Adoptive Immunotherapy of Human Cancer: The Cytokine Cascade and Monocyte Activation following High-Dose Interleukin 2 Bolus Treatment


ABSTRACT

Serum concentration kinetics of γ-interferon (IFN-γ), neopterin, 2'-5' A synthetase and tumor necrosis factor α were determined in five cancer patients undergoing adoptive immunotherapy with high-dose interleukin 2 (IL-2) bolus infusion and lymphokine-activated killer cells according to the National Cancer Institute, NIH protocol.

In all cases a significant increase of these markers was observed after IL-2 treatment. This suggests that the antitumor effect of high-dose IL-2 bolus administration may be in part mediated by activation of a cascade of endogenous cytokines including IFN-γ and tumor necrosis factor α. After IL-2 bolus injection, the kinetics of neopterin was similar but delayed when compared to that of IFN-γ. The specific immune responses of lymphocytes and monocytes were studied by measuring soluble IL-2 receptor and β2-microglobulin during adoptive immunotherapy with rIL-2 and LAK cells.

INTRODUCTION

Adoptive cancer immunotherapy involves (a) the selective in vitro activation of lymphocyte subpopulations (LAK cells) from a patient with tumor by rIL-2 and (b) their subsequent administration to the ill. LAK cells exhibit the in vitro capacity of lysing fresh tumor cells in a non-major histocompatibility-restricted manner.

In our study we followed the National Cancer Institute adoptive immunotherapy protocol, which has been devised for the management of patients with advanced metastatic malignant disease (mainly melanoma and renal and large intestine carcinomas), after the failure or lack of standard therapy (1-3). The protocol comprises three phases: (a) In phase I, high doses (4.5 or 6 x 10⁶ units/m² in 3 doses/day) of rIL-2 are administered by i.v. bolus injection; (b) In phase II, after 2 days of rest, patients undergo daily leukapheresis for 4 days. Autologous lymphocytes are collected and cultured with rIL-2 to generate LAK cells; (c) In phase III, patients are reinfused with LAK cells, together with high dose rIL-2, in three daily administrations for 5 days.

Phase I is associated with considerable toxic effects, including fever and rigors, nausea, vomiting, diarrhea, stomatitis, rash, oliguria, hypotension, and massive fluid retention (3-5); these reactions subside in phase II but may reappear during the reinfusion period. It has been shown that in vivo infusion of IL-2 produces several biological effects, ranging from induction of LAK cell activity markers (i.e., IL-2 receptor) (7) to alterations in the differential leukocyte count (i.e., eosinophilia, neutropenia, etc.) presumably caused by the secretion of other cytokines (8).

We have recently reported a marked increase in serum levels of soluble IL-2 receptor and β₂-microglobulin during adoptive immunotherapy with rIL-2 and LAK cells (9).

In the present study, we have monitored the levels of IFN-γ, TNF-α, and neopterin in the sera of five patients undergoing adoptive immunotherapy with high dose rIL-2 bolus injection and LAK cells. We have also evaluated the 2'-5' A levels in four of these patients. Additionally, we measured the concentrations of these substances in the supernatants of LAK cells and stimulated lymphocytes or monocytes from a normal donor.

MATERIALS AND METHODS

Patients. Five patients were submitted to adoptive immunotherapy in the present study: three had melanoma; one had colon carcinoma; and one had carcinoma of the adrenal cortex. Their Karnofsky performance status ranged from 70 to 100. Oliguria, hypotension, tachycardia, and weight gain due to fluid retention were the most common toxic effects. The patients' response to therapy shall be reported separately.

Adoptive Immunotherapy Protocol. The adoptive immunotherapy protocol consists of three phases (Fig. 1).

Phase I (days 1-5) involves the i.v. administration of human rIL-2 (Roche Laboratories, Nutley, NJ) by means of a central catheter. The IL-2 dosage was 4.5 x 10⁶ units/m² in 3 doses/day for patients 1, 2, and 3, and 6 x 10⁶ units/m² for patients 4 and 5.

Phase I, after a 48-h rest period, patients underwent daily leukapheresis (days 8-11). Harvested cells were centrifuged through a Ficoll-Hypaque density gradient. The resulting lymphocyte preparations were cultured in RPMI 1640 (Flow Laboratories, Glasgow, United Kingdom) containing 2% heat-inactivated AB serum, penicillin (10 units/ml), streptomycin sulfate (10 ng/ml), gentamicin sulfate (5 ng/ml), rIL-2 was then added to the cultures at a final concentration of 1500 units/ml for patients 1, 2 and 3, and 2000 units/ml for patients 4 and 5.

Phase III (days 12-16) entails the infusion of IL-2-activated cells (LAK cells) together with IL-2 every 8 h as tolerated.

Clinical Specimens. Serum plasma samples were obtained at sequential times after IL-2 injections in phase I. Blood samples for neopterin, IFN-γ, TNF-α, and 2'-5' A evaluation, collected in 5-ml tubes containing 10.5 mg EDTA and 3.5 tretin inhibitor units (aprotinin; Sigma, St. Louis, MO), were centrifuged at 750 x g for 10 min. After removal of plasma, samples were centrifuged at 10,000 x g for 4 min to eliminate residual platelets and stored at −80°C until assayed.

Cultures of Normal Peripheral Blood Mononuclear Cells. Heparinized whole blood from a healthy donor was diluted 1:2 with RPMI 1640 (Flow), stratified on Ficoll-Hypaque (Nyegaards, Oslo, Norway) liquid, and centrifuged for 40 min at 1600 rpm at room temperature. Following centrifugation, collected cells were washed three times in Hanks' saline solution (Flow), counted, resuspended in RPMI 1640, and plated on Petri dishes for 1 h at 37°C to allow monocytes adherence. Lymphocyte-containing supernatants were then removed and the plates were washed three times with RPMI 1640. Adherent cells (monocytes) were grown in the same medium containing either 10% FCS alone or combined

Received 1/2/90; accepted 6/4/90.

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1. This study was supported by a grant from Istituto Superiore di Sanità, Rome, Italy ('Italy-USA Project on Therapy of Tumors').

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3. The abbreviations used are: LAK, lymphokine-activated killer; rIL-2, recombinant human interleukin 2; IL-2, interleukin 2; IFN, interferon; TNF, tumor necrosis factor; 2'-5' A synthetase; FCS, fetal calf serum; PHA, phytohemagglutinin.

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ADOPTIVE IMMUNOTHERAPY OF HUMAN CANCER

with IL-2 (Hoffmann-La Roche Inc., NY) at a final concentration of 10^2 units/ml, or IFN-γ (Boehringer Ingelheim, Wren, Austria) at a final concentration of 10^3 units/ml.

After two additional adherence cycles for removal of residual monocytes, lymphocytes were grown in flasks with RPMI 1640 supplemented with 10% FCS; PHA or IL-2 was added to some flasks, at a final concentration of 2 μg/ml and 10^3 units/ml, respectively.

Control immunofluorescence experiments showed that: (a) adherent cells were composed of 98% CD14+ cells; nonadherent cells were composed of a majority of T-lymphocytes (68% CD3+ cells) and NK lymphocytes (16% CD56+ cells) and a minority of lymphocytes (8% CD19+ cells) and residual monocytes (0.8% CD14+ cells).

Cell cultures were maintained at 5% CO2 in a 37°C humidified atmosphere. Culture supernatants were collected by centrifugation at 4000 rpm for 10 min and stored at −80°C.

For evaluation of neopterin, TNF-α, IFN-γ, and 2′-5′AS concentrations in tissue culture supernatants, RPMI 1640 containing 10% FCS was used as a negative control in addition to the negative controls provided by the manufacturer.

IFN-γ Assay. IFN-γ in patients’ sera and culture supernatants was measured by a sensitive radioimmunoassay (Centalcor, Philadelphia, PA), which can detect as low as 0.1 unit/ml.

Neopterin Assay. A solid-phase radioimmunoassay was adopted (Henning, Berlin, West Germany), the sensitivity of which is 0.3 ng/ml neopterin.

2′-5′AS Assay. 2′-5′AS concentration was evaluated by solid-phase radioimmunoassay (Eiken Chemical Co., Ltd., Tokyo, Japan) with a measurable range of 10–810 pmol/dl.

TNF-α Assay. The levels of TNF-α in serum and tissue cultures supernatants were measured by an enzyme sandwich immunoassay (T Cell Sciences, Inc., Cambridge, MA). The detection limit is 1 pg TNF/ml.

RESULTS

IFN-γ, Neopterin, 2′-5′AS, and TNF-α Levels in Serum of Adoptive Immunotherapy Patients Receiving Bolus IL-2 Infusion

IFN-γ. IFN-γ was virtually undetectable in patients' sera before treatment. Following IL-2 administration, its level promptly rose, reaching a peak on the third day for patients 1, 3, and 4 and on the fifth day for patients 2 and 5. The concentration then dropped, reaching minimum level on day 8 (the end of the 2-day therapeutic rest period), but progressively increased during LAK cell administration; a second peak was usually observed on days 15–16 (Figs. 2 and 3).

IFN-γ levels varied in different patients and apparently were not strictly related to IL-2 dosage; in fact, the highest values were observed in two patients on the lower-dose schedule.

We also evaluated the serum levels of IFN-γ in four patients immediately after bolus injection of IL-2: the results of these cases are shown in Table 1. The level increased 60–120 min after injection and peaked at 240 min. The peak value observed after the seventh administration of IL-2 was significantly higher than those detected after the first and fourth IL-2 injection.

It is emphasized that peak levels of IFN-γ after IL-2 bolus injection are particularly high, i.e., they are at least 100 times (>20 units/ml) higher than pre-IL-2 infusion values (0.2 units/ml).

Neopterin. All patients showed a marked increase in serum neopterin concentration during the 16-day cycle of therapy. Absolute values were heterogeneous; peak levels showed a 4–20-fold increase over basal levels. Overall, the kinetics of neopterin is similar to that of IFN-γ (Figs. 2 and 3). However, nadir and peak levels were asynchronous; peak concentrations for neopterin were usually observed 2 days later than IFN-γ, thus suggesting that the rise in IFN-γ concentration was responsible for neopterin secretion by monocytes.

We also investigated the serum levels of neopterin at early times following the first, fourth and seventh bolus injections of IL-2 (Table 2). After the first one, values were within the normal range until 360 min. After the fourth and seventh IL-2 infusion, the starting values of serum neopterin were significantly higher than before adoptive immunotherapy but did not increase following IL-2 infusion (Table 2).

2′-5′AS. 2′-5′AS levels rose significantly during IL-2 adoptive immunotherapy, although to a lesser extent than IFN-γ and neopterin concentrations (Figs. 2 and 3). Here again, substantial differences were observed in the absolute peak values monitored in different patients.

TNF-α. In all five patients TNF-α levels remained very low during the 16-day cycle of IL-2 and LAK cells therapy (data not shown).

To determine whether IL-2 could rapidly induce in vivo TNF-α release, TNF-α levels were evaluated in four patients at early times after bolus IL-2 infusion. Before treatment, TNF-α was very low or undetectable in serum. After IL-2 injection two peaks of TNF-α were observed: the first occurred at the end of IL-2 bolus infusion or 15 min later; the second occurred 240 min after the end of IL-2 injection (Table 3). This phenomenon was particularly evident for three of the four patients studied, whereas the fourth one exhibited only a moderate increase of plasma TNF-α concentration following IL-2 bolus infusion. Furthermore, peak levels were higher at the fourth and seventh IL-2 infusions, as compared to the first one. In all cases, TNF-α levels rapidly decreased after reaching peak level (Table 3).

IFN-γ, Neopterin, 2′-5′AS, and TNF-α Levels in Normal Peripheral Blood Mononuclear Cell Culture Supernatants

Aliquots of cell culture supernatants were collected at 2, 24, 48, and 65 h for evaluation of IFN-γ, neopterin, and 2′-5′AS levels (Fig. 4). Supernatants derived from monocyte cultures were also harvested at 1, 2, 4, 17, and 25 h after the start of
ADOPTIVE IMMUNOTHERAPY OF HUMAN CANCER

Fig. 3. Kinetics of serum IFN-γ, neopterin, and 2'-5' A synthetase concentrations during adoptive immunotherapy with IL-2 and LAK cells in two cancer patients. The IL-2 dosage was $6 \times 10^6$ units/m² for 3 doses/day. •, IFN-γ; △, neopterin; ○, 2'-5' A.

Table 1 Plasma IFN-γ levels (units/ml) after bolus injection of rIL-2
Serial plasma samples were obtained from four representative patients before and 0, 15, 30, 60, 120, 240, and 360 min after a 30-min infusion of $6 \times 10^6$ units/m² rIL-2.

<table>
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Table 2 Plasma neopterin levels (mg/ml) after bolus injection of rIL-2
Serial plasma samples were obtained from four patients before and 0, 15, 30, 60, 120, 240, or 360 min after a 30-min infusion of $6 \times 10^6$ units/m² rIL-2.

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<th>Patient No.</th>
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Table 3 Plasma TNF-α levels (mg/ml) after bolus injection of rIL-2
Serial plasma samples were obtained from four representative patients before and 0, 15, 30, 60, 120, 240, and 360 min after a 30-min infusion of $6 \times 10^6$ units/m² rIL-2.

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* ND, not detectable.

culture and used for the measurement of TNF-α (Fig. 5).

Supernatants from resting lymphocyte cultures showed very low levels of IFN-γ. As expected, lymphocytes grown in the presence of PHA exhibited a striking increase in the level of this lymphokine. Supernatants from IL-2-stimulated cells exhibited lower levels than those monitored in PHA-treated cultures.

In monocyte culture supernatants, neopterin concentration was strongly augmented by IFN-γ, whereas no effect was observed by addition of IL-2. A modest increase of this parameter was observed in lymphocyte supernatants, which may be related to a slight monocyte contamination.

Finally, we tested 2'-5' A production by lymphocytes grown in the presence of PHA or IL-2; in both cases, a rapid rise in 2'-5' A levels was observed, which occurred as early as 2 h after the start of culture. Higher values were observed in supernatants from IL-2-treated cells. The concentration of 2'-5' A also significantly increased in monocyte cultures stimulated by IFN-γ or IL-2.

TNF-α was detected in supernatants of adherent monocytes grown without additives. The addition of IFN-γ elicited a significant stimulation of TNF-α release (Fig. 5).

DISCUSSION

Macrophages play an important role in the inflammatory process elicited by noxious agents (e.g., microorganisms and foreign bodies). After migration to the site of infection, macrophages are exposed to a variety of stimuli (e.g., cytokines produced by activated T-cells, lipopolysaccharides derived from...
the cell walls of Gram-negative microorganisms), which induce a sequence of functional changes leading to optimal microbical potency (activated macrophages) (10–16).

Macrophage activation is thus achieved through lymphokines released by T-lymphocytes specifically sensitized to a microbe in the setting of infection or responding to a lectin stimulus. Increasing evidence indicates that IFN-γ is the most potent of the macrophage-activating factors, which thus far include granulocyte-macrophage-colony-stimulating factor (17, 18), IFN-α, TNF-α, and IL-4 (19).

In experimental animals IFN-γ injection induces macrophage activation and resistance to infection (20–24). Similar results have been reported after administration of a small glycopeptide, muramyl dipeptide, derived from the cell wall of bacteria (23, 24).

In the present study we have evaluated the serum concentrations of IFN-γ, neopterin, 2'-5'AS, and TNF-α in cancer patients undergoing adoptive immunotherapy with high dose IL-2 bolus injection and LAK cells. The possibility was considered that IL-2 release of IFN-γ by activated lymphocytes may lead to the in vivo activation of monocytes: this activation was assessed by evaluation of the serum concentrations of neopterin and TNF-α, which are selectively released by activated monocytes.

All patients showed a significant increase in IFN-γ concentrations which peaked on days 3–5 of IL-2 infusion (first week of therapy) and rapidly declined when IL-2 administration was discontinued (second week of therapy). An increase of IFN-γ levels was again observed during the reinfusion period. LAK cells are presumably the source of IFN-γ, as suggested by the higher concentration of this lymphokine in LAK cell culture supernatants. Evaluation of plasma IFN-γ levels early after IL-2 bolus injection showed a high peak occurring 240 min after the end of the injection. The levels then slowly decline. It is of interest that the early IFN-γ peak was observed in patients receiving IL-2 bolus injection but not in those receiving a constant IL-2 infusion at lower dosages (3 × 10⁶ units/m²/day) (data not shown here). This clearly suggests that an elevated serum IL-2 concentration (bolus injection) induces stimulation of IFN-γ production by responsive lymphocytes, while this phenomenon is not observed at lower IL-2 serum levels (continuous IL-2 infusion).

Neopterin is a pteridine derived from an intermediate compound in the biosynthetic pathway leading to the formation of tetrahydrobiopterin from GTP (25, 26). In vitro studies have shown that it is specifically released by monocytes-macrophages upon stimulation by IFN-γ (27). Other blood cells (i.e., B- and T-lymphocytes, natural killer cells, and granulocytes), do not produce neopterin. In addition to IFN-γ, only high-dose IFN-α2 has thus far been reported to stimulate neopterin production (17, 28). Thus, the monocyte-macrophage represents the specific source of neopterin and IFN-γ the key lymphokine inducing its secretion. Indeed, neopterin release occurs during IL-2-dependent T-cell activation, as a result of increased IFN-γ production (28, 29). Moreover, serum values of neopterin are elevated during activation of cell-mediated immune responses (29), e.g., allograft rejection (30, 31), viral (including human immunodeficiency virus) (32–34), intracellular bacterial infections (35), autoimmune or other inflammatory diseases (36), and various neoplasias, especially of hematological type (37–39).

The patients considered in our study, except for the case with colon cancer, showed serum levels of monocyte-derived neopterin within the normal range before treatment. During adoptive immunotherapy, all patients showed a multifold increase in neopterin levels, although with marked differences among cases. These results indicate that neopterin derives from patients’ monocyte systems upon stimulation by IFN-γ. Indeed: (a) monocytes are the natural source of neopterin (Ref. 27 and
this paper); (b) the kinetics of neopterin and IFN-γ levels are very similar, in that they show two peaks during phases I and III of immunotherapy and a negative deflection during the therapeutic rest phase; more importantly (c) IL-2 infusion rapidly induces a marked IFN-γ rise, whereas neopterin levels remain initially unmodified and rise only at later days. Moreover, neopterin peaks fall later than those of IFN-γ, suggesting that neopterin release occurs after IFN-γ stimulation. Finally, the time interval between the peak level of IFN-γ and neopterin in patients’ sera is similar to the period required by in vitro monocye cultures to exhibit a significant rise in neopterin secretion after stimulation with IFN-γ, i.e., 2 or 3 days.

Brown et al. (40) have recently reported increased levels of neopterin in the urine of cancer patients treated with rIL-2. However, no significant increase in plasma IFN-γ concentration was observed in these patients (40); this may be due to the low sensitivity of the assay for IFN-γ.

TNF-α is selectively produced by monocytes exposed to bacterial endotoxin, as well as to some viruses or other infectious agents, provided that they have been “primed” by IFN-γ (41, 42).

The effects of TNF-α can be metabolic, toxic, or immunoregulatory and comprise: (a) suppression of lipoprotein lipase and several anabolic enzymes in adipocytes in vitro; (b) a lytic effect on some neoplastic cell lines (e.g., U937); (c) activation of neutrophils, eosinophils, primed T-lymphocytes, and macrophages themselves (e.g., increased response to IL-2); (d) other effects on hematopoietic progenitor, endothelial, synovial, bone, cartilage, and muscle and fibroblasts cells (such as stimulation of class I HLA antigen, inhibition of cell proliferation, and stimulation of cellular differentiation and/or maturation) (41, 42).

Evaluation of plasma TNF-α levels immediately after IL-2 infusion in four patients showed two peaks occurring at 0 and 240 min. However, the levels are low (≤30 pg/ml) and rapidly decline.

In our patients, we observed lower levels of serum TNF-α after IL-2 infusion than those reported by Mier et al. (43). This discrepancy may be attributed to the different types of rIL-2, i.e., from the Roche Co. for the present study and from the Cetus Corp. by Mier. A significant increase in plasma TNF-α level in a minority of patients undergoing rIL-2 bolus treatment has also been reported (44).

Thus, in vivo release of IFN-γ although sufficient to induce neopterin synthesis and release, as well as macrophage activation, only slightly stimulates production of TNF-α. This suggests that a strong induction of this cytokine also requires stimulation by bacterial endotoxin or other invasive stimuli, as suggested by in vivo observations in humans and primates (45, 46).

2'-5'AS is a cellular enzyme widely distributed in mammalian cells (47), which is induced by IFN-α, -β, and -γ. It acts by converting ATP, in the presence of double-stranded RNA, into a series of oligonucleotides called 2'-5'-oligoadenylate (2'-5' A). These in turn activate a latent endogenous RNase, which cleaves m- and rRNA, thereby blocking viral protein synthesis in infected cells (48). Thus, the 2'-5' A complex is considered one of the mechanisms responsible for the antiviral effect of IFN; measurement of 2'-5'AS activity has proved useful for the diagnosis of acute viral diseases and for the control of antiviral IFN therapy (49–51).

As expected, we observed a rise of 2'-5'AS in the sera of our patients during adoptive immunotherapy. It was not possible, however, to establish a positive correlation between the levels of the enzyme and those of IFN-γ (data not shown).

The possible role of monocyte activation in adoptive immunotherapy deserves consideration.

A large pool of data supports the concept that macrophages play an important role in antitumor defense mechanisms (52, 53) by presenting antigens to cytotoxic T-lymphocytes and/or directly carrying out tumor lysis. In this last regard, “resting” macrophages are not able to destroy tumor cells in vitro but develop cytolytic properties when activated by IFN-γ (52, 53).

Further evidence in favor of a possible antitumor role of these cells stems from the observation that macrophages isolated from growing tumors generally do not exhibit tumoricidal activity in vitro, whereas those from tumors in regression do (52, 53). Although the precise mechanism underlying direct tumor recognition and lysis by activated macrophages is not yet understood, it has been demonstrated that close cell-to-cell contact is necessary; antibody dependent cellular cytotoxicity is hence possibly involved.

We show that during adoptive immunotherapy with high dose IL-2, bolus injection and LAK cells the sharp rise of circulating IFN-γ level apparently induces macrophage activation, as indicated by the increased serum levels of the macrophage-specific compound, neopterin. We suggest that activated macrophages may add to the antitumor effect of LAK cells in standard adoptive immunotherapy protocols with high dose IL-2 bolus injection.

ACKNOWLEDGMENTS

We are grateful to Roche Laboratories for generously providing rIL-2 and to Boehringer Ingelheim for TNF-α and IFN-γ.

We thank C. Miracco, M. Ganci, and M. Blasi for secretarial assistance.

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