

INTERLEUKIN 2 ACTIVATION OF NATURAL KILLER CELLS RAPIDLY INDUCES THE EXPRESSION AND PHOSPHORYLATION OF THE Leu-23 ACTIVATION ANTIGEN

BY LEWIS L. LANIER, DAVID W. BUCK, LORI RHODES, ATHENA DING,
ELIZABETH EVANS, COLLEEN BARNEY, AND JOSEPH H. PHILLIPS

From the Becton Dickinson Monoclonal Center, Inc., Mountain View, California 94043

IL-2 potentiates both growth and cytotoxic function of T lymphocytes and NK cells. Resting peripheral blood NK cells can respond directly to rIL-2, without requirement for accessory cells or cofactors, and enhanced cytotoxicity can be measured within 4 h after exposure to this lymphokine (1–3). The majority of NK cells enter the cell cycle after exposure to IL-2; however, only a minor proportion sustain continued growth after this initial response (4). By contrast, most resting peripheral blood T cells do not respond directly to IL-2, but require additional signals (e.g., IL-1 or activators of protein kinase C) to induce proliferation (4–6). A subset of low buoyant density T cells, isolated by Percoll gradient centrifugation, can respond directly to IL-2 (4), presumably since these T cells are in a “primed” state and require only IL-2 for proliferation.

Activation and proliferation of T cells is accompanied by the *de novo* expression of new antigens on the plasma membrane (7–13). With respect to NK cells, activation by IL-2, or in mixed lymphocyte cultures, induces the expression of transferrin receptors, HLA-DR and the CD25 IL-2-R (4, 14–16). However, whereas IL-2-induced augmentation of NK cell-mediated cytotoxicity can be detected within 4 h and is maximal by 18 h after activation, HLA-DR and CD25 are not detectable on the majority of NK cells during this time period (2–4, 16). Thus, there is no direct correlation between the expression of these activation antigens and lytic activity. In this study, we describe an activation antigen, Leu-23, that is rapidly induced and phosphorylated after IL-2 stimulation of NK cells and a subset of low buoyant density T lymphocytes. Induction of this glycoprotein on NK cells is IL-2 dependent and closely parallels the acquisition of lytic activity.

Materials and Methods

Cells. Mononuclear cells from normal peripheral blood (Stanford Blood Center, Stanford, CA) were isolated using Ficoll/Hypaque. After plastic adherence and passage through nylon wool to remove monocytes and B cells, respectively, lymphocytes were fractionated by centrifugation on discontinuous gradients consisting of 30 and 40% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in PBS (0.01 M phosphate, 150 mM NaCl, pH 7.3) containing 10% serum (17). Low buoyant density and high buoyant density lymphocytes were isolated from the interface and bottom of the Percoll gradients,

respectively. Normal thymocytes were obtained from children undergoing cardiac surgery.

Culture. Lymphocytes and thymocytes were cultured in RPMI 1640 medium (M.A. Bioproducts, Walkersville, MD) containing 1 mM glutamine (Gibco Laboratories, Grand Island, NY), 100 µg/ml gentamicin (Gibco Laboratories) and either FCS (KC Biological, Inc., Lenex, KS) or heat-inactivated horse serum (KC Biological, Inc.). Lymphocytes were activated and cultured using rIL-2 (Cetus Corp., Emeryville, CA). In experiments to establish conditions necessary for the induction of activation antigens, serum-free HB101 medium (Hana Biologicals, Alameda, CA) was used to exclude potential nonspecific stimulation by heterologous serum.

Antibodies. Leu series mAbs were produced by the Becton Dickinson Monoclonal Center, Inc., Mountain View, CA. Anti-Leu-23 is an IgG1 mAb secreted by the L78 hybridoma cell line. L78 hybridoma was generated by immunizing BALB/c mice with a CD8⁺ alloantigen-directed CTL cell line. Immune lymph node cells were fused with SP2/0 Ag14 myeloma cells, and hybridomas were selected in azaserine (18). Rabbit anti-IL-2 serum was purchased from Genzyme, Boston, MA. Goat anti-IL-2 serum (16) was generously provided by Dr. Giorgio Trinchieri, Wistar Institute, Philadelphia, PA.

Immunofluorescence and Flow Cytometry. Methods of immunofluorescence, flow cytometry, and data analysis have been described previously (19). Fluorescence was measured by using a FACS 440 and data were analyzed by using a Consort 40 Data Analysis System (Becton Dickinson Immunocytometry Systems).

Cytotoxic Assay. ⁵¹Cr-labeled tumor cells were used as targets in a 4-h radioisotope release assay (20).

Immunoprecipitation and Electrophoresis. Cells were labeled with ¹²⁵I (Amersham Corp. Arlington Heights, IL) by using the glucose oxidase/lactoperoxidase method (21). Cells were solubilized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, pH 8.0) containing 5 mM 3[3-cholamidopropyl-dimethylammonio]-1-propane sulfonate (CHAPS)¹ detergent, 20 KI U/ml aprotinin (Sigma Chemical Co., St. Louis, MO) and 1 mM PMSF (Sigma Chemical Co.). Unincorporated ¹²⁵I was removed using Dowex 1X8-400 ion exchange resin (Sigma Chemical Co.), and lysates were precleared by incubation with formalin-fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., San Diego, CA) coated with rabbit anti-mouse Ig serum (Pel-Freez Biologicals, Rogers, AR) (22). Immunoprecipitation was performed using rabbit anti-mouse Ig-coated *S. aureus*, as described (22). Samples were analyzed by SDS-PAGE (23), two-dimensional "diagonal" (1st dimension nonreducing; 2nd dimension, reducing) (24), and two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE) using 3.5–10% ampholines (LKB Instruments, Inc., Gaithersburg, MD) (25).

Deglycosylation. Antigen was eluted by adding 20 µl of phosphate buffer (0.1 M, pH 6.1) containing 50 mM EDTA, 1% 2-ME, and 1% SDS to the *S. aureus*/immune complex. After incubation at 56°C for 20 min, the *S. aureus* was pelleted by centrifugation at 13,000 g for 15 s. The eluate was removed and diluted 10-fold in phosphate buffer (0.1 M, pH 6.1) containing 50 mM EDTA and 1.0% NP-40. Aliquots of equal volume (25 µl) were placed into microfuge tubes. Endo F (New England Nuclear, Boston, MA) was added to a final concentration of 23 U/ml, and samples were incubated for 16 h at 37°C. An equal volume (25 µl) of 2X sample buffer containing 10% 2-ME was added to each sample to quench the digestion and samples were incubated in a boiling water bath for 5 min. Samples were analyzed by SDS-PAGE and NEPHGE.

Phosphorylation. Cells were washed and incubated for 1 h at 37°C in phosphate-free buffer (modified MEM phosphate-free Earle's salts with 25 mM Hepes (Irvine Scientific, Santa Ana, CA) supplemented with 1 mM glutamine, nonessential amino acids, 100 µg/ml gentamicin, and 2% heat-inactivated horse serum [dialyzed against modified MEM phosphate-free Earle's salts]). Cells were resuspended in phosphate-free buffer at 5 × 10⁷ cells/ml containing 1.25 mCi/ml [³²P]orthophosphate (carrier-free; Amersham

¹Abbreviations used in this paper: CHAPS, 3[3-cholamidopropyl-dimethylammonio]-l-propane sulfonate; NEPHGE, nonequilibrium pH gradient electrophoresis; PE, phycoerythrin.

Corp.) and 800 U/ml rIL-2. Cells were incubated for 3 h at 37°C, and then washed three times in cold PBS containing 0.1 mM Na₃VO₄, 0.4 mM EDTA, 10 mM Na₄P₂O₇, 10 mM NaF, and 0.1% NaN₃. Cells were solubilized in 0.5 ml 5 mM CHAPS lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, pH 8.0) containing 1 mM PMSF, 10 mM iodoacetamide, and 10 mM NaF. After incubation at 4°C for 45 min, nuclei were removed by centrifugation at 13,000 *g* for 3 min. Lysates were precleared twice by incubation with packed formalin-fixed *S. aureus* (Pansorbin; Calbiochem-Behring Corp.) coated with rabbit anti-mouse Ig serum (Pel-Freez Biologicals) (22). Immunoprecipitation was performed using anti-Leu-23 mAb bound to rabbit anti-mouse Ig-coated *S. aureus*, as described (22). After immunoprecipitation, samples were transferred to fresh microfuge tubes and antigen was eluted using sample buffer containing 2.3% SDS. Immunoprecipitates were analyzed by two-dimensional "diagonal" gel electrophoresis (24). After second-dimension electrophoresis, gels were stained in 0.1% Coomassie Blue R-250, 50% methanol, 7.5% acetic acid for 20 min, and then incubated overnight at room temperature in 50 mM NaH₂PO₄, 10% TCA solution. Gels were subsequently destained twice for 20 min in 5% methanol, 7.5% acetic acid solution, and finally washed for 20 min in distilled water containing 5% glycerol. Gels were dried and autoradiographed at -70°C on Kodak XR film using intensifying screens (Cronex Lightening Plus; DuPont Co., Wilmington, DE).

Results

Expression of Leu-23 on IL-2-activated Lymphocytes. Anti-Leu-23 specifically reacted with essentially all IL-2-activated NK cells, but reacted only weakly with a minor proportion of resting peripheral blood NK cells (Table I). Similarly, anti-Leu-23 was negative or reacted only weakly with most resting peripheral blood T and B lymphocytes. However, expression of Leu-23 antigen is not restricted to activated NK cells. As summarized in Table I, Leu-23 is also present

TABLE I
Cellular Distribution of Leu-23

Cell type	Reactivity
Normal peripheral blood	
T lymphocytes (CD3 ⁺)	0-5% positive*
B lymphocytes (CD19 ⁺)	0-5% positive*
NK lymphocytes (CD16 ⁺)	0-15% positive*
Monocytes	Weakly positive*
Granulocytes	<5% positive
Normal thymocytes	~30% positive
Normal splenocytes	~10% positive
Normal bone marrow	~10%
IL-2-dependent cell lines	
CD8 ⁺ alloantigen-directed CTL cell lines	4/4 positive [‡]
CD4 ⁺ tetanus toxoid helper T clones	5/5 positive [‡]
Thymocyte cell lines	4/4 positive [‡]
NK cell lines	5/5 positive [‡]
PHA-activated T lymphoblasts (3 d)	~50% positive

Binding was assessed by direct or indirect immunofluorescence using flow cytometric analysis.

* Based on analysis of 10 normal, adult blood donors.

[‡] Indicates that at least 50% of cells within the cell line expressed detectable amounts of Leu-23 antigen.

on most IL-2-dependent CD8⁺ CTL cell lines, CD4⁺ tetanus-toxoid-specific helper T cell clones, IL-2-dependent thymocyte cultures, and mitogen-activated T lymphocytes. The antigen does not simply correlate with proliferation per se, since it could not be detected on several cultured T leukemia cell lines (HPB-ALL, PEER, CCRF-CEM) and an EBV-transformed B lymphoblastoid cell line (CCRF-SB).

Biochemical analysis of the Leu-23 antigen was conducted to determine whether the different cell types expressing the antigen would demonstrate similar structural properties. As shown in Fig. 1 A, an essentially identical structure was immunoprecipitated from IL-2-dependent cell lines of peripheral blood NK cells, peripheral blood T lymphocytes, and thymocytes. Anti-Leu-23 immunoprecipitated a disulfide-bonded 50–60-kD glycoprotein that dissociated into subunits of 32 kD and 28 kD after reduction. Two-dimensional “diagonal” SDS-PAGE analysis confirmed disulfide bonding of the subunits, and the relative mobility of the subunits below the diagonal suggested that these proteins may form dimers of 32 + 32 kD, 32 + 28 kD, and 28 + 28 kD proteins (Fig. 1 B). Deglycosylation of the antigen with a predetermined optimal concentration of Endo F enzyme revealed a single protein of 24 kD. In experiments to optimize Endo F digestion conditions, a partially deglycosylated species was observed with a relative mobility of 28 kD. Since Endo F cleaves both high mannose and complex types of carbohydrates linked through asparagine to the peptide, the Leu-23 antigen is apparently a protein of 24 kD with at least two sites for N-linked glycosylation. Two-dimensional NEPHGE analysis of the intact and deglycosylated Leu-23 antigen further indicated that the 32- and 28-kD subunits likely represent the same polypeptide that differs by the addition of a single N-linked carbohydrate (Fig. 2 B). The deglycosylated 24-kD protein was resistant to further digestion with N-glycanase, an enzyme that hydrolyzes asparagine-linked oligosaccharides that may be resistant to Endo F treatment (not shown). The microheterogeneity observed in the deglycosylated 24-kD protein may have resulted from carbamylation of the protein by urea during NEPHGE analysis, or alternatively could be due to the presence of enzyme-resistant carbohydrates or O-linked oligosaccharides.

Induction of Leu-23 Antigen. Experiments were conducted to determine the conditions necessary to induce expression of Leu-23 on NK cells and T lymphocytes. Peripheral blood lymphocytes were separated into low buoyant density and high buoyant density fractions using Percoll gradient centrifugation. As previously reported (17), the high buoyant density cells were >95% small, resting T lymphocytes and the low buoyant density fraction contained both NK cells and T lymphocytes. Lymphocytes were cultured in 500 U/ml IL-2 and aliquots were removed after 2, 6, and 18 h of culture. Cells were stained by two-color immunofluorescence with phycoerythrin-(PE)-conjugated anti-Leu-23 and either FITC-conjugated anti-Leu-11 (CD16) to identify NK cells or FITC-conjugated anti-Leu 4 (CD3) to identify T lymphocytes. Before stimulation with IL-2, Leu-23 was not detectable on low buoyant density (large) T cells or high buoyant density (small) T cells, and was presently only in low amounts on a minor proportion of NK cells (Fig. 3). However, Leu-23 was detected by 6 h after IL-2 stimulation on the majority of NK cells and a subset of the T lymphocytes in the

low buoyant density fraction. Within 18 h, essentially all IL-2-stimulated NK cells expressed Leu-23. By contrast, only a minor proportion of IL-2-stimulated NK cells expressed transferrin receptors, HLA-DR or CD25 (Fig. 4). Since all NK cells expressed Leu-23 within 18 h after exposure to IL-2, this indicates that

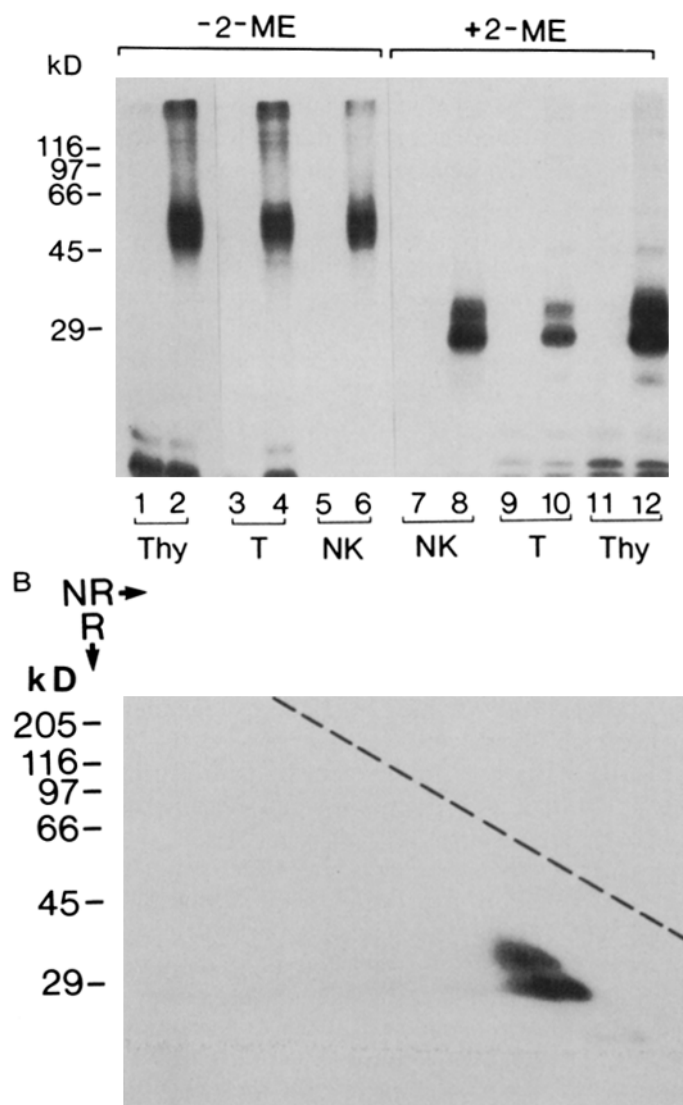


FIGURE 1. Immunoprecipitation of Leu-23. (A) IL-2-dependent lines of NK cells, thymocytes (Thy), and peripheral blood T lymphocytes were radiolabeled with ^{125}I and were detergent solubilized. Lysates were immunoprecipitated using *S. aureus* coated with control immunoglobulin or anti-Leu 23. Immune complexes were eluted in sample buffer containing 2.3% SDS with or without 5% 2-ME. Samples were analyzed using 10% acrylamide gels. (B) An aliquot of the anti-Leu-23 immunoprecipitate of IL-2-cultured thymocytes shown in A was analyzed by two-dimensional "diagonal" SDS-PAGE (1st dimension, nonreducing (NR), 7.5% acrylamide gels; 2nd dimension, reducing (R), 10% acrylamide gels).

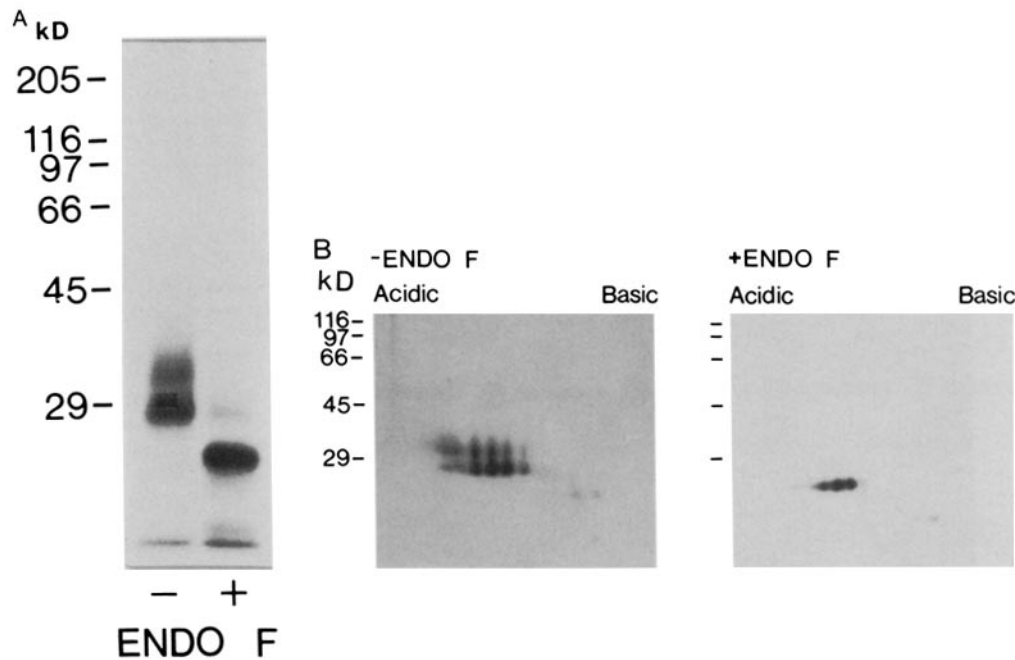


FIGURE 2. Deglycosylation of Leu-23. (A) An aliquot of the anti-Leu-23 immunoprecipitate of IL-2-cultured thymocytes shown in Fig. 1 A was de-glycosylated using Endo F and analyzed by SDS-PAGE (A) and two-dimensional NEPHGE (B) using 10% acrylamide gel. (–) No Endo F digestion; (+) Endo F.

all NK cells are IL-2 responsive. NK cells maintained in culture with IL-2 for 72 h remained uniformly Leu-23⁺ (not shown). Whereas all NK cells acquired Leu-23, even after 72 h of culture with IL-2 only a subset of the T lymphocytes in the low buoyant density fraction expressed this antigen (not shown). In contrast to the T lymphocytes in the low buoyant density fraction, IL-2 did not induce Leu-23 expression on a substantial proportion of the small, resting T

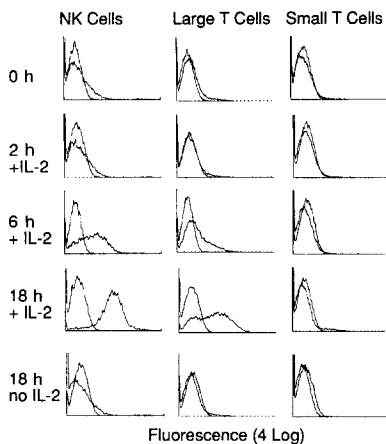


FIGURE 3. Kinetics of induction. Low buoyant density and high buoyant density peripheral blood lymphocytes were cultured in serum-free medium in the presence or absence of 500 U/ml IL-2. At 0, 2, 6, and 18 h, aliquots of cells were stained with: FITC-conjugated IgG and PE-conjugated anti-IgG control antibodies; FITC-conjugated anti-CD16 (Leu 11) and PE-conjugated anti-Leu-23; and FITC-conjugated anti-CD3 (Leu-4) and PE-conjugated anti-Leu-23. Samples were analyzed by flow cytometry. Electronic gates were set on low buoyant density CD16⁺ cells (NK cells), low buoyant density CD3⁺ cells (large T cells), and high buoyant density CD3⁺ cells (small T cells) and data were reprocessed to determine the amount of Leu-23 antigen expression on each population. Histograms of anti-Leu-23-stained cells are superimposed over the histograms of cells stained with the IgG1 control antibody. The control is the histogram nearest the ordinate in each plot.

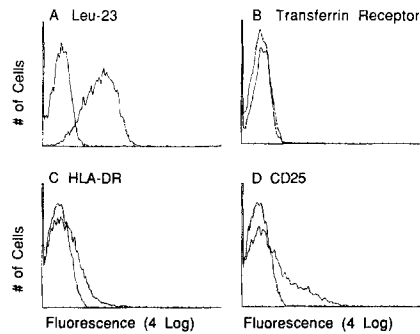


FIGURE 4. Activation antigens expressed on IL-2-stimulated NK cells. Low buoyant density peripheral blood lymphocytes were cultured in serum-free medium in the presence or absence of 500 U/ml IL-2. After 18 h, cells were stained with: FITC-conjugated IgG and PE-conjugated IgG control antibodies; FITC-conjugated anti-Leu-23 (A) and PE-conjugated anti-CD16 (Leu-11), FITC-conjugated anti-transferrin receptor (B) and PE-conjugated anti-CD16 (Leu-11); FITC-conjugated anti-CD16 (Leu-11) and PE-conjugated anti-HLA-DR (C); and FITC-conjugated anti-CD16 (Leu-11) and PE-conjugated anti-CD25 (IL-2-R) (D). Cells were analyzed by flow cytometry. An electronic gate was placed on CD16⁺ NK cells and data were reprocessed to determine the amount of each activation antigen coexpressed on NK cells. Data are presented as described in Fig. 3.

lymphocytes in the high buoyant density fraction (Fig. 3), even after 72 h of culture (not shown).

Induction of Leu-23 antigen by IL-2 was dose dependent (Fig. 5). After 18 h of culture, low levels of Leu-23 antigen were induced on NK cells at 3 U/ml IL-2, and 100 U/ml IL-2 was sufficient for induction of the antigen on a majority of NK cells. IL-2 was necessary and sufficient to induce expression of Leu-23 on NK cells. As shown in Fig. 6., NK cells, purified by isolating CD16⁺ lymphocytes from the low buoyant density fraction using a FACS, acquired expression

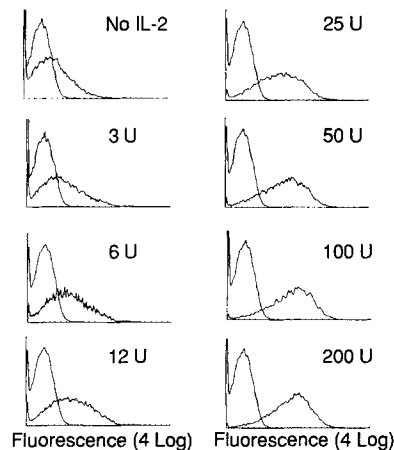


FIGURE 5. IL-2 Concentration required for induction. Low buoyant density peripheral blood lymphocytes were cultured in serum-free medium in the presence or absence of the indicated concentration of IL-2. After 18 h, cells were stained with: FITC-conjugated IgG and PE-conjugated IgG control antibodies; and FITC-conjugated anti-CD16 (Leu-11) and PE-conjugated anti-Leu-23. Cells were analyzed by flow cytometry. An electronic gate was placed on CD16⁺ NK cells and data were reprocessed to determine the amount of Leu-23 antigen coexpressed on NK cells. Data are presented as described in Fig. 3.

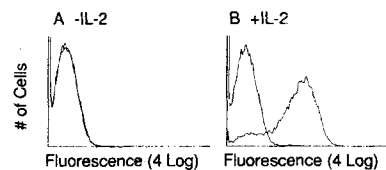


FIGURE 6. Induction of Leu-23 on purified NK cells. Low buoyant density peripheral blood lymphocytes were stained with FITC-conjugated anti-CD16 (Leu-11) and CD16⁺ NK cells were isolated to >95% purity using a FACS. NK cells were cultured in serum-free medium in the presence or absence of the indicated concentration of IL-2. After 18 h, cells were stained with: PE-conjugated Ig control antibody and PE-conjugated anti-Leu-23. Cells were analyzed by flow cytometry. Data are presented as described in Fig. 3.

of Leu-23 after 18 h of culture with IL-2. T lymphocytes, monocytes, or other accessory cells were not required for IL-2 to induce the expression of Leu-23 on NK cells.

Correlation of Leu-23 Antigen and IL-2-induced Cytotoxicity. We have previously demonstrated that NK cells can lyse NK-resistant tumor cell targets, including autologous tumors, after activation by IL-2 (3). The kinetics and dose requirements for IL-2-enhanced cytotoxicity are quite similar to those required for the induction of Leu-23 antigen (2, 3). Thus, further studies were conducted to determine whether IL-2 enhancement of cytotoxicity would correlate with expression of Leu-23. In these experiments, low buoyant density cells were cultured with 100 U/ml IL-2 for 18 h in the presence or absence of neutralizing anti-IL-2 serum. As shown in Table II, both IL-2-enhanced cytotoxicity against a colon carcinoma cell line and induction of Leu-23 on NK cells were comparably inhibited by the neutralizing antiserum. Note the quantitative relationship between inhibition of cytotoxicity and inhibition of Leu-23 antigen induction. These results demonstrate that both phenomena are IL-2 dependent, and possibly, coregulated events.

Experiments were performed to determine whether or not the level of Leu-23 antigen expression would correlate with lytic activity. Low buoyant density cells were cultured with 6 and 12 U/ml for 18 h, and were then stained with FITC-conjugated anti-CD16 and PE anti-Leu-23. As shown in Fig. 3, low levels of Leu-23 were induced on the CD16⁺ NK cell population under these conditions. Although no distinct negative or positive subsets of Leu-23 were evident, it was possible to identify CD16⁺ NK cells that were negative or expressed low amounts of Leu-23 antigen and CD16⁺ NK cells that expressed relatively high levels of Leu-23 antigen. Leu-23 negative (or "dim" positive) and Leu 23

TABLE II
IL-2 Induction of Leu-23 and Cytotoxicity

Antiserum	IL-2	Cytotoxicity		Inhibition of Colo- 205 lysis	Inhibition of Leu-23 antigen
		K562	Colo-205		
		%		%	%
None	—	43	0		
None	+	97	60		
Normal serum	+	100	65	0	0
Rabbit anti-IL-2	+	69	20	69	50
Goat anti-IL-2	+	41	0	100	96

Low buoyant density peripheral blood lymphocytes were cultured in serum-free medium in the presence or absence of 100 U/ml IL-2 with or without normal rabbit serum (1/100 dilution), rabbit anti-IL-2 (1/100 dilution), or goat anti-IL-2 (1/100 dilution). Goat anti-IL-2 serum was used at a dilution determined to completely neutralize 100 U/ml IL-2. Rabbit anti-IL-2 was used at a concentration to achieve 50% inhibition of 100 U/ml IL-2. After 18 h of culture, cells were harvested and assayed for cytotoxicity against ⁵¹Cr-labeled K562 and Colo-205 tumor cell targets at an effector/target ratio of 12:1. Cells were also stained with FITC-conjugated IgG control antibody or FITC-conjugated anti-Leu-23 and were analyzed by flow cytometry. Percent inhibition of Leu-23 antigen was determined by comparison of the mean fluorescence of cells cultured with IL-2 in the presence or absence of neutralizing antiserum.

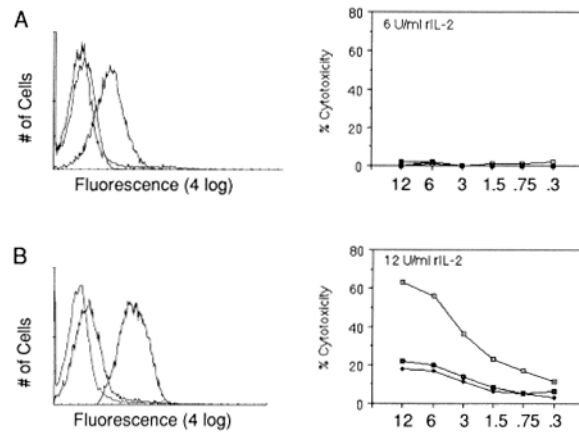


FIGURE 7. Cytotoxicity mediated by NK cells expressing Leu-23. Low buoyant density peripheral blood lymphocytes were cultured in serum-free medium containing 6 U/ml IL-2 (A) or 12 U/ml IL-2 (B). After 18 h, cells were harvested and stained with FITC anti-Leu-23 and PE anti-CD16 (Leu-11). CD16⁺, Leu-23⁻ (or dim Leu-23) and CD16⁺, Leu-23⁺ cells were isolated to >95% purity using a FACS. Reanalysis of the sorted fractions is presented. The histogram of FITC-conjugated IgG control stained cells is nearest the ordinate in each plot, whereas the histograms of Leu-23⁻ or Leu-23 dim population and Leu-23 bright positive cells are to the right

of the control histogram. Unsorted low buoyant density cells (\diamond), CD16⁺, Leu-23⁻ (or dim) (\blacksquare), and CD16⁺, Leu-23⁺ (\square) cells were assayed for cytotoxicity against ⁵¹Cr-labeled Colo-205 colon carcinoma cell targets (x-axis, effector/target ratio; y = axis, percent cytotoxicity).

“bright” CD16⁺ NK cells were isolated using a FACS; reanalysis of the purified populations is presented in Fig. 7. Using 6 U/ml IL-2 for induction, no augmentation of cytotoxicity was observed against an NK-insensitive colon carcinoma cell line, even though low levels of Leu-23 antigen could be detected on the NK cell population (Fig. 7 A). These results indicate that Leu-23 antigen can be expressed before detection of cytotoxic activity. Using 12 U/ml IL-2 for induction, cytotoxicity was mediated by NK cells that expressed both “dim” and “bright” levels of Leu-23 antigen (Fig. 7 B). Comparison of the cytotoxic activity mediated at several effector/target ratios clearly indicated that NK cells express-

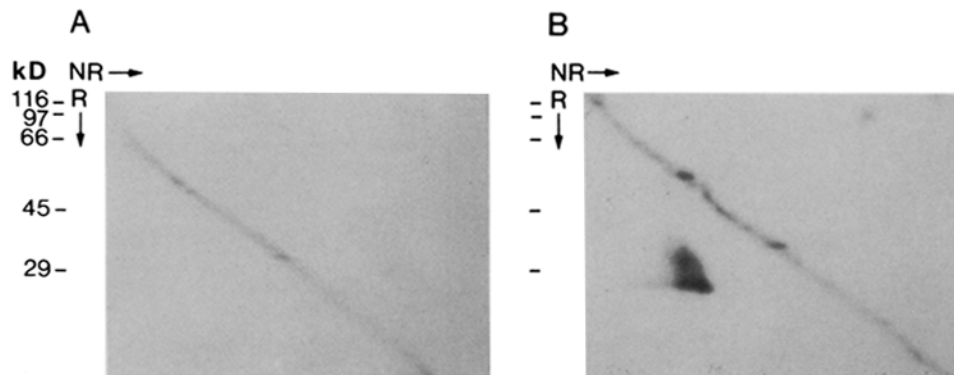


FIGURE 8. Phosphorylation of Leu-23. Low buoyant density peripheral blood lymphocytes were cultured for 18 h with 800 U/ml IL-2 and were labeled with [³²P]orthophosphate during the last 3 h of culture. Cells were detergent solubilized and lysates were immunoprecipitated with control Ig (A) or anti-Leu-23 (B). Samples were analyzed two-dimensional “diagonal” SDS-PAGE using 10% acrylamide gels for both dimensions (1st dimension, nonreducing [NR]; 2nd dimension, reducing [R]). After 15 h of culture with IL-2 (immediately before addition of [³²P]orthophosphate), all CD16⁺ NK cells in the culture expressed the Leu 23 antigen (~40% of the total cells), and ~20% of the low buoyant density CD3⁺ T lymphocytes expressed Leu-23 (not shown).

ing higher levels of Leu-23 antigen were more lytic than the population of NK cells lacking Leu-23 or expressing low levels of this antigen.

IL-2-induced Phosphorylation of Leu-23. Lymphocyte stimulation results in activation of intracellular protein kinases, which in turn phosphorylate growth factor receptors, components of the TCR complex, and certain other membrane glycoproteins (26–31). Experiments were conducted to determine whether Leu-23 is phosphorylated, as well as induced by IL-2. In these experiments, low buoyant density lymphocytes were cultured with IL-2 for 20 h and were labeled with [32 P]orthophosphate during the last 3 h of culture. Cells were detergent solubilized, the Leu-23 glycoprotein was immunoprecipitated, and samples were analyzed by two-dimensional “diagonal” SDS-PAGE (Fig. 8). Disulfide-bonded [32 P]-labeled protein dimers composed of 32 and 28 kD subunits were detected, identical in structure to the 125 I-labeled membrane proteins presented in Fig. 1 B. Therefore, the Leu-23 glycoprotein is both induced and phosphorylated as a consequence of IL-2 stimulation.

Discussion

Peripheral blood NK cells can be activated by IL-2, without requirement for exogenous cofactors or accessory cells (1–4, 16). IL-2-induced activation results both in cellular proliferation and enhancement of NK cytotoxic activity (3, 4). Within a few hours after exposure to IL-2, NK cells acquire the capacity to lyse NK-resistant tumor cell targets, including fresh autologous tumor (1–3, 32, 33). Using purified NK effector cells, cytotoxicity is maximal within 18 h after exposure to IL-2 and requires protein synthesis, but not cell division (2, 3). IL-2 also induces the expression of certain activation antigens on the plasma membrane of NK cells; however, most of these antigens are not detectable until substantially later than detection of IL-2-enhanced cytotoxicity (1, 2, 4, 16). In the present study, we have demonstrated that the Leu-23 antigen is detectable on the surface of NK cells with a few hours after exposure to IL-2. Previously, it has been uncertain whether all NK cells or only a subset are responsive to IL-2. Since within 18 h after exposure to IL-2, essentially all NK cells express Leu-23, these findings indicate that all peripheral blood NK cells are responsive to stimulation by IL-2. The kinetics of Leu-23 antigen induction closely parallel the acquisition of cytolytic activity. Both expression of Leu-23 antigen and enhanced NK cell-mediated cytotoxicity can be induced directly by exposure to IL-2, without requirement for additional activation signals. Moreover, both events were neutralized by anti-IL-2 antiserum to a comparable extent. These results suggest that the two events may be coregulated.

The ability of IL-2 to induce Leu-23 antigen on a subset of low buoyant density T lymphocytes, but not a substantial proportion of small, resting T lymphocytes, suggests that this subset of T lymphocytes may be “primed” in vivo, requiring only IL-2 for activation. Recently, London et al. (4) have reported that a minor subset of low buoyant density T lymphocytes in peripheral blood enter the cell cycle after exposure to IL-2. It should be noted that we have observed expression of Leu-23 antigen on a small subpopulation of circulating peripheral blood T and NK lymphocytes in some normal individuals. Whether these cells represent in vivo activated lymphocytes will be examined in further studies.

It is possible that the Leu-23 glycoprotein is involved in the IL-2 activation of NK and T lymphocytes. However, we have been unable to directly demonstrate involvement of the Leu-23 antigen with cell function. Anti-Leu-23 mAb did not inhibit IL-2-induced NK cell-mediated cytotoxicity or IL-2-induced proliferation of NK or T lymphocytes (unpublished observations). It is unlikely that the Leu-23 glycoprotein is involved directly with the cytolytic process, since the antigen is also present on noncytotoxic lymphocytes (e.g., CD4⁺ tetanus-toxoid-specific T cell clones, mitogen-activated T lymphoblasts, and normal thymocytes). Expression of Leu-23 on lymphocytes with diverse immune functions suggests that this glycoprotein may be involved in more general processes involved with cellular activation.

Structural features of the antigen are consistent with the possibility that Leu-23 is a membrane receptor for growth or differentiation factors. Leu-23 is a disulfide-linked homodimer composed of 24-kD subunits with at least two N-linked carbohydrates. Of particular interest was the observation that the glycoprotein is both induced and phosphorylated via an IL-2-dependent process. Many growth factor receptors, including somatomedin C, epidermal growth factor receptor, insulin receptor, and IL-2, are phosphorylated subsequent to cellular activation (26–29). The present studies indicated that for NK cells and a subset of low buoyant density T cells, IL-2 is the only exogenous factor necessary to induce expression of Leu-23. Further studies will be necessary to elucidate the intracellular events that occur subsequent to IL-2 binding and result in the induction and phosphorylation of Leu-23.

The EA-1 (31) and MLR3 (34) activation antigens have structural properties and a cellular distribution similar to Leu-23. Whether EA-1, MLR3, and Leu-23 are the same antigen, distinct epitopes on the same antigen or different glycoproteins will require comparative binding and biochemical analysis. Hara et al. (31) previously demonstrated that the EA-1 antigen is rapidly induced on T lymphocytes after activation by mitogens or antigens and that the glycoprotein is phosphorylated after stimulation with phorbol ester; however, in these experiments the role of IL-2 in the process was not investigated. Similarly, expression of MLR-3 is rapidly induced by mitogen activation of T lymphocytes (34). Cosulich et al. (34) have reported that mAb against MLR3 inhibits IL-1-dependent stimulation of resting T cells using anti-CD3. Although we have not determined the functional effect of anti-Leu-23 mAb on IL-1-dependent T cell activation, IL-1 did not affect expression of Leu-23 antigen on NK cells (unpublished observation).

In conclusion, the Leu-23 antigen is rapidly induced on peripheral blood NK cells and a unique subset of T lymphocytes after exposure to IL-2, without the requirement for exogenous cofactors or accessory cells. The kinetics of IL-2-induced expression of Leu-23 on NK cells closely paralleled the acquisition of cytotoxic function against solid tumor cell targets, thereby providing a useful marker of NK cell activation. The rapid induction and phosphorylation of this glycoprotein suggest that the structure may be involved in IL-2-dependent activation mechanisms. Further studies directed at defining the molecular properties of this membrane antigen may provide valuable insight into function of this glycoprotein.

Summary

IL-2 potentiates both growth and cytotoxic function of T lymphocytes and NK cells. Resting peripheral blood NK cells can respond directly to rIL-2, without requirement for accessory cells or cofactors, and enhanced cytotoxicity can be measured within a few hours after exposure to this lymphokine. In this study, we describe an activation antigen, Leu-23, that is rapidly induced and phosphorylated after IL-2 stimulation of NK cells and a subset of low buoyant density T lymphocytes. Previously, it has been uncertain whether all NK cells or only a subset are responsive to IL-2. Since within 18 h after exposure to IL-2, essentially all NK cells express Leu-23, these findings indicate that all peripheral blood NK cells are responsive to stimulation by IL-2. The Leu-23 antigen is a disulfide-bonded homodimer, composed of 24-kD protein subunits with two *N*-linked oligosaccharides. Appearance of this glycoprotein on NK cells is IL-2 dependent and closely parallels IL-2-induced cytotoxicity against NK-resistant solid tumor cell targets.

We thank Dr. Jim Allison for invaluable discussion and advise relating to the phosphorylation experiments; Drs. Brett Gemlo and Anthony Rayner for providing thymocytes; and Dr. Anne Jackson for providing the phycoerythrin-conjugated anti-Leu-23 reagent.

Received for publication 2 December 1987.

References

1. Trinchieri, G., M. Matsumoto-Kobayashi, S. C. Clark, J. Seehra, L. London, and B. Perussia. 1984. Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* 160:1147.
2. Lanier, L. L., C. J. Benike, J. H. Phillips, and E. G. Engleman. 1985. Recombinant interleukin 2 enhanced natural killer cell-mediated cytotoxicity in human lymphocyte subpopulations expressing the Leu 7 and Leu 11 antigens. *J. Immunol.* 134:794.
3. Phillips, J. H., and L. L. Lanier. 1987. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. *J. Exp. Med.* 164:814.
4. 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.
5. Manger, B., A. Weiss, C. Weyand, J. Gornozzy, and J. D. Stobo. 1985. T cell activation: differences in the signals required for IL 2 production by nonactivated and activated T cells. *J. Immunol.* 135:3669.
6. Mizel, S. B. 1982. Interleukin 1 and T cell activation. *Immunol. Rev.* 63:51.
7. Cotner, T., J. M. Williams, L. Christenson, H. M. Shapiro, T. B. Strom, and J. Strominger. 1983. Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. *J. Exp. Med.* 157:461.
8. Maino, V., and M. E. Janszen. 1986. Monoclonal antibodies, L-35 and L-36, define novel T cell activation antigens. *J. Immunol.* 137:3093.
9. Uchiyama, T., S. Broder, and T. A. Waldmann. 1981. A monoclonal antibody (Anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. *J. Immunol.* 126:1393.
10. Haynes, B. F., M. E. Hemler, D. L. Mann, G. S. Eisenbarth, J. Shelhamer, H. S.

- Mostowski, C. A. Thomas, J. L. Strominger, and A. S. Fauci. 1981. Characterization of a monoclonal antibody (4F2) that binds to human monocytes and to a subset of activated lymphocytes. *J. Immunol.* 126:1409.
11. Evans, R. L., T. J. Faldetta, R. E. Humphreys, D. M. Pratt, E. J. Yunis, and S. F. Schlossman. 1978. Peripheral human T cells sensitized in mixed leukocyte culture synthesize and express Ia-like antigens. *J. Exp. Med.* 148:1440.
 12. Omary, M. B., I. S. Trowbridge, and J. M. Minowada. 1980. Human cell-surface glycoprotein with unusual properties. *Nature (Lond.)*. 286:888.
 13. Hemler, M. E., J. Jacobson, M. B. Brenner, D. Mann, and J. L. Strominger. 1985. VLA-1: a T cell surface antigen which defines a novel late stage of human T cell activation. *Eur. J. Immunol.* 15:502.
 14. 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.
 15. Phillips, J. H., and L. L. Lanier. 1985. A model for the differentiation of human natural killer cells. Studies on the in vitro activation of Leu 11+ granular lymphocytes with a natural killer-sensitive tumor cell, K562. *J. Exp. Med.* 161:1464.
 16. London, L., B. Perussia, and G. Trinchieri. 1985. Induction of proliferation in vitro of resting human natural killer cells: expression of surface activation antigens. *J. Immunol.* 134:718.
 17. Phillips, J. H., N. L. Warner, and L. L. Lanier. 1984. Correlation of biophysical properties and cell surface antigenic profile of Percoll gradient-separated human natural killer cells. *Nat. Immun. Cell Growth Regul.* 3:73.
 18. Fong, S. K. H., D. T. Sasaki, C. F. Grumet, and E. G. Engleman. 1982. Production of functional T-T hybridomas in selection medium lacking aminopterin and thymidine. *Proc. Natl. Acad. Sci. USA.* 79:7484.
 19. Parks, D. R., L. L. Lanier, and L. A. Herzenberg. 1986. Flow cytometry and fluorescence activated cell sorting (FACS). In *Handbook of Experimental Immunology*. Fourth ed., D. M. Weir, L. A. Herzenberg, C. C. Blackwell, and L. A. Herzenberg, editors. Blackwell Publications, Ltd., Oxford, United Kingdom. 29.1-29.21.
 20. Lanier, L. L., A. M. Le, J. H. Phillips, N. L. Warner, and G. F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu 11 (NK-15) antigens. *J. Immunol.* 131:1789.
 21. Keski-Oja, J., D. F. Mosher, and A. Vaheri. 1977. Dimeric character of fibronectin, a major cell-surface associated glycoprotein. *Biochem. Biophys. Res. Commun.* 74:699.
 22. Lanier, L. L., N. A. Federspiel, J. J. Ruitenberg, J. H. Phillips, J. P. Allison, D. Littman, and A. Weiss. 1987. The T cell antigen receptor complex expressed on normal peripheral blood CD4⁺, CD8⁺ T lymphocytes: a CD3-associated disulfide-linked γ chain heterodimer. *J. Exp. Med.* 165:1076.
 23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680.
 24. Allison, J. P., B. W. McIntyre, and D. Bloch. 1982. Tumor-specific antigen of murine T lymphoma defined with monoclonal antibody. *J. Immunol.* 129:2293.
 25. Jones, P. P. 1980. Analysis of radiolabeled lymphocyte proteins by one- and two-dimensional polyacrylamide gel electrophoresis. In *Selected Methods in Cellular Immunology*. B. B. Mishell and S. M. Shiigi, editors. Freeman Publications San Francisco, CA. 398-440.
 26. Shackelford, D. A., and I. S. Trowbridge. 1984. Induction of expression and phosphorylation of the human interleukin 2 receptor by a phorbol diester. *J. Biol. Chem.* 259:11706.

27. Hunter, T., and J. A. Cooper, 1985. Protein-tyrosine kinases. *Annu. Rev. Biochem.* 54:897.
28. Jacobs, S., N. E. Sahyoun, A. R. Saltiel, and P. Cuatrecasas. 1983. Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C. *Proc. Natl. Acad. Sci. USA.* 80:6211.
29. Iwashita, S., and C. R. Fox. 1984. Epidermal growth factor and potent phorbol tumor promoters induce epidermal growth factor receptor phosphorylation in a similar by distinctively different manner in human epidermoid carcinoma A431 cells. *J. Biol. Chem.* 259:2559.
30. Samelson, L. E., J. B. Harford, and R. D. Klausner. 1985. Identification of the components of the murine T cell antigen receptor complex. *Cell.* 43:223.
31. Hara, T., L. K. L. Jung, J. M. Bjorndahl, and S. M. Fu. 1986. Human T cell activation. III. Rapid induction of a phosphorylated 28 kD/32 kD disulfide-linked early activation antigen (EA-1) by 12-o-teradecanoyl phorbol-13-acetate, mitogens, and antigens. *J. Exp. Med.* 164:1988.
32. Itoh, K., A. B. Tilden, and C. M. Balch. 1986. Lysis of human solid tumor cells by lymphokine-activated natural killer cells. *J. Immunol.* 136:3910.
33. Ortaldo, J. R., A. Mason, and R. Overton. 1986. Lymphokine-activated killer cells: analysis of progenitors and effectors. *J. Exp. Med.* 164:1193.
34. Cosulich, M. E., A. Rubartelli, A. Risso, F. Cozzolino, and A. Bargellesi. 1987. Functional characterization of an antigen involved in an early step of T-cell activation. *Proc. Natl. Acad. Sci. USA.* 84:4205.