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Increased whole blood chemiluminescence in patients with chronic renal failure independent of hemodialysis treatment

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Abstract

Introduction: The luminol-enhanced whole blood chemiluminescence (LBCL) assay is a rapid assay for the measurement of reactive oxygen species (ROS) generation by circulating phagocytes. This study's aim was to determine if patients on maintenance hemodialysis (HD) and non-dialyzed patients with chronic renal failure (CRF) have altered LBCL and if dialysis itself affects ROS production in the blood.
Materials and Methods: Twenty-six HD patients, 11 non-dialyzed patients with CRF, and 20 gender- and age-matched healthy controls were studied. Resting (rCl) and 2×10^{-5} M n-formyl-methionyl-leucyl-phenylalanine-stimulated LBCL (peak chemiluminescence: pCl, total light emission after agonist addition: tCl) calculated per 10^4 phagocytes present in the 3-µl blood samples were measured with a Bio-Orbit® 1251 luminometer at 37°C for 11 min.
Results: Prior to the HD session, median rCL, pCL, and tCL were 1.5, 3.0, and 2.8 times higher in HD patients than in healthy controls ($p<0.01$) and tended to increase at the end of the session. Significant increases in tCl were observed at 30 min and 240 min (end) of HD (1023.5 vs. 1810.6 vs. 2006.8 arbitrary units×s/10^4 phagocytes, $n=9$, $p<0.05$). Median pCl and tCl were 5.0 and 4.3 times higher in non-dialyzed patients with CRF than in healthy controls ($p<0.001$). However, no significant differences were found between pre- and post-HD LBCL of HD patients and the LBCL of non-dialyzed patients with renal failure.
Conclusions: Blood from patients with renal failure generates elevated amounts of oxidants independently of HD treatment. This may add to the understanding of the nature of oxidative stress and suggests the need of anti-oxidant treatment in these patients.

Key words: uremia, hemodialysis, whole blood chemiluminescence, reactive oxygen species.

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INTRODUCTION

Hemodialysis (HD) in patients with end-stage renal failure is associated with continuous systemic oxidative stress [12, 21, 26]. This may result from the inflammatory response that accompanies uremia [10, 31] and the bio-incompatibility of the dialyzer membrane [27, 28], as both may stimulate the formation of reactive oxygen species (ROS). Circulating phagocytes, especially polymorphonuclear leukocytes (PMNs), seem to be the most crucial sources of ROS in HD patients. However, conflicting research data demonstrate activation [4, 45], lack of relevant changes [14, 44], as well as inhibition of PMN respiratory burst [33, 34, 38] in uremia. Similarly, studies on the effect of HD on ROS generation by isolated PMNs have yielded conflicting results, with this function being reportedly suppressed [17, 43], normal [28, 33, 42], or increased [4, 14, 20, 28, 33, 36, 42, 46].

These discrepancies may result from differences in patients' clinical status, protocols of HD and blood sampling, and various procedures of cell isolation and phagocyte respiratory burst stimulation. It is well known that isolation procedures may enhance resting cell activity and prime phagocytes for enhanced respiratory burst in response to agonist stimulation [16, 35, 47]. Therefore, changes in phagocyte activity related to their isolation could interfere with the effect of uremia and HD on ROS production in human phagocytes.

Luminol-enhanced whole blood chemiluminescence (LBCL) assay is a rapid and reliable technique that can be used for measuring ROS generation by circulating phagocytes. This method is as sensitive as isolated PMN luminol-enhanced chemiluminescence and eliminates the risk of cell priming during the preparation procedure [23, 24]. Moreover, LBCL allows for monitoring phagocyte ROS generation under conditions that more closely resemble the *in vivo* situation than the assays with isolated PMNs [23].

Therefore, this study was aimed at determining the intensity of resting LBCL and LBCL stimulated by n-formyl-methionyl-leucyl-phenylalanine (fMLP), a synthetic analogue of chemotactic bacterial peptides [40], in blood specimens collected from uremic patients before, at 30 and 60 min of the HD session, and after HD and compare these with healthy controls and non-hemodialyzed patients with chronic renal failure (CRF). We found that resting and fMLP-stimulated LBCL were elevated in uremic patients independently of treatment with HD compared with healthy subjects, while HD itself had only a moderate enhancing effect on fMLP-stimulated LBCL.

MATERIALS AND METHODS

Study subjects

The study involved 26 (HIV- and HBV-negative) patients (age, median M: 52, range R: 27–85 years; body mass index M: 21.4, R: 18.6–24.7 kg/m^2; plasma parathormone M: 508, R: 132–1657 pg/ml; 11 women) with end-stage renal disease who had been on regular maintenance HD (three times a week, using an AK-100 GAMBRO, Lund, Sweden, with a Clirens C-101 single-use cuprophane dialyzer from Terumo Corp, Tokyo, Japan) for 1.8–17.3 years (M: 7.6 years). Also studied were 11 patients with CRF never treated with HD (age M: 67, R: 43–80 years; body mass index M: 23.7, R: 20.2–24.8 kg/m^2; plasma parathormone M: 312, R: 158–618 pg/ml, 4 women) and 20 healthy individuals as controls (age M: 53, R: 27–72 years; body mass index M: 27.2, R: 23.4–28.6 kg/m^2, 10 women; Table 1). No subject was a current cigarette smoker or had suffered from any infectious disease during at least two months preceding the study. The controls were not on any medications and their routine physical examinations showed no abnormalities. All medications were stopped one month prior to the study (including vitamins with anti-oxidant properties and blood transfusions), but recombinant human erythropoietin continued to be administered (Neo-Recormon, Roche, 4000 units twice a week) to maintain the hematocrit fraction between 30 and 35% (n=23), as were the combinations of beta-blocker (atenolol, n=17; metoprolol, n=4) and angiotensin-converting enzyme inhibitor (enalapril maleate) required for hypertension control (n=21) in patients on maintenance HD. Non-hemodialyzed patients with CRF received enalapril maleate (n=7), calcium-channel blockers (amlodipine, n=5), and diuretics (furosemide, n=9). Patients with plasma parathormone concentrations higher than 300 pg/ml received alfacalcidol (n=28). Glomerulonephritis, polycystic renal disease, and essential hypertension caused renal failure in 11, 9, and 6 uremics on HD and in 5, 4, and 2 non-hemodialyzed patients with CRF, respectively. All subjects involved in the study gave informed consent and the study protocol was approved by the local Bioethics Committee at the Medical University of Łódź.

Study protocol

Three-ml blood specimens were drawn from the fistula into Vacuette® tubes (K3EDTA, Greiner bio-one, GmbH Krensmunster, Austria) before (0 min) and just after HD (240 min) and also in 9 patients at 30 and 60 min of HD and LBCL was measured within the 30 min. HD always started between 8 and 9 a.m. Venous blood samples from patients with CRF and healthy controls

Table 1. Selected blood parameters in uremic patients and healthy controls

Parameter	HD patients		CRF patients	Healthy subjects
	before HD	after HD		
Urea [mM]	28.4[a] (16.4–47.9)	11.5[a, b] (7.4–24.1)	17.5[a] (10.90–27.10)	5.23 (4.30–6.30)
Creatinine [μM]	876[a] (432–1468)	367[a, b] (231–933)	248[a] (195–481)	93 (80–110)
Uric acid [μM]	397[a] (278–523)	215[b] (114–353)	367[a] (296–466)	195 (156–57)
Hemoglobin [g/dl]	9.5[a] (6.60–12.70)	10.8[a, b] (6.5–14.4)	12.3[a] (8.90–16.20)	14.5 (12.7–17.7)
Erythrocytes [×10^6 cells/μl]	3.14[a] (2.01–4.96)	3.52[a] (2.34–5.89)	3.60[a] (2.88–4.55)	4.68 (4.12–5.34)
Phagocytes [PMNs + MCs; ×10^3 cells/μl]	4.4 (1.30–9.60)	4.7 (2.00–8.70)	4.5 (2.70–9.30)	4.0 (2.70–6.20)

MCs – monocytes; [a] $p<0.05$ vs. healthy controls, [b] $p< 0.05$ vs. value before HD.

were assayed parallel to the specimens from the HD patients. Preliminary studies with 7 healthy subjects revealed no significant differences in LBCL parameters between blood specimens collected at 8:30 and at 12:30 (240 min later). Therefore, blood specimens from healthy control subjects were collected only at one time, between 8 and 9 a.m. Since various blood dilutions were applied in the chemiluminescence assay in the past [13, 24, 32, 33, 40], additional preliminary experiments with blood from 10 dialyzed uremics were performed to find the optimal blood dilution giving the maximal LBCL signal. Of the three tested blood specimen dilutions (30, 100, and 330 times), the maximal signal was noted in the highest (Table 2). Similar results were obtained with blood from 10 healthy donors (data not shown). Therefore, a blood dilution of 330 times was chosen for further studies. To confirm the repeatability of uremia- and HD-induced LBCL changes, the experiments with the same group of uremics were repeated two months later.

LBCL assay

The resting chemiluminescence (rCL) level, i.e. without any agonist stimulation, and the fMLP-induced LBCL were measured according to Kukovetz et al. [24] with some modifications [40]. A 3-μl blood specimen was added to 947 µl of mixture solution, placed in the 1251 luminometer (Bio-Orbit®, Turku, Finland), and incubated for 30 min at 37°C. Then the rCL was recorded continuously for 1 min and 50 µl of fMLP solution was added by an automatic dispenser to a final concentration of 2×10^{-5} M and the measurement was continued for 10 min. The final concentration of fMLP was 100 times higher in the LBCL assay than in the case of isolated neutrophils due to non-specific fMLP binding to serum proteins and the surface of erythrocytes [24]. The mixture solution was prepared freshly before the assay by adding 1 ml of sterile Ringer solution, 5 ml of 0.025% w/v luminol, and 0.2 ml of 5% w/v glucose solution to 3.6 ml of de-ionized pyrogen-free water (resistance >18 MΩcm, HPLC Water Purification System USF ELGA, England) [24]. fMLP was dissolved in dimethyl sulfoxide to a final concentration 2×10^{-2} M and stored at -80^0C until assay. This solution was diluted with sterile 0.9% NaCl to an fMLP concentration of 4×10^{-4} M just before use. Luminol solution was prepared by dissolving 25 mg of luminol in 90 ml of 0.1 M Na_2HPO_4, then the pH was adjusted to 7.4 with 1 N HCl and the volume filled to 100 ml with distilled water [24]. Afterwards, the solution was filtered through a 0.2-μM Millipore filter and stored at 4°C in the dark for no longer than two weeks. The following LBCL parameters were calculated: mean rCl, peak chemiluminescence (pCl, maximal chemiluminescence signal reached after fMLP addition), the increase in chemiluminescence after addition of fMLP (dCl=pCl–rCl), total light emission after addition of fMLP (tCl, the area under the chemiluminescence intensity curve after addition of fMLP until returning to the baseline), and peak-time (the time from fMLP addition to the maximal chemiluminescence signal). In all cases the chemiluminescence signal returned to baseline after 6–9 min of recording. Individual results were obtained from triplicate experiments and rCl, dCl, and pCl were expressed in arbitrary units per 10^4 phagocytes (PMNs and monocytes) present in the assayed blood sample (aU/10^4p, where p is phagocytes). tCl was expressed in units of aU×seconds per 10^4 phagocytes (aU×s/10^4p).

To exclude any platelet contribution to fMLP-induced LBCL response, blood specimens from three healthy donors and uremics were centrifuged (13 min, 200×g, at room temperature) to obtain platelet-containing plasma ($228\pm47\times10^3$ platelets/µl, without leukocyte contamination). Then 3 µl of blood and the corresponding platelet-containing plasma specimens were stimulated with fMLP while chemiluminescence was recorded. The increase in chemiluminescence (dCL) reached 0.53 ± 0.09 aU, 0.07 ± 0.01 aU and 3.12 ± 0.24 aU, 0.06 ± 0.02 aU for blood and plasma specimens from healthy donors and for blood and plasma from uremics, respectively (results calculated per 3 µl of assayed sample). This indicates that the contribution of platelets to fMLP-induced LBCL was negligible.

Additional measurements

Part of the blood specimens were used for determining the blood cell count, including the number of PMNs and monocytes (ABX MICROS analyzer, AVL, Montpellier, France) and the plasma concentrations of uric acid, urea, and creatinine (Diagnostic Laboratory of

Table 2. Effect of specimen dilution on LBCL parameters in 10 patients on maintenance HD

LBCL parameter	Blood specimen dilution		
	30 times	100 times	330 times
rCl [aU /10^4p]	0.08[a] (0.04–0.45)	0.30[a] (0.13–2.10)	1.07[a] (0.42–6.71)
pCl [aU /10^4p]	1.30[a] (0.20–4.90)	4.0[b] (0.50–10.50)	5.3 (1.00–30.20)
dCl [aU /10^4p]	1.10 (0.20–4.70)	2.8[b] (0.40–10.10)	4.5 (0.50–28.80)
tCl [aU×s /10^4p]	368.5[a] (48.0–1266.2)	1137.9[a] (124.9–2814.5)	1570.6[a] (274.2–8598.9)
Peak-time [s]	256 (110–279)	252 (120–287)	253 (116–290)

aU – arbitrary units, p – blood phagocytes (PMNs + monocytes). Data from healthy subjects are not shown. In parentheses range. [a] $p<0.01$, [b] $p<0.05$ vs. dilution 330 times.

Medical University Hospital of Łódź). Plasma parathormone level was measured with the electrochemiluminescence immunoassay ECLIA (Elecsys PTH kit Cat. No. 11972103, Roche Diagnostics GmbH, Mannheim, Germany; method sensitivity: 1.20 pg/ml, normal range: 15–65 pg/ml) only in patients with renal failure.

Statistical analysis

Data are expressed as median and range. The differences between the groups were computed with the Mann-Whitney U-test. The effect of HD on the measured variables was analyzed with Friedman ANOVA followed by the Student's or the Wilcoxon matched pairs tests depending on the sample distribution. Correlation coefficients were calculated by the Pearson or Spearman tests. A p value of <0.05 was considered significant.

RESULTS

Comparison of LBCL in patients with renal failure and healthy controls

The LBCL assay performed before and just after HD revealed elevated levels of rCl, indicative of increased resting ROS production by the circulating phagocytes of hemodialyzed patients. Alternatively, rCl did not differ significantly between non-dialyzed patients with renal failure and healthy controls (Table 3). Three (pCL, dCl, and tCL) of the four analyzed parameters of fMLP-induced LBCL were significantly elevated in HD patients (both the pre-HD and the post-HD values) and in non-dialyzed patients with CRF compared with healthy controls. The highest difference, almost 13-fold ($p<0.001$), was observed for median dCl between non-dialyzed patients with CRF and healthy controls. Total ROS production after stimulation with fMLP expressed as tCl was 2.8 (pre-HD), 4.1 (post-HD), and 4.3 (non-HD) times higher in dialyzed and non-dialyzed patients than in healthy controls. Non-dialyzed patients did not differ significantly from those on regular HD with respect to fMLP-induced LBCL. Although, median dCl, pCl, and tCl in hemodialyzed patients before the HD session were 1.6, 2.0, and 1.5 times lower than the corresponding parameters in non-dialyzed CRF patients, these differences did not reach statistical significance (Table 3).

Effect of HD on LBCL in patients with end-stage CRF

In the first series of experiments that involved 26 hemodialyzed patients, LBCL parameters (excluding peak-time) tended to increase after the HD session compared with the values prior to HD. The higher difference, reaching a 1.5-fold increase, was observed for median tCl, but did not reach statistical significance (Table 3). However, analysis of the time-course of LBCL changes during HD revealed significant tCl changes (Table 4 and Fig. 1). Median tCl at 30 min and at 240 min (end) of HD ($n=9$) were almost 1.8 and 2.0 times higher than the pre-HD value ($p<0.05$). Furthermore, experiments performed two months later with the same group of patients to assess the effect of a single HD session on LBCL revealed significant increases in pCl and dCl, demonstrating enhanced LBCL induced by fMLP in this setting (Table 5).

Correlation between LBCL and clinical and laboratory parameters

There was no significant correlation between any parameter of LBCL and the number of erythrocytes, hemoglobin level, or serum concentrations of uric acid, urea, and creatinine in healthy controls, non-dialyzed patients with CRF (data not shown), or patients before and after HD. For instance, the correlation coefficients (r) between any of the parameters of fMLP-induced LBCL and plasma pre- and post-HD variables ranged from –0.33 to 0.30. No parameter of LBCL correlated with the duration of maintenance of HD treatment (data not shown) nor with plasma parathormone concentration in both non-dialyzed and HD patients.

Positive correlation was noted between rCL and fMLP-elicited light emission (pCl and tCl) in the blood specimens of healthy controls, patients before and after the HD session, and non-dialyzed patients with CRF (Table 6). The strongest associations ($r=0.77$ and $r=0.79$, $p<0.001$) were found between rCl and tCl in healthy individuals and patients before HD. On the other hand, no parameter of LBCL response before HD

Table 3. Comparison of LBCL parameters in HD patients, non-dialyzed patients with CRF, and healthy subjects

LBCL parameters	HD patients		CRF non-dialyzed patients	Healthy controls
	before HD	after HD		
rCl [aU /10^4p]	1.08[a] (0.41–10.42)	1.12[a] (0.41–4.90)	0.84 (0.52–3.78)	0.74 (0.18–1.31)
pCl [aU /10^4p]	3.6[b] (1.3–47.7)	4.7[b] (0.70–14.90)	6.0[b] (1.80–22.2)	1.2 (0.40–3.70)
dCl [aU /10^4p]	2.5[b] (0.6–37.3)	3.1[b] (0.30–0.90)	5.1[b] (1.10–19.7)	0.4 (0.10–2.40)
tCl [aU×s /10^4p]	1232.1[b] (305.0–16381.4)	1787.3[b] (271.2–5188.3)	1879.1[b] (582.8–6685.6)	438.1 (146.6–270.3)
Peak-time [s]	339 (91–417)	247 (88–376)	288[c] (225–626)	230 (163–270)

aU – arbitrary units, p – phagocytes (PMNs + monocytes). In parentheses range. [a] $p<0.01$, [b] $p<0.001$ vs. healthy controls, [c] border of significance: $0.06<p<0.08$.

Table 4. Time course of blood phagocyte count, resting and fMLP-stimulated LBCL in 9 patients during HD

Variable	Time of HD session				p value
	0 min	30 min	60 min	240 min	
Phagocytes [$\times 10^3$cells/μl]	5.1 (4.2–9.6)	4.2 (2.7–5.9)	4.9 (3.9–7.5)	4.8 (2.9–10.2)	0.006
rCl [aU /10^4p]	0.92 (0.65–4.59)	1.49 (0.94–6.48)	1.62 (0.72–7.77)	1.98 (0.74–8.01)	0.08
pCl [aU /10^4p]	3.2 (0.9–20.2)	5.9 (1.1–16.9)	4.4 (0.9–25.4)	5.9 (1.0–22.1)	0.11
dCl [aU /10^4p]	2.2 (0.2–15.6)	4.7 (0.1–11.6)	3.4 (0.2–19.7)	4.0(0.2–14.1)	0.23
tCl [aU×s/10^4p]	1023.5 (334.6–5716.3)	1810.6 (412.7–5268.7)	1424.5 (338.4–8070.7)	2006.8 (385.9–7140.8)	0.03
Peak-time [s]	224 (91–417)	255 (98–480)	254 (93–450)	263 (94–445)	0.23

aU – arbitrary units, p – blood phagocytes (PMNs + monocytes). Results were obtained from triplicate experiments. Blood specimens for phagocyte number determination and LBCL measurement were collected just before (0 min) and at 30, 60, and 240 min (end) of HD. Phagocyte number and tCL changed significantly during the HD session. Value-p in relation to variable changes over whole HD session. Also see Fig. 1.

Table 5. LBCL in HD patients before and after a single session

LBCL parameter	HD patients	
	before HD	after HD
rCl [aU /10^4p]	1.03 (0.56–1.48)	0.94 (0.37–2.87)
pCl [aU /10^4p]	3.10 (1.80–12.6)	5.10 (1.30–19.60)[a]
dCl [aU /10^4p]	2.01 (0.70–7.2)	4.30 (1.40–12.70)[a]
tCl [aU×s/10^4p]	1152.2(668.5–2929.5)	1264.3 (648.5–6412.1)
Peak-time [s]	241 (96–415)	236 (102–451)

aU – arbitrary units, p – phagocytes (PMNs + monocytes). Results of the series of experiments performed 2 months later. Results obtained from 20 patients because 6 patients were excluded from the second series of experiments due to symptoms of upper respiratory tract infection. In parentheses range. [a] $p<0.05$ vs. value before HD.

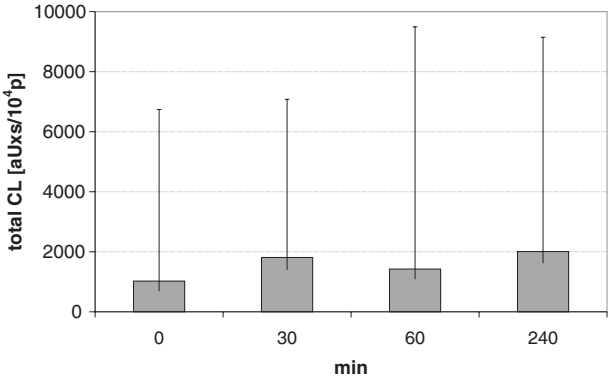

Fig. 1. Total light emission after addition of fMLP in the course of an HD session. Results were obtained from triplicate experiments. tCL changed significantly during HD session ($p=0.03$). Blood specimens for phagocyte number determination and LBCL measurement were collected just before (0 min) and at 30, 60, and 240 min (end) of a single HD session.

correlated with the respective LBCL values found after the HD session. The highest, but not significant ($r=0.40$ and $r=0.39$, $p>0.05$), correlation was between pre- and post-HD pCl, and pre- and post-HD tCl, respectively. However, if LBCL response was not expressed in relation to 10^4 phagocytes, significant correlation between light emission from 3-μl blood sample was found

Table 6. Correlation between selected parameters of LBCL in HD patients, non-dialyzed CRF patients, and healthy controls

Correlated LBCL parameters	HD patients (n=26)		Non-dialyzed CRF patients (n=11)	Healthy controls (n=20)
	before HD	after HD		
rCl and pCL	0.55[b]	0.75[a]	0.57[c]	0.69[a]
rCl and tCL	0.79[a]	0.40[c]	0.59[b]	0.77[a]
rCl and peak-time	0.50[b]	0.71[a]	0.62[b]	–0.125
pCl and tCl	0.83[a]	0.42[c]	0.99[a]	0.96[a]
pCl and peak-time	0.53[b]	0.89[a]	0.26	0.23
tCL and peak-time	0.69[a]	0.42[c]	0.27	0.16

[a] $p<0.001$; [b] $p<0.05$, [c] border of significance $0.06<p<0.08$.

(r=0.61 for pre- and post-HD pCl, r=0.62 for pre- and post-HD tCl, $p<0.01$). This may result from a strong correlation between the number of circulating phagocytes at 0 and 240 min of HD ($r=0.82$, $p<0.0001$) and also from the correlation between light emission (pCl and tCl) from the blood sample and the number of circulating phagocytes at these time-points ($r=0.50$ and $r=0.51$, $p<0.05$, at 0 min; $r=0.75$ and $r=0.67$, $p<0.001$, at 240 min). Analysis of individual patient results obtained from the second series of experiments showed similar associations. However, no significant correlation was identified between LBCL parameters before and after HD obtained from the first and second series of experiments (data not shown).

DISCUSSION

Increased fMLP-stimulated LBCL was found in uremic patients on maintenance HD and non-dialyzed patients with CRF compared with healthy individuals. HD itself caused a moderate increase in LBCL; however, this effect was unsettled as it was assessed with two measurements performed over a 2-month period. These findings suggest that circulatory phagocytes of patients with renal failure have increased ability to produce ROS

and that HD moderately primes cell response to fMLP, an analogue of bacterial chemotactic peptide.

The advantage of the LBCL method in comparison to the commonly applied ROS generation in pure PMN and/or monocyte suspensions is that the assay can be performed just after blood collection, without the leukocyte isolation procedure, which can possibly alter the oxidative metabolism of blood phagocytes [35, 47]. However, blood specimens for the LBCL assay should be diluted many times to avoid significant interference from erythrocytes, plasma proteins, and low-molecular-weight anti-oxidants, including uric acid [23, 24]. In this study, the dilution magnitude appeared to affect LDCL parameters consistently in all the study groups and we used blood specimen diluted 330-times, which yielded the highest chemiluminescence signals. In addition, at this dilution no parameter of LBCL response revealed significant association with red blood cell count, hemoglobin concentration, and plasma levels of uric acid, urea, and creatinine. This ensured that plasma and erythrocyte anti-oxidants and/or uremic toxins did not significantly affect the results of the LBCL assay. This is important due to the significant changes in some biochemical compounds, including plasma uric acid and hemoglobin levels, in the course of HD.

Few data exist on the intensity of whole blood chemiluminescence in uremic patients and its changes during HD with cuprophane and other types of dialyzer [4, 8, 13, 32, 33, 44]. In the majority of previous reports the possible association of blood chemiluminescence with circulatory anti-oxidants had not been analyzed [4, 8, 32, 33, 44]. Moreover, in five of these studies, only pCl was measured, while assessment of the total light emission (tCl), which reflects total ROS generation in a blood specimen, was omitted [8, 13, 32, 33, 44]. One study reported decreased fMLP-induced LBCL in uremic patients on HD [32]. In this study, blood samples were diluted about four times with autologous plasma, thus a strong suppressive effect of plasma and erythrocyte anti-oxidants on LBCL could not be excluded. Other authors, using a maximal dilution of 160 times in blood specimens, described decreased or normal pre-HD agonist-provoked LBCL (including fMLP-induced LBCL) [33] that did not change significantly at the end of HD [8, 33]. Ueda [44] described increased agonist-induced LBCL in uremics, while Chen et al. [5] found increased resting blood superoxide anion activity in uremics that was augmented by HD. The study protocol of Eckhardt et al. [13] included blood specimens diluted 250 times from uremics and healthy controls. They found normal resting and enhanced phorbol myristate acetate- and zymosan-induced LBCL in uremics. On the other hand, they described significant negative correlation between pCL and hemoglobin and creatinine levels [13]. This endorses the possibility of LBCL inhibition by blood anti-oxidants and uremic toxins if the sample dilution is lower than 330 times [23, 24, 50].

Apart from the high specimen dilution that minimizes the effect of blood anti-oxidants and uremic toxins on LBCL, our study protocol involved measurement of pre- and post-HD LBCL at two occasions within a two-month interval and estimation of the time-course of LBCL during the HD session. Therefore, our results clarify the above-mentioned discrepancies in LBCL in uremics and clearly show that patients on maintenance HD have increased resting and fMLP-stimulated ROS production. Moreover, HD with the cuprophane membrane (of relatively high bio-incompatibility) increased to a moderate extent the ability of circulating phagocytes to produce ROS after stimulation with fMLP. It is interesting that non-dialyzed patients with renal failure also had increased LBCL, which did not differ from patients on maintenance HD. This suggests that some agents (e.g. TNF-α, LTB_4), released in consequence of blood contact with the dialyzer cuprophane membrane [18, 19], are not the main factors responsible for increased pre-HD LBCL. In addition, their catabolism in plasma and diffusion through the dialyzer membrane into the dialyzing fluid [29, 41] can strongly limit the duration of the stimulatory effect on blood phagocytes. Perhaps other factors that are generated and/or accumulated in the blood and tissues of patients with renal failure, such as circulating adhesion molecules and advanced glycation end-products, can be responsible for enhanced LBCL [15, 48]. Advanced glycation end-products are able to prime PMNs and monocytes to enhanced ROS production (via activation of cytosolic phospholipase A_2) upon stimulation with various agonists, including fMLP [35]. Due to their relatively high molecular weight, they are hardly dialyzable and, therefore, the increased ability of uremic phagocytes to produce ROS in response to agonist stimulation is not corrected by HD [22]. Recently we described increased H_2O_2 exhalation in uremics that did not change significantly in response to HD [37]. Our present results are consistent with this finding especially in light of the view that alveolar macrophages and PMNs seem to be the main source of exhaled H_2O_2 [40] and tissue accumulation of advanced glycation end-products in uremic patients may prime these cells to enhanced ROS production [15, 16, 48].

Although no parameter of LBCL correlated with plasma parathormone level, the influence of this peptide on fMLP-induced phagocyte ROS production cannot be excluded. Parathormone increased the resting intracellular calcium (Ca^{2+}) concentration in PMNs and stimulated neutrophil elastase release [2, 30]. Elevated resting intracellular Ca^{2+}, which is also observed in PMNs of uremic patients [2], primes these cells for enhanced ROS production in response to stimulation with fMLP [40]. On the other hand, preincubation of human monocytes with parathormone decreased the ROS production associated with the phagocytosis of latex particles [39]. Thus the effect of parathormone on fMLP-induced LBCL remains open and requires further studies.

Treatment with high dose of alfacalcidol (3 µg/day) increased superoxide radical production by peripheral

blood monocytes, but not by PMNs in uremic patients undergoing continuous ambulatory peritoneal dialysis [9]. *In vitro* furosemide decreased ROS production by PMNs stimulated with fMLP, but only at concentrations at least 100-times higher than that observed in plasma after intravenous drug infusion [3]. Oral administration of enalapril in patients with moderate heart failure as well as *in vitro* cell incubation with the drug had no influence on ROS production by human PMNs [6, 49]. Similarly, atenolol did not affect PMNs' ability to produce ROS *in vitro* [11]. Thus one may conclude that these drugs had no significant effect on resting and fMLP-induced LBCL in our patients.

Almost all the uremics involved in our study received recombinant human erythropoietin. Erythropoietin was reported to elevate ROS production by resting and agonist-activated blood phagocytes in uremics on maintenance HD [47, 48]. Administration of amlodipine normalized basal cytosolic Ca^{2+} concentration and the Ca^{2+} transient in response to 3G8 monoclonal antibody in PMNs isolated from uremics [1]. Since Ca^{2+} is involved in PMN activation with fMLP, it is possible that amlodipine can influence fMLP-induced LBCL; however, the effect of this drug on ROS production by PMNs has not been reported so far. On the other hand, the similar LBCL intensity in uremics on maintenance HD and non-dialyzed patients with renal failure suggests that treatment with erythropoietin and amlodipine also has no significant influence on ROS production by circulating phagocytes.

Analysis of correlation between LBCL parameters showed marked differences between healthy controls and uremics. In contrast to patients with end-stage renal failure on maintenance HD, no parameter of light emission from the sample (rCl, pCl, tCl) correlated with peak-time in healthy subjects. This suggests a different shape of the LBCL response in uremics than in healthy controls. Altered conditions of signal transduction, including changes in the number of membrane receptors on blood PMNs [25], could be an acceptable explanation of these differences. PMNs from non-dialyzed patients with CRF behaved indirectly and revealed significant correlation between rCl and peak-time. The number of fMLP receptors on the outer membrane of PMNs was reported to change significantly in response to HD [7]. This may explain the lack of correlation between LBCL parameters before and after HD in uremics. Although there was a strong correlation between pre- and post-HD leukocyte count, these cells behaved as separate populations and pre-dialysis LBCL did not associate with the corresponding values after treatment.

In conclusion, we found that peripheral blood phagocytes from patients with CRF had increased ability to produce ROS as evaluated with the fMLP-elicited LBCL assay. Chronic maintenance HD with a cuprophane dialyzer did not distinguish patients with renal failure with regard to the intensity of ROS generation in blood specimens; however, the single HD procedure itself caused a moderate increase in LBCL. This may add to the understanding of the nature of oxidative stress in patients with CRF and may imply considering early intervention with anti-oxidant treatment in uremic patients.

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