Apoptosis of human polymorphonuclear neutrophils accelerated by dialysis membranes via the activation of the complement system

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Abstract

Background. Haemodialysis (HD) with bioincompatible cellulosic membranes like Cuprophan (CU) is considered to influence negatively the clinical outcome of acute and chronic renal failure. In this effect, apart from the disturbance of phagocytosis or oxygen species production by leukocytes, increased apoptosis also has been implicated recently. The objective of this study was to study the effect of HD membranes on apoptosis induction in polymorphonuclear neutrophils (PMN).

Methods. PMN from healthy donors and uraemic patients were isolated and apoptosis was induced by co-incubation with CU, Hemophan or polyamide hollow fibres in the presence of serum from healthy or uraemic humans. Apoptosis was quantified by flow cytometry using Annexin V–FITC and propidium iodide staining and was confirmed by the detection of DNA fragmentation on gel electrophoresis. The deposition of immunoglobulins (Ig) and complement factors on hollow fibres was detected by direct immunofluorescence.

Results. Heat inactivation or the depletion of complement components or Ig significantly reduced apoptosis, indicating its dependence on classical complement activation. The detection of IgG on hollow CU fibres and the restored acceleration of apoptosis by the appropriate replenishment of Ig-deficient sera additionally confirmed these findings. Inhibition experiments revealed that caspases were necessary mainly, but not exclusively, for apoptosis to occur after complement activation. Uraemia led to increased PMN apoptosis in the presence of bioincompatible, but not biocompatible, membranes.

Conclusions. Our results suggest that the acceleration of PMN apoptosis in the presence of CU is mediated via an antibody-dependent activation of the classical complement pathway mobilizing both caspase-dependent and -independent pathways.

Keywords: apoptosis; biocompatibility; complement system; dialysis membranes; neutrophils

Introduction

Haemodialysis (HD) with bioincompatible cellulosic membranes is known to activate polymorphonuclear neutrophils (PMN) [1]. Despite the increasing use of biocompatible synthetic dialysis membranes, biocompatibility is still an intensely debated topic in nephrology [2]. In recent years, controversial studies have been published about the possible negative influences of bioincompatible membranes on clinical outcomes in chronic dialysis patients, including on mortality, infection rates and amyloidosis (reviewed in [3]). A recent meta-analysis demonstrated that the use of bioincompatible cellulose-based membranes leads to higher mortality in patients with acute renal failure when compared with synthetic membranes [4].

In patients with end-stage renal disease, the essential functions of leukocytes—including phagocytosis, oxygen species production, upregulation of specific cell surface receptors or apoptosis—are disturbed (reviewed in [1]). They are further altered as a result of complement activation by the HD procedure, particularly when bioincompatible cellulosic membranes are used. To date, conflicting results describing both delay and acceleration of apoptosis in dialysis patients have been published and they do not acknowledge the influence of uraemic toxins on the rate of apoptosis in PMN [5,6]. Studies by Kettritz et al. [7] suggested that superoxide release is required for both the
spontaneous and N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced apoptosis of PMN. Based on our findings that reactive oxygen species in concert with complement play an important role in HD-induced side effects [8], we became interested in the induction or acceleration of apoptosis in human PMN exposed to different dialyser membranes. A recent experiment by our group showed that apoptosis is accelerated when human PMN are co-incubated with the bioincompatible membrane Cuprophan (CU) and to a significantly lesser extent when synthetic membranes, such as polysulphone, are used. We also found that apoptosis was dramatically reduced in the absence of an active complement system [9]. Recently, Lhotta and coworkers [10] extended the concept of complement activation through CU membranes, showing that this activation is based on four steps: the binding of anti-polysaccharide antibodies, followed by the classical, the alternative and the terminal pathways of activation. A controversy still exists on whether complement can induce apoptosis or if this process might not involve necrosis characterized by apoptotic phenomena, such as DNA fragmentation and chromatin condensation (reviewed in [11]). Recently, Nauta and coworkers [12] described the induction of apoptosis in mesangial cells dependent on the activation of the terminal complement pathway. We now demonstrate for the first time that the induction of apoptosis in PMN is dependent on the classical complement pathway and also that this phenomenon is mainly dependent on the activation of caspases.

Subjects and methods

Cell preparations

After informed consent, whole blood from normal volunteers or uraemic patients was drawn into heparinized tubes at the beginning of dialysis and centrifuged on a Ficoll–Paque density gradient (Pharmacia, Uppsala, Sweden). After separation, PMN were isolated from the pellet by hypotonic lysis of red blood cells using an ammonium chloride buffer (157 mM NH4Cl, 10 mM KHCO3 and 0.1 mM ethylenediaminetetraacacetate Na2). After lysis, the PMN were collected by centrifugation at 4°C and washed with saline. The cells were resuspended in phosphate-buffered saline (PBS) without calcium and magnesium to give a concentration of 1 × 107/ml and were kept at 6°C until use. The purity of the PMN preparation always exceeded 95% and the viability of the PMN, as determined by propidium iodide staining, was ≥98%.

Serum preparation

Phlebotomy was performed in healthy volunteer controls or uraemic patients at the beginning of dialysis, the venous blood being collected into glass tubes to avoid complement activation. After incubation at room temperature for 30 min, followed by a 30 min incubation on ice to allow coagulation, serum was separated by centrifugation at 4°C and stored at −80°C until further use. Some aliquots of serum were heated at 56°C for 30 min immediately after separation from whole blood; this preparation is referred to as heat-inactivated serum. Other aliquots were incubated three times for 30 min each with 14 mg of CU hollow fibres at 4°C, using fresh fibres each time to ensure maximum absorption of the serum. This preparation is referred to as CU-pretreated serum. C1q-, C3- and complement factor B-depleted serum was purchased from Calbiochem (Basel, Switzerland). The qualities of these sera were ensured by showing absolute non-reactivity in CH50 assays (as described in [8]). Furthermore, serum taken from a patient (with Guillain–Barré syndrome) after 10 immunoadsorption sessions was used as immunoglobulin (Ig)-deficient serum, in which IgG, IgM and IgA levels were below their detection limits. C4-deficient serum was obtained from a patient with an inherited C4-complement deficiency as described previously [10].

Reagents

Propidium iodide was obtained from Molecular Probes (Eugene, OR, USA); Annexin-V [human, recombinant, fluorescein isothiocyanate (FITC)] from Alexa (Carlsbad, CA, USA); TRI ReagentTM as well as Poly-HEMA from Sigma-Aldrich (St Louis, MO, USA); Ac-DEVD-CMK, Z-VAD-FMK, human IgG and IgM from Calbiochem (Basel, Switzerland); NOR Partigen IgG MC plates for radial immunodiffusion from Dade Behring (Deerfield, IL, USA); FITC-conjugated IgG-, IgM- and C3-antibody from The Binding Site (Birmingham, UK); and C1q-, C4c-antibody from DAKO (Aarhus, Denmark).

Assessment of apoptosis in PMN

Isolated PMN (4 × 107 cells/ml) were incubated in Dulbecco’s modified Eagle’s medium, low glucose (DMEM low glucose; Biowhittaker, Brussels, Belgium) containing 5% fetal bovine serum. For stimulation, fMLP at 10−8 M or 14 mg of hollow fibres of the biocompatible membrane polyanide (PA) (Gambro, Hechingen, Germany) or the bioincompatible membranes CU (Fresenius, Bad Homburg, Germany) or Hemophan (Cobe, Dransfeld, Germany) were added to the respective serum preparations. Incubations were performed in triplicate in 96-well, flat-bottomed microtitre plates for 30 min in 37°C water baths. For the caspase blocking experiments, PMN were pre-treated with the respective caspase inhibitors at 37°C for 1 h. Afterwards, PMN were transferred onto 24-well dishes previously coated with Poly-HEMA to prevent adhesion. After 4 h of incubation, cells were collected for viability and apoptosis assessment. Induction of apoptosis was proven by three different methods. First, for light microscopic evaluation, cytospin preparations were fixed in methanol, stained with Wright–Giemsa and, finally, examined at a magnification of ×400. Apoptotic PMN were identified using morphological criteria of apoptosis (chromatin condensation and fragmented nuclei). For all samples, 200–400 cells per slide were evaluated by an examiner blinded to the prior processing of the samples. Second, we performed DNA laddering to identify DNA cleavage that occurs during apoptosis. DNA was isolated using a commercially available DNA isolation kit (QIAamp DNA Blood Midi Kit; Valencia, CA, USA). Next, we did an electrophoresis with a 1.5% agarose gel containing ethidium bromide. Third, the assessment of PMN apoptosis was performed by flow
cytometry using an FITC-conjugated Annexin-V and propidium iodide to differentiate between early apoptosis and necrosis. The numbers of necrotic cells never exceeded 1% of the total population in all experiments.

**Demonstration of Ig and complement deposition on hollow fibres**

To show the binding of Ig and complement cleavage products to CU, the hollow fibres were washed with saline, incubated in the presence of normal human serum, Ig-deficient serum (from the Guillain–Barre´ patient) and PBS for 30 min at 37°C. After washing twice with PBS, the fibres were incubated with FITC-conjugated anti-IgG, anti-IgM, anti-C1q, anti-C3 or anti-C4c antibody for 30 min at room temperature. The fibres were washed again with PBS and prepared for immunofluorescence microscopy.

**Statistical analysis**

Statistical analysis was performed using the unpaired t-test. All results are expressed as means ± SD. Differences were considered significant at \( P < 0.05 \).

**Results**

**CU hollow fibres accelerate apoptosis in human PMN**

We have shown previously that CU membranes are able to accelerate apoptosis in PMN, as determined by morphological criteria [9]. To prove these results formally, we extended our evaluation using three different techniques of apoptosis assessment. All experiments were controlled with normal human serum. Using Annexin V-staining, we detected a significant increase in the percentage of apoptotic PMN after 4 h of co-incubation of PMN with hollow fibres of CU, compared with unstimulated cells (referred to as medium control in the following) (Figure 1, representative picture in Figure 2C). The percentage of apoptotic cells after CU exposure was also significantly higher, compared with exposure to the biocompatible PA membrane. PMN stimulated with other biocompatible membranes, namely polysulphone and polymethylmethacrylate, exhibited percentages of apoptosis similar to PMN activated by the PA membrane (data not shown). Additionally, the percentage of apoptotic cells increased significantly when PMN were incubated with fibres of the modified cellulosic membrane Hemophan, but not to the extent induced by CU (Figure 1). Apoptosis was further confirmed by DNA laddering (Figure 2A) and the evaluation of morphological criteria using light microscopy (Figure 2B). The numbers of necrotic cells never exceeded 1% of the total population in all experiments.

**The need for specific complement components for the acceleration of apoptosis induced via CU membrane**

Since the activation of the complement system is dependent on heat-sensitive components and complement factor C3 [9], using complement-deficient sera we investigated the contribution of the classical or alternative pathways of complement to the apoptosis of PMN accelerated by CU. When C1q-, C3- and C4-deficient sera were substituted for normal human serum, CU-induced apoptosis was profoundly reduced (Figure 3; \( P < 0.05 \)). To evaluate the role of the alternative pathway of the complement system, factor B-deficient serum was used instead of normal serum, and significant decrease in apoptosis was observed (reduction by 74.04 ± 14.58%; \( n = 3; \ P < 0.05 \)). This demonstrates the involvement of both the classical and the alternative pathways in the apoptosis of PMN induced by CU.

**Requirement of Ig for accelerated apoptosis of PMN co-incubated with hollow fibres of CU**

Since the classical pathway is triggered by an antigen-antibody complex, we hypothesized that the acceleration of apoptosis in the presence of CU is induced via antibodies deposited on the CU hollow fibres before the formation of the cleavage products of the complement system. Therefore, PMN were incubated with CU hollow fibres in the presence of Ig-deficient serum, which significantly diminished apoptosis (−87.04 ± 8.06% compared with CU; \( n = 3; \ P < 0.001 \); Figure 4). Furthermore, we speculated that Ig can be removed by pre-incubating CU hollow fibres with normal human serum. Using the serum thus pre-treated in co-incubation experiments with fresh CU fibres and PMN, a reduction in apoptosis comparable with that produced by Ig-deficient serum was seen (−60.09 ± 13.04%; Figure 4). These experiments were confirmed by co-incubating those pre-treated CU fibres (based on speculation that they have the deposited Ig on their surface) and Ig-deficient serum, which restored the accelerated
Furthermore, we were able to detect a significant reduction of IgG after incubation with CU hollow fibres using radial immunodiffusion (human serum: 14.31±1.9 g/l; Ig-depleted serum: 4.39±0.3 g/l; n=3; P<0.05). To visualize the suspected Ig deposition on the CU hollow fibres, we stained those fibres incubated with normal human serum or sera deficient in human IgG and IgM. The staining revealed an immunofluorescence signal only if the serum was not Ig-depleted or deficient (Figure 5).

To show that the manipulated serum (Ig-deicient serum from an immuno adsorption patient) has still intact complement components, we stained for the deposition of C1q, C3 and C4, all of which were clearly positive (Figure 5).

To determine which Ig class is involved in the CU-induced acceleration of apoptosis of PMN,
CU-pre-treated serum was replenished with human IgG (10 mg/ml) or IgM (1 mg/ml). PMN incubated with CU in the presence of IgG-replenished serum and PMN incubated with CU in the presence of normal human serum showed similar degrees of apoptosis (CU: 15.8±0.8%; IgG-replenished: 12.7±2.62%; P = NS).

In contrast, the apoptosis of PMN incubated with CU in the presence of IgM-replenished serum was significantly decreased compared with the CU-stimulated PMN (IgM-replenished: 5.43±2.03%; n = 3; P < 0.01; Figure 6). These data suggest that it is mainly IgG, in combination with the complement system, that plays a major role in the induction of CU-induced apoptosis in PMN.

**Activation of caspases in PMN undergoing apoptosis after stimulation by CU**

To evaluate the involvement of caspases in the apoptosis of PMN after incubation with CU, we studied the effect of caspase inhibition. PMN were incubated with CU in the presence of normal human serum after pre-treatment with 50 μmol/l of the specific caspase-3 inhibitor, Ac-DEVD-CMK, and the broad spectrum caspase inhibitor Z-VAD-FMK. After 4h of incubation, both caspase inhibitors reduced apoptosis in the presence of CU (control: 7.53±2.46%, CU: 17.27±2.35%, Ac-DEVD-CMK:
Acceleration of apoptosis by complement

**Fig. 7.** Caspase inhibitors counter the CU-induced acceleration of apoptosis in PMN. PMN were pre-treated with cell-permeable caspase-3 inhibitor (Ac-DEVD-CMK, 50μM) or a general caspase inhibitor (z-VAD-FMK, 50μM) prior to addition to CU. The cells were further cultured and after 4h apoptosis was quantitated by flow cytometry. Data are presented as means±SD (n=3). *P<0.05.

**Table 1.** Effect of uraemia on CU-, Hemophan- or PA-mediated death of PMN. PMN of healthy volunteers were incubated without a stimulus (control) or with CU, Hemophan and PA in the presence of normal (n) or uraemic (u) human serum for 30 min. Afterwards, cells were transferred to Poly-HEMA-coated plates and incubated for 4h. The percentage of apoptotic PMN was measured using flow cytometry.

<table>
<thead>
<tr>
<th>PMN Serum</th>
<th>Normal</th>
<th>Normal</th>
<th>Uraemic</th>
<th>Normal</th>
<th>Uraemic</th>
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<tbody>
<tr>
<td>Control</td>
<td>13.08±3.7</td>
<td>7.03±2.48</td>
<td>9.4±1.74</td>
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</tr>
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<td>CU</td>
<td>23.27±5.31a</td>
<td>32.45±4.83b</td>
<td>31.95±10.41a</td>
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<tr>
<td>Hemophan</td>
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<td>24.08±7.37a</td>
<td>20.75±4.59a</td>
<td>25.9±9.5a</td>
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<tr>
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<td>14.07±3.97</td>
<td>11.65±3.38</td>
<td>7.4±1.75</td>
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The data are presented as means±SD (n=4).

aThe percentage of apoptotic cells was significantly increased, compared with the appropriate control (P<0.05).
bUnder uraemic conditions the percentage of apoptotic cells (uraemic PMN/uraemic serum) was significantly increased compared with the respective stimulation of normal PMN in the presence of normal serum.

11.97±2.87%, P=NS, Z-VAD-FMK: 10.47±1.37%, n=3, P<0.05; Figure 7). These data demonstrate, therefore, that the activation pathway is mainly, but not exclusively, caspase-dependent.

**Biocompatibility in uraemic patients**

Uraemic PMN are known to exhibit accelerated apoptosis in the presence of autologous plasma or 10% fetal calf serum. Moreover, PMN from healthy donors exposed to uraemic plasma exhibit a higher apoptotic rate compared with PMN exposed to healthy plasma. The apoptosis of PMN in uraemic patients was significantly elevated by CU and Hemophan in comparison with polysulphone membranes (reviewed in [1]). Therefore, we were interested in comparing the effect on PMN apoptosis of uraemia in combination with the bioincompatible membranes CU or Hemophan or the biocompatible membrane PA. PMN of healthy donors were incubated with the fibres of different membranes in the presence of healthy or uraemic sera for 4h. Compared with normal human serum, uraemic serum increased further the apoptosis of healthy PMN already accelerated by CU and by Hemophan. The apoptosis of PMN mediated by PA was not differently regulated by uraemic or healthy human sera (Table 1). Compared with normal PMN, uraemic PMN also showed higher percentages of apoptosis induced by CU and Hemophan in the presence of normal human serum (Table 1). Uraemic serum was not able to further increase apoptosis in uraemic PMN activated by CU or Hemophan (Table 1). These data confirm previous studies that show that uraemic patients have higher apoptotic rates of their PMN incubated with bioincompatible membranes (reviewed in [1]) and that this effect is even worse in the presence of bioincompatible membranes.

**Discussion**

This study explored the mechanisms leading to the acceleration of apoptosis in human PMN after exposure to the hollow fibres of the bioincompatible membrane CU. To exclude the well-known pro-apoptotic capacity of uraemic toxins [6], sera and PMN isolated from healthy donors were also used. The influence of uraemia on biocompatibility was tested using uraemic PMN and uraemic serum. Our results demonstrate for the first time that cellulosic membranes accelerate apoptosis in human PMN, the process originating in the antibody-dependent classical pathway of complement, involving mainly the activation of caspases.

Haemodialysis leads to an array of side effects—via the activation of the complement cascade, release of proteases, production of cytokines and oxygen radicals, all of which could influence the clinical courses of dialysis patients (reviewed in [1]). Their survival is dramatically reduced—on the one hand, probably by uraemic factors that accelerate cardiovascular disease and mortality and, on the other, possibly by the renal replacement therapy itself. Therefore, the dialysis community is still searching for new mechanisms responsible for the reduced survival of dialysis patients. Little is known about the factors that influence apoptosis of peripheral blood leukocytes in uraemia and dialysis. Preliminary findings suggested that dialyser membranes can affect apoptosis of leukocytes in vitro (reviewed in [1]). However, these studies have produced contradicting results. The experiments done to date were in the presence of uraemic plasma, which itself can have a dramatic impact on apoptosis, as demonstrated by the group of Cendoroglo [6]. If done in the absence of uraemic plasma, studies have shown an increase [13] or a decrease in apoptosis [14]. These two studies might be biased by testing PMN harvested shortly after the start of dialysis. In that period of time, PMN are functionally defective, compared with cells harvested before the start of dialysis. Our in vitro data on uraemic PMN and uraemic serum further underline the importance of biocompatibility...
of dialysis membranes in vivo, since under ‘uraemic’ conditions apoptosis of PMN was even more accelerated (Table 1).

Our group published preliminary data, showing an acceleration of apoptosis of non-uraemic PMN in the presence of the bioincompatible membrane CU but not the biocompatible membrane polysulphone [9]. We now present evidence that the classical pathway of complement is heavily involved in the CU-induced acceleration of apoptosis. The activation of the classical pathway requires the binding of C1q to the CU membrane. The acceleration of apoptosis needed C1q, for C1-depleted serum abolished this effect. The classical pathway is usually activated by the binding of C1q through its globular non-collagenous heads to IgG or IgM. Since IgG is a monomer, multiple IgG molecules must be aggregated, bringing multiple Fc regions together, before C1 can be activated. Such aggregation typically occurs if IgG antibodies bind to a multideterminant antigen, such as ones on bacterial surfaces (in this case CU mimics a bacterial surface).

A decade ago, anti-dextran antibodies of the IgG isotype, which are present in normal serum, were shown to bind to CU, with their titre correlated with complement activation [15]. The classical pathway may be activated by these anti-dextran antibodies or other natural polyreactive anti-polysaccharide antibodies of the IgG or IgM isotypes. Our replenishment experiments, adding IgG or IgM to IgG-decient or -depleted sera in the presence of PMN and CU fibres, demonstrate that these antibodies are probably of the IgG subtype. The existence of preformed antibodies—each individual having a different amount [15]—would explain why the ‘first-use syndrome’, an anaphylactic reaction in dialysis patients using CU for the first time, is of different severity in different patients, leading to severe reactions only in ~10% of dialysis patients [16]. A recent study has shown that the initial deposition of complement C3b on the CU membrane, necessary for the activation of the amplification loop of the alternative pathway, is mediated by the classical C3-convertase C4b2a [10]. The researchers extended the concept of complement activation through CU, postulating the binding of anti-polysaccharide antibodies to the membrane and the activation of the classical pathway, followed by the activation of the alternative pathway, before the activation of the terminal pathway leads to cell lysis [10]. This stepwise activation also can be seen in our model, since blocking the alternative pathway of complement leads also to a dramatic decrease in apoptosis in the presence of CU.

It is well known that the complement pathway is heavily involved in many autoimmune diseases, like systemic lupus erythematoses, leading to cell death via so far only vaguely described pathways. Therefore, we were interested to further dissect the course of programmed cell death in human PMN. PMN have been reported to express a variety of regulatory and effector caspases, including caspase-1, -3, -4 and -8 [17]. In general, apoptosis is divided into three different steps. The initiation phase, which allows the intracellular signalling, precedes the effector phase, which includes the regulation of apoptosis by the bcl-2 family. Subsequently, irreversible cell death occurs upon activation of caspases, which function as executors of apoptosis. The present study, in agreement with previous findings [18], gives evidence that caspase-3 may be involved in the final degradation process in apoptosis of PMN. With respect to complement-mediated apoptosis, a controversy exists on whether complement can induce apoptosis or if this process involves necrosis characterized by apoptotic phenomena, such as DNA fragmentation [11]. Recently, Nauta et al. [12] demonstrated that the induction of apoptosis by complement is dependent on the activation of the terminal complement pathway and the activation of caspases. The mechanism may be similar to that involved in the induction of apoptosis by other pore-forming proteins, such as perforin. Perforin is a constituent of the granules of natural killer cells and cytotoxic lymphocytes and, together with granzymes, it induces apoptosis of target cells by caspase-dependent pathways [19]. Although the data of Nauta et al. are based on in vitro experiments with mesangial cells, they are in line with our findings in tests using exactly the same caspase inhibitor [12].

Taken together, our studies suggest the following hypothesis for the initiation of accelerated apoptosis in human PMN activated by the CU dialyser membrane (Figure 8), as described next. The contact of human serum with cellulosic membranes leads to the deposition of preformed anti-polysaccharide antibodies, mainly IgG antibodies. This enables C1q to bind to these Ig and C4 is cleaved into C4a and C4b. C4b probably binds covalently to the amino

Fig. 8. Model of CU-induced cell death. The contact of human serum with biocompatible membranes leads to the deposition of preformed anti-polysaccharide antibodies, followed by C1q-binding and formation of the classical pathway C3 convertase (C4b2a), which in turn proceeds to proteolytic cleavage into C3a and C3b, also deposited on the membrane. This leads to C3 convertase formation consisting of C3b and factor Bb, members of the alternative pathway. So far it is not clear if C3bi, by binding to Mac-1 or forming the MAC complex on the activated PMN, is finally leading to PMN apoptosis.
groups of the antibodies. After binding and cleavage of C2, the classical pathway C3-convertase C4b2a is formed, which activates C3 by proteolytic cleavage into C3a and C3b and which is deposited on the membrane. The binding and activation of factor B creates the alternative pathway C3-convertase C3bBb, which leads to explosive complement activation by the amplification loop of the alternative pathway. So far, it is not clear if the terminal complement complex—whether acting via the formation of pores or the direct contact of the cell with the membrane or the deposited complement cleavage products as well as the Ig—is necessary to initiate the cell death pathway via caspase-dependent and -independent pathways. We think, therefore, that apoptosis is an important health threat in patients on dialysis. Besides uraemic factors leading to accelerated apoptosis, we have identified a new source which can dramatically decrease the half-life of human PMN in vivo.

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Conflict of interest statement. None declared.

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