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Detection of endotoxin-like interleukin-1-inducing activity during in vitro dialysis

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Detection of endotoxin-like interleukin-1-inducing activity during in vitro dialysis. In order to study the integrity of dialysis membranes to pyrogens, the dialysate side of a closed loop hemodialysis (HD) circuit was challenged with *E. coli* microfiltrate containing 500 ng/ml endotoxin. Three solutions, a) tissue culture medium/saline, b) 5% human serum albumin, and c) 10% fresh human plasma, were circulated in the blood loop for five hours. Samples drawn from the blood side were assayed for interleukin-1 (IL-1)-inducing activity on human mononuclear cells (MNC) in vitro. No IL-1-inducing substances were detected when saline or culture medium was circulated in the blood loop. Circulating 5% human serum albumin revealed IL-1-inducing activity in the samples drawn only after five hours of HD. However, the addition of 10% fresh human plasma to the blood side resulted in the appearance of an IL-1-inducing substance(s) after 15 minutes of HD. After 30 minutes, maximum IL-1-inducing activity was observed (control stimulation index, 3.30 ± 0.67 SEM vs. 7.59 ± 1.50, $P < 0.02$). The IL-1-inducing activity of the samples was completely inhibited by polymyxin B, a cationic antibiotic which blocks the IL-1-inducing activity of endotoxin. Additional experiments demonstrated that in vitro MNC IL-1-production induced by the same *E. coli* microfiltrate is enhanced in the presence of 10% plasma. These studies demonstrate that: (a) in the presence of plasma, IL-1-inducing factors pass into the blood compartment of a dialysis system challenged with bacterial pyrogen; and (b) MNC production of IL-1 is enhanced in the presence of plasma. Since the Limulus test is influenced by plasma, in vitro MNC-IL-1-production provides a more reliable and relevant assay to determine dialysis membrane permeability for pyrogens.

Fever and symptomatic hypotension are well known phenomena complicating routine hemodialysis (HD) therapy [1–8]. The fall in blood pressure during HD is frequently accompanied by nausea, myalgia, headache, lassitude, and sleepiness. Some of these symptoms also occur in patients suffering from injury, infection, or inflammatory diseases and are known as acute phase responses [9]. This first step in host response is mediated by a family of polypeptides called interleukin-1 (IL-1) with molecular weights of approximately 17000 daltons. Two distinct IL-1 gene products have been cloned [10, 11] and recombinant human IL-1 has been used to confirm its multiple biological properties [12]. IL-1 causes decreased plasma zinc and iron levels, increased hepatic synthesis of several acute phase proteins, induces PGE_2 production resulting in fever, and stimulates PGI_2 and platelet activating factor production which are potent vasodilators [13, 14]. Many of these changes can occur following hemodialysis, including symptomatic hypotension, and it has been proposed that these acute phase responses may be due to IL-1 produced during HD [15–17].

Although the dialysis membranes are considered to be impermeable for endotoxin (ET), fever during HD is associated with bacterial contamination in the dialysate [4–8]. Humans are most sensitive to the IL-1-inducing property of endotoxin in vivo (3 ng/kg of *E. coli* ET produce fever) and human monocytes produce IL-1 in vitro when incubated with ET concentrations as low as 50 pg/ml [18]. We have recently demonstrated that fresh human blood circulated through the blood side of a closed-loop dialysis circuit is stimulated to produce IL-1 when ET is present on the dialysate side [19]. In contrast, the Limulus amebocyte lysate (LAL) test has repeatedly failed to demonstrate the passage of ET into the blood compartment under various conditions despite highly contaminated dialysate [20, 21]. Since these studies [19–21] found positive results using whole blood but not saline in the blood compartment, it seems likely that proteins or other plasma components are necessary to demonstrate the presence of endotoxin-like substances in the fluid phase of the blood compartment. Although the LAL test is able to detect ET in water or saline at concentrations as low as 5 to 10 pg/ml, assaying plasma samples with the Limulus test is not useful due to false positive reactions caused by plasma components, such as thrombin [22]. In addition, human monocytes respond also to pyrogenic substances such as muramyl peptides which are negative in the Limulus assay. Therefore, in vitro human monocyte-IL-1 production seems to be a more reliable assay than the LAL test to determine dialysis membrane permeability for pyrogens [23]. In addition, if dialysate is contaminated, it consists of live bacteria and enzymatic degradation products of ET rather than purified endotoxin as we and others have used it previously [19–21].

Therefore, the present study was designed to clarify the mechanism of IL-1-induction during in vitro dialysis with contaminated dialysate. To test the effect of plasma components, in vitro experiments were performed using three different solutions to fill the blood side: (a) saline/cell culture medium; (b) 5%

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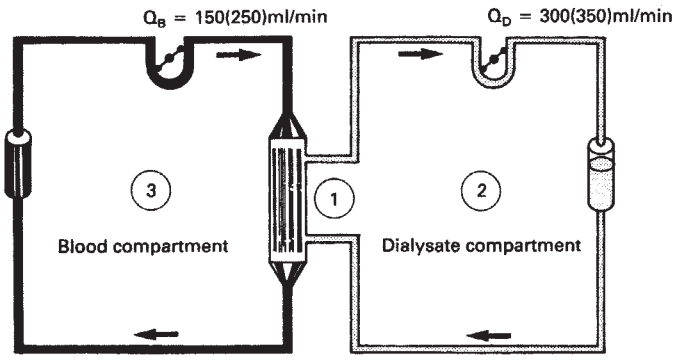

Fig. 1. *Flow diagram of the in vitro dialysis circuit.* Abbreviations are: QB, "blood" flow, QD, "dialysate" flow; 1, hollow fiber dialyzer (regenerated cellulose); 2, dialysate compartment (DC); 3, blood compartment (BC). Two different dialyzers were used: (**A**) 12 μm membrane dialyzer, 0.8 m^2 surface area. DC volume = 150 ml containing MEM without or with *E. coli* filtrate, maintained at 37°C, Q_D = 300 ml/min. BC volume = 150 ml containing MEM, 5% albumin or 10% plasma, 37°C, Q_B = 150 ml/min. (**B**) 15 μm membrane dialyzer, 0.6 m^2 surface area. DC volume = 1200 ml containing saline without or with purified endotoxin, maintained at 37°C, Q_D = 350 ml/min. BC volume = 1100 ml containing saline or 5% albumin, 37°C, Q_B = 250 ml/min.

human serum albumin in saline; and (c) 10% fresh human donor plasma in cell culture medium. We also used two different, regenerated cellulosic membranes to test the influence of membrane thickness. In addition, to simulate in vivo conditions, we used an *E. coli* culture microfiltrate (0.22 μm) rather than purified ET to challenge the dialysate side.

Methods

Preparation of E. coli culture microfiltrate

E. coli were grown in brain-heart infusion broth in a shaking water bath at 37°C for 24 hours. The cells were pelleted by centrifugation and the supernatant was filtered through a 0.22 μm filter. Sterility was confirmed on blood agar plates. The ET concentration in the filtrate was approximately 50 μg/ml measured by the Limulus amebocyte lysate (LAL) test (Pyrotell, Associates Cape Cod, Woods Hole, Massachusetts, USA). The filtrate was stored at 4°C until it was used to challenge the dialysate side of the in vitro dialysis circuit.

The in vitro dialysis circuit

We used a closed loop dialysis system as shown in Figure 1. The blood lines were standard hemodialysis line sets (G555, Gambro Inc., Newport News, Virginia, USA). Two different, regenerated cellulose capillary dialyzers were used: (a) 12 μm membrane dialyzer with a surface area of 0.8 m^2 (Terumo Corp, Tokyo, Japan); and (b) 15 μm membrane dialyzer with a surface area of 0.6 m^2 (Asahi Medical Co Ltd, Tokyo, Japan). Figures in this paper employ "A" for conditions of the 12 μm and "B" for the 15 μm dialyzer. The system was assembled under aseptic conditions and the blood and dialysate compartments were each rinsed with 2 liters of sterile, pyrogen free saline. In experiments using the 12 μm dialyzer, the saline in the dialysate side was replaced by sterile minimal essential medium, pH 7.4 (MEM, Microbiological Associates, Walkerville, Maryland, USA) containing 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. LAL testing in these media detected 10 to 100 pg/ml ET. The sterile medium in the dialysate loop (volume = 150 ml) was circulated at a flow rate of QD = 300 ml/min. After one hour of sterile HD, 1.5 ml of *E. coli* culture microfiltrate was added to the dialysate loop (dilution factor = 1:100). Fifteen minutes later, the LAL test detected an ET concentration of approximately 500 ng/ml in the dialysate. The *E. coli* microfiltrate was circulated for an additional four hours. The blood compartment (150 ml) contained one of the following: (a) sterile MEM; (b) 5% human serum albumin (Plasbumin-5, Cutter Biological, Berkely, California, USA); or (c) 1:10 dilution of fresh human plasma (heparinized with 10 U/ml) in MEM. As control, 10% plasma in MEM was circulated for five hours against sterile MEM in the dialysate compartment. In all experiments, the solution in the blood side was circulated at a flow rate of QB = 150 ml/min. Both loops were kept at 37°C and circulated in a countercurrent manner for five hours.

Samples from the dialysate loop were drawn at 15 minutes of sterile HD and 75 minutes (15 minutes after the addition of the *E. coli* microfiltrate). Samples from the blood loop were drawn at 15, 30, 45, 60 minutes (sterile HD) and 75, 90, 105, 120, 300 minutes (*E. coli* microfiltrate) of circulation. Every sample (3 ml) was replaced by an equal volume of the corresponding medium to maintain a constant circulating volume in both compartments. All samples, including a relevant medium control (no contact to the dialysis circuit), were pre-incubated for two hours at 37°C in the presence or absence of 5 μg/ml polymyxin B (PmB) (Pfizer Inc., New York City, New York, USA), a inhibitor of ET-mediated monocyte stimulation. Following this, all samples were assayed for IL-1-inducing activity on human mononuclear cells (MNC). In plasma experiments, the same donor provided both plasma and cells.

Using a 15 μm dialyzer (surface area 0.6 m^2), similar experiments were performed with saline as dialysate. The blood compartment (volume: 1100 ml, QB = 250 ml/min) contained either saline or 5% human serum albumin in saline. After a short time of sterile HD (30 minutes in saline experiments, 5 minutes in albumin experiments) the dialysate side (volume: 1200 ml, QD = 350 ml/min) was challenged with 25 mg purified endotoxin (phenol extracted lipopolysaccharide from *E. coli*, Sigma, St. Louis, Missouri, USA). One hour later, an ET concentration of 28 μg/ml was measured in the dialysate by the LAL test (Pyrogent, Buk-Mallickrodt, Dietzenbach, FRG). With saline present in both compartments, in vitro HD was performed for 90 minutes and samples were drawn from the circuit after 30 minutes (blood side) and 90 minutes (both sides). Using 5% albumin in the blood compartment, HD was performed for five hours and samples were drawn after five minutes (blood side), 10 minutes and five hours (both sides). All samples were assayed for IL-1-inducing activity on human MNC and for ET in the LAL test.

IL-1 measurement

Thymocyte assay. MNC were separated by Ficoll Hypaque gradient centrifugation and resuspended in MEM containing 2% heat-inactivated human AB serum, at a concentration of 5×10^6 cells/ml. Samples drawn from the in vitro dialysis system were incubated in triplicate (0.1 ml/well) with equal volumes of MNC

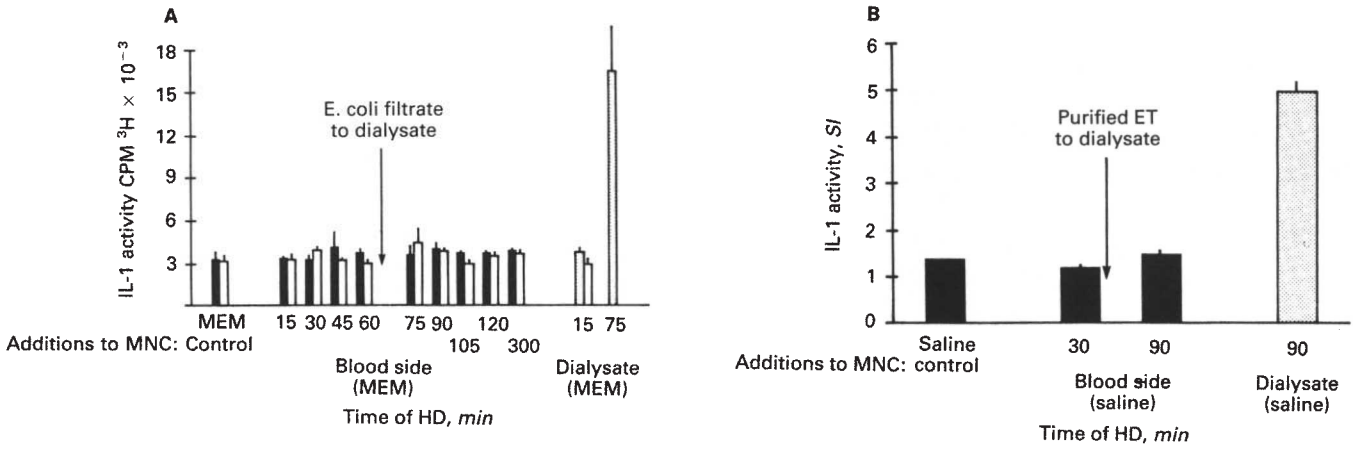

Fig. 2A. *IL-1 activity in samples drawn from the circuit containing MEM in both compartments (12 μm membrane).* Solid bars depict IL-1 activity induced by BC samples without PmB; open bars depict BC samples with 5 μg/ml PmB. Stippled bars depict IL-1 activity induced by DC samples without PmB (open bar = 5 μg/ml PmB). IL-1 production from control MNC is indicated as MEM. The numbers under the horizontal axis indicate the time (minutes of in vitro HD) when samples were removed and added to MNC. The amount of IL-1 is expressed as mean of triplicate CPM of ^{3}H-thymidine incorporation into thymocytes ± SD. The results represent a single experiment. **(B)** IL-1 activity by samples drawn from the circuit containing saline in both compartments (dialyzer: 15 μm, 0.6 m^2 surface area). Solid bars = BC samples, stippled bars = DC sample. Saline = Control IL-1 production from MNC. The numbers under the horizontal axis indicate the time (minutes of in vitro HD) when samples were removed and added to MNC. The amount of IL-1 is expressed as SI and represents the mean (± SEM) of three separate experiments.

suspension in 96 well, flat-bottom microtiter plates (A/S Nunc, Roskilde, Denmark). During 24 hours of incubation, the cells were kept at 37°C and exposed to an atmosphere containing 5% CO_2. After this period, the supernatants of the triplicate samples were removed, pooled in sterile 1.5 ml microcentrifuge tubes and spun for five minutes at 4°C. The samples were diluted 1:10 in RPMI 1640 containing 10 mM HEPES (both from Microbiological Associates, Walkersville, Maryland, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Grand Island Biological Co. Grand Island, New York, USA) and 5 × 10^{-5} M 2-mercaptoethanol (Eastman Kodak, Rochester, New York, USA) and then assayed for IL-1 activity on murine thymocytes. ET-resistant C3H/HeJ mice (4 to 6 weeks old, Jackson Laboratories, Bar Harbor, Maine, USA) were used as the source of thymocytes. The preparation of the murine cells and the IL-1 assay were performed as described previously [24]. The thymocytes were resuspended in RPMI 1640 containing 5% fetal calf serum (Sterile Systems, Logan, Utah, USA) and used in a final concentration of 2.5 × 10^6 cells/ml. Just before use, 1 μg/ml of the mitogen phytohemagglutinin (PHA, Burroughs-Wellcome Co. Research Triangle Park, North Carolina, USA) was added to the cells. Triplicate samples of each MNC supernatant (0.1 ml/well) were added to equal volumes of the thymocyte suspension in microtiter plates (NUNC). Following incubation for 48 hours in a humidified 5% CO_2 atmosphere at 37°C, the samples were pulsed with 1 μCi/well ^{3}H-thymidine (6.7 Ci/mM, New England Nuclear, Boston, Massachusetts, USA) and incubated for an additional 18 hours. Lysed cellular material was collected on glass fiber filter paper with a cell harvester, added to organic scintillation solvent (Ready-solv., Beckman Instruments, Fullerton, California, USA) and incorporated radioactivity was determined with a liquid scintillation counter (Beckman Inst.). Since responsiveness to both PHA and an IL-1 standard varies from one thymocyte preparation to the next, all experimental and associated control samples were tested simultaneously using the same cell preparation, identical media composition and incubation conditions.

In single experiments, IL-1 activity is expressed as mean of triplicate counts per minute (CPM) of incorporated ^{3}H-thymidine ± standard deviation (SD). To pool the results of several experiments, IL-1 activity is expressed as a stimulation index (SI) where the background activity of ^{3}H-thymidine incorporation into murine thymocytes incubated with PHA alone was assigned a value of 1. Data from the latter experiments are given as the mean ± standard error of the mean (SEM). Significance was assessed using the Student's *t*-test for paired values.

Radioimmunoassay for IL-1 beta. We used an IL-1 beta (^{125}I) RIA Kit (Cistron Biotechnology, Pine Brook, New Jersey, USA). The sensitivity of the assay was 50 pg/ml IL-1 beta within a confidence limit of 95%.

Results

Effect of non-protein solution in the blood compartment

As depicted in Figure 2A, there was no IL-1-inducing activity in the fluid phase of the blood side from the 12 μm dialyzer within one hour of HD against sterile MEM or during four hours of HD against *E. coli* filtrate (data represent a single experiment). This finding is similar to results of experiments in which there was no IL-1-inducing activity in the blood loop of the dialyzer with dialysate containing approximately 28 μg/ml of purified endotoxin (Fig. 2B, $N = 3$). In the latter study, all samples drawn from the blood side were negative in the LAL test (Pyrogent, sensitivity: 15 pg/ml).

Effect of albumin in the blood compartment

When the dialyzer was challenged with *E. coli* filtrate, IL-1-inducing activity on MNC was detected in samples drawn from the blood side containing 5% human serum albumin (Fig.

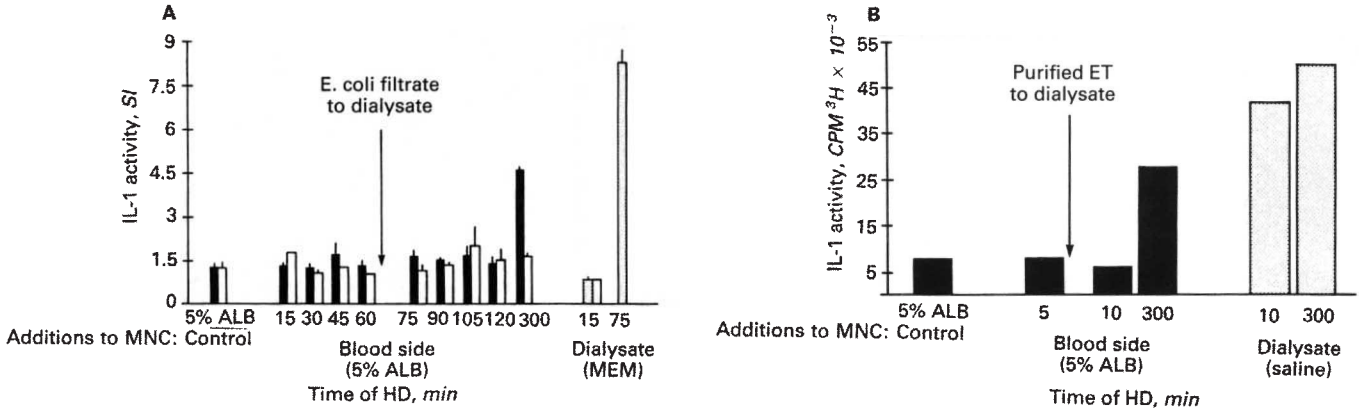

Fig. 3A. *IL-1 activity by samples drawn from the circuit filled with 5% human serum albumin in saline (5% ALB) in BC and MEM in DC (12 μm membrane).* Solid bars depict stimulation of IL-1 production by BC samples without PmB; open bars depict those samples with 5 μg/ml PmB. Stippled bars depict IL-1 activity induced by DC samples without PmB (open bar = 5 μg/ml PmB). Five percent ALB depicts control IL-1 production from MNC. The numbers under the horizontal axis indicate the time (minutes of in vitro HD) when samples were removed and added to MNC. IL-1 activity is expressed as SI and represents the mean (± SD) of two separate experiments. **(B)** IL-1 activity induced by samples drawn from the circuit containing 5% human serum albumin in saline (5% ALB) in BC and saline in DC (dialyzer: 15 μm, 0.6 m^2 surface area). Solid bars = BC samples, stippled bars = DC samples. Five percent ALB = control IL-1 production from MNC. The numbers under the horizontal axis indicate the time (minutes of in vitro HD) when samples were removed and added to MNC. IL-1 activity is expressed as mean of triplicate CPM (SD in all samples <10%), and represents a single experiment.

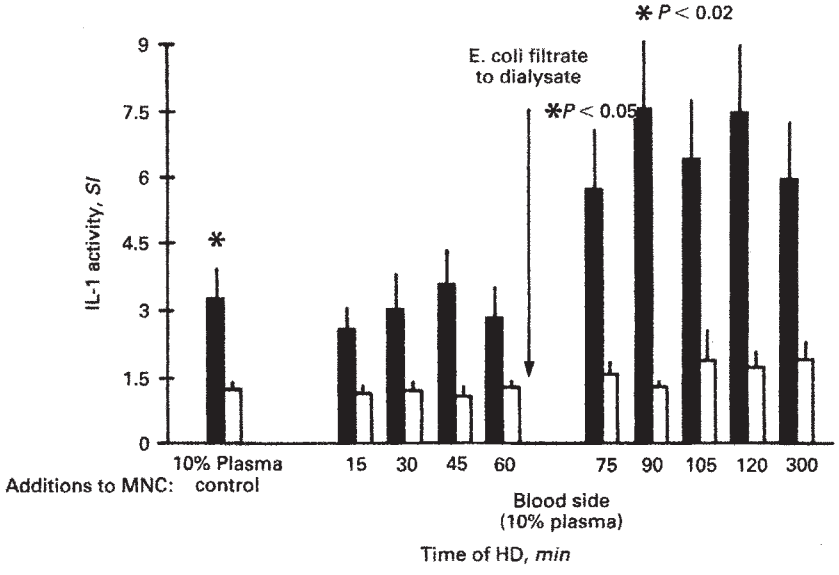

Fig. 4. *IL-1 production from human MNC incubated with samples taken from BC containing 10% fresh human plasma in MEM (12 μm membrane).* Solid bars depict stimulation of IL-1 production by samples without PmB; open bars depict those samples with 5 μg/ml PmB. Control IL-1 production from MNC is indicated as 10% plasma. The numbers under the horizontal axis indicate the time (minutes of in vitro HD) when samples were removed and added to MNC. The amount of IL-1 is expressed as SI (±SEM) and represents the mean of 7 separate experiments.

3A, $N = 2$). Compared to the control (5% albumin), only the sample drawn after 300 minutes of HD induced a significant amount of IL-1. This IL-1-induction was blocked by PmB. In another experiment using 5% albumin in the blood loop of a 15 μm dialyzer and purified ET (28 μg/ml in saline) in the dialysate, we observed comparable results (Fig. 3B, a single experiment). In contrast, the Limulus assay did not detect any positive material in the 300 minute, blood loop sample.

Effect of 10% human plasma in the blood compartment

We next circulated *fresh* human plasma in the blood compartment. Figure 4 compares IL-1 production by MNC incubated with samples drawn from the blood compartment containing a 1:10 dilution of *fresh* human plasma in MEM to IL-1 production by MNC incubated with the control solution of 10% plasma in MEM (no contact to the dialysis circuit, $N = 7$). All plasma samples, including the control, induced MNC-IL-1-production. IL-1 induction was blocked by PmB. Compared to the control (SI = 3.30 ± 0.67), no significant IL-1 production was induced by the samples drawn during one hour of sterile HD. In contrast, after the dialysate side was challenged with *E. coli* filtrate, the IL-1-inducing activity in the blood side increased to 5.77 ± 1.37 ($P < 0.05$) within 15 minutes (75 minutes of HD) and to 7.59 ± 1.50 ($P < 0.02$) within 30 minutes (90 minutes of HD). The samples drawn after 105, 120 and 300 minutes of HD induced a similar amount of IL-1 ($P < 0.02$). When 10% plasma

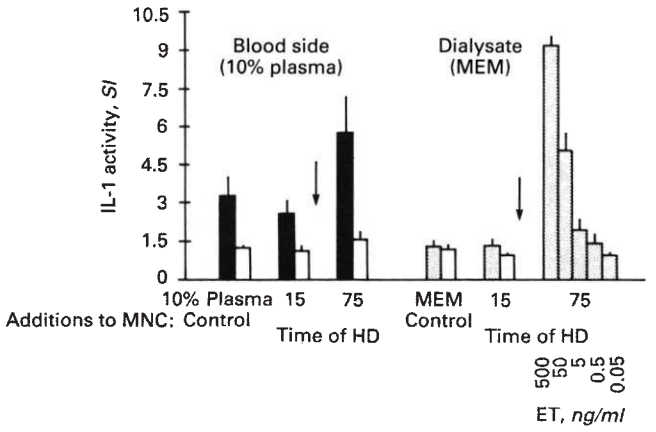

Fig. 5. *IL-1 activity induced by samples drawn from both compartments at two time points.* BC samples including the control are the same as shown in Figure 4 (10% plasma, 15 and 75 minutes). Symbols are: (■) without PmB; (□) with 5 μg/ml PmB; (□) DC samples containing MEM (↓) E. coli filtrate to dialysate. The 15 minute DC sample is depicted without (stippled bar) and with (open bar) PmB. The 75 minute DC sample is depicted in log dilutions. MEM indicates control IL-1 production by MNC relevant for DC. The amount of IL-1 is expressed as SI (± SEM) and represents the mean of 7 separate experiments.

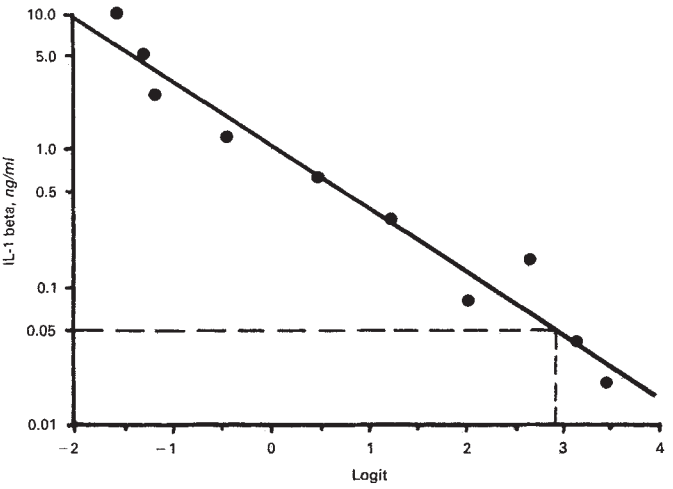

Fig. 6. *Standard curve for the IL-1 beta RIA.* The standards were prepared in MEM containing 10% plasma. The gamma CPM of each standard were converted into x = % zero standard. On the horizontal axis, x is expressed as logit x = LN (x/(100−x)). The concentration of IL-1 beta standards is depicted on the y axis in ng/ml (logarithmic scale). The detection limit within a 95% confidence is indicated by the broken line.

in the blood compartment was circulated against sterile MEM as the dialysate for five hours, no significant IL-1 production was induced by any sample (data not shown).

Figure 5 shows the IL-1 production induced by blood side samples and dialysate samples drawn at the same time ($N = 7$). Compared to the corresponding control, no significant IL-1 production was induced by the samples drawn after 15 minutes of HD. After 75 minutes of HD (15 minutes after challenging the dialysate compartment with *E. coli* filtrate), significant IL-1-inducing activity was detected in both blood side and dialysate side samples.

To estimate the amount of IL-1-inducing activity penetrating the dialysis membrane, various concentrations of *E. coli* filtrate diluted in MEM were compared to the activity in the 10% plasma sample drawn at 75 minutes (right side of Fig. 5). The amount of IL-1-inducing activity in the 10% plasma sample was similar to the activity found in the 1:10 dilution of the *E. coli* filtrate. To determine the influence of 10% plasma in the MNC assay, log dilutions of *E. coli* filtrate in either MEM or MEM containing 10% *fresh* human plasma where tested on MNC. After incubation, 10% plasma was added to the plasma-free samples to reach comparable conditions in the IL-1 assays. Figure 6 shows the standard curve of the RIA in the presence of 10% plasma. As measured in the RIA, ET concentrations of 5 to 5000 ng/ml induced up to 10 times as much IL-1 beta under the conditions of plasma present in the MNC incubation (Fig. 7A). In addition, 50 ng/ml ET in MEM containing 10% plasma and 5000 ng/ml ET in MEM alone induced a similar amount of IL-1 beta. Since the detection limit of the RIA was 50 pg/ml IL-1 beta, we assayed MNC supernatants also on murine thymoctes. As shown in Figure 7B, IL-1 activity is induced by ET concentrations as low as 50 pg/ml. Again, MNC IL-1 production is enhanced in the presence of plasma: 50 pg/ml ET in 10% plasma induced as much IL-1 as 500 pg/ml ET in MEM alone. Therefore, the human monocyte response to the *E. coli* culture filtrate seems to be enhanced one hundred-fold under the conditions of fresh human plasma present in the incubation.

In addition, MNC were incubated in the presence or absence of *E. coli* filtrate (50 pg/ml LAL-reactive material) in: (a) MEM alone; (b) MEM containing 10% fresh plasma; or (c) MEM containing 10% fresh serum (same donor's blood was used as a source for plasma and serum). Compared to the relevant control, an increased IL-1 production only occurred in the presence of *plasma plus ET.*

Discussion

These results demonstrate that ET-like IL-1-inducing substances penetrate the seemingly intact, regenerated cellulose membrane. However, in order to demonstrate this, plasma needs to be present in the blood loop.

In this study, we investigated the influence of various in vitro conditions on the ability of pyrogens present in the dialysate to penetrate dialyzer membranes. To simulate contamination conditions which occur in dialysate, we used a filtrate of an *E. coli* bacterial culture. The concentration of 500 ng/ml, as measured by the LAL test, was used to challenge the dialyzer and this amount of ET has been demonstrated in contaminated dialysate used in hemodialysis units [6]. Using protein free solutions such as saline or cell culture medium in the blood side, neither IL-1-inducing activity nor LAL-positive materials could be isolated from the fluid phase of the blood compartment. This agrees with previous studies by other investigators [21] who used the kinetic LAL method. Since the LAL test is known to detect low concentrations of ET in water or saline (5 to 10 pg/ml), evidence is provided that under rinsing conditions as performed prior to routine hemodialysis therapy less than 5 pg/ml ET is present in the fluid phase of the blood loop.

Circulating 5% human serum albumin in the blood loop resulted in the detection of IL-1-inducing activity. After either four or five hours of in vitro HD against ET-contaminated

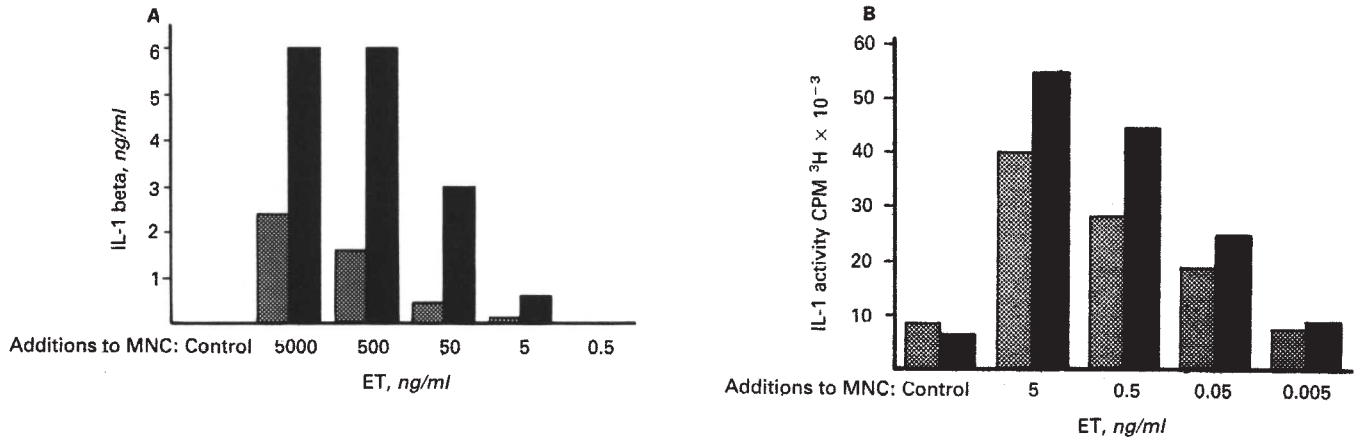

Fig. 7A. *IL-1 production from human MNC incubated with various concentrations of E. coli filtrate (ET), by RIA measurement in duplicate.* Closed bars depict IL-1 beta induced by ET diluted in MEM containing 10% *fresh* human plasma; stippled bars depict those dilutions in MEM alone. Control indicates IL-1 from MNC in medium alone (10% plasma or MEM) (below level of detection). Data represent one single experiment. **(B)** IL-1 production from human MNC incubated with various concentrations of *E. coli* filtrate (ET), as measured in the thymocyte assay. Closed bars depict IL-1 activity induced by ET diluted in MEM containing 10% plasma; stippled bars depict those dilutions in MEM alone. Control indicates IL-1 production induced by medium alone (10% plasma or MEM). The amount of IL-1 is expressed as mean of triplicate CPM of ^{3}H-thymidine incorporation into thymocytes.

dialysate, IL-1-inducing activity was isolated from the fluid phase of the blood compartment. Consistent with other studies [20], the LAL test did not detect any positive material in these samples. In spite of negative LAL results we assume that the IL-1-inducing activity is due to an ET-like substance because the IL-1 induction in these samples is blocked by PmB. The most plausible explanation is that ET-like materials penetrate the membrane in very low concentrations and become concentrated over time by binding to albumin. In addition, no difference was observed dependent on membrane thickness or ET preparation (Fig. 3A and B). The albumin-bound ET is not easily detected in the Limulus assay [25, 26] but recognized by human MNC. These data support previous studies emphasizing the difficulties in attempting to demonstrate ET in protein-containing solutions using the LAL test [22].

In the experiments performed with *fresh* human plasma as the blood replacement and *E. coli* filtrate in the dialysate, IL-1-inducing activity was isolated from the blood side as early as 15 minutes after challenging the dialysate side. This rapid appearance of IL-1-inducing substances in the fluid phase of the blood loop may be explained by several factors. It is known that ET purification with hot phenol results in the formation of large molecular aggregates (10^6 to 10^7 daltons) which are excluded by the dialysis membrane. However, after five hours of recirculation at 37°C, desintegration of the large ET aggregates may take place and smaller subunits of ET such as lipid A penetrate the membrane. Several investigators have described ET subunits less than 1000 daltons isolated from human body fluids [27]. These smaller fragments may be generated enzymatically in the *E. coli* filtrates. Size exclusion chromatography and gel electrophoresis have been used to detect small ET subunits with biological activity [27]. The *E. coli* filtrate used in the present studies was subjected to high performance liquid chromatography which revealed a peak of LAL reactive material eluting between 10 and 20,000 daltons.

Although the size of ET subunits in *E. coli* filtrates may play

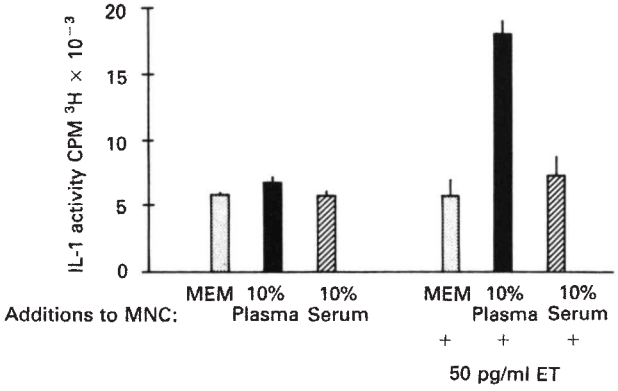

Fig. 8. *IL-1 production from MNC incubated with 50 pg/ml E. coli filtrate (ET) (on the right side).* Culture medium contained MEM alone (stippled bars), 10% *fresh* plasma in MEM (closed bars) or 10% *fresh* serum (striped bars). IL-1 induction by media alone (control) is depicted on the left side. The amount of IL-1 is expressed as mean of triplicate CPM of ^{3}H-thymidine incorporation into thymocytes ± SD.

a role in these results, the effect of plasma in the blood side seems to be of major importance. The data shown in Figure 5 suggest that approximately 10% of the IL-1-inducing activity in the dialysate compartment is detectable in the blood compartment. Since *fresh* plasma itself augments the IL-1-inducing activity of *E. coli* filtrate in the order of 100-fold (Fig. 7A and B), the results depicted in Figure 5 represent penetration of less than 0.1% (less than 500 pg/ml ET). It is unclear what is responsible for this enhancing effect of *fresh* plasma. Since *fresh serum* drawn from the same donor does not contain this enhancing activity (Fig. 8), it seems unlikely, the effect is due to complement activation. Since the IL-1-inducing activity recovered in the plasma from the blood side was blocked by PmB, this activity is probably related to endotoxin related substances

or lipid A-containing fragments. The reduction of IL-1-inducing activity in control (no contact to dialysis) samples suggest that plasma likely enhances the small amount of ET present in the tissue culture medium.

The reason plasma samples drawn from the blood side during sterile HD also stimulate IL-1 remains to be explained. Since this IL-1 induction is also blocked by PmB, it is possible that small amounts of ET remaining in the blood lines or the dialyzer membrane after sterilization are solubilized by the 10% plasma solution but not by rinsing the system with saline. Although we can not exclude accidental contamination of the circuit by drawing the samples, this is unlikely because there is no difference in the IL-1-inducing activity found in the control and the samples drawn during sterile HD and, in addition, all MNC cultures were bacteriologically sterile. Although there is evidence that the regenerated cellulosic membrane itself stimulates C5a which may result in IL-1 production [28–31], the amount of C5a which would be present during MNC incubation in the 5% plasma solution (final concentration) is below that which was necessary to stimulate IL-1 production. However, we cannot exclude complement activation during in vitro dialysis with *fresh* plasma as playing a role in IL-1 induction. Since plasma enhances IL-1-induction by substances entering the blood compartment, evidence is provided that plasma components may play a role in the onset of the acute phase response during hemodialysis [16, 17]. Under in vivo conditions, plasma is present in the blood loop at a concentration of about 60% rather than 10%, and thus efforts should be directed to reducing the pyrogen content of dialysate used in routine hemodialysis.

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