Hemodialysis leukopenia and complement function with different dialyzers

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Hemodialysis leukopenia and complement function with different dialyzers. The relationship between leukopenia and the complement system during hemodialysis was re-examined by studying not only the in vivo effects of four different dialyzer membranes (cellulose hydrate, cuprophan, cellulose acetate, and polyacrylonitrile) on leukocyte counts and complement levels, but especially by investigating the effects of these membranes on complement function in vitro. Whereas from in vivo studies no definite conclusions could be drawn, in vitro investigations provided clear-cut information. When more sophisticated technical approaches were undertaken, it became evident that hemodialysis leukopenia has to be thought of in terms of chemotactic factor generation. In fact, a strict correlation was demonstrated between the degree of leukopenia induced by the dialyzers tested and the ability of the relative membrane to generate chemotactic activity in vitro. Moreover, the previously observed ability of polyacrylonitrile membrane to induce a decrement in complement function was due to the ability of polyacrylonitrile to adsorb complement activity and did not correspond to effective complement consumption. This finding explained why polyacrylonitrile dialysis is not accompanied by a decrease in circulating granulocytes. Taken together, our data strongly point to a pivotal role of complement system in the pathogenesis of hemodialysis leukopenia.

Leucopénie de l’hémodialyse et fonction du complément avec différents dialyseurs. La relation entre la leucopénie et le système complémentaire pendant l’hémodialyse a été réexaminée en étudiant non seulement les effets in vivo de quatre membranes de dialyse différentes (hydrate de cellulose, cuprophan, acétate de cellulose, et polyacrylonitrile) sur les numérotations leucocytaires et les niveaux du complément, mais surtout en étudiant les effets de ces membranes sur la fonction complémentaire in vitro. Alors que des études in vivo aucune conclusion définitive ne pouvait être tirée, les études in vitro ont apporté des informations précises. Quand des approches techniques plus sophistiquées ont été utilisées, il est apparu évident que la leucopénie de l’hémodialyse doit être pensée en termes de génération de facteurs chimiotactiques. De fait, une corrélation stricte a été démontrée entre le degré de leucopénie induite par le dialyseur testé et la capacité de la membrane à générer une activité chimiotactique in vitro. En outre, la capacité préalablement observée de la membrane en polyacrylonitrile d’induire une diminution de la fonction complémentaire était due à la capacité du polyacrylonitrile d’absorber l’activité complémentaire, et ne correspondait pas à une consommation effective du complément. Ce résultat a expliqué pourquoi la dialyse au polyacrylonitrile n’était pas accompagnée par une diminution des granulocytes circulants. Prises dans leur ensemble, nos données mettent fortement l’accent sur un rôle-clé du système complémentaire dans la pathogenie de leucopénie de l’hémodialyse.

It has long been known that early hemodialysis is accompanied by a profound, transient leukopenia [1, 2]. This leukocytopenia is due to a selective cell sequestration in the pulmonary microvasculature [3, 4] and is accompanied by a progressive decrease of arterial PO₂ [5, 6]. The phenomenon specifically involves granulocytes and monocytes, cells sharing plasma membrane reactivity toward activated complement components. Many data, obtained both in experimental models [7] and humans [8–11] strongly support the hypothesis that complement activation plays a pivotal role in the pathogenesis of hemodialysis-induced leukopenia through the release of chemotactic factors, as C5a, C567, and perhaps C3a [12–14].

Recently, however, some doubt has been cast on the central role of complement activation in the dialysis-mediated leukopenia and hypoxemia. Main criticism of this hypothesis came from the finding that polyacrylonitrile membranes caused a reduction in complement function without inducing significant neutropenia, whereas polycarbonate dialyzers provoked a sizeable drop in leukocyte counts without detectable complement depletion [15, 16]. Moreover, when individual patients are considered, no relationship is found between the intensity of complement depletion and induced neutropenia [17]. Finally, leukopenia and hypoxemia seem to be unrelated effects of hemodialysis, the latter occurring with dialyzers that do not cause any significant change in leukocyte counts [18].

The aim of the present study is to re-assess the relationship between complement system and neutropenia during hemodialysis. For this purpose, the behavior of four different dialyzer membranes with regard to in vivo and in vitro effects on complement function will be examined by using sensitive techniques for the detection of chemotactic factors putatively responsible for pulmonary leukocyte trapping [19, 20].

Methods

Patients. Four groups of eight patients undergoing routine hemodialysis for endstage renal failure were studied by using four different types of dialyzers. None of them had clinical evidence of infection or was taking drugs known to affect leukocyte counts or complement function. Age and sex distribution was comparable between groups. Dialyzer membranes used were cellulose hydrate (Sifra Secon 104, Ravizza, Italy),...
cuprophan (UF 3.2 AB Gambro, Lund, Sweden), cellulose acetate (CDAK 3500, Cordis Dow Corp., Miami, Florida), and polyacrylonitrile (Hospital RP 610 Dasco, Bologna, Italy). The dialyzer membranes had not been used previously and were primed with saline.

Blood samples were collected from the afferent line, just proximal to the point of heparin infusion, before (time 0) and at 10 min and 1 and 4 hr of dialysis. Leukocyte counts were performed in duplicate using an automatic cell counter (Coulter Harpenden, Herts, England). Leukocyte differential counts were determined by standard methods. Blood gas determinations were obtained with commercially available instruments. Aliquots of plasma for complement studies were stored in liquid nitrogen and thawed only once for testing. Normal plasma or serum samples were collected from laboratory personnel.

Complement assays

CH 50. Classical pathway hemolytic activity was determined by the method of Kabat and Mayer [21]. To obviate as far as possible interassay variability, plasma samples from each patient were always tested all at once in the same experiment, and patients undergoing dialysis with different membranes were grouped in a single test. Results were expressed in CH 50 units (50% hemolysis point), and the percentage of pre-dialysis values was calculated. With regard to the intra-test variability, the coefficient of variation (SD × 100/mean) was evaluated by the replicate testing of a sample in the same experiment, and confidence limits were set at twice the coefficient of variation (6%). For individual patients, any variation from the basal value exceeding 6% was considered significant (P < 0.05).

AP 50. Alternative pathway hemolytic activity was determined by the method of Platts-Mills and Ishizaka [22]. Experiments were planned as described for the CH 50 test, and the percentage of pre-dialysis values was calculated from the results expressed in AP 50 U. For each patient, any variation from the basal value exceeding twice the coefficient of variation (6%) was considered significant.

Factor B assay. Functional molecule titration was used for the evaluation of factor B (Bf) in the serum. As a finishing reagent containing all the components other than the one under test, serum heated for 20 min at 50°C (R-Bf) was used [23]. A standard serum was tested as a source of Bf, and functional molecule titration was performed according to Kabat and Mayer [21]. The results of the titrations with polyacrylonitrile (PAN)- and hydrate-incubated R-Bf were compared with that obtained in control R-Bf (serum incubated alone).

Detection of granulocyte-aggregating activity

Detection of chemotactic factors in the patient plasma or in in vitro activated plasma was performed by using granulocyte aggregometry according to Hammerschmidt et al [20], with minor modifications.

Peripheral blood from normal donors was collected in EDTA 20 mm. Theophylline (Sigma Chemical Company, St. Louis, Missouri) was added to the blood to achieve a final concentration of 10 mm. Red blood cell sedimentation was obtained by adding equal volumes of ice-cold 3% dextran (Clinical Grade, Sigma Chemical Company) in saline. Buffy-coat cells were collected after 60 min sedimentation at 4°C. Contaminating erythrocytes were lysed by hypotonic shock. After restoration of isotonicity, the suspension was centrifuged at ×400g for 30 min at 4°C on a Ficoll-Urovison gradient. Cells at the bottom were recovered and adjusted to 15 × 10^9/ml with ice-cold final buffer (isotonic saline, 0.5% albumin-theophylline 2.5 mM). The resultant cell suspension was composed of greater than or equal to 99% granulocytes (GC), as judged by May Grünwald-Giemsa staining. Viability, assessed by trypan blue exclusion, always exceeded 95%.

Granulocyte aggregometry was performed on a standard platelet aggregometer (Elvi 840), by adding sequentially in the cuvettes 200 μl of the GC suspension, 20 μl of 10 mm Ca++, 20 μl of 10 mm Mg++, and 20 μl of a 50 μg/ml solution of Cytochalasin B (Sigma Chemical Company). After adaptation for 2 min at 37°C, 20 μl of the sample to be tested were added to the stirred cell suspension. The aggregometer/recording system was calibrated with a fresh GC suspension diluted 1:1 with final buffer (100%), and the ΔT between the initial cell preparation and 100% was set at 16 cm. Aggregation waves were recorded as increments in light transmission on an arbitrary scale (ΔT).

In vitro plasma/serum incubation with dialyzer membranes

Normal plasma anticoagulated with 2 U/ml of heparin was preincubated for 60 min at 37°C in revolving plastic tubes (Falcon 2001) with different dialyzer membranes at a ratio of 20 cm² membrane/ml of plasma. Negative controls were fresh plasma, plasma incubated alone, and decomplemented plasma incubated with membranes. Positive control for plasma complement activation was provided by the use of Zymosan.

In a set of experiments, plasma was first incubated with hydrate or PAN for 60 min. At the end of the incubation period, hydrate-incubated plasma was re-incubated with a new PAN membrane, whereas PAN-incubated plasma was transferred on a new hydrate membrane, and incubation was carried out for an additional 60 min. Moreover, normal serum was incubated with hydrate or PAN in the presence of 20 mm EDTA, to prevent complement activation. After a 60-min incubation, serum was removed and Ca++ and Mg++ concentration was restored. Sera were tested in AP 50 assay, and comparisons were done between membrane-incubated sera and the serum incubated alone.

Removal of GC-aggregating activity by PAN

Normal serum activated by Zymosan was heated at 56°C to prevent further possible complement activation by the membranes and was subsequently incubated with PAN or hydrate for 60 min at 37°C. In a set of experiments, after the removal of serum, PAN membrane was briefly washed with isotonic saline and treated at room temperature for 60 min with one of the following: (1) distilled water; (2) NaCl 3 m; (3) glycine-HCl-NaCl buffer pH 2.0. After recovery and reconstitution of isotonicity and pH, the supernatants were tested for the ability to induce GC aggregation.

In vitro "re-use" of hydrate membrane

In our Dialysis Unit, we do not re-use dialyzers routinely. However, we tried to reproduce re-use of dialyzer membrane in vitro by the following procedure. Hydrate membrane was incubated with normal plasma as previously described. After removal of plasma, the membrane was extensively washed with isotonic saline and finally stored at room temperature with 3%
Table 1. Arterial-blood neutrophil counts in four groups of patients undergoing hemodialysis with different dialyzers

<table>
<thead>
<tr>
<th>Dialyzer</th>
<th>Time of dialysis, min</th>
<th>0</th>
<th>15</th>
<th>60</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrate</td>
<td>3556 ± 380</td>
<td>301 ± 60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3241 ± 398</td>
<td>4400 ± 504</td>
<td></td>
</tr>
<tr>
<td>Cuprophan</td>
<td>3224 ± 361</td>
<td>692 ± 244&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2653 ± 373</td>
<td>3392 ± 412</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>3511 ± 344</td>
<td>1765 ± 353&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3557 ± 358</td>
<td>4200 ± 461</td>
<td></td>
</tr>
<tr>
<td>Polyacrylonitrile</td>
<td>3431 ± 268</td>
<td>3574 ± 372</td>
<td>4032 ± 372</td>
<td>3993 ± 390</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are expressed as absolute numbers of circulating neutrophils/mm<sup>3</sup>. Mean values ± SEM for each group of eight patients are given before (time 0) and at different times of dialysis.

Table 2. Changes in total hemolytic complement activity in four groups of eight patients undergoing hemodialysis with different dialyzers

<table>
<thead>
<tr>
<th>Dialyzer</th>
<th>Complement pathway</th>
<th>Time of dialysis, min</th>
<th>15</th>
<th>60</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrate</td>
<td>C</td>
<td>98 ± 3</td>
<td>104 ± 2</td>
<td>114 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>97 ± 2</td>
<td>102 ± 2</td>
<td>108 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cuprophan</td>
<td>C</td>
<td>86 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97 ± 3</td>
<td>114 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>93 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>101 ± 4</td>
<td>113 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>C</td>
<td>86 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>101 ± 2</td>
<td>123 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>96 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103 ± 3</td>
<td>122 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Polyacrylonitrile</td>
<td>C</td>
<td>90 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105 ± 4</td>
<td>123 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<tr>
<td></td>
<td>A</td>
<td>98 ± 2</td>
<td>108 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>115 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Classical (C) and alternative (A) pathway activities are expressed as a percentage of pre-dialysis values at different times of dialysis. Mean values ± SEM for each group of patients are given.

Fig. 1. Effect on total hemolytic complement activity of in vitro incubation of normal plasma with different dialyzer membranes and Zymosan. Results are expressed as a percentage of values obtained in the control (plasma incubated alone), and mean values ± SEM of six consecutive experiments are depicted. Open columns represent CH 50 activity and closed columns AP 50 activity. All values are significant in comparison with control (P < 0.01).

### Results

**In vivo studies**

Changes in circulating leukocytes in patients undergoing hemodialysis. Table 1 shows the results of monitoring the number of circulating GC at different times of dialysis. All the dialyzers tested but PAN induced an early fall in neutrophil counts, followed by a return to pre-dialysis levels after 4 hr from the start of dialysis. At 15 min, hydrate dialyzer were more effective in provoking neutropenia (92% mean decrease), followed by cuprophan (79% decrease), and cellulose acetate (50% decrease) dialyzers.

Changes in complement activity in patients undergoing hemodialysis. Hemolytic complement levels during hemodialysis are shown in Table 2. At group level, a significant mean decrease in both CH 50 and AP 50 activity was observed after 15 min of dialysis with acetate and cuprophan membranes. On the contrary, PAN dialyzers induced a significant reduction at 15 min only via the classical pathway, whereas hydrate dialyzers did not cause an early change.

However, the behavior of individual patients was extremely different: The same type of dialyzer affected in some cases
either the classical or alternative pathway (or both), whereas in some subjects no significant change in hemolytic complement titer was detectable at all. However, no correlation was found between the degree of complement depletion and neutropenia in individual patients.

After 60 min of dialysis, complement activity did not show any significant difference in comparison with pre-dialysis values. At the end of the dialysis, a significant increase in serum complement activity was evident in all the patients studied. Such an increase, besides rebound hyperproduction, could be related in part to the hemo-concentration occurring at the end of dialysis.

When plasma samples were tested for the presence of circulating GC-aggregating activity (GC-AA), no C5a-like activity could be detected at any time of dialysis (not shown).

In vitro studies

Effect of dialyzer membranes on total hemolytic complement.

In vitro incubation of normal plasma with different dialyzer membranes significantly affected total hemolytic complement activity (Fig. 1). Complement depletion was more intense with regard to the alternative pathway, particularly with hydrate and PAN, but also cuprophan and acetate membranes were able to induce a significant decrement of activity. As concerns the classical pathway, complement depletion was less pronounced. In six separate experiments, hydrate caused a mean decrement of 30% and acetate a decrement of 25%, whereas cuprophan and PAN provoked a reduction of activity of about 15%.

In vitro generation of GC-aggregating activity in normal plasma by dialyzer membranes. In our experimental model plasma incubated at 37°C for 60 min induced a slight increase in light transmission, owing to spontaneous complement decay and chemotactic activity generation, whereas fresh plasma did not induce any GC aggregation (Fig. 2A). When normal plasma was incubated with dialyzer membranes, a sharp aggregation wave was observed with all the membranes tested but PAN (Fig. 2B). In six consecutive experiments, maximal GC-AA generation occurred with hydrate (14.9 ± 1.3 cm), but the ability to generate such an activity was progressively lost on sequential in vitro "re-use." Also, the cuprophan membrane showed a strong generation of GC-AA (11.8 ± 2.1 cm), whereas cellulose acetate induced less pronounced, although still significant, GC-AA generation (6.6 ± 1.1 cm). On the other hand, GC challenged with PAN-incubated plasma were still able to generate a sharp aggregation wave upon re-stimulation with hydrate-incubated plasma (not shown).

Immunoelectrophoretic analysis of membrane-activated plasma. Plasma incubated in vitro with hydrate and PAN membranes was analyzed for C3 conversion. As shown in Figure 3, a sizeable degree of C3 conversion was present also in control plasma. More marked conversion, similar to the pattern obtained in Zymosan-incubated plasma, was present in plasma incubated with hydrate membrane. Surprisingly, the incubation of plasma with PAN did not induce any increase in the C3c peak. On the contrary, a striking reduction of both $\beta_1\alpha$(C3c) and $\beta_1\alpha$C(C3) peaks, in comparison with control plasma, was evident. Moreover, when Zymosan-activated serum was incubated with PAN, a sharp reduction was observed in the concentration of both C3 and C3c peaks, whereas the incubation with hydrate did not induce any significant change (not shown).

Evidence for the adsorption of complement components by PAN. To provide additional support for the hypothesis of an adsorption of complement components by PAN, several experimental approaches were undertaken. First of all, we showed that incubation of plasma with PAN, but not with hydrate, even in the presence of complement inhibitors (EDTA), completely removed hemolytic activity. Analogously, when functional molecule titration was performed on a standard serum, the ability of the serum depleted in factor B to act as a "finishing reagent" was prevented by prior incubation with PAN but not with hydrate (data not shown). These data were consistent with the finding that pre-incubation of plasma with PAN prevented the generation of GC-AA on subsequent contact with hydrate membrane (not shown).

Additional evidence came from the finding that GC-AA was removed completely from Zymosan-activated serum by incubation with PAN (Fig. 4). Any attempt to elute GC-AA from the membrane by molar shock or acid buffer was ineffective.

Finally, direct evidence was provided by the adsorption of a significant amount of $^{125}$I-labelled Clq by PAN membrane (Fig. 5). The binding did not take place with PAN previously incubated with normal serum or in the presence of excess "cold" Clq molecules (not shown in the figure).
Discussion

The results reported in this paper help to explain apparent contradictions in the literature. Whereas the in vivo observations provide no clear answers, confirming contradictory results previously reported [16, 17], from in vitro investigations two definite conclusions can be stated reasonably. The first one is that the decrement in total hemolytic complement function reported with PAN does not correspond to effective complement activation. The starting-point for this hypothesis was the puzzling finding that the sharp complement depletion by PAN observed in AP 50 test was not followed by in vitro chemotactic activity generation, as well as the fact that we could never show an electrophoretic conversion of C3 during hemodialysis with PAN dialyzers. To explain these findings, we considered the possibility of an adsorption and/or inactivation of complement components by PAN. We have developed a large body of evidence, both functional and immunochemical, favoring this hypothesis.

Obviously, it is beyond the limits of the present paper to extensively investigate the mechanism(s) of the adsorption of complement activity by PAN. We merely suggest that the
The second inference that we are able to draw from our studies concerns the strict relationship between complement system and hemodialysis leukopenia. In this paper, we demonstrate that the problem of the role of the complement system in hemodialysis-induced leukopenia should be thought of in terms of the chemotactic factor generation by using a steady in vitro model. In fact, when we plot the capability of in vitro generation of GC-AA by the different membranes versus the degree of neutropenia induced by the membranes tested seems convincing enough, and the previously observed inability of polycarbonate dialyzers to cause a decrement of complement activity likely resides in the low sensitivity of in vivo complement studies. Actually, we feel that in vivo investigations are inadequate to the study of complement function during hemodialysis, in that they simply reflect the balance between the rates of consumption and de novo synthesis of complement components, which can be extremely different in individual patients. Neither the more sensitive technical approaches, as detection of chemotactic factors and studies of C3 conversion (not shown), afford definite conclusions. The inability to show circulating GC-AA may result from dilution of the activated components into the blood volume and/or rapid clearing of chemotactic peptides from the circulation.

Although favoring the hypothesis of a pivotal role of chemotactic factors in the pathogenesis of hemodialysis-induced leukopenia, we do not exclude the possibility that other mechanisms play a role. The finding that cuprophan membranes are able to directly release from leukocytes cationic proteins, substances known to be capable of mediating GC aggregation [28, 29], as well as the finding that PAN dialyzers are able to provoke a significant drop in arterial PO2 (confirmed in many laboratories including our own), seem to suggest that other physiopathologic mechanisms could be involved [18].

Acknowledgments

The studies were supported in part by research grants from Ministero della Pubblica Istruzione, Cap. 10-02-01853.

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Fig. 6. Correlation between the ability of different membranes to generate GC-aggregating activity upon in vitro incubation with normal plasma and the degree of neutropenia induced by the corresponding dialyzer. Mean increases in light transmission (ST) were calculated in six consecutive experiments by testing the ability to generate GC-aggregating activity of normal plasma incubated in vitro with hydrate (○), cuprophan (●), cellulose acetate (□), and polyaclronitrile (■) membranes. Mean ΔT ± SEM are plotted against the ability of the relative dialyzer to induce neutropenia after 15 min of hemodialysis, expressed as mean percent decrease (± SEM) from the basal value in eight individual patients (r = 0.993, P < 0.01).