

# IDENTIFICATION AND PURIFICATION OF NATURAL KILLER CELL STIMULATORY FACTOR (NKSF), A CYTOKINE WITH MULTIPLE BIOLOGIC EFFECTS ON HUMAN LYMPHOCYTES

By MICHIKO KOBAYASHI,<sup>†‡</sup> LORI FITZ,<sup>\*</sup> MARY RYAN,<sup>\*</sup>  
RODNEY M. HEWICK,<sup>\*</sup> STEVEN C. CLARK,<sup>\*</sup> SUSAN CHAN,<sup>‡</sup>  
ROBERT LOUDON,<sup>‡</sup> FREDERICK SHERMAN,<sup>‡</sup>  
BICE PERUSSIA,<sup>‡</sup> AND GIORGIO TRINCHIERI<sup>‡</sup>

*From the <sup>\*</sup>Genetics Institute, Inc., Cambridge, Massachusetts 02140; and  
<sup>†</sup>The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104*

Human B lymphoblastoid cell lines facilitate the growth in vitro of human NK cells and of T cell clones (1-4), and together with a source of IL-2, have been successfully used to maintain both NK and T cell clones in culture (1, 2). We have shown that irradiated B lymphoblastoid cell lines induce proliferation of purified human NK cells only in synergy with IL-2 (3). They also facilitate continued proliferation and enhance the cloning efficiency of purified human NK cells in limiting dilution assays in the presence of IL-2 without increasing the proportion (>50%) of NK cells entering the cell cycle in response to IL-2 (4). During culture of total PBMC with irradiated B cell lines, NK cells become activated, as shown by increased cytotoxic activity, by proliferation, and by expression of surface activation antigens such as class II HLA antigens, transferrin receptors, and IL-2 receptors (5, 6). In these cultures, a preferential proliferation of CD16<sup>+</sup> CD56(NKH-1)<sup>+</sup> CD3<sup>-</sup> NK cells is observed (6): in 10-d cultures, NK cell number is increased 25-fold, whereas T cell number is increased only 3-fold. Elimination of CD4<sup>+</sup> cells or the presence of an anti-IL-2 antiserum completely prevents NK cell proliferation (6), suggesting that this probably depends on the production of IL-2 by CD4<sup>+</sup> T cells upon allogeneic stimulation. However, the B cell lines also contribute directly to the proliferation of NK cells because in the absence of B cell lines neither high doses of IL-2 alone nor stimulation by allogeneic PBMC induce preferential proliferation of NK cells (6).

The mechanism by which B lymphoblastoid cell lines affect lymphocyte proliferation is not known. Studies with both human and murine lymphocytes (7, 8) suggest a role for immune interferon (IFN- $\gamma$ ) in NK and T cell proliferation, although other studies (4) have shown that IFN- $\gamma$  production is not required. IFN- $\gamma$  is produced in cultures of thymocytes with irradiated B lymphoblastoid cell lines (9). Low density murine spleen B cells (10) and certain human B cell lines (Cassatella, M. A.,

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B. Perussia, G. Trinchieri, unpublished observation) directly stimulate purified NK cells to produce IFN- $\gamma$ . B lymphoblastoid cell lines also produce a variety of other lymphokines, including IL-1, IL-6, IFN- $\alpha$ , IFN- $\beta$ , lymphotoxin (LT)<sup>1</sup>, and B cell growth factors (11-14). However, if soluble factors are involved in the effect of B cell lines, those factors are probably either active only at a short range or are produced only when the B cells are in contact with NK or T cells, since lymphocyte proliferation requires cell contact or at least contiguity (6, 15).

In this paper we report that certain B lymphoblastoid cell lines, constitutively and after phorbol ester stimulation, produce a novel lymphokine that induces IFN- $\gamma$  production, augments NK cell-mediated cytotoxicity, and enhances the T cell responses to the mitogens PHA and phorbol-12,13-dibutyrate (PDBu) when tested on PBL. This lymphokine, designated NK cell stimulatory factor (NKSF), was purified as a 70-kD glycoprotein from the supernatant fluid of the PDBu-induced, EBV-transformed B lymphoblastoid cell line RPMI 8866. Purified NKSF biological activities on NK and T cells can be demonstrated at concentrations as low as 0.1-1 pM.

### Materials and Methods

**Cell Lines.** The human B lymphoblastoid RPMI 8866 (16) and ADP (2), the erythromyeloid leukemic K562, the Burkitt lymphoma-derived Daudi, and the rhabdomyosarcoma-derived RDRC cell lines were maintained in culture in RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 10% FCS (Flow Laboratories). All cell lines were free of mycoplasma contamination on repeated testing.

**Preparation of Serum-free Supernatant Fluid from Cell Lines.** Cultured cells were washed and suspended ( $10^6$  cells/ml) in serum-free RPMI 1640 medium with or without 100 nM PDBu (Chemical for Cancer Research, Inc., Eden Prairie, MN) and cultured for 48 h at 37°C in a 5% CO<sub>2</sub> atmosphere. The cell-free supernatant fluids were harvested by filtration through a 0.2- $\mu$ m filter (Durapore hydrophilic cartridge filter; Millipore, Bedford, MA). Tween-20 (0.02%) and PMSF (0.1 mM) were added to the supernatant fluid of PDBu-induced RPMI 8866 cells to be used for biochemical purification. The fluid was then concentrated 50-fold under pressure using a Pellicon Cassette System (Millipore) with Pellicon Disc Ultrafilters (PTGG 00005, Millipore) or a Diaflow hollow-fiber cartridge (HIP 10-20; Amicon Corp., Danvers, MA). Unless otherwise stated, all buffers used for subsequent purification contained 0.02% Tween-20 and 0.1 mM PMSF.

**Peripheral Blood Lymphocytes.** Peripheral blood was obtained by venipuncture from adult healthy donors using heparin as anticoagulant. PBMC were separated on Ficoll-Hypaque density gradient (Lymphoprep; Nygaard and Co., Oslo, Norway). PBL were prepared from PBMC after partial depletion of monocytes by adherence to plastic surfaces (45 min, 37°C).

**Cytokines and Cytokine Assays.** Human rIFN- $\alpha$  (rIFN-A,  $2 \times 10^8$  U/mg) was kindly provided by Hoffmann-LaRoche (Nutley, NJ); rIL-2 ( $10^7$  U/mg), by Dr. T. Taguchi, Osaka University and Takeda Chemical Industry, Inc., Osaka, Japan; rIFN- $\gamma$  ( $10^7$  U/mg), rTNF, and rLT, by Dr. H. M. Shepard (Genentech Inc., South San Francisco, CA); rIFN- $\beta$ , by Dr. J. S. Price (Cetus Corp., Emeryville, CA); rIL-1 by Dr. P. T. Lomedico (Hoffmann-LaRoche); rIL-1 $\beta$  and rIL-4 were purchased from Genzyme Co. (Boston, MA); rIL-3, rIL-5, rIL-6, and recombinant granulocyte/macrophage CSF (rGM-CSF) were produced at Genetics Institute. Inactivating calf anti-IFN- $\beta$  antiserum was provided by Dr. J. Vilcek (New York University, New York, NY); sheep anti-IFN- $\alpha$  antiserum was purchased from Interferon Sciences, Inc. (New Brunswick, NJ); goat anti-IL-2, anti-IL-6, anti-LT antisera, and murine anti-TNF and anti-IFN- $\gamma$  mAbs were produced at the Wistar Institute; rabbit anti-IL-3 and anti-GM-CSF antisera were produced at Genetics Institute.

IFN antiviral activity was assayed on human diploid fibroblasts and typed using specific antibodies, as described (17). IFN- $\gamma$  was measured by RIA as described (18) or with a commercially available kit (Centocor, Malvern, PA). TNF and LT were measured by cytotoxic assay on murine L cells, and TNF was also tested in radioimmunoassay as described (18).

IL-1, IL-6, and GM-CSF were measured in biological assays as described (19–21). The specificity of each biological assay was confirmed by inhibition of the biological activity using neutralizing antibodies specific for the various cytokines: expression of lymphokine genes in the RPMI 8866 cell line was also confirmed by Northern blotting analysis.

*IFN- $\gamma$  Induction Assay.* 100  $\mu$ l of the samples to be tested were added in triplicate to PBL ( $10^6$  cells/100  $\mu$ l RPMI 1640/10% FCS) in U-bottomed microtiter plates (96-2311; Costar, Cambridge, MA) and incubated (37°C, 5% CO<sub>2</sub>) for 18 h. After incubation, 100  $\mu$ l of cell-free supernatant was collected from each well and the IFN- $\gamma$  produced was measured by RIA. This assay was used for standardization of NKSF activity: 1 U of NKSF per milliliter is defined as the amount required to induce one-half of the maximal IFN- $\gamma$  production induced by optimal concentrations of NKSF.

*Cell-mediated Cytotoxicity.* PBL were incubated ( $5 \times 10^6$  cells/ml RPMI-10% FCS for 18 h at 37°C) in the presence or absence of different concentrations of rIFN- $\alpha$ , rIL-2, rIFN- $\gamma$ , or a source of NKSF. PBL were then washed and added at different concentrations to  $10^4$  target cells in a 3-h <sup>51</sup>Cr-release assay. 1 lytic unit (LU) is the number of lymphocytes mediating one-half of the maximum experimental <sup>51</sup>Cr release (usually 90% of the specific <sup>51</sup>Cr release measured by detergent lysis) in the 3-h assay period, as calculated using a modified Von Krogh equation (22).

*Lymphocyte Proliferation.* PBL were cultured ( $10^5$  cells/200  $\mu$ l RPMI 1640–10% FCS at 37°C, 5% CO<sub>2</sub>) in triplicate in flat-bottomed 96-well plates for 3 or 6 d in the absence or presence of 1% PHA-M (Wellcome Research Laboratories, Beckenham, England) or 100 nM PDBu with or without different concentrations of a source of NKSF. Cultures were pulsed for 6 h with [<sup>3</sup>H]TdR (5  $\mu$ Ci/ml; New England Nuclear, Boston, MA) on day 3 or 6; cells were harvested onto glass fiber filters and assayed for [<sup>3</sup>H]TdR incorporation by liquid scintillation counting.

*SDS-PAGE.* SDS-PAGE was performed according to the method of Laemmli (23) on 10% polyacrylamide slab gels (0.75-mm thickness). After electrophoresis, gels were either stained using silver staining reagents (Bio-Rad Laboratories, Richmond, CA) or cut into 2-mm slices, extracted in 0.5 ml RPMI medium for 4 h at 24°C, and assayed for NKSF activity. Apparent molecular weight was determined using the protein standards: phospholipase B (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD) and lactalbumin (14 kD). <sup>125</sup>I-labeled proteins (chloramine-T iodination method, followed by separation of iodinated protein from free sodium iodide by chromatography on a PD10 column; Pharmacia Fine Chemicals, Piscataway, NJ) were visualized in the dried gels after exposure to X-ray film.

*Anion-Exchange Cartridge Chromatography.* The 50-fold concentrated PDBu-induced RPMI 8866 cell-free supernatant fluid (2 liters) was dialyzed against 0.1 M Tris-HCl buffer (pH 8.0) and applied at a flow rate of 150 ml/min, to four QAE Zetaprep 250 cartridges (Pharmacia Fine Chemicals) connected in parallel and previously equilibrated with the same buffer. The cartridges were washed with three liters of 0.1 M Tris-HCl buffer (pH 6.8), and NKSF activity was eluted with 0.5 M NaCl in 0.1 M Tris-HCl buffer (pH 6.8) in five 300-ml fractions.

*Lentil Lectin-Sepharose Chromatography.* Pooled NKSF-containing fractions were applied to a column (2.5 × 15 cm) of lentil lectin-Sepharose 4B (Pharmacia Fine Chemicals) that had been equilibrated with 20 mM Tris-HCl buffer (pH 7.2). The column was washed with five column volumes of the same buffer and bound material was eluted with three column volumes of buffer containing 0.2 M  $\alpha$ -methyl-D-mannopyranoside (Sigma Chemical Co., St. Louis, MO) and 0.5 M NaCl. Approximately half of the NKSF activity was bound to the column and was recovered in the fractions eluted with  $\alpha$ -methyl-D-mannopyranoside.

*Hydroxylapatite Chromatography.* The concentrated pool of NKSF activity bound/eluted from the lentil lectin-Sepharose column was dialyzed against 1 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM CaCl<sub>2</sub> and 0.15 M NaCl, and applied to a hydroxylapatite column (2 × 5 cm, Biogel HT; Bio-Rad Laboratories) previously equilibrated with the same buffer. The column was washed with five column volumes of equilibration buffer and eluted with 100 ml of a linear gradient of potassium phosphate buffer (pH 6.8) from 1 to 400 mM containing 0.15 M NaCl. Fractions (4 ml) were collected and tested for NKSF activity. NKSF activity eluted in a single peak between ~200 and 300 mM potassium phosphate.

*Heparin-Sepharose Chromatography.* Eluted NKSF-containing fractions from the Biogel HT

column were pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.2) and applied to a heparin-Sepharose column (1 × 10 cm, Pierce Chemical Co., Rockford, IL). The column was washed with five column volumes of 20 mM sodium phosphate buffer (pH 7.2) and eluted with 100 ml of a linear gradient from 0 to 1 M NaCl in the same buffer. Fractions (3 ml) were collected. Essentially all NKSF activity bound to the heparin column and eluted at ~0.6–0.7 M NaCl.

**Mono-Q Chromatography.** Pooled fractions from the heparin-Sepharose column were dialyzed against a 20 mM Tris-HCl buffer (pH 6.8) containing 1% ethylene glycol and 1.1 mM PMSF without Tween-20 and concentrated to 2 ml using a YM 10 membrane in a stirred cell (Amicon Corp.). The sample was applied to a Mono-Q column (5/5) in a FPLC apparatus (Pharmacia Fine Chemicals) and eluted with a linear gradient of 0–1 M NaCl in the same buffer (pH 6.8). Fractions (0.5 ml) were collected and tested for NKSF activity. The activity eluted from the column as a single peak between ~220 and 270 mM NaCl. The same procedure was used to fractionate concentrated crude supernatant fluid from PDBu-induced RPMI 8866 cells.

**Reverse-Phase HPLC (RP-HPLC).** Pooled fractions from the Mono-Q column were applied to a RP-HPLC column (Aquapore 2.1 × 30 mm column 0711-0056 connected to a 130A Separation System; Applied Biosystems, Inc., Foster City, CA) and eluted with a linear gradient of 0 to 75% (vol/vol) acetonitrile containing 0.1% trifluoroacetic acid.

**Protein Determination.** Protein concentration was determined either using the Bio-Rad Laboratories protein assay or by measuring absorbance at 280 nm. BSA was used as a standard.

## Results

**Identification of NKSF.** Cells from the EBV-transformed cell line RPMI 8866 were cultured for 48 h in serum-free medium in the presence of 10<sup>-7</sup> M PDBu. The supernatant fluid from PDBu-induced RPMI 8866 cells contained various cytokines, including LT, IL-6, GM-CSF, and IL-1 as detected in biological assays (not shown). Extensive dialysis to remove contaminant PDBu from the supernatant fluid reduced the concentration of PDBu to <10<sup>-11</sup> M (as determined by scintillation counting after adding [<sup>3</sup>H]PDBu to the sample), a level at which PDBu did not affect any of the bioassays. When added to freshly separated PBL, the supernatant fluid mediated three activities (Table I and Fig. 1) that could not be readily associated with

TABLE I  
*Production of IFN- $\gamma$ -inducing Activity by Human B Cell Lines*

Cell line	PDBu	IFN- $\gamma$ at supernatant fluid dilution of:		
		1/18	1/6	1/2
		U/ml		
RPMI 8866	–	2	23	41
RPMI 8866	+	40	160	335
ADP	–	<1	<1	5
ADP	+	2	22	38
Raji	–	<1	<1	<1
Raji	+	<1	<1	<1

Cell-free supernatant fluids were collected from cell lines cultured for 48 h (10<sup>6</sup> cells/ml serum-free RPMI 1640 medium) in the presence (+) or absence (–) of 10<sup>-7</sup> M PDBu. Dilutions of extensively dialyzed supernatant fluid were added to PBL, and IFN- $\gamma$  production was measured after 18 h by RIA.

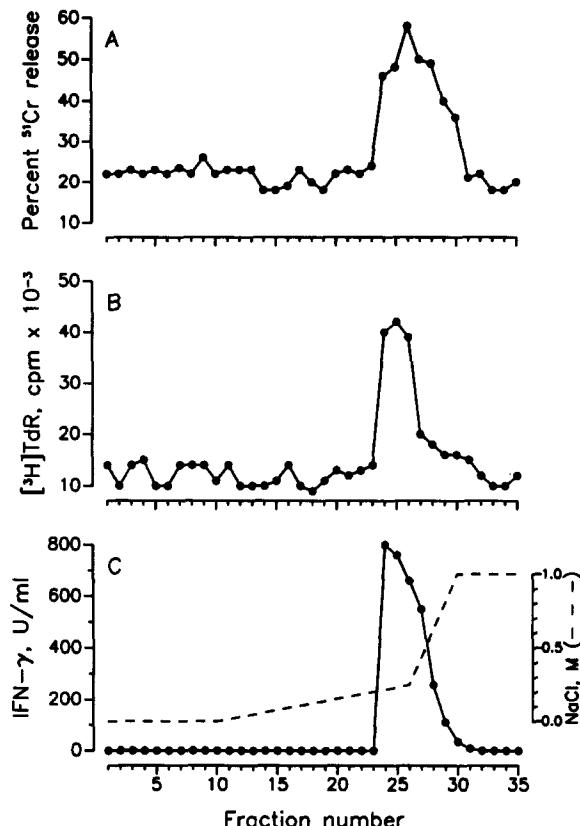


FIGURE 1. Mono-Q fractionation of concentrated cell-free supernatant fluid from PDBu-induced RPMI 8866 cells. Fractions eluted at the indicated NaCl concentration were dialyzed, sterile filtered, and tested on PBL for ability to: (A) augment cell-mediated spontaneous cytotoxicity (as tested against RDMC target cells at an E/T cell ratio of 25:1); (B) enhance proliferation in the presence of PHA (6-d culture); (C) induce IFN- $\gamma$  production.

any of the known cytokines present in the supernatant fluid: (a) it induced IFN- $\gamma$  production; (b) it augmented spontaneous cytotoxic activity against a variety of target cell lines; (c) it enhanced proliferation of PBL in response to PHA or PDBu. IFN- $\gamma$ -inducing activity was also detected in the supernatant of unstimulated RPMI 8866 cells, but at levels  $\sim$ 10-fold lower than in supernatants of PDBu-induced cells (Table I). A similar, but lower activity, was detected in the supernatant fluid of the EBV-transformed cell line ADP (Table I), whereas no activity was detected in the supernatant fluids of other human cell lines tested, including four B, six myeloid, and five T cell lines, regardless of PDBu induction (Table I and not shown).

Previously characterized lymphokines, either present in the supernatant fluid or not, were analyzed for ability to mediate the three activities on PBL. Because this analysis consistently gave negative results, those data are only summarized here. Recombinant IL-1 $\alpha$ , IL-1 $\beta$ , IL-3, IL-4, IL-5, IL-6, IFN- $\alpha$ , IFN- $\beta$ , GM-CSF, TNF, and LT, tested in a wide range of concentrations, did not induce production of significant amounts of IFN- $\gamma$  by PBL. rIL-2 consistently induced IFN- $\gamma$  production, but the level of IFN- $\gamma$  induced by doses of rIL-2 as high as 1000 U/ml was always lower than that induced by supernatant fluids diluted 1:2 from PDBu-stimulated RPMI 8866 cells. Anti-IL-2, IL-3, IL-6, TNF, LT, GM-CSF, IFN- $\alpha$ , and IFN- $\beta$  neutralizing antibodies did not inhibit the ability of RPMI 8866 supernatant to in-

duce IFN- $\gamma$  production. The supernatant fluid of PDBu-stimulated RPMI 8866 cells contains  $\sim$ 5 ng/ml of LT (24). LT, purified to homogeneity from this supernatant, has no IFN- $\gamma$ -inducing, NK cell-augmenting, or comitogenic activity when tested on PBL (24). Supernatant fluids from PDBu-induced RPMI 8866 cells also contained  $\sim$ 50 antiviral units of IFN, which was inhibited partially by antiserum against IFN- $\alpha$  or IFN- $\beta$  and completely by a combination of the two. Such an IFN concentration is sufficient to induce a modest increase in NK cell-mediated cytotoxicity (25). However, the two anti-IFN antisera did not suppress the ability of RPMI 8866 supernatant to enhance NK cell-mediated cytotoxicity.

Serum-free medium from PDBu-induced RPMI 8866 cells was concentrated by membrane filtration under pressure using capillary tubings or filters with a 10-kD exclusion limit. The concentrated material was fractionated by gel filtration on Sephadex G100, by ion-exchange chromatography on a Mono Q column, or by hydrophobic chromatography on a phenyl-Sepharose column. Fractions were tested for biological activity on PBL. Fig. 1 illustrates the results obtained with the fractions from the Mono-Q column. With this column, and also with the other two (not shown), the IFN- $\gamma$ -inducing activity, the NK cell-enhancing activity, and the comitogenic activity eluted in the same fractions, suggesting that the three activities were mediated by a single anionic protein of  $\sim$ 70 kD. This protein, designated NKSF, was further purified on the basis of its IFN- $\gamma$ -inducing activity.

*Determination of NKSF Molecular Mass by SDS-PAGE.* Concentrated supernatant from PDBu-induced RPMI 8866 cells was separated by SDS-PAGE under nonreducing and reducing conditions (Fig. 2). The NKSF activity was recovered from the un-

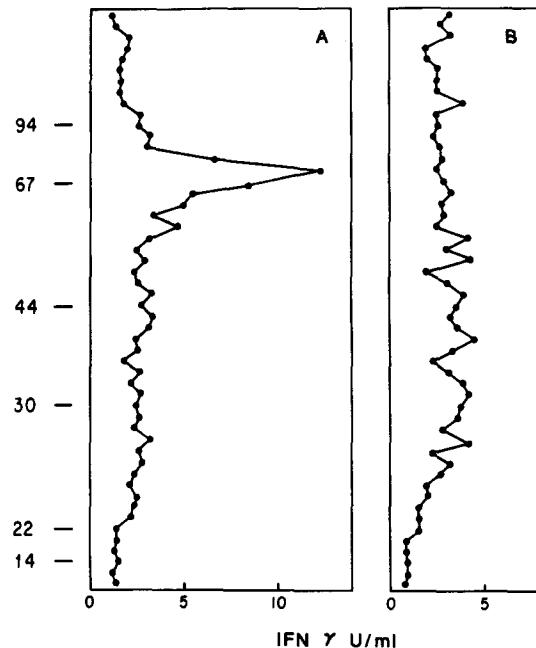


FIGURE 2. Size determination of NKSF in RPMI 8866-conditioned medium. NKSF was incubated with sample buffer at 37°C for 30 min and subjected to SDS-PAGE (10% gel) under (A) nonreducing and (B) reducing conditions. After electrophoresis, the gel was cut into 2-mm slices and extracted with 0.5 ml of RPMI 1640 medium at 24°C for 4 h. Each extract was sterile filtered and assayed for NKSF activity, expressed as U/ml of IFN- $\gamma$  induced in the PBL cultures.

reduced gel as a single species with apparent molecular mass of 70 kD. Thus, most NKSF activity produced by RPMI 8866 cells appears to reside in a 70-kD protein. However, because not all cytokines can be recovered from gels in active form after SDS treatment, the possibility remains that other SDS-sensitive molecules with NKSF activity are produced by RPMI 8866 cells but were not detected in this experiment.

**Purification of NKSF** The low level of NKSF activity in the serum-free conditioned medium (10 U/ml) necessitated the purification of the NKSF activity from large amounts (192 liters) of conditioned medium in order to obtain sufficient material for detailed biochemical and biological analysis. The purification scheme, summarized in Table II, included the following sequential steps: filtration through a QAE-Zetaprep cartridge that selectively binds anionic proteins; affinity chromatography on a lentil lectin-Sepharose column; hydroxylapatite column chromatography of the activity recovered in the bound fraction from the lentil lectin column; affinity chromatography on heparin-Sepharose; anion-exchange chromatography on a Mono-Q resin column; and RP chromatography on an Aquapore column.

A substantial amount of NKSF activity was lost during the early steps of the purification protocol. Approximately half of NKSF activity bound to lentil lectin-Sepharose, indicating that the NKSF molecule is, at least in part, glycosylated. Specific activity of NKSF recovered in the lectin-unbound fraction was much lower than that of the bound fraction. For practical purposes purification of only the lectin-bound fraction was pursued, although in this way characterization of an NKSF species with lower or no glycosylation might have been missed. Essentially all of the NKSF activity recovered in the bound fractions from the lentil lectin and hydroxylapatite columns bound to heparin-Sepharose with intermediate to high affinity. NKSF was released from the heparin-Sepharose column at 0.6–0.7 M NaCl. Recovery of total NKSF activity increased substantially both after the lentil lectin and heparin-Sepharose fractionation steps: in the former case, the yield of activity in the bound fraction was 100% despite the fact that half of the recovered activity was found in the unbound fraction; in the latter case the yield of activity was 450% with a 16-fold purification. Possibly, inhibitory substances were concentrated along with the NKSF activity during the early steps of purification but were removed during the later steps.

TABLE II  
*Purification of NKSF*

Purification steps	Total protein mg	Total activity $U \times 10^{-5}$	Specific activity $U/mg \times 10^{-3}$	Yield %	Purification -fold
1. Cell supernatant (192 liters)	4,350	20	0.45	100	1
2. Concentrated supernatant (4 liters)	4,200	9.6	0.23	48	<1
3. QAE-Zetaprep cartridge	670	1.4	0.21	7	<1
4. Lentil lectin-Sepharose	31.5	1.5	4.70	8	10
5. Hydroxylapatite	10.0	0.94	9.40	5	20
6. Heparin-Sepharose	3.17	4.5	140	22	320
7. Mono-Q-FPLC	0.072	3.0	4,160	15	9,200
8. RP-HPLC	0.018	0.03	167	0.15	371

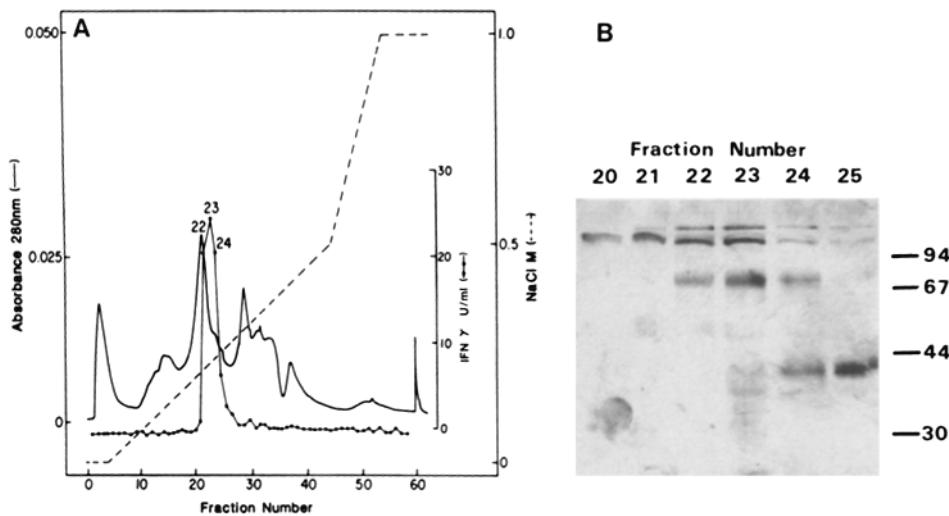


FIGURE 3. Analysis of the Mono-Q fractionation (Step 7) of the NKSF purification. (A) Profiles of NKSF activity (●) in units IFN- $\gamma$  induced per milliliter and protein concentration (—) ( $A_{280}$ ) eluted from the Mono-Q column with the indicated (---) salt gradient. (B) Fractions 20–25 from the Mono-Q column were analyzed by SDS-PAGE (10% gel). The relative positions of molecular mass reference proteins are indicated.

NKSF activity eluted as a single peak from a Mono-Q column with 0.25 M NaCl (Fig. 3). The Mono-Q purification step resulted in a 30-fold purification of NKSF with 70% recovery of total activity (Table II). Recovery of biologic activity after the Mono-Q step was 15% and the specific activity of the NKSF protein at this point was  $4.2 \times 10^6$  U/mg. This purified NKSF did not contain significant amounts of IL-1, IL-6, TNF, LT, GM-CSF, or IFN, as determined in biological assays (not shown).

Fig. 3 shows the SDS-PAGE analysis under nonreducing conditions of each of the NKSF-containing and several adjacent fractions from the Mono-Q-FPLC fractionation (Step 7). A prominent species with apparent molecular mass of 70 kD was present in each of the NKSF-containing fractions but absent from the adjacent fractions. The size estimate for this protein species is in agreement with that of the activity eluted from the SDS gel shown in Fig. 2. Furthermore, the 70-kD species eluted from an unstained gel was active in the IFN- $\gamma$  induction assay (not shown). These results are consistent with the identification of the 70-kD species visible in the gel shown in Fig. 3 as NKSF. Assuming a molecular mass of 70 kD for NKSF, and based on the specific activity of the Step 7 material, we calculated that a final concentration of 3.5 pM NKSF yielded half of the maximal response in the IFN- $\gamma$ -induction assay (1 U NKSF).

The 70-kD protein was further separated from contaminating proteins in the preparation using RP-HPLC (Fig. 4). NKSF was highly sensitive to the solvents used in the RP fractionation and exposure to them resulted in profound reduction of biologic activity (not shown). The presence of the 70-kD species in the fractions from the RP-HPLC column correlated well with the presence of NKSF activity in the same fractions, although the total recovery of activity from the column was only

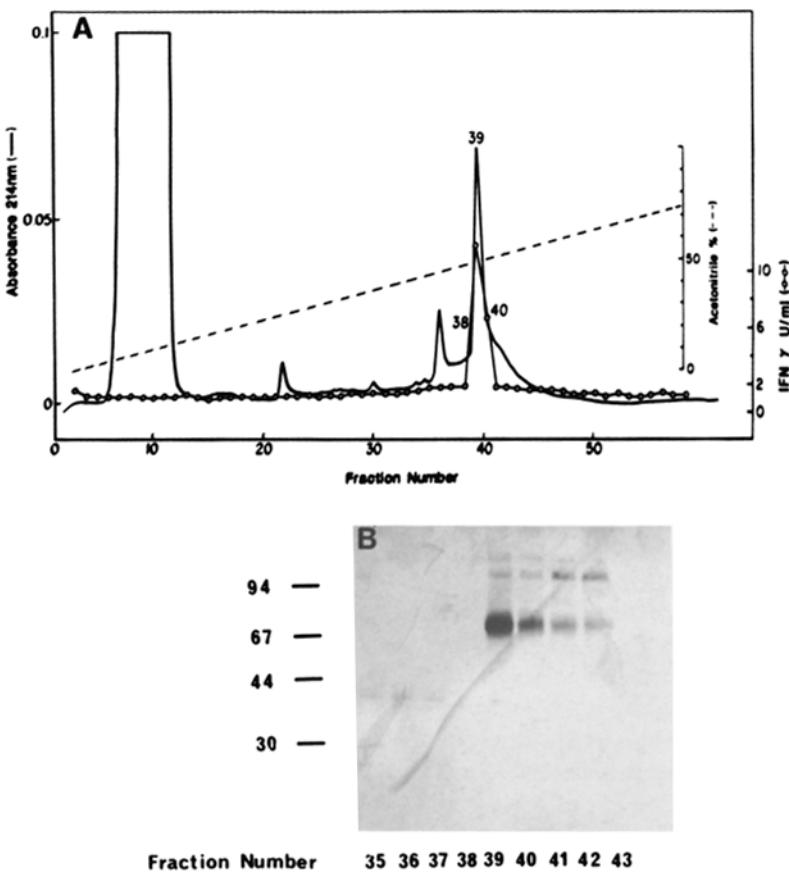


FIGURE 4. Analysis of the RP-HPLC fractionation (Step 8) of the NKSF purification. (A) Profiles of NKSF activity (○) and protein concentration ( $A_{214}$ , ---) eluted from the RP-HPLC column with the indicated gradient of acetonitrile were as shown. (B) Fractions 35–43 from the RP-HPLC fractionation were analyzed by SDS-PAGE in parallel with the indicated reference proteins.

1% (Fig. 4). Therefore, the RP-HPLC step resulted in the inactivation of 99% of the NKSF activity but yielded an estimated 90% of NKSF protein (Fig. 4). Thus, biologic studies were performed using NKSF from the penultimate purification step (Step 7), and the most highly purified, but largely inactive material from the final RP column (Step 8) was used for biochemical characterization. Final recovery of protein at this step was 18  $\mu$ g, obtained from 192 liters of supernatant fluid.

**SDS-PAGE Analysis of the Purified NKSF Protein.** To further analyze the structure of the NKSF protein, the Step 7 material was labeled with  $^{125}\text{I}$  and subjected to SDS-PAGE under nonreducing conditions. The 70-kD species was eluted from the gel and fractionated on a reducing gel. As illustrated in Fig. 5, reduction of the gel-purified,  $^{125}\text{I}$ -labeled NKSF resulted in the disappearance of the 70-kD species and the appearance of two new polypeptides with apparent molecular masses of 40 and 35-kD. Thus, NKSF appears to have a heterodimeric structure comprising two disulfide-linked monomers with masses of 40 and 35 kD, respectively. However, the

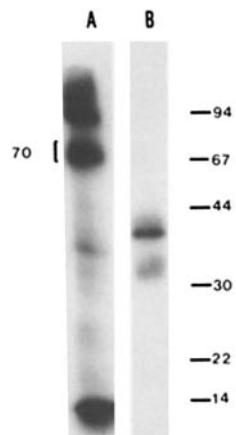


FIGURE 5. SDS-PAGE analysis of the 70-kD NKSF protein after reduction. (A) Radioiodinated (Step 7) NKSF subjected to SDS-PAGE under non-reducing conditions. (B) The 70-kD species eluted from gel A was reanalyzed by SDS-PAGE after reduction with 2-ME. The relative mobilities of reference proteins in the second gel are indicated.

inactivation of NKSF upon reduction has prevented the ultimate demonstration that both polypeptides are required to yield the active NKSF species.

*IFN- $\gamma$  Induction by Purified NKSF.* Purified NKSF (Step 7) induces IFN- $\gamma$  production by human PBL. The levels of IFN- $\gamma$  produced using PBL preparations from different donors were variable. In the experiment depicted in Fig. 6, 18-h treatment of PBL with 2 U/ml NKSF (7 pM) yielded up to 300 U/ml IFN- $\gamma$ , whereas 1,000 U/ml of IL-2 ( $\sim$ 20 nM) yielded only 200 U/ml IFN- $\gamma$ . When tested in combination, the two factors acted synergistically. With optimal concentrations of both NKSF and IL-2, IFN- $\gamma$  levels higher than 1,000 U/ml were observed with PBL of most

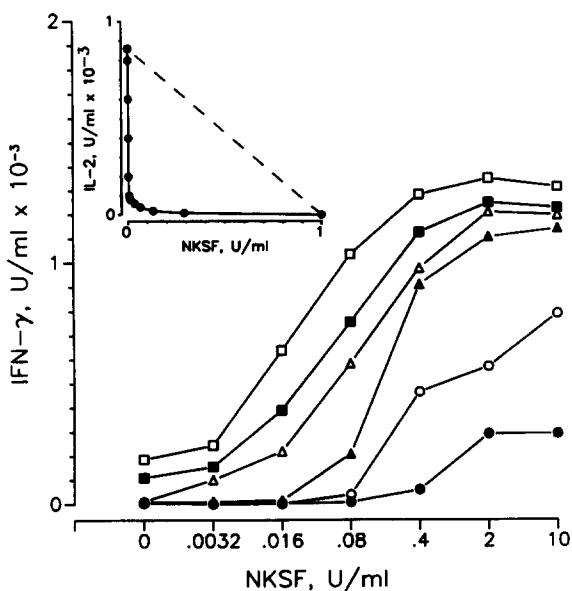
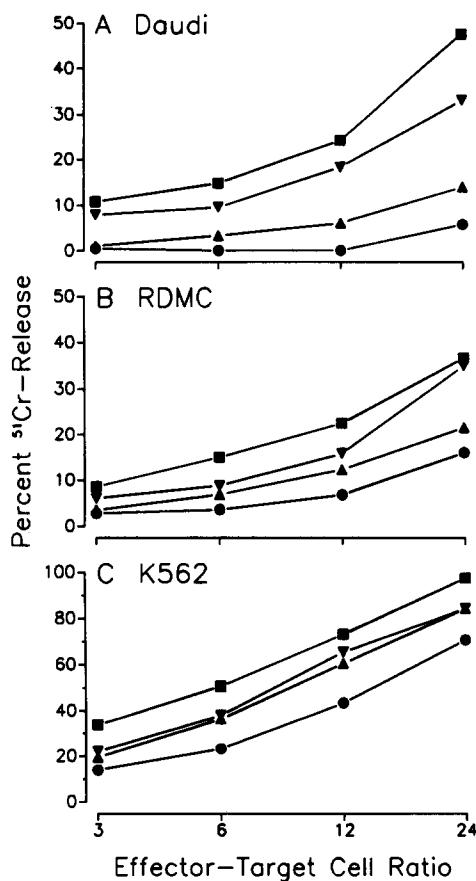


FIGURE 6. Induction of IFN- $\gamma$  production by NKSF and IL-2. PBL were incubated (18 h, 37°C) in the presence of the indicated concentrations of NKSF and the following concentrations of rIL-2: (●) none; (○) 10 U/ml; (▲) 40 U/ml; (△) 100 U/ml; (■) 400 U/ml; (□) 1,000 U/ml. Supernatant fluids were collected and tested for IFN- $\gamma$  content by RIA. The isobologram in the inset shows the concentrations of the two lymphokines needed to induce 150 U/ml IFN- $\gamma$  when used in combination.

donors. The combination of 10 U/ml IL-2 (200 pM) and 0.4 U/ml NKSF ( $\sim$ 1.5 pM), concentrations that by themselves were almost ineffective in the assay, induced production of as much as 500 U/ml IFN- $\gamma$  by PBL. The interaction between these two factors was evaluated by isobologram analysis in which the concentration of IL-2 required to induce production of 150 U/ml IFN- $\gamma$  was plotted as a function of NKSF concentration (Fig. 6, inset). The experimental results strikingly deviated from the expected results for an additive effect, indicating strong synergistic interaction between the two cytokines.

**Enhancement of PBL-mediated Spontaneous Cytotoxicity by Purified NKSF.** Spontaneous cytotoxicity mediated by PBL against three target cell lines was augmented by culturing PBL in the presence of NKSF for 18 h (Fig. 7). This enhancement of spontaneous cytotoxicity was dose dependent over the range of 0.08–4 U/ml of NKSF (0.29–14.4 pM). The observed enhancement was most pronounced using as target Daudi cells, which are largely resistant to killing by unstimulated NK cells. The effector cells mediating cytotoxicity were CD16 $^+$ , CD3 $^-$ , CD5 $^-$  NK cells, as demonstrated by cell separation experiments using specific mAbs (not shown).

NKSF appears to be the most potent of the known NK cell-activating cytokines



**FIGURE 7.** Enhancement of PBL-mediated spontaneous cytotoxicity by NKSF. PBL were incubated (18 h, 37°C) in the presence of: (●) medium only; (▲) 0.08 U/ml NKSF; (▼) 0.4 U/ml NKSF; (■) 2 U/ml NKSF. PBL were washed and used as effector cells in a 3-h <sup>51</sup>Cr-release assay against <sup>51</sup>Cr-labeled Daudi, RDMC, and K562 target cells, as indicated.

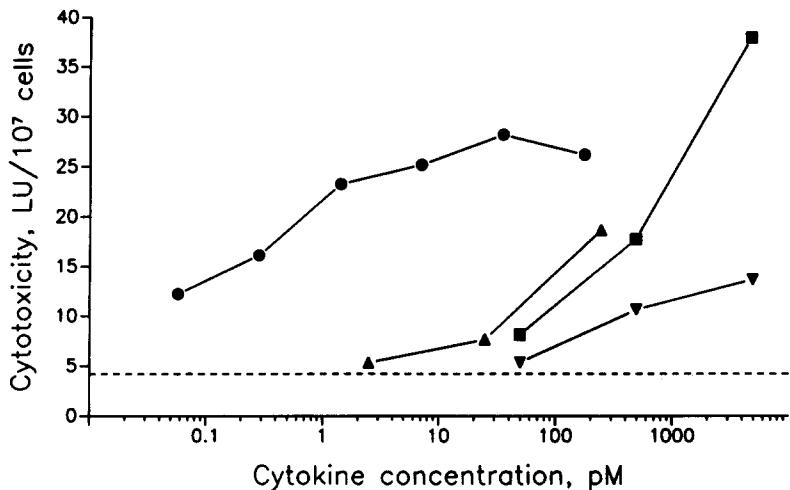


FIGURE 8. Enhancement of PBL-mediated spontaneous cytotoxicity on RDMC target cells. PBL were incubated (18 h, 37°C) in the presence of the indicated concentrations of (●) NKSF; (■), rIL-2; (▲) rIFN- $\alpha$ ; (▼) rIFN- $\gamma$ . PBL were then washed and used as effector cells in a 3-h  $^{51}\text{Cr}$ -release assay. The discontinuous line indicates the cytotoxicity mediated by PBL incubated in medium alone.

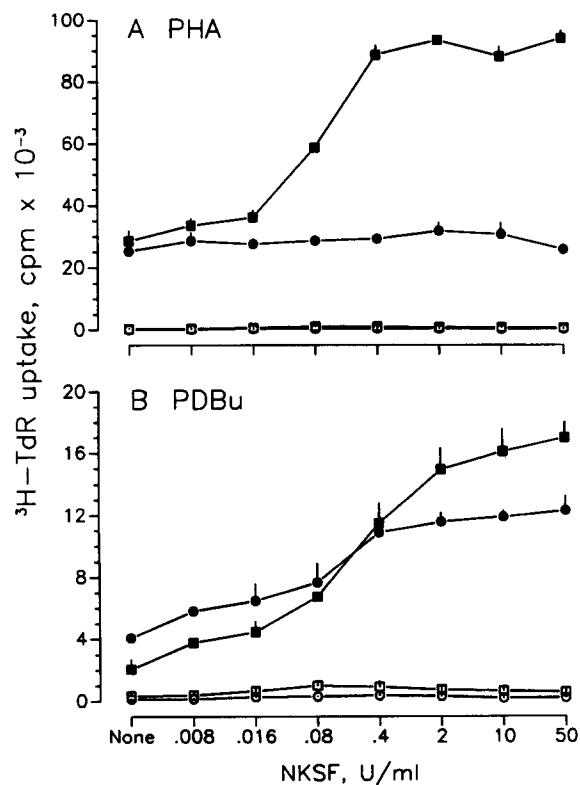


FIGURE 9. Effect of NKSF on PBL proliferation induced by PHA (A) or PDBu (B). PBL were cultured in the presence of the indicated concentrations of NKSF and (○, □) medium; (●, ■) 1% PHA (A) or 100 nM PDBu (B); [ $^3\text{H}$ ]TdR uptake was measured at: (○, ●) day 3; (□, ■) day 6. Vertical bars indicate 1 SD of triplicates.

tested (Fig. 8). A significant enhancement of PBL-mediated cytotoxicity was observed in PBL treated for 18 h with concentrations of NKSF between 0.10 and 10 U/ml (0.36–36 pM). The generation of similar levels of cytotoxicity required concentrations of IL-2 >50 U/ml (>1 nM) or of either IFN- $\alpha$  or IFN- $\gamma$  >1,000 U/ml (>100 pM).

**Comitogenic Activity of Purified NKSF.** PBL were cultured in the presence of purified NKSF (Step 7) alone or in the presence of NKSF and either PHA or PDBu (Fig. 9). NKSF alone did not induce detectable levels of proliferation as measured by incorporation of [<sup>3</sup>H]TdR on either day 3 or 6 of culture. In the presence of either PHA or PDBu, NKSF induced a significant dose-dependent proliferative response detectable on day 6 in PHA-stimulated culture and on both days 3 and 6 in the PDBu-stimulated cultures. The half-maximal activity of NKSF in the comitogenic assay was observed at NKSF concentrations (~0.35 pM) ~10-fold lower than those effective in the INF- $\gamma$ -inducing assay (Figs. 6 and 9).

### Discussion

We have identified and purified from the conditioned medium of an EBV-transformed B cell line a novel 70-kD glycoprotein that when added to cultures of resting PBLs, induces production of IFN- $\gamma$ , augments NK cell-mediated cytotoxicity, and enhances proliferation induced by mitogenic lectins and phorbol diesters. The identification of this 70-kD protein as the active component in these assays rests in: (a) the elution of biologic activity from SDS gels with an apparent molecular mass of 70 kD; (b) the co-purification of this protein with these three biologic activities through a 9,200-fold purification; and (c) the high specific activity of the purified protein with half-maximal activity in the IFN- $\gamma$ -induction assay at 3.5 pM. Upon reduction, the most highly purified preparation of 70-kD NKSF yielded two polypeptides with approximate molecular masses of 40 and 35 kD, suggesting that the native molecule is a disulfide-linked heterodimer. The purification results strongly suggest that a single major 70-kD protein is present in the purified NKSF preparation and that this protein mediates the three biological activities. However, because even the most purified NKSF preparations still contain traces of other peptides, it cannot be excluded that the biological activities reside in different minor components of ~70 kD present in the preparations or that the two bands in the reduced preparation originate from different 70-kD components comigrating at the same position in nonreducing gels. Only amino acid sequencing and molecular cloning efforts, which are now in progress, will allow us to exclude these unlikely possibilities.

The biologic activities of NKSF and its biochemical characteristics distinguish it from other known cytokines. A role for other lymphokines produced by RPMI 8866 cells in NKSF biologic activities is excluded because the lymphokines identified in the supernatant fluid were eliminated during purification. Moreover, antibodies against the identified lymphokines or others, not detectable in the NKSF preparation but that might be expected to mediate similar biologic effects, do not inhibit NKSF activities. Finally, the high specific activity of purified NKSF argues against the possibility of low level contamination of the preparations with other lymphokines.

Relatively few of the known cytokines induce IFN- $\gamma$  production by resting PBL. Reem et al. (9) reported that B lymphoblastoid cell lines, different from the one used in the present study, and their supernatant fluids induce IFN- $\gamma$  production

by human thymocytes, but those authors did not further characterize this activity. Among the cytokines that we have tested, only IL-2 consistently induces significant levels of IFN- $\gamma$  production by PBL (26). The strong synergism between NKSF and rIL-2 in inducing IFN- $\gamma$  production and the inability of anti-IL-2 antibodies to significantly inhibit the effect of NKSF exclude that IL-2 is responsible for the NKSF activity. The synergistic effect of these two cytokines also suggests that IL-2 and NKSF induce IFN- $\gamma$  production by PBL acting through different receptors and probably different intracellular signal transduction pathways. Both rIL-1 (27) and rIL-6 (not shown) occasionally induced low level IFN- $\gamma$  production in our assay system but failed to synergize with IL-2. IL-6 has been shown in other systems to induce IL-2 production, which might account for its IFN- $\gamma$ -inducing activity (28). Recently, IFN- $\gamma$ -inducing activity has also been described in the conditioned medium from galactose oxidase-treated monocytes (29, 30). The biologic activities of this factor are in part similar to those of NKSF. However, the distinct biochemical properties of the 70-kD NKSF clearly distinguish it from the monocyte-derived lymphocyte stimulator. Moreover, the latter factor does not synergize with IL-2, but rather appears to induce IFN- $\gamma$  indirectly, via induction of IL-2 (29).

The culture system that we have used to study the induction of IFN- $\gamma$  production by unfractionated lymphocytes is a complex biologic system with many possible direct and indirect interactions between different cells and cytokines (26, 27). The production of IFN- $\gamma$  in response to IL-2 requires HLA-DR $^+$  accessory cells (27, and our unpublished results) and is due largely to NK cells (26). Preliminary experiments with NKSF indicate that the action of this cytokine also requires an HLA-DR $^+$  accessory cell population and that both NK and T cells might produce IFN- $\gamma$  in response to NKSF, although the relative contribution of the two cell types remains to be established.

Several lymphokines enhance NK cell-mediated cytotoxicity. Among those identified in RPMI 8866 supernatant fluid, IFN (25) and, to a much lower extent, LT (31) are known to be NK cell activators. However, the absence of these lymphokines in purified NKSF preparations and the inability of specific inactivating antisera to block the NK cell-enhancing effect of both crude and purified NKSF preparations exclude a role for either IFN or LT in this effect of NKSF. IL-2 is also a potent activator of NK cell-mediated cytotoxicity (26, 32). Upon addition of IL-2 to cultures of resting PBL, a dose-dependent increase in spontaneous NK cytotoxicity is observed within a few hours; this increase both precedes and is independent of IFN- $\gamma$  production in the same cultures (26). NKSF was able to enhance spontaneous cytotoxicity of resting PBL at molar concentrations at least two orders of magnitude lower than the concentrations of IFN or IL-2 required to achieve similar responses. Preliminary observations suggest that the augmenting effect of NKSF on NK cell-mediated cytotoxicity, as in the case of IL-2, is mostly independent of the IFN- $\gamma$  produced in the culture. The relatively high concentrations of IL-2 required for both IFN- $\gamma$  production by PBL and augmentation of NK cell-mediated cytotoxicity is explained by the finding that IL-2 acts on resting PBL through the intermediate-affinity p70 chain of the IL-2 receptor and not through the high-affinity receptor that includes the p55 Tac chain (26, 33-35). The ability of NKSF to mediate the same biologic activities at a much lower concentration than IL-2 suggests the possi-

bility of a high-affinity NKSF receptor expressed on resting NK cells and possibly on all resting PBL.

NKSF alone is not mitogenic for resting PBL and does not synergize with rIL-2 in inducing proliferation. However, NKSF at very low molecular concentrations has a potent comitogenic effect with two T cell mitogens, PHA and PDBu. The effect of NKSF together with PHA is not demonstrable during the peak of the PHA response, but becomes significant at later times when PBL proliferation in cultures with PHA alone usually declines; in the presence of both PHA and NKSF, proliferation continues to increase for at least 6 d. The effect of NKSF added at the beginning of the culture is therefore observed after several days. By contrast, the comitogenic effect of NKSF with PDBu is clearly observed at both 3 and 6 d of culture. Preliminary characterization of the cells proliferating in response to PHA or PDBu, with or without NKSF, showed that virtually all of them are T cells. Because PHA and PDBu are mitogenic for T cells but not for NK cells, this finding suggests that the cellular specificity of the induced proliferation is determined by the specificity of the mitogens used. The comitogenic effect on T cells also confirms that, as in the case of induction of IFN- $\gamma$  production, NKSF acts not only on NK cells, but also has important regulatory functions on T lymphocytes. Further analyses should reveal whether NKSF is acting directly as a T cell growth factor or whether its growth-promoting effects are mediated by induction of secondary growth factor production in culture.

Lymphocyte proliferation in different systems has been shown to require IFN- $\gamma$  (7, 8) and IFN- $\gamma$  might play a role in the effect of NKSF on proliferation. By inducing IFN- $\gamma$ , and possibly other lymphokines, NKSF might expand the range of its possible biologic effects both in vitro and in vivo. Because NKSF has been identified at this time only as a product of in vitro established virus-transformed B cell lines, one can only speculate on its possible physiological role in the interaction of normal B cells with T and NK cells. In the mouse, activated spleen B cells induce NK cells to produce IFN- $\gamma$ , which inhibits the polyclonal Ig production induced by LPS (10). In nude mice IFN- $\gamma$  production by NK cells is a necessary requirement for a B cell response to thymus-independent antigens (36). Similarly, NK cells, probably through IFN- $\gamma$  production, are necessary for recovery of B cell function of patients in the early times after bone marrow transplantation (37). Induction of IFN- $\gamma$  production is therefore an important aspect of the functional interaction in vivo between activated B cells and both NK and T lymphocytes. The possibility then arises that normal B cells produce NKSF or other IFN- $\gamma$ -inducing factors.

Although NKSF has potent effects on NK cell cytotoxicity and on lymphocyte proliferation, it does not account completely for the various effects mediated by irradiated B lymphoblastoid cell lines on PBL cultures. Furthermore, we could not demonstrate NKSF production by the Daudi cell line, which is a powerful inducer of preferential NK cell proliferation in PBMC cultures (6). However, Daudi and other cell lines can induce NK cells to produce IFN- $\gamma$  when directly added to the culture, suggesting the possibility that NKSF is produced by these cell lines at a low concentration acting only on contiguous cells or that it is expressed in a non-secreted membrane-bound form, as shown for other cytokines such as IL-1 or TNF. Although NKSF alone does not account for the whole spectrum of stimulatory and

accessory functions of B cell lines added to PBL cultures, it may represent an important and possibly necessary mediator for some of these functions.

IL-2 can activate different lymphocyte populations to kill tumor cells both in animals and in humans (26, 32, 38-40). A variety of therapeutic approaches using IL-2 have been attempted, including treatment of patients with IL-2-activated lymphocytes obtained from ex vivo cultures of the patient's own lymphocytes in the presence of high concentrations of IL-2 (39). These approaches have occasionally shown some promise, but substantial improvement is clearly necessary before they have a significant impact on cancer therapy. The ability of NKSF to augment lymphocyte cytotoxicity, to induce IFN- $\gamma$  production, and to enhance proliferation of resting PBL at concentrations much lower than those at which IL-2 is effective, as well as the synergistic effect with IL-2 in some of these functions, might make this factor a promising candidate for clinical use, alone or in combination with other cytokines, in the therapy of cancer and other diseases.

### Summary

We have identified and purified a novel cytokine, NK cell stimulatory factor (NKSF), from the cell-free supernatant fluid of the phorbol diester-induced EBV-transformed human B lymphoblastoid cell line RPMI 8866. NKSF activity is mostly associated to a 70-kD anionic glycoprotein. The purified 70-kD protein, isolated from an SDS-PAGE gel, yields upon reduction two small species of molecular masses of 40 and 35 kD, suggesting that this cytokine is a heterodimer. When added to human PBL, purified NKSF preparations induce IFN- $\gamma$  production and synergize with rIL-2 in this activity, augment the NK cell-mediated cytotoxicity of PBL preparations against both NK-sensitive and NK-resistant target cell lines, and enhance the mitogenic response of T cells to mitogenic lectins and phorbol diesters. The three activities remain associated through different purification steps resulting in a 9,200-fold purification, and purified NKSF mediates the three biological activities at concentrations in the range of 0.1-10 pM. These data strongly suggest that the same molecule mediates these three activities, although the presence of traces of contaminant peptides even in the most purified NKSF preparations does not allow us to exclude the possibility that distinct biologically active molecules have been co-purified. The absence of other known cytokines in the purified NKSF preparations, the unusual molecular conformation of NKSF, the high specific activity of the purified protein, and the spectrum of biological activities distinguish NKSF from other previously described cytokines.

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