Reduction of Granulocyte Activation during Hemodialysis with Regional Citrate Anticoagulation: Dissociation of Complement Activation and Neutropenia from Neutrophil Degranulation

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ABSTRACT
Neutropenia and degranulation of neutrophils during hemodialysis with cellulosic membranes have been linked to complement activation, whereas in the synthetic polymethyl methacrylate (PMMA) membrane, degranulation occurs without notable complement activation. The mechanisms of neutrophil degranulation under these conditions have not yet been elucidated. Ionized calcium is an important prerequisite of granulocyte activation during in vitro blood contact with both types of artificial surfaces. This study compared the effect of normal ionized calcium during heparin anticoagulation with the effect of extracorporeal calcium depletion during regional citrate anticoagulation on activation of blood components. Because ionized calcium is reduced only in the extracorporeal circuit, citrate anticoagulation in addition helps to differentiate between extracorporeal and systemic activation phenomena. Twelve chronic hemodialysis patients were dialyzed with polymethyl methacrylate (PMMA, 16 treatments) or cuprophane (CUP, 16 treatments) membranes either during regional citrate anticoagulation or while anticoagulated with heparin. During hemodialysis with CUP, anticoagulation with citrate significantly reduced neutropenia, C3a levels, and lactoferrin release. Elastase concentrations, however, were not reduced by citrate, probably because elastase release occurred not locally in the cuprophane dialyzer, but mostly in the systemic circulation of the patient. PMMA did not elevate C3a levels, and neutropenia was only mild. Both parameters were not influenced by citrate anticoagulation. However, PMMA profoundly induced elastase and lactoferrin release during heparin anticoagulation. Depletion of ionized calcium markedly reduced PMMA-mediated neutrophil degranulation in the extracorporeal circuit. The results indicate that ionized calcium is a requirement for neutrophil degranulation during hemodialysis. In PMMA membranes, neutrophil degranulation occurs independent of high complement levels, occurs at least partially inside the dialyzer, and requires the presence of ionized calcium in the extracorporeal circuit. In cuprophane membranes, degranulation was uncoupled from neutropenia and did not correlate with the degree of complement activation. Even in cuprophane dialysis, degranulation of secondary granules was markedly dependent on ionized calcium levels in the extracorporeal circuit.

Key Words: Biocompatibility, calcium, cuprophane, granulocytopenia, polymethylmethacrylate

Temporary granulocytopenia during hemodialysis with cellulosic membranes is the most prominent sign of bioincompatibility (1). In cuprophane membranes, complement activation occurs in parallel with leukopenia and is associated with the degranulation of neutrophils (2). This close association between complement activation, neutropenia, and degranulation and the elegant studies of Craddock et al. (3) have lead to the hypothesis that granulocyte activation is secondary to complement activation in cellulosic membranes (4), and that this activation takes place partly in the systemic circulation of the patient. More recently, Cheung et al. (5) pointed out, however, that even in cuprophane membranes, only a small part of neutrophil degranulation can be attributed to complement activation, whereas other mechanisms, including β2-integrins and divalent cations, appeared to be more important.

We previously demonstrated (6,7) that some synthetic membranes activate granulocytes without notable complement activation. In addition, changes in intracellular calcium during hemodialysis correlate well with degranulation of neutrophils (8), and calcium channel blockers reduce cytosolic calcium and degranulation during dialysis (9,10). In an in vitro model of hemodialysis using citrate anticoagulation, ionized calcium was a prerequisite of granulocyte activation during contact with artificial surfaces (11). In clinical hemodialysis, the effect of citrate anticoag-
ulation may differ because citrate provides anticoagulation by chelation of calcium ions only in the extracorporeal circulation. Thus, only calcium-dependent activation processes occurring inside the dialyzer will be inhibited by regional citrate anticoagulation.

This study investigates the effect of regional citrate anticoagulation during clinical hemodialysis with complement activating cuprophane (CUP) and during treatment with more biocompatible polymethyl methacrylate (PMMA) membranes on granulocyte activation. The results demonstrate that degranulation of specific or unspecific granules can be separated from complement activation and granulocytopenia depending on the "milieu exterieur" in the extracorporeal circuit, and that these activation phenomena do not necessarily occur together.

METHODS

Patients

Twelve stable chronic hemodialysis patients (9 men, 3 women, mean age 59.3 ± 3.5 yr) were studied. Causes of renal failure were diabetic nephropathy (five patients), interstitial nephritis (one patient), bilateral nephrectomy as a result of hypernephroma (one patient), retroperitoneal lipomatosis (one patient), and unknown cause (four patients). Four patients were dialyzed with cuprophane membranes (CUP 1.25 m²; E3 Fresenius, Oberursel, Germany) and four patients were treated with dialyzers made from polymethyl methacrylate (PMMA 1.2 m²; Filtryzer B2-1.2H Toray, Tokyo, Japan). Four patients were investigated with both membranes. All patients gave informed consent in accordance with the Declaration of Helsinki.

Hemodialysis and Anticoagulation

Details of the hemodialysis and anticoagulation regimen are given in Table 1. In brief, hemodialysis with heparin anticoagulation (HHD) followed standard procedures with an initial heparin bolus followed by continuous heparin infusion. An acetate-based calcium containing dialysate was used (K33; Salvia, Homburg/Saar, Germany). In hemodialysis with regional citrate anticoagulation (CHD), a 9% solution of trisodium citrate was infused into the arterial line at a rate of 7.4 mmol citrate per liter of blood passing through the extracorporeal system. In order to recalcify the blood before it was returned to the patient, a 0.5 molar CaCl₂ solution was infused into the venous line. In CHD, an acetate-based but calcium-free dialysate (KG 82, Salvia) was used. Blood flow and dialysate flow were comparable in both treatments, but ultrafiltration rate was higher in CHD because approximately 290 mL/h of citrate and CaCl₂ infusion had to be removed.

The effect of anticoagulation was monitored by measuring activated clotting time (ACT-tester; Tri-Med, Huntington Beach, CA) in the venous line before CaCl₂ substitution. Ionized calcium was measured in blood samples from the arterial and the venous line (before CaCl₂ infusion) by an ion-selective calcium electrode (Ionometer; Fresenius).

Blood Sampling

Blood samples were obtained before dialysis and repeatedly during the procedure after 15, 60, 120 and 180 min of treatment from the arterial and venous line before CaCl₂ infusion. Samples were anticoagulated with EDTA and processed immediately for complete blood count or centrifuged at 2000 rpm at 4°C. Plasma samples were stored at -20°C or -70°C as appropriate until analysis.

Analytical Techniques

Complete differential blood count and hematocrit were measured by using an automatic counter (Technicon H 6000; Technicon, Tarrytown, NY). Chemical differential blood counts were repeatedly confirmed by microscopic examination of conventionally stained blood smears.

Desargamminated C3a (C3a-desArg) was measured by ELISA (Progen, Heidelberg, Germany) in samples stored at -70°C. Elastase in complex with α₁ proteinase inhibitor (E-α₁PI, from here on called "elastase") was assayed by ELISA (Merck, Darmstadt, Germany). Lactoferrin was measured by using the ELISA technique as previously described (12). Citrate was measured enzymatically by using citrate lyase as described by Möllering and Gruber (13).

Statistical Analysis

Data are expressed as mean ± SE. Granulocyte counts were corrected for hemoconcentration or hemodilution by using the quotient of baseline hematocrit and hematocrit of the sample. The t test for paired samples or Wilcoxon's rank sum test were used as appropriate to compare differences between anticoagulation regimens. A P value < 0.05 was considered significant.

RESULTS

Citrate Levels and Ionized Calcium

In arterial samples, a mean citrate concentration of 1.9 ± 0.2 mmol/L was measured, and 7.4 mmol citrate per liter blood was infused into the extracorporeal circuit. However, the venous concentration in-

| TABLE 1. Hemodialysis with regional citrate and heparin anticoagulation |
|---------------------------------|-----------------------------|-----------------------------|
| Variable                        | Conventional anticoagulation with sodium-heparin (HHD) | Regional citrate anticoagulation with 9% trisodium-citrate (CHD) |
| Anticoagulation                 | Initial bolus, 5000 U of heparin; maintenance during HD, 4218 ± 497 U of heparin | Citrate infusion at 7.4 mmol per L of blood |
| Recalcification                 | 221 ± 5.4 mL/min            | CaCl₂ 1.6 mmol per L of blood |
| Blood flow                      | 221 ± 5.4 mL/min            | 207.5 ± 4.1 mL/min           |
| Dialysate flow                  | 500 mL/min                  | 500 mL/min                  |
| Ultrafiltration                 | 628.8 ± 31.5 mL/h           | 906.3 ± 35.8 mL/h           |
| Acetate-buffered dialysate      | K33 Salvia® (Ca 1.75 mmol/L) | KG 82 Salvia® (calcium free) |
increased only to 5.6 ± 0.3 mmol/L because about 50% of the infused citrate was immediately removed into the dialysate and did not reach the patient.

Changes in ionized calcium (Figure 1) were not influenced by the type of membrane. Therefore, calcium data obtained during dialysis treatment with cuprophane or PMMA membranes were combined. During heparin anticoagulation, the ionized calcium concentration of the blood increased because of calcium influx from the dialysate containing 1.75 mmol/L of calcium. Before dialysis, the ionized calcium measured 1.01 ± 0.06 mmol/L. During heparin dialysis, ionized calcium in the venous line increased to 1.71 ± 0.13 mmol/L. In the arterial line, the calcium load increased calcium concentration to 1.56 ± 0.11 mmol/L.

During citrate anticoagulation, ionized calcium was measured in the arterial line before infusion of CaCl₂ for recalcification. Ionized calcium measured 1.01 ± 0.06 mmol/L before dialysis and decreased in the venous line to 0.24 ± 0.01 mmol/L. Ionized calcium in the arterial line (0.95 ± 0.05 mmol/L) did not change significantly compared with the baseline before dialysis. Citrate infusion effectively lowered ionized calcium in the dialyzer but CaCl₂ infusion prevented a decrease of ionized calcium in the systemic circulation.

**Granulocyte Count**

With use of heparin anticoagulation, cuprophane membranes (H-CUP) induced a marked granulocytopenia in the systemic circulation. Granulocyte count in the arterial line decreased from baseline values before HD (4453 ± 537/µL) to 696 ± 276/µL after 15 min and rebounded to 5613 ± 986/µL after 180 min of treatment (Figure 2A). During regional citrate anticoagulation (C-CUP), granulocytopenia and rebound were blunted. After 15 min, neutrophils had decreased to 2657 ± 901/µL, significantly less than with heparin (P < 0.01). After 180 min of C-CUP, the granulocyte count measured 4851 ± 737/µL, not significantly different from the baseline before HD.

When using PMMA membranes (Figure 2B) with heparin anticoagulation (H-PMMA), a moderate but significant granulocytopenia was seen, much less than with cuprophane membranes: 0 min, 3584 ± 174/µL; H-PMMA: 15 min, 1829 ± 197/µL; 180 min, 3,455 ± 389/µL. Citrate anticoagulation (C-PMMA) did not result in significant differences compared with H-PMMA: 15 min, 2390 ± 297/µL; 180 min, 3128 ± 267/µL.

**Complement Activation**

In H-CUP dialysis (Figure 3), a marked complement activation was noted in the systemic circulation, measured as C3a concentration in the arterial line. Levels increased from baseline values of 292.8 ± 21.9 ng/mL to 1505 ± 207 ng/mL after 15 min and further to 1718 ± 197 ng/mL after 60 min of treatment (Figure 3A). In the venous line, C3a concentrations peaked after 15 min of H-CUP at 3726 ± 635 ng/mL. Samples taken from the venous line (Figure 3B) showed always higher C3a levels than arterial samples.

In C-CUP (regional citrate anticoagulation), complement activation was blunted. C3a concentrations in the arterial line (Figure 3A) increased to only 933.8 ± 194 ng/mL after 15 min, significantly less than during heparin anticoagulation (P < 0.001). After 60 min, C3a concentrations reached 1361 ± 140 ng/mL. C3a concentrations in venous samples (Figure 3B) were always higher than arterial samples (Figure 3A). However, the effect of citrate on C3a levels was present only during the first 60 min of cuprophane hemodialysis both in arterial and venous samples.

When using PMMA membranes (Figure 4A and B)
with heparin anticoagulation (H-PMMA), no significant complement activation was seen in arterial or venous lines compared with baseline (0 min, 292.8 ± 21.9 ng/mL; 15 min arterial, 374.4 ± 52.4 ng/mL). Citrate anticoagulation (C-PMMA) did not result in significant differences compared with H-PMMA, and C3a levels were not elevated.

Neutrophil Degranulation: Elastase Release

Elastase in complex with α1-proteinase inhibitor increased during H-CUP dialysis from 61.8 ± 3.7 ng/mL to 117 ± 11.7 ng/mL after 120 min (Figure 3C). Levels in venous samples (Figure 3D) were not higher than in samples from the arterial line (Figure 3C). Citrate anticoagulation with use of cuprophane membranes (C-CUP) did not reduce elastase release from granulocytes (124 ± 10.7 ng/mL after 180 min (Figure 3C and D).

In PMMA membranes, elastase showed a different pattern: Arterial levels (Figure 4C) during heparin anticoagulation peaked at 135 ± 14.1 ng/mL after 180 min. Venous concentrations (Figure 4B) were always higher than arterial levels. Citrate anticoagulation (C-PMMA) resulted in venous levels that were no longer higher than arterial levels. In addition, arterial levels were higher with heparin than with citrate anticoagulation, peaking at 96.4 ± 11 ng/mL.
Neutrophil Degranulation: Lactoferrin Release

Lactoferrin in heparin-anticoagulated cuprophane dialysis (H-CUP) increased from 137.2 ± 25.5 ng/mL to 411.6 ± 98.7 ng/mL (Figure 3E). Venous values (Figure 3F) were only slightly higher than arterial samples. With citrate anticoagulation (C-CUP), increases in arterial lactoferrin levels (Figure 3E) were significantly less than during heparin anticoagulation.

With the use of PMMA membranes (Figure 4E), lactoferrin concentrations increased to 461.5 ± 155.2 ng/mL with heparin anticoagulation (H-PMMA). During citrate anticoagulation (C-PMMA), lactoferrin levels increased only minimally to 181.8 ± 46.6 ng/mL, significantly less than during heparin anticoagulation. Venous levels (Figure 4F) always measured higher than arterial samples. Citrate markedly reduced lactoferrin release in all arterial and venous samples.

DISCUSSION

The poor biocompatibility of cuprophane compared with PMMA dialysis membranes has recently been linked to reduced renal recovery rates and patient survival in patients with acute renal failure (14). PMMA may be protective because it induces less complement activation and less reactive oxygen species (ROS) production than cuprophane (15). However, previous results by us (11,16) and others (17) have shown that PMMA is not entirely biocompatible because degranulation of neutrophils is as pronounced with PMMA as with cuprophane. In agreement with previous observations (6,18), we found a mild but significant granulocytopenia during dialysis with PMMA. The stimulus for this granulocytopenia may be a slight increase in C5a (18) and C5b-9 levels (8). Himmelfarb et al. (15), however, suggested that the dissociation of complement, ROS production, and degranulation points to different mechanisms of activation. For the clinical relevance of dialyzer-induced blood cell activation, it may be important, whether activation occurs mainly inside the dialyzer or in the patients’ body. Because most granulocyte products such as oxygen radicals or elastase are inactivated within milliseconds, degranulation in the microcirculation of the patient, e.g., from granulocytes marginated to the vessel wall, may be more detrimental than activation of granulocytes limited to the extracorporeal circuit. This study gives insights into the mechanisms and location of granulocyte activation during clinical use of hemodialyzers made from PMMA or cuprophane.

Regional citrate anticoagulation has long been advocated to reduce the risk of bleeding in dialysis patients prone to bleeding complications (19–21). In addition, citrate anticoagulation offers the unique opportunity to selectively influence blood-membrane interactions inside the dialyzer, while activation phenomena occurring within the patients’ body are not inhibited. Activation of polymorphonuclear cells (PMN) was considered to occur mainly in the patient's systemic circulation, if venous concentrations of elastase or lactoferrin were not higher than levels in the arterial line. Degranulation of PMN was suspected to occur at least in part locally in the dialyzer, if venous concentrations of elastase or lactoferrin were consistently higher than levels in the arterial line, and if citrate anticoagulation markedly reduced this arteriovenous difference as well as systemic levels. However, if activation occurs in both the dialyzer and systemically, but to a greater extent in the dialyzer than in the systemic circulation, the venous concentration would still be higher than the arterial concentration and a firm conclusion about the site of activation can not be reached. In addition, hemocoagulation occurs in the dialyzer as a result of ultrafiltration, which influences concentrations of elastase and lactoferrin. At a blood flow of 207 to 221 mL/min an ultrafiltration rate of 10 to 15 mL/min (Table 1), hemocoagulation is expected to elevate venous levels by 4.5 to 7.5% compared with arterial levels, even without cell activation in the dialyzer.

Depending on the polymer, regional citrate anticoagulation had different effects on granulocytes. In cuprophane membranes, (1) complement activation inside the dialyzer was blunted by citrate, but markedly elevated levels of C3a were still present; (2) granulocytopenia was reduced by citrate in parallel with lowered C3a levels; (3) elastase release from granulocytes occurred primarily in the patients' circulation and was not influenced by citrate or lowered C3a levels; and (4) lactoferrin release from granulocytes was markedly reduced by citrate. In PMMA membranes, (1) notable complement activation was not detectable regardless of anticoagulant used; (2) the mild granulocytopenia seen with PMMA was not influenced by citrate; (3) elastase and lactoferrin release occurred at least partially inside the dialyzer and both were reduced by citrate.

The effect of sodium citrate is the result of chelation of the divalent cations in the extracorporeal circuit (22). We previously showed in vitro (11) that reconstitution of physiologic ionized calcium levels by the addition of CaCl₂ allowed uninhibited activation. This indicates that any inhibition occurring in the presence of sodium citrate is not related to citrate toxicity and that depletion of ionized calcium is the predominant cause of inhibition. Depletion of ionized calcium is a likely mechanism to reduce plasmatic or cellular activation, because many of them are known to be calcium dependent (23). Wiegmann et al. (24) noted that granulocytopenia but not hypoxemia was reduced with citrate anticoagulation. Complement is activated during hemodialysis with cellulose membranes by the alternative pathway, which is believed not to depend on ionized calcium but on magnesium. Thus, lower C3a levels during cuprophane hemodialysis with citrate anticoagulation may be the result of low ionized magnesium levels in the extracorporeal...
circuit, because magnesium is also chelated by citrate (22). If less activation is noted during citrate anticoagulation, compared with heparin, a direct activating effect of heparin on plasmatic or cellular blood components needs to be considered. Low doses of heparin may result in clot formation and enhanced complement activation but neutrophil degranulation does not increase (25). High heparin concentrations increased myeloperoxidase but not lactoferrin or lysozyme release (26). Thus, in the usual therapeutic doses, neutrophil degranulation appears not to be markedly affected by heparin itself.

Citrate anticoagulation provided several lines of evidence that neutrophil degranulation during cuprophane hemodialysis is partly independent of complement activation. First, degranulation of primary granules was uncoupled from C3a levels because elastase concentrations did not decrease when C3a levels were lowered. However, failure to observe a decrease in elastase release when complement activation was decreased could be explained by complement activation exceeding the level required for maximal stimulation of this response. Second, during citrate anticoagulation, C3a levels, although reduced, were still high. These high C3a levels, however, were unable to maintain lactoferrin levels in the same range as with heparin anticoagulation despite a normal serum calcium level in the patients' systemic circulation. Third, although a dose-response study between C3a and degranulation was not performed, it is noteworthy that the reduction of lactoferrin release was much more pronounced than expected from the 40 to 50% decrease of complement levels at 15 min. Thus whereas the degree of neutropenia during cuprophane hemodialysis appeared to be linked to C3a levels in this experimental setting, degranulation of neutrophils was only partly related to complement activation.

Other techniques to reduce activation of blood components inside cuprophane hemodialyzers have also dissociated complement activation from neutrophil degranulation. In a previous study (27), we observed that reuse of cuprophane membranes considerably reduced complement levels and elastase release, but failed to affect degranulation of secondary granules. Thus, under these conditions, lactoferrin release appeared to be independent of complement activation. In contrast to reuse, pretreatment of cuprophane dialyzers with 25% albumin solution did not lower complement activation or lactoferrin levels, but reduced elastase release (27). Thus, neither lactoferrin nor elastase levels were consistently linked to complement activation. Recently, Neveceiral et al. (28) primed cuprophane dialyzers either with total plasma proteins, albumin, or immunoglobulins. This approach completely dissociated complement and granulocyte activation. Although complement activation was not influenced by these maneuvers, leukopenia was prevented by priming with total plasma proteins. Moreover, neutrophil oxygen radical production and chemotaxis were normalized with all three plasma preparations despite continuously high C3a levels, indicating that C3a by itself is not sufficient to induce degranulation.

In cuprophane membranes, special techniques such as citrate anticoagulation, reuse, or protein pretreatment are required for demonstration of the independence of neutrophil degranulation from complement activation, because under routine operating conditions, all activation phenomena occur simultaneously. Synthetic PMMA membranes, however, induced marked neutrophil degranulation without elevation of C3a levels, even during standard operating conditions. In vitro, complement independent neutrophil activation by PMMA membranes required direct contact of the cells with the polymer and occurred only during blood flow but not during incubation of blood with membrane material without blood flow (29). The mechanisms of complement independent neutrophil degranulation need to be clarified.

Our data indicate that ionized calcium is a requirement for neutrophil degranulation during dialysis with cuprophane or PMMA membranes. In PMMA and cuprophane membranes, lactoferrin release from secondary granules was inhibited by extracorporeal calcium depletion, probably because a significant part of lactoferrin release occurs inside the dialyzer and is therefore vulnerable to extracorporeal calcium depletion. PMMA induced elastase release, which is possibly the result of frustrated phagocytosis (30), was blunted by regional citrate anticoagulation, probably because it occurs at least in part inside the dialyzer. In cuprophane hemodialysis, elastase is released from neutrophils primarily outside the dialyzer (Figure 3C and D) and consequently, regional citrate anticoagulation did not affect this response. In contrast to clinical hemodialysis, citrate anticoagulation in an in vitro model of hemodialysis with cuprophane membrane (11) also prevented elastase release, probably because in this model, ionized calcium concentrations were low in all of the recirculating blood. Thus, normal systemic calcium levels in regional citrate anticoagulation may explain the failure to lower elastase release during clinical hemodialysis. These observations are supported by the in vitro data of Cheung et al. (5) that show that in cuprophane membranes, complement activation accounts for only a small part of neutrophil degranulation. They demonstrated in vitro that complement-independent neutrophil degranulation requires divalent cations. Degranulation of primary or secondary granules is preceded by a rise in intracellular calcium. Primary granules degranulate at a higher intracellular calcium level than secondary granules. Interestingly, intracellular calcium not only rises in response to binding of chemoattractant mediators to specific receptors, but also in response to neutrophil adherence to nude or fibronectin-coated surfaces (23). Therefore, it appears possible that complement-independent but calcium-dependent neutro-
Granulocyte Activation During Regional Citrate Anticoagulation

Cell membrane degranulation is brought about by direct cell contact with the dialysis membrane.

Abnormally high levels of intracellular calcium have been found in neutrophil dysfunction during uremia and hemodialysis. Impaired phagocytosis and the higher rate of infection in dialysis patients have been linked to chronically elevated levels of intracellular calcium in PMN of hemodialysis patients. Vanholder et al. (32) and Ward and McLeish (33) pointed out that the dialysis procedure more than uremia itself causes an impairment of phagocytosis. Intracellular calcium of PMN rises acutely during hemodialysis with PMN-activating membranes (8). This rise in intracellular calcium, the degranulation of PMN (9), and metabolic abnormalities (31,34) can be blunted if the patient is treated with calcium channel blockers. Calcium channel blockers lower intracellular calcium, the degranulation of PMN (9), and improve phagocytosis (31). Neutrophil activation during dialysis treatment may contribute to chronically elevated intracellular calcium levels and impaired phagocytosis in hemodialysis patients.

In summary, ionized calcium is a requirement for neutrophil degranulation during hemodialysis with cuprophane and PMMA membranes. Activation occurring inside the dialyzer is more likely to be blunted by regional citrate anticoagulation and extracorporeal depletion of ionized calcium than activation that takes place in the systemic circulation. Whether repeated calcium-mediated neutrophil degranulation contributes to chronic neutrophil dysfunction and increases the risk of infection in hemodialysis patients remains to be determined.

References