In Vivo Induction of Gamma Interferon and Tumor Necrosis Factor by Interleukin-2 Infusion Following Intensive Chemotherapy or Autologous Marrow Transplantation

By Helen E. Heslop, David J. Gottlieb, Alessandra C.M. Bianchi, Anthony Meager, H. Grant Prentice, Atul B. Mehta, A. Victor Hoffbrand, and Malcolm K. Brenner

Interleukin-2 (IL-2) therapy may improve immune reconstitution and reduce the risk of leukemic relapse in the setting of minimal residual disease by augmenting cytotoxic effector mechanisms directed at residual malignant cells. In addition, IL-2 in vitro promotes the release of cytokines including γ-interferon (γ-IFN) and tumor necrosis factor (TNF), which also possess antileukemic activity and can enhance granulocyte function. To determine if IL-2 infusion induces release of γ-IFN and TNF in vivo in sufficient quantity to mediate these effects, we have measured serum levels of these cytokines and secretion by lymphocytes obtained from patients receiving this cytokine in a phase 1 trial. Serum γ-IFN was undetectable pre-IL-2 and increased to 1.5 to 17 U/mL during IL-2 infusion (P < .05). Culture of patient lymphocytes for 48 hours produced 1.2 U γ-IFN/2 × 10^6 cells/mL pre-IL-2 rising to 50 U/2 × 10^6 cells/mL when the lymphocytes were obtained during therapy (P < .05). Lymphocyte subset analysis showed that both CD3+ and CD16+ cells secreted γ-IFN in response to IL-2. TNF secretion by lymphocytes also rose during IL-2 infusion from a mean of 5 U/mL to 14.4 U/mL (P < .01) although no rise was seen in serum levels. The material secreted by IL-2-stimulated lymphocytes is bioactive as addition of supernatants from lymphocytes obtained during IL-2 therapy to cultures of myeloid blasts significantly inhibited clonogenic growth. IL-2-induced secretion of these cytokines mediated this inhibition as it could be partially blocked by either anti-γ-IFN or anti-TNF antibodies. Preincubation of granulocytes with the same supernatants produced enhanced oxidative metabolism, measured by chemiluminescence in response to N-formyl-methionyl-leucyl-phenylalanine (FMLP). This effect also could be partially abrogated by anti-γ-IFN and anti-TNF antibodies. Therefore, secondary cytokine secretion may boost granulocyte function and contribute to the antileukemic effects of IL-2 infusion in patients following bone marrow transplantation or chemotherapy.

In THE SETTING of minimal residual hematologic malignancy, interleukin-2 (IL-2) therapy may be of value as adjuvant therapy to augment cytotoxic effector mechanisms directed at residual malignant cells. During the period of immunodeficiency that follows chemotherapy or bone marrow transplantation (BMT) for hematologic malignancy, IL-2 may be of additional value by increasing cell-mediated cytotoxic activity directed at virus-infected cells and by improving B-cell reconstitution, thereby reducing the high incidence of infection. This direct cytotoxicity of peripheral blood lymphocytes (PBLs) from BMT and chemotherapy recipients can be enhanced in vivo by infusion of IL-2.

We have investigated whether IL-2 may also generate a second antileukemic/antinfective mechanism in these patients. In normal donors, IL-2 induces secondary cytokine release in vitro from CD3+ and CD16+ cells. If effector cells stimulated by IL-2 infusion were able to secrete γ-interferon (γ-IFN) and tumor necrosis factor (TNF) in vivo following BMT or chemotherapy, then these cytokines could benefit the patient by further reducing the survival of myeloid blasts and by stimulating mature granulocyte function. However, patients recovering from autografts or chemotherapy have reduced numbers of CD8+, CD4+, and CD16+ lymphocytes and are regenerating these cells from progenitors that may have been damaged by chemotherapy; therefore, the target cells able to secrete cytokines in response to IL-2 may not be present. To investigate whether IL-2 induced secondary cytokine secretion occurs in vivo in these patients, we have measured TNF and γ-IFN secretion by lymphocytes obtained before and during IL-2 infusions, and measured cytokine levels in serum. We have also investigated whether the secondary cytokines produced are bioactive and able to mediate antileukemic activity and enhance granulocyte function.

MATERIALS AND METHODS

Trial design and patient detail. With the approval of the Hospital Ethical Practices Committee and after obtaining written informed consent, 13 patients aged between 18 and 67 years were given 21 courses of recombinant IL-2 (rIL-2). Ten suffered from acute myeloid leukemia (AML) and three from multiple myeloma. Six patients were treated after autologous BMT and seven following combination chemotherapy. Patients with relapsed AML received either MACE (m-amsacrine 100 mg/m² daily, cytarabine 100 mg/m² daily, etoposide 100 mg/m² daily) and methylprednisolone 1 g/m² twice daily all for five days, high dose cytarabine 1 g/m² twice daily for five days or mitoxantrone plus high dose cytarabine (mitoxantrone 10 mg/m² × 5 plus cytarabine 0.5 to 1 g/m² twice daily × 5). Patients receiving autografts for AML were in first or second remission and were conditioned with cyclophosphamide 60 mg/kg × 2 and 750 cGy single dose total body irradiation. Patients with myeloma received three to six courses of VAMP (vincristine 0.4 mg/m² daily, Adriamycin [doxorubicin, Adria Laboratories, Columbus, OH] 8 mg/m² daily and methyl prednisolone 1 g/m² daily all for four days) at monthly intervals before transplant and high dose melphalan 200 mg/m² as conditioning.

Recombinant IL-2 cloned in Escherichia coli with a specific activity between 1.7 × 10^6 U/mg and 3.2 × 10^6 U/mg protein was provided by Glaxo IMB, Geneva. Lyophilized material was reconsti-

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INDUCTION OF CYTOKINES DURING IL-2 INFUSION

Two patients, one following autograft and one following chemotherapy, received IL-2 infusions commencing 48 hours posttreatment at a dose of 50 μg/m2/d doubling every two days to 400 μg/m2 by day 10. The remaining 11 patients received fixed doses of the cytokine escalating from 160 to 700 μg/m2/d over three- to five-day courses commencing when the granulocyte count reached 0.5 x 109/L.

Clinical effects of this administration schedule have been described in detail elsewhere. Briefly, this regimen was well tolerated with major toxicity confined to hypotension (two courses) that responded rapidly to treatment cessation. No patient required Intensive Therapy Unit level support. IL-2 did not adversely affect marrow regeneration and the major biochemical abnormality was hypokalemia, which responded to supplementation. Immunomodulation as assessed by cytotoxic activity directed at natural killer (NK) and lymphokine activated killer (LAK) targets was induced in all cases. Clinical details are shown in Table 1.

Recombinant DNA-derived cytokines. Human IL-2 (specific activity 3.6 x 106 U/mg) granulocyte-macrophage colony stimulating factor (GM-CSF) (specific activity, 1.1 x 105 U/mg) IL-3 (specific activity, 1.4 x 105 U/mg) and γ-IFN (specific activity, 3.3 x 105 U/mg) were a gift of Glaxo 1MB. Human TNF (specific activity 6.63 x 104 U/mg) was a gift of Knoll Chemische Fabriken AG, Ludwigshafen, West Germany.

Phenotype. The surface antigen phenotype of PBLs was determined by indirect immunofluorescence using a microplate method. First layer antibodies used were RFT1 (CD5), RFT8 (CD8) (both gifts from Prof G. Janossy, Immunology Department, Royal Free Hospital, London), Leu M3 (CD14), Leu 11b (CD16) (both from Becton Dickenson, Mountain View, CA) and Tac (CD25) (a gift of Dr Hans Drexler, Haematology Department, Royal Free Hospital). Second layer antibody was fluorescein or rhodamine conjugated goat antimouse immunoglobulin (lg) (Southern Biotechnology, Birmingham, AL). One hundred to 500 cells were analyzed under an epimmunofluorescent microscope.

PBL culture. Sixty to 120 mL of blood were collected on two to four occasions from patients receiving IL-2 and from age-matched normal controls. The blood was defibrinated and diluted 1:1 with RPMI 1640 (Flow Laboratories) supplemented with L-glutamine (2 mmol/L) and penicillin/streptomycin (100 U/mL) and layered in 10 mL aliquots on 10 mL lymphoprep (Nyegaard, Oslo, Norway). After centrifugation at 400 g for 30 minutes, the mononuclear layer was washed, resuspended at 5 x 106 cells/mL in RPMI/10% fetal calf serum (FCS) and adherent cells were depleted by incubation at 37°C for 90 minutes in 50 mm Petri dishes (Nunc, Roskilde, Denmark). PBLs were cultured for 48 hours at 2 x 106 cells/mL in serum-free medium. Supernatants were filtered through a 0.22 U membrane and stored at -70°C before testing.

Lymphocyte subset depletion. PBLs were incubated for 15 minutes at room temperature with Leu 11b (CD16) or OKT3 (CD3) added in saturating amounts (1/20 dilution for each antibody). A one third dilution of rabbit complement (Buxted) was added and the cells incubated at 37°C for 45 minutes. A second round of complement-mediated lysis was performed after washing. The efficacy of depletion was assessed by phenotyping and the CD3−-populations contained <3% CD+ cells and the CD16−-populations <2% CD16+ cells.

γ-Interferon assay. γ-IFN was measured using a commercially available immunoradiometric assay (Sucrose IRMA Boots-CellTech Diagnostics, Slough, UK). The minimum detection limit of the assay was 1 U/mL γ-IFN.

TNF ELISA. Immunoassay was performed using a double antibody sandwich technique. The assay was calibrated with an interim TNF standard (86/659) and had a detection limit of one reference unit (25 pg TNF)/mL.

TNF bioassay. Murine L929 cells were incubated in 96-well microtiter plates (Nunc) at 4 x 104 cells/well in serum-free medium containing actinomycin D 1 μg/mL and dilutions of TNF standards of supernatants. After 24 hours viability was assessed by Amido Black staining with absorbance read at 620 mm in a Titerx multiwell plate reader (Flow Laboratories, Rickmansworth, UK). One unit of TNF activity was defined as causing 50% cytotoxicity. Internal standards of TNF were included in each assay.

Chemiluminescence. Granulocytes were separated on a discontinuous Percoll gradient as previously described and suspended at 2 x 105 mL in Hanks’ buffered saline solution (HBSS) without phenol red (pH 7.4) containing 2 x 10-8 mol/L Luminol (Sigma Chemical, St Louis) and 0.1% bovine serum albumin (BSA). Cells were incubated alone or with PBL supernatants from patients receiving IL-2 in vivo added at 10% final culture volume. In some cases supernatants were preincubated with a neutralizing anti-TNF monoclonal antibody provided by Dr A. Shaw, Glaxo IMB, Geneva (10 mg/mL with 2,600 neutralizing U/μg) added at 1 per 20,000 final dilution or a polyclonal anti-γ-IFN antibody provided by Dr Hans Drexler, Haematology Department, Royal Free Hospital.

Table 1. Clinical Details

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Disease</th>
<th>Treatment</th>
<th>IL-2 Dose µg/m2/d</th>
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<td>45</td>
<td>M</td>
<td>AML</td>
<td>ABMT</td>
<td>Escalating</td>
</tr>
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<td>Ara-C</td>
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<td>AML</td>
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<td>AML</td>
<td>Ara-C+ Mitozantrone</td>
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<td>ABMT</td>
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<td>MM</td>
<td>ABMT</td>
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<td>58</td>
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<td>55</td>
<td>F</td>
<td>MM</td>
<td>ABMT</td>
<td>600</td>
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</table>

Abbreviations: Ara-C, cytosine arabinoside; ABMT, autologous bone marrow transplantation; MM, multiple myeloma.
Boots-Celltech (27.5 mg/mL with 2,000 neutralizing U/mg) added at 1 per 100 final dilution. Aliquots were prewarmed to 37°C and chemiluminescence (CL) measured in a luminometer (LKB Wallace). FMLP (Sigma) $10^{-7}$ mol/L was added as a stimulus and peak light emission measured in millivolts at 10-second intervals. Results are expressed as maximum light emission after subtraction of background.

**Myeloid blast cell inhibition assay.** To determine if supernatants could affect blast colony growth, blasts were cultured in supplemented McCos medium containing 15% FCS and 0.3% agar. Colony-stimulating activity was provided in medium containing 15% FCS and 0.3% BMT was associated with the appearance of detectable colonies. Colony-stimulating activity was provided in medium containing 15% FCS and 0.3% BMT was associated with the appearance of detectable colonies. Colony-stimulating activity was provided in medium containing 15% FCS and 0.3% BMT was associated with the appearance of detectable colonies. Colony-stimulating activity was provided in medium containing 15% FCS and 0.3% BMT was associated with the appearance of detectable colonies.

Statistical analyses. Data or log-transformed data were analyzed using parametric methodology including paired and unpaired t testing. Statistical analyses were undertaken using the Rubycrete Beebstat program (London) running on a BBC Master computer.

**RESULTS**

**γ-Interferon in serum.** IL-2 infusion after chemotherapy and BMT was associated with the appearance of detectable levels of γ-IFN in serum. Figure 1 shows that serum γ-IFN reached peak levels by the first day of infusion and rapidly returned to baseline when infusion was halted. Figure 2 shows the serum γ-IFN levels before and during IL-2 infusion. γ-IFN was only detectable in serum from one of nine patients preinfusion but was present in all nine individuals studied during the course of infusion. The mean level of γ-IFN preinfusion was <1 U/mL: During infusion it reached 4 U/mL (P < .01) (Fig 1).

**γ-Interferon secretion by PBLs.** Circulating PBL contributed to the serum γ-IFN detected. Figure 3 shows that IL-2 infusion increased or induced γ-IFN production from circulating PBL in the majority of patients in both autograft and chemotherapy groups. Low levels of γ-IFN are spontaneously secreted by circulating PBL after autografting and these are increased in five of seven courses by up to two logs from a mean of 2.5 U/mL/2 x 10⁶ cells to a mean of 43 U/mL/2 x 10⁶ cells. Following chemotherapy, there was no spontaneous secretion of γ-IFN but infusion of IL-2-induced secretion from the PBLs of eight of ten patients and γ-IFN levels rose from a mean level of <1 U/mL to >1 U/mL. Overall secretion rose from a mean of 1.2 U/mL pre-IL-2 to 50 U/mL during IL-2 infusion (P < .01).

**Phenotype of cells producing γ-interferon.** Table 2 shows that autograft or chemotherapy patients pre-IL-2 have lower numbers of peripheral blood mononuclear cells (PBMs) and of CD4, CD8, and CD16 subsets than normal controls although the difference in subset numbers was only significant for CD4.

To determine whether IL-2-dependent γ-IFN production was produced exclusively by CD3+ T cells or by CD16+
During IL-2 infusion, granulocytes from patients 3 to 6 weeks postautograft. Preincubation with these supernatants resulted in an increased response to FMLP compared with granulocytes incubated in medium alone; preincubation in rIL-2 alone did not enhance the response. Preincubation of supernatant with anti-TNF antibody (Fig 5) reduced stimulation, and anti-γ-IFN monoclonal antibody had a similar effect (data not shown).7

Figure 6 shows the inhibition of myeloid blast cell clonogenic precursors, assayed by colony and cluster formation in semisolid media. Addition of PBL supernatants generated during IL-2 therapy produced the same modest but consistent inhibition of the growth of myeloid blast cell clusters and colonies (CFU-L) previously reported for TNF/γ-IFN.

Table 2. Phenotype of Patient Cells Pre–IL-2

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>WBC × 10⁹/L</th>
<th>PBM × 10⁹/L</th>
<th>CD4 × 10⁹/L</th>
<th>CD8 × 10⁹/L</th>
<th>CD16 × 10⁹/L</th>
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<td>Postchemotherapy</td>
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<tr>
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<td>6</td>
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<tr>
<td>13</td>
<td>1.6</td>
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<td>Postautograft</td>
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<td>5.9</td>
<td>3.8</td>
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<td>2.4</td>
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<tr>
<td>Mean ± SE pre–IL-2</td>
<td>(n = 13)</td>
<td>1.2 ± 0.26</td>
<td>0.28 ± 0.06</td>
<td>0.47 ± 0.18</td>
<td>0.12 ± 0.04</td>
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<tr>
<td>Mean ± SE post–IL-2</td>
<td>(n = 11)</td>
<td>3.7 ± 1.08</td>
<td>1.5 ± 0.62</td>
<td>0.71 ± 0.08</td>
<td>0.78 ± 0.23</td>
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<tr>
<td>Normal ± SE</td>
<td>(n = 14)</td>
<td>2.6 ± 0.35</td>
<td>1.4 ± 0.13</td>
<td>0.8 ± 0.09</td>
<td>0.3 ± 0.09</td>
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Table 3. γ-IFN and TNF Secretion: Effect of Depleting Lymphoid Subpopulations

<table>
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<tr>
<th>Condition</th>
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<th>CD16 Depleted</th>
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<tr>
<td>γ-IFN</td>
<td>34 ± 18 U/mL</td>
<td>9 ± 5 U/mL</td>
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<tr>
<td>TNF</td>
<td>99 ± 50 U/mL</td>
<td>38 ± 46 U/mL</td>
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</table>

Fig 4. Spontaneous TNF secretion by PBLs. PBLs were obtained from patients preinfusion and during IL-2 infusion and cultured as described in the Materials and Methods section. Solid bars represent the log of spontaneous secretion in units per milliliter pre–IL-2 infusion and shaded bars secretion during IL-2 infusion.
CFU-L were reduced from a mean of 223 (±68 SEM n = 5) to a mean of 133 (±61 SEM) when cultured in the presence of supernatant. Again the effects of patient PBL supernatant could be reduced if the supernatant was preincubated with anti-γ-IFN or anti-TNF antibody (Fig 6), demonstrating that the inhibition was mediated by secondary cytokines.

Although both TNF and γ-IFN contribute to the actions of the IL-2-induced supernatants, they do not mediate all of the observed effect. When supernatants of PBLs cultured with IL-2 in vitro were preincubated with neutralizing antibodies to both γ-IFN and TNF, neither the inhibitory effects of the supernatant on CFU-L growth nor the stimulatory effect on granulocyte CL were completely abrogated. These data suggest that other cytokines may be induced by IL-2 and contribute to the observed effect.

**DISCUSSION**

We have shown that infusion of IL-2 into patients whose marrow is regenerating following chemotherapy or BMT for hematologic malignancies induces or enhances in vivo production of secondary cytokines affecting the growth and function of both normal and malignant myeloid cells.

The regimen these patients received produced immunomodulation as measured by changes in circulating lymphocytes with an initial lymphopenia followed by a rebound lymphocytosis with significant increases in NK and CD25 subsets. Significant increases in NK and LAK activity were also generated. These immunostimulatory effects are similar to those found by others using similar or slightly higher doses of IL-2 in patients with solid tumors. However, these patients differed from those in our series in that they had a normal lymphocyte subset profile pre-IL-2. The ability of IL-2 to induce secondary cytokine secretion in vivo was not examined in any of these studies.

Although an increase in serum γ-IFN has previously been documented in patients receiving higher doses of IL-2 as treatment for solid tumors, these patients had normal mononuclear cell subset profiles pre-IL-2 infusion. In contrast, patients recovering from intensive chemotherapy or autograft have reduced numbers of circulating CD4, CD8, and CD16 lymphocytes (Table 2) and following autografting, a reversed CD4/CD8 ratio. In addition, the regenerating lymphoid cells are derived from progenitors that may have been damaged by exposure to intensive chemotherapy. Despite this abnormal phenotypic pattern, circulating lymphocytes were able to secrete cytokines in response to IL-2 infusion, producing increased amounts of γ-IFN and TNF. Circulating CD3+ and CD16+ cells both produce secondary cytokines as depletion of either subset alone only partially abrogates secretion, whereas depletion of both subsets almost completely blocks secretion.

Although increased γ-IFN production by PBLs was accompanied by the appearance of γ-IFN in patient serum, serum TNF levels did not rise. This discrepancy between TNF production by PBL and detection in serum may relate to the instability of TNF in the circulation due to rapid polymerization by serum proteases. In addition, TNF effects in some cases may be mediated by an integral transmembrane form, so TNF content in PBL supernatants may underestimate the biologic effect of TNF. Therefore, local secretion of TNF or production of the cell surface form may not be reflected by an increase in serum TNF.

IL-2-dependent γ-IFN and TNF release may have three benefits in the setting of minimal residual disease following BMT or chemotherapy for haematologic malignancy. First, TNF and γ-IFN can directly reduce the survival of myeloid blasts and thereby may help to eliminate residual leukemic cells, reducing the risk of relapse. Second, TNF and γ-IFN are active against virus-infected target cells and thereby may help to limit viral reactivation/dissemination.
during the period of immunosuppression that follows BMT/chemotherapy. These further increments in host antileukemic and antiviral effector function occur over and above the enhancement of cell-mediated cytoxicity induced directly by IL-2. Finally, TNF and γ-IFN enhance granulocyte, monocyte, and B-cell function, and thereby promote humoral mechanisms important in resistance to bacterial, fungal, and protozoal disease.

All the patients treated had received either autografts or chemotherapy, but the potential benefits could also apply to recipients of marrow allografts. One potential hazard in such an application is that IL-2 could enhance the growth of mature alloreactive T cells in the graft inoculum and thereby accelerate and augment graft-versus-host disease (GVHD). However, murine studies suggest that IL-2 does not induce GVHD after T-cell depletion of allografts, so that further exploration of the combination of T-cell-depleted allografts and IL-2 infusion may be justified.

IL-2-induced secondary cytokines such as TNF, may have adverse effects. Infusion of IL-2/LAK cells is associated with the capillary leak syndrome and induction of adult respiratory distress syndrome; anti-TNF antibodies can protect against their development. Although these effects of TNF may be particularly harmful in the posttransplant situation where pneumonitis is a significant cause of morbidity and mortality, the levels of IL-2 infused were associated with significant lung problems in only one of 21 courses of IL-2 administered.

There is already circumstantial evidence to suggest that secondary cytokine release in vivo has a significant clinical impact. One of the major differences between autologous/allogeneic BMT and chemotherapy is that the incidence of relapse is higher after chemotherapy. One factor contributing to these observed differences may be the different pattern of cytokine production following BMT or chemotherapy. Thus, activated CD3+ and CD16+ cells spontaneously secreting TNF and γ-IFN circulate in the peripheral blood after both autologous and allogeneic BMT. Spontaneous cytokine secretion does not occur following chemotherapy alone.

We have shown that infusion of IL-2 in the setting of minimal residual disease increases production of cytokines of potential therapeutic benefit and increases LAK function. If this activated pattern of PBL recovery is important in eradicating minimal residual disease, then we would predict that the effects of infusion of IL-2 in inducing cytokine secretion in patients recovering from chemotherapy and enhancing production after autologous BMT will reduce the risk of relapse.

ACKNOWLEDGMENT

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In vivo induction of gamma interferon and tumor necrosis factor by interleukin-2 infusion following intensive chemotherapy or autologous marrow transplantation

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