

# Induction of Interferon $\gamma$ Production by Natural Killer Cell Stimulatory Factor: Characterization of the Responder Cells and Synergy with Other Inducers

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## Summary

We previously reported that natural killer cell stimulatory factor (NKSF), a heterodimeric lymphokine purified from the conditioned medium of human B lymphoblastoid cell lines, induces interferon  $\gamma$  (IFN- $\gamma$ ) production from resting peripheral blood lymphocytes (PBL) and synergizes with interleukin 2 in this activity. In this study, we show that human NKSF induces IFN- $\gamma$  production from both resting and activated human PBL and from freshly isolated murine splenocytes. Human T and NK cells produce IFN- $\gamma$  in response to NKSF, but resting PBL require the presence of nonadherent human histocompatibility leukocyte antigens DR<sup>+</sup> (HLA-DR<sup>+</sup>) accessory cells to respond to NKSF. The mechanism(s) by which NKSF induces IFN- $\gamma$  production results in accumulation of IFN- $\gamma$  mRNA, is insensitive to cyclosporin A, and synergizes with those mediated by phytohemmagglutinin, phorbol diesters, anti-CD3 antibodies, and allogeneic antigens, but not by Ca<sup>2+</sup> ionophores. The ability of NKSF to directly induce IFN- $\gamma$  production and to synergize with other physiological IFN- $\gamma$  inducers, joined with the previously described ability to enhance lymphocyte cytotoxicity and proliferation, indicates that this lymphokine is a powerful immunopotentiating agent.

Natural killer cell stimulatory factor (NKSF)<sup>1</sup> is a cytokine that was identified and purified from the conditioned medium of B lymphoblastoid cell lines. The NKSF protein is a 70-kD disulfide-linked heterodimer composed of a 35-kD and a 40-kD chain. NKSF induces IFN- $\gamma$  production from PBLs, augments NK cell-mediated cytotoxicity, and enhances T cell proliferation induced by lectins and phorbol diesters (1). We have cloned and sequenced the two genes encoding NKSF and have shown that the biological activity of recombinant NKSF is identical to those we reported for purified natural NKSF, confirming that NKSF is a novel cytokine (1a). Recently, cytotoxic lymphocyte maturation factor (CLMF) was purified by Stern et al. (2) from the con-

ditioned medium of the human B lymphoblastoid cell line NC-37; this factor appears to have physicochemical characteristics, biological activity, and NH<sub>2</sub>-terminal amino acid sequences identical to those of NKSF.

Regulation of IFN- $\gamma$  production during inflammation or an immune response is of central importance to the mechanisms of both adaptive and nonadaptive resistance (3–6). Thus, the ability of NKSF to induce IFN- $\gamma$  production from PBL may represent one of the most biologically significant functions of this lymphokine. IFN- $\gamma$  is not constitutively produced by resting PBL. However, both NK and T cells are induced to secrete IFN- $\gamma$  in response to IL-2 stimulation (7–9). NKSF synergizes with IL-2 in inducing IFN- $\gamma$  production from PBL (1). T cells also produce IFN- $\gamma$  upon specific ligand interaction with TCR (7); this interaction can be partly replaced by antibodies to TCR or to the TCR-associated CD3 complex (10). NK cells produce IFN- $\gamma$  upon crosslinking of the type III receptor for Fc of IgG (Fc $\gamma$ RIII or CD16) (11). Similar to NKSF, TCR-CD3 and CD16 ligands syner-

<sup>1</sup> Abbreviations used in this paper: CLMF, cytotoxic lymphocyte maturation factor; CsA, cyclosporin A; EMC, encephalomyocarditis; F/H, Ficoll-Hypaque; LT, lymphotoxin; MLC, mixed leukocyte culture; NKSF, natural killer cell stimulatory factor; PDBu, phorbol dibutyrate; PKC, protein kinase C; TPA, tetradecanoylphorbol acetate.

gize with IL-2 to induce IFN- $\gamma$  production from T and NK cells, respectively. The effect of these IFN- $\gamma$  inducers can be mimicked by lectins such as PHA (12–14), by phorbol diesters (12, 15, 16), and by Ca<sup>2+</sup> ionophores (17, 18).

In this study, we have characterized the IFN- $\gamma$ -inducing activity of NKSF and compared it to other inducers of IFN- $\gamma$ . We report that human NKSF induces IFN- $\gamma$  production from resting and activated human T and NK cells, as well as from mouse lymphocytes. NKSF-induced IFN- $\gamma$  production in human PBL is mediated via a mechanism that is insensitive to cyclosporin A (CsA), synergizes with several other IFN- $\gamma$  inducers, and requires the participation of nonadherent HLA-DR<sup>+</sup> accessory cells.

## Materials and Methods

**Cytokines and Reagents.** rIL-2 (10<sup>7</sup> U/mg) was generously provided by Dr. T. Taguchi (Osaka University, Osaka, Japan) and Takeda Chemical Industrial Industry, Inc. (Osaka, Japan). The following reagents were purchased from commercial sources: PHA-M (Wellcome Diagnostics, Dartford, England); 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma Chemical Co., St. Louis, MO); phorbol-12,13-dibutyrate (PDBu; Chemsyn Science Laboratories, Lexena, KS); Ca<sup>2+</sup> ionophore A23187 (Sigma Chemical Co., St. Louis, MO); cyclosporin A (CsA; Sandoz Ltd., Vienna, Austria).

**Preparations of NKSF.** NKSF was purified as described in detail by Kobayashi et al. (1) from serum-free conditioned medium of 48-h PDBu-induced RPMI 8866 cell cultures. The IFN- $\gamma$  induction assay (described below) was used to quantitate NKSF activity in semi-purified and purified preparations. 1 U of NKSF activity is defined as the amount required to induce half-maximal IFN- $\gamma$  production. The purified NKSF preparation used in this study had a specific activity of 4  $\times$  10<sup>6</sup> U/mg protein. In many of the experiments described, we used semi-purified preparations of NKSF obtained after a two-step purification procedure: (a) hydroxylapatite chromatography and (b) Mono Q ion exchange chromatography performed as previously described (1).

**Antibodies.** mAbs B36.1 (anti-CD5, IgG2b), B67.1 (anti-CD2, IgG2a), B73.1 (anti-CD16, IgG1), B52.1 (anti-CD14, IgM), B33.1 (anti-HLA-DR, nonpolymorphic determinant, IgG2a), B133.1 and B133.5 (anti-IFN- $\gamma$ , IgG1) were produced and characterized in our laboratory. Antibody OKT3 (anti-CD3, IgG2a) was produced from hybrid cells purchased from American Type Culture Collection (ATCC) (Rockville, MD) and antibody 3G8 (anti-CD16, IgG1) was kindly provided by Dr. J. Unkeless (Mount Sinai School of Medicine, New York, NY). Culture supernatants or antibodies purified from ascitic fluids were used at predetermined optimal concentrations. Goat anti-IL-2 antiserum (>10<sup>5</sup> neutralizing U/ml) and the affinity-purified goat anti-mouse IgG were produced in our laboratory. An NKSF neutralizing antiserum was obtained from a rabbit immunized with the purified recombinant 40-kD chain of NKSF. FITC-labeled goat F(ab')<sub>2</sub> anti-mouse Ig was purchased from Cappel Laboratories (Cochranville, PA) and used after adsorption to human IgG.

**Cell Preparations.** Peripheral blood was obtained by venipuncture from adult healthy donors using heparin as anticoagulant. PBMC were separated on Ficoll-Hypaque (F/H) density gradient (Lymphoprep; Nyegaard and Co., Oslo, Norway). PBL were prepared from PBMC after partial depletion of monocytes by adherence to plastic (45 min, 37°C). Different PBL subsets and accessory cells were purified from the PBL preparations by (a) positive and negative selection, as indicated, using mAbs specific for leuko-

cyte differentiation antigens, indirect antiglobulin rosetting, and F/H gradient separation, as described (19), or (b) fluorescence-activated cell sorting with a FACS IV<sup>®</sup> flow cytometry system (Becton Dickinson & Co., Mountain View, CA), after staining by indirect immunofluorescence using FITC-labeled goat F(ab')<sub>2</sub> anti-mouse Ig. PHA blasts were prepared by culturing PBMC in 1% PHA-M for 5 d at a starting concentration of 10<sup>6</sup> cells/ml. NK cells (CD3<sup>-</sup> CD5<sup>-</sup> CD14<sup>-</sup>) and T cells (CD16<sup>-</sup> CD56<sup>-</sup> and CD14<sup>-</sup>) were purified by negative selection using the indirect antiglobulin rosetting method from 10-d cultures of PBMC with  $\gamma$ -irradiated (50 Gy) RPMI 8866 cells, as described (20). The human B lymphoblastoid cell line, RPMI 8866, was maintained in RPMI 1640 (Flow Laboratories Inc., Rockville, MD) supplemented with 10% FCS (Flow Laboratories Inc.); it was free of mycoplasma contamination on repeated testing. Murine splenocytes were obtained from spleens of 4–6-wk-old BALB/c female mice and depleted of erythrocytes by lysis with hypotonic medium. Surface Ig<sup>+</sup>-depleted cells were obtained by negative selection using antiglobulin rosetting and F/H gradient separation as described (21).

**IFN- $\gamma$  Induction Assay.** The different leukocyte preparations (10<sup>6</sup> cells/200  $\mu$ l/well) were incubated (37°C, 5% CO<sub>2</sub>) for 18 h with various inducers in round-bottomed 96-well microtiter plates (Costar, Cambridge, MA). Stimulation with anti-CD3 antibodies was performed in wells coated with various dilutions of purified OKT3 antibody. The antibody diluted in 100  $\mu$ l of 0.1 M carbonate buffer, pH 9.4, was incubated overnight at 4°C in flat-bottomed bacteriological 96-well microtiter plates; the wells were washed three times with PBS immediately before the different leukocyte preparations were added and the plates incubated (37°C, 5% CO<sub>2</sub>, 18 h). After incubation, triplicate 50  $\mu$ l of cell-free supernatant was collected from each well and IFN- $\gamma$  was measured as described below.

**Human Mixed Leukocyte Culture (MLC).** 5  $\times$  10<sup>4</sup> responder PBMC were mixed with 10<sup>5</sup> irradiated (45 Gy) stimulator PBMC from autologous or unrelated allogeneic donors in 200  $\mu$ l of RPMI 1640 supplemented with 10% heat-inactivated human AB serum in round-bottomed microtiter plates. After 6 d of incubation at 37°C, cell-free supernatant fluids were collected and IFN- $\gamma$  was measured by RIA.

**IFN Assays.** RIA for human IFN- $\gamma$  was performed as previously described (22). Sensitivity of the assay is 0.5 U/ml IFN- $\gamma$  and no crossreaction with TNF, lymphotoxin (LT), or other species of IFN is observed. Murine IFN- $\gamma$  was measured using a commercial ELISA (Amgen, Thousand Oaks, CA): sensitivity of the assay is 12 U/ml IFN- $\gamma$ . Antiviral activity in the cell-free supernatants of murine cell cultures was assessed by inhibition of the cytopathic effect of encephalomyocarditis (EMC) virus on the mouse fibroblast cell line, LF2, as described (23); assays were quantitated using the NIH mouse IFN reference as standard.

**In Situ Hybridization.** Cell preparations were deposited on microscope slides using a cytocentrifuge. <sup>35</sup>S-labeled riboprobes were prepared from plasmids containing a 5' 80-bp fragment of the human IFN- $\gamma$  cDNA inserted into the PstI restriction site of the pGem3 vector. Plasmids were linearized at the EcoRI and HindIII restriction sites and transcribed into mRNA-complementary (antisense) and mRNA-like (sense) probes using <sup>35</sup>S-UTP (New England Nuclear, Wilmington, DE) and a riboprobe kit (Promega Biotec, Madison, WI) according to the manufacturer's suggestions. Hybridization was carried out overnight at 45°C as described by Harper et al. (24). Slides were washed twice (10 min at room temperature) in 2 $\times$  SSC (1 $\times$  = 0.15 M NaCl, 0.015 M NaCitrate, pH 7.0) and four times (15 min, 55°C) in 0.25 $\times$  SSC, 1 mM EDTA, 1 mM DTT. Nonhybridized probe was digested with 40  $\mu$ g/ml RNase

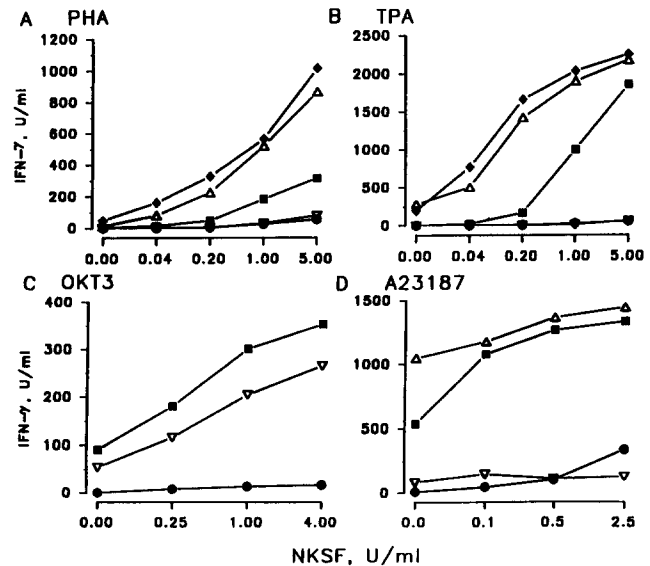
A (Sigma Chemical Co.) in 10 mM tris, pH 8, 0.3 M NaCl for 30 min at 37°C. The slides were then washed (30 min, 55°C) twice in 50% formamide, 2× SSC, 1 mM DTT, and finally, in 2× SSC, 1 mM DTT. After dehydration in graded ethanol solutions containing 0.3 M ammonium acetate and air drying, the slides were dipped in NTB3 nuclear track emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with 0.6 M ammonium acetate. After exposure for 3–7 d at 4°C, the slides were developed with Kodak D-19 developer, fixed with Kodak Rapidfix, and counterstained with hematoxylin and eosin.

**Northern Blot Hybridization.** Cytoplasmic RNA samples were prepared from induced and uninduced cells using the NP-40 lysis method as described (25). RNA samples were fractionated in a 1% agarose-formaldehyde gel. Briefly, the fractionated RNA was transferred to nylon membrane filters (Schleicher & Schuell, Inc., Keene, NH) by capillary action and crosslinked to filters by UV irradiation. The human IFN- $\gamma$  cDNA probe (restriction fragment at the PstI site from a construct in pSWIF) and the human  $\beta$ -actin cDNA probe in pBR322 (grown in plasmid form, kindly provided by Dr. Roberto Weinmann, Wistar Institute), were labeled with  $^{32}\text{P}$ - $\alpha$ CTP ( $\sim 3000$  Ci/mmol; New England Nuclear) using a random priming kit (Amersham Corp., Arlington Heights, IL). Filter-bound RNA was hybridized according to Singh and Jones (26) at 42°C for 16 h with labeled probe at  $1\text{--}2 \times 10^6$  cpm/ml hybridization solution: 4× SET (0.1 M NaCl, 6 mM tris-Cl, pH 7.5, 0.4 mM EDTA, pH 7.4), 0.1% sodium pyrophosphate, 0.2% SDS, 10% dextran sulfate, 500  $\mu\text{g}/\text{ml}$  heparin sodium salt grade II from porcine intestinal mucosa (Elkins-Sinn, Inc., Cherry Hill, NJ), and 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA. After hybridization, the filters were washed three times at 37°C with 2× SSC, 0.1% SDS, once at 65°C with 0.1× SSC, 0.1% SDS for 45 min, and then exposed to X-Omat AR film (Eastman Kodak Co.) between double intensifying screens (DuPont Co., Wilmington, DE) at  $-70^\circ\text{C}$ .

## Results

**IFN- $\gamma$  Production by PBL Induced with NKSF.** We (1) previously reported that purified NKSF induces IFN- $\gamma$  production in freshly isolated PBL in a dose-dependent manner and synergizes with rIL-2 in this activity. To determine if NKSF synergizes with other inducers of IFN- $\gamma$ , PBL were incubated (18 h) with increasing concentrations of purified NKSF in the absence or presence of PHA, phorbol diesters,  $\text{Ca}^{2+}$  ionophores, or anti-CD3 (OKT3) antibodies; IFN- $\gamma$  was quantitated by RIA in the cell-free supernatants. NKSF, in a dose-dependent manner, synergized with PHA (Fig. 1 A), the phorbol diesters TPA (Fig. 1 B) and PDBu (not shown), and anti-CD3 antibodies (Fig. 1 C) at all concentrations tested, to induce IFN- $\gamma$  secretion. rIL-2, which synergizes with NKSF in inducing IFN- $\gamma$  production, did not synergize with PHA or phorbol diesters (not shown). The  $\text{Ca}^{2+}$  ionophores, A23187 (Fig. 1 D) and ionomycin (not shown), efficiently synergized with phorbol diesters in inducing IFN- $\gamma$  production, but the same concentrations did not synergize with NKSF and inhibited IFN- $\gamma$  production induced with high NKSF concentrations.

To determine whether PHA or TPA synergizes with NKSF via the induction of IL-2 production, PBL were stimulated with NKSF, alone, or in combination with PHA or TPA,

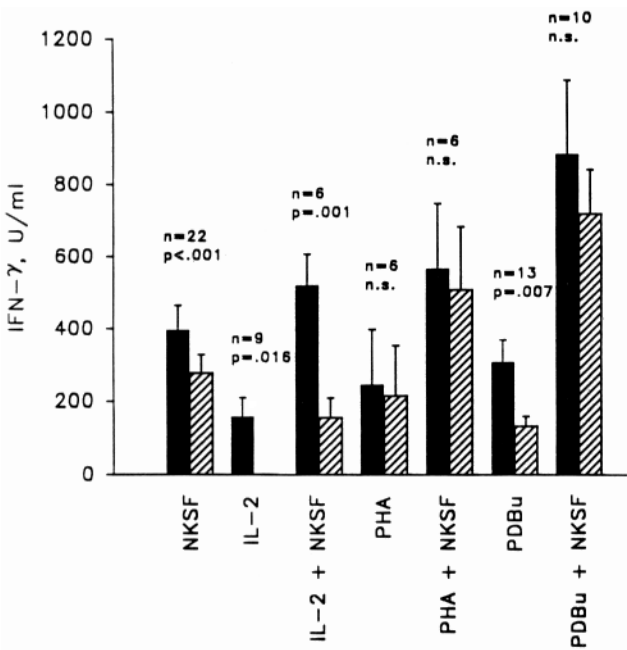


**Figure 1.** IFN- $\gamma$  production by PBL induced with NKSF. Cells were incubated with the indicated concentrations of purified NKSF for 18 h at 37°C in the absence (●) or presence of (A) PHA at (▽) 0.4  $\mu\text{g}/\text{ml}$ ; (■) 1.1  $\mu\text{g}/\text{ml}$ , (△) 3.3  $\mu\text{g}/\text{ml}$ , (◆) 10  $\mu\text{g}/\text{ml}$ ; (B) TPA at (▽)  $10^{-10}$  M, (■)  $10^{-9}$  M, (△)  $10^{-8}$  M, (◆)  $10^{-7}$  M; (C) OKT3 plated at (▽) 0.2  $\mu\text{g}/\text{well}$ , (■) 0.8  $\mu\text{g}/\text{well}$ ; and (D) (▽) 0.2  $\mu\text{g}/\text{ml}$  A23187, (■)  $10^{-7}$  M PDBu, (△)  $10^{-7}$  M PDBu and 0.5  $\mu\text{g}/\text{ml}$  A23187. IFN- $\gamma$  was measured by RIA in cell-free supernatant fluids.

in the presence of a goat anti-IL-2 antiserum (Fig. 2). The antiserum completely suppressed IFN- $\gamma$  production in response to 100 U/ml of rIL-2 and partially (22%), but significantly reduced IFN- $\gamma$  production induced by NKSF alone. IFN- $\gamma$  production in response to NKSF and rIL-2 was inhibited to the levels observed with NKSF alone. No inhibition of IFN- $\gamma$  production by anti-IL-2 antiserum was observed when PBL were stimulated with PHA, with or without NKSF, or with phorbol diesters plus NKSF. However, significant inhibition was observed when PBL were stimulated by phorbol diesters alone. Preimmune goat serum did not inhibit IFN- $\gamma$  production induced by any of the stimuli used (not shown).

To determine whether CsA, which suppresses anti-CD3 and lectin-induced IFN- $\gamma$  and IL-2 production (27) in T cells, affects NKSF-induced IFN- $\gamma$  production, PBL were stimulated with NKSF and other stimuli in the presence of CsA. As expected, CsA (1  $\mu\text{g}/\text{ml}$ ) significantly inhibited IFN- $\gamma$  production in PBL stimulated with anti-CD3 (OKT3) antibodies but not with rIL-2 (Table 1). At the same concentration, CsA did not inhibit IFN- $\gamma$  production induced by NKSF with or without rIL-2 (Table 1), but partially inhibited IFN- $\gamma$  production induced by PHA or TPA, alone or in synergy with NKSF (not shown).

**Neutralization of IFN- $\gamma$ -inducing Activity by Rabbit Antiserum Against Recombinant NKSF.** Results in Table 2 show that a 1:160 dilution of the anti-40-kD chain antiserum completely blocks the IFN- $\gamma$  production induced by semi-purified NKSF, alone or in synergy with rIL-2, without significantly affecting



**Figure 2.** Effect of goat anti-IL-2 on IFN- $\gamma$  induction by NKSF in PBL. PBL were incubated for 18 h at 37°C with 2.5 U/ml semi-purified NKSF alone or in combination with 100 U/ml rIL-2, 10  $\mu$ g/ml PHA, or  $10^{-7}$  M PDBu in the absence (solid bars) or in the presence (hatched bars) of a 1/100 dilution of goat anti-human IL-2 antiserum. IFN- $\gamma$  production was measured by RIA. Results are expressed as mean  $\pm$  SE of results obtained with *n* different donors. *p* values (IFN- $\gamma$  production in cultures in the absence versus presence of anti-IL-2) were calculated using the Student *t* test.

IFN- $\gamma$  production induced by rIL-2. Preimmune serum had no effect. These results show that NKSF is the only factor with IFN- $\gamma$ -inducing activity present in the semipurified preparations of NKSF.

**Table 1.** Effect of CsA on IFN- $\gamma$  Production by Human PBL

Inducer*	IFN- $\gamma$ (U/ml)		<i>p</i> <sup>†</sup>
	No CsA	CsA (1 $\mu$ g/ml)	
None	11.1 $\pm$ 6.4 <sup>§</sup>	2.33 $\pm$ 1.3	NS
Anti-CD3 (1 $\mu$ g/well)	1,567.3 $\pm$ 244.7	268.2 $\pm$ 152.5	0.01
rIL-2 (100 U/ml)	1,298.7 $\pm$ 379.9	1,052.5 $\pm$ 235.1	NS
NKSF (2.5 U/ml)	1,482.3 $\pm$ 374.6	1,183.0 $\pm$ 267.0	NS
NKSF + rIL-2	3,023.3 $\pm$ 328.2	2,997.7 $\pm$ 190.2	NS

\* IFN- $\gamma$  was measured by RIA in the cell-free supernatant fluids collected from PBL cultured (18 h, 37°C) in the presence of the indicated inducers, with or without CsA.

<sup>†</sup> *p* of the comparison between IFN- $\gamma$  levels in cell-free supernatant fluids from PBL cultured with or without CsA, Student *t* test. NS, not significant, *p* > 0.05.

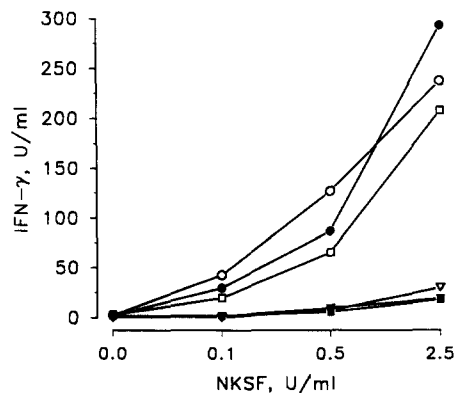
<sup>§</sup> Mean  $\pm$  SD of results obtained with three different PBL preparations.

**Table 2.** Inhibition of NKSF Activity by Rabbit Antiserum Against Recombinant NKSF 40-kD Chain

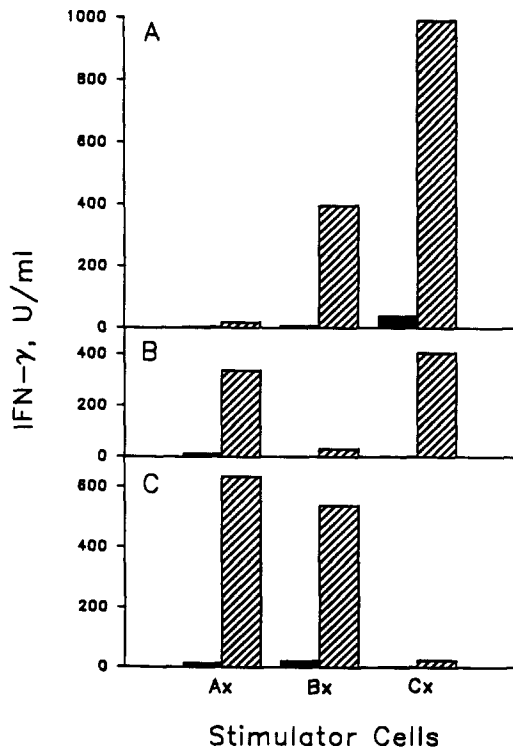
Inducer*	IFN- $\gamma$		
	Medium	Preimmune Serum	Anti-40-kD Serum
		U/ml	
None	<0.5	1.9	<0.5
rIL-2, 100 U/ml	3.4	3.0	4.0
NKSF <sup>†</sup> , 1 U/ml	28.0	47.0	<0.5
rIL-2 + NKSF	744.0	540.0	20.0

\* PBL from one donor were incubated 18 h at 37°C with the indicated inducer in the presence or not of a 1:160 dilution of preimmune rabbit serum or rabbit antiserum against purified recombinant 40-kD chain of NKSF. The results are representative of four experiments performed. <sup>†</sup> The NKSF used was a semi-purified preparation, as described in Materials and Methods.

**Requirement of Accessory Cells for IFN- $\gamma$  Production by PBL in Response to NKSF.** Plastic adherent PBMC, total nonadherent PBL, and the HLA-DR<sup>+</sup> and DR<sup>-</sup> PBL populations prepared by indirect rosetting were cultured with increasing concentrations of NKSF. As shown in Fig. 3, similar levels of IFN- $\gamma$  were produced by PBL or by PBL depleted of monocytes after a second adherence or by removal of CD14<sup>+</sup> cells. PBL depleted of HLA-DR<sup>+</sup> cells produced significantly lower levels of IFN- $\gamma$ , but removal of CD20<sup>+</sup>, CD21<sup>+</sup> B cells from PBL did not result in decreased IFN- $\gamma$  production in response to NKSF (not shown). Adherent cells, containing >90% CD14<sup>+</sup> monocytes (28), were unable to reconstitute IFN- $\gamma$  production (Fig. 3). IFN- $\gamma$  production was restored



**Figure 3.** Requirement of HLA-DR<sup>+</sup> accessory cells for IFN- $\gamma$  production by PBL in response to NKSF. The different subpopulations were separated from nonadherent PBMC by indirect rosetting and incubated with the indicated concentrations of semi-purified NKSF for 18 h at 37°C. (○) PBL, (●) CD14<sup>-</sup> cells, (□) HLA-DR<sup>-</sup> cells with added 10% HLA-DR<sup>+</sup> cells, (▽) HLA-DR<sup>-</sup> cells, (▼) HLA-DR<sup>-</sup> and 10% adherent PBMC, and (■) HLA-DR<sup>+</sup> cells.



**Figure 4.** Effect of NKSF on IFN- $\gamma$  production in MLC. PBMC were cultured with irradiated PBMC ( $10^5/200 \mu\text{l}$ ) from autologous or allogeneic donors for 6 d at 37°C in the absence (solid bars) or presence (hatched bars) of 0.5 U/ml purified NKSF. Cell-free supernatant fluids were collected and IFN- $\gamma$  was measured by RIA. Results obtained with responder cells from three donors, A, B, and C, are shown in A, B, or C, respectively. Ax, Bx, Cx indicate the irradiated stimulator cells from the same three donors.

only when the HLA-DR<sup>-</sup> population was reconstituted with 10% nonadherent HLA-DR<sup>+</sup> cells. Neither the nonadherent HLA-DR<sup>+</sup> cells nor the adherent cell populations produced IFN- $\gamma$  when stimulated with NKSF. Northern blotting (not shown) of RNA extracted from HLA-DR<sup>-</sup> and HLA-DR<sup>+</sup> cells, purified by indirect rosetting from total PBL after stimulation for 18 h with NKSF, showed IFN- $\gamma$  mRNA accumulation only in the HLA-DR<sup>-</sup> subset, demonstrating that the HLA-DR<sup>-</sup> cells are the INF- $\gamma$ -producing cells, and that the HLA-DR<sup>+</sup> cells act as accessory cells.

**Production of IFN- $\gamma$  in Human MLC in the Presence of NKSF** NKSF was added to MLC at the beginning of culture and IFN- $\gamma$  in the supernatants was measured after 6 d (Fig. 4). In the absence of added NKSF, cultures stimulated by allogeneic but not autologous cells produced IFN- $\gamma$  at levels lower than 40 U/ml. In the presence of NKSF, allogeneic cultures produced between 300 and 1,000 U/ml of IFN- $\gamma$ , whereas autologous cultures produced no more than 30 U/ml of IFN- $\gamma$ . In addition, when cells collected from a primary MLC at day 14 were restimulated with the specific allogeneic cells in a secondary MLC, INF- $\gamma$  production in cultures stimulated in the presence of NKSF were higher than in control cultures (not shown).

**Table 3.** IFN- $\gamma$  mRNA Accumulation in Peripheral Blood T and NK Cells as Detected by In Situ Hybridization

PBL subpopulation*	Inducer	Percent positive	
		cells (>10 grains/cells) <sup>†</sup>	Grains per positive cell
Total	None	0	mean $\pm$ SD N/A
CD16 <sup>+</sup>	NKSF (2.5 U/ml)	1.8	38.3 $\pm$ 25.5
CD16 <sup>-</sup>	NKSF (2.5 U/ml)	1.9	64.7 $\pm$ 38.1
CD3 <sup>+</sup>	NKSF (2.5 U/ml)	1.6	52.6 $\pm$ 29.7
CD3 <sup>-</sup>	NKSF (2.5 U/ml)	6.2	57.7 $\pm$ 34.1
CD16 <sup>+</sup>	rIL-2 (100 U/ml)	4.1	24.2 $\pm$ 36.7
CD16 <sup>-</sup>	rIL-2 (100 U/ml)	2.1	51.5 $\pm$ 67.6
CD3 <sup>+</sup>	rIL-2 (100 U/ml)	0.9	53.3 $\pm$ 50.0
CD3 <sup>-</sup>	rIL-2 (100 U/ml)	1.7	30.9 $\pm$ 20.5
CD16 <sup>+</sup>	NKSF + rIL-2	10.3	81.5 $\pm$ 56.8 <sup>§</sup>
CD16 <sup>-</sup>	NKSF + rIL-2	14.2	89.4 $\pm$ 67.4 <sup>§</sup>
CD3 <sup>+</sup>	NKSF + rIL-2	6.4	82.7 $\pm$ 69.0 <sup>§</sup>
CD3 <sup>-</sup>	NKSF + rIL-2	16.2	70.6 $\pm$ 45.2 <sup>§</sup>

\* PBL were cultured for 18 h at 37°C with the indicated inducers and the different subsets were separated by fluorescence activated cell sorting after indirect immunofluorescence with antibodies anti-CD3 (OKT3) or anti-CD16 (3G8) and FITC-labeled goat F(ab')<sub>2</sub> anti-mouse Ig. Sorted cells were cytocentrifuged on slides for in situ hybridization.

<sup>†</sup> 150 cells in three replicate slides hybridized with antisense <sup>35</sup>S-labeled IFN- $\gamma$  riboprobe were counted. Control hybridization slides with sense probe showed  $2.2 \pm 2.1$  grains/cells, and no cells with more than 10 grains/cells.

<sup>§</sup> A proportion of cells contained > 100 grains per cell and their exact number could not be accurately determined. These cells have been included in the calculation of average values as having 100 grains/cell; therefore, these numbers represent a minimum estimate of the average number of grains per cell.

**IFN- $\gamma$  Production by Peripheral Blood T and NK Cells Stimulated with NKSF.** Preparations enriched in T and NK cells were obtained from PBL by positive or negative selection using anti-CD3 or anti-CD16 antibodies and indirect rosetting. The cells were stimulated with NKSF, alone or in combination with rIL-2. Positively selected cells with either antibody were poor producers of IFN- $\gamma$ , probably because of the absence of accessory cells in the preparations. However, when HLA-DR<sup>+</sup> accessory cells were added to the enriched cell preparations, similar levels of IFN- $\gamma$  were detected in both the enriched T (CD3<sup>+</sup> or CD16<sup>-</sup>) and NK cell (CD3<sup>-</sup> or CD16<sup>+</sup>) preparations (not shown).

IFN- $\gamma$  production in PBL was analyzed at the single cell level by in situ hybridization. Total PBL were stimulated for 18 h with NKSF, rIL-2, or NKSF plus rIL-2. CD3<sup>+</sup> or CD3<sup>-</sup> and CD16<sup>+</sup> or CD16<sup>-</sup> cell populations were separated by fluorescence activated cell sorting after staining by indirect immunofluorescence with antibodies OKT3 and 3G8, respectively. Accumulation of IFN- $\gamma$  mRNA in these populations was analyzed by in situ hybridization (Table 3). IFN- $\gamma$

**Table 4.** IFN- $\gamma$  Production by Cultured T and NK Cells

Inducer*	IFN- $\gamma$	
	T cells <sup>†</sup>	NK cells
	U/ml	
None	1.9 $\pm$ 0.5 <sup>§</sup>	0.8 $\pm$ 0.4
rIL-2, 100 U/ml	77.3 $\pm$ 17.5	74.0 $\pm$ 41.0
NKSF, 1 U/ml	44.0 $\pm$ 10.5	41.0 $\pm$ 22.5
rIL-2 + NKSF	875.3 $\pm$ 63.0	930.0 $\pm$ 116.0

\* Cells were cultured ( $5 \times 10^6$ /ml, 200  $\mu$ l/well) with the indicated inducers for 18 h at 37°C, and IFN- $\gamma$  in the cell-free supernatant fluid was quantitated by RIA.

<sup>†</sup> T cells (CD16<sup>-</sup>, CD56<sup>-</sup>, CD14<sup>-</sup>) and NK cells (CD3<sup>-</sup>, CD5<sup>-</sup>, CD14<sup>-</sup>) were purified using indirect antiglobulin rosetting and gradient centrifugation from 10-d culture of PBL stimulated with irradiated RPMI 8866 cells. T cells preparation were >95% pure and NK cell preparation >98% pure.

<sup>§</sup> Mean  $\pm$  SD of three experiments.

mRNA was detected in a small proportion of T and NK cells induced with NKSF or rIL-2 (Table 3). The number of positive cells (> 10 grains per cell) in cultures treated with NKSF or rIL-2 alone ranged from 1.5 to 6.5%; the number of positive cells detected in cultures from both cell types treated with NKSF plus rIL-2 ranged from 7.5 to 16%. In addition, the highly positive cells in cultures stimulated with NKSF and rIL-2 contained a much greater number of grains than the highly positive cells in cultures stimulated with NKSF or rIL-2 alone (not shown).

Lastly, IFN- $\gamma$  production was measured in T and NK cell preparations purified from 10-d cocultures of PBL with the irradiated B lymphoblastoid cell line, RPMI-8866; these populations were treated with NKSF, in the absence or presence of rIL-2, for 18 h. As shown in Table 4, both T and NK cells produced IFN- $\gamma$  in response to NKSF and/or rIL-2 stimulation.

**IFN- $\gamma$  mRNA Accumulation in PBL and PHA-activated Blasts Stimulated with NKSF.** To determine if resting and activated lymphocytes respond to NKSF with the same kinetics, we tested the ability of NKSF to induce IFN- $\gamma$  in PBL versus PHA-activated T cell blasts. PHA blasts contain >98% CD3<sup>+</sup> T cells with variable proportions of CD4<sup>+</sup> and CD8<sup>+</sup> cells, as detected by indirect immunofluorescence. More than 90% of these cells are CD25<sup>+</sup>, CD71<sup>+</sup>, and HLA-DR<sup>+</sup> (not shown); PHA blasts do not proliferate but accumulate in the G1 phase of the cell cycle after 5 d in culture. However, they are rapidly induced to proliferate in response to IL-2 (not shown).

PBL and PHA blasts were stimulated for 1, 2, 4, and 24 h in the presence of NKSF and/or rIL-2. Cytoplasmic RNA, extracted at each time point, was probed for IFN- $\gamma$  mRNA by Northern blot hybridization and IFN- $\gamma$  was measured in

**Table 5.** NKSF-induced Production of IFN- $\gamma$  by Total and sIg<sup>-</sup> Mouse Splenic Lymphocytes

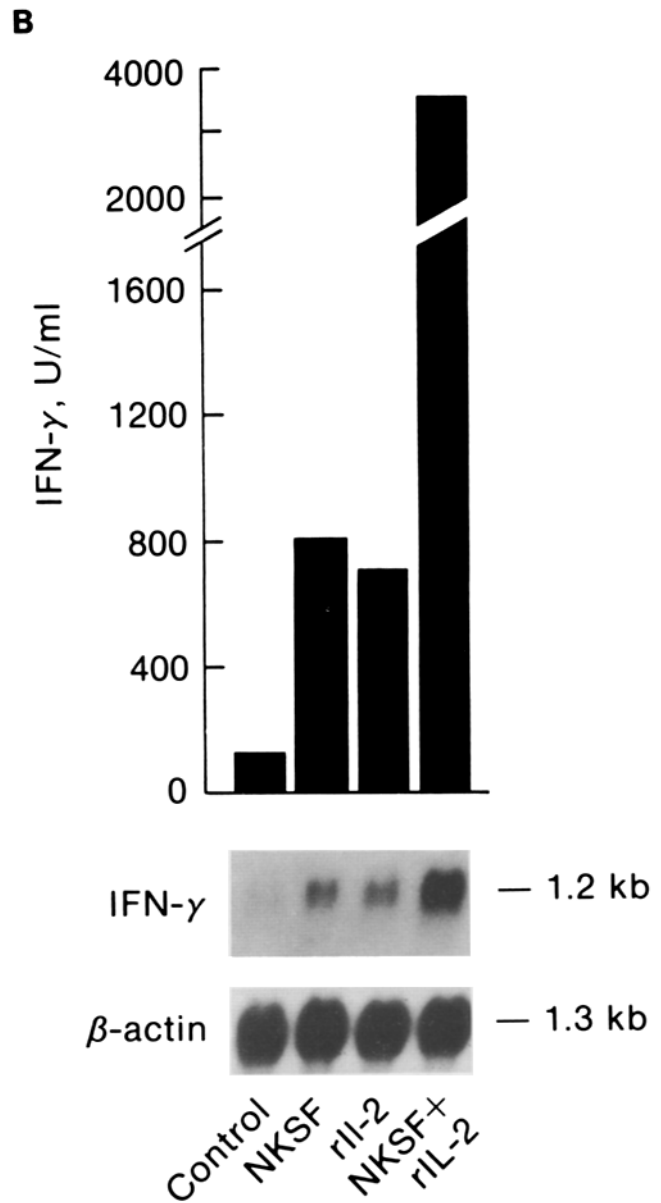
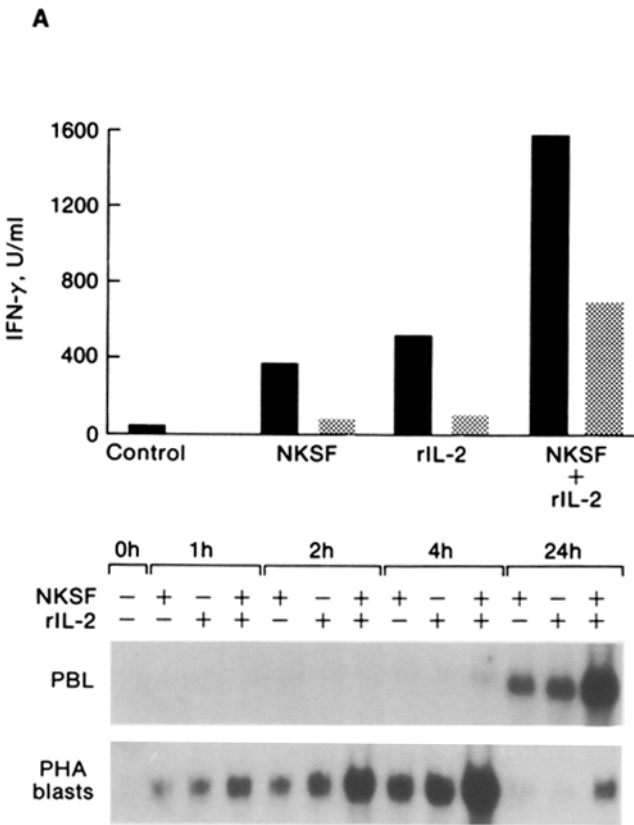
NKSF*	rIL-2 (10 <sup>3</sup> U/ml)	Antiviral activity (Producer cells)			sIg <sup>-</sup> spleno- cytes
		Total splenocytes			
		Exp. 1	Exp. 2	Exp. 3	
		U/ml <sup>†</sup>			
None	-	0	0	0	4
	+	8	4	4	2
0.2 U/ml	-	8	2	8	2
	+	32	32	8	32
0.02 U/ml	-	8	8	8	32
	+	64	32	128	64
$2 \times 10^{-3}$ U/ml	-	0	0	0	2
	+	32	2	16	16
$2 \times 10^{-4}$ U/ml	-	0	0	0	2
	+	16	2	2	4

\* Total unseparated or sIg<sup>-</sup> splenocytes from BALB/c mice ( $5 \times 10^6$  cells/ml) were incubated for 18 h at 37°C in the presence of the indicated inducers.

<sup>†</sup> Antiviral activity in the cell-free supernatant fluids was measured by inhibition of the cytopathic effect of EMC virus on LF<sub>2</sub> murine fibroblasts.

the cell-free supernatant fluids of the 24-h cultures. NKSF, alone or in combination with rIL-2, induced PHA blasts to secrete IFN- $\gamma$  albeit at consistently lower levels than observed with PBL (Fig. 5 A). Both PBL and PHA blasts accumulated IFN- $\gamma$  mRNA in response to NKSF and/or rIL-2 stimulation, although the activated cells displayed significantly earlier kinetics of mRNA accumulation than PBL (Fig. 5 A). On average, IFN- $\gamma$  mRNA was detectable in PHA blasts after 1 h of stimulation and reached peak levels 4–8 h post-induction whereas maximal levels of IFN- $\gamma$  mRNA were detected in PBL after 24-h culture. Identical results were obtained in PHA blasts using purified (Fig. 5 B) NKSF at 4 h of induction.

**IFN- $\gamma$  Production by Human NKSF-treated Mouse Lymphocytes.** To test whether human NKSF is effective across species barriers on mouse lymphocytes, total splenocytes and spleen cells depleted of surface Ig<sup>+</sup> (sIg<sup>+</sup>)-B cells by antiglobulin indirect rosetting and density gradient were obtained from adult BALB/c mice and incubated for 18 h with increasing concentrations of human NKSF, alone or in combination with 1,000 U/ml of human rIL-2. The supernatants were tested for antiviral activity, as measured by inhibition of the cytolytic effect of EMC virus on mouse fibroblast LF<sub>2</sub> cells (Table 5) and for IFN- $\gamma$  concentration by ELISA (not shown). NKSF induced antiviral activity and IFN- $\gamma$  production by both total and sIg<sup>-</sup> splenocytes in a dose-dependent manner and synergized with rIL-2 in this effect.



**Figure 5.** IFN- $\gamma$  mRNA accumulation in PBL and PHA-activated blasts stimulated with NKSF. (A) (Top panel) PBL (solid bars) and PHA blasts (dotted bars) were incubated with 2.5 U/ml semi-purified NKSF, 100 U/ml rIL-2, or NKSF plus rIL-2. Cell-free supernatants were assayed for IFN- $\gamma$  by RIA. (Bottom panel) RNA were extracted from cells at various time points after incubation with NKSF and/or rIL-2 and analyzed by Northern blot using a cDNA probe for human IFN- $\gamma$ . (B) PHA blasts ( $5 \times 10^6$ /ml) were stimulated for 18 h with 1.25 U/ml purified NKSF, 100 U/ml rIL-2, or NKSF plus rIL-2. Cell-free supernatants were assayed for IFN- $\gamma$  production by RIA. Cytoplasmic RNA were analyzed by Northern blot.

## Discussion

The possibility that human B lymphoblastoid cell lines produce factor(s) affecting lymphocyte functions was suggested by their ability to facilitate IL-2-dependent proliferation of both T and NK cells *in vitro* (29–31). During studies in which lymphotoxin production by these cell lines was characterized (22), we identified a novel cytokine that we defined NKSF (1). NKSF was purified 9,200-fold to near homogeneity from the supernatant fluid of phorbol diester-induced cells of the EBV-transformed human lymphoblastoid cell line, RPMI 8866, on the basis of its ability to induce IFN- $\gamma$  production by human PBL (1).

NKSF, when added to freshly isolated nonadherent PBL in an 18-h culture, induces IFN- $\gamma$  production in a dose-dependent manner that is only partially inhibited (22% on average) by anti-IL-2 antiserum. In addition, as reported ear-

lier, NKSF strongly synergizes with IL-2 in inducing IFN- $\gamma$  at all concentrations of rIL-2 tested (1). These observations exclude the possibility that IFN- $\gamma$  is indirectly induced by NKSF via the induction of IL-2. Since IFN- $\gamma$  production induced by NKSF was partially inhibited by anti-IL-2 antiserum, this inhibition is probably due to minimal concentrations of IL-2 produced constitutively by PBL which enhances the IFN- $\gamma$  production induced by NKSF. We did not detect significant IL-2 message by standard Northern blotting techniques in induced and uninduced lymphocytes. NKSF also synergizes with other IFN- $\gamma$  inducers such as PHA and phorbol diesters; the quantities of IFN- $\gamma$  induced in these cultures range between 3–10-fold over levels expected for an additive effect. In addition, endogenously produced IL-2 does not appear to be responsible for this synergy. However, a partial

inhibition of the phorbol diester-induced IFN- $\gamma$  production by anti-IL-2 antiserum suggests that IL-2 production may play some role in inducing IFN- $\gamma$  production in these cultures.

Solid-phase linked anti-CD3 antibodies induce IFN- $\gamma$  production in PBL and this effect is synergistic with NKSF during an 18-h culture. The ability of NKSF to synergize with signals transduced through the TCR is also indicated by the powerful enhancement of IFN- $\gamma$  production observed in allogeneic but not in autologous MLC in the presence of NKSF. A signal transduction pathway activated through TCR-CD3 results in increased  $[Ca^{2+}]_i$  and protein kinase C (PKC) activation (32). The  $Ca^{2+}$  ionophores, A23187 and ionomycin, which are strongly synergistic with phorbol diesters (33), do not synergize with NKSF, suggesting that mechanisms other than increased  $[Ca^{2+}]_i$  are responsible for the synergistic effect of TCR-CD3 stimulation with NKSF. Since anti-CD3 antibodies, antigens, and PHA (34) act via the TCR-CD3 complex, and phorbol diesters activate PKC (35), a major mechanism by which these inducers synergize with NKSF may be through activation of PKC. The mechanism of synergy between NKSF and IL-2 is, however, probably dependent on other pathways of signal transduction. Indeed, we (Chan, S.H., M. Kobayashi, D. Santoli, B. Perussia, and G. Trinchieri, manuscript submitted for publication) have observed major differences in the molecular mechanisms by which IL-2 and phorbol diesters synergize with NKSF in inducing IFN- $\gamma$ .

CsA has been proposed to suppress early events governing T cell activation, such as IL-2 transcription, by inhibiting the activity of DNA binding proteins in response to antigen receptor triggering (27). CsA does not inhibit IFN- $\gamma$  production by PBL induced with NKSF or IL-2, alone or in combination, but almost completely abolishes the IFN- $\gamma$  production induced by anti-CD3 and partially inhibits the IFN- $\gamma$  production induced by PHA or phorbol diester, as well as the synergy between NKSF and these inducers. Taken together, these results suggest that NKSF functions through a different cell surface receptor and intracellular signal transduction pathway than each of the inducers tested. The ability of human NKSF to induce IFN- $\gamma$  production by murine splenocytes suggests that receptors crossreacting with the human cytokine are also present on murine lymphocytes.

Previous studies have shown that T and NK cells are the only producers of IFN- $\gamma$  during an immune response (7-9). NKSF induces IFN- $\gamma$  from both T and NK cells and synergizes with rIL-2 in both cell types. To mediate this activity on fresh PBL, NKSF requires the presence of nonadherent HLA-DR<sup>+</sup> accessory cells. If HLA-DR<sup>-</sup> cells are reconstituted with HLA-DR<sup>+</sup> cells, which do not make IFN- $\gamma$ , they become responsive to NKSF. The accessory cells are not monocytes because monocyte-depleted cells can still respond to NKSF and HLA-DR<sup>-</sup> cells reconstituted with adherent monocytes do not produce IFN- $\gamma$  when stimulated with NKSF. This observation is consistent with those by others (36-41) which demonstrate that IFN- $\gamma$  induction by other stimuli also requires the presence of HLA-DR<sup>+</sup> accessory cells. Various cell types have been implicated as accessory cells.

Antigen (42) or mitogen-induced (36, 43) activation of T lymphocytes generally requires the participation of accessory cells such as macrophages/monocytes (37, 38), dendritic cells (39, 44), or fibroblasts (40). Additionally, production of IFN- $\gamma$  from resting T cells and CD16<sup>+</sup> large granular lymphocytes in response to IL-2 stimulation requires the presence of HLA-DR<sup>+</sup> accessory cells; these cells have been described as nonadherent, esterase-negative monocytes and/or dendritic cells (41). The nature of the accessory cells in our system is unclear. Dendritic cells are necessary for specific antigen-induced IFN- $\gamma$  production by T cells (our unpublished observation); they are loosely adherent cells and are present in the adherent leukocyte population that is unable to support NKSF action. We had identified a nonadherent HLA-DR<sup>+</sup> light density cell type which, in addition to producing IFN- $\alpha$  in response to virus infection, acts as accessory cells for NK cell cytotoxicity against virus-infected cells (45, 46). The reported characteristics of these cells are consistent with those of the accessory cells required for NKSF activity. The role played by the accessory cells is unclear. We have not determined whether NKSF acts on freshly isolated PBL directly (i.e., via receptors for NKSF expressed on the IFN- $\gamma$ -producing cells which require additional signals from HLA-DR<sup>+</sup> cells), or indirectly (i.e., via receptors for NKSF expressed on HLA-DR<sup>+</sup> accessory cells which in turn activate the IFN- $\gamma$ -producing cells). However, the apparent lack of accessory cell requirement in activated T or NK cell preparations and, more convincingly, the ability of a leukemic T cell line to respond to NKSF (Chan, S.H., M. Kobayashi, D. Santoli, B. Perussia, and G. Trinchieri, manuscript submitted for publication) strongly suggest that NKSF acts directly on the IFN- $\gamma$ -producing cells.

NKSF induces IFN- $\gamma$  production in both T and NK cells, as clearly shown by the results obtained using enriched and purified preparations of the two cell types from both fresh and cultured PBL. The results of *in situ* hybridization performed on T (CD3<sup>+</sup> CD16<sup>-</sup>) and NK (CD3<sup>-</sup> CD16<sup>+</sup>) cells, positively and negatively selected from PBL after stimulation with NKSF and/or IL-2, confirm the ability of both cell types to respond to NKSF. T and NK cells stimulated with NKSF plus IL-2 contain much higher numbers of grains per cell than cells stimulated with either cytokine alone, suggesting that synergy between NKSF and IL-2 is largely due to the synergistic accumulation of IFN- $\gamma$  mRNA in individual cells. A higher number of cells accumulating IFN- $\gamma$  mRNA is observed, however, in samples stimulated with both inducers than with one alone. This observation may depend on the sensitivity of the detection system which may not have detected cells expressing very low levels of IFN- $\gamma$  mRNA in cells induced with one lymphokine alone.

The observation, by *in situ* hybridization analysis, that <20% of PBL accumulate detectable levels of IFN- $\gamma$  mRNA in response to NKSF and/or IL-2, suggests that only a small proportion of PBL are induced to accumulate mRNA and secrete IFN- $\gamma$  at a given time. Our results are consistent with those of Wilson et al. (41), who demonstrated through immunofluorescent staining for IFN- $\gamma$  that only 5 to 20% of

normal lymphocytes synthesize IFN- $\gamma$  in primary cultures with rIL-2. Qui et al. (13) also showed that <20% of PBMC accumulated IFN- $\gamma$  mRNA when stimulated with different stimuli, as detected by in situ hybridization. The observation that only a small proportion of lymphocytes respond to different stimuli with IFN- $\gamma$  production is difficult to explain. At present, no specific subset of human T and NK cells has been demonstrated to preferentially produce IFN- $\gamma$ . The small proportion of IFN- $\gamma$ -producing cells may be due to discrete PBL subsets which express receptors for the various IFN- $\gamma$  inducers, a heterogeneity in the ability of PBL to express receptors or to produce IFN- $\gamma$ , or a proportion of activated lymphocytes present in PBL which either express increased numbers of receptors or an increased efficiency in IFN- $\gamma$  production. The latter possibility is unlikely, however, because similarly small proportions of IFN- $\gamma$ -producing cells were detected by in situ hybridization of PHA-activated blasts stimulated with NKSF and IL-2 (our unpublished observation).

Although both quiescent PBL and PHA-activated blasts are stimulated by NKSF to produce IFN- $\gamma$ , overall IFN- $\gamma$  production is lower in PHA blasts than in fresh PBL. However, PHA blasts respond to NKSF accumulating IFN- $\gamma$  mRNA with significantly faster kinetics than fresh lymphocytes. Our results are consistent with studies performed by Siggins et al. (47) who compared the kinetics of IFN- $\gamma$  mRNA accumulation between fresh PBMC and cultured lymphoblasts after stimulation with staphylococcal enterotoxin A and mezerein and showed that growing lymphoblasts achieve peak IFN- $\gamma$  mRNA levels much earlier than resting lymphocytes. The lower production of IFN- $\gamma$  by PHA blasts in the presence of rapid and abundant accumulation of IFN- $\gamma$  mRNA suggests that, in addition to transcriptional or post-transcriptional mechanisms that regulate mRNA accumulation, IFN- $\gamma$  production and secretion could be differentially regulated by translational or posttranslational mechanisms.

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