Induction of interleukin-1 and interleukin-1 receptor antagonist during contaminated in-vitro dialysis with whole blood

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Abstract

Background. Previous studies on the permeability of cellulosic and synthetic dialysers for bacterial-derived cytokine-inducing substances gave conflicting results. We tried to study this issue as close to the in-vivo situation as possible.

Methods. An in-vitro dialysis circuit with whole human blood present in the blood compartment of cuprophane (Cup), polysulphone (PS), and polyamide (PA) dialysers was employed; sterile filtrates derived from Pseudomonas aeruginosa cultures were added to the dialysate. We studied the induction of interleukin-1β (IL-1β) by plasma samples taken from the blood side of all dialysers; IL-1β and interleukin-1 receptor antagonist (IL-1Ra) in mononuclear cells separated from whole blood after circulation by radioimmunoassay and polymerase chain reaction.

Results. Plasma samples from the blood side of all dialysers induced IL-1β from non-circulated mononuclear cells after addition of pseudomonas filtrates to the dialysate; the maximal amount of IL-1β induced by samples from the blood compartment was 4.8±1.2 ng/ml for Cup, 1.9±0.5 ng/ml for PS, and 2.0±0.6 ng/ml for PA. Mononuclear cells separated after contaminated dialysis with all types of dialysers expressed increased mRNA levels for IL-1β and IL-1Ra. Production of IL-1Ra by cells separated after contaminated dialysis was determined after Cup and PS dialysis; there was increased production of IL-1Ra by these cells (Cup, 10.3±4.2; PS, 7.3±2.5 ng/ml) compared to cells separated after sterile dialysis (Cup, 5.6±2.1, P<0.05; PS, 4.5±1.1 ng/ml, n.s.) or from non-circulated blood (Cup experiments, 4.7±1.5, P<0.05; PS experiments, 4.1±1.2 ng/ml, n.s.).

Conclusions. These data suggest penetration of cytokine-inducing substances through both cellulosic and synthetic dialysers. Differences between dialysers may exist regarding extent and time course of penetration.

The detection of cytokine mRNA as well as the measurement of IL-1Ra synthesis is a more sensitive marker for the transfer of cytokine-inducing substances through dialyser membranes than the measurement of IL-1β protein synthesis.

Key words: cytokines; haemodialysis; interleukin-1; interleukin-1 receptor antagonist; Pseudomonas aeruginosa

Introduction

There have been a number of studies investigating the penetration of cytokine-inducing substances derived from bacteria through dialyser membranes. Studies using the limulus-amoebocyte-lysate test for detection of LPS in the blood compartment of Cup or PS dialysers failed to demonstrate passage of purified LPS added to the dialysate into the blood compartment [1, 2]. Only when samples from the blood side were tested for cytokine induction by mononuclear cells could passage of bacterial substances through the dialyser membrane be demonstrated. In the first of these studies, purified LPS from E. coli was added to the dialysate compartment of an in-vitro dialysis circuit using Cup dialysers and whole blood circulating in the blood compartment [3]. Mononuclear cells (PBMC) separated after circulation produced increased IL-1 bioactivity when LPS was added to the dialysate compartment compared to sterile dialysis. In subsequent studies, substances that induced IL-1 or TNF production from PBMC could be detected in the blood compartment of Cup and certain high-flux dialysers containing 10% human plasma when filtrates from E. coli or Pseudomonas maltophilia were added to the dialysate [4, 5]. Using radiolabelled purified LPS fragments from E. coli, Neisseria meningitides, and Pseudomonas testosteroni, two studies reported transfer of LPS fragments through Cup, polyacrylonitrile, and PS [6, 7] with saline or albumin present in the blood compartment. However, IL-1-inducing activity did not
parallel the appearance of radiolabelled LPS in the blood compartment but was increased only in the first 10 min of recirculation; in contrast, levels of radiolabelled LPS fragments remained elevated throughout the experiment [6].

However, the cited studies employed experimental designs not fully comparable to the in-vivo situation with whole blood in the blood compartment and unfractionated bacterial products in the dialysate compartment. In the studies using unfractionated bacterial products in the dialysate compartment, either saline or diluted plasma components was recirculated in the blood compartment [4,5,8]. The studies employing whole blood in the blood compartment used purified E. coli LPS in the dialysate compartment [3,7]. Purified LPS consists of higher-molecular-weight species than natural LPS and might not easily penetrate dialyser membranes; in addition to LPS, other bacterial products may induce cytokines such as exotoxins [9]. The presence of plasma and cellular elements in the blood compartment may also influence the penetration of bacterial products: we recently demonstrated that the generation of a plasma protein layer on PS membranes reduces the penetration of cytokine-inducing substances derived from pseudomonas [10]. Neutrophils activated during haemodialysis release products such as bacterial/permeability increasing protein that may bind bacterial products [11,12] and prevent them from stimulating PBMC.

We therefore conducted experiments with whole human blood in the blood compartment of an in-vitro dialysis circuit using a low-flux cellulose and two high-flux synthetic dialysers. Crude bacterial cultures derived from pseudomonas were employed to challenge the dialysate. Moreover, since it has been suggested that the induction of IL-1Ra is a better marker for the induction of the inflammatory response than IL-1 itself [13], we also investigated the possible stimulation of this cytokine during contaminated in-vitro dialysis.

Subjects and methods

Challenge material

Pseudomonas aeruginosa PA 103 [14] was grown in bicarbonate dialysate until LOG-phase of growth. The culture was filtered consecutively through filters (Millipore) of 5, 1.2, 0.8, 0.45 μm pore size, and finally through a 300-kDa filter. Since the cytokine-inducing potency of these filtrates was found to be unstable when culture filtrates were stored at 4°C or −20°C, we lyophilized the bacterial cultures to maintain a stable, reproducible challenge material over prolonged periods of time. The filtrate was desalted using EconoPac 10DG columns (Biorad, Richmond, CA), lyophilized and stored at room temperature. Before use, it was reconstituted with distilled water, adjusted to pH 7.4 and again sterile filtered. The challenge material contained approximately 100 ng ml LPS as determined in a chromogenic limulus-amoebocyte-lysate test (QCL 1000, BioWhitaker, Walkersville, MD) with a sensitivity of 1 pg/ml. The challenge material induced IL-1β and IL-1Ra production by PBMC from three different donors significantly at a 1/400 dilution, as shown in Table 1.

<table>
<thead>
<tr>
<th>Challenge material</th>
<th>IL-1Ra (ng/ml)</th>
<th>IL-1β (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (RPMI)</td>
<td>3.3 ± 0.1</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>Undiluted</td>
<td>4.5 ± 0.9*</td>
<td>17.7 ± 1.6*</td>
</tr>
<tr>
<td>1/10</td>
<td>24.6 ± 1.6*</td>
<td>13.6 ± 1.7*</td>
</tr>
<tr>
<td>1/100</td>
<td>17.2 ± 0.2*</td>
<td>5.0 ± 0.7*</td>
</tr>
<tr>
<td>1/200</td>
<td>14.7 ± 0.7*</td>
<td>3.3 ± 0.6*</td>
</tr>
<tr>
<td>1/400</td>
<td>11.8 ± 3.4*</td>
<td>1.8 ± 0.4*</td>
</tr>
<tr>
<td>1/800</td>
<td>6.6 ± 0.1*</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>1/1600</td>
<td>5.0 ± 0.4</td>
<td>0.3 ± 0.12</td>
</tr>
<tr>
<td>1/3200</td>
<td>4.5 ± 0.2</td>
<td>0.8 ± 0.06</td>
</tr>
<tr>
<td>1/6400</td>
<td>4.8 ± 1.1</td>
<td>0.13 ± 0.03</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n = 3; * = P < 0.05 vs RPMI.

in Table 1. The same lyophilized stock of challenge material was used for all experiments and was used undiluted to challenge the dialysate.

The whole blood in-vitro dialysis circuit

Cup (GFS12, 1.2 m², Gambro, Hechingen, FRG), PS (F60, 1.25 m², Fresenius, Bad Homburg, FRG) or PA (Polyflux 130, 1.3 m², Gambro, Hechingen, FRG) dialysers were aseptically connected to standard blood tubing. Both compartments were rinsed with 2 litres of sterile saline each. Blood was drawn from healthy male volunteers into heparinized syringes (10 U/ml). 20 ml of blood was diluted 1:3 with saline and was left standing at room temperature for the duration of the experiment; this sample served as the non-circulated control. Sixty millilitres of blood was put into the blood compartment, replacing an equal volume of saline. The total volume of the blood compartment was approximately 220 ml, that of the dialysate compartment approximately 250 ml. Two dialysers of the same type (either Cup/PS or PS/PA) were run in parallel at the same time, one with sterile dialysate, the other one with contaminated dialysate. The blood was recirculated for 5 min before challenging the dialysate compartment. Two hundred and fifty millilitres of undiluted pseudomonas filtrate was placed in the dialysate compartment, replacing an equal volume of saline. After challenging the dialysate compartment, recirculation was performed for 2 h at room temperature using flow rates of 150 ml/min in both compartments. Both compartments were tightly closed to reduce net ultrafiltration.

Samples were taken from the circulating system from the blood and the dialysate compartments before challenge and at 5, 30 and 120 min after challenging the dialysate compartment. All samples were placed on ice. Samples from the blood compartment were centrifuged immediately at 2000 g, plasma was obtained and kept on ice.

Preparation of mononuclear cells

PBMC were separated after dialysis from circulated or non-circulated (that was left standing throughout the experiment) whole blood by centrifugation through Ficoll and Hypaque made from powder (Ficoll Type 400; sodium- and meglumine diatrizoate, Sigma, Munich, FRG). The water for preparation of Ficoll and Hypaque and all other fluids used were subjected to ultrafiltration using PA filters (U 2000, Gambro, Hechingen, FRG) to remove cytokine-inducing substances [15]. For incubation, PBMC were washed twice with normal
saline, resuspended at 5 x 10^9/ml in serum-free RPMI 1640 culture medium (Gibco, Paisley, UK), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin; 400 μl of cell suspension were incubated in 24-well plates (Nunc, Denmark) for 18–20 h with 400 μl of the samples from the blood and dialysate compartments at 37°C in a humidified atmosphere containing 5% CO₂. After incubation the plates were subjected to three freeze/thaw cycles to lyse the cells.

**Assay procedures**

Total production (cell-associated plus secreted) of IL-1β and IL-1Ra was determined in PBMC lysates by RIA as described previously [16,17]. All samples were assayed in duplicates at two different dilutions (undiluted and 1:10) in order to calculate cytokine concentrations in the linear part of the standard curve. The detection limit of the RIA for IL-1β was 50–100 pg/ml and 80–100 pg/ml for IL-1Ra. Antibodies against IL-1Ra as well as recombinant IL-1Ra were a kind gift of Dr. A. Dinarello, Boston, MA. The antibody against IL-1β was a kind gift of Dr D. Boraschi, Sclavo, Italy. Recombinant IL-1β was a kind gift of Dr A. Shaw, Glaxo, Switzerland.

Bacterial/cytoskeleton-increasing protein (BPI) was measured by sandwich ELISA using antibodies kindly provided by Dr M. N. Marra, Incyte Pharmaceuticals Inc., Palo Alto, USA. All other reagents were purchased from Sigma.

Ninety-six-well polystyrene-plates were coated with 1 μg/well primary antibody in 25 mM sodiumbore buffer, pH 9.5, for 14–20 h. All incubations were performed at 37°C. Plates were blocked with 200 μl 5% BSA. After incubation of 100 μl sample or standard for 2 h, plates were washed with 50 mM Tris-HCl; 150 mM NaCl; 1 mg/ml BSA; 0.05% Tween; 1 μg/ml Polymyxin B, as after each of the following steps.

After incubation of 100 μl of secondary antibody, 100 μl/well streptavidin-conjugated alkaline phosphatase, diluted 1:1000 (Amersham) was added. The chromogenic reaction of the substrate (p-Nitrophenyl Phosphat, Sigma 104-105) was started by staining with anti-digoxigenin antibody conjugated to alkaline phosphatase (DIG Luminescent Detection Kit, Boehringer Mannheim, FRG). Luminescence of the substrate was measured by sandwich with anti-digoxigenin antibody conjugated to alkaline phosphatase (DIG Luminescent Detection Kit, Boehringer Mannheim, FRG).

**Statistics**

Cytokine concentrations were compared using ANOVA for multiple comparisons followed by the paired test after logarithmic transformation of the data. Results are expressed as mean ± standard error of the mean (SEM).

**Results**

**Appearance of IL-1β-inducing substances in the blood compartment during whole-blood circulation**

When blood was drawn from the blood compartment, centrifuged, and the plasma was added to PBMC separated from non-circulated blood, no significant induction of IL-1β was observed during sterile dialysis with both Cup and PS (Figure 1). During contaminated dialysis, there was an increase in IL-1β-inducing substances on the blood side with time that peaked at the end of the experiment with Cup; in contrast, with PS the peak was found 5 min after challenging the dialysate compartment. The maximal induction of IL-1β was higher in the case of Cup compared to PS, the difference between both membranes was statistically significant (P<0.01) at t = 120 min.

Samples from the dialysate compartment during sterile dialysis did not induce IL-1β above background levels: Cup, at 5 min 58 ± 12 pg/ml; at 120 min 49 ± 6 pg/ml; PS, at 5 min 147 ± 92 pg/ml; at 120 min 127 ± 60 pg/ml. Dialysate samples during contaminated dialysis induced large amounts of IL-1β even when added to PBMC in a 1/10 dilution: Cup, at 5 min 4915 ± 750 pg/ml; at 120 min 4640 ± 661 pg/ml; PS, at 5 min 4458 ± 295 pg/ml; at 120 min 3794 ± 108 pg/ml.

When whole blood from the same donors was recirculated in parallel through either PS or PA dialysers, a similar increase in IL-1β-inducing substances could be detected in the blood compartment of both dialysers after contamination of the dialysate (Figure 2).

**Cytokine content in PBMC**

PBMC were separated after dialysis from blood that had been left standing at room temperature during the experiment, or were separated from blood that had
Fig. 1. Appearance of IL-1β-inducing substances in the blood compartment during whole-blood circulation using Cup or PS dialysers. Samples were drawn from the blood compartment containing human whole blood at various time points after challenging the dialysate. Blood samples were centrifuged and 400 μl of plasma obtained was added to an equal volume of mononuclear cell (PBMC) suspension separated from non-circulated blood of the same donor. After 20 h incubation, total IL-1β production (cell-associated plus secreted) was determined by RIA. The dialysate consisted either of sterile saline or of pseudomonas filtrates. Experiments were performed using blood from the same donor for both dialysers. Results are means±SEM, n = 6; * P<0.02 vs predialysis; § P<0.02 vs sterile dialysis. # indicates that the difference between both membranes was statistically significant (P<0.01) at t = 120 min.

Fig. 2. Appearance of IL-1β-inducing substances in the blood compartment during whole-blood circulation using PS or PA dialysers. Experiments were performed as described for Figure 1 except that different dialysers were used. Experiments were performed using blood from the same donor for both dialysers and for both conditions (sterile and contaminated dialysate). Results are means±SEM, n = 4; * P<0.05 vs predialysis; § P<0.05 vs sterile dialysis.

Fig. 3. IL-1β production by PBMC. PBMC were separated from donor blood after 2 h of in-vitro dialysis with sterile or contaminated dialysate. Control cells (non-circulated) were separated after incubating whole blood for 2 h at room temperature in polypropylene tubes. Cells were further incubated for 20 h either without stimulus (RPMI) or were stimulated with 10 ng/ml LPS. Total IL-1β production was determined by RIA. Results are means±SEM, n = 6.
spontaneously produced significantly more IL-1Ra than non-circulated cells ($P=0.02$). PBMC separated after contaminated dialysis with PS also produced more IL-1Ra than non-circulated cells, but this difference was not significant (Figure 4). IL-1Ra production after contaminated dialysis was higher compared to sterile dialysis, this difference was significant only for the experiments with Cup. Spontaneous IL-1Ra production after contaminated dialysis was not significantly higher with Cup compared to PS ($10378\pm4207$ vs $7306\pm2561$ pg/ml, $P=0.17$). When non-circulated PBMC and PBMC separated after sterile or contaminated dialysis were stimulated with LPS, no differences in IL-1Ra production were observed (data not shown).

Expression of IL-1β and IL-1Ra mRNA after dialysis

Non-circulated PBMC and PBMC separated after sterile or contaminated dialysis were lysed immediately after circulation. RNA was extracted and analysed for GAPDH, IL-1β and IL-1Ra mRNA by PCR. As shown in Figure 5, mRNA for IL-1β was induced even after sterile dialysis with Cup, but not with PS. Contamination of the dialysate resulted in a similar expression of IL-1β mRNA with both membranes. mRNA for IL-1β was readily detectable by semiquantitative PCR (Figure 6). Again, contamination of the dialysate resulted in a similar expression of IL-1β and
Cuprophan

Sterile HD

Contaminated HD

Polysulfone

Sterile HD

Contaminated HD

Fig. 7. Plasma levels of BPI during whole-blood circulation. Samples were drawn at the indicated time points from the blood compartment during sterile and contaminated dialysis with Cup and PS. Means±SEM, n = 5, * P<0.05 vs pre HD. The difference between sterile and contaminated dialysis with each membrane was not significant.

IL-1Ra with both Cup and PS. Expression of GAPDH was unchanged.

When blood from the same donor was recirculated in parallel through PA and PS, mRNA for IL-1β, but not IL-1Ra was increased after sterile dialysis compared to non-circulated cells with both dialysers (Figure 7). After contaminated dialysis with both dialysers, mRNA for IL-1β and IL-1Ra was increased compared to non-circulated cells and to cells separated after sterile dialysis. There were no differences in mRNA expression for these cytokines between the two dialysers (Figure 7). Expression of mRNA for GAPDH was unchanged.

Plasma levels of BPI

Plasma levels of BPI in the blood compartment increased to a similar extent during dialysis with Cup and PS and were maximal at the end of the experiment (Figure 8). Although levels of BPI were higher during contaminated dialysis compared to sterile dialysis, the difference was not significant.

Discussion

The present study demonstrates the passage of cytokine-inducing substances derived from Pseudomonas aeruginosa through Cup, PS, and PA dialysers when whole human blood is recirculated in the blood compartment. Especially because of the possibility of back-filtration with high-flux membranes [18,19], there has been concern that high-flux membranes are more permeable than low-flux cellulosic membranes for low-molecular-weight bacterial products due to the larger effective pore size of high-flux membranes. Investigating the induction of cytokine transcription and production, we did not observe that the high-flux PS is more permeable for cytokine-inducing substances than the low-flux Cup membrane in a model of diffusive transport; indeed, regarding the appearance of IL-1β-inducing substances on the blood side, it appeared that the Cup membrane was more permeable than the PS membrane. However, it is also possible that Cup is not more permeable for bacterial products, but that complement activated with Cup acts synergistically with bacterial products from the dialysate on cytokine induction by PBMC, as we reported previously [20]. In any case, the net result is a larger cytokine induction with Cup.

PBMC separated after contaminated dialysis did not produce detectable IL-1β spontaneously above levels in non-circulated cells (Figure 3), although increased IL-1β mRNA was detected in these cells after contaminated circulation. It is possible that given a weak stimulus the detection of mRNA is more sensitive than the detection of IL-1β protein by RIA. As shown in Figure 3, PBMC after sterile or contaminated dialysis are still capable of producing IL-1β when stimulated with LPS. Therefore it seems unlikely that the PBMC separated from blood after 2 h recirculation are damaged by the mechanical shear stress during pumping. LPS-induced IL-1 production by PBMC in Cup experiments was less after contaminated in-vitro dialysis than after sterile dialysis or by PBMC without contact to the dialysis circuit. The same tendency was observed with PS. These data are in agreement with a recent publication by Granovitz et al. [21], who showed that PBMC separated from the blood of healthy volunteers after systemic LPS injections are suppressed in their in-vitro LPS-induced cytokine production. These data suggest that pyrogens penetrating the dialyser membrane induce cytokines in PBMC but may also render PBMC less responsive (or tolerant) to a second LPS stimulus.

In contrast to IL-1β, we observed transcription as well as translation of IL-1Ra after contaminated dialysis contrary to Cup and PS dialysers. This finding can be explained by differences in the mRNA structure between IL-1β and IL-1Ra. Several cytokines including IL-1β, GMCSF and TNF possess the pentanucleotide sequence AUUUA in the 3'-untranslated region of their mRNA that regulates mRNA stability and translational efficiency of the mRNA [22,23]. Transcription of mRNA of these cytokines without translation into protein has been reported [23,24]. The mRNA for IL-1Ra lacks this sequence [25]; transcription of IL-1Ra is therefore expected to be regularly followed by translation. We suggest that the detection of cytokine mRNA as well as the measurement of IL-1Ra synthesis is a more sensitive marker for the transfer of
cytokine-inducing substances through dialyser membranes than the measurement of IL-1β protein synthesis. IL-1Ra has been previously suggested to be a better parameter for the induction of the inflammatory response [26], possibly also because of the fact that it is produced in larger quantities than IL-1 or TNF itself that are easier to measure. For instance, after LPS-injections into normal volunteers, plasma levels of IL-1Ra are about a hundredfold higher than those of IL-1β [27].

In previous studies [5], there appeared to be no significant penetration of TNF-inducing substances through PS and PA. There are, however, several differences between the previous and the present study. In the cited experiments, 10% human plasma had been recirculated in the blood compartment 60 min before challenging the dialysate; in the present study the bacterial filtrate was added to the dialysate 5 min after neutrophils on blood circulation. Therefore, the proteolysis layer formation before contaminating the dialysate was qualitatively and quantitatively different. We recently reported that the protein layer generated on PS membranes reduces the penetration of cytokine-inducing substances from the dialysate [10]. Second, in the previous study, TNF- but not IL-1β-induction by samples from the blood side was determined. Finally, the previous study used a different strain of pseudomonas (Ps. maltophilia) than the present study, and there are bacteria-specific differences regarding the penetration of bacterial products through high-flux membranes [8].

Plasma levels of BPI increased continuously during dialysis and were similar for both Cup and PS. We recently reported that plasma levels of BPI in vivo were higher after dialysis with Cup compared to dialysis with PS in the same patients [12]. This apparent difference may be explained by persistent stimulation of neutrophils during the 2 h of recirculation in the present study, resulting in maximal release of BPI even with the less-activating membrane. Plasma levels of BPI were higher after contaminated dialysis compared to sterile dialysis. Although this difference was not significant (p = 0.07), it is tempting to speculate that bacterial substances from the dialysate caused neutrophil activation and release of BPI on the blood side; bacterial products such as LPS are well known stimuli for BPI release [28]. Despite of the continuously increasing levels of BPI during dialysis, there was also an increase in IL-1β-inducing substances on the blood side (Figure 1), suggesting that the BPI released from neutrophils on the blood side was not able to block all cytokine-inducing substances penetrating from the dialysate. It is possible that the levels of BPI were not sufficient and that greater amounts of BPI would have blocked all cytokine-inducing activity on the blood side. On the other hand, it is possible that the cytokine-inducing substances penetrating through the membrane are not of LPS-origin and therefore do not bind to BPI. The latter possibility is supported by the observation that cytokine-inducing substances derived from *Pseudomonas maltophilia* that appeared on the blood side of Cup dialysers could not be blocked by Polymyxin B [5], an antibiotic that binds to and inactivates LPS.

It should be noted that the amount of cytokine-inducing substances detected in the blood compartment was less than that added to the dialysate. The applied PBMC-test to detect substances that induce cytokines is a sensitive bioassay but is not a quantitative test system. In addition, it is difficult to compare the amount of cytokine-inducing substances in the challenge material and in plasma samples from the blood side because of the presence of factors such as LPS-binding protein in plasma that enhance cytokine induction by LPS. In any case, the cytokine-inducing potency on the blood side was less than that in the dialysate. Nevertheless, a recent study suggests that even moderate degrees of dialysate contamination influences cytokine production in vivo [29]: PBMC-associated levels of IL-1Ra in patients on chronic haemodialysis decreases when non-ultrafiltered dialysate is used compared to non-ultrafiltered dialysate (with a median of 148 colony-forming units/ml) [29]. This observation together with the finding in the present study that all tested dialysers are permeable for cytokine-inducing substances suggest that it is highly advisable to use sterile dialysate free from cytokine-inducing substances. When considering the use of a biocompatible dialyser (regarding activation of complement and cells) one should not underestimate the role of contaminated dialysate in biocompatibility.

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**References**

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