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Original Article

Whole blood production of monocytic cytokines (IL-1β, IL-6, TNF-α, sIL-6R, IL-1Ra) in haemodialysed patients

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Abstract

Background. The production of monocytic cytokines by isolated mononuclear cells after stimulation by phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) is generally increased in haemodialysed (HD) patients. We performed whole blood (WB) cultures to evaluate cytokine production by blood cells inside their complex cellular and humoral network.

Methods. Diluted whole blood from HD patients (collected before dialysis) and controls was cultured alone with PHA (2.5 μg/ml) or LPS (1 and 3 μg/ml). Supernatants were collected after 24 and 48 h of culture, and concentrations of IL-1 β, IL-6, TNF-α, sIL-6R and IL-1Ra were determined by ELISA.

Results. The low spontaneous production of IL-1β, IL-6 and TNF-α in both patients and controls was not significantly modified by PHA. The lower dose of LPS (1 μg/ml) induced a significant but lower increase in production of IL-1β, IL-6 and TNF-α in patients than in controls. In contrast, while it did not further increase their production in controls, the higher concentration of LPS (3 μg/ml) still increased their production in patients to the same level than in controls. The plasma concentrations of sIL-6R were higher in patients than in controls. In both groups, the sIL-6R concentration did not vary during the culture period whether the cells were stimulated or not with LPS or PHA. This suggests that the increased plasma levels of sIL-6R were not produced by blood cells. Despite a similar significant LPS and PHA induced production of IL-1Ra, the IL-1Ra/IL-1β ratio was always higher in patients than in controls.

Conclusion. Monocytes from HD patients in WB cultures are hyporesponsive to PHA and LPS for their IL-1β, TNFα and IL-6 production in contrast to isolated monocytes that demonstrate signs of activation. If it reflects the *in vivo* situation it could partly explain the immune defect in uraemic and haemodialysed patients. Higher sIL-6R/IL-6 and IL-1Ra/IL-1β ratios could also participate to the complex immune disturbances of HD patients by reducing the biological activity of two cytokines playing a major role in the immune and inflammatory network.

Key words: cytokines; haemodialysis; monocytes; whole blood

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Introduction

Abnormalities of the immunocompetent cells have been extensively studied in uraemic and especially haemodialysed (HD) patients [1]. Clinically, these patients elicit poor antibody responses to hepatitis B vaccines [2], have prolonged survival of skin allografts, and increased morbidity and mortality related to a high incidence of infection and malignant tumours [3,4]. This immunodeficiency is characterized by multiple and complex defects of both cellular and humoral defense mechanisms. Several findings indicate that monocytes are chronically activated in patients with chronic renal failure (CRF) whether they are dialysed or not. First, serum concentrations of interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor α (TNF-α) and their soluble receptors are increased [5–7]. Secondly, expression of mRNA for IL-1, IL-6, and TNF-α is increased during the dialysis session [8–10] and there is enhanced spontaneous and/or stimulated production of these cytokines by monocytes in culture [11–14]. Thirdly, serum levels of neopterin and interleukin-1 receptor antagonist (IL1-Ra), which are considered as good markers of chronic activation of monocytes and macrophages, are increased [5,15,16]. Fourthly, serum levels of macrophage colony stimulating factor (M-CSF) in uraemic undialysed, as well as HD or continuous ambulatory peritoneal dialysis patients, are increased [17]. In contrast, reduced or normal predialysis IL-1β production by monocytes has also been reported [18–20]. These discrepancies are probably due in part to the differences in culture techniques.

In most cases, *in vitro* cytokine production has been studied with mononuclear cells isolated from peripheral blood (PBMC). This method eliminates numerous mediators present in plasma and modifies the ratio and intercellular relationships between immunocompetent cells, including polymorphonuclear cells. By contrast, cultures of whole blood (WB) allow one to evaluate cytokine production by cells inside their complex cellular and humoral network [21]. Since most immune defects of uraemic patients are probably related to complex functional alterations of the 'cytokine network' [1,5] we used WB cultures to evaluate in HD patients the production of IL-1β, IL-6, TNF-α, soluble interleukin-6 receptor (sIL-6R) and IL-1Ra, that are most likely produced by activated monocytes and macrophages.

Methods

Patients

Informed consent was obtained from each participant. Ten HD patients (six men and four women) were studied and compared with 10 healthy volunteers (five men and five women) as controls. The HD patients had been haemodialysed three times per week for at least 6 months prior to the study, using the same membrane and the same dialysis procedure for at least 3 months: polyacrilonitrile AN 69 (Hospal, France, four patients), cellulose diacetate (Hospal, France, three patients), polyamide (Gambro, Sweden, three patients). The underlying diseases consisted of a vascular nephropathy (three patients), chronic glomerulonephritis (two patients), diabetic glomerulopathy (one patient), chronic interstitial nephritis (two patients), undetermined nephropathy (two patients). At the time of the study no patient had clinical evidence of acute infection, autoimmune disease or had received drugs known to interfere with the immune response such as calcium channel blockers. Blood samples were collected in heparinized tubes and rapidly processed for culture and/or plasma collection. In HD patients, blood was drawn just prior to the routine haemodialysis session 3 days after the previous one.

Cell counts and viability

The total white blood cell count and differential was obtained for each blood sample using a Coulter STKS counter. Cell viability in culture was assessed by the trypan blue dye exclusion method for each culture period (24, 48 and 72 h).

Whole blood culture

Blood diluted 1/10 in RPMI 1640 (Gibco, Ghent, Belgium) was distributed in 24-wells plates (Nunc, Roslike, Denmark) (2 ml/well) and stimulated with phytohaemagglutinin (PHA) 2.5 μg/ml (PHA L-9132 from Phaseolus vulgaris, Sigma, St Louis, USA), or lipopolysaccharide (LPS) 1 or 3 μg/ml (LPS L6761 from *Salmonella enteritidis*, Sigma, St Louis, USA). Plates were incubated at 37°C with 5% CO_2. After controlling cell viability, well contents were collected after 24, 48, and 72 h of culture, and centrifuged at 900 *g* for 5 min. Supernatants were then harvested and frozen at −20°C until use for cytokine measurements.

Cytokine assays

Cytokines were measured with specific immunoassays according to the manufacturer's instructions. The detection limits were 10 pg/ml for IL-1 β (Cistron Biotechnology, Pine Brook, USA), 14 pg/ml for IL1-Ra (Quantikine R&D Systems, Abingdon, Great Britain), and 25 pg/ml for TNF-α, 15 pg/ml for IL-6, and 125 pg/ml for sIL-6R (Diaclone, Besançon, France). All samples of supernatants were tested simultaneously in duplicate at appropriate dilutions. Plasma sIL-6R was also measured in the patients and controls of this study as well as in 30 HD, 27 CRF and 20 controls.

Statistical analysis

Student's unpaired and paired *t*-tests or Mann–Whitney's test were used when appropriate to compare data. Differences were considered to be significant when $P<0.05$. Coefficients of correlation between the productions of cytokines were calculated using the linear regression analysis.

Results

Cells

Mean numbers (±SD) of white blood cells, polymorphonuclear cells (PMN), monocytes and lymphocytes are shown in Table 1. The HD patients had significantly lower lymphocyte counts than controls ($P<0.01$). The viability of cultured cells was 95% in HD patients and 94% in controls at 24 h, 90 and 92% at 48 h, 84 and 86% at 72 h.

Production of IL-1 β, IL-6 and TNF-α by cultured whole blood

The kinetic study of the production of the three cytokines showed that maximal concentrations occurred in the culture medium at 24 h and remained stable or decreased after 48 and 72 h (data not shown). The spontaneous production (mean ± SE) of IL-1β, IL-6 and TNF-α by unstimulated WB at 24 h was low, respectively at 176 ± 45, 1106 ± 337, 150 ± 27 pg/ml in controls and 137 ± 15, 1364 ± 251, 150 ± 20 pg/ml in patients (Figure 1) without any significant difference between the two groups. Results were quite similar at 48 h. Stimulation with 2.5 μg/ml of PHA did not modify the release of IL-1β. In contrast, production of IL-6 and TNF-α was slightly, but significantly, increased in HD patients and controls, without significant differences between the two groups. LPS (1 μg and 3 μg/ml) significantly increased the production of IL1-*β*, IL-6 and TNF-α in controls and HD patients (Figure 1). As shown in Table 2 the production of each of these three cytokines was strongly correlated with the production of the two others. Interestingly, while WB from HD patients produced significantly less IL1-*β*, IL-6 and TNF-α than WB from controls when stimulated with 1 μg/ml of LPS, the difference was no longer significant when stimulated with 3 μg/ml of LPS. The results observed for WB cultures at 48 h are not shown since they remained quite similar to

Table 1. Haematological parameters in patients and controls

	White blood cells (10^9/l)	Lymphocytes (10^9/l)	Monocytes (10^9/l)	PMN (10^9/l)
Controls ($n=10$)	7.20±1.7	2.60±0.3	0.49±0.02	3.49±1.4
HD patients ($n=10$)	6.70±1.8	1.29±0.3[a]	0.58±0.03	3.95±1.3

Results are expressed as means±SD. [a]A significant difference between HD patients and controls ($P<0.05$).

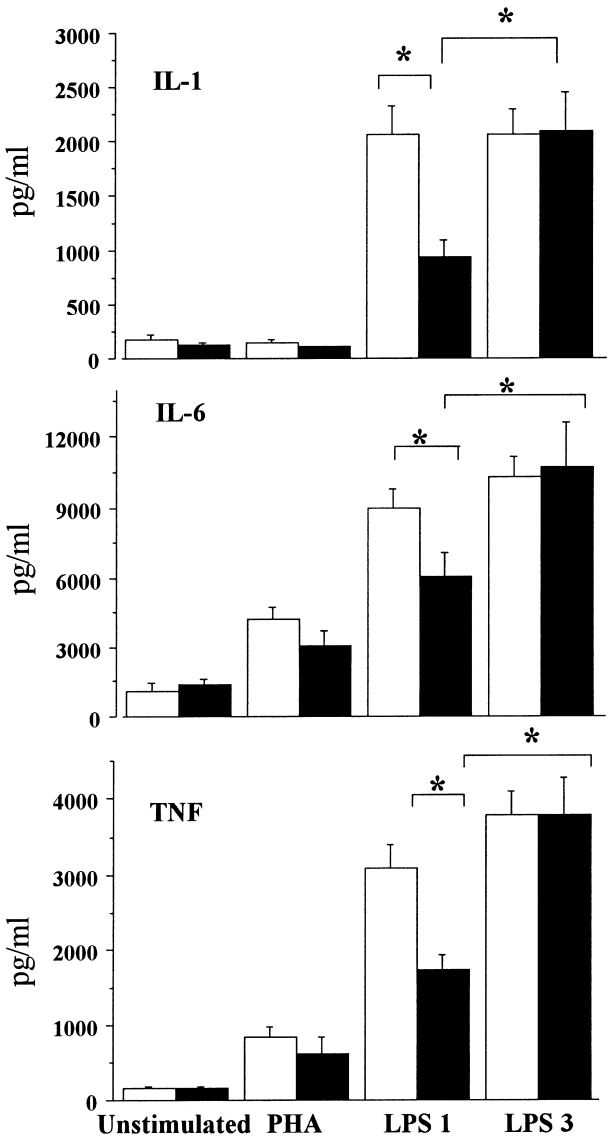

Fig. 1. WB production of IL-1 β, IL-6, and TNF α in 10 controls (□) and in 10 HD patients (■) at 24 h of culture. The cultures were either unstimulated or stimulated with 2.5 μg/ml of PHA, 1 or 3 μg/ml of LPS (see methods). Results are expressed as mean±SE of cytokine production. Significant differences between HD patients and controls ($P<0.05$) were noted with an asterisk.

those observed at 24 h. This difference in cytokine production between controls and HD patients with the lower concentration of LPS (1 μg/ml) cannot be accounted for by the number of monocytes in culture, which was slightly but not significantly higher in HD patients than controls (Table 1). When expressed per number ($pg/10^4$) of monocytes in culture, the production of these cytokines by WB remained significantly lower in HD patients than in controls (data not shown). Cytokine production was similar regardless of the type of membrane used during the haemodialysis session (data not shown).

Table 2. Linear regression analysis of the correlation between the production of IL-1 β, IL-6, and TNF α by WB cultures stimulated with 1 μg/ml of LPS in a group of 20 subjects including 10 haemodialysed patients and 10 controls

	r	*P*
IL-1 β and IL-6	0.708	0.0006
IL-1 β and TNF α	0.727	0.0004
IL-6 and TNF α	0.636	0.0036

Concentrations of the soluble gp 80 IL-6 receptor (sIL-6R) in plasma and cultured WB supernatants

The high levels of sIL-6R in the culture supernatants from HD patients and controls at $T=0$ decreased after 24 and 48 h of culture (Figure 2). At all time points they were significantly higher in HD patients than in controls (Figure 2). The high levels of sIL-6R were not significantly modified by PHA and LPS stimulation. The dosages of the sIL-6R plasma levels in these patients and controls as well as in 30 additional HD patients, 27 CRF undialysed patients and 20 additional controls confirmed that sIL-6R concentrations were higher in HD patients (223±74 ng/ml, $P=0.0002$) and CRF undialysed patients (215±54 ng/ml, $P=0.001$) than in controls (151±33 ng/ml). These results confirm that sIL-6R levels in the culture supernatants are related to the high plasma concentrations. In addition, it is interesting to note that the sIL-6R plasma concentrations did not correlate with the degree of CRF as calculated by the Cockroft formula ($r=-0.182$, $P=0.36$).

Production of IL-1Ra by cultured WB

The production of IL-1Ra by unstimulated cells was higher, however not significantly, in HD patients than in controls at 24 h (3.01±1.4 ng/ml *vs* 2.0±0.4 ng/ml). Stimulation by PHA or LPS (1 and 3 μg/ml) induced a significant and similar increase in the production of IL-1Ra (reaching approximately 6.50 ng/ml) in both patients and controls (Figure 3) at 24 h. Similar results were obtained at 48 h (data not shown).

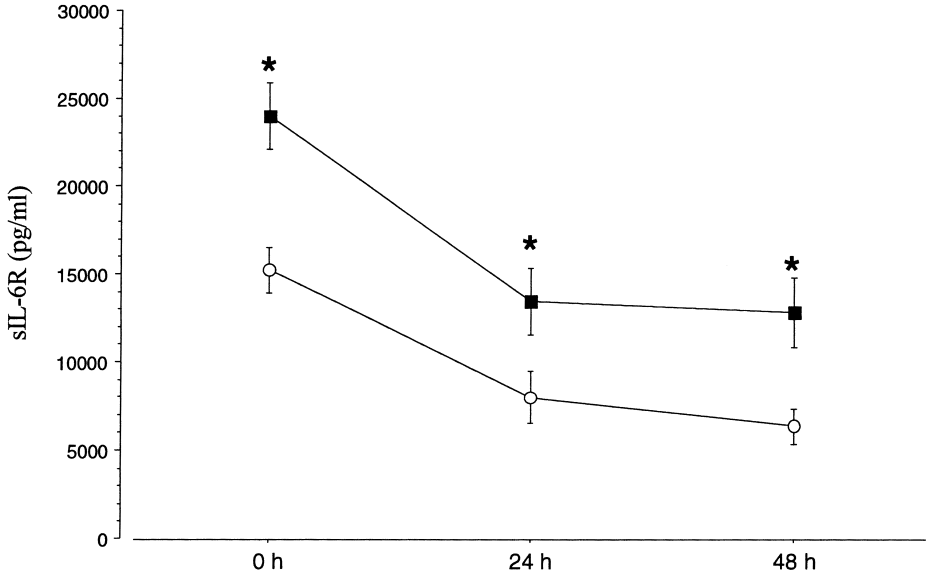

Fig. 2. Concentrations of sIL-6R in the WB supernatants of 10 controls (○) and of 10 HD patients (■) at time 0 and after 24 h and 48 h of unstimulated culture. Results are expressed as mean ± SE of soluble receptor concentration. Significant differences between HD patients and controls ($P < 0.05$) were noted with an asterisk.

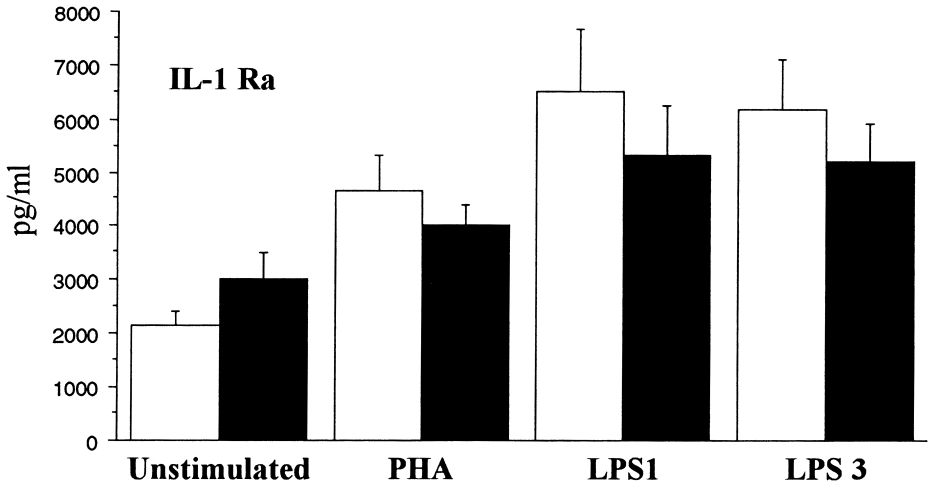

Fig. 3. WB production of IL-1Ra in 10 controls (□) and in 10 HD (■) patients at 24 h of culture. The cultures were either unstimulated or stimulated with 2.5 μg/ml of PHA, 1 or 3 μg/ml of LPS (see methods). Results are expressed as mean ± SE of receptor antagonist production.

IL-1Ra/IL-1 and sIL-6R/IL-6 ratios

These molar ratios were calculated in unstimulated and LPS (1 and 3 μg/ml)-stimulated cultures. The IL-1Ra/IL-1 ratio was significantly higher in unstimulated and LPS (1 μg/ml)-stimulated cultures of HD patients when compared with controls (15.0 *vs* 8.3 in unstimulated condition, 4.1 *vs* 2.1 with 1 μg/ml of LPS). By contrast, in LPS (3 μg/ml)-stimulated cultures the ratio was similar in both groups. The sIL-6R/IL-6 ratio was similar in unstimulated cultures of HD patients and controls. By contrast, it was significantly higher ($P < 0.0005$) in LPS (1 and 3 μg/ml)-stimulated cultures of HD patients than controls (1.9 *vs* 0.8 with 1 μg/ml of LPS; 1.6 *vs* 0.6 with 3 μg/ml of LPS).

Discussion

De Groote *et al.* [21] compared the production of five cytokines (IL-1 β, TNF-α, IL-6, interferon γ and GM-CSF) by PBMC and WB from healthy controls. As their results suggest, WB may be the most appropriate milieu in which to study cytokine production *in vitro*, in both physiological and pathological situations. We used it to compare the production of three cytokines (IL-1β, IL-6 and TNF-α), one soluble receptor (sIL-6R), and one receptor antagonist (IL-1Ra) in HD patients and controls. In order to assess baseline cytokine production of blood cells in HD patients, WB was collected just before a dialysis session, so that cells activated during the previous session 3 days earlier had left the vascular compartment.

The spontaneous production of IL-1β, IL-6 and TNF-α was similar in HD patients before dialysis and controls. By contrast, it was significantly lower in HD patients than controls when WB cells were stimulated by the lower concentration of LPS (1 μg/ml), that induced the plateau production of these three cytokines in healthy controls. The higher concentration of LPS (3 μg/ml) did not modify the production of IL-1β, IL-6 and TNF-α by control cells but increased their production by HD cells to the same level than in controls. Our results contradict those of other reports showing an increased production of IL-1β, IL-6 and TNF-α by PBMC of uraemic patients [11–14]. A number of authors have studied cytokine production during a dialysis session. Monocytes may become activated during dialysis by endotoxins originating from contaminated dialysis fluid [22–24], complement components activated by contact with dialysis membranes [25] or direct contact of monocytes with the membrane [26]. When monocytes are collected and tested just prior to a dialysis session to assess their baseline activity, the production of IL-1 has been found to be increased [13,14], decreased [18,19], or unchanged [15,20] as compared with controls. It is noteworthy that the experimental culture conditions varied in terms of medium (RPMI, DMEM), the presence or absence of fetal calf serum (FCS), culture duration (0–48 h), dosage techniques of these cytokines (RIA, ELISA, bio-assays), and the type or doses of activators used (without, LPS from 10 ng to 100 μg/ml, Con A, PHA), which, in several studies including ours were supraphysiologic as already mentioned by Tielemans *et al.* [27]. Some authors measured IL-1α which is mostly intracellular while others measured IL-1β which is secreted by living cells. In addition, it has been shown that cytokine production by PBMC varies depending on the type of dialysis membrane used [12,28,29]. Lastly, Pereira *et al.* [15] showed reduced production of IL-1β from PBMC of undialysed uraemic patients suggesting that 'haemodialysis could mask this defect by priming these cells to produce more IL-1' [30]. Our results obtained using WB harvested 3 days after a haemodialysis session agree with this hypothesis.

In complex pathological situations such as CRF and haemodialysis, the use of WB instead of PBMC simulates more reliably the '*in vivo*' situation for several reasons. The PBMC preparation procedure (generally using Ficoll) eliminates PMN cells and platelets that may play a role in intercellular communication regulating cytokine release [31]. This procedure also decreases the monocyte/lymphocyte ratios when compared with WB [21] and could activate monocytes through their binding and phagocytosis of silica particles contained in Ficoll. Furthermore, the use of FCS in the majority of PBMC cultures may alone modify the production of cytokines [32]. The PBMC procedure also eliminates the effects of potentially important regulatory molecules present in the plasma of HD patients at abnormal levels. For example, even in healthy controls, IL-1 production was higher in PBMC than in WB cultures seeded with identical concentrations of mononuclear cells, suggesting a downregulation by WB components [21].

The lower production of IL-1β, IL-6 and TNF-α, observed with 1 μg/ml of LPS in HD patients when compared with controls, suggest that these cells were hyporesponsive to exogenous stimuli, either due to intrinsic alterations of one or several signalling pathways, or to complex cellular or humoral inhibitory interactions. Our findings also suggest that the direct and indirect role played by PMNs, platelets and plasma in the production of these monocytic cytokines has probably been underestimated.

The soluble IL-6 receptor (50 kDa) is present in the serum and urine of healthy controls [33] and its levels are increased in patients with haematological disorders such as lymphoid malignancies and multiple myeloma [34,35]. The high plasma levels of sIL-6R in undialysed and HD CRF patients are not due to its impaired renal excretion, as shown by the absence of correlation between plasma sIL-6R and creatinine clearance in CRF patients. In addition, our results show that it is not produced by WB cells even when stimulated with PHA or LPS. Then, the cellular sources and mechanisms inducing elevated levels of plasma sIL-6R in CRF remain to be elucidated. It is also interesting to notice that the sIL-6R/IL6 ratio is increased in the WB culture supernatants from HD patients in comparison with controls. The biological significance of this finding should be explored in the light of recent results. When sIL-6R and IL-6 are linked, the complex binds and stimulates the signal transducer sub-unit gp130 on responsive cells [36]. The gp130 sub-unit also exists as a soluble truncated form in human blood [37]. It was recently shown that when IL-6 is linked to soluble gp130, the presence of sIL-6R increases the antagonist activity of gp130 in this soluble complex [38]. In CRF, the role of increased sIL-6R concentrations should be investigated by measuring the plasma concentrations, cell production and presence at the cell surface of the two other partners IL-6 and gp130.

IL-1 Ra acts as a competitive inhibitor of IL-1 by binding to IL-1 cell surface receptors without triggering a biological response. While it has been shown *in vitro* that a 100-fold molar excess of IL-1Ra is necessary to block the effects of IL-1β, similar data are unavailable *in vivo* [39]. Increased production by isolated monocytes and increased plasma concentrations of IL-1Ra in HD patients [15,40] have suggested that 'IL-Ra could be the better indicator of monocyte activation' [16]. Spontaneous production of IL-1Ra by WB was higher (however not statistically significant) in our HD patients and probably reflected the well-known elevation in plasma concentrations [40]. In contrast, when stimulated by PHA and LPS the production of IL-1Ra was not different in the two groups. Finally, the Il-1Ra/Il-1β molar ratio was higher in HD patients without stimulation and with stimulation using 1 μg/ml of LPS. Thus, the biological significance of this finding and its role in the disturbances of the cytokine network in our HD patients remains uncertain.

In vitro, monocytes of uraemic patients demonstrate signs of activation that contrast with their depressed chemotactic, phagocytic and bactericidal capacities [41–43] and their ability to inhibit T-cell proliferation [44]. Our results with WB cells of HD patients studied at a distance from the dialysis session, contradict those obtained by others with PBMC or isolated monocytes. This paradox could be explained by the differences of cellular and humoral environment between these culture techniques. By using the WB method, we also evidenced for the first time, disturbances of the IL-1Ra/IL-1β and sIL-6R/IL-6 ratios, that could play a role in the immune unbalance of CRF patients. As a conclusion, we suggest that the WB culture method better mimicks the abnormal '*in vivo*' humoral and cellular environment of monocytes during uraemia. These hyporesponsive monocytes could be unable to normally fulfill some of their major physiological functions such as fighting infection and activation of B- and T-cell functions.

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