

# Cytokine Production During *In Vitro* Hemodialysis With New and Formaldehyde- or Renalin-Reprocessed Cellulose Dialyzers<sup>1</sup>

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## ABSTRACT

Critics of reuse have suggested that patients treated with reprocessed dialyzers are exposed to pyrogen trapped from the water or solutions used during the reprocessing cycle, thereby triggering the synthesis and release of proinflammatory cytokines, resulting in cachexia. To test this hypothesis, the production of interleukin (IL)-1 $\alpha$  by peripheral blood mononuclear cells (PBMC) during *in vitro* dialysis with new or reprocessed cellulose dialyzers was compared. An *in vitro* closed-loop dialysis circuit was created with standard hemodialysis blood lines and either new cellulose dialyzers or dialyzers reprocessed 10 times with either formaldehyde/bleach (formaldehyde) or peracetic acid/hydrogen peroxide mixture (Renalin). The circuit was rinsed with 2 L or more of pyrogen-free normal saline before the start of *in vitro* dialysis until the blood compartment tested negative for residual formaldehyde/Renalin. Heparinized whole blood from healthy volunteers was circulated for 3 h in the blood compartment at 100 mL/min at 37°C. The dialysate compartment was sealed. Peripheral blood mononuclear cells (PBMC) were harvested from the blood compartment before and at the end of 3 h of *in vitro* dialysis. Total IL-1 $\alpha$  synthesis (cell associated plus secreted) by unstimulated and endotoxin-stimulated PBMC was measured by a specific, non-cross-reactive radioimmunoassay. After 3 h of *in vitro* dialysis, IL-1 $\alpha$  production (in picograms per 2.5 million PBMC) by unstimulated PBMC increased from 354  $\pm$  63 at baseline to 454  $\pm$  57 with new dialyzers ( $P = 0.25$ ), from 453  $\pm$  101 to 450  $\pm$  67 with formaldehyde-

reprocessed dialyzers ( $P = 0.98$ ), and from 360  $\pm$  61 to 538  $\pm$  144 with Renalin-reprocessed dialyzers ( $P = 0.23$ ). IL-1 $\alpha$  production by endotoxin-stimulated PBMC increased from 5,214  $\pm$  996 to 9,237  $\pm$  929 with new dialyzers ( $P < 0.001$ ), from 6,395  $\pm$  955 to 9,636  $\pm$  1,058 with formaldehyde-reprocessed dialyzers ( $P = 0.006$ ), and from 7,561  $\pm$  1,000 to 10,092  $\pm$  2,470 with Renalin-reprocessed dialyzers ( $P = 0.32$ ). However, there were no significant differences among groups with respect to IL-1 $\alpha$  production by unstimulated or endotoxin-stimulated PBMC either before or after 3 h of *in vitro* dialysis. These data argue against the suggestion that exposure to reprocessed dialyzers results in enhanced synthesis of proinflammatory cytokines. In fact, reprocessed dialyzers probably induce less cytokine production than do new cellulose dialyzers.

Key Words: Dialyzer reprocessing, cytokines, interleukin

The reuse of hemodialyzers was first introduced by Shaldon and colleagues in refrigerated coil dialyzers (1). Although dialyzer reuse was originally developed to reduce costs (2), the reprocessing of dialyzers with formaldehyde or peracetic acid was shown to attenuate complement activation induced by cellulose membranes, an effect believed to be due to plasma coating of the complement-activating hydroxyl residues (3-7). However, many centers that reprocess dialyzers with formaldehyde or glutaraldehyde also use sodium hypochlorite (bleach) as a cleansing agent. Bleach not only improves the appearance of the dialyzer, but also increases the number of reuses obtained because it strips the plasma proteins deposited on the membrane (8). Consequently, the use of bleach in the reuse process restores the complement-activating potential of cellulose membranes (6,9).

Recently, reuse practices in the United States have come under severe criticism, and enhanced cytokine production during dialysis with reprocessed dialyzers has been incriminated as a potential cause for the increased mortality in these patients (10,11). During hemodialysis, complement activation leads to the transcription of mRNA for several proinflammatory cytokines (12,13), but in the absence of a "second stimulus," the mRNA for these cytokines may not get translated into protein (13). L-Fucose residues on cellulose membranes have also been incriminated as a signal for cytokine production by mononuclear cells (14). The presence of small quantities of endotoxin can amplify cytokine production by these "primed" cells

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(12). Critics of reuse have suggested that patients treated with reprocessed dialyzers are exposed to pyrogen trapped from the water used during the reprocessing cycle or from the reprocessing solution itself (10,11). This exposure to pyrogen has been postulated to induce the synthesis and release of cytokines, resulting in cachexia. In order to test this hypothesis, we compared the production of interleukin (IL)-1 $\alpha$  during *in vitro* dialysis with new cellulose dialyzers, with dialyzers reprocessed with formaldehyde/bleach (formaldehyde) or a peracetic acid/hydrogen peroxide mixture (Renalin).

## METHODS

### Preparation of Dialyzers

Three conditions were studied.

### New Dialyzers

New dialyzers were rinsed with 2 L of pyrogen-free normal saline before the start of *in vitro* dialysis.

### Formaldehyde-Reprocessed Dialyzers

Dialyzers were procured from Dialysis Clinics Inc. (New England Medical Center, Boston, MA). The reprocessing procedure consisted of cleansing with reverse osmosis water, processing with sodium hypochlorite (<1%) with an automated system (DRS4; Seratronics Inc., Concord, CA), and disinfection by incubation with formaldehyde (0.75%) for 24 h at 40°C. Each dialyzer had been used on a patient 10 times and consequently was studied after being reprocessed 10 times. Before *in vitro* dialysis, the dialyzer was rinsed by an automated system with a dilute bicarbonate solution at 650 mL/min for 15 min (Quick-Rinse™).

### Renalin-Reprocessed Dialyzers

Dialyzers were procured from the Goddard Brockton Kidney Center (Stoughton, MA). The reprocessing procedure consisted of cleansing with reverse osmosis water and processing and disinfection with a peracetic acid/hydrogen peroxide mixture (Renalin; Renal Systems, Minneapolis, MN) with an automated system (Renatron II; Renal Systems). Each dialyzer had been used on a patient 10 times and consequently reprocessed 10 times.

### Water Treatment

The water treatment systems at the dialysis units from which the formaldehyde-reprocessed dialyzers and the Renalin-reprocessed dialyzers were procured were similar. The Dialysis Clinics Inc. (formaldehyde/bleach reprocessing) uses a water softener followed by reverse osmosis. The dialysis unit affiliated with Goddard Brockton Kidney Center (Renalin reprocessing) uses a water softener followed by reverse osmosis and UV light. Throughout the study, the water, reprocessing water, and dialysate water quality met the Association for Advancement of Medical Instrumentation (AAMI) requirements (15).

### *In Vitro* Dialysis

*In vitro* dialysis was carried out at 37°C with a water bath according to the protocols established in our laboratory. Briefly, an *in vitro* closed-loop dialysis circuit was created with standard hemodialysis blood lines (G555; Gambro Inc.,

Newport News, VA) and either a new or reprocessed 1.5-m<sup>2</sup> cellulose dialyzer (Clirans T150L; Terumo Medical Corporation, Somerset, NJ). The entire circuit was rinsed with 2 L of pyrogen-free normal saline before the start of *in vitro* dialysis or until the blood compartment tested negative for residual formaldehyde/Renaline. Of the 2 L used to rinse the dialyzer, 1 L was run through the blood compartment and 1 L was ultrafiltered. A total of 180 mL of heparinized blood (10 U/mL) was obtained from healthy volunteers. Of this, 30 mL was used to harvest peripheral blood mononuclear cells (PBMC) at baseline (baseline control) and 30 mL was set aside as a 3-h stationary control. The remaining 120 mL of heparinized blood was circulated for 3 h in the blood compartment at 100 mL/min. The dialysate compartment was sealed. At the end of 3 h, PBMC were harvested from the blood compartment as well as from the 3-h stationary control.

### *In Vitro* Production of IL-1 $\alpha$ by PBMC

The water and tissue culture media used in this study were subjected to ultrafiltration with a polyamide hollow-fiber ultrafilter (U2000; Gambro AB, Hechingen, Germany) to remove cytokine-inducing agents (16). PBMC were harvested as previously described (17,18). Briefly, each 10-mL sample of blood was diluted with 10 mL of sterile pyrogen-free normal saline (Abbott Laboratories, Rockford, IL) and underlayered with 10 mL of Ficoll-Hypaque. The tube was centrifuged at 450 *g* for 45 min at room temperature. The PBMC layer was harvested, washed in saline, and centrifuged at 400 *g* for 10 min. The cells were then resuspended in ultrafiltered tissue culture medium (RPMI 1640; pH 7.4; Sigma Chemical Co., St. Louis, MO), containing 10 mmol/L L-glutamine, 24 mmol/L NaHCO<sub>3</sub> (Mallinckrodt, Paris, KY), 10 mmol/L *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Irvine Scientific, Santa Ana, CA). PBMC were counted with a standard hemocytometer, a suspension of 5  $\times$  10<sup>6</sup> PBMC/mL was prepared in RPMI, and a 0.5-mL suspension of PBMC was aliquoted into two 12  $\times$  75 mm polypropylene tubes. To one tube, 0.5 mL of the RPMI was added (unstimulated PBMC), and to the second tube, RPMI containing 20 ng/mL endotoxin (*Escherichia coli*, serotype 055:B5, Sigma) was added (endotoxin stimulated; final concentration, 10 ng/mL). All tubes were incubated upright at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. At the end of 24 h, the tubes were frozen at -70°C. The cell suspensions were then subjected to two additional freeze-thaw cycles for total IL-1 $\alpha$  synthesis (cell-associated plus secreted) measurements. Lysates were added to radioimmunoassay (RIA) buffer (0.01 mol/L phosphate-buffered saline [pH 7.4], 0.25% bovine serum albumin, and 0.05% sodium azide) and total IL-1 $\alpha$  synthesis was measured by a specific, non-cross-reactive RIA (19). The IL-1 $\alpha$  concentrations were read from a logit plot of percent specific binding versus the log concentration of the serially diluted IL-1 $\alpha$  from the linear portion of the curve (usually between 35 and 85% specific binding). The lower limit of detection of the RIA for IL-1 $\alpha$  was 160 pg/mL.

To ensure uniformity of conditions, (1) the same healthy donor was used for each of the three experimental conditions; (2) the same membrane (cellulose) and same manufacturer (Terumo) were used in all experiments; (3) the dialyzers were reprocessed exactly 10 times; (4) the water treatment systems involved in the two methods of reprocessing were similar; and (5) experiments were performed by a single individual under identical laboratory conditions.

## Statistical Analysis

Statistical analysis was performed with the SYSTAT software package (SYSTAT Inc., Evanston, IL). Analysis of variance of repeat measures was used to detect differences between groups. All data are expressed as mean  $\pm$  standard error. Total IL-1 $\alpha$  production by PBMC is expressed as picograms per 2.5 million PBMC. Differences were considered significant if  $P < 0.05$ .

## RESULTS

A total of 13 healthy donors were recruited for the study. Of these, all 13 completed the "new dialyzer" limb of the study, 11 completed the "formaldehyde limb," and 9 completed the "Renalin limb."

### IL-1 $\alpha$ Production by Unstimulated PBMC

At baseline, IL-1 $\alpha$  production by unstimulated PBMC was  $354 \pm 63$ ,  $453 \pm 101$ , and  $360 \pm 61$ , respectively, with new dialyzers, formaldehyde-reprocessed dialyzers, and Renalin-reprocessed dialyzers (Figure 1). These differences were not statistically significant ( $P = 0.18$ ). After 3 h of *in vitro* dialysis, IL-1 $\alpha$  production by unstimulated PBMC was  $454 \pm 57$  with new dialyzers ( $P = 0.25$ ),  $450 \pm 67$  with formaldehyde-reprocessed dialyzers ( $P = 0.98$ ), and  $538 \pm 144$  with Renalin-reprocessed dialyzers ( $P = 0.23$ ). The increase in IL-1 $\alpha$  production by unstimulated PBMC was  $90 \pm 25\%$  with new dialyzers,  $31 \pm 26\%$  with formaldehyde-reprocessed dialyzers, and  $57 \pm 35\%$  with Renalin-reprocessed dialyzers. However, after 3 h of *in vitro* dialysis, IL-1 $\alpha$  production by unstimulated PBMC was not significantly different among groups ( $P = 0.50$ ). The increase in IL-1 $\alpha$  production by unstimulated PBMC from the stationary control was not significantly different from baseline in blood used for *in vitro* dialysis with new dialyzers (0.54), formaldehyde-reprocessed dialyzers (0.68), or Renalin-reprocessed dialyzers (0.10).

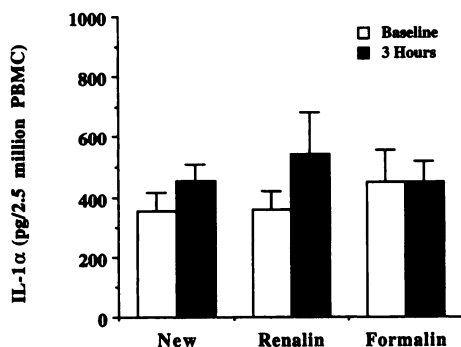


Figure 1. IL-1 $\alpha$  production by unstimulated PBMC before and after *in vitro* dialysis with new and reprocessed cellulose dialyzers. After 3 h of *in vitro* dialysis, there was an increase in IL-1 $\alpha$  production by unstimulated PBMC with new dialyzers, formaldehyde-reprocessed dialyzers, and Renalin-reprocessed dialyzers, but none of these increases was statistically significant.

### IL-1 $\alpha$ Production by Endotoxin-Stimulated PBMC

At baseline, IL-1 $\alpha$  production by endotoxin-stimulated PBMC was  $5,214 \pm 996$ ,  $6,395 \pm 955$ , and  $7,561 \pm 1,000$ , respectively, with new dialyzers, formaldehyde-reprocessed dialyzers, and Renalin-reprocessed dialyzers (Figure 2). These differences were not statistically significant ( $P = 0.11$ ). After 3 h of *in vitro* dialysis, IL-1 $\alpha$  production by endotoxin-stimulated PBMC increased to  $9,237 \pm 929$  with new dialyzers ( $P < 0.001$ ), to  $9,636 \pm 1,058$  with formaldehyde-reprocessed dialyzers ( $P = 0.006$ ), and to  $10,092 \pm 2,470$  with Renalin-reprocessed dialyzers ( $P = 0.32$ ). The increase in IL-1 $\alpha$  production by endotoxin-stimulated PBMC was  $169 \pm 57\%$  with new dialyzers,  $85 \pm 32\%$  with formaldehyde-reprocessed dialyzers, and  $48 \pm 35\%$  with Renalin-reprocessed dialyzers. However, after 3 h of *in vitro* dialysis, IL-1 $\alpha$  production by unstimulated PBMC was not significantly different among groups ( $P = 0.87$ ).

In contrast to the significant increase in IL-1 $\alpha$  production by endotoxin-stimulated PBMC after 3 h of *in vitro* dialysis with new and formaldehyde-reprocessed dialyzers, IL-1 $\alpha$  production by endotoxin-stimulated PBMC from the stationary control was not significantly different from baseline in blood used for *in vitro* dialysis with new dialyzers (0.48), formaldehyde-reprocessed dialyzers (0.42), or Renalin-reprocessed dialyzers (0.35).

## DISCUSSION

Over the last two decades, the use of reprocessed dialyzers in the United States has steadily increased from 18% of centers and 18% of patients in 1976 to 73% of dialysis centers and 79% of patients in 1993 (20,21). In the United States, the germicides currently used for the reprocessing of dialyzers include the

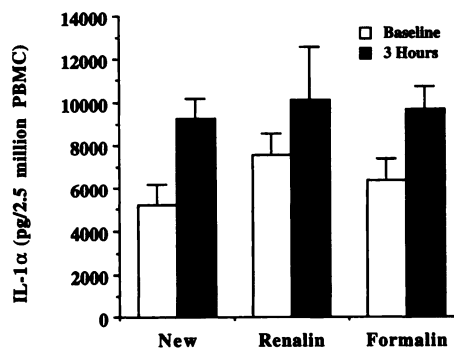


Figure 2. IL-1 $\alpha$  production by endotoxin-stimulated PBMC before and after *in vitro* dialysis with new and reprocessed cellulose dialyzers. After 3 h of *in vitro* dialysis, there was a significant increase in IL-1 $\alpha$  production by endotoxin-stimulated PBMC with new dialyzers ( $P < 0.001$ ) and formaldehyde-reprocessed dialyzers ( $P = 0.006$ ). Although there was also an increase in IL-1 $\alpha$  production by endotoxin-stimulated PBMC with Renalin-reprocessed dialyzers, the increase was not statistically significant ( $P = 0.32$ ).

peracetic acid and hydrogen peroxide-based Renalin (51% of centers), formaldehyde (40% of centers), glutaraldehyde (8% of centers), and heat as a physical germicide (1% of centers) (21). However, several authors have questioned the safety of reprocessed dialyzers and have suggested that the specific techniques or germicides used during reprocessing could be associated with a higher mortality in hemodialysis patients (10,11,22). In an analysis of 33,000 U.S. Medicare hemodialysis patients treated at 845 free-standing units during the years 1989 and 1990, Held and colleagues (22) observed that compared with units that did not reprocess dialyzers, the relative risk of death for patients treated in units that reprocessed dialyzers with glutaraldehyde, Renalin, and formaldehyde was 1.17, 1.13, and 1.06, respectively. However, the specific causes for this increased mortality in units that reprocess dialyzers are unclear.

During hemodialysis, human blood leukocytes come into contact with several exogenous challenges including the surface material of the dialyzer membrane, plasma products activated by the dialyzer materials, and substances (solutes and microbial products) from the dialysis bath. These interactions can trigger the synthesis of proinflammatory cytokines such as IL-1 and tumor necrosis factor (23–25). Indeed, during dialysis, new gene expression for cytokines occurs, and the intracellular content of IL-1 is elevated; plasma levels of cytokines are increased after dialysis. These cytokines have been implicated as the cause of several acute intradialytic symptoms as well as chronic dialysis-related morbidity (23,24). Unlike new dialyzers, reprocessed dialyzers do not carry a guarantee of sterility because the solutions used in the reuse process are not sterile (11). Consequently, there is a theoretical risk that fragments of endotoxin or other bacterial products could be retained in the blood compartment, perhaps providing a potent stimulus for the production of proinflammatory cytokines by blood mononuclear cells. Indeed, pyrogen reactions are more frequently encountered in hemodialysis units that reprocess dialyzers than in those that do not (20). On the basis of these observations, some authors have suggested that repeated stimulation of PBMC by bacterial products retained in reprocessed dialyzers and its consequences could be the cause of the increased mortality observed in patients treated in dialysis units that reprocess dialyzers (10,11).

To test the above hypothesis, we circulated whole blood through new and formaldehyde or Renalin-reprocessed dialyzers and compared the cytokine production by unstimulated and endotoxin-stimulated PBMC before and after *in vitro* dialysis. We observed that after 3 h of *in vitro* dialysis, the increase in IL-1 $\alpha$  production by unstimulated PBMC was not statistically significant with either new or reprocessed dialyzers. In contrast, a statistically significant increase in IL-1 $\alpha$  production by endotoxin-stimulated PBMC was observed with new dialyzers and formaldehyde-reprocessed dialyzers. Although an increase in IL-1 $\alpha$  pro-

duction by endotoxin-stimulated PBMC was also observed with Renalin-reprocessed dialyzers, this increase was not statistically significant. Exposure to low concentrations of endotoxin probably amplifies the differences between groups that were not apparent in unstimulated PBMC. Consequently, the modestly lower increase in IL-1 $\alpha$  production by endotoxin-stimulated PBMC with reprocessed dialyzers could be due to the partial plasma coating of cytokine-activating sites such as L-fucose residues. Although agents such as bleach are known to strip the plasma coating of dialyzers (6,8,9), this stripping may not be complete. Nonetheless, the results of this study argue against the suggestion that exposure to reprocessed dialyzers results in the enhanced synthesis of proinflammatory cytokines. In fact, reprocessed dialyzers induced less cytokine production than did new cellulose dialyzers.

In a recent study by Luft and colleagues, *in vitro* dialysis was performed with whole blood and new polyamide dialyzers, dialyzers reprocessed with solutions contaminated with 6 ng/mL *Pseudomonas putilla* filtrate (more than sixfold above the upper limit of the AAMI standard of 1 ng/mL), or dialyzers reprocessed with pyrogen-free solutions (26). After 2 h of *in vitro* dialysis, IL-1 $\alpha$  production by unstimulated PBMC was significantly higher in dialyzers reprocessed with solutions contaminated with *P. putilla* filtrate compared with dialyzers reprocessed with pyrogen-free solutions or new dialyzers (26). In contrast, we observed that new cellulose dialyzers stimulated more IL-1 $\alpha$  production in unstimulated or endotoxin-stimulated PBMC compared with reprocessed dialyzers. The differences between that study and ours could be that hydrophobic polyamide membranes are more likely to adsorb bacterial toxins than cellulose membranes (16). Further, the reprocessing solutions used by Luft and colleagues contained bacterial toxin levels that were more than sixfold above the upper limit of AAMI standards (15). Although neither of the dialysis units that provided reprocessed dialyzers for our study took any additional precautions to ensure the safety of water, both adhered to the standards recommended by the AAMI.

In summary, the results of our study argue against the suggestion that exposure to reprocessed dialyzers results in enhanced synthesis of proinflammatory cytokines. In fact, reprocessed dialyzers probably induce less cytokine production than do new cellulose dialyzers.

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