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Production of Natural Killer Cell Stimulatory Factor (Interleukin 12) by Peripheral Blood Mononuclear Cells

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Summary

Natural killer cell stimulatory factor (NKSF), or interleukin 12 (IL-12), is a 70-kD heterodimeric cytokine composed of two covalently linked chains, p40 and p35. NKSF/IL-12 has multiple effects on T and NK cells and was originally identified and purified from the supernatant fluid of Epstein-Barr virus (EBV)-transformed human B lymphoblastoid cell lines. We have produced a panel of monoclonal antibodies against both chains of NKSF/IL-12. Some of these antibodies have neutralizing activity, and several combinations of them have been used to establish sensitive radioimmunoassays detecting the free p40 chain, the free p35 chain, or the p70 heterodimer. Using these reagents, we have determined that most EBV-transformed human B lymphoblastoid cell lines constitutively produce low levels of the p70 heterodimer and an excess of the free p40 chain, whereas Burkitt lymphoma–derived, T, myeloid, and many solid tumor–derived cell lines produce neither. Production of both p40 and p70 is increased several-fold upon stimulation of the EBV-transformed cell lines with phorbol diesters. The ability of supernatant fluids from unstimulated and phorbol diester–stimulated cell lines to induce interferon γ (IFN-γ) production from T and NK cells, one of the effects of NKSF/IL-12, parallels the levels of production of the p70 heterodimer, known to be the biologically active form of NKSF/IL-12. *Staphylococcus aureus* Cowan I strain (SAC) and other stimuli induce accumulation of p40 mRNA and production of both p40 and p70 by peripheral blood mononuclear cells (PBMC). The producer cells appear to include both adherent cells and nonadherent lymphocytes, possibly B cells. The supernatant fluids from SAC-stimulated PBMC mediate the typical functions of NKSF/IL-12 (i.e., IFN-γ induction, mitogenic effects on T/NK blasts, enhancement of NK cell cytotoxicity) at concentrations of p70 similar to those at which recombinant NKSF/IL-12 mediates the same functions. Moreover, these activities are significantly inhibited by anti-NKSF/IL-12 antibodies. The neutralizing anti-NKSF/IL-12 antibodies also inhibit 85% of the IFN-γ production in response to SAC, an NKSF/IL-12 inducer, and approximately 50% of the IFN-γ production in response to non-NKSF/IL-12-inducers such as IL-2, phytohemaglutinin, and anti-CD3 antibodies. These results indicate that induced or constitutively produced NKSF/IL-12 has a major role in facilitating IFN-γ production by peripheral blood lymphocytes. Our findings that NKSF/IL-12 is both spontaneously produced and inducible in adherent PBMC and lymphocytes suggest that NKSF/IL-12 might be a major physiological regulator of T and NK cell function during an immune response and inflammation.

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Natural killer cell stimulatory factor (NKSF)[2] is a heterodimeric cytokine originally identified and purified from the conditioned medium of EBV-transformed human B lymphoblastoid cell lines (1). The terms cytotoxic lymphocyte maturation factor (CLMF) and IL-12 have also been used to describe this cytokine (2, 3). The genes encoding the two chains of NKSF/IL-12 (p40 and p35) have been cloned in humans (3, 4) and recently in the mouse (5). The two chains are covalently linked to form a biologically active p70 heterodimer, whereas no biological activity has been shown to be mediated by either recombinant chain separately (4). However, B cell lines, producing biologically active NKSF/IL-12 p70, also produce a large excess of free p40 chain, the exact significance of which is not known (1, 3). The p35 chain has a structure similar to other α helix–rich cytokines, whereas the p40 chain has homology not with other cytokines but with the extracellular portion of proteins of the hemopoietin receptor family, particularly with the receptor for ciliary neurotrophic factor and with the receptor for IL-6, suggesting that the two chains of NKSF/IL-12 might be derived from a primordial cytokine-receptor complex (5–7).

NKSF/IL-12 has multiple effects on both T and NK cells. Specifically, NKSF/IL-12: (*a*) induces production of IFN-γ and other cytokines (e.g., TNF) by both resting and activated T and NK cells (1, 8, and Chehimi, J., N. M. Valiante, A. D'Andrea, M. Rengaraju, Z. Rosado, M. Kobayashi, B. Perussia, S. F. Wolf, S. E. Starr, and G. Trinchieri, manuscript submitted for publication); (*b*) synergizes with other IFN-γ inducers (e.g., IL-2, mitogenic lectins, anti-CD3 antibodies, alloantigenic stimulation, and phorbol diesters) in inducing IFN-γ gene expression acting both at the transcriptional and posttranscriptional levels (8, 9); (*c*) enhances the cytotoxic activity of both resting NK and T cells (1, 10, and Chehimi et al., manuscript submitted for publication); (*d*) directly induces and synergizes with IL-2 in the generation of lymphokine-activated killer (LAK) cells (2); (*e*) has a comitogenic effect with mitogenic lectins, anti-CD3, antigenic stimulation, and phorbol diesters on the proliferation of resting T cells (1, 10a); (*f*) directly induces proliferation of activated T and NK cells (10a, 11); and (*g*) antagonizes the IL-2-induced proliferation of NK cells and both normal and leukemic T γ/δ cells with a mechanism dependent on TNF production (10a).

Although expression of p40 and p35 mRNA has been detected in normal murine tissues (5), nothing was known about the ability of normal cells to produce the biologically active NSKF/IL-12. In this paper, we utilize newly generated mAbs against either the p40 or the p35 chains of NKSF/IL-12 to investigate the production of NKSF/IL-12 by normal human peripheral blood cells. Using these antibodies, RIAs detecting the NKSF/IL-12 free p40 chain, the free p35 chain, or the p70 heterodimer have been established. Most EBV-transformed cell lines, constitutively or upon phorbol-diester stimulation, produce the biologically active p70 and usually a large excess of the free p40 chain, whereas production of the free p35 chain was not demonstrated. PBMC are induced to produce high levels of both the free p40 chain and the p70 heterodimer by stimulation with fixed *Staphylococcus aureus* Cowan I strain (SAC); a lower constitutive production was also observed. Both adherent cells and monocyte-depleted nonadherent lymphocytes produced NKSF/IL-12 in response to SAC stimulation. The NKSF/IL-12 produced by PBMC has biological activities similar to those of recombinant or EBV-transformed cell line–derived NKSF/IL-12, e.g., IFN-γ induction, enhancement of NK cell cytotoxicity, and mitogenic effects on T and NK cells. The use of neutralizing antibodies against NKSF/IL-12 allowed us to demonstrate that constitutive or induced NKSF/IL-12 production plays a major role in the IFN-γ production by PBMC stimulated by various IFN-γ inducers.

Materials and Methods

NKSF/IL-12 Preparations and Assay. Recombinant NKSF/IL-12 p40 and p35 chains were produced as IlvE fusion proteins and purified by SDS-PAGE as described (4). Recombinant NKSF/IL-12 free p40 chain was also purified from the supernatant fluid of Cos-1 cells transfected with p40 cDNA, as described (4). The cDNAs encoding the p40 and p35 subunits of NKSF were subcloned into the mammalian expression vector pEMC-DHFR (12) to generate plasmids useful for transfecting monkey Cos-1 cells. Cells were transfected with either cDNA or with a combination of the two; biological NKSF/IL-12 activity was observed only in the supernatants of the Cos-1 cells transfected with both cDNA (4). Pure recombinant NKSF, in biologically active heterodimeric p70 form, was purified from the supernatant of stably transfected CHO cells.

NKSF activity was measured in the IFN-γ induction assay (1), as well as in the blast proliferation assay (11) and the NK cell cytotoxicity enhancement assay (1). 1 U of NKSF/IL-12 is defined as the concentration of NKSF/IL-12 inducing half-maximal induction of IFN-γ production and was determined to correspond to 3.6 pM of purified RPMI 8866–derived NKSF/IL-12 (1). The supernatant fluids of Cos-1 cells transfected with both cDNAs contained between 300 and 1,000 U/ml of NKSF/IL-12. The purified CHO cell–derived rNKSF/IL-12 had a specific activity of 5×10^6 U/mg.

mAb Preparation. 6-wk-old female BALB/c mice were immunized subcutaneously in CFA with 5–15 μg of rNKSF/IL-12 or isolated chains, followed by two to three weekly injections of antigen subcutaneously in IFA. The animals were then injected intravenously with the same amount of purified proteins in saline, and after 3 d the animals were killed, a spleen cell suspension was prepared, and then was fused with the BALB/c myeloma cell line PX63.Ag8.6.5.3. The following antigens were used: purified *Escherichia coli*–derived p40 (fusions C2 and C3); purified *E. coli*–derived p35 (fusion C7); purified Cos-1 cell–derived p40 (fusion C8); and purified CHO cell–derived rNKSF/IL-12 p70 heterodimer (fusion C11). Supernatant fluids of growing hybrid cells were screened for production of anti-NKSF/IL-12 antibodies by indirect RIA. Antigens (1 μg/ml purified recombinant proteins or 3 U/ml semipurified RPMI 8866–produced NKSF/IL-12) were added to 96-well vinyl plates (Serocluster; Costar, Cambridge, MA) at 100 μl/well in 0.1 M carbonate buffer, pH 9.5. After overnight incubation, the plates were washed with PBS containing 0.05% Tween 20 (Bio-Rad Laboratories, Richmond, CA), 100 μl of supernatant fluid from hybrid cells were added, and antibody binding was detected by using

[2] *Abbreviations used in this paper:* NKSF, natural killer cell stimulatory factor; PDBu, phorbol dibutyrate; SAC, *Staphylococcus aureus* Cowan I strain.

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an affinity-purified ^{125}I-labeled goat IgG anti–mouse Ig as second antibody.

Positive wells were cloned by limiting dilutions. Isotypes of the antibodies were determined by immunodiffusion in agar using 20-fold concentrated supernatant fluids and isotype-specific antisera (Sigma Chemical Co., St. Louis, MO). All the anti-NKSF/IL-12 antibodies described in this paper are of the IgG1 isotype. Antibodies were purified from ascites fluid using a protein G column (Pharmacia-LKB, Piscataway, NJ).

Double Determinant RIAs. Anti-NKSF/IL-12 antibodies were absorbed (5 μg/ml, 100 μl/well, in 0.1 M carbonate buffer, pH 9.5) for 24 h or longer at 4°C to 96-well vinyl plates. The plates were washed three times with PBS 0.05% Tween-20, dilutions of standard antigen solutions or supernatant fluids to be tested were added to the plates (100 μl/well, 18 h, 4°C), the plates were again washed, and 0.1 μg/ml of a different mAb, labeled with ^{125}I (chloramine T method, 1 mCi ^{125}I/20 μg of protein) was added to the plates. After an 18-h incubation at 4°C, the plates were washed, dried, the wells were removed with a hot wire, and radioactivity was quantitated in a gamma counter (Packard Instruments Co., Downers Grove, IL). In addition to the antibodies described in this paper, the rat IgG mAb 20C2, reacting with NKSF/IL-12 heterodimer but not with isolated p35 or p40 chains (Chizzonite et al., manuscript in preparation), was utilized as a ^{125}I-labeled antibody in an RIA.

Cell Lines and PBMC Preparations. The human EBV-transformed B lymphoblastoid cell lines RPMI 8866, RPMI 7766, RPMI 1788, NC37, Bristol 8, CESS, and SK-W64 (all obtained from American Tissue Culture Collection [ATCC], Rockville, MD), RA-EB, RW-EB, and SF-EB (established in our laboratory); the Burkitt lymphoma cell lines Raji and Daudi, the T leukemia cell line Jurkat J32, and the myeloid leukemia cell lines HL-60 and U937 were grown in RPMI 1640 supplemented with 10% FCS (Flow Laboratories, Inc., Rockville, MD). For stimulation of NKSF/IL-12 production by phorbol diester, the cell lines were incubated (24 h, 37°C) at 10^6 cells/ml in serum-free medium containing or not 10^{-7} M phorbol dibutyrate (PDBu; Chemsy Science Lab, Lexeno, KS). The cell-free supernatant fluids used for biochemical or immunological analyses were in some experiments concentrated 10-fold under pressure using a Diaflo ultrafiltration system with PM10 membranes (Amicon, Danvers, MA). Supernatant fluids from PDBu-stimulated cells, used in IFN-γ induction assays, were dialyzed for 24 h against several changes of 0.15 M NaCl followed by 24-h dialysis against RPMI 1640. Using [^{3}H]PDBu as a tracer, this treatment was previously shown to be able to reduce the concentration of PDBu to levels without any detectable effects in the various biological assays (1).

Peripheral blood obtained from healthy donors was anticoagulated with heparin. PBMC were separated on Ficoll-Hypaque (F/H) density gradient (Lymphoprep; Nyegaard and Co., Oslo, Norway). Lymphocytes (PBL) were obtained after adherence of PBMC to plastic flasks (1 h, 37°C). Adherent cells were obtained by scraping the flasks with a rubber policeman after carefully rinsing out nonadherent cells with three washings with PBS; these preparations contained >95% CD14$^+$ monocytes. In some experiments, monocyte-free PBL preparations were obtained after two adherence cycles on plastics, sensitization with the IgM mAb B52.1 (anti-CD14), elimination of agglutinated cells by filtration through nylon wool, and depletion of the remaining antibody-sensitized CD14$^+$ monocytes by indirect rosetting with antiglobulin-coated sheep erythrocytes and F/H gradient separation, as described (13). These PBL preparations contained <1% (usually 0.1–0.5%) monocytes, as determined on each preparation by nonspecific esterase staining.

PHA blasts were from 6-d PBL cultures (10^6 cells/ml) containing 1% PHA-M (Wellcome Diagnostics, Dartford, England). These preparations were composed of >98% T cells, as determined by immunofluorescence with antileukocyte subset mAb.

NK cells (blasts) were purified using indirect antiglobulin rosetting and negative selection (using mAb OKT3 [anti-CD3], B36.1 [anti-CD5], and B52.1 [anti-CD14]) from PBMC cultured for 8 d with 50-Gy irradiated RPMI 8866 cells, as described (14). These preparations contained >95% CD3$^-$, CD16$^+$, and/or CD56$^+$ NK cells.

Stimulation of NKSF/IL-12 production from peripheral blood cells with fixed SAC was performed by adding 0.0075% (wt/vol) of SAC (Pansorbin; Calbiochem-Behring Co., La Jolla, CA) to the cells (10^6/ml), followed by an 18–24-h incubation at 37°C. Cell- and SAC-free medium was collected by centrifugation and filtering through 0.2-μm filters. This procedure completely removed SAC, and in control experiments no residual SAC activity in any of the biological or immunological assays was determined.

For stimulation of IFN-γ, the different inducers were added to PBL, PHA blasts, or purified NK cells (5 × 10^6/ml, 200 μl/well in 96-well plates) for 18 h, then the supernatant fluids were collected, and IFN-γ release was determined in triplicates using an IFN-γ-specific RIA, as described (15). The following inducers were used: various supernatant fluid containing NKSF/IL-12; various preparations of natural and recombinant NKSF/IL-12, rIL-2 (100 U/ml, 10^7 U/mg, kindly provided by Dr. J. Taguchi, Osaka University, Osaka, Japan), OKT3 IgG2a anti-CD3 antibody (3 μg/ml absorbed to flat-bottomed 96-well plates, Costar); SAC (0.0075%; wt/vol); and PHA-M (1%).

The ability of NKSF/IL-12 and IL-2 to induce proliferation of PHA blasts and NK cells was analyzed in a 48-h [^{3}H]thymidine incorporation assay as described (10a). The NK cell–mediated cytotoxicity-enhancing activity of NKSF/IL-12 was tested by incubating PBL for 18 h with various concentrations of NKSF/IL-12-containing supernatant fluids; PBL were then washed and tested against ^{51}Cr-labeled Daudi target cells, as described (1). In some experiments sheep anti-IFN-α serum (Interferon Sciences Inc., New Brunswick, NJ), cow anti-IFN-β serum (kindly provided by Dr. Jan Vilček, New York University), or goat anti-IL-2 (produced in our laboratory) were added to PBL during the 18-h culture before the NK cell–mediated cytotoxicity assay.

Northern Blot Hybridization. Northern blots were performed as previously described (16). Total RNA was extracted from induced and uninduced cell lines and peripheral blood cells by the guanidine isothiocyanate method. Equal amounts of RNA (15 μg/lane) were size fractionated in a 1% agarose-formaldehyde gel. 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 transferred to nitrocellulose paper was then hybridized to ^{32}P-cDNA probes for NKSF/IL-12 p40 and β-actin. The filters were autoradiographed and quantitated by densitometry on the exposed film and the values of the induced cells were normalized with those of the uninduced cells on the basis of hybridization with the β-actin probe.

SDS-PAGE and Western Blotting. 1 ml of 10-fold concentrated serum-free supernatant fluids from uninduced and induced RPMI 8866 cells (PDBu stimulation) or PBMC (SAC stimulation) were immunoprecipitated with mAb C11.79 coupled to Sepharose 4B (Pharmacia/LKB). Immunoprecipitated material was loaded onto 5–20% continuous gradient polyacrylamide slab gels and SDS-PAGE was performed according to the method of Laemmli (17). After electrophoresis, resolved proteins were transferred to nitrocellulose paper using a semi-dry transblotter (Bio-Rad Laboratories). NKSF/

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IL-12 p70 and NKSF/IL-12 p40 were detected by incubation with ^{125}I-labeled anti-NKSF/IL-12 mAb C8.6 followed by autoradiography.

Results

Production of mAbs against Human NKSF/IL-12 and Development of Specific Quantitative RIAs. After immunization of BALB/c mice with various preparations of NKSF/IL-12 p35, p40, and p70, a large panel of mAbs was obtained. The reactivity of some of these antibodies is illustrated in Fig. 1. By indirect RIA, the C2 and C3 series of mAbs, generated using *Escherichia coli*–derived p40 chain as antigen, react with both *E. coli*–derived and Cos-derived p40, as well as with CHO-derived p70. The C8 series of antibodies, generated using as antigen Cos-derived p40, have a reactivity similar to that of the C2 and C3 antibodies, with Cos-derived p40 and CHO-derived p70, but do not detect *E. coli*–derived p40 (Fig. 1). The C11.5 and C11.79 antibodies, generated against CHO-derived p70, react with the p40 chain and have a reactivity similar to that of the C8 series (not shown). The antibodies of the C7 series, generated using as antigen *E. coli*–derived p35, react with *E. coli* p35 and with the p70 heterodimer, but not with either p40 preparation (Fig. 1). The low or absent reactivity of C7 antibodies against semipurified RPMI 8866–derived nNKSF/IL-12 (Fig. 1 *D*) is probably due to the fact that this preparation contains prevalently the free p40 chain and a lower concentration of the p70 heterodimer.

None of the antibodies produced against *E. coli*–derived p40 or p35 had a neutralizing activity, whereas a proportion of the mAbs generated using as antigens Cos-derived p40 or CHO-derived p70 were able to neutralize the biological activities of NKSF/IL-12. Fig. 2 illustrates the ability of two of these neutralizing mAbs, C8.1 and C8.6, to inhibit three different biological activities of rNKSF/IL-12, i.e., IFN-γ induction, mitogenic effects on PHA blasts, and enhancement of NK cell–mediated cytotoxicity. Both antibodies neutralized all three biological activities, but C8.6 was more efficient at lower concentrations than C8.1. By competitive binding, it was demonstrated that C8.1 and C8.6 bind to two different determinants on the p40 chain (data not shown). Similar neutralizing activity was mediated by C8.1 and C8.6 mAbs on natural and recombinant NKSF/IL-12 (data not shown).

To establish the specificity of the neutralizing effect of mAb C8.6, its ability to inhibit proliferation of PHA blasts and NK cells (blasts) induced by rNKSF/IL-12 and rIL-2 was assayed (data not shown). The mitogenic effect of rNKSF/IL-12 on both T and NK cells was completely abolished by 10 μg/ml of C8.6, whereas no effect was observed on IL-2-induced proliferation.

All possible combinations of >30 mAbs were tested in double-determinant RIA for detection of NKSF/IL-12 or its chains. Several combinations were effective, and the most sensitive and useful combinations are illustrated in Fig. 3. The combination of the two anti-NKSF/IL-12 p40 antibodies, C11.79 and ^{125}I-labeled C8.6, detects both the free p40 chain and the p70 heterodimer with approximately equal efficiency. The combination of the anti-p40 mAb C8.3 and ^{125}I-C8.6

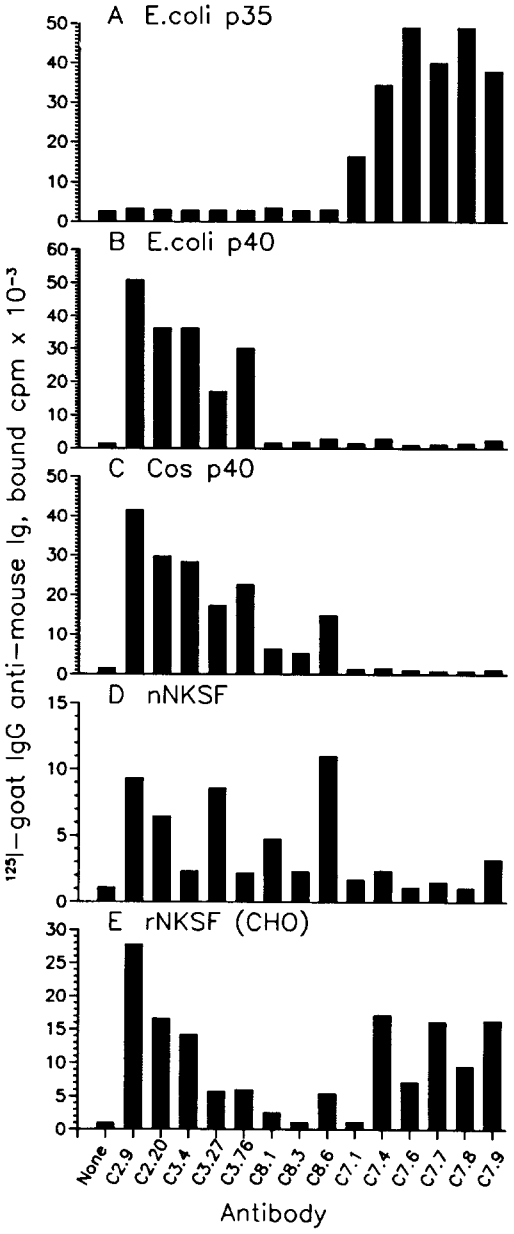

Figure 1. Indirect RIA of representative anti-NKSF/IL-12 mAb tested against various preparations of NKSF/IL-12 or its free chains. Purified *E. coli*–derived p35 chain (*A*), *E. coli*–derived p40 chain (*B*), Cos-derived p40 chain (*C*), semipurified RPMI 8866–derived nNKSF/IL-12 (both p40 and p70) (*D*), and pure CHO-derived rNKSF p70 heterodimer (*E*) were adsorbed to 96-well vinyl plates, and binding of the indicated mAb was detected with a second ^{125}I-labeled affinity-purified goat antibody anti–murine Ig.

detects only the free p40 chain and not the p70 heterodimer, probably due to the inability of C8.3 to efficiently bind to the p40 chain when complexed with the p35 chain, as indicated by the results of indirect RIA in Fig. 1. A combination of two anti-p35 mAbs, C7.4 and ^{125}I-C7.8, was very sensitive in detecting the free p35 chain, but not the p70 heterodimer. All of the combinations of anti-p35 mAb with anti-p40 mAb were tested in an attempt to obtain a RIA specific

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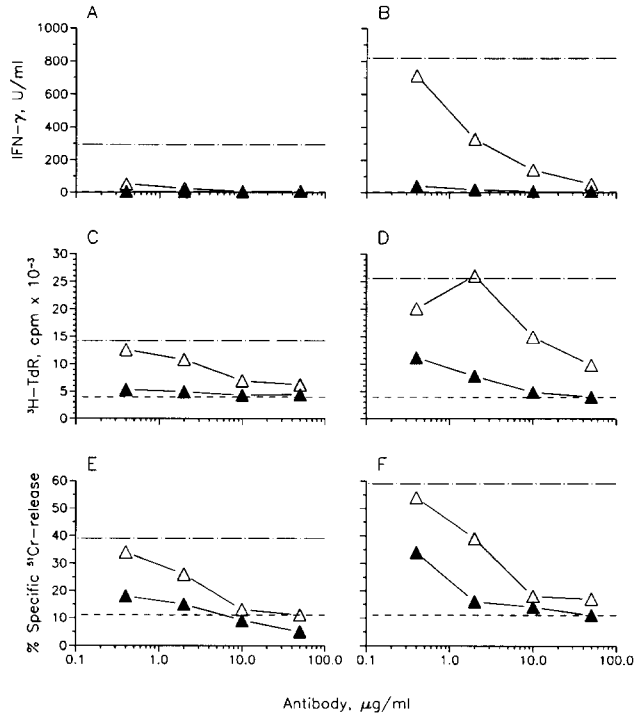

Figure 2. Ability of anti-NKSF/IL-12 mAb to neutralize rNKSF/IL-12 biological activities. The indicated concentrations of purified IgG1 mAbs C8.1 (△) and C8.6 (▲) were added to rNKSF/IL-12 (0.2 ng/ml, *A*, *C*, and *E*; 1 ng/ml, *B*, *D*, and *F*), and the ability of rNKSF/IL-12 to induce IFN-γ production from PBL (*A* and *B*), induce proliferation of PHA blasts (*C* and *D*), and enhance PBL-mediated spontaneous cytotoxicity of Daudi-target cells (E/T cell ratio of 50:1, *E* and *F*) was tested as described. In each panel the dashed line indicates the activity of cells not treated with rNKSF/IL-12, and the dash-dot-dash line indicates the activity of cells treated with rNKSF/IL-12 in the absence of mAb.

for the p70 heterodimer, but unexpectedly, none of these combinations were effective. This was surprising because in indirect RIA (Fig. 1) or by Western blotting (not shown) most of these antibodies were able to react with the p70 heterodimer. However, the combination of the anti-p40 mAb C11.5 with the ^{125}I-labeled mAb 20C2 (reacting with the p70 heterodimer but not with either free chain; Chizzonite et al., manuscript in preparation), was relatively specific for the p70 heterodimer. A limited crossreactivity with free p40 chains was observed, however, with a plateau at 1,000–4,000 cpm reached at concentrations of p40 >1 ng/ml. To quantitate p70 in the presence of free p40, this RIA was performed in parallel with the C8.3/^{125}I-C8.6 RIA to measure free p40. The counts per minute obtained in the C11.5/^{125}I-20C2 assay were corrected by subtracting the crossreactive amount expected on the basis of the determined free p40 chain concentration and used to determine p70 concentration. Because of this necessary correction, the assay was only able to accurately quantitate p70 in the presence of free p40 when the concentration of p70 was higher than at least 100 pg/ml. A 10-fold concentration of the supernatant fluid from cell lines or peripheral blood cells was usually required in order to quantitate p70.

Production of NKSF/IL-12 by Human Cell Lines. The supernatant fluids of a large number of cell lines were analyzed for production of the free p40 chain and the p70 heterodimer. Results with representative cell lines are illustrated in Fig. 4. Most EBV-transformed human B cell lines produced the free p40 chain and this production was significantly enhanced by stimulation with phorbol diesters. Only a low level of p70 was constitutively produced by RPMI 8866 and NC37 cell lines, but a larger group of EBV-transformed B cell lines produced p70 upon phorbol diester stimulation. Although

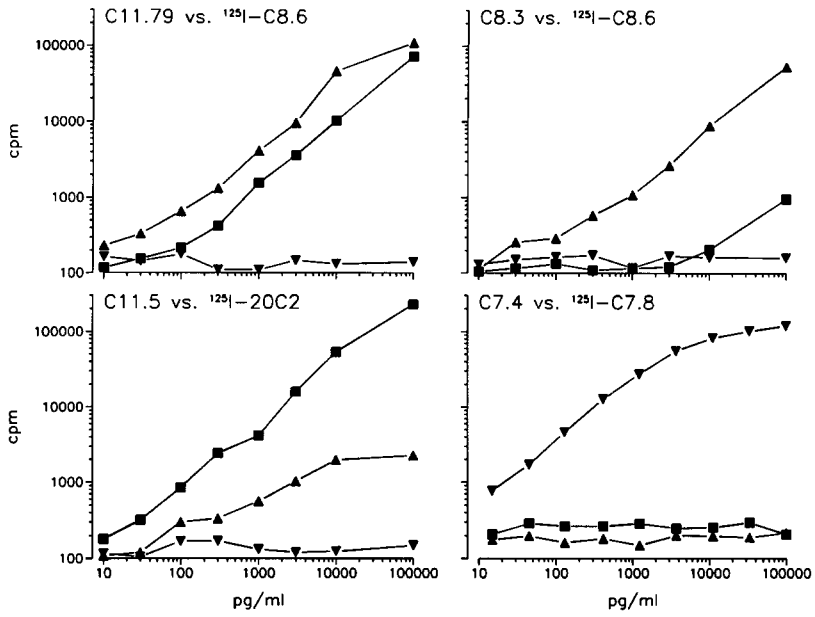

Figure 3. Double determinant RIAs with anti-NKSF/IL-12 antibodies. The indicated different pairs of mAbs were used in RIA as described using as standards purified CHO-derived p70 NKSF/IL-12 heterodimer (■), Cos-derived p40 (▲), and *E. coli*-derived p35 (▼).

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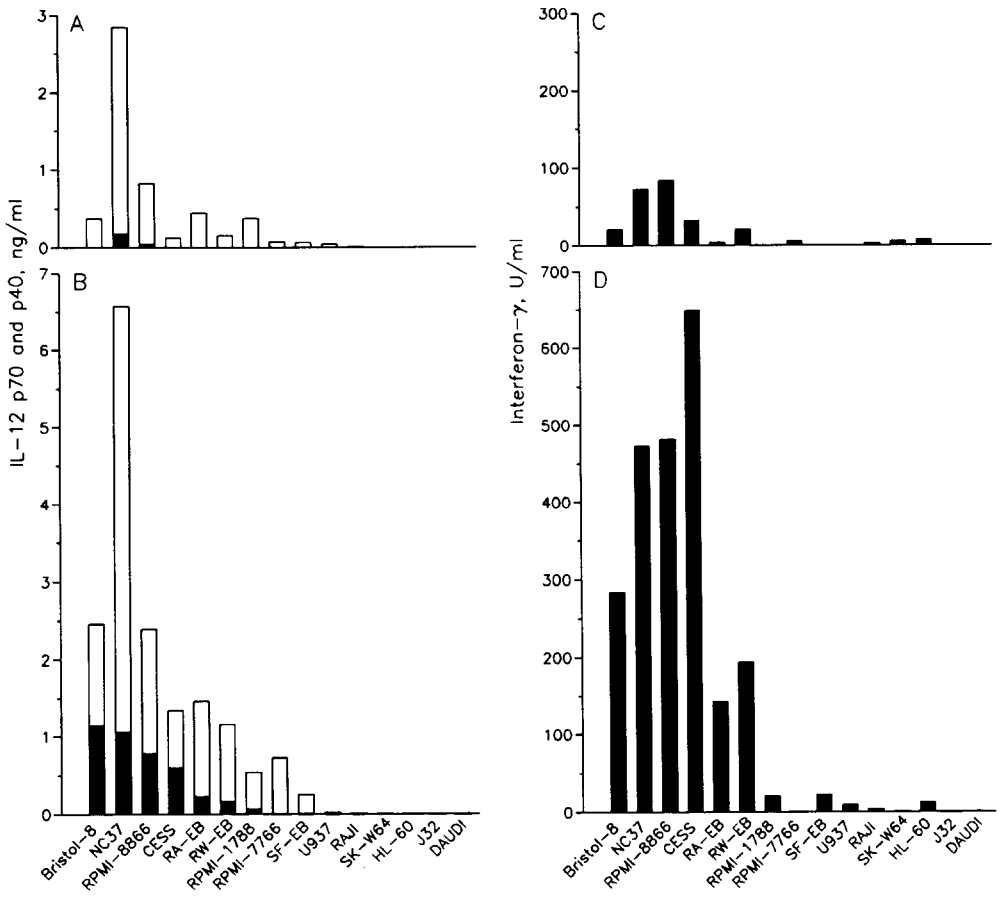

Figure 4. Constitutive and phorbol diester–induced production of free p40 chains and biologically active p70 heterodimers by human cell lines. Cell-free supernatant fluids from the indicated cell lines were obtained after an 18-h incubation in the absence (*A* and *C*) or presence (*B* and *D*) of 10^{-7} M PDBu. The concentration of the p70 heterodimer (C11.5/^{125}I-20C2 RIA, *filled portion of the bars*, *A* and *B*) and of the free p40 chain (C8.3/^{125}I-C8.6 RIA, *open portion of the bars*) was determined. For determination of IFN-γ-inducing activity (*C* and *D*), the supernatant fluids (after extensive dialysis of those containing PDBu) were added at dilutions of 1:2 to purified NK cells (blasts) in the presence of 100 U/ml rIL-2. The values reported are the difference between IFN-γ production in the presence and in the absence of 10 μg/ml C8.6 neutralizing anti-NKSF/IL-12 mAb.

most in vitro EBV-transformed cell lines produce at least the free p40 chain, almost no production of NKSF/IL-12 was observed from the two Burkitt lymphoma–derived cell lines, Daudi and Raji. Several T cell lines (Jurkat J32, Fig. 4; and others, not shown), myeloid leukemic cell lines (HL-60 and U937, Fig. 4; and ML-3 and THP-1, not shown) and many solid tumor-derived cell lines (melanoma, coloncarcinoma, and others, not shown) did not produce NKSF/IL-12 constitutively or upon stimulation with phorbol diesters. The relative production of p40 and p70 by the stimulated and unstimulated RPMI 8866, CESS, Raji, and Daudi cell lines was confirmed by Western blotting (data not shown). Significant production of the free p35 chain was never observed with any cell line (not shown). The presence of transcripts for the p40 and p35 genes was analyzed by reverse transcriptase (RT) PCR; whereas the accumulation of the p40 transcript was restricted to the cell lines producing p40 and p70, the transcripts of the p35 gene were much more ubiquitous and present in many cell lines, including most T, myeloid, and solid tumor cell lines (data not shown, and 18).

The presence of the p70 heterodimer in the supernatant fluids from either PDBu-stimulated or unstimulated cells paralleled the presence of IFN-γ-inducing activity (Fig. 4, *C* and *D*). The ability of C8.6 mAb anti-NKSF/IL-12 to almost completely abolish the IFN-γ-inducing ability of the supernatant fluids of the cell lines confirms that this activity was due to NKSF/IL-12.

Production of NKSF/IL-12 by Normal Peripheral Blood Cells. As shown in Table 1, PBMC constitutively produced in culture low but detectable levels of the free p40 chain and the p70 heterodimer. Monocyte-free PBL and adherent cells (>95% monocytes) produced equivalent amounts of NKSF/IL-12. Unlike B cell lines, stimulation with phorbol diester did not significantly increase NKSF/IL-12 production by peripheral blood cells. Among many different possible inducers tested, SAC presently appears to be the most powerful, increasing production of both the free p40 chain and the p70 heterodimer. No production of the free p35 chain was detected by RIA (data not shown). After SAC stimulation, adherent cells were more efficient than PBL in producing NKSF/IL-12 p40, but less efficient in producing the p70 heterodimer. Monocyte contamination (<1% as determined by nonspecific esterase staining) could not account for the level of NKSF/IL-12 production by the PBL preparations. Other inducers of NKSF/IL-12 from PBMC were LPS (*E. coli*, serotype 0127:B8; 1 μg/ml) and *Mycobacterium tuberculosis* (H37 RA nonviable desiccated; 1 μg/ml). On average, LPS and *M. tuberculosis* induced 6% and 8%, respectively, of the SAC-induced NKSF/IL-12 p40 production in the same PBMC preparations. Various recombinant cytokines tested (IL-1α and

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Table 1. *Production of the Free NKSF/IL-12 p40 Chain and the p70 Heterodimer by PBMC in Response to SAC Stimulation*

	Stimulation			
	None		SAC 0.0075% (wt/vol)	
Responder cells*	p40	p70	p40	p70
	pg/ml (mean ± SE)			
PBMC	774 ± 297	31 ± 19	4,953 ± 1,844	623 ± 252
PBL	221 ± 109	25 ± 7	3,557 ± 1,556	557 ± 209
Adherent cells	288 ± 88	10 ± 2	7,109 ± 1,995	304 ± 139

* PBMC, monocyte-free PBL, and plastic-adherent cells (>95% monocytes) from eight donors were cultured (10^6 cells/ml, 18 h, 37°C) in the presence or absence of SAC. Supernatant fluids were collected, and the p40 chain and the p70 heterodimer concentrations were determined by RIA.

β, 1 μg/ml; IL-2, 1,000 U/ml; IL-4, 100 U/ml; IL-6, 200 U/ml; IFN-γ and -β, 1,000 U/ml; TNF α and β, 100 ng/ml; GM-CSF, 100 ng/ml) were unable to enhance NKSF/IL-12 p40 production from PBMC.

The ability of SAC to induce NKSF/IL-12 production was confirmed at the mRNA level by Northern blot analysis (Fig. 5). SAC increased the accumulation of p40 mRNA two- to three-fold in monocytes and induced it from undetectable levels in monocyte-free PBL. Production in response to SAC of the p40 chain was also observed by RIA and by Northern blotting in 7-d cultures of monocyte-derived macrophages (not shown). PHA blasts (mostly activated T cells) and purified cultured NK cells showed neither significant accumulation of p40 mRNA (Fig. 5) nor secretion of p40 or p70 (not shown).

The ability of SAC to induce both p40 and p70 production was confirmed by Western blotting using immunoprecipitated supernatant fluids from both unstimulated and SAC-stimulated PBMC (Fig. 6). Western blotting analysis with anti-p40 antibody C8.6 did not detect constitutive production of p40 or p70 by PBMC from two donors, whereas these proteins were observed in the supernatant fluids of the same

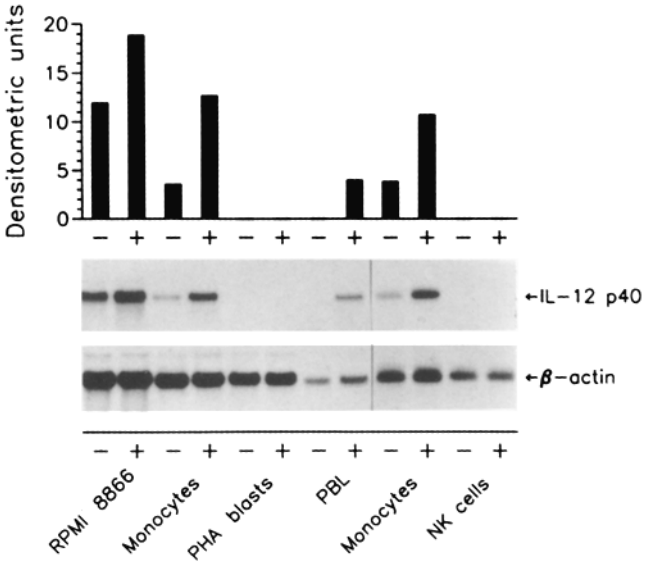

Figure 5. The accumulation of NKSF/IL-12 p40 mRNA is enhanced by phorbol diester stimulation of the RPMI 8866 cell line and by SAC stimulation of adherent PBMC and PBL. Total RNA was extracted from RPMI 8866 unstimulated (−) or stimulated with 10^{-7} M PDBu (+) and from two preparations of plastic-adherent PBMC (indicated as *Monocytes* in the figure and containing >95% $CD14^+$ cells) and one preparation of monocyte-depleted PBL, PHA blasts, and NK cells unstimulated (−) or stimulated with 0.0075% (wt/vol) SAC(+). The Northern blot was hybridized with ^{32}P-cDNA for p40 NKSF/IL-12 and for β-actin. For densitometric analysis of the hybridization with p40 cDNA (*top*), the values of the induced and uninduced cells are corrected according to the relative intensity of β-actin hybridization.

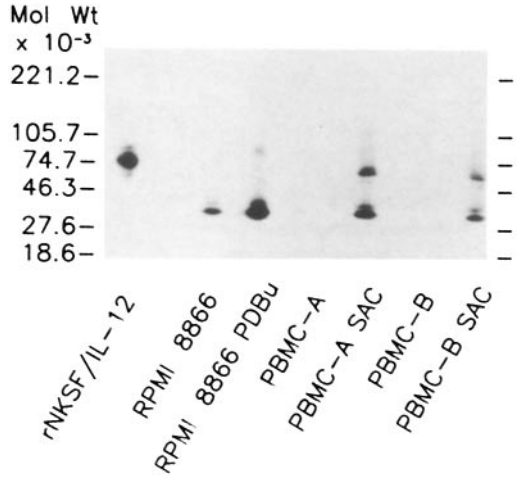

Figure 6. Western blotting analysis of recombinant and natural NKSF/IL-12. Western blotting was performed as described using 1 ng CHO cell-derived rNKSF/IL-12 and immunoprecipitates with antibody C11.79 (anti-p40) from the supernatant fluid (1 ml, 10-fold concentrated) of RPMI 8866 cells, stimulated or not with 10^{-7} M PDBu, or of PBMC from two donors (*A* and *B*), stimulated or not with 0.0075% SAC. ^{125}I-labeled antibody C8.6 was used for detection of the blotted NKSF/IL-12. The supernatant fluids from SAC-stimulated PBMC from donors A and B were the same as those used for the experiments in Fig. 7.

PBMC preparations stimulated with SAC for 18 h. In the 40-kD region, two bands were observed in supernatants from both PBMC and RPMI 8866. These multiple bands are probably explained by differential glycosylation of the p40 chain.

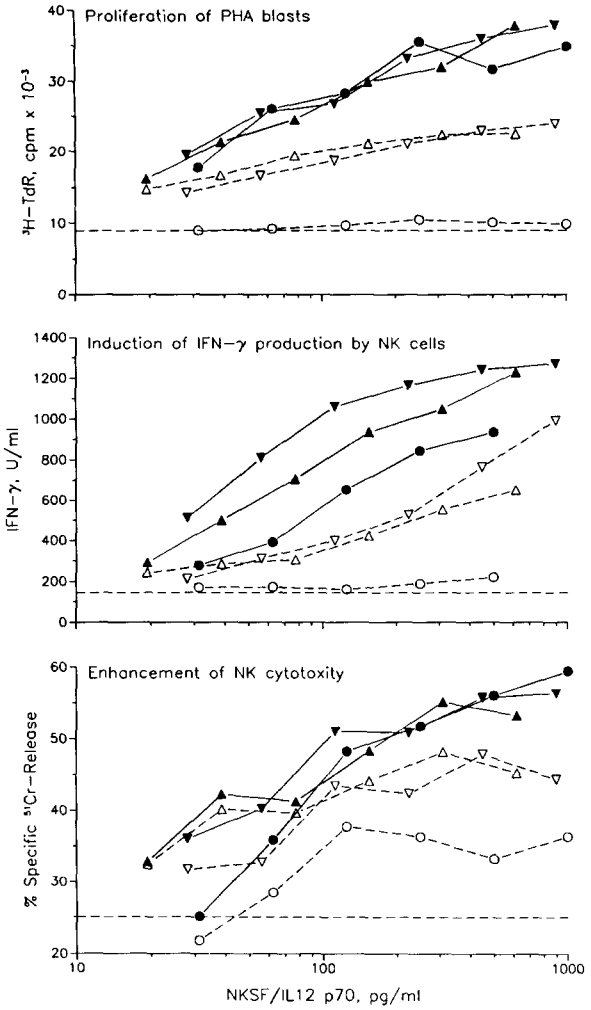

Figure 7. Biological activity of SAC-induced NKSF/IL-12 produced by PBMC. PBMC from healthy donors A and B were cultured (10^6 cells/ml, 1% FCS, 18 h at 37°C) in the presence of 0.0075% SAC, supernatant fluids were collected, and concentrated 10-fold. The concentration of the p70 heterodimer, as determined by RIA, in 10-fold concentrated supernatant fluids from donors A and B, were 1.8 and 1.2 ng/ml, respectively. Twofold dilutions of the concentrated fluids were then tested for their ability to induce proliferation of PHA blasts ([H]TdR incorporation, 48-h cultures), induction of IFN-γ production by purified NK cells (5×10^6 cells/ml, 18-h cultures, in the presence of 100 U/ml rIL-2), and enhancement of NK cell-mediated cytotoxicity. 2×10^5 PBL were incubated for 18 h in round-bottomed 96-well plates in the presence of dilutions of supernatant fluid or rNKSF/IL-12 and in the presence of anti-IFN-α, anti-IFN-β, and anti-IL-2 antibodies; ^{51}Cr-labeled Daudi cells were then added (10^4/well) and supernatant fluid for determination of ^{51}Cr-release were collected after 3 h. All experiments were performed in the presence (*dashed lines* and *open symbols*) or absence (*solid lines* and *filled symbols*) of anti-NKSF/IL-12 antibodies C8.6 and C8.1 (10 μg/ml each). The continuous horizontal dashed line indicates the control values in the absence of supernatant fluids or rNKSF/IL-12. (● and ○) rNKSF/IL-12; (▼ and q) supernatant fluid of donor A; (▲ and △) supernatant fluid of donor B.

In the 70-kD region, presumably corresponding to the heterodimer, two bands with slightly faster and slower migrations, respectively, than the main band observed with CHO cell-derived p70 heterodimer were observed in the supernatant fluid of PDBu-stimulated RPMI 8866 cells and SAC-stimulated PBMC. The larger band was predominant in the supernatant fluid from RPMI 8866 cells, whereas the smaller band was predominant in the supernatant fluid from PBMC. The exact chemical basis of this microheterogeneity and its possible functional relevance remain to be investigated.

As shown in Fig. 7, the SAC-stimulated supernatant fluids from SAC-stimulated PBMC were able to mediate enhancement of NK cell cytotoxicity, production of IFN-γ, and mitogenic effects on NK cells (blasts) at concentrations of p70 roughly equivalent to the concentrations of rNKSF/IL-12 with similar activity. The activities mediated by the supernatant fluids were partially abolished by neutralizing anti-NKSF/IL-12 antibodies, suggesting that, in addition to NKSF/IL-12, other factors produced by PBMC might be effective in these assays. However, the inability of the mAbs to inhibit completely the three biological functions could also be due in part to the presence in the supernatant fluid not only of the p70 chain,

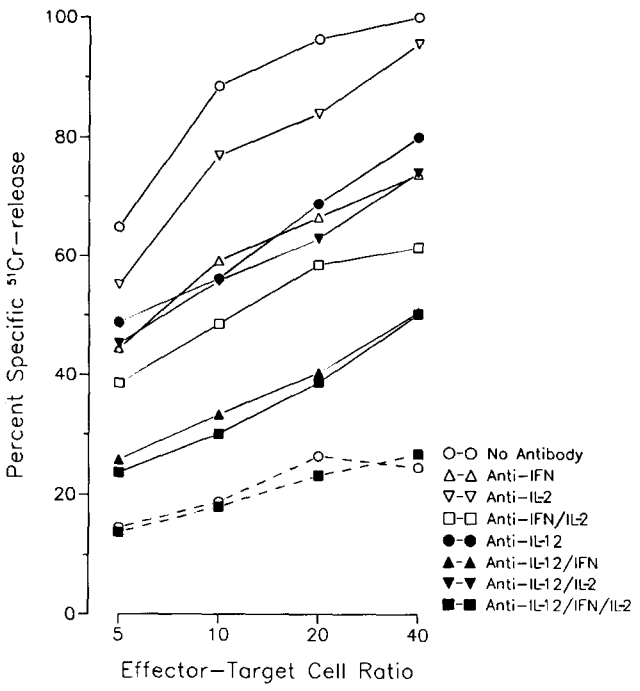

Figure 8. NK cell-mediated cytotoxicity-enhancing activity of SAC-induced supernatant fluid from PBMC. PBL from a normal donor were cultured (5×10^6 cells/ml, 18 h, 37°C) in medium (*dashed lines*) or in the presence (*solid lines*) of supernatant fluids from SAC-stimulated PBMC (same donor A as in Figs. 6 and 7) at a dilution corresponding to a concentration of 0.3 ng/ml NKSF/IL-12 p70. The following antibodies were present during the culture: (○ and ●) no antibody; (△ and ▲) sheep anti-IFN-α (1:200) and cow anti-IFN-β (1:200); (q and ▼) goat anti-IL-2 (1:200); (□ and ■) anti-IFN and anti-IL-2. Open symbols refer to cultures in the absence, and filled symbols to cultures in the presence, of anti-NKSF/IL-12 C8.6 and C8.1 antibodies (10 μg/ml each). PBL were then washed and tested at the indicated E/T cell ratio against ^{51}Cr-labeled Daudi target cells in a 3-h cytotoxic assay.

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but also of an excess of the free p40 chain, competing with the biologically active heterodimer for binding to the anti-p40 antibodies. The presence of other factors in the supernatant fluid able to mimic NKSF/IL-12 function was directly evaluated for NK cell–mediated cytotoxicity enhancement. As shown in Fig. 8, anti-IFN α/β, anti-NKSF/IL-12, and, to a lesser extent, anti-IL-2 antibodies partially blocked the enhancing ability of the supernatant fluid of SAC-stimulated PBMC to enhance NK cell–mediated cytotoxicity. An almost complete inhibition was observed using a combination of anti-IFN α/β and anti-NKSF/IL-12 antibodies. These results indicate that NKSF/IL-12 and IFN α/β represent the two predominant factors contained in the supernatant fluid with the ability to enhance NK cell–mediated cytotoxicity, with a possible minor role for IL-2 and other nonidentified factors.

Role of Endogenous NKSF/IL-12 in the IFN-γ Production by Peripheral Blood Cells in Response to Various Inducers. As shown in Table 2, the mAb C8.6 at a concentration of 10 μg/ml inhibits >95% of the IFN-γ production induced by 1 ng/ml of rNKSF/IL-12. The NKSF/IL-12 inducer SAC was also a powerful stimulator of IFN-γ production by PBL. SAC-induced IFN-γ production was inhibited >80% by the mAb C8.6, suggesting that it was mostly dependent on SAC-induced NKSF/IL-12. However, the IFN-γ-inducing ability of stimuli such as rIL-2, PHA, and anti-CD3 antibodies that do not induce NKSF/IL-12 was also inhibited ~50% by the mAb C8.6. Similar results were obtained with other neutralizing anti-NKSF/IL-12 p40 antibodies, whereas in several experiments nonneutralizing anti-p40 and anti-p35 antibodies as well as irrelevant isotype-matched antibodies did not inhibit IFN-γ production. It is likely that the inhibition reflects a role for low levels of constitutively expressed NKSF/IL-12 acting synergistically with the three stimuli to induce IFN-γ production. It is of interest that when IFN-γ production was analyzed using purified NK cells (Table 2) or PHA blasts (not shown), which are unable to produce NKSF/IL-12, SAC did not induce IFN-γ production, whereas the IFN-γ-inducing activity of rIL-2, unlike that observed with PBMC, was not inhibited by C8.6 mAb. As expected, however, C8.6 mAb almost completely inhibited the rNKSF/IL-12-mediated IFN-γ induction in both NK cells and PHA blasts.

Table 2. *Ability of Anti-NKSF/IL-12 Antibodies to Inhibit IFN-γ Production by Human PBL and Purified Cultured NK Cells*

Producer cells	Stimulus	Concentration	Percent inhibition (*n*)
			mean ± SE
PBL*	rNKSF/IL-12	1 ng/ml	95.2 ± 0.8 (16)
PBL	rIL-2	100 U/ml	64.9 ± 4.0 (16)
PBL	PHA	1%	42.5 ± 4.1 (6)
PBL	OKT3	3 μg/ml	42.7 ± 11.8 (9)
PBL	SAC	0.0075% (wt/vol)	83.1 ± 3.0 (14)
NK	rNKSF/IL-12	1 ng/ml	90.0 ± 2.2 (4)
NK	rIL-2	100 U/ml	5.8 ± 3.9 (4)

* Fresh PBL or purified cultured NK cells were cultured for 18 h with the various inducers at the indicated concentration in the presence or absence of 10 μg/ml C8.6 antibody, and percent inhibition of IFN-γ production was evaluated.

Discussion

In the last two years, we and other laboratories have characterized many of the biological functions of NKSF/IL-12 on T and NK cells. These studies have shown that NKSF/IL-12 has important regulatory effects on the proliferation, cytotoxic ability, and lymphokine production from these two cell types. However, the lack of information on whether NKSF/IL-12 was produced by normal cells and by which cell types precluded the determination of a possible physiological in vivo role of NKSF/IL-12.

In this paper, we have addressed the question of the production of NKSF/IL-12 by cell lines and normal peripheral blood cells utilizing newly generated mAbs as well as cDNA probes. mAbs to NKSF/IL-12 have been previously described (19) and two anti-p40 antibodies were used for the establishment of an ELISA (19). However, the sensitivity of the immune assay was low and the interpretation of the results was complicated by the fact that it was not clear whether the assay measured the biologically active p70 heterodimer, the free p40 chain, or both. Because cell lines (1, 3) and, as shown in this paper, PBMC consistently produce an excess of the free p40 chain over the p70 heterodimer, assays able to separately quantitate the p40 and p35 free chains and the p70 heterodimer were necessary. Testing various combinations of mAbs, we were able to establish four sensitive RIAs that specifically quantitated: (*a*) the free p40 chain, (*b*) the free p35 chain, (*c*) both the free p40 chain and the p70 heterodimer, and (*d*) the p70 heterodimer only. The specificity and the ability of different antibodies to be effective in these RIAs could not be determined on the basis of their known reactivity in other assays (e.g., indirect RIAs with plastic-bound antigens, immunoprecipitation, or Western blotting), indicating that the different conditions of the assay (e.g., the use of plastic-bound or soluble, unlabeled or ^{125}I-labeled antibodies, soluble or plastic-bound antigens) might affect the specificity and affinity of the antibodies. In particular, it was surprising that pairs of anti-p40 and anti-p35 antibodies were unable to detect the p70 heterodimer. To establish a RIA selectively detecting the p70 heterodimer, we used the anti-p40 mAb C11.5 bound on plastic and soluble ^{125}I-labeled 20C2 mAb for detection. This latter antibody detects only the p70 heterodimer and not the free p35 or p40 chains. Its reactivity with human/mouse chimeric rNKSF/IL-12, in which the p35 chain was of human origin but not with those with the p40 chain of human origin, suggests that 20C2 mAb reacts with human p35 only when complexed with either human or murine p40 in the heterodimer (Chizzonite et al., manuscript in preparation). However, our results, showing a low but definite cross-reactivity with purified Cos-derived p40, support the alternative possibility that 20C2 mAb reacts with a conformational

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determinant on both human and murine p40, expressed only when they are in association with human, but not murine, p35.

All the neutralizing antibodies against NKSF/IL-12 described by us (4, and this paper) or by others (19), with the possible exception of the 20C2 antibody described above, are specific for the p40 chain. Although this may be due to trivial reasons (such as the higher antigenicity of p40 or the use of conformationally altered bacteria-derived proteins for production of the anti-p35 antibodies), it is possible that either p40 or both p40 and p35 are involved in receptor binding. The latter possibility is supported by the data indicating that p35 determines the specificity of the p70 heterodimer for binding to the human or murine receptor (5). However, it is possible that the p35 chain does not directly participate in the binding but is responsible for determining in p40 conformational modifications, possibly recognized by 20C2 mAb, responsible for binding to either the human or the murine receptor.

We used the RIA to analyze the ability of different human cell lines to produce NKSF/IL-12. As expected from the previously published results, showing that three EBV-transformed (20) cell lines (RPMI 8866, ADP, and NC37) produced NKSF/IL-12 (1, 2), we observed that most EBV-transformed cell lines produce NKSF/IL-12, constitutively, and that this production is increased several-fold by stimulation with phorbol diesters. Within the sensitivity of our RIA, only two cell lines (RPMI 8866 and NC37) were found to produce p70 constitutively, but a large number of the cell lines were induced to produce it upon stimulation with phorbol diester. All these cell lines also produced an excess of the free p40 chain over the p70 heterodimer. As expected, on the basis of the knowledge that only the p70 heterodimer has biological activity (1, 4), the ability of the supernatant fluids of the cell lines to induce IFN-γ production paralleled the level of production of the p70 heterodimer.

The significance of the production of the free p40 chain by cell lines, and, as discussed below, by normal cells, is unclear. It is possible that the p40 chain serves as a carrier for proteins other than p35, to which it might associate in a noncovalent way and from which it dissociates in SDS. Alternatively, the free p40 chain might have biological functions different from those mediated by the p70 heterodimer and not yet determined. Unlike p40, we have been unable to demonstrate secretion of free p35 from cell lines or normal cells, although the p35 mRNA was found to be expressed in a much more ubiquitous way than p40 and it accumulates, for example, not only in B cell lines, but also in T and myeloid cell lines as well as in normal T and NK cells, all cell types not expressing p40 mRNA and not producing NKSF/IL-12 (18).

When PBMC were analyzed for production of NKSF/IL-12, it was found that these cells constitutively produced the p70 heterodimer and the free p40 chain. Unlike B cell lines, phorbol diester was not an inducer of NKSF/IL-12 production in PBMC. A preliminary search of many different compounds able to induce cytokine production in B and other cell types identified SAC as the most powerful inducer of NKSF/IL-12 production by PBMC. Constitutive and SAC-induced production of p40 and p70 was observed both in adherent cell preparations, composed mostly of monocytes, and in monocyte-depleted PBL preparations. The inability of purified activated T and NK cell preparations to accumulate p40 mRNA and to produce NKSF/IL-12 proteins, even when stimulated by phorbol diesters, IL-2 and/or anti-CD3 or anti-CD16 antibodies, suggests that normal B cells, present in the PBL preparation, similarly to the transformed B cell lines, might be, together with adherent PBMC, one of the NKSF/IL-12 producer cell types. Preliminary experiments of cell depletion showed that removal from PBL of $CD19^+$, $CD20^+$ B cells significantly reduced, and removal of HLA class II^+ cells almost completely suppressed, NKSF/IL-12 p40 production in response to SAC stimulation. The ability of SAC to stimulate both B cells (possibly because of binding of protein A to VHIII-associated crossreactive idiotypes) and monocytes is consistent with previously reported observations (20, 21). It is, however, of interest that protein A or Staphylococcus enteroxin A or B did not substitute for the intact SAC in stimulating NKSF/IL-12 production. In addition to SAC, various other stimuli were shown to induce NKSF/IL-12 production, although less efficiently. These inducers, which are presently being investigated in detail, include LPS and *M. tuberculosis*. The ability of SAC to stimulate NKSF/IL-12 was paralleled by induction of p40 mRNA in PBL and an enhancement of the constitutive expression of the p40 mRNA in adherent PBMC. It should be pointed out, however, that the experiments presented in this paper have not been performed in strictly endotoxin-free medium, and it is possible that the accumulation of p40 mRNA and production of p40 and p70 by uninduced adherent PBMC might be dependent on the stimulation by endotoxin present in the culture medium.

The p70 heterodimer produced by SAC-stimulated PBMC has the same biological activities and it is active at similar concentrations as either rNKSF/IL-12 or B cell line–derived NKSF/IL-12. When stimulated by SAC, PBMC, at a cellular concentration of 10^6/ml, below that present in vivo in peripheral blood or in other lymphoid organs, produced biologically active NKSF/IL-12 at concentrations several-fold above those required for biological functions, suggesting that NKSF/IL-12 has important physiological roles in vivo. It is of interest that, similarly to B cell lines, PBMC also produce an excess of the free p40 chain, indicating that production of the isolated chain is not due to a dysregulation in transformed cells and renewing the question about the significance of this finding.

The use of neutralizing antibodies against NKSF/IL-12 has clearly shown that NKSF/IL-12 is required for optimal IFN-γ production by PBL, not only in response to NKSF/IL-12-inducing stimuli such as SAC, but also to noninducing stimuli such as IL-2, anti-CD3 antibodies, or PHA. In the case of these three inducers, the ability of the constitutively produced NKSF/IL-12 to participate in the induction of IFN-γ is probably due to the ability of NKSF/IL-12 to synergize with those stimuli in inducing IFN-γ production (1, 8).

The ability of monocytes/macrophage and possibly B cells to secrete NKSF/IL-12 at biologically active concentrations strongly suggests that this cytokine may play an important role in the regulation of T and NK cells during a localized immune response and during inflammation. It is important to point out that although monocytes and B cells produce several cytokines (e.g., IL-1, IL-6, TNF, TGF-β) with modulating effects on T and NK cell functions, NKSF/IL-12 is the only cytokine known to be produced by these cells that has a direct effect on proliferation, cytotoxicity, and cytokine production, acting at much lower molar concentrations than other cytokines (1). The ability of bacteria to induce NKSF/IL-12 production suggests that this molecule could play a role in determining the immune response to bacterial infection. It has been shown that in SCID mice, IFN-γ production by NK cells in response to Listeria (22, 23) or Toxoplasma (A. Sher, personal communication) requires the interaction of both NK cells and macrophages with the microorganisms. Although TNF plays some role in this interaction (22), TNF by itself does not induce IFN-γ, and other factors, possibly including NKSF/IL-12, are required. It is of interest that although the effect of NKSF/IL-12 in inducing production of IFN-γ from T and NK cells is direct, antibodies to TNF abolish the antiproliferative effects of NKSF/IL-12 in NK and T$\gamma\delta$ cells (10a) and partially prevent IFN-γ production induced in vitro by NKSF/IL-12 (our unpublished results).

The ability of NKSF/IL-12 to induce IFN-γ might be particularly important in vivo, because NKSF/IL-12 synergizes with other stimuli in inducing this lymphokine. Early production of IFN-γ during an infection may significantly affect resistance to pathogenic microorganisms by inducing prevalent differentiation of Th1 cells, which favor a cell-mediated response, vs. Th2 cells, which favor a humoral, often insufficient immune response to infection (24). NKSF/IL-12, if produced in vivo during infection, may play a significant role in these regulatory immune mechanisms.

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References

1. Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170:827.
2. Stern, A.S., F.J. Podlaski, J.D. Hulmes, Y.E. Pan, P.M. Quinn, A.G. Wolitzky, P.C. Familletti, D.L. Stremlo, T. Truitt, R. Chizzonite, and M.K. Gately. 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA.* 87:6808.
3. Gubler, U., A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitzky, P.M. Quinn, P.C. Familletti, and M.K. Gately. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA.* 88:4143.
4. Wolf, S.F., P.A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R.M. Hewick, K. Kelleher, S.H. Herrmann, S.C. Clark, L. Azzoni, S.H. Chan, G. Trinchieri, and B. Perussia. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and Natural Killer cells. *J. Immunol.* 146:3074.
5. Schoenhaut, D.S., A.O. Chua, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, W. McComas, P.C. Familletti, M.K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. *J. Immunol.* 148:3433.
6. Cassatella, M.A., F. Bazzoni, F. Calzetti, I. Guasparri, and G. Trinchieri. 1991. Interferon-γ (IFN-γ) transcriptionally modulates the expression of the genes for the high affinity IgG-Fc receptor (FcγR-I) and the 47 kD cytosolic component of NADPH oxidase in human polymorphonuclear leukocytes. *J. Biol. Chem.* 266:22079.
7. Merberg, D.M., S.F. Wolf, and S.C. Clark. 1992. Sequence similarity between NKSF and the IL-6/G-CSF family. *Immunol. Today.* 13:77.
8. Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Popsísil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark, and G. Trinchieri. 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173:869.
9. Chan, S.H., M. Kobayashi, D. Santoli, B. Perussia, and G. Trinchieri. 1992. Mechanisms of IFN-γ induction by natural killer cell stimulatory factor (NKSF/IL-12): role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J. Immunol.* 148:92.
10. Chehimi, J., S. Starr, I. Frank, M. Rengaraju, S.J. Jackson, C. Llanes, M. Kobayashi, B. Perussia, D. Young, E. Nickbarg, S.F. Wolf, and G. Trinchieri. 1992. Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells

from both healthy donors and human immunodeficiency virus-infected patients. *J. Exp. Med.* 175:789.

10a. Perussia, B., S. Chan, A. D'Andrea, K. Tsuji, D. Santoli, M. Pospisil, D. Young, S. Wolf, and G. Trinchieri. 1992. Natural killer cell stimulatory factor or Interleukin-12 has differential effects on the proliferation of TCR$\alpha\beta^+$, TCR$\gamma\delta^+$ T lymphocytes and NK cells. *J. Immunol.* In press.

11. Gately, M.K., B.B. Desai, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, F.J. Podlaski, P.C. Familletti, F. Sinigaglia, R. Chizzonite, U. Gubler, and A.S. Stern. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol.* 147:874.

12. Kaufman, R.J. 1990. Selection and coamplification of heterologous genes in mammalian cells. *Methods Enzymol.* 185:537.

13. Perussia, B., S. Starr, S. Abraham, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. *J. Immunol.* 130:2133.

14. Perussia, B., C. Ramoni, I. Anegón, M.C. Cuturi, J. Faust, and G. Trinchieri. 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. *Nat. Immun. Cell Growth Regul.* 6:171.

15. Murphy, M., R. Loudon, M. Kobayashi, and G. Trinchieri. 1986. γ interferon and lymphotoxin, released by activated T cells, synergize to inhibit granulocyte-monocyte colony formation. *J. Exp. Med.* 164:263.

16. Cassatella, M.A., L. Hartman, B. Perussia, and G. Trinchieri. 1989. Tumor necrosis factor and immune interferon synergistically induce cytochrome b_{-245} heavy chain gene expression and NADPH oxidase in human leukemic myeloid cells. *J. Clin. Invest.* 83:1570.

17. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.

18. Wolf, S., D. Seiburth, B. Perussia, J. Yetz-Adalpe, A. D'Andrea, and G. Trinchieri. 1992. Cell sources of natural killer cell stimulatory factor (NKSF/IL-12) transcripts and subunit expression. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:A1335.

19. Chizzonite, R., T. Truitt, F.J. Podlaski, A.G. Wolitzky, P.M. Quinn, P. Nunes, A.S. Stern, and M.K. Gately. 1991. IL-12: monoclonal antibodies specific for the 40-kDa subunit block receptor binding and biologic activity on activated human lymphoblasts. *J. Immunol.* 147:1548.

20. Shokri, F., R.A. Mageed, B.R. Maziak, and R. Jefferis. 1991. Expression of VHIII-associated cross-reactive idiotype on human B lymphocytes. Association with staphylococcal protein A binding and staphylococcus aureus Cowan I stimulation. *J. Immunol.* 146:936.

21. Devalon, M.L., G.R. Eliot, and W.E. Regelmann. 1987. Oxidative response of human neutrophils, monocytes, and alveolar "macrophages" induced by unopsonized surface adherent Staphylococcus aureus. *Infect. Immun.* 55:2398.

22. Bancroft, G.J., K.C. Sheehan, R.D. Schreiber, and E.R. Unanue. 1989. Tumor necrosis factor is involved in the T cell independent pathway of macrophage activation in scid mice. *J. Immunol.* 143:127.

23. Wherry, J.C., R.D. Schreiber, and E.R. Unanue. 1991. Regulation of gamma interferon production by natural killer cells in scid mice: role of tumor necrosis factor and bacterial stimuli. *Infect. Immun.* 59:1709.

24. Scott, P. 1991. IFN-γ modulates the early development of Th1 and Th2 response in a murine model of cutaneous Leishmaniosis. *J. Immunol.* 147:3149.