Lack of Plasma Interleukin-1β or Tumor Necrosis Factor-α Elevation During Unfavorable Hemodialysis Conditions

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(J. Am. Soc. Nephrol. 1991; 2:1007-1013)

ABSTRACT
Plasma interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were determined by ELISA in 17 healthy controls, 23 HD patients, 10 continuous ambulatory peritoneal dialysis patients, and 15 chronic renal failure patients, as well as in 2 HD patients experiencing pyrogenic reactions. Another group of 10 chronic HD patients were dialyzed for 2.5 h, 5 with first-use Cuprophane® membranes and 5 with first-use high-flux cellulose triacetate membranes. The mean bacterial and endotoxin concentrations of the dialysate used for HD treatments during the study period were 18,440 ± 530 CFU/mL (mean ± SEM) and 976 ± 205 pg/mL, respectively. Blood specimens were obtained intradialysis and postdialysis for cytokine assay and were incubated to augment cytokine production. There was no difference in plasma IL-1β or TNF-α concentrations among the healthy controls, continuous ambulatory peritoneal dialysis patients, chronic renal failure patients, or HD patients. Neither cytokine increased significantly during or after HD. Two patients experiencing pyrogenic reactions had plasma TNF-α concentrations of 537 and 413 pg/mL, compared with matched controls of 6 and 0 pg/mL. IL-1β concentration did not differ from controls. We conclude that: (1) plasma IL-1β and TNF-α are not chronically elevated in chronic renal failure, continuous ambulatory peritoneal dialysis, or HD patients; (2) HD with new Cuprophan or cellulose triacetate membranes and high concentrations of dialysate endotoxin and bacteria does not cause elevation of circulating IL-1β or TNF-α; and (3) pyrogenic reactions might be mediated by TNF-α.

Key Words: Cytokines, hemodialysis, ESRD, pyrogenic reactions, endotoxin

In 1983, Henderson et al. proposed the "Interleukin Hypothesis" implicating interleukin-1β (IL-1β) as the mediator of acute and chronic sequelae occurring in chronic hemodialysis (HD) patients (1). More recently, tumor necrosis factor (TNF) has been suggested to induce similar sequelae (2,3). These cytokines share an array of physiologically mediated activities including fever, fatigue, lethargy, headache, excessive sleepiness, osteopenia, lipid abnormalities, and increased catabolism of fat, muscle, and connective tissue (2-5). The exact role of cytokines in the clinical problems of the ESRD patient has not been elucidated. However, recent technological advances in cytokine assay procedures have dramatically improved the ability to investigate this area.

Measurement of IL-1β and TNF-α by ELISA has been shown to be sensitive and specific and lacking the problems associated with other cytokine assays (6-8). In this study, we measured IL-1β and TNF-α plasma concentrations by ELISA in healthy volunteers, patients receiving chronic ambulatory peritoneal dialysis (CAPD), chronic renal failure (CRF) patients not on dialysis, HD patients experiencing pyrogenic reactions, and patients undergoing HD with conditions purported to favor cytokine release.

MATERIALS AND METHODS
Study Population
These studies were approved by the Human Investigation Committee of Emory University School of Medicine. Informed consent was obtained.
A total of 65 subjects in four groups was enrolled in this study and included the following: (1) 23 patients with ESRD receiving chronic HD three times a week for more than 6 months; (2) 10 ESRD patients receiving CAPD; (3) 15 patients with CRF (serum creatinine >5 or creatinine clearance <20 mL/min)
not receiving dialysis; and (4) 17 healthy controls. Patients with recent sepsis, fever, or active connective tissue disease or those taking immunosuppressive drugs were excluded. Plasma from three septic, nondialysis patients were also included for assay.

**Blood Collection**

Five milliliters of blood was aseptically collected via venipuncture or from the venous side of an arteriovenous graft before HD. All glassware was made endotoxin free by being baked at 180°C for 4 h. Collection tubes contained 1.5 mg/mL of EDTA (Sigma Chemical Co., St. Louis, MO) and 0.67 trypsin inhibitory units (TIU)/mL of aprotinin (Sigma) as described by Cannon et al. (9). Specimens were immediately placed on ice. Plasma was separated by centrifugation (1,000 x g for 10 min) and stored at -70°C.

**Cytokine Assays**

ELISA for IL-1β and TNF-α were used according to the recommendations of the manufacturer (Cistron Biotechnology, Pinebrook, NJ). The assay for IL-1β has been shown to be specific for IL-1β with no cross-reactivity with IL-1α, IL-2, TNF, or interferon gamma. The assay for TNF-α has been shown to be specific for TNF-α with no cross-reactivity with TNF-β, IL-1α, IL-1β, IL-2, IL-3, IL-4, or IL-6. The lowest concentration of either cytokine detectable by this assay is reportedly 20 pg/mL at a 95% confidence limit (personal communication, Cistron Biotechnology). We found that standard curves for IL-1β and TNF-α from 12.5 to 200 pg/mL revealed correlation coefficients of 0.995 and 0.998 curves. Determinations of TNF-α and IL-1β concentrations in plasma from 1 to 20 pg/mL were reproducible.

Pooled uremic plasma was used as the standard curve matrix for samples from uremic patients. Pooled plasma was heated to 56°C for 1 h to inactivate any cytokines that might be present. Nonuremic plasma was similarly prepared for control samples. To evaluate the effect of lipid or protein factors on plasma was similarly prepared for control samples. Determinations of TNF-α and IL-1β concentrations in plasma from 1 to 20 pg/mL were reproducible.

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**Cytokines During and After HD**

Ten adult chronic HD patients were dialyzed for 2.5 h. Five of these patients were treated with first-use nonreprocessed high-flux cellulose triacetate (CTA) membranes (CTA-190; Baxter Healthcare Corp., Deerfield, IL) as part of their usual dialysis regimen. The remaining five patients were changed from their usual CTA dialyzer to first-use nonreprocessed Cuprophan® membranes (CF 23-08; Baxter Healthcare Corp.). Drake-Willock model 480 ultrafiltration controlled dialysate delivery units (Drake-Willock, Inc., Portland, OR) and bicarbonate concentrate naturally contaminated with high concentrations of bacteria and endotoxin were used. Water containing <200 CFU/mL was used to dilute the bicarbonate concentrate. Dialysate endotoxin concentrations and bacterial counts were measured at the start of dialysis. Oral temperatures were obtained predialysis, middialysis, and end of dialysis with an electronic thermistor (Model 2000; IVAC Thermometers, San Diego, CA). Arterial blood was obtained from the arteriovenous HD fistula intradialysis after heparin bolus at 0, 1.5, and 2.5 h. Postdialysis blood was obtained at 5, 24, and 48 h. Hemoglobins were measured to correct cytokine plasma levels for hemocencentration. By using a laminar flow biological safety cabinet, collected whole blood was separated into three equal aliquots. Plasma was immediately obtained from one aliquot. The remaining two aliquots were incubated in an attempt to augment cytokine production, if cytokine-inducing material (CIM) was present. One was incubated at 37°C for 2 h, and the other was incubated at 37°C for 2 h plus an additional 24 h at 25°C. A previous in vitro study demonstrated that IL-1 concentrations were greater after a 26-h incubation and TNF concentrations were maximal after a 2-h incubation (11). After incubation, plasma was obtained for assay.

**Microbial and Endotoxin Assays**

Dialysate endotoxin concentrations were determined by using the Limulus amebocyte lysate (LAL) turbidimetric assay system (LAL-5000; Associates of Cape Cod, Woods Hole, MA) with Pyrotell GT lysate (Associates of Cape Cod). Dialysate was cultured by using membrane filtration (0.45-μm pore size), and membranes were directly plated on Trypticase soy agar (BBL, Becton-Dickinson Microbiology Systems, Cockeysville, MD) (10). Cultures were incubated at 30°C for 72 h, and colony counts were obtained at 24, 48, and 72 h.

**Cytokines and Pyrogenic Reactions**

Oral temperatures were obtained at the beginning, middle, and end of HD in approximately 210 patients treated in three dialysis units during a 30-day period. A pyrogenic reaction (PR) was defined as a rise in patient temperature to ≥37.8°C or rigors in a patient afebrile at the initiation of dialysis. Arterial blood was immediately obtained from any patient experiencing a PR. Control specimens were obtained from patients concurrently dialyzed with the same dialysate and dialyzer type.
Statistical Analysis

Results are expressed as the mean sample level ± SE. Significance was assessed by using Kruskal-Wallis for nonparametric paired data and the t test for group means for paired data. Analysis of variance was used for parametric, paired data.

RESULTS

Baseline Cytokines in Study Groups

There was a reduced sensitivity of the TNF-α ELISA for uremic plasma samples compared with nonuremic normal plasma (Figure 1). Chloroform extraction did not alter the TNF-α sensitivity in either uremic or nonuremic plasma (data not shown). However, the blunted sensitivity was eliminated by using uremic plasma as the standard curve matrix. Consequently, all samples from ESRD patients in this study were assayed with uremic plasma as the reference standard curve matrix. IL-1β sensitivity was identical in both uremic and nonuremic plasma.

Baseline IL-1β or TNF-α plasma concentrations from 17 healthy controls, 10 CAPD patients, 15 CRF patients, and 33 HD patients were not significantly different (Table 1). Isolated elevations in the various groups were not associated with clinical symptoms.

Two HD patients experiencing pyrogenic reactions during HD treatments had elevated concentrations of TNF-α when compared with matched controls. IL-1β concentrations in these patients did not differ from those of matched controls (Table 1).

Samples of plasma from three nondialysis patients who succumbed to septic shock after receiving packed erythrocyte units contaminated with Yersinia enterocolitica and high concentrations of endotoxin were also assayed for TNF-α and IL-1β. Plasma samples were obtained 1 to 4 h post-transfusion, and all contained high concentrations of TNF-α (mean of 2,255 pg/mL). Plasma from one of the three patients had detectable IL-1β (2,196 pg/mL) (Table 1).

Cytokines During and After HD

Ten patients, six male and four female, with a mean age of 55.3 ± 4.7 yr were studied. The mean dialysate bacterial count at the beginning of dialysis was 18,440 ± 530 CFU/mL (range, 15,100 to 20,100), and the mean dialysate endotoxin concentration was 976 ± 205 pg/mL (range, 640 to 2,700). Although there was an overall rise in temperature during dialysis (mean predialysis, 36.6 ± 0.16°C to postdialysis, 36.9% ± 0.17°C), no individual experienced a rise in temperature exceeding 37.4°C or rigors. First-use syndrome was not observed during any dialysis.

TNF-α concentrations were below detectable limits in all 60 samples, and IL-1β was not detected in 59 out of 60 plasma samples (Table 2). One sample contained 10 pg/mL of IL-1β, which is below the reported sensitivity of the assay. HD with either Cuprophan or high-flux CTA dialysis membranes did not increase cytokine production. Comparison of preincubation and postincubation cytokine levels over time within each group and between groups did not indicate significant differences (Table 3).

TABLE 1. IL-1β and TNF-α plasma concentrations in healthy controls, CAPD patients, CRF patients, HD patients, PR and three septic nondialysis patients

<table>
<thead>
<tr>
<th>Study Group</th>
<th>TNF-α +/Test pg/mL</th>
<th>IL-1β +/Test pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls</td>
<td>1/17</td>
<td>2/17</td>
</tr>
<tr>
<td>CAPD Patients</td>
<td>0/0/10</td>
<td>0/0/10</td>
</tr>
<tr>
<td>CRF Patients</td>
<td>1/15</td>
<td>3/15</td>
</tr>
<tr>
<td>HD Patients</td>
<td>0/33</td>
<td>3/33</td>
</tr>
<tr>
<td>PR Reactors</td>
<td>2/2 (413, 537)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Matched controls</td>
<td>1/2 (17, 25)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Septic, Nondialysis</td>
<td>3/3 (2,102, 2,370)</td>
<td>1/3 (0)</td>
</tr>
</tbody>
</table>

* +/Test, number of tests positive/total number of tests.

TABLE 2. IL-1β and TNF-α plasma concentrations in 10 HD patients predialysis, intradialysis, end of dialysis, and 5, 24, and 48 h postdialysis

<table>
<thead>
<tr>
<th>Sample Collection Period</th>
<th>TNF-α +/Test pg/mL</th>
<th>IL-1β +/Test pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predialysis (0 h)</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Intradialysis (1.25 h)</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>End of dialysis (2.5 h)</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>5 h postdialysis</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>24 h postdialysis</td>
<td>0/10</td>
<td>1/10</td>
</tr>
<tr>
<td>48 h postdialysis</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

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TABLE 3. Number of positive samples and concentrations of TNF-α and IL-1β in plasma from nonincubated versus incubated whole blood collected from 10 patients during HD

<table>
<thead>
<tr>
<th>Sample Collection Period</th>
<th>TNF-α Nonincubated</th>
<th>Incubateda</th>
<th>IL-1β Nonincubated</th>
<th>Incubatedb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/Test (pg/ml)</td>
<td>+/Test</td>
<td>+/Test (pg/ml)</td>
<td>+/Test</td>
</tr>
<tr>
<td>Predialysis</td>
<td>0/10</td>
<td>2/10</td>
<td>2.20</td>
<td>0/10</td>
</tr>
<tr>
<td>Middialysis</td>
<td>0/10</td>
<td>2/10</td>
<td>5.5</td>
<td>0/10</td>
</tr>
<tr>
<td>End of dialysis</td>
<td>0/10</td>
<td>2/10</td>
<td>1.3</td>
<td>0/10</td>
</tr>
<tr>
<td>5 h postdialysis</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>24 h postdialysis</td>
<td>0/10</td>
<td>0/10</td>
<td>1.10</td>
<td>4/10</td>
</tr>
<tr>
<td>48 h postdialysis</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
<td>4/10</td>
</tr>
</tbody>
</table>

a Whole blood incubated for 2 h at 37°C.
b Whole blood incubated for 2 h at 37°C plus an additional 24 h at 25°C.

DISCUSSION

The study presented here demonstrates that IL-1β and TNF-α plasma concentrations are similar in healthy controls, HD patients, CAPD patients, and CRF patients. There was no demonstrable cytokine release during or after a HD treatment of 10 chronic HD patients, despite conditions favorable for cytokine induction (i.e., high-flux or complement-activating dialyzer membranes plus high concentrations of dialysate endotoxin and bacteria). Incubation of whole blood from these patients failed to significantly increase plasma cytokines, which suggests that clinically significant amounts of CIM were not released into the dialyzer blood compartment. PR during HD of two patients were associated with TNF-α elevation, which may act as a primary indicator of these reactions.

Several mechanisms for cytokine induction during HD have been suggested (12). First-use Cuprophan membranes consistently activate complement (13). Activated components of complement are known to cause mononuclear cell release of IL-1 and TNF (14–16). Although mononuclear cells incubated in the presence of activated complement readily release cytokines in vitro, the brief exposure of these cells to activated complement during clinical HD might not be an adequate stimulus for cytokine release. Yet, intradialytic increases in monocyte-associated or plasma IL-1 activity during HD when Cuprophan membranes are used have been described (17,18).

The dialysis membrane, independent of complement activity, has been shown to induce IL-1 and TNF production in the monocyte (15,17). One recent study differs from those results. Schindler et al. measured IL-1β and TNF-α by RIA and used DNA probes to assess mononuclear cell transcription. They demonstrated evidence for cytokine mRNA synthesis that was greater in the presence of Cuprophan than polysulfone membranes. However, cytokine peptide synthesis only occurred when stimulated by lipopolysaccharides (19).

Small-molecular-weight microbial products in the dialysate can theoretically cross into the blood compartment through membrane defects or convective transport by back-filtration with highly permeable membranes, thereby inducing cytokine release (20). This risk, clinically, might be overstated because a number of studies have demonstrated dialyzer membranes to be an effective barrier to the transfer of bacteria and endotoxin (21,22). Tierno and Aboody examined bacterial transfer in the presence of blood leaks and were unable to detect transfer of bacteria into the blood compartment (23). We previously reported that after approximately 27,000 HD treatments with dialysate containing high levels of endotoxin and a mean bacterial count greater than 19,000 CFU/mL, the incidence of febrile reactions was considered low at 0.9 per 1,000 treatments and did not significantly differ by conventional, high-efficiency, or high-flux dialysis (24). Other investigators have demonstrated that endotoxin does not readily transfer into the blood compartment of patients dialyzed with large quantities of endotoxin in the dialysate (25,26). Without a specific assay, the status of endotoxin fragment transfer remains uncertain (12).

Another potential mechanism for dialysis cytokine induction is acetate exposure. Acetate has been implicated in causing peritoneal fibrosis when used as a peritoneal dialysate (27). Monocytes incubated in the presence of sodium acetate have been shown to have an increase in IL-1 activity (28). However, the clinical importance of cytokine production during acetate HD remains unknown.

This study incorporated conditions purported to induce cytokine production during chronic HD: (1) complement-activating membranes; and (2) high-flux dialysis with high concentrations of microbial products in the dialysate. During the study presented here, we did not measure C3a and C5a but assumed
complement activation with first-use Cuprophan membranes (13). Despite these conditions, we were not able to demonstrate cytokine elevation during or after HD. Our findings agree with those of Holmes et al. who examined IL-1β and TNF-α plasma levels, by an ELISA, during HD with high-flux dialysis and dialysate with microbial concentrations within the current Association for the Advancement of Medical Instrumentation (AAMI) limits (29). These results differ from those of Herbelin et al., who, by using a similar assay, noted low levels of plasma IL-1β in chronic HD patients that increased during dialysis (6). However, most of the patients in that study were treated with acetate dialysate. The low level of acetate (3 mEq/L) used with bicarbonate dialysate during this study did not induce cytokine release (Table 2).

Several reports have demonstrated increased IL-1β plasma concentrations or monocyte-associated IL-1β activity in HD patients (12,17,18,30,31). However, bioassays of IL-1β are confounded by interfering substances in human plasma (9), lack of specificity from cross-over with other cytokines (19), and possible contamination of bioassay medium with lipopolysaccharide (9). RIA increase the sensitivity of cytokine measurements in vitro but have been plagued with difficulties when assaying human plasma because of precipitation of radioactivity (6), reducing both sensitivity and specificity. Assays which demonstrate increased monocyte-associated IL-1β activity are of uncertain clinical significance because circulating cytokines behave as hormones (17). The lack of specificity of bioassay techniques can also lead to higher than expected titers of cytokines (32). In a review by Meager et al., it was concluded that bioassays are not the ideal system for measuring biological activity of cytokines because endogenous and environmental factors may distort the results to such a degree that inappropriate conclusions could be drawn regarding cytokine activity (33). Similarly, Desch et al. found the ELISA superior to biological assays because the former avoids monocyte activation that can occur during monocyte isolation procedures required in biological assays (34). Others have reported that the ELISA provides a highly specific assay that measures cytokines directly in the plasma without cross-reactivity (35,36).

The cytokine ELISA that we used proved highly sensitive (Figure 1), but intrinsic uremic factors reduced the sensitivity for TNF-α but not for IL-1β. Although the precise cause of this aberrancy is unknown, acidosis or increased osmolality might interfere with substrate utilization or immunoglobulin binding with the TNF-α ELISA. Use of uremic plasma matrix for the development of standard curves ameliorates this phenomenon and should be considered for TNF-α assays of uremic subjects.

Until recently, in vitro testing of cytokine production had been cumbersome, often requiring serum-free conditions that made the test difficult (9). In the study presented here, whole-blood from HD was incubated in an effort to examine transfer of CIM during clinical HD. CIM can include endotoxin fragments or other bacterial cell wall products that may be unreactive to the Limulus amebocyte lysate assay. If one assumes substantial transfer of CIM during HD, incubation of blood from HD patients should induce a greater rise in plasma cytokines than that seen in healthy controls or CRF patients. Our attempt to induce cytokine release through incubation of the blood of hemodialyzed patients did not induce a greater increase in either TNF-α or IL-1β. If no synthesis of IL-1 or TNF occurs in the absence of endotoxin as previously suggested (19), then the lack of change in the incubated blood cytokine concentration implies that transfer of substantial amounts of endotoxin or CIM does not occur.

Cytokine measurements associated with PR during HD have not previously, to our knowledge, been reported. The two patients experiencing PR on HD in our study had high elevations of TNF-α, but not IL-1β. Lonnemann et al. reported a patient, experiencing fever and chills during hemofiltration with an endotoxin-contaminated hemofilter, who had a rise in TNF-α but not in IL-1β (37). The transient nature by which TNF-α rises after an endotoxin challenge, as demonstrated by Michie et al. (38), suggests that TNF levels are higher during the prodromal chill. Interestingly, both of our patients experienced only rigors as manifestations of their PR. Plasma from three septicemic nondialysis patients also displayed high elevations of TNF-α, and one had a high elevation of IL-1β.

Although there was an overall insignificant rise in temperature during HD in the 10 HD patients, the lack of cytokine elevation might suggest a mechanism other than cytokines to explain the low-grade rise in temperature during HD. This is in contrast to the two patients experiencing symptomatic febrile reactions in this study that had fever and chills most likely mediated through acute elevations of TNF-α.

The proposed biological effects of IL-1 or TNF in the chronic HD patient such as osteopenia, anorexia, and cachexia (1,2,5) can be explained by the physiology of ESRD or chronic disease per se. Indeed, the current investigation failed to find evidence of circulating cytokines in the ESRD patient. Despite our findings, the role of increased cell-associated cytokines in HD (12) remains uncertain. Furthermore, the significance of elevated cytokine mRNA in the mononuclear cells of HD patients remains to be determined.

In conclusion, the investigation presented here does not demonstrate any evidence to support chronic or recurrent elevations of plasma IL-1β or TNF-α during or after HD. However, HD-induced symptomatic PR appear to be mediated by TNF-α.
ACKNOWLEDGMENTS

This research was supported in part by funding from Dialysis Clinic, Inc., Nashville, Tennessee. The authors thank Dr. William Mitch for reviewing the manuscript. The tireless support of the nursing, technical, and secretarial staff at West Peachtree DCI (Atlanta) is very much appreciated.

REFERENCES


