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Adsorption of complement factor D by polyacrylonitrile dialysis membranes

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Adsorption of complement factor D by polyacrylonitrile dialysis membranes. Factor D, an essential enzyme of the alternative pathway (AP) of complement, accumulates in end-stage renal failure (ESRF). Polyacrylonitrile (PAN) membrane activates complement poorly and has been shown to adsorb C3a, the main anaphylatoxin released during complement activation. In the present work we investigated whether factor D might be adsorbed on PAN. *In vitro* there was a loss of hemolytic factor D when normal human serum (NHS) was incubated with PAN dialysis fibers, whereas no loss was observed with cuprophan (Cu) fibers. There was a dose and time dependent binding of purified radiolabeled factor D to PAN but not to Cu. The hemolytic function of factor D released from PAN by 2 M NaCl was normal. By contrast, factor D was inactive while adsorbed to PAN fibers. When 50 ml of NHS or 100 ml of whole blood were passed through a new hollow fiber PAN dialyzer 95% of factor D was adsorbed. The eluate from a PAN filter which had been used for dialysis in a patient with ESRF contained 38.4 mg of hemolytic factor D, representing 34% of the proteins eluted. By immunoblotting, antigenic factor D from the PAN eluate was identical to purified human factor D. In six patients there was a 81.4% decrease in hemolytic factor D in blood after dialysis with PAN, contrasting with a 9.6% decrease in those dialyzed with cellulose acetate. No factor D was found in the dialysis fluid of PAN dialyzers, indicating that PAN removed factor D mainly by adsorption. In conclusion, PAN has a significant capacity to adsorb factor D, a reaction that might contribute to the diminished capacity of PAN membrane to activate the AP of complement. Whether the efficient removal of large amounts of factor D might be beneficial in uremic patients remains to be defined.

The contact of blood with biomaterials triggers the coagulation and complement cascades [1–8]. Dialysis membranes have been selected on the basis of minimal activation of both to avoid thrombosis and adverse reactions related to the formation of complement fragments. Complement activation occurs mainly through the alternative pathway (AP) and is due to the direct interaction between complement proteins and the chemical residues on the dialysis membrane [9–11]. The activation of complement depends on a fine balance between various interactions which either favor or inhibit amplification of the AP. For instance, AP activation is promoted on crushed Sephadex whereas no amplification occurs on carboxymethyl Sephadex which allows factor H to bind avidly to C3b [11, 12]. In addition, it has been shown recently that specific antibodies can also influence the degree of AP activation induced by cellulosic biomaterials *in vitro* [13, 14]. This might be important in determining complement activation *in vivo* during dialysis.

Another type of interaction between biomaterials and blood proteins is adsorption. The PAN membrane, which is made of a copolymer of polyacrylonitrile and methallylsulfonate has important adsorptive properties [15]. For instance, 400 to 600 mg of β_2-microglobulin are removed weekly with a PAN dialyzer by adsorption and mass transfer to the dialysis fluid [16, 17]. Since the accumulation of β_2-microglobulin plays an important role in the dialysis-related amyloidosis, this property of PAN might be beneficial for patients undergoing chronic hemodialysis [18]. The production of interleukin 1 (IL-1), a cytokine with multiple physiologic and pathologic effects, is induced during hemodialysis. IL-1 has also been shown to be adsorbed to PAN [19, 20]. A small amount of different complement proteins such as C1q, C3 and C5 are adsorbed to PAN as well [21, 22]. However, most impressive has been the almost total adsorption of C3a and possibly C5a, the powerful anaphylatoxins released during complement activation by dialysis membranes [23]. This observation might explain why hemodialysis with PAN increases C3a and C5a only minimally and why there is almost no transient drop in neutrophils at the start of dialysis.

A series of intriguing observations have been made by Amadori et al [21] some years ago. The incubation of serum with PAN induced a decrease in total hemolytic complement which did not correspond to complement activation, since no C3 activation could be detected. It was hypothesized that PAN removed and/or inactivated an essential complement component. More recently in a preliminary study, Moore et al [24] have examined the effect of dialysis circuits which incorporate in series a cuprophan (Cu) and a PAN membrane. They showed that PAN adsorbed C3a released by Cu, as expected. More interesting was that PAN placed upstream of Cu reduced complement activation by Cu, which again suggested that plasma exposed to PAN lost a factor responsible for complement activation.

Factor D, a highly specific serine protease, cleaves factor B bound to C3b, generating the C3bBb enzyme which is the AP C3 convertase [25]. Factor D is the rate limiting enzyme of the AP, and is present in a low concentration (2 μg/ml) in plasma [26, 27]. However, factor D levels are increased 10-fold in patients with end-stage renal failure (ESRF) since factor D is catabolized by the kidney under normal conditions [27–29]. The function of factor D is not modified in renal failure. As shown in

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various *in vitro* assays, this high level of functionally active factor D is responsible for enhanced activation of the AP [29, 30].

The present study was undertaken to determine whether factor D might be lost or inactivated when plasma is exposed to PAN.

Methods

Hemodialysis membranes

PAN (Hospal, AN69, Lyon, France), a copolymer of polyacrylonitrile and sodium methallyl sulfonate, and Cu (Gambro, Lund, Sweden) fibers were prepared by cutting short fragments of fibers (<4 mm) of new hollow fiber dialyzers.

PAN (surface: 1.2 m^2) and cellulose acetate (CA, Baxter, France, surface: 1.7 m^2) hollow fiber dialyzers which had been used to dialyze patients with end-stage renal failure (ESRF) (3.5 hr dialysis sessions, flow rate of 200 to 250 ml/min, dialysate flow rate of 500 ml/min) were immediately washed with one liter of 15 mM NaCl. The dialysis compartment was filled with 15 mM NaCl and closed. The membrane-associated proteins were eluted as described below.

Factor D

Human factor D was purified from the peritoneal fluid of patients on chronic ambulatory peritoneal dialysis employing three successive chromatographic steps: Bio-Rex 70 (Bio-Rad Laboratories, Richmond, California, USA), Heparin-Sepharose CL 6B and Mono-S FPLC (Pharmacia, Uppsala, Sweden) [31].

The protein was radiolabeled with ^{125}I-Bolton Hunter reagent (Amersham, Buckinghamshire, UK) [32] to a specific activity of 0.95 μCi/μg of factor D. Free iodine was removed by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden). Trichloroacetic acid precipitation (TCA, final concentration 10%) showed that 95% of the radioactivity was protein-bound. The ^{125}I-factor D was dialyzed against PBS and aliquots were kept frozen in liquid nitrogen until used. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [33] followed by autoradiography and quantification of the blots by Phosphorimager (Image Quant Software FAST SCAN, Molecular Dynamics Ltd, Kemsing, UK) revealed one major band of 23 Kd, which represented 95% of the radioactivity, and two minor bands representing each 2.5%.

Factor D-depleted serum (RD) was prepared by immunoaffinity using rabbit IgG anti-factor D as previously described [34].

In vitro assays with the dialysis membranes

Veronal buffered saline containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (VBS^{++}) was used in all *in vitro* assays. Before incubation with serum or with ^{125}I-factor D, the dialyzer membranes (PAN and Cu) were hydrated and equilibrated by incubation with VBS^{++} for one hour at 37°C, washed twice and resuspended in VBS^{++}. Normal human serum (NHS) was obtained by allowing freshly drawn venous blood from normal donors to clot at room temperature for 30 minutes before separation by centrifugation. The fresh serum was frozen in liquid nitrogen and stored in aliquots at −80°C until used. This procedure prevented significant complement activation *in vitro*.

Complement activation by PAN and Cu membranes. This was assessed by measuring the C3 conversion to C3c (% of C3 activation induced by the membranes) using crossed immunoelectrophoresis [35]. Mixtures containing 75 μl of NHS and 225 μl of VBS^{++} were incubated with 15 mg of PAN or Cu membranes for 30 minutes at 37°C. Controls included spontaneous C3 conversion of NHS diluted 1 to 4 in VBS^{++}, and C3 conversion induced by 10 mg of crushed Sephadex (Pharmacia; provided by Prof. D. Labarre) which is known to be a potent activator of the AP of complement [11, 12].

The concentrations of C3, C4 and factor B were measured by single radial immunodiffusion using monospecific antisera (Lucerna, Lucerne, CH).

Removal of hemolytic factor D from serum. Various amounts of membranes (5 to 45 mg) pre-equilibrated in VBS^{++} were added to 75 μl of NHS and 225 μl of VBS^{++}. The mixtures were incubated for 60 minutes at 37°C with gentle agitation. Residual factor D hemolytic activity in the supernatants was measured using a hemolytic plate assay and human RD serum [34] (limit of sensitivity of the assay: 2.5% of the normal serum concentration of factor D). Note that 100% of hemolytic factor D corresponds to 2 μg/ml of factor D (normal serum concentration).

The functional activity of factor D bound to the PAN membrane was assessed in a C3 converting assay. PAN fibers (40 mg) were first incubated with 50 μl of NHS diluted 1/4 in VBS^{++} for 60 minutes at 37°C to adsorb factor D. Approximately 100 ng of factor D were adsorbed by this incubation, as calculated from the disappearance of factor D from the NHS and the known serum concentration of factor D (2 μg/ml). Then, 50 μl of RD serum were incubated with 150 μl of VBS^{++} and 5 μl of cobra venom factor (CVF; 0.3 mg/ml, 100 inhibitory U/ml; Cordis, Miami, Florida, USA) at 37°C in the presence of either 0.5 ng of purified factor D (positive control), 40 mg of PAN membrane only (negative control) or 40 mg of PAN membrane which had bound approximately 100 ng of factor D by preincubation with NHS. EDTA (10 mmol) was added after incubation for 60 minutes and the percentage of C3 conversion determined.

Binding of ^{125}I-factor D. The binding of factor D to PAN and Cu membranes was measured by adding 3 μl (12.5 ng) of ^{125}I-factor D to various amounts of membranes (5 to 45 mg) in a final volume of 300 μl of VBS^{++} (with 10 μl of NHS to avoid nonspecific binding). The mixtures were incubated for 60 minutes at 37°C. The membranes were then washed three times in VBS^{++}, and the percentage of ^{125}I-factor D bound to the membranes was counted.

Adsorption of factor D by PAN dialyzers

Fifty milliliters of NHS and 100 ml of whole heparinized blood (1 U/ml) freshly drawn from a normal donor were run at a flow rate of 1 ml/min through a hollow fiber PAN dialysis filter, that had been rinsed with sterile isotonic saline via the blood and dialysate compartments. The dialysate compartment filled with NaCl 15 mM was kept closed during the experiment. Fractions of 4 ml were collected from the outlet and the residual serum or plasma factor D in the fractions after the passage through the filter were measured by the hemolytic plate assay. In the experiment with heparinized blood, the total protein content, C3 and C4 were also determined before and after the

passage through the filter, respectively, by optical densitometry and single radial immunodiffusion.

PAN and CA dialyzers were collected immediately at the end of a regular dialysis session, and extensively washed with 15 mM NaCl. Proteins from the membranes were eluted with 2 M NaCl, and the presence of hemolytic factor D in the eluates was determined and quantitated. Total protein content in the eluates was determined by optical densitometry.

The presence of antigenic factor D in the eluates of dialysis membranes was also assessed by immunoblots after separation of the proteins by SDS-PAGE. In brief, 40 ml of the eluates were concentrated eightfold by using a YM5 (43 mm) membrane with a cut-off of 10 Kd (Amicon, Lausanne, Switzerland). The concentrated eluate (40 μl) and plasma (0.25 μl) from a patient with ESRF on hemodialysis were applied to 5 to 15% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. The concentrated eluate from a CA filter was used as control. The nitrocellulose was incubated overnight with 100 μg of anti-factor D mAb 72-96-25 [34]. Biotinylated sheep IgG against mouse IgG (Amersham, H. Rahn and Co, Zürich, Switzerland) was used as the second antibody. Finally, streptavidin-horseradish peroxydase was added and revealed with 4-chloro-1-naphtol (BioRad, Zürich, Switzerland). On similar nitrocellulose blots beta-2-microglobulin was detected using specific rabbit polyclonal antibodies (Dakopatts, Denmark), followed by ^{125}I-protein G (Amersham) and revealed by autoradiography.

Plasma factor D levels in chronically hemodialyzed patients before and after dialysis

EDTA-anticoagulated blood samples were collected before (pre) and after (post) completion of dialysis in six patients with ESRF dialyzed with PAN and in five patients dialyzed with CA cellulose hollow fiber dialyzers. After centrifugation, plasma samples were stored at −80°C until measurement of hemolytic factor D. A normal plasma pool of 32 healthy blood donors was used to define the normal concentration of hemolytic factor D. IgM levels were measured by laser nephelometry, and used to correct results for changes in hemoconcentration after dialysis.

Statistical analysis

The Student's *t*-test for paired samples was used to compare the factor D levels before and after dialysis. The unpaired *t*-test was used to compare pre-dialysis plasma factor D levels of patients on PAN or CA, and to analyze data from in vitro experiments. All values from *in vitro* experiments are reported as the mean ± SEM.

Results

Complement activation by dialysis membranes in vitro

First, we studied the activation of C3 in NHS incubated with PAN fibers, Cu fibers or crushed Sephadex. The formation of C3c, the major breakdown product of C3, was maximal with crushed Sephadex, intermediate with Cu, and minimal with PAN (Fig. 1). In fact, the proportion of C3c formed in the presence of PAN was not different from the control serum incubated without any fibers. Different experiments were performed to determine whether the absence of C3c formation in serum incubated with PAN was due to adsorption of C3 and/or its major fragment C3c to the PAN fibers. Serum was first exposed to crushed Sephadex which generated maximal formation of C3c. This serum was then incubated for 60 minutes at 37°C and overnight at 4°C with fresh PAN fibers. The total concentration of C3 and the proportion of C3c present after exposure to crushed Sephadex were not modified by the incubation with PAN. These data indicate that, although some C3 or C3b might bind to dialysis membranes, the proportion bound was too low to be detected in our *in vitro* assays, and thus the large differences observed between the three materials tested were not due to loss of C3 or C3c by adsorption. Finally, the absence of significant C3c formation induced by PAN *in vitro* was in accordance with previous results [21] and with the generally accepted reduced ability of PAN to activate complement as compared to cellulosic membranes [1].

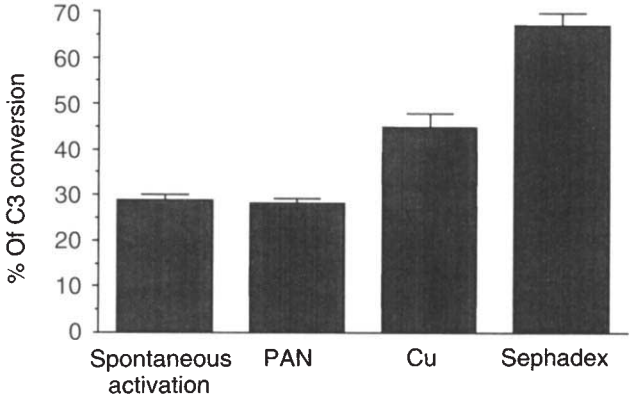

Fig. 1. *Percentage of C3 conversion to C3c after incubation for 30 minutes at 37°C of NHS (spontaneous activation), or NHS in the presence of PAN fibers (15 mg), Cu fibers (15 mg) or crushed sephadex (10 mg).* Results are depicted as the mean ± SEM ($N = 3$). The % of C3 conversion was significantly higher in the Cu group as compared to the PAN group ($P < 0.01$).

Removal by PAN of a factor necessary for complement activation by crushed Sephadex

To see whether PAN removed a factor necessary for complement activation, normal serum was incubated with PAN overnight at 4°C. This serum was then incubated for 30 minutes at 37°C with crushed Sephadex. Under such conditions no significant C3c formation was observed, which contrasted with the result obtained using the NHS which had not been preincubated with PAN (Table 1). The factor removed by PAN was unlikely to be C3, since antigenic C3 concentration was not modified, and similarly we could not demonstrate a significant modification of factor B concentration after the pre-incubation with PAN (the loss of factor B was only 10%). The third factor tested was factor D. The addition of purified factor D restored most of the capacity of serum to generate C3c in the presence of crushed Sephadex.

Loss of functional factor D from serum exposed to PAN

PAN was responsible for a dose dependent loss of hemolytic factor D from normal serum (Fig. 2). No such loss was observed with cuprophan. The disappearance of factor D suggested adsorption to PAN. Thus, the PAN fibers were washed with PBS and then exposed to 2 M NaCl. Hemolytic factor D was

Table 1. Loss of alternative pathway function by pre-incubating NHS with PAN membrane

	Percentage of C3c formation after incubation of NHS with 10 mg of crushed Sephadex[a]
NHS incubated overnight[b]	73.0 ± 3.4%
NHS incubated overnight with PAN (40 mg)	10.4 ± 0.9%
NHS incubated overnight with PAN (40 mg) + 150 ng factor D	58.9 ± 4.7%

Results are reported as the mean ± SEM (N = 3).
[a] Assay: 75 μl of NHS diluted 1/4 in VBS^{++}, for 30 min at 37°C; C3c in NHS before the assay <8%
[b] Overnight incubation at 4°C

Table 2. Factor D adsorbed to PAN is not functional

	Percentage of C3c formation
RD serum[a]	6.9 ± 0.4%
RD serum + CVF	7.0 ± 0.9%
RD serum + CVF + 0.5 ng factor D[b]	63.9 ± 5.8%
RD serum + PAN/factor D[c]	8.2 ± 1.0%
RD serum + CVF + PAN/factor D	7.5 ± 0.5%

Results are reported as the mean ± SEM (N = 3).
[a] Assay: 50 μl of RD diluted 1/4 in VBS^{++}, incubated for 60 min at 37°C
[b] Factor D: 0.5 ng corresponds to 0.5% of the normal factor D concentration
[c] PAN/factor D: 40 mg of PAN precoated with approximately 100 ng of factor D

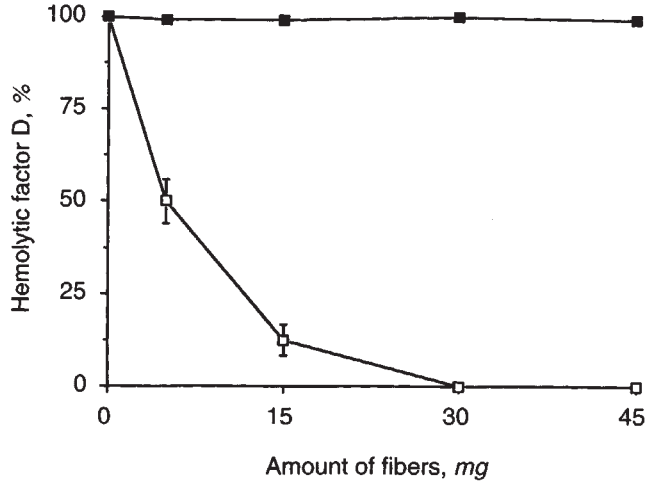

Fig. 2. *Percentage of hemolytic factor D remaining in solution after incubation of NHS with various amounts of PAN (□) or Cu (■) fibers.* Results are depicted as the mean ± SEM (N = 2).

recovered in this eluate but not in the control experiment in which PAN had been preincubated with serum immunochemically depleted of factor D (RD).

To see whether the factor D adsorbed to the PAN was functional, RD was incubated with PAN fibers to which factor D had been adsorbed by preincubation of PAN with NHS. This RD + PAN/factor D did not behave differently from RD as the addition of cobra venom factor (CVF), a potent activator of the AP, did not generate significant amount of C3c (Table 2). In the control experiment, RD was supplemented with purified factor D to 0.5% of its normal concentration. This minimal amount of factor D was sufficient to allow C3c formation by CVF. Thus, factor D adsorbed to PAN had lost more than 99.5% of its functional activity.

Binding of radiolabeled factor D to PAN

When ^{125}I-factor D was incubated with various amounts of PAN fibers in the presence of minimal amounts of normal serum, there was a dose dependent binding of ^{125}I-factor D to the fibers (Fig. 3A). As shown by SDS-PAGE and autoradiography analysis, all the ^{125}I-factor D bound to the PAN fibers when 30 mg or more fibers were used (not shown). The binding of radiolabeled factor D to the fibers was very rapid since 70% was bound in five minutes (Fig. 3B). No significant binding was observed when Cu was used. Finally the binding of ^{125}I-factor D was not modified by 10 mM EDTA or by 10 U/ml of heparin.

Adsorption of factor D by PAN dialysis filters

When 50 ml of NHS or 100 ml of whole heparinized blood (1 U/ml) were run for 60 minutes through a new PAN hollow fiber dialyzer *in vitro*, the disappearance of factor D was almost complete, since less than 3% of the hemolytic factor D could be recovered after passage through the filter. Functional factor D could be eluted with 2 M NaCl. No factor D was found in the dialysate (60 ml) even after a 10-fold concentration using YM5 Amicon membranes. Since the factor D hemolytic assay could detect concentrations as low as 50 ng/ml, we calculated that under these conditions more than 99% of factor D disappearance was due to adsorption. As expected, there was also a non-specific adsorption of many proteins under these conditions. Indeed, by measuring the plasma total protein content, C3 and C4 before and after passage through the PAN filter (experiment with heparinized blood), only 78% of total protein, 69% of C3 and 79% of C4 were recovered after passage through the filter.

To see whether a similar phenomenon occurred *in vivo* during dialysis with PAN, we studied two PAN dialyzers immediately after the end of a dialysis session. After extensive washing with 15 mM NaCl, elution of membrane-associated proteins was performed with 2 M NaCl. The proteins recovered were analyzed by SDS-PAGE and compared to the plasma of the two patients with ESRF. The eluates contained many small molecular weight proteins in addition to albumin (Fig. 4). One protein was particularly abundant at a molecular wt of 24 Kd corresponding to the expected molecular wt of factor D. By immunoblotting this protein was shown to be factor D (Fig. 5). Another protein known to be adsorbed by PAN, that is, β_2-microglobulin, was shown to be present in the eluates as well (Fig. 6). Quantitative analysis indicated that factor D was a major component of the eluate since 38.4 mg of hemolytic factor D were recovered from one PAN dialyzer, representing 34% of the total proteins. Only 2 mg of β_2-microglobulin were recovered in this eluate, suggesting that the elution conditions did not allow complete release of all the membrane-associated proteins because higher amounts of β_2-microglobulin have been reported to be adsorbed by PAN dialyzers [16]. No factor D could be recovered from two CA filters.

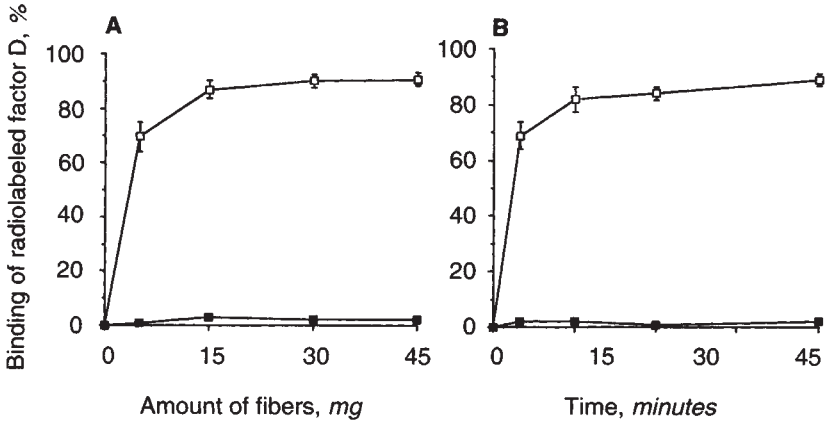

Fig. 3. A. *Percentage of binding of radiolabelled factor D to various amounts of PAN (□) and Cu (■) fibers, after 60 minutes at 37°C.* **B.** Kinetics of binding of radiolabelled factor D to 45 mg of PAN and Cu fibers. The experiments were performed in the presence of 10 μl of NHS so as to avoid non-specific binding. Results are depicted as the mean ± SEM ($N = 2$).

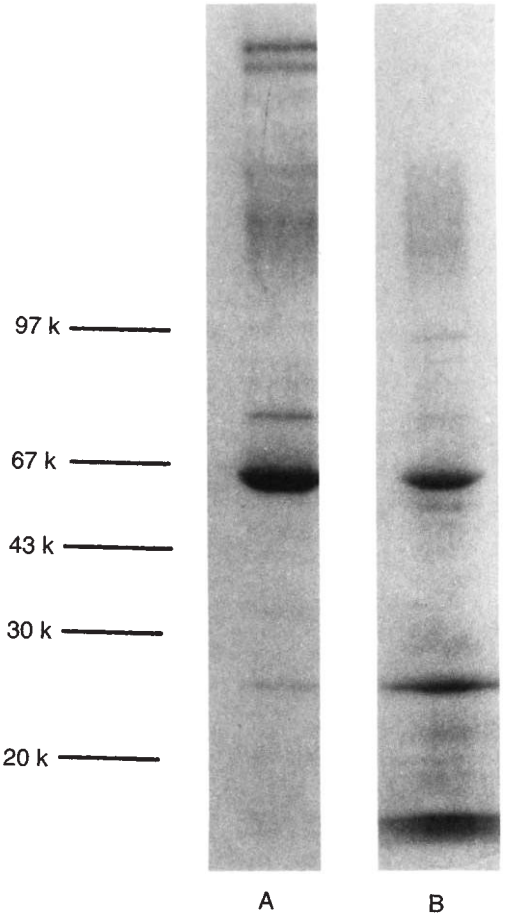

Fig. 4. *SDS-PAGE analysis of proteins eluted from a PAN dialysis filter.* The filter was taken after a regular dialysis session of a patient with ESRF, washed with 15 mM NaCl and the proteins eluted with 2 M NaCl. Lane A: plasma proteins of the patient; lane B: proteins eluted from the PAN filter.

Modifications of factor D concentration during hemodialysis with PAN

To see whether the *in vitro* findings of factor D adsorption to PAN membranes might also be relevant for patients on chronic hemodialysis, hemolytic plasma levels of factor D were measured before and at the end of dialysis sessions (Fig. 7). In six patients dialyzed with PAN, there was a 81.4 ± 9.1% decrease in plasma factor D after dialysis ($P < 0.001$). To determine whether factor D might also have been lost by mass transfer into the dialysis fluid, we measured hemolytic factor D in six samples of dialysis fluid taken during dialysis (at 30 min intervals) in two patients. Although these samples were first concentrated 10-fold, none contained any hemolytic factor D. Thus less than one milligram of hemolytic factor D was lost by mass transfer given the sensitivity of the hemolytic assay, and a dialysate flow rate of 500 ml/min for 3.5 hours (total: 105 liters of dialysis fluid). By calculation it was estimated that dialysis on PAN removed at least 48 mg of factor D per dialysis session. Thus, PAN removed factor D mainly by adsorption. In five patients dialyzed with CA, there was a 9.6 ± 3.5% decrease in factor D that could be explained by mass transfer of the protein to the dialysis fluid, since significant amounts (approximately 10 mg of hemolytic factor D) were recovered in this compartment in one patient. The mean predialytic level of factor D in plasma was slightly but significantly lower in the patients dialyzed with PAN as compared to CA (18.2 ± 5.6 μg/ml versus 25.1 ± 3.2 μg/ml, $P < 0.05$).

Discussion

The main finding of this work was the very significant adsorption of complement factor D by PAN dialysis membrane. The second interesting finding was that factor D bound to PAN was inactive, that is, incapable of enhancing complement activation on the dialysis membrane. The third and possibly most relevant observation was that in patients with ESRF, the plasma concentration of factor D dropped several-fold, reaching almost normal values after dialysis with PAN.

The interactions between PAN and the complement cascade are not simple. Based on the measurements of complement fragments (C3a, C3d, C5a, C5b-9) and neutrophil counts, PAN membrane has been classified as a weak activator of complement as compared to cellulosic membranes [1–8, 36, 37]. However, in view of its important adsorptive properties in particular for the anaphylatoxins C3a and C5a, it has been difficult to assess the real degree of complement activation induced by PAN [15, 23]. For this reason, we performed a series of *in vitro* experiments to compare PAN with Cu fibers. Fresh human serum was exposed to the two biomaterials, and the activation of C3 was measured by its cleavage to C3c. Thus, the results provided only indications on the relative capacity of

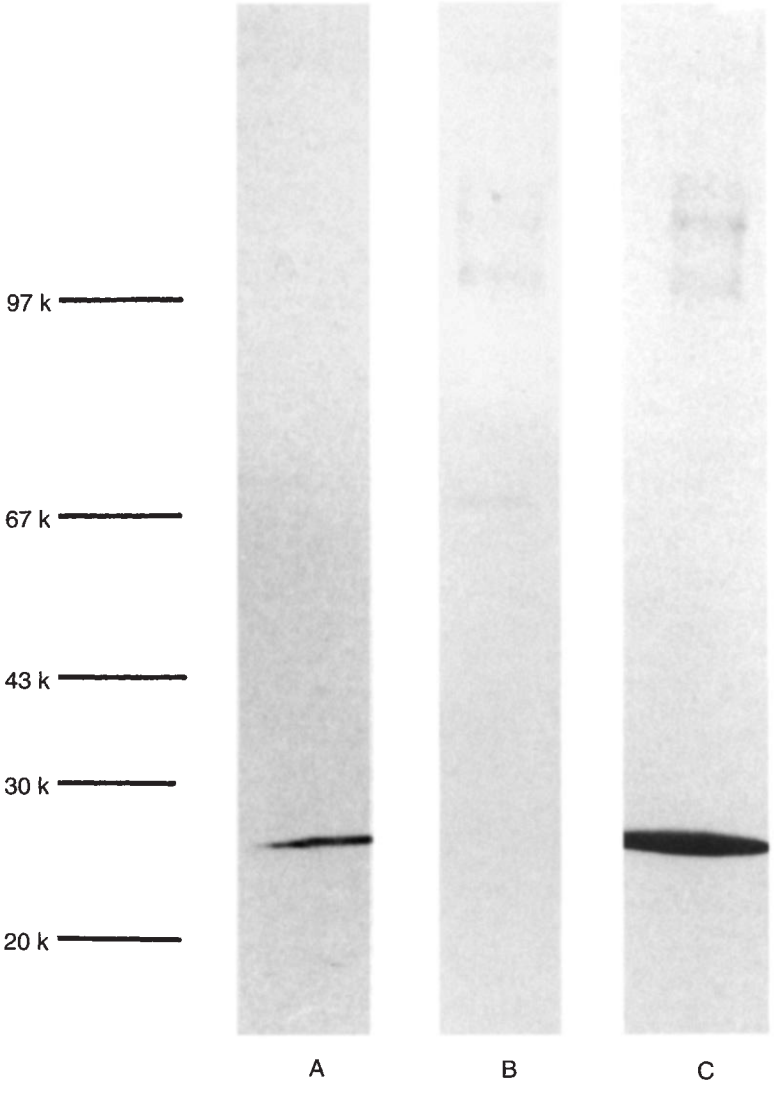

Fig. 5. *Detection of factor D in the proteins eluted from the PAN filter by immunoblotting.* Lane A: purified factor D (500 ng); lane B: plasma (0.25 μl) from the patient; lane C: proteins eluted from the PAN filter. To note is that factor D concentration was too low in plasma to be detected by immunoblotting.

PAN and Cu to activate complement. However, they were unequivocal: PAN is a weaker activator of complement than Cu, and this result was not due to adsorption of the complement fragment measured (C3c).

The second point was to confirm the observations of Amadori et al and Moore et al [21, 24]. Indeed, incubation of normal serum with PAN removed a factor essential for complement activation by the AP, and this factor was shown to be factor D. The experiments reported here concurred to demonstrate an efficient adsorption of factor D by PAN. *In vitro*, hemolytic factor D was lost from whole serum exposed to PAN and was recovered by elution with 2 M NaCl. This adsorption could be studied directly by incubating purified radiolabeled factor D with PAN fibers: there was a dose and time dependent binding of radiolabeled factor D to PAN which was not observed with cellulosic fibers. A similar efficient adsorption of factor D was demonstrated using whole PAN dialyzers *in vitro*. Finally, when PAN filters which had been used for dialysis were eluted with 2 M NaCl, it was possible to recover intact antigenic and functional factor D in the eluate. Almost 40 mg of factor D were present in one PAN eluate, representing a third of all proteins eluted. The striking enrichment in factor D might be an overestimate because factor D may be released more readily than other proteins by 2 M NaCl. However, the 2 M NaCl eluate of a PAN filter is a convenient starting material for the purification of human factor D. Indeed, purification of factor D has been hampered by its low concentration in biological fluids [26, 31, 38].

Other proteins essential for AP function might have been adsorbed by PAN as well. However, the demonstration that the AP function of serum exposed to PAN was restored by purified factor D suggested that adsorption of other proteins plays only a minor role. Under conditions which depleted hemolytic factor D by more than 95%, the losses of C3 and factor B were minimal. From the eluates of PAN dialyzers, it was, however, apparent that many other small molecular wt proteins bound specifically to PAN. They included β_2-microglobulin as expected [16, 17], and possibly C3a. It would be interesting to define the remaining proteins as well to see which other biologically active proteins are removed specifically by PAN.

The binding of factor D might enhance and focus complement activation on the dialysis membrane. On the other hand, factor

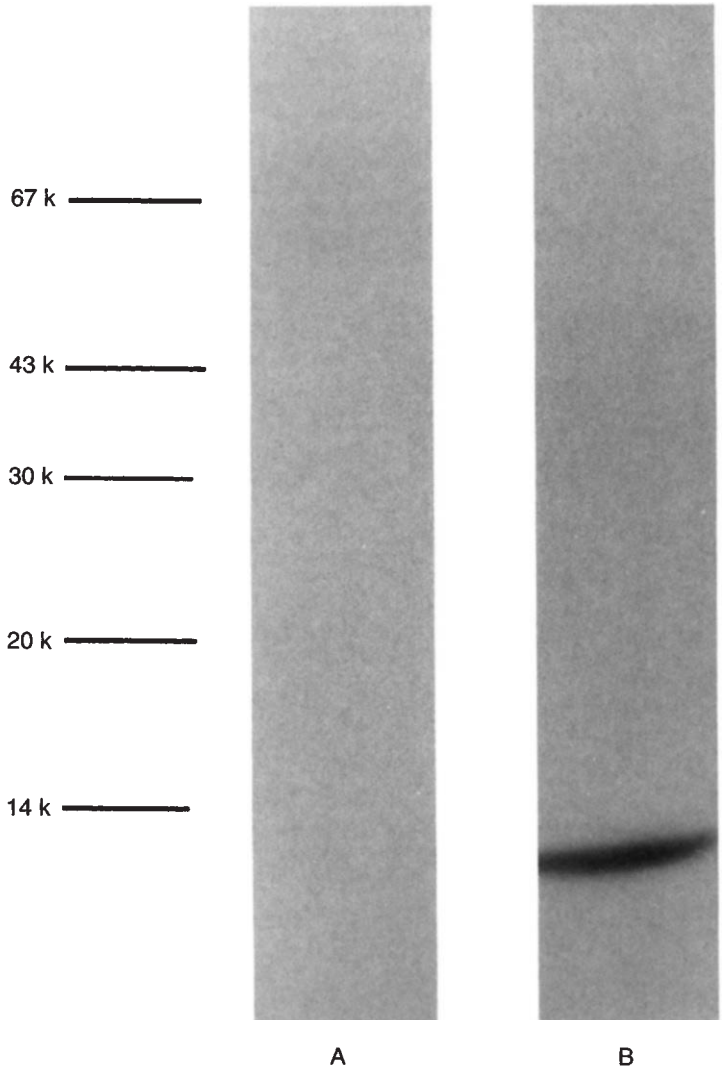

Fig. 6. *Detection of β_2-microglobulin in the proteins eluted from the PAN filter by immunoblotting.* Lane A: plasma from the patient; lane B: proteins eluted from the PAN filter. β_2-microglobulin concentration was too low in plasma to be detected by immunoblotting.

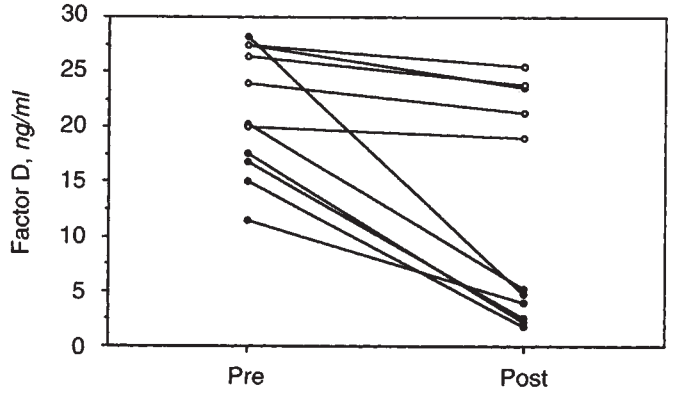

Fig. 7. *Plasma levels of factor D before (pre) and after (post) dialysis in six patients dialyzed with PAN filters (closed circles) and in five patients dialysed with CA filters (open circles).*

D bound to the membrane might be inactive because the binding involves or modifies the site responsible for its enzymatic activity. Using a very sensitive assay which detected 0.5% of functional factor D, it was not possible to show any functional factor D while bound to PAN. However, factor D hemolytic activity was recovered after elution of factor D from PAN fibers, suggesting that the binding to PAN involves the serine protease enzymatic site, but does not modify it. Adsorption or inhibition of factor D function on PAN might result in complement inhibition at the blood/membrane interface and contribute to the reduced ability of such membranes to activate complement. Whether anionic sulfonate groups are critical for factor D adsorption, as proposed for other substances such as anaphylatoxins [22], remains to be defined.

The drop in factor D concentration during hemodialysis with PAN was substantial since more than 80% of circulating factor D was removed at the end of dialysis. Considering the lower detection limits of the hemolytic assay, it was estimated that less than one milligram of factor D was present in the PAN dialysate, that is, the removal of factor D by PAN occurred mainly by protein adsorption. In patients dialyzed with CA, the small reduction in hemolytic factor D after dialysis could be explained by mass transfer of the protein to the dialysis fluid. The large decrease in plasma factor D after dialysis with PAN

should decrease the hyperactivability of the AP of complement observed in the plasma of patients with ESRF [30, 39, 40]. However, the effect on factor D levels was only transient since just prior to the next dialysis session the levels were increased again to very high values. The absence of a sustained effect could be anticipated from the high rate of synthesis of factor D and its almost exclusive renal catabolism [29]. However, even a transient decrease of factor D with PAN might be beneficial in patients with end-stage renal disease undergoing hemodialysis, since there are several reasons to suggest that the hyperactivability of the AP secondary to high factor D levels is detrimental in ESRF [30, 40].

The adsorptive characteristics of dialysis membranes merit further investigation to develop new biomaterials with even better adsorptive properties for proteins of low molecular weight, such as β_2-microglobulin and factor D, for which an efficient removal might be beneficial in ESRF. Uremic toxins, which include several small molecular wt proteins, might also be removed more efficiently by biomaterials combining high diffusing and adsorbing properties.

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Appendix. Abbreviations

AP	Alternative pathway
CA	Cellulose acetate
CVF	Cobra venom factor
Cu	Cuprophan
ESRF	End-stage renal failure
PAN	Polyacrylonitrile
RD	Normal serum immunochemically depleted of factor D
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
VBS^{++}	Veronal buffered saline

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