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Effects of ultrapure and non-sterile dialysate on the inflammatory response during *in vitro* hemodialysis

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Effects of ultrapure and non-sterile dialysate on the inflammatory response during *in vitro* hemodialysis. Several studies support the hypothesis that bacterial contamination of the dialysate stimulates the inflammatory response to hemodialysis (HD) and increases the long-term morbidity of HD patients; this phenomenon could also be modulated by the nature of the HD membrane. Therefore, this study was designed to compare the effects of non-sterile (NSBD, mean endotoxin content ± SEM 97 ± 22 EU/ml) and ultrapure bicarbonate dialysate (UPBD, sterile and pyrogen-free, obtained by ultrafiltration through polyamide) on several aspects of the inflammatory reaction during *in vitro* HD. The HD sessions (7 in each experimental group) were performed using miniaturized new cuprophane (CU) and polyacrylonitrile (PAN) hollow fiber dialyzers, and closed dialysate and blood circuits (the latter filled with heparinized blood from healthy donors). Plasma C3aDesarg levels were significantly increased after 15 minutes (t1) and increased further after three hours (t2) of CU HD, while during PAN dialysis they decreased from t0 to t1 and t2; however, no difference appeared between experiments with NSBD and UPBD. Granulocyte (PMN) and monocyte (MNC) expression of LFA-1, Mac-1, and CD45 at the start (t0), t1 and t2 was quantitated by flow cytometry analysis, after staining of the cells with specific fluorescinated monoclonal antibodies. In contrast with published data of *in vivo* HD, LFA-1 was overexpressed at t1 and peaked at t2, which suggests that the leukocytes expressing more LFA-1 leave the systemic circulation during *in vivo* HD. During CU HD, Mac-1 and CD45 on PMN and MNC were significantly increased at t1, and still more at t2. During PAN HD, Mac-1 and CD45 remained unchanged at t1, but increased significantly at t2 on PMN as on MNC. Again, no significant difference was found between NSBD and UPBD in LFA-1, Mac-1 and CD45 expression on PMN and MNC, during both CU and PAN HD. After three hours of dialysis, plasma levels of TNF-α, but not of IL-6, were significantly increased with CU and PAN. Again, no difference appeared when NSBD and UPBD were compared. Moreover, the lack of influence of bacterial contamination of the dialysate on TNF-α production was confirmed when MNC were cultured up to 24 hours after the end of the HD session. We conclude that complement activation products, either in plasma (CU) or those adsorbed on the HD membrane (CU and PAN) play the major role in the overexpression of β2-integrins and CD45 by PMN and MNC during HD. Also, bacterial products (at the levels that can be found in clinical conditions) do not influence either β2-integrin overexpression or TNF-α production induced by the dialysis membrane.

The concept that bacterial products in the dialysate could contribute to the inflammatory reaction that is associated with hemodialysis (HD) is born with the so-called "interleukin hypothesis" [1]. This concept has now found some support in several experimental and clinical data. So, during acute *in vitro* experiments, HD with non-sterile dialysate has been shown to be associated with a greater interleukin-1 (IL-1) production than with sterile dialysate [2–5]. Moreover, in a group of HD patients shifted for one month from non-sterile (NSBD) to ultrapure bicarbonate dialysate (UPBD), plasma levels of IL-1 and tumor necrosis factor (TNF) were found to decrease significantly [6]. Also, some data suggest that both serum levels of β2 microglobulin [7] and the incidence of the carpal tunnel syndrome [8] are lower in patients dialyzed with ultrapure dialysate. Consequently, the use of sterile, endotoxin-free dialysate has been advocated by some to minimize the inflammatory response to HD [5, 6], and several manufacturers have already integrated ultrafilters in their dialysate generators to provide UPBD. The present study was performed to further analyze the influence of bacterial contamination of the dialysate (and also a possible modulation according to the nature of the HD membrane) on several components of the inflammatory response. The effects of UPBD and NSBD were thus compared during *in vitro* cuprophane (CU) and polyacrylonitrile (PAN) HD on complement activation, monokines secretion, and on the expression by granulocytes (PMN) and monocytes (MNC) of several surface molecules involved in cell adhesion and activation.

Methods

In vitro hemodialysis

We performed *in vitro* HD sessions of three hours using closed loop blood and dialysate circuits. The dialyzers consisted in miniaturized new ethylene oxide-sterilized CU or PAN hollow fiber dialyzers, provided by Hospal (Meyzieu, France). The blood circuit was filled with heparinized blood from healthy donors (2 females, 1 male; ages 30 to 42 years). The dialysate consisted either in non-sterile bicarbonate dialysate (NSBD) or ultrapure bicarbonate dialysate (UPBD). Blood and dialysate were maintained at 37°C and recirculated with peristaltic pumps at 10 ml/min in a countercurrent manner, with no net ultrafiltration (volumetric control by means of reservoirs fluid level). The HD circuits were assembled using a careful aseptic technique. The apyrogenicity of the blood and of the dialysate circuits (in the experiments with UPBD) was confirmed at the start and by the

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Table 1. Cytokines inducing capacity of non-sterile (NSBD) and ultrapure bicarbonate dialysate (UPBD) as compared to RPMI 1640

		NSBD		UPBD		RPMI	
	No.	5%	10%	5%	10%	5%	10%
TNF-α *pg/ml*	1	3974	9124	≤ 15	≤ 15	≤ 15	≤ 15
	2	577	1614	≤ 15	≤ 15	≤ 15	≤ 15
Mean ± SEM		3822 ± 1905		≤ 15		≤ 15	
IL-6 *pg/ml*	1	2084	14863	≤ 30	81	≤ 30	60
	2	6972	12220	34	86	≤ 30	≤ 30
Mean ± SEM		9034 ± 2838		58 ± 15		39 ± 7	

NSBD, UPBD, and a cell culture medium serving as control (RPMI 1640) were mixed with whole blood of 2 healthy donors to final concentrations of 5 volume % (5%) and 10 volume % (10%). After 5 hours of incubation at 37°C, soluble TNF-α and IL-6 concentrations were measured in plasma. No. is identification number of the healthy blood donors.

end of each HD session by the absence of detectable endotoxin levels.

Preparation and characteristics of UPBD and NSBD

Both NSBD and UPBD were prepared from the standard bicarbonate dialysate (SBD) in use in our in-hospital HD facility. The NSBD consisted in the SBD that had been left overnight at room temperature to allow further bacterial proliferation. The most prevalent bacteria isolated from dialysate were *Pseudomonas* strains (*paucimobilis, cepacia, putida*) and *Xanthomonas maltophilia*. Chemical composition of NSBD was found unchanged after this delay (per liter: Na 145, K 3, Cl 111, acetate 3, bicarbonate 32, Mg 1, and Ca 3 mEq; dextrose 1.5 g). The final endotoxin levels of this NSBD were: (a) CU and PAN experiments together, mean ± SEM 97 ± 22 EU/ml, range 1.5 to 225, geometric mean 43; (b) CU experiments, 104 ± 33, 1.5 to 225, and 40, respectively; (c) PAN experiments, 88 ± 32, 6 to 225, and 48, respectively. The UPBD consisted in the SBD that had been previously ultrafiltered through a polyamide membrane (Ultrafilter U2000, Gambro, Sweden), a procedure known to retain endotoxin [9, 10], which was repeatedly confirmed by our own studies (data not shown). In other preliminary studies, we had also assessed the cytokines inducing capacity of UPBD and NSBD as compared to a cell culture medium serving as a negative control (RPMI 1640, Gibco, Life Technologies, Paisley, UK; no detectable endotoxin, that is, < 0.015 EU/ml). This was done as follows: a sample of NSBD (endotoxin concentration of 161 EU/ml), UPBD, and RPMI was diluted to final concentrations of both 5 and 10 volume% with whole blood of two different healthy donors. After five hours of incubation at 37°C, these samples were centrifuged and the concentrations of IL-6 and TNF-α were determined in the supernatants, using the assays described below. Results are presented in Table 1. By contrast with NSBD, UPBD compared with RPMI by having no residual monokines inducing capacity, confirming previous observations of Dinarello and colleagues [10].

Comparison of UPBD and NSBD

Both with CU and PAN, we compared the effects of UPBD and NSBD (7 experiments in each situation) on the expression of Mac-1, LFA-1, and CD45 at the surface of PMN and MNC. The expression of these molecules was evaluated at the start (t0), after 15 minutes (t1), and after 180 minutes (t2) of *in vitro* HD. We also compared the effect of UPBD and NSBD on complement activation, by measuring plasma C3aDesarg concentrations on several occasions at t0, t1, and t2. All the samples were obtained from the afferent blood line.

Also, the plasma levels of TNF-α and IL-6 were measured at t0 and t2 during CU and PAN dialysis, comparing the respective influence of UPBD and NSBD (7 experiments in each condition). Moreover, as MNC could be only primed during HD but produce monokines only several hours after the end of the session, we also studied the production of TNF-α by MNC maintained in culture up to 24 hours after the HD procedure.

Measurement of endotoxin levels

Endotoxin levels were measured using a chromogenic endotoxin specific assay (Seikagaku Corporation, Tokyo, Japan). This assay was chosen to get rid of the false positive results frequently observed with other assays while measuring endotoxin concentrations in the blood circuits of cuprophane hollow-fiber dialyzers, and which are due to non-pyrogenic cellulose-derived reactive material [11]. Results are expressed as EU/ml. The sensitivity of the assay is 0.15 and 0.015 EU/ml in blood and dialysate, respectively.

Measurement of plasma C3aDesarg concentrations

To avoid any further complement activation after sampling, blood was collected to tubes with EDTA, kept on ice and centrifuged immediately. Plasma was removed and frozen at −80°C. The concentrations of C3aDesarg were measured subsequently with a commercial radioimmunoassay (Amersham, Buckinghamshire, UK).

Measurement of plasma TNF-α and IL-6 concentrations

Plasma TNF-α and IL-6 levels were measured using commercial immuno-enzymetric assays (TNF-α-EASIA and IL-6-EASIA, respectively, Medgenix, Brussels, Belgium).

Delayed monokines production after in vitro HD

To study whether HD–in particular using NSBD–could prime MNC to produce monokines after the end of the procedure, the "delayed" TNF-α production was studied as follows. Blood was harvested after three hours of *in vitro* HD and the peripheral mononucleated cells (PBMC) were separated by density gradient centrifugation (Lymphoprep®, Nycomed, Oslo, Norway). The proportion of MNC among the PBMC was counted manually on 100 cells after staining with the May-Grünwald-Giemsa stain. The PBMC were then resuspended (2×10^6 cells/ml) in non-adherent polypropylene Falcon tubes (Becton Dickinson, Plymouth, UK) with RPMI 1640 containing fetal calf serum (5 vol%), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM (non essential amino acids), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5×10^{-5} M mercapto-ethanol (all from Gibco, Paisley, UK); the cells were cultured at 37°C in a humidified atmosphere with 5% CO_2, either for 3 ($t_{2+3\ hr}$) or for 24 hours ($t_{2+24\ hr}$). Controls consisted in PBMC obtained at the start of the HD sessions (t0), handled and cultured in the same manner, respectively, for 3 ($t_{0+3\ hr}$) and 24 hours ($t_{0+24\ hr}$). The TNF-α levels were measured both in the MNC ("intra-cellular TNF-α") and in the culture supernatants with the assay used for plasma TNF-α determinations. By the end of the culture periods, the samples were centrifuged and the culture supernatants and the PBMC pellets were separated; the latter were resuspended in 1 ml RPMI for the determinations

Table 2. Plasma C3aDesarg levels (ng/ml) during *in vitro* hemodialysis (HD)

Conditions	*N*	t0	t1	P[a]	t2	P[b]	P[c]
(A) CU/NSBD	7	331 ± 68	2390 ± 450	0.003	14170 ± 3430	0.007	0.011
(B) CU/UPBD	7	389 ± 1	3554 ± 1334	0.043	7563 ± 2819	0.042	NS
A vs. B[d]		NS	NS		NS		
(C) PAN/NSBD	3	220 ± 75	117 ± 45	NP	69 ± 7	NP	NP
(D) PAN/UPBD	4	264 ± 100	101 ± 34	NP	88 ± 13	NP	NP
PAN (C + D)[e]	7	245 ± 61	108 ± 25	0.018	80 ± 8	0.022	NS

Abbreviations are: CU, cuprophane; PAN, polyacrylonitrile; NSBD, non-sterile bicarbonate dialysis; UPBD, ultrapure bicarbonate dialysate; *N*, number of experimental HD sessions; t0, start of HD; t1 and t2, after 15 and 180 minutes of HD, respectively; NS, not significant; NP, analysis not performed due to too small size of data sets.

[a] t1 vs. t2, [b] t2 vs. t0, and [c] t2 vs. t1 respectively, by Student's *t*-test for paired data

[d] A vs. B by Student's *t*-test for unpaired data

[e] All data with PAN were pooled (NSBD and UPBD) to describe evolution of C3aDesarg levels during HD

of the intracellular TNF-α content of the MNC. Therefore, the PBMC were lysed by three successive freeze-thaw cycles at −80°C as previously described by N. Haeffner-Cavaillon et al [12]. The MNC lysates were then centrifuged at 2000 g for 10 minutes at 4°C and the TNF-α levels were measured in the supernatants ("intracellular TNF-α levels"). Both intracellular TNF-α levels and TNF-α concentrations in the culture supernatants were expressed as pg/per 2×10^5 MNC. Three experimental HD sessions were performed using PAN and NSBD; as no TNF-α production was observed no additional experiments were made with PAN and UPBD. On the contrary, because CU dialysis was associated with a significant TNF-α production, three experiments were performed with NSBD and three with UPBD to compare the effects of both dialysates. In all the studies on delayed TNF-α production, the endotoxin content of the NSBD was adjusted to 125 EU/ml.

Table 3. Plasma TNF-α and IL-6 levels (pg/ml) at the start (t0) and after 3 hours (t2) on *in vitro* hemodialysis

	TNF-α at t0	TNF-α at t2	t2 vs. t0[a]	IL-6 at t0	IL-6 at t2	t2 vs. t0[a]
CU						
(a) NSBD	< 15	127 ± 38	0.033	< 20	40 ± 16	NS
(b) UPBD	< 15	134 ± 44	0.042	< 20	34 ± 12	NS
a vs. b[b]		NS			NS	
PAN						
(a) NSBD	< 15	42 ± 10	0.041	< 20	20 ± 13	NS
(b) UPBD	< 15	49 ± 13	0.044	< 20	25 ± 5	NS
a vs. b[b]		NS			NS	

Abbreviations are: CU, cuprophane; PAN, polyacrylonitrile; NSBD and UPBD, non-sterile and ultrapure bicarbonate dialysate respectively; NS, not significant. TNF-α and IL-6 were always undetectable at t0.

Data are mean values ± SEM.

Student's *t*-test for paired ([a]) and unpaired ([b]) data, respectively

Measurement of Mac-1, LFA-1, and CD45 expression by PMN and MNC

Mac-1, LFA-1, and CD45 expression was assessed using two-color direct immunofluorescence staining of PMN and MNC, as previously detailed [13]. Briefly, 100 μl of blood is incubated for 30 minutes at 4°C with selected monoclonal IgG fluorescinated antibodies at saturating concentrations, in a double labeling procedure. These antibodies were anti-CD45 fluorescein isothiocyanate (FITC) - anti-CD14 phycoerythrin (PE) (Becton Dickinson, Mountain View, CA, USA), anti-CD11a FITC (Immunotech, Marseille, France) - anti-CD14 PE (Becton Dickinson), and anti-CD14 FITC (Becton Dickinson) - anti-CD11b PE (Becton Dickinson). As negative controls we used monoclonal isotopic IgG purchased from DAKO (Glostrup, Denmark). Red blood cells were lysed after staining, using a commercial solution (FACS lysing solution, Becton Dickinson). The samples were centrifuged, washed in phosphate-buffered saline (PBS) and resuspended in PBS for flow cytometric analysis on a FACstar Plus analyzer (Becton Dickinson). PMN and MNC were identified using their morphological characteristics (forward vs. side scatter resolution); discrimination between both cell types was further confirmed by the intensity of their CD14 expression. Acquisition was made on 10,000 cells. The green and red fluorescence was quantitated using dual-laser excitation of FITC and PE with logarithmic amplification (band pass filter 530-30 nm for FITC and 575-26 nm for PE separation). Results were expressed as mean fluorescence intensity channel (MFC).

Data presentation and statistical analysis

All data are expressed as mean values ± SEM. The evolution of Mac-1, LFA-1, and CD45 expression on leukocytes during the HD sessions were analyzed using the Wilcoxon's test for coupled differences (intra-experiments analysis). However, as baseline expression of Mac-1, LFA-1, and CD45 may vary rather widely according to the donor and from day to day, comparison between experiments with UPBD and NSBD was performed as follows. The differences in MFC values (ΔMFC) between t0, t1 and t2 were calculated as $\Delta 1$ = t1 − t0 and $\Delta 2$ = t2 − t0. Statistical analysis between experiments with UPBD and NSBD was made by comparing the values of the $\Delta 1$ and $\Delta 2$ by the Wilcoxon's test (inter-experiment analysis). For statistical analysis, plasma concentrations of TNF-α and IL-6 below the detection limit were assumed to be equal to the detection limit. Comparison of C3aDesarg, TNF-α, and IL-6 levels was performed by the Student's *t*-test for unpaired or paired data, as required. The tests were considered significant when α or $P \leq 0.05$.

Results

Endotoxin was never detectable in the blood circuits, even by the end of the HD sessions with NSBD

Complement activation. During CU HD, plasma C3aDesarg levels were elevated at t1, and were further increased at t2 (Table 2). On the contrary, during PAN dialysis, plasma C3aDesarg concentrations were lower at t1 and t2 than at the start of HD. The level of complement activation was never different with NSBD as compared to UPBD (Table 2).

Table 4. Delayed production of TNF-α by MNC obtained before (t0) and after 3 hours (t2) of *in vitro* hemodialysis and maintained in culture for 3 (t_{0+3h} and t_{2+3h}) and 24 hours (t_{0+24h} and t_{2+24h}), respectively

			Intracellular TNF-α *pg/per 2 × 10⁵ MNC*				TNF-α in culture supernatants *pg/per 2 × 10⁵ MNC*			
Membrane	Dialysate	Experiment	t_{0+3h}	t_{2+3h}	t_{0+24h}	t_{2+24h}	t_{0+3h}	t_{2+3h}	t_{0+24h}	t_{2+24h}
PAN	NSBD	1	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
		2	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
		3	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
CU	NSBD	1	< 20	< 20	< 20	< 20	< 20	40	< 20	854
		2	< 20	< 20	< 20	< 20	< 20	< 20	< 20	142
		3	< 20	< 20	< 20	< 20	< 20	38	< 20	64
CU	UPBD	1	< 20	< 20	< 20	< 20	< 20	< 20	< 20	328
		2	< 20	< 20	< 20	< 20	< 20	23	< 20	1066
		3	< 20	< 20	< 20	< 20	< 20	92	< 20	70

Abbreviations are: CU, cuprophane; PAN, polyacrylonitrile; NSBD and UPBD, non-sterile (125 EU/ml) and ultrapure bicarbonate dialysate, respectively; MNC, monocytes.
See **Methods** section for detailed protocol.

Table 5. Expression of Mac-1, LFA-1, and CD45 at the surface of PMN during *in vitro* HD

Glycoprotein	Time	CU/UPBD	CU/NSBD	PAN/UPBD	PAN/NSBD
Mac-1	t0	324 ± 92	139 ± 33	176 ± 52	285 ± 107
	t1	625 ± 132[c]	400 ± 134[c]	161 ± 29 NS	284 ± 59 NS
	t2	982 ± 157[c]	961 ± 166[c]	467 ± 79[c]	718 ± 133[c]
LFA-1	t0	45 ± 5	41 ± 3	42 ± 4	47 ± 5
	t1	50 ± 3[a]	45 ± 3[b]	44 ± 9[a]	49 ± 6 NS
	t2	50 ± 4[a]	47 ± 3[b]	49 ± 4[b]	52 ± 6[b]
CD45	t0	58 ± 14	43 ± 5	48 ± 10	57 ± 9
	t1	92 ± 16[c]	74 ± 15[b]	44 ± 5 NS	59 ± 9 NS
	t2	138 ± 15[c]	116 ± 12[c]	78 ± 8[c]	102 ± 16[c]

Abbreviations are: CU, cuprophane; PAN, polyacrylonitrile; UPBD, ultrapure bicarbonate dialysate; NSBD, non-sterile bicarbonate dialysate; t0, start of hemodialysis session; t1 and t2, after 15 and 180 minutes of dialysis, respectively.
Statistical significance is given as compared to: [a] $\alpha < 0.05$, [b] $\alpha < 0.025$, [c] $\alpha < 0.01$ (Wilcoxon test for coupled differences)

Table 6. Expression of Mac-1, LFA-1, and CD45 at the surface of MNC during *in vitro* HD

Glycoprotein	Time	CU/UPBD	CU/NSBD	PAN/UPBD	PAN/NSBD
Mac-1	t0	251 ± 74	175 ± 52	221 ± 71	394 ± 134
	t1	518 ± 121[c]	341 ± 92[c]	233 ± 51 NS	411 ± 120 NS
	t2	789 ± 154[c]	727 ± 136[c]	541 ± 92[c]	754 ± 146[c]
LFA-1	t0	164 ± 76	80 ± 8	79 ± 12	102 ± 15
	t1	184 ± 73[b]	102 ± 12[c]	90 ± 14[c]	122 ± 19[a]
	t2	224 ± 70[c]	139 ± 12[c]	198 ± 18[c]	168 ± 26[b]
CD45	t0	174 ± 72	93 ± 9	95 ± 12	129 ± 18
	t1	223 ± 75[c]	133 ± 20[c]	108 ± 8 NS	144 ± 20 NS
	t2	287 ± 67[c]	198 ± 18[c]	163 ± 16[c]	223 ± 32[c]

Abbreviations are: CU, cuprophane; PAN, polyacrylonitrile; UPBD, ultrapure bicarbonate dialysate; NSBD, non-sterile bicarbonate dialysate; t0, start of hemodialysis session; t1 and t2, after 15 and 180 minutes of dialysis, respectively.
Statistical significance is given as compared to: [a] $\alpha < 0.05$, [b] $\alpha < 0.025$, [c] $\alpha < 0.01$ (Wilcoxon test for coupled differences).

HD-associated production of monokines

TNF-α and IL-6 were never detectable in plasma at the start of the experimental HD sessions (Table 3). Both with CU and with PAN, the plasma levels of TNF-α had significantly increased after three hours of HD compared to t0 (Table 3). However, no difference appeared with the use of UPBD compared to NSBD, whatever the membrane (Table 3). In addition, the levels of TNF-α by the end of dialysis were higher with CU than with PAN (experiments with UPBD and NSBD together: CU versus PAN, 131 ± 28 versus 45 ± 26, $P < 0.001$. The IL-6 plasma levels were slightly increased at the end of dialysis as compared to t0, as well with CU as with PAN; this increase, however, did not reach the level of significance. Again, when experiments with NSBD and UPBD were compared, there was no significant difference in the plasma concentrations of IL-6 at t2, no matter which membrane was used.

Production of TNF-α by MNC harvested at the start and by the end of HD and cultured for 3 or 24 hours can be found in Table 4. With PAN, despite the use of NSBD, no TNF-α was found either within MNC nor in culture supernatants. With CU dialysis, no intracellular TNF-α was detected. However, low levels of TNF-α were found in the supernatants of MNC cultured for three hours (Table 4, $t_{2+3\ hr}$ vs. $t_{0+3\ hr}$) and, most especially, significant levels of TNF-α were found in the culture supernatants after 24 hours (Table 4, $t_{2+24\ hr}$ vs. $t_{0+24\ hr}$). However, this CU-induced delayed production of TNF-α was obviously not different with NSBD compared to UPBD.

Expression of Mac-1, LFA-1, and CD45 by PMN and MNC

The evolution during CU and PAN dialysis of Mac-1, LFA-1, and CD45 at the surface of PMN and MNC are detailed in Tables 5 and 6, respectively. The behavior of Mac-1 and of CD45 were strictly identical. During CU dialysis, there was an up-regulation of Mac-1 and CD45 on both PMN and MNC, that was already present at t1, and still increased at t2, whatever the dialysate. During PAN dialysis, with both UPBD and NSBD, Mac-1 and CD45 remain unchanged at t1 but increased significantly by t2, as well on PMN as on MNC. The expression of LFA-1 also increased at t1 and peaked at t2, whatever the cell type, the HD membrane, or type of dialysate (Table 6). However, the increase of LFA-1 expression on PMN at t1 during PAN dialysis with NSBD failed to reach significance.

Comparison between UPBD and NSBD for the Δ1 and Δ2 of Mac-1, LFA-1, and CD45 did not show any significant difference, neither for PMN nor for MNC, whether or not the HD was run on CU or PAN (data not shown).

Discussion

Bacterial products such as endotoxins and muramyl peptides are potent stimuli of inflammatory reaction, namely resulting in

Table 7. Experimental conditions of the studies that have assessed the role of bacterial contamination of the dialysate on monokines production during *in vitro* hemodialysis

Ref.	BC	DC	EXT source	ETX concentration *μg/ml*	Monokines studies
[2]	B	PBS	Purified ETX	1	IL-1 activity in the supernatants of MNC cultures
[4]	MEM/S 5%sAlb/S 10%FHP/S	MEM	*E. coli* Microfiltrate	0.5	IL-1 inducing activity of "blood" samples on MNC
[5]	S	S S/Bic	Purified ETX	0.8–1.1	MNC-associated IL-1 activity
[18]	S S/sAlb	S	Purified ETX	10.8	IL-1/TNF-α in plasma/cell culture supernatants

Abbreviations are: BC and DC, fluid used in the blood and dialysate circuits respectively; ETX, endotoxin; B, blood; MEM, minimal essential medium; S, saline; sAlb, seralbumin; FHP, fresh human plasma; PBS, phosphate buffered saline, Bic, sodium bicarbonate, IL-1, interleukin-1, TNFα, tumor necrosis factor α, MNC, monocytes.

complement activation [14], increased monokines production [15], and up-regulation at the surface of leukocytes of molecules involved in cell adhesion and/or activation, such as Mac-1 [16] and CD45 [17]. Also, it has been suggested that bacterial products in the dialysate could participate in the inflammatory reaction to hemodialysis [1–8]. Therefore, we performed this *in vitro* study to compare the effects of UPBD and NSBD on several aspects of the inflammatory response during cuprophane and polyacrylonitrile hemodialysis.

First, it must be emphasized that the limulus amoebocyte lysate (LAL) assay never detected endotoxin on the blood side of our experimental hemodialysis circuits, even after three hours of cuprophane or polyacrylonitrile hemodialysis with NSBD. This is consistent with other data in the literature. Even those groups whose results indirectly suggest that endotoxin can permeate the hemodialysis membrane, were unable to detect endotoxin on the blood side with the LAL-assay, despite the use of very high endotoxin concentrations in the dialysate [4, 5, 18].

During cuprophane hemodialysis, we observed a significant increase of C3aDesarg levels (Table 2) reflecting the well-known complement activation occurring with this membrane [19]. However, there was no difference in the level of complement activation between cuprophane hemodialysis with NSBD and UPBD. C3aDesarg levels were somewhat higher after three hours of hemodialysis with NSBD as compared to UPBD, but the difference was far from significant ($P = 0.16$, NS). No complement activation was observed in the blood phase during polyacrylonitrile hemodialysis, which is consistent with the classical data in the literature [19]. On the contrary, plasma C3aDesarg levels even decreased significantly during our *in vitro* experiments, which is presumably due to C3aDesarg adsorption onto the polyacrylonitrile membrane [19]. Again, C3aDesarg plasma levels were not different whether polyacrylonitrile hemodialysis was performed with NSBD or UPBD.

Both TNF-α and IL-6 plasma levels were elevated by the end of hemodialysis, either with cuprophane or polyacrylonitrile (Table 3). However, TNF-α and IL-6 plasma concentrations were not higher with NSBD as compared to UPBD, using cuprophane or polyacrylonitrile. Moreover, MNC were found to be primed during CU (but not PAN) dialysis to produce TNF-α while maintained in culture for 24 hours. Again, this effect was not enhanced by the use of NSBD containing 125 EU/ml of endotoxin as compared to UPBD. Our results contrast with the findings of several other groups, who reported higher IL-1 [2, 4, 5, 18] or TNF-α [18] production when hemodialysis was performed with an endotoxin-containing dialysate. However, it must been emphasized that the experimental conditions of these studies were quite different from the conditions in this study (reviewed in Table 7), which can explain the contrasting results. (1) All these groups used *E. coli* endotoxin, either purified [2, 5, 18] or an *E. coli* microfiltrate [4] added to the dialysate. Now, the source of the endotoxin might influence the monokines production associated with hemodialysis, since different endotoxins affect the inflammatory reaction to different levels [14]. Recently, endotoxins present in the bicarbonate dialysate were shown to be about 100 times less potent as compared to endotoxins from *E. coli* in their ability to stimulate monokines synthesis [21]. (2) Most important, all these groups used huge concentrations of endotoxin (0.8 to 10.8 μg/ml, that is, about 4,000 to 54,000 EU/ml), which are at least one hundred times higher than the endotoxin contamination of dialysate fluids used for clinical dialysis [22]. In the present study, the endotoxin concentrations obtained in the NSBD were far less, while remaining higher than those found in most clinical conditions, where 88% of the values are below 5 EU/ml [22], and the worst being around 50 EU/ml [23]. Taken together, our results and those in the literature are consistent with the concept that it is not the presence, but rather the concentration of endotoxin in the dialysate that is the relevant issue. According to our experiments, the levels of endotoxin generally present in the dialysate for clinical use do not stimulate the synthesis of monokines. (3) Moreover, only one group [2] used blood in the blood circuit and none used bicarbonate dialysate. It has been shown that the nature of the fluids used in the blood and dialysate circuits affect the transport of the cytokines-inducing products through the dialysis membranes [4, 18]. For instance, the transmembrane passage of cytokine-inducing substances has been studied by most other groups using *in vitro* models in which saline or saline supplemented with cell culture medium, 5% albumin or 10% plasma rather than whole blood was used in the blood compartment (Table 7). However, the recent characterization of the bactericidal/permeability increasing factor (BPI) question the relevance of these models. Indeed, the BPI, which is released during hemodialysis [24], may bind to endotoxin and neutralize its effect. Hence, *in vitro* models that do not use blood may overestimate the effect of cytokine-inducing bacterial substances that cross the HD membrane. For all these reasons, we believe that our experimental conditions, using whole blood and bicarbonate dialysate with endotoxins levels found in clinical conditions, are a much closer representation of the conditions present during clinical hemodialysis. Applied to the *in vivo* situation, our results

are consistent with the finding in recent prospective studies that bacterial contamination of the dialysate is not associated with a significantly increased risk of pyrogenic reactions [23]. Both cuprophane and complement activation products have been shown to prime monocytes to respond to endotoxin by cytokines production [25–27]. Hence, if significant amounts of endotoxin had been able to cross the dialysis membranes, we would have expected that NSBD hemodialysis would have been associated with significantly higher production of TNF-α as compared to dialysis with UPBD. At last, it must also be noticed that TNF-α production was higher with cuprophane than polyacrylonitrile, which is consistent with previous results in other *in vitro* [28] and *in vivo* [29] experiments.

Leukocytes express at their surface several glycoproteins such as the integrins, selectins and CD45, which are involved in numerous immune functions and in the regulation of the cell activation state. The expression of these molecules can be up- or down-regulated under the influence of various stimuli.

Integrins are a family of α/β heterodimeric glycoproteins. Mac-1 and LFA-1 belong to the $\beta 2$ subfamily of integrins, characterized by the nature of its β chain, which is called CD18. The α chain is variable and characteristic of each individual integrin: CD11b in the case of Mac-1 and CD11a in that of LFA-1. Mac-1 and LFA-1 are also known as leukocyte integrins since their expression is restricted to white blood cells [30].

Mac-1 (also called Mo 1, CR3, C3bi receptor, and CD11b/CD18) is expressed mainly at the surface of PMN, MNC, and natural killer cells. Upon stimulation, Mac-1 is recruited from a preformed intracellular pool [31, 32] and is quickly up-regulated at the cell surface. Known stimuli include C5a, fMLP, leukotriene B4 [33], and endotoxin [16]. Ligands comprise the intercellular cell adhesion molecule 1 (ICAM-1) [34], C3bi, various microorganisms and endotoxin [reviewed in 33]. Mac-1 is involved in numerous immune functions such as cell adherence and aggregation, chemotaxis, phagocytosis, and cell lysis (antibody-dependent cytotoxicity). LFA-1 (leukocyte function antigen 1, also known as gp 180/95 and CD11a/CD18) is strongly expressed on leukocytes. Counter receptors are ICAM-1 and ICAM-2. LFA-1 is involved in T cell proliferation and activation, and in leukocyte adherence and aggregation [reviewed in 33].

CD45 (leukocyte common antigen or T200) is a transmembrane glycoprotein expressed on all nucleated hematopoietic cells. The extracellular domain is probably involved in specific interaction with proteins. The intracellular domain has a tyrosine phosphatase activity and appears to be involved in signal transduction after stimulation through a number of surface receptors. CD45 might function to dephosphorylate a tyrosine residue on protein tyrosine kinases involved in receptor-mediated second messenger formation [reviewed in 35]. In the case of MNC, CD45 seems to be implicated namely in tumor necrosis factor-α and interleukin-1β release [36] and might play anti-adhesive functions [37].

Several *in vivo* studies have shown that hemodialysis, either with cuprophane or polyacrylonitrile, is associated with increased expression on peripheral PMN and MNC of Mac-1 [13, 38–42] and CD45 [13, 43]. However, the time course of leukocytes integrins and CD45 expression is dependent on cell type and on the nature of the dialysis membrane, suggesting that different triggering mechanisms might be implicated [13]. The pattern of Mac-1 and CD45 up-regulation during *in vivo* hemodialysis has led to the hypothesis that both complement activation and the transfer across the membrane of bacterial products arising from the dialysate might be implicated [13]. However, the results of the present study invalidate the hypothesis that bacterial contamination of the dialysate–even those encountered in the worst clinical conditions–could induce an increased expression of Mac1, LFA-1 or CD45 on PMN and MNC (Tables 5 and 6). Of course, this does not exclude that much higher concentrations of endotoxin, but less than what should been encountered in the conditions of clinical dialysis, could influence the expression of integrins and CD45.

The behavior of Mac1 and CD45 at the surface of PMN and MNC during our *in vitro* hemodialysis experiments on cuprophane and polyacrylonitrile was very similar to the one reported previously during *in vivo* hemodialysis [13, 38–41, 43]. The only notable difference is the fact that during *in vitro* hemodialysis, the expression of Mac1 and CD45 on PMN still increased from 15 to 180 minutes. This difference might be easily explained by the different profile in the concentration of complement activation products and might emphasize the major role of these products in the up-regulation on PMN of both Mac1 [31, 32] and CD45 [17]. Indeed, during *in vivo* dialysis, complement activation was maximal around 15 minutes [13]; on the contrary, during close circuit *in vitro* hemodialysis, the concentration of complement activation products are even higher after 180 minutes (Table 2), presumably because their biological clearance is limited. At the surface of MNC, Mac1 and CD45 behaved similarly during *in vitro* as compared to *in vivo* cuprophane hemodialysis, being already increased at 15 minutes and peaking at the end of the session. As *in vivo*, during polyacrylonitrile *in vitro* dialysis, the levels of Mac1 and CD45 on both PMN and MNC remained unchanged after 15 minutes but were much increased after three hours. These similarities between *in vivo* hemodialysis (where many leukocytes leave the systemic circulation to be trapped in the lung circulation [44]) and *in vitro* hemodialysis (where the leukocytes are confined to the closed blood circuit) also suggest that the expression of Mac1 and CD45 on the circulating leukocytes *in* vivo is representative of the expression of these molecules by the whole leukocyte population.

In contrast with *in vivo* hemodialysis [13, 40], there was a mild but significant increase in the expression of LFA-1 on PMN and MNC, both with cuprophane and polyacrylonitrile, and whatever the dialysate. This discrepancy between *in vivo* and *in vitro* hemodialysis might suggest that the cells expressing more LFA-1 leave the systemic blood circulation during *in vivo* dialysis.

In summary, our *in vitro* hemodialysis experiments show that the bacterial contamination of bicarbonate dialysate, at least at the levels observed in clinical hemodialysis, neither enhances the level of complement activation nor the secretion of the monokines TNF-α and IL-6 associated with cuprophane and polyacrylonitrile hemodialysis. Our results also suggest that cuprophane and polyacrylonitrile membranes themselves, and especially their complement activation properties, but not bacterial contamination of the dialysate, play the major role in the overexpression of Mac-1, and CD45 observed at the surface of PMN and MNC. In the case of cuprophane hemodialysis, we speculate that the complement activation products present in the plasma and on the membrane are a most important trigger [45]. In the case of polyacrylonitrile dialysis, this role can be allocated to the complement activation products which are massively adsorbed on the

membrane [20]. At least with cuprophane, the complement activation products that are membrane bound are known to remain functionally active and involved in the adherence of leukocytes to the membrane [45]. Whether bicarbonate dialysate for clinical use should be ultrapure and pyrogen-free will probably constitute an important debate of the next years in the field of dialysis. To solve such a complex issue all aspects of the inflammatory reaction that can be triggered by bacterial products will need to be investigated. In this setting, although negative, our data on complement, monokines, β2-integrins, and CD45 constitute a contribution to be added to an intricate file.

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