RESPONSE OF RESTING HUMAN PERIPHERAL BLOOD NATURAL KILLER CELLS TO INTERLEUKIN 2

By GIORGIO TRINCHIERI, MICHIKO MATSUMOTO-KOBAYASHI, STEVEN C. CLARK,* JASBIR SEEHRA,* LUCILLE LONDON, AND BICE PERUSSIA

*From The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104; and the *Genetics Institute, Boston, Massachusetts 02115*

Resting T cells can be activated to proliferate by antigens and mitogenic lectins, but their proliferation depends on a hormone-like lymphokine, T cell growth factor or interleukin 2 (IL-2)[1] (1–2). Activation of T cells by a mitogenic stimulus induces both secretion of minute quantities of IL-2 and the appearance of a high-affinity receptor for IL-2 on the T cell surface (3–4). This receptor has been identified as a 50,000 dalton protein, not detectable on resting T cells, that is recognized by the monoclonal antibody anti-Tac (4–6). Several lines of evidence suggest that resting natural killer (NK) cells can respond directly to IL-2, with enhanced cytotoxic activity and eventually proliferation, with no additional requirement for other activation stimuli. IL-2-containing preparations, free of antiviral activity, enhance NK cell activity in mouse spleen cells (7–9) and in human peripheral blood lymphocyte (8, 10, 11) (PBL) cultures in a manner similar to that of interferon (IFN). IL-2 induces production of IFN-γ in leukocyte cultures (11–14) by a mechanism that, in the mouse, depends upon cooperation between macrophages and cells with NK characteristics (14). It has been suggested that the enhancement of NK activity induced by IL-2 is mediated by the IFN-γ produced in the culture, since anti-IFN-γ antisera abolish the effect (8). IL-2 has also been shown to induce proliferation and production of IFN-γ by low density human PBL purified on discontinuous Percoll gradients (11, 15). However, those studies, based on the use of gradient separation and of monoclonal antibodies of only relative specificity for NK cells, do not allow a distinction between the possibilities that proliferation and IFN-γ production are confined to NK cells, or are in part or entirely a property of in vivo activated T cells, copurifying with NK cells in low density Percoll fractions. In addition to enhancing NK activity in short-term cultures, IL-2 can induce, in in vitro cultures of

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[1] *Abbreviations used in this paper:* C, complement; CdL, complement-dependent lysis; CMC, cell-mediated cytotoxicity; CTLL, cytotoxic T lymphocyte line; E, erythrocytes; ER, receptor for sheep erythrocytes; FBS, fetal bovine serum; FcR, receptor for the Fc fragment of IgG; F/H, Ficoll/Hypaque; FITC, fluorescein isothyocyanate; [^{3}H]TdR, [^{3}H]thymidine; IFN, interferon; IL-2, interleukin 2; LAK, lymphokine-activated killer; LU, lytic unit; NK, natural killer; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; r, recombinant; SDS, sodium dodecyl sulfate; VSV, vesicular stomatitis virus.

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three or more days, cells cytotoxic for NK-insensitive tumor target cells (16). These effector cells, termed lymphokine-activated killer (LAK) cells, bear markers of cytotoxic T cells but derive from precursor cells that lack markers of either T or NK cells and copurify with NK cells on a Percoll gradient (17).

Recently (18, 19), the gene for human IL-2 has been cloned in several laboratories and this has allowed purification of homogeneous recombinant IL-2 (rIL-2) from transfected *Escherichia coli.* With this material, it becomes possible to assay the effect of IL-2 on human NK cells and exclude any effect of contaminants such as lectins, phorbol esters, IFN, or other lymphokines.

In the present paper, we show that rIL-2 induces a rapid and potent enhancement of the spontaneous cytotoxicity of human lymphocytes. The IL-2-induced cytotoxic effector cells have surface markers of NK cells and are generated from the same PBL subset mediating spontaneous NK activity. Like IFN, IL-2 induces increased cytotoxicity against a large panel of NK-sensitive or relatively resistant target cells. rIL-2 induces IFN-γ production in lymphocyte cultures and, in short-term cultures, NK cells appear to be the major producer cells, whereas T cells are unable to produce IFN-γ in response to rIL-2. The kinetics of enhancement of cytotoxicity and production of IFN-γ and the inability of monoclonal antibodies to IFN-γ to suppress the IL-2-dependent NK enhancement suggest that the effect of rIL-2 on NK cells is independent of IFN-γ. The enhancement of NK cell activity by rIL-2 precedes any proliferative response of the lymphocytes. By contrast, proliferation is observed in longer cultures in cells with both NK or T cell markers.

Materials and Methods

Lymphokines. IL-2 was produced at high levels by *E. coli* transfected with an expression plasmid containing the IL-2-coding region cloned from human lymphocytes (18). Harvested *E. coli* were resuspended (40 g packed cells in 100 ml final volume) in 50 mM Tris-HCl (pH 7.5), 2% (vol/vol) Triton X-100 and sonicated at 20 kHz for 10 min. Vesicles containing IL-2 were collected by centrifugation, washed twice with phosphate buffer (0.1 M, pH 4.5), and dissolved in the same buffer supplemented with 1% (wt/vol) sodium dodecyl sulfate (SDS) and 5% (vol/vol) 2-mercaptoethanol. The protein solution was purified by high pressure liquid chomatography first on a gel permeation column and then by reverse phase chromatography. Protein concentration was determined by a dye-binding assay using bovine plasma gamma globulins as a standard (Bio-Rad Laboratories, Richmond, CA). The purity of the IL-2 preparations was >99% and the specific activity was 3–10 $\times$ 10^6 U/mg (1 U being the amount of IL-2 giving 50% of the maximal [^{3}H]-thymidine ([^{3}H]TdR) incorporation in the cytotoxic T lymphocyte line (CTLL) assay, as described below). Contamination with endotoxins was excluded using the limulus amebocyte lysate assay (E-Toxate; Sigma Chemical Co., St. Louis, MO). Affinity-purified IFN-γ (10^6 U/mg) and purified IFN-α (Ultra-pure IFNα, 10^8 U/mg) were obtained from Interferon Sciences, New Brunswick, NJ.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide vertical slab gels according to a modification of the Laemmli procedure (20). After electrophoresis, gels were stained by the silver staining method (Bio-Rad Laboratories). Molecular weight standards were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

IL-2 Biologic Assay. IL-2 growth-promoting activity was determined by the IL-2 concentration–dependent stimulation of proliferation of CTLL-2, a cloned murine cytotoxic T lymphocyte line (2, 21). CTLL (5 $\times$ 10^4 cells/ml) proliferation was monitored by [^{3}H]TdR incorporation (5 μCi/ml, 2 Ci/mmol specific activity; New England Nuclear,

Boston, MA) during the last 6 h of a 24-h culture in the presence of serial dilutions of the IL-2 preparation.

Monoclonal Antibodies and Antisera. Two monoclonal antibodies, specific for the FcR for aggregated IgG on NK cells and neutrophils, were used in this study: B73.1 (IgG1), produced in our laboratory (22, 23) and VEP13 (IgM) (24), kindly provided by Dr. H. Rumpold (Institute of Experimental Pathology, Vienna, Austria). The two antibodies react with two different epitopes of the same surface molecule. The reactivity of antibody B73.1 on neutrophils is absent or reduced as compared with that of VEP13 (25, 26), and only antibody VEP13 is cytotoxic with complement (C). Among peripheral blood mononuclear cells, virtually all NK cells, but not T cells, B cells, or monocytes are detected by the two antibodies (22–26). Antibody HNK-1 (IgM) (27), which reacts with a variable proportion of NK and T cells in the peripheral blood (22, 28), was produced from cell cultures obtained from American Type Culture Collection, Rockville, MD. Antibody Leu-4 (IgG1), reacting with the 20,000 mol wt surface molecule present on all peripheral blood T cells, was kindly provided by Dr. N. Warner (Becton Dickinson Monoclonal Center, Sunnyvale, CA). Monoclonal antibody anti-Tac (IgG2a) (5) was kindly donated by Dr. T. Waldman (NIH, Bethesda, MD). Antibody OKM1 (IgG2a), reacting with the heavy chain of a complex of two polypeptide chains of 95,000 and 177,000 daltons, corresponding to the receptor for C3bi (29) present on monocytes, granulocytes, and most NK cells, was kindly provided by Dr. G. Goldstein (Ortho Pharmaceutical, Raritan, NJ). Antibodies B67.1 (IgG2a), specific for the 45,000 mol wt receptor for sheep erythrocytes (ER) (23); B36.1 (IgG2b), of specificity identical to that of anti-Leu-1 (22) and detecting a 69,000 mol wt molecule present on all peripheral blood T cells; and B33.1 (IgG2a), detecting a nonpolymorphic determinant of the HLA-DR molecule (23), were produced in our laboratory. An IgG2a monoclonal antibody (X-400) with neutralizing activity against IFN-γ was obtained from Meloy Laboratories, Inc., Springfield, VA as a stock solution neutralizing 20,000 U of the Gg23-901-530 NIH IFN-γ standard per milliliter. Monoclonal antibodies B133.1 and B133.3 (both IgG1) were produced in our laboratory and react specifically with human IFN-γ and not with either IFN-α or -β. The tissue culture supernatant fluids from B133.1 and B133.3 used in the present study neutralized 3,600 and 32,400 U of the NIH IFN-γ standard per milliliter, respectively. A sheep antiserum against human IFN-α (10^5 neutralizing U/ml) was obtained from Interferon Sciences. A neutralizing rabbit antiserum anti-human IL-2 was obtained by immunization with homogeneous rIL-2: the antiserum did not show any neutralizing activity against IFN. The preparation used inhibited the growth-promoting activity of 1.25 μg of rIL-2 per milliliter.

PBL Preparations. Venous peripheral blood was obtained from healthy donors and was anticoagulated with heparin. Mononuclear cells were prepared by Ficoll-Hypaque (F/H) gradient centrifugation and, unless otherwise indicated, were partially depleted of monocytes by adherence on plastic petri dishes (No. 3024, Falcon Plastics, Becton Dickinson & Co., Cockeysville, MD) for 1 h at 37°C. These monocyte-depleted preparations are referred to in the text as PBL. Lymphocyte cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere (5×10^6 cells/ml) in RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories).

Cell Lines. The human erythro-myeloid leukemia cell line K562, the promyelocytic lines HL-60 and ML3, the macrophage line U937, the B cell lines Raji and Daudi, the T cell lines Molt 4 and Jurkat, the fetal skin fibroblast strains HF-28 and Detroit 532, the rhabdomyosarcoma-derived line RDMC, and the melanoma-derived line SK-Mel/23, were maintained in culture in RPMI 1640 medium supplemented with 10% FBS.

Spontaneous Cell-mediated Cytotoxicity (CMC) Assays. The spontaneous CMC assay was performed in round-bottom microtiter plates as described, using ^{51}Cr-labeled target cells (10^4 cells/well) in a 3-h assay (30). Adherent target cells were labeled with ^{51}Cr as a monolayer and trypsinized immediately before use as a target cell suspension. All CMC assays were always performed using at least four different effector cell concentrations. Standard deviation of triplicate wells was usually <1%. Results were quantitated at various

effector-to-target cell ratios by calculating the number of lytic units (LU) per 10^7 cells at 45% specific lysis with the use of the linear regression to a modified von Krogh's equation (31–33). One LU is the number of effector cells necessary to lyse 45% of the target cells during the assay period.

Complement-dependent Lysis (CdL). Rabbit complement (C) from baby rabbits was obtained from Cedarlane Laboratories Ltd., Hornby, Ontario, Canada and screened among several lots for high lytic titer and low toxicity. PBL were incubated (30 min at 4°C) with an optimal predetermined concentration of the monoclonal antibodies. Rabbit C was then added (10^{-1} dilution) and the incubation continued for 1 h at 37°C. Dead cells were scored immediately by erythrosin B dye exclusion. Cells were then washed twice, resuspended in culture medium at the original volume, and used for cytotoxic tests or IFN production. We did not correct cell concentration for the number of remaining live cells to allow a quantitative evaluation of the decrease in cytotoxicity due to the eliminated subpopulation. A slight decrease in NK cell cytotoxicity was sometimes observed in C-treated control populations in comparison with the untreated ones.

Separation of Lymphocyte Subpopulations. Cells were incubated with the monoclonal antibodies (1 μg/ml, 30 min at 4°C), washed three times in phosphate-buffered saline, pH 7.2 (PBS), and incubated for 30 min at 4°C as pellets with $CrCl_3$-treated sheep erythrocytes (E) (22) coated with a goat $F(ab')_2$ antibody anti-mouse Ig (E to PBL ratio, 50:1). The goat $F(ab')_2$ anti-mouse Ig (Cappel Laboratories, Cochranville, PA) was preabsorbed on a human Ig-Sepharose 4B (Pharmacia Fine Chemicals) column to eliminate antibodies cross-reactive with human Ig, and then affinity-purified on a mouse Ig-Sepharose 4B (Pharmacia Fine Chemicals) column. The use of affinity-purified antibodies completely prevents background in the absence of an antibody reacting with the cells, and increases efficiency of the separation method. No spontaneous rosette formation was ever observed in the control population under the same experimental conditions, since $CrCl_3$ treatment of sheep E and the experimental conditions used do not allow E rosette formation by T cells. The proportion of rosette-forming cells was evaluated by scoring at least 200 cells by light microscopy.

Rosetting and nonrosetting cells were then separated on a one-step F/H gradient. Contaminating E in the antibody-positive (rosetting) fraction were lysed by treatment with hypotonic medium. A portion of cells was tested before F/H separation for the effect of antibody and rosetting. The values of cytotoxic activity observed in this control and in the total untreated lymphocyte preparation were similar, although occasionally NK cell cytotoxicity decreased slightly in the control unseparated population.

Thymidine Incorporation and Simultaneous Detection of Thymidine Incorporation and Surface Antigen Expression. PBL or separated subsets were cultured (10^6 cells/ml) and [^{3}H]TdR (1 mCi/ml, 2 Ci/mmol sp act; New England Nuclear) was added to the culture during the last 2 h (1 μCi/well). Cells were collected on glass fiber filters using an automatic cell harvester (Skatron Inc., Sterling, VA) and the incorporated [^{3}H]TdR was assayed by liquid scintillation. For the simultaneous detection of cells incorporating [^{3}H]TdR and cells expressing surface antigens, after the [^{3}H]TdR pulse, the cells were washed, sensitized with a monoclonal antibody and allowed to react with anti-mouse Ig-coated sheep E, according to the technique of indirect rosetting described above. Cytocentrifuge smears were prepared, fixed with absolute methanol for 30 min, covered with NTB-2 emulsion (Eastman Kodak Company, Rochester, NY) and developed after a 2-d exposure. Cells reacting with the antibody were identifiable on the slide by formation of rosettes with sheep E. The proportion of cells having incorporated [^{3}H]TdR was scored within antigen-positive and -negative cells.

Indirect Immunofluorescence Assay. Cells were incubated with the monoclonal antibody (30 min at 4°C), washed three times, and incubated with a 1:80 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse Ig (Cappel Laboratories) absorbed on a column of human Ig-Sepharose 4B. All washes were done in PBS containing 0.1% gelatin and 0.1% NaN_3. An Ortho Cytofluorograf System H50 connected to a Data General MP/200 miroprocessor (Ortho Diagnostic Systems, Inc., Westwood, MA) was used for analysis of the fluorescent cells. Cells were considered positive when their

fluorescence intensity exceeded the level at which 99% of the cells in the negative control, treated with the second immunofluorescent reagent only, had lower fluorescence intensity (34).

Interferon Assay. Antiviral activity was tested in supernatant fluids by inhibition of the cytopathic effect of vesicular stomatitis virus (VSV) on human fibroblast strain Detroit 532, derived from a subject with trisomy 21. The IFN concentration inducing 50% protection of the cytopathic effect on Detroit 532 cells corresponded to ~1 U of the NIH IFN-γ standard Gg23-901-530 and to 0.1 U of the NIH IFN-α standard Gg23-901-527.

Results

Characterization and Growth-promoting Activity of rIL-2. The rIL-2 preparation appears as a homogeneous silver-stained band of 15,000 mol wt on SDS gel under reducing conditions (Fig. 1, *left*), and monoclonal antibodies to IL-2 bound to the band as demonstrated by immunoblotting (not shown). The faint band of 30,000 apparent mol wt is probably a dimer of the IL-2 molecule, because it is not resolved when the sample is boiled extensively in the presence of SDS, and because it is stained with monoclonal anti-IL-2 antibodies (not shown). These data are consistent with previous reports (35) that functionally active dimers of natural IL-2 can be demonstrated on SDS gels. Growth-promoting activity (as tested on CTLL cells) was concentrated in proteins eluting at the 15,000 mol wt position (not shown). The dose of rIL-2 that induced 50% of the maximum [^{3}H]TdR incorporation by CTLL cells was ~3 ng/ml in this preparation of rIL-2 (Fig. 2). Considering some variability in different preparations and the lack of standardized methods for determination of IL-2 activity and protein content,

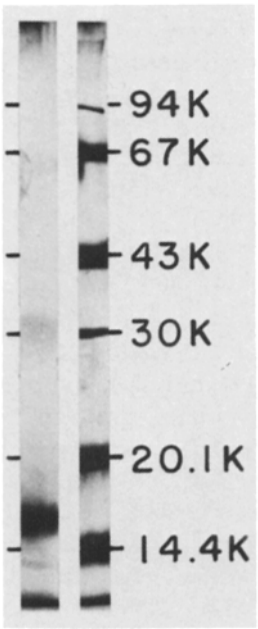

FIGURE 1. Electrophoresis ot purified rIL-2. 1 μg rIL-2 was run on an SDS–10% polyacrylamide slab gel under reducing conditions (*left*). Molecular weight markers were run on an adjacent lane in the same slab gel (*right*). Protein bands were detected using silver staining.

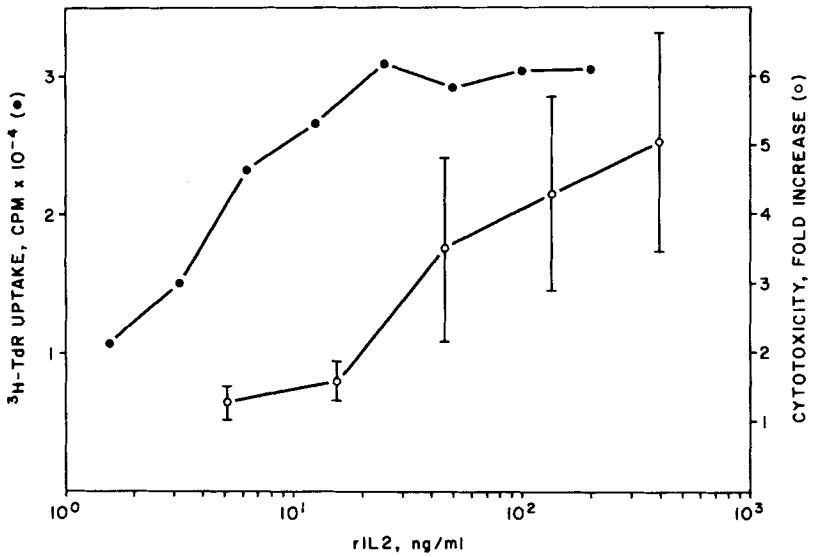

FIGURE 2. Growth-inducing and cytotoxicity-enhancing ability of rIL-2. rIL-2 at various concentrations was tested for its ability to induce [^{3}H]TdR uptake in murine CTLL cells (48 h culture, 5 × 10^4 cells/ml) (●) and to enhance the spontaneous cytotoxicity of human PBL (pretreated for 18 h at 37°C at 5 × 10^6 cells/ml) against RDMC target cells in a 3-h ^{51}Cr-release assay. The increase of cytotoxicity is expressed as the ratio between LU of the PBL cultured in the presence or absence of rIL-2; the open circles and the bars represent mean ± standard error of the results obtained with four different donors.

the specific activity of rIL-2 is comparable to that reported for natural IL-2.

Enhancement of Spontaneous Cytotoxic Activity of Human PBL. A dose-dependent enhancement of spontaneous cytotoxicity was observed in PBL cultured for 18 h in the presence of rIL-2, at concentrations >20 ng/ml (Fig. 2). The effect was demonstrable, at the same concentrations of rIL-2, with target cells that are either very sensitive (Fig. 3*a*) or relatively resistant (Fig. 3*b*) to NK cell–mediated lysis. Optimal concentrations of IFN-α (500 U/ml) and rIL-2 (0.2 μg/ml) induced a similar pattern of enhancement of PBL-mediated cytotoxicity against 10 human cell lines of different histological origin, although cytotoxicity was consistently enhanced to higher levels with rIL-2 (Table I). Both IFN-α and rIL-2 enhanced the cytotoxicity of PBL against target cell lines susceptible to spontaneous cytotoxicity and also induced high levels of cytotoxicity against relatively resistant target cells that were not significantly lysed by PBL from most donors in a 3-h cytotoxic assay (Table I). The enhancement of PBL cytotoxicity observed with effective doses of rIL-2 was significant in assays performed after a 2–5-h preincubation in the presence of rIL-2 and reached a maximum after a 12–18-h preincubation (Fig. 4, *top*).

Phenotypic Characterization of the IL-2-induced Cytotoxic PBL. To identify the surface phenotype of the cytotoxic cells induced by rIL-2, PBL were treated with various antilymphocyte subset monoclonal antibodies and C either before or after culturing in the presence of rIL-2 and the level of cytotoxicity mediated by the PBL was measured. Fig. 5 shows the results of a representative experiment. The anti-HLA-DR antibody B33.1 and the anti–pan T cells B36.1 did not

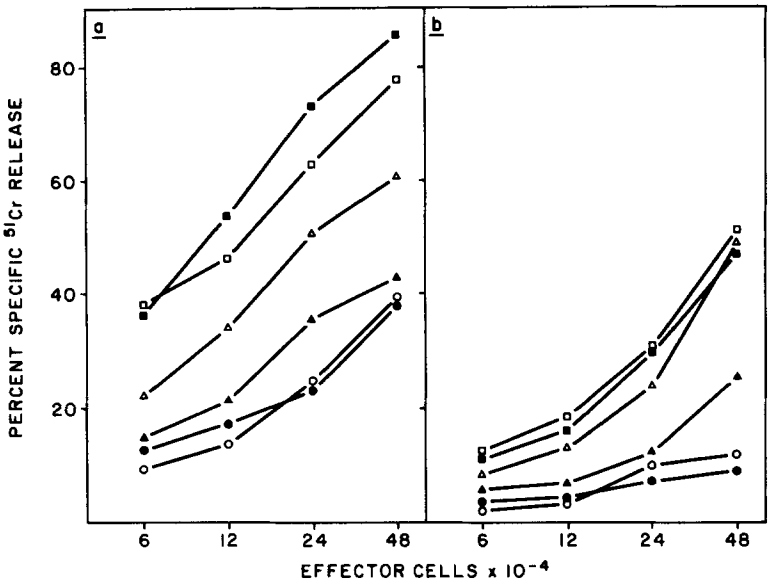

FIGURE 3. rIL-2-induced enhancement of spontaneous cytotoxicity by human PBL. PBL were incubated (18 h at 37°C, 5 × 10^6 cells/ml) in culture medium, in the presence or absence of different concentrations of rIL-2, washed, and tested as effector cells against 10^4 K562 (*a*) or RDMC (*b*) target cells, in a 3-h ^{51}Cr-release cytotoxicity assay. Pretreatment of PBL was: none (●); rIL-2 at (○) 5 ng/ml, (▲) 15 ng/ml, (△) 44 ng/ml, (■) 133 ng/ml, (□) 400 ng/ml.

significantly affect cytotoxicity. Both spontaneous cytotoxicity and cytotoxicity enhanced by rIL-2, either before or after CdL, were partially inhibited and almost completely abrogated after treatment with the anti-ER antibody B67.1 and with the anti-FcR antibody VEP13, respectively. Antibody HNK-1 and the anti-C3biR antibody OKM1 inhibited to some extent the cytotoxicity of PBL from different donors: the extent by which the two antibodies inhibited spontaneous cytotoxicity approximated that by which they inhibited rIL-2-induced cytotoxicity of PBL from the same donor (experiments not shown). To confirm the results obtained by depletion of cell subsets with antibody and C, PBL were positively and negatively selected after sensitization with the anti-FcR antibody B73.1 and the anti–pan T cell antibody B36.1, cultured for 18 h in the presence or absence of rIL-2, and assayed for cytotoxicity. As shown in Fig. 6, which depicts the results of one representative experiment out of six performed, both spontaneous cytotoxicity of PBL and cytotoxicity induced by culture with three different concentrations of rIL-2 were almost completely restricted to B73.1$^+$ or B36.1$^-$ cells. Thus, cytotoxic PBL induced by rIL-2 in a short-term (18 h) culture have a surface phenotype corresponding to that of spontaneous NK cells; furthermore, the cytotoxic cells induced derive from mature cells.

Effect of rIL-2 on the Expression of Lymphocyte Surface Markers in Short-term (18 h) Cultures of PBL. A panel of antilymphocyte monoclonal antibodies was tested by indirect immunofluorescence on PBL cultured in the presence of rIL-2 at doses optimal for enhancing cytotoxicity. The proportion of positive cells and the antigen density (fluorescence intensity) were determined by flow cytofluo-

TABLE I

Effect of Treatment with IFN-α or rIL-2 on the Spontaneous Cytotoxicity of PBL Against Various Human Cell Lines

Target cells	Origin	Treatment of PBL*		
		None	IFN-α	rIL-2
K562	Chronic myeloid leukemia	32.7‡	73.1	120.2
Daudi	Burkitt lymphoma	13.3	36.4	77.8
Raji	Burkitt lymphoma	<2	3.6	11.0
Molt 4	T lymphoma	4.4	8.2	20.7
Jurkat	T lymphoma	13.9	74.8	169.5
HL-60	Acute myeloid leukemia	<2	2.4	9.3
U937	Acute monocytic leukemia	78.1	147.8	262.1
HF-28	Skin fibroblasts	<2	3.1	6.5
RDMC	Rhabdomyosarcoma	<2	23.2	51.4
SK-Mel 23	Melanoma	<2	4.1	12.1

* PBL were incubated for 18 h at 37°C in culture medium or in medium containing 500 U/ml IFN-α or 0.2 μg/ml rIL-2.

‡ Lytic units 45% per 10^7 PBL, in a 3-h ^{51}Cr-release assay.

rometric analysis. rIL-2 treatment did not affect the expression of HLA-DR, pan T antigens (Leu-4 and B36.1), ER, and markers of the helper and suppressor T cell subsets. Also, neither the intensity of fluorescence nor the proportion of reactive cells changed when monoclonal antibodies reacting with all or a proportion of NK cells, including OKT10, B73.1, HNK-1, and N901, were assayed. On the other hand, expression of C3biR, as detected by antibody OKM1, was consistently increased: the number of $OKM1^+$ cells was not changed (on PBL from five different donors, 17.0 ± 6.1% of the PBL cultured in medium and 16.7 ± 6.2% of the PBL cultured for 18 h in the presence of 400 ng/ml rIL-2 were $OKM1^+$, but the intensity of fluorescence on the positive cells of five out of five donors was increased. Using arbitrary units (channels) of fluorescence from 1 to 200, the fluorescence intensity of $OKM1^+$ PBL, cultured in medium, was 85.7 ± 14.2 and that of PBL cultured in rIL-2, 95.8 ± 10.3

Production of Immune IFN by PBL Cultured in the Presence of rIL-2. The supernatant fluid from PBL cultured in the presence of rIL-2 contained antiviral activity. This was induced by rIL-2 in a dose-dependent fashion and could be detected in the supernatant fluid of the PBL cultured in the presence of rIL-2 at 12 h of incubation or longer (Fig. 4, *bottom*). The IFN titer on Detroit 532 cells in experiments with PBL from a total of 23 donors (18 h incubation with 0.4 μg/ml rIL-2) was 34 ± 38 ($\bar{x}$ ± SD). The ability of lymphocyte subsets to produce IFN in response to rIL-2 was analyzed: $B73.1^+$ and $B36.1^-$ cells consistently produced high titers of IFN, whereas $B36.1^+$ T cells produced none (Fig. 7). $B73.1^-$ PBL from some donors were unable to produce IFN, whereas those from other donors produced some, although less than unseparated PBL. The antiviral activity produced by $B73.1^+$ and $B36.1^-$ cells was characterized using anti-IFN antibodies, and was found to be almost exclusively IFN-γ (Table II).

Effect of Anti-IFN-γ, Anti-IL-2, and Anti-Tac Antibodies on rIL-2-induced PBL

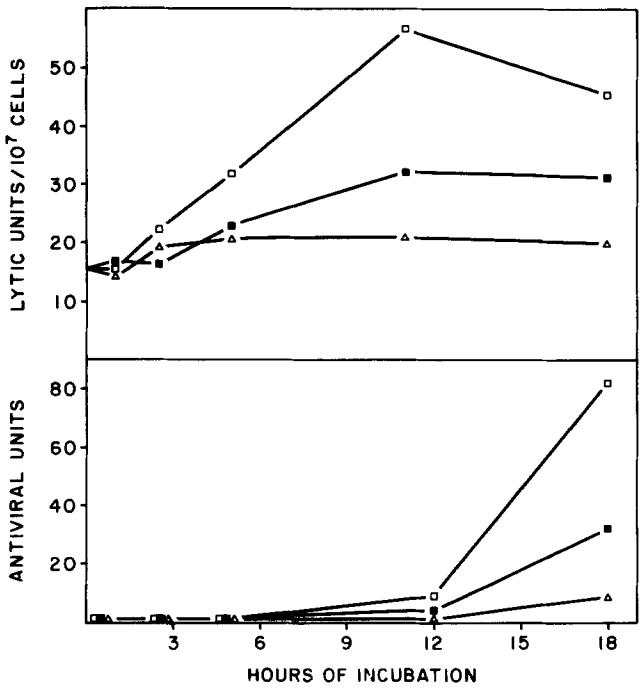

FIGURE 4. Kinetics of enhancement of spontaneous cytotoxicity and IFN production induced by rIL-2. PBL were cultured (37°C, 5×10^6 cells/ml) for different times in the presence or absence of rIL-2. Supernatant fluids were collected for IFN assay and PBL were washed and tested as effector cells against RDMC target cells in a 3-h ^{51}Cr-release cytotoxicity assay (*top*). The antiviral titer was assayed on Detroit 532 cells (*bottom*). rIL-2 concentrations in the culture were: (△) 44 ng/ml, (■) 133 ng/ml, (□) 400 ng/ml.

Cytotoxicity. The enhancement of PBL cytotoxicity and the IFN-γ production induced by rIL-2 were completely abrogated by rabbit anti-IL-2 antibodies (Fig. 8 and Table III). Three different anti-IFN-γ monoclonal antibodies, at concentrations able to completely neutralize the antiviral titer in the culture supernatant fluids and to prevent the enhancement of NK activity by doses of IFN-γ much higher than those produced in the rIL-2-containing PBL culture, were unable to prevent the enhancement of PBL cytotoxicity induced by rIL-2, although a modest decrease of the cytotoxic efficiency of the rIL-2-treated PBL was occasionally observed (Fig. 8 and Table III). The IL-2 receptor antibody anti-Tac (ascitic fluid dilutions, 1:200 and 1:600) was also unable to inhibit the rIL-2-mediated enhancement of PBL cytotoxicity (Table III). Spontaneous or IFN-γ-induced cytotoxicity was not significantly affected by either antibody concentrations used in these experiments (not shown).

Induction of PBL Proliferation by rIL-2. B73.1-positive and -negative PBL from three different donors were cultured for 6 d in the presence or absence of 20 ng/ml rIL-2, and the incorporation of [^{3}H]TdR in the last 6 h of the culture was evaluated (Table IV). The purity of the separated cells was confirmed by evaluating surface markers and by testing the NK cytotoxic activity of the two fractions (not shown). rIL-2 induced proliferation in both B73.1-positive and

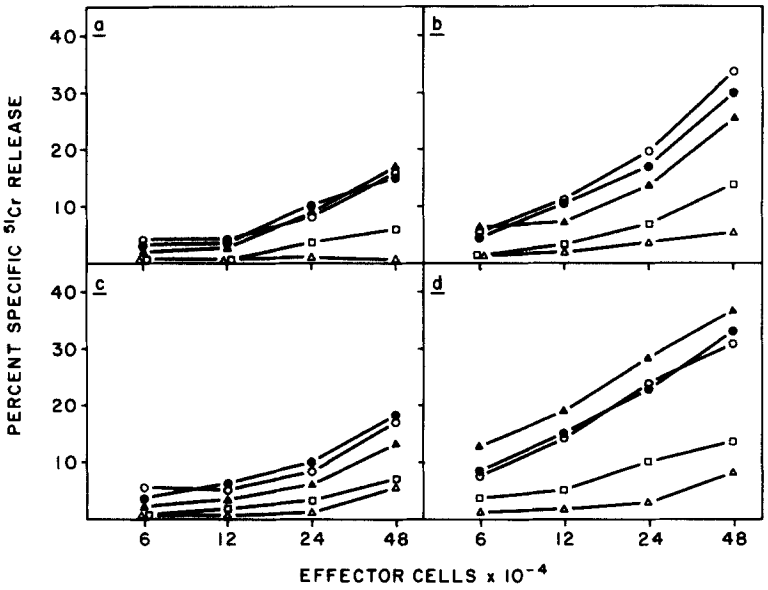

FIGURE 5. Surface markers on IL-2-induced effector cells, evaluated by treatment of a PBL preparation with monoclonal antibodies and C, before or after culture in the presence of rIL-2. The figure depicts the cytotoxicity (RDMC target cells, 3-h ^{51}Cr-release assay) of the following effector cell preparations: PBL first treated with antibodies and C and then cultured (18 h, 37°C) in the absence (*a*) or presence (*b*) of 133 ng/ml rIL-2; PBL cultured (18 h, 37°C) in the absence (*c*) or presence (*d*) of 133 ng/ml rIL-2 and then treated with antibodies and C. Antibodies used were: (●) none (C only), (○) B33.1 and C, (▲) B36.1 and C, (△) VEP13 and C, (□) B67.1 and C.

-negative PBL in two donors, but only in the B73.1$^+$ cells from a third donor. The rIL-2-induced proliferation was abolished if rabbit anti-IL-2 antibodies were present in the culture (Table IV). To determine whether this rIL-2-induced proliferation could play a role in the enhancement of cytotoxicity in short-term cultures, PBL from three other donors were cultured for 1 or 4 d in the presence and absence of rIL-2, and [^{3}H]TdR was added to the cultures during the last hour of incubation (Table V). The cytotoxic ability of the cells was measured against RDMC and K562 target cells; [^{3}H]TdR incorporation and expression of the NK antigen recognized by antibody B73.1, or of the T cell antigen, recognized by antibody Leu-4, were simultaneously detected using autoradiography and indirect rosetting. rIL-2 induced a strong enhancement of cytotoxicity, detectable on both target cells at days 1 and 4. An increase in the levels of cytotoxicity, especially in control PBL cultured in the absence of rIL-2, was apparent on day 4, although caution should be used in comparing values obtained in cytotoxicity experiments performed on different days. On day 1, no Leu-4$^+$ or B73.1$^+$ cells incorporating [^{3}H]TdR were detected, even though >1,000 cells per experimental condition were scored (Table V). On day 4, a small proportion of the PBL cultured with medium only, and a much larger proportion of those cultured in the presence of rIL-2, incorporated [^{3}H]TdR. [^{3}H]TdR-incorporating cells were detected in both the Leu-4$^+$ and the B73.1$^+$ subsets in all three donors, with donor to donor variation in the relative frequency.

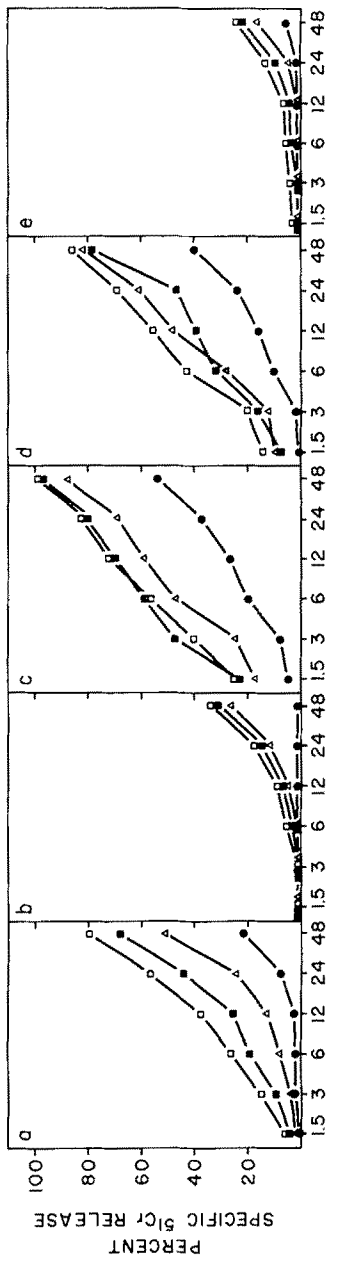

FIGURE 6. Effect of rIL-2 on the spontaneous cytotoxicity of PBL subsets positively and negatively selected. Unseparated PBL (*a*) and B73.1$^-$ (*b*), B73.1$^+$ (*c*), B36.1$^-$ (*d*), and B36.1$^+$ cells (*e*), obtained by separation on F/H gradient of cells after sensitization with the monoclonal antibodies and formation of indirect rosettes, were cultured for 18 h at 37°C (5×10^6 cells/ml) in the presence or absence of rIL-2, washed and tested as effector cells against RDMC target cells in a 3-h ^{51}Cr-release assay. PBL were incubated in medium without rIL-2 (●) or in the presence of: (△) 44 ng/ml, (■) 133 ng/ml, or (□) 400 ng/ml of rIL-2.

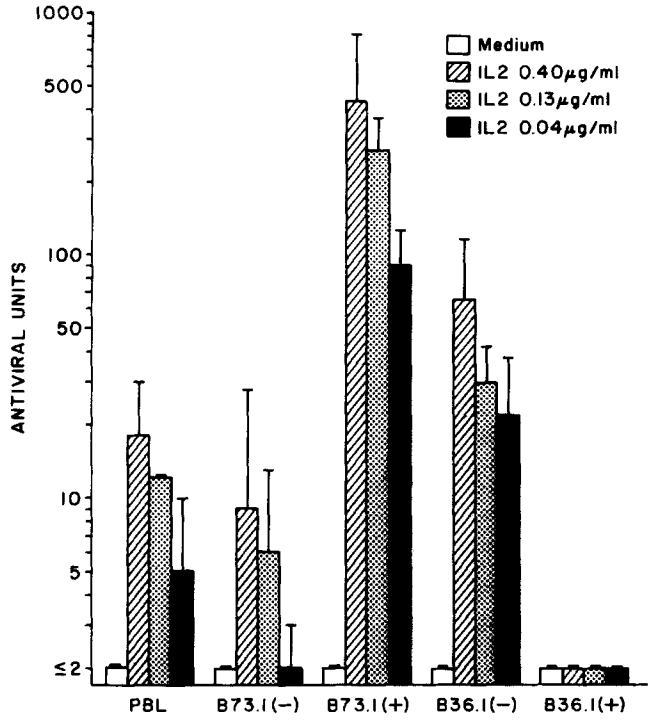

FIGURE 7. IFN production by different PBL subsets upon exposure to rIL-2. B37.1 (+) and (−) and B36.1 (+) and (−) cells were obtained by separation on an F/H gradient of cells after sensitization with the monoclonal antibodies and formation of indirect rosettes. PBL or PBL subsets were incubated 18 h at 37°C (5×10^6 cells/ml) in medium or in the presence of the indicated concentrations of rIL-2. IFN was measured in the culture supernatant fluid. Bars indicate the means of experiments with four different PBL donors; error bars are standard deviation.

TABLE II

Characterization of the Antiviral Activity Produced by Human Lymphocytes upon Induction with rIL-2

IFN preparation	IFN assay* in the presence of:			
	No Ab	Anti-IFN-α	Anti-IFN-γ	Anti-IFN-α + anti-IFN-γ
IFN-α	2187‡	<1	2187	ND§
IFN-γ	81	81	<1	ND
Donor 1. B73.1$^+$ cells + rIL-2‖	81	81	1	<1
Donor 2. B73.1$^+$ cells + rIL-2	243	243	1	<1
Donor 2. B36.1$^-$ cells + rIL-2	27	9	1	<1

* Samples were mixed 1:1 with medium, anti-IFN-α sheep antiserum (1:10 dilution), anti-IFN-γ monoclonal antibody X-100 (1:10 dilution), or with a mixture of the antibodies, and assayed by serial dilutions on Detroit 532 cells for antiviral activity.

‡ Reciprocal of the highest dilution protecting at least 50% of the fibroblasts monolayer from the cytopathic effect of VSV.

§ Not done.

‖ Cell-free supernatants from 18-h rIL-2-containing (133 ng/ml) cultures of the indicated cell types.

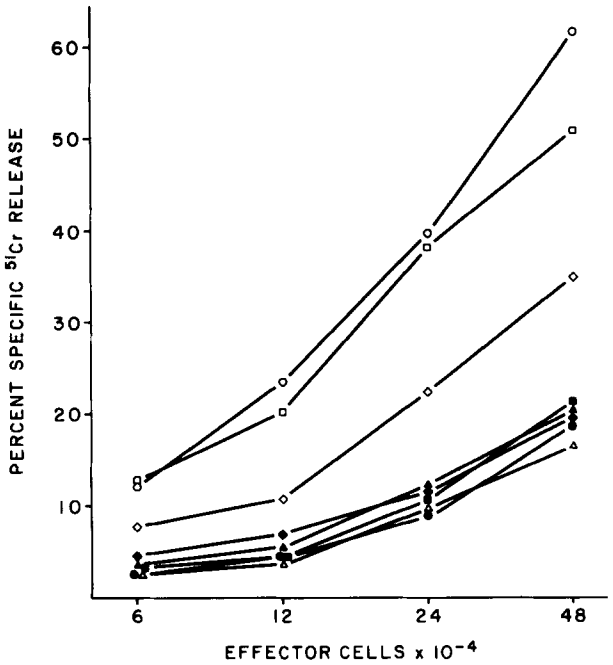

FIGURE 8. Effect of anti-IL-2 and anti-IFN-γ antibodies on the enhancement of PBL-mediated cytotoxicity induced by rIL-2 or IFN-γ. PBL were cultured (18 h at 37°C, 5×10^6 cells/ml) in the presence or absence of rIL-2 (133 ng/ml) or IFN-γ (1,000 U/ml), washed, and tested as effector cells against RDMC target cells in a 3-h ^{51}Cr-release cytotoxicity assay, in the presence or absence of the anti-IFN-γ monoclonal antibody B133.3 (culture supernatant fluid, 1:10) or rabbit anti-IL-2 serum. PBL cultured in medium alone were tested for cytotoxicity in the presence of: (●) medium alone, (■) antibody B133.3, (▲) anti-IL-2 antibody. PBL cultured in the presence of rIL-2 were tested for cytotoxicity in the presence of: (○) medium alone (□) antibody B133.3, (△) anti-IL-2 antibodies. PBL cultured in the presence of IFN-γ were tested for cytotoxicity in the presence of: (◇) medium alone, (◆) antibody B133.3

Discussion

The lineage of human NK cells is still open to question, insofar as these cells share phenotypic markers with both myeloid cells and lymphocytes, and reliable progenitor cell assays have not yet been established. Clones with characteristics of NK cells have been described that are dependent for their growth on IL-2 (36–40), although other factors or stimuli might be required for optimal growth (40, 41). Resting lymphocytes do not express a significant number of IL-2 receptors (200 per cell) (4), and reactivity with anti-Tac cannot be demonstrated with either peripheral blood T cells or NK cells (5). Growing NK cells, on the contrary, express the high-affinity receptor for IL-2 recognized by the monoclonal antibody anti-Tac.[2] To respond to IL-2, T cells require a stimulus, antigen or mitogen, able to induce the expression of the receptor for IL-2 (2, 42). Instead, a proportion of NK cells have been shown to initiate clonal proliferation in response to IL-2, and the T cell mitogen phytohemagglutinin does not increase the proportion of proliferating NK cells (38). Other studies (43) have shown that, among human peripheral blood cells reacting with antibody HNK-1, only

[2] London, L., B. Perussia, and G. Trinchieri. Induction of proliferation of resting human NK cells in vitro: expression of surface activation antigens. Manuscript in preparation.

TABLE III

Effect of Antibodies to IFN-γ, IL-2, or IL-2 Receptor on the Enhancement of PBL Cytotoxicity by rIL-2

Exp.	No. of donors	Inducers*	Antibody‡	Cytotoxicity§	IFN-γ‖
A	7	None	B133.1	0.98 ± 0.25¶	<1**
		None	B133.3	0.91 ± 0.23	<1
		rIL-2	None	3.23 ± 1.89	249 ± 337
		rIL-2	B133.1	2.87 ± 1.14	6 ± 13
		rIL-2	B133.3	2.53 ± 0.95	<1
		IFN-γ	None	1.78 ± 0.44	ND‡‡
		IFN-γ	B133.1	0.76 ± 0.33	ND
		IFN-γ	B133.3	0.89 ± 0.25	ND
B	3	None	X-100	1.08 ± 0.29	<1
		rIL-2	None	3.80 ± 1.01	96 ± 32
		rIL-2	X-100	4.83 ± 3.02	<1
C	3	None	Anti-Tac, 1:200	1.04 ± 0.08	ND
		None	Anti-Tac, 1:100	1.07 ± 0.04	ND
		rIL-2	None	3.47 ± 1.17	ND
		rIL-2	Anti-Tac, 1:200	3.12 ± 0.39	ND
		rIL-2	Anti-Tac, 1:600	2.88 ± 0.64	ND
D	3	None	Anti IL-2	0.96 ± 0.26	<1
		rIL-2	None	4.41 ± 2.33	27 ± 0
		rIL-2	Anti IL-2	1.02 ± 0.21	<1

* PBL were cultured for 18 h (37°C, 5 × 10^6 cells/ml) in medium with or without rIL-2 (133 ng/ml) or IFN-γ (500 U/ml).

‡ Antibodies were added to the PBL cultures at the following concentrations: B133.1 and B133.3, 20% supernatant fluid; X-100, 1,000 neutralizing units/ml; anti-Tac, ascites fluid, 1:200 or 1:600; rabbit anti-IL-2 serum, 1:5.

§ After culture, PBL were washed and tested as effector cells in a 3-h ^{51}Cr-release assay against RDMC.

‖ IFN titer was assayed on Detroit 532 cells in the supernatant fluid of the PBL cultures.

¶ Numbers represent $\bar{x} \pm$ SD of the ratio between LU of PBL cultured under the indicated experimental conditions and LU of PBL cultured in medium without inducers or antibodies.

** Antiviral units ($\bar{x} \pm$ SD).

‡‡ Not done.

those with the mature T cell phenotype, and not the mature NK cell phenotype, are induced to express IL-2 receptors (as detected by anti-Tac) upon treatment with mitogens. Although these data have been interpreted on the assumption that expression of the IL-2 receptor is induced on putative NK cell precursors, they also show that T cell mitogens are unable to induce IL-2 receptors on mature NK cells. Other soluble factors, such as IFN (44) and cellular stimuli (45), reportedly play some role in conferring on NK cells the ability to respond to IL-2. There are other reports (7–11, 16, 17, 46, 47) indicating that IL-2 regulates or induces NK cell growth, and still others showing that IL-2 can induce enhancement of cytotoxic activity of NK cells or of cells with NK-like activity. This effect was attributed, in short-term cultures (<1 d), to a rapid induction of IFN-γ in lymphocyte cultures in the presence of IL-2 (8), or, in long-term cultures (3–6 d), to an IL-2-induced growth of NK cells (11). Several

TABLE IV

rIL-2-induced [^{3}H]TdR Incorporation in PBL Preparations Enriched for or Depleted of NK Cells

Donor	Lympho-cytes	[^{3}H]TdR incorporation*		
		Medium‡	rIL-2§	rIL-2 + anti-IL-2‖
1	B73.1$^+$	76	14,839	35
	B73.1$^-$	2,082	29,816	897
2	B73.1$^+$	86	11,360	62
	B73.1$^-$	9,307	20,041	8,466
3	B73.1$^+$	332	11,336	54
	B73.1$^-$	319	76	159

* PBL from three donors were separated in B73.1 (+ and −) cells by the indirect rosetting method and cultured for 6 d (2.5×10^5 cells/ml) in flat-bottom microtiter plates in RPMI 1640 medium together with 10% human serum. In the last 6 h of culture, [^{3}H]TdR (1 μCi/well) was added. Cells were collected on glass fiber filters and radioactivity was measured by liquid scintillation.

‡ Lymphocytes cultured in medium only.

§ Lymphocytes cultured in the presence of rIL-2 (20 ng/ml).

‖ Lymphocytes cultured in the presence of rIL-2 and rabbit anti-IL-2 antibodies.

TABLE V

Cytotoxicity and [^{3}H]TdR Uptake in PBL Cultured in the Presence of rIL-2

Donor	Culture condition	Day 1*				Day 4			
		Cytotoxicity‡		[^{3}H]TdR uptake§		Cytotoxicity		[^{3}H]TdR uptake	
		K562	RDMC	Leu-4$^+$	B73.1$^+$	K562	RDMC	Leu-4$^+$	B73.1$^+$
1	Medium	6‖	3	0.0¶	0.0	39	14	1.2	0.0
	rIL-2	61	34	0.0	0.0	61	56	6.4	5.5
2	Medium	8	3	0.0	0.0	22	23	0.6	0.5
	rIL-2	19	8	0.0	0.0	52	56	3.2	10.8
3	Medium	10	2	0.0	0.0	22	25	0.4	0.9
	rIL-2	57	23	0.0	0.0	89	90	2.1	3.1

* PBL from three donors were cultured for 1 and 4 d (37°C, 2×10^6 cells/ml) in RPMI 1640, 10% FBS medium with or without 133 ng/ml rIL-2.

‡ The cytotoxic activity of cultured PBL was assayed in a 3-h ^{51}Cr-release assay using K562 or RDMC target cells.

§ The number of Leu-4$^+$ and B73.1$^+$ cells incorporating [^{3}H]TdR was detected by simultaneous use of autoradiography and indirect rosetting techniques.

‖ LU/10^7 cells.

¶ Percent cells incorporating [^{3}H]TdR.

of these investigations, however, were hampered by either the use of incompletely purified IL-2 or by an inadequate identification of the effector cells as NK cells. Although natural IL-2 purified to apparent homogeneity was used in some studies, in other studies contamination with small amounts of potent activators of NK cells such as IFN or phorbol esters, was difficult to exclude. The use of homogeneous rIL-2 almost rules out the possibility of contaminant factors. Much more serious concern is raised about the criteria used to identify NK cells. Cytotoxicity against NK-sensitive target cells is not sufficient to identify NK cells, because other cell types, e.g., activated T cells, may share this property (48, 49).

In most other studies, the identification of NK cells has been based on separation of cells on Percoll discontinuous gradients and on their reactivity with antibody HNK-1 (11). The use of Percoll gradients (38) has greatly helped in identifying NK cells as large granular lymphocytes (LGL) and in obtaining NK cell–enriched preparations; however, it is now clear that purification of light density cells cannot be used for positive identification of NK cells. A clearcut example is given by the identification of the cells producing IFN-α as NK cells on the basis of results of Percoll separation (50), whereas a more detailed analysis of the surface markers of those cells established that they belong to a cell type copurifying with NK cells on Percoll gradient, but devoid of cytotoxic activity and separable from NK cells by use of monoclonal antibodies (51). In addition to the contamination with IFN-α-producing cells, the low-density Percoll fractions are also likely to contain a small number of activated T cells or immature lymphoblasts that are able to respond to IL-2. The use of the HNK-1 antibody is also not discriminating because recent studies have clearly established that this antibody (*a*) reacts only with a variable proportion of cytotoxic NK cells (from 20 to 60%) (26, 28), (*b*) does not react with presumably immature NK cells in human cord blood (22, 52), and (*c*) reacts with a significant proportion of cells with the surface phenotype of mature T cells, either OKT8- or OKT4-positive (53 and unpublished data), suggesting that the antigen detected may be present at some stage of differentiation and/or activation of T cells and of other hematopoietic and nonhematopoietic cell types. The use of monoclonal antibodies to the low-affinity FcR of NK cells and neutrophils (such as B73.1 and VEP13) appears at present to be the most specific way to identify the NK cell subset within the peripheral blood mononuclear cells because (*a*) B73.1 (and the other antibodies) recognizes a lymphocyte subset that does not express any T cell markers except the ER (on 80–90% of cells) and OKT8 antigen (at low density on 30–50% of the cells), which have been shown to be present on an equivalent proportion of cytotoxic NK cells (22, 23, 25, 26, 54); (*b*) virtually all NK activity is contained in the B73.1$^+$ subset (22); (*c*) >80% of the IFN-treated B73.1$^+$ cells on average, and 100% in some experiments, bind to K562 target cells (22); and (*d*) in single-cell cytotoxicity assays in agarose, 60–70% of the B73.1$^+$ cells that are bound to K562 cells lysed the target cell during a 3-h incubation (55).

In the present study, we analyzed the ability of pure recombinant IL-2 to enhance the activity of human peripheral blood NK cells in a short-term (18 h) culture and compared the ability of rIL-2 to induce proliferation of NK cells in those short-term cultures with that observed in longer term (4–6 d) cultures. The rIL-2 was a very potent activator of NK cell activity, both against target cells susceptible to fresh NK cells or relatively resistant to their cytotoxic effect. At optimal concentrations, rIL-2 was as effective or more effective than IFN-α in enhancing NK activity, but no significant difference in the relative efficiency of IL-2-activated (18-h culture) vs. IFN-activated NK cells against various target cells was observed. The kinetics of IL-2-mediated enhancement was rapid and similar to that observed with IFN. Enhancement of NK activity preceded any proliferation induced by IL-2 in the resting NK cells. The lymphocyte subset responsible for the enhancement of cytotoxic activity was clearly identified as that containing spontaneous and IFN-inducible NK activity, using monoclonal

antibodies and C in negative selection procedures and also in negative and positive selection procedures using indirect rosetting. The facts that (*a*) identical results were obtained using PBL treated with antibody and C either before or after IL-2 treatment, and (*b*) 50% target cell lysis is observed at an effector-to-target cell ratio of 3:1 or less, after IL-2 treatment of $B73.1^+$ cells, argue against the possibility that the observed enhancement of cytotoxicity is due to a small proportion of immature NK cells induced to differentiate and/or proliferate. The possibility that these results could depend on the presence of a minor cytotoxic population with very high recycling ability seems unlikely, due to the limited recycling ability (56) and rapid exhaustion of both resting and activated NK cells (57). The observation that IL-2 treatment induces differentiation of $OKM1^-$ to $OKM1^+$ NK cells (10) was not supported in the present study. Although the OKM1 antigen (C3bi receptor) was the only surface antigen of T or NK cells whose expression was significantly enhanced by rIL-2 treatment, no difference in the number of $OKM1^+$ cells before or after IL-2 treatment was observed in our study. It should be pointed out that the expression of the OKM1 antigen on NK cells is extremely heterogeneous, with some cells expressing it at very low density. If less sensitive conditions in the immunofluorescence technique and cytofluorometric analysis are used, and if a threshold for distinction between positive and negative cells is set that fails to detect low-intensity $OKM1^+$ cells, then an apparent increase in the number of $OKM1^+$ cells, actually reflecting the increase in OKM1 antigen density induced by IL-2, could be easily misinterpreted as the induction of differentiation of $OKM1^-$ into $OKM1^+$ cells. Our results, therefore, show that, in short-term cultures, rIL-2 induces an enhancement of cytotoxic activity in a cell subset with phenotypic characteristics of mature NK cells, although no information is yet available at the single cell level about whether IL-2 potentiates the activity of already cytotoxic cells, increases the proportion of mature NK cells with cytotoxic ability, or, like IFN, does both.

The concentration of rIL-2 required to enhance NK cytotoxicity is ~10-fold that required for half-optimal proliferation of CTLL cells in a standardized assay. However, the cell concentration in the proliferation assay (5×10^4 cells/ml) is 100-fold lower than that of PBL (5×10^6/ml) used in the present experiments. The concentration of rIL-2 required for proliferation has been shown to quantitatively correlate with the density of the responder cells (2). In the PBL culture, we are unable to determine the relative density of rIL-2-responder cells vs. bystander cells. However, if one assumes that all or most of NK cells (on average, 15% of PBL) are potential IL-2 responders, the quantity of IL-2 required for CTLL proliferation (and for proliferation of human T cells, data not shown) is very close or identical to that required for enhancement of NK cell activity. Considering that the specific activity of rIL-2 is comparable to that of natural IL-2, it is possible to envision an enhancing effect of IL-2 on NK cell cytotoxicity in physiological conditions. Human lymphocytes stimulated with mitogens pro duce 3–8 ng of IL-2 per milliliter per 10^6 cells. These concentrations of IL-2 are sufficient to enhance NK cell activity, even if not to optimal levels. It is conceivable that higher local concentrations of IL-2 can be reached in vivo and that other factors such as IFN act synergistically with IL-2 to lower the threshold of NK cell responsiveness. On the basis of the level of IL-2 able to enhance NK

activity in vitro, therefore, a physiological role for IL-2 in regulating the cytotoxic activity of mature NK cells seems a reasonable postulate.

Treatment of human PBL with rIL-2 induces a dose-dependent production of IFN-γ. Both human and murine T (58) and NK cell clones (11) have been shown to produce IFN-γ in response to antigens or IL-2. Several authors reported that IL-2 acts synergistically with other stimuli to induce IFN-γ (59), and some studies have demonstrated that IL-2 alone can induce IFN-γ production (13, 60). Production of IFN-γ in 3-d cultures of human PBL in the presence of IL-2 has been attributed to T cells or Percoll-separated, low-density lymphocytes. IFN-γ has been shown to be produced by NK-like cells, with the participation of macrophages, in short-term (24 h) cultures of mouse spleen cells (60). In this paper, we show that the major or only producers of IFN-γ in 18-h cultures in the presence of rIL-2 are $B73.1^+$, $B36.1^-$ NK cells. In a series of four cell separation experiments, unseparated PBL produced, on average, <20 U/ml of IFN-γ, whereas $B73.1^+$ cells produced >400 U/ml. This discrepancy with a report (13) showing production by T cells is not clear, although, as discussed below, in the 3-d incubation period used in that study, proliferation of IFN-γ-producing T cells might have occurred. Although in our hands, IFN-γ is not as potent as IFN-α in activating NK cells, the possibility exists that the enhancement of NK activity induced by IL-2 is mediated by the IFN-γ produced. Weigent et al. (8) showed that antisera to IFN-γ could completely block IL-2-induced enhancement of NK activity. In our experiments and in those of Weigent et al. (8), production of IFN-γ preceded or paralleled the increase in NK activity, a result difficult to interpret, as the secreted IFN-γ should act secondarily on NK cells to enhance their activity. IFN-γ was reported to enhance NK activity with a kinetics slower than that observed with IFN-α (61). Moreover, three different monoclonal antibodies to IFN-γ, at concentrations able to completely neutralize the antiviral activity produced in the supernatant fluid of the IL-2-containing PBL culture and to block the enhancement of NK activity induced by exogenous IFN-γ, failed to prevent the enhancement of NK activity by rIL-2. These data suggest that IFN-γ does not play a major direct role in the IL-2-induced enhancement of NK activity. The discrepancy between our results and those reported by Weigent et al. (8) might rest in the more restricted specificity of the monoclonal antibodies in comparison with the polyclonal antisera. However, it is also possible that concentrations of IFN-γ higher than those that can be immediately inactivated by the antibodies are reached within the IFN-producing cells and NK cells in the cell pellet. Moreover, a direct cell-to-cell transfer of IFN-γ-induced NK-enhancing activity, not inhibitable by anti-IFN antibodies, could be responsible for the enhancement of NK cell activity, in a way analogous to that demonstrated for cell-to-cell transfer of antiviral activity (62).

The enhancement of NK cell activity observed in our experiments precedes any proliferation induced by rIL-2, as shown in autoradiography by the absence of any NK or T cells incorporating [^{3}H]TdR at the end of the 18-h incubation. However, if the culture of $B73.1^+$ NK cells and $B73.1^-$ cells in the presence of irradiated monocytes is continued for 4–6 d, proliferation, as evaluated by [^{3}H]-TdR incorporation, is observed in both cell subsets. These results, showing proliferation of both NK and of $B73.1^-$ cells, devoid of NK activity, contrast

with a recent preliminary report by Shiiba et al. (11) that attributes the proliferation induced by IL-2 in fresh PBL entirely to NK cells, based on the copurification of proliferating cells in low-density Percoll gradient fractions and on the partial blocking of proliferation by treatment of the cells with antibody HNK-1 and C. To determine whether cells with both NK ($B73.1^+$) and T cell phenotype ($Leu\text{-}4^+$) were induced to proliferate by rIL-2, we analyzed the phenotype of the cells incorporating [^{3}H]TdR after a 4-d culture in the presence of rIL-2. We have previously[2] shown that antibodies B73.1 and Leu-4 recognize two separate nonoverlapping subsets of lymphocytes both in fresh (22) and in proliferating PBL. rIL-2 induced a significant increase of [^{3}H]TdR-incorporating cells after 4 d of cultures. [^{3}H]TdR-incorporating lymphocytes could be identified both in $B73.1^+$ and $Leu\text{-}4^+$ cell subsets. These results show that rIL-2 can directly induce a proportion of NK cells to proliferate, but can also induce cells expressing specific T cell markers. The results reported by Shiiba et al. (11) could be possibly due to the presence of cells other than NK in the light-density cell fraction, e.g., immature or activated T cells, or to the absence of accessory cells, such as monocytes, in the unresponsive high-density cell fractions. Grimm et al. (16) have shown that the LAK cells, generated in 3–5-d cultures by pure IL-2 (17) or by rIL-2 (19), express the Leu-4 antigen, proliferate in culture, but are derived from $Leu\text{-}4^-$ immature T precursors that are distinct from NK cells, but copurify with them on a Percoll gradient. A portion of the $Leu\text{-}4^+$ cells incorporating [^{3}H]TdR observed in our 4-d culture could possibly be LAK cells.

Unlike activated proliferating T or NK cells, resting PBL reveal only a very limited number of high-affinity IL-2 receptors as demonstrated either by IL-2 binding or by reactivity with antibody anti-Tac. The question emerges of which type of surface IL-2 receptor allows resting mature peripheral blood NK cells to respond rapidly and efficiently to IL-2 with enhanced cytotoxic activity and IFN production. In the present study, anti-Tac was unable to reduce the IL-2-mediated enhancement of NK activity in short-term cultures. Even much higher dilutions of anti-Tac have been reported to block generation of LAK cells in longer cultures as well as IL-2-dependent human T cell proliferation (17). However, a role for the IL-2 receptor recognized by anti-Tac in NK cell enhancement cannot be excluded. The binding affinity of IL-2 to its receptor (5, 6) is much higher than that of an antibody, and, especially when relatively high IL-2 concentrations and cell densities are used, as in our experiments, anti-Tac may fail to compete for IL-2 binding, even when the recognized receptor is responsible for the binding. We are currently analyzing the binding of rIL-2 to highly purified preparations of resting NK cells in an effort to define the number of receptors on NK cells, their binding affinity, and their biochemical and antigenic relationship with the Tac antigens.

Summary

The present study shows that recombinant interleukin 2 (IL-2) purified to homogeneity induces a rapid and potent enhancement of spontaneous cytotoxicity of human peripheral blood lymphocytes. The cells mediating cytotoxicity after 18-h treatment with IL-2 have surface markers of natural killer (NK) cells and are generated from the peripheral blood subset containing spontaneous

cytotoxic cells. A parallel production of gamma interferon (IFN-γ) is induced by recombinant IL-2 (rIL-2), and NK cells appear to be the major producer cells, whereas T cells are unable to produce IFN-γ under these experimental conditions. However, the kinetics of the enhancement of cytotoxicity are faster than those of IFN-γ production, and monoclonal anti-IFN-γ antibodies do not suppress this effect, making it unlikely that the IFN-γ produced is responsible for the enhancement. The enhancement of NK cell activity induced by rIL-2 precedes any proliferative response of the lymphocytes, which is instead observed in longer-term cultures of both NK and T cells.

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