The Effect of Uremia on Tumor Necrosis Factor-Alpha Release After an In Vitro Whole-Blood Endotoxin Challenge

Carl W. Oettinger, Lee A. Bland, Jamie C. Oliver, Matthew J. Arduino, Sigrid K. McAllister, and Martin S. Favero

ABSTRACT

Uremia has been associated with immunologic aberrations, including anergy, increased susceptibility to infections, and reduced phagocytic activity of polymorphonuclear leukocytes. In this study, cytokine release in uremic and nonuremic blood after in vitro endotoxin stimulation was studied. Blood from nonuremic controls, chronic renal failure patients not on dialysis, and chronic hemodialysis patients predialysis and postdialysis was spiked with 10 ng/mL of Escherichia coli endotoxin and incubated for 26 h. Plasma tumor necrosis factor-alpha (TNFα) concentrations were determined by ELISA after each incubation period. To further study which uremic blood component may be responsible for enhanced release of TNFα, plasma and cellular components of chronic renal failure patients and controls were switched and then given an in vitro endotoxin stimulation (1 ng/mL). It was found that (1) TNFα release is enhanced by uremia and is exacerbated with progressive declines in renal function, (2) enhanced TNFα release is related to a blood cellular phenomenon induced by uremia, and (3) enhanced TNFα release in hemodialysis patients is associated with a prolonged stimulation and/or reduced plasma elimination of TNFα.

Key Words: Endotoxin, hemodialysis, chronic renal failure, cytokines, ELISA

The uremic state is characterized by a variety of immunologic abnormalities, including anergy (1–3), increased susceptibility to mycobacterial infections (4), and reduced responsiveness to certain vaccinations such as influenza (5) and hepatitis B (6). Reduced phagocytic activity of polymorphonuclear (PMN) leukocytes has also been described in uremia (7), as has a higher incidence of infections in patients with renal failure (8,9).

Aberrations in cytokine release may also play a role in the infectious complications associated with uremia. Several reports have characterized increased concentrations of tumor necrosis factor binding protein (TNF-BP) in both urine and plasma of patients with chronic renal failure (CRF) (10–13). It has been observed that the concentration of TNF-BP increases as renal function declines (14). We have previously reported an increase in tumor necrosis factor-alpha (TNFα) release in uremic whole blood compared with nonuremic whole blood after an in vitro endotoxin challenge (15). As a follow-up to this observation, we evaluated the effect of progressive declines in renal function on endotoxin-stimulated TNFα release. In addition, studies were performed to ascertain if cellular blood components or substances retained in uremic plasma were responsible for this enhanced TNFα release.

MATERIALS AND METHODS

The study was divided into two parts; the first evaluated the effect of progressive declines in renal function on endotoxin-stimulated TNFα release, and the second determined if cellular blood components or plasma substances are responsible for enhanced TNFα release in blood from uremic patients. An in vitro whole-blood model was used in both parts to analyze TNFα release (16). All studies were approved by the Human Investigations Committee of Emory University and the Institutional Human Subjects Re-
view Board of the Centers for Disease Control and Prevention. Informed consent was given by all subjects before venipuncture. Subjects with recent sepsis, infection, fever, or active connective tissue disease and those taking cyclooxygenase inhibitors or immunosuppressive drugs were excluded from the study.

PART 1. DECLINING RENAL FUNCTION AND TNFα RELEASE

Four groups were enrolled to examine the effect of declining renal function on TNFα release: 10 nonuremic controls, 10 hemodialysis patients both predialysis (HDpre) and postdialysis (HDpost), 6 patients with a creatinine clearance (Ccr) from 30 to 50 mL/min (CRF group 1; CRF1), and 11 patients with a Ccr from 10 to 30 mL/min (CRF group 2; CRF2). All hemodialysis patients had been receiving high-flux dialysis (blood flow, Qb = 400 mL/min; dialysate flow, Qd = 500 mL/min) with cellulose triacetate hemodialyzers (Model CT-190; Baxter Healthcare, Deerfield, IL) for an average of 165 min (range, 135 to 195 min). Bicarbonate dialysate containing an average bacterial and endotoxin burden of <15 CFU/mL and 15 pg/mL, respectively, was used in all patients for more than 1 yr. Hemodialysis patients were anticoagulated with sodium heparin (<4,000 U/dialysis), 60% at hook-up and 40% at the midpoint of dialysis. The last dose of heparin was administered at least 68 min before postdialysis TNFα sampling. Ccr was calculated by the formula (17):

\[ Ccr = \frac{(140 - \text{age})(IBW)}{(72)(Scr)} \]

where Ccr is milliliters per minute, IBW is ideal body weight in kilograms, and Scr is serum creatinine in milligrams per deciliter.

Twenty-milliliter blood samples were obtained aseptically either by venipuncture from nonuremic controls and CRF patients or just after graft cannulation, before heparinization, from hemodialysis patients. The samples were collected in endotoxin-free glassware containing 1.5 mg/mL of EDTA (Sigma Chemical Co., St. Louis, MO) (Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.) and 0.67 trypsin inhibitory units (TIU)/mL of aprotinin (Sigma) (18) and were subsequently divided into five aliquots. Plasma for baseline data determination was removed from one aliquot after centrifugation at 13,000 \( \times g \) for 3 min and stored at \(-70^\circ C\) until assayed. Of the four remaining aliquots, two were spiked with 10 ng/mL of Escherichia coli O113 endotoxin (Associates of Cape Cod, Woods Hole, MA), and two served as unspiked controls. The four aliquots were placed on an orbital rotator for 2 h at 35°C. One spiked and one unspiked aliquot from each pair was centrifuged, and the plasma was stored at \(-70^\circ C\). The remaining two aliquots were kept at 25°C for an additional 24 h before plasma was removed and stored.

TNFα concentrations were determined by an ELISA. Assays were performed in duplicate on each sample. Commercial TNFα ELISA kits (Cistron Biotechnology, Pinebrook, NJ) were used according to the manufacturer’s recommendations. The manufacturer’s stated sensitivity is 20 pg/mL; however, we previously reported reproducible detection of concentrations as low as 1 pg/mL (15). Undetectable concentrations were assigned a value of zero (0 pg/mL); otherwise, the actual value measured was reported. A plasma matrix for the standard curve was prepared from pooled plasma that was heated at 56°C for 1 h to inactivate endogenous cytokines, as recommended by the manufacturer.

PART 2. BLOOD COMPONENTS AND ENHANCED TNFα RELEASE IN UREMIA

Ten nonuremic controls and 10 chronic hemodialysis patients not previously studied in Part 1 were enrolled in Part 2 of the study. All control subjects and hemodialysis patients met the criteria for eligibility as described in Part 1.

Each hemodialysis patient was matched to a nonuremic control by blood type. Figure 1 contains a diagram of the study groups. Briefly, 25-mL blood samples were obtained aseptically either by venipuncture from nonuremic controls or just after graft cannulation, before heparinization, from hemodialysis patients. The samples were put into endotoxin-free glassware as previously described.

Blood from each patient in a matched pair was divided into four equal samples. The plasma and cellular components were divided by centrifugation and were resuspended by gentle agitation to construct the following groups: Group 1, nonuremic cells and uremic plasma; Group 2, uremic cells + non-

![Figure 1. Schematic representation of the group construction in Part 2. All groups were analyzed for baseline TNFα and endotoxin (ET)-spiked and ET-unspiked TNFα concentrations at 2 and 26 h. UP, uremic plasma; NP, nonuremic plasma; UC, uremic cells; NC, nonuremic cells.](image-url)
uremic plasma: Group 3, nonuremic cells and nonuremic plasma: Group 4, uremic cells plus uremic plasma.

One aliquot from each group served as a baseline. Plasma was separated and stored for analysis as in Part 1. Two aliquots from each group were spiked with 1 ng/mL of E. coli O113 endotoxin (Associates of Cape Cod), and the remaining two aliquots served as unspiked controls. TNFα concentrations were measured at baseline, 2 h, and 26 h as previously described.

DATA ANALYSIS

All data are reported as median (range) unless otherwise noted. TNFα concentrations reported are the actual concentrations measured and in some cases are below the manufacturer’s stated limit of sensitivity. Baseline cytokine elevations, if present, were subtracted from study concentrations before analysis. The data were tested for normality of distribution before analysis, to determine if nonparametric statistics should be used with a univariate procedure on SAS 6.06 (SAS Institute, Cary, NC). Differences between the groups were assessed by the use of Kruskal-Wallis nonparametric analysis of variance, and Wilcoxon sign-rank was used to compare differences between predialysis and postdialysis samples on the same patient. The use of nonparametric tests reduces the statistical power but prevents errors that would occur with an inappropriate parametric test.

RESULTS

Part 1. TNFα concentrations were undetectable in any baseline or unspiked sample. Significant differences in TNFα were seen only when the nonuremic control group was compared with CRF2 (P ≤ 0.02) or HDpre (P ≤ 0.02) groups 2 h postendotoxin exposure and with HDpre (P = 0.01) and HDpost (P ≤ 0.005) groups 26 h after endotoxin exposure (Table 1). The median TNFα concentration was 0 pg/mL (undetected) in controls, CRF1, and CRF2 after 26 h of incubation (Table 1). However, TNFα concentrations in HDpre and HDpost samples remained significantly elevated (Table 1). It should be noted that the HDpost samples did contain small amounts of heparin (=1 U/mL); however, the concentration of heparin present postdialysis is dramatically lower than previously demonstrated to produce errors in measured TNFα concentrations (18,19). In addition, the increase in TNF-BP induced by heparin is short lived (≈60 min) and may not interfere with postdialysis concentrations of TNFα (20).

TNFα concentrations at 2 h were not significantly different between HDpre and HDpost or between HDpost and controls, but were significantly different

<table>
<thead>
<tr>
<th>TABLE 1. Median TNFα concentrations in nonuremic controls, CRF patients, and HDpre and HDpost patients 2 and 26 h after an in vitro 10 ng/mL E. coli endotoxin challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>(N = 10)</td>
</tr>
<tr>
<td>CRF1</td>
</tr>
<tr>
<td>(Range)</td>
</tr>
<tr>
<td>(N = 6)</td>
</tr>
<tr>
<td>CRF2</td>
</tr>
<tr>
<td>(Range)</td>
</tr>
<tr>
<td>(N = 11)</td>
</tr>
<tr>
<td>HDpre</td>
</tr>
<tr>
<td>(Range)</td>
</tr>
<tr>
<td>(N = 10)</td>
</tr>
<tr>
<td>HDpost</td>
</tr>
<tr>
<td>(Range)</td>
</tr>
<tr>
<td>(N = 10)</td>
</tr>
</tbody>
</table>

* CRF1 are patients with Ccr = 30 to 50 mL/min.  
* CRF2 are patients with Ccr = 10 to 30 mL/min.  
* Significantly greater (P ≤ 0.02) than controls at some postexposure time. 0 pg/mL undetected.

(P ≤ 0.02) between controls and HDpre. Also, an increasing trend in TNFα release was observed as Ccr declined (analysis of variance for trend; P = 0.03). The use of actual concentrations instead of setting all undetectable samples to 20 pg/mL does not alter the statistical outcome.

Part 2

TNFα concentrations were not significantly different in the nonuremic cell matrix, regardless of the plasma matrix at either 2 or 26 h. Median TNFα concentrations were undetectable in samples containing nonuremic cells and either uremic or nonuremic plasma 26 h after endotoxin exposure (Table 2). Uremic cells with either a uremic or nonuremic plasma matrix released significantly greater (P < 0.001) concentrations of TNFα both at 2 and 26 h than did either nonuremic cell matrix combination. TNFα concentrations in uremic cells in combination with nonuremic plasma were significantly greater than in uremic cells with uremic plasma (P < 0.03) at 2 h but not at 26 h.

DISCUSSION

These studies demonstrate an enhanced release of TNFα in uremic versus nonuremic whole blood after
TABLE 2. Median TNFα concentrations in nonuremic plasma (NP) or uremic plasma (UP) combined with nonuremic blood cells (NC) or uremic blood cells (UC) 2 and 26 h after a 1.0 ng/mL E. coli endotoxin challenge

<table>
<thead>
<tr>
<th>Cell + Plasma Matrix</th>
<th>Median TNFα Concentrations (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>NC + NP (N=10)</td>
<td>121 (0-568)</td>
</tr>
<tr>
<td>NC + UP (N=10)</td>
<td>220 (0-466)</td>
</tr>
<tr>
<td>UC + UP (N=10)</td>
<td>717a (134-942)</td>
</tr>
<tr>
<td>UC + NP (N=10)</td>
<td>1009bc (496-1,736)</td>
</tr>
</tbody>
</table>

a Significantly greater (P < 0.001) than either 2-h NC matrix.
b Significantly greater (P < 0.001) than either 26-h NC matrix: 0 pg/mL.
c Significantly greater (P < 0.001) than either 2-h NC matrix or UC + UP.

an in vitro endotoxin challenge. This effect is exacerbated as renal function declined and appeared to be related to cellular factors contained in uremic whole blood.

These observations expand on our previous studies of enhanced TNFα release in blood from CRF patients in response to in vitro stimulation with endotoxin (15). To the best of our knowledge, enhanced release of TNFα in Ccr-graded CRF patients not on hemodialysis has not been previously reported. As renal function progressively declined, stimulation with E. coli endotoxin produced a progressive and significant elevation in TNFα release. This effect was also observed in patients on chronic hemodialysis. Because TNFα concentrations after endotoxin stimulation were not significantly different in patients on hemodialysis compared with patients with a Ccr from 10 to 30 mL/min, enhanced release of TNFα does not seem to be related to dialysis therapy in patients using cellulose triacetate membranes in this whole-blood model. It should be noted that others have demonstrated an increase in cytokine release in peripheral blood mononuclear cell cultures in dialysis patients (21,22). A recent study by Friedlander et al. demonstrated a blunted release of TNFα from blood monocytes in culture when stimulated by endotoxin in patients receiving peritoneal dialysis or hemodialysis with cellulose acetate membranes compared with healthy controls (23). It appears that these differences may be, in part, methodologic. Our study uses whole blood and closely reproduces the cytokine release kinetic patterns observed in baboons during an experimental gram-negative bacteremia (24,25). The kinetics of cytokine release observed by Friedlander et al. using blood monocyte cell cultures are dramatically different from the expected in vivo response. This may be because of the lack of lymphocytes or polymorphonuclear (PMN) cells in a cell culture. This observation is supported by significantly lower TNFα and interleukin-1β release from peritoneal cells containing lymphocytes and PMN, challenged with endotoxin (23). The exact mechanism of the observed differences between whole-blood and cell culture cytokine kinetics are not yet apparent; however, the results obtained from whole blood in vitro may be more representative of an in vivo response.

In our model, elimination of TNFα occurred within 26 h of endotoxin exposure in blood from nonuremic controls and CRF patients not on dialysis. However, blood from chronic hemodialysis patients (HDpre and HDpost) still had measurable concentrations of TNFα 26 h after exposure (Table 1). The approximate half-life of TNFα in nonuremic whole blood is 18 min, but it was obviously greatly increased for the hemodialysis patients (16). Because this phenomenon was not observed in CRF patients, it may relate to kidney function or lack thereof. Although no clear elimination mechanism is present in whole blood, other investigators have demonstrated a decline in TNFα concentration in whole blood similar to that observed in this study (26,27). A possible mechanism for the in vitro elimination of TNFα from the model may be the observed effect that 125I-labeled TNFα bound to L929 cell surface receptors is rapidly internalized and degraded, as evidenced by the release of 125I into the culture media (28).

A potential mechanism of the augmented response of uremic cells to endotoxin may be the result of bacterial overgrowth in the small intestine of uremic patients not observed in nonuremic patients (29). The increase in the bacterial flora may be responsible for low-grade stimulation of the resident macrophage/monocytes by endotoxin entering the portal circulation through the intestinal wall, which results in increased mRNA transcription for TNFα synthesis without elevated baseline concentrations of TNFα.

A similar finding of increased transcription of mRNA for TNFα synthesis without concomitant TNFα blood concentrations has been reported in hemodialysis patients (30). A stimulus for TNFα release, such as endotoxin secondary to a septic episode, may then trigger an exaggerated TNFα release. An additional factor that may participate in enhanced TNFα release in patients with CRF may be a decrease in the phagocytic ability of white blood cells as renal function declines. Several studies have demonstrated a decrease in the phagocytic ability of PMN cells as
renal failure progressed into a Cr range of 0 to 10 mL/min (7,31). If PMN cells do not remove endotoxin as rapidly as when renal function declines, then macrophages might become primed to release TNFα when a more potent endotoxin challenge is presented. It has been demonstrated that receptors for endotoxin are present on leukocytes (32-35); however, specific abnormalities for endotoxin binding or metabolism have not yet been investigated in the uremic state. Our study, which demonstrates a protracted elevation in TNFα concentrations 26 h after an endotoxin challenge in chronic hemodialysis patients compared with nonuremic controls, would be consistent with either a prolonged stimulation of macrophages by endotoxin or a reduced plasma clearance of TNFα.

In Part 2 of our study, blood from hemodialysis patients and nonuremic controls was separated into plasma and cellular components and mixed together in order to determine if cellular or plasma-soluble factors were responsible for the enhanced TNFα release noted in uremic blood. Uremic cells, not plasma factors, appear to be responsible for the enhanced TNFα release after an endotoxin challenge. The augmented TNFα release was only observed in those combinations that contained uremic cells. Of interest was the significantly higher concentration of TNFα from uremic cells with nonuremic plasma compared with concentrations from uremic cells with uremic plasma. Although TNF-BP was not measured, it has been demonstrated that the plasma and urine from uremic patients contain TNF-BP (10,13). The elevation of TNF-BP in patients with CRF also appears to progressively increase as renal function declines (14). The lower TNFα concentrations in uremic cells/uremic plasma compared with those in uremic cells/nonuremic plasma in the 2-h sample may be due to increased TNF-BP in the uremic plasma, which would bind and mask a portion of TNFα from detection by ELISA (12). At 26 h, there was no difference in TNFα concentrations between the uremic cells/uremic plasma or uremic cells/nonuremic plasma groups. Because the group containing uremic cells/nonuremic plasma would be expected to contain less TNF-BP, it is unclear why there are no differences in these groups. However, TNFα has been shown to stimulate the production of TNF-BP (36). There are no current data evaluating the possibility that TNFα may stimulate higher concentrations of TNF-BP from normal plasma than uremic plasma already containing TNF-BP.

In summary, TNFα release by whole blood is greatly augmented by uremia. This effect is accentuated by progressive declines in renal function and is related to cellular phenomena induced by the uremic state. Although the physiologic consequence of this observation is unclear, it may play a role in the severity of septic complications in patients with renal disease.

REFERENCES