New indicators to evaluate bacteriological quality of the dialysis fluid and the associated inflammatory response in ESRD patients

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Introduction

The problem of bacterial contamination of dialysis fluid is well recognized since the beginning of routine haemodialysis therapy. The wide use of reverse osmosis greatly improved the bacteriological quality of water in dialysis units. However, there is still detectable bacterial growth in water, the bicarbonate concentrate, and the dialysate fluid used in haemodialysis centres today. The American Association of Medical Instrumentation (AAMI) proposed standards of bacteriological quality which are widely accepted. According to these standards the bacterial growth should not exceed 200 CFU/ml in water and 2000 CFU/ml in dialysate. There are no recommendations concerning the frequency of bacteriological tests on water and dialysate samples. In many centres, bacterial growth is measured in water, but not dialysate, every 2–3 months. However, bacterial counts in dialysate fluid are very variable and hard to predict, not only in different dialysis centres but also within the same centre. In addition, there are several reports about clusters of pyrogenic reactions due to contaminated dialysate in centres which had before documented good bacteriological quality of water and dialysate [1]. Reasons for the difficulty of predicting clusters of bacterial growth in dialysate and the associated risk of pyrogenic reactions in ESRD patients may reside in the low frequency of dialysate sampling as well as in the use of inadequate bacteriological methods to detect bacterial growth in dialysate. Studies by Klein et al. demonstrated that Gram-negative bacteria—predominantly Pseudomonas species—are found routinely in water and dialysate [2]. These dialysate-born microorganisms grow better or even exclusively on salt-containing, nutrition-poor agars [3] at room temperature. In contrast, standard bacteriological methods employing nutrition-rich culture media such as Columbia agars or tryptic soy agars, which are used to detect enterobacteria such as Escherichia coli, failed to adequately determine the growth of microorganisms contaminating dialysate. Using optimized test conditions, several studies suggest that approximately 20% of all dialysate samples may exceed the recommended limit of 2000 CFU/ml.

Pyrogens released from microorganisms cultured from dialysate

Recent studies suggest that microorganisms growing in water and dialysate produce and release biologically active substances which are different from those of enterobacteria such as E. coli. The pyrogenic activity of bacterial products was described by the ability to induce the production of cytokines, e.g. interleukin-1 (IL-1) and tumour necrosis factor (TNFα), in human peripheral blood mononuclear cells (PBMC). PBMC can be isolated from donor blood and incubated in vitro with samples from dialysers challenged with bacterial culture filtrate. An inhibitory effect of polymyxin B added to the PBMC incubation indicates the presence of the classical endotoxin lipopolysaccharide (LPS) or lipid A-containing fragments of LPS, because the antibiotic polymyxin B inactivates the cytokine-inducing activity of LPS by specific binding to lipid A. Using the PBMC incubation as a test system in the presence of polymyxin B, we found that approximately 90% of the cytokine-inducing activity of E. coli culture filtrates is provided by LPS. In contrast, culture filtrates of Pseudomonas maltophilia and Alcaligenes species were only inhibited approximately 50% by polymyxin B, indicating that bacterial products different from LPS contribute significantly to the pyrogenic activity derived from these bacteria growing in water and dialysate (Figure 1). These pyrogens are not detected by the Limulus amoebocyte lysate (LAL) assay. Most of the non-LPS pyrogens have not yet been identified. However, there is ample evidence that growing Pseudomonas species secrete exotoxin A which has been shown to induce cytokines [4]. We performed in vitro studies using a goat antiserum raised against exotoxin A from P. aeruginosa to inhibit the cytokine-inducing activity of P. aeruginosa culture filtrate. Due to antibody inhibition, our preliminary data suggest that approximately 50% of the cytokine-inducing activ-
In order to investigate whether bacterial contamination of dialysate fluid is harmful to ESRD patients, several groups tested the permeability of intact dialyser membranes for substances derived from bacteria contaminating dialysate fluid [7–13]. The results of these studies are difficult to compare because of major differences in the design of the *in vitro* dialysis experiments, in the source and preparation of bacterial products used for test material, and in the assays to detect the transmembrane passage of the pyrogenic substances. Earlier studies using the LAL which is specific for the classical endotoxin LPS failed to demonstrate the permeability of low-flux cellulose [10] or high-flux polysulfone membranes [7] challenged with phenol-extract, purified LPS. Studies using radiolabelled small molecular fragments of LPS suggested that low-flux cellulose as well as high-flux polysulfone and AN69 membranes are permeable to LPS fragments. This substance induced IL-1 production in PBMC but were negative in the LAL assay [9]. Further studies by our group as well as others used the *in vitro* incubation of PBMC to detect the transmembrane passage of cytokine-inducing substances across low- and high-flux membranes challenged with bacterial culture filtrates derived from dialysate-borne bacteria such as *Pseudomonas maltophilia* or *P. aeruginosa* [8,11]. In these experiments, samples from the blood compartment containing 10% plasma were taken after contamination of the dialysate with for example *P. maltophilia* culture filtrate and added to donor PBMC in culture. In this system, *P. maltophilia*-contaminated dialysate led to increased cytokine-inducing activity in the blood compartment of low-flux cellulose, high-flux cellulose triacetate and high-flux AN69 dialyser membranes. In contrast, no increased activity was detectable in the blood compartment of high-flux polysulfone and polyamide membranes [11]. Most recently, studies getting as close as possible to the *in vivo* situation were performed, circulating whole donor blood in the blood compartment of an *in vitro* dialysis system with a filtrate of *P. aeruginosa* in the dialysate compartment [14]. In contrast to our earlier studies with 10% plasma in the blood compartment, increased IL-1-inducing activity was detectable in blood side samples of polysulfone and polyamide dialysers. In summary, it has to be concluded that all dialyser membranes can be demonstrated to be permeable to cytokine-inducing substances derived from contaminated dialysate. However, there are microorganism- and dialyser membrane-dependent differences in the pyrogen permeability.

**Microorganism-dependent differences in pyrogen permeability of dialyser membranes**

Microorganism-dependent differences in the pyrogen permeability of dialyser membranes are demonstrated in Figure 1. When cellulose triacetate membranes were challenged with a culture filtrate of *E. coli*, the cytokine-inducing activity appearing in the blood side was significantly inhibited by polymyxin B. These data indicate that LPS and LPS-derived fragments are the predominant pyrogens in dialysate and the blood compartment of cellulose membranes if *E. coli* culture filtrate is the challenge material. In contrast, only 50% of the cytokine-inducing activity of dialysate-born...
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**Pseudomonas** bacteria and *Alcaligenes* species is provided by LPS, the remaining activity being due to non-LPS pyrogens. However, the pyrogenic activity appearing in the blood compartment was never inhibited by polymyxin B, suggesting that predominantly non-LPS pyrogens penetrate dialyser membranes if *Pseudomonas* or *Alcaligenes* culture filtrate is the challenge material (Figure 1). Preliminary data from our laboratory suggest that *Pseudomonas*-derived pyrogens are partially inhibited by an antiserum raised against exotoxin A. We therefore propose that exotoxin A and related fragments play a role in the induction of cytokines in response to contaminated dialysate whereas the role of LPS seems to be negligible. Exotoxin A and related fragments are not detectable by the LAL assay, but are inducers of IL-1 in PBMC. Therefore, the measurement of cytokines in PBMC as an indicator of cell activation may offer the most adequate approach to determine pyrogen permeability of intact dialyser membranes in *vitro* as well as in *vivo*.

**Membrane-dependent differences in the pyrogen permeability of dialyser membranes**

In *vitro* studies comparing the pyrogen permeability of various dialyser membranes revealed that synthetic high-flux membranes are less permeable than low- and high-flux cellulosic membranes [11,14]. Therefore, reduced pore size of low-flux membranes is not a limiting factor reducing or preventing pyrogen permeability.

There are membrane-dependent differences in the permeability of high-flux membranes suggesting that specific properties of the synthetic membrane material may reduce transmembrane passage of pyrogens. Because synthetic high-flux membranes differ from cellulosic membranes in that the former contain hydrophobic domains in the membrane polymer, adsorption of lipid-like pyrogens (such as LPS and lipid A) to the hydrophobic domains by hydrophobic interactions has been proposed as an important mechanism reducing pyrogen permeability of synthetic dialyser membranes. This hypothesis is supported by experiments in which ultrafilters containing polysulfone and polyamide membranes were successfully used to remove cytokine-inducing bacterial substances from contaminated fluids such as dialysate [15–18]. The capacity of polysulfone and polyamide membranes to reject pyrogens differed depending on the type of bacterial culture filtrate used to challenge the ultrafilters. The retention was more effective for pyrogens derived from *E. coli* than for those derived from *P. aeruginosa* [18]. As previously described, LPS is the predominant pyrogen released by *E. coli* whereas approximately 50% of pyrogens released from *Pseudomonas* species are non-LPS substances. An important fraction of these non-LPS substances consists of exotoxin A or exotoxin A-related fragments which are peptides [19]. Taken together, results of the ultrafiltration experiments support the concept that adsorption of lipid-like pyrogens such LPS and lipid A via hydrophobic interactions with the respective domains in the membrane polymer of polysulfone and polyamide is the most effective mechanism reducing penetration of LPS-related substances across synthetic high-flux membranes. Although there is detectable permeability of these membranes for peptide-like pyrogens, i.e. exotoxin A and related fragments, rejection of these substances is significant with retention factors of approximately 100. In other words, a concentration of *Pseudomonas* culture filtrate which induces significant cytokine production in PBMC has to be increased at least 100-fold before breakthrough of cytokine-inducing material occurs. In conclusion, synthetic high-flux membranes can be used as ultrafilters to reduce the content of pyrogens including non-LPS substances in contaminated dialysate.

**Effect of ultrafiltered dialysate on haemodialysis-dependent activation of PBMC in ESRD patients**

Based on the results of the *in vitro* studies mentioned above, we performed *in vivo* studies comparing routine bicarbonate dialysate with polysulfone-ultrafiltered dialysate on the content of IL-1 receptor antagonist (IL-1Ra) in non-incubated PBMC from ESRD patients treated with low-flux cuprophan [20].

**IL-1 receptor antagonist is an indicator of PBMC activation**

Although IL-1Ra is a functional antagonist of the proinflammatory cytokine IL-1, IL-1Ra can be measured as an indicator of PBMC activation for the following reasons. IL-1Ra is produced in parallel with IL-1 by the same cell but in much greater quantities (ng/ml) than IL-1 itself (pg/ml) and is therefore easier to measure by immunological methods. In addition, there are two forms of IL-1Ra, a secreted form and a cell-associated form [21], which are both biologically active and detected by the same antibody [22]. Therefore, RIA determinations in PBMC lysates will measure the activity of both forms of IL-1Ra as indicators of cell activation. An additional argument to measure IL-1Ra as opposed to IL-1 in PBMC lysates is that IL-1Ra is always translated following transcription whereas post-transcriptional blocks have been described for IL-1 [23]. The half-life of IL-1Ra is much longer than that of IL-1β [24]. In summary, to study the long-term effect of contaminated dialysate on PBMC activation in ESRD patients, IL-1Ra measured in freshly isolated PBMC appears to be the parameter of choice.

With the measurement of IL-1Ra in cell lysates of freshly isolated PBMC from ESRD patients on cuprophan haemodialysis, we have recently demonstrated that the use of ultrafiltered dialysate (1.9 m² polysulfone filters, Fresenius, Bad Homburg, Germany), compared to routine dialysate, prevented bacterial growth and reduced endotoxin contamination of the dialysate significantly [20]. Although the contamination of routine dialysate was very moderate with a median of
148 CFU/ml, the use of ultrafiltered dialysate reduced PBMC activation in ESRD patients as indicated by cell-associated IL-1Ra levels. This study demonstrates that even a moderate degree of dialysate contamination, meeting the recommended standards, induces a systemic inflammatory response in ESRD patients on cuprophan haemodialysis. The use of polysulfone-ultrafiltered dialysate reduces PBMC activation in ESRD patients.

We extended the study described above and measured the cell-associated content of IL-1Ra in PBMC of ESRD patients treated in three consecutive study periods: (a) with routine dialysate and cuprophan dialyzers, (b) with routine dialysate and high-flux polysulfone dialyzers (F60, Fresenius, Bad Homburg, Germany) and (c) with ultrafiltered dialysate (1 m² polysulfone filters, Fresenius, Bad Homburg, Germany) and F60 dialyzers. Preliminary data of this study also suggest that ultrafiltration of dialysate using polysulfone filters prevents bacterial growth and reduces endotoxin contamination of dialysate significantly. We thereby confirmed our previously published data [20]. PBMC activation due to moderately contaminated dialysate may be reduced by the use of high-flux polysulfone dialyzers compared to haemodialysis with low-flux cuprophan. The use of ultrafiltered dialysate in combination with high-flux synthetic dialyzers may further reduce PBMC activation in ESRD patients and thus provide the most effective way to reduce the haemodialysis-dependent induction of an inflammatory response in ESRD patients.

In conclusion, non-LPS pyrogens derived from contaminated dialysate are able to penetrate low- and high-flux dialyser membranes and are probably the most important inducers of PBMC activation during haemodialysis. There is a growing number of studies suggesting that repeated activation of PBMC with the consequence of cytokine expression may contribute to chronic pathology associated with long-term haemodialysis therapy such as β2-microglobulin amyloidosis [25] or muscle protein degradation [26]. In addition, repeated haemodialysis-dependent stimulation of PBMC may lead to impaired function of these chronically activated cells. There are reports of cell defects in ESRD patients with respect to phagocytosis [27], antigen presentation, or IL-1 and IL-2 secretion [28,29]. Because of the potential impact on haemodialysis-associated pathology in ESRD patients, PBMC activation should be minimized by the use of ultrafiltered dialysate.

References