PRODUCTION OF HEMATOPOIETIC COLONY-STIMULATING FACTORS BY HUMAN NATURAL KILLER CELLS

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A role for NK cells in hematopoietic homeostasis was originally suggested by Cudkowicz and co-workers (1, 2), who observed that NK cells are the major effector cells of hybrid resistance to parental bone marrow transplants in irradiated mice. NK cells differentiate in the bone marrow (3), from where they migrate at an early stage of differentiation so that mature NK cells are rare in normal bone marrow (4). However, during experimental virus infection in mice, activated NK cells that suppress hematopoiesis can be demonstrated (5). NK cell depletion in vivo in mice using mAbs results in increased numbers of myeloid precursor cells in the spleen, suggesting that in physiological conditions NK cells serve as regulators of extramedullary myelopoiesis (6). Several lines of evidence in human and experimental pathology suggest a role for NK cells in suppression of hematopoiesis (for a review see reference 7).

Human NK cells have been shown to suppress the growth of hematopoietic colonies in vitro (8–10). All or most of this suppressor activity is mediated through release of a soluble factor that we identified as TNF (9, 11). TNF is produced by NK cells upon contact with their target cells or with precursor cell–enriched bone marrow cell preparations (11); it cooperates with IFN-γ, another product of NK and T cells (12), in suppressing hematopoietic colony formation (11, 13). However, in some cases, NK cells stimulate colony formation by releasing CSF (14–17); thus, the effect of NK cells on hematopoiesis, like that of T cells, may be complex.

Production of a large number of factors has been attributed to NK cells. NK cells have been shown to produce burst-promoting activity (BPA)[1] (14, 15, 18). Several factors mediate such activity, and the possibility that NK cells produce IL-3 and granulocyte/macrophage CSF (GM-CSF), the two lymphokines with known BPA activity, has not been tested directly. Several types of CSF activity that sustain the growth of GM colonies (16), enhance GM colony formation from human peripheral blood (17), or induce growth of megakaryocytes (19) have been detected in supernatants from mitogen-stimulated large granular lymphocytes (LGL) or from cell preparations enriched for NK cells. Large quantities of IL-1 (hemopoietin-1), a cytokine

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[1] *Abbreviations used in this paper:* BPS, burst promoting activity; CML, chronic myeloid leukemia; G-CSF, granulocyte CSF; GM-CSF, granulocyte/macrophage CSF, LGL, large granular lymphocytes; PDBu, phorbol dibutyrate.

synergizing with various CSF to induce growth of early precursor cells of several lineages, have also been suggested to be produced by NK cells (20, 21). However, in many of these reports, the factor mediating the activity produced was not identified and/or NK cells were not positively demonstrated to be the activity-producer cells, and no CSF activity has been detected by Hermann et al. (10) in supernatant fluids from NK cell clones.

We recently developed a method by which very large numbers of NK cells, >99% pure, can be obtained from cocultures of PBMC with irradiated lymphoblastoid cells (22). Using these cell preparations, we demonstrated that NK cells can be induced to transcribe IFN-γ and TNF genes upon specific stimulation with rIL-2 in combination with ligands for the NK cell receptor for IgG-Fc (FcR), CD16 antigen, or upon nonspecific stimulation with phorbol-diester and calcium ionophore (23). These advances in the methodologies to purify and study NK cells and the availability of cDNA probes for all the major CSF types allowed us to analyze in detail CSF production by NK cells.

In this paper, we demonstrate that activated NK cells produce large amounts of GM-CSF; IL-3 and CSF-1 transcripts were demonstrable in NK cells under certain conditions of stimulation. No evidence of G-CSF, IL-1α, or IL-1β transcripts was found in any NK cell preparation, independent of the stimulus used.

Materials and Methods

Cell Lines. The human B-lymphoblastoid RPMI 8866 (22) and bladder carcinoma 5637 (21, 24) cell lines, and the murine mAb-producing hybrid cell clones were maintained in culture in RPMI 1640 (Flow Laboratories, Rockville, MD) supplemented with 10% FCS (Flow Laboratories). All cell lines were free of mycoplasma contamination on repeated testing.

Monoclonal and Polyclonal Antibodies. mAbs B73.1 (IgG1, anti-CD16) (4), B36.1 (IgG2b, anti-CD5) (25), B52.1 (IgM, anti-CD14 on monocytes), and B13.4 (IgM, anti-monocyte granulocyte) (25, 26) were produced and characterized in our laboratory. 3G8 (IgG1, anti-CD16) (27) was produced from cells kindly provided by Dr. J. Unkeless (Mount Sinai Medical School, New York, NY); N901 (IgG1, anti-NKH-1 antigen) (28) was kindly provided by Dr. J. Griffin (Dana Farber Cancer Institute, Boston, MA). OKT3 (IgG2a, anti-CD3) was produced from cells obtained from the American Type Culture Collection (Rockville, MD). The anti-IFN-γ B133.1 and B133.5 and the anti-TNF B154.7 and B154.9 antibodies used for the IFN-γ and TNF RIA were produced and characterized in our laboratory (12, 29). Antibody B133.3 (IgG1) was used to inactivate the biological activity of IFN-γ (1 ml of ascites fluid neutralizes $>10^7$ U of rIFN-γ). mAbs to GM-CSF were obtained from Olympus Co. (Media, PA) and, after labeling with biotin, were used for immunoprecipitation. A polyclonal sheep antiserum to human rGM-CSF (30) (neutralizing titer >6,000 U/ml) and a rabbit polyclonal antiserum to rIL-3 (31) were used in inactivation experiments. When indicated, purified antibodies were labeled with biotin or linked to CNBr-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to routine procedures. The polyclonal FITC-goat $F(ab')_2$ anti-mouse Ig and the rabbit IgG anti-bovine erythrocytes (E) were purchased from Cooperbiomedical (Malvern, PA). The goat $F(ab')_2$ anti-mouse IgG used to prepare the E for indirect rosetting was produced in our laboratory, absorbed on human IgG and affinity-purified on a mouse IgG-Sepharose 4B column (Pharmacia Fine Chemicals).

Peripheral Blood Lymphocytes, NK Cells and T Cells. Peripheral blood was obtained by venipuncture from adult healthy donors using heparin as anticoagulant. PBMC were separated on Ficoll-Hypaque density gradient (Lymphoprep, Nyegaard and Co., Oslo, Norway). PBL were prepared from PBMC depleted of monocytes after two cycles of adherence on plastic surfaces (45 min, 37°C) and depletion of B52.1/B13.4$^+$ monocytes and mature myeloid cells by indirect antiglobulin rosetting (4). Fresh NK and T cells were prepared by negative selection using the indirect rosetting method as described (32). To obtain large numbers of NK cells,

short-term bulk cultures of these cells were prepared as previously described in detail (22, 33). Briefly, PBMC were cultured at 37°C with 50-Gy irradiated RPMI 8866 lymphoblastoid cells for 10 d (5:1 PBMC/B lymphoblastoid cells, 2.5×10^5 PBMC/ml RPMI/10% FCS) in tissue culture plates in a 5% CO_2 humidified atmosphere. The total number of cells recovered after a 10-d culture is increased, on average, 10-fold, and the cell population comprises 60–80% $CD16^+/NKH\text{-}1^+/CD3^-$ NK and 20–40% $CD3^+/CD16^-/NKH\text{-}1^-$ T cells. Homogeneous populations of NK and T cells were negatively selected and purified by density gradient centrifugation after sensitization of the lymphocytes with a mixture of antimonocyte B52.1 and B13.4, and either anti-CD3 and anti-CD5, or anti-CD16 and anti-NKH-1 antibodies, respectively, and indirect rosetting with $CrCl_3$-treated goat anti-mouse Ig-coated E (4). Both the NK and the T cells purified from these cultures have morphologic, phenotypic, genotypic, and functional properties identical to those of the two subsets freshly obtained from PBL (22, 33, and data not shown). However, unlike fresh cells, the majority of these cells express HLA-DR antigens. Monocytes and B cells were not detected in the cultures on day 10 (22). The purity of the various lymphocyte preparations (on average >98%) was always determined by indirect immunofluorescence (flow cytofluorometry) performed as described (4) using a panel of mAbs.

Lymphocyte Stimulation. The purified fresh PBL, and the NK and T cell subpopulations obtained from the bulk cultures, were cultured (37°C, 5×10^6 cells/ml RPMI/10% FCS) for the indicated periods of time with different inducers. FcR(CD16) ligands were anti-CD16 antibody (3G8) linked to CNBr-Sepharose 4B (5 mg antibody/ml Sepharose, 5μl beads/10^6 cells) and particulate immune complexes, i.e., IgG-sensitized bovine E (EA7S) prepared as previously described (34). Sepharose-linked irrelevant antibodies (anti-CD3 OKT3, anti-HLA class I W6.32) and E were used as controls. When indicated, purified human rIL-2 was present (100 U/ml) (rIL-2, 5×10^6 U/mg in a standard CTLL assay; Takeda Chemical Industry, Inc., Osaka, kindly provided by Dr. T. Taguchi, Osaka University, Osaka Japan). Alternatively, lymphocytes were stimulated with phorbol dibutyrate (PDBu; Peter Borchert, Eden Prairie, MN), 2.5×10^{-7} M, and the Ca^{2+} ionophore A23187 (0.2 μg/ml; Sigma Chemical Co., St. Louis, MO). After culture, the cell-free supernatant fluids and/or cells were collected, Sepharose beads were detached from the cells by vigorous vortexing, and they were eliminated from the cell suspension after a 1-min centrifugation at 500 rpm. Particulate immune complexes were removed by lysis with hypotonic medium. Ligand-free cells were washed and used for different assays. Cell viability was >95% in all conditions and recovery was ~80% of the original input without loss of lymphocyte subsets.

IFN-γ and TNF RIA. These were performed as previously described in detail (29, 35) on cell-free supernatants obtained from the cells induced as above. Purified human natural IFN-γ (Interferon Sciences, New Brunswick, NJ) and purified human rTNF (5×10^7 U/mg on L929 cells, kindly provided by Dr. H. M. Shepard, Genentech Inc., South San Francisco, CA) were used in each assay to construct the standard curve. Sensitivity of the RIA for IFN-γ and for TNF was 0.2 U/ml and 0.1–0.2 U/ml, respectively.

Northern Blot Hybridization. Northern blots were performed as previously described in detail (29). Briefly, total cytoplasmic RNAs were extracted from uninduced and induced cells. Equal amounts of RNA (20 μg/lane, as quantified by spectrophotometric reading at 260 and 280 nm) were size fractionated in a 1% agarose-formaldehyde gel. When indicated, RNA was poly(A) selected and the amount of poly(A)$^+$ RNA derived from 200 μg of total RNA was loaded per lane. Ethidium bromide–stained gels were visualized to assess integrity of RNA and to verify that equal amounts of total RNA were loaded in each lane. RNA was transferred by capillarity to nitrocellulose paper. The cDNA probes used for mRNA detection, all isolated at the Genetics Institute, were: human GM-CSF probe pCSF-1 (36), human CSF-1 probe p3A CSF-69 (37); human IFN-γ probe pSWIF; human G-CSF probe; gibbon IL-3 probe pCSF-MLA, hybridizing with human IL-3 mRNA (30); human IL-1α and IL-1β probes. The human TNF cDNA probe was kindly provided by Dr. H. M. Shepard (Genentech Co.) The phosphoglycerate kinase (PGK) cDNA probe (38), detecting an mRNA constitutively expressed in lymphocytes, was obtained through the American Type Tissue Collection and used as control. Probes were excised from their vectors, purified by gel electrophoresis, labeled with hexanucleotide primers and [^{32}P]dCTP, and used for hybridization. After hybridization and washing, the filters were autoradiographed using XAR film

(Eastman Kodak Co., Rochester, NY) and an intensifying screen (DuPont Co., Wilmington, DE).

GM-CSF Immune Precipitation. Purified NK cells were cultured (16 h, 37°C) in methionine-free RPMI 1640 medium (Flow Laboratories) supplemented with 5% dialyzed FCS and [^{35}S]methionine (100 μCi/ml, 5 × 10^6 cells/ml; sp act 1,245 Ci/mMol, Amersham International, Arlington Heights, IL) with or without added PDBu (2.5 × 10^{-7}M) and A23187 (0.2 μg/ml). The cell-free supernatant was collected and precleared with streptavidin-Agarose beads (Bethesda Research Life Technologies, Inc., Gaithersburg, MD), 25 μl/ml supernatant, 2 h at 4°C). 2-ml aliquots of supernatants were then incubated with anti-GM-CSF mAb or an irrelevant isotype-matched antibody used as control (15 μg). Both antibodies were biotin labeled according to standard procedures. After a 2-h incubation at 4°C, 15 μl of streptavidin-agarose beads were added, and the incubation was continued for 2 h. The beads were washed six times with 0.15 M NaCl containing 4 mM EDTA, 0.2 mM PMSF, 0.05% NaN_3, 10 mM Hepes, 0.1 Tween-20, resuspended in acrylamide sample buffer with added DTT (4 mM; Sigma Chemical Co.) as a reducing agent, and boiled for 10 min. The samples and the ^{14}C-labeled molecular weight standards (Amersham International) were electrophoresed on a 12.5% polyacrylamide-SDS gel (SDS-PAGE). After drying, the gels were exposed to a Kodak XAR film with intensifying screen for 5 d.

Cell Lines and Chronic Myeloid Leukemia (CML) Blast Proliferation. The human T cell leukemia line TALL-101 was kindly provided by Dr. G. Rovera (Wistar Institute). TALL-101 requires GM-CSF for growth and was maintained in medium supplemented with 5 ng/ml rGM-CSF. rCSF, in pure form or in the supernatant fluid of transfected COS cells, were produced at the Genetics Institute. TALL-101 growth is not supported by G-CSF and only minimally by IL-3, but both lymphokines synergize in inducing TALL-101 growth (39). Proliferation was determined in short-term cultures measuring [^{3}H]TdR incorporation in TALL-101 cells seeded at 10^4/well in 96-well round-bottomed microtiter plates (Falcon Labware, Oxnard, CA) in the presence of the indicated concentrations of recombinant cytokines or conditioned media from NK and T cells. After a 5-d incubation at 37°C, 2 μCi of [^{3}H]TdR (Amersham International) was added to each well and the cells were harvested 18 h later onto fiberglass filters using an automated harvester (Skatron, Inc., Sterling, VA). Isotope incorporation was measured with a liquid scintillation counter. All data are presented as mean cpm from triplicate cultures.

The CML assay was performed according to previously described procedures (40). Briefly, frozen primary CML blast cells were thawed and cultured overnight. Viable mononuclear cells were purified by centrifugation through a Ficoll-Hypaque gradient and seeded at 2 × 10^4/well in microtiter wells in the presence of the indicated concentrations of recombinant cytokines or conditioned media. Proliferation was measured by [^{3}H]TdR incorporation after a 72-h culture, as described above for TALL-101 cells. The assay is sensitive to GM-CSF, IL-3, and IFN-γ (30, 31, 40).

Results

Accumulation of mRNA for CSF in Human NK Cells. Total cytoplasmic RNA was extracted from NK cells purified from the 10-d cultures of PBMC with irradiated B-lymphoblastoid cell lines and incubated for 2 h in medium alone or supplemented with 100 U/ml rIL-2, CD16 FcR ligands (Sepharose-linked 3G8 antibody or EA7S), rIL-2 and CD16 ligands, or PDBu (2.5 × 10^{-7}M) and A23187 (0.2 μg/ml). Northern blots were hybridized with cDNA probes for the various cytokines. NK cells cultured in medium did not constitutively express transcripts for any of the cytokines tested. Accumulation of mRNA for GM-CSF (Fig. 1) was detected in NK cells stimulated with IL-2 and 3G8-Sepharose. The two stimuli together acted synergistically in inducing mRNA accumulation but significantly greater accumulation was observed upon stimulation with PDBu and A23187 (Fig. 1). Stimulation with immune complexes (EA7S) gave results equivalent to those obtained with 3G8-Sepharose (not

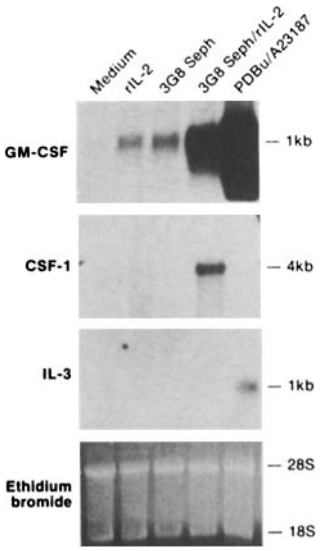

FIGURE 1. Accumulation of GM-CSF, CSF-1, and IL-3 mRNA in human NK cells stimulated with 100 U/ml rIL-2, 3G8-Sepharose, rIL-2 and 3G8-Sepharose, or 2.5×10^{-7} M PDBu and 0.2 μg/ml A23187. Total cytoplasmic RNA was extracted from NK cells (CD3/CD5$^-$ cells from 10-d cultures of PBMC with irradiated RPMI 8866 cells and incubated [2 h, 37°C] in the presence of various inducers). After electrophoresis and transfer to nitrocellulose, RNA was hybridized to the indicated cDNA probes. Amount and integrity of RNA was checked by ethidium bromide staining. This experiment is representative of at least four experiments performed for each cytokine, with identical results.

shown). Accumulation of mRNA for CSF-1 was observed in NK cells stimulated with rIL-2 and 3G8-Sepharose, but not in NK cells stimulated with either stimulus alone or in those stimulated with PDBu and A23187 (Fig. 1). On the contrary, IL-3 transcripts were detected in NK cells only when they were stimulated with PDBu and A23187. Transcripts for G-CSF, IL-1α, or IL-1β were never detected in any NK cell preparations under the conditions tested.

To confirm that the GM-CSF transcripts detected were from NK cells and not from possible contaminant T cells, mRNA was isolated from NK and T cells purified from 10-d PBMC cultures and stimulated with CD16 ligands (3G8-Sepharose) as an NK-specific stimulus, and with CD3 ligands (OKT3-Sepharose) as a T-specific stimulus (Fig. 2). rIL-2 induced accumulation of GM-CSF transcripts in both NK and T cells; 3G8-Sepharose induced accumulation of GM-CSF transcripts by itself or in synergy with rIL-2, only in NK cells, and OKT3-Sepharose only in T cells. Stimulation with W6.32-Sepharose was ineffective both on NK and T cells (not shown). Upon comparable stimulation, the accumulation of GM-CSF mRNA observed in NK cells was always higher than that observed in the T cells from the same donors.

To confirm that NK cells, under the stimulation conditions used, do not transcribe G-CSF, IL-1α, and IL-1β genes, poly(A)$^+$ RNA was isolated from cultured NK cells either unstimulated or stimulated with 3G8-Sepharose/rIL-2 or with PDBu/A23187, and was analyzed by Northern blotting. Total cytoplasmic RNA from the 5637 cell line (21, 24) was used as positive control. As expected, stimulation with 3G8 Sepharose/rIL-2 induced accumulation of GM-CSF transcripts in NK cells and PDBu/A23187 induced it both in NK and in T cells (Fig. 3). GM-CSF transcripts are present in low abundance in 5637 cells (24) and were detectable after a longer exposure time than that used in Fig. 3 (not shown). IL-3 transcripts were detectable in NK and T cells when stimulated with PDBu/A23187. G-CSF, IL-1α, and IL-1β transcripts were not detected in either NK or T cell poly(A)$^+$ RNA. The efficiency

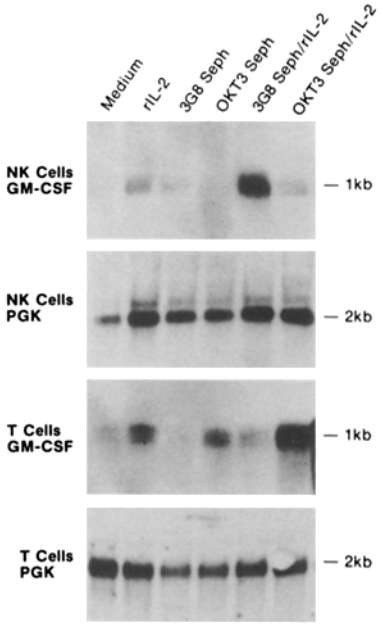

FIGURE 2. Effect of CD16 ligands and CD3 ligands, in synergy with rIL-2, on GM-CSF mRNA accumulation in NK and T cells. Total cytoplasmic RNA was extracted from NK and T cells, purified as described in Materials and Methods, and stimulated with 100 U/ml rIL-2, 3G8-Sepharose, 3G8-Sepharose and rIL-2, OKT3-Sepharose, or OKT3-Sepharose and rIL-2. After electrophoresis and transfer to nitrocellulose, RNA was sequentially hybridized to the GM-CSF cDNA probe and as a control for amount and integrity of RNA, to the PGK cDNA probe, after stripping the first probe.

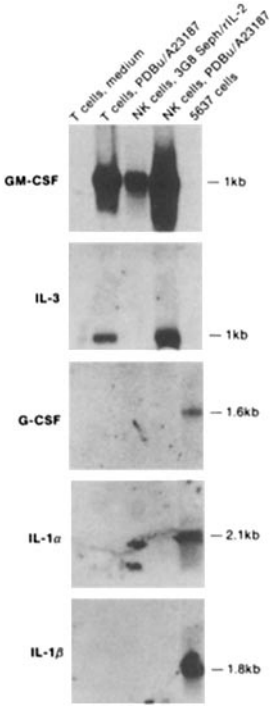

FIGURE 3. Northern blot analysis of the presence of cytokine gene transcripts in poly(A)$^+$ RNA from NK and T cells. Total cytoplasmic RNA was isolated from cultured T cells stimulated for 2 h with PDBu/A23187 or from cultured NK cells stimulated for 2 h with 3G8-Sepharose/rIL-2 or PDBu/A23187. Poly(A)$^+$ RNA, purified from 200 μg of total RNA, or 20 μg total cytoplasmic RNA from the cell line 5637, were loaded in each lane as indicated.

of the hybridization of these probes was demonstrated by the positive signal obtained with total RNA from 5637 cells (Fig. 3).

Kinetics of Cytokine Transcript Accumulation in Fresh and Cultured NK Cells. Total cytoplasmic RNA was isolated from cultured NK cells stimulated for different times with 3G8-Sepharose/rIL-2. Accumulation of IFN-γ, TNF, GM-CSF, and CSF-1 transcripts was analyzed by Northern blot (Fig. 4 *A*). As previously reported (23), IFN-γ and TNF mRNA accumulation peaked at 1 h after stimulation. The peaks of both GM-CSF and CSF-1 accumulation were at 2–4 h. Kinetics of expression of TNF and GM-CSF genes were different in fresh (Fig. 4 *B*) compared with cultured NK cells. Because of the difficulty in obtaining large numbers of fresh NK cells, only RNA from cells stimulated for 2 or 18 h with 3G8-Sepharose/rIL-2 or for 2 h with PDBu/A23187 was analyzed. TNF mRNA accumulation in fresh NK cells at 2 h was higher than at 18 h, whereas the reverse was observed for GM-CSF mRNA accumulation.

Immunoprecipitation of GM-CSF from NK Cell Supernatant Fluid. Purified (10-d culture) NK cells from two donors were cultured for 18 h in the presence of [^{35}S]methionine in culture medium with or without PDBu/A23187. Supernatant fluids were collected and precipitated with biotin-labeled mAbs to human GM-CSF and streptavidin-agarose. The precipitate was analyzed by SDS-PAGE (Fig. 5). No specific precipitates were obtained from the supernatant fluids of unstimulated NK cells, whereas a major protein of 23×10^3 apparent molecular weight, corresponding to that expected of human GM-CSF, was precipitated from the supernatant fluids of both NK cell preparations stimulated with PDBu/A23187.

Biological Activity of GM-CSF in NK Cell Supernatants. The biological activity of GM-CSF in 18-h cell-free supernatant fluids of purified (10-d culture) NK cells cultured in medium, rIL-2, EA7S, or EA7S and rIL-2, was measured by its ability to support proliferation of the TALL-101 cell line. Fig. 6 shows results of experiments performed on supernatant fluids from NK cells of two different donors (out of six analyzed). Supernatant fluids from unstimulated NK cells did not support TALL-101 cell proliferation. Supernatant fluids from NK cells stimulated with ei-

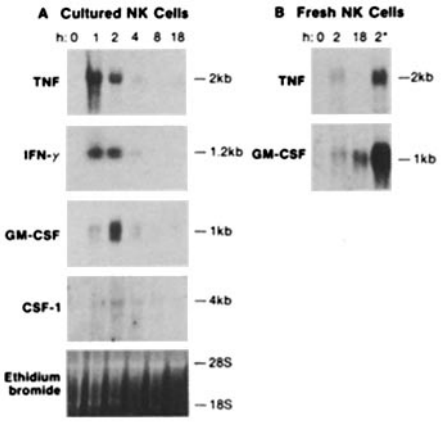

FIGURE 4. Kinetics of cytokine mRNA accumulation in cultured (*A*) and fresh (*B*) NK cells upon stimulation with 3G8-Sepharose/rIL-2. Total cytoplasmic RNA was isolated from cultured or fresh NK cells after the indicated time of stimulation with 3G8-Sepharose/rIL-2 or after 2-h stimulation of fresh NK cells with PDBu/A23187 (right lane in *B*, indicated with 2*). Northern blots were performed as described. The experiments shown are representative of five different experiments with cultured NK cells and three with fresh NK cells.

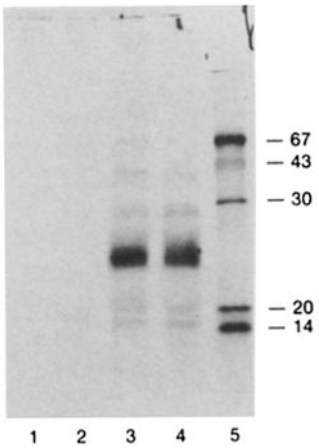

FIGURE 5. Immunoprecipitation of GM-CSF from NK cell supernatant fluid. Purified NK cells (10-d cultured cells) from two donors were cultured for 18 h in the presence of [^{35}S]methonine in medium (lanes *1* and *2*) or in medium containing 2.5 × 10^{-7} M PDBu and 0.2 μg/ml A23187 (lanes *3* and *4*). GM-CSF was precipitated from the supernatant fluids using biotin-labeled mAbs to human GM-CSF and streptavidin-agarose. The precipitates were analyzed by 12.5% SDS-PAGE. Lane *5*: mol wt standard (× 10^{-3}).

ther EA7S or rIL-2 induced low levels of proliferation only when used at high concentrations, whereas supernatant fluids from NK cells stimulated with both EA7S and rIL-2 supported TALL-101 proliferation even when used at concentrations $<10^{-2}$. Addition of a 1:200 dilution of a polyclonal sheep anti-GM-CSF serum completely abolished proliferation induced by any supernatant fluid (Fig. 6 and data not shown), whereas anti-IL-3 antiserum (1:200) was ineffective (not shown). On the basis of titration curves with rGM-CSF (not shown), the proliferation-inducing activity contained in the supernatant fluid from NK cells stimulated with 3G8-Sepharose/rIL-2 was equivalent to that of 5–10 ng/ml rGM-CSF. As shown in Table I, the NK cell supernatant fluids also contained TNF and IFN-γ. However, mAbs neutralizing TNF or IFN-γ neither inhibited nor facilitated proliferation of TALL-101 cells induced by the supernatants (not shown).

The supernatant fluids derived from the same two donors used for the above experiments were also analyzed for CSF-1 biological activity by testing their ability

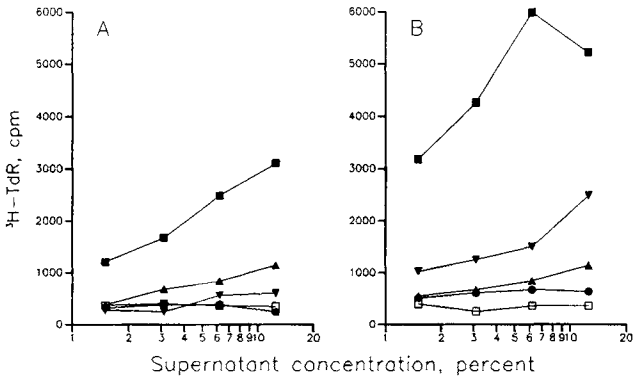

FIGURE 6. Ability of supernatant fluids from stimulated NK cells (*A* and *B*, two donors) to induce proliferation of the GM-CSF-dependent TALL-101 cell line. Supernatant fluids were added to the culture at the indicated concentrations and the assay was performed as described in Materials and Methods. Supernatant fluids were collected from NK cells cultured for 18 h in the presence of: ●, medium; ▼, rIL-2 100 U/ml; ▲, EA7S; ■, rIL-2 and EA7S; □, rIL-2 and EA7S, tested in the presence of sheep anti-GM-CSF (1:200).

TABLE I
IFN-γ and TNF in Supernatant Fluids from Stimulated NK Cells

Stimulus*	Donor A‡		Donor B	
	IFN-γ	TNF	IFN-γ	TNF
		U/ml		
Medium	<0.2	<0.1	1.3	0.1
EA7S	10.9	1.0	8.1	0.4
rIL-2	1.8	<0.1	27.6	0.2
EA7S + rIL-2	153.7	42.7	220.4	36.3

* NK cells (from 10-d cocultures of PBMC and RPMI 8866 cells) were cultured (5×10^6/ml, 18 h, 37°C) in the presence of the indicated stimuli; the cell-free supernatant fluids were collected and IFN-γ and TNF measured by RIA.
‡ Supernatant fluids from NK cells of donor A and B are those shown in Fig. 6 *A*, and in Figs. 6 *B* and 7, respectively.

to induce monocyte colonies from mouse bone marrow. Low but significant activity was demonstrated in the supernatant fluids from NK cells stimulated with IL-2 and EA7S in combination (data not shown).

The ability of the NK cell supernatant fluids to support CML blast proliferation, an assay which is sensitive to GM-CSF, IL-3, and IFN-γ, was also tested. The ability of the supernatant fluids to induce proliferation in this assay paralleled that observed with the TALL-101 cells. The supernatant fluids used in the CML blast assay (Fig. 7 *A*) were the same used in the TALL-101 assay reported in Fig. 6 *B*. Maximal proliferative activity (detectable at dilutions of the supernatants $<10^{-2}$) was present in the supernatant fluids from NK cells stimulated with 3G8-Sepharose/rIL-2. CML blast proliferation induced by these supernatant fluids was partially inhibited by a poly-

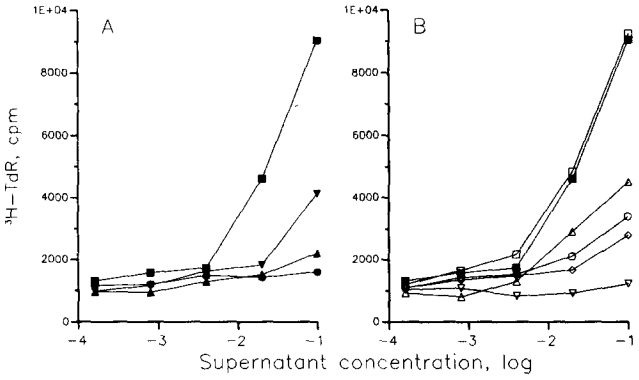

FIGURE 7. Ability of supernatant fluids from stimulated NK cells to induce proliferation of CML blasts (*A*) and inhibition by antibodies to cytokines (*B*). Supernatant fluids from NK cells of donor B (in Table I and Fig. 6 *B*), obtained as described in Table I, were added to the assay at the indicated concentrations. (*A*) Supernatant fluids were from NK cells stimulated with: ●, medium; ▼, rIL-2 100 U/ml; ▲, EA7S; ■, rIL-2 and EA7S. (*B*) Supernatant fluids from NK cells stimulated with rIL-2 and EA7S were tested in the presence of: ■, medium; □, rabbit anti-IL-3 (1:100); △, anti-IFN-γ mAb B133.3 (ascites 1:100); ○, sheep anti-GM-CSF (1:200); ◇, anti-IL-3 and anti-GM-CSF; ▽, anti-GM-CSF and anti-IFN-γ.

clonal sheep antiserum to GM-CSF and by an mAb to IFN-γ, and completely abrogated using the two antibodies together (Fig. 7 *B*). Rabbit anti-IL-3 did not significantly affect proliferation when used either alone or in combination with anti-GM-CSF (Fig. 7 *B*) or anti-IFN-γ antibodies (not shown). Identical results were obtained when the neutralizing antibodies were added to the supernatant fluids from NK cells stimulated with either EA7S or rIL-2 (not shown). Each of the three antibody preparations, at the dilutions used in this assay, completely and specifically suppressed CML blast proliferation induced with quantities of the homologous cytokines resulting in proliferation greater than that observed using the supernatant fluids (not shown).

Supernatants from NK cells stimulated for 18 h with PDBu and A23187 were extensively dialyzed and tested in the CML blast proliferation assay. They induced proliferation that was largely inhibited by anti-GM-CSF antibodies (Table II). The remaining activity, unlike that observed with the supernatants from NK cells stimulated with EA and rIL-2, was significantly inhibited by anti-IL-3 antibodies (Table II).

Discussion

We have analyzed the ability of purified homogeneous preparations of human NK cells to produce CSF using optimal stimulation protocols. Our data clearly show that, upon activation, NK cells produce GM-CSF, with similar or higher efficiency than T cells. We have shown that GM-CSF mRNA is accumulated in appropriately stimulated NK cells, and documented the presence of GM-CSF in the supernatant fluids by precipitation of the protein and detection of its biological activity. The possibility that contaminant T cells, and not NK cells, were the producers of GM-CSF in our purified NK cell preparations was excluded by the observation that CD16 ligands, which stimulate GM-CSF production in NK cells, are inactive in T cells, and that CD3 ligands, potent stimulators of T cells, are inactive in our NK cell preparations. GM-CSF could then be the molecule responsible, at least in part, for the BPA activity and for the activity of a factor(s) enhancing myeloid precursor colony formation reportedly detected in NK cell supernatants (14, 15).

Transcripts of the IL-3 gene were detectable in NK cells, though at lower abundance than those of GM-CSF, upon stimulation with phorbol diester and calcium

TABLE II

Ability of Supernatant Fluids from NK Cells Stimulated with PDBu and A23187 to Induce Proliferation of CML Blasts

Antibodies	[^{3}H]TdR uptake with supernatant fluids:*		
	None	NK cells, donor 1	NK cells, donor 2
		cpm	
None	614	5,515	8,461
Anti-GM-CSF	595	2,772	4,174
Anti-GM-CSF + anti-IL-3	584	983	2,123

* Supernatant fluids from NK cells (donors 1 and 2) stimulated for 18 h with PDBu and A23187 were extensively dialyzed against PBS and RPMI 1640 medium and assayed in the CML blast proliferation assay at a 1:10 final dilution in the presence or absence of the indicated antibodies. Sheep anti-GM-CSF was used at 1:200 final dilution, rabbit anti-IL-3 at 1:100.

ionophore. Whereas CD16 ligands/rIL-2 might also induce IL-3 gene expression but to undetectable levels, this possibility seems unlikely because we detected no IL-3 transcripts in CD16 ligands/rIL-2-stimulated NK cells even using poly(A)$^+$ RNA. Alternatively or concomitantly, accessory cells, absent in our NK cell preparations, may be required for IL-3 production by NK cells, and such a requirement might be bypassed using phorbol ester and calcium ionophore for stimulation. Antibody inhibition studies in the CML blast proliferation assay show that NK cells stimulated by PDBu/A23187 but not by CD16 ligands/rIL-2 produce biologically active IL-3. A quantitative measurement of IL-3 in the supernatant fluids is presently difficult because of the presence, in the same supernatant fluids, of various lymphokines active in the CML blast proliferation assay and their possible synergistic or antagonistic interaction. Because IL-3 transcripts are present at higher abundance in NK cells than in comparably stimulated T cells, NK cells might be one of the major producers of human IL-3. IL-3 produced by NK cells could account, in addition to GM-CSF, for at least some of the BPA activity detected in NK cell supernatants (14, 15, 18) and also for the megakaryocyte-CSF activity attributed to NK cells (19).

CSF-1 transcripts were detected, also at much lower abundance than those of GM-CSF, in NK cells stimulated with CD16 ligands/rIL-2 but not in NK cells stimulated with phorbol diester/rIL-2. The observation that GM-CSF, CSF-1, and IL-3 are differentially induced using distinct, though both optimal, stimulation protocols suggests that the genes encoding these cytokines are independently regulated. Low levels of CSF-1 biological activity can be demonstrated in NK cell supernatant fluids.

Transcripts for G-CSF, IL-1α, and IL-1β were not detectable in NK cells even when poly(A)$^+$ RNA from optimally stimulated NK cells was used. Although the conditions and time of stimulation we used might not have been optimal for detection of mRNA of these three cytokines, our results strongly suggest that they are not products of NK cells. By contrast, Scala et al. (41) reported that human LGL are potent producers of IL-1. However, the cell preparations used in that study, although carefully purified, were not homogeneous preparations of NK cells, and it is possible that a contaminant cell population was the source of IL-1. Alternatively, NK cells might produce molecules with IL-1 activity but that differ from IL-1α or IL-1β.

The kinetics of mRNA accumulation for GM-CSF (and CSF-1) in NK cells is slower than that for TNF and IFN-γ, suggesting that NK cells, upon stimulation, first secrete factors with an inhibitory effect on hematopoiesis and subsequently stimulatory factors. Thus, if stimulated NK cells produce suppressor factors such as TNF and, in some cases, IFN-γ, before or simultaneously with CSF, it might have been difficult in previous studies to identify CSF activity. We (35) have shown that, in order to demonstrate in vitro the presence of GM-CSF activity in supernatants from activated T cells, it is necessary to deplete them of lymphotoxin, TNF, and IFN-γ, all factors synergizing to inhibit growth of the colony types induced by GM-CSF. Depending on the type of colonies analyzed, the suppressor or stimulator factors might have dominated and this might also explain why, when BPA activity was detected, it was often observed in the absence of a GM-CSF activity. The two biological assays used here for analysis of CSF biological activity are not significantly affected by TNF and/or IFN-γ. Proliferation of TALL-101 cells is not affected by these two factors and elimination of TNF and IFN-γ from NK cell supernatant fluids does

not lead to increased proliferation of these cells (results not shown). In the case of CML blast proliferation, IFN-γ itself acts as a proliferation-inducing factor, although not as efficiently as GM-CSF or IL-3 (31).

The results presented in this paper show that NK cells have the potential to both suppress and enhance hematopoiesis through release of distinct cytokines. However, it is difficult to extrapolate these in vitro results to the in vivo situation. Although TNF and IFN-γ have direct inhibitory effects on hematopoietic precursor cells, they can induce CSF production from monocytes, fibroblasts, and endothelial cells (42, 43) and can therefore stimulate hematopoiesis indirectly.

The three major cytokines produced by NK cells (TNF, IFN-γ, and GM-CSF) can all function as extremely powerful activators of phagocytic cells i.e., neutrophils, eosinophils, and monocyte/macrophages (44–46). NK cells are important effector cells in nonadaptive resistance and, as such, play a role in the first line of defense against virus infection and tumor metastasis. The ready release of cytokines by NK cells broadens the action of these cells in nonadaptive resistance by enabling recruitment of the other relevant effector cells. Production of such cytokines by NK cells may also be important during an immune response through the interaction of the FcR(CD16) with immunocomplexed Igs and IL-2. IFN-γ and GM-CSF production by NK cells in response to immune complexes is a specific function of NK cells, not shared with other cell types. This may represent the main in vivo function of NK cells, and their effects on bone marrow hematopoiesis in physiological conditions might be only secondary. A more significant role is probably played by NK cells in the regulation of extramedullary hematopoiesis. In pathological conditions, activation, proliferation, and changes in anatomical distribution of NK cells, including their persistence or migration in the bone marrow, might bring NK cells in contact with hematopoietic precursor cells, thereby mediating suppression or regulation of hematopoiesis.

Summary

We have analyzed the ability of highly purified preparations of human NK cells to produce CSF. NK cells, purified by negative selection from 10-d cultures of PBMC incubated with irradiated B-lymphoblastoid cell lines, were stimulated with rIL-2, FcR(CD16) ligands (particulate immune complexes or anti-CD16 antibodies bound to Sepharose), a combination of CD16 ligands and rIL-2, or the phorbol diester phorbol dibutyrate (PDBu) together with the Ca^{2+} ionophore A23187. Both rIL-2 and CD16 ligands induce accumulation of GM-CSF mRNA in NK cells and the combined effect of the two stimuli is synergistic. Maximal accumulation of GM-CSF mRNA is observed after PDBu/A23187 stimulation. The participation of contaminant T cells in the observed expression of the GM-CSF gene is excluded because CD16 ligands do not stimulate T cells and CD3 ligands, powerful stimulators of T cells, are inactive on NK cells. Accumulation of CSF-1 mRNA is observed only in NK cells stimulated with both CD16 ligands and rIL-2, whereas accumulation of IL-3 mRNA is observed only in NK cells stimulated with PDBu/A23187. Transcripts of the G-CSF, IL-1α, and IL-1β genes were never detected in NK cells in these experiments. The kinetics of accumulation of GM-CSF and CSF-1 mRNA in NK cells stimulated with CD16 ligands and rIL-2 peaked at 2–4 h and was slower than that of TNF and IFN-γ mRNA, which peak at 1 h. GM-CSF was precipitated from the supernatant fluids

of NK cells stimulated with PDBu/A23187 and its biological activity was demonstrated by the ability of the supernatants to sustain proliferation of the TALL-101 cell line or CML blasts. Biological activity of IL-3 and CSF-1 was demonstrable in supernatant fluids of NK cells stimulated with PDBu/A23187 and CD16 ligands/rIL-2, respectively.

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