Circulating Cytokines in Patients with Metastatic Cancer Treated with Recombinant Interleukin 2 and Lymphokine-activated Killer Cells

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ABSTRACT

Treatment with recombinant interleukin 2 and lymphokine-activated killer cells (rIL-2/LAK) has produced a clinical antitumor effect in preliminary human trials. The cytokines γ-interferon (IFN-γ), tumor necrosis factor α (TNF-α), and tumor necrosis factor β (TNF-β, lymphotoxin) have potent in vitro antitumor activity and some clinical toxicities similar to interleukin 2 (IL-2)/LAK. This study sought to determine whether these cytokines were detectable in sera of IL-2/LAK-treated patients. Ten patients were treated with a protocol of 5-day i.v. rIL-2 bolus priming (10⁶ units/kg, every 8 h), followed by 5 daily phereses with harvested lymphocytes cultured in vitro to generate LAK, and 5 days of rIL-2 bolus with infusion of LAK cells. Five patients were treated with a protocol modified to a 3-day rIL-2 prime and 6-day continuous infusion rIL-2 (3 x 10⁶ units/m²/day) with infusion of LAK cells. Serum specimens were obtained before, 4 h, 12 h, and 24 h after rIL-2 or LAK cell administrations. IFN-γ was detected by enzyme-linked immunosorbent assay, TNF-α by WEHI 164 bioassy or enzyme-linked immunosorbent assay, and TNF-β by WEHI 164 cell bioassay. During the prime, few patients manifested in vivo detectable serum cytokines: IFN-γ, three of ten, 5-day prime (1.03 ± 0.46 ng/ml), and zero of five, 3-day prime; TNF-α, one of ten, 5-day prime, and one of three, 3-day prime; TNF-β, one of ten, 5-day prime. The supernatants of in vitro LAK generation cultures had detectable levels of cytokines at 24 h which increased progressively until culture harvest at Day 4 (IFN-γ, 2.56 ± 0.34 ng/ml; TNF-α, 356 ± 110 pg/ml; TNF-β, 8.2 ± 4.4 units/ml). The highest levels of in vivo serum cytokines occurred following LAK cell infusion and were more often elevated in patients receiving rIL-2 by bolus than by continuous infusion: IFN-γ, four of six bolus, zero of three continuous infusion; TNF-α, six of six bolus (maximum 679 pg/ml) versus two of three continuous infusion (maximum, 106 pg/ml). LAK cells in vitro responded with cytokine release on stimulation by tumor cell lines (IFN-γ, 0.88 ± 0.06 ng/ml; TNF-α, 426 ± 16 pg/ml; TNF-β, 0.64 ± 0.06 units/ml). In summary, this preliminary study has detected circulating cytokines in sera of patients receiving IL-2/LAK therapy. The greatest cytokine elevations followed LAK cell infusion. Further studies are warranted to delineate the relative effects of various therapeutic regimens on cytokine levels and possible correlation with toxicity and clinical response.

INTRODUCTION

Incubation of PBL³ with IL-2 results in the generation of non-major histocompatibility complex-restricted cytolytic activity for fresh human tumors in vitro. This activity is mediated by a subpopulation of cells which have been termed LAK cells (1). These effector cells have recently been identified as activated natural killer cells (2-4). Murine models have demonstrated the potent antitumor activity of combination rIL-2 and LAK cell therapy (5). The successful application of this phenomenon to adoptive immunotherapy of human cancer has recently been reported (6). Notable toxicity including fever, chills, and decreased pulmonary function has been associated with infusion of activated killer cells (7). Similar toxicity has been noted with rIL-2 infusion alone, and at maximal tolerated doses capillary leak syndrome, hypotension, and multisystem dysfunction occur as well (8).

The cytokines TNF-α, IFN-γ, and TNF-β (lymphotoxin) have each been described to possess antitumor activity, as well as some toxicities at highest doses similar to those seen with rIL-2/LAK therapy (9-11). It has also been shown that IL-2 activation of PBL results in the expression of mRNA for IL-1 α and β, IFN-γ, and TNF-α (12). It is possible that the clinical antitumor effects of rIL-2/LAK therapy or the associated toxicity is mediated in part through the release of these cytokines. The goal of this preliminary study was to determine whether detectable elevations of cytokines occurred during IL-2/LAK cell immunotherapy and to identify optimal sampling time points for further study.

MATERIALS AND METHODS

Treatment Protocol. Following informed consent, patients with metastatic renal cell carcinoma, melanoma, or colon carcinoma were treated according to the National Cancer Institute Extramural rIL-2/LAK Working Group protocols at the University of California-San Francisco Cancer Research Institute (13). Patients were treated according to the protocols illustrated in Fig. 1. For the protocol in Fig. 1A, Days 1 to 5 comprised the initial 5-day priming course of i.v. bolus rIL-2 (100,000 units/kg every 8 h; Cetus Corp., Emeryville, CA). On Days 8 to 12, PBL were harvested by 4 h of lymphopheresis (COBE 2997 pheresis unit; COBE Laboratories, Inc., Lakewood, CO). In vitro LAK activation was performed as previously described (14). Briefly, mononuclear cells were separated using Ficoll-Hypaque (800 x g for 20 min). Following two washes, the cells were incubated with 1500 units/ml of rIL-2 in complete medium consisting of RPMI-1640 supplemented with 100 µg/ml of penicillin, 100 µg/ml of streptomycin, 300 µg/ml of glutamine, and 2% human AB-positive converted plasma (Whittaker-M. A. Bioproducts, Walkersville, MD). The incubation was performed at a cell concentration of 1.5 x 10⁶/ml in sealed roller bottles at 37°C. On Days 12, 13, and 15 the LAK cultures were harvested (Day 3 or 4 of culture). The LAK cultures were washed extensively with balanced salt solution, resuspended in 5% human AB-positive plasma (American Red Cross, Washington, DC) in saline, and reinfused on the 3 separate days, with a rest day between the second and third cell infusions (Day 14). Concomitantly, on Day 12, i.v. bolus rIL-2 administration was resumed (100,000 units/kg every 8 h). The first dose of rIL-2 was given immediately following the initial cell infusion. The rIL-2 was continued for 2 days after the last cell infusion (Days 12 to 16).

The second series of patients were treated according to the modified protocol in Fig. 1B. This regimen incorporated the following changes: 3 rather than 5 days of initial bolus rIL-2 priming; pheresis for 4 rather than 5 days; and the second course of rIL-2 given as a continuous infusion (3000 units/kg/h). The LAK cell infusion schedule remained the same.

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3 The abbreviations used are: PBL, peripheral blood lymphocytes; IL-1, interleukin 1; IL-2, interleukin 2; rIL-2, recombinant interleukin 2; LAK cell, lymphokine-activated killer cell; TNF-α, tumor necrosis factor α; TNF-β, tumor necrosis factor β; IFN-γ, γ-interferon; ELISA, enzyme-linked immunosorbent assay; rHuIFN-γ, recombinant human γ-interferon; rTNF-α, recombinant tumor necrosis factor α; rHuLT, recombinant human lymphotoxin; NK, natural killer.

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Patient Population. Patients 1 to 10 were treated with the regimen in Fig. 1A (bolus rIL-2 including a 5-day priming course). The malignancies of these patients included 7 with melanoma (one who did not receive LAK cells, priming phase only analyzed), 1 with renal cell carcinoma, and 2 with colon carcinoma. Five patients were treated with the regimen in Fig. 1B (3 days of bolus rIL-2 priming with continuous infusion of rIL-2 during LAK culture; 3 with melanomas, 2 with renal carcinomas).

Sampling of Peripheral Blood and Culture Supernatants. Peripheral blood was drawn into Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) and allowed to clot at room temperature. The tubes were centrifuged at 800 x g for 10 min, and the serum was aspirated and stored in Cryuves (Wheaton Scientific, Millville, NJ) at -20°C for later analysis. Sampling was performed before and 0.5, 2, 3, and 5 h after the morning IL-2 dose on Days 1, 3, and 5 of the initial priming phase or after LAK infusions 1 and 3 (Fig. 1) and at 8 h after completion of the entire treatment course. All time points could not be obtained for analysis on each patient. Supernatant samples of the LAK cultures were obtained at 24-h intervals during the 4 days of in vitro incubation and frozen at -20°C in vials for later analysis.

Assay for γ-Interferon. IFN-γ was assayed by an IFN-γ-specific ELISA with a detection limit of 0.40 ng/ml as previously described (15). By this assay, rHuIFN-γ has a specific activity of 1 x 10⁹ units/ml.

Assay for Tumor Necrosis Factor α. TNF-α in patient serum was determined by a specific ELISA with a detection limit of 40 pg/ml (16). The assay for in vitro production of TNF-α was performed using a fibrosarcoma cell line (WEHI 164, clone 13 (17)). Cells were seeded in 96-well microtiter plates (Costar, Cambridge, MA) (2 x 10⁵ cells/well) and grown at 37°C in 100 ml of medium consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 30 mg/ml of gentamicin, 0.1 nmol/liter of glutamine, and 10% heat-inactivated fetal calf serum. LAK culture supernatant were serially diluted and added to the wells in the presence or absence of rabbit antiserum to rTNF-α. After 20 h of culture, cell survival was assessed colorimetrically using a vital dye. The detection level of this assay was 0.10 pg/ml for recombinant human TNF-α.

Assay for TNF-β. To determine the concentration of TNF-β, all TNF-α activity was first neutralized by adding a 3-fold excess of rabbit polyclonal anti-TNF-α antibody for at least 30 min prior to assay. This antiserum has a neutralizing titer of 2.9 x 10⁴ L929 units/ml. The polyclonal anti-TNF-α antibody for at least 30 min prior to assay. This analysis reached statistical significance.

RESULTS

Priming Phase. In vivo circulating cytokines were detected during the rIL-2 priming phase of the protocol in 6 of 15 patients. For IFN-γ, there was detectable elevation in 3 of 10 patients on Day 5 of the 5-day rIL-2 priming, but no elevations were seen prior to that point. Fig. 2 demonstrates the time course of IFN-γ induction following Day 2 rIL-2 bolus in the three patients who developed detectable serum levels. IFN-γ was not detected in patients primed for 3 days. Serum TNF-α levels were elevated in 1 of 10 patients primed for 5 days and in 1 of 3 patients primed for 3 days. Increased levels of TNF-β were seen in 1 of 10 patients given a 5-day prime (not the same patient who had elevated TNF-α levels). TNF-β levels were not measured during the 3-day prime.

LAK Cell Infusion Phase. The highest elevation in serum cytokine levels occurred following LAK cell infusion (Fig. 3).
The cytokine response to LAK cell infusion varied according to treatment group. Those patients who received bolus rIL-2 during the cell reinfusion more frequently manifested elevations in cytokine levels than those who received continuous infusion. Four of 6 bolus patients had elevated IFN-γ levels as opposed to 0 of 3 continuous infusion patients. Most patients had elevated serum levels of TNF-α following LAK cell infusion: 7 of 7 bolus patients and 2 of 3 continuous infusion patients. The magnitude and duration of TNF-α elevation were more pronounced in the bolus patients. For bolus patients, 16 of 41 samples had elevated TNF-α levels as opposed to 3 of 20 for continuous infusion. The highest TNF-α level in the bolus group was 697 pg/ml versus 106 pg/ml for continuous infusion. None of the bolus patients had elevated TNF-β levels during cell reinfusion. Continuous infusion patients were not assayed for TNF-β levels.

The difference in serum cytokine levels for bolus versus continuous infusion patients is unlikely to be due to the number of cells administered during the reinfusion phase. The bolus patients received 6.29 x 10¹² ± 8.63 x 10¹² (mean ± SEM) cells during the first LAK cell infusion, and 2.14 x 10¹³ ± 3.69 x 10¹³ cells during the third LAK cell infusion. The patients treated with a 3-day prime and continuous infusion rIL-2 received 5.19 x 10¹⁰ ± 9.07 x 10¹⁰ cells with the first reinfusion, and 1.96 x 10¹¹ ± 3.71 x 10¹⁰ with the third. The total cells received over three infusions were 1.01 x 10¹¹ ± 1.37 x 10¹⁰ and 9.51 x 10¹⁰ ± 1.59 x 10¹⁰.

At the completion of therapy IFN-γ and TNF-α were undetectable. However, 4 of 6 patients given a 5-day prime did have detectable levels of TNF-β.

LAK Culture Supernatants. In vitro elaboration of cytokines in the LAK cultures was evident in 14 of 15 patients. Detectable levels of all three cytokines were present after 24 h of culture and increased during the duration of culture until cell harvest on Day 4 (Fig. 4). Elevated IFN-γ levels were generated in 6 of 9 patients given a 5-day prime and 5 of 5 patients given a 3-day prime. Eight of nine 5-day-prime and five of five 3-day-prime patients had elevated TNF-α levels in their culture supernatants. There were no significant differences in the level of in vitro cytokine production between the 3-day- and 5-day-prime groups by t test. Five of nine 5-day patients had elevated TNF-β levels in culture. TNF-β levels were not tested in the supernatants of 3-day-prime patients.

In Vitro LAK Cell Cytokine Release. A possible mechanism for the elevation in serum cytokine levels could be the release of cytokines from LAK cells following in vivo stimulation with tumor cells. K562 (NK sensitive), DAUDI (LAK sensitive), or COLO (LAK sensitive) target cells were added to in vitro LAK cultures from one patient (Table 1). Addition of K562 or COLO stimulated release of TNF-α and IFN-γ into LAK cell supernatants. Only DAUDI cells were capable of inducing low levels of TNF-β; however, DAUDI cells themselves have been noted to secrete this cytokine, which has not been observed in the other cell lines used.

DISCUSSION

The characterization of rIL-2/LAK therapy has, to date, focused on the cytolytic potential of the LAK cells for fresh human tumors. These functional studies have been the basis for the development of murine and human protocols for the treatment of metastatic cancer. The role of rIL-2/LAK therapy as an in vivo stimulator of other cytokines, or the dependence on other cytokines for the in vivo effects of the therapy has not been delineated.

We have demonstrated in vivo generation of elevated levels of cytokines in the serum of patients receiving rIL-2/LAK therapy. This appears to be a cumulative dose-related phenomenon. Administration of parenteral rIL-2 every 8 h as a bolus also leads to in vivo generation of LAK activity. In previous work we have demonstrated that this also appears to be a cumulative dose-related phenomenon (2). During the priming phase of rIL-2/LAK therapy there is no detectable production of IFN-γ, TNF-α, or TNF-β after the initial dose of rIL-2. Elevated TNF-β levels after 3 days were seen in only one patient. Increased levels of IFN-γ were seen in 3 patients after 5 days of bolus therapy. There was no measurable induction of IFN-γ earlier in the treatment course or in patients primed for only 3 days.

The highest serum levels of circulating cytokines were detected following the infusion of LAK cells. The first LAK cell infusion invariably represented the largest of the three cell infusions and produced the highest levels of circulating cytokines. The cells were washed and suspended in infusion medium just prior to administration which minimized the likelihood of infusion of supernatant cytokines. Considering the low frequency of elevated cytokines during rIL-2-alone therapy in the priming phase, it is possible that the reinfused LAK cells are a major source of cytokines measured in vivo. Cytokines are possibly released due to in vivo stimulation of the LAK cells by tumor.

TNF-α and IFN-γ levels differed in kinetics after cell infusion. TNF-α levels were highest 30 min after the completion of cell infusion (the first point measured) and fell to base line by 5 h. IFN-γ levels peaked at 3 h. This difference may represent

### Table 1

<table>
<thead>
<tr>
<th>Target cell</th>
<th>IFN-γ (ng/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>TNF-β (units/ml)</th>
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</thead>
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<tr>
<td>K562</td>
<td>0.88 ± 0.06</td>
<td>426 ± 16</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>DAUDI</td>
<td>0.90 ± 0.08</td>
<td>185 ± 19</td>
<td>1.30 ± 0.02</td>
</tr>
<tr>
<td>COLO</td>
<td>0.68 ± 0.01</td>
<td>613 ± 13</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.39</td>
<td>93 ± 14</td>
<td>0.50 ± 0.26</td>
</tr>
</tbody>
</table>

* PBL from a representative patient were incubated with 1500 units/ml of rIL-2 in a 96-well microplate at a concentration of 10⁶ cells/ml. After 3 days, the medium was removed, and target cells (10⁶/well) were added. Supernatants were removed after 24 h and assayed for IFN-γ, TNF-α, and TNF-β as described in "Materials and Methods."

* Mean ± SEM of 6 determinations.

M. A. Palladino, unpublished observation.
more prolonged or delayed in vivo production of IFN-γ.

The administration of bolus rIL-2 in conjunction with the infusion of LAK cells generated more frequent and higher in vivo cytokine levels than did continuous infusion rIL-2. It is possible that LAK cells require higher blood levels of rIL-2 to produce these products than are provided by continuous infusion and that the transient high levels of rIL-2 may be more efficient at eliciting cytokine production.

The in vitro LAK generation cultures elaborated elevated levels of cytokines demonstrated by supernatant analysis, as has been noted previously in normal donors (18). Activation for 4 days with rIL-2 resulted in a serial increase in IFN-γ levels. TNF-α was also measured, reaching greater than 350 pg/ml by Day 4. Bioassay for TNF-β revealed a less pronounced serial increase, but levels did double between 1 and 4 days of culture. It is of interest that the one patient who failed to demonstrate any elevated in vitro cytokine levels in the 5-day prime group was a responder to therapy (>50% reduction in measurable tumor burden).

The elevated levels of multiple cytokines in these patients may mediate, in part, the antitumor effects as well as toxicity. Recent studies have indicated that TNF-α can enhance LAK cell as well as cytotoxic T-cell activity, suggesting that the immunoregulatory function of TNF-α may be an important component of its in vivo mechanism of action (19, 20). It has also been demonstrated that NK cells will release TNF-α when exposed to NK-sensitive targets (21). We have shown LAK cultures release cytokines in response to NK-resistant as well as NK-sensitive cell lines.

The goal for IL-2/LAK therapy is to dissociate toxicity while maintaining clinical efficacy. Our initial experience is that there is less cytokine elevation during continuous infusion rIL-2, and reports by other indicate that continuous infusion rIL-2/LAK therapy may produce clinically meaningful antitumor response at reduced toxicity (22). The current analysis has utilized serum samples for demonstration of elevated circulating levels of cytokines at the end of the priming phase and following LAK cell infusion. Further studies are now in progress by the National Cancer Institute Extramural IL-2/LAK Working Group. These studies will provide data for bolus and continuous infusion IL-2/LAK-treated patients to assess potential correlation of circulating cytokine levels with toxic manifestations and clinical antitumor effect.

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REFERENCES

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