In-vitro Biocompatibility of alternative CAPD fluids; comparison of bicarbonate-buffered and glucose-polymer-based solutions

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Abstract. Evidence is accumulating that conventional dialysis fluids for CAPD are incompatible with peritoneal host defence. We therefore investigated the effect of alternative CAPD fluids on mononuclear leukocyte (PBMC) viability and cytokine production in vitro. Fluids tested were bicarbonate-buffered solutions containing 1.5% or 4.25% glucose, 7.5% glucose polymer dialysis fluid (GPDF), and conventional 1.5% glucose fluid (Gl.5%). PBMC were stimulated (2 h, 37°C) in the different test fluids with a clinical isolate of Staphylococcus epidermidis or Escherichia coli lipopolysaccharide. The cytokines TNFα and IL-6 in PBMC supernatants were measured by specific enzyme immunoassays. Induction of cytokine messenger RNA was evaluated by reverse transcription-polymerase chain reaction. Conventional Gl.5% (pH 5.5) inhibited cytokine release from activated PBMC by >95%, whereas cell responses in low-glucose bicarbonate fluid were not significantly reduced. In contrast, high-glucose bicarbonate fluid exerted >80% inhibition despite its neutral pH. GPDF was inhibitory at its initial low pH, whereas cytokine release was restored following pH neutralization. Cytokine mRNA expression was suppressed by conventional Gl.5% fluid and by high-glucose bicarbonate fluid. These data indicate that pH neutralization leads to a substantial improvement of dialysis fluid biocompatibility; however, hyperosmolality and/or high glucose content inhibit cell responsiveness even at normal pH. Replacement of glucose by glucose polymer might prove beneficial provided that the initial low pH is neutralized.

Key words: biocompatibility; CAPD fluids; continuous ambulatory peritoneal dialysis; cytokines; IL-6; polymerase chain reaction; TNFα

Introduction

An increasing body of evidence indicates that conventional peritoneal dialysis fluids are incompatible with peritoneal host defence. Earlier reports demonstrated that these fluids are not only cytotoxic, but also impair the function of phagocytic cells, including their capacity to phagocytose and kill micro-organisms [1-5] as well as their ability to secrete cytokines [6-8]. The inhibitory components of dialysis fluids associated with these inhibitory effects have been identified as their low pH in combination with their lactate content [9,10], and also their hyperosmolality and high glucose concentration [11]. These negative aspects of conventional glucose-based dialysis fluids have focused interest on the search for alternative solutions and osmotic agents for use in CAPD.

Such alternative dialysis fluids which are presently under consideration for clinical use are based on two different principles: (1) the replacement of lactate as the buffer by bicarbonate to achieve physiological pH, and (2) the substitution of glucose with an alternative osmotic agent to achieve physiological osmolality and to enhance ultrafiltration, e.g. with glucose polymer [12]. In addition, two recent in-vitro studies suggested that glucose polymer dialysis fluids might be less detrimental to certain phagocyte functions (phagocytosis, metabolic burst activation and bacterial killing) than hyperosmolar conventional CAPD fluids, however, also potentially negative aspects such as improved bacterial survival data were reported [13,14]. In the meantime, both bicarbonate-buffered glucose solutions and glucose polymer dialysis fluid (GPDF) are now also being clinically evaluated in ongoing multicenter trials.

More recently research in the area of peritoneal inflammation has focused on the mechanisms of cell activation and cell interactions in the peritoneal cavity. It is becoming increasingly clear that intraperitoneal inflammation is regulated by a network of immune mediators derived from resident peritoneal cells (peritoneal macrophages and mesothelial cells), but also
from infiltrating neutrophils [15-17]. Peritoneal macrophages form the first line of defence against invading micro-organisms and it is assumed that these cells provide the initial signals for the recruitment of leukocytes and the activation of resident peritoneal cell populations [18].

The current study therefore aimed at characterizing the effects of newly formulated bicarbonate-buffered or glucose-polymer-containing dialysis fluids on the synthesis and secretion of two major cytokines, tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6), as a biocompatibility index of these alternative CAPD solutions. For this purpose we employed an established in-vitro model assessing cytokine gene transcription and release by peripheral blood mononuclear leukocytes (PBMC) activated with either Staphylococcus epidermidis (S. epidermidis) or Escherichia coli (E. coli) lipopolysaccharide (LPS) [6-8].

Our results demonstrate that the suppression of cytokine production associated with conventional CAPD fluids is related to an inhibition of cytokine gene transcription. Bicarbonate-buffered low-glucose fluids and pH-neutralized glucose polymer fluids potentially restore cell responsiveness to both inflammatory stimuli; however, excessive hyperosmolality and glucose content as well as the low pH of glucose polymer fluids may reverse these potentially beneficial effects.

**Subjects and methods**

**Composition of test fluids**

The composition of test fluids is indicated in Table 1. All fluids used were manufactured and supplied by Fresenius, Bad Homburg, Germany.

**Leukocyte separation**

PBMC were isolated from the peripheral blood of healthy volunteers by Ficoll density gradient centrifugation (Ficoll–Hypaque, Pharmacia, Uppsala, Sweden) under strictly sterile conditions (laminar air flow). All reagents used for cell separation and incubation experiments were LAL-negative. The PBMC fraction consisted of 15-30% monocytes, 60-70% lymphocytes, <10% granulocytes, and few erythrocytes and thrombocytes, as established by light-microscopy and dual-colour flow cytometry [6,8]. Cell viability by trypan blue exclusion test was >95% before incubation.

**Incubation procedures**

Aliquots obtained from one individual donor, each containing 5 x 10⁶/ml PBMC, were used for each series of the incubation experiments. All materials used were cell culture grade (Nunc, Wiesbaden, Germany). PBMC were incubated (2 h, 37°C) in RPMI-1640 medium culture (Sigma, Deisenhofen, Germany) in the presence of 100 ng/ml endotoxin from E. coli strain O55:B5 (Sigma). Parallel experiments were performed challenging the cells with a clinical isolate of S. epidermidis (DM) [17] (50 μl/ml of suspension S. epidermidis grown in nutrient broth No. 2 (Oxoid Ltd, Basingstoke, UK) adjusted to an optical density of 1.0 at 560 nm). For each set of experiments, an unstimulated control (5 x 10⁶/ml PBMC in RPMI medium without challenge) as well as a positive control (5 x 10⁶/ml PBMC in RPMI medium containing LPS or S. epidermidis respectively) were run.

The incubations were terminated by rapid centrifugation, the cell supernatants were stored at −70°C until cytokine content was determined. For assessment of cytokine gene transcription, cells were lysed and subjected to messenger RNA extraction and reverse transcription into cDNA (see below).

**Determination of TNFα and IL-6 in cell supernatants**

TNFα and IL-6 were determined by specific enzyme immunoassays as described previously [8].

**RNA extraction and reverse transcription**

Total RNA was extracted from the cell pellets of the incubations by the RNAzol method as described by the manufacturer (Cinna-Biotex Laboratories, Houston, TX, USA). One microgram total RNA was heat denatured at 95°C for 3 min in the presence of 100 pmol random hexamer (Pharmacia, Freiburg, Germany) and cooled on ice for 2 min. The RNA was...
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reverse transcribed in a final volume of 20 μl of 1 × PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin) and 625 μM of each dNTP (Boehringer, Mannheim, Germany), 20 U of RNAsin (Promega, Heidelberg, Germany), 10 mM dithiothreitol, and 200 U of MMuLV reverse transcriptase (Gibco-BRL, Berlin, Germany). The reaction mixture was incubated for 10 min at room temperature, 45 min at 42°C, and 5 min at 95°C.

Amplification of cytokine cDNA by polymerase chain reaction

PCR amplification of cytokine cDNA was carried out in a total volume of 50 μl (2 μl of reverse transcription product and 48 μl master mix [36.25 μl H₂O, 1.25 μl 3'-primer (20 μmol/l), 1.25 μl 5'-primer (20 μmol/l), 4 μl nucleotide triphosphate, 5 μl 10 × PCR buffer, and 0.25 μl Taq polymerase (2.5 U, Amplitaq, ILS Ltd, London, UK)] using a Perkin-Elmer model 480 thermocycler (Perkin-Elmer, Uberlingen, Germany). The PCR protocol was as follows: first cycle, 94°C for 3 min, 55°C for 1 min, 72°C for 1 min; second through 24th cycles 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. The final cycle was 94°C for 1 min and 60°C for 10 min. PCR was performed for 25 cycles with α-actin, for 35 cycles with TNFα, and for 30 cycles with IL-6.

One-tenth of the PCR reaction products was separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and photographed. The negatives were scanned by laser densitometry, and the relative density of the cytokine transcripts was compared to the actin controls.

Oligonucleotide primers

Oligonucleotide primers for PCR were generously provided by Dr Peter Scholz, Schering AG, Berlin, Germany. The sequences of the amplification primers are shown in Table 2.

Results

Effect of alternative dialysis fluids on cytokine release from PBMC

Control PBMC in RPMI released 902 ± 212 pg/ml TNFα and 1154 ± 317 pg/ml IL-6 upon stimulation with S. epidermidis (mean ± SEM, n = 7) and 1486 ± 161 pg/ml TNFα and 1000 ± 136 pg/ml IL-6 upon stimulation with E. coli LPS (mean ± SEM, n = 7). Exposure of cells to conventional G1.5% reduced the release of both cytokines back to unstimulated control levels (P < 0.01). PBMC cytokine response was not significantly impaired in low-glucose Bic20, but substantially suppressed (>80% compared to controls) following exposure to high-glucose Bic30 (P < 0.01). PBMC cytokine response was not restored with low-pH glucose polymer dialysis fluid, but was significantly improved following exposure to bicarbonate-buffered GPDF (P < 0.01) (Figures 1, and 2).

Effect of alternative CAPD fluids on cytokine gene transcription

Unstimulated PBMC in RPMI control medium showed weak expression of specific transcripts for both TNFα and IL-6. Expression of both cytokines was markedly enhanced following activation of the cells with E. coli LPS. Both conventional low-glucose and bicarbonate-buffered high-glucose CAPD fluids resulted in weak cytokine expression similar to unstimulated controls, whereas cytokine gene transcription was unaltered in cells exposed to bicarbonate-buffered, low-glucose fluid or glucose polymer dialysis fluid (irrespective of pH). The expression of the control gene, actin, remained unchanged with either of the incubation media (Figures 3 and 4).

Table 2. Sequences of the amplification primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product size</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>ACACCATGAGCAGCTGAAAGC</td>
<td>525 bp</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>TGATGCCAGAGGAGGAGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>TACATCTCGACGCGCATTC</td>
<td>465 bp</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>GCTGATTTGCGAAGAAGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Actin</td>
<td>GGAGCAATGTCTTGATCTT</td>
<td>204 bp</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>TCTGAGGTACGGGCTTCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Effect of alternative CAPD fluids (Bic20, Bic30, GPDF) on the release of IL-6 (hatched columns) and TNFα (open columns) from PBMC activated with *E. coli* lipopolysaccharide (mean ± SEM, n=7). Statistical significance by repeated measures one-way analysis of variance (ANOVA) between positive controls and experimental fluids (P<0.05) is indicated by a single asterisk above the columns, significance between GPDF at low and neutral pH by double asterisks.

Fig. 3. Effect of alternative peritoneal dialysis fluids on cytokine gene transcription for IL-6, TNFα, and α-actin in PBMC activated with *E. coli* lipopolysaccharide. PCR amplification products were separated on agarose gels, stained with ethidium bromide, and photographed.

- A: 123 base pair cDNA standards
- B: unstimulated control (RPMI medium without LPS)
- C: stimulated control (RPMI with 100 ng/ml *E. coli* LPS)
- D: Conventional G1.5%
- E: Bic20
- F: Bic30
- G: GPDF pH 5.5
- H: GPDF pH 7.4

(D to H all stimulated with 100 ng/ml *E. coli* LPS).

Fig. 4. Effect of alternative peritoneal dialysis fluids on steady-state messenger RNA expression for IL-6 and TNFα in PBMC activated with *E. coli* LPS. Negatives of gels were scanned by laser densitometry, and the ratios for relative density of cytokine transcripts to actin controls were calculated. Mean (±SEM) cytokine/actin ratios of five experiments are shown.
Effect of CAPD fluids on cell viability

Cell viability as assessed by trypan blue exclusion was significantly reduced only following exposure of PBMC to conventional CAPD fluids containing 1.5% (Gl.5%) and 4.25% glucose (G4.25%), whereas both bicarbonate-buffered and glucose polymer dialysis fluids did not display significant cytotoxicity (Figure 5).

Discussion

The current study employed an in-vitro model for the preclinical characterization of the effects of alternative peritoneal dialysis fluids on the generation of cytokines by monocytes/macrophages. The cell type chosen for study were peripheral mononuclear cells (PBMC) from healthy donors rather than peritoneal macrophages obtained from CAPD patients, since (1) the latter have previously been exposed to the dialysis fluids which are the subject of the study, and (2) it has been shown earlier in a similar model assessing the effects of conventional CAPD fluids that healthy PBMC are not significantly different from uraemic PBMC in terms of their stimulated cytokine (TNFα and IL-6) production [8].

Two different types of fluids were tested: (1) bicarbonate-buffered fluids with physiological pH (7.4), but still containing high glucose concentrations as the osmotic agent, with a resulting hyperosmolality of between 358 and 511 mosmol/kg, and (2) glucose polymer dialysis fluid with physiological osmolality, but containing high lactate concentrations (40 mmol/l) and with an initial pH of 5.5.

Both mechanisms, low pH in combination with lactate and hyperosmolality related to high glucose content, have previously been identified as critical factors leading to the inhibition of cytokine release by monocytes/macrophages as well as reduced phagocytosis and respiratory burst activation of phagocytes [6,9,10,11,22]. It was thus expected that both types of alternative fluids might bring about an improved responsiveness of leukocytes to inflammatory stimuli when compared to conventional low-pH, hyperosmolar CAPD fluids.

As parameters of intact cell responsiveness to inflammatory stimuli such as S. epidermidis and E. coli lipopolysaccharide the production and release of the cytokines TNFα and IL-6 were chosen. TNFα is a cytokine with pleiotropic proinflammatory properties [23,24], including the activation of fibroblasts [25] and mesothelial cells [15]. In the setting of CAPD peritonitis, TNFα (in addition to IL-1) is believed to play an important role as the initial signal for the activation of the peritoneal mesothelial cells which respond to this signal with secretion of biologically significant quantities of the chemotactic peptide IL-8, thus contributing to the recruitment of polymorphonuclear leukocytes [16]. Interleukin-6 on the other hand may act in both pro- and anti-inflammatory manner [26]. Although high levels of IL-6 are generated within the inflamed peritoneal cavity [27], its precise role in peritoneal infection is unknown. It is, however, believed that one of the important actions of IL-6 is to limit the extent of peritoneal inflammation, since it has been shown to downregulate the production of TNFα in activated monocytes [28,29].

In order to make a first step towards the elucidation of the cellular mechanisms by which dialysis fluids may interfere with the production of these mediators, we not only assessed the effect of the fluids on released cytokines but also evaluated their effect on cytokine messenger RNA accumulation. In this respect our results indicate that the subtotal inhibition of TNFα and IL-6 release observed following exposure of activated PBMC to conventional CAPD fluid is, at least in part, related to a reduction of steady-state cytokine messenger RNA levels. A similar reduction of cytokine mRNA was observed with high-glucose, bicarbonate-buffered fluid, in which case the release of cytokine proteins was slightly greater as compared to conventional dialysis fluid. On the other hand low-glucose bicarbonate fluid neither significantly interfered with cytokine gene transcription nor with the release of cytokine proteins. In contrast, glucose polymer dialysis fluid, when tested at its initial low pH, showed no clear effect on cytokine mRNA, but significantly reduced the release of both TNFα and IL-6, suggesting that its suppressive effect is most probably related to a post-transcriptional event. The precise mechanism of this inhibition is currently being investigated.

In summary, the pattern of inhibition observed with the different alternative dialysis fluids suggests that:

1. the replacement of lactate by bicarbonate, thus resulting in a physiological fluid pH, is sufficient to allow for a normal cytokine response provided that hyperosmolality and/or glucose concentrations are moderate (i.e. Bic20);

2. high-glucose, hyperosmolar bicarbonate fluid (i.e. Bic30) is severely suppressive despite its physiological pH;
the potential benefit of the physiological osmolality of glucose polymer dialysis fluid is reversed by its initial low pH.

In conclusion, replacement of conventional lactate-buffered glucose fluids by bicarbonate-buffered solutions may potentially result in an improved cell responsiveness in cases where only moderate glucose concentrations and hyperosmolality are needed. In patients with ultrafiltration problems and the requirement of high glucose/hyperosmolar CAPD exchanges, the glucose polymer fluid might be beneficial provided that its initial low pH is neutralized. In this respect it may be predicted that the biocompatibility of the glucose polymer fluid could be clearly enhanced by replacement of lactate by bicarbonate as the buffer system.

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