In Vivo Induction of the Lymphokine-activated Killer Phenomenon: Interleukin 2-dependent Human Non-Major Histocompatibility Complex-restricted Cytotoxicity Generated in Vivo during Administration of Human Recombinant Interleukin 2


ABSTRACT

The availability of purified human recombinant interleukin 2 (IL-2) has enabled clinical trials to test its in vivo effects. We report here the immunological effects of 7 consecutive days of IL-2 treatment given to 25 patients with cancer in a clinical Phase I study. Peripheral blood lymphocytes obtained from patients following therapy with IL-2 had enhanced proliferative responses to IL-2 and enhanced direct cytotoxic activity on K562 target cells. This lytic activity was further augmented by the addition of IL-2 during the 51Cr release assay. Fresh peripheral blood lymphocytes from some patients who had just completed treatment at the higher IL-2 dose levels were able to kill both the natural killer-resistant Daudi cell line and fresh tumor cells while pretreatment samples and peripheral blood lymphocytes from healthy controls were not. This lytic activity was best detected when IL-2 was present in the in vitro effector assay. These results demonstrate that the administration of IL-2 to patients with cancer induces a population of effector cells able to directly destroy natural killer-resistant target cells, when assayed in the presence of IL-2.

INTRODUCTION

Following 3–6 days of in vitro incubation with IL-2, human peripheral blood lymphocytes and mouse splenic lymphocytes are capable of lysing autologous and allogeneic tumor cells (1–4) and a variety of other targets, including normal lymphocytes (5, 6). These IL-2 expanded leukocytes consist of a heterogeneous population of effectors which mediate non-major histocompatibility complex restricted cytotoxicity (7, 8) which has been designated the LAK phenomenon (2). In vivo, these IL-2 expanded leukocytes can prevent growth of murine and human tumors when combined with additional IL-2 (9–12). Systemic administration of IL-2 at high doses to normal mice leads to the in vivo generation of cells able to mediate NRC of tumor targets (13). In vivo treatment of lymphocytes with IL-2 also increases natural killer activity (14–16) and induces T-cell proliferation (17). Systemic administration of IL-2 at high doses to tumor-bearing animals can induce regressions of a variety of tumors (18). The relative roles of in vivo activation of NRC and the direct action of γ-interferon (or other immune mediators released) in enabling these antitumor responses following high dose IL-2 therapy remains unclear (19).

The availability of purified recombinant human interleukin 2 (20) has enabled clinical testing over a wide dose range (12, 21, 22). Patients receiving IL-2 treatment have been reported to show changes in proliferative responses to IL-2, and changes in circulating LAK precursors, but circulating LAK cells could not be detected (21–23). We have examined the effects of IL-2 administered by either bolus or continuous i.v. infusions in a Phase I clinical trial for 25 patients with cancer. We report here the effects of in vivo IL-2 therapy on lymphocyte function measured in vitro in IL-2-induced proliferation assays and direct cytotoxicity assays on NK-sensitive and -resistant target cells.

MATERIALS AND METHODS

Clinical Study. The data used in this analysis were generated in a Phase I clinical trial of recombinant interleukin 2 given by i.v. bolus or continuous infusion (24). Twenty-five patients with refractory malignancies who met eligibility criteria were treated on this Phase I trial. Each patient received either 7 i.v. bolus injections or a 7-day continuous i.v. infusion of recombinant IL-2 (provided by the National Cancer Institute Biologic Response Modifiers Program via Hoffmann-La Roche Inc., Nutley, NJ) at doses ranging from 1 × 105 to 1 × 106 units/m²/day. All IL-2 units listed are based on the National Cancer Institute Biologic Response Modifiers Program standard IL-2 unit determination. Additional samples for cytotoxicity experiments were obtained from patients on a second clinical trial where patients received 4 cycles of IL-2 (1–3 × 106 units/m²/day) over 28 days. Each cycle consisted of 4 days of IL-2 given by continuous i.v. infusion separated by 3 days of observation.

Blood Samples. Blood samples for determination of immunological responses were obtained prior to, during, and after IL-2 therapy. PBL were isolated from patients’ heparinized blood samples by Ficoll-Hypaque centrifugation and were directly tested in all assays.

Cryopreservation of PBL from Healthy Control Donors and Patients. Normal donors were leukapheresed and their lymphocytes were cryopreserved by controlled rate freezing in 10% dimethyl sulfoxide and stored in liquid N₂. These samples were tested in parallel with freshly isolated patients’ PBL in all in vitro assays. On occasion the yield of patients’ cells was greater than that needed to perform all scheduled tests; these “excess cells” were also cryopreserved. Informed consent forms, approved by the University of Wisconsin Human Subjects Committee were obtained from patients participating in this clinical trial, and from control PBL donors.

Time Line for Obtaining Blood Samples. Fig. 1 presents the schedule of IL-2 administration as seven consecutive daily bolus (15 min) or continuous (24 h) i.v. infusions of IL-2, and the times at which blood samples were obtained for in vitro analysis from all patients. The “on-study” sample (sample 1) was obtained 4 to 14 days before the patient received IL-2. The “pretreatment” sample (sample 2) was obtained on the day IL-2 therapy began, just prior to the first IL-2 injection. Data from these first 2 blood samples were pooled for determination of baseline values with the exception of 3 patients where only 1 sample was...
available and data from this sample were used for the base-line determination. Blood sample 3 was obtained at the completion of one full dose of IL-2. This was at 1 h after the IL-2 dose for patients receiving daily bolus therapy and 24 h after starting IL-2 therapy for patients receiving continuous infusion of IL-2. Blood sample 4 was obtained just before the last bolus injection was given, or less than 1 h before the continuous infusion of IL-2 was stopped. Blood samples 5 and 6 were obtained 1 and 24 h, respectively, after the last dose of IL-2 had been infused. Additional samples were obtained for cytotoxicity experiments from patients on a subsequent clinical trial. These were obtained prior to initiating IL-2 therapy and 24 h following the stop of a continuous i.v. infusion of IL-2 (data in Table 3).

**Proliferative Assay.** PBL (1 x 10^5/well) were incubated in 0.2-ml U-bottomed microplates (Costar, Cambridge, MA) in HS-RPMI. Recombinant IL-2 (Hoffmann-La Roche) at a final concentration of 100 units/ml was added. Cultures were incubated at 37°C in 5% CO_2 for 54 or 126 h, then pulsed with 1 μCi 3H thymidine (New England Nuclear, Boston, MA) for 18 h. Cultures were harvested with a MASH harvester (Otto Hiller, Madison, WI) and counted by liquid scintillation. Median cpm of quadruplicate samples are reported.

**Cytotoxic Assay.** Target cells were labeled with 250 μCi 51Cr for 2 h at 37°C in 5% CO_2. Effector cells (5 x 10^5) in 0.1 ml HS-RPMI were serially diluted in 96-well U-bottomed microplate wells. Effectors were incubated at 37°C in 5% CO_2 for 1 h in either HS-RPMI alone or HS-RPMI containing 200 units/ml recombinant IL-2. Target cells (5 x 10^6) in 0.1 ml HS-RPMI were then added to quadruplicate test wells resulting in effector/target ratios of 50/1, 25/1, 12/1, and 6/1. Plates were centrifuged at 200 x g for 5 min and then incubated for 4 h at 37°C. The plates were then centrifuged at 500 x g for 10 min and harvested with a Skatron harvesting system (Skatron, Sterling, VA). Samples were counted for 1 min in a γ counter. Percentage of cytotoxicity was calculated by using the formula

\[
\% \text{ of cytotoxicity} = \frac{\text{Exp. cpm} - \text{Spon. cpm}}{\text{Max. cpm} - \text{Spon. cpm}} \times 100
\]

where Exp. is the experimental number of counts obtained from target cells incubated with the effectors; Spon. is the spontaneously released counts obtained with targets incubated in medium alone; and Max. is the maximum counts obtained with targets lysed with a 2% cetrimide solution (Sigma Chemical Co., St. Louis, MO). When a single percentage of cytotoxicity value is reported this represents the mean of the 4 values obtained with the 4 effector/target ratios tested, and reflects the interpolated percentage of cytotoxicity that would be expected for an effector/target ratio of 17.7/1. 51Cr release data from all 4 effector/target ratios were also converted to lytic units (25). One lytic unit was defined for these studies as the number of harvested effector cells resulting in 20% lysis of 5 x 10^5 target cells; lytic units are expressed as LU/10^7 effector cells harvested.

Target cells were the K562 erythroleukemia line, or the Daudi Burkitt's lymphoma line, obtained from Dr. A. Maluish of the National Cancer Institute Biologic Response Modifier Program (Frederick, MD), or freshly cryopreserved neoplastic cells obtained from leukemia marrow aspirates, malignant effusions, or minced solid tumor specimens.

**RESULTS**

**Patient Entry and Clinical Tolerance.** Patients were treated for 7 consecutive days with IL-2 by either continuous i.v. infusion or by i.v. bolus injections. Six patients received 10^3 units/m^2/day (3 as bolus and 3 as continuous infusion), and 6 patients similarly received 10^4 units/m^2/day, all without any detectable clinical toxicity. The next 2 patients received 10^2 units/m^2/day (1 each as bolus and continuous), and exhibited dose-limiting toxicities requiring cessation of their IL-2 treatment after approximately 4 days. Six patients then received IL-2 at 10^3 units/m^2/day, and 1 of the 3 bolus patients required cessation of IL-2 after 4 days. Five patients then received IL-2 at 3 x 10^4 units/m^2/day; both of the patients receiving continuous infusion of IL-2 required cessation of therapy after 4 days. The clinical tolerance, toxicity, and clinical laboratory evaluation of the 20 patients who tolerated 7 days of IL-2, and the 5 who required early stoppage of the IL-2 are beyond the scope of this immunological report and is published separately (24).

A blood sample was obtained from 3 of the 5 patients who stopped IL-2 prior to schedule approximately 24 h after stopping the IL-2, and was used in all in vitro assays for the posttherapy blood sample (Fig. 1, sample 6) assessment.

**Enhanced in Vitro Proliferative Response to IL-2.** Fig. 2 presents the 3-day proliferative responses to IL-2 for all five patients receiving 3 x 10^4 units IL-2/m^2/day. The “base-line” value was determined by averaging values obtained from the on-study and pretreatment blood samples (samples 1 and 2). The blood sample obtained after the first dose of IL-2 therapy for each patient was sample 3; the sample obtained 24 h after stopping the course of IL-2 treatment was sample 6. At the completion of one dose of IL-2, lymphocytes from all five patients mediated very poor proliferative responses to IL-2. In contrast, lymphocytes obtained 24 h after completion of IL-2 therapy showed markedly enhanced proliferative responses to IL-2; these responses far exceeded the base-line value for each patient. For these five patients the mean base-line response to

![Graph](https://example.com/graph2.png)

Fig. 1. Times at which blood samples were obtained for in vitro analysis relative to the 7 days of continuous i.v. IL-2 infusion, or 7 consecutive days of i.v. bolus IL-2 injections. Sample 1 was obtained from 4 to 14 days before in vitro IL-2 treatment. Sample 3 was obtained just prior to administration of IL-2. Data from assays of these 2 samples was averaged to obtain base-line values. The third blood sample was obtained just after completion of one dose of IL-2; this was at 1 h after the dose for patients receiving bolus injections, and at 24 h after starting the IL-2 for patients receiving the continuous infusion. Sample 4 was obtained just before stopping the IL-2 for the continuous infusion patients, or just before the last bolus injection. Samples 5 and 6 were obtained 1 and 24 h after stopping IL-2. In some instances it was possible to obtain additional samples.

![Graph](https://example.com/graph1.png)

Fig. 2. The 3-day in vitro proliferative response to IL-2 of 5 patients receiving IL-2 at 3 x 10^4 units/m^2/day. Base-line values are the mean of values obtained from blood samples 1 and 2. Sample 3 was obtained upon completion of one dose and sample 6 at 24 h after completion of therapy. Values reported are median cpm of quadruplicate wells.
IL-2 was 5,523 cpm while the mean response to IL-2 by PBL taken 24 h after stopping IL-2 therapy was 22,186 cpm.

The summary of IL-2 responses for 22 evaluable patients indicates that there was a significant drop in the response to IL-2 by PBL from sample 3, obtained following the first dose of IL-2 (Table 1). PBL from sample 6, obtained 24 h after completion of therapy, showed an IL-2-induced proliferative response in the 3-day assay that was significantly enhanced above base-line values. It is important to note that these proliferative responses were measured after 3 days of in vitro culturing with IL-2. Lymphocytes from healthy control individuals (26) and these same patients' pretreatment samples gave maximal responses to IL-2 after 6 days, not 3 days of in vitro culturing with IL-2. In contrast, PBL obtained after completion of the IL-2 therapy often reached peak responses by day 3 and then maintained these high levels through day 6. These data suggest that in vivo IL-2 initially causes a loss from the circulation of cells responsive to IL-2 in vitro; following the 4- to 7-day course of IL-2 treatment, the peripheral blood contains cells that show a more rapid, stronger proliferative response to IL-2, indicative of in vivo activation of IL-2-responsive cells.

Effect of in Vivo IL-2 on Lysis of K562 Target Cells by Fresh PBL. Fresh PBL populations contain effector cells that readily kill K562 target cells. This cell-mediated NRC is enhanced by adding IL-2 (100 units/ml) to the effector lymphocytes 1 h prior to (and during) their 4-h interaction with the 51Cr-labeled target cells. Fig. 3 presents data from the 6 separate cytotoxicity assays performed with fresh PBL obtained at the 6 study time points from one patient receiving 3 x 10^6 units/m2 IL-2 by daily i.v. bolus injections. At both of the base-line time points (samples 1 and 2) the in vitro IL-2 incubation enhanced the percentage of cytotoxicity at all effector/target ratios tested.

![Table 1 Three-day in vitro proliferative response to IL-2 enhanced by in vivo IL-2 administration](image)

<table>
<thead>
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<th>Lymphocytes obtained</th>
<th>Mean cpm [^{3}Hjthymidine]</th>
<th>P (\leq 0.002)</th>
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<tr>
<td>Blood sample 6</td>
<td>14,604</td>
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</table>

\(^{a}\) Based on 22 evaluable patients, one patient did not have a blood sample 3 and two patients did not have a sample 6 obtained for these assays.

\(^{b}\) P, based on paired t-tests.

![Fig. 3. Summary of the direct cytotoxicity on K562 targets assayed at 4 effector/target (E/T) ratios, using PBL from the 6 separate blood samples from 1 patient receiving IL-2 at 10^6 units/m2/day by the bolus route.](image)

This boosting effect by the in vitro IL-2 treatment was still clearly evident for PBL obtained just before and 24 h following the last IL-2 dose (samples 4 and 6). This indicates that the IL-2 treatment in vivo did not prevent this enhancement of cytotoxicity by in vitro IL-2 incubation; in fact, the IL-2 treatment in vivo enhances this in vitro effect of IL-2 (see below). Finally, the cytotoxicity by PBL obtained 1 h after each bolus injection (samples 3 and 5) was too low to detect any boosting effect by IL-2 in vitro.

Fig. 4 compares the destruction of K562 targets by fresh PBL from one patient receiving IL-2 at 3 x 10^6 units/m2/day by bolus infusions (Fig. 4A) and from one receiving IL-2 at 10^6 units/m2/day by continuous infusions (Fig. 4B). Again, the in vitro IL-2 incubation (beginning 1 h before the 4-h \(^{51}Cr\) release assay) enhanced the lytic activity of cells obtained from both patients prior to their in vivo IL-2 treatment, and enhanced the NRC mediated by cryopreserved lymphocytes from the control donor. In each of the 7 samples (Fig. 4A) or 6 samples (Fig. 4B) separate assays performed, the responses by the cryopreserved PBL from a control donor (all obtained from a single leukapheresis of a volunteer donor) remained relatively constant, with a small yet significant enhancement of cytotoxicity with the in vitro IL-2 incubation. The relatively minor variation in these values reflects the day to day variability in this assay system. In contrast, the multiple blood samples from these 2 patients (before, dur-
ing, and after IL-2 treatment) tested in parallel to these control specimens show dramatic fluctuations. Fresh cells obtained 1 h after a bolus injection of IL-2 (Fig. 4A, samples 3 and 5) had nearly lost all detectable cytotoxic activity, whether tested in the presence or absence of in vitro IL-2. In the absence of in vitro IL-2, NRC by PBL from samples 4 and 6 (24 h after the previous injection of IL-2) rebounded well above the pretreatment levels. When these same lymphocytes from samples 4 and 6 were incubated with IL-2 during the cytotoxicity assay, there was a dramatic enhancement of K562 target cell lysis. These results suggest that the cells responsible for this K562 killing may be leaving the peripheral circulation immediately after the bolus IL-2 therapy (samples 3 and 5), but return within 24 h (samples 4 and 6), at which time these cells demonstrate an enhanced cytotoxic response that is further augmented by in vitro incubation with IL-2.

The cells obtained from patients receiving continuous infusions of IL-2 show some similar effects (a representative patient's assays are shown in Fig. 4B). After the first 24 h of continuous IL-2 therapy (sample 3), the PBL mediate little or no detectible lysis of K562 targets, even when tested with IL-2 in vitro. However, 6 days into the IL-2 infusion, while IL-2 is still being infused, cells with direct lytic function are readily detected in the PBL (sample 4). These cells also demonstrate an enhanced level of cytotoxicity if they are incubated with IL-2 during the in vitro release assay. The levels of direct and IL-2-augmented cytotoxicity by PBL from the next two samples (samples 5 and 6, corresponding to 1 h and 24 h after the cessation of IL-2) are also greater than that mediated by PBL obtained prior to IL-2 treatment.

Table 2 presents the summary of all cytotoxicity assays on K562 target cells, with and without in vitro IL-2 incubation, using fresh PBL from all patients in this clinical trial who had evaluable base-line, sample 3 and sample 6 assays. For patients receiving >1×10^9 units/m^2/dose, the cytotoxicity mediated by lymphocytes from sample 6 was greater than that mediated in the base-line assays. This increase appeared more pronounced when IL-2 was added to this in vitro assay. The levels of cytotoxicity by cells from sample 3 were dramatically decreased for patients receiving the higher doses of IL-2 (>1×10^9 units/m^2/day); the degree of K562 destruction mediated by these samples was not detectably increased by the in vitro incubation with IL-2. Examining changes in percentage of cytotoxicity (both with and without the in vitro IL-2 incubation) from the base-line samples to samples 3 and 6 indicate that the cytotoxic function of lymphocytes obtained following the first dose of IL-2 (sample 3) was significantly less than that mediated by lymphocytes obtained before (12 versus 19% in medium, P < 0.05; 18 versus 30% in IL-2, P ≤ 0.05 for base-line samples) or 24 h after the full course of IL-2 treatment (12 versus 29% in medium, P ≤ 0.01; 18 versus 49% IL-2, P ≤ 0.01 for sample 6). Furthermore, the cytotoxic function mediated by cells from sample 6 was significantly greater than that from the base-line samples (29 versus 19% in medium, P ≤ 0.05; 49 versus 30% in IL-2, P ≤ 0.05). A least-squares regression analysis revealed a significant in vivo IL-2 dose effect on the change in percentage of cytotoxicity from base-line samples to sample 6 (F_{1,20} = 10.6, P = 0.004 without IL-2 incubation; F_{1,20} = 28.3, P ≤ 0.0001 with IL-2 incubation). This indicates that patients receiving higher doses of IL-2 had a greater increase in cytotoxicity following their in vivo IL-2 treatment.

Following the completion of the IL-2 infusions, lymphocytes obtained from these patients showed an enhanced augmentation in cytotoxic activity if given the in vitro IL-2 incubation. Fig. 5 shows the augmentation of cytotoxicity by lymphocytes obtained at base line and lymphocytes obtained from sample 6, and presents the data as change of percentage of cytotoxicity due to inclusion of IL-2 in the 4-h 51Cr release assay. Analysis of the difference in augmentation by IL-2 of PBL from base line and sample 6 demonstrates that the IL-2 incubation augments K562 destruction more for PBL from sample 6 than for base-line PBL. There was an average boost of 10.7% cytotoxicity with IL-2 incubation of PBL from the base-line samples (average of all doses), and 20.3% cytotoxicity with PBL obtained following in vivo IL-2 (sample 6). The difference of 9.6% cytotoxicity in the IL-2-enhanced cytotoxicity (sample 6 IL-2 augmentation minus base-line IL-2 augmentation for all 22 patients) was significant (P < 0.01). A least-squares regression analysis of these data demonstrated a statistically significant dose response relationship (F_{1,20} = 18.0, P < 0.0005), indicating that higher doses of IL-2 therapy in vivo enabled a more striking augmentation by the in vitro IL-2 incubation. The in vitro...
treatment with IL-2 is thus able to further augment the cytotoxic potential of PBL that have already been activated in vivo to express enhanced cytotoxicity in vitro against the K562 target.

In Vivo Generation of Effector Cells Able to Destroy Daudi and Fresh Tumor Targets. Sufficient lymphocytes were cryopreserved from six patients prior to IL-2 therapy and from sample 6 for these same patients to repeat the direct cytotoxicity testing of all these samples in a single in vitro assay (Fig. 6). This replicate assay for these PBL samples included the K562 target and the relatively NK-resistant Daudi target cell in order to determine if these populations demonstrated any direct destruction of NK-resistant targets (the LAK phenomenon) (Fig. 6B). Cryopreserved lymphocytes from these six patients were thawed simultaneously and assayed. The cells obtained following IL-2 infusions from four of these six patients (Patients B, C, D, and E) mediated potent direct destruction (>100 LU/10^7 cells) of the K562 target cells; this cytotoxic effect was boosted by the in vitro addition of IL-2. These same four patients’ lymphocytes from blood sample 6 were also able to kill the Daudi target cells (>10 LU/10^7 cells) when IL-2 was added to the effector cells in the ^51Cr release assay. In contrast, there was little detectable killing of the Daudi target cells by base-line time point lymphocytes or by PBL from the control donor, with or without the in vitro IL-2 incubation. This indicates that cells with LAK-like killer activity are generated in vivo during IL-2 treatment, but their in vitro detection in a direct cytotoxicity assay (not requiring ≥3 days of in vitro IL-2 activation) is facilitated by brief in vitro IL-2 incubation.

Due to the lack of additional posttherapy cells from patients on this Phase I protocol, these results have been replicated with fresh lymphocytes from additional patients receiving multiple 7-day cycles of IL-2 therapy in a subsequent Phase I study (4 days of continuous infusion IL-2 at 10^6 or 3 × 10^6 units/m^2/day followed by 3 days without IL-2 treatment, clinical results to be reported separately). Sufficient PBL were obtained from these patients to assay them against fresh tumor target preparations. Assays from representative patients after the first and second cycles of in vivo IL-2 therapy assayed on the K562 and Daudi cell lines and on target cells from a fresh ovarian carcinoma tumor preparation are presented in Table 3. The control donor’s and the patients’ fresh PBL kill the K562 target and this killing is enhanced by the inclusion of IL-2 in the 4-h assay. The control PBL mediate very low level cytotoxicity against the relatively NK-resistant Daudi cell line and do not kill the fresh tumor cells; the IL-2 incubation does not enhance this killing. In contrast, inclusion of IL-2 in the assay with the patients’ cells obtained following in vivo IL-2 enabled the PBL to kill the Daudi and fresh tumor targets.

DISCUSSION

In vivo administration of IL-2 to normal or tumor-bearing mice induces the activation of cells mediating the LAK phenomenon which can be detected by testing fresh spleen cells from these animals in an in vitro ^51Cr release cytotoxicity assay (13). Infusions of high dose IL-2, without any other treatment can induce regression of some pulmonary metastases and tumor implants (18). When IL-2 has been tested in clinical trials as a single agent, occasional (rare) patients have shown tumor regression (22); however, published data suggest that circulating “LAK cells” cannot be detected (21, 22).

In this report we document that cells able to destroy fresh tumor cells and Daudi cells can be found in the PBL of some patients who received IL-2 at doses of at least 1 × 10^6 units/m^2/day for 4-7 days. In order to reproducibly demonstrate this cytotoxicity by fresh PBL, it was necessary to include IL-2 in the culture medium for the short term assay. In previous clinical trials where in vivo generation of direct LAK activity was not demonstrated, IL-2 was not present in the short term ^51Cr release assay (17, 18). In the in vitro studies shown here (Fig. 6; Table 3), and in others we have performed with fresh PBL

<table>
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Table 3 In vivo induction of LAK cells capable of killing fresh tumor cells in vitro

Freshly obtained PBL from 2 different patients completing 1 or 2 7-day cycles of IL-2 (4 days constant infusion IL-2 at 10^6 units/m^2/day followed by 3 days without IL-2) were tested in a 4-h ^51Cr release assay (with or without the 1-h IL-2 preincubation) at 4 effector/target (E/T) ratios. Shown are the 2 highest ratios (50/1 and 25/1). Assays were run in HS-RPMI or HS-RPMI containing IL-2 at 100 units/ml. The fresh tumor target was a freshly cryopreserved ovarian carcinoma obtained from ascites.
from patients who received IL-2, the cytotoxicity by PBL on the Daudi target cells was best detected when PBL were obtained 24 hours after cessation of in vivo IL-2 treatment, and was dramatically greater when the \( ^{51} \)Cr assay was performed with IL-2 present in vitro. Little or no LAK activity was seen when PBL from control donors, or from these patients prior to their IL-2 infusions, were tested on Daudi target cells or fresh tumor targets in the presence of in vitro IL-2. These data indicate that the inclusion of IL-2 was not rendering the target cells more susceptible to lysis, rather that the IL-2 was affecting the function of the cytotoxic cells and suggest that LAK activity was activated by the in vivo IL-2 administration, as has been shown for IL-2 administration in animals (9). The need for in vitro IL-2 during the direct cytotoxic assay suggests that cells mediating the LAK activity are “dependent” on IL-2 for their in vitro cytotoxic function. This may be consistent with the relatively rapid loss of lytic activity by in vitro IL-2-activated effector cells (or IL-2-dependent T-cell lines) when they are placed in tissue culture medium not containing IL-2. The kinetics of effector cell generation in vitro with PBL obtained following IL-2 therapy in vivo suggests that a population of IL-2-responsive effector cells are activated in vivo and that subsequent reexposure to IL-2 in vitro rapidly activates and expands the effector cells that were “primed” in vivo.

In a clinical trial of IL-2 treatment, previously reported by Lotze et al. (21), in vitro proliferative responses by PBL were quantitated. They saw no consistent change in the proliferative response to phytohemagglutinin; however, there were consistent changes in the proliferative response to IL-2. Following initiation of in vivo IL-2 therapy, the proliferative response to IL-2 decreased. Subsequently, the response returned to baseline levels, and at times exceeded base-line values. Our data confirm that there is a statistically significant drop in the IL-2 response by PBL circulating after the first dose of IL-2. Furthermore, there is a rebound proliferative response by PBL obtained following cessation of 4 to 7 days of IL-2 therapy that exceeds the IL-2 response by PBL obtained before in vivo IL-2 administration. These changes in response are dependent on the dose of IL-2 given in vivo. We have reported that peak proliferative responses to IL-2 by PBL from control donors are observed after 6 days in vitro (26). In this study, the 6-day in vitro proliferative responses to IL-2 by control’s PBL, and by PBL from patients prior to IL-2 therapy, far exceeded their 3-day in vitro proliferative responses. In contrast, PBL obtained following the in vivo IL-2 treatment at higher doses showed 3-day proliferative responses that were comparable to the 6-day responses. This suggests that IL-2 exposure in vivo expands the number of cells that proliferate in response to IL-2, such that an earlier (“secondary-like”) response is seen when these PBL are stimulated with IL-2 in vitro. This is consistent with the increased number of IL-2 receptor-bearing lymphocytes detected by flow cytometry that are circulating in these patients following IL-2 therapy (21), and with our own unpublished in vitro results detecting more rapid proliferative responses to IL-2 by lymphocytes that had been first primed by in vitro culturing with IL-2.

In this present clinical trial, lymphocyte counts in the peripheral blood showed a significant drop after the first dose of IL-2 for patients receiving \( \geq 10^6 \) units/m\(^2\)/day (24). These counts returned to base line, or exceeded it, following the course of IL-2 therapy. We speculate that in vivo administration of IL-2 at high doses causes a rapid change in the membranes of lymphocytes triggered by IL-2. This membrane modification causes them to leave the circulation and, in all likelihood, remain transiently in lymphoid organs. This could account for the reports of increased lymphocyte numbers (and increased NRC mediated by them) in the spleens of mice receiving IL-2; there has as yet been no report of T-cell or LAK activity in the peripheral blood of these same animals immediately following IL-2 (27). Our present data would predict that murine lymphocytes would also leave the circulation following the initiation of IL-2. This postulate is also consistent with the documentation of rapid clearance from the circulation of i.v. infused lymphocytes that had been activated in vitro (28–30). Furthermore, we postulate that continued treatment with IL-2 in vivo causes the in vivo activation (and proliferation) of lymphocytes with lytic activity. This would parallel the murine reports (13), and is consistent with the present studies in which PBL from blood obtained at sample 4 [just prior to the last of 7 IL-2 bolus injections (Fig. 4A), or following 6 of the 7 days of constant infusion IL-2 (Fig. 4B)] show dramatically increased direct destruction of K562 targets over base-line values. Finally, once IL-2 therapy has stopped, we speculate that the membranes of IL-2-responsive lymphoid cells may return toward normal so that they are no longer preferentially sequestered in lymphoid organs. At this point there is a dramatic endogenous infusion of these activated lymphocytes into the peripheral blood, with a dramatic rebound in circulating lymphocyte counts, often exceeding 10,000 lymphocytes/mm\(^3\) (24).

Because initial findings have shown the combination of IL-2 and IL-2-expanded leukocytes may cause shrinkage of some human malignancies, and IL-2 alone can be effective in some animal tumor models, it is important to generate and test clinical protocols designed to optimize conditions for beneficial immune effects, yet simultaneously try to minimize treatment-related toxicity. The in vitro generation and expansion of LAK cells is costly and technically difficult. With IL-2 alone leading to the in vivo generation of effector cells mediating NRC of tumor targets, modifications of treatment schedules might enable sufficient activation of in vivo tumor-reactive lymphocytes to allow IL-2 alone to be effective without necessarily needing infusions of leukocytes that have been activated in vitro (22, 27). Additionally, IL-2 at doses currently shown to have a clinical antitumor effect when combined with in vitro-activated cells is associated with life-threatening toxicity (12, 22). If alternate administration schedules can be devised that can maintain the dramatic biological changes presently observed, yet not induce such severe toxicity, there may be potential for greater application of IL-2 therapy. Schedules of IL-2 administration like the ones tested here, which induce effector function by endogenous lymphocytes, would be expected to maintain the in vivo function of exogenously activated autologous (and allogeneic) IL-2-dependent cytotoxic cells (31–33). Clinical trials testing these possibilities are now in progress (34–35).

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