NATURAL KILLER (NK) CELL-DERIVED HEMATOPOIETIC COLONY-INHIBITING ACTIVITY AND NK CYTOTOXIC FACTOR

Relationship with Tumor Necrosis Factor and Synergism with Immune Interferon

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Growing evidence suggests that control of hematopoiesis is one of the functions exerted in vivo by natural killer (NK)[1] cells (1), and cells with NK cell characteristics have been shown to inhibit different types of hematopoietic colonies in vitro (2, 3). We have shown (4) that the human peripheral blood cells responsible for this inhibition bear the surface markers of cytotoxic NK cells, and that this inhibitory effect is mediated by a soluble factor, NK cell-derived colony-inhibiting activity (NK-CIA), released by NK cells upon culture with NK-sensitive target cell lines, or with bone marrow cells. A soluble factor, the NK-derived cytotoxic factor (NKCF) (5), might mediate the cytotoxic effect of NK cells. Like NK-CIA, NKCF is released by lymphocytes cultured with NK-sensitive target cells. The sensitivity of different cell lines to the cytotoxic effect of NKCF correlates generally with their sensitivity to NK cell-mediated cytotoxicity (6), although some cell lines are sensitive to NKCF but not to NK cell cytotoxicity because they fail to induce NKCF production by NK cells, whereas other cell lines induce NKCF but are not sensitive to it (7). Interferon (IFN)-treated target cells, for example, are much less susceptible to NK cell-mediated cytotoxicity (8), and they do not induce NKCF production (7); on the other hand, IFN enhances the cytotoxicity of NKCF (7). Based on these observations, two recognition events to allow NK killing have been proposed: the first allows binding of NK to the target cells, and the second allows the release of NKCF (7, 9). The relationship of NKCF with other lymphotoxins (LT) produced by T cells upon antigen- or lectin-stimulation has not been completely clarified (10). The enhancing effect of IFN on NKCF activity, reminiscent of the phenomenon described with LT (11), and the fact that some anti-LT antisera partially inhibit NK killing (10),

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[1] *Abbreviations used in this paper:* CFU-GM, granulocyte-macrophage colony-forming unit; CIA, colony-inhibiting activity; CSF, colony-stimulating factor; FBS, fetal bovine serum; F/H, Ficoll/Hypaque; FPLC, fast protein liquid chromatography; IFN, interferon; LT, lymphotoxin; mAb, monoclonal antibody; NK, natural killer; NKCF, NK cell-derived cytotoxic factor: PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; r, recombinant; TNF, tumor necrosis factor; VSV, vesicular stomatitis virus.

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suggest a similarity between LT and NKCF; however, preliminary biochemical characterization of NKCF (12) seems to exclude its complete identity with LT, at least on the basis of molecular weight. The genes encoding LT and tumor necrosis factor (TNF), a cytotoxic factor produced mostly by monocyte/macrophages and with amino acid sequence homology with LT, have been recently cloned (13–15). Monoclonal antibodies (mAb) specific for LT (13) and TNF are now available, making possible a more accurate analysis of the relationship of NKCF and NK-CIA with LT and TNF.

Various soluble factors produced by leukocytes have been described that inhibit bone marrow colony formation. Various types of IFN (16, 17) and prostaglandins (18) have proved to be effective inhibitors of various types of colonies. An 80 kilodalton (kD) molecule with high inhibitory activity on hematopoietic precursor cells and toxicity on HLA-DR$^+$ cell lines has been recently described (19) that is produced by a hybrid between peripheral blood T cells and the Jurkat T cell line. Other factors, such as lactoferrin or acidic isoferritin, are produced by normal or leukemic cells, and inhibit hematopoietic colonies mostly by blocking production of colony-stimulating factors (CSF) by macrophages (20, 21).

In the present study, we analyzed the relationship of NK-CIA with NKCF and IFN and show that NKCF and the factor responsible for NK-CIA are simultaneously produced by NK cells, and appear indistinguishable in a series of functional and biochemical analyses. IFN do not account for NK-CIA activity, but recombinant IFN-γ acts synergistically with NK-CIA, reversing the normal resistance of late GM precursor cells to NK-CIA. mAb against TNF but not LT completely block both NKCF and NK-CIA activity, and recombinant TNF mediates both NKCF and NK-CIA activity.

Materials and Methods

Cell Lines. The human erythromyeloid leukemic cell line K562, the macrophage line U937, the B cell line Raji, the Detroit 539 fibroblast, and the murine P815y mastocytoma, and L929 cells of the α subline were maintained in culture in RPMI 1640 medium (Flow Laboratories, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, Inc.). All cell lines used were repeatedly tested and constantly found free of mycoplasma contamination by Hoechst fluorescent staining for cytoplasmic DNA, and by direct anaerobic agar plate cultivation.

Monoclonal Antibodies. Antibody B73.1 (IgG1) reacts with the receptor for the Fc fragment of IgG present on human NK cells and neutrophilic granulocytes, and detects virtually all resting and IFN-induced NK cells (22, 23). B36.1 (IgG2b) reacts with the 69 kD surface molecule (T1 antigen) present on all peripheral blood T cells (23). These antibodies, and the anti-HLA-DR common determinant antibody B33.1 (IgG2a) (23, 24) have all been produced in our laboratory. The anti-NK antibody N901 (IgG1) (25) was kindly provided by Dr. J. Griffin (Dana-Farber Cancer Institute, Boston, MA). Supernatants from the hybrid cell lines were used throughout this study.

Peripheral Blood Lymphocytes (PBL) and PBL Subpopulations. Peripheral blood was obtained by venipuncture from healthy donors, and anticoagulated with preservative-free heparin. Mononuclear cells were prepared by density gradient centrifugation on Ficoll/Hypaque (F/H) (1.077 ± 0.01 g/ml), and depleted of monocytes by adherence to plastic for 45 min at 37°C. PBL subpopulations were obtained by indirect rosetting of antibody-sensitized PBL with $CrCl_3$-treated, anti–mouse Ig–coated sheep erythrocytes, according to our previously described technique (22, 24). The goat $F(ab')_2$ anti–mouse Ig (Cappel Laboratories, Cochranville, PA) used was preabsorbed with human Ig and immunopurified on a mouse Ig–CNBr–Sepharose (Pharmacia Fine Chemicals, Uppsala,

Sweden) column, as described (22). Both antibody-positive (rosetting) and -negative (nonrosetting) populations were recovered after F/H density gradient centrifugation, and contaminating erythrocytes were lysed hypotonically.

Bone Marrow Cell Preparations. Rib bone marrow cells were purified from specimens (kindly provided by Dr. P. Addonizio, Dept. of Surgery, University of Pennsylvania, Philadelphia, PA) that are routinely removed during thoracic surgery. The marrow was collected in sterile RPMI 1640 with 10% FBS, sequentially flushed with medium through 18-, 23-, and 27-gauge needles, and the cell suspension was layered on a F/H density gradient (1.077 g/ml). Mononuclear cells collected at the interface were washed three times with RPMI 1640 supplemented with 10% FBS. In the indicated experiments, the mononuclear cells were depleted of mature monocytes and other adherent cells by two cycles of adherence to plastic surfaces. The resulting population was further separated into HLA-DR^+ and HLA-DR^- cells by indirect rosetting (as described above), after sensitizing the cells with mAb B33.1. ~10–15% of the bone marrow cells were recovered in the HLA-DR^+ fraction, which contained virtually all colony-forming units of granulocytes and macrophages (CFU-GM).

Bone Marrow Colonies. The CFU-GM assay was performed according to the method of Pike and Robinson (26), with slight modification. Medium conditioned by the Detroit 554 cell line (kindly provided by Dr. R. K. Shadduck, Montefiore Hospital, University of Pittsburgh School of Medicine, Pittsburgh, PA) was used as a source of CSF (26). Bone marrow cells were resuspended (10^5 cells/ml) in 0.3% agar (Difco Laboratories, Inc., Detroit, MI) in supplemented McCoy's medium (Gibco Laboratories, Grand Island, NY), containing 10% Detroit cell-conditioned medium. 1-ml aliquots of the cell suspension were seeded into 35-mm Petri culture dishes (Lux Plastics, Flow Laboratories, Inc., McLean, VA), and cultured at 37°C in a 5% CO_2 humidified atmosphere in air. Colonies (aggregates containing >50 cells) were scored on days 7 and 14 of culture. Colony growth on day 7 evaluates differentiation and proliferation of more mature GM precursor cells (late CFU-GM), colony growth on day 14 evaluates these parameters in more primitive cells (early CFU-GM) (27). All cultures were performed in triplicate. The results of CFU-GM are expressed as number of colonies per 10^5 plated cells. The same lots of FBS and Detroit cell-conditioned medium were used throughout.

Production of Supernatant Fluids from PBL Cocultured with Cell Lines or with Bone Marrow Cells. PBL and the different PBL subpopulations were resuspended (5×10^6 cells/ml) in RPMI 1640 with 10% FBS, but with or without 10^3 U/ml partially purified IFN-α (10^6 U/mg; Interferon Sciences, New Brunswick, NJ) and incubated for 18 h at 37°C in a 5% CO_2 humidified atmosphere in tissue culture flasks (Falcon Labware, Oxnard, CA). After culture, the cells were washed three times with phosphate-buffered saline (PBS, pH 7.2), and resuspended (3×10^6 cells/ml) in RPMI with 10% FBS containing K562, Raji, or P815y cells (6×10^5 cells/ml final concentration). After a 6-h incubation at 37°C, the cells were centrifuged, and cell-free supernatants were harvested. Control supernatants were prepared using either effector or target cells alone under the same experimental conditions as above. In the indicated experiments, supernatants were obtained using as inducer total mononuclear bone marrow cells, nonadherent mononuclear bone marrow cells, or HLA-DR^+ and HLA-DR^- bone marrow subpopulations (3×10^6 cells/ml, final concentration). Supernatants were either used immediately, or kept frozen at −70°C until used. The effect of soluble factors on colony formation was tested by adding supernatants (100 μl or the indicated quantities) at the time of addition of the bone marrow cells, or at the indicated time after seeding to triplicate dishes.

[^{3}H]Thymidine ([^{3}H]TdR) Incorporation. Cell lines (Raji, U937) were resuspended in RPMI with 10% FBS supplemented or not with different concentrations of recombinant TNF (rTNF). 200 μl of the cell suspension were plated in each well of flat-bottomed microtiter tissue culture plates (5×10^3 cells/well) and cultured for 4 d at 37°C. 6 h before harvesting, the cells were pulsed with [^{3}H]TdR (sp act, 2 Ci/mmol; New England Nuclear, Boston, MA), at 2 μCi/well. The cells were collected on glass-fiber filters using an automatic cell harvester (Skatron Co., Sterling, VA), and cell-incorporated [^{3}H]TdR was assayed by liquid scintillation counting.

Assay for NKCF. NKCF in the supernatants was tested in an 18-h ^{51}Cr-release assay using several different target cells, as indicated. Different concentrations of supernatant were added to ^{51}Cr-labeled target cells (U937, 2.5×10^3 cells/well; K562 and the other cell lines, 5×10^3 cells/well) in 100 μl final volume, in triplicate wells of flat-bottomed microtiter plates. After an 18-h incubation, 100 μl of PBS were added to each well, and 100 μl of supernatant were collected after spinning. Percent specific ^{51}Cr release = 100 × [(cpm released from experimental samples) − (spontaneous cpm released from target cells alone)]/[(maximum cpm released from target cells with Triton X-100) − (spontaneous release)].

NK Cytotoxicity Test. This was performed as previously described (28) using ^{51}Cr-labeled K562 cells (10^4 cells/well) as targets in a 3-h assay. Cytotoxicity was quantitated from the cytotoxicity curves obtained using at least four different effector/target cell ratios, in terms of lytic units, calculated using a modified von Krogh equation, as described (29). One lytic unit is defined as the number of effector cells required to mediate 45% specific ^{51}Cr release from the target cells during the assay period.

Absorption of Supernatants with Different Cell Types. Cell-free supernatants were incubated with the indicated cell lines or bone marrow cell populations (5×10^6 cells/ml) for 6 h at 20°C with intermittent shaking. Cell-free supernatant was then harvested after spinning the cells (2,000 rpm for 5 min). D-Mannose-6-phosphate (Sigma Chemical Co., St. Louis, MO) was dissolved in RPMI 1640 supplemented with 10% FBS, and used at a final concentration of 25 or 50 mM in assays of NKCF and NK-CIA activity and absorption. Supernatants produced or absorbed in the presence of D-mannose-6-phosphate were dialyzed for 18 h at 4°C against RPMI 1640 and sterilized by 20,000 rad irradiation before use.

Chromatographic Fractionation of NK-CIA-containing Supernatants. NK-CIA-containing supernatant was prepared from IFN-α (10^3 U/ml per 5×10^6 cells)-stimulated B73.1$^+$ PBL cultured with K562 cells as inducers. All stimulations were performed in RPMI 1640 supplemented with 1% FBS. The cell-free supernatant was concentrated 50-fold by an Amicon Model 8050 stirred cell equipped with a PM-10 membrane, and fractionated by gel filtration chromatography using a fast protein liquid chromatography (FPLC) system (Pharmacia Fine Chemicals) with a Superose 12 column. Samples (200 μl) were applied to the column and eluted with 0.1% polyethylene glycol 8000, 0.1 M β-lactose, and 0.1 mM phenylmethylsulfonyl fluoride in PBS, pH 7.4, at a flow rate of 0.6 ml/min. Fractions of 500 μl were collected, dialyzed against medium, and sterilized by filtration. Alternatively, the samples were dialyzed against 20 mM Tris buffer, pH 7.8, and applied to a Mono Q anion-exchange column (Pharmacia Fine Chemicals). The column was eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris buffer, pH 7.8, at a flow rate of 0.8 ml/min. Fractions of 1 ml were collected, dialyzed, and sterile-filtered as above.

Lymphokines and Antilymphokine Antibodies. Human recombinant IFN-γ (rIFNγ) from *E. coli* was kindly provided by Drs. J. C. Sevastopoulos and H. M. Shepard (Genentech, Inc., South San Francisco, CA) and has a antiviral activity titer of 7×10^7 U/mg on HeLa cells. No significant CSF activity was demonstrable in the rIFN-γ preparation using normal bone marrow cells. Recombinant type A IFN-α (rIFNA) from *E. coli* (2×10^8 U/mg) was kindly provided by Hoffman-LaRoche, Inc. (Nutley, NJ). Purified human IFN-γ (10^6 U/mg) was obtained from Interferon Sciences, Inc. (New Brunswick, NJ). Human rIFN-β (sp act, 1.5×10^8 U/mg) and human rTNF (90% pure, 10^7 U/mg on L-929 cells) were kindly provided by Dr. J. S. Price (Cetus Corp., Emeryville, CA). Sheep antiserum against human IFN-α (10^5 neutralizing U/ml) was obtained from Interferon Sciences. mAb B133.3 (IgG1), which reacts specifically with human IFN-γ and with neither IFN-α nor -β (30), was used as ascites; it had a titer of 1.5×10^7 neutralizing U/ml. mAb specific for human LT (LTB) (5.5×10^2 inhibiting U/mg antibody) and to human TNF (TNFD) (2.7×10^3 inhibiting U/μg antibody) were kindly provided by Dr. H. M. Shepard (Genentech, Inc.) and used at a final concentration of 5 μg/ml to pretreat (6 h at 4°C) NKCF- or NK-CIA-containing supernatant.

Assay of Cytotoxins on L Cells. The cytotoxic activity of the supernatants on mouse L-929 cells (α subline) was assayed in flat-bottomed 96-well microtiter plates by adding serial

dilutions of supernatant fluid to 4×10^4 L cells/well in the presence of 1 μg/ml actinomycin D (Calbiochem Behring Corp., La Jolla, CA). Cytotoxicity was scored microscopically after incubation for 24 h at 37°C. 1 U of cytotoxin is defined as the highest dilution producing 50% cytotoxicity.

IFN Test. Antiviral activity in the supernatants was tested by inhibition of the cytopathic effect of vesicular stomatitis virus (VSV) on human fibroblast strain Detroit 539, derived from a subject with trisomy 21. The IFN concentration inducing 50% protection from the cytopathic effect on Detroit cells corresponded to ~1 U of the National Institutes of Health (NIH) IFN-γ standard Gg-23-901-530 and to 0.1 U of the NIH IFN-α standard G-023-901-527.

Results

Correlation Between Production of NK-CIA and NKCF. NKCF activity, as detected in an 18-h ^{51}Cr-release assay on the NK-sensitive K562 or U937 target cell line, was present in the cell-free supernatant fluid containing NK-CIA. A highly significant positive correlation between cytotoxic activity on U937 cells and inhibition of CFU-GM was seen (Fig. 1) at 14 d in 32 different supernatant fluids produced by various PBL subsets from different donors stimulated by culture with K562 cells. Different PBL subsets, separated by indirect rosetting using various mAb, were tested for cell-mediated cytotoxicity on K562 target cells and for their ability to produce NKCF and NK-CIA when cultured with K562 cells. Table I gives the results obtained with some of the antibodies tested. NK activity and production of both NKCF and NK-CIA segregate in the same PBL subset, i.e., B73.1/N901$^+$, and B36.1/B33.1$^-$ (Table I). Like cytotoxic NK cells, a proportion of both NKCF and NK-CIA-producing PBL reacted with anti-T8 antibody B116.1 and with antibody HNK-1, whereas PBL reacting with anti-T4 antibody B66.6 and with OKT3 never produced either factor (not shown). Production of both NKCF and NK-CIA (Table I), as well as NK activity (not shown), are increased upon IFN-α treatment of the PBL subsets containing NK

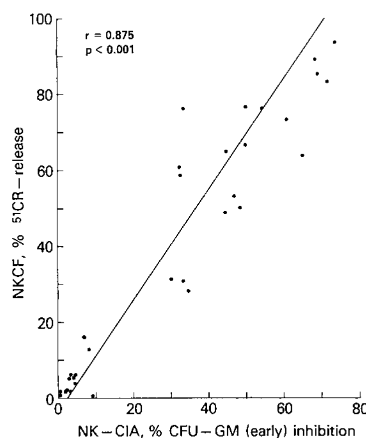

FIGURE 1. Correlation between NKCF and NK-CIA. Different supernatants, obtained by induction of various PBL subsets from different donors with K562 cells, were simultaneously tested for NKCF activity (^{51}Cr release from U937 target cells, incubated in a final supernatant concentration of 50%) or for NK-CIA (100 μl supernatant per dish). Each dot represents the values obtained with a separate supernatant.

TABLE I
Cell-mediated NK Activity and K562-induced Production of NKCF and NK-CIA by PBL Subsets

Exp.	PBL subset*	NK (lytic units per 10^7 cells)‡	NKCF (percent ^{51}Cr release)§		NK-CIA (percent inhibition)‖	
		PBL	PBL	IFN-PBL	PBL	IFN-PBL
1	Unseparated	78.9	16.9	25.1	36.8	52.3
	B73.1$^+$	659.7	33.2	42.6	59.9	81.2
	B73.1$^-$	3.9	2.5	2.8	14.9	17.8
2	Unseparated	37.3	29.4	51.5	33.8	46.9
	B36.1$^+$	1.3	1.2	2.3	2.3	6.7
	B36.1$^-$	326.5	74.5	87.7	54.5	68.3
	B33.1$^+$	1.2	2.3	3.0	0.9	3.9
	B33.1$^-$	28.3	45.2	56.5	31.1	44.5
	N901$^+$	276.8	68.6	88.5	63.4	72.8
	N901$^-$	0.2	3.4	9.3	1.7	5.6

PBL or PBL subsets were incubated for 18 h at 37°C in the presence (IFN-PBL) or absence (PBL) or 1,000 U/ml of IFN-α before coculture with K562 cells. Data are average of results obtained with two donors.
* PBL subsets were separated by indirect rosetting after sensitization with the indicated mAb.
‡ NK activity was tested in cell-mediated cytotoxicity assay on K562 target cells.
§ NKCF activity, induced during 6-h culture with K562 cells, was tested on K562 cells (Exp. 1) or U937 cells (Exp. 2) (50% supernatant final concentration).
‖ NK-CIA, induced during 6-h culture with K562 cells, was tested for inhibition of 14-day (early) CFU-GM (100 μl supernatant per dish).

TABLE II
Production of NKCF and NK-CIA Induced by Different Cell Types

B73.1$^+$ PBL cocultured with inducer cells	NKCF (percent ^{51}Cr release)		NK-CIA (percent CFU-GM [early] inhibition)	
	PBL	IFN-PBL	PBL	IFN-PBL
None	1.3, 1.0	2.0, 2.0	−4.5, 0.0	−3.0, 3.1
K562	28.8, 29.0	37.7, 41.0	47.0, 63.7	63.7, 78.8
Raji	0.7, 0.0	1.0, 0.0	0.0, 0.0	0.0, 0.6
Bone marrow, HLA-DR$^+$	34.7, 38.5	51.7, 47.9	41.9, 43.9	55.2, 56.2
Bone marrow, HLA-DR$^-$	5.6, 2.8	5.4, 4.8	6.2, 4.1	7.2, 2.1

Following coculture for 6 h at 37°C with the indicated inducer cells, supernatants were assayed for NKCF (U937 target cells, final supernatant concentration 50%) or for NK-CIA (100 μl supernatant per dish). B73.1$^+$ producer PBL were incubated for 18 h at 37°C in the presence (IFN-PBL) or absence (PBL) of 1,000 U/ml of IFN-α before coculture with inducer cells. Data are given separately for two independent experiments.

cells, but the same treatment does not induce production of either lymphokine by the NK cell–depleted PBL subsets.

The ability of different cell lines and bone marrow cells to induce NKCF and NK-CIA production was tested using PBL preincubated or not with IFN-α. Total (not shown) and B73.1$^+$ PBL (Table II) produced both NKCF and NK-CIA when cultured with the NK-sensitive target cells K562 or with HLA-DR$^+$ bone

TABLE III

Absorption of NK-CIA and NKCF by Different Cell Types

Absorbing cells	NKCF (percent ^{51}Cr release [U937])	NK-CIA (percent CFU-GM [early] inhibition)
None, −70°C	53.7, 70.2	62.8, 45.7
None, 6 h at 20°C	50.2, 67.1	62.8, 47.4
K562	19.6, 27.6 (59.8)	2.8, 8.8 (89.5)
Raji	53.2, 66.5 (−1.0)	61.1, 49.2 (0)
Bone marrow, nonadherent	23.1, 22.9 (60.8)	27.4, 19.3 (57.7)
Bone marrow, HLA-DR$^+$	23.0, 16.8 (66.1)	12.7, 12.3 (77.4)
Bone marrow, HLA-DR$^-$	46.4, 49.4 (18.3)	62.8, 47.4 (0)

Two preparations of NK-CIA-containing supernatants (produced by IFN-α-treated B73.1$^+$ PBL induced by K562 cells) were tested for NKCF (50% supernatant final dilution) and NK-CIA (100 μl supernatant per dish) as described, immediately after defrosting (−70°C) or after absorption for 6 h at 20°C (5 × 10^6 cells/ml) with the indicated cell types. Data are given separately for two independent experiments. Numbers in parentheses are average percent absorptions compared to the control kept for 6 h at 20°C.

marrow cells, and the production was enhanced by preincubation of PBL with IFN-α. PBL, pretreated or not with IFN-α, produced neither NKCF nor NK-CIA when cultured alone or in the presence of NK-insensitive Raji cells, or with HLA-DR$^-$ bone marrow cells. B73.1$^-$ PBL did not produce NKCF or NK-CIA in response to any of the tested inducer cells, regardless of IFN-α pretreatment (not shown).

Absorption of NK-CIA and NKCF on Cell Lines and on Bone Marrow Cells. The ability of different cell types to absorb NK-CIA and NKCF was then tested. Bone marrow and K562, but not Raji cells, absorb most of the NK-CIA and NKCF activity from the supernatants in 6 h at 20°C (Table III). Among the bone marrow cells, only HLA-DR$^+$ cells were able to absorb both NK-CIA and NKCF, whereas HLA-DR$^-$ cells did not absorb either activity significantly. When total bone marrow or HLA-DR$^+$ cells were washed after the absorption and plated for colony formation, inhibition of 14-d CFU-GM was observed (not shown).

Ability of D-Mannose-6-phosphate to Block Both NK-CIA and NKCF Activity and their Absorption by Target Cells. As previously reported (31) for several monosaccharides, D-mannose-6-phosphate inhibited the NKCF cytotoxic effect on U937 cells when present during the ^{51}Cr-release assay. The inhibition was complete at a 50 mM concentration of D-mannose-6-phosphate, and partial at 25 mM (Fig. 2). Addition of the sugar at these two concentrations during the CFU-GM assay did not affect colony formation, but prevented the inhibition of early CFU-GM by NK-CIA. An almost complete reversion of the inhibition was observed when D-mannose-6-phosphate was used at a 50 mM concentration, and a partial reversion resulted when a 25 mM concentration was used (Fig. 2). To test whether the sugar inhibited binding of the factors to the target cells, a supernatant produced by IFN-α-pretreated B73.1$^+$ cells cultured with K562 cells was absorbed on K562 cells in the presence or absence of 50 mM D-mannose-6-phosphate, dialyzed to eliminate the sugar, and tested for residual NK-CIA and NKCF activity (Fig. 3). Addition of 50 mM D-mannose-6-phosphate to the supernatant, followed by dialysis, did not alter NK-CIA and NKCF activity. The

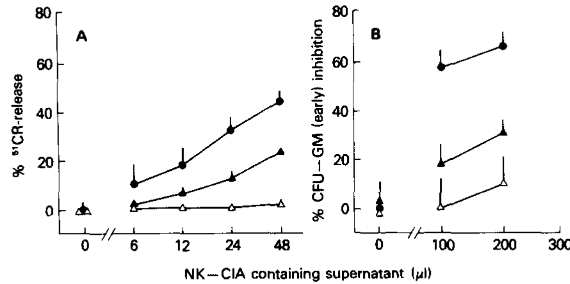

FIGURE 2. Inhibition of NKCF and NK-CIA by D-mannose-6-phosphate. Supernatant fluid from IFN-α-pretreated B73.1^{+} PBL induced by K562 cells was tested, using the indicated quantity per assay, for (*A*) NKCF activity (U937 target cells) or (*B*) NK-CIA in medium only (●) or in the presence of 25 mM (▲) or 50 mM (△) D-mannose-6-phosphate. Error bars are SD of the results obtained with three separate supernatants in *A*, and of triplicate dishes in *B*.

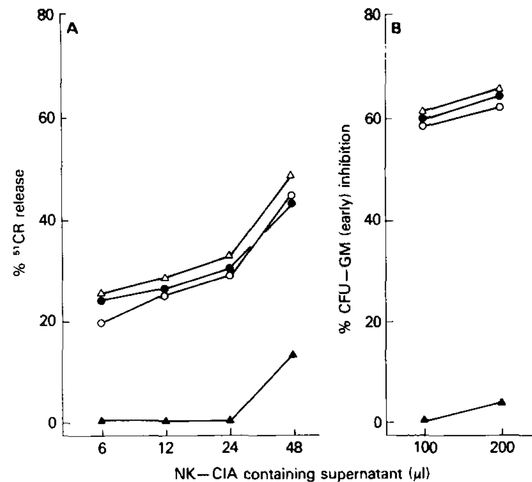

FIGURE 3. D-Mannose-6-phosphate inhibits absorption of NKCF and NK-CIA to K562 cells. Supernatant fluid from IFN-α-pretreated B73.1^{+} PBL induced by K562 cells was absorbed on K562 cells in the presence or absence of 50 mM D-mannose-6-phosphate, and tested, at the indicated quantity per assay, for (*A*) NKCF activity (U937 target cells) or (*B*) NK-CIA activity. (●), unabsorbed supernatant; (▲), supernatant absorbed on K562 cells; (○), unabsorbed supernatant to which 50 mM D-mannose-6-phosphate was added; (△), supernatant absorbed on K562 cells in the presence of 50 mM D-mannose-6-phosphate. All supernatants were dialyzed against RPMI 1640 medium before testing in order to eliminate D-mannose-6-phosphate.

presence of the sugar, however, completely prevented absorption of both NK-CIA and NKCF activity on K562 cells.

Interaction Between NK-CIA and IFN. The presence of IFN in the NK-CIA-containing supernatants was tested by inhibition of the cytopathic effect of VSV on human fibroblasts. No detectable IFN (<1 U) was ever found in the supernatants produced by untreated PBL, whereas titers of IFN up to 10 U/ml were occasionally found in supernatants produced by PBL pretreated with IFN-α (not shown). The antiviral activity detected in these supernatants was partially blocked by either a sheep antiserum to IFN-α or by an anti-IFN-γ mAb, and it was completely inhibited by a mixture of the two antibodies. To test the possibility

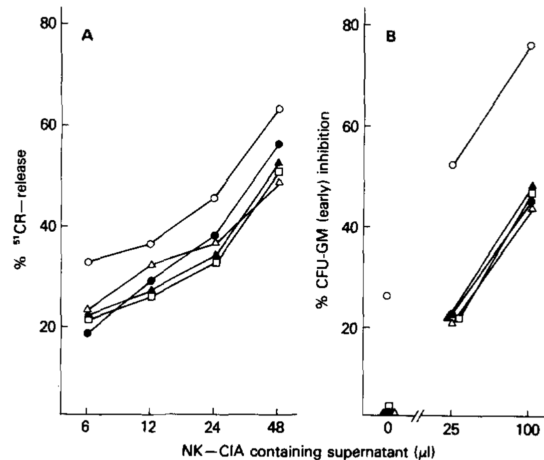

FIGURE 4. Inability of anti-IFN antibodies to suppress the activity of NKCF and NK-CIA. Supernatant fluid from IFN-α-pretreated B73.1$^+$ PBL induced by K562 cells was tested, at the indicated quantity per assay, for (*A*) NKCF activity (U937 target cells) or (*B*) NK-CIA activity in the presence or absence of rIFNγ or various anti-IFN antibodies. Assays were performed in: (●), medium alone; (○), 1,000 U/ml rIFNγ; (△), sheep anti-IFN-α antiserum (10^{-2} final dilution); (▲), B133.3 anti-IFN-γ mAb (ascites 10^{-3} final dilution); (□), anti-IFN-α and anti-IFN-γ antibodies.

that IFN play a role in either the NKCF or NK-CIA activity of the supernatants, the ability of anti-IFN antibodies to affect NKCF or NK-CIA activity was tested. Results of one representative experiment out of three performed with identical results are shown in Fig. 4. NKCF or NK-CIA activity was not affected by the anti-IFN-α antiserum, by the anti-IFN-γ mAb, or by a mixture of the two antibodies during the assay. Addition of rIFN-γ during the assay had an additive effect on the inhibition of CFU-GM (early) growth by NK-CIA and, like IFN-α (not shown), had a synergistic effect on the cytotoxicity of NKCF on U937 cells.

The effect of simultaneous addition of IFN and NK-CIA-containing supernatants on early (14-d) and late (7-d) CFU-GM formation by bone marrow cells was tested in three separate experiments. Representative results are reported in Fig. 5. Homogenous preparations of rIFN-α, -β or -γ were added to the culture, in the presence or absence of supernatant fluid of B73.1$^+$ cells (not IFN-α-pretreated) cultured with K562 cells. No antiviral activity was detectable in the NK-CIA-containing supernatant. Both rIFN-α and -β alone each induced a dose-dependent inhibition of CFU-GM, which was more apparent on day 7 than on day 14 colonies. ≤1,000 antiviral U/ml of rIFN-γ did not inhibit or only moderately inhibited CFU-GM. The NK-CIA-containing supernatant alone did not inhibit day 7 colonies, and inhibited day 14 colonies by ~50%. When rIFN-α and -β were added to NK-CIA, the degree of inhibition of day 7 colonies was the same as that observed using the two IFN types alone; rIFN-α and -β and NK-CIA had a less than additive effect on day 14 colonies. On the contrary, the simultaneous presence of rIFN-γ and NK-CIA resulted in a dramatic synergistic inhibition of day 7 CFU-GM, and almost complete inhibition of colonies was obtained with 1,000 U/ml rIFN-γ. This synergistic effect was still present on

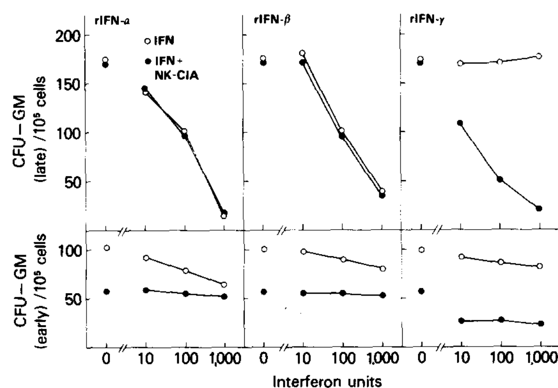

FIGURE 5. Effect of simultaneous addition of IFN and NK-CIA to CFU-GM colonies. Normal bone marrow cells were plated in the presence or absence of the indicated IFN types and NK-CIA-containing supernatant (supernatant of B73.1$^+$ PBL induced by K562 cells). Colonies were scored on day 7 (late CFU-GM) and on day 14 (early CFU-GM). (○), cultures in medium only; (●) cultures to which 100 μl/dish of NK-CIA-containing supernatant was added.

day 14 CFU-GM, but it was masked in part by the effective inhibition due to NK-CIA alone.

Fractionation of NK-CIA and NKCF-containing Supernatant Fluid by Gel Filtration and Anion-exchange Chromatography. The NKCF and NK-CIA-containing supernatants contained <1–8 U of cytotoxin when assayed on L-929 target cells (results not shown). When the supernatant fluids were fractionated on gel filtration columns using an FPLC apparatus, NKCF (U937 target cells) and cytotoxin (L-929 target cells) activities coeluted in a broad peak between 15 and 40 kD, which resolved in most experiments as two partially overlapping peaks of 15–20 and 30–35 kD (Fig. 6). NK-CIA activity also eluted in the same broad peak (results not shown). NKCF and cytotoxin activities were also coeluted from a Mono Q column at ~0.15 M NaCl (Fig. 6).

Effect of Anti-LT and Anti-TNF mAb on NKCF and NK-CIA Activity. Six different supernatant fluids obtained by incubation of NK cell–enriched preparations with K562 cells, and four obtained by incubation with allogeneic bone marrow cells were incubated for 6 h at 4°C in the presence of 5 μg/ml of LTB or of TNFD, then tested for NKCF activity on U937 cells and for NK-CIA activity on early and late CFU-GM, in the presence or absence of 1,000 U/ml of rIFN-γ (Table IV). In all cases, the anti-TNF antibody completely abolished NKCF and NK-CIA activity, whereas the anti-LT antibody had no effect.

NKCF and NK-CIA Activity Mediated by rTNF. rTNF in concentrations ranging from 0.06 to 64 U/ml was tested for cytotoxic (18-h ^{51}Cr-release assay) and cytostatic (48-h [^{3}H]TdR-incorporation assay) effects on U937 cells, and for inhibition of colony formation by early and late CFU-GM precursor cells, in the presence or absence of 1,000 U/ml of rIFN-γ (Fig. 7). TNF induced a significant and dose-dependent cytotoxic effect on U937 cells in the range of concentrations tested, and the effect was increased by the presence of rIFN-γ. IFN-γ alone was not cytotoxic for U937 cells. Percent inhibition of [^{3}H]TdR incorporation at both 24 (not shown) and 48 h (Fig. 7) was higher than percent ^{51}Cr release at 18 h, but most of the decreased incorporation of [^{3}H]TdR was probably due to cell

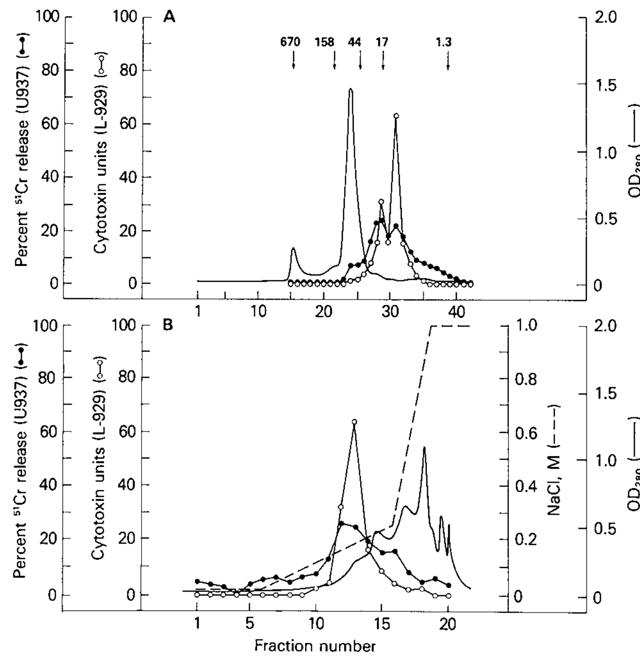

FIGURE 6. Gel filtration (*A*) and anion-exchange chromatography (*B*) of NKCF-containing supernatant produced by B73.1$^+$ PBL induced with K562 cells. Separations were performed as described using an FPLC apparatus and a Superose 12 column for gel filtration, or a Mono Q column for anion-exchange chromatography. Each fraction was tested for cytotoxic activity (○) on L-929 cells and for NKCF activity (●) on U937 target cells (50% final concentration).

TABLE IV
Effect of mAb LTB and TNFD on NKCF and NK-CIA Activity

Activity tested	Target cells	Inducer cells	Number of supernatants tested	rIFN-γ (1,000 U/ml)	mAb added*		
					None	LTB	TNFD
NKCF	U937	K562	6	–	51.4 ± 23.2‡	49.1 ± 22.8	5.9 ± 7.4
NK-CIA	CFU-GM (late)	K562	6	–	−1.3 ± 3.9	−1.5 ± 2.8	−0.7 ± 3.1
		K562	6	+	38.5 ± 22.8	39.7 ± 19.9	−6.7 ± 12.6
		Bone marrow	4	–	−1.5 ± 3.1	0.5 ± 1.7	−1.8 ± 2.4
		Bone marrow	4	+	28.5 ± 10.5	29.8 ± 5.6	−17.7 ± 6.4
NK-CIA	CFU-GM (early)	K562	6	–	39.5 ± 18.3	40.2 ± 15.3	3.7 ± 6.6
		K562	6	+	40.2 ± 16.8	37.5 ± 16.2	2.7 ± 6.1
		Bone marrow	4	–	53.0 ± 9.1	50.0 ± 12.2	5.8 ± 5.9
		Bone marrow	4	+	46.5 ± 17.5	46.0 ± 16.1	−0.3 ± 0.6

Supernatant fluids, obtained from NK-enriched PBL incubated for 6 h with the indicated inducer cells, were tested for NKCF activity and for NK-CIA activity on the indicated target cells, as described.

* Supernatant fluids were incubated for 6 h at 4°C with the indicated mAb (5 μg/ml) before being tested for NKCF and NK-CIA activity.

‡ Values indicate percent ^{51}Cr release (NKCF) or percent colony inhibition (NK-CIA) (mean ± SD). Number of colonies (four separate experiments, mean ± SD) was: CFU-GM (late), 160.2 ± 29.6; CFU-GM (late), 1,000 U/ml rIFN-γ, 123.1 ± 36.0; CFU-GM (late), 5 μg/ml LTB, 152 ± 42.5, CFU-GM (late), 5 μg/ml TNFD, 150.5 ± 33.4; CFU-GM (early), 65.5 ± 10.1; CFU-GM (early), 1,000 U/ml rIFN-γ, 65.3 ± 11.9; CFU-GM (early), 5 μg/ml LTB, 65.7 ± 10.0; CFU-GM (early), 5 μg/ml TNFD, 65.3 ± 9.5.

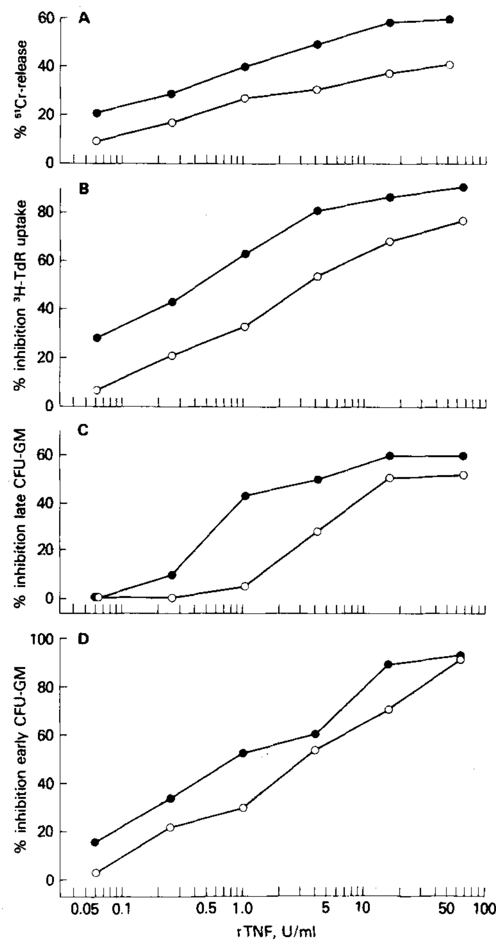

FIGURE 7. Effect of rTNF on U937 cells and on CFU-GM colonies. Recombinant TNF at the indicated concentrations was tested on U937 target cells in (*A*) a 18-h ^{51}Cr-release assay, and (*B*) a 48-h [^{3}H]TdR incorporation assay, or was tested for its ability to inhibit (*C*) late (day 7) CFU-GM colonies and (*D*) early (day 14) CFU-GM colonies, in the presence (●) or absence (○) of 1,000 U/ml of rIFN-γ. Number of colonies per 10^5 bone marrow cells was: late CFU-GM, 183.7 ± 1.7; late CFU-GM, 1,000 U/ml IFN-γ, 92.3 ± 2.7; early CFU-GM, 68.0 ± 12.2; early GFU-GM, 1,000 U/ml of rIFN-γ, 62.0 ± 7.3.

death. A similar inhibition of [^{3}H]TdR uptake, but a much lower ^{51}Cr release, was observed on other myeloid cell lines, suggesting a predominantly cytostatic effect of TNF on those lines (not shown). The presence of 1,000 U/ml of rIFN-γ alone resulted in 50% inhibition of [^{3}H]TdR incorporation, and induced a synergistic inhibition of [^{3}H]TdR in conjunction with rTNF (Fig. 7). Formation of colonies by late (day 7) CFU-GM precursor cells was inhibited by doses of rTNF ≥4 U/ml, and in the presence of 1,000 U/ml of IFN-γ, by doses ≥0.25 U/ml. Formation of colonies by early (day 14) CFU-GM precursor cells was inhibited by doses of rTNF ≥0.25 U/ml, and in the presence of 1,000 U/ml of IFN-γ, by doses ≥0.06 U/ml.

Discussion

In this study, we have compared the factor responsible for the activity of NK-CIA-containing supernatants wih NKCF, and on the basis of functional, biochemical, and antigenic similarities, have identified the factor responsible for NK-CIA as NKCF. All supernatants containing NK-CIA also contained NKCF, and a highly significant positive correlation between the two activities was demonstrated. Previous studies (5, 32) have identified NK cells as the human NKCF producer cells on the basis of separation of PBL on a discontinuous Percoll gradient. However, because NK cell–enriched fractions on Percoll gradients are always contaminated with cell types other than NK, the presence of cells that produce a given lymphokine in light-density PBL preparations is not sufficient to identify NK cells as the producers. The use of mAb has made it possible to distinguish and separate NK cells from the cells that produce IFN-α upon virus infection, and that copurify with NK cells on Percoll gradients (33). In this study, we used PBL preparations highly enriched for cells with NK activity and obtained, using anti–NK cell–specific antibodies (B73.1 and N901), and a series of antilymphocyte antibodies reacting with antigens present or absent on a proportion of mature NK cells. Results of all cell separation experiments were consistent, allowing the unambiguous identification of the cells producing NKCF as PBL bearing all and only the known surface markers of cytotoxic NK cells.

IFN-treatment of PBL enhances both NKCF and NK-CIA production. The same cell types, i.e., NK-sensitive cell lines and bone marrow cells enriched for HLA-DR$^+$ precursor cells, induce NK cells to produce both NK-CIA and NKCF. Similarly, these same cell types absorb both NK-CIA and NKCF activity from PBL supernatants. The activity of NK-CIA and NKCF, and the ability of K562 cells to absorb them are both blocked by D-mannose-6-phosphate. Both NK-CIA and NKCF are inactivated by incubation at 37°C for 48 h.

An 80 kD factor has been described recently (19) that is produced by a T cell hybrid line, and that inhibits bone marrow colony formation. Unlike NK-CIA and NKCF, this factor is specifically cytotoxic for HLA-DR$^+$ cell lines, independent of their sensitivity to NK cells, and its effect is prevented by anti-HLA-DR mAb (19, 34). NK-CIA can be distinguished from other factors mediating colony-inhibiting activity, such as prostaglandin E, lactoferrin, and acidic isoferritin on the basis of different molecular mass, different producer cells, and different mechanisms of inhibition.

The absence of antiviral activity in supernatant fluids containing strong NK-CIA activity, and the inability of antibodies against IFN-α and -γ to block inhibition of hematopoietic colonies by NK-CIA exclude the possibility that IFN accounts for the inhibition. The inhibitory effect of IFN-α and -β on CFU-GM we observed using homogeneous IFN preparations is consistent with previous reports (16, 17). However, unlike recent reports with natural IFN-γ (17, 35), we find little or no inhibition of CFU-GM colonies by rIFN-γ, even at doses up to 1,000 U/ml. This result does not rest in loss of activity of the IFN-γ preparation used, as repeated controls indicated intact antiviral activity, and the ability to induce or enhance HLA-DR antigen expression on sensitive cell lines. In other studies (17, 35), anti-IFN-γ mAb blocked the inhibition of colony formation, and on this basis, IFN-γ was identified as the colony-inhibiting activity contained

in the supernatant fluid of phytohemagglutinin-stimulated human PBL (35). However, IFN-γ has a strong synergistic effect with LT in preventing growth inhibition of various cell lines (36, 37); the effect of IFN-γ is probably more dramatic than that previously described for IFN-α (11), and a mixture of LT and IFN-γ can completely prevent growth of cell lines that are not sensitive to the growth-inhibiting activity of LT or IFN-γ alone. LT are common contaminants of purified preparations of natural IFN-γ (36). We have evidence that the inhibition of colony formation by the supernatant fluid of phytohemagglutinin-stimulated PBL is due to the synergistic action of LT and IFN-γ (M. Murphy, manuscript in preparation). Thus it is possible that contaminant LT in the purified preparations of IFN-γ are responsible for the inhibition of colony formation, and that anti-IFN-γ antibodies prevent this inhibition by abrogating the synergistic effect of the two lymphokines. As suggested in a recent study (38), it is also possible that IFN-γ acts only indirectly on the precursor cells, through activation of other cell types, such as NK cells, T cells, or mature granulocytes. These cell types may not be present in our bone marrow samples because of the low initial contamination with peripheral blood cells (NK and T cells), and because mature granulocytes are depleted on F/H gradients. Alternatively, as suggested by studies (39) on murine bone marrow precursor cells, the discordant results on the effects of IFN-γ might be due to the use of different types or different concentrations of CSF.

Our data indicate that IFN-γ, similar to the effect described for LT on cell lines (36, 37), has a strong synergistic effect with NK-CIA. Whereas NK-CIA in the absence of IFN-γ is active only on early myeloid colony precursors, it becomes highly effective on late GM precursor cells, which are nonresponsive to NK-CIA alone, when IFN-γ is present during the culture period. Because the activity of NK-CIA is lost after 2 d of culture at 37°C, and NK-CIA and IFN-γ were added simultaneously to the cultures, IFN-γ must act on the GM precursor cells within the first 48 h of culture. The inability of NK-CIA to affect late CFU-GM may rest in the loss of the receptors for NK-CIA during maturation of GM precursor cells. IFN-γ, on the other hand, might induce the expression of these receptors. It is impossible, at present, to determine whether this effect of IFN-γ is linked to its ability to induce HLA-DR antigen expression and monocytic differentiation on immature myeloid cells, but a direct effect on HLA-DR antigens seems unlikely, due to the independence of NKCF activity and HLA-DR expression on target cells. The synergistic effect of IFN-γ and NK-CIA is of particular interest because NK cells are also efficient producers of IFN-γ, for example, in response to interleukin 2 (30). IFN-γ produced by NK or T cells induces differentiation of immature myeloid cells (40) and B cells (41), and is a potent activator of cells such as macrophages (42) and neutrophils (43 and our unpublished results), which are involved in the regulation of hematopoiesis and immune responses through secretion of both stimulating and suppressing factors.

The NK-CIA-containing supernatants contain <1–8 cytotoxin units, as assayed on L-929 cells. NKCF and NK-CIA activities coelute with the L cell cytotoxic activity from gel filtration and anion-exchange columns. Also, the molecules mediating the three activities fail to bind to a lentil lectin–Sepharose column (not shown). A series of soluble factors has been described that exert cytotoxic or

cytostatic activity on hemopoietic cell lines or bone marrow cells. Classes of LT had been defined based on differing molecular mass (44), but recent evidence (36) suggests that the higher molecular mass forms observed under nondenaturing conditions are oligomers of the two basic forms, because sodium dodecyl sulfate gel electrophoresis reveals only a 25 and a 20 kD form of pure LT (36). NKCF elutes upon gel filtration in two peaks of activity that may represent different molecules or aggregates (dimers?) of a single molecule of 15–20 kD. However, unlike NKCF (12), the 20 and the 25 kD monomeric forms of LT are not shown under nondenaturing conditions. A transient and unstable peak of LT activity of 10–15 kD (LT) has also been reported, but its molecular mass is lower than that noted for NKCF or NK-CIA (44). Under our separation conditions, LT, produced by mitogenic stimulation of peripheral blood T cells (44) and by phorbol ester stimulation of B cell lines (45), elutes in gel filtration as a major peak of 60 kD, and binds to lentil lectin columns and B cell–derived LT elutes from anion-exchange columns at a lower molarity than NKCF.

TNF, another cytotoxin produced by leukocytes, has been characterized in the serum of endotoxin-treated rabbits as a 68 kD molecule on sodium dodecyl sulfate (SDS) electrophoresis (46). TNF produced by human monocytes and myeloid cell lines resolves as a 30 kD peak by gel filtration, and as a 17 kD species on SDS gels (14, 15, and J. Vilcek, personal communication). The cDNA for human TNF, cloned from HL-60 cells, encodes a protein of 156 amino acids with a calculated molecular weight of 17,356, with no *N*-glycosylation sites (14, 15). The TNF sequence shows 28% homology with the LT sequence (13, 14). The biochemical characteristics of NKCF/NK-CIA and of the cytotoxin present in these preparations are not inconsistent with those reported for monocyte-derived TNF. The presence of the activity in the 15–20 kD peak in gel filtration could be due to the presence of a monomeric form found in NK-derived supernatant fluids, but not found in the monocyte-derived supernatant. Alternatively, the low molecular mass peak could be due to a hydrophobic interaction with the matrix of the FPLC columns used in our separations, whereas such interaction might not occur with conventional gel filtration columns. Preliminary evidence suggesting the identification of NKCF as TNF has been reported (47) based on the ability of anti-TNF mAb to block NKCF activity (47). We tested both anti-LT and anti-TNF for their ability to block NKCF and NK-CIA activity in our supernatant fluid. Only anti-TNF antibodies completely blocked the cytotoxic effect of several supernatants on U937 target cells, and blocked their ability to inhibit CFU-GM. The inhibition of early GM precursor cells by supernatants alone, and the inhibition of late GM precursor cells by supernatants plus rIFN-γ were both blocked by the anti-TNF antibody. These results suggest the possibility that NK cells, in response to target cell lines or immature bone marrow cells, produce TNF. This possibility is supported by the observation that purified rTNF is able to mediate NKCF and NK-CIA type activity in a range of concentrations corresponding to the titer of cytotoxins present in the NK cell–derived supernatant, as measured on L-929 cells. However, in light of the partial sequence homology between LT and TNF, and the existence of lymphokines such as IFN that represent a group of homologous and only partially antigenically similar molecules, the reactivity of one mAb with both TNF and NKCF/NK-

CIA might not constitute definitive evidence for the identification of the cytotoxin produced by NK cells with the TNF produced by monocytes. This identification will require detailed comparative biochemical and antigenic studies of the cytotoxins produced by the different cell types.

Summary

We characterize the natural killer (NK) cell colony-inhibiting activity (CIA) produced in supernatants from cultures of human peripheral blood lymphocytes (PBL) with NK-sensitive target cell lines, and study its relationship with NK cell–derived cytotoxic factor (NKCF). Using monoclonal antibodies (mAb) specific for NK cells or other lymphocyte populations, we unambiguously identify NK cells as the only PBL subset able to produce both NKCF and NK-CIA. We present functional and biochemical data suggesting that NKCF and NK-CIA represent the same molecule: (*a*) a highly significant positive correlation exists between the quantity of NKCF and NK-CIA in supernatants independently produced by different PBL subsets; (*b*) both NK-CIA and NKCF are induced by culture of PBL with NK-sensitive, but not with NK-insensitive cell lines, and with HLA-DR$^+$ bone marrow cells; (*c*) both NKCF and NK-CIA are absorbed on the same cell lines or bone marrow cell types; (*d*) the two activities coelute in the same gel filtration fractions; (*e*) D-mannose-6-phosphate blocks both NKCF and NK-CIA activity, and prevents their absorption by K562 cells; and (*f*) both NKCF and NK-CIA activity are lost after 2 d at 37°C.

The NK-CIA-containing preparations are devoid of antiviral activity, and antiinterferon (anti-IFN) antibodies do not block the inhibitory activity of NK-CIA. The effect of NK-CIA on day 14 (early) colony-forming units of granulocytes and macrophages (CFU-GM) is synergistic with that of IFN-γ, and this synergy is also evident on day 7 (late) CFU-GM growth. A combination of NK-CIA and IFN-γ suppresses late CFU-GM, at concentrations of the two lymphokines that are completely ineffective when used independently. No synergy between NK-CIA and IFN-α or -β was observed, due to a direct inhibitory effect of these two IFN types on late CFU-GM. Antibodies specific for tumor necrosis factor (TNF), but not those specific for lymphotoxins, inhibit both NK-CIA and NKCF activity in the NK cell–derived supernatant. Recombinant TNF, in the range of concentrations corresponding to that of the cytotoxic activity on L-929 cells present in supernatants, mediated both NKCF and NK-CIA activity. Together, our data suggest that the molecules responsible for both the cytotoxic effect of NK cell supernatants on NK-sensitive cell lines and the cytotoxic or cytostatic effect of the same supernatants on bone marrow cells are identical, and that these molecules are antigenically, functionally, and biochemically similar to or identical with the TNF produced by human monocytes and myeloid cell lines.

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