Production of Cytokines in Hemodialysis

C. Tetta\textsuperscript{a}, G. Camussi\textsuperscript{a,c}, E. Turello\textsuperscript{a}, M. Salomone\textsuperscript{a}, G. Aimo\textsuperscript{b}, G. Priolo\textsuperscript{b}, G. Segoloni\textsuperscript{a}, A. Vercellone\textsuperscript{a}

\textsuperscript{a}Laboratorio di Immunopatologia, Cattedra di Nefrologia, Ospedale Molinette, Torino;
\textsuperscript{b}Divisione di Nefrologia e Dialisi, Laboratori Centrali 'Baldi e Riberi', Ospedale Maggiore San Giovanni Battista e della Città di Torino;
\textsuperscript{c}Cattedra di Nefrologia Sperimentale, Dipartimento di Biochimica e Biofisica, Facoltà di Medicina e Chirurgia, Università di Napoli, Italia

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Abstract. Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-1 (IL-1\(\beta\)) are cytokines primarily produced by monocytes/macrophages when stimulated by endotoxin, complement-derived anaphylatoxins and the specific antigen. In the present study, the plasma levels of TNF-\(\alpha\) and IL-1\(\beta\) were evaluated before and after hemodialysis with cuprophane membrane (in 9 patients) and hemodiafiltration (in 9 patients) using three high-permeability membranes such as polymethylmethacrylate, polyacrylonitrile (AN-69) and polysulfone. In vitro spontaneous production of TNF-\(\alpha\) and IL-1\(\beta\) was evaluated in the supernatants from short-term cultured monocytes obtained before and after treatment. The predialytic levels of TNF-\(\alpha\) and IL-1\(\beta\) were significantly higher (\(p < 0.05\)) in the uremic population than in 1 healthy subjects taken as controls. The analysis of the uremic population regarding the mode of therapy indicated that in hemodialysis the predialytic plasma levels of TNF-\(\alpha\) and IL-1\(\beta\) did not significantly differ from those of healthy subjects. In contrast, in hemodiafiltration with polymethylmethacrylate and AN-69, but not with polysulfone, the predialytic plasma levels of both cytokines were significantly (\(p < 0.05\)) increased. No significant variation in plasma levels of both cytokines was observed after hemodialysis with cuprophane membranes. Hemodiafiltration with polymethylmethacrylate and AN-69, but not with polysulfone, brought about a consistent reduction in plasma levels of both cytokines. Detectable amounts of TNF-\(\alpha\) and IL-1\(\beta\) were spontaneously produced by peripheral-blood monocytes 6 h after the end of hemodialysis but not of hemodiafiltration. These studies suggest a possible role of TNF-\(\alpha\) and IL-1\(\beta\) in the biocompatibility of different extracorporeal treatments.
Introduction

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) participate in the so-called polypeptide mediator network, which defines a complex array of interacting signals that regulates growth, differentiation and function of the cells involved in immunity, hematopoiesis and inflammation [1]. IL-1β and TNF-α are produced primarily by mononuclear phagocytes when these cells are stimulated by a variety of agents including microbes, microbial products, inflammatory agents such as C5a, C3a and their carboxypeptidase-B-derived des-arginated products, plant lectins and antigens [2, 3]. Furthermore, TNF-α is a stimulus for IL-1β production from blood monocytes and vascular endothelial cells [4, 5]. Endotoxin is the most potent soluble inducer of IL-1β and TNF-α production in vitro requiring concentrations of picograms to nanograms per milliliter. TNF-α plays a primary role in the alterations associated with endotoxemia and septic shock. IL-1β and TNF-α are recognized as potent mediators of inflammation [6-9].

Considerable growth of organisms in the dialysis fluid and high levels of endotoxin especially when bicarbonate concentrate is used as dialysate buffer are known to occur and have been implicated in the induction of synthesis and release of IL-1β in vitro and clinical hemodialysis [reviewed in ref. 2, 10]. Microbial contamination of the dialysis fluid has drawn increasing interest since backfiltration of dialysate into the blood compartment indeed occurs with today’s high-permeability membranes [reviewed in ref. 10]. As first outlined by Henderson et al. [11] and Shaldon et al. [12], several of the overlapping biological activities of IL-1β and TNF-α [reviewed in ref. 13] may be of pathogenetic relevance in at least some of the acute and chronic clinical symptoms and biohumoral alterations observed in hemodialysis patients such as fever, hypotension, low plasma zinc levels, increased plasma levels of acute-phase response proteins and late occurrence of amyloidosis. However, only few studies have so far examined the in vivo production of TNF-α in hemodialysis patients.

The present study was undertaken to evaluate in patients on hemodialysis with complement-bioincompatible cuprophane and in patients on hemodiafiltration with high-permeability membranes [polymethylmethacrylate, polyacrylonitrile (AN-69), polysulfone] their pre- and postdialytic plasma levels and in vitro spontaneous production of TNF-α and IL-1β from monocytes in short-term culture.

The results of the present study suggest an in vivo production of IL-1β and TNF-α in the interdialytic period in patients on hemodiafiltration with high-permeability membranes as inferred from the increased predialytic plasma levels of both cytokines. However, hemodiafiltration with polymethylmethacrylate and AN-69, but not polysulfone, drastically reduces the plasma levels of both cytokines. Interdialytic production of IL-1β and TNF-α is markedly reduced in patients on hemodialysis with cuprophane.

Materials and Methods

Patients

Eighteen chronically uremic patients entered this study. All patients reported no febrile reaction during or after hemodialysis or at home and they had been free of intercurrent disease for the last 4 weeks. Exclusion criteria were: present or past history of diabetes; autoimmune diseases; Australia antigen serum positivity; intolerance to the hemodialysis membranes in study; therapy with corticosteroids; β-receptor-block-
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The primary renal disease was polycystic kidney disease in 2 patients, multicystic kidney in 1, congenital malformation in 1, chronic glomerulonephritis in 2, nephroangiosclerosis in 8, chronic pyelonephritis in 3 and Henoch-Schönlein disease in 1. Patients were categorized in two groups on the basis of the hemodialysis technique used: 9 patients (mean age 59.3 ± 13.7 years, mean dialytic age 9.5 ± 6.5 years) were on hemodialysis with cuprophane (Bravo 501), and 9 patients (mean age 53.8 ± 19.1 years, mean dialytic age 6.1 ± 4.3 years) underwent hemodiafiltration, a simultaneous application of hemofiltration to hemodialysis [14, 15], using polymethylmethacrylate (Bi-1.6U, Hoechst-Toray, Kyoto, Japan), AN-69 (Filtral 16, Hospal) and polysulfone (BL632, Bellco). The order of high-flux membranes was randomized. The apparatus used was a Hospal hemodiafiltration system, with an ultrafiltration rate controller and Equalizer. The substitution fluid was infused into a venous line to compensate for water and solute removal (after dilution) and had the following composition (in mEq/l): Na⁺ 140; K⁺ 1.5; Ca²⁺ 4.5; Mg²⁺ 1; Cl⁻ 105; lactate 42; glucose (5.5 mg%); osmolality (297 mosm/l), pH 5-6. Dialysate was counter-currently delivered simultaneously at a flow rate of 500 ml/min. Ultrafiltrate removed during each treatment ranged from 9-10 liters, which was approximately 2-3 liters more than the infused substitution fluid. Pre- and postdialytic hematocrit was evaluated. All patients were dialyzed with the same membrane for 2 running sessions before the study. Blood (5 ml) was drawn in sterile heparinized glass tubes, centrifuged and the plasma was aspirated, filtered sterile (Acrodisc, 0.2 μm) and stored at 4 °C before TNF-α and IL-1β assays. Samples for determination of plasma C3a des Arg were drawn in Na-EDTA glass tubes and immediately centrifuged (700 g, 15 min, 4 °C) at time 0, 15 min and at the end of treatment. The plasma was recentrifuged for 5 min at 4 °C in a Beckman Microcentrifuge B (Beckman Analytical, Milano, Italy) and stored at −80 °C until assayed for C3a des Arg by radioimmunoassay (Amity, Milano, Italy).

Control subjects were 21 healthy members of our institution (age range 32-42 years).

Preparation of Peripheral-Blood Monocytes
Peripheral-blood monocytes were purified from pre- and postdialysis heparinized blood drawn from the arteriovenous fistula of 9 patients on hemodialysis with cuprophane membranes and of patients on hemodiafiltration with polymethylmethacrylate (9), AN-69 (9) and polysulfone (9). Separation of mononuclear cells was achieved after centrifugation (700 g, 20 min) using sterile lymphocyte separator tubes (FAR Italia, Verona, Italy) and undiluted Lymphoprep (Nycomed, Oslo, Norway). The light density fraction contained mainly lymphocytes and 10% monocytes. After 3 washes in RPMI 1640 (Gruppo Flow, Opera, Italy), the cells were allowed to adhere (1 h, 37 °C, 5% CO₂) in 6-well plates at the concentration of 2 X 10⁶ cells/ml of RPMI 1640 supplemented with 10% fetal calf serum (Gibco, Paisley, UK), 100 IU/ml penicillin and 100 μg/ml streptomycin. Nonadherent cells were eliminated by 3 washes and the adherent cells were identified as monocytes (90%) on the basis of their staining with nonspecific esterase, using a-naphthol-AS-D-chloroacetate as substrate, combined with staining abrogation with sodium fluoride. RPMI contained no detectable endotoxin as assessed by the Limulus assay. Spontaneous release of TNF-α and IL-1β was assessed in the supernatants of adherent monocytes after 6 h culture.

TNF-α Assay
A sensitive biological assay was used to quantitate TNF-α in plasma and in the supernatants of cultured monocytes that was based on its cytotoxic activity in the presence of an inhibitor of protein synthesis [16]. Two-fold serial dilutions of samples were added together with 0.1 mg/ml of cycloheximide to cultures of human (SK-MEL-109) melanoma cells that are sensitive to the cytotoxic activity of TNF-α in concentrations as low as 20 pg/ml. These cells were grown as monolayers in 24-well cluster plates, incubated with appropriate dilutions of samples and after 20 h washed with phosphate-buffered saline before staining with crystal violet which was eluted and measured as described earlier [16]. A calibration curve was constructed with human recombinant TNF-α (0.01, 0.1, 1 ng/ml) to convert the cytotoxic activity of biological samples into nanograms per milliliter of TNF-α. In order to assess specificity for TNF-α-induced cytotoxicity, samples which exerted > 30% cytotoxicity on the TNF-α-sensitive SK-MEL-109 cell line were assayed on TNF-α-resistant cells (designated R4) selected from SK-MEL-109 cells. The lack of cytotoxicity of TNF-α-resistant R4 cells in such control experiments showed that cell death observed with TNF-α-sensitive SK-MEL-109 cells was specifically caused...
by TNF-α. In addition, TNF-α-dependent cytotoxicity was blocked by a rabbit polyclonal antihuman recombinant TNF-α antibody (Cetus, Emeryville, Calif., USA). The cytotoxicity assays were carried out in triplicate and gave a standard error of <5%.

IL-1β Assay
An enzyme-linked immunosorbent assay (ELISA) was used for the quantitative measurement of IL-1β in the plasma and in the supernatants from monocytes (T Cell Sciences). The procedure consisted of a 4-step test carried out in a microtitration well plate which was coated with a monoclonal antibody specific for IL-1β. All samples were run in duplicate. Sensitivity was 20 pg IL-1β/ml. No cross-reaction was detectable with TNF-α. Intra-assay variation for varying concentration of IL-1β was 6.7% (at 990 pg/ml), 4.9% (at 280 pg/ml) and 5.3% (at 55 pg/ml). Interassay variation for varying concentrations of IL-1β was 6.6% (730 pg/ml), 9.4% (130 pg/ml) and 9.4% (30 pg/ml).

Statistical Studies
All data were corrected for the variation in the hematocrit, were entered in a database using IBM personal computer and analyzed for statistical significance (p < 0.05) using BMDP3D one-sample and two-sample t tests (BMDP Statistical Software, Los Angeles, Calif., USA).

Results
Statistically significant differences (p < 0.05) were found in predialytic plasma TNF-α (fig. 1a) and IL-1β (fig. 1b) between healthy subjects and patients on substitutive treatment for chronic renal failure. In patients on hemodialysis with cuprophane, the predialytic plasma levels of TNF-α and IL-1β were not statistically different from those recorded in healthy subjects (TNF-α = 1.08 ± 1.49 vs. 0.9 ± 1.8 ng/ml, p > 0.05, fig. 2a; IL-1β = undetectable in both groups, p > 0.05, fig. 3a). At the end of hemodialysis with cuprophane, the plasma levels of TNF-α (fig. 2a) and IL-1β (fig. 3a) did not signifi-

![Fig. 1. Plasma levels of TNF-α (a) and IL-1β (b) in healthy subjects and in patients before treatment. Squares indicate the means ± 1 SD.](image-url)
Fig. 2. Pre- and postdialytic plasma levels of TNF-α in patients undergoing hemodialysis with cuprophane (a), and hemodiafiltration with polymethylmethacrylate (b), AN-69 (c) and polysulfone (d). Squares indicate the means ± 1 SD. The statistical significance is reported in 'Results'.

Fig. 3. Pre- and postdialytic levels of IL-1β in patients undergoing hemodialysis with cuprophane (a), and hemodiafiltration with polymethylmethacrylate (b), AN-69 (c) and polysulfone (d). Squares indicate the means ± 1 SD. The statistical significance is reported in 'Results'.
cantly change (1.82 ± 1.49 ng/ml and undetectable, respectively, p > 0.05). At the end of hemodiafiltration with polymethylmethacrylate and AN-69, the plasma levels of TNF-α (fig. 2b, c) and IL-1β (fig. 3b, c) were consistently reduced in respect to the predialytic values relative to each membrane (for polymethylmethacrylate, TNF-α = 1.22 ± 2.5 ng/ml, p < 0.05, and IL-1β = 20.33 ± 18.27 pg/ml, p < 0.001; for AN-69, TNF-α = 0.55 ± 1.16 ng/ml, p < 0.001, and IL-1β = 60.69 ± 21.45 pg/ml, p < 0.001). However, in patients on hemodiafiltration with polysulfone, the postdialytic plasma levels of both cytokines (fig. 2d, 3d) did not statistically differ from the predialytic values (TNF-α = 2.47 ± 3.47 ng/ml, p > 0.05, and IL-1β = 157.37 ± 188.35 pg/ml, p > 0.05).

In order to test the possibility that extracorporeal treatment with different membranes could stimulate monocytes to produce either TNF-α or IL-1β, we examined the in vitro spontaneous release of both cytokines by peripheral-blood monocytes in short-term cultures obtained before and after 1 session for each membrane. Cultured monocytes obtained from patients after hemodialysis with cuprophane released significant (p < 0.005) amounts of TNF-α and IL-1β as compared to cultured monocytes from healthy subjects used as controls (see 'Materials and Methods'; fig. 4). On the contrary, no increase in either cytokine was detectable in the supernatants of adherent monocytes obtained after hemodiafiltration with polymethylmethacrylate, AN-69 and polysulfone and incubated for 6 h (fig. 4).

The plasma concentrations of C3a antigen were also measured. As expected, C3a des Arg increased significantly (p < 0.001) at 15 min (550 ± 98 ng/ml) from a predialytic plasma value of 54 ± 14 ng/ml in the efferent samples from patients on hemodialysis with cuprophane membranes, but to a much lesser extent in patients on hemodiafiltration with polymethylmethacrylate (210 ± 92 ng/ml), AN-69 (205 ± 110 ng/ml) and polysulfone (231 ± 43 ng/ml).

Discussion

The present study shows that the predialytic plasma levels of TNF-α and IL-1β in our dialytic population taken as a group are consistently increased in respect to healthy subjects. In agreement with these data are the results of Blumenstein et al. [17] and Haeffner-Cavaillon et al. [18] who showed a significant increase in predialytic plasma levels of IL-1β in patients dialyzed with polyacrylonitrile. Factors other than the membrane such as the device and the mode of therapy may account for the differences in predialytic levels of cytokines. The possible mechanism underlying the increased production of IL-1β and TNF-α during the interdialytic period may be dependent on an endotoxin stimulation of monocytes/macrophages when endotoxin-containing dialysate diffuses back into the blood compartment, rather than on the interaction between dialysis membranes and monocytes [19]. Using an ELISA specific for antibodies to Escherichia coli endotoxin and E. coli J-5 antigen, Yamagami et al. [20] showed that hemodialysis with high-flux, high-permeable membranes is associated with the highest percentage of patients positive for endotoxin antibody as compared to that observed with low-flux membranes. Laude et al. [21] have shown that lipopolysaccharide (LPS) may indeed cross polyacrylonitrile membranes at least in vitro. However, the fate of IL-1β and
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Fig. 4. Spontaneous release of TNF-α (a) and IL-1β (b) in the supernatants from short-term (6 h) cultures of peripheral-blood monocytes obtained from blood samples drawn before (□) and after (■) hemodialysis with cuprophane (CU) or hemodiafiltration with polymethylmethacrylate (PMMA), AN-69 and polysulfone (PS). The results are means ± 1 SD. The statistical significance is reported in 'Results'.

TNF-α is different and this may greatly influence the plasma levels in the interdialytic period. Whilst in the absence of renal function IL-1β may accumulate in the body since more than 90% of the molecule is eliminated intact in the urine [22], only 5% of TNF-α is filtered by glomeruli and the major part of TNF-α is degraded at the receptor level expressed on tissues and circulating cells [23]. Therefore, if back-diffusion of LPS from the dialysate in the blood compartment is the predominant mechanism of stimulation, TNF-α rather than IL-1β would appear to be a more sensitive indication for such a response. Furthermore, there is evidence that the plasma levels of TNF-α but not of IL-1β
increase in healthy subjects exposed to endotoxin [24, 25]. The increased predialytic plasma levels of IL-1β and TNF-α in patients on hemodiafiltration using high-permeability membranes are consistent with the data reported by Haeffner-Cavaillon et al. [18]. These authors have suggested that monocytes from patients dialedyzed with high-permeability membranes may be chronically stimulated in vivo to produce IL-1β. Therefore, it may be suggested that, upon an appropriate stimulation, patients in hemodiafiltration with high-permeability membranes of the polymethylmethacrylate and AN-69 type retain an intact or enhanced ability to produce and release cytokines. However, the results concerning the predialytic TNF-α levels and postdialytic values of both cytokines obtained in patients on hemodiafiltration with polysulfone indicate that patients on hemodiafiltration using this membrane behave rather differently from other patients on polymethylmethacrylate and AN-69. The reason for such discrepancies is not clear at the moment and will require further investigation. In hemodialysis with cuprophane membranes, the plasma levels of cytokines were not significantly modified. Our data are at variance with those reported by Herbelin et al. [26] who showed significant increases in plasma IL-1 activity after hemodialysis with both cuprophane and polycrylonitrile membranes. However, patient selection and the different assays used may explain such discrepancies. Furthermore, our studies indicate that hemodiafiltration, a simultaneous application of hemofiltration to hemodialysis, drastically reduces the plasma levels of TNF-α and IL-1β with all membranes except with polysulfone. Such a consistent reduction may be explained on the basis of a clearance of the molecules by the dialyzer. Furthermore, absorption of IL-1β and TNF-α to all membranes may occur, as suggested by Lonneman et al. [27], and may contribute to removing both cytokines from the blood compartment. With cuprophane membranes, the plasma levels of TNF-α during hemodialysis remained rather constant. This might be explained by the relative inefficacy of this membrane to eliminate middle molecules such as TNF-α having a molecular weight of 17 kilodaltons and its ability to trigger the production of TNF-α as observed in short-term culture of monocytes obtained after the hemodialytic session. However, the postdialytic plasma levels of IL-1β increased significantly as also reported previously [28]. Both the in vivo induction of IL-1β synthesis and release of TNF-α at a later stage may be related to well-known complement bioincompatible phenomena also described in these studies. Schindler et al. [29] demonstrated that recombinant C5a triggers the transcription of mRNA for IL-1β and TNF-α but not the synthesis of both cytokines. Our results on short-term cultured peripheral-blood monocytes from patients on hemodialysis with cuprophane showed that these cells were capable of spontaneously releasing consistent amounts of both cytokines, a fact in apparent discordance with the lack of variation of plasma levels of both cytokines in these patients. One possible explanation may be related to plasma factors inhibiting both cytokine production in vivo but not in vitro as short-term cultures were performed in the absence of the patients' sera. Future studies will have to define whether patients on hemodialysis associated with repeated complement activation may have a defective production of cytokines. No detectable amounts of TNF-α or IL-1β could be detected in the supernatants of monocytes
from patients dialyzed with high-permeability membranes. However, we cannot exclude that both cytokines were indeed produced but remained cell-associated as reported earlier [18]. Future studies will have to define whether an increased cytokine production in patients on substitutive treatment may be detrimental or beneficial. Many physiological effects of the cytokines such as the enhancement of in vivo cell-mediated immune responses [30] may play an important role in various aspects of host defense.

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Prof. G. Camussi, MD
Laboratorio di Immunopatologia
Ospedale Molinette
Corso Polonia, 14
1–10126 Torino (Italy)