Continuous Dialysis with Bicarbonate/Lactate-Buffered Peritoneal Dialysis Fluids Results in a Long-Term Improvement in Ex Vivo Peritoneal Macrophage Function

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Abstract. To circumvent the potentially negative consequences of long-term exposure to unphysiologic acidic lactate-buffered peritoneal dialysis fluids (PDF), neutral pH solutions buffered with bicarbonate/lactate have recently been introduced in phase 2 and 3 clinical trials. This study examines the longitudinal changes in peritoneal macrophage (PMØ) function in patients dialyzed continuously with either lactate (LPD; 40 mM lactate, pH 5.2)-buffered or bicarbonate/lactate (TBL; 25 mM/15 mM bicarbonate/lactate, pH 7.3)-buffered PDF. Before the study, during the run in period of a phase 3 clinical trial, all patients had been taking LPD for at least the previous 18 wk. At the beginning of the study (day 0), both constitutive and serum-stimulated zymosan (STZ) stimulated tumor necrosis factor alpha (TNF-α) synthesis were assessed in PMØ isolated from 12-h dwell effluent (with 1.36% glucose) in all patients. The patients were subsequently randomized to either continuous TBL or LPD therapy and PMØ function was assessed after further 3- and 6-mo periods in all patients. At all time points measured STZ induced a dose-dependent increase in PMØ TNF-α secretion (P = 0.043 versus control for doses greater than 100 µg/ml). In patients continuously dialyzed with LPD, constitutive PMØ TNF-α synthesis levels (mean ± SEM, pg/10⁰ PMØ per18 h, n = 5 patients) were 154 ± 65, 261 ± 60, and 101 ± 99 at 0, 3, and 6 mo, respectively. Stimulated STZ (1000 µg/ml) levels were 1340 ± 519, 1046 ± 586, and 758 ± 250 at 0, 3, and 6 mo, respectively. In patients dialyzed with TBL, constitutive PMØ TNF-α synthesis levels (pg/10⁰ PMØ per 18 h, n = 5 patients) were 300 ± 136, 106 ± 35, and 213 ± 62 at 0, 3, and 6 mo, respectively. Stimulated STZ (1000 µg/ml) levels were 1969 ± 751, 1541 ± 330, and 2670 ± 671 at 0, 3, and 6 mo, respectively. At 6 mo, STZ-stimulated PMØ TNF-α synthesis was significantly higher in patients treated with TBL compared with those treated with LPD (P = 0.0035).

These data suggest that in patients continuously dialyzed with a neutral pH solution, there is a long-term improvement in PMØ function compared with patients on conventional therapy. Better PMØ function suggests improved host defense status and may affect the peritoneum’s susceptibility to infection and potentially reduce the negative consequences of repeated intraperitoneal inflammation on long-term membrane function.

Peritoneal dialysis (PD) has been successfully used as a form of renal replacement therapy for more than two decades. During this period, both in vitro and in vivo reports have demonstrated that conventional lactate-buffered PD solution formulations have bioincompatible characteristics. These are related to the solution acidity, its buffer composition, glucose content, and hyperosmolality (1).

One of the major inhibitory pathways by which conventional PD solutions modulate cell function is related to the combination of their acidity and lactate concentration (2). To address this issue, neutral pH solutions buffered with bicarbonate or bicarbonate/lactate mixtures have been introduced into clinical practice (3,4). The introduction of these solutions has been facilitated by the development of dual container systems (5). By use of a two-chambered design, bicarbonate and calcium are separated during the sterilization process, thus avoiding precipitation of calcium carbonate. In addition, the glucose compartment is maintained at low pH, which reduces the amount of glucose degradation product (GDP) formation (6).

Both in vitro and animal infusion studies with bicarbonate-based formulations have consistently shown improvements in almost all biocompatibility measurements compared with acidic, lactate-buffered solutions (7–9). Fewer data are available, however, regarding the effect of improved solution biocompatibility on parameters in vivo in PD patients. Ex vivo approaches have demonstrated changes indicative of improvements in both host defense status and membrane longevity in patients exposed in vivo to solutions designed to enhance biocompatibility (10–13). These studies have either assessed peritoneal macrophage (PMØ) function in cells isolated from acute dwell effluents or have assessed changes in peritoneal effluent markers (such as cancer antigen 125 and hyaluronic acid).
Previous ex vivo studies of PMØ function have been limited to assessment of cell function in effluents isolated after short in vivo dwell periods (usually 30 to 90 min). Although these experiments have provided evidence of improved cell function with more biocompatible solutions, they do not provide any indication of whether these potentially positive changes in host defense parameters are maintained during long-term, continuous exposure to different dialysis solutions. In addition, because the solutions are infused for only short periods of time, it has not yet been established whether the changes observed are maintained in the dialed peritoneum throughout the whole dwell period. To address this issue, we here evaluate the function of PMØ, as assessed by tumor necrosis factor alpha (TNF-α) release, isolated from overnight effluent during a 6-mo period of continuous dialysis with either lactate-buffered PD fluid (LPD) or bicarbonate/lactate-buffered PD fluid (TBL).

Our results demonstrate that in patients continuously dialyzed with TBL, PMØ function of cells isolated from equilibrated effluent show an increase in function after a 6-mo period of dialysis. In contrast, patients continuously dialyzed with LPD showed no temporal changes in either constitutive or stimulated TNF-α synthesis. These observations suggest that dialysis with more biocompatible solution is associated with a time-dependent improvement of peritoneal cell function, indicative of improved host defense and normalization of peritoneal homeostasis.

Materials and Methods

Patients

Ten patients in this study center (University of Wales College of Medicine, Cardiff, Wales) participating in a multicenter phase 3 bicarbonate/bicarbonate-lactate clinical trial were enrolled (14). All patients used the System 2 Freeline disconnect Y-system (Baxter Healthcare, Newbury, UK). All patients provided informed consent, and the study was performed under local ethical approval guidelines (Bro Taf Health Authority). Patient details are presented in Table 1. All patients had been on continuous ambulatory peritoneal dialysis for at least 5 mo before the beginning of the study. Six men and 4 women entered the study; the ages in the groups were (mean ± SD) 60.4 ± 11.9 and 62.6 ± 7.6 yr for the TBL and LPD groups, respectively. The duration of PD was 16 ± 13 and 19 ± 9 mo for the TBL and LPD groups, respectively. There were no significant differences in age or time on therapy between the different patient groups. During the study, there were three episodes of peritonitis, which occurred in two patients in the LPD group and in one patient in the TBL group (Table 1). The incidence of peritonitis was not significantly different between the patient groups. No side effects were observed that could be attributed to the use of any of the types of PDF used.

Study Design and Test Solutions

Patients were randomly allocated to each treatment group. Ten patients completed the study, five in the TBL group and five in the LPD group. In the run-in phase, all patients received their standard dialysis regimen (Table 1) with LPD (Dianeal PD4, 40 mM lactate, pH 5.2 to 5.3; Baxter Healthcare, Deerfield, IL). On day 0, the patients were randomized to each of the treatment groups for the 6-mo trial period. All patients received their standard dialysis regimen with either the LPD or TBL (25 mM bicarbonate/15 mM lactate, pH 7.3, pCO₂ = 48.2 ± 3.5 mmHg) containing either 1.36 or 3.86% dextrose. At time 0 (before randomization) and at 3 and 6 mo in the trial phase, ex vivo PMØ function was assessed as described below.

Ex Vivo Evaluation of Cell Function in Overnight Effluent

Previous experiments ex vivo studies have only been performed on short dwell effluents (11,12,15). To assess the effect of long-term exposure to the different dialysis solutions on PMØ function, rather than the previously described acute inhibitory effects, we elected to measure PMØ function in cells isolated from timed (12 h) overnight effluent. Each patient performed one overnight dwell PMØ function assessment immediately before randomization (time 0) when all patients were being dialyzed with LPD; this was repeated at both 3 and 6 mo in both the LPD and TBL arms of the study. All overnight dwells were performed with the appropriate solution (containing 1.36% glucose) for that arm of the study.

Serum-Treated Zymosan

Previous studies had identified that serum-treated zymosan (STZ) was an appropriate stimulus for PMØ TNF-α activation studies over the dose range examined. Boiled and washed zymosan (Sigma-Aldrich Ltd., Poole, UK) (40 mg) was incubated with 75% v/v pooled normal human serum (Sigma-Aldrich) in Hank’s balanced salt solution (Sigma-Aldrich) for 30 min at 37°C (16). After incubation, the STZ was washed three times with PBS and resuspended to 4 ml in RPMI 1640. This stock solution was formed into aliquots, stored at −70°C, and used as a stimulus at the final concentrations indicated. Fresh aliquots of the same batch of STZ were thawed immediately before each PMØ activation experiment (12).

Isolation and Stimulation of PMØ

PMØ were isolated from chilled overnight (12 h) peritoneal effluents and maintained in culture as described previously (12,17,18). Total cell numbers were calculated after automatic cell counting (Coulter ZM, Coulter Electronics, Luton, UK). The cells were washed in PBS, pH 7.3, and resuspended in RPMI 1640 tissue culture medium (Life Technologies Ltd., Paisley, UK) containing 0.2% w/v bovine serum albumin to a cell density of 1 × 10⁷/ml. Two milliliters of cell suspension was plated and PMØ was purified by 90-min adherence to 60-mm tissue-culture-grade dishes ( Falcon: Becton Dickinson, Oxford, UK). More than 95% of the adherent cells were consistently found to be PMØ, as assessed by their morphology, after staining withNeal differential hematology stain (Guest Medical Ltd., Sevenoaks, UK). The viability of isolated PMØ always exceeded 95% as assessed by cellular ATP levels (data not shown).

Before addition of the stimulus (STZ, 10 to 1000 μg/ml), the adherent cells were washed 3 times with warm tissue culture medium (RPMI 1640, Life Technologies). Washes were collected and pooled for calculation of total adherent cell numbers. PMØ were incubated, in duplicate, for 18 h at 37°C in an atmosphere of 5% CO₂ in the presence or absence of STZ. At the end of the incubation period, cell supernatants were removed, centrifuged (150 × g), formed into aliquots, and stored at −70°C before evaluation of cytokine content.

Cell Differentials

Before plating of peritoneal leukocytes, cytospin preparations of total peritoneal cells were prepared (Cytospin II, Life Sciences Ltd., Basingstoke, UK), air dried, and stained with Neal differential hematology stain (Guest Medical). Differential cell counts were performed by at least two independent blinded observers.
Table 1. Patient details

<table>
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<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Treatment Group</th>
<th>Time on Peritoneal Dialysis (mo)</th>
<th>Previous Episodes of Peritonitis</th>
<th>Peritonitis Episodes during Study</th>
<th>No. of Ex Vivo Dwells</th>
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a PTH, hyperparathyroidism; IHD, ischaemic heart disease; BP, hypertension; PO₄, hyperphosphataemia; ESI, exit site infection; PTx, parathyroidectomy; CVA, cardiovascular accident; 4 \times 1.36, 4 exchanges with 1.36% glucose solution; TBL, 25 mM bicarbonate/15 mM lactate peritoneal dialysis fluid (pH 7.4); LPD, 40 mM lactate peritoneal dialysis fluid (pH 5.2).
**TNF-α Assay**

TNF-α synthesis was quantitated in appropriate dilutions of PMØ supernatants by commercial enzyme-linked immunosorbent assay (Biotrak, Amersham-Pharomic Biotech [UK] Ltd., Amersham, UK) according to the manufacturer’s instructions. All determinations were performed with assay kits from the same batch. Minimal detectable concentration was 3 pg/ml (0 standard ± 2 SD). Intra-assay coefficient of variation was between 3.7 to 5.2%, and the interassay precision was between 7.8 to 8.3%. All TNF-α data are presented as picograms per $10^6$ PMØ after correction of cell numbers based on the total numbers of adherent cells calculated before stimulation. Final TNF-α values were determined by measuring the four individual values obtained in the duplicate stimulation experiments. Where dilution of samples was required, to obtain values within the standard range, serial dilution were performed to confirm the linearity of dilution.

**Statistical Analyses**

Statistical analysis was performed by the SAS (version 8.0; SAS, Cary, NC) procedure GLM (19). Factors in the model included the group effect, month effect, stimulation dose effect, stimulation time effect, and all two-way, three-way, and four-way interactions. Patients were treated as the random factor with repeated measures of month-dose-hour effects. The log TNF-α values were analyzed because the distribution was highly skewed. Values below 10 pg/mL were treated as 10 µg/mL when taking the log transformation.

It was apparent that the increase from zero dose depended on the treatment group, stimulation hours, and evaluation month. This was confirmed by the significant ($P = 0.0335$) 4-way interaction effect. Therefore, the analysis was performed by each combination of treatment group, stimulation hours, and evaluation month. Analysis focused on the treatment difference in terms of geometric mean ratios of TNF-α values at the 1000 µg/mL over 0 µg/mL stimulation doses (group by dose interaction).

**Results**

**Activation of PMØ TNF-α Synthesis by STZ**

As in previous studies, irrespective of the origin of the isolated PMØ or the trial time point, STZ (10 to 1000 µg/ml) induced a dose-dependent induction of PMØ TNF-α synthesis (12). These increases in PMØ TNF-α synthesis were significantly different to unstimulated controls at doses greater than 100 µg/ml ($P = 0.043$; data not shown).

In patients continuously dialyzed with LPD, constitutive PMØ TNF-α synthesis levels (mean ± SEM, pg/10^6 PMØ per 18 h, n = 5 patients) were 154 ± 65, 261 ± 60, and 101 ± 99 at 0, 3, and 6 mo, respectively. Stimulated STZ (1000 µg/ml) levels were 1340 ± 519, 1046 ± 586, and 758 ± 250 at 0, 3, and 6 mo, respectively (Figure 1).

In patients dialyzed with TBL, constitutive PMØ TNF-α synthesis levels (pg/10^6 PMØ per 18 h, n = 5 patients) were 300 ± 136, 106 ± 35, and 213 ± 62 at 0, 3, and 6 mo, respectively. Stimulated STZ (1000 µg/ml) levels were 1969 ± 751, 1541 ± 330, and 2670 ± 671 at 0, 3, and 6 mo, respectively. At 6 mo, STZ-stimulated PMØ TNF-α synthesis was significantly higher in patients treated with TBL compared with those treated with LPD ($n = 5, P = 0.0035$) (Figure 1).

**Differential Cell Counts**

Total cell numbers and the percentage of PMØ within the isolated peritoneal cell populations showed a large degree of...
variability both with time and between and within individuals during the study. There was, however, no significant relationship with time in any of the patient groups (data not shown). No significant differences were observed with time, between or within individuals, or between treatment groups in the phenotype of peritoneal effluent cell populations (PMØ, polymorphonuclear leukocytes, mesothelial cells, lymphocytes, or red blood cells) throughout the study (data not shown).

**Discussion**

In this study, we have examined the *ex vivo* function of PMØ isolated from the overnight dwell effluent of patients continuously treated with either LPD or TBL dialysis solutions for periods up to 6 mo. Previous *in vitro* studies have identified potential biocompatibility advantages of neutral pH PD fluids (PDF) buffered with bicarbonate or with combinations of bicarbonate with other buffers (7,8). The clinical relevance of these studies is, however, difficult to ascertain because the majority of these studies used a static design that does not mimic the equilibration that occurs in PDF once it is infused into the peritoneal cavity (20). De Fijter et al. (15,21) were the first group to demonstrate that when peritoneal cells were exposed *in vivo* to test solutions that their function was modulated after isolation and *in vitro* assessment. These pioneering *ex vivo* studies demonstrated that the composition of the infused solution (both pH and glucose concentration) could modulate PMØ function.

More recently, by means of a similar approach, we have compared the effects of acute *in vivo* exposure to lactate-, bicarbonate-, and bicarbonate/lactate-buffered PDF on PMØ (11,12). In these studies, we elected to use an acute *ex vivo* approach, followed by measurement of cell functional parameters. Initial short-term, randomized, single-dwell experiments (performed during a 2-wk period with both 1.36 and 3.86% glucose) demonstrated that PMØ function in neutral pH bicarbonate solutions—and to a greater extent bicarbonate-lactate solutions—was significantly enhanced compared with acidic lactate-buffered PDF (11). In subsequent experiments carried out during a 12-wk phase 2 clinical trial, these data were confirmed and extended in patients continuously dialyzed with test solutions (12). These latter data confirmed that PMØ function isolated from acute dwellswells in patients continuously dialyzed with bicarbonate/lactate-buffered solutions showed enhanced responsiveness compared with acidic lactate-buffered solutions.

These experiments have provided evidence of improved PMØ function with more biocompatible solutions; however, they do not provide any indication of the time scale over which these improvements occur or whether these potentially positive changes in host defense parameters are maintained during long-term continuous exposure to different dialysis solutions. In addition, because the solutions are infused for only short periods (30 min in each case), equilibration of pH, osmolality, and lactate concentration is not complete, particularly in acidic lactate-buffered solutions (22). Under these circumstances, it is not completely clear whether the differences observed in PMØ function are simply the result of differential equilibration, rather than an overall normalization of the peritoneal environment in patients treated with more biocompatible solutions. In this respect, measurements in isolated overnight effluent in patients treated with theoretically more biocompatible solutions has demonstrated changes indicative of reduced peritoneal inflammation and a normalization of mesothelial cell turnover (10,13).

To address these issues, in this study, we have evaluated the function (as assessed by TNF-α release) of PMØ isolated from overnight effluent in patients dialyzed continuously with the test solutions for extended periods. Previous studies have demonstrated that PMØ function increases with dwell time, reaching a maximum between 6 and 12 h dwell (11,12,15). These data suggest that in overnight effluent, any inhibitory effects observed are related to the overall effect of the solution on the cells within the peritoneal cavity rather than the acute inhibitory effects of solution components such as low pH. In addition, modulatory effects observed over time on dialysis may be related to the longer-term normalization of the peritoneal environment as a result of more biocompatible solution exposure (23).

In this study, continuous exposure to acidic lactate-buffered solutions was not associated with any time-dependent changes in either constitutive or STZ-stimulated PMØ TNF-α synthesis. In the stimulated PMØ, although there was a tendency toward decreasing TNF-α release with time of therapy, this change was not statistically significant. In patients treated with TBL, no changes were observed in constitutive PMØ TNF-α release during the trial period. In contrast, at all time points, PMØ function in patients continuously treated with TBL was elevated. At the 6-mo time point, this increase between the TBL and the LPD groups was highly significant. Significant variability was observed within each sampling period; thus, additional studies will be needed to confirm the results of this study.

Nevertheless, these data are in broad agreement both with our previous acute *ex vivo* studies and also with *in vitro* observations showing enhanced cell function in neutral pH bicarbonate- and bicarbonate/lactate-buffered solutions (11,12). These data suggest that in addition to the acute effects observed in short-dwell effluents, which appear to be directly related to the time-dependent equilibration process, that effects of exposure to more biocompatible solutions can be manifested during longer treatment periods. Whether the significant enhancement in cell function in the TBL group would be maintained for longer periods remains to be determined from ongoing studies. Experiments with intraperitoneal markers of inflammation, fibrosis, and mesothelial cell integrity, however, suggest that the observed changes are present during extended periods of dialysis with theoretically more biocompatible solutions (24).

The majority of the observed effects on cell function would appear to be related to the neutral pH of the TBL solutions; however, TBL also contains reduced levels of GDP that can modulate cell function *in vitro* (6,25,26). Whether reduced GDP levels contribute to the increased cell function seen in this study remains to be determined. Clinical and *ex vivo* marker
studies that used pH >6.0 lactate-buffered solutions with reduced GDP levels do modulate the peritoneal environment (13).

PD is now a major form of renal replacement therapy; its increased use has focused attention on those factors that limit technique and patient survival. Exposure to bioincompatible solutions is presumed to be one of the factors that affect the host’s resistance to infection, and long-term exposure is assumed to negatively affect peritoneal membrane structure and function (27–30). Data demonstrating direct effects of solution components on peritoneal host defense and peritoneal membrane structure/function relationships are limited; ex vivo effluent measurements of presumed markers of membrane integrity and inflammation, cell function, and the results of animal studies suggest that solution components are capable of modulating cell structure and function in vivo (9,10,13,31,32). The results of the study presented here suggest that the introduction of neutral pH bicarbonate/lactate-buffered solution is associated with long-lasting improvements in PMO function. Larger studies are now needed to confirm these observations.

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