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Removal of Interleukin-1β and Tumor Necrosis Factor from Human Plasma by *in Vitro* Dialysis with Polyacrylonitrile Membranes

PILAR BARRERA, ELLY M. JANSSEN, PIERRE N.M. DEMACKER, JACK F.M. WETZELS, JOS W.M. VAN DER MEER

ABSTRACT

We studied the suitability of *in vitro* dialysis with polyacrylonitrile (PAN) membranes to remove small amounts of interleukin-1β (IL-1β) and tumor necrosis factor (TNF) from plasma to be used as diluent for the standards in radioimmunoassays (RIA). Incubation of PAN membrane fragments with radiolabeled IL-1β or TNF yielded a significant binding of both cytokines to the membrane (percentage of membrane-bound cytokine after incubation in saline or plasma was 14–17% and 23–46%, respectively). Dialysis of plasma (containing radiolabeled cytokine) against plasma (initially devoid of cytokine) resulted in a binding percentage of IL-1β and TNF to the PAN membranes of 44 and 28%, respectively. When plasma was dialyzed against saline the percentage of membrane-bound IL-1β and TNF was 63 and 37%, respectively. After dialysis of plasma against either plasma or saline the percentage IL-1β recovered from the dialysate was approximately 16% in contrast with 1–2% TNF. The results confirm the capacity of *in vitro* dialysis with PAN membranes to remove IL-1β and to a lesser extent TNF from plasma. Removal is most marked in the first minutes of dialysis (suggesting saturation of the membrane) and less effective for TNF due to its low diffusion across the membrane.

INTRODUCTION

In radioimmunoassays, the selection of an appropriate diluent for the standards is essential. To avoid loss of parallelism and to improve sensitivity, the diluent should possess a matrix similar to the sample. In the assay of circulating cytokines as interleukin-1β (IL-1β) and tumor necrosis factor (TNF), cytokine-free plasma should preferably be used as diluent. As normal pooled plasma usually contains variable amounts of these cytokines, its purification before use as diluent for the standards is indicated.

Previous studies by Lonnemann et al. (1,2) have shown that *in vitro* dialysis with polyacrylonitrile (PAN) membranes can remove IL-1 and TNF from protein-free tissue culture medium or whole blood. This prompted us to evaluate the suitability of *in vitro* dialysis for the depletion of cytokines from plasma to be used as diluent in cytokine radioimmunoassays. Whether dialysis procedures are able to effectively remove circulating IL-1β and TNF or not could also be important in clinical situations such as renal failure, septic shock, and adult respiratory distress syndrome (ARDS) where dialysis or continuous arteriovenous hemofiltration (CAVH) is often used as therapeutic measure.

MATERIALS AND METHODS

Iodination of human IL-1β and TNF

Recombinant human IL-1β (kindly provided by Glaxo, Geneva, Switzerland) and TNF (kindly provided by Dr. G.R. Adolf, Boehringer, Vienna, Austria) were labeled with a modification of the chloramine-T method as described elsewhere (5 μg of protein was labeled with 0.5 mCi ^{125}I) (3). Free iodine

was removed by Sephadex G-25 filtration (Pharmacia, Upsala, Sweden) and the labeled cytokines were stored at 4°C for up to 2 months.

Prior to use, any remaining free iodine was again removed by Sephadex G-25 M column chromatography. Fractions were collected and two peaks of ^{125}I activity were found. Fractions in the first peak containing ^{125}I-bound cytokine were used.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to screen the radiolabeled cytokines for the presence of polymers or decay products. Samples containing 20,000 cpm of radiolabeled IL-1β, TNF, and calibration markers (14, 24, and 60 kDa) were diluted with buffer containing sodium dodecyl sulfate (SDS). A discontinuous 9–13% separation gel and a 4.5% stacking gel were used. The gel was stained with Coomassie Brilliant Blue to estimate the molecular weight of the samples by comparison with the molecular weight calibration markers. Dried gels were subjected to autoradiography using Kodak films (Kodak X-OMAT(R) AR).

The relative amount of radioactivity in each lane was estimated by densitometric scanning (Ultroscan XL laser densitometer, Pharmacia, Upsala, Sweden).

Incubation of PAN membrane fragments with radiolabeled IL-1β or TNF

PAN membranes removed from a new dialyzer were cut in fragments and rinsed with saline. Incubation was performed in polystyrene Petri dishes (50 mm diameter; Nunc, Denmark) and in chambers previously described as the Teflon film dish. This film dish consists of a reusable aluminum holder with a Teflon ring (inner diameter 35 mm) in which a membrane can be mounted (4).

In the Petri dishes, PAN membrane fragments of 13.5 cm^2 were immersed in the fluid phase. In the film dish only one surface of the membrane fragment (9.62 cm^2) was in contact with the fluid phase. Each Petri dish and film dish was filled with 4 ml human plasma or saline to which ^{125}I-labeled IL-1β or TNF was added (approximately 1000 to 200,000 cpm). After 6 h shaking incubation at room temperature, radioactivity in the fluid phase and adhered to the membrane was determined.

The results are expressed as percentage of radioactivity recovered after incubation. The sum of radioactivity recovered in the fluid phase and membrane after correction for blanks, was set at 100%.

In vitro dialysis

A plate dialyzer with polyacrylonitrile (PAN) membrane (AN 69s; Biospal SCU/CAVH Al-U Kit, Hospal NL 5400 AC Uden, The Netherlands) was used. Dialysis was performed in a closed-loop dialysis circuit as shown in Fig. 1.

Both blood and dialysate compartments were rinsed with 2 liters of pyrogen-free saline and flushed with air before filling. To avoid gradients across the membrane, dialysis of plasma against plasma was performed in six experiments; frozen
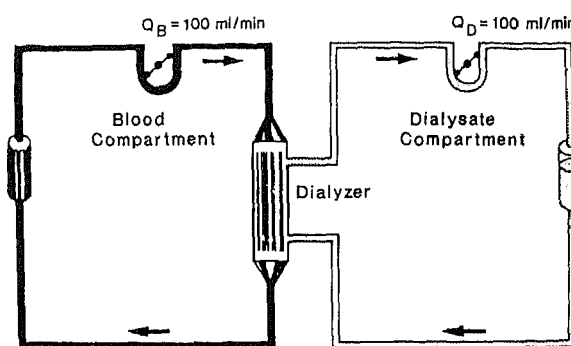

FIG. 1. Diagram of *in vitro* dialysis circuit. Q_B and Q_D, flow in blood and dialysate compartment. Mean fill volumes of the blood and dialysate compartment were 117 and 100 ml, respectively.

plasma from the blood bank was thawed and used to fill both compartments. The blood compartment contained ^{125}I-labeled recombinant human IL-1β or TNF (approximately 10^4 cpm/ml plasma equivalent to less than 30 ng per dialysis), while the plasma in the dialysate compartment did not contain radiolabeled cytokine. Dialysis of plasma against saline was performed in two experiments: the blood compartment contained plasma with radiolabeled IL-1β or TNF and the dialysate compartment was filled with saline free of radiolabeled cytokine. The solutions in both compartments were circulated for 1 h in a countercurrent manner at a flow rate of 100 ml/min. The dialysis was performed under sterile conditions at room temperature. To determine time-dependent removal of the cytokines, samples (0.5 ml) were drawn every 5 min from both compartments. After dilution with 2 ml saline the samples were treated with an equal volume of 20% trichloroacetic acid (TCA), centrifuged, and the precipitates measured in a gamma counter. In all experiments, the non-TCA precipitable radioactivity during the dialysis procedure was negligible (<5%). The percentage of TCA precipitable radioactivity in the samples after correction for blank represents cytokine bound [125]I. (Radioactivity in the sample from the blood compartment before dialysis was set at 100%.)

Recovery of radiolabeled cytokine from dialyzer compartments and PAN membranes after dialysis

After 1 h of circulation as described above, the dialyzer was emptied by flushing air. The void volumes from both compartments were collected separately and the radiolabeled cytokine present in each compartment was calculated.

After rinsing the system with saline, the PAN membranes were mechanically removed and the membrane-bound radioactivity was determined before and after a washing step with 0.1% Triton X-100. Results are expressed as percentage of radioactivity recovered from dialyzer and membranes. The total
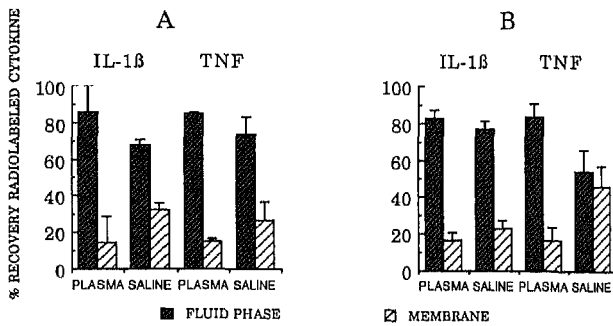
FIG. 2. Recovery of radiolabeled IL-1β and TNF after incubation with PAN membrane fragments. Results are expressed in percentage of radiolabeled cytokine recovered from the fluid phase (plasma or saline) and membrane fragment after incubation. Each bar represents 8 experiments (mean ± SD). The sum of cpm in fluid phase and membrane fragment after blank subtraction was set at 100%. (A) Incubation in Petri dishes. (B) Incubation in film dishes.

amount of radioactivity initially added to the blood compartment was set at 100%. Results are corrected for blanks.

RESULTS

Incubation of PAN membrane fragments with radiolabeled IL-1 and TNF

Results of incubation of PAN membrane fragments in Petri dishes are shown in Fig. 2A. The recovery of radiolabeled IL-1β in the fluid phase was 85.8% (SD ± 14.2) after incubation in plasma (n = 8) and 67.4% (SD ± 3.2) after incubation in saline (n = 8). Consequently the membrane-bound radioactivity was 14.1 and 32.6%, respectively.

Similar experiments with TNF yielded a recovery in the fluid phase of 84.6% (SD ± 1.1) (n = 8) and 73.3% (SD ± 9.7) (n = 8) after incubation with plasma or saline, respectively. The remaining membrane-bound activity was 15.4 and 26.7% for plasma and saline incubation, respectively.

When incubation was performed in the film dishes (Fig. 2B), the percentage of IL-1β recovered in the fluid phase was 83% (SD ± 3.7) for plasma (n = 8) and 76.7% (SD ± 4.3) for saline (n = 8) incubation. The percentage of membrane-bound IL-1β was 16.9 and 23.2%, respectively. Similar experiments with TNF yielded a recovery in the fluid phase of 83.5% (SD ± 7.1) for plasma (n = 8) and 54.3% (SD ± 11.3) for saline (n = 8) incubation while the percentage of membrane-bound TNF was 16.5 and 45.7% after incubation in plasma and saline, respectively.

After incubation, the Petri dishes and film dishes did not contain radioactivity above the background levels. The membrane-bound radioactivity measured both before and after washing twice with saline and 0.1% Triton X-100, respectively, was essentially unchanged.

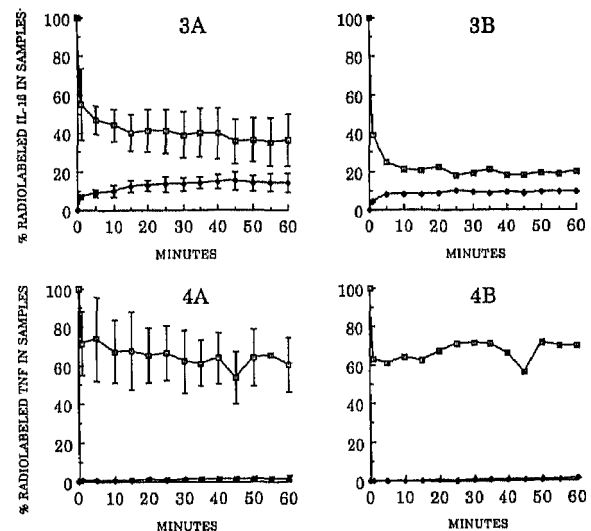*Time-dependent removal of radiolabeled IL-1β and TNF during in vitro dialysis with PAN membranes*

The distribution of ^{125}I-labeled IL-1β over both compartments during 1 h of *in vitro* dialysis is shown in Fig. 3. When dialysis of plasma against plasma was performed (n = 3), the amount of ^{125}I-labeled IL-1β in the blood compartment fell rather abruptly to 54.8% in the first minutes and decreased further gradually to 36.6% of the initial value after 1 h. In the dialysate, which initially did not contain radiolabeled cytokine, ^{125}I-labeled IL-1β gradually reached a plateau. After 1 h, 14.3% of ^{125}I-labeled IL-1β had crossed the hemodialysis membrane and was present in the dialysate compartment (Fig. 3A).

Dialysis of plasma against saline (n = 1) yielded an even more pronounced decrease in radioactive IL-1β content of the blood compartment in the first minutes with a concentration of 19.5 and 9.1% in blood compartment and dialysate, respectively, after 1 h dialysis (Fig. 3B).

The results of similar experiments performed with ^{125}I-labeled TNF are shown in Fig. 4. The amount of ^{125}I-labeled TNF in the blood compartment decreased to 67.6 and 64.2% after 10 min dialysis with a final concentration of 60.5 and 69.5% after 1 h dialysis of plasma against plasma (Fig. 4A) and saline (Fig. 4B), respectively. In contrast with IL-1β, less than 2% radiolabeled TNF was found in the dialysate after 1 h dialysis either against plasma or saline.

Recovery of radiolabeled cytokine from dialyzer compartments and PAN membranes after dialysis

From the initial amount of ^{125}I-labeled IL-1β present in the blood compartment an average of 84.5% was recovered after 1 h

FIGS. 3 and 4. Time-dependent distribution of ^{125}I-labeled cytokine during *in vitro* dialysis with PAN membranes. Horizontal axis: minutes of dialysis. Percentage radiolabeled cytokine in the samples of the blood compartment (open symbols), or dialysate (closed symbols). Fig. 3. Results for ^{125}I-labeled IL-1β. Fig. 4. Results for ^{125}I-labeled TNF. (A) Dialysis of plasma against plasma (n = 3 for each cytokine). (B) Dialysis of plasma against saline (n = 1 for each cytokine).
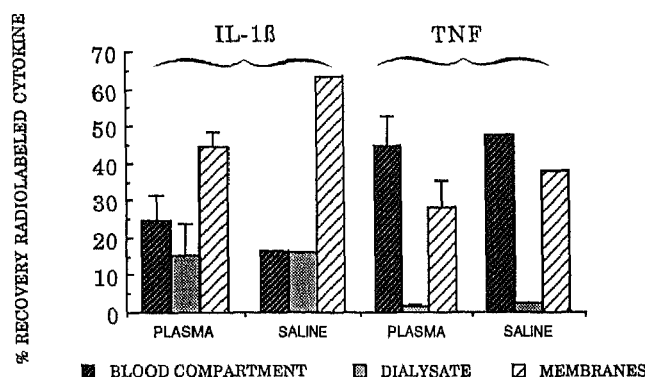
FIG. 5. Recovery of ^{125}I-labeled cytokine from the blood and dialysate compartments and membranes of the PAN dialyzer after 1 h of *in vitro* dialysis. The total amount of radiolabeled cytokine present in the blood compartment before dialysis was set at 100%. Horizontal indicates dialysis of plasma against either plasma or saline. The six bars on the left represent recovery of ^{125}I-labeled IL-1β, the six bars on the right recovery of ^{125}I-labeled TNF.

dialysis of plasma against plasma ($n = 3$): 24.7% was found in the blood compartment, 15.3% in the dialysate, and 44.5% was membrane-bound. Dialysis of plasma against saline ($n = 1$) yielded a total recovery of 95.8% radiolabeled IL-1β distributed as follows: 16.6% was recovered from blood compartment, 16.1% from dialysate, and 63.1% from membranes (Fig. 5).

In similar experiments with ^{125}I-labeled TNF an average of 84% was recovered after 1 h dialysis of plasma against plasma ($n = 3$): 54.2% was found in the blood compartment, 1.7% in the dialysate, and 28.1% was membrane-bound. Dialysis of plasma against saline ($n = 1$) yielded a total recovery of 87.9% TNF with the following distribution: 47.6% from blood compartment, 2.6% from dialysate, and 37.7% from membranes (Fig. 5).

The radioactivity not recovered in these experiments is partly due to loss in sampling (mean 5%); the rest probably remained bound to the nonmembrane parts of the dialyzer and the tubing.

In all experiments the saline used to rinse the dialyzer after dialysis contained no radioactivity above blank levels. The membranes recovered from the dialyzer were washed with Triton X-100; only 1.4 and 1.9% (respectively, for IL-1β and TNF) of the membrane-bound radioactivity was released after the washing step.

SDS-PAGE

Results of autoradiography after SDS-PAGE of radiolabeled TNF and IL-1β are shown in Fig. 6. For both TNF and IL-1β several weak bands were observed in the molecular weight range of 65–90 kDa. More than 95% of IL-1β and 80% of TNF was present in a molecular weight range under 20 kDa. Two strong bands with molecular weights around 40 and 55 kDa were present only in the TNF sample and comprised 19% of the total radioactivity as revealed by densitometric scanning. These probably represent TNF oligomers or complexes of TNF and other plasma proteins. Samples from the dialysate compartment were not analyzed in view of the low recovery TNF in the dialysate compartment.

DISCUSSION

In the present study we confirmed previous findings of a significant binding of IL-1β to PAN membranes (1,2) and extended these observations to TNF, using small amounts of these cytokines such as are likely to be found in pooled plasma. The removal of IL-1α was not studied since this cytokine is known to be mostly bound to cell membrane and not circulating (5).

Incubation of PAN membrane fragments either in Petri dishes or film dishes resulted in a 14–17% of membrane-bound
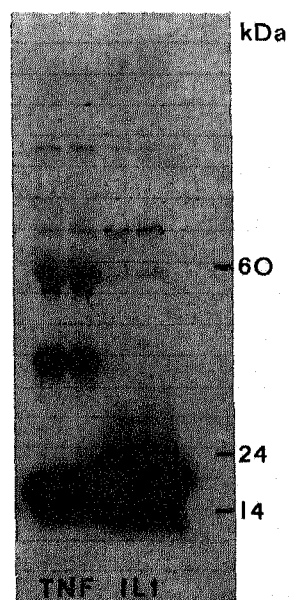

FIG. 6. Autoradiogram of a native polyacrylamide gel. The numbers on the right show the migration of molecular weight markers.

IL-1β or TNF when these cytokines were diluted with plasma. When diluted with saline, binding increased almost 2-fold independent of the cytokine studied. This suggests that cytokine binding to the membrane is not specific and probably has to compete with other plasma proteins. In general, experiments with membrane fragments yielded a lower percentage of membrane-bound radioactivity than *in vitro* dialysis. This can be explained by the differences in surface exposed, the stationary versus recirculatory incubation, and the diffusive clearance (in addition to adsorption) that takes place during dialysis. In view of these results, *in vitro* incubation of plasma with PAN membranes seems less suitable than *in vitro* dialysis to remove IL-1β and TNF from plasma.

The results of the *in vitro* dialysis with PAN membrane demonstrate effective removal from plasma of a significant amount of IL-1β; the removal of TNF is less pronounced.

When the time-dependent distribution of cytokine during dialysis is examined, the decrease of radiolabeled cytokine content in the blood compartment is most marked during the first minutes of dialysis and is more pronounced for IL-1β than for TNF. The plateau reached thereafter suggests saturation of the PAN membrane either by cytokines themselves or, more probably, by adsorption of other plasma proteins. In the dialysate compartment IL-1β appears gradually, in contrast with TNF, which hardly diffuses through the membranes.

After 1 h dialysis, the amount of TNF remaining in the blood compartment is more than 2-fold higher for IL-1β while the amount recovered from the dialysate is 6-fold higher for IL-1β than for TNF. This underscores the low capacity of TNF to cross the PAN membranes.

Dialysis of plasma against saline yields a higher cytokine binding to the membrane (specially for IL-1β) than dialysis of plasma against plasma. This suggests that the presence of saline in the dialysate compartment leads to an increase in the passage of the cytokines through the membrane and in the binding to the dialysate side of the membrane, which is not in contact plasma proteins.

Taken together the results show that removal of IL-1β from the blood compartment is due to adsorption to the membrane and filtration to the dialysate compartment, while removal of TNF is less effective and mostly due to adsorption. Similar results have been recently reported by Lonnemann et al. (6). The low degree of diffusion of TNF through the PAN membrane was not unexpected. The PAN membrane has a high transmittance coefficient for molecular sizes between 1, 3, and 44 kDa (7), which should allow filtration of both cytokines in monomeric form (17 kDa), however, active human natural and recombinant TNF is known to consist of trimers (8) and dimers (9). Our data on SDS-PAGE of samples containing recombinant radiolabeled TNF confirmed the presence of TNF aggregates. Besides by molecular size exclusion, other factors such as unspecific adsorption of plasma proteins to the membrane which could complex with TNF, the tertiary structure of the TNF molecule, and the negative surface charge of the PAN membrane, may also play a role in the low filtration of TNF through the membrane.

The proportion of cytokine that remains membrane-bound after 1 h dialysis is lower for TNF than for IL-1β. These results contrast with the similar membrane binding observed for both cytokines after incubation with membrane fragments in plasma.

An explanation could be that due to the low TNF fraction present in the dialysate compartment, the PAN membrane is mainly exposed to TNF in the blood compartment while exposure to IL-1β (which crosses the membrane readily) takes place at both compartments.

This lower membrane binding of TNF is also in contrast with a recent study by Lonnemann et al. (6). In that study human blood with recombinant or natural TNF or IL-1 was circulated against saline and a higher membrane binding was found for TNF than for IL-1. In that study, the amount of membrane-bound cytokine was calculated from the difference between the amount added and recovered from both dialyzer compartments, while our data show a direct measurement of the membrane-bound cytokine; furthermore it is possible that a greater TNF load in the blood compartment or a greater proportion of monomeric TNF could explain the differences.

Although we did not investigate removal of larger quantities of radiolabeled cytokine, the early saturation of the membrane (suggested by the plateau invariably present in the blood compartment after some minutes dialysis) with the relative low cytokine load, which we investigated, could imply decreasing efficiency of the cytokine removal with increasing amounts of cytokine. Lonnemann et al. (6), however, found similar percentage of cytokine absorbed to the PAN membrane during *in vitro* dialysis with increasing cytokine load (75 ng and 1.5 μg). Together with our data, this suggests that after some minutes of dialysis, an equilibration between the circulating cytokine and the fraction that is adsorbed to the membrane has taken place, and to reduce further the circulating load the use of a new dialyzer would be necessary.

The mechanism of binding IL-1β and TNF by PAN is not elucidated here; binding curves similar to ours have been reported for complement components and cytochrome *c* (10), and therefore we do not believe that the binding is cytokine-specific. Washing the PAN membranes with saline and 0.1% Triton X-100 did not lead to significant release of membrane-bound cytokine.

In vitro dialysis with PAN membranes seems to be a useful method to remove IL-1β from plasma (specially with dialysis of plasma against saline) to be used for standards in immunoassays. Removal of TNF is less effective and other methods such as affinity chromatography (11) may prove more suitable.

Our results may also have implications for clinical settings where these dialyzers are used. Continuous arteriovenous hemofiltration (CAVH) used as adjunctive therapy in septic shock and ARDS is aimed to remove deleterious exogenous and endogenous mediators (such as TNF and IL-1). These cytokines are probably associated with acute and chronic complications of *in vivo* hemodialysis (12,13). The presumed rapid saturation of these membranes, the low filtration of TNF, and the induction of cytokine production that may occur during *in vivo* dialysis (14–20) casts doubts on the usefulness of such membranes for removal of these cytokines *in vivo*.

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Address reprint requests to:
Dr. Pilar Barrera
University Hospital Nijmegen
Department of Internal Medicine
Geert Grooteplein Zuid 8
6500 HB Nijmegen, Postbus 9101
The Netherlands