12.5% horse serum (Hyclone Laboratories, Logan, UT), 2.9 mmol/L L-glutamine, penicillin 1,000 U/mL, streptomycin 100 U/mL (GIBCO Laboratories, Grand Island, NY), and 10^-8 mol/L hydrocortisone (A-hydrocort; Abbott Laboratories, North Chicago, IL). Once confluent, stroma was irradiated with 1,000 cGy and stroma cells were recovered by sheap cell rosetting. Cells were cultured in NK media consisting of Roswell Park Medical Institute media (RPMI 1640 supplemented with 10 or 1,000 U/mL recombinant interleukin-2 (rIL-2) as indicated (a generous gift from Hoffmann-La Roche, Nutley, NJ). 10% human heat-inactivated AB serum (North American Biologicals, Miami, FL), 2 mmol/L L-glutamine, and penicillin 1,000 U/mL plus streptomycin 100 U/mL (GIBCO).

**Maintenance of LTBMC.** Cultures were maintained in a humidified atmosphere at 37°C and 5% CO₂. At day 7, the culture volume was doubled with fresh NK media. Subsequently, at weekly intervals, the cultures were fed by carefully removing half the supernatant to avoid removing nonadherent cells and replacing it with fresh NK media. At week 5, cultures were washed thoroughly and all cells were collected and analyzed for expansion, cytotoxicity, and phenotype characteristics. All cell populations were counted in a hemocytometer to determine fold expansion. Fold expansion was derived by dividing number of cells present at week 5 by the number of sorted cells inoculated on day 0.

**Phenotype.** Cell surface antigen expression was determined by direct staining of cells with mouse monoclonal antibodies. FITC- or PE-coupled antibodies (Becton Dickinson) were directed at CD2, CD3, CD5, CD7, CD8, CD14, CD15, CD16, CD19, CD25, CD33, CD54, CD56, T-cell receptor (TCR) (α/β and γ/δ), HLA-DR, and MY8 (uncoupled; Coulter Immunology, Hialeah, FL). Phenotypic analysis was performed with a FACS Star Plus flow cytometer (Becton Dickinson) equipped with a consort 40 computer. FITC- and PE-coupled isotype-matched lgs were used as controls.

**Cytotoxicity assays.** Cultured NK populations were tested for cytotoxicity against the NK-sensitive cell line K562 (American Tissue Type Collection [ATCC], Rockville, MD) and the NK-resistant cell line Raji (ATCC) in a 4-hour Cr⁵¹ release assay. Effector to target (E:T) ratios ranged from 20:1 to 0.082:1. Target cells were labeled with 200 mCi sodium chromate-Cr⁵¹ (DuPont, Wilmington, DE) for 60 to 90 minutes. All determinations were performed in triplicate, and percentage lysis was determined using the following equation:

\[
\text{Experimental Mean cpm} - \text{Spontaneous Release Mean cpm} \\
\times 100 = \% \text{Lysis}
\]

**Limiting dilution assays.** Irradiatedstromal cells were subcultured at 3.75 × 10⁴/well into 96-well plates (Costar). After 3 to 5 days, all LTBMC media was removed and CD34+/DR- cells were plated with NK media to 28 to 2,250 cells/well with 24 replicates per concentration. Media was doubled at day 7 and then half changed with fresh IL-2 media weekly until week 5. Wells were determined positive for NK cells if greater than 10% specific lysis was observed after the addition of 5,000 C⁶¹-labeled K562 targets in a 4-hour release assay as described above. Lysis was calculated as described above; 48 wells were used to determine total lysis and 48 wells for spontaneous release (spontaneous release was always <15% of total). The frequency of NK progenitors in the CD34⁺/DR⁻ population was calculated as the reciprocal of the concentration of cells that resulted in 37% negative wells using Poisson statistics24 and the weighted mean method.25

**Statistics.** Results of experimental points obtained from multiple experiments were reported as mean ± 1 standard error of the mean (SEM). Significance levels were determined by the two-sided Student's t-test analysis.

**RESULTS**

**Characteristics of BM progenitors.** A BM population enriched for primitive progenitors was obtained by sequential enrichment of BMMNC by counterflow elutriation and depletion of T-lymphocyte and NK by sheep cell rosetting. This population was evaluated by tricolor FACs analysis for antigens coexpressed with the CD34 antigen. CD34⁺ progenitors showed no evidence of lineage commitment, with less than 0.4% of the population expressing CD34 and coexpressing lymphoid-specific antigens CD2, CD5, CD7, CD8, CD16, and CD56. Primitive CD34⁺/DR⁻ progenitors were further purified for culture experiments using four-parameter FACs to obtain cells with low vertical and very low horizontal light scatter properties which expressed
high-density CD34 antigen, but not the HLA-DR antigen as previously described.\textsuperscript{11-13}

**Proliferation of cells in LTBMC.** CD34\textsuperscript{+}/DR\textsuperscript{-} primitive progenitors were plated directly onto allogeneic irradiated stroma with RPMI-based media containing 1,000 U/mL rIL-2 (NK media) for 5 weeks. No cells proliferated when irradiated stroma was maintained in NK media without progenitors, and no proliferation was observed when progenitors were plated in NK media in the absence of a stromal layer. When progenitors were plated in the absence of rIL-2 or in the presence of hydrocortisone, no proliferation was observed. Yet, when 15,000 CD34\textsuperscript{+}/DR\textsuperscript{-} cells were cultured on allogeneic irradiated stroma with rIL-2 and in the absence of hydrocortisone, significant proliferation resulted (147- ± 21-fold expansion, n = 14). In four experiments, progenitors were cultured with 10 U/mL rIL-2 and the 120- ± 60-fold expansion observed was not significantly different from progenitors cultured with 1,000 U/mL rIL-2. Wright-Giemsa-stained cytospins of week 5 cultured cells exhibited the morphology of large granular lymphocytes (Fig 1). As NK cells proliferated, progressive stromal cell damage was observed that eventually resulted in the destruction of the stromal layer. In the absence of progenitor cells, in the absence of rIL-2 or in the presence of hydrocortisone stromal layers remained intact throughout the 5-week culture period.

**Phenotype of cultured cells.** Cells recovered at week 5 from cultures initiated with CD34\textsuperscript{+}/DR\textsuperscript{-} primitive progenitors under the above described conditions were evaluated for the expression of antigens associated with NK, myeloid, and other lymphoid cells. A percentage of cells (84.8% ± 1.5%) were CD56\textsuperscript{+}/CD3\textsuperscript{-} and a majority of these were CD56\textsuperscript{+} (bright)/CD3\textsuperscript{-}. A population of cultured CD56\textsuperscript{+} cells coexpressed CD2 (7.8% ± 1.2%), CD7 (19.5% ± 2.8%) CD8 (3.1% ± 1.0%), or CD16 (4.5% ± 1.3%) (Fig 2). No cultured cells expressed CD3, CD5, TCR \(\alpha/\beta\), or TCR \(\gamma/\delta\) T-cell markers. Similarly, no cells expressed evidence of myeloid or monocytoid lineage (CD14, CD15, MY8, or CD33) or B-lymphoid lineage (CD19). The CD34 antigen highly expressed on all starting cells could no longer be identified on cells after the 5-week culture period.

**Function of cells after LTBMC.** Culture of FACS-purified CD34\textsuperscript{+}/DR\textsuperscript{-} progenitors for 5 weeks in the presence of 1,000 U/mL rIL-2 in contact with allogeneic irradiated stroma resulted in the proliferation of NK cells with significant cytotoxicity. When tested against the NK-sensitive target K562 and NK-resistant target Raji in a 4-hour chromium release assay, 79% ± 1.9% K562 and 38% ± 5.7% Raji targets were lysed at an E:T of 20:1 (Fig 3). Cells cultured for 5 weeks with 10 U/mL of rIL-2 killed K562 targets significantly less than those cultured with 1,000 U/mL (E:T 6.6:1, 51% ± 6.1% vs 79% ± 5.0%; n = 4; \(P = .013\)).

**Limiting dilution analysis.** To examine the NK progenitor frequency in the CD34\textsuperscript{+}/DR\textsuperscript{-} population, we performed experiments in which progenitors were plated in limiting dilution assays on allogeneic irradiated stroma subcultured in 96-well plates. CD34\textsuperscript{+}/DR\textsuperscript{-} progenitors were plated in NK media at concentrations of 28 to 2,250 cells/well with 24 replicates per concentration. To determine the presence of functional NK cells, a Cr\textsuperscript{51} release assay against the NK-sensitive target K562 was performed at the end of the 5-week culture period. Well positivity was defined as greater than 10% specific lysis 4 hours after the addition of 5,000 chromium-labeled K562 targets. Analysis by poisson statistics\textsuperscript{24,25} showed that the frequency of NK progenitors was 1:169 ± 50 initially plated CD34\textsuperscript{+}/DR\textsuperscript{-} cells.

**Comparison of BM CD34\textsuperscript{+}/DR\textsuperscript{-} Primitive progenitors with PB CD56\textsuperscript{+}/CD3\textsuperscript{-} NK cells.** We performed a final set of experiments to examine the possibility that a small number of mature NK cells may have contaminated our BM derived CD34\textsuperscript{+}/DR\textsuperscript{-} population and could account for the ob-

![Fig 1. CD34\textsuperscript{+}/DR\textsuperscript{-} cells obtained from normal BM by FACS were plated on allogeneic, irradiated stroma and cultured for 5 weeks with NK media containing rIL-2 and human serum. Wright-Giemsa-stained cytospin preparations exhibited the characteristic morphology of large granular lymphocytes.](image-url)
served results. A CD56+/CD3− PB NK population was obtained using FACS. CD56+/CD3− and CD34+/DR− cells obtained from the same donor were plated in stroma-dependent cultures under identical circumstances and evaluated for proliferation, cytotoxicity, and phenotype characteristics.

PB CD56+/CD3− NK (PBNK) were plated at a concentration of 1,600 to 15,000 cells/well directly onto allogeneic irradiated stroma in NK media and maintained under identical conditions as those described for BM primitive progenitors. The absolute number of cells obtained after 5 weeks from 15,000 PBNK was significantly less than equivalent cultures initiated with 15,000 CD34+/DR− cells (0.63 ± 0.1 × 10^6 vs 1.96 ± 0.4 × 10^6; n = 8; P = .019). Even if significant contamination were present, the yield from 5,000 initially plated PBNK (0.43 ± 0.1 × 10^6 cells) or 1,600 initially plated PBNK (0.24 ± 0.06 × 10^6 cells) could not account for the cell yield obtained from the CD34+/DR− population. The phenotype of cells derived from CD34+/DR− cells differed from cultured populations derived from PBNK (Table 1). CD56+ cultured cells derived from the CD34+/DR− population coexpressed less CD2, CD7, and CD8 than CD56+ cells obtained from cultures initiated with PBNK. Finally, cells obtained from cultures initiated with
CD34+/DR− cells killed K562 and Raji targets less than cells derived from cultures initiated with PBNK (Fig 4).

**DISCUSSION**

We show that CD34+/DR− cells known to contain primitive human hematopoietic progenitors can differentiate into a cell population with morphologic, phenotypic, and functional characteristics of NK in a modified LT-BMC system. Although the starting CD34+/DR− population does not express antigens associated with lymphoid-lineage commitment, the majority of cultured cells express the CD56+/CD3− phenotype associated with NK1−3 and some cells coexpress CD2 and CD7, further suggesting lymphoid lineage.2,4,5 Cytotoxicity against NK-sensitive and NK-resistant tumor targets serves as further evidence that cells derived from the CD34+/DR− population are NK cells.4,6 Although previous investigators have shown the origin of myeloid, B-lymphoid, and T-lymphoid cells from populations similar to the CD34+/DR− population,13,15 the data presented here show that human NK can originate from this same BM population.

We performed several experiments to exclude the possibility that small numbers of PB NK contaminated the CD34+/DR− fraction and proliferated in modified LT-BMC. Culture of PB NK under the same conditions used to cultivate NK from CD34+/DR− cells did result in the proliferation of a CD56+/CD3− population. However, this NK cell population exhibited quantitative differences in the coexpression of lymphoid antigens and cytotoxic capacity when compared with NK obtained from CD34+/DR− populations. Diminished proliferative capacity of PB NK also suggested that contamination with this population could not account for the marked expansion of CD34+/DR− derived NK in LT-BMC.

Differentiation of NK from the CD34+/DR− population requires BM stroma in our culture system. Several other studies support an important function for stroma in the progression of progenitor to committed lymphoid or myeloid lineages. Van der Brink et al20 have shown that pure populations of rat NK can be generated from BM cells depleted in vitro or in vivo of mature NK only when stroma was present in the culture system. Cumano et al27 have cultivated both B-lymphoid cells and macrophages from single murine primitive progenitor cells when plated on stroma. Billips et al28 have shown that while IL-7 and kit ligand can stimulate murine pre-B lymphocytes to proliferate, stroma must be present in the culture system to induce progeny from the pro-B− to the pre-B−lymphocyte stage. These data, taken together with the numerous reports implicating BM stroma in the differentiation of myeloid progenitors,11−14,29 suggest an important role for stroma in the initiation and support of both myeloid and lymphoid hematopoiesis.

This study provides evidence that cells within the human CD34+/DR− population that do not express lineage-specific antigens can differentiate into NK cells and implicates human BM marrow stroma in this process. Further studies using the modified LT-BMC system described here will be used to explore the ontogeny of human NK and other lymphoid lineages.

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