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Cytokine Profiles During Clinical High-Flux Dialysis: No Evidence for Cytokine Generation by Circulating Monocytes

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Abstract. Secretion of cytokines by monocytes has been implicated in the pathogenesis of dialysis-related morbidity. Cytokine generation is presumed to take place in two steps: induction of mRNA transcription for cytokines by C5a and direct membrane contact, followed by lipopolysaccharide (LPS)-induced translation of mRNA (priming/second signal theory, *Kidney Int* 37: 85–93, 1990). However, the *in vitro* conditions on which this theory was based differed markedly from clinical dialysis. To test this postulate for routine hemodialysis, 13 patients were studied cross-over with high-flux cuprammonium (CU), cellulose triacetate (CTA), and polysulfon dialyzers, using standard bicarbonate dialysate, as well as CTA with filtered dialysate (fCTA). Besides leukocytes, C3a, C5a, and limulus amebocyte lysate reactivity, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-1RA, soluble TNF receptors, and IL-1 β mRNA were assessed. Only during dialysis with CU did C5a increase significantly (561 to 8185 ng/ml, $P < 0.001$). Endotoxin content of standard bicarbonate

was higher than filtered dialysate (median, 24.3 and <5 pg/ml respectively, $P = 0.002$), whereas limulus amebocyte lysate reactivity was not detected in the blood, except in the case of CU. TNF- α levels were elevated before, and remained stable during, dialysis, independent of the modality used. IL-1 β , IL-6, and mRNA coding for IL-1 β could not be demonstrated. IL-1RA and soluble TNF receptors (p55/p75) were markedly elevated compared with normal control subjects, but showed no differences between fCTA and CTA. To summarize, no evidence was found for production and release of cytokines by monocytes during clinical high-flux bicarbonate hemodialysis, neither with complement-activating membranes nor with unfiltered dialysate. Therefore, this study sheds some doubt on the relevance of the “priming/second signal” theory for clinical practice. The data presented suggest that reluctance to prescribe the use of high-flux dialyzers, as advocated in many reports, may not be warranted. (*J Am Soc Nephrol* 8: 1745–1754, 1997)

Numerous reports have been published about the unfavorable interactions between peripheral blood and dialyzer membrane in hemodialysis (HD) patients (1,2). One of the first phenomena described was a transient decline in the number of circulating neutrophils (3,4), related to alternative pathway complement activation (5,6). The complex of HD-induced acute and chronic side effects has been termed bioincompatibility (7). More recently, evidence has been obtained that proinflammatory cytokines are involved in this process (8–10). The close similarities between their biological effects and the clinical symptoms in HD patients (11,12) have led to the proposal of the interleukin hypothesis (13). This theory suggested that the release of proinflammatory cytokines acts as an underlying pathophysiologic event in HD-related acute-phase responses, such as fever and hypotension. A potential role of cytokines

(14) in chronic dialysis-related morbidity, such as amyloidosis and muscle wasting (15), was also suggested.

However, both the nature of and the factors responsible for the activation of peripheral blood mononuclear cells (PBMC) (15) are controversial issues. Besides a normal basal activation state (16–18), an increase (19,20), as well as a decline (21,22), have been described. Moreover, both an elevated response to exogenous stimuli (21–23) and a decrease have been observed (24). The discrepant findings may be due to the various test systems used (25). For the activation of PBMC and subsequent cytokine release, various factors have been implicated, such as complement activation (26–29), acetate dialysate (30), direct cell-dialyzer interaction (31), and backtransport of bacterial-derived material from the dialysate to the blood compartment (32).

In 1990, an attractive hypothesis was presented defining the nature of HD-induced PBMC cytokine generation more precisely (28): Primarily complement activation, but also direct PBMC adherence to the dialyzer, serves as a priming event and results in the transcription of mRNA coding for the cytokines interleukin (IL)-1 β and tumor necrosis factor (TNF)- α . Thereafter, a second signal is required, such as backtransport of

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bacterial-derived substances, resulting in mRNA translation and subsequent production and release of cytokines.

However, although fascinating, the above-mentioned theory has been based primarily on data obtained under highly unphysiological conditions that may have confounded the outcome for several reasons. First, the *in vitro* dialysis circuit was perfused for 2 h with 100 ml of saline, supplemented with 80 ml of donor blood. Obviously, the repeated exposure of a small amount of diluted test blood may amplify cellular activation, at least in comparison with clinical HD. Second, donor blood was obtained from healthy human volunteers, most likely exhibiting normal immune responses, in contrast to uremic patients (8). Third, in clinical HD the entire immune system will be involved in the inflammatory response caused by HD. Hence, *in vitro* both the participation of neutralizing factors, such as lipopolysaccharide (LPS)-binding proteins (33) and inhibitors of cytokines (34), and the influx of new cells originating from the marginating pool (35), will markedly differ from the clinical situation. Fourth, PBMC were incubated with LPS doses, up to 1000-fold larger than those found in clinical HD (36,37). Fifth, *Escherichia coli*, a notably uncommon bacterial contaminant of HD systems, was used for the preparation of LPS (37). Finally, acetate-containing dialysate was used, which is known to induce PBMC activation (30). Hence, the question arises whether conclusions, based on results obtained from *in vitro* recirculation systems, are relevant for clinical bicarbonate dialysis.

In the present study, 13 stable patients underwent bicarbonate HD cross-over on three dialyzers (cuprammonium [CU], cellulose triacetate [CTA], and polysulfon [PS]), differing in membrane material, degree of complement activation, pore-size, and absorptive characteristics (38). In the case of CTA, standard as well as filtered dialysate was used. The objective of our study was twofold: (1) to assess the production and release of cytokines by PBMC in routine bicarbonate HD and, more specifically, the impact of complement activation, membrane material, and contamination of the dialysate on this process; and (2) to evaluate the validity and relevance of the "priming/second signal" theory for clinical HD.

Materials and Methods

Patients

Thirteen stable patients (three women and 10 men undergoing HD for at least 5 mo [median, 35 mo; range, 6 to 224] and with a median age of 68 yr [range, 27 to 88 yr]) participated in the study after giving informed consent. Exclusion criteria were comorbidity (malignancy, autoimmune disease) or medication that might interfere with the immune system (nonsteroidal anti-inflammatory drugs, cytostatics, prednisone).

Dialyzers

Dialyzers were selected according to the following criteria:

Complement Activation. As outlined in the introductory remarks, dialyzers were selected first on the basis of their known complement-activating properties. Therefore, low- (PS), low-average (CTA), and high- (CU) complement-activating membranes were cho-

sen to offer different levels of complement products as priming event (Table 1).

Membrane Material. Direct contact between blood and dialyzer might stimulate PBMC to cytokine mRNA transcription, depending on the type of membrane used (28). Furthermore, dialyzers differ in their ability to adsorb proteins and pyrogens onto their membrane surface (39). CU and PS appear to behave quite opposite in this respect. Elegant studies showed that radioactive LPS was eluted in larger quantities from PS than from CU (40). Other investigators demonstrated that plasma coating significantly reduced the pyrogen permeability in the case of PS, but not in the case of CU (41). Hence, based on these criteria, one synthetic (PS) and two cellulosic (CU, CTA) membranes were selected.

Pore Size. According to the priming/second signal theory, the transfer of cytokine-inducing substances across the membrane offers the second signal for primed PBMC. The molecular weight of LPS is approximately 100 kD, and that of exotoxins 25 to 78 kD (37,42). Because the molecular size exclusion of dialysis membranes is below these molecular weights (PS and CTA: ± 30 kD; CU: ± 27 kD; reference 43), only fragments of these substances are likely to permeate the membrane. Hence, dialyzers with large pore sizes, and consequently high ultrafiltration (UF) factors, were selected (PS: 40; CTA: 35 ml/mmHg per h). In the case of CU, the highest UF factor available was 20 ml/mmHg per h.

Dialyzers with Filtered Dialysate. To avoid the second signal as much as possible, one additional HD session was performed with dialysate that had been passed through an endotoxin filter (41). On the basis of the combination of modest complement activation (4) and potential backtransport (high UF factor), CTA dialyzers were selected for this extra experimental session (filtered CTA [fCTA]).

Study Design

A diagram of the dialysis modalities used is depicted in Figure 1. All patients were dialyzed for 3 wk with each dialyzer in a cross-over study design. In each third week, blood samples were collected during a single HD session. In the case of CTA dialysis, blood samples were collected twice in 1 wk (once with standard and once with filtered dialysate).

Samples drawn from the afferent line before dialysis and from the efferent line several times afterward (see following sections) were analyzed for leukocyte count, C3a and C5a, limulus amoebocyte lysate (LAL) reactivity, IL-1 β , IL-6, TNF- α , IL-1RA, soluble TNF receptors (sTNFR), and mRNA coding for IL-1 β . Dialysate samples taken at 180 min afterward were cultured for bacterial growth and analyzed for LAL reactivity.

Table 1. Dialyzer characteristics^a

Dialyzer	CT 150 G	AM-UP-75	F 60 S
Material	Cellulose triacetate	Cellulose	Polysulfon
Surface area	1.5 m ²	1.5 m ²	1.3 m ²
Sterilization	Gamma	Gamma	Steam
Structure	Capillary	Capillary	Capillary
UF factor (ml/mmHg per h)	35	20	40

^a UF, ultrafiltration. CT 150 G was from Baxter (Osaka, Japan), AM-UP-75 was from Asahi (Tokyo, Japan), and F 60 S was from Fresenius (Bad Homburg, Germany).

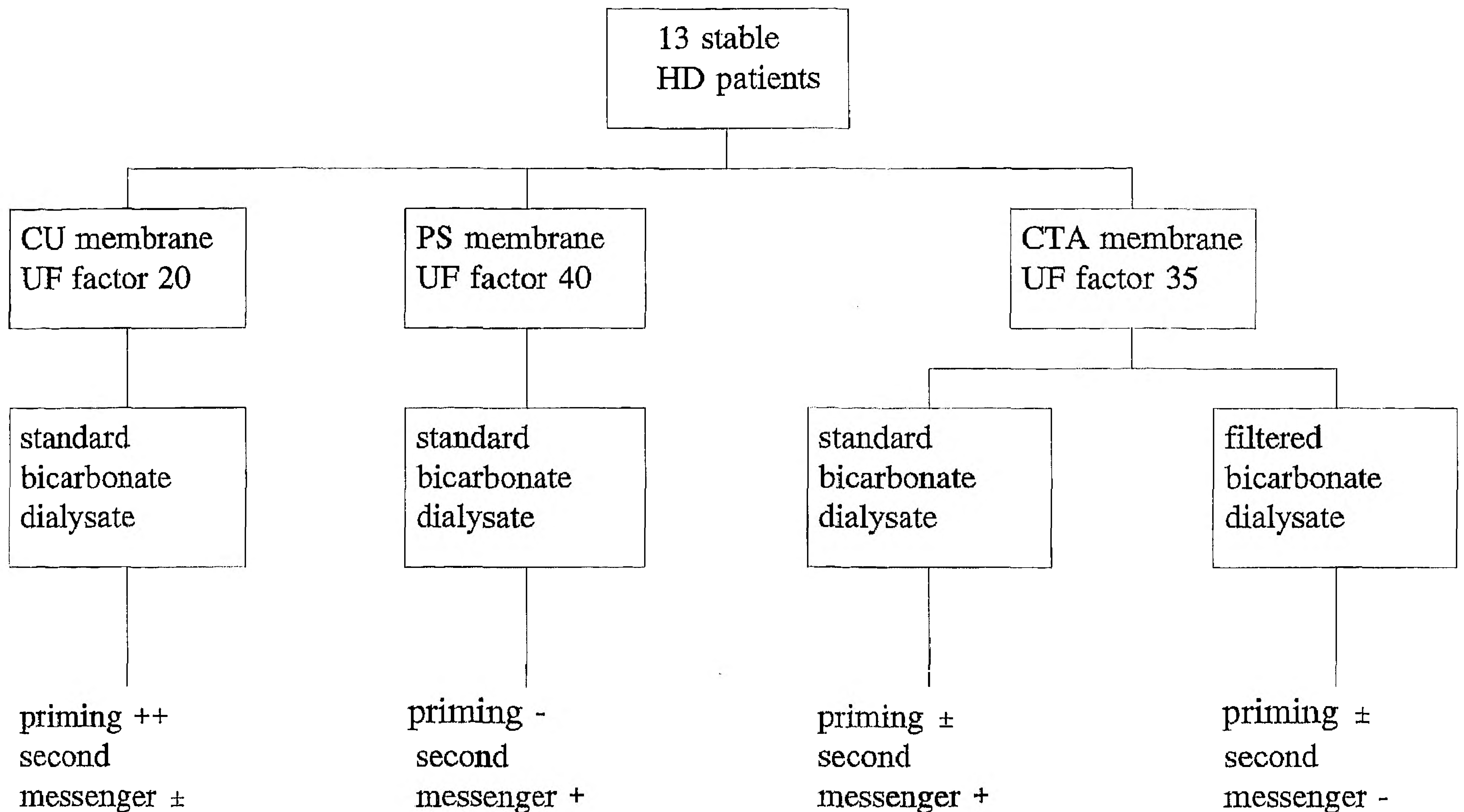


Figure 1. Study design. Thirteen patients were dialyzed according to the four modalities, as shown in the diagram. Selection of dialyzers was based principally on their complement-activating properties (priming event) and potential transmembrane permeation of cytokine-inducing substances (second signal).

Dialysis Procedure and Materials

Before the study, all patients underwent dialysis with low-flux PS. All dialyzers (CTA: CT 150 G, Baxter, Osaka, Japan; PS: F 60 S, Fresenius, Bad Homburg, Germany; CU: AM-UP-75, Asahi, Tokyo, Japan) were comparable in surface area, *in vitro* performance, and sterilization procedure (ethylene oxide-free). The UF coefficient differed slightly between the devices (Table 1).

Only first-use dialyzers, prerinsed with 1000 ml of NaCl 0.9% containing 5000 IU of heparin, were used. For dialysate preparation, tap water, purified by reversed osmosis, was used for dilution of a concentrated bicarbonate solution (138 mM Na⁺, 2 mM K⁺, 1.75 mM Ca²⁺, 0.5 mM Mg²⁺, 109.5 mM Cl⁻, 3 mM CH₃COO⁻, and 32 mM HCO₃⁻). Dialysate flow was 500 ml/min. Filtered dialysate was obtained by the interposition of a PS filter (SPS 600, Fresenius) (44).

The dialysis sessions lasted 3 to 4 h, depending on the previous prescription of the patients. All assessments were performed within the first 3 h, during which blood flow and UF rates were kept constant, according to the individual needs of the patients (blood flow, 200 to 250 ml/min; UF rate, 300 to 1000 ml/h). Anticoagulation was achieved by heparin with a priming dose of 1250 to 5000 IU and 250 to 1500 IU/h continuously. Individual conditions (blood flow, heparin dose, and UF) were maintained stable throughout the study period.

Analytical Methods

Leukocytes. Leukocyte counts and differentiation were determined from samples drawn in tripotassium ethylenediamine tetraacetic acid (EDTA) before dialysis (t0) and at 7.5 (t7.5), 15 (t15), 30 (t30), and 180 (t180) min afterward, using a Sysmex NE-8000 cell analyzer (TOA Medical Electronics, Kobe, Japan).

Complement Activation Products. On the basis of a pilot study, sample times for complement determination were fixed at t0, t15, and t180. Samples were collected in tubes containing tripotassium EDTA, kept on ice, and centrifuged immediately at 4°C for 10 min at 1500 g. The plasma was then stored at -70°C until required for testing. One hundred microliters of plasma were treated with 50 μl of 30% polyethylene glycol 6000 (BDH Poole, United Kingdom) in phosphate-buffered saline containing 10 mM EDTA, pH 7.2 to 7.5 (45). After centrifugation, the supernatant was collected for determination of C3a and C5a with an inhibition RIA, as has been described for the detection of IL-2 (46). In short, a monospecific polyclonal rabbit antibody against C3a or C5a was incubated overnight with the test sample. After that, iodinated C3a or iodinated recombinant C5a was added, and a second overnight incubation was performed. Complexed labeled C3a or C5a was precipitated with goat anti-rabbit antiserum in the presence of 5.6% polyethylene glycol 6000. Inhibition of the precipitation of hot C3a or C5a indicated the presence of these substances. The assays were calibrated using standard curves obtained with recombinant C5a (Sigma, St. Louis, MO) or purified C3a. The range of detection was 62 to 1000 ng/ml for C3a and 781 to 12,500 pg/ml for C5a. The average intra-assay coefficient of variance for C3a and C5a was 2 to 4% and 2 to 6%, respectively.

Endotoxin Assay. Blood samples for endotoxin determinations were collected at t0 and t180 in heparinized and pyrogen-free materials (Monovette[®], Sarstedt, Nümbrecht, Germany); dialysate samples were collected only at t180. The blood samples were centrifuged at 190 g for 10 min at 4°C to obtain platelet-rich plasma (PRP). All samples were stored at -20°C until determination.

Endotoxin activity was quantified by a kinetic chromogenic

method based on LAL by measurement of the activated enzyme by the sensitive chromogenic substrate S 2422 (Kinetic-QCL, Boehringer Ingelheim Bioproducts, Verviers, Belgium). Software was modified to allow detection of 1 pg of LPS per milliliter of PRP.

All determinations were performed in duplicate. After 1:10 dilution, PRP was heated for 10 min at 75°C (47,48). Standard series of purified *Escherichia coli* 055:B5 endotoxin (Boehringer Ingelheim Bioproducts) were made in PRP obtained from a healthy volunteer and endotoxin-free water (LAL-reagent water). All standard series showed linearity from 1 to 25 pg of LPS per milliliter of PRP. Inhibition and interference testing was performed on each sample by an endotoxin spike (*Escherichia coli* 055:B5 endotoxin, Boehringer Ingelheim Bioproducts) equal to 5 pg of LPS per milliliter of PRP. Recoveries of spikes between 50 and 150% were accepted.

Microbiological Evaluation of Dialysate. Dialysate samples were drawn in sterile tubes. Total plate counts were performed on glucose yeast extract agar (Merck, Darmstadt, Germany; Oxoid, Basingstoke, United Kingdom) and on Columbia agar (Oxoid) with sheep red blood cells after 48 h of incubation at 37°C. Identification of micro-organisms was achieved using Analytical Profile Index identification (Analytical Profile Index system S.A., Montalieu-Vercieu, France).

Cytokines. The cytokines IL-1 β and IL-6 were determined at t0, t30, and t180, and TNF- α at t0 and t180, in serum by sandwich-type enzyme immunoassays, according to the manufacturers' procedures (IL-1 β and TNF- α [Immunotech, Marseille, France], and IL-6 [Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands] (49). After centrifugation (10 min, 1500 g), the serum samples were stored immediately at -70°C until required for testing. All determinations were performed in undiluted samples and in duplicate. Lower limits of detection for IL-1 β , TNF- α , and IL-6 were 5, 10, and 4 pg/ml, respectively. The average intra-assay coefficient of variation was 5.1% for IL-1 β , 10% for TNF- α , and 7.6% for IL-6. Data at t180 were corrected for changes in hematocrit (Ht): corrected value_{t180} = (Ht_{t0}/Ht_{t180}) \times value_{t180}. Serum samples of septic patients served as positive control subjects ($n = 9$, median [range]: IL-1 β 5 (0 to 218) pg/ml; TNF- α 9 (0 to 102) pg/ml; IL-6, 105 (3 to >750) pg/ml).

IL-1RA and sTNFR. IL-1RA was measured at t0 and t180 in 100 μ l of EDTA plasma with an RIA, using polyclonal rabbit antibodies as described by Drenth *et al.* (50). The lower detection limit of this assay was 80 pg/ml. Normal values, as measured in 97 healthy volunteers, were 156 \pm 78 pg/ml. sTNFR were measured at t0 and t180, using an enzyme-linked immunobinding assay (Hoffmann-La Roche, Basel, Switzerland) (51). Normal values, as measured in 19 healthy volunteers, were 1470 \pm 190 pg/ml for sTNFR-p55 and 2520 \pm 660 pg/ml for sTNFR-p75. Data at t180 were corrected for changes in Ht.

mRNA Coding for IL-1 β . PBMC were isolated from EDTA blood samples by density gradient centrifugation on Ficoll-Paque ($d = 1.077$ g/cm³, Pharmacia, Uppsala, Sweden). Total cellular RNA from these cells was isolated with RNAzol (Biotech Laboratories, Ives Houston, TX), according to the procedure described by Chomczynski and Sacchi (52), and reverse-transcribed into cDNA by oligo-dT priming. Deoxyoligonucleotide primers were constructed from the published cDNA sequences of IL-1 β (53) and β -actin (54). The primers were synthesized on a DNA synthesizer (Cyclone Millipore, Bedford, MA) by the phosphoramidite method (55).

The amplification of cDNA by PCR was performed by a modified procedure of Saiki (56). Ten microliters of cDNA, 50 pmol of each primer, and 0.5 U of Taq DNA polymerase were added to a final volume of 100 μ l (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.0 mM

MgCl₂, 2 mg/ml bovine serum albumin, and 0.25 mM of each dNTP). The mixture was heated to 95°C, 1.5 min at 55°C, and 1.0 min at 72°C. After termination of the last cycle, the samples were chilled to 4°C. Ten microliters of the amplified DNA was electrophoresed on 1% agarose gels and analyzed for the appearance of bands of 388 and 527 bp for IL-1 β and β -actin, respectively.

Statistical Analyses

Data are expressed as mean \pm SD, or median and range when appropriate. Analysis was performed with the Statistical Package for Social Sciences/PC+ software system, using multivariate ANOVA and paired *t* test. In the case of the dialysate data, a Kolmogorov-Smirnov test was performed. Differences are considered statistically significant at $P < 0.05$.

Results

Leukocytes

At t15, all three membranes (four modalities) showed a decrease in the number of white blood cells (WBC), but only significantly in the case of CU (Figure 2). Comparing CTA with CU, multivariate analysis with a difference contrast showed an interaction between membrane and time (Hotelling's T² test: $F = 20.87$; df (2,9); $P < 0.001$). Univariate analysis revealed a significant effect of membrane; the WBC dip was more pronounced with CU than with CTA ($F = 38.44$; df (12,1); $P < 0.001$) or PS ($F = 19.51$; df (12,1); $P = 0.001$). The effect of time will not be discussed.

As expected, the WBC decline was mainly due to a drop in the number of polymorphonuclear cells (CTA from 3.96 to 2.97 $\times 10^9$ /L; PS from 4.14 to 3.72 $\times 10^9$ /L; CU from 3.76 to 0.29 $\times 10^9$ /L).

Complement

As shown in Figure 2, only in the case of CU was a significant increase in C5a observed. Multivariate analysis as described above showed an interaction between membrane and time (comparison between CTA and CU: Hotelling's T² test: $F = 26.45$, df (1,11); $P < 0.001$), and further univariate analysis revealed a highly significant membrane-dependent effect on C5a levels (comparison between CTA and CU: $F = 45.40$, df (12,1), $P < 0.001$; likewise, between PS and CU: $P < 0.001$). No marked differences were observed between PS, CTA, and fCTA. With respect to C3a, results were comparable to C5a measurements (data not shown).

Endotoxin Content of Dialysate and Blood

Dialysate endotoxin content during HD with filtered and unfiltered dialysate is shown in Table 2. The difference between fCTA (filtered dialysate) and the average of the other treatment groups combined (standard dialysate) was highly significant (filtered, <5 [<5 to 14] pg/ml; standard, 24.3 [<5 to 132] pg/ml; $P = 0.002$). No marked variations were observed between CTA, CU, and PS.

Neither during HD with fCTA nor during standard CTA and PS dialysis could endotoxins be detected in the blood (all samples, <5 pg of LPS per milliliter of PRP). However, during CU dialysis, blood samples showed surprisingly high LAL

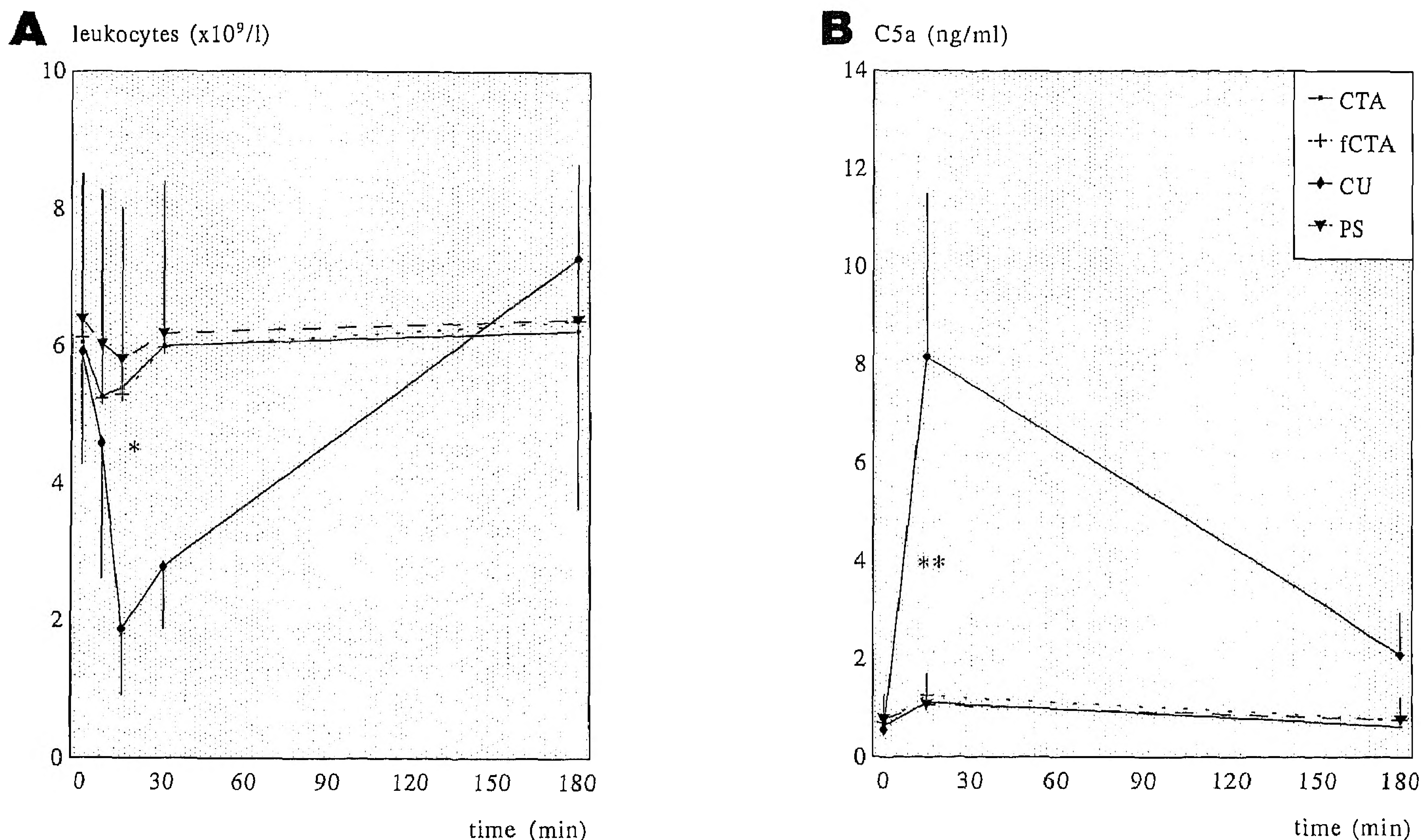


Figure 2. (A) Leukocyte count ($\times 10^9/L$, mean and SD) during standard bicarbonate hemodialysis (HD) with cellulose triacetate (CTA), cuprammonium (CU), and polysulfon (PS) membranes, as well as with CTA and filtered bicarbonate dialysate (fCTA). HD with CU dialyzers induced a significant leukocyte dip after 15 min ($*P < 0.001$). (B) C5a levels (ng/ml) during HD with the above-mentioned modalities. Only HD with CU induced a significant increase in C5a after 15 min ($**P < 0.001$).

Table 2. Dialysate cultures and endotoxin levels^a

Characteristic	Unfiltered Dialysate ^b (n = 39)	Filtered Dialysate (n = 13)
Dialysate culture (CFU/ml)	170(0 to 7500) ^c	30(0 to 40) ^{c,d}
Gram stain (number)		
positive		
negative	15	
positive and negative	5	
none	19	13
Endotoxins (pg/ml)	24.3(<5 to 132)	<5(<5 to 14.5) ^e

^a CFU, colony-forming unit.

^b Total of three modalities with unfiltered bicarbonate dialysate (CTA, PS, CU).

^c Median (range).

^d $P < 0.001$, filtered *versus* unfiltered dialysate.

^e $P = 0.002$, filtered *versus* unfiltered dialysate.

reactivity before and after HD (all samples, >250 pg of LPS per milliliter of PRP), which was markedly higher than concomitant dialysate levels (all samples, <36 pg/ml).

Dialysate Cultures

Results of the bacterial cultures from the dialysate are shown in Table 2. Gram-positive organisms were cultured in five

cases, but only when gram-negative organisms were demonstrated as well. When comparing fCTA with the total of non-filtered modalities, a highly significant difference was observed (filtered dialysate fCTA, 30 [0 to 40] colony-forming units/ml; standard dialysate, 170 [0 to 7500] colony-forming units/ml; $P < 0.001$).

TNF- α , IL-1 β , and IL-6

Compared with healthy control subjects ($n = 10$; TNF- α below the detection limit 10 pg/ml), mean TNF- α levels were moderately elevated (Figure 3) before and after 3 h of HD with all four dialysis modalities (t0: CTA 31.5, fCTA 22.1, PS 22.9, CU 20.5 pg/ml; t180: CTA 19.5, fCTA 14.0, CU 24.4, PS 17.1 pg/ml). None of the four modalities showed marked differences between t0 and t180.

In the samples from the first six patients on CTA and PS dialysis (24 samples) and from all patients on CU dialysis (39 determinations), IL-1 β and IL-6 levels appeared to be below the detection limit (5 and 4 pg/ml, respectively) at all sampling times. Therefore, these cytokines were not determined in the remaining cases.

sTNFR and IL-1RA

Compared with healthy control subjects, both sTNFR-p55 and sTNFR-p75 were markedly elevated in all four modalities

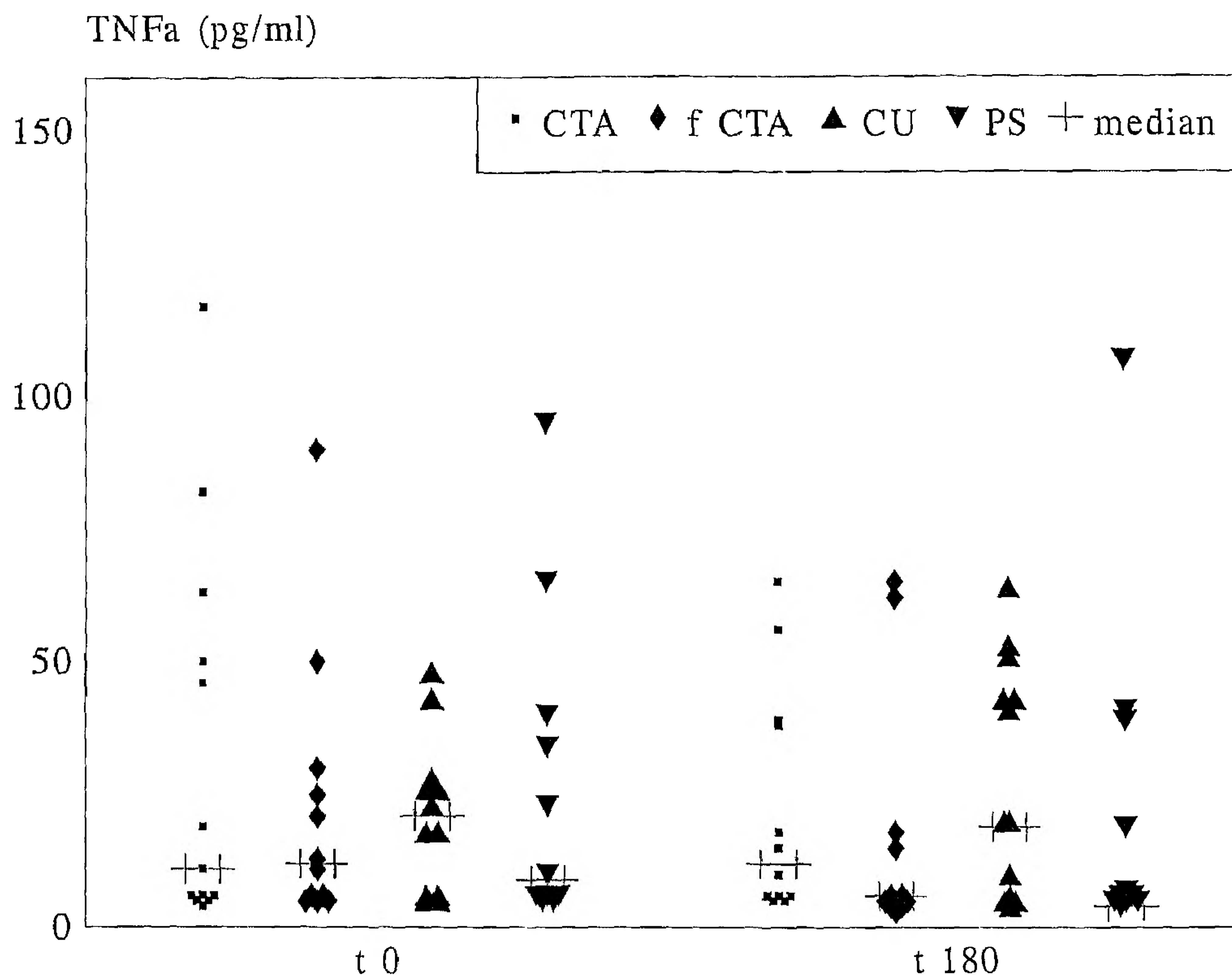


Figure 3. Levels of tumor necrosis factor (TNF)- α (pg/ml) before (t0) and after (t180) dialysis with the four different modalities (Figure 1). Median values are indicated with a +. Serum levels were markedly elevated compared with normal control subjects. No significant changes were observed during HD or among the four modalities themselves.

at all sampling times (mean p55: 23.9 ± 10.1 ng/ml; mean p75: 31.2 ± 7.4 ng/ml; control subjects: 1.5 ± 0.2 and 2.5 ± 0.7 ng/ml, respectively, Figure 4). During HD with fCTA and CTA, a decrease in sTNFR-p55 was observed, whereas levels remained stable during HD with CU and PS. The difference between these two modalities and CTA or fCTA was significant. Multivariate analysis, comparing CTA and CU, showed an interaction between time and membrane ($F = 9.82$; df (1,11); $P = 0.01$); further univariate analysis only showed a significant effect of membrane ($F = 18.24$; df (1,11); $P = 0.001$). In contrast, sTNFR-p75 increased during CU HD, whereas it remained stable during HD with the other modalities (effect of membrane, CU versus CTA: $F = 12.06$; df (1,9); $P < 0.01$; CU versus fCTA and PS: $P < 0.01$).

Figure 4 shows the levels of IL-1RA at t0 and t180 with the four HD modalities. Although the measured quantities appeared to be approximately threefold higher compared with a group of healthy control subjects (mean CTA at t0: 490 ± 360 pg/ml; mean control subjects: 156 ± 79 pg/ml), no marked differences were observed among the four modalities or between the various sample times. Neither dialysate endotoxin levels nor dialysate cultures showed an influence on intradialytic changes in IL-1RA or sTNFR (p55 and p75) levels.

mRNA coding for IL-1 β

Neither mononuclear cells isolated after HD with LPS-free dialysate (fCTA) nor samples isolated after the three other modes of HD (CU, CTA, and PS) showed mRNA coding for

IL-1 β (for a total of 52 determinations). Correct PBMC and RNA extractions were confirmed by agarose electrophoresis, showing 5S, 18S, and 28S mRNA. PCR accuracy was confirmed by the assessment of β -actin in all samples.

Discussion

The present study was designed to elucidate the mechanism of cytokine generation by PBMC in routine bicarbonate HD, in particular with respect to the priming/second signal theory, as outlined in the introductory remarks (28). As for the *priming event*, dialyzers were used, differing both in membrane characteristics and degree of complement activation. Potential backtransport of bacterial-derived substances in the dialysate, the so-called *second signal*, was facilitated by using only dialyzers with large pore sizes (57,58). In addition, the dialyzers selected differed in their protein and pyrogen adsorptive capacities: CU low versus PS high (40,41). Finally, in one of the experimental settings, the bicarbonate dialysate was filtered (fCTA), with the objective of excluding the second signal, LPS (endotoxins), and/or non-LPS (exotoxins) (42), as much as possible. According to a recent report, UF through pyrogen-adsorbing high-flux membranes such as PS improves the bacteriological quality by a factor of 10^2 to 10^3 (41).

Priming of PBMC was assessed by the analysis of mRNA coding for IL-1 β . Sequelae of the so-called second-signal were quantified by the assessment of blood cytokines IL-1 β , IL-6, and TNF- α , as well as the neutralizing factors IL-1RA, sTNFR-p55 (p55), and sTNFR-p75 (p75).

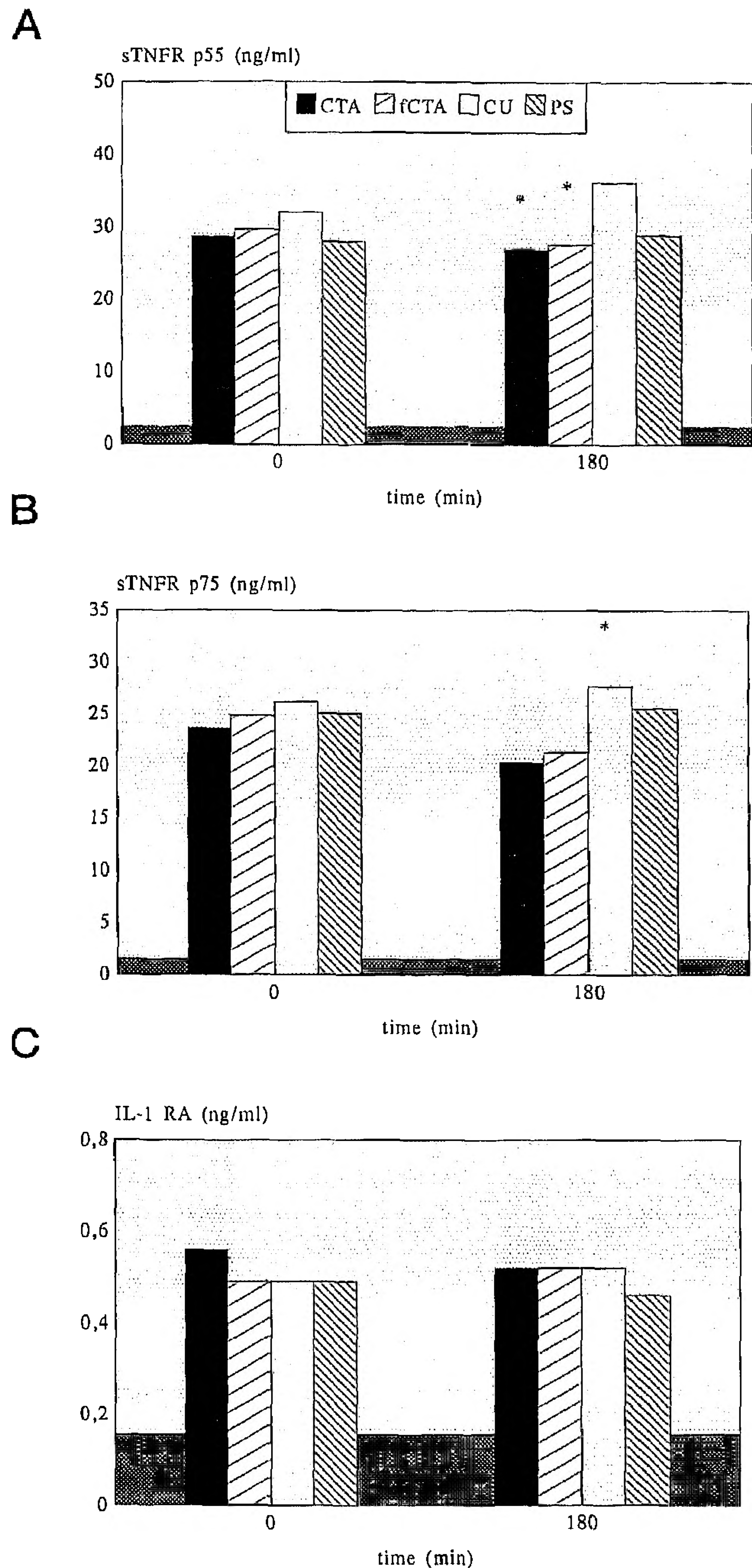


Figure 4. Soluble TNF receptor (sTNFR) levels before (t0) and after (t180) dialysis with the four different modalities (Figure 1). Both sTNFR-p55 (ng/ml, Panel A) and sTNFR-p75 (ng/ml, Panel B) were markedly elevated compared with healthy control subjects (grey area). A significant decrease in p55 was noted during fCTA and CTA HD ($*P < 0.01$), whereas p75 was significantly higher during CU dialysis compared with fCTA, CTA, and PS ($**P < 0.01$). As for interleukin-1 receptor antagonist (IL-1RA) levels (ng/ml, Panel C), average serum levels before HD were approximately threefold higher than in healthy control subjects (grey area). No marked changes were observed during HD or among the four modalities themselves.

A transient leukocyte drop, accompanied by marked complement activation (the proposed priming event), was demonstrated only during CU dialysis. The second signal, bacterial

dialysate contamination as assessed by cultures and LAL determinations, was markedly reduced in the case of fCTA HD compared with the other modalities. In peripheral blood, all LAL determinations were negative, except in the case of CU, in which plasma levels were approximately 10-fold higher than concomitant dialysate levels before as well as after HD. However, despite the high LAL reactivity in blood, neither mRNA for IL-1 β nor serum cytokines could be detected. Therefore, in our opinion, it is highly unlikely that the presence of LAL-positive material in blood was due to backtransport of bacterial-derived substances from the dialysate. The release of soluble, LAL-reactive membrane constituents, as has been described (59), seems a more plausible explanation for these findings.

With respect to TNF- α , average serum levels were moderately elevated compared with healthy control subjects before, as well as after, HD, without notable differences between the four modalities. Hence, our data seem to be in line with reports indicating that plasma TNF- α levels are only slightly influenced by HD (29,60) and are more or less similar to those found in continuous ambulatory peritoneal dialysis patients (34), although both an HD-induced increase (61) and decrease (34) have been reported as well. Nonetheless, our findings suggest that complement activation, membrane characteristics, and the quality of the dialysate did not influence the production of this cytokine (62).

Compared with healthy control subjects, all four modalities showed markedly elevated values of both sTNFR in HD patients, as has been described in uremic subjects (63). With respect to p55, HD with both CTA and fCTA showed a significant decline over time, whereas no notable variations were observed during HD with CU or PS. As for p75, dialysis with CU generated the highest levels, significantly different from fCTA, CTA, and PS. The above-mentioned differences, both for p55 and p75, persisted after correction for changes in hematocrit. On the basis of these data, a causative effect of complement activation and/or dialyzer characteristics, such as release of LAL-positive membrane material in the case of CU (64), cannot be ruled out. Pooled data on CTA and CU membranes also showed raised levels of sTNFR at the end of HD (34), which was attributed to acute shedding rather than to increased gene expression (65). Yet, our data indicate that the bacterial quality of the dialysate did not influence serum sTNFR levels.

With respect to IL-1 β and IL-6, none of our experiments showed values higher than the detection limit, neither at the start nor at the end of HD. Hence, in our study, secretion was not influenced by complement activation, the type of membrane, or the bacterial quality of the dialysate. In several recent studies, IL-1 β and IL-6 levels appeared similar in continuous ambulatory peritoneal dialysis patients and pre- and post-HD patients (34,60,66), suggesting that HD treatment itself does not stimulate PBMC to release notable amounts of these cytokines, which is obviously in line with our findings.

In accordance with recent data (34), average IL-1RA levels appeared about three times as high as those from healthy control subjects, before as well as after HD. No marked differences were observed between the various modalities. Again,

these observations indicate that complement activation, membrane characteristics, and the bacterial quality of the dialysate did not exert enhancing effects on serum IL-1RA levels. Recent findings showed that IL-1RA even tended to decrease at the end of HD (34). These data seem even more striking, as endotoxin-stimulated PBMC from HD patients produce significantly more IL-1RA than IL-1 β , indicating that IL-1RA might be a better indicator of cytokine-inducing stimuli than IL-1 β (22,67). Finally, mRNA coding for IL-1 β could not be demonstrated in any of the experiments performed, indicating that complement activation, different membrane characteristics, and type of dialysate used did not act as demonstrable priming events.

Taken together, our data shed some doubt on the clinical relevance of the concept of a complement and/or membrane-induced transcriptional signal, followed by an endotoxin-induced translational stimulus in routine bicarbonate HD. This skepticism is consistent with other observations. In an *in vitro* study, the transfer of cytokine-inducing substances was shown in the case of high-flux CTA and polyacrylonitrile membranes, but only after challenge doses of ± 1000 pg/ml, which is approximately 30- to 50-fold larger than the unfiltered dialysate used in our analysis (37). Others demonstrated that the presence of bacterial products in the dialysate, at the levels found in clinical conditions, did not influence cytokine profiles during *in vitro* HD with polyacrylonitrile and CU membranes (68).

In conclusion, neither transcription of mRNA coding for IL-1 β nor HD-induced release of IL-1 β , IL-6, and TNF- α could be demonstrated in our study, despite considerable differences in complement activation, membrane characteristics, and bacterial contamination of the dialysate between the four modalities tested. With respect to IL-1RA and sTNFR, comparable results were obtained, although an increase in sTNFR levels was observed during CU dialysis, possibly related to complement activation (34) and/or release of membrane-derived LAL-positive material. Although the present data do not permit definite conclusions, our results shed some doubt on the relevance of the priming/second signal theory for clinical practice. Therefore, the reluctance to prescribe the use of high-flux dialyzers, as advocated by several authors (57,62,69,70), may not be warranted, at least as far as backtransport of cytokine-inducing substances is concerned.

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