IL-10 synthesis and secretion by peripheral blood mononuclear cells in haemodialysis patients

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Abstract

Purpose of study. IL-10 may explain the paradox between immunodeficiency and oversecretion of cytokines in chronic haemodialysis (HD) patients. We analysed the secretion of IL-10 by PBMC and the expression of IL-10 mRNA in 10 long-term HD patients (108–276 months), 10 short-term HD patients (3–18 months), and 10 healthy controls.

Results. Spontaneous IL-10 secretion was higher in HD patients than in controls (15 pg/ml vs 2 pg/ml, P = 0.004). It was detected in 13 of 20 patients and in 1 of 10 controls (P = 0.01). IL-10 mRNA expression was also higher in HD patients than in controls. Spontaneous secretions of IL-10 and IL-6 were positively correlated in patients. IL-10 secretion in response to LPS was higher than the upper limit of control range in 4 of 10 long-term HD patients and in no short-term HD patients (P = 0.04). IL-10 mRNA expression was also higher in long-term than in short-term HD patients.

Conclusions. This study demonstrates that IL-10 is spontaneously synthesized and secreted in HD patients, supporting an immunomodulating role in this setting. The greater IL-10-producing capacity in long-term HD patients indicates a chronic effect of haemodialysis on PBMC responsiveness.

Key words: haemodialysis; immunodeficiency; immunomodulation; interleukin-10; interleukin-6; peripheral blood mononuclear cells

Introduction

Chronic haemodialysis (HD) patients present an immunodeficiency that is related to abnormal phagocytosis and to a major T cell defect (reviewed in [1]). This immunodeficiency is paradoxically associated with functional signs of T cell and monocyte activation, including an oversecretion of inflammatory cytokines such as tumour necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6, and increased spontaneous production of these cytokines by isolated monocytes [2]. This paradox could be partly explained by the recent identification of IL-10, a cytokine with macrophage and lymphocyte suppressive properties, and previously designated as cytokine synthesis inhibitor factor. IL-10 can inhibit the secretion of IL-1, TNF-α and IL-6 [3] and can constrain the antigen-presenting function of monocytes, leading to a decrease in T cell activation [4]. IL-10 may exert a direct inhibitory effect on T cell populations through a specific impairment of IL-2 secretion by the responding T cells [4]. In vivo data also support an immunosuppressive role for IL-10: in leishmaniasis, elevated levels of IL-10 mRNA transcripts in bone marrow aspirates were found in the visceral form, which is the most severe [5]; in leprosy, IL-10 mRNA from skin biopsy specimens predominated in the lepromatous multibacillary form [6]; intradermal injection of recombinant IL-10 facilitates induction of in vivo hapten-specific tolerance [7]; administration of anti-IL-10 antibodies accelerates rejection of allogenic skin grafts [8].

For HD patients, Girndt et al. [9] reported that some patients showed an increase in IL-10 secretion by peripheral blood mononuclear cells (PBMC) stimulated with bacterial lipopolysaccharide (LPS) but not by unstimulated PBMC. In that study, IL-10 secretion was associated with response to hepatitis B vaccination. Lonnemann et al. [10] reported similar IL-10 secretion in whole blood and similar IL-10 levels in plasma of HD patients and controls. However, they demonstrated an above normal increase in IL-10 plasma levels in 30% of HD patients.

In the present work, we analysed the production of IL-10 by PBMC of HD patients. We measured in the same time cytokine mRNA synthesis and cytokine secretion. This study was conducted in 20 patients dialysed in the same center, with the same type of membrane, and with the same type of carefully treated...
osmeded water. Another purpose of the study was to verify whether an increased production of IL-10 could contribute to the regulation of monocyte response capacity. At this prospect, we analysed the balance between IL-10 and IL-6 secretions. We chose IL-6 because it is considered a key member of the cytokine network [11] and because its expression has been directly linked to the pathogenesis of well-identified diseases such as myeloma, Castleman’s disease, septic shock [12,13], and to beta-2 microglobulin overssecretion [14]. Finally, since some studies have stressed on the need to consider the influence of HD treatment duration on cytokine secretion by PBMC [15–17], we compared IL-10 synthesis and secretion in short and long-term HD patients.

**Subjects and methods**

**Subjects**

Twenty patients on regular haemodialysis treatment participated in the study. Informed consent was obtained from each person. To analyse the influence of length of time on haemodialysis treatment on cytokine secretion, we selected 10 long-term HD patients, all treated for more than 100 months (mean 174 months, range 108–276). These patients were compared with 10 short-term HD patients, treated for less than 24 months (mean 10 months, range 3–18). The clinical and biological characteristics of the two patient groups are shown in Table 1. There were no significant differences between them.

The complete HD population (short-term and long-term HD patients) had the following characteristics: 13 were women and 7 were men; mean age was 59 years (range 26–81). Mean length of time on haemodialysis was 92 months (range 3–276). Weekly dialysis time was 14 h at 37°C. IL-10 and IL-6 were measured in supernatants by immunoenzymetric assays. IL-10 was measured by IL-10 EASIA (Medgenix, Fleurus, Belgium), which is based on a sandwich method with a capture anti-IL-10 monoclonal antibody coated on a plastic well and an acetylcholinesterase-labelled anti-IL-10 monoclonal antibody. The minimum detectable concentration of IL-10 was estimated to be 2 pg/ml. IL-6 was measured by an enzyme immunoassay kit (Immunotech, Marseille, France) based on a sandwich method with a capture anti-IL-6 monoclonal antibody coated on a plastic well and a horseradish peroxidase-labelled anti-IL-6 monoclonal antibody. The minimum detectable concentration of IL-6 was 0.5 pg/ml.

**PBMC**

Blood was drawn into heparinized tubes. PBMC and adherent cells were prepared as previously described [19]. PBMC were separated by Ficoll gradient centrifugation (MSL, Eurobio, Les Ulis, France). One aliquot of PBMC was saved for RNA analysis (see below) and the other was used for cytokine secretion analysis. PBMC and adherent cells were incubated in RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, during 18 h at 37°C, with or without LPS (E. coli extract, 055:B5, Sigma, St Louis, MO) at a concentration of 1 μg/ml. Supernatants were collected and stored at −70°C before assay.

**Immunoaassays for IL-10 and IL-6**

IL-10 and IL-6 were measured in supernatants by immunoenzymetric assays. IL-10 was measured by IL-10 EASIA (Medgenix, Fleurus, Belgium), which is based on a sandwich method with a capture anti-IL-10 monoclonal antibody coated on a plastic well and a horseradish peroxidase-labelled anti-IL-10 monoclonal antibody. The minimum detectable concentration of IL-10 was estimated to be 2 pg/ml. IL-6 was measured by an enzyme immunoassay kit (Immunotech, Marseille, France) based on a sandwich method with a capture anti-IL-6 monoclonal antibody coated on a plastic well and an acetylcholinesterase-labelled anti-IL-6 monoclonal antibody. The minimum detectable concentration of IL-6 was 0.5 pg/ml.

**Semiquantitative PCR**

PBMC (5 × 10⁶ cells/assay) in 0.5 ml supplemented RPMI 1640 were stimulated or not with LPS at the concentration of 1 μg/ml during 4 h at 37°C. RNA was extracted by acid guanidinium thiocyanate/phenol chloroform method as previously described [20]. RNA (0.5 μg) was then incubated in 40 μl buffer containing 0.25 mM each dATP, dCTP, dGTP, dTTP (Boehringer Mannheim, Germany), 0.5 μg oligo(dT)₁₂₋₁₈ (Pharmacia, Uppsala, Sweden), 400 U reverse transcriptase (Superscript, Life Technologies, Ergany, France), 80 U ribonuclease inhibitor (RNasin, Promega, Charbonnières, France) and 10 mM dithiothreitol, and maintained for 60 min at 37°C. The reaction was stopped by heat

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**Table 1. Characteristics of the two patient groups**

<table>
<thead>
<tr>
<th></th>
<th>Short-term HD patients</th>
<th>Long-term HD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean time on haemodialysis (months)</td>
<td>10±4</td>
<td>174±60</td>
</tr>
<tr>
<td>Sex ratio (men/women)</td>
<td>5/5</td>
<td>2/8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.5</td>
<td>62.6</td>
</tr>
<tr>
<td>Kt/V urea</td>
<td>1.48</td>
<td>1.50</td>
</tr>
<tr>
<td>Haemodialysis duration (h/week)</td>
<td>13.6</td>
<td>14.7</td>
</tr>
<tr>
<td>Serum PTH (pg/ml)</td>
<td>85</td>
<td>154</td>
</tr>
<tr>
<td>Serum ferritin (μg/l)</td>
<td>426</td>
<td>546</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>Serum albumin (g/l)</td>
<td>39.1</td>
<td>37.7</td>
</tr>
<tr>
<td>Serum fibrinogen (g/l)</td>
<td>3.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Patients treated by erythropoietin (n)</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Patients treated by high-flux haemodialyser (n)</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Kt/V represents the dialysis dose index: K is clearance, t is duration of dialysis session, V is the patient’s size.
inactivation for 5 min at 95°C. Then, cDNA products were amplified by an automated thermocycler (Perkin Elmer, Saint Quentin, France). The reaction was performed in a 100 μl reaction volume consisting of 5 μl cDNA specimens, 100 mM Tris–HCl pH 8.3, 2 mM MgCl₂, 50 mM KCl, 2.5 U Taq polymerase (Perkin Elmer), 0.2 mM each dATP, dCTP, dGTP, dTTP in the presence of 100 ng of 5’ and 3’ primers (Eurogentec, Brussels, Belgium). Negative controls were performed by omitting RNA from cDNA synthesis and PCR amplification. The sequences of primers of glyceraldehyde 3-phosphate dehydrogenase (G3PDH), IL-10 and IL-6 are described elsewhere [20,21]. The temperature of amplification cycles was 94°C for 1 min, 60°C (G3PDH) or 65°C (IL-6, IL-10) for 1 min, and 72°C for 2 min. PCR products were electrophoresed in 2% agarose gels and their size was determined by DNA molecular-weight markers (Boehringer Mannheim). The bands were visualized with ethidium bromide staining and photographs were taken with polaroid films.

**Quantitative PCR**

IL-10 and IL-6 mRNA expression was also quantitated with competitive PCR detection kit (CytoXpress, BioSource, Camarillo, CA). Briefly, a known number of copies of an exogenous DNA (Internal Calibration Standard, ICS) was mixed with the sample cDNA prior to amplification. ICS contains PCR primer binding sites identical to the IL-10 (or IL-6) cDNA and a capture binding site that allows the ICS amplicon to be distinguished from cytokine amplicon. One of IL-10 (or IL-6) primers is biotinylated and incorporated into IL-10 and ICS amplicons (or IL-6 and corresponding ICS). The amplicons were then hybridized to either IL-10 or ICS (or IL-6 and corresponding ICS) sequence-specific capture oligonucleotides, which were pre-bound to microtiter wells. The capture sequences were detected by addition of a streptavidin-peroxidase conjugate followed by chromogen substrate. The generated signal was proportional to the amount of amplicon. Since the amplification efficiency of ICS was identical to the IL-10 (or IL-6) cDNA, it is used as a standard for cytokine cDNA quantitation.

**Statistical analysis**

Data were not normally distributed, so results were expressed as median values and presented as individual points. Comparisons were performed with the non parametric Mann–Whitney two-tailed U-test, with the Chi 2 test or with the Fisher’s exact test. Correlations were analysed by non parametric methods (Spearman rank-order correlation coefficient). Calculations were performed using the SPSS v 6.1 software package (SPSS inc, Chicago, IL, 1996).

**Results**

**IL-10 secretion by PBMC**

The secretion of IL-10 by unstimulated PBMC was detected in 13 of 20 HD patients and in only 1 of 10 controls (P=0.01). Median values of spontaneous IL-10 secretion were 15 pg/ml in HD patients and 2 pg/ml in healthy controls (P=0.004) (Figure 1a).

The stimulation of PBMC with 1 μg/ml LPS led to a marked increase in IL-10 secretion in all HD patients (median 515 pg/ml) and controls (425 pg/ml) (Figure 1b). LPS-stimulated secretion of IL-10 was not significantly different in HD patients and controls (P=0.31). However, in four HD patients we found that stimulated IL-10 secretion was higher than the upper limit of control range, defined as mean ± 2 SD (1230 pg/ml).

Note that spontaneous IL-10 secretion by adherent cells accounted for 20% of total PBMC secretion; LPS-stimulated IL-10 secretion by adherent cells represented 25% of total PBMC secretion (data not shown).

**IL-6 secretion by PBMC**

To assess the equilibrium among cytokines, we measured IL-6 secretion in supernatants from PBMC. The median value of spontaneous IL-6 secretion by PBMC was significantly greater in HD patients than in healthy controls (1700 pg/ml vs 250 pg/ml, P=0.001) (Figure 1c). We found a significant positive correlation between spontaneous IL-6 and IL-10 secretions in HD patients (r=0.62; P=0.01), but not in controls.

On the other hand, the median value of LPS-stimulated secretion of IL-6 was similar in HD patients and in healthy controls (39 200 pg/ml vs 31 500 pg/ml, P=0.94) (Figure 1d). There was no correlation between LPS-stimulated IL-10 and LPS-stimulated IL-6 secretions in HD patients.

**Effect of length of time on haemodialysis treatment on IL-10 and IL-6 secretions**

The number of patients with spontaneous IL-10 secretion was equivalent in both short-term and long-term HD groups (7/10 in short-term HD patients and 6/10 in long-term HD patients). On the other hand, IL-10 secretion in response to LPS was higher than the upper limit of control range in 4 of 10 long-term HD patients, but in none of short-term HD patients (P=0.04) (Table 2). The number of patients with spontaneous IL-6 secretion over the upper limit of control range was similar in short-term and long-term HD groups. The secretion of IL-6 in response to LPS was in the control range for all patients (Table 2). This observation suggests that length of time on haemodialysis influences LPS-stimulated secretion of IL-10.

**Table 2. Repartition of patients with elevated cytokine secretion values in short-term or long-term HD groups**

<table>
<thead>
<tr>
<th>Secretion</th>
<th>Short-term HD patients (n=10)</th>
<th>Long-term HD patients (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated spontaneous IL-10</td>
<td>7</td>
<td>4</td>
<td>0.36</td>
</tr>
<tr>
<td>Elevated stimulated IL-10</td>
<td>0</td>
<td>4</td>
<td>0.04</td>
</tr>
<tr>
<td>Elevated spontaneous IL-6</td>
<td>5</td>
<td>4</td>
<td>0.99</td>
</tr>
<tr>
<td>Elevated stimulated IL-6</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

Elevated cytokine secretion values were defined as values greater than the upper limit of control range, calculated as the mean ± 2 SD.
Fig. 1 a–c. Concentrations of IL-10 and IL-6 in the culture supernatant of PBMC from haemodialysis (HD) patients and healthy controls. (a) Spontaneous secretion of IL-10; (b) LPS-stimulated secretion of IL-10; (c) spontaneous secretion of IL-6; (d) LPS-stimulated secretion of IL-6.
IL-10 synthesis and secretion by PBMC in haemodialysis

Fig. 2. Semiquantitative analysis of IL-10 mRNA expression. IL-10 mRNA expression was analysed in PBMC of subjects which did not spontaneously secrete IL-10. The IL-10 mRNA expression started to increase after 32 amplification cycles in long-term HD (P1), whereas it started to increase after only 34 amplification cycles in short-term HD (P2). IL-10 mRNA were not expressed in control PBMC (C). This figure is representative of three distinct subjects in each group.

Table 3. IL-6 and IL-10 synthesis measured by quantitative PCR (competitive method) in unstimulated PBMC from 3 healthy controls, 3 short-term HD, and 3 long-term HD

<table>
<thead>
<tr>
<th>Subjects</th>
<th>IL-6 cDNA copies/μg RNA</th>
<th>IL-10 cDNA copies/μg RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>6168</td>
<td>131</td>
</tr>
<tr>
<td>Control 2</td>
<td>8446</td>
<td>335</td>
</tr>
<tr>
<td>Control 3</td>
<td>5544</td>
<td>532</td>
</tr>
<tr>
<td>Short-term HD 1</td>
<td>8782</td>
<td>1737</td>
</tr>
<tr>
<td>Short-term HD 2</td>
<td>6528</td>
<td>1467</td>
</tr>
<tr>
<td>Short-term HD 3</td>
<td>8924</td>
<td>3464</td>
</tr>
<tr>
<td>Long-term HD 1</td>
<td>5786</td>
<td>2869</td>
</tr>
<tr>
<td>Long-term HD 2</td>
<td>7964</td>
<td>3978</td>
</tr>
<tr>
<td>Long-term HD 3</td>
<td>9404</td>
<td>5723</td>
</tr>
</tbody>
</table>

Analysis of IL-10 and IL-6 mRNA levels in PBMC by semiquantitative PCR

The expression of cytokine mRNA was investigated in PBMC from control, short-term and long-term groups, at the basal state, and after stimulation by LPS for 4 h. Similar amounts of PCR products were generated with G3PDH primers, used as internal standard.

In HD patients with spontaneous IL-10 secretion, IL-10 mRNA transcripts were found in PBMC (data not shown). In HD patients without spontaneous IL-10 secretion, IL-10 mRNA expression could be detected after increasing the number of PCR cycles, whereas no IL-10 transcripts were detected in controls whatever the number of PCR cycles (Figure 2). Differences were found between short-term and long-term HD patients: IL-10 mRNA expression was observed after 32 cycles in long-term HD patients, whereas it was observed only after 34 cycles in short-term HD patients (Figure 2).

The comparison of IL-10 and IL-6 mRNA expression in PBMC with and without stimulation by LPS is shown in Figure 3. Without LPS stimulation, IL-10 mRNA was only detected in long-term HD patients. With LPS stimulation, IL-10 mRNA was detected in all subjects. No difference in IL-6 mRNA expression was found between short-term and long-term HD patients.

Analysis of IL-10 and IL-6 mRNA levels in PBMC by quantitative PCR

The measurement of IL-10 and IL-6 mRNA by quantitative PCR method confirmed the preceding data (Table 3). The number of IL-10 transcripts was significantly higher in HD patients compared to controls. In HD patients, the highest values were observed in long-term HD patients. In contrast, the number of IL-6 cDNA copies was similar in the three groups.

Discussion

In this study, the measurement of IL-10 synthesis and secretion by PBMC in 20 HD patients and 10 healthy controls gives the following informations: (1) PBMC from HD patients spontaneously synthesized and secreted more IL-10 than PBMC from healthy controls. (2) PBMC from HD patients and controls, stimulated with LPS, secreted similar amounts of IL-10, demonstrating that the ability of PBMC from HD to mount an IL-10 response to a triggering agent was fully conserved. (3) IL-10 secretion by adherent cells stimulated or not by LPS accounted for respectively 25 and 20% of total PBMC production. (4) IL-10 and IL-6
secretions by unstimulated PBMC were positively correlated in HD patients. (5) The comparison of short-term and long-term HD patients showed a significantly higher secretion capacity of IL-10 in response to LPS in long-term HD patients.

This report is to our knowledge the first observation of a spontaneous increase in IL-10 secretion by PBMC in HD patients. It is unlikely that this secretion could be induced by cell isolation procedure because experiments in patients and controls were performed at the same time in the same conditions.

The secretion of IL-10 was detected in 13 of 20 patients. In addition, in patients without spontaneous IL-10 secretion, PCR amplification could detect IL-10 transcripts, and quantitative PCR confirmed that IL-10 mRNA expression was higher in HD patients than in controls. This increased synthesis of IL-10 is another evidence of the well-documented state of activation of monocytes and lymphocytes in HD patients.

The secretion of IL-10 by PBMC from HD patients was previously reported by Girndt et al. [9]. They found no increase in IL-10 production from unstimulated PBMC. This discrepancy with our results may be due to differences in the sensitivity of the measurement methods, to the selection of patients, or to factors influencing IL-10 production. In the same study, Girndt et al. showed that PBMC stimulated with LPS secreted higher levels of IL-10 in patients responders to hepatitis B vaccination than in non responders and in controls [9]. We could not confirm these findings since all the HD patients studied in the present study were responders to hepatitis B vaccination.

It is possible that the increased production of IL-10 represents an autoregulatory response to the increased production of pro-inflammatory cytokines by monocytes. We found a positive correlation between IL-10 and IL-6 secretions by unstimulated PBMC from HD patients. This result was unexpected because IL-10 is known to inhibit the secretion of inflammatory cytokines such as IL-6 [3]. The inhibitory role of IL-10 on IL-6 secretion was demonstrated in vitro by the addition of exogenous human recombinant IL-10 to PBMC [3,22]. In fact, the positive correlation between IL-10 and IL-6 secretions does not rule out a regulatory effect of IL-10 on IL-6 secretion. Indeed, a concomitant secretion of cytokines and cytokine inhibitory substances in response to inflammation has been reported [23], and the simultaneous secretion of IL-10 and inflammatory cytokines was observed also in septic shock [24].

This study gives an insight into the mode of secretion of IL-10. IL-10 can be produced by a variety of cell types, including monocytes, T and B lymphocytes, keratinocytes and mast cells [4]. We showed here a strong secretion of IL-10 by total PBMC in response to LPS. On the other hand, IL-10 secretion by adherent cells stimulated or not by LPS accounted for only 25 and 20% of the total PBMC secretion. Since LPS is known to essentially stimulate the secretion of cytokines by monocytes, our results suggest that IL-10 secretion by monocytes requires a lymphocyte-monocyte interaction. These results could be related with the evidence of the stimulation effect of T cell-monocyte contact on IL-10 secretion by monocytes, recently provided in in vitro experiments [25].

The comparison of short-term and long-term HD patients reported here is one of the few studies on cytokine secretion in HD patients treated for nearly 10 years. There are several arguments for the greatest synthesis and secretion of IL-10 by PBMC from long-term HD patients. First, all the patients with great values of IL-10 secretion in response to LPS were in the long-term HD group. Second, in patients without spontaneous IL-10 secretion, IL-10 transcripts could be detected after 34 PCR cycles in short-term HD and only after 32 cycles in long-term HD. Third, quantitative PCR showed higher values of IL-10 transcripts in long-term HD than in short-term HD. These findings suggest a chronic effect of either HD treatment or uraemic state on PBMC responsiveness. Other studies have also shown the influence of HD treatment duration on immune function and cytokine secretion by PBMC [15–17,26]. PBMC may be stimulated by membrane-activated complement products, by the entry of endotoxins or acetate into circulation, or by uraemia per se [27,28].

In summary, in the present study we demonstrated the spontaneous synthesis and secretion of IL-10 by PBMC in HD patients. We found that the spontaneous secretion of IL-10 was positively correlated with that of IL-6. Finally, we showed that PBMC from long-term HD patients had greater synthesis of IL-10, and greater secretion in response to LPS stimulation, compared to short-term HD patients.

References
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