Annals of Surgical Oncology, **7**(4):268–275
Published by Lippincott Williams & Wilkins

Systemic Toxicity and Cytokine/Acute Phase Protein Levels in Patients After Isolated Limb Perfusion With Tumor Necrosis Factor-α Complicated by High Leakage

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Background: Since the introduction of high-dose tumor necrosis factor-α (TNFα) in the setting of isolated limb perfusion (ILP) in the clinic, prevention of leakage to the body of the patient is monitored with great precision for fear of TNF-mediated toxicity. That we observed remarkably little toxicity in patients with and without leakage prompted us to determine patterns of cytokines and acute phase proteins in patients with high leakage and in patients without any leakage.

Methods: TNFα, interleukin (IL)-6, IL-8, C-reactive protein, and secretory (s)-phospholipase A_2 were measured at several time points during and after (until 7 days) ILP in 10 patients with a leakage to the systemic circulation varying in percentage from 12% to 65%. As a control, the same measurements, both in peripheral blood and in perfusate, were performed in nine patients without systemic leakage.

Results: In patients with systemic leakage, levels of TNFα increased during ILP, reaching values to 277 ng/ml. IL-6 and IL-8 peaked 3 hours after ILP with values significantly higher compared with patients without systemic leakage. C-reactive protein and s-phospholipase A_2 peaked at day 1 in both patient groups, s-phospholipase A_2 with significant higher levels and C-reactive protein, in contrast, with lower levels in the leakage patients.

Conclusions: High leakage of TNFα to the systemic circulation, caused by a complicated ILP, led to 10-fold to more than 100-fold increased levels of TNFα, IL-6, and IL-8 in comparison with patients without leakage. The increase of the acute phase proteins was limited. Even when high leakage occurs, this procedure should not lead to fatal complications. The most prominent clinical toxicity was hypotension (grade III in four patients), which was easily corrected. No pulmonary or renal toxicity was observed in any patient. It is our experience that, even in the rare event of significant leakage during a TNFα-based ILP, postoperative toxicity is usually mild and can be easily managed by the use of fluid and, in some cases, vasopressors.

Key Words: Isolated limb perfusion—TNFα—Leakage—Toxicity—Secondary cytokine response—Acute phase response.

The technique of regional isolated limb perfusion (ILP) with the use of an extracorporeal circuit was pioneered by Creech and co-workers.[1] The advantage of this treatment modality is that a high dose of cytostatic drug can be delivered to the tumor-bearing extremity without producing systemic side effects. ILP permits regional cytostatic concentrations 15 to 20 times higher than those reached after systemic administration.[2] The standard drug in this setting is melphalan (L-phenyl-alaninemustard). In patients with multiple melanoma in-transit metastases, an ILP with melphalan results in about 50% of the patients in a complete remission.[3] Addition of tumor necrosis factor-α (TNFα) to melphalan has proven most effective in terms of response rate, yielding a 80% to 90% complete response rate and an overall response of about 100%.[4,5] In locally advanced soft-tissue sarcomas, the use of TNFα in combination with melphalan has

Received September 14, 1999; accepted January 14, 2000.

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proven remarkably effective in rendering irresectable tumors resectable and thereby preventing amputations.[5,6] The efficacy of TNF against the drug-resistant soft-tissue sarcomas has led to the approval of TNF by the EMEA (European Medicine Evaluation Agency) for its use in combination with melphalan.[7]

In the ILP system, TNFα is administered in a 10-fold higher dose compared with the maximum tolerable dose in systemic administration. The maximum tolerable dose of TNFα in single-dose intravenous or intramuscular administration is limited by toxicity at 400 $\mu g/m^2$.[8,9] Toxicity consists of fever, hypotension, chills, and transient leukopenia. Hardly any tumor response has been reported after systemic administration of TNFα.[9–11]

Despite careful precautions, systemic leakage of more than 10% appeared in 10 patients during the last 6 years in our hospital. These patients had a remarkably mild clinical course. To get more insight into the cause of this discrepancy between the high systemic concentration of TNFα and the mild clinical symptoms, we studied cytokine levels and the acute phase response.

PATIENTS AND METHODS

Patients

From the 212 patients who underwent an ILP with TNFα and melphalan in the past 8 years in our hospital, 10 patients were selected because of very high systemic levels of TNFα caused by leakage from the perfusate. These patients were treated because of an irresectable sarcoma (n = 6) or melanoma with multiple in-transit metastases (n = 4). Demographic and treatment characteristics are summarized in Table 1. As a control group, we studied nine comparable patients undergoing ILP without systemic leakage. These patients were all sarcoma patients, who underwent a 90-minute ILP with 3 to 4 mg of TNFα. The mean age was 48 years (range, 21–77 years).

Treatment Schedule

The procedure of ILP has been described previously.[5] In brief, ILP consisted of a 90-minute-long perfusion with 3 to 4 mg of recombinant human TNFα (Boehringer Ingelheim, Alkmaar, The Netherlands) and 10 to 13 mg/liter perfusion tissue of melphalan (Alkeran; Burroughs Wellcome, London, UK) at mild hyperthermia (39°C to 40°C). Composition of the perfusate was as follows: priming volume of 700 to 850 ml consisted of 400 to 500 ml of blood (50% red blood cells/50% plasma), 200 to 400 ml of 5% dextran-40 in glucose 5% (Isodex; Pharmacia, Uppsala, Sweden), 10 to 30 ml of 8.4% sodium bicarbonate, and 0.5 ml of 2500 to 5000 IU heparin. TNFα was injected as a bolus into the arterial line if limb tissue temperature was >38°C. Melphalan was administered 30 minutes later at limb temperatures between 39°C to 40°C. At the end of perfusion, the limb was washed with at least 2 liters of 6% dextran-70 (Macrodex; Pharmacia). During and after ILP, vital signs of the patients, including body temperature, heart rate, blood pressure, and fluid balance, were recorded. Toxicity was registered according to the World Health Organization criteria.[12]

Leakage Monitoring

During ILP, there was a dynamic balance between two pressure compartments, the systemic vasculature and the isolated circuit, which could be influenced by adjusting the systemic blood pressure and/or the extracorporeal flow rate. Throughout the perfusion period, any potential leakage of the drugs was monitored by using a radioactive tracer. A small calibration dose of human serum albumin radiolabeled with iodine 131 or technetium 99m was injected into the systemic circulation and a 10-fold higher dose of the same isotope into the isolated extremity. Continuous monitoring was performed with a precordial scintillation probe. Systemic leakage was ex-

TABLE 1. *Patient characteristics*

Patient no.	Age, y	Sex	Diagnosis	Arm/leg	Duration of perfusion, min	Dose (mg of TNFα)	Leak, %	Maximum systemic TNF (ng/ml)
1	55	F	Sarcoma	Leg	90	2	23	169
2	52	F	Melanoma	Leg	90	4	20	178
3	66	M	Sarcoma	Arm	90	3	12	30
4	61	F	Sarcoma	Arm	30	3	65	277
5	71	F	Melanoma	Leg	90	2	24	112
6	56	M	Sarcoma	Leg	90	2	15	77
7	65	M	Melanoma	Leg	90	2	32	108
8	64	F	Melanoma	Leg	90	4	13	104
9	83	M	Sarcoma	Leg	75	4	19	174
10	55	F	Sarcoma	Leg	90	4	16	90

TNF, tumor necrosis factor.

pressed quantitatively as a percentage such that 100% leakage represented a homogeneous distribution of the isotope in the body.

Blood Sampling Procedure

Venous blood samples were collected at several time points, i.e., the day before ILP, just before administration of TNFα in the perfusate, halfway perfusion, and just before release of the tourniquet (after completion of the washout procedure at the end of the perfusion); then after ILP, 5, 10, and 30 minutes, and 3 and 7 hours after release of the tourniquet, and once a day until 7 days after ILP. Samples from the perfusate were obtained at the following times: 0 (just before administration of TNFα) and 10, 30, 60, and 90 minutes after administration of TNFα. Blood samples were immediately centrifuged; plasma was collected and stored at -70°C until tested.

Assays for Cytokine and Acute Phase Protein Analysis

Cytokine and acute phase protein (APP) levels were measured by using an enzyme-linked immunosorbent assay (ELISA). Used antibodies were obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). For measuring TNFα, as described previously,[13] flat-bottomed microtiter plates (Nunc, Kamstrup, Denmark) were coated overnight with purified monoclonal antibody (mAb) against TNFα (CLB-TNF/7). After washing, serial dilutions of TNF-containing samples were added. Bound TNFα was detected by biotinylated sheep anti-TNFα. The detection limit of the assay was 5 pg/ml. Healthy controls were at less than 5 pg/ml. The IL-6-specific ELISA has been described previously.[14] A coat of CLB-IL-6/16 was applied overnight, and bound IL-6 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-6. A lower detection limit was 1 pg/ml, and normal healthy control subjects were at less than 10 pg/ml.

For IL-8, a coat of CLB-IL-8/1 mAb was applied overnight and bound IL-8 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-8. The lower detection limit of this assay was 8 pg/ml. Normal values were at less than 20 pg/ml.[15]

C-reactive protein (CRP) levels were measured by a sandwich ELISA, using polyclonal rabbit anti-human CRP Abs as catching Abs and biotinylated mAb anti-CRP (CLB anti-CRP-2) as a detecting Ab. Results were referred to a standard (Behringwerke AG, Marburg, Germany) and expressed as milligrams per liter. The detection limit was 10 ng/liter.[16]

The ELISA used for measuring s-phospholipase A_2 ($sPLA_2$) has been described before.[17] Two different mAbs against human $sPLA_2$ were used as coating and catching antibodies respectively. The lower limit of detection was 0.1 ng/ml. Normal healthy volunteers were at less 5 ng/ml.

Statistics

Median values are expressed with range. Comparison between the cytokine and APP levels in the two groups (with and without leakage) were made by the Mann-Whitney *U* test. Values of $P = .05$ were considered to be statistically significant.

RESULTS

Systemic Toxicity

Ten patients with a systemic leakage percentage of more than 10% were entered onto this study. Leakage varied from 12% to 65% (mean, 24%; Table 1).

Because of expected toxicity, all patients are well monitored at our intensive care unit postoperatively. Systemic toxicity is summarized in Table 2. During ILP, blood pressure and pulse rate remained stable with adequate fluid management. The body temperature did not increase above 38°C. After ILP, all patients received indomethacin to suppress flu-like symptoms. Eight of 10 patients developed fever grade II (38°C to 40°C) within

TABLE 2. *Systemic toxicity after ILP in the 10 patients with leakage to the systemic circulation compared with 9 patients without leakage*

	Toxicity (WHO grade)			
	Grade 0–I	Grade II	Grade III	Grade IV
Leakage				
Fever	2	8	0	0
Hypotension	3	3	4	0
Leukocytes[a]	8	0	1	1
Platelets[b]	6	3	0	1
Bilirubin[c]	3	3	4	0
ASAT/ALAT[d]	7	3	0	0
Nausea	8	2	0	0
Nonleakage				
Fever	6	3	0	0
Hypotension	9	0	0	0
Leukocytes	9	0	0	0
Platelets	9	0	0	0
Bilirubin	9	0	0	0
ASAT/ALAT	9	0	0	0
Nausea	8	1	0	0

WHO, World Health Organization; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

[a] Grade III: $0–1.9 \times 10^9$/liter; grade IV: $<1.0 \times 10^9$/liter.

[b] Grade II: $50–74 \times 10^9$/liter; grade IV: $<25 \times 10^9$/liter.

[c] Grade II: $2.6–5 \times$ N; grade III: $5.1–10 \times$ N (N = upper limit of normal value).

[d] Grade II: $2.6–5 \times$ N.

a few hours after ILP (mean maximal temperature, 38.9°C). In the patients without detectable leakage, the mean maximal temperature was 38.1°C. In four leakage patients, the heart rate increased to more than 110 beats per minute (range, 120–132 beats per minute). Four patients had a hypotension, which was not quickly restored to normal values by fluid administration alone, and required additional treatment with dopamine (3–6 μg/kg/min) for 2 to 3 days. From the start of surgery, a mean of 8 liters was administered to the leakage patients during the first 16 hours vs. 5 liters for nonleakage patients. Leukopenia and thrombocytopenia was absent or mild. Grade IV leukopenia and thrombocytopenia in one patient was induced by leakage of melphalan. No transfusion was required. Patients without leakage did not develop hematological toxicity. All leakage patients had a hyperbilirubinemia after 2 days, and the transaminases increased (grade I-II). In the no-leakage group, only 1 patient had a mild hyperbilirubinemia.

No pulmonary or renal toxicity was observed in any patient.

Plasma Cytokine and APP Levels

In Fig. 1, median values with ranges were represented for the cytokines TNFα and IL-6 and the APPs CRP and $sPLA_2$. Median peak levels of all measured cytokines and APPs in both patient groups, depicted in Table 3, were significantly different (*P* values in Table 3). Because we know the curves of IL-8 and $sPLA_2$ from previous published experiments,[18,19] we restricted the determinations to the preoperative and the maximum level time points.

In the leakage group, very high circulating concentrations of TNFα are found during perfusion (at −45 minutes), in contrast to the nondetectable TNFα levels in patients without leakage. Plateau circulating concentrations are measured at the end of the perfusion, lasting up to 30 minutes after ILP. The small amount of TNFα that remains in the limb after the washout procedure does not increase the colossal systemic levels any further in these patients, in contrast to what is observed in patients without leakage. There we observed a brief peak of systemic levels more than 100-fold less than in leakage patients. Moreover, the peak occurs typically at 5 to 10 minutes after ILP and represents the TNFα that was left behind in the limb after the washout. TNF levels decreased already after 30 minutes, because rapid clearance of this cytokine with a short half-life time of 17 minutes is operational. Thus, in leakage patients, very high TNF concentrations are present for about 4 hours, whereas a very short peak of 20 to 30 minutes of "moderate" increased TNF levels is present in leakage-free patients. There was a strict correlation between the degree of leakage estimated by isotope monitoring, the (adjusted) dose of TNFα, and the measured maximum systemic levels of TNFα, depicted in Table 1.

IL-6 increased already during perfusion in leakage patients, immediately induced by TNFα. In the nonleak patients, IL-6 increased 5 to 10 minutes after ILP, i.e., after TNFα from the washed out limb appeared in the systemic circulation. Maximum values of IL-6 were reached 3 hours after ILP. IL-8 showed the same pattern as IL-6 (data not shown). In the control group, values of IL-6 and IL-8 were 10 to 60 times lower than in the leakage patients.

The APP CRP was increased from 3 hours after ILP until more than 7 days after ILP. Peak levels occurred at day 1. The CRP curve in patients without leakage was comparable, but the peak value was higher. Levels of $sPLA_2$ were very different for each patient. However, the pattern was consistent, with the start of the increase at 3 hours after ILP and the peak at day 1. Levels were still increased after 7 days. Levels in the nonleakage patients were factor 6 lower.

Levels of Cytokines and APPs in Perfusate

No significant difference was observed in perfusate levels of cytokines and APPs in patients who underwent ILP with systemic leakage and without leakage. From the ILP patients with systemic leakage, only five series of perfusate samples were available. Curves are presented in Fig. 2. TNF levels remained stable at approximately 7.5 μg/ml. IL-6 increased after 10 minutes of perfusion, to 4.3 ng/ml at the end of perfusion. IL-8 increased from 65 to 1600 pg/ml during perfusion. CRP did not change during perfusion, with values hardly detectable or at less than the detection limit. In three of five patients, $sPLA_2$ increased during perfusion; the median value at the end of perfusion was 14 ng/ml and the range, 8.5 to 266 ng/ml.

DISCUSSION

The aim of our study was to quantify cytokine levels and acute phase response in patients who underwent an ILP with high-dose TNFα complicated by high leakage, compared with the same variables in patients without leakage, and correlate findings with clinical toxicity. In this study, we measured TNFα plasma levels up to 277 ng/ml. Nevertheless, most of the patients needed only extra intravenous fluid administration. Systemic levels of this magnitude have been described before in the same setting[20,21] and once after a 30-minute intravenous infusion of recombinant TNFα.[22]

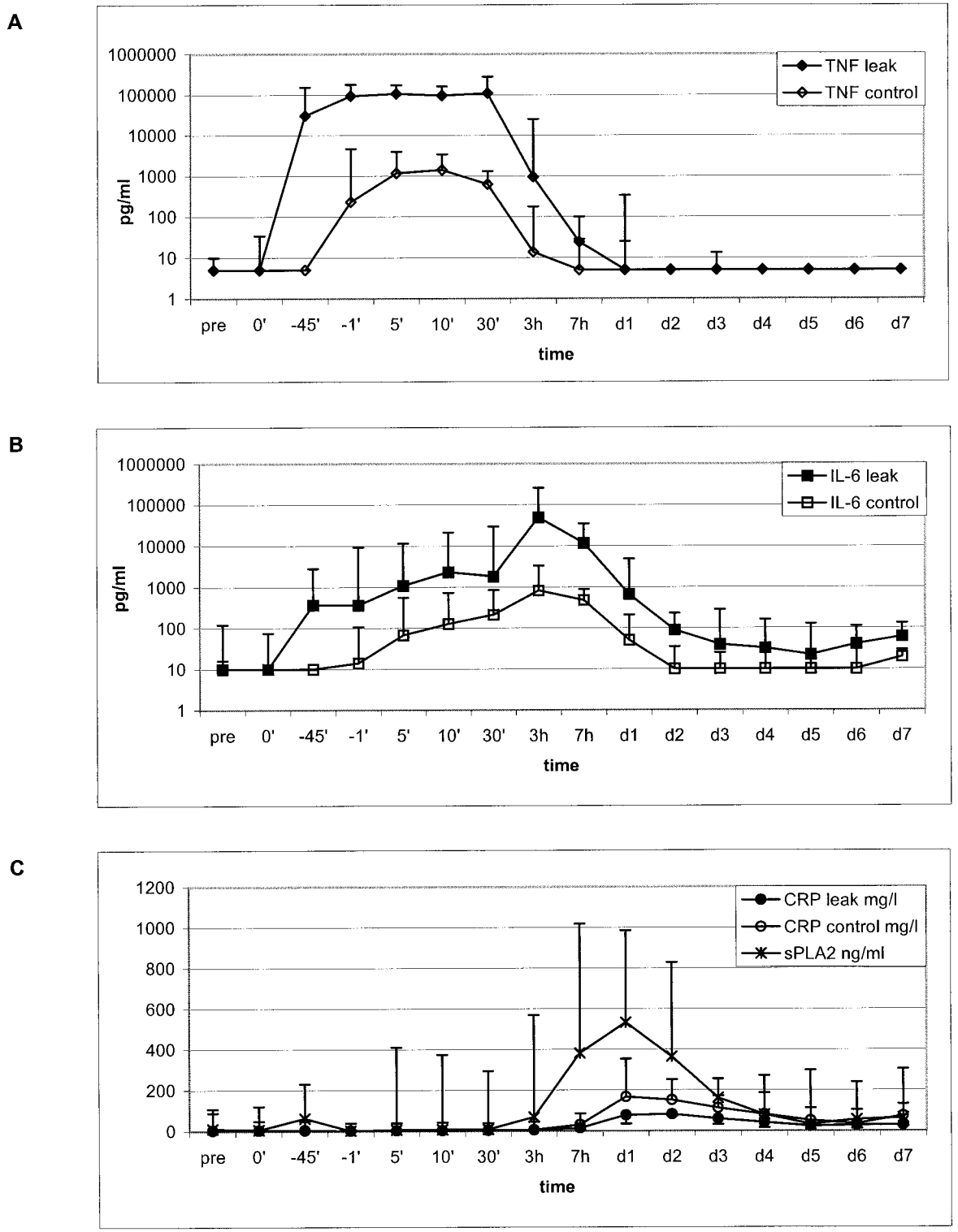

FIG. 1. Median (with range) of tumor necrosis factor (TNF) (A), interleukin (IL)-6 (B), and C-reactive protein (CRP) and s-phospholipase A_2 ($sPLA_2$) (C) in plasma from 10 patients who underwent an isolated limb perfusion (ILP) complicated by high leakage (>12%) to the systemic blood circulation. For TNF (◇; A), IL-6 (□; B), and CRP (○; C), the curves of the nonleakage group are also depicted. Time points were as follows: pre, preoperative; 0′, just before perfusion; −45′, halfway perfusion; −1′, just before release of the tourniquet; 5′ (10′ and 30′), 5 (10 and 30) minutes after release of the tourniquet; 3h and 7h, 3 and 7 hours after ILP; d1 to d7, number of days after ILP.

TABLE 3. *Median and range of peak levels of cytokines and acute phase proteins in 10 patients undergoing ILP with more than 10% leakage compared with 9 patients without leakage in the systemic circulation*

Cytokine/acute phase protein	Time point	Leakage patients, median (range)	No leakage, median (range)	*P*
TNFα, ng/ml	10 min after ILP	108 (26–277)	1.4 (0.3–3.4)	<.001
IL-6, ng/ml	3 h after ILP	49 (13–257)	0.8 (0.3–3.3)	<.001
IL-8, ng/ml	3 h after ILP	14 (1.3–49)	0.2 (0.01–1.7)	<.001
CRP, mg/l	Day 1	76 (34–419)	166 (93–350)	<.01
$sPLA_2$, ng/ml	Day 1	568 (123–986)	84 (20–390)	<.01

ILP, isolated limb perfusion; TNFα, tumor necrosis factor-α; IL-6 and IL-8, interleukin-6 and interleukin-8; CRP, C-reactive protein; $sPLA_2$, s-phospholipase A_2.

In our patients, the necessity for dopamine administration was not related to the highest levels of TNFα in the systemic circulation. This finding is in accordance with previous studies in which no correlation between maximum TNFα concentrations in the peripheral blood of an individual and the side effects could be found,[20,23] which indicates that patients vary in their sensitivity to TNFα.

Systemic toxicity seems to be determined by the duration of exposure to high levels of TNFα. Our data demonstrate this clearly, with levels of 1,000 to more than 100,000 pg/ml for 4 hours in high leakage patients, and only "moderate" levels (~1,000 pg/ml for 20 minutes) in nonleakage patients. In nonleakage patients, 20 minutes of "moderate" TNF levels were not enough to cause hypotension. This is in accordance with the findings reported previously in this journal by Vrouenraets et al.,[24] who described minimal toxicity after leakage-controlled ILP in 20 patients. The IL-6 curves demonstrate the effect of prolonged exposure to high TNF levels even more pungently. Even at 24 hours after ILP, IL-6 levels are still higher in the leakage patients than the peak IL-6 levels observed in ILP patients without leakage. IL-6 levels remained elevated for at least 3 whole days.

In the 10 patients with high leakage, 4 hours of exposure to very high levels of TNFα resulted in three patients with grade II and four patients with grade III hypotension. Four patients required dopamine support temporarily with good response. In phase I–II studies on the systemic administration of TNFα, dose-limiting toxicity was observed at TNF concentrations similar to those observed in our 10 patients described here. For instance, Schaadt et al.[22] reported dose-limiting hypotension in 32% of the patients after administration of 650 $\mu g/m^2$ intravenously, resulting in a systemic TNF peak concentration of approximately 270 ng/ml. Moreover, grade II hepatotoxicity was observed in 80% of the patients. This is quite different from the relative lack of toxicity observed in our 10 ILP patients who had similar systemic TNF peak concentrations. In other studies, hypotension was dose limiting at lower doses, with serum TNF levels of 10 ng/ml.[8,9]

In comparison with septic shock, the duration of exposure to elevated levels of TNFα in the leakage patients is relatively short. The prolonged exposure in septic shock, despite concentrations many times lower than the short peak levels after ILP, results in the typically unresponsiveness of the hypotension to fluid challenge in the septic shock patients.[25–28] Adequate diuresis plays a key role to keep the period of high circulating TNF levels as short as possible, to prevent a septic shock-like state in perfusion patients. That patients are well hydrated at the time of exposure to TNF, and that their blood pressure is optimally maintained by fluid challenge, and only if necessary also by the use of dopamine, prevents a septic shock-like situation. This explains why these patients have little toxicity in view of the very high circulating TNF levels. It is a fundamental difference, with often poorly hydrated patients with metastatic cancer who received intravenous TNFα in phase I-II studies in the past. Moreover, septic patients are infected and have significant levels of endotoxin, which has been shown to be synergistic with TNFα for toxicity (in rats).[29,30] In addition, Feelders et al.[31] have shown that, in patients who underwent ILP, cortisol is already increased before the TNF peak as a result of surgery and anesthesia. The cortisol response may have a down-regulatory effect on TNFα.

The increased TNFα levels in the patients with systemic leakage were followed by significantly higher levels of IL-6 and IL-8. This is in accordance with previous studies.[18,20,32] In our study, we also determined CRP and $sPLA_2$ as variables of the acute phase response. $sPLA_2$ levels were more increased in the leakage patients; CRP, in contrast, had significantly lower levels in these patients at the time points of maximum values. Lower levels of CRP than expected were also observed in patients who underwent an isolated hepatic perfusion.[33] That CRP had even lower levels in leakage patients could be ascribed to a higher expenditure of CRP in the

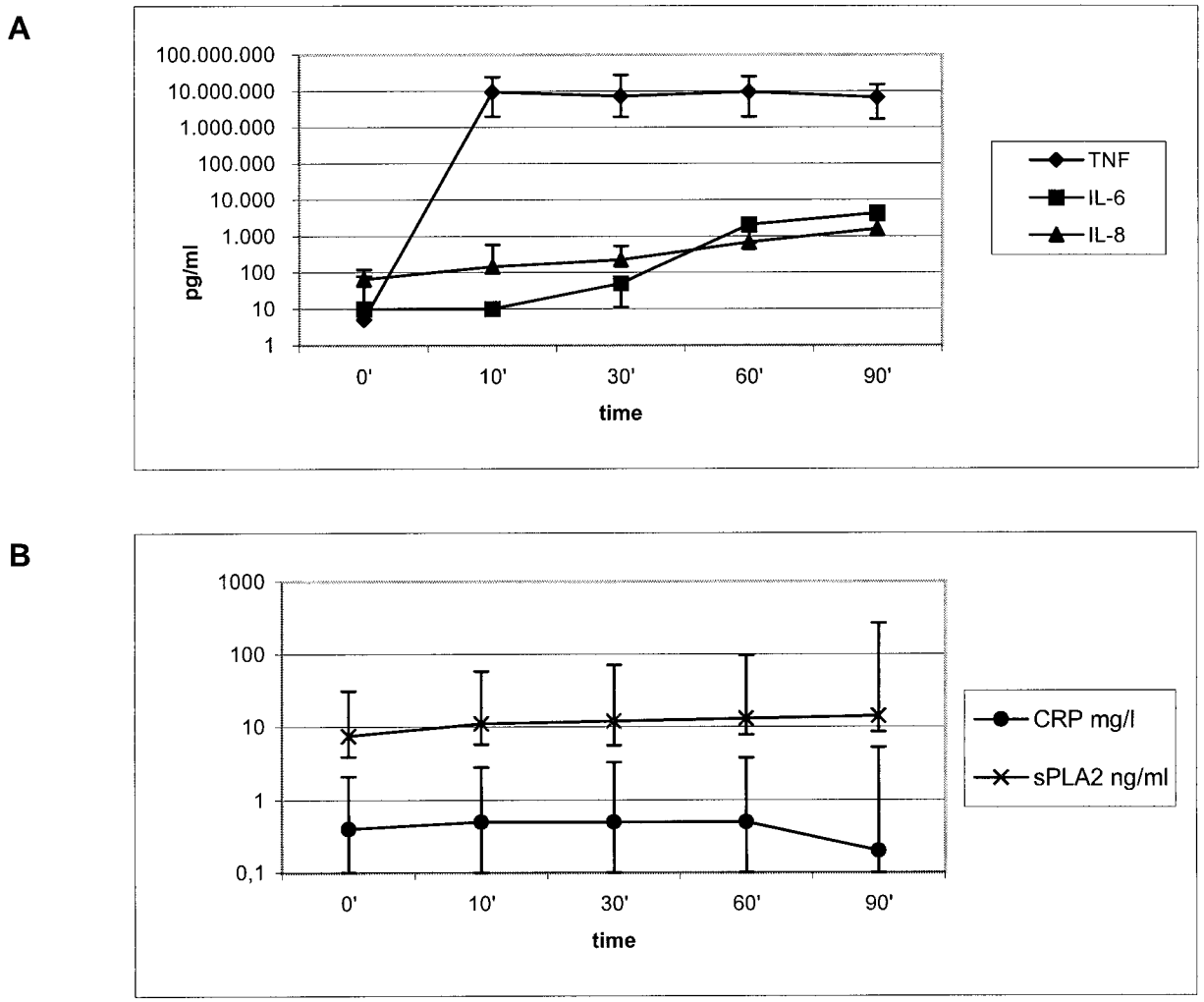

FIG. 2. Median (with range) of cytokine (A) and acute phase protein (B) profile in perfusate from five patients who underwent an isolated limb perfusion complicated by high leakage (>12%) to the systemic blood circulation. Time points were as follows: 0′, just before administration of tumor necrosis factor (TNF) in the perfusate; 10′ (30′ and 60′), 10 (30 and 60) minutes after start of perfusion; 90′, end of perfusion, just before release of the tourniquet. IL-6, interleukin-6; CRP, C-reactive protein; $sPLA_2$, s-phospholipase A_2.

neutralization of the effects of exposure to higher levels of TNFα.[34–36]

In conclusion, ILP, complicated by high leakage to the systemic circulation, resulted in high systemic levels of TNFα up to 277 ng/ml. IL-6 and IL-8 followed with significantly higher levels compared with values measured in patients without leakage. The patterns of the APPs CRP and $sPLA_2$ resembled each other, except that CRP had significantly lower maximum levels in leakage patients compared with patients without leakage. Overall, the patients with high systemic leakage had a marked mild clinical course. It is our experience that, even in patients with very high leakage, life-threatening reactions have not occurred and that temporary hypotension can be easily dealt with by fluid challenge and sometimes by temporary vasopressor support. Our observations support the need for further study of the potential use of TNFα systemically.

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