Abstract. The objective of the study was to evaluate the contribution of reactive oxygen intermediate formation for receptor modulation on neutrophils by the cellulose dialyzer membrane cuprophan (CU). In patients dialyzed with CU, CD11b and CD66b upregulation on neutrophils (by 104.3 \pm 37.9\% and 85.7 \pm 31.1\%, respectively), and a downregulation of \( \alpha \)-selectin (by 44.9 \pm 26.9\%) was seen, whereas expression of CD11a remained unaltered. Hemodialysis with polysulfone did not bring about major changes in surface receptor expression. In vitro incubation of isolated neutrophils in the presence of serum with hollow fibers of CU or polysulfone showed similar results: Only CU resulted in upregulation of CD11b and CD66b expression (by 65.5 \pm 18.7\% and 60.1 \pm 24\%) and a decrease in CD62L expression (by 60.6 \pm 18.2\%). In contrast to receptor alterations, generation of reactive oxygen intermediate by CU occurred in the absence of serum. Inhibition experiments with soluble complement receptor 1, which produced only partial inhibition of receptor up-/down-regulation, indicated the existence of also other than alternate complement-dependent mechanisms for neutrophil activation. By using C5-depleted serum instead of normal human serum, up-/down-regulation of CD11b, CD62L, and CD66b by CU was dramatically reduced, whereas C3-depleted serum did not produce that effect. C5-deficient serum repleted with purified C5, as well as purified C5 alone, was able to induce receptor modulation by CU comparable to normal human serum. \( \alpha \)-Methionine, a specific inhibitor for the oxidative activation of C5, blocked the modulatory effect of CU in assays with purified C5 as well as with serum. As a result, in addition to the alternative pathway of complement, a C5-dependent mechanism probably activated by neutrophil-derived reactive oxygen intermediate leads to receptor modulation and subsequent generation of the well known side effects of bioincompatible dialyzer membranes.

Hemodialysis with cellulose membranes is associated with an array of adverse reactions, including leukopenia, pulmonary sequestration, and dysfunction of leukocytes (1,2). Activation of the alternative pathway of complement due to direct contact of plasma with cellulose membranes like cuprophan (CU), a commonly used hemodialysis membrane, is considered responsible for the induction of these side effects (1,3–7). On the cell surface of polymorphonuclear leukocytes (PMN), upregulation of particular receptor proteins, including CD35 (8), CD11b (9), and CD66b (10), and downmodulation of \( \alpha \)-selectin (CD62L) (11) and sialophorin (CD43) (12) has been reported, and these changes in expression have been correlated with the development of granulocytopenia during dialysis. Shedding of \( \alpha \)-selectin was also reflected by the detection of soluble \( \alpha \)-selectin after dialysis with cuprophan membranes (13). Dialysis in the same patients with a non-complement-activating membrane showed no significant changes in receptor expression, and therefore, complement-split products were speculated to be responsible for up-/down-regulation of integrins and selectins on neutrophils (11).

Although other mechanisms have also been postulated to be involved in the generation of a number of acute and chronic potentially harmful effects, their relationship is less well established. Products of monocyte activation such as interleukin-1 and tumor necrosis factor-\( \alpha \) have been detected in some studies, but their serum concentration did not seem to correlate with the type of dialysis membrane (14–18). The release of elastase during dialysis with cuprophan has been detected but did not correlate with the generation of early complement-split products (19,20). Reactive oxygen intermediates (ROI) were reported to be produced in patients dialyzed with cuprophan membranes (19,21–23) and were suspected to contribute to an increased rate of malignancies and atherosclerosis (22). Recently, we were able to show that ROI production also occurs independently of complement activation (24), i.e., in the absence of serum, cellulose membranes induced ROI formation in neutrophils in vitro. Additionally, we demonstrated a direct correlation between the nadir of leukocyte counts and ROI formation in vivo but not with the release of the complement-split product Bb. This finding indicates that other mechanisms...
in addition to direct complement activation by contact of serum with cellulosic membranes are responsible for the generation of early side effects.

There is recent evidence that PMN-derived ROI are able to oxidatively convert the complement component C5 to a functionally active C5b-like conformation (25). The present study investigates the contribution of ROI elaborated by cuprophan-stimulated PMN as mediators in this nonenzymic complement-dependent pathway for the activation of neutrophils by cellulosic membranes. Modulation of neutrophil surface receptors during dialysis in vivo was compared with the effect of cellulosic membranes in vitro, and tested under conditions that allowed us to study the influence of particular complement components. By using this approach, we were able to show for the first time that cuprophan-stimulated neutrophils appear to activate, probably by means of the release of ROI, a C5-dependent mechanism leading to receptor modulation comparable to in vivo changes during dialysis with cellulosic membranes.

Materials and Methods

Patient Characteristics
Six chronic hemodialysis patients (four men and two women), who had given informed consent, entered the study. The mean age was 61.2 yr (range, 40 to 74 yr). Mean duration of dialysis was 20.6 mo (range, 4 to 50 mo). The chronic dialysis protocol consisted of 4 h three times weekly by means of an arteriovenous fistula. Membrane material used for chronic dialysis was polysulfone with a surface area of 1.25 m² (F60; Fresenius, Bad Homburg, Germany). Dialyzers and lines were steam-sterilized, and no patient had dialyzer reuse. All had bicarbonate dialysis and anticoagulation with low molecular weight heparin. Blood flow was adjusted between 200 and 300 ml/min, and dialysate flow was 500 ml/min. Additional therapy consisted of phosphate binders and active vitamin D in all patients. Four patients had calcium antagonists, two digitoxin, and three water-soluble vitamins.

To test the possible difference between polysulfone and cuprophan membranes in up-/down-regulation of surface membrane receptors in human neutrophils, patients normally on polysulfone membrane were switched once to cuprophan hollow fiber dialyzers (Fresenius E3, surface area 1.25 m², steam-sterilized). During this and the following session, again by using polysulfone membrane, neutrophil activation was studied. Blood samples were taken from the arterial line of the dialyzer before starting dialysis treatment, after 5, 10, 20, 30, and 60 min, and at the end of the dialysis procedure.

Reagents
1-Methionine, zymosan A, and N-formyl methionyl-leucyl-phenylalanine (fMLP) were purchased from Sigma Chemical Co. (St. Louis, MO). 2′,7′-Dichlorofluorescin-diacetate (DCFH-DA) was obtained from Molecular Probes (Junction City, OR). DCFH-DA was dissolved in ethanol at a concentration of 2.5 mg/ml and stored in the dark at −20°C. Complement component C3- and C5-depleted human serum as well as purified C5 was obtained from Calbiochem Corp. (Cambridge, MA). Recombinant soluble complement receptor 1 (sCR1; 35.06 lot 92M026) was a kind gift of Dr. Henry Marsh (T Cell Sciences, Inc., Needham, MA). FITC-conjugated monoclonal antibodies (mAb) anti-CD11a (clone 253.3.1), anti-CD11b (clone BEAR 1), anti-CD62L (clone Dreg 56) were purchased from Immunotech (Marseille, France), and anti-CD66b (clone 80H3) was from Serotec (Oxford, United Kingdom), as were purified anti-CD88 antibodies (clones S5/1 and W17/1). Purified fragment of anti-CD16 (clone 3G8) and anti-CD32 (clone IV.3) were obtained from Medarex, Inc. (Lebanon, NH). As isotype control, a combi reagent of IgG1 and IgG2 by An der Grub, Scandic (Vienna, Austria) was used.

Determination of Surface Receptor Expression In Vivo

For analysis of surface receptor expression, blood from hemodialyzed patients was obtained at specified time points, drawn into ethylenediaminetetraacetate tubes, and then immediately stored at 4°C. A 50-μl aliquot from whole blood was taken and then incubated for 30 min on ice with 20 μl of the respective mAb, followed by washing twice with phosphate-buffered saline/bovine serum albumin 1%. All samples were then analyzed within 2 h by flow cytometry using a FACScan (Becton Dickinson, Sunnyvale, CA). Data analysis was performed by first displaying forward scatter versus side scatter and gating on the neutrophil population. Data on at least 10,000 events were collected in list mode file, the histograms were generated for each population, and the mean fluorescence level was calculated.

Cell Preparations
Polymorphonuclear neutrophils (PMN) were isolated from peripheral blood of healthy donors by using discontinuous Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. After separation, erythrocytes were removed from the pellet by hypotonic lysis with ammonium chloride buffer (157 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM ethylenediaminetetraacetate Na₂). Purity of PMN preparations always exceeded 95%; cell viability as determined by propidium iodide staining was at least 98%.

Oxidative Burst Measurements
Oxidative burst formation by PMN was assessed as described previously (26) by measuring the oxidation of DCFH-DA at a single-cell level (10,000 cells analyzed). Briefly, isolated PMN (5 × 10⁵) were incubated in the presence of DCFH-DA with hollow fibers (14 mg) of the respective dialysis membrane, with medium control or fMLP (10⁻⁷ M) as positive control for 30 min. After incubation, PMN were separated by resuspension with a pipette from the hollow fibers and transferred onto ice (4°C), and the mean fluorescence intensity (MFI), reflecting neutrophil ROI production, was immediately assessed by flow cytometry. Production of reactive oxygen intermediates was expressed by the following formula: x-fold increase = (MFIₜₚₑᵣₑ瘼 − MFIₜₑᵣₑₘᵣₑₚ)/MFIₜₑᵣₑₘᵣₑₚ, where MFIₜₑᵣₑₘᵣₑₚ represents mean fluorescence intensity of cells incubated in the presence of hollow fibers of dialysis membranes, and MFIₜₑᵣₑₘᵣₑₚ represents the mean fluorescence intensity of cells incubated with phosphate-buffered saline. More than or equal to twofold increase of MFI was considered positive.

Determination of Surface Receptor Expression In Vitro
PMN (5 × 10⁵) were incubated with the respective hollow fibers (14 mg) in the presence or absence of normal human serum, C3- or C5-depleted serum (final concentration 25%) for 15 min at 37°C. Incubations were performed in duplicate on 96-well flat-bottom microtiter plates (Costar, Cambridge, MA). In blocking/inhibition experiments, the following agents were used: purified anti CD88 mAb (20 μg/ml), sCR1 (30 μg/ml), and 1-methionine (10 and 50 mM). To avoid preterm PMN activation and to obtain full capacity inhibition in our system, hollow fibers were mixed with the respective blocking agents and serum at 4°C. Finally, isolated PMN were added. After incubation for 15 min in a 37°C waterbath, PMN were intensively
resuspended by using a pipette, and then 100 μl of the cell suspension was transferred onto ice and incubated with 20 μl of the respective FITC-conjugated mAb for 20 min, followed by washing twice with phosphate-buffered saline/bovine serum albumin 1%. Cells were analyzed by using EPICS-XL flow cytometry (Coulter, Miami, FL).

**Determination of Complement**

Complement-split product C3a generation was determined by using the commercially available Complement C3a desArg Biotrak RIA System (Amersham, Buckinghamshire, United Kingdom). The total hemolytic complement activity was assessed by testing the ability of the respective sera to lyse 50% of a standard suspension of sheep erythrocytes coated with optimal amounts of rabbit antibody in a reaction that includes the entire classic activation pathway as well as the terminal sequence (CH50 assay).

**Statistical Analyses**

Statistical analysis for the evaluation of a possible difference in receptor expression after PMN stimulation under different conditions was performed by using unpaired *t* test. All results are expressed as the mean ± SD and were considered significantly different at *P* ≤ 0.05.

**Results**

**Receptor Modulation on PMN during Hemodialysis with Cuprophan and Polysulfone Membranes In Vivo**

Activation of neutrophils after hemodialysis has been associated with activation of the alternative pathway of complement leading to increased adhesiveness and aggregation and subsequent neutropenia (1,2). In the present study, expression on PMN of surface receptors central to an inflammatory reaction, L-selectin (CD62L), and CD11b, was investigated in patients during hemodialysis with a cuprophan or a polysulfone membrane. Furthermore, analysis of CD66b expression was included as an example of a granulocyte-specific activation antigen stored in specific neutrophil granules and rapidly moved to the cell surface upon activation (27–29). Evaluation of CD11a expression served as negative control, because alterations have not been described during hemodialysis.

In patients dialyzed with CU membranes, at the time of maximal activation (30 to 60 min) CD11b was upregulated by 104.3 ± 37.9% (*P* < 0.001) and CD62L expression decreased by 44.9 ± 26.9% (*P* = 0.017) (Figure 1). CD66b was upregulated by 85.7 ± 31.1% (*P* = 0.001). No major changes in receptor expression of these molecules were detected in patients dialyzed with polysulfone (PS), reflecting the biocompatibility of this material. Modulation of CD11a was not observed in CU- or in PS-dialyzed patients.

**Receptor Modulation on PMN by Hollow Fibers of Cuprophan and Polysulfone Membranes In Vitro Is Serum-Dependent**

To test the possible influence of the complement system on the expression of neutrophil surface receptors modulated by CU or PS membranes, isolated neutrophils were stimulated with the respective hollow fibers in the presence or absence of fresh normal human serum. As shown in Figure 2, *in vivo* findings could be reproduced *in vitro* in the presence of serum.

In cultures with CU and serum, CD11b and CD66b were upregulated by 65.5 ± 18.7% (*P* < 0.007) and 68.9 ± 27.9% (*P* < 0.01), respectively, and CD62L expression was decreased...
No significant alterations were seen for CD11a. In the absence of serum, only CD62L shedding exceeded 40%, whereas all other receptors showed changes below 20%. Receptor modulation on PMN during incubation with PS remained insignificant (up-/down-regulation less than 20% in all experiments; data not shown).

**Influence of the Recombinant Soluble Complement Receptor 1 on Receptor Modulation In Vitro**

sCR1, a specific inhibitor of alternate/classic complement activation, was used to determine the influence of complement on receptor expression. Applying concentrations of sCR1 (30 µg/ml) previously demonstrated to completely prevent complement activation (30), we found a partial inhibition of CD11b and CD66b upregulation and CD62L shedding (Figure 3). Higher concentrations of sCR1 (up to 120 µg/ml) did not improve the inhibition. Because inhibition by sCR1 was at best 40%, our results suggest that receptor modulation is under the control of mechanisms other than the conventional complement-dependent pathways.

**Dissection of the Role of Specific Complement Components on Cuprophan-Induced Receptor Modulation in PMN**

Because CU membranes activate complement and activated complement fragments are known to stimulate neutrophils (31), the effect of complement on cuprophan-induced receptor modulation was studied by using sera depleted of C3 or C5. The quality of these sera was ensured by showing absolute nonreactivity in CH50 assays. Furthermore, in contrast to normal human serum or C3-depleted serum, in C3-depleted serum no C3a generation was achieved after stimulation with either zymosan A or CU as determined by C3a desArg RIA.

When C5-depleted serum was substituted for normal human serum, up-/down-regulation of receptors by CU was profoundly inhibited (Figure 4), whereas C3-depleted serum did not show effects that were different from normal human serum. Repeating the C5-depleted serum with purified C5 completely restored the capacity of the serum to synergize with CU membranes in receptor modulation. Moreover, stimulation of PMN by CU in the presence of purified C5 (70 µg/ml) alone resulted in receptor alterations of comparable magnitude to serum, with significant upregulation of CD11b and CD66b as well as shedding of CD62L (Figures 4 and 5). In the absence of CU, C5 by itself was not able to induce substantial changes in receptor expression (Figure 5). These data identify C5 as an essential element in cuprophan-induced adhesion molecule regulations.

The role of C5 in receptor modulation seems not to involve C5a receptor-mediated signaling, because two blocking mAb (clones S5/1 and W17/1) against C5a receptor (32) were unable to interfere with CU-induced alterations in expression of CD11b, CD66b, or CD62L (data not shown).

**Oxidation of C5 Is Involved in Receptor Modulation on PMN in the Presence of Cuprophan**

Several reports describe an effect of oxygen radicals on receptor expression in PMN (33), a direct role for ROI in...
activating complement (34), and specifically a nonenzymatic activation of C5 (35). We have shown that substantial radical formation occurs both in vivo and in vitro after contact with cuprophan membrane (24). Incubation of PMN with hollow fibers of cuprophan for 30 min resulted in significant production of ROI (Figure 6). In contrast, polysulfone membranes did not elicit radical formation. fMLP-stimulated neutrophils served as positive control and showed a stimulation index comparable to cuprophan-stimulated PMN. We tested our hypothesis that CU-induced ROI were responsible for C5-dependent receptor modulation. The addition of excess L-methionine, a chloramine scavenger known as the target amino acid on the C5 molecule for oxidative conversion (25), led to a significant dose-dependent inhibition of CD11b modulation by C5 and CU membrane (Figure 7). A similar effect was detected when using serum instead of purified C5. PMN stimulation by fMLP was not influenced by L-methionine, indicating no direct interference of this amino acid with receptor modulation and underscoring its competitive oxygen scavenger function for C5.

Discussion

In this study, the role of ROI formation and complement activation in receptor modulation on PMN by the cellulosic membrane cuprophan was evaluated. Our results demonstrate a self-augmenting activation loop, consisting of cuprophan-stimulated neutrophil respiratory burst probably activating a C5-dependent pathway, which in turn is able to stimulate PMN, as shown by CD11b and CD66b upregulation, and CD62L shedding.

As already shown by many investigators (9–11), dialysis of patients with CU led to a significant upregulation of CD11b and CD66b, and shedding of l-selectin CD62L, whereas no major changes were detected in patients dialyzed with the biocompatible polysulfone membrane. This difference in neutrophil stimulation has thus far been attributed mainly to activation of the alternative complement pathway by cellulosic membranes (4,36). Our previous investigation revealed that during hemodialysis with CU the peak of ROI formation coincided with the nadir of PMN counts in the circulation (24), leading to the speculation that other mechanisms might be operative in hemodialysis-associated neutrophil activation. In our present in vitro experiments, the cuprophan-induced modulation of neutrophil adhesion molecules appeared to be highly dependent on normal human serum component(s), which points to the contribution of alternative complement activation. However, inhibition experiments based on the use of sCR1 indicated the involvement of (an)other mechanism(s) in receptor modulation. Recombinant sCR1 has been described as a potent inhibitor of the activation of the complement system in vitro and in vivo (37–39). sCR1 regulates complement activation by means of its decay accelerating activity for the C3 and C5 convertases of both the classic and alternative pathway. Second, it serves as a cofactor for the enzymatic degradation of C3b and C4b by factor I (37). Himmelfarb et al. described a complete inhibition by sCR1 of C3a desArg generation and of up-/down-regulation of PMN adhesion molecules during ex vivo hemodialysis (30). On the other hand, Finn et al. found no...
inhibition of adhesion receptor modulation in an extracorporeal circuit setting (cardiopulmonary bypass), despite abolishing C3a levels (40). Cheung et al. showed an incomplete inhibition of elastase release, besides a total inhibition of C3a, suggesting that dialysis-induced neutrophil activation is mediated in part by non-complement-dependent mechanisms (41). In our experiments, the use of recombinant sCR1 showed a significant but only partial inhibition of receptor up-/down-regulation on human PMN, also indicating C3/C5 convertase-independent processes.

By using C5-depleted human serum instead of normal human serum, we identified the C5 molecule as the central complement component involved in PMN receptor modulation. In contrast, in assays with selective depletion of C3, only a modest inhibitory influence on these neutrophil surface receptor changes was observed. These findings cannot be explained by what is currently known about complement-activating properties of cuprophan, which involve the C3/C5 convertase of the alternative pathway necessitating functional C3 and not C5 availability. The essential role for C5 with regard to cuprophan-related neutrophil surface receptor modulation was confirmed in PMN/cuprophan incubations with purified human C5, which resulted in profound CD11b and CD66b upregulation and CD62L shedding, comparable to normal human serum containing the entire set of complement components. We conclude that direct C5 activation, presumably by PMN-derived factors, had occurred and exerted stimulating effects on granulocytes.

Reactive oxygen species elaborated by PMN are known protagonists for nonenzymic complement activation. We have previously demonstrated a strong oxidative burst reaction of PMN on contact with cuprophan both in vivo and in vitro (24). Furthermore, there are several reports on the direct complement-activating potency of reactive oxygen intermediates (34), with emphasis on the oxidation-sensitive complement factor C5 (35,42–44). This novel third pathway of complement activation bypasses the early enzymic reactions of both the classic and the alternative pathway. It does not require complement components C1, C4, C2, C3, and B, to allow late events such as C5 activation and the consecutive assembly of the membrane attack complex, C5–9 to occur. Vogt recently showed that stimulated neutrophils are capable of C5 activation by highly reactive products of the myeloperoxidase-halide system (25). Taurine dichloramine and NH₂CL, the monochloramine of ammonia, have been implicated as PMN-derived oxidants activating C5 (25,45). Indeed, this ROI-induced pathway of complement activation seems to be responsible for neutrophil activation in our studies. L-Methionine, well documented as an inhibitor of the oxidative conversion of native C5 to activated C5b-like C5 (42–44,46), abolished modulation of neutrophil adhesion receptor expression induced by cuprophan and C5. Also, in whole-serum assays a significant dose-dependent inhibition of neutrophil activation was observed, strongly suggesting that this oxidative pathway is operative under physiologic conditions.

So far, however, it is not clear how signaling of CU-induced receptor modulation is brought about by oxidatively altered C5. ROI-related C5 activation leads to conformational changes in the C5 molecule, which enables C6 binding (44) and does not involve cleavage of C5 into C5a and C5b (42). ROI alter the C5 molecular conformation by oxidation of methionyl residues to the respective sulfoxides (44), thereby yielding C5b-like activity. We propose the spontaneous assembly of C5b-like C5 with C6 and C7 molecules, resulting in formation of a C5b67 complex that was previously shown to insert into plasma membrane lipid bilayers and to directly activate G proteins without the requirement for a specific cell surface receptor (47). Indeed, C6 and C7 are stored in considerable amounts by neutrophils (48), and, therefore, probably present even in our serum-free experiments. In addition, recent studies by Wang et al. identified hemolytically inactive C5b67 as chemotactic agonist for PMN, with evidence for the existence of a distinct iC5b67 receptor (49,50).

Any C5a-like chemotactic activity generated by radicals in whole serum (34,51) is secondary and is caused by enzymic cleavage of the C5b-like C5 molecule by plasma proteases (46), because oxidatively modified C5 seems to be more susceptible to enzymic digestion (52). To assess a possible C5a-mediated contribution, whether originating from alternative complement activation or from secondary cleavage of oxidized C5, to the observed cuprophan-dependent adhesion molecule modulations on neutrophils (53), we performed inhibition studies by using anti-C5a receptor monoclonal antibodies S5/1 and
W17/1 (32). However, no significant influence of C5aR blockade could be observed on isolated PMN incubated with cuprophan and purified C5 or normal human serum. This finding supports our idea of oxidative C5 alteration to C5b-like conformation as a dominant mechanism for complement activation by cuprophan in the presence of neutrophils.

To our knowledge, our group is the first to investigate the influence of PMN-activated C5b-like C5 on PMN surface receptor modulation. Our observation of a novel C5-dependent neutrophil-activating mechanism probably attributable to the release of oxygen radicals in normal human serum, in addition to the expected complement activation by means of enzymic C5/C5 convertase activity make a similar situation in dialysis possible. On the basis of this investigation and our previous in vivo data on reactive oxygen intermediate formation during dialysis with a cuprophan membrane (24), we postulate that both the alternative and the oxidative pathway of complement activation synergistically contribute to leukocyte activation and related untoward effects of dialysis, such as leukocyte accumulation in the pulmonary microvasculature (1,2).

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