INDUCTION OF ENDOGENOUS CYTOKINE-mRNA IN CIRCULATING PERIPHERAL BLOOD MONONUCLEAR CELLS BY IL-2 ADMINISTRATION TO CANCER PATIENTS

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The lymphokine IL-2 plays a central role in immune regulation. Recent clinical trials have shown that when administered systemically either alone, or in combination with lymphokine-activated killer cells, IL-2 can cause regression of metastatic tumors in some patients with a variety of otherwise refractory cancers. To evaluate the mechanism of in vivo action of IL-2, as well as the toxicity associated with its administration, we have studied the in vivo cytokine-mRNA expression of circulating PBMC in cancer patients undergoing treatment with high dose IL-2. Before IL-2 administration, we found low level or no evidence of cytokine-mRNA expression in PBMC. After IL-2 infusion, circulating PBMC showed enhanced proliferative activity and contained significant levels of mRNA for TNF-α and IL-6 as well as mRNA for the p55 IL-2R, Tac, but no mRNA coding for granulocyte-monocyte-CSF and TNF-β [lymphotokine]. IL-1β mRNA was expressed at very low levels in circulating PBMC after IL-2 infusion. Each of these cytokine-mRNA was, however, inducible in vitro by stimulation of PBMC with IL-2 alone. The results of these in vivo studies suggest that IL-2 may be a physiologic inducer of TNF and IL-6 which, because of their pleiotropic effects, may be important endogenous signals in the body's immune response and account for some of the physiologic changes seen in patients receiving high dose IL-2.

IL-2 is a lymphokine produced by lectin- or Ag-stimulated T cells that plays a central role in immune regulation (1). The administration of IL-2 alone or in conjunction with the adoptive transfer of LAK cells is capable of mediating the regression of established metastases from a variety of experimental murine tumors and from selected patients with advanced cancer as well (2, 3). The mechanism by which IL-2 mediates tumor regression in vivo is unknown and the pathogenesis of the toxic side effects associated with IL-2 administered is not well understood. In vitro activation of human PBMC by IL-2 results in the generation of LAK cells and in the production of several cytokines (4–10) including TNF-α, TNF-β, LT, and IFN that may be involved in the anti-tumor effects of IL-2. However, these in vitro effects may not reflect the activity of PBMC in vivo in patients administered i.v. IL-2. We have, therefore, performed studies of cytokine mRNA expression of circulating PBMC in cancer patients before and after receiving high dose rIL-2 as a part of therapeutic protocols, and we have found that the administration of high dose IL-2 to cancer patients leads to the in vivo induction of mRNA coding for TNF, IL-6, and Tac in circulating PBMC.

MATERIALS AND METHODS

Human PBMC. Peripheral blood or leukocytes were obtained from leukophereses performed before and after infusion of high dose rIL-2 to cancer patients. The PBMC were isolated by Ficoll-Hypaque density sedimentation. The purified PBMC were either directly processed for RNA isolation or in vivo studies or cultured for 1 to 2 days in RPMI 1640 medium (2% heat inactivated human AB serum) with or without the addition of rIL-2 (1000 U/ml) for in vitro studies. RNA isolation and Northern blot analysis. Total cellular RNA was isolated from both cultured and uncultured PBMC by the guanidinium isothiocyanate/cesium chloride ultracentrifugation method (11). For Northern blots, RNA (25 μg) was electrophoresed on 1% agarose/2.2 M formaldehyde gels followed by blot transfer to nitrocellulose paper. Prehybridization of the blots was carried out for 4 to 6 h at 42°C in 50% formamide, 5 × SSC, 3 × Denhardt's solution, 10 mM Tris (pH 7.4), and 20 μg/ml sheared and denatured salmon sperm DNA. Filters were hybridized for 16 to 20 h at 42°C in the above buffer containing 10% dextran sulfate. After hybridization with 32P-labeled probes, the filters were washed three times in 1 × SSC and 0.25% SDS at 55°C for 90 min, followed by a high stringency wash in 0.1 × SSC at 55°C to 60°C for 30 min. Autoradiography was carried out at −70°C by using Kodak XAR 5 (Kodak Corporation, Rochester, NY) films with intensifying screens. The quality, equivalent loading, and uniform RNA transfer were assured by ethidium bromide staining of the gels before and after the Northern transfer and rehybridization of RNA blots with 32P-labeled human γ-actin probes (12).

Human probes used were: TNF-α (13), the 800-bp EcoRI cDNA fragment; LT, (14), the 950-bp EcoRI cDNA fragment; GM-CSF (15), the 800 bp Xhol cDNA fragment; IL-6 (G. G. Wong and S. C. Clark, Genetics Institute), the 1.2-kb Xhol cDNA fragment; Tac (16), the 937-bp EcoRI cDNA fragment; IL-1β (D. Carter, Upjohn Company, Kalamazoo, MI), the 810-bp Ps1 cDNA fragment; IFN-γ (17), the 960 bp HindIII/BsrE II cDNA fragment; γ-actin cDNA, pHHγ-A-1 (12). The fragments were excised, purified, and 32P-labeled by random priming method (18).

Proliferation assay. PBMC were plated in replicate at a density of 10^5 cells/well in 96-well tissue culture plates and pulsed with 1 μCi of [3H]thymidine (80 μCi/ml, NEN, Boston, MA) for 4 h at 37°C. The cells were then harvested and the amount of incorporated [3H] thymidine was assessed by liquid scintillation counting. The viability of cells was determined by trypan blue exclusion.

RESULTS AND DISCUSSION

Six patients with cancer (three with melanoma and three with colon cancer) were evaluated for in vivo and in vitro cytokine mRNA expression before and after IL-2 administration. Patients were initially treated with high dose rIL-2 (kindly supplied by the Cetus Corporation, 0022-1767/89/1432-0736$02.00/0
Emeryville, CA) alone given i.v. at a dose of 10^6 U/kg body weight every 8 h for 4 to 5 days. Leukaphereses were performed 1 to 2 days before IL-2 treatment and 2 days after cessation of IL-2 treatment. PBMC were analyzed for cytokine mRNA expression and also cultured with IL-2 to generate LAK cells in vitro. Previous results have shown that IL-2 treatment induces an initial drop in PBMC within the first 24 h, followed by a rebound lymphocytosis resulting in PBMC numbers to two six times the pretreatment values. 1 to 2 days after discontinuing IL-2 (3).

PBMC obtained pre- and post-IL-2 infusion were immediately pulsed for 4 h at 37°C with [3H]thymidine for in vitro culture (3,560 dpm/10^6 cells for pre-IL-2 PBMC vs 16,285 ± 2,742 dpm/10^6 cells post-IL-2 PBMC). For mRNA expression studies, PBMC obtained before or after IL-2 treatment were processed for RNA isolation either in vivo or after in vitro culture for 1 to 2 days in the absence or presence of 1000 U/ml IL-2 (in vitro studies). The Northern blots were successively hybridized with 32P-labeled cytokine cDNA probes. The data from six patients are summarized in Table I and a representative profile of lymphokine-mRNA expression in one patient is presented in Figure 1.

Endogenous expression of TNF (panel A) and IL-6 mRNA (panel D) was induced in PBMC after in vivo IL-2 infusion (lane 4), whereas the mRNA were undetectable before IL-2 treatment (lane 1; panels A and D). Low levels of TNF-mRNA were detectable in the basal state of pre- and post-IL-2 PBMC on longer exposure of the x-ray films. LT, which is partially homologous to TNF and shares nearly identical biologic activities (19-22), was undetectable in vivo in PBMC obtained from both pre- and post-IL-2 infused patients (lanes 1 and 4; panel B). GM-CSF, which is a T lymphocyte-derived hemopoietic growth factor (23) remained undetectable and IL-1β mRNA which is a monokine of multiple biologic activities (24), was detectable at very low levels in vivo (lanes 1 and 4; panels C and E, respectively). Likewise there was no in vivo expression of IFN-γ mRNA either before or after IL-2 treatment (data not shown). Although we were unable to detect by Northern blotting significant expression of IL-1β mRNA in circulating PBMC after IL-2 infusion, high level expression of IL-1β mRNA consistent with previous reports (24) was detectable by in situ hybridization (data not shown). There was no inherent defect in patient PBMC to express LT, GM-CSF, and IL-1β mRNA because the cells were functionally responsive in vitro to IL-2 for the expression of these mRNA (lanes 3 and 6; panels B, C, and E) as well as TNF and IL-6 mRNA (lanes 3 and 6; panels A and D). However, when the cells were cultured in the absence of IL-2, the cytokine-mRNA were not detectable (lanes 2 and 5). The in vivo expression of TNF and IL-6 mRNA was less compared with the in vitro expression. For all the cytokines, the size of the induced mRNA was consistent with the size of the mRNA reported by others. The 2.1-kb actin-mRNA was expressed almost uniformly in all lanes (panel G).

The high affinity IL-2R complex is composed of at least two distinct IL-2-binding subunits, the 55-kDa α-subunit, also called CD 25 or Tac Ag, and the more recently identified 70-kDa IL-2R β-subunit (25, 26). Resting LAK precursor cells most often do not express the Tac Ag and therefore lack the high affinity IL-2R, but are induced to express the Tac Ag after activation (27, 28). Endogenous Tac-mRNA (two transcripts, 3.5 and 1.5 kb) were seen in PBMC-stimulated in vivo with IL-2 (panel F), suggesting in vivo activation. However, in two patients low level expression of Tac-mRNA occurred before IL-2 treatment (Table I).

It thus appears that the administration of IL-2 to humans leads to the in vivo induction of mRNA expression coding for TNF, IL-6, and Tac in circulating PBMC. Previous studies have shown increase in Tac + mononuclear cells and serum IFN-γ levels in patients treated with IL-2 (29). In the present study the failure to detect IFN-γ mRNA in vivo may be related to the time of measurement after cessation of IL-2 treatment in vivo or to the small percentage of cells expressing IFN-γ mRNA in response to IL-2. Under normal circumstances, little or no TNF is present within PBMC. Although spontaneous production of cytotoxic activity consistent with TNF by PBMC from normal donors and cancer patients has been previously reported (30, 31), other studies have failed to detect such activity in the sera of tumor-bearing patients (32-34). In our study we have detected low or no endogenous basal levels of TNF mRNA in patients before IL-2 treatment. It is likely that blood monocytes and natural cytotoxic cells in cancer patients after IL-2 treatment may be in an activated state, and possibly such cells when in contact with tumor cells may locally synthesize and release TNF. The notion that toxicity of TNF may be localized is consistent with the recent finding of Kriegler et al. (35) who have identified a novel cytotoxic transmembrane form of TNF. Thus TNF may be an important lymphokine involved in the antitumor effects of IL-2. Another mechanism by which TNF could act is by its capacity to induce a number of positive and negative regulators including IL-1, IL-6, and MHC Ag expression. IL-6 has multiple biologic effects including T and B cell activation, up-regulation of MHC expression, and hemopoietic stimulation (36). It has been demonstrated recently that administration of high dose TNF to cancer patients induces circulating biologically active IL-6 (37). Because TNF may be a direct activating signal for IL-6, it is likely that endogenous production of IL-6 seen in IL-2-treated patients may be related to the initial production of TNF.

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**Table 1**

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<th>Cytokine-mRNA</th>
<th>In vivo expression</th>
<th>In vitro expression</th>
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*In two patients only post-IL-2 samples were available.

**Abbreviations**

- **IL-1β**: Interleukin-1β
- **IFN-γ**: Interferon-γ
- **TNF**: Tumor necrosis factor
- **IL-6**: Interleukin-6
- **GM-CSF**:Granulocyte macrophage colony stimulating factor
- **LAK**: Lymphokine-activated killer cells
- **MHC**: Major histocompatibility complex
- **Tac**: The 55-kDa α-subunit of IL-2 receptor

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* IL-1β mRNA levels were very low compared with TNF- or IL-6 mRNA levels.
The in vivo induction of TNF and IL-6 may also be associated with some of the side effects characteristic of IL-2 therapy including fever, chills and the appearance of hepatic acute phase reactants (3).

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REFERENCES


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