Short Bacterial DNA Fragments: Detection in Dialysate and Induction of Cytokines

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Abstract. A number of bacterial cytokine-inducing substances (CIS) such as lipopolysaccharides (LPS) and exotoxins have been detected in dialysate and may contribute to inflammation in hemodialysis patients. Short DNA fragments, oligodeoxynucleotides (ODN) of 6 to 20 nucleotides, are able to bind to Toll-like receptors and are stimulatory on immune cells. ODN induce natural killer cell activity and induce IFN-γ, TNF-α, and IL-6 from mononuclear cells. The presence of ODN in dialysate samples and bacterial cultures was investigated. ODN were extracted from fluids by adsorption to reverse-phase columns. ODN were detected in 18 of 20 investigated dialysate samples, in eight of 10 reverse-osmosis water samples, and in all cultures from various bacterial strains. The presence of bacterial DNA in dialysate was confirmed by PCR specific for bacterial tRNA gene sequences. Saline for intravenous use contained 0.02 ± 0.01 µg/ml DNA, dialysate samples contained 0.28 ± 0.02 µg/ml, and Pseudomonas cultures contained 1.0 ± 0.03 µg/ml DNA. ODN from bacterial cultures were only partially removed by ultrafiltration and were able to diffuse through regular high-flux dialyzer membranes. Synthetic cytosine-guanosine dinucleotide–containing ODN were able to induce IL-6 in human mononuclear cells. It is concluded that short bacterial-derived DNA fragments are present in clinically used fluids, e.g., dialysate. These fragments are of sufficient small size to pass through dialyzer membranes. Bacterial DNA fragments may be an overlooked factor contributing to inflammation in hemodialysis patients.

Since the introduction of hemodialysis (HD) into clinical practice, there has been concern about the transfer of bacterial cytokine-inducing substances (CIS) from the dialysate into the blood compartment and subsequent deleterious effects on the patient. A number of bacterial products such as lipopolysaccharides (LPS), exotoxins, and peptidoglycans share the ability to induce cytokines and are known activators of immune functions. A number of in vitro and in vivo studies have been performed to investigate the permeation of these substances through dialysis membranes. In many studies, the biologic test of cytokine induction in peripheral blood mononuclear cells (PBMC) has been used to detect these substances. However, the exact chemical natures of bacterial CIS as well as the interactions between different types of CIS are not completely understood, and most likely CIS consist of a mixture of bacterial products. LPS is not the only and maybe not the most important product in dialysate that induces cytokines. This is supported by the observation that the cytokine-inducing activity of Pseudomonas products that appear on the blood side of dialyzers cannot entirely be blocked by polymyxin B (1) and are negative in the limulus-amebocyte-lysate (LAL) test (2).

The immune system can be triggered by “adaptive” and by “innate” immune responses. Whereas the adaptive immune response relies on the specific recognition of foreign pathogens by receptors on T and B cells, the innate response is triggered by nonspecific so-called pattern recognition receptors and generates unspecific “danger” signals. A family of Toll-like receptors (TLR) belong to these pattern recognition receptors, are involved in activating immune cells, and are able to induce NF-κB–dependent gene expression [for review, see (3)]. Ten different TLR have been described so far. They are triggered by ligands such as LPS (TLR4) or viral RNA (TLR3). The signaling pathways by which bacterial ODN activate cells involve TLR9. Macrophages and dendritic cells from TLR9-deficient mice do not respond to ODN (4). It seems that after endocytosis of ODN, cytosine-guanosine dinucleotide (CpG) motifs become engaged with TLR9 and activate mitogen-activated protein kinases and NF-κB (5).

These bacterial-derived short DNA fragments are new pyrogenic candidates that may pass dialyzer membranes. The structural requirement for immunostimulation by ODN was defined to be a cytosine-guanosine (CG) core that had to be unmethylated to stimulate mammalian cells (6). There seems to be strict sequence requirement of two 5’ purines and two 3’ pyrimidines flanking the CpG for full cytokine induction (7). This CpG motif is one of the features that distinguish bacterial from mammalian DNA and allows mammalian phagocytic cells to recognize and to be activated by bacterial DNA.
CpG-ODN of six to 20 nucleotides are able to induce natural killer cell activity and induce IFN-γ, TNF-α, and IL-6 from PBMC (8–10). When injected intraperitoneally, CpG-ODN induce IL-6, TNF, and IL-12 in mice and may even lead to septic shock (4).

How does the mammalian immune system distinguish between bacterial and self-DNA? Vertebrate and bacterial DNA differ markedly in their CpG content owing to CpG suppression: CpG dinucleotides in vertebrate genomes occur only approximately one quarter as frequently as would be predicted if base utilization were random. Furthermore, the bases that flank CpG in vertebrate genomes are not random: Most often the base preceding a CpG is a C, and most often the base following a CpG is a G in vertebrates. These types of CpG motifs do not induce strong stimulation of immune cells. In addition to these differences in CpG content, ODN need to be unmethylated to stimulate mammalian immune cells. CpG dinucleotides are not methylated in bacterial DNA but are routinely methylated at the 5’ position of ~70% of the cytosines in vertebrates (6). However, despite CpG suppression and increased methylation, vertebrate DNA contains many unmethylated CpG motifs, and still vertebrate DNA does not stimulate immune cells. In addition, vertebrate DNA that has been almost completely unmethylated still has no immune stimulatory effects on CpG-responsive cells. Thus, vertebral DNA must contain immune neutralizing sequences that block the effects of the unmethylated CpG motifs (6). Taken together, there are several differences between vertebrate and bacterial DNA that allow the mammalian immune system to distinguish them and to react only to the potentially dangerous material.

ODN of only five to six nucleotides are able to induce cytokines in human mononuclear cells (11). Bacterial ODN can stimulate immune cells both in single-stranded and in double-stranded form (5). Thus, cytokine-inducing ODN could be of sufficient small size (5 nucleotides ~1250 Da) to pass through dialyzer membranes. Bacterial ODN may be a factor contributing to cytokine induction during HD. We therefore quantified the presence of ODN in dialysate as a new type of dialysate contaminant and investigated their effect on cytokine production in human PBMC.

Materials and Methods

PBMC Preparation

PBMC were separated from whole blood by centrifugation through Ficoll and Hypaque made from powder (Ficoll Type 400; sodium-diatriazote; Sigma, St. Louis, MO). The water for preparation of Ficoll and Hypaque and all other fluids used were subjected to ultrafiltration using polyflux filters (PF14S; Gambro, Hechingen, Germany) to remove cytokine-inducing substances (12). For incubation, PBMC were washed twice with normal saline and resuspended at 5 × 10^6/ml in serum-free RPMI 1640 culture medium (Life Technologies, Paisley, UK) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml Streptomycin. A total of 250 µl of cell suspension was incubated in 24-well plates (Nunc, Roskilde, Denmark) for 18 h with 250 µl of stimulus at 37°C in a humidified atmosphere that contained 5% CO₂. After incubation, PBMC were subjected to three freeze/thaw cycles to lyse the cells.

Bacterial Cultures and Synthetic ODN Sequences

Clinical isolates of Pseudomonas aeruginosa were grown in standard dialysate. Bacteria were pelleted by centrifugation, and the culture supernatant was filtered through 0.45-µm cellulose acetate filters (Nalgene, Wiesbaden, Germany) to remove any residual bacteria. Dialysate samples were taken from the waste dialysate outlet of regular dialysis machines and also filtered through 0.45-µm filters. When indicated, dialysate samples were taken from the dialysate inlet of the dialyzer as well as from the waste dialysate outlet. In some experiments, dialysate and Pseudomonas cultures were subjected to ultrafiltration through polyflux (PF14S; Gambro) or polysulphone (F60S; Fresenius, Bad Homburg, Germany) dialyzers. Both pre- and postultrafilter samples were subjected to DNA extraction.

DNA was extracted from fluids by adsorption onto reverse-phase columns (SepPak C18; Waters, Taunton, MA). Columns were rinsed with acetonitrile (Fluka, Buchs, Germany), distilled water, and 10 mM ammonium acetate. Two to 50 ml of dialysate or bacterial supernatant was passed through the column, and DNA fragments were eluted with 2 ml of 60% methanol in water. DNA was precipitated with cold ethanol and dried under vacuum, and the concentration of DNA was determined by measuring absorbance at 260 and 280 nm. DNA was labeled with digoxigenin (DIG) by means of a DIG-oligonucleotide–tailing kit using terminal transferase (Boehringer Mannheim, Mannheim, Germany). This enzyme labels DNA fragments of various sizes from ~10 to 100 nucleotides with high specificity. The labeling products were separated by molecular size on a standard 2% agarose gel, and the products were transferred to nylon membranes (Genescreen; NEN Life Science, Zaventem, Belgium) by capillary blotting. Labeled ODN were visualized by detection with anti-DIG antibodies using a DIG detection kit (Boehringer) after exposure to x-ray films (Biomax ML; Kodak, Rochester, NY).

ODN were synthesized by TIB-Molbiol (Berlin, Germany) according to sequences published by Takeshita et al. (13). The sequence was ATG GAC TCT CGA GCG TTC TC. The endotoxin content in these ODN were <0.01/µg DNA as determined in the LAL test.

In Vitro HD Circuit

To investigate the diffusive transport of DNA fragments through dialyzer membranes, we used a well-characterized in vitro HD circuit. We previously reported large differences in the permeability of three polysulfone-based high-flux dialyzers for LPS (14). Therefore, we also used these three dialyzers for the present experiment. Dialyzers that contained polysulfone (F60S; Fresenius Medical Care, Bad Homburg, Germany), helixone (FX60; Fresenius Medical Care), and DIAEPES (BLS814SD; Bellco, Mirandola, Italy) were aseptically connected to standard blood tubing. The three different dialyzers were tested in parallel using the same material to challenge the dialysate. Whereas the blood compartments were connected individually, the dialysate compartments were serially connected with blood tubing to produce a single dialysate compartment. The order of the serially connected dialyzers was varied at random. All compartments were rinsed with 1 L of sterile saline each. A total of 0.5 ml of 20% sterile human serum albumin was added to the blood compartments. The fluid in the blood compartment was recirculated for 15 min before challenging the dialysate compartment.

The dialysate compartment was contaminated with sterile filtrates of Stenotrophomonas maltophilia isolated from clinically used dialyzer. LPS was diluted in sterile saline that contained 0.02% human serum albumin, resulting in a stock concentration of 10 µg/ml LPS. Bacteria were grown overnight in bicarbonate dialysate in a shaking water bath. Bacteria were sonicated for 30 min and pelleted by...
centrifugation, and the supernatant was filtered through 0.45-μm cellulose acetate filters (Nalgene). Bacterial filtrates were stored at 4°C and were used within 2 wk after preparation. After challenging the dialysate compartment with 50 ml of *Stenotrophomonas maltophilia* filtrate, recirculation was performed for 30 min at room temperature using flow rates of 150 ml/min in both compartments. The blood compartments were tightly closed to reduce net ultrafiltration. Samples were taken from the circulating system from the blood and the dialysate compartments at 30 min after challenging the dialysate compartment and subjected to DNA extraction.

**Assay Procedures**

IL-6 was determined by ELISA technique. Primary and biotinylated antibodies against IL-6 were purchased from R&D Systems (Wieshaden, Germany). Cytokines were determined after two freeze/thaw cycles. Ninety-six-well plates (Maxisor; Nunc) were coated overnight with 50 μl/well of the primary antibody in coating buffer (0.2 M NaHCO₃/Na₂CO₃ [pH 10.5]). Wells were blocked with 0.2% casein (Sigma) in PBS for 1 h, and 50 μl of standards or samples were added to the wells and incubated overnight. All dilutions were made in PBS that contained 0.05% Tween (Sigma); after each incubation step, wells were washed with PBS that contained 0.05% Tween. A total of 50 μl/well of the appropriately diluted biotinylated secondary antibody was added and incubated for 1 h. After incubation with peroxidase-Streptavidin-biotin complexes (Amersham, Braunschweig, Germany) for 1 h, plates were developed with TMB (240 μg/ml 3,3',5,5' tetramethylbenzidine; Fluka Chemicals, Buchs, Switzerland) in Gallati buffer (42 μg/ml citric acid [pH 3.95]/0.01% H₂O₂). Optical density was determined at 450 and 630 nm on a spectrophotometer (Model 9700; Perkin-Elmer-Cetus). Aliquots (10 μl) were taken after different numbers of cycles (usually after 15, 20, and 25 cycles) and analyzed on 1.5% agarose gels, and the PCR products were stained with ethidium bromide. The PCR products were transferred to nylon membranes (Amersham) by capillary blotting using 20× SSC as blotting buffer. The nylon membranes were fixed by UV light, and the DIG-UTP that had been incorporated into the PCR products was visualized by staining with anti-DIG antibody conjugated to alkaline phosphatase (DIG Luminescent Detection Kit; Boehringer Mannheim). Luminescence of the substrate (Lumigen PPD) was documented by short exposure to x-ray film (Kodak).

**Statistical Analyses**

The differences in cytokine concentration were compared using *t* test for paired samples. Significance was defined as *P* < 0.05. Results are expressed as means ± SEM. Analysis was performed using the Software program Instat (Graphpad Software, San Diego, CA).

**Results**

**Short DNA Fragments Are Present in Bacterial Cultures**

*Pseudomonas* cultures were grown in dialysate, and DNA present in 10 ml of supernatant was precipitated with cold ethanol without previous column extraction. Agarose gel electrophoresis and staining DNA with ethidium bromide revealed short DNA fragments present in two different *Pseudomonas* cultures (Figure 1). Besides presumably genomic, high-molecular weight DNA (gel top), there are abundant DNA fragments well below 100 nucleotides (arrow). Short DNA fragments were also detected in cultures from other clinically isolated bacteria. Five milliliters of culture

![MW Marker](image)

Figure 1. Agarose gel showing the size distribution of DNA from two *Pseudomonas aeruginosa* cultures. There are abundant DNA fragments well below 100 nucleotides (nt; arrow).
supernatant from *E. coli*, *P. maltophilia*, and *Enterococcus fecalis* were passed over C18 columns; the bound DNA was eluted, precipitated, and labeled with DIG. In all three tested bacterial strains, DNA fragments could be found (data not shown). The molecular size of the labeled DNA was ~20 nucleotides.

**Short DNA Fragments Are Present in Dialysate**

DNA from 50 ml of dialysate obtained from the dialysate inlet of six different dialysis machines was purified using C18 columns, and the eluted DNA was labeled. DNA fragments could be demonstrated in dialysate samples in different amounts (Figure 2). For comparison, DNA extracted from 50 ml of two intravenous saline preparations and from 2 ml of three *Pseudomonas* cultures are also shown. All together, DNA fragments were detected in 18 of 20 dialysate samples investigated that were different from the samples used for agarose gels.

To quantify the amount of DNA present in various fluids, DNA was extracted by adsorption onto C18 columns. After elution, DNA was precipitated with cold ethanol and dried under vacuum, and the concentration of DNA was determined by measuring absorbance at 260 and 280 nm. Saline for intravenous use (*n* = 9) contained 0.02 ± 0.01 µg/ml DNA, dialysate samples collected at the dialysate inlet (*n* = 25) contained 0.28 ± 0.02 µg/ml, and *Pseudomonas* cultures (*n* = 3) contained 1.0 ± 0.03 µg/ml DNA (Figure 3). RPMI culture medium samples (*n* = 9) contained 0.039 ± 0.01 µg/ml.

**Experiments Investigating the Source of DNA Fragments**

The presence of short DNA fragments in dialysate does not prove their bacterial origin. It may be possible that human DNA fragments penetrate from the blood onto the dialysate side during HD. In this case, more DNA should be observed at the dialysate outlet compared with the dialysate inlet. Therefore, dialysate samples were taken from the dialysate inlet and outlet of various dialysis machines, and DNA was extracted. As shown in Figure 4, DNA fragments could be demonstrated before and after the dialysis machines. It therefore seems unlikely that the DNA fragments are of human origin. To specifically detect bacterial DNA, we performed PCR for the gene coding for the 16S-tRNA common for all gram-negative bacteria. As shown in Figure 5, gram-negative bacterial DNA could be detected in two dialysate samples to a greater degree than in reverse-osmosis water and in saline for intravenous use. The largest amount of bacterial DNA was observed in cultures from *Pseudomonas* (Figure 5).
Removal of Short DNA Fragments by Ultrafiltration and Diffusive Transfer through High-Flux Membranes

Cultures from *Pseudomonas* were diluted 1/10 in saline for intravenous use, and the fluid was ultrafiltered using polysulfone or polyflux dialyzers. Pre- and postfilter samples were passed over C18 columns, and bound DNA was eluted and visualized after labeling with DIG. The results are shown in Figure 6. DNA in *Pseudomonas* cultures was partially removed by ultrafiltration with polyflux and polysulfone. However, DNA could be detected even in postultrafiltration samples with both dialyzer membranes. There was no consistent difference between the tested membranes. Thus, ultrafiltration removes some but not all DNA fragments present in aqueous solutions.

To investigate the diffusive transport of ODN through different high-flux membranes, we used an *in vitro* HD circuit as described (14). When the dialysate was contaminated with filtrates from *Stenotrophomonas maltophilia*, ODN could be detected after 30 min of recirculation on the blood side of all three membranes tested (Figure 7). Before dialysate contamination, there were no detectable ODN in the blood compartment.

Cytokine Induction by Synthetic ODN

To investigate cytokine induction by synthetic ODN, we cultured human PBMC with medium alone or with increasing amounts of ODN (Figure 8). Spontaneous production of IL-6 from PBMC cultured without stimulus was $21 \pm 7$ pg/ml ($n = 9$). ODN induced significant IL-6 production at concentrations...
of 50 µg/ml (390 ± 89 pg/ml) at 5 µg/ml (215 ± 78) and at 0.5 µg/ml (52 ± 17; all P < 0.05 versus medium).

Discussion

To our knowledge, the present report is the first description of the occurrence of bacterial DNA fragments in clinically used solutions, especially dialysate. Penetration of even a fraction of these bacterial ODN through dialysis membranes onto the blood side is likely to have widespread effects on the immune system of dialysis patients. The innate immune system recognizes and identifies the type of invading pathogen by using so-called "pattern-recognition receptors" (3). The best characterized pattern-recognition receptors are the Toll-like receptors TLR-1 to TLR-10 which are expressed on a variety of cell types, such as dendritic cells, macrophages, monocytes, and B and T cells (7). These receptors trigger cell activation when they recognize microbial-specific molecules such as LPS, peptidoglycans, zymosan, and unmethylated oligonucleotides. Signals initiated by the interaction between TLR and their specific ligands direct and specify the resulting inflammatory response.

Although we did not provide direct evidence that transfer of ODN onto the blood side really takes place during clinical HD (e.g., by demonstrating the emergence of bacterial ODN in the blood of dialysis patients during HD), it seems likely that transfer will occur when ODN are present in the dialysate. ODN of only five to six nucleotides are able to induce cytokines in human mononuclear cells (11). We demonstrated that small ODN are able to pass high-flux dialyzer membranes and are only partially removed by ultrafiltration using regular ultrafilters. Moreover, we observed that ODN appear on the blood side of high-flux dialyzers under diffusive conditions. Thus, regular high-flux membranes seem not to be a safe barrier against these bacterial products.

It could be argued that the detected DNA fragments originated from human rather than bacterial sources. Sequence comparison of the short DNA fragments would have revealed their origin, but we were unable to sequence the short DNA fragments because of their small size. However, ODN were detected in dialysate samples that entered the dialyzer (before any contact with blood) as well as in dialysate that left the dialyzer (Figure 4). In addition, we performed PCR specific for bacterial DNA and detected larger bacterial DNA in all dialysate samples by this technique.

It may be argued further that the concentration of ODN in dialysate is too low to stimulate a considerable clinical response. The average DNA concentration in dialysate was 0.28 ± 0.02 µg DNA/ml, corresponding to ~0.1 µM. However, this is only a minimal estimation because we cannot be sure that all ODN present in dialysate had bound to the columns used. Thus, the accurate concentration of ODN in dialysate may well be higher. Moreover, we observed induction of IL-6 production from human PBMC at 0.5 µg/ml. Other authors reported induction of IFN-γ-inducible protein IP-10 by as little as 0.1 µg/ml of certain ODN (17) and IL-6 production in human monocytes by 0.062 µM of certain ODN (18). Thus, the concentrations of bacterial DNA found in dialysate are well within the range reported to activate immune cells. Moreover, dialysis patients are exposed to 18,000 to 23,000 L of dialysate per year, making the clinical relevance most likely, considering the failure of dialysis membranes to hinder passage of ODN into the blood stream. However, not all of the naturally occur-
ring bacterial DNA fragments are immunologically active. There are certain structural requirements ("CpG-motif") for binding to TLR-9 and for cell activation. In mice, the optimal activating sequence is GACGTT, whereas in human cells, the optimal sequence is GTCGTT (19). Recently, two different types of ODN were identified for optimal activity in human cells: "K-type" ODN for induction of IL-6 and/or IgM in types of ODN were identified for optimal activity in human cells; "K-type" ODN for induction of IL-6 and/or IgM in human monocytes and B cells and "D-type" ODN for induction of IFN-γ in NK cells (18). Thus, only a fraction of the ODN found in dialysate may activate immune cells in dialysis patients. The magnitude of this fraction is unknown; sequencing the bacterial ODN found in dialysate could reveal the percentage of immune-stimulatory ODN in dialysate. Alternatively, investigating the stimulatory effect of dialysate or bacterial cultures before and after specific removal of ODN will further clarify the biologic relevance of ODN, and we are currently working on techniques to remove ODN from aqueous solutions.

Because induction of cytokines by ODN is low compared with other stimuli, such as LPS, the biologic effects of the presence of ODN in dialysate on HD patients is unclear. However, CpG-ODN put forth a variety of effects in vivo, and there is a potential interaction between ODN and LPS. LPS and ODN exert their effects by stimulation of TLR4 and TLR9, and both receptors activate NF-κB. Both LPS and ODN induce TNF, but they show synergistic induction when used together (20). Mice that received injections of sublethal doses of LPS before or at the same time as sublethal doses of ODN tolerated treatment. When ODN were given before sublethal doses of LPS, a high mortality was observed (6). However, when ODN were given 48 h or earlier before challenge with Listeria monocytogenes, ODN pretreatment even protected against mortality (6). Thus, although still much has to be learned regarding the effects of ODN on the immune system, bacterial ODN can prime the host for an inflammatory response. Even when dialysate contains only substimulatory concentrations of LPS and ODN, there might be a significant biologic effect in dialysis patients as a result of the synergism between both stimuli. It should be noted that there is also a synergism between activated complement products such as C5a and LPS on cytokine induction (21). In the clinical situation, various stimuli (activated complement, LPS, DNA fragments) may act synergistically on cytokine production by PBMC on the blood side of dialyzers.

It may be surprising that regular ultrafilters were unable to significantly remove ODN. However, normal ultrafilters are designed to remove predominantly hydrophobic substances such as LPS by adsorption. Hydrophilic substances such as toxic shock syndrome toxin from Staphylococcus (12) or DNA are removed to a lesser extent. Although other water treatment devices such as reverse osmosis or charcoal filters can be expected to remove small DNA fragments, bacteria that grow in the water distribution line may release ODN (similar to other substances such as LPS) that are transported to the dialysis machines. Additional devices or improved ultrafilters with enhanced capacity to adsorb DNA fragments are required to remove ODN before entering the dialysis machine.

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References


