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Induction of Circulating Tumor Necrosis Factor (TNFα) as the Mechanism for the Febrile Response to Interleukin-2 (IL-2) in Cancer Patients

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Fever is frequently observed in cancer patients treated with high-dose recombinant human interleukin-2 (rIL-2). The preincubation of rIL-2 with polymyxin B, an antibiotic that inhibits the biologic effects of endotoxins, did not diminish the pyrogenicity of IL-2 in New Zealand rabbits, indicating that IL-2-induced fever is not due to contaminating endotoxins. In contrast to interleukin-1 (IL-1), tumor necrosis factor (TNF), and interferon α, which cause fever through their effects on arachidonic acid metabolism in the hypothalamus, IL-2 was unable to induce prostaglandin E_2 synthesis in hypothalamic cells or fibroblasts *in vitro*, suggesting that IL-2 is not intrinsically pyrogenic. To determine if IL-2-induced fever is mediated indirectly through the generation of pyrogenic cytokines, culture supernatants from IL-2-stimulated human peripheral blood mononuclear cells were screened for the presence of pyrogens by direct injection into rabbits and by measuring the amounts of IL-1α, IL-1β, and TNFα by specific radioimmunoassays (RIA). All three cytokines were readily detected by RIA in these supernatants, which in turn caused fever when injected into rabbits. Furthermore, in six of six cancer patients treated with rIL-2, elevated levels of TNFα were detected in the plasma by RIA 2 hr after IL-2 administration. Plasma TNF levels increased from pretreatment values of 14 ± 7 to 765 ± 150 pg/ml 2 hr after an IL-2 injection. These results strongly implicate IL-2-induced pyrogenic cytokines, in particular TNFα, as a major cause of the fever and possibly other aspects of the acute-phase response associated with IL-2 therapy.

KEY WORDS: Interleukin-2; tumor necrosis factor; fever.

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INTRODUCTION

Cancer patients undergoing treatment with high-dose recombinant interleukin-2 (rIL-2) develop an array of potentially life-threatening side effects including chills, fever, and hypotension beginning approximately 2 hr after initiating therapy (1–5). Although the concomitant administration of antipyretics usually suppresses IL-2-induced fever, temperatures in excess of 42°C have been recorded in some patients. The administration of IL-2 also causes marked elevations in the serum levels of hepatic acute-phase proteins and stress-related pituitary hormones (1, 6). Whether these diverse systemic effects can be attributed directly to the IL-2, to detergents or contaminating endotoxins in the IL-2 preparations, or to cytokines generated in response to IL-2 is uncertain. The late onset of fever after an IL-2 injection; the ability of IL-2 to stimulate the synthesis of interferon γ, lymphotoxin, and tumor necrosis factor (TNF) in cultured peripheral blood mononuclear cells (PBMC) (7, 8); and the ability of TNF to induce fever in both experimental animals (9) and humans (10) support the view that IL-2-induced pyrogenic cytokines may be responsible for at least some of the toxic effects attributed to IL-2.

To assess the contribution of IL-2-induced pyrogens to the toxicity of IL-2 treatment, we employed

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radioimmunoassays for TNFα and IL-1α and IL-1β to quantitate these cytokines both in culture supernatants of IL-2-activated PBMC and in the plasma of cancer patients treated with IL-2. Our studies indicate that IL-2 is not intrinsically pyrogenic, nor is its ability to cause fever in experimental animals due to contaminating endotoxins. On the other hand, our data demonstrate that the administration of IL-2 results in the prompt release of TNFα into the circulation and suggest that this inducible pyrogen may be responsible for the fever and other side effects associated with IL-2 treatment.

MATERIALS AND METHODS

Cytokine Preparations

Human rIL-2 was provided as a lyophilized powder by Cetus Corporation, Emeryville, CA. The preparation utilized in the *in vitro* studies and in the animal pyrogen experiments was identical to the material undergoing evaluation in cancer patients at our institution. The biological activity of the IL-2 was confirmed in our laboratory using a standard ^{3}H-thymidine incorporation assay with phytohemagglutinin (PHA)-activated human T lymphoblasts (11), and the concentration yielding 50% maximal incorporation (1 U/ml) was 0.3 ng/ml. All preparations contained less than 0.01 ng of endotoxin/mg protein as determined by the Limulus assay. The IL-2 was reconstituted immediately prior to use in pyrogen-free sterile water and diluted to the appropriate concentration in sterile saline or tissue culture medium. A proprietary mixture of sodium dodecyl sulfate and mannitol present in the IL-2 formulation, henceforth referred to as excipient, was also provided by Cetus Corporation to serve as an experimental control. Interleukin-1 (IL-1) was purified to homogeneity from the conditioned media of human monocytes exposed to heat-killed *Staphylococcus albus* (12). Recombinant IL-1α and IL-1β were kindly provided by Hoffman–LaRoche and Cistron Biotechnology, Inc., Pine Brook, NJ, respectively. TNFα was obtained from Genentech, Inc., South San Francisco, CA.

Preparation of PBMC Conditioned Media

Human PBMC were obtained from the heparinized venous blood of healthy donors by density-gradient centrifugation with lymphocyte separation medium (Litton), washed, and resuspended at a density of 3×10^6 cells/ml in minimal essential medium (MEM) containing 1% heat-inactivated human AB serum (MA Bioproducts, Walkersville, MD). The cells were incubated overnight at 37°C in a 5% CO_2 atmosphere in the presence of 100 ng/ml endotoxin (Sigma) or 10 μg/ml polymyxin B (Pfizer) with various concentrations of rIL-2 ranging from 0 to 10^3 U/ml in 5-ml aliquots in 50-ml polypropylene centrifuge tubes (Corning). The cells were then removed by centrifugation at 500*g* for 10 min and the supernatants frozen at −70°C until used.

Pyrogen Assays

Female albino New Zealand rabbits weighing approximately 2 kg were obtained from a single supplier and trained for pyrogen testing as previously described (13). Core temperatures were measured using indwelling rectal thermistors (Yellow Spring Instruments, Yellow Spring, OH) and recorded at 1-min intervals on a Digistrip II printer (Kaye Instruments, Bedford, MA). Baseline rabbit rectal temperatures were determined by computing the mean temperature over an interval ranging from 30 min before to 10 min after the injection of the material being assayed. Fever was measured relative to the mean baseline temperature. Rabbits were baseline temperatures varying more than 0.3°C were not used. All materials administered as an intravenous bolus injection were warmed to 39°C before injection and injected volumes did not exceed 20 ml. In several experiments, rIL-2 was administered as a prolonged intravenous infusion into a lateral ear vein utilizing a syringe infusion pump (Harvard Apparatus Co., Dover, MA) adjusted to inject at a rate of 6 ml/hr. In some experiments, the rabbits received ibuprofen (Upjohn), 10 mg/kg, as a bolus injection followed by a continuous infusion at a rate of 1.0 mg/kg/hr in addition to IL-2. Recombinant IL-2 was also preincubated for 2 hr at 39°C with 10 μg/ml polymyxin B to inactivate endotoxins before injection into rabbits (14). In these studies, the IL-2 preparation was diluted to a concentration of less than 40,000 U/ml before adding the antibiotic.

Prostaglandin E_2 (PGE_2) Assays

Rabbit hypothalamic minces prepared as previously described (15) as well as human foreskin fibroblasts and PBMC were used to test the ability

of rIL-2 to induce prostaglandin E_2 (PGE_2) synthesis *in vitro*. The fibroblasts were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and were split every 4 days. The fibroblasts were utilized as indicator cells after 3 weeks in culture and were at 50% confluence at the time of the assays. The cells were washed in MEM and incubated at 37°C for 4 hr in MEM containing 10 μg/ml polymyxin B and the appropriate dilution of rIL-2 or other cytokine. The supernatants were collected and frozen at −70°C until assayed. The PGE_2 assays were performed in duplicate using a specific radioimmunoassay (Seragen, Boston, MA) and the results are expressed as picograms of PGE_2 per 10^4 fibroblasts. Human PBMC were cultured overnight at a density of 10^6 cells/ml in MEM containing 1% AB serum and either rIL-2 or a suspension of heat-killed *Staphylococcus albus*. These supernatants were similarly assayed and the results are expressed as picograms of PGE_2 per milliliter of culture medium.

Clinical Specimens

Serial plasma samples for the human studies were obtained from cancer patients enrolled in an ongoing Phase II clinical trial with rIL-2 and autologous lymphokine-activated killer (LAK) cells. Patients were initially treated with rIL-2 alone at a dose of 100,000 U/kg by brief intravenous infusions every 8 hr for 3 consecutive days. Unless otherwise stated, the plasma samples for this investigation were obtained after the first injection. After a 36-hr respite, the patients were subjected to four consecutive daily leukaphereses and the harvested PBMC were cultured in rIL-2-supplemented medium as described by Rosenberg *et al.* (3). The IL-2 treatment was then resumed as a continuous infusion administered in conjunction with the LAK cells. This trial was previously approved by the Human Investigation Review Board at the New England Medical Center, and all study participants gave prior informed consent.

Cytokine Radioimmunoassays (RIAs)

Blood samples for the TNF and IL-1 RIAs were collected in 7-ml vacuum-sealed tubes containing 10.5 mg of EDTA and 3.5 trypsin inhibitor units of aprotinin (Sigma). The samples were first centrifuged at 500*g* for 15 min, the plasma was removed, and then the samples were subjected to a second centrifugation at 10,000*g* for 4 min to eliminate residual platelets. All plasma samples were stored at −20°C until assayed.

TNFα levels in plasma and culture supernatants were measured by a competitive inhibition RIA (16). Recombinant TNFα standards were prepared at concentrations ranging from 5 pg/ml to 5 ng/ml in an RIA buffer containing 0.01 *M* Na phosphate, 0.25% bovine serum albumin (BSA), and 0.05% Na azide in 0.15 *M* NaCl, pH 7.4. Triplicate 100-μl aliquots of plasma, culture supernatants, TNF standards, or buffer alone were mixed with 300 μl of 0.3% normal rabbit serum (NRS) and 100 μl of diluted rabbit anti-TNF antiserum in 10 × 75-mm polystyrene tubes. The anti-TNF antiserum used in these assays was generated in rabbits by repeated immunization with recombinant human TNFα. Samples were incubated for 24 hr at room temperature. Radiolabeled TNF was prepared using a modified chloramine T technique. One hundred microliters of ^{125}-I-TNF (7×10^5 cpm/ml, 10 μCi/μg) was then added to the assay tubes. After another 24-hr incubation, 700 μl of a 6% polyethylene glycol (6000 MW) solution containing 1.4% sheep anti-rabbit IgG (Sigma) was added to each sample. Precipitable immune complexes were collected by centrifugation for 15 min at 1000*g*. Following the decantation of the supernatants, the radioactivity of the pelleted material was determined with a Beckman 4000 gamma counter. All samples were assayed in triplicate and standard errors were less than 6%. This RIA does not detect IL-1 or TNFβ (lymphotoxin) (16). The concentrations of IL-1α and IL-1β in plasma and culture supernatants were also measured by RIA (17, 18).

IL-1 Bioassay

Plasma IL-1 levels were measured as previously described (19). Heparinized blood was obtained prior to and 2, 4, and 8 hr after the first IL-2 injection and the plasma separated and frozen at −70°C. Plasma (0.3 ml) was applied to a 1 × 30-cm column of autoclaved packed Sephadex G50-fine (Pharmacia) previously equilibrated with sterile RPMI 1640 medium containing 0.1% BSA and 10 μg/ml polymyxin B. Fractions of 0.6 ml were collected and assayed in quadruplicate for proliferative activity in a ^{3}H-thymidine incorporation assay using as indicator cells a subclone of the murine D10.G4.1

helper T-cell line specifically selected for its high level of sensitivity to IL-1 (20). The active fractions (numbers 16–19) from the gel filtration column were pooled. In order to reduce the IL-2 concentration, the pooled fractions were then passed over an IL-2 affinity column. The anti-human rIL-2 antibody used in the preparation of the affinity gel was raised in a New Zealand rabbit by repeated multiple-size subcutaneous injections of rIL-2 suspended in Freund's adjuvant. Hyperimmune IgG was isolated from a 47% ammonium sulfate precipitate of serum using protein A agarose (Genzyme, Boston, MA) affinity chromatography and immobilized by cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ). This neutralizing anti-human rIL-2 IgG was readily detected in a rIL-2 enzyme-linked immunosorbent assay (ELISA) using a goat anti-rabbit IgG antibody (Hyclone, Logan, UT) as the indicator antibody. The affinity gel contained 5 mg IgG/ml gel. Columns containing 0.5 ml of gel were prepared in Pasteur pipettes. Pooled fractions 16–19 from both pretreatment and posttreatment plasma samples were slowly passed over the columns. Nonbinding material was free of IL-2 as determined by ELISA (InterTest-2, Genzyme, Boston). The nonbinding, IL-2-depleted, material (flow-through) was then incubated overnight with 1% NRS or an antiserum generated against human monocyte-derived IL-1 (12) and subsequently assayed as above for proliferative activity with D10.G4.1 cells. All ^{3}H-thymidine incorporation assays were performed in quadruplicate and standard deviations were consistently less than 5%.

RESULTS

Pyrogenicity of rIL-2 in Rabbits

As shown in Fig. 1, a bolus injection of 400,000 U (120 μg)/kg of rIL-2 produced a monophasic fever in rabbits which began after a latent period of 60 min and reached maximal elevation 90 to 120 min after the injection. The fever was unaffected by prior incubation of the rIL-2 with polymyxin B. In contrast, rIL-1 β (100 ng/kg) induced an immediate fever which reached peak elevation 50 min after the injection.

As shown in Fig. 2, the administration of rIL-2 at a rate of 100,000 U (30 μg/kg/hr induced a progressive rise in core temperature in rabbits beginning approximately 2 hr after initiating and continuing throughout the infusion. Temperature increments

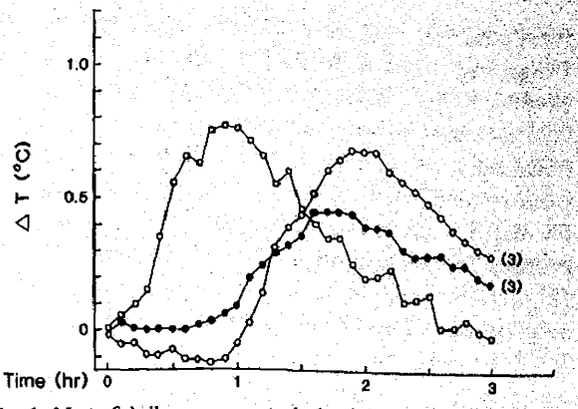

Fig. 1. Mean febrile response to bolus injections of rIL-2. New Zealand rabbits were treated with 400,000 U/kg rIL-2 alone (●) or IL-2 preincubated with the endotoxin-binding antibiotic polymyxin B (○) or with monocyte-derived IL-1 (□). IL-2-associated fever is delayed relative to that induced by IL-1 and is not diminished by preincubation of the IL-2 with polymyxin B. Numbers in parentheses indicate the number of animals in each treatment group.

ranging from 0.35 to 2.85°C were recorded in individual rabbits 4 hr after beginning the infusion. The induction of fever was prevented by intravenous ibuprofen. The infusion of excipient material at a rate equivalent to the dose received by the IL-2-treated animals did not cause fever. Infusions of rIL-2 at a dose of 10,000 U/kg/hr were likewise nonpyrogenic.

Efforts to detect circulating pyrogens by direct plasma transfer were unsuccessful. In these experiments, 20 ml of heparinized plasma taken from

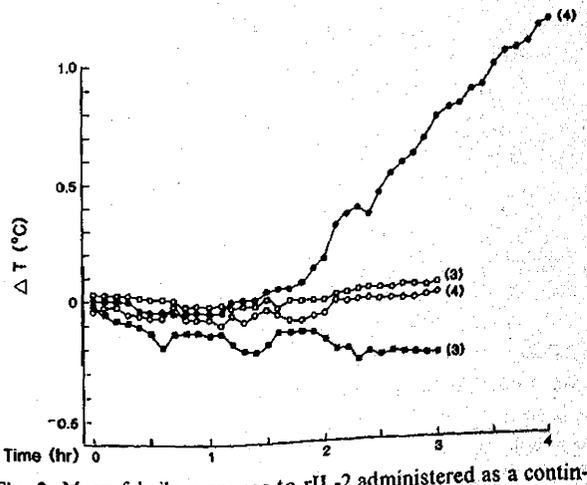

Fig. 2. Mean febrile response to rIL-2 administered as a continuous infusion. New Zealand rabbits treated with 100,000 U/kg/hr rIL-2 developed fever (●) which could be prevented by the simultaneous administration of ibuprofen (■). Animals treated with 10,000 U/kg/hr (○) or with excipient (□) remained afebrile.

febrile rabbits 2 hr after an IL-2 injection (400,000 U/kg) was injected into other rabbits. Only one of seven rabbits so treated developed more than a 0.25°C temperature increase. Conversely, plasma obtained 2 hr after a bolus injection of endotoxin from animals with temperature elevations comparable to those of the IL-2-treated rabbits produced prompt febrile responses in the recipient rabbits characteristic of the fever resulting from endogenous pyrogens, (data not shown).

Cytokine-Induced Prostaglandin E_2 (PGE_2) Synthesis

We previously demonstrated that recombinant human IL-2 at concentrations up to 500 U/ml does not induce PGE_2 synthesis in cultured rabbit hypothalamus cells (21). Identical results were obtained with the rIL-2 preparations currently in use in the various IL-2/LAK-cell clinical trials under way at our institution (data not shown). To determine if these negative results with human IL-2 and rabbit indicator cells were a consequence of an unsuspected species specificity, human foreskin fibroblasts and PBMC were exposed to various concentrations of IL-2 and the conditioned media assayed for PGE_2. As shown in Table I, rIL-2 at concentrations ranging up to 10,000 U/ml failed to induce PGE_2 synthesis in the fibroblasts, whereas IL-1 was consistently positive in these assays. Human PBMC, on the other hand, produced PGE_2, but only in response to high concentrations (1000 U/ml) of rIL-2 or to opsonized *Staphylococcus albus*. As demonstrated below, elevated levels of PGE_2 from PBMC stimulated with high concentrations of rIL-2 for 24 hr are likely secondary to the coinduction of IL-1 and TNF.

Table I. Induction of PGE_2 Synthesis

Human foreskin fibroblasts		
	PGE_2 (pg/ml/10^4 cells)	
Culture medium	Expt 1	Expt 2
Control	52	13
IL-1 (100 pg/ml)	2600	900
IL-2 (U/ml)		
1	105	0
10	72	32
100	70	53
1,000	86	50
10,000	53	68
Excipient	ND	0
Human PBMC		
	PGE_2 (pg/ml)	
Culture medium	Expt 1	Expt 2
Control	0	
Staphylococcus albus	140	520
IL-2 (U/ml)		
1	0	0
10	0	0
100	0	0
1,000	390	0
10,000	680	360

IL-2-Induced Pyrogenic Cytokines in PBMC Culture Supernatants

Human PBMC produce several cytokines when exposed to high concentrations of rIL-2 (7, 8). In the present studies, we injected rabbits with rIL-2-stimulated PBMC culture supernatants and observed rapid-onset monophasic fevers characteristic of the response to endogenous pyrogens (Fig. 3). In these experiments, the PBMC were incubated for 18 hr in the presence of 500 U/ml of rIL-2 and the amount of rIL-2 contained in the injected supernatant was less than 5000 U, well below the amount of IL-2 required to produce fever when injected alone (21).

The pyrogenic culture supernatants were also assayed for IL-1α, IL-1β, and TNFα using RIAs specific for each cytokine. As depicted in Table II, the unstimulated PBMC from three human donors produced low or undetectable levels of the three cytokines, but after exposure to 1000 U/ml of rIL-2, the PBMC secreted large quantities of TNFα and IL-1β. IL-2-induced secretion of IL-1α was consistently less than that of IL-1β and TNFα. These increases were comparable to those induced by 10 ng/ml of bacterial endotoxin. These cytokines induced by rIL-2 may explain the elevated PGE_2 levels in the culture supernatants of PBMC stimulated with high concentrations of rIL-2 (Table I).

IL-2-Induced Circulating Pyrogens in Humans

Because of the relative insensitivity of the rabbit pyrogen assay, RIAs specific for various human cytokines were employed in an effort to detect pyrogenic substances in the plasma of cancer patients participating in ongoing IL-2 clinical trials. Plasma samples obtained before and 2, 4, and 8 hr

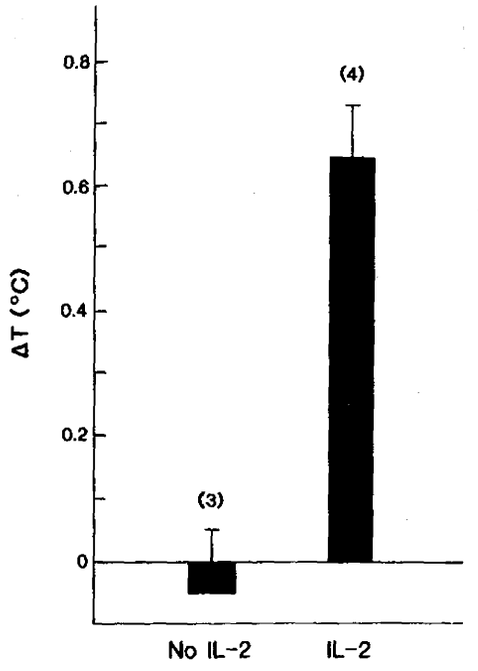

Fig. 3. Pyrogenicity of PBMC culture supernatants. Ten milliliters of culture supernatant from unstimulated PBMC or from cells incubated at 3×10^6 cells/ml in medium containing 500 U/ml rIL-2 was injected into rabbits. Animals treated with the supernatant from rIL-2-activated PBMC developed a prompt febrile response, whereas those treated with the conditioned medium from the unstimulated cells remained afebrile. ΔT was recorded at 1 hr postinjection.

after an injection of 100,000 U/kg rIL-2 were assayed for IL-1α and IL-1β and TNFα using the RIAs described under Materials and Methods. Plasma concentrations of TNFα rose from pretreatment levels of 14 ± 7 to 765 ± 150 pg/ml within 2 hr after an IL-2 injection and subsequently declined toward baseline in each of the patients studied (Fig. 4). This time course is similar to that of the febrile response to IL-2 reported in earlier Phase I studies, in which antipyretics were not routinely administered (1, 2). In some patients, additional plasma samples were obtained at 24, 48, and 72 hr after the first IL-2 injection, and in none of these samples did the TNFα levels revert to baseline, indicating that TNF was present in the circulation throughout the entire 3 days of IL-2 treatment. In contrast, plasma levels of IL-1α remained below the limits of detection of the RIA (data not shown).

Table II. IL-2-Induced Secretion of IL-1 and TNF by PBMC[a]

	Cytokine concentration (ng/ml)		
	Medium control[b]	IL-2 (1000 U/ml[b]	Endotoxin (10 ng/ml)
Expt 1			
IL-1α	<0.1	0.7	1.2
IL-1β	0.8	6.1	12.0
TNFα	0.4	6.6	3.1
Expt 2			
IL-1α	<0.1	3.9	0.8
IL-1β	<0.1	6.8	5.2
TNFα	<0.1	4.8	3.6
Expt 3			
IL-1α	<0.1	5.3	2.7
IL-1β	<0.1	16.0	22.0

[a]Cells were cultured for 18 hr at a density of 5×10^6/ml.
[b]PBMC cultures with medium alone or with IL-2-supplemented medium contained 5 μg/ml polymyxin B.

As shown in Fig. 5, IL-1β (>100 pg/ml) was detected in the pretreatment plasma of all study participants. However, in contrast to the TNFα levels, the plasma concentration of IL-1β changed only minimally during the 8-hr sampling period. The high pretreatment plasma levels of IL-1β and the failure of IL-2 injections to increase the plasma IL-1β levels to the same extent as the TNFα levels also contrast with the *in vitro* data, which failed to demonstrate IL-1 production by unstimulated PBMC and which showed comparable amounts of IL-1β and TNFα in PBMC culture supernatants after 24 hr of exposure to IL-2.

To verify that the immunoreactive material detected in the plasma with the IL-1β RIA was biologically active, a highly sensitive bioassay for IL-1 using a subclone of the murine D10.G4.1 helper T-cell line was employed (20). This assay detects concentrations of IL-1 100-fold lower than the threshold for the RIA. Although highly sensitive to IL-1, these cells also respond to IL-2, IL-4, and possibly other cytokines (22). High molecular weight inhibitors of IL-1-induced T-cell proliferation (19, 23) were first removed from the plasma samples by gel filtration. Bioassays of gel filtration fractions from plasma obtained from these patients at various intervals after an injection of rIL-2 revealed a peak of activity in fractions corresponding to a molecular weight range of 15–25 kD (Fig. 6). Because of the sensitivity of the D10.G41 cells to IL-2, the active gel filtration fractions (numbers 16–19) were pooled and then passed over an IL-2 affinity column, which reduced the IL-2 concentration to undetectable levels in the IL-2 ELISA. The IL-2-depleted material was incubated with normal rabbit serum or a neutralizing anti-IL-1 rabbit antiserum and then assayed for proliferative activity with the D10.G4.1 cells. Despite the marked difference between the gel filtration profile of the pre-

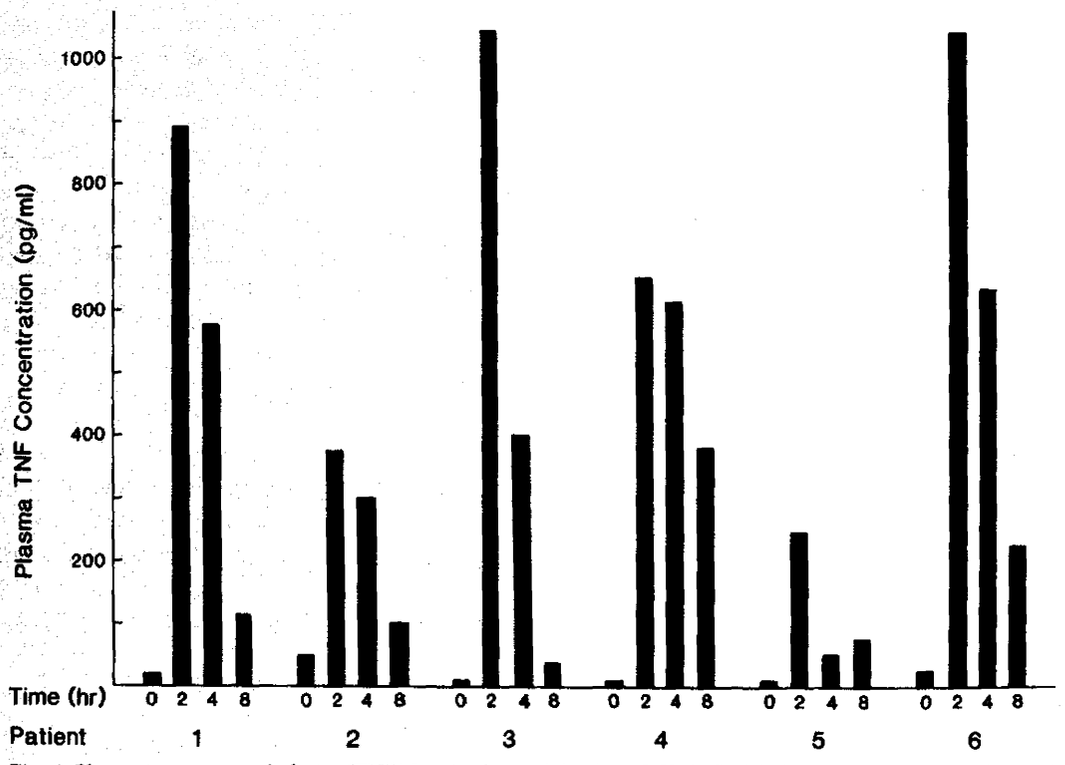

Fig. 4. Plasma tumor necrosis factor (TNF) levels after an injection of rIL-2. Serial plasma samples were obtained before and 2, 4, and 8 hr after an injection of 100,000 U/kg rIL-2 from six consecutively treated patients, and TNF levels in the plasma determined by RIA. In each patient, the IL-2 injection gave rise to a prompt increase in the plasma concentration of TNF.

treatment plasma and those obtained after an IL-2 injection (Fig. 6), the proliferative activities of the affinity gel flow-through fractions were relatively constant throughout the 8 hr of plasma sampling (Fig. 7). Nearly all of the biological activity of the pooled gel filtration fractions derived from the 2-, 4-, and 8-hr plasma samples was eliminated by the affinity gel, indicating that most of the activity of these fractions in the bioassay was due to residual IL-2 and not to IL-1 induced by the treatment. Pretreatment samples were active in the bioassay and their proliferative activity was partially neutralized by an IL-1 antibody (Fig. 7). These results indicate that small amounts of IL-1 may be produced constitutively, independent of a specific inducer such as IL-2.

The affinity gel flow-through fractions assayed in Fig. 7 contained TNF by RIA and were free of exogenous IL-2 and high molecular weight inhibitors of lymphocyte proliferation. The failure of the bioassay to demonstrate an increase in the IL-1 concentration of the fractions generated from postinjection plasma that is comparable with the increase in plasma TNF levels corroborates the RIA data. In contrast to TNF, which is released promptly into the circulation after an IL-2 injection, IL-1 levels may not appreciably increase or do so only several hours after an IL-2 injection.

DISCUSSION

Despite the efficacy of IL-2 as an antineoplastic agent, the initial enthusiasm for its widespread use in cancer patients has somewhat abated as a consequence of its severe toxicity. Although low doses of IL-2 are generally well tolerated, high doses are associated with a multitude of side effects including chills, fever, and confusion as well as potentially lethal effects such as hypotension, myocardial infarction, renal and hepatic failure, and fluid retention resulting in massive weight gain and pulmonary edema (1–5). Patients treated with high doses of IL-2 also develop increased serum levels of hepatic acute-phase reactants and hypothalamic–pituitary hormones otherwise associated with the metabolic response to infection and inflammation (2, 6).

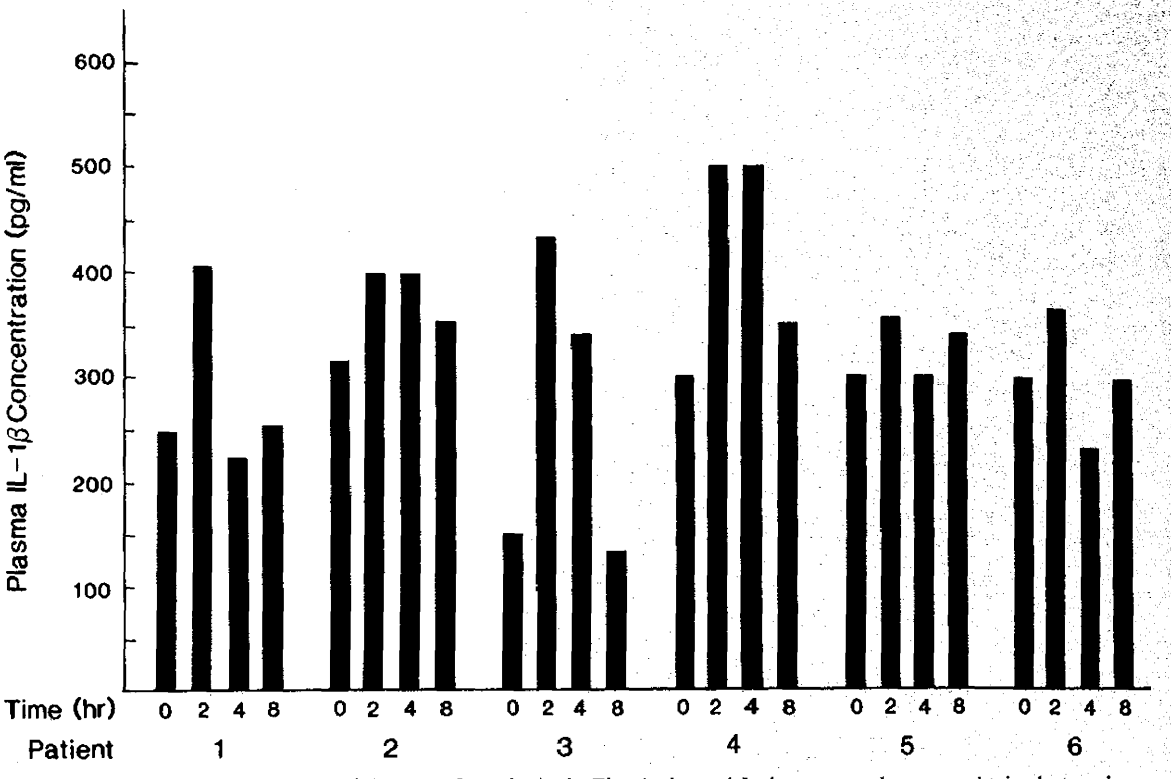

Fig. 5. Plasma IL-1β levels after an injection of rIL-2. As in Fig. 4, the serial plasma samples were obtained at various intervals after an injection of 100,000 U/kg rIL-2. The IL-1β levels were determined by RIA. Pretreatment levels of IL-1β were much higher than those of TNFα. However, the plasma concentrations of IL-1β were much less affected than the TNF levels by the administration of rIL-2.

These toxic effects of rIL-2 could be attributed to residual endotoxin, although only extremely low levels (<0.01 ng/mg protein) have been detected with the Limulus assay. Several investigators have reported that endotoxin at concentrations below the threshold of the Limulus assay is capable of activating monocytes to produce the pyrogen interleukin-1 (24, 25). Therefore, to determine if residual endotoxin in the IL-2 preparations might be responsible for IL-2-associated fever, IL-2 was preincubated with polymyxin B prior to injection. The inability of polymyxin B to reduce the pyrogenicity of IL-2 in New Zealand rabbits suggests that the fever associated with IL-2 is due to the lymphokine itself or to IL-2-induced pyrogens rather than contaminating endotoxins.

The fever associated with IL-2 is clearly dose dependent. In a recently completed dose escalation Phase I clinical trial in cancer patients, fever was not observed after a single bolus injection of IL-2 until doses in excess of 250,000 U/M^2 (approximately 6500 U/kg) were reached (2). In New Zealand rabbits, neither a bolus injection of 10,000 U/kg (21) nor a continuous infusion of 10,000 U/kg/hr induced fever, whereas 100,000 U/kg/hr was pyrogenic. An injection of 400,000 U (120 μg)/kg generally induces a temperature increase of less than 1°C, an increment readily achieved with only 100 ng/kg of IL-1 (26), indicating that IL-2 is less than 1/1000 as potent a pyrogen as IL-1.

We previously demonstrated that rIL-2 does not induce PGE_2 synthesis in cultured rabbit hypothalamus cells (21). These findings were felt to indicate that IL-2 was not an intrinsic pyrogen capable of interacting directly with the hypothalamus, as is the case with IL-1, IFNα, and TNF (9, 15, 27). Although human rIL-2 will support the growth of activated T cells from virtually all species, it is conceivable that its effects on arachidonic acid metabolism might be species specific. Therefore, our previous experiments were repeated employing human fibroblasts. These studies showed that IL-1-treated, but not IL-2-treated, fibroblasts synthesized PGE_2 (Table I). Studies with human PBMC yielded similar results except when high concentrations of IL-2 were employed. PBMC stimulated

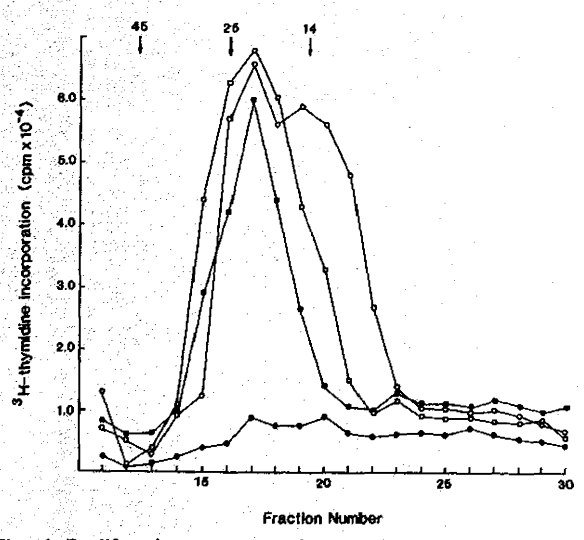

Fig. 6. Proliferative response of D10 cells to cytokines in the plasma of patients treated with rIL-2. Plasma samples obtained before (●) and 2 hr (○), 4 hr (□), and 8 hr (■) after an injection of 100,000 U/kg rIL-2 were fractionated on a Sephadex G50 column and the gel filtration fractions assayed in a ^{3}H-thymidine incorporation assay with murine D10 lymphoblasts as indicator cells. A 15- to 25-kilodalton peak of proliferative activity was evident in all of the postinjection plasma samples. The elution points and molecular weights (kilodaltons) of the various standards are indicated by arrows.

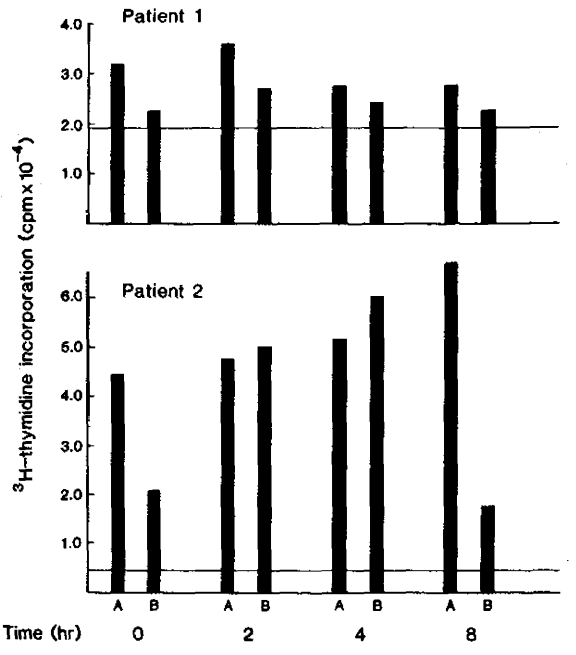

Fig. 7. Proliferative response of D10 lymphoblasts to IL-2-depleted gel filtration fractions from the serial plasma samples of two IL-2 recipients. Pooled biologically active fractions from the Sephadex G50 column were depleted of IL-2 by passage over an affinity column of Sepharose-immobilized IgG from a rabbit hyperimmune to rIL-2. The fractions were incubated overnight in 1% normal rabbit serum (A) or IL-1 antiserum (B) and assayed for proliferative activity in the D10.G4.1 bioassay. Background ^{3}H-thymidine incorporation in unstimulated cells is indicated by the horizontal line. The partial neutralization of the activity of the IL-2-depleted fractions by an anti-IL-1 antibody indicates that at least a portion of the remaining activity was due to IL-1. However, no consistent increase in proliferative activity or in the extent to which the activity was neutralized by the IL-1 antibody was observed in IL-2-depleted fractions derived from plasma serially obtained after an IL-2 injection.

with either opsonized *Staphylococcus albus* or 1000 U/ml IL-2 synthesized PGE_2; however, these supernatants were pyrogenic in rabbits and contained both TNFα and IL-1, both of which are potent inducers of PGE_2 synthesis (9), suggesting that the effect of IL-2 on PGE_2 synthesis by PBMC may have been mediated by TNF and IL-1 generated in response to IL-2.

Compared with the fever associated with IL-1, TNF, or IFNα, that induced by IL-2 is uniquely delayed in onset, beginning approximately 1 hr after a bolus injection in rabbits and reaching peak elevation 30–40 min later. Fever is evident within 10 min and reaches maximal levels 45 min after an injection of IL-1 or TNF (9, 26). The delayed onset of IL-2-induced fever and the inability of IL-2 directly to induce PGE_2 synthesis argue against its being an endogenous pyrogen; on the other hand, the data suggest that IL-2-associated fever may be mediated through the induction of bona fide pyrogenic cytokines.

Several experiments were carried out to determine if pyrogenic substances could be detected in the plasma after an IL-2 injection. The transfer of plasma from febrile endotoxin-treated rabbits is known to cause fever in the recipient animals. Our transfer experiments with IL-2-treated animals serving as the plasma donors failed to demonstrate circulating pyrogenic material, presumably because of the low sensitivity of the rabbit pyrogen assay. For these reasons, we employed highly sensitive RIAs to study IL-2-induced pyrogens. As shown in Table II, both IL-1 and TNF (8, 28) are generated by PBMC in response to IL-2 in the absence of endotoxin and could therefore play a causative role in IL-2-induced fever. Because the antibodies to IL-1 and TNF utilized in the respective RIAs were raised in rabbits by immunization with recombinant human cytokines, we were unable to employ these RIAs to detect circulating pyrogens in rabbits with fever due to IL-2. However, in human subjects, we

clearly detected circulating immunoreactive TNF following IL-2 administration; furthermore, the kinetics of the TNF release into the circulation were consistent with onset of fever in IL-2-treated humans not receiving antipyretics. Conversely, the RIAs for IL-1α and IL-1β failed to demonstrate a comparable increase in the plasma level of either cytokine, despite their readily demonstrable inducibility *in vitro*.

Cannon *et al.* reported that the low molecular weight fractions of gel-filtered plasma from healthy human donors were active in thymocyte proliferation assays and that the biological activity of these fractions was inhibited by anti-IL-1 antibodies, results suggesting that IL-1 is produced constitutively *in vivo* (29). The constitutive expression of IL-1 genes has also been demonstrated by Northern analysis of RNA extracted from lymphoid tissue from pathogen-free mice (30). Although our investigation did not specifically compare plasma IL-1 levels of healthy donors with those of cancer patients, both the RIA and the bioassay results indicate that at least a modest amount of IL-1β is produced constitutively in patients with disseminated malignancy.

As shown in Fig. 5, we were able to document only a modest increase in immunoreactive IL-1β levels in the plasma after a IL-2 injection. Likewise, as shown in Fig. 7, we were unable to show that IL-2 treatment altered the proliferative activity of fractionated plasma that could be attributed to IL-1 to the same degree that it increased the TNF levels. Given the low levels of proliferative activity on the highly IL-1-sensitive D10.G4.1 cells, we conclude that IL-1 may not be one of the cytokines rapidly released into the circulation following high-dose IL-2 therapy in cancer patients. This conclusion is supported by the observation that PBMC stimulated with IL-2 *in vitro* rapidly release TNFα into the culture supernatant, whereas the secretion of IL-1 is scarcely detectable in the first few hours (31). Both IL-1α and IL-1β are initially produced as 35-kD precursors, which are subsequently cleaved to generate the 17-kD species found in culture supernatants. The delay associated with the processing of IL-1 as well as the fact that IL-1 (especially IL-1α) remains, to a large extent, cell associated regardless of the inducing agent (31) may account for our failure to detect IL-1 in the plasma within a few hours of an IL-2 injection, when circulating TNFα is readily demonstrable and the patient already febrile and hypotensive.

We conclude from these studies that IL-2 does not directly stimulate the hypothalamic fever mechanism but rather induces circulating TNF and that this cytokine may be primarily responsible for the initial febrile response to IL-2 treatment. The dramatic and sudden increase in circulating TNF levels 2 hr following an IL-2 injection is temporally consistent with the onset of fever as well as some of the other toxic manifestations of IL-2. The contribution of other pyrogenic cytokines such as TNFβ (lymphotoxin) to the toxicity of IL-2 treatment is yet to be determined. These studies await the availability of highly sensitive and specific assays.

In addition to its potential role as a direct mediator of IL-2 toxicity, TNF may also contribute to the antineoplastic effects of IL-2 treatment. TNF increases the expression of IL-2 receptors on large granular lymphocytes and enhances their tumoricidal activity (32). TNF affects the interaction of leukocytes with endothelial cells (33) and may therefore influence the trafficking of IL-2-activated lymphocytes into tumor nodules. It is therefore unclear to what extent the antitumor effects of IL-2 can be dissociated from its toxicity, much of which may be mediated by inducible pyrogens such as TNF.

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