

INTERACTION OF Fc RECEPTOR (CD16) LIGANDS INDUCES
TRANSCRIPTION OF INTERLEUKIN 2 RECEPTOR (CD25)
AND LYMPHOKINE GENES AND EXPRESSION OF THEIR
PRODUCTS IN HUMAN NATURAL KILLER CELLS

BY IGNACIO ANEGÓN, MARIA CRISTINA CUTURI, GIORGIO TRINCHIERI,
AND BICE PERUSSIA

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Receptors for the Fc domain of IgG (FcR) are present on a variety of cell types and mediate interactions between the intracellular and humoral compartments. Of the three FcR types identified on human leukocytes, the low-affinity FcR for immune complexed IgG (CD16) is present on NK cells and neutrophils (1, 2) and is biochemically similar to the low-affinity FcRII for murine IgG1/IgG2b detected on murine macrophages and B cells by antibody 2.4G2 (3), which also detects an Fc on murine NK cells (Perussia, B., Tutt, M., Trinchieri, G., Kumar, V., manuscript in preparation). CD16 on human NK cells and neutrophils are, like the FcR on murine B cells and macrophages, antigenically crossreactive (1, 4). These observations suggest the possibility that CD16 is the human equivalent of FcRII. Two genes have been identified that encode murine FcRII: one expressed in macrophages, and one in both macrophages and lymphocytes (5); cloning of human FcR gene(s) has not been reported yet. The sequences obtained for these FcRII genes indicate that they belong to the Ig supergene family (5). The extracellular domains of their products are 95% homologous, independent of the cell type on which they are expressed, but transmembrane and cytoplasmic domains in the different cell types bear no homology. Most proteins encoded by members of the Ig supergene family are surface molecules or receptors involved in recognition of membrane-associated or soluble molecules and transduce signals intracellularly upon ligand binding.

Unlike monocytes and neutrophils (6, 7), human NK cells bear only one type of FcR (i.e., CD16), which represents, at present, the only defined receptor expressed on NK cells and not on other lymphocyte subsets. It is through this surface molecule that the activity of NK cells can be specifically targeted toward cells sensitized with IgG antibodies. We showed that interaction of the NK cell FcR with immune complexes, with anti-CD16 antibodies, or with IgG antibody-sensitized target cells, all induce similar down-modulation of FcR (CD16) (4, 8) and trigger cellular events that result in exhaustion of cytolytic potential of the cells (9). Enhancement of NK cell activity upon interaction of the cells with anti-

This work was supported, in part, by National Institutes of Health grants CA-10815, CA-20833, CA-32898, CA-37155, CA-40456, and CA-45284. B. Perussia is a Scholar of the Leukemia Society of America.

CD16 antibodies has also been reported (10). The effects observed in NK cells upon FcR ligand binding are reminiscent of those observed in CD3⁺ T cells upon interaction of antigens with TCR or of antibodies with the TCR-associated molecule CD3. CD3 antigen modulation is induced in these conditions (11) and T cells undergo a series of metabolic events that include both activation (12–14) and inactivation or refractoriness to further stimulation (15, 16). In the case of T cells, two distinct stimuli are necessary to induce maximal activation (17).

NK cells bear the gp75 molecule that binds IL-2 with low affinity (18) and, upon stimulation with high doses of IL-2, are activated to secrete lymphokines (e.g., IFN- γ) (19), to express activation antigens, e.g., Tac(CD25), and eventually to undergo proliferation (20, 21). Upon recognition and binding of target cells through a putative NK cell receptor, NK cells release cytotoxic molecules (22) and our data indicating that TNF is released in the supernatant from NK target cell cocultures (23) suggest the possibility that NK cells produce this cytokine upon specific stimulation.

Detailed studies of the intracellular events after NK cell stimulation with specific (immune complexes, target cells) or nonspecific (IL-2) ligands have been hampered by the difficulty of obtaining sufficient numbers of cells either from fresh peripheral blood or from clonally expanded cultures. We developed an in vitro short-term bulk culture system that allows us to obtain large numbers of homogeneous NK cell preparations with all characteristics of fresh NK cells but activatable with faster kinetics (24, 25). Here, we used these cells to study the role of FcR(CD16) in NK cell activation and the mechanisms on which such activation depends.

Materials and Methods

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

Monoclonal and Polyclonal Antibodies. mAbs B73.1 (IgG1, anti-CD16) (26), B36.1 (IgG2b, anti-CD5) (27), B33.1 (IgG2a anti-HLA-DR nonpolymorphic determinant) (8), B52.1 (IgM, anti-CD14 on monocytes), and B13.4 (IgM, anti-monocyte/granulocytes) (27, 28) were produced and characterized in our laboratory. 3G8 (IgG1, anti-CD16) (2) was produced from cells kindly provided by Dr. J. Unkeless (Mount Sinai Medical School, New York, NY); N901 (IgG1, anti-NKH-1 antigen) (29) and Tac (IgG1, anti-IL-2R, CD25) (30) were kindly provided by Drs. J. Griffin (Dana Farber Cancer Institute, Boston, MA) and T. Waldmann (National Institutes of Health, Bethesda, MD), respectively. OKT3 (IgG2a, anti-CD3), 5E9 (IgG1, anti-transferrin receptor (Tf-R)), 4F2 (IgG1, anti-lymphocyte activation antigen), and W6.32 (IgG2a, anti-HLA class I nonpolymorphic determinant) were produced from cells obtained from the American Tissue Culture Collection (Rockville, MD). The anti-IFN- γ B133.1 and B133.5 and the anti-TNF B154.7 and B154.9 used for the IFN- γ and TNF RIA were produced and characterized in our laboratory (19, 31). FITC-labeled antibodies Leu-19 (IgG1, anti-NKH-1) and Leu-1 (IgG2b, anti-CD5) (32) were kindly provided by Dr. L. Lanier and N. Warner (Becton Dickinson & Co., Mountain View, CA). When indicated, purified antibodies were labeled with biotin or linked to CNBr-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to routine procedures. The polyclonal FITC-goat F(ab')₂ anti-mouse Ig and the rabbit IgG antiovine erythrocytes (E) were purchased from Cappel Laboratories (Cochranville, PA). The goat F(ab')₂ anti-mouse IgG used to prepare the E for indirect

rosetting was produced in our laboratory, absorbed on human IgG, and affinity purified on mouse IgG-Sepharose 4B column (Pharmacia Fine Chemicals).

Peripheral Blood Lymphocytes, NK, and T Cells. Venous peripheral blood was obtained by venipuncture from adult healthy donors and anticoagulated with heparin. PBMC were separated on Ficoll-Hypaque density gradient (Lymphoprep; Nyegaard and Co., Oslo, Norway). PBL were obtained from PBMC depleted of monocytes after two cycles of adherence on plastic surfaces (45 min, 37°C) and depletion of B52.1/B13.4⁺ cells by indirect antiglobulin rosetting (26). To obtain large numbers of NK cells, short-term bulk cultures of these cells were prepared as previously described in detail (24, 25). Briefly, PBMC were cultured at 37°C with 50 Gy-irradiated RPMI 8866 or Daudi B lymphoblastoid cells for 10 d (5:1 PBMC/B lymphoblastoid cells, 2.5×10^5 PBMC/ml RPMI-10% FCS) in tissue culture plates in a 5% CO₂ humidified atmosphere. The total number of cells recovered after a 10-d culture is increased, on average, 10-fold, and the cell population is ~60–80% CD16⁺/NKH-1⁺/CD3⁻ NK and 20–40% CD3⁺/CD16⁻/NKH-1⁻ T cells. Homogeneous populations of NK and T cells were negatively selected and purified by density centrifugation after sensitization of the lymphocytes with a mixture of antimonocyte B52.1 and either anti-CD3 and anti-CD5, or anti-CD16 and anti-NKH-1 antibodies, respectively, and indirect rosetting with CrCl₃-treated goat anti-mouse Ig-coated E (26). Both the NK and the T cells purified from these cultures have morphologic, phenotypic, genotypic, and functional properties identical to those of the two subsets freshly obtained from PBL (24, 25). Unlike fresh cells, however, the majority of these cells express HLA-DR antigens. A small proportion (5–10%) of the NK cells bear Tac(CD25) at low density and the cells can be induced to proliferate with fast kinetics in response to rIL-2 without added accessory cells. Similarly, T cells respond with fast kinetics to rIL-2, and a proportion of them bear Tac at higher density than the NK cells purified from the same cultures. Monocytes and B cells were not detected in the cultures on day 10 (25).

Lymphocyte Stimulation. The purified fresh PBL, the NK and T cell subpopulations obtained from the bulk cultures were cultured (37°C, 5×10^6 cells/ml RPMI-10% FCS) for the indicated periods of time with different inducers. FcR(CD16) ligands were: (a) anti-CD16 antibodies (3G8 or B73.1) used in either soluble form (1 µg IgG1/ml) or linked to CNBr-Sepharose 4B (5 mg antibody/ml Sepharose, 10 µl beads/ 10^6 cells), (b) particulate immune complexes, i.e., IgG-sensitized bovine E (EA7S) prepared as previously described (4). Sepharose-linked irrelevant antibodies (anti-CD3 OKT3, anti-HLA class I W6.32) and E were used as controls. When indicated, purified human rIL-2 was present (100 U/ml) (rIL-2, 5×10^6 U/mg in a standard CTLL assay; Takeda Chemical Industry, Inc., Osaka; kindly provided by Dr. T. Taguchi, Osaka University, Osaka, Japan). In some cases the stimuli were added to cells pretreated with cycloheximide (10 µg/ml, 1 h at 37°C) (Calbiochem-Behring Corp., La Jolla, CA); cycloheximide was present throughout the culture period. After culture, the cells were collected, Sepharose beads were detached by vigorous vortexing, and eliminated from the cell suspension after a 1-min centrifugation at 500 rpm. Particulate immune complexes were removed by lysis with hypotonic medium. Ligand-free cells were washed and used for the different assays. Cell viability was >95% in all conditions and recovery was ~80% of the original input without loss of lymphocyte subsets.

IFN-γ and TNF RIA. These were performed as previously described in detail (31, 33) on cell-free supernatants obtained from the cells induced as above. Purified human natural IFN-γ (Interferon Sciences, New Brunswick, NJ) and purified human rTNF (5×10^7 U/mg on L929 cells, kindly provided by Dr. H. M. Shepard, Genentech Inc., South San Francisco, CA) were used in each assay to construct the standard curve. Sensitivity of the RIA for IFN-γ was 0.2 U/ml, of that for TNF, 0.1–0.2 U/ml.

Immunofluorescence. Indirect and direct immunofluorescence were performed as previously described in detail using an FITC-goat F(ab')₂ anti-mouse Ig (Cappel Laboratories) preabsorbed on human IgG (26), or predetermined saturating concentrations of

biotin-labeled antibodies detected with phycoerythrin (PE)¹-labeled avidin (Becton Dickinson & Co.) and FITC-labeled antibodies (4), respectively. Irrelevant antibodies of matched isotypes were used as negative controls. The samples were analyzed on an Ortho Cytofluorograf 50H connected to a 2150 Data Handling System (Ortho Diagnostic Systems Inc., Westwood, MA). Intensity of fluorescence was measured on a logarithmic scale.

Northern and Dot Blot Hybridization. Northern blots were performed as previously described in detail (31). Briefly, total cytoplasmic RNAs were extracted from uninduced and induced cells. Equal amounts of RNA (20 µg/lane, as quantified by spectrophotometric reading at 260 and 280 nm) were size fractionated in a 1% agarose-formaldehyde gel. Ethidium bromide-stained gels were visualized to assess integrity of RNA and to verify the loaded amounts. RNA were transferred by capillarity to nitrocellulose paper and hybridized with nick-translated purified cDNA fragments bearing human IL-2-R, IFN-γ, or TNF sequences. When dot blots were used, sequential 1:3 dilutions of RNA (starting from 2 µg) were applied to nitrocellulose using a dot blot apparatus (Schleicher and Schuell, Keene, NH). After hybridization and washing, the filters were autoradiographed using XAR film (Eastman Kodak, Rochester, NY) and an intensifying screen (Dupont Co., Wilmington, DE). The cDNA probes used were: IL-2R2 for IL-2-R, kindly provided by Dr. W. Green (NIH, Bethesda, MD), TNF provided by Dr. H. M. Shepard (Genentech Co.), and pSWIF for IFN-γ provided by Dr. S. Clark (Genetics Institute, Boston, MA). Human β-actin probe, pBR322, pB_{AE} probe for 28S ribosomal RNA, and pSP64 were used as controls and were kindly provided by Dr. R. Weinmann and P. Curtis (Wistar Institute).

Nuclear Transcription Analysis (Run-On Assays). Nuclei from 20 min-stimulated and unstimulated cells were isolated as described by Krönke et al. (34). Briefly, cells were lysed in 10 mM Tris buffer (pH 7.5) containing 2 mM MgCl₂, 3 mM CaCl₂, 3 mM DTT, 300 mM sucrose, and 0.3% NP-40. Nuclei were pelleted by centrifugation over a 750-mM sucrose gradient. In vitro transcription was according to Groudine et al. (35) with slight modifications. Nuclei were resuspended in a solution containing 1 mM MgCl₂, 70 mM KCl, glycerol 15%, 2.5 mM DTT, 0.25 mM each ATP, GTP, and CTP (Sigma Chemical Co., St. Louis, MO), and 400 µCi [³²P]UTP (sp act ~400 Ci/mM; Amersham International, Amersham, United Kingdom). After a 25-min incubation at 26°C, the reaction was stopped adding sequentially RNase-free DNase I (Promega, Madison, WI) and Proteinase K (Calbiochem-Behring Corp., San Diego, CA). Nuclear RNA (nRNA) was isolated as described by Gariglio et al. (36) by extraction with phenol/chloroform and chloroform, and isopropyl-alcohol precipitation at -70°C. The pellet was resuspended in 10 mM Tris (pH 8.0) 1 mM EDTA and free ³²P was eliminated on a Sephadex G-50 spin column (Boehringer-Mannheim Biochemicals, Indianapolis, IN). After repeating the DNA and protein digestion and RNA extraction and precipitation, the samples were washed in ethanol 80%, dried, and resuspended in hybridization solution (20 mM sodium phosphate buffer, pH 6.5, 50% formamide, 5× SSC [1×: 0.15 M NaCl, 0.015 M sodium citrate pH 7.0], 1× Denhardt's solution [0.02% polyvinylpyrrolidone, 0.02% BSA, 0.02% Ficoll 400], 100 µg/ml denaturated salmon sperm DNA, 0.1% SDS). Incorporated radioactivity was determined by liquid scintillation counting and equal amounts of radioactivity per sample were heat denaturated and resuspended (5–7.5 × 10⁶ cpm/ml) in hybridization solution. The same number of ³²P-labeled nRNA cpm per sample were hybridized (3 d) at 42°C with prehybridized nitrocellulose filters on which 10 µg of linearized plasmid cDNAs had been immobilized using a slot-blot apparatus (Schleicher & Schuell, Inc., Keene, NH). Filters were washed twice with 2× SSC and 0.1% SDS at 37°C and, after rinsing with 2× SSC, were incubated (30 min, at 37°C) in 2× SSC with 10 µg/ml RNase. After two washes in 0.1× SSC and 0.1% SDS at 65°C for 30 min, filters were dried and exposed to Kodak XAR film with intensifying screens. Extent of hybridization was quantitated by scanning densitometry with an LKB laser densitometer (Ultrascan XL; LKB Instruments, Inc., Gaithersburg, MD) on films exposed for the appropriate length

¹ Abbreviations used in this paper: EA7S, IgG antibody-sensitized E; LGL, large granular lymphocyte; PE, phycoerythrin; Tf, transferrin.

of time to produce bands with absorption within the linear response range. To calculate the rate of transcription, background hybridization to pBR322 or pSP64, whichever applicable, was subtracted from the densitometric values and these were normalized relative to values obtained for the 28S rRNA probe pBAE.

Results

FcR(CD16) Crosslinking at the NK Cell Membrane Induces Expression of Tac (CD25) Antigen in Fresh Peripheral Blood NK Cells. Total PBL populations were cultured with insolubilized CD16 ligands and the proportion of T and NK cells induced to express Tac antigen was analyzed in two-color immunofluorescence. 5.4% of resting PBL (average of results from four experiments performed, representative results with PBL from one donor are reported in Fig. 1) express Tac antigen at minimal density (Fig. 1, *top panels*), and this proportion is unchanged upon an 18-h incubation with rIL-2 (not shown). After 18 h of culture in the presence of particulate immune complexes (EA7S, not shown) or Sepharose-linked B73.1 antibody (Fig. 1, panels in third row) an average of 9.8% of PBL were Tac⁺. Tac was expressed on an average of 23.9% of the NKH-1(Leu-19⁺) NK cells, but only on 4.3% of the CD5(Leu-1⁺) T cells (or CD3 (OKT3⁺) cells, not shown). The proportion of Tac⁺ PBL increased, on average, to 14.7% and 23% when PBL were stimulated with EA7S (Fig. 1, panels in second row) or with Sepharose-linked anti-CD16 antibody B73.1 (Fig. 1, *bottom panels*), respectively, in the presence of rIL-2 (100 U/ml). In these cases, Tac was expressed on 31.3% and 40.8% of the NKH-1⁺ cells, and on 8.2% and 11.8% of the CD5⁺ T cells, respectively. The proportion of NKH-1⁺ or CD5⁺ cells (average, 16.5% and 68.0%, respectively) and that of HLA-DR⁺ cells were unchanged under any of the experimental conditions. No Tac antigen expression was induced on PBL upon culture with soluble anti-CD16 antibodies, Sepharose-linked anti-HLA class I antibodies, or nonsensitized E, independently of the presence of rIL-2 (data not shown).

Interaction of FcR(CD16) Ligands with Cultured NK Cells Induces Expression of Surface Activation Antigens. Negatively selected CD3/CD5⁻ NK cells were obtained from the 10-d coculture of PBMC with irradiated B lymphoblastoid cells. The proportion of cells expressing different surface antigens was detected by indirect immunofluorescence (Table I and Fig. 2). The majority of the CD3/CD5⁻, CD16/NKH-1⁺ cells were HLA-DR⁺, an average of 12% of them expressed Tac antigen or TfR at low density, and ~3% of the cells expressed 4F2 antigen (Table I). No significant antigenic change was detected upon culture of the cells (18 h, 37°C) in the presence of rIL-2. Soluble anti-CD16 antibodies (not shown), Sepharose-linked anti-CD3 or anti-HLA class I antibodies, or E either in the presence or absence of rIL-2 (Table I) did not affect the expression of Tac, TfR, and 4F2. As we previously demonstrated using fresh PBL (4, 8), interaction of NK cells with FcR ligands induced down-modulation of the CD16 antigen, and this effect was not reversed in the presence of rIL-2. When NK cells were incubated with Sepharose-linked anti-CD16 antibody B73.1 the proportion of Tac⁺ cells increased, on average, to 27% and, when rIL-2 was present during the incubation, to 54% (Table I). In the presence of rIL-2 the density of Tac antigen on the positive cells, as evaluated from fluorescence intensity, was much higher than in cells treated with Sepharose-linked anti-CD16 antibodies

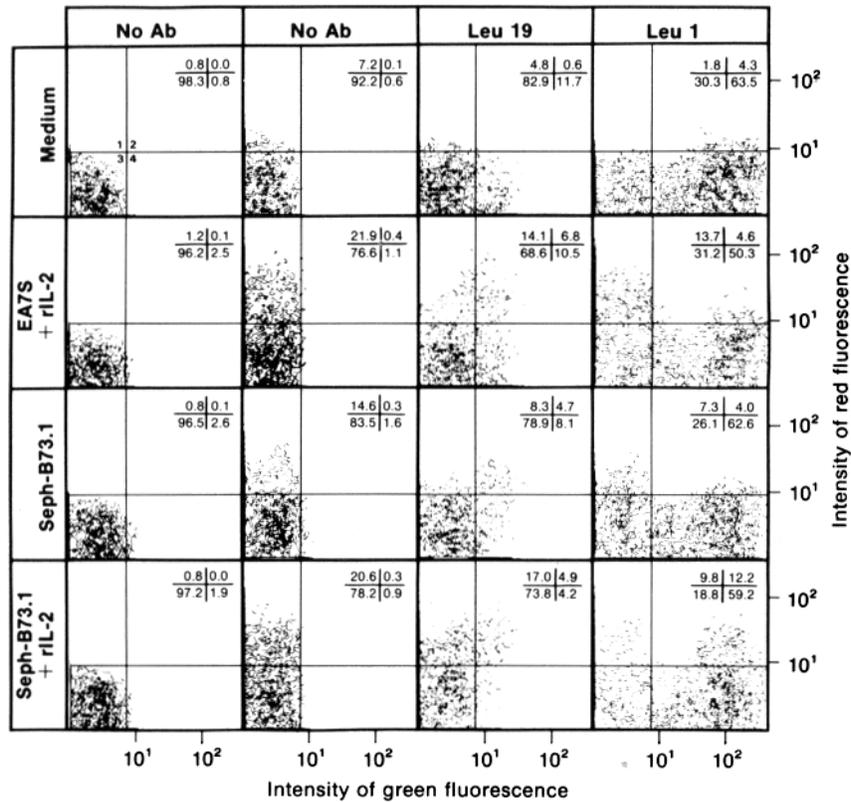


FIGURE 1. Induction of expression of Tac antigen in fresh PBL stimulated with FcR(CD16) ligands. PBL from one donor were incubated (18 h, 37°C) in the presence of the indicated stimuli. Surface antigen expression was evaluated by two-color immunofluorescence. Tac was detected using biotin-labeled anti-Tac and PE-avidin (red fluorescence; second, third, and fourth columns of contour plots), NKH-1 and CD5 were detected using the FITC-labeled antibodies indicated on the top of the columns (green fluorescence). Correlate measurements of green (x-axis, log scale) and red (y-axis, log scale) fluorescence are displayed as two-dimensional contour plots. Based on the negative control samples in the absence of antibodies (contour plots in the first column), the contour plots were divided into quadrants, indicating: (a) cells with red fluorescence (binding PE-avidin only); (b) double-positive cells; (c) double-negative cells; (d) cells with green fluorescence (binding FITC-labeled antibody only). Numbers in the top right corner of each scattergram indicate the proportion of cells in the respective quadrants. The numbers on the scales refer to arbitrary units of fluorescence intensity.

only (Fig. 2). The proportion of Tfr⁺ and of 4F2⁺ cells increased similarly under the same conditions, whereas no significant changes were detected in the expression of HLA-DR antigens under any condition. Analogously to the stimulation with anti-CD16 antibodies, stimulation of NK cells with particulate (EA7S) immune complexes induced increased expression of Tac antigen (Table I) and Tfr and 4F2 antigens (not shown), and the presence of rIL-2 potentiated this effect. In all these experimental conditions, the proportion of NK cells, as detected by expression of NKH-1 antigen, was unchanged and that of CD3⁺ (or CD5⁺, not shown) cells remained <2%.

CD16⁻ T cells were also purified from the 10-d PBMC cultures and analyzed under conditions identical to those used for NK cells (Fig. 2, bottom panel,

TABLE I
FcR(CD16) Ligands Induce Expression of Activation Antigens on NK Cells

Inducer*	Percent expressing (mean ± SD, n experiments) [‡] surface antigens [§] (mAbs):						
	CD16 (3G8)	NKH-1 (N901)	CD3 (OKT3)	CD25 (Tac)	Tf-R (5E9)	4F2	HLA-DR (B33.1)
None	90.9 ± 3.0	86.6 ± 7.6	1.9 ± 0.1	12.3 ± 6.3	10.8 ± 7.0	3.3 ± 0.5	82.8 ± 4.8
rIL-2 (100 U/ml)	86.0 ± 5.6	86.4 ± 5.0	1.1 ± 1.1	7.1 ± 1.1	7.5 ± 3.0	4.4 ± 0.4	78.2 ± 3.7
Sepharose-B73.1	14.9 ± 3.0	75.2 ± 7.0	0.5 ± 0.7	27.3 ± 7.4	23.4 ± 11.3	15.6 ± 7.5	82.6 ± 3.6
Sepharose-B73.1 + rIL-2	10.2 ± 3.4	78.0 ± 9.8	2.0 ± 1.0	53.9 ± 12.0	42.4 ± 12.5	38.2 ± 17.7	88.2 ± 1.3
Sepharose-W6.32 + rIL-2	92.3 ± 4.7	90.5 ± 3.0	4.9 ± 4.1	10.7 ± 2.8	10.8 ± 2.0	8.1 ± 2.5	86.7 ± 3.0
Sepharose-OKT3 + rIL-2	89.0 ± 8.0	92.0 ± 2.0	2.4 ± 1.8	10.3 ± 1.0	10.5 ± 3.3	5.7 ± 3.8	90.0 ± 4.0

	CD16 (3G8)	NKH-1 (N901)	CD3 (OKT3)	CD25 (Tac)
None	81.7 ± 7.7	85.2 ± 6.0	2.5 ± 0.3	9.2 ± 6.7
rIL-2 (100 U/ml)	88.2 ± 1.2	90.0 ± 4.0	1.5 ± 1.5	3.8 ± 2.2
E	73.0 ± 10.5	74.7 ± 1.2	2.1 ± 1.6	11.5 ± 8.5
E + rIL-2	89.1 ± 5.3	90.6 ± 5.0	1.6 ± 1.5	4.2 ± 3.4
EA7S	11.2 ± 7.0	67.2 ± 8.8	1.8 ± 1.8	13.8 ± 5.0
EA7S + rIL-2	5.0 ± 5.0	71.6 ± 8.1	2.2 ± 5.0	40.5 ± 14.0

* CD3/CD5(-) NK cells purified from 10-d cultured PBMC were incubated (18 h, 37°C) in the presence of the indicated inducers.
[‡] In the top part of the table, n = 6, in the bottom part, n = 4.
[§] Surface antigens were detected by indirect immunofluorescence, using the indicated mAbs: The values of background fluorescence in control samples treated with isotype-matched irrelevant antibody and FITC-goat anti-mouse Ig (1-2% in the different conditions) have been subtracted.

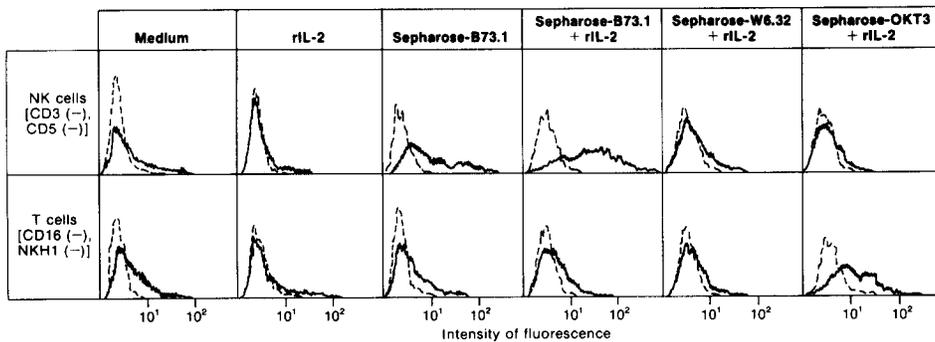


FIGURE 2. Interaction of anti-CD16 antibody with cultured NK cells induces expression of Tac(CD25) antigen. CD3/CD5⁻ NK cells and CD16/NKH-1⁻ T cells purified from 10-d cultures of PBMC were incubated (18 h, 37°C) with the indicated Sepharose-linked antibodies with or without rIL-2 (100 U/ml). CD25 antigen expression was detected in indirect immunofluorescence with anti-Tac (solid line in each histogram). The broken line is the histogram of the fluorescence of cells treated with FITC-labeled second antibody only. x-axis, intensity of fluorescence (exponential scale); y-axis, number of cells.

representative of eight experiments). Tac antigen expression (both proportion of positive cells and intensity of fluorescence) was higher on the purified T cells than on the NK cells. Enhanced expression of Tac antigen was induced on T cells stimulated with Sepharose-linked anti-CD3 antibody in the presence of rIL-2, but not with rIL-2 alone or with Sepharose-linked anti-CD16 or anti-HLA class I antibodies in the presence or absence of rIL-2.

FcR(CD16) Ligands Induce Production of Cytokines by NK Cells: Synergy with rIL-2. NK cells obtained from the 10-d cultures of PBMC with irradiated B lymphoblastoid cells do not spontaneously produce significant levels of either

TABLE II
FcR(CD16) Ligands Induce NK Cells to Produce Lymphokines

Inducer*	rIL-2 (100 U/ml)	Lymphokine [‡]	
		IFN- γ	TNF
U/ml			
None	-	0.2 \pm 0.1	1.1 \pm 0.4
	+	10.6 \pm 3.4	1.7 \pm 0.6
Sephарose-B73.1	-	43.3 \pm 17.1	8.0 \pm 3.5
	+	313.0 \pm 124.1	56.5 \pm 26.2
Sephарose-OKT3	-	3.8 \pm 2.1	4.0 \pm 1.2
	+	3.4 \pm 0.8	0.7 \pm 0.3
Sephарose-W6.32	-	2.3 \pm 2.2	2.0 \pm 1.9
	+	0.6 \pm 0.6	0.3 \pm 0.2
E	-	7.2 \pm 7.2	1.0 \pm 0.3
	+	14.7 \pm 4.9	1.3 \pm 0.6
EA7S	-	8.4 \pm 4.0	7.7 \pm 2.7
	+	271.5 \pm 88.5	22.6 \pm 10.8
PDBu/A23187	-	525.0 \pm 110.8	68.9 \pm 29.4

* CD3/CD5(-) NK cells purified from 10-d cultures of PBMC were incubated (18 h, 37°C) with the indicated inducers, without (-) or with (+) rIL-2 added. IFN- γ and TNF in the cell-free supernatants were tested by RIA.

[‡] Mean \pm SE. *n* = 9.

IFN- γ or TNF (Table II) and respond to rIL-2 stimulation with production of low levels of IFN- γ (~10 U/ml), but not TNF. Culture of the cells in the presence of CD16 ligands (either Sepharose-linked anti-CD16 antibodies or EA7S) resulted in production of low levels of both IFN- γ (~40 U/ml) and TNF (~8 U/ml). When both rIL-2 and FcR ligands were present during the 18-h culture, levels of IFN- γ and TNF significantly higher than the sum of those induced by the two stimuli separately were reproducibly detected (on average 300 U/ml and 50 U/ml, respectively). As reported in Table II and Fig. 3 (A and B), neither Sepharose-linked irrelevant antibodies (anti-CD3 OKT3 and anti-HLA class I W6.32) nor E, in the presence or absence of rIL-2, induced cytokine production. T cells obtained from the same cultures were induced to produce both IFN- γ (on average 150 U/ml) and TNF (on average 100 U/ml) when stimulated with Sepharose-linked anti-CD3 antibodies and rIL-2 (Fig. 3, C and D; experiment reported in Fig. 3 representative of six performed), but not under conditions that induced cytokine production from NK cells.

Kinetics of Induction of Tac(CD25) Antigen Expression and Cytokine Production by NK Cells Stimulated with FcR(CD16) Ligands. Sepharose-linked anti-CD16 (3G8) antibody or immune complexes (EA7S) both in the presence or absence of rIL-2 (Fig. 4A) induced Tac antigen expression in a proportion of NK cells higher than that present in untreated NK cells starting at 7-h incubation. As shown in Fig. 2, however, at any time point the density of the antigen on the cells incubated with both stimuli was significantly higher than that observed using the CD16 ligands alone. The proportion of Tac⁺ cells (and density of antigen expression, not shown) increased to 50–60% at 18 h and remained constant up to 24 h of culture. IFN γ (Fig. 4, panel B) and TNF (Fig. 4, panel C) were not produced at

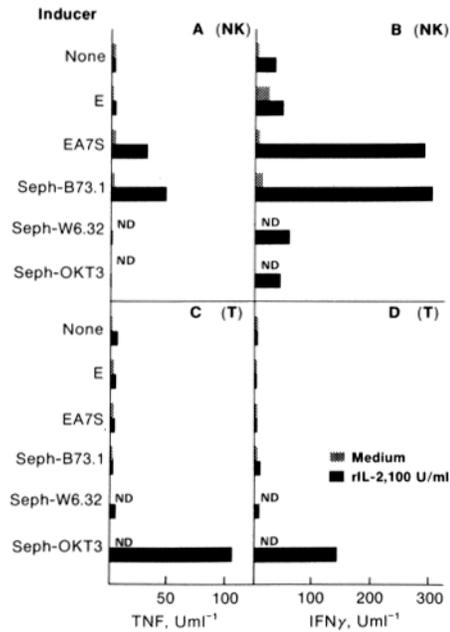


FIGURE 3. Interaction of NK cells with FcR(CD16) ligands induces lymphokine production. CD3/CD5⁻ NK cells (A and B) and CD16/NKH-1⁻ T cells (C and D) were purified and stimulated as in Fig. 2. Cell-free supernatants were collected after 18 h of culture at 37°C and tested for the presence of TNF (A and C) and IFN- γ (B and D) by RIA.

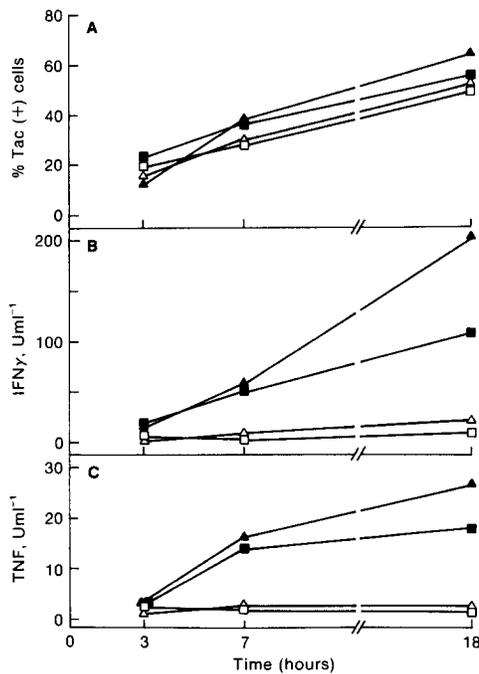


FIGURE 4. Kinetics of induction of Tac (CD25) antigen expression and lymphokine production by NK cells stimulated with FcR(CD16) ligands. CD3/CD5⁻ NK cells were purified and stimulated as in Fig. 2. Cells were collected at the indicated time points and tested for Tac expression by direct immunofluorescence (A); IFN- γ (B) and TNF (C) in the cell-free supernatants were tested by RIA. The stimuli used were: (O) none, cells cultured in medium only; (●) rIL-2, 100 U/ml; (Δ) Sepharose-linked 3G8 antibody; (▲) Sepharose-linked 3G8 and rIL-2, 100 U/ml; (□) EA7S; (■) EA7S and rIL-2, 100 U/ml. Proportion of Tac⁺ cells before culture was 9.8% and after culture, in medium or rIL-2, 10.4%. Cell-free supernatants from cells cultured 18 h in medium contained 0.4 U/ml IFN- γ and 0.8 U/ml TNF, those from cells cultured with rIL-2 contained 5.4 and 1.0 U/ml, respectively.

significant levels in supernatants of NK cells stimulated with rIL-2 alone or with either type of FcR(CD16) ligands in the absence of rIL-2. When FcR(CD16) ligands and rIL-2 were simultaneously present during the culture, significant levels of both IFN γ (50 U/ml in the experiment in Fig. 4, representative of 4

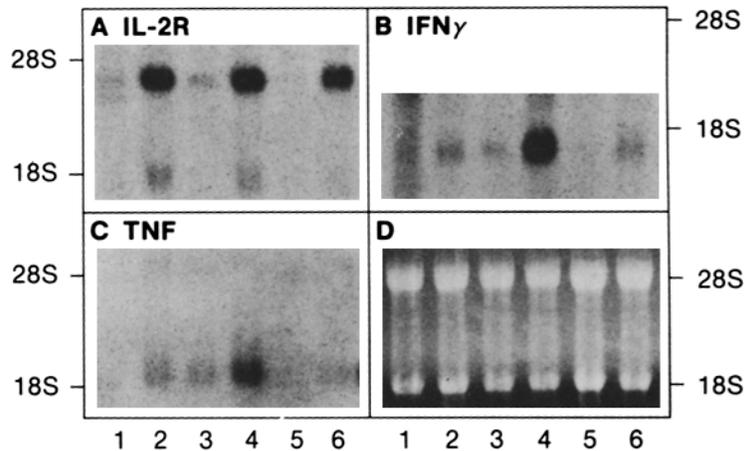


FIGURE 5. Accumulation of IL-2-R (A), IFN- γ (B), and TNF (C) mRNA in human NK cells stimulated with FcR(CD16) ligands. Total cytoplasmic RNA, extracted from NK cells (CD3/CD5⁻ cells from 10-d cultures of PBMC with irradiated RPMI 8866 cells and incubated [2.5 h, 37°C] in the presence of various inducers) were electrophoresed on 1% agarose-formaldehyde gel (20 μ g mRNA per lane). After transfer to nitrocellulose, RNA was hybridized to the indicated cDNA probes. Amount and integrity of RNA was checked by ethidium bromide staining (D). Cells were cultured in the presence of: (Lane 1) medium; (lane 2) rIL-2 (100 U/ml); (lane 3) Sepharose-B73.1 antibody; (lane 4) Sepharose-B73.1 and rIL-2; (lane 5) Sepharose-W6.32; (lane 6) Sepharose-W6.32 and rIL-2.

performed) and TNF (15 U/ml) were detected at 7-h incubation, and these levels increased further at 18-h incubation.

FcR(CD16) Antigen Crosslinking at the NK Cell Surface Induces Accumulation of mRNA for Cytokines and IL-2-R. Northern blot analysis was performed on cytoplasmic RNA obtained from NK cells purified from 10-d cultures and stimulated for 2.5 h with Sepharose-linked anti-CD16 antibody B73.1 with or without rIL-2 added. The degree of mRNA accumulation induced under the different conditions was quantitated spectrophotometrically. As shown in Fig. 5, IL-2-R (A), IFN- γ (B), and TNF mRNA (C) were barely detectable in NK cells immediately after purification. Stimulation with rIL-2, and, to a lesser extent, with CD16 ligands, induced accumulation of the mRNA for IL-2-R. The values obtained for IL-2-R mRNA levels induced by CD16 ligand and rIL-2 together were higher (as determined by densitometric scanning of the autoradiograms) than the sum of those obtained using either stimulus alone in two of eight experiments performed and equal to the sum in the other six. rIL-2 and, to a higher degree in most experiments, CD16 ligands alone induced accumulation of both IFN- γ and TNF cytoplasmic mRNA. mRNA accumulations with the combined stimuli were always higher than 2-fold the sum of those obtained with either stimulus along in the case of IFN- γ (average 4-fold in eight experiments), and in five of seven experiments in the case of TNF (average 3.5-fold in five experiments). Identical results were obtained using particulate immune complexes (EA7S) in place of the Sepharose-linked anti-CD16 antibody (not shown). Sepharose-linked anti-HLA class I antigens (Fig. 5), soluble anti-CD16 antibody, or E (not shown) did not induce significant accumulation of any of the mRNA tested. Sepharose-linked anti-CD3 antibodies did not induce mRNA accumula-

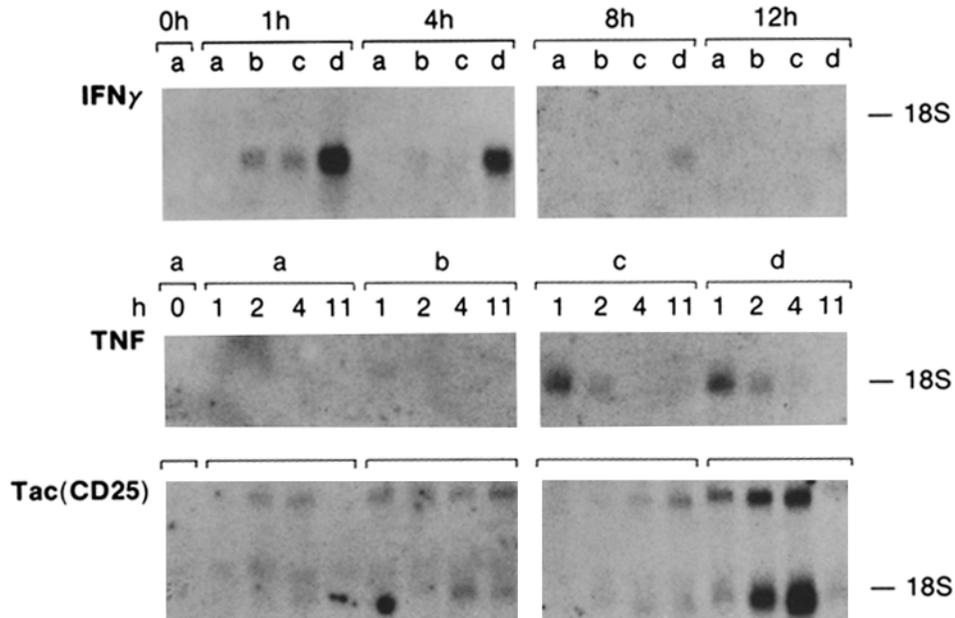


FIGURE 6. Kinetics of accumulation of IFN- γ , TNF, and IL-2-R mRNA in human NK cells stimulated with FcR(CD16) ligands. Total cytoplasmic RNA was extracted from NK cells, prepared as in Fig. 5, immediately after separation (0 h) or incubated for 1, 2, 4, 8, and 11 h in medium (a), in the presence of rIL-2 (100 U/ml) (b), EA7S (c), or EA7S and rIL-2 (d). RNA was electrophoresed (20 μ g per lane), transferred to nitrocellulose, and hybridized to the indicated cDNA probes.

tion for IL-2-R or for the two cytokines in NK cells but, unlike CD16 ligands, induced it in T cells purified from the same cultures (not shown).

IFN- γ and TNF mRNA accumulation in NK cells (Fig. 6) occurred within 0.5 h of stimulation (not shown), reached maximal levels at 1 h, and declined after 2 h of stimulation. Accumulation of IL-2R mRNA (Fig. 6) was detectable at 2 h, maximal at 4 h, and undetectable or, in four of eight experiments, detectable at low levels at 11 h of incubation. The kinetics of induction of mRNA accumulation was unchanged using FcR(CD16) ligands and rIL-2 in combination. Hybridization of the same filters with the β -actin probe indicated that β -actin mRNA was detectable at high levels in the same cells, but it did not change significantly at any time after stimulation (not shown).

Induction of mRNA Accumulation by CD16 Ligands Does Not Require Protein Synthesis. NK cells purified from the 10-d cultures were stimulated with EA7S in the presence of cycloheximide after 1 h of pretreatment with the inhibitor. As shown in Fig. 7, both accumulation of IFN- γ mRNA and the higher degree of accumulation observed upon combined stimulation with CD16 ligands and rIL-2 were evident also under these conditions. Such effects were still present at 3 h of culture (not shown) and were also observed for TNF and IL-2R mRNA (not shown). Cycloheximide treatment resulted in superinduction of IFN- γ (Fig. 7) and TNF mRNA (not shown).

Stimulation of NK Cells with FcR(CD16) Ligands and rIL-2 Induces Transcription of IL-2-R and Lymphokine Genes. Nuclear run-on experiments were performed

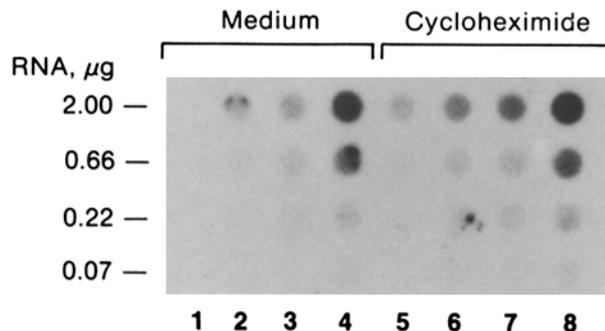


FIGURE 7. Accumulation of IFN- γ mRNA in human NK cells stimulated with FcR ligands is independent of protein synthesis. NK cells (prepared as described in Fig. 5) were preincubated (1 h, 37°C) in the absence or presence of cycloheximide (10 μ g/ml). Medium (dots in 1 and 5), rIL-2 (100 U/ml, dots in 2 and 6), EA7S (dots in 3 and 7), or EA7S and rIL-2 (dots in 4 and 8) were then added and incubation was continued for 2.5 h, when cells were

collected and total cytoplasmic RNA was isolated. The indicated amounts of RNA were hybridized to the IFN- γ cDNA probe pSWIF. Hybridization of the same filter with the cDNA probe pB_{AE} (for 28S rRNA) after elution of the IFN- γ cDNA probe and ethidium bromide staining of a 1% agarose-formaldehyde gel of the same RNA confirmed that each dot contained equivalent amounts of RNA and that this was not degraded (not shown).

TABLE III
FcR(CD16) Ligands Induce Transcription of IL-2-R, IFN- γ and TNF Genes in NK Cells

Stimulus*	IL-2-R	IFN- γ	TNF
None	0.92 \pm 0.23 [‡]	3.70 \pm 1.50	2.25 \pm 0.85
rIL-2 (100 U/ml)	4.92 \pm 1.28	5.85 \pm 3.02	5.60 \pm 1.28
Sepharose-B73.1	3.10 \pm 0.71	9.37 \pm 3.40	16.87 \pm 4.34
Sepharose-B73.1 + rIL-2	3.77 \pm 0.32	10.55 \pm 4.06	10.72 \pm 2.53

* CD3/CD5⁻ NK cells purified from 10-d cultures of PBMC were incubated (20 min, 37°C) with the indicated stimuli. Nuclear RNA was extracted and the rate of transcription of the indicated genes analyzed in run-on experiments as described in Materials and Methods.

[‡] Values are nuclear RNA transcripts (arbitrary densitometric units) normalized to the amount of ribosomal RNA transcription, mean \pm SE of four experiments, calculated as described in Materials and Methods.

to define whether the accumulation of RNA transcripts for IL-2-R, IFN- γ , and TNF observed in NK cells stimulated with the FcR(CD16) ligands in the presence or absence of rIL-2 is due to induction of transcription of their genes. Nuclear RNA was prepared from NK cells purified from the 10-d cultures and stimulated with the different inducers for 20 min; the results obtained in four experiments performed are summarized in Table III. IFN- γ and, to a lesser extent, TNF and IL-2-R genes were constitutively transcribed at low rates by the 10-d cultured NK cells. Upon stimulation with rIL-2, the relative rate of transcription for IL-2-R, IFN- γ , and TNF genes was increased 5.3-, 1.6-, and 2.5-fold, respectively. Stimulation of the cells with Sepharose-linked B73.1 antibody induced a 3.4-, 2.6-, and 17.5-fold increase, respectively (Table III and Fig. 8), and these values were similar to those obtained when the rate of transcription was measured in cells stimulated with FcR ligands in the presence of rIL-2. The rate of transcription of the β -actin mRNA was not significantly affected by either stimulus (Fig. 8).

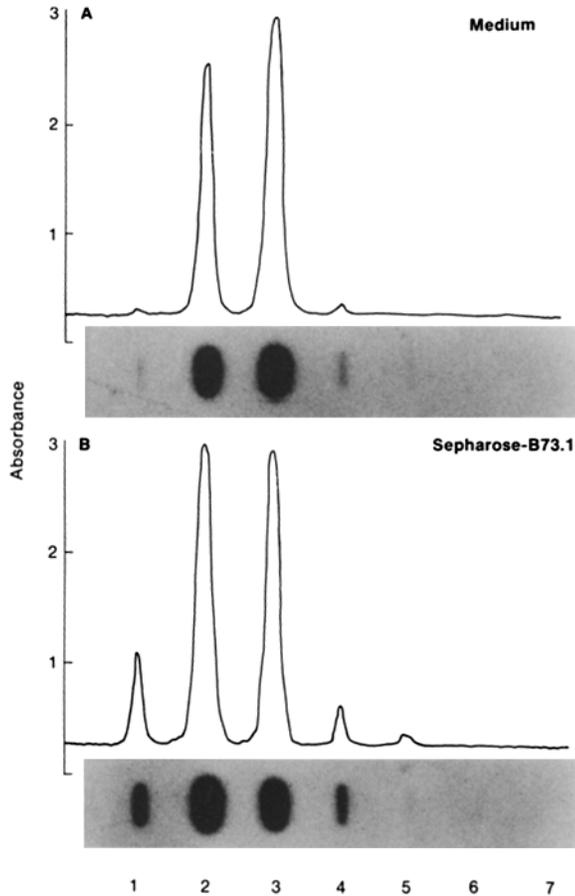


FIGURE 8. FcR(CD16) ligands induce transcription of IL-2-R, IFN- γ , and TNF genes. NK cells (obtained as in Fig. 5) were cultured for 20 min at 37°C in medium in the absence (A) or in the presence of Sepharose-B73.1 (B). Nuclear RNA was extracted and used in nuclear run-on transcription reactions as described in Materials and Methods. The labeled transcripts from each reaction were hybridized to excess denatured cDNA immobilized on nitrocellulose filter. The cDNA used to hybridize to 32 P-labeled nRNA were: 1, TNF; 2, β -actin; 3, pB_{AE} for 28S RNA; 4, pSWIF for IFN γ ; 5, IL-2R2 for IL-2R; 6, pBR322; 7, pSP64. The histograms are densitometric readings of the bands in the respective films.

Discussion

In this study we demonstrate that interaction of FcR(CD16) on human NK cells with ligands induces transcription of genes encoding surface molecules and cytokines (IFN- γ and TNF) relevant to NK cell biology and functions. Our results indicate that the FcR-mediated regulation of expression of surface activation antigens and of cytokine production by NK cells follows pathways in part distinct but synergistic with those induced by other stimuli (e.g., IL-2) at the post-transcriptional or translational level.

To obtain a sufficient number of cells for molecular analysis, in most experiments we used CD16⁺ NK cells purified by negative selection from short-term bulk cultures of PBL. These NK cell preparations have markers and functions of fresh NK cells (24, 25) and are thus unlikely to represent only one NK cell subset, are >95% pure, and are not contaminated with monocytes, as evaluated using mAbs or enzymatic staining. Lymphocyte preparations expanded in culture for a few days have been widely used in studies of T cell physiology. In the case of both NK and T cells, these preparations are composed of cells that, after rapid activation and proliferation *in vitro*, have reverted to a resting state and lost almost completely expression of activation antigens (24). Unlike quiescent

peripheral blood T or NK cells, which require a lag period before they can be induced to proliferate or to express new markers, these cells respond rapidly to external stimuli, reentering the cell cycle or synthesizing specific products, and are therefore convenient to study the mechanisms regulating activation, proliferation, and functions of lymphocytes.

Recent studies showed that rIL-2 induces IL-2-R (37) and IFN- γ (38) mRNA accumulation in fresh Tac⁻ large granular lymphocytes (LGL) upon 2-d stimulation. Our observations using highly purified homogeneous NK cell preparations that, unlike LGL, are devoid of monocytes and other immature CD3⁻/CD16⁻ T cells, are consistent with those reports and provide evidence that rIL-2 acts directly on NK cells, inducing not only IL-2-R and IFN- γ , but also TNF mRNA transcription and accumulation. The experiments showing mRNA accumulation in the presence of cycloheximide exclude the possibility that other *de novo* synthesized lymphokines or proteins (produced by the NK cells or, in the case of LGL stimulation, by other contaminating cell types) mediate the effect of rIL-2. Our observation that rIL-2 induces efficiently both transcription and accumulation of mRNA for these proteins but only minimally induces their expression and secretion suggests that the latter events are regulated at a translational or post-translational level by factors not induced by rIL-2 alone in purified NK cells. Unlike fresh PBL (19), the 10-d cultured NK cells respond to rIL-2 with minimal IFN- γ production. Induction of IFN- γ production in fresh PBL by rIL-2 has been shown to depend upon the presence of accessory HLA-DR⁺ dendritic cells (39); a role for these accessory cells, highly enriched in LGL preparations, in regulating expression of IFN- γ at a post-transcriptional level might explain these contrasting results using cell preparations of different purity.

The molecular effects induced in FcR-bearing cells with FcR ligands have not been previously investigated. Stimulation of purified cultured NK cells with FcR(CD16) ligands alone, unlike stimulation with rIL-2, consistently induced expression of Tac(CD25) and of other activation markers within 18 h in the absence of enhanced expression of other antigens endogenously expressed on cultured NK cells (e.g., HLA-DR, NKH-1, and CD2) and induced production of low but significant levels of IFN- γ and TNF. NK cell activation was specifically induced by FcR(CD16) ligands because neither anti-HLA class I nor anti-CD3 antibodies nor E were effective. FcR(CD16) ligands induced neither activation antigens nor lymphokine production by CD16⁻ T cells purified from our bulk cultures. The ready induction of Tac(CD25) expression, cytokine production, and IL-2-R, IFN- γ , and TNF mRNA accumulation upon interaction with Sepharose-linked anti-CD3 antibodies and rIL-2 in our purified T cell preparations demonstrates the ability of these cells to respond to T cell-specific stimuli.

We previously showed that resting NK, but not T cells, are induced to produce TNF upon target cell recognition (23), whereas stimulation of PBL with mitogens and phorbol esters results in TNF production and accumulation of TNF mRNA mostly in T cells (31). Our present results directly prove that NK cells produce TNF upon specific stimulation with FcR(CD16) ligands that are unable to stimulate CD16⁻ T cells. Thus, NK cells respond with TNF production to activation through FcR, as it occurs upon recognition and killing of antibody-sensitized target cells, and also to activation through surface molecules other

than FcR involved in recognition and killing of NK-sensitive target cells. Interaction of NK cells with FcR(CD16) ligands or antibody-coated target cells, and with NK-sensitive target cells results in similar inactivation/exhaustion of cytotoxic activity (9, 40). A complete understanding of the activation pathways involved in the two types of target cell recognition and cytotoxicity by NK cells awaits a better characterization of the receptor(s) involved in spontaneous NK cytotoxicity.

When total unseparated fresh PBL were stimulated with FcR ligands, Tac(CD25) was induced only on a proportion of NK cells and not on T cells. In the presence of rIL-2, induction of Tac(CD25) by FcR ligands was enhanced on NK cells and some expression was also observed on T cells, although the proportion of CD25⁺ NK cells was always significantly higher than that of CD25⁺ T cells. The T cells induced to express Tac among PBL treated with FcR(CD16) ligands may be the CD16⁺/CD3⁺ T cell subset reported by Lanier et al. (41). However, we cannot exclude that Tac induction on NK and/or on T cells is indirectly mediated, at least in part, through release of cytokines, e.g., TNF, lymphotoxin, or IL-1, acting on T and NK cells and produced by contaminating monocytes, by NK cells, or by T cells present in the total PBL preparations. TNF has been reported to synergize with rIL-2 to induce Tac(CD25) expression on NK cells (42) and on activated T cells (43).

Induction of activation antigens and secretion of lymphokines upon FcR(CD16) ligand interaction at the NK cell surface occur only under conditions that induce FcR crosslinking at the cell membrane, i.e., using particulate immune complexes (EA7S) or Sepharose-linked anti-CD16 antibodies. The observation that treatment of NK cells with soluble anti-CD16 antibodies does not result in any of the effects studied in this report does not exclude the possibility that signal transduction occurs but at a level insufficient to induce the complete series of intracellular events leading to cell activation. This possibility is supported by the finding (10; Perussia, B., and G. Trinchieri, unpublished data) that soluble anti-CD16 antibodies enhance cytotoxic activity of NK cells and by our preliminary observation that they induce increase of $[Ca^{2+}]_i$. Interaction of FcR with monomeric anti-CD16 antibodies and its crosslinking by particulate ligands induce down-modulation (4, 8) and probably internalization (44) of FcR on NK cells, although with different kinetics. The mechanisms by which different conditions of FcR(CD16) ligand interaction result in distinct biological responses remain to be investigated.

In cell types other than NK, binding of ligands to the membrane receptors induces redistribution of an intracellular antigen pool to the membrane or release of preformed cytokines. For example, CD21 is redistributed from intracellular pools in neutrophils upon binding of phenyl-Met-Leu-Phe (45), and preformed pharmacologically active mediators are released by basophils upon binding of antigen to FcεR-bound IgE (46). We did not test for the possible role of FcR-induced redistribution of an intracellular antigenic pool or transduction of secretagogue stimuli for preformed cytokines in mediating effects observed at the level of protein expression. However, we clearly showed that FcR ligand-induced IFN- γ and TNF production and Tac(CD25) expression depend, at least in part, on transduction through CD16 of intracellular signals inducing accu-

mulation of mRNA for these proteins. The results of the experiments performed in the presence of cycloheximide indicate that no *de novo* synthesis is required for signal transduction. The results of nuclear run-on assays indicate that the observed increases of mRNA induced with FcR ligands alone are due, at least in part, to enhanced transcription.

Both induction of activation antigen expression and cytokine production are synergistically enhanced when rIL-2 and FcR ligands are used in combination. IFN- γ and TNF mRNA accumulation was synergistically increased using CD16 ligands and rIL-2 together, and, on average, equal to fourfold the sum of those observed using each stimulus alone. This value is in the same order of magnitude observed at the protein level, i.e., ~5.8-fold increase in IFN- γ and TNF production using rIL-2 and FcR ligands compared to either stimulus alone. The observations that the kinetics of mRNA accumulation of both cytokines are similar and that mRNA accumulation occurs, with any of the stimuli used, after inhibition of protein synthesis, argue against the possibility that either or both cytokines are involved in regulating mRNA accumulation for the other.

Unlike the observations for IFN- γ and TNF, Tac(CD25) mRNA accumulation was always significantly induced by rIL-2 but only minimally by CD16 ligands. An additive rather than synergistic effect on Tac(CD25) mRNA accumulation was observed in most experiments using the two stimuli in combination. These results at mRNA level are difficult to reconcile with the observations that expression of Tac(CD25) antigen at the NK cell surface is efficiently induced by CD16 ligands, but not by rIL-2, and that the two stimuli synergize in inducing Tac(CD25) expression. The kinetics of accumulation of IL-2-R mRNA induced by rIL-2 is slower (maximal at 4 h) than that of IFN- γ and TNF mRNA induced by either or both rIL-2 and FcR ligands. Both IFN- γ and TNF have been reported to synergize with IL-2 in inducing Tac(CD25) expression on NK cells (42, 47), and we cannot exclude that IFN- γ and/or TNF produced by NK cells upon FcR ligand interaction are involved in expression of CD25. However, we could not detect IL-2 mRNA transcription in NK cells stimulated by FcR(CD16) ligand alone or under any of our experimental conditions (data not shown). A role for TNF is unlikely since it does not reproducibly induce TAC(CD25) expression and/or mRNA accumulation in NK cells, and anti-TNF antibodies do not abolish Tac(CD25) induction on FcR(CD16) ligand-treated NK cells (our unpublished data). An indirect role of IFN- γ or of other unidentified cytokines cannot yet be excluded, although the inability of cycloheximide to prevent Tac(CD25) mRNA accumulation indicates that any cytokines with such a role should be preformed in the cells.

The synergistic effect of FcR ligands and rIL-2 observed at the protein and cytoplasmic mRNA level was not observed at the level of transcription. The persistence of synergism in determining accumulation of mRNA in the presence of cycloheximide and the superinduction of mRNA observed in all experimental conditions after cycloheximide treatment suggest that both IFN- γ and TNF production are suppressed by inhibitory proteins in resting NK cells, as reported for IFN- γ and TNF regulation in other cell types (48, 49). Greater accumulation of IFN- γ and TNF mRNA occurs in NK cells upon FcR(CD16) ligand binding than upon rIL-2 stimulation, and mRNA accumulation induced by the combined

stimuli is still evident at time points at which accumulation induced by either stimulus alone is not present (4 h or, in several experiments, 11 h). These data are consistent with the possibility that part of the effect of the FcR ligand interaction and the synergistic effect of rIL-2 and FcR ligands in NK cells depend on regulation of inhibitory proteins and/or on stabilization of the mRNA for both cytokines.

Although both rIL-2 and FcR(CD16) ligands induced transcription of Tac(CD25), IFN- γ and TNF within 20 min, Tac(CD25) transcription was induced at higher levels by rIL-2 than by the CD16 ligands. The reverse was true for TNF and IFN- γ transcription, which were not induced by rIL-2 in two of the four experiments performed, but were significantly induced by FcR ligands in all experiments. This observation, although based on a limited number of experiments and genes, is consistent with the hypothesis that signals transduced through NK cell-specific receptors result in transcription of genes specifically involved in NK cell functions, whereas stimuli transduced by factors, such as IL-2, that affect several distinct cell types, result in preferential activation of genes (e.g., IL-2-R) relevant to the overall physiology of the cells. Only combined stimulation would result in complete cell activation through mechanisms, not completely elucidated, regulating post-transcriptional events. Similar studies on other cell types (e.g., FcR-bearing B cells and monocytes) could provide significant information about the relationship between the mechanisms through which lineage-specific receptors or functionally similar receptors on different cell types activate effectors of nonadaptive and adaptive immunity.

Summary

We report evidence that FcR(CD16) on human NK cells are signal-transducing molecules that, upon ligand binding, induce transcription of genes encoding surface activation molecules [IL-2-R(CD25)] and cytokines (IFN- γ and TNF) relevant to NK cell biology and functions. Homogeneous NK and T cell populations purified from short-term bulk cultures of PBMC with irradiated B lymphoblastoid cell lines were cultured in the presence of FcR ligands (particulate immune complexes or immobilized anti-CD16 antibodies) alone or with rIL-2. Upon 18 h of stimulation, NK cells express Tac, Tfr, and 4F2 antigens and produce IFN- γ and TNF; both effects are synergistically enhanced in the presence of rIL-2, which is itself ineffective. Treatment of NK cells with FcR(CD16) ligands induces accumulation of mRNA for IFN- γ and TNF and, to a lesser extent, IL-2-R with fast kinetics also in the absence of *de novo* protein synthesis. rIL-2 and FcR(CD16) ligands synergize to induce mRNA accumulation. mRNA accumulation and transcription of TNF and IFN- γ genes induced by FcR(CD16) ligands are greater than those induced by rIL-2, and the reverse is true for IL-2-R. The two stimuli do not synergize at the transcriptional level. These observations indicate that the mechanisms through which FcR(CD16) ligands and rIL-2 induce NK cell activation are, in part, distinct. Both operate at the transcriptional level, although other mechanisms are probably induced by the FcR ligand stimulus per se or in combination with other lymphokines and synergize at a post-transcriptional or translational level to enhance NK cell activation.

We thank J. Faust and J. Urffer for assistance at the cytofluorograph, M. Hoffman for editing, and M. Kaplan for secretarial assistance.

Received for publication 8 October 1987.

References

1. Perussia, B., and G. Trinchieri. 1984. Antibody 3G8, specific for the human neutrophil Fc receptor, reacts with natural killer cells. *J. Immunol.* 132:1410.
2. Fleit, H. G., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fc receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79:3275.
3. Unkeless, J. C. 1977. The presence of two Fc receptors on mouse macrophages: evidence from a variant cell line and differential trypsin sensitivity. *J. Exp. Med.* 145:931.
4. Perussia, B., G. Trinchieri, A. Jackson, N. L. Warner, J. Faust, H. Rumpold, and L. L. Lanier. 1984. The Fc receptor for IgG on human natural killer cells: phenotypic, functional and comparative studies using monoclonal antibodies. *J. Immunol.* 133:180.
5. Ravetch, J. V., A. D. Luster, R. Weinshank, J. Kochan, A. Pavlovec, D. A. Portnoy, J. Hulmes, Y-C. E. Pan, and J. C. Unkeless. 1986. Structural heterogeneity and functional domains of murine immunoglobulin G Fc receptors. *Science (Wash. DC).* 243:718.
6. Anderson, C. L., P. M. Guyre, J. C. Whitin, D. H. Ryan, R. J. Looney, and M. W. Fanger. 1986. Monoclonal antibodies to Fc receptors for IgG on human mononuclear phagocytes: antibody characterization and induction of superoxide production in a monocyte cell line. *J. Biol. Chem.* 261:12856.
7. Vaughn, M., M. Tayler, and T. Mohanakumar. 1985. Characterization of human IgG Fc receptors. *J. Immunol.* 135:4059.
8. Perussia, B., O. Acuto, C. Terhorst, J. Faust, R. Lazarus, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking FcR functions. II. Studies of B73.1 antibody-antigen interaction on the lymphocyte membrane. *J. Immunol.* 130:2142.
9. Perussia, B., G. Trinchieri, and J. C. Cerottini. 1979. Functional studies of Fc receptor-bearing human lymphocytes: effect of treatment with proteolytic enzymes. *J. Immunol.* 123:681.
10. Jondal, M., C. Kullman, M.-B. Alter, and K. Ljunggren. 1986. Monoclonal antibodies against the NK cell. FcR and the T₃-complex potentiate normal lymphocyte killing. *Cell. Immunol.* 100:158.
11. Ledbetter, J. A., C. H. June, P. J. Martin, C. E. Spooner, J. A. Hansen, and K. E. Meier. 1986. Valency of CD3 binding and internalization of the CD3 cell surface complex control T cell responses to second signals: distinction between effects on protein kinase C, cytoplasmic free calcium, and proliferation. *J. Immunol.* 136:3945.
12. Weiss, A., J. Imboden, K. Hardy, and J. Stobo. 1987. The role of the antigen receptor/T3 complex in T-cell activation. In *Mechanisms of Lymphocyte Activation and Immune Regulation*. S. Gupta, W. E. Paul, and A. S. Fauci, editors. Plenum Press, New York. 45.
13. Meuer, S. C., R. E. Hussey, D. A. Cantrell, J. C. Hodgdon, S. F. Schlossman, K. A. Smith, and E. L. Reinherz. 1984. Triggering of the T3-Ti antigen receptor complex results in clonal T cell proliferation via an interleukin 2 dependent autocrine pathway. *Proc. Natl. Acad. Sci. USA.* 81:1509.
14. Scheurich, P., L. Ucer, M. Wrann, and K. Pfizenmaier. 1985. Early events during primary activation of T cells: antigen receptor crosslinking and interleukin 1 initiate proliferative response of human T cells. *Eur. J. Immunol.* 15:1091.

15. Kammer, G. M., R. Kurrasch, and J. J. Scillian. 1984. Capping of the surface OKT3 binding molecule prevents the T-cell proliferative response to antigens: evidence that this molecule conveys the activation signal. *Cell. Immunol.* 87:284.
16. Pantaleo, G., D. Olive, A. Poggi, T. Pozzan, L. Moretta, and A. Moretta. 1987. Antibody-induced modulation of the CD3/T cell receptor complex causes T cell refractoriness by inhibiting the early metabolic steps involved in T cell activation. *J. Exp. Med.* 166:619.
17. Weiss, A., J. Imboden, R. Wiskocil, and J. Stobo. 1984. The role of T3 in the activation of human T cells. *J. Clin. Immunol.* 4:165.
18. Tsudo, M., C. K. Goldman, K. G. Bongiovanni, W. C. Chan, E. F. Winton, M. Yagita, E. A. Grimm, and T. A. Waldmann. 1987. The p75 peptide is the receptor for the interleukin 2 on large granular lymphocytes and is responsible for the interleukin 2 activation of these cells. *Proc. Natl. Acad. Sci. USA.* 84:5394.
19. Trinchieri, G., M. Matsumoto-Kobayashi, S. C. Clark, J. Sheehra, L. London, and B. Perussia. 1984. Response of resting human peripheral blood natural killer cells to interleukin-2. *J. Exp. Med.* 160:1147.
20. Phillips, J. H., A. M. Le, and L. L. Lanier. 1984. Natural killer cells activated in a human mixed lymphocyte response culture identified by expression of Leu-11 and class II histocompatibility antigens. *J. Exp. Med.* 159:993.
21. London, L., B. Perussia, and G. Trinchieri. 1986. Induction of proliferation *in vitro* of resting human natural killer cells: IL-2 induces into cell cycle most peripheral blood NK cells, but only a minor subset of low density T cells. *J. Immunol.* 137:3845.
22. Wright, S. C., and B. Bonavida. 1982. Studies on the mechanism of natural killer (NK) cell-mediated cytotoxicity (CMC). I. Release of cytotoxic factors specific for NK-sensitive target cells (NKCF) during coculture of NK effector cells with NK target cells. *J. Immunol.* 129:433.
23. Degliantoni, G., M. Murphy, M. Kobayashi, M-K. Francis, B. Perussia, and G. Trinchieri. 1985. Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. *J. Exp. Med.* 162:1512.
24. Liu, C.-C., B. Perussia, A. Cohn, and J. D.-E Young. 1986. Identification of a pore-forming protein of human peripheral blood NK cells. *J. Exp. Med.* 164:2061.
25. Perussia, B., C. Ramoni, I. Anegon, 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.
26. 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.
27. Perussia, B., G. Trinchieri, D. Lebman, J. Jankiewicz, B. Lange, and G. Rovera. 1982. Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells. *Blood.* 59:382.
28. Perussia, B., D. Lebman, S. H. Ip, G. Rovera, and G. Trinchieri. 1981. Terminal differentiation surface antigens of myelomonocytic cells are expressed in human promyelocytic leukemia cells (HL60) treated with chemical inducers. *Blood.* 58:836.
29. Griffin, J. D., T. Hercend, R. Beveridge, and S. F. Schlossman. 1983. Characterization of an antigen expressed on human natural killer cells. *J. Immunol.* 130:2947.
30. Leonard, W. J., J. M. Depper, T. Uchiyama, K. A. Smith, T. A. Waldmann, and W. C. Green. 1982. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor: partial characterization of the receptor. *Nature (Lond.).*

- 300:267.
31. Cuturi, M. C., M. Murphy, M. P. Costa-Giomi, R. Weinmann, B. Perussia, and G. Trinchieri. 1987. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J. Exp. Med.* 165:1581.
 32. Lanier, L. L., A. M. Le, C. I. Civin, M. R. Loken, and J. H. Phillips. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* 136:4480.
 33. Murphy, M., R. Loudon, M. Kobayashi, and G. Trinchieri. 1986. Gamma interferon and lymphotoxin, released by activated T cells, synergize to inhibit granulocyte-monocyte colony formation. *J. Exp. Med.* 164:263.
 34. Krönke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Woong-Staal, R. C. Gallo, T. A. Waldman, and W. C. Greene. 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc. Natl. Acad. Sci. USA.* 81:5214.
 35. Groudine, M., M. Peretz, and H. Weintraub. 1981. Transcriptional regulation of hemoglobin switching in chicken embryos. *Mol. Cell. Biol.* 1:281.
 36. Gariglio, P., M. Bellard, and P. Chambon. 1981. Clustering of RNA polymerase B molecules in the 5' moiety of the adult β -globin gene of hen erythrocytes. *Nucleic Acids Res.* 9:2589.
 37. Yamada, S., F. W. Ruscetti, W. R. Overton, R. B. Herberman, M. C. Birchenall-Sparks, and J. R. Ortaldo. 1987. Regulation of human large granular lymphocyte and T cell growth and function by recombinant interleukin 2: induction of interleukin 2 receptor and promotion of growth of cells with enhanced cytotoxicity. *J. Leukocyte Biol.* 41:505.
 38. Young, H. A., and J. R. Ortaldo. 1987. One-signal requirement for interferon- γ production by human large granular lymphocytes. *J. Immunol.* 139:724.
 39. Le, J., J. S. Yao, D. M. Knowles, and J. Vilcek. 1986. Accessory function of thymic and tonsillar dendritic cells in interferon gamma production by T lymphocytes. *Lymphokine Res.* 5:205.
 40. Perussia, B., and G. Trinchieri. 1981. Inactivation of natural killer cell cytotoxic activity after interaction with target cells. *J. Immunol.* 126:754.
 41. Lanier, L. L., T. J. Kipps, and J. H. Phillips. 1985. Functional properties of a unique subset of cytotoxic CD3⁺ lymphocytes that express Fc receptors for IgG (CD16/Leu11 antigen). *J. Exp. Med.* 162:2089.
 42. Ostensen, M. E., D. L. Thiele, and P. E. Lipsky. 1987. Tumor necrosis factor- α enhances cytolytic activity of human Natural Killer cells. *J. Immunol.* 138:4185.
 43. Scheurich, P., B. Thoma, U. Ucer, and K. Pfizenmaier. 1987. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)- α : induction of TNF receptors on human T cells and TNF- α -mediated enhancement of T cell responses. *J. Immunol.* 138:1786.
 44. Perussia, B., and G. Trinchieri. 1988. Structure and functions of NK cell Fc receptor. *EOS. J. Immunol. Immunopharmacol.* In press.
 45. Miller, L. J., D. F. Bainton, N. Borregaard, and T. A. Springer. 1987. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. *J. Clin. Invest.* 80:535.
 46. Metzger, H., G. Alcaraz, R. Hohman, J.-P. Kinet, V. Pribluda, and K. Quarto. 1986. The receptor with high affinity for immunoglobulin E. *Annu. Rev. Immunol.* 4:419.
 47. Olabuenaga, S. E., C. G. Brooks, S. Gillis, and C. S. Henney. 1983. Interleukin 2 is not sufficient for the continuous growth of cloned NK-like cytotoxic cell lines. *J. Immunol.* 131:2386.

48. Lebendiker, M. A., C. Tal, D. Sayar, S. Pilo, A. Eilon, Y. Bonai, and R. Kaempfer. 1987. Superinduction of the human gene encoding immune interferon. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:585.
49. Collart, M. A., D. Belin, J.-D. Vassalli, S. De Kossodo, and P. Vassalli. 1986. Interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. *J. Exp. Med.* 164:2113.