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Abstract

The immunologic consequences of prolonged infusions of rIL-2 in doses that produce physiologic serum concentrations of this cytokine were investigated. rIL-2 in doses of 0.5–6.0 × 10^6 U/m^2 per d (3.3–40 μg/m^2 per d) was administered by continuous intravenous infusion for 90 consecutive days to patients with advanced cancer. IL-2 concentrations (25±25 and 77±64 pM, respectively) that selectively saturate high-affinity IL-2 receptors (IL-2R) were achieved in the serum of patients receiving rIL-2 infusions of 10 μg/m^2 per d and 30 μg/m^2 per d. A gradual, progressive expansion of natural killer (NK) cells was seen in the peripheral blood of these patients with no evidence of a plateau effect during the 3 mo of therapy. A preferential expansion of $CD56^{bright}$ NK cells was consistently evident. NK cytotoxicity against tumor targets was only slightly enhanced at these dose levels. However, brief incubation of these expanded NK cells with IL-2 in vitro induced potent lysis of NK-sensitive, NK-resistant, and antibody-coated targets. Infusions of rIL-2 at 40 μg/m^2 per d produced serum IL-2 levels (345±381 pM) sufficient to engage intermediate affinity IL-2R p75, which is constitutively expressed by human NK cells. This did not result in greater NK cell expansion compared to the lower dose levels, but did produce in vivo activation of NK cytotoxicity, as evidenced by lysis of NK-resistant targets. There was no consistent change in the numbers of $CD56^-$ $CD3^+$ T cells, $CD56^+$ $CD3^+$ MHC-unrestricted T cells, or B cells during infusions of rIL-2 at any of the dosages used. This study demonstrates that prolonged infusions of rIL-2 in doses that saturate only high affinity IL-2R can selectively expand human NK cells for an extended period of time with only minimal toxicity. Further activation of NK cytolytic activity can also be achieved in vivo, but it requires concentrations of IL-2 that bind intermediate affinity IL-2R p75. Clinical trials are underway attempting to exploit the differing effects of various concentrations of IL-2 on human NK cells in vivo. (*J. Clin. Invest.* 1993. 91:123–132.) Key words: IL-2 • natural killer cells • immunotherapy • IL-2 receptor • cytotoxicity

Introduction

IL-2 plays a central role in the vertebrate immune response (1). Produced by T lymphocytes activated through exposure to cognate antigen, IL-2 promotes proliferation and differentiation of helper T cells, cytotoxic T cells, and B cells (1, 2). Consequently, IL-2 participates in both cell-mediated immunity and humoral immunity, and is probably necessary for both primary and secondary adaptive immune responses. IL-2 may also augment innate or "natural" immunity by stimulating natural killer (NK)[1] cells (3, 4) and monocytes (5, 6). Furthermore, IL-2 can induce the classic signs of delayed-type hypersensitivity even in the absence of specific antigen (7). Commensurate with the crucial involvement of IL-2 in immune responses, congenital absence of IL-2 production in humans causes a severe combined immune deficiency with life-threatening infections (8, 9). Replacement therapy with recombinant IL-2 (rIL-2) can be effective treatment for this disorder (8).

The diverse effects of IL-2 are mediated through specific cell surface receptors on lymphocytes, monocytes, and other cell types (10). The IL-2R is comprised of at least two subunits (10). The intermediate affinity IL-2R is a 70–75-kD protein that binds IL-2 with an equilibrium K_d of ~ 1 nM; IL-2R p75 can initiate intracellular signal transduction after IL-2 binding (11). The low affinity IL-2R (p55 or CD25) is a 55-kD protein that binds IL-2 with a K_d of ~ 10 nM. IL-2R p55 has not been shown to mediate internalization of IL-2 or signal transduction by itself. Expressed together on the cell surface, the p75 and p55 chains can associate noncovalently to form a heterodimer that binds IL-2 with high affinity (K_d, ~ 10 pM). Most resting T cells and B cells express neither IL-2R p75 nor p55 and do not respond to exogenous IL-2 (10). However, after activation by antigen or mitogens, T cells and B cells express high affinity IL-2R heterodimers and proliferate in an IL-2–dependent fashion (10, 11). By comparison, almost all resting NK cells in human peripheral blood constitutively express isolated IL-2R p75 chains (12–14). Thus, NK cells can be activated by nanomolar concentrations of IL-2 in the absence of additional stimuli. Since NK cells comprise ~ 10% of PBL, as many as 10^9 circulating lymphocytes are potentially immediately responsive to IL-2.

The predominant therapeutic use of IL-2 to date has been as an immunostimulant in patients with cancer (15, 16). Based on the paradigms of cytotoxic chemotherapy, IL-2 has been administered in progressively higher doses to define the maximally tolerated dose (17, 18), and bolus intravenous infusions of 15–150 × 10^6 U (1–10 mg) of IL-2 have been used in most subsequent clinical trials (15, 16). Approximately 15–20% of patients with advanced renal cell carcinoma or melanoma dem-

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1. *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; E/T, effector to target (ratio); NK, natural killer (cells); PE, phycoerythrin; rIL-2, recombinant interleukin 2.

onstrate objective tumor responses after "high dose" IL-2 therapy. Unfortunately, such treatment is associated with life-threatening toxicities, including severe hypotension, pulmonary edema, renal failure, cardiac arrhythmias, and neurologic dysfunction (19, 20). It is unclear whether beneficial responses can be induced by lower, more physiologic doses of IL-2 that would not lead to such severe systemic toxicity.

We recently published the preliminary results of a phase I trial of rIL-2 given in low doses ($0.5–6.0 \times 10^5$ U/m^2 per d; 3.3–40 μg/m^2 per d) by continuous intravenous infusion for 3 mo to patients with advanced cancer (21). Toxicity was modest and generally did not impair the normal daily activities of these ambulatory patients. Almost all patients receiving between 1.5×10^5 U/m^2 per d (10 μg/m^2 per d) and 6.0×10^5 U/m^2 per d (40 μg/m^2 per d) demonstrated significant peripheral blood lymphocytosis caused by the selective expansion of circulating NK cells. In this report, we describe in detail the immunologic consequences of prolonged low-dose rIL-2 infusion. Our results define a range of IL-2 doses that produces serum IL-2 concentrations sufficient to saturate high affinity IL-2 receptors and induces a marked selective expansion of circulating NK cells.

Methods

Description of the clinical study. 21 patients with advanced cancer received prolonged continuous intravenous infusions of rIL-2 at doses that ranged from 0.5×10^5 U/m^2 per d (3.3 μg/m^2 per d) to 6.0×10^5 U/m^2 per d (40 μg/m^2 per d) (specific activity 1.5×10^7 U/mg protein). At least three patients received ≥ 10 wk of rIL-2 infusion at each of the four dose levels tested. Recombinant human IL-2 was supplied by Hoffmann-LaRoche (Nutley, NJ). This clinical trial was carried out with the approval of the Human Subjects Protection Committee, Dana-Farber Cancer Institute (Boston, MA). All patients gave informed consent for participation in this study. Patient characteristics and toxicities have been described elsewhere (21). Blood specimens were collected at weekly or biweekly intervals in heparinized 60-cm^3 syringes, and PBMC were immediately separated by Ficoll-Hypaque density gradient centrifugation. Cells were washed three times and resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% human antibody serum for phenotypic and functional assays. Aliquots of PBMC were also cryopreserved in liquid nitrogen for later use in proliferation and cytotoxicity assays. Blood specimens were also obtained periodically for determinations of complete blood counts, serum chemistries, and serum IL-2 levels.

Analysis of cell surface antigens. Single- and two-color immunofluorescence analysis was performed using FITC- and phycoerythrin (PE)-conjugated murine mAb as previously described (22). CD2, CD3, CD4, CD5, CD8, CD20, CD25, CD56, and anti-IL-2R p75 antibodies were supplied by Coulter Immunology (Hialeah, FL). FITC-conjugated anti-IL-2Rp75 mAb TU-27 was kindly provided by Dr. Kazuo Sugamura, Tohoku School of Medicine (Sendai, Japan). CD16 mAb 3G8 was kindly provided by Dr. Jay Unkeless, Mount Sinai School of Medicine (New York). Flow cytometry was performed on an Epics V or Epics Elite (Coulter Electronics, Hialeah, FL) and results were displayed as single color histograms or as two-color orthographic projections plotting log green fluorescence versus log red fluorescence. Background fluorescence was determined by incubating cells with fluorochrome-conjugated murine mAb of irrelevant specificity.

Cytotoxicity assays. NK cytolytic activity was measured using standard 4-h ^{51}Cr-release assays as previously described (23). 5,000 ^{51}Cr-labeled target cells were added to wells of V-bottom 96-well microtiter plates, and effector cells were plated in triplicate to yield various effector to target (E/T) ratios. Patient PBMC were assayed for cytotoxicity immediately after separation from peripheral blood or after overnight (18-h) incubation at 37°C in media alone or in media containing various concentrations (10 pM–10 nM) of rIL-2. Target cells included K562 (an NK-sensitive human myeloid leukemia cell line), COLO 205 (an NK-resistant human colon adenocarcinoma cell line), and P815 (an NK-resistant murine mastocytoma cell line). Antibody-dependent cellular cytotoxicity (ADCC) assays were performed against P815 cells preincubated with either medium alone or with a 1:100 dilution of polyclonal rabbit anti-mouse lymphocyte serum (Accurate Chemical & Scientific Corp., Westbury, NY) as previously described (23).

Proliferation assays. Cryopreserved patient PBMC were thawed, stained with PE-conjugated CD56 mAb, and sorted into CD56$^+$ and CD56$^-$ subsets by standard methods using flow cytometry. PBMC from healthy volunteer donors were isolated by Ficoll-Hypaque density gradient centrifugation, enriched for NK cells by negative selection using immunomagnetic beads, and sorted into CD56^{dim} and CD56^{bright} subsets by flow cytometry as previously described (23). Lymphocyte subsets were plated at 30,000 cells per well in 96-well U-bottom microtiter plates and cultured at 37°C; 1 μCi of tritiated thymidine was added to each well after 4 d. Samples were collected 18 h later using a cell harvester, and thymidine incorporation was determined using a liquid scintillation counter as previously described (23).

Measurement of serum IL-2 concentrations. Serum samples were assayed for IL-2 using an IL-2 enzyme immunoassay kit from Advanced Magnetics, Inc. (Cambridge, MA). Each sample was assayed in duplicate (undiluted or diluted 1:2, 1:4, and 1:8) and compared to a standard curve using homogeneous rIL-2 (Takeda Chemical Industries, Ltd., Osaka, Japan). The lower limit of detection in this assay is 10 pM.

Results

Changes in lymphocyte subsets during continuous infusion rIL-2. Phenotypic analyses of T cell, B cell, and NK cell populations were performed every 7–14 d while patients were receiving rIL-2. Quantitative changes in circulating NK (CD56$^+$) cells are summarized in Fig. 1 *A*. At the lowest dose level, (0.5×10^5 U/m^2 per d or 3.3 μg/m^2 per d), there was no appreciable change in NK cell number. However, at dose levels of 1.5, 4.5, and 6.0×10^5 U/m^2 per d (10, 30, and 40 μg/m^2 per d, respectively), there were consistent increases in the NK cell population during the course of IL-2 infusion. In the peripheral blood of three patients who completed ≥ 10 wk of therapy at 1.5×10^5 U/m^2 per d, the absolute number of total NK cells increased from a mean of 134/μl at week 0 to 410/μl at week 12. In patients who completed ≥ 10 wk of therapy at 4.5×10^5 U/m^2 per d, the absolute number of total NK cells increased from a mean of 165/μl at week 0 to 2,425/μl at week 12. Similarly, at a dose of 6.0×10^5 U/m^2 per d, the absolute number of NK cells increased from 247/μl at week 0 to 1,337/μl at week 12. In these patients, the increase in circulating NK cells occurred gradually throughout the entire period of IL-2 infusion, and there was no evidence to suggest decreased responsiveness over time. It is also noteworthy that a dose-dependent increase in NK cells was observed in patients who received 1.5×10^5 U/m^2 per d or 4.5×10^5 U/m^2 per d, while a further increase in dose to 6.0×10^5 U/m^2 per d did not result in any additional expansion in the NK cell population. In fact, only a five to sixfold increase in NK cells was noted at 6.0×10^5 U/m^2 per d compared to an average 15-fold increase in patients treated with 4.5×10^5 U/m^2 per d.

The absolute number of CD3$^+$ T cells remained essentially the same for all patients at each of the final three dose levels, with a mean value of 1,170/μl at week 0 and a mean value of 1,030/μl at week 12 (Fig. 1 *B*). Additional studies were per-

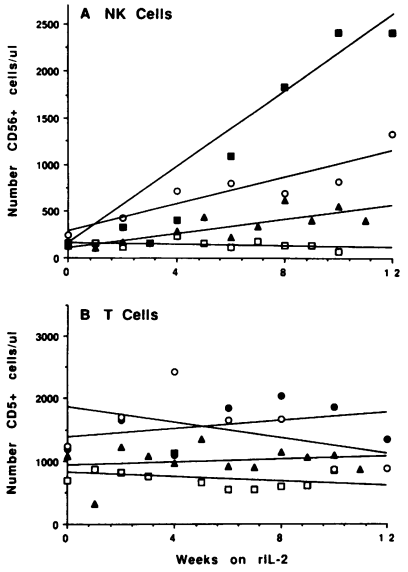

Figure 1. (*A*) Absolute number of NK cells in peripheral blood of patients receiving continuous infusion of rIL-2 at each of four dose levels. Values were obtained by multiplying the absolute lymphocyte count by the percent $CD56^+$ cells within the lymphocyte population identified by flow cytometric analysis. Each point represents the mean value for at least three patients completing ≥ 10 wk of treatment at a specific dose level. The dose levels used in this study were □, 0.5×10^5 U/m² per d; ▲, 1.5×10^5 U/m² per d; ■, 4.5×10^5 U/m² per d; ○, 6.0×10^5 U/m² per d. (*B*) Absolute number of T cells in peripheral blood of patients receiving continuous infusion of rIL-2 at each of four dose levels. Values were obtained by multiplying the absolute lymphocyte count by the percent $CD5^+$ cells identified by flow cytometric analysis. Each point represents the mean value for at least three patients completing ≥ 10 wk of treatment at a specific dose level. The dose levels used in this study were □, 0.5×10^5 U/m² per d; ▲, 1.5×10^5 U/m² per d; ■, 4.5×10^5 U/m² per d; ○, 6.0×10^5 U/m² per d.

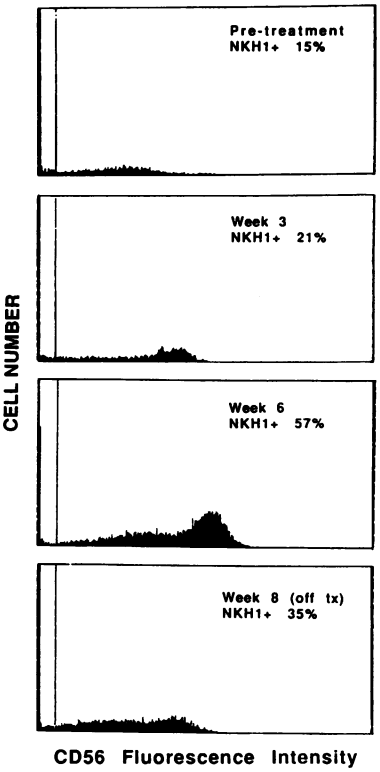

Figure 2. Expression of the CD56 antigen by PBL obtained from an individual patient at indicated time points before, during, and after the continuous infusion of 4.5×10^5 U/m² per d of rIL-2. Freshly isolated nonadherent PBL were stained with PE-conjugated mAb NKH1(CD56) and analyzed by flow cytometry. Percent $CD56^+$ cells is indicated in the upper right. Note the prominent expansion of $CD56^{bright}$ cells during the rIL-2 infusion, and subsequent regression of the $CD56^{bright}$ population after discontinuation of IL-2. The last analysis was performed after a 2-wk interruption in IL-2 therapy.

formed to assess whether T cell activation occurred in vivo during infusion of low dose rIL-2. Proliferation studies performed in vitro failed to demonstrate any spontaneous proliferation of patient T cells, yet showed normal responses after in vitro activation with either PHA or anti-CD2 antibodies (data not shown). Although no patient exhibited a persistent increase in circulating T cells, transient increases in T cells were noted in several patients during rIL-2 infusion. For example, at the final two doses, a transient increase in absolute T cell number (arbitrarily defined by a twofold increase from pretherapy value) was noted in four patients, followed by a return to near baseline values within 14 d. Each episode of T cell increase occurred within the first 5 wk of initiating the infusion, with the exception of one patient who demonstrated a second increase during weeks 9 and 10. Phenotypic analyses performed during these increases did not reveal any selective changes in either CD4 or CD8 T lymphocyte subsets. Absolute numbers of B lymphocytes ($CD20^+$ cells) remained relatively unchanged during the entire period of IL-2 infusion. The mean value for absolute numbers of B cells at the three highest dose levels was 90/μl at week 0 and 120/μl at week 12. In addition, there were no significant changes in serum IgG, IgM, or IgA levels either during or after the continuous in vivo infusion of rIL-2 (data not shown).

IL-2 induced expansion of $CD56^{bright}$ NK cells. The $CD56^{bright}$ subset of NK cells represents only ~ 10% of human NK cells and ≤ 1% of all resting human PBL, yet is the only lymphocyte population that constitutively expresses the high affinity IL-2R and exhibits a profound proliferative response to low concentrations of rIL-2 in vitro (24–26). As shown in Fig. 2 for a representative patient, phenotypic examination of peripheral blood NK cells during IL-2 infusion at doses of 1.5–6.0 $\times 10^5$ U/m² per d revealed a gradual increase in the $CD56^{bright}$ population compared to the $CD56^{dim}$ cells. While the $CD56^{bright}$ cells accounted for < 10% of $CD56^+$ cells in all patients before the infusion of rIL-2, this population accounted for the majority of $CD56^+$ cells after the 12-wk continuous infusion of rIL-2 at the two highest doses. In some cases, there was a ≥ 100-fold increase in the absolute number of $CD56^{bright}$ cells. The discontinuation of rIL-2 infusion was invariably followed by a decrease in the number of $CD56^{bright}$ cells in peripheral blood within days to weeks (Fig. 2, A–D).

CD16 (FcγRIII) is expressed by the majority of human NK cells and is the receptor that triggers NK cell ADCC (27, 28). In normal peripheral blood, the $CD56^{bright}$ population expresses little or no CD16 and mediates ADCC relatively poorly (25, 29). In patients receiving continuous infusion IL-2, the expanded $CD56^{bright}$ cells demonstrated little change in CD16 antigen density, and the vast majority coexpresses CD16 (Fig. 3). Nevertheless, a significant fraction of the $CD56^+$ cells in all patients were also CD16 negative. Additional two-color immunofluorescence analysis demonstrated that the $CD56^+$ cells consistently lacked CD3, CD4, and CD5, while > 95% of these lymphocytes expressed CD2 and ~ 40% expressed CD8 (data not shown).

Expression of IL-2 receptors on expanded NK cells. During the course of rIL-2 infusion we also examined expression of both p55 and p75 subunits of the IL-2 receptor by NK cells and T cells using direct two-color immunofluorescence analysis. As shown for a representative patient in Fig. 4, few NK cells expressed IL-2R p55 by flow cytometric analysis either before or during rIL-2 infusion. In contrast, expression of IL-2R p75 was evident on almost all $CD56^+$ cells before treatment, and there appeared to be an increase in antigen density during the period of IL-2 infusion. Simultaneous analysis of T cells failed to dem-

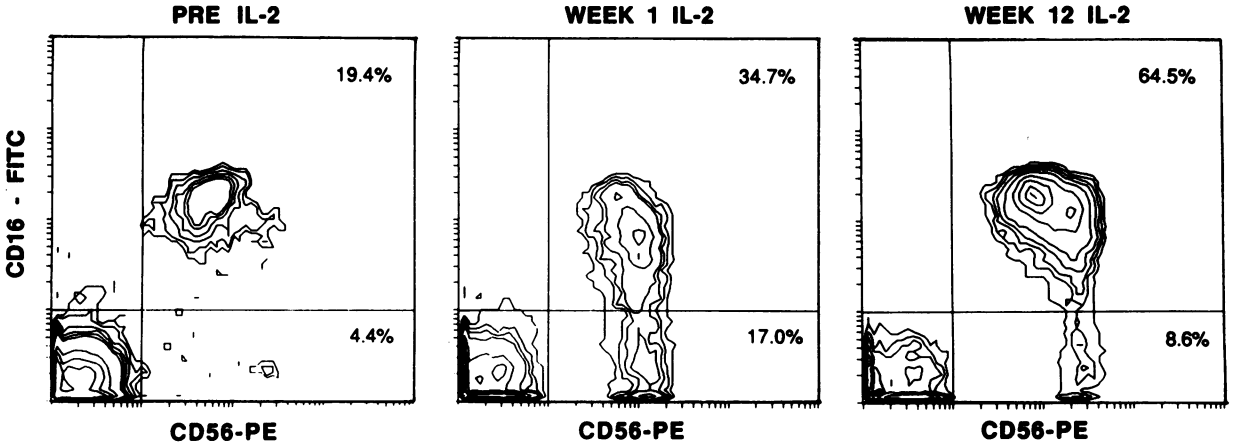

Figure 3. Coexpression of CD56 and CD16 (Fcγ RIII) by peripheral blood NK cells in a representative patient receiving continuous IL-2 infusion of 4.5×10^5 U/m^2 per d. Nonadherent PBL obtained at the indicated time points were stained with PE-conjugated anti-CD56 mAb and FITC-conjugated anti-CD16 mAb and analyzed by flow cytometry. Note the predominance of CD16$^+$ CD56^{dim} NK cells prior to therapy and the expansion of both CD16$^+$ CD56^{bright} and CD16$^-$ CD56^{bright} cells during the rIL-2 infusion.

onstrate significant expression of either IL-2R p55 or IL-2R p75 on CD3$^+$ cells as a result of IL-2 infusion (data not shown). Furthermore, Northern blot analysis performed on purified populations of T cells from selected patients receiving low dose rIL-2 infusion showed only minimal expression of IL-2R p75 mRNA when compared with NK cells isolated under identical conditions, or compared with T cells after CD3 activation (data not shown).

Effects of rIL-2 infusion on non-MHC-restricted cytotoxic T lymphocytes. A small subset of resting CD3$^+$ cells expresses the CD56 antigen at low density; these unique T cells comprise ~ 2% of normal PBL (30). CD56$^+$ CD3$^+$ cells exhibit the morphology of large granular lymphocytes, mediate spontaneous non-MHC-restricted cytotoxicity when freshly isolated, and demonstrate augmented lysis of NK-resistant tumor cell lines after short-term incubation in nanomolar concentrations of rIL-2 (30). While detected in virtually all patients in varying percentages before the initiation of therapy, this population did not expand during continuous low dose rIL-2 infusion. This can be best appreciated in one patient with an unusually high percentage of CD56$^+$ CD3$^+$ non-MHC-restricted T cells before treatment (Fig. 5). The percentage of CD56$^+$ T cells progressively diminishes as the absolute number of CD56$^+$ CD3$^-$ NK cells selectively expands during continuous infusion low dose rIL-2 therapy. Similar results were obtained with all patients treated on this study (not shown). Thus CD56$^+$ CD3$^+$ lymphocytes, like conventional CD56$^-$ CD3$^+$ T cells, do not appear to express high affinity IL-2R or to proliferate in vivo in the presence of low concentrations of IL-2.

Effects of IL-2 infusion on cytolytic activity of NK cells. The cytolytic activity of PBMC against both NK sensitive (K562) and NK resistant (COLO 205) target cells was examined periodically during the course of rIL-2 infusion. A gradual increase in cytotoxicity against K562 target cells was detected during IL-2 therapy, generally correlating with the degree of expansion of circulating NK cells (21). Patients treated at the highest dose level (6.0×10^5 U/m^2 per d) exhibited low but significant killing of COLO 205 target cells during rIL-2 infusion. NK cells incubated in vitro with nanomolar concentrations of IL-2 develop non-MHC-restricted cytotoxicity against tumor cell targets generally resistant to lysis by resting NK cells lymphokine-activated killer (LAK activity) (31, 32). Further studies were undertaken to determine whether the cytolytic activity of NK cells expanded in vivo by low dose rIL-2 infusion could be further enhanced by incubation with rIL-2 in vitro. Fig. 6 demonstrates results obtained using PBMC from a representative patient after 8 wk of continuous rIL-2 infusion at 6.0×10^5 U/m^2 per d. PBMC incubated overnight in medium alone demonstrated only minor cytotoxicity against NK-resistant target cells, and the presence of low concentrations of IL-2 (10 pM) did not enhance cytotoxicity. Cytotoxicity was enhanced after incubation of PBMC with 100 pM IL-2, but this was evident only at the higher effector/target cell ratios. Incubation with higher concentrations of IL-2 (1–10 nM) produced markedly enhanced killing of the NK-resistant target, even at low effector/target ratios.

ADCC by PBMC was also tested during the course of rIL-2 infusion. PBMC isolated before low dose rIL-2 infusion failed to demonstrate any significant lysis of the P815 murine mastocytoma cell line in the absence (Fig. 7 *A*) or presence (Fig. 7 *B*) of the polyclonal rabbit anti-mouse lymphocyte serum; further incubation with varying concentrations of exogenous rIL-2 also failed to induce killing of P815 target cells. After in vivo expansion of CD56$^+$ cells by low dose rIL-2 infusion for 6–8 wk, no significant cytotoxicity was seen in the absence of polyclonal rabbit anti-mouse lymphocyte serum. Despite further stimulation with rIL-2 in vitro, minimal cytotoxicity occurred even at the highest concentration of IL-2 (10 nM) (Fig. 7 *C*). After addition of polyclonal rabbit anti-mouse lymphocyte serum and exogenous IL-2 (Fig. 7 *D*), however, the expanded CD56$^+$ cells exhibited a high degree of ADCC. As with NK activity and lymphokine-activated killer activity, high concentrations of IL-2 (1–10 nM) were required to demonstrate enhanced ADCC.

Proliferative responses of NK cells expanded in vivo by rIL-2 infusion. Since NK cell numbers progressively increased in patients receiving low dose continuous infusion rIL-2, it seemed likely that these expanded NK cells would proliferate vigorously to exogenous IL-2. Surprisingly, CD56$^+$ cells isolated by flow cytometric cell sorting from the blood of patients

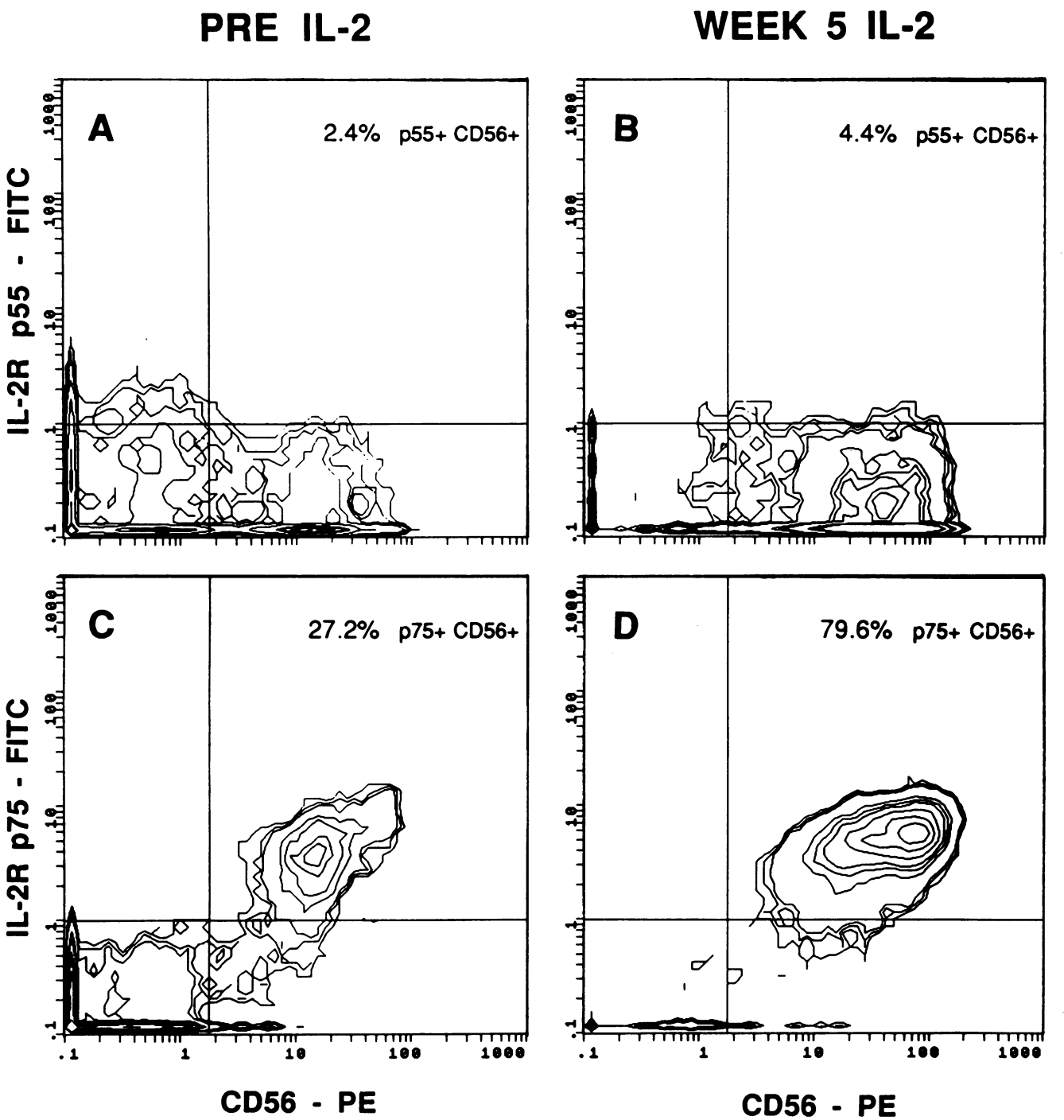

Figure 4. Expression of p55 and p75 IL-2R by peripheral blood CD56$^+$ NK cells from a representative patient before and during (week 5) continuous rIL-2 infusion of 4.5×10^5 U/m^2 per d. Nonadherent PBL were stained with PE-conjugated anti-CD56 mAb and either FITC-conjugated anti-IL-2R p55 (*A* and *B*) or FITC-conjugated anti-IL-2R p75 (*C* and *D*) and analyzed by flow cytometry. Actual values for percent double-positive cells is indicated in the upper right quadrant of each histogram.

on low dose IL-2 therapy showed very little proliferation to exogenous IL-2 in vitro (Table I). These expanded NK cells demonstrated almost no proliferation to IL-2 in concentrations of 10 pM (Table I), and anti-IL-2R p55 mAb failed to abrogate their proliferation to higher concentrations of IL-2 (not shown). Although some proliferation was consistently seen when expanded NK cells were cultured with 1 nM or 10 nM IL-2, the degree of proliferation was much less than that seen with CD56^{bright} NK cells freshly isolated from normal donors (Fig. 8). Indeed, the proliferative response of NK cells expanded in vivo closely resembles that of normal CD56^{dim} NK cells.

Serum concentrations of IL-2 during rIL-2 infusion. Serial measurements of serum IL-2 concentrations were carried out in 11 patients while they were receiving continuous infusions of IL-2. As shown in Fig. 9, IL-2 was undetectable (concentra-

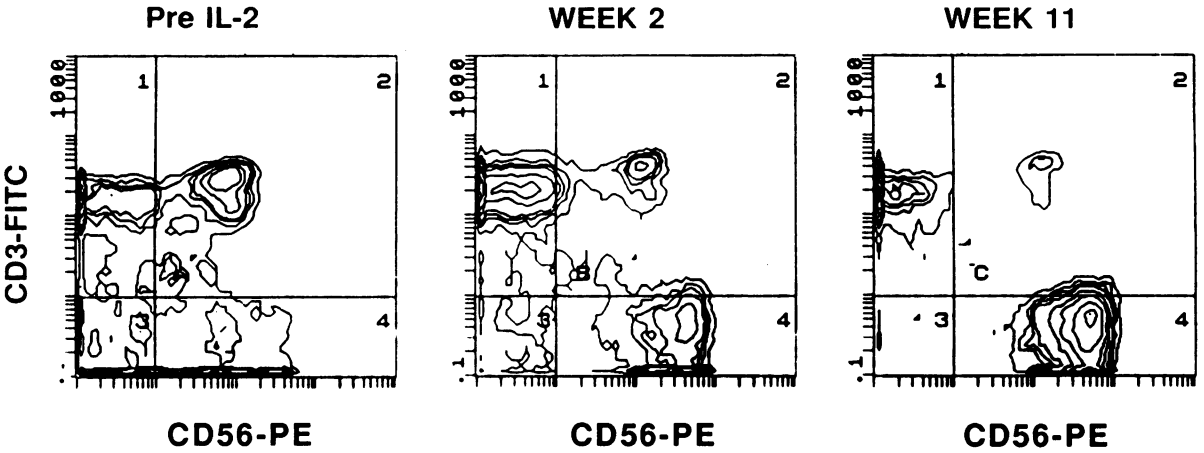

Figure 5. Relative decrease in peripheral blood CD56$^+$ CD3$^+$ cytotoxic T cells in a single patient receiving continuous rIL-2 infusion at 4.5×10^5 U/m^2 per d. Nonadherent PBL were obtained at the indicated time points during rIL-2 therapy, stained with PE-conjugated anti-CD56 mAb and FITC-conjugated anti-CD3 mAb, and analyzed by flow cytometry. Despite an unusually high percentage of CD56$^+$ CD3$^+$ cells in the peripheral blood of this patient before IL-2 therapy, this lymphocyte population failed to demonstrate any expansion during prolonged rIL-2 infusion, while CD56$^+$ CD3$^-$ NK cells were markedly increased (*lower right quadrant of each histogram*).

tion < 10 pM) in the serum of patients treated at the first dose level of 0.5×10^5 U/m^2 per d. At a dose of 1.5×10^5 U/m^2 per d, the mean serum IL-2 concentration during infusion was 25±25 pM, but one of three patients did not have consistently detectable levels. At a dose of 4.5×10^5 U/m^2 per d, the mean serum IL-2 concentration during infusion was 77±64 pM. At the highest dose used, 6.0×10^5 U/m^2 per d, there was greater variation in serum IL-2 concentration seen between patients. One patient had a mean concentration of 39±4 pM, a second patient had a mean concentration of 383±216 pM, and the third patient treated at this dose had a mean concentration of 776±336 pM. Of these three patients, the one with the lowest serum IL-2 concentration had the most profound increase in NK cell number during rIL-2 infusion, while the one with the highest serum IL-2 concentration showed the least increase in NK cells.

During the course of this trial, one patient failed to show any increase in NK cell number as seen in the majority of patients treated at similar doses. The patient was treated at 1.5×10^5 U/m^2 per d, yet had undetectable (< 10 pM) serum concentrations of rIL-2 throughout the infusion. This is in contrast to the two other patients treated at this dose, who showed measurable serum levels of rIL-2 and had more than a sevenfold increase in the absolute NK cell number during the 3 mo of rIL-2 therapy. A second patient demonstrated increases in NK cell number during the infusion, but at a rate which was significantly slower than the majority of patients. This patient was treated at the highest dose level (6.0×10^5 U/m^2 per d), and had serum rIL-2 concentrations that were the highest in the study, ranging between 419 and 1,230 pM. In addition, this patient uniquely exhibited a profound increase in both neutrophil and eosinophil counts, as well as a significant decrease in platelet count, suggesting that additional activation via the IL-2R p75 may have resulted in a multitude of stimulatory and inhibitory hematologic effects, possibly through the release of additional cytokines (33–35).

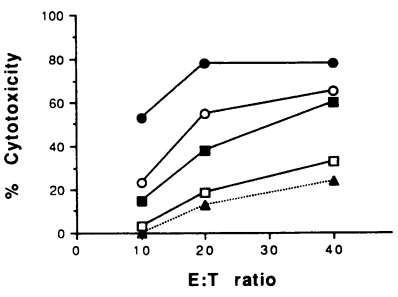

Figure 6. Lysis of NK-resistant target cells by lymphocytes expanded in vivo by rIL-2 infusion. Nonadherent PBL (64% CD56$^+$ cells) were isolated from the blood of a representative patient after 8 wk of rIL-2 infusion at 6.0 $\times 10^5$ U/m^2 per d, cultured for 18 h in either media alone or media containing 10 pM, 100 pM, 1 nM, or 10 nM rIL-2 as indicated, and tested for lysis of ^{51}Cr-labeled COLO 205 target cells at various E/T ratios. Results represent the mean percent lysis for triplicate samples. COLO target cells: --- ▲ ---, media; — □ —, 10 pM; — ■ —, 100 pM; — ○ —, 1 nM; — ● —, 10 nM.

The relationship between the IL-2 dose administered, serum IL-2 levels attained, and the calculated percentage of IL-2 receptor occupancy are shown in Table II. Since the K_d for the three classes of IL-2 binding sites differ by three orders of magnitude, there is a marked difference in the proportion of receptors occupied at each IL-2 dose level. Thus, the high affinity IL-2 receptor (K_d, 10 pM) is > 90% saturated when the IL-2 concentration is 100 pM, while < 10% of the intermediate affinity receptors are occupied at this concentration. However, 26% of the intermediate affinity receptors are occupied at a serum IL-2 level of 345 pM.

Discussion

We have investigated the immunologic consequences of maintaining near-physiologic serum concentrations of IL-2 for extended periods by prolonged intravenous infusions of the cytokine. The doses of rIL-2 administered could produce serum IL-2 concentrations (10–100 pM) that selectively saturate high affinity IL-2R heterodimers, as well as concentrations (~ 1 nM) that can engage isolated intermediate affinity IL-2R p75. Such therapy induced a profound and selective expansion of NK cells without any substantial change in the absolute number of T cells, B cells, or monocytes. A clear dose-response effect was seen between 1.5×10^5 U/m^2 per d and 4.5×10^5

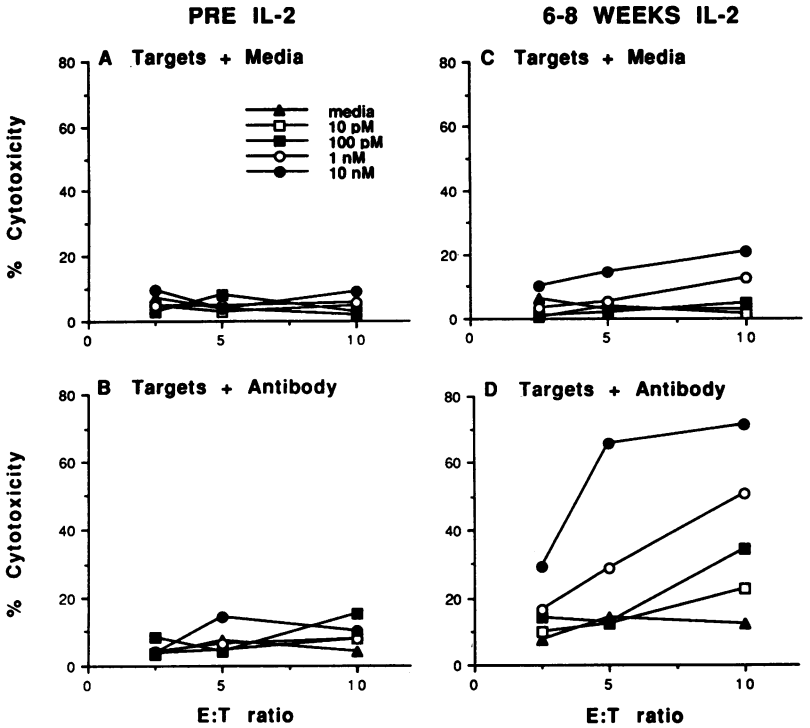

Figure 7. ADCC mediated by lymphocytes expanded in vivo by rIL-2 infusion. PBL obtained from three patients before (*A* and *B*) or after (*C* and *D*) IL-2 therapy were cultured for 18 h in media alone or media containing IL-2 at the indicated concentrations and then tested for cytotoxicity against P815 cells preincubated with media alone (*A* and *C*) or polyclonal rabbit anti-mouse lymphocyte serum reactive with P815 (*B* and *D*). PBL before therapy contained 14%, 13%, or 26% $CD56^+$ cells, respectively. PBL obtained after 6–8 wk of rIL-2 infusion at 6.0×10^5 U/m^2 per d contained 50%, 77%, or 79% $CD56^+$ cells. Each point represents the mean value of percent lysis by PBL from the three patients.

U/m^2 per d, which resulted in serum IL-2 concentrations of ~ 25 pM and ~ 77 pM, respectively. Increasing the IL-2 dose from 4.5×10^5 U/m^2 per d to a slightly higher dose of 6.0×10^5 U/m^2 per d did not result in further NK cell expansion. Serum IL-2 levels at this highest dose were variable, which may in part reflect poor renal excretion of IL-2 as a consequence of previous chemotherapy-induced nephrotoxicity. In two of three patients, this dose produced serum IL-2 levels sufficient to engage intermediate affinity IL-2R (K_d, 1 nM). Commensurate with this, the circulating NK cells from these patients demonstrated higher levels of NK cytotoxicity compared with NK cells from patients receiving the lower doses of IL-2. The observation that absolute peripheral blood NK cell numbers were actually lower after continuous infusion of 6.0×10^5 U/m^2 per d of rIL-2 than after infusion of 4.5×10^5 U/m^2 per d is intriguing. Activation of NK cells through IL-2R p75 causes the upregulation of certain NK cell adhesion molecules (22), and thus the higher serum IL-2 concentrations produced by the 6.0×10^5 U/m^2 per d dose might have induced the extravasation of expanded NK cells into tissues. Alternatively, secondary cytokines may have been produced by NK cells or monocytes stimulated through IL-2R p75, resulting in both systemic toxicity and an inhibitory effect on NK cell expansion. Of note, the number of circulating cytotoxic effectors induced by chronic low dose IL-2 therapy in some instances equaled the number of effectors that can be generated ex vivo using high dose IL-2 (15). Furthermore, this expanded NK population was evident for months and showed no sign of diminution throughout the course of IL-2 infusion. Despite the dramatic increase in NK cell numbers, NK cytotoxicity against tumor targets was only slightly enhanced at dose levels of 1.5×10^5 U/m^2 per d and 4.5×10^5 U/m^2 per d. Thus, prolonged low-dose IL-2 infusion

Table I. Proliferation of NK Cells Expanded In Vivo by rIL-2 Infusion

Patient	Week on IL-2	Proliferation (cpm) in the presence of IL-2 concentration of: None	10 pM	100 pM	1 nM	10 nM
1	6	231	149	448	4,351	N.D.
2	12	238	230	403	8,391	13,102
3	11	284	467	485	2,834	2,948
4	12	580	295	3,528	10,680	4,664

Total $CD56^+$ NK cells from the peripheral blood of patients receiving rIL-2 infusions of 6×10^5 U/m^2 per d for 6–12 wk were cultured in vitro with medium alone or medium containing various concentrations of IL-2 as indicated. Tritiated thymidine incorporation was determined as described in Methods. Results are mean cpm from triplicate wells. N.D., not done.

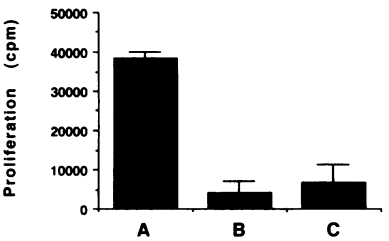

Figure 8. IL-2-induced proliferation of normal $CD56^{bright}$ NK cells, normal $CD56^{dim}$ NK cells, and NK cells expanded in vivo during rIL-2 infusions. Sorted $CD56^{bright}$ (*A*) and $CD56^{dim}$ (*B*) NK cell subsets from healthy donors or sorted total $CD56^+$ NK cells (*C*) from patients receiving rIL-2 infusions at 6.0×10^5 U/m^2 per d for 6–12 wk were isolated by flow cytometry and cultured for 5 d in medium containing 1 nM IL-2. Tritiated thymidine incorporation was determined as described in Methods. Data represent mean±SD of cpm for NK cells derived from four normal donors (*A* and *B*) or four IL-2 patients (*C*). Background cpm in the presence of medium alone (278±35 for *A*, 322±119 for *B*, and 245±27 for *C*) have been subtracted.

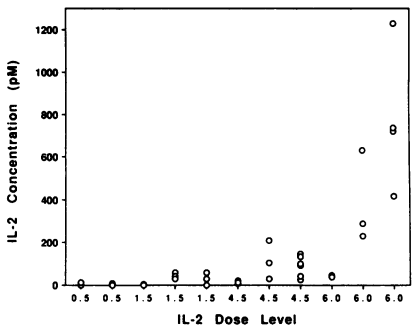

Figure 9. Serum rIL-2 concentrations in 11 patients receiving continuous intravenous infusions of rIL-2 for 90 d. Patients 1 and 2 received 0.5×10^5 U/m^2 per d; patients 3, 4, and 5 received 1.5×10^5 U/m^2 per d; patients 6, 7, and 8 received 4.5×10^5 U/m^2 per d; and patients 9, 10, and 11 received 6.0×10^5 U/m^2 per d of rIL-2. Each point represents a separate measurement. Serum levels were drawn randomly throughout the infusions. IL-2 was not detectable in the serum of any patient before the initiation of rIL-2 therapy.

can produce prominent NK cell expansion without activation of substantial NK cytolytic activity. However, brief in vitro incubation of expanded NK cells with concentrations of IL-2 sufficient to bind IL-2R p75 resulted in marked cytotoxicity against NK-sensitive, NK-resistant, and antibody-coated target cells. This is consistent with the expression of isolated intermediate affinity p75 detected on expanded NK cells by flow cytometry. The ability to expand the NK population at low, nontoxic doses of rIL-2 and then to activate highly specific antibody-directed target cell killing using a higher dose of rIL-2 (Fig. 7) suggests future strategies for immunotherapy.

Despite inducing up to 15-fold increases in the absolute number of circulating NK cells, low dose continuous infusion IL-2 caused no significant increase in B cells, CD56$^-$ CD3$^+$ conventional T cells, or CD56$^+$ CD3$^+$ MHC-unrestricted T cells in most patients. The selective NK cell expansion reported here was expected based on the known distribution of IL-2R on normal resting human lymphocytes (24). The vast majority of T cells and B cells express neither intermediate nor high affinity IL-2R binding sites until activated by antigen, and these lymphocytes are therefore largely unresponsive to exogenous rIL-2

Table II. Relationship between IL-2 Dose, Serum IL-2 Concentration, and IL-2R Occupancy

IL-2 dose	Serum (IL-2)	Percent receptor occupancy		
		High	Intermediate	Low
μg/m² per d	*pM*			
3.3	<10	—	—	—
10	25	71	2	<1
30	77	88	7	1
40	345	97	26	3

Calculation based on the K_d of each class of receptor: high affinity, K_d = 10 pM; intermediate affinity, K_d = 1 nM; low affinity, K_d = 10 nM (11).

Percent $R_{occ} = \frac{(\text{IL-2})}{K_d + (\text{IL-2})} \times 100$, where R_{occ} is the receptor occupancy.

(10, 24). In contrast, ~ 90% of NK cells express IL-2R p75 in the absence of IL-2R p55 and demonstrate enhanced cytolytic activity in response to concentrations (~ 1–10 nM) of rIL-2 that half-saturate intermediate affinity IL-2R. A novel subset of NK cells, identified by high density expression of the CD56 antigen, appears to express constitutively high affinity IL-2R p55/75 heterodimers, as well as an excess of isolated intermediate affinity IL-2R p75. These CD56^{bright} NK cells can proliferate to concentrations (~ 10–100 pM) of rIL-2 that saturate only high affinity IL-2R. In contrast, although brief increases in T cell number occurred in several patients during the infusion of rIL-2, the number of T cells generally returned to baseline levels within 2–3 wk; a cumulative increase in T cell number was not observed. Transient proliferation of T cells during rIL-2 infusion is not unexpected, since T cells are presumably activated intermittently in vivo after encountering cognate antigen. After antigen stimulation, T cells express high affinity IL-2R p55/p75 heterodimers and proliferate in the presence of picomolar concentrations of IL-2. However, high affinity IL-2R are downregulated on T cells 10–14 d after activation (10), and thus T cell expansion would not be expected to persist despite the continued presence of exogenous rIL-2. In contrast to activated T cells, activated NK cells do not appear to produce significant amounts of IL-2 (27, 36); thus NK cells are probably incapable of autocrine stimulation through the IL-2 pathway. However, the expanded NK cells in our patients demonstrate stable or even enhanced surface staining with IL-2R p75 mAb, and their cytolytic activity can be augmented by nanomolar concentrations of IL-2 in vitro. Thus, we have no evidence to suggest that these NK cells downregulate IL-2R p75 after prolonged exposure to exogenous IL-2.

Most previous cancer immunotherapy trials have involved very high doses of rIL-2 (~ 15–150 $\times$ 10^6 U or 1–10 mg) administered intravenously over several days or intermittently over several weeks (15, 16). The immunologic and clinical effects of high dose IL-2 therapy contrast markedly with those seen in our trial. High dose therapy commonly causes a profound lymphopenia followed by rebound lymphocytosis comprised of both T cells and NK cells (17, 37, 38). Most human NK cells (12–14) and monocytes (6) constitutively express isolated IL-2R p75 chains, and nanomolar concentrations of IL-2 can induce the secretion of several cytokines by these cells (6, 28, 36, 39). The T cell activation detected in patients receiving high dose IL-2 is likely caused by secondary cytokines derived from activated NK cells and monocytes, rather than directly caused by rIL-2 itself. Indeed, PBMC from patients receiving high dose IL-2 infusions demonstrate enhanced expression of mRNA for several cytokines, including IL-1, IL-5, IL-6, INF-γ and tumor necrosis factor, and elevated blood levels of these cytokines can be detected in some patients after IL-2 therapy (33–35). Furthermore, such cytokines may contribute to the severe toxicities of high dose IL-2 therapy, including hypotension (40) and the vascular leak syndrome (41). In this regard, the selective NK cell modulation and mild toxicity that we describe after infusion of low dose IL-2 may delineate the direct effects of IL-2 as opposed to the confounding effects of secondary cytokines.

Although selective NK cell expansion was expected in this trial, the physiologic basis for the markedly increased NK cell number during low dose rIL-2 infusion is not entirely clear. A preferential expansion of CD56^{bright} NK cells was seen in this study, as well as other IL-2 trials (42, 43). Since freshly isolated

CD56^{bright} NK cells from normal donors express high affinity IL-2R and proliferate vigorously to low concentrations of IL-2 in vitro, we initially speculated that CD56^{bright} circulating NK cells also selectively proliferated in vivo during IL-2 administration. However, several observations argue against this hypothesis. CD56^{bright} NK cells in the peripheral blood of healthy persons express little or no surface CD16, while the expanded CD56^{bright} NK cells from our IL-2 patients are largely CD16-positive. Furthermore, CD56^{bright} NK cells from normal persons express functional high affinity IL-2R, whereas most expanded CD56^{bright} NK cells appear to lack high affinity IL-2R, both by flow cytometric analysis and by in vitro functional studies using various concentrations of IL-2. Finally, CD56^{bright} NK cells from normal persons proliferate vigorously to low concentrations of exogenous IL-2 in vitro, while NK cells expanded in vivo demonstrate very little proliferation even in response to nanomolar concentrations of IL-2.

It is possible that both the CD56^{bright} and CD56^{dim} NK cells that accumulate in the peripheral blood of patients receiving prolonged infusions of low dose IL-2 arise from immature NK cell progenitors that can proliferate to low concentrations of IL-2. The regulation of human NK cell differentiation remains poorly understood. The earliest NK cell progenitors are almost certainly generated in the bone marrow, but it is uncertain whether these progenitors differentiate into fully mature effector cells in the marrow, or whether NK precursors leave the marrow and undergo differentiation elsewhere (27, 28). However, human and animal data indicate that immature NK cell progenitors can be stimulated with exogenous IL-2 to produce mature NK cells (44–47). Thus, the expanded NK cells in patients receiving prolonged infusions of low dose IL-2 could reflect proliferation of both mature peripheral blood CD56^{bright} NK cells and immature NK cell precursors possessing high affinity IL-2R. Of note, NK cells in these patients progressively expanded during continuous rIL-2 infusion and subsequently decreased in number after discontinuation of IL-2 treatment. Furthermore, reinstitution of IL-2 therapy resulted in recurrent NK cell expansion. Thus, the NK cell subset of lymphocytes appears to respond to IL-2 in vivo in a manner similar to the response of certain myeloid cell subsets to hematopoietic growth factors, such as erythropoeitin or granulocyte colony-stimulating factor (48). Extending this speculation further, NK cell production under physiologic conditions might also be regulated in a fashion more analogous to myeloid cells than to T and B lymphocytes. Monocytes, granulocytes, and erythrocytes are terminally differentiated cells, so a need for increased numbers of mature myeloid cells is met by increased marrow production of these cells. In contrast, adaptive immune responses require the clonal expansion of specific T cells and B cells, and these lymphocytes proliferate extensively after activation via their antigen-specific, clonotypic receptors. There is currently little evidence to suggest that NK cells have clonally distributed, highly variable receptors (27, 28), so that there is no obvious advantage to expanding individual clones of mature NK cells. Therefore, the demand for increased numbers of NK cells could be met by increasing marrow production from immature progenitors. This might also explain the apparently limited proliferative potential of the vast majority of mature peripheral blood NK cells (29, 36).

Our results indicate that small subpopulations of lymphocytes can be selectively and profoundly expanded by prolonged infusion of low dose rIL-2. This study strongly suggests that T cells express functional high affinity IL-2R for only a limited time after antigen activation, while NK cells can respond to IL-2 without known previous antigen activation and for an apparently unlimited period of time. Attempts must now be made to identify patients who might benefit from the expansion of these cytotoxic lymphocytes. For example, patients with certain hematologic malignancies have a high risk of recurrent disease despite myeloablative chemoradiotherapy and bone marrow transplantation. Recent studies have shown that prolonged low dose rIL-2 therapy can be well tolerated after either autologous or T cell–depleted allogeneic bone marrow transplantation (49). Intermittent infusions of higher doses of IL-2 might also be administered to activate expanded NK cells via IL-2R p75, in hopes of improving the antitumor activity of these cells. Furthermore, this approach could be combined with serotherapy using mAb reactive with tumor cells or viruses, since most of the expanded NK cells express the FcγRIII (CD16) and can mediate ADCC. Clinical trials to evaluate these approaches are currently underway. The design of our IL-2 regimen was based on the pharmacokinetics of IL-2, the binding affinities of the IL-2R, and the known distribution of IL-2R on lymphocyte subsets. Further rational clinical trials with IL-2 and other cytokines could be undertaken using fundamental principles of immunology and endocrinology rather than the paradigms of cytotoxic chemotherapy.

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