

Interleukin 2 Enhances Natural Killer Cell Activity Through Induction of Gamma Interferon

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Highly purified interleukin 2 (IL 2), free of interferon activity, enhanced natural killer (NK) cell activity against tumor cells in mouse spleen cell cultures and in human peripheral lymphocyte cultures in a manner similar to that of interferon (IFN). We determined that IL 2 enhanced NK activity indirectly in a cascade manner by the induction of gamma IFN (IFN- γ) in the cultures, which actually mediated the enhanced killing. Accordingly, lymphocyte cultures treated with IL 2 alone produced 10 to 100 U of IFN per ml in 6 to 24 h of culture. The IFN was typed as IFN- γ by specific antibodies. Specific antibodies either to natural IFN- γ or to a synthetic peptide corresponding to the human IFN- γ N-terminal amino acids, when added to cultures treated with IL 2, completely blocked IL 2 enhancement of NK cell activity for both the mouse and human systems. IL 2-induced proliferation was not affected by the antibodies. Thus, the enhancement of NK cell activity by IL 2 is completely mediated by IL 2-induced IFN- γ . The findings clearly indicate a cascade effect whereby one lymphokine (IL 2) induces the production of another. The latter lymphokine (IFN- γ) then mediates an important biological effect (natural killing).

Natural killer (NK) cells are a heterogeneous group of effector cells that are capable of cytotoxicity against a variety of target cells without obvious prior sensitization (8). NK cells are particularly cytotoxic against tumor cell lines and virus-infected cells (4, 18), and thus they may play an important role in host defense. NK cell cytotoxic activity has been shown to be dramatically enhanced by treatment with interferon (IFN) α/β in the mouse (3, 6) and IFN- α and IFN- β in humans (20) before the addition to target cells. Recently, interleukin 2 (IL 2) or T-cell growth factor that was devoid of antiviral activity was also shown to be capable of enhancing NK cell cytotoxic activity in the mouse system (7, 12). The independence of IL 2 enhancement of NK activity from that of IFN- α/β was demonstrated by showing that the concomitant addition of these two lymphokines to NK cells resulted in the augmentation of NK activity to levels not obtainable with either lymphokine alone (7, 22). It is possible that a given lymphokine, in addition to having a direct effect on cells, may induce lymphocytes to produce other lymphokines which may be responsible for biological effects attributable to the original lymphokine. We have shown, for example, that IL 2 alone can induce mouse lymphocytes to make IFN- γ (22). Thus, the possibility exists that the NK-cell-enhancing effects of IL 2 may be partially or totally attributable to IFN- γ . The data

presented here demonstrate that this is, in fact, the case and that studies comparing IL 2 and IFN- α/β enhancement of NK cell activity were actually comparing IL 2-induced IFN- γ and IFN- α/β . We show that there is no evidence that IL 2 is capable of enhancing NK cell activity in either the mouse or human system, except by its ability to induce IFN- γ .

MATERIALS AND METHODS

Cell preparations. C57BL/6 female mice, 4 to 8 weeks old (Jackson Laboratories, Bar Harbor, Maine) were sacrificed, the spleens were aseptically removed, and cell suspensions prepared in Eagle minimum essential medium (EMEM) supplemented with 2% fetal bovine serum, 0.1% penicillin-streptomycin, 40 μ g of gamycin per ml, and 0.075% sodium bicarbonate. Human leukocytes were prepared from peripheral blood by the Ficoll-Hypaque gradient separation method (2). Plastic adherent cells from both mouse and human cell preparations were removed by incubating 5 ml of a 1×10^7 cells per ml suspension in plastic tissue culture dishes for 1 h at 37°C. For IFN- γ or IL 2 treatment, 0.3 ml of a lymphocyte suspension (10×10^6 cells per ml) was added to various dilutions of IFN- γ or IL 2 and incubated for various times at 37°C. The cells were washed twice by centrifugation, suspended in EMEM, and added to target cells as described below. Control lymphocytes were incubated in EMEM without IFN or IL 2 and otherwise treated the same as the test lymphocytes.

IFN- γ and IL 2 preparations. Mouse and human IFN- γ (10^5 U per mg of protein) were prepared by

previously described methods (13, 16). Highly purified mouse IL 2 was produced and purified by gel filtration, hydrophobic chromatography, and electrofocusing as previously described and kindly provided by W. R. Benjamin and J. J. Farrar (9). Highly purified human IL 2 was produced and purified as previously described and kindly provided by J. J. Oppenheim and T. Kasahara (19). Mouse and human IL 2 prepared in this manner did not contain any detectable IFN.

Antibodies to mouse IFN- γ . Rabbit antibodies to partially purified natural mouse and human IFN- γ , as well as antibodies to a synthetic peptide corresponding to 20 N-terminal amino acids of human IFN- γ based on cDNA data, were prepared as previously described (11, 14, 17). The antibodies against mouse IFN- γ were absorbed against C57BL/6 spleen cells (5×10^6 cells per ml of sera), and antibodies to human IFN- γ were absorbed against human peripheral blood lymphocytes (PBL). The neutralization of IFN- γ in preparations containing IFN- γ or IL 2 or both was accomplished by adding serial dilutions of anti- γ antibody. The suspensions were mixed in a total volume of 100 μ l and incubated at room temperature for 1 h before the supernatants were assayed for antiviral activity and enhancement of NK cell activity. Antibodies to both mouse and human IFN- γ did not neutralize the ability of IL 2 to stimulate DNA synthesis in an IL 2-dependent T-cell line (CT6), did not inhibit the activity of purified IL 1 on murine thymocytes, or inhibit colony-stimulating factor activity when tested in standard laboratory assays. The antibodies were therefore highly specific for IFN- γ .

Proliferation assay. Both mouse and human IL 2 were assayed on an IL 2-dependent T-cell line (CT6) in a 48-h incubation (9). The cultures were incubated in triplicate, and 1 μ Ci of [3 H]thymidine ([3 H]TdR; specific activity, 20 Ci/mmol) was added to each well for the last 4 h of incubation. At harvest, 10% trichloroacetic acid was added. The cells were harvested with a multiple automatic sample harvester, washed with 5% trichloroacetic acid, and counted for [3 H]TdR incorporation in a liquid scintillation counter.

IFN assay. We assayed mouse IFN by a microplaque reduction method, using approximately 40 PFU of vesicular stomatitis virus per well in mouse L cells as described previously (10). Human IFN was assayed on WISH cells by a cytopathic assay using Sindbis virus as described elsewhere (1). Each point represents the mean of three assays, each performed in triplicate. In our studies, a concentration of 1 U of IFN- γ per ml is defined as the concentration required to decrease the number of PFU/well, or the cytopathic effect, by 50%. One unit of our IFN- γ inhibits virus replication about as much as 1 U of NIH reference fibroblast IFN.

Assay of NK cell activity. Mouse L cells were propagated in 96-well microtiter plates (Falcon Plastics, Oxnard, Calif.) in EMEM with antibiotics and 2% fetal bovine serum and prelabeled with sodium chromate (51 Cr) at a concentration of 10 μ Ci/ 2×10^5 cells per 4 h at 37°C. After incubation, the target cells were washed three times with EMEM, and human or mouse lymphocytes were added at an effector-to-target-cell ratio of 10:1. Specific 51 Cr release was determined from triplicate cultures after incubation at 37°C for 18 h. The standard deviation of triplicate assays was less than 5%. The percent specific 51 Cr release was calculated as $R = (E - S/M - S) \times 100$ where E is the counts per

minute in the experimental wells, S is spontaneous release, and M is the maximal release in the presence of 1% saponin.

RESULTS

IL 2 induction of IFN- γ and enhancement of NK cell activity. Since the role of induced IFN- γ in the IL 2 enhancement of NK cell activity was the question addressed, it seemed appropriate to first examine the ability of highly purified IL 2 to induce IFN- γ and to enhance NK cell activity in both the mouse and human systems. Mouse IL 2 was evaluated by incubating mouse spleen cells for 18 h with various concentrations of IL 2 (Fig. 1A). Spleen cells treated with IL 2 (1 to 150 U) containing no detectable IFN activity produced detectable levels of IFN after treatment with as little as 1 U of IL 2. Peak titers of IFN activity were observed after treatment with 60 U of IL 2. All of the IFN activity induced by IL 2 was demonstrated to be IFN- γ by neutralization with specific antibody (data not shown) (17). The level of IL 2-enhanced NK cell activity was proportional to the amount of IFN- γ produced. The incubation of human IL 2 with human PBL also resulted in the production of IFN- γ and enhancement of NK cell activity (Fig. 1B). As with the mouse system, NK cell activity was proportional to the amount of IFN- γ produced. The data from both systems are suggestive of a possible role for IFN- γ in the mediation of IL 2 enhancement of NK cell activity.

Kinetics of IFN- γ production and enhancement of NK cell activity by lymphocytes treated with IL 2. Having established that a dose-response relationship existed between the extent of IL 2 enhancement of NK cell activity and the amount of IFN- γ produced by lymphocyte cultures, we next addressed the question of the relationship of the kinetics of IFN- γ production and enhancement of NK cell activity. We first determined the kinetics of IL 2-induced IFN- γ production and enhancement of NK cell activity in mouse spleen cells incubated with 30 U of IL 2 during a 30-h period (Fig. 2A). Detectable levels of IFN and NK cell activity were observed as early as 6 h after culture initiation. The induced NK cell activity profile peaked at 24 h and was similar to and closely associated with increased IFN- γ production, suggesting a role for IFN- γ in the mediation of the IL 2 effects. The incubation of human IL 2 with human PBL also resulted in the early production of IFN, with peak levels at 24 h (Fig. 2B). The type of IFN at all stages of production was γ as determined by neutralization tests (data not shown). Human NK cell activity quantitatively paralleled IFN- γ production, again suggesting a role for IFN- γ in the mediation of IL 2 enhancement of NK cell activity.

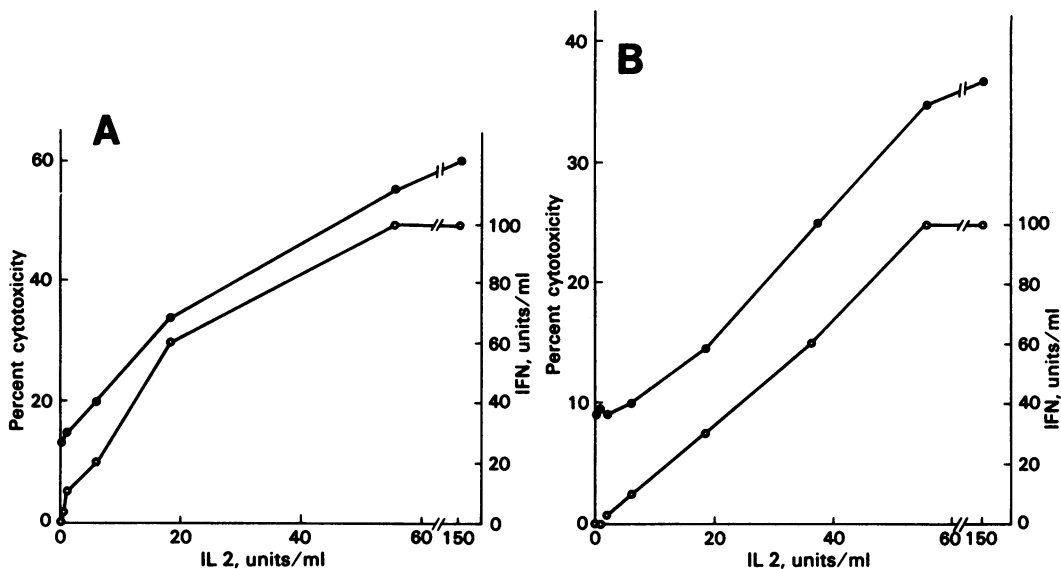


FIG. 1. Dose-response curves of mouse (A) and human (B) IL 2 induction of IFN- γ and enhancement of NK cell activity. Various concentrations of IL 2 were added to macrophage-depleted spleen cells or human PBL (1×10^7 /ml). IFN (\circ) and NK cell activity (\bullet) were measured 24 h after the initiation of culture.

Inhibition of mouse IL 2 enhancement of NK cell activity by antibodies to mouse IFN- γ . To determine more directly whether IL 2 induction of IFN- γ was responsible for IL 2 enhancement of NK cell activity, we treated mouse spleen cell cultures with antibodies to IFN- γ at the time of IL 2 treatment. The rationale was to neutralize IFN- γ as it was produced and to determine how this affected IL 2 enhancement of NK cell

activity. Representative data (Fig. 3A) showed a marked inhibition of cytotoxic activity in such IL 2-treated cultures. The dose-response profile of the inhibition of cytotoxicity with antibodies to IFN- γ corresponded to the neutralization of IFN- γ activity (data not shown). In the same experiment, a control IFN- γ preparation was added to cultures containing antibodies, and a similar dose-response profile of the inhibition of

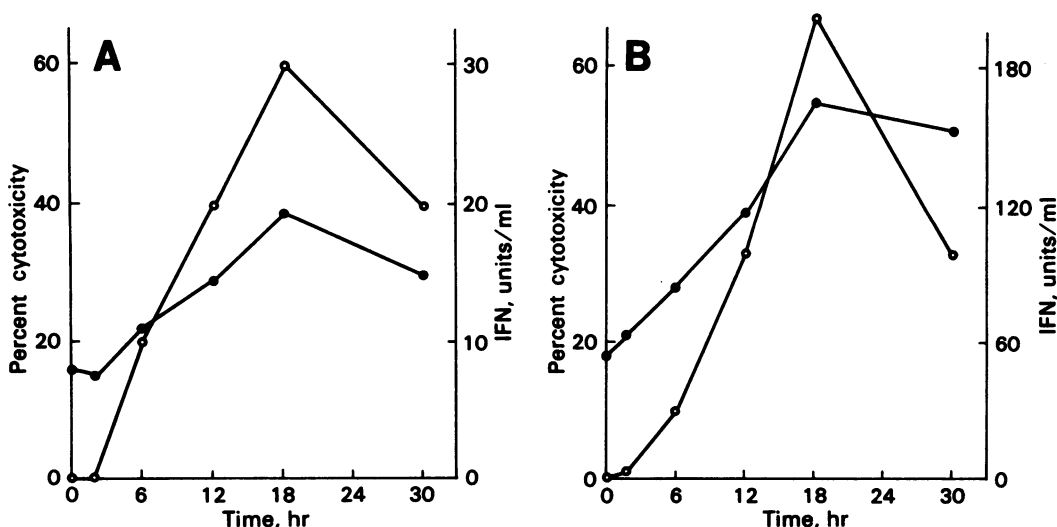


FIG. 2. Kinetics of IFN production and enhancement of NK cell activity by mouse spleen cells (A) and human PBL (B) treated with IL 2. Mouse or human IL 2 (30 U each) was added to PBL, IFN- γ (\circ) production and enhancement of NK cell activity (\bullet) from IL 2-treated cultures were measured during a 36-h period.

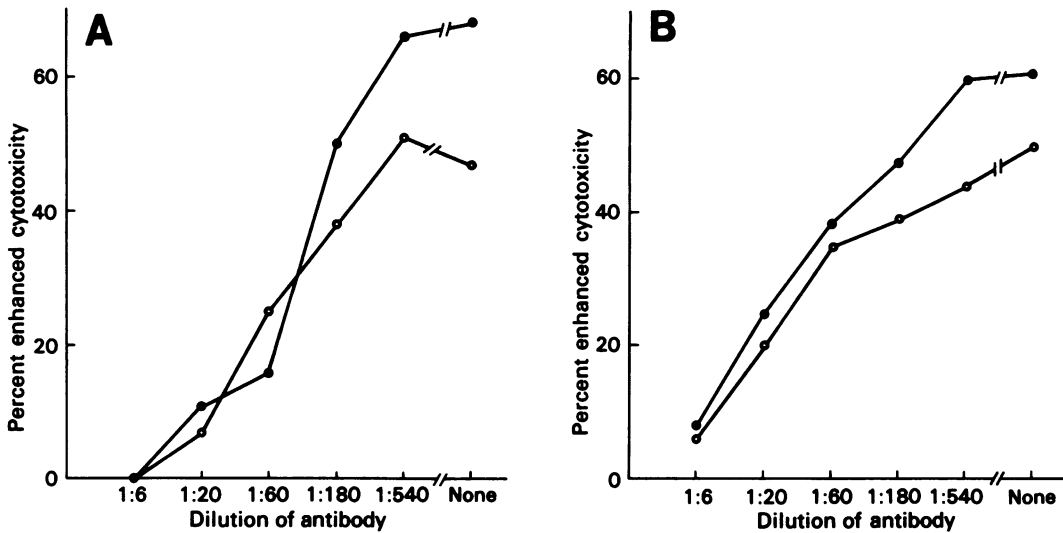


FIG. 3. Inhibition of mouse (A) and human (B) IL 2 enhancement of NK cell activity by antibodies to mouse and human IFN- γ , respectively. Approximately 50 U of IL 2 per ml or 30 U of IFN- γ per ml was incubated with equal volumes of various dilutions of antibodies to IFN- γ (1:6 dilution; neutralizes 500 U of IFN- γ per ml) for 1 h. The mixtures were incubated with PBL for 18 h, after which the cells were washed and assayed for NK cell activity. The unenhanced reactivity of mouse spleen cells and human PBL was 12 and 7%, respectively. Symbols: ●, IFN- γ plus anti-IFN- γ ; ○, IL 2 plus anti-IFN- γ .

enhanced natural killing and IFN- γ activity was seen. The same antibodies used above had no effect on the ability of IL 2 to stimulate DNA synthesis in an IL 2-dependent T-cell line, whereas the antiviral activity of IFN- γ was inhibited by >97%. We conclude that antibodies to mouse IFN- γ probably blocked the enhancement of NK cell activity by sequestering the IFN- γ that was induced by IL 2.

Inhibition of human IL 2 enhancement of NK cell activity by antibodies to human IFN- γ . We next investigated whether the IL 2 enhancement of NK cell activity in the human system could also be similarly regulated by antibodies to IFN- γ . Accordingly, various concentrations of rabbit antibodies to human IFN- γ were added to PBL cultures that had been treated with IL 2. As with the mouse system, the enhancement of human NK cell activity by IL 2 and the appearance of IFN- γ activity were blocked in a dose-response manner by the antibodies (Fig. 3B). Again, the antibodies did not block the direct effect of human IL 2 on the stimulation of DNA synthesis in an IL 2-dependent T-cell line, whereas they inhibited IFN- γ activity by >99% (Table 1).

Finally, highly specific antibodies to human IFN- γ were tested to determine whether they could block the enhancement of NK cell activity as did antibodies to natural IFN- γ . These antibodies were prepared in rabbits against a synthetic peptide comprising the inferred 20 N-terminal amino acids of human IFN- γ based on

cDNA sequence (11). The results show that these antibodies also neutralized the ability of IL 2 to enhance killing (Table 2). This latter observation with antibodies to a chemically defined

TABLE 1. Effect of antibodies in IFN- γ on IFN- γ induction of antiviral state and IL 2 induction of proliferation in an IL 2-dependent cell line

Lymphokine	Treatment ^a	Residual activity (U/ml)	% Neutralization
Human IFN- γ	None	300	— ^b
	NRS	300	0
	Anti-HuIFN- γ	<3	>99
Human IL 2	None	55	—
	Anti-HuIFN- γ	49	11
Mouse IFN- γ	None	100	—
	NRS	100	0
	Anti-MoIFN- γ	<3	>97
Mouse IL 2	None	300	—
	Anti-MoIFN- γ	275	8

^a Normal rabbit serum (NRS) and rabbit antibodies to mouse (Mo) or human (Hu) IFN- γ were incubated with the appropriate IL 2 (50 U each) for 1 h at room temperature before being added to mouse spleen cells or human PBL. After this, the effect of the antibodies on the antiviral state and IL 2-induced proliferation was measured as described in the text.

^b —, None.

TABLE 2. Inhibition of enhanced NK cell activity induced by human IL 2 with specific antibodies to a synthetic peptide corresponding to the N-terminal region of IFN- γ

Lymphokine	Treatment ^a	% Cytotoxicity above control	% Neutralization
Human IL 2	None	36	— ^b
	NRS	34	6
	Antipeptide (1:10)	13	64
	Antipeptide (1:30)	21	42
	Antipeptide (1:100)	34	6
Human IFN- γ	None	51	—
	NRS	49	4
	Antipeptide (1:10)	14	73
	Antipeptide (1:30)	35	32
	Antipeptide (1:100)	44	14

^a Normal rabbit serum (NRS) and rabbit antibodies to a synthetic peptide of human IFN- γ were incubated with IL 2 or IFN- γ (50 U each) for 1 h at room temperature before being added to human PBL. Eighteen hours later, the cells were washed twice and added to mouse L cell targets where chromium release was determined in an 18-h assay.

^b —, None.

synthetic antigen provides compelling evidence that IFN- γ is responsible for the enhanced killing activity associated with IL 2.

DISCUSSION

Based on the accumulating data, NK cells may play a pivotal role in host defense, particularly against intracellular infections and cancer (8). It is, therefore, an important observation that IFN can enhance NK cell activity (3, 6, 20). Recently, IL 2 has also been shown to be capable of enhancing NK cell activity (7, 12). Three observations appear to be the basis for attributing NK cell-enhancing properties to IL 2 that are distinct from IFN enhancement. First, IL 2 free from antiviral activity was capable of enhancing NK cell activity (7, 12). Second, the addition of both IL 2 and IFN- α/β to NK cells in the mouse system resulted in the augmentation of NK cell activity to levels not obtainable with either lymphokine alone (7, 12). Lastly, subclasses of NK cells in the mouse system responded differentially to the enhancement of activity by IL 2 versus IFN- α/β (15).

Two observations on our part suggested the possibility that the NK cell-enhancing effect of

IL 2 may be due to IFN- γ . First, IL 2 induces T cells to make IFN- γ without additional obvious stimulation, and thus the produced IFN- γ could actually enhance killing (22). Second, when IFN- α/β and IFN- γ were added together to the appropriate cultures, we have previously noted an augmentation of or potentiation effect on both the antiviral state and the enhancement of NK cell activity to levels that were not obtainable with either IFN alone (5, 21).

We have shown here that, in both the mouse and human systems, the IL 2 enhancement of NK cell activity is totally due to the ability of highly purified IL 2 to induce IFN- γ in lymphocyte cultures. The dose-response and kinetic curves for IL 2 enhancement of NK cell activity and the induction of IFN- γ were similar, suggesting that the induced IFN- γ was responsible for the NK cell activity enhancement. More definitively, we were able to completely block IL 2-induced enhancement of NK cell activity in both the mouse and human systems by adding well-defined specific antibodies to IFN- γ to cultures at the time of IL 2 addition. These antibodies blocked NK cell activity enhancement and antiviral activity in a dose-response manner, while having no significant effect on the ability of IL 2 to stimulate DNA synthesis in an IL 2-dependent T-cell line. The antibodies were also nonreactive with IL 1 and colony-stimulating factor. It is of particular interest that one of the human antibodies that significantly blocked IL 2 enhancement of NK cell activity was produced by the immunization of rabbits with a synthetic peptide encoded by the 5' end of human IFN- γ cDNA (11), thus precluding the possibility that contaminating IL 2 or other lymphokines were in the immunogen. The same antibodies had no effect on IL 2 stimulation of DNA synthesis in an IL 2-dependent T-cell line. The data indicate that highly purified IL 2 enhances NK cell activity through its induction of IFN- γ . In a general sense, the data presented here point out the interrelationship of lymphokines and functions: a given lymphokine, in addition to exerting a direct effect on cells, may also induce lymphocytes to produce other lymphokines which may be responsible for the biological events of interest.

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