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Stimulation of Proliferative Activity of Human Natural Killers ($CD16^+CD56^+$ Cells) by Recombinant Interleukin-3 *In Vitro*

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The proliferative activity of human natural killers ($CD16^+CD56^+$ cells) in the presence of 100 and 1000 IU/ml human recombinant interleukin-3 is investigated *in vitro*. It is shown that recombinant interleukin-3 reliably enhances natural killer proliferation, causing a 9-15.2-fold increase of ^{3}H-thymidine uptake by $CD16^+CD56^+$ cells both in complete culture medium and in conditioned medium. The effect of the factor is 3.9-6.4 and 3.6-8.9-fold more potent than that of recombinant interleukin-2 and granulocyte-macrophage colony-stimulating factor, respectively, in the same doses.

Key words: *natural killers; interleukin-3; proliferation*

Interleukin-2 (IL-2) is regarded as a factor of differentiation for natural killers from bone marrow and peripheral precursors and as a growth factor of mature cells [2,11]. IL-2 can induce the maturation of undifferentiated cell forms into cytotoxic natural killers *in vitro* and the proliferation of human large granular lymphocytes with an increase of the number of $HNK\text{-}1^+$ cells in the culture. However, not all natural killer precursors in the mononuclear cell fraction (MNC) are able to respond to IL-2 action [4], the most potent cells in this case being $T3^-$, Leu 7^-, and $Fc\gamma R^+(^-)$ [4,6]. There are two different populations of precursors of natural killers derived from mouse bone marrow: precursors which can be transformed into cytotoxic cells in the presence of IL-2, and non-transformed cells [12].

Although the fraction enriched with large granular lymphocytes (more than 90%), expresses the maximal proliferative response to IL-2 in an MNC culture [13], the presence of IL-2 alone is not sufficient for latent natural killers to undergo optimal proliferation *in vitro* [15]. It is thought that IL-2 can only support a given proliferation level [15], while other costimulative cytokines are needed for proliferation increase [14]. One such costimulator may be colony-stimulating factor-1 (CSF-1) [5].

The aim of the present study was to investigate the proliferative activity of natural killers ($CD16^+CD56^+$ cells) in the presence of IL-3, which is known as an inhibitor of cellular cytotoxic ac-

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tivity [1,8], which can probably enhance nonspecific lymphocyte proliferation. Proliferation in control cultures was induced using IL-2 and granulocyte-macrophage (GM) CSF.

MATERIALS AND METHODS

MNC were isolated from peripheral blood samples (200 ml), obtained from 2 healthy donors, by centrifugation at 2000 rpm, 20 min at 20°C on a one-step Ficoll-Paque gradient (Pharmacia Fine Chemicals) in a Beckman GP centrifuge. Cells were collected from the interphase ring and washed twice in phosphate buffer solution for 10 min at 20°C using 2000 rpm and 1200 rpm centrifugation.

MNC were cultured in plastic Petri dishes (Costar) for 1 h at 37°C in a Heraeus CO_2 incubator in 10 ml of RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 5 mM glutamine, 100 U/ml penicillin, 1 μg/ml streptomycin, and 2 ml of iron-containing agent (Technicon) for the removal of adhesive and phagocytic cells. B cells and some monocytes were removed on nylon padded columns during 45 min at 37°C in a Heraeus CO_2 incubator. MNC culture enrichment with light fraction cells was carried out on a three-step percoll density gradient (Pharmacia Fine Chemicals), for which 60.6, 45, and 30.3% percoll was prepared. Two ml of MNC suspension was layered onto 9 ml of percoll and was centrifuged in the "off" mode during 20 min at 2500 rpm, 20°C in a Beckman GP centrifuge. MNC enriched with large granular lymphocytes were collected from the 30.3/45% percoll interphase. T lymphocytes were removed using immunomagnetic separation with reagent containing magnetic microbeads M450 Pan T (CD2) (Dynal), covered with anti-CD2 antibodies. The procedure was carried out during 1 h in the cold with continuous stirring. Cells that bound with beads were removed using an MPC-1 magnetic concentrator (Dynal) during 15 min in the cold.

Each step of cell isolation was accompaned by a visual cell count in a microchamber and with sample collection for estimation of the concentration of $CD16^+CD56^+$ lymphocytes, which are mature natural killers derived from a common precursor of natural killers and T lymphocytes [10]. Cells were stained using Simultest IMK Plus Reagents testkits (Becton Dickinson), containing fluorescent-labeled mouse monoclonal anti-Leu 4/11+19 (CD3/CD16+CD56). Three μl of antibodies were added to a sample of 5×10^5 MNC cells in 50 μl of complete incubation medium and incubated 45 min in the cold. After incubation, the cells were resuspended and washed twice for 5 min at 1500 rpm and 20°C. Estimation of the $CD16^+CD56^+$ cell concentration was carried out in a Facscan flow cytofluorimeter (Becton Dickinson) with a Helwett-Packard 9153 B computer system which can sum fluorescent signals of 5×10^3 living cells in each test.

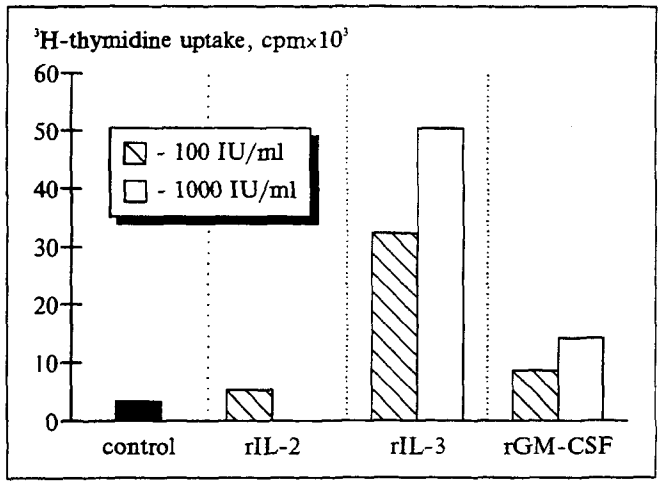

Fig. 1. Proliferation of human natural killers ($CD16^+CD56^+$ cells) in the presence of rIL−2, rIL−3, and rGM−CSF *in vitro*.

According to double flow cytofluorimetry data, the final MNC suspensions, obtained from donor 1 and donor 2 and enriched with natural killers, contained 69.8 and 75.68% of $CD16^+CD56^+$ cells (6.14×10^6 and 12.1×10^6 natural killers, respectively). Other cell populations represented: T lymphocytes 1.6 and 2.04%, B lymphocytes 13.08 and 10.58%, and monocytes 21.64 and 12.22%, respectively. Drops of natural killer suspensions in complete growth medium (0.1 ml) were introduced into 96-well flat-bottom microplates (Nunc), and 0.1 ml of medium (control) or investigated factor solution was added to each well. The cultures were incu-

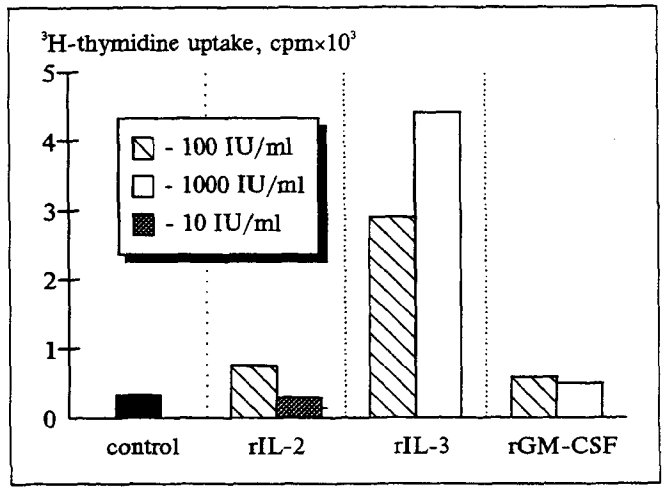

Fig. 2. Proliferation of human natural killers (10^6 $CD16^+CD56^+$ cells/ml) in the presence of rIL−2, rIL−3, and rGM−CSF in conditioned medium *in vitro*.

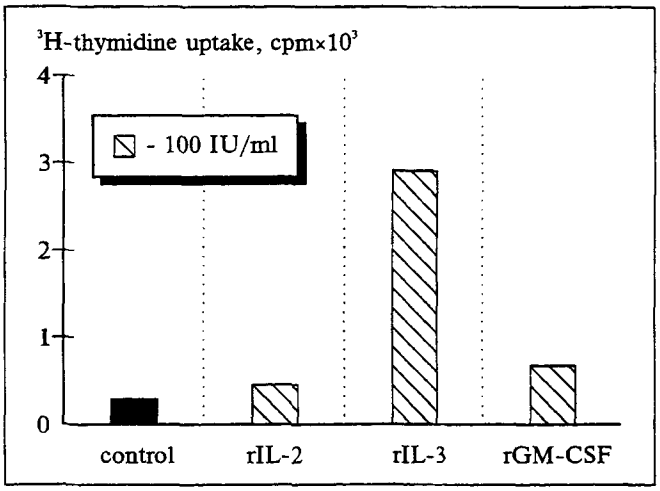

Fig. 3. Proliferation of human natural killers (7.5×10^5 $CD16^+CD56^+$ cells/ml) in the presence of rIL-2, rIL-3, and rGM-CSF in conditioned medium *in vitro*.

bated 72 h at 37°C in a CO_2 incubator (Heraeus). The final concentrations of natural killers per well were: 10^6/ml (test 1) and 10^6/ml or 7.5×10^5/ml (test 2). Preparations of human IL-2 (rIL-2), IL-3 (rIL-3), and GM-CSF (rGM-CSF), a gift of Dr. A. Emmendorffer (Institute of Toxicology of Fraunhofer's society, Hanover), were used in doses of 10, 100, and 1000 IU/ml. After incubation the culture medium was changed (test 1) or not discarded (test 2), and ^{3}H-thymidine in a dose of 1 μCi/ml was added to each well. The suspension was incubated 24 h in the previously described conditions. After culturing the well content was sedimented on fiberglass filters with 2.5 μ pores using a Scatron multichannel harvester. Residual radioactivity of the filters was estimated using a scintillation β-counter (Beckman LS-1801). The reliability of differences of the means was assessed with Student's *t* test.

RESULTS

In a dose of 100 IU/ml rIL-3 caused a 9.7-fold increase of human natural killer proliferative activity *in vitro* ($p<0.02$) (Fig. 1). ^{3}H-thymidine uptake by $CD16^+CD56^+$ was increased from 3322±217 to 32185±6715 cpm ($n=3$). The presence in the culture of 1000 IU/ml IL-3 caused a 15.2-fold proliferation increase ($p<0.01$), and ^{3}H-thymidine uptake increased to 50321±7564 cpm ($n=2$). In a dose of 100 IU/ml rIL-2 had no reliable effect on natural killer proliferation *in vitro*. The presence of rGM-CSF in doses of 100 and 1000 IU/ml in the culture medium caused an increase of ^{3}H-thymidine uptake by $CD16^+CD56^+$ cells to 8465±2147 cpm ($p<0.1$, $n=3$) and 14050±2769 cpm ($p<0.05$, $n=2$), respectively (Fig. 1). Thus, rIL-3 efficiency in the stimulation of natural killer proliferation is 6.2-fold higher then that of rIL-2 ($p<0.05$) and 3.6-3.8-fold higher than that of rGM-CSF ($p<0.1$).

In conditioned medium rIL-3 a in dose of 100 IU/ml caused a 9-fold increase of human natural killer proliferation activity *in vitro* when the concentration of $CD16^+CD56^+$ cells was 10^6/ml ($p<0.02$). ^{3}H-thymidine uptake was increased from 332±72 to 2989±559 cpm ($n=3$, Fig. 2). The presence of 1000 IU/ml IL-3 in the culture medium caused a 13-fold increase of proliferation ($p<0.001$), and ^{3}H-thymidine uptake increased to 4352±179 cpm ($n=3$). rGM-CSF in doses of 100 and 1000 IU/ml had no reliable effect on natural killer proliferation *in vitro*. IL-2 in doses of 10 and 100 IU/ml induced ^{3}H-thymidine uptake by $CD16^+CD56^+$ cells at levels 296±36 and 754±118 cpm ($p<0.1$, $n=3$), respectively (Fig. 2). Thus, in conditioned medium rIL-3 is 5.2-8.9-fold more active than rGM-CSF ($p<0.05$ and $p<0.001$) and 3.9-5.8-fold more active than rIL-2 ($p<0.05$ and $p<0.001$) in stimulating natural killer proliferation.

In conditioned medium at a $CD16^+CD56^+$ concentration of 7.5×10^5 cells/ml rIL-3 in a dose of 100 IU/ml caused a 10-fold increase of human natural killer proliferation ($p<0.001$), and ^{3}H-thymidine uptake was increased from 286±27 to 2893±64 cpm ($n=3$, Fig. 3). rIL-2 at 100 IU/ml had no reliable effect on $CD16^+CD56^+$ cell proliferation. rGM-CSF in a dose of 100 IU/ml increased ^{3}H-thymidine uptake to 666±88 cpm ($p<0.05$, $n=3$, Fig. 3). Thus, in this case rIL-3 is 4.3-fold more active than rGM-CSF ($p<0.001$) and 6.4-fold more active than rIL-2 ($p<0.01$) in stimulating natural killer proliferation.

It is reported that IL-3 is not active in the process of maturation of natural killers from mouse bone marrow cells [11] and is not involved in the positive regulation of cytotoxic effector differentiation. Nevertheless, IL-3 can act on cloned $CD3^-CD16^+$ natural killer cells [7] by blocking the killer activity of the cells and the appearance of cells with the natural killer phenotype in the culture [8]. The results of the present study suggest that the depressive action of IL-3 on natural killer maturation from bone marrow precursors [2,8] may be due to its ability to induce proliferation of natural killers or their precursors, which is associated with the transformation of cells into blast forms. As we can see in Figs. 1-3, the effect of the factor is much more pronounced than that of rIL-2, which is known as a stimulator of human and animal natural killer proliferation [2,3,11,13].

IL-3 inhibits natural killers but does not affect IL-2-induced cytotoxic activity of MNC [9]. IL-3

itself may be considered as factor of progression which can directly trigger natural killer proliferation not only in the presence of IL-2 [7], but independently as well. Comparison of the results presented in Figs. 2 and 3 shows that a decrease of the $CD16^+CD56^+$ cell concentration from 10^6 to 7.5×10^5/ml abolishes the effect of rIL-2. Under the same conditions rIL-3 action remains at the same level.

Taking into account data [14,15] concerning the inability of IL-2 to induce stable proliferation of natural killers (only a few precursors can respond to it [4,12]), our results indicate that IL-3 may be considered as a costimulative signal which is necessary for human natural killer proliferation [14]. It is logical to link this with its inhibiting efffect on human natural killer cytotoxicity [1,8] and on the differentiation of natural killers from bone marrow precursors [2,8,9].

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