Induction, Transcription, Synthesis, and Adsorption of Interleukin-1 by Dialyzer Membranes

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ABSTRACT
This study was designed to dissect the direct effect of dialyzer membrane on interleukin-1 (IL-1) induction from those of complement activation, mechanical stimulation, acetate/bicarbonate and endotoxin diffusion, and cell type interactions. To this end, a suspension of P388D1 murine macrophages in a complement-free culture medium containing 10% heat-inactivated serum, a closed-loop system consisting of tubing alone or with a hollow-fiber cuprammonium cellulose (CU) or polyacrylonitrile (PAN) dialyzer, and a roller pump were used. The dialysate compartment was filled with the same medium and capped. Cell suspension was recirculated at 300 mL/min for 3 h. Cells and supernates were separated, and adhering proteins were eluted. All samples tested negative for endotoxin. IL-1 mRNA was greatest with CU, followed by PAN and tubing alone. IL-1 in the supernate was greatest with CU than with either tubing alone or PAN (P<0.005; analysis of variance), which showed comparable values. IL-1 eluted from loops was greatest with PAN dialyzers, followed by sets with CU dialyzers and tubing alone (P<0.001; analysis of variance). Thus, both CU and PAN membranes directly induce IL-1. However, avid adsorption by PAN attenuates the rise in circulating IL-1.

Key Words: Hemodialysis, renal failure, macrophage activation, polyacrylonitrile, cuprophan

Interleukin-1 (IL-1) induction by hemodialysis has been implicated in the pathogenesis of certain acute and long-term complications of hemodialysis such as febrile reaction, hypotension, headache, lassitude, osteoporosis, muscle atrophy, hyper-β-2-microglobulinemia, and dialysis amyloidosis (1–8). Several events may trigger cytokine production during hemodialysis. These include back filtration of endotoxin fragments (9–14), membrane-induced complement activation (15–18), leukocyte adhesion (19,20), acetate diffusion (21), and mechanical stress associated with the roller pump (22). In addition, products of platelet and granulocyte activation may influence cytokine induction. Accordingly, because of the inherent multiplicity of factors involved in the course of in vitro and in vitro hemodialysis, it has been difficult to dissect the direct role of dialyzer membrane on IL-1 induction in circulating monocytes. Likewise, because of the nonspecific effect of cell adhesion on cytokine induction (19,20), data derived from in vitro cell-membrane incubation experiments are inconclusive. Similarly, interpretation of IL-1 kinetics reported by Donati et al. (23) using in vitro dialysis of a peripheral blood mononuclear (PBMN) cell suspension is complicated by the heterogeneity of the PBMN cell population. This is because the IL-1 released from the monocytes can be avidly removed by numerically predominant lymphocytes possessing IL-1 receptors (24,25), thereby obscuring the potential rise in extracellular IL-1.

This study was designed to examine the direct effect of dialyzer membrane on IL-1 induction, independent of other compounding influences. To this end, we examined the transcription, synthesis, and release of IL-1α in P388D1 murine macrophages after in vitro dialysis with cuprophan (CU) and polyacrylonitrile (PAN) dialyzers. We used a suspension of P388D1 cells in a complement-free culture medium and a closed-loop system consisting of either tubing sets alone or a tubing set plus a dialyzer. The dialysate compartment was filled with the same medium and capped to avoid ultrafiltration. The following features of our experimental design helped to isolate the membrane-target cell interaction from the secondary influences: 1) the use of a homogeneous cell population eliminated the interindividual variations in response to various components of dialysis; (2) the cellular homogeneity of the suspension precluded possible interaction of macrophages with, or removal of IL-1 by, other cell types, e.g., endothelial cells, lymphocytes, platelets, or granulocytes; (3) the use of a complement-free culture medium eliminated the effect of complement activation; and finally, (4) the use of the same medium in both dialysate and blood
compartments precluded the effect of dialysate components such as acetate.

**MATERIALS AND METHODS**

**Cell Line**

P388D1 murine macrophages (ATCC, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM 1-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma, St. Louis, MO) at 37°C in 5% CO₂ and 95% air. The media were renewed two to three times weekly, and the cells were subcultured every 7 to 10 days.

P388D1 cells are murine macrophages known to secrete IL-1. The cell line was originally isolated from a methylcholanthrene-induced lymphoid neoplasm in DBA/2 mice. Because of their IL-1-inducible property, these cells are commonly used in studies concerned with IL-1 induction and regulation. We, therefore, used P388D1 cells, which constituted a sensitive and well-characterized system for these studies.

**In Vitro Dialysis Experiments**

The experiments were carried out by the use of a closed-loop system, which consisted of a reservoir containing cell suspension and arterial and venous blood lines (tubing) attached either to a dialyzer or to one another and powered by a roller pump (Figure 1). Hollow-fiber dialyzers made of CU (CS 1511; Baxter Inc., McGaw, IL) or PAN (AN69; Hospal Inc., Leon, France) membranes were used. In the preparation of the experiments, the system was rinsed with pyrogen-free saline. P388D1 cells were suspended in 300 mL of culture medium at a concentration of 2 x 10⁵ cells/mL. The fetal calf serum used in these experiments was heat Inactivated (56°C for 45 mm) in order to prevent cytokine induction by products of complement activation. The cell suspension was placed in a reservoir that was thermostatically controlled at 37°C and continuously agitated to prevent cell adhesion and to ensure proper mixing. The dialysate compartment was filled with the cell-free medium and capped to prevent ultrafiltration. The suspension was pumped out of the reservoir through the dialyzer, when present, and back to the reservoir at a rate of 300 mL/min for 3 h. Throughout the experiments, the cell suspension in the reservoir was continuously gassed with oxygen to avoid hypoxia. Samples were collected at 0 and 180 min. In other experiments, samples were obtained at 0, 30, 60 and 180 min. After centrifugation, the supernatant was removed and saved for IL-1α measurement, and the cells were used for RNA extraction and northern blot analysis.

**Elution Procedures**

Elution procedures were carried out to measure IL-1α adsorbed to dialyzer membrane and the tubing sets by a fractionated elution method as follows. In brief, the dialyzers were sequentially washed with the following eluents (150 mL each): saline (twice), 1 M Tris (pH 7.4) (twice), and 1% sodium dodecyl sulfate (SDS) in saline (once). Each eluent was circulated through the closed-loop system for 30 min. The solutions were then collected, concentrated to small volumes, and stored at -20°C until assayed for IL-1α measurement.

**Endotoxin Test**

All samples obtained before, during, and after each experiment were collected by sterile techniques. Samples were tested for the presence of endotoxin with Limulus Amebocyte Lysate (LAL), a pyrogen multitest system, which has a minimum sensitivity of 0.06 endotoxin unit (EU)/mL (Whittaker Bioproducts, Inc., Walkersville, MD). Briefly, each assay included serial twofold dilutions of the standard endotoxin, test sample, and LAL reagent water (negative control). A 0.1-mL aliquot of each of the above preparations of the test samples and standards was added to 0.1 mL of the LAL preparation. The contents were gently mixed and incubated at 37°C in a water bath for 1 h. The tube was then inverted 180 degrees and inspected for the presence of a gel clot. A firm clot adherent to the base of the tube was defined as a positive test. All specimens were run in duplicate, and experiments yielding positive test results were discarded.

**RNA Isolation, Northern Blot Analysis, and Densitometry**

RNA was isolated from P388D1 cells by a single-step method using acid guanidinium thiocyanate-phenol-chloroform extraction (26). Cells stimulated with lipopolysaccharide (LPS), 10 µg/mL, were used.
as positive controls, and cells collected before the experiments were used as negative controls. A 30-µg aliquot of total RNA from each sample was electrophoresed in a 1.2% agarose–2.2 M formaldehyde gel at 3 to 4 V/cm for 2 to 3 h. The gel was blotted for 18 to 24 h to a Gene Screen membrane (Dupont, NEN Research Products, Boston, MA) by capillary elution. Equivalency of the samples and lack of degradation were verified by ethidium bromide staining of the 28S and 18S rRNA bands. The blot was baked for 2 h at 80°C before prehybridization in 50% formamide, 5× sodium chloride–sodium citrate solution (SSC), 2× Denhardt’s reagent, 125 µg/mL denatured salmon sperm DNA, and 0.1% SDS for 3 h at 42°C. Mouse IL-1α probe was derived from a 2-kilobase BamH1-HindIII fragment of cDNA inserted into a PBluescript SK+ plasmid (ATCC) (27). A heat-denatured, 32P-labeled probe was added into the prehybridization solution and hybridized at 42°C with constant agitation for 16 to 20 h. The filter was washed twice in 2× SSC–0.1% SDS at room temperature for 10 min each and twice in 0.1× SSC–0.1% SDS at 60°C for 30 min each, followed by washing in 2× SSC–0.1% SDS at room temperature for 15 min. An autoradiograph of the blot was developed after a 3- to 5-day exposure to Kodak AR film (Eastman Kodak, Rochester, NY) at −70°C. The density of each band was measured with a laser densitometer (PD121: Molecular Dynamics, Sunnyvale, CA). On each occasion, the optical density of IL-1 mRNA was normalized to that of the constitutively released actin gene expression.

IL-1α Measurement

Mouse IL-1α concentration in both the supernate and eluate was determined with a commercial ELISA kit with a minimum sensitivity of 15 pg/mL (Genzyme, Cambridge, MA) according to the manufacturer’s protocol. Briefly, a 96-well microplate was pre-coated with antimouse IL-1α monoclonal antibody. Blocking solution was added and incubated at 37°C for 40 min. The samples and standards, a biotinylated polyclonal antimouse IL-1α antibody, and avidin-peroxidase conjugate were added successively. Each incubation was carried out at 37°C for 40 min, and in between the incubations, the plate was washed four times with washing buffer. Finally, the substrate tetramethylbenzidine solution was added, and absorbance was measured 4 to 5 min later at 450 nm by an ELISA reader (ICN, Costa Mesa, CA). A standard curve was constructed by plotting the concentrations of the mouse IL-1α in serial dilutions of the standard against the resulting absorbances. The IL-1α concentration in the test sample was determined by interpolation of the absorbance values into the standard curve. In an attempt to avoid interassay variations, all samples were tested in a single session. The intra-assay variation of the assay was 5.6%.

Data Analysis

Analysis of variance (ANOVA) and t test were used in analysis of the data, which are expressed as mean ± SE. P values equal to or less than 0.05 were considered significant.

RESULTS

mRNA Response

The autoradiograph of a representative blot illustrating the bands obtained with LPS-stimulated (positive control) and unstimulated cell suspensions (negative control) and those subjected to a 3-h recirculation through the closed loops with PAN and CU dialyzers and tubing alone is shown in Figure 2A. As can be seen, the visual density of the band obtained with the CU dialyzer is slightly greater than that observed with the PAN dialyzer. The bands observed with both dialyzers were denser than that found with the tubing alone. These visual observations were confirmed by densitometry, which allowed comparative quantitation of the optical densities of mRNA bands for IL-1α normalized against those of the corresponding actin mRNA bands for all experiments (Figure 2B).

IL-1α Levels

IL-1α levels found in the supernates, the eluates, and the sum thereof (total IL-1α) for all experiments are displayed in Table 1. The amount of IL-1α present in the supernates obtained from the CU dialyzer experiments was significantly greater than that observed with experiments using either PAN or the tubing alone (P < 0.005; ANOVA). No significant
The optical densities of mRNA bands obtained at 30, 60, and 180 min of Cu dialysis relative to that observed at time 0 (given an arbitrary value of 1) were 1.06, 1.65, and 2.13, respectively.

**DISCUSSION**

In this study, we attempted to examine the direct effect of dialyzer membrane on IL-1 induction, independent of that of the secondary processes inherent to in vivo and in vitro hemodialysis and in vitro incubation experiments. The results showed that both Cu and Pan dialyzers markedly promote transcription, synthesis, and release of IL-1α by P388D1 cells. In addition, recirculation through the tubing set and roller pump alone was associated with a low-grade induction of this cytokine.

Endotoxin fragments have been shown to diffuse from dialysate to blood compartment and promote cytokine production (9–14). Endotoxin and other bacterial products did not play a part in IL-1 induction here because we used contaminant-free media/ reagents and used strict aseptic measures in the course of the experiments. In addition, we tested samples from the blood and dialysate compartments for LAL-reactive material and discarded all experiments that yielded a positive test.

Exposure to the dialyzer membranes, particularly those made of cellulose derivatives, can activate the complement system (26–32). Products of complement activation, particularly C5a, cause granulocyte/monocyte aggregation, pulmonary leukostasis, and transient leukopenia early in the course of hemodialysis (30). Simultaneously, complement activation triggers cytokine induction (15–18). In this study, we excluded the effect of complement activation by substituting plasma with a complement-free culture medium. In addition, the small amount of fetal calf serum used to supplement the medium was heat inactivated. Therefore, IL-1 induction observed in this study was not related to membrane-induced complement activation.

High concentrations of acetate present in acetate-based dialysates have been shown to stimulate cytokine production by blood monocytes (21). In this study, we filled the dialysate compartment with the same medium as that used in the cell suspension, thereby excluding the role of dialysate constituents.

Mechanical stresses associated with the roller pump may cause monocyte activation and cytokine induction (22). Because roller pumps were present in the closed-loop systems used in our experiments, it may have, partly, contributed to the observed IL-1 induction. In fact, the low-grade induction seen with the tubing set alone may have been largely due to this phenomenon. It should be noted, however, that because a roller pump was common to all experiments here, it cannot account for differences between data obtained from experiments with Cu, Pan, or no dialyzer. Moreover, the magnitude of IL-1 induction with the tubing set alone was relatively small as compared with that seen when either Cu or Pan dialyzers were present, suggesting the predominant effect of the dialyzer.

Membrane interaction and mechanical stimulation during hemodialysis cause platelet and granulocyte activation, adhesion, and degranulation (33). These events may potentially contribute to monocyte/mac-

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<tr>
<th>Supematant</th>
<th>Eluate</th>
<th>Total</th>
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<tr>
<td>Tubing</td>
<td>359.7 ± 89.1</td>
<td>10.7 ± 12.3</td>
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<tr>
<td>PAN</td>
<td>333.9 ± 88.0</td>
<td>182.3 ± 48.6</td>
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<tr>
<td>CU</td>
<td>551.0 ± 32.7</td>
<td>81.7 ± 32.0</td>
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ANOVA P < 0.005 P < 0.000 P < 0.000

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* N = 4 for all experiments. Data are given as picograms per 10^7 cells.

* P < 0.05 tubing versus Cu.

* P < 0.01 tubing versus Cu.

**TABLE 1. Amounts of IL-1α in the supernate, eluate, and the combination thereof (total) recovered after 3 h of continuous recirculation of a suspension of P388D1, murine macrophages in a closed loop consisting of hemodialysis tubing alone (tubing) or tubing and either Pan or Cu dialyzers.**
rophage activation and cytokine production. The use of a single-cell population in this study practically excluded the possibility of such cell-type interactions. In addition, the system used here precluded the use of anticoagulants, minimized the removal of IL-1 by target cells, and helped to maximize cell-membrane interaction by minimizing membrane coating by plasma proteins. Moreover, continuous recirculation of the cell suspension in the \textit{in vitro} system precluded the sequestration of the activated cells and the metabolic clearance of IL-1, which occur \textit{in vivo}. Accordingly, the system used here served to maximize the cumulative actions of the membrane while minimizing the sequestration of the activated cells and the elimination of the IL-1 produced.

We emphasize that this study was solely designed to examine the direct effect of membrane-macrophage interaction on IL-1 induction. Therefore, the design advantages noted above are meant to apply only to the delineation of this narrow objective as opposed to that of the much more complex interaction between blood and dialysis membrane.

In a recent study, Donati et al. reported a marked increase in the PBMN cell contents of IL-1\(\beta\) during \textit{in vitro} hemodialysis with both CU and polysulfone dialyzers (23). These observations were confirmed by \textit{in vitro} dialysis experiments using a suspension of PBMN cells (23). The dialysis membrane–induced rise in IL-1 mRNA in the macrophage cell line shown in this study is consistent with the rise in PBMN cell-associated IL-1 reported by Donati et al. However, despite increased cell-associated IL-1, those investigators found no rise in IL-1 concentration in the circulating medium \textit{in vitro} and a paradoxical fall in plasma IL-1 \textit{in vivo} (23). In contrast to those observations, the rise in IL-1 mRNA in the macrophage cell population used in this study was associated with the appearance of substantial amounts of extracellular IL-1, indicating synthesis and release of this cytokine.

The reason for the observed disparity between the two studies is uncertain and may be related to the species differences, \textit{i.e.}, the use of a mouse macrophages in this study versus human PBMN cells in the latter study. However, an alternative and more plausible explanation may lie in the use of a pure IL-1-producing cell population here and that of a mixture of IL-1-producing (monocytes) and IL-1-receiving cells (T and B lymphocyte) in the study reported by Donati et al. (23). By virtue of possessing high- and low-affinity IL-1 receptors, the numerically predominant T and B lymphocytes (24,25) present in the PBMN cell mixture can avidly capture IL-1 (released by the activated monocytes) from the surrounding medium, thereby obscuring the potential rise in extracellular IL-1 concentration. This can account for the reported lack of IL-1 rise in the circulating medium, despite its elevated total cell-associated pool shown in the latter study (23). In fact, an associated up-regulation of IL-1 receptors on lymphocytes and endothelial cells may, partly, account for the paradoxical fall in plasma IL-1 level reported by those authors during \textit{in vivo} hemodialysis (23). Thus, the mere lack of a rise in circulating IL-1 may not necessarily exclude the occurrence of IL-1 release and as such may not be a reassuring sign.

Although a consensus exists as to the direct or indirect cytokine-inducing properties of such complement-activating membranes as CU, the available data on more biocompatible membranes (e.g., PAN, polysulfone, polyamide, and hemophane) are contradictory. In this regard, cell-associated IL-1 has been shown to rise during hemodialysis with polysulfone dialyzers by some investigators, suggesting cytokine induction (23). In contrast, other investigators have found no rise in IL-1\(\beta\) mRNA in blood samples obtained 5 min after the onset of clinical hemodialysis with polysulfone, PAN, hemophane, or polyamide dialyzers (34). In yet another study, a rise in cell-associated IL-1\(\beta\) was noted after the completion of, but not during, hemodialysis with PAN dialyzers and was attributed to the back filtration of endotoxin fragments (35). The results of this study clearly demonstrate that the biocompatible PAN membrane can directly induce transcription and translation of the IL-1 gene, leading to IL-1 release.

It was of interest that the amount of IL-1 detected in the supernates from the PAN experiments was similar to that seen with the tubing set alone, despite a marked mRNA production with this membrane. However, the recovery of large amounts of IL-1 in the eluates from the sets containing PAN dialyzers excluded the possibility of a translation or secretion block. Instead, avid adsorption to the PAN membrane of IL-1\(\alpha\) accounted for the apparent discrepancy. In contrast, a lower adsorption combined with a greater induction by CU membrane resulted in much higher supernatant IL-1 than that seen in the PAN experiments. These observations are consistent with previous studies that demonstrated avid adsorption by PAN membrane of radiolabeled IL-1 (20) and complement activation products (32).

In conclusion, this study has demonstrated that both CU and PAN membranes directly induce IL-1 transcription, synthesis, and release by P388D1 cells, independent of complement activation, cell-type interaction, endotoxin exposure, dialysate constituents, and pump-related mechanical stresses. However, avid adsorption by PAN membrane prevented the rise in circulating IL-1 above that seen with the tubing set alone. Therefore, the distinction between CU and PAN membranes with respect to their effect on IL-1 relates more to their difference in adsorption rather than to the induction of this cytokine.
REFERENCES


