Influence of Polysulfone and Hemophan Hemodialysis Membranes on Phagocytes

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Abstract. The aim of the study was to compare the effect of hemophane and polysulfone membranes on the phagocyte-derived production of reactive oxygen species (ROS) as well as on neutrophil CD11b and CD62L expression in patients undergoing regular hemodialysis. The effects of hemodialysis membranes were also studied in *in vitro* conditions after coincubating them with differentiated HL-60 cells. ROS production was measured using chemiluminometric and flow cytometric methods. Expression of CD11b, CD62L and mitochondrial membrane potential were detected by monoclonal antibodies and by the JC-1 fluorescent probe, respectively. Depressed ROS production was observed in patients already before dialysis. Further decrease in ROS production and an increase in CD11b expression were observed especially in patients after hemophan hemodialysis. Decreased ROS production and increased CD11b expression were observed also after incubation of HL-60 cells with hemophan membranes. Mitochondrial membrane potential dropped only after incubating cells with hemophan membranes proving its more serious adverse effects in comparison with the polysulfone membrane. In conclusion, deleterious effects of hemodialysis on the metabolic activity of phagocytes were proved. Combining chemiluminescent and flow cytometric methods for the detection of ROS production and determining mitochondrial membrane potential can be useful tools for the analysis of material biocompatibility.

Key words: Hemodialysis — Hemophan — Polysulfone – Reactive oxygen species — CD11b

Abbreviations: ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; HE, hydroethidine; PMA, phorbol myristate acetate; FMLP,

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N-formyl-L-methionyl-L-leucyl-L-phenylalanine; OZP, zymosan opsonized in human serum; MRF, median of relative fluorescence.

Introduction

Hemodialysis is a life sustaining procedure for hundreds of thousand patients world wide. Due to the non-self nature of dialysis membrane materials which come to contact with various effector systems in blood, various humoral and cellular inflammatory reaction cascades can be triggered (Kormoczi et al. 1999). Although cellulose based membranes are frequently used in clinical practice, their use is associated with transient marked neutropenia during the initial phase of dialysis (Nguyen et al. 1985; Himmelfarb et al. 1992; Rosenkranz et al. 1994) followed by rebound neutrophilia (Descamps-Latscha et al. 1991; Iijima et al. 1999). Cellulose membranes also induce marked complement activation, which is proposed to be a dominant mechanism for granulocyte activation during hemodialysis (Descamps-Latscha et al. 1991; Himmelfarb et al. 1995).

Since neutrophils are effector cells of the immune system, their proper numbers and function are essential for a defense against infective agents. Selectins, which are expressed on their surface mediate initial tethering and rolling of leukocytes along the endothelial lining of the postcapillary venules in inflamed tissue. CD62L (L-selectin) is constitutively expressed on leukocytes and rapidly shed from plasma membranes upon cell activation. β_2-integrins CD11a/CD18 and CD11b/CD18 (also known as MAC-1) are responsible for polymorphonuclear cell anchoring to the endothelium. Moreover, CD11b/CD18 and CD11c/CD18 also function as complement receptor 3 (CR3). The transformation of CD11b/CD18 from an inactivated to a transiently activated state with concomitant cell surface upregulation can be observed during leukocyte activation (Kormoczi et al. 1999). Complement activation caused by cellulose membranes is associated with a rapid and dramatic upregulation of CD11b/CD18 receptors (Himmelfarb et al. 1992). Increased cell surface expression of CD11b (Himmelfarb et al. 1992) and shedding of CD62L (Iijima et al. 1999) may be involved in increased granulocyte adhesiveness to the endothelium resulting in granulocytopenia (Kormoczi et al. 1999).

A major function of activated phagocytic cells in host defense is the production of ROS which play a major role in killing pathological microorganisms, but can also cause damage to the surrounding tissue (Bauer and Bauer 1999). It was proved that increased amounts of neutrophil-derived ROS are produced in patients dialyzed with cellulose membranes (Nguyen et al. 1985; Haag-Weber et al. 1989; Descamps-Latscha et al. 1991; Himmelfarb et al. 1993; Rosenkranz et al. 1999). A direct correlation between the nadir of leukocyte counts and ROS formation was shown by Rosenkranz et al. (1999). On the other hand, it was shown that cellulose membrane-induced neutrophil-derived ROS formation can occur *in vitro* (Rosenkranz et al. 1999) as well as during hemodialysis procedure (Kormoczi et al. 1999) independently of complement activation.

A growing body of evidence has accumulated suggesting that oxidative stress may be one of the important complications occurring in hemodialysis (Galli and Ronco 2000). A significantly increased oxidative stress associated with an insufficient activity of extracellular antioxidant mechanisms was observed in hemodialysed patients in comparison with healthy subjects in our previous studies (Lojek et al. 1998; Soška et al. 1995). However, these studies did not differentiate the effects of the hemodialysis procedure due to the type of membrane used. The aim of the present study was to examine and compare the effects of the hemodialysis procedure performed using hemophan (a frequently used cellulose membrane) and polysulfone (a synthetic membrane) on the phagocyte-derived production of ROS as well as on the expression of blood phagocyte surface antigens (CD11b and CD62L). To clarify the effects of the membranes, they were also evaluated *in vitro* while co-incubating the membranes with promyelocytic HL-60 cells differentiated to cells with neutrophil-like morphology.

Materials and Methods

Reagents

Phorbol myristate acetate (PMA), N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), zymosan from Saccharomyces cerevisiae, dimethyl sulfoxide, RPMI-1640 medium, gentamycin sulfate, heat inactivated fetal calf serum and luminol were obtained from Sigma-Aldrich (USA). Lysing solution Cal-Lyse, fluorescein isothiocyanate-labeled anti-human CD11b murine monoclonal antibody, phycoerythrin-labeled anti-human CD62L murine monoclonal antibody and appropriate control isotype murine antibodies were purchased from Caltag Laboratories (USA). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and hydroethidine (HE) were purchased from Molecular Probes (USA). All other chemicals were purchased in the highest grade p.a. from local distributors.

Patients and related analyses

Patients: Twenty one patients undergoing chronic hemodialysis (8 men and 13 women), who had given informed consent, were included into the study. The mean age was 64.7 years (range of 45 to 85 years). The chronic dialysis protocol consisted of 4 h three times a week. The membrane materials used for chronic dialysis was polysulfone with surface area of 1.6 m^2 (F7HPS, Fresenius, Germany) and hemophan with surface area of 1.7 m^2 (GFS Plus 16, Gambro Dialysatoren, Germany). Dialyzers and lines were steam sterilized, and no patients had a dialyzer reuse. All patients underwent bicarbonate dialysis and anticoagulation with heparin (the mean dose being 4200 ± 1800 IU). Twenty five healthy volunteers (10 men and 15 women) were used as age matched control subjects.
Blood collection: Heparinized blood samples (50×10^3 IU/l) were drawn at the beginning and at the end of the hemodialysis procedure from the arterial line. Pre- and post-hemodialysis samples from each patient were examined 3 times for

the hemophan membrane (the beginning of the study, and 3 and 6 months after that) and 3 times for the polysulfone membrane (9, 12 and 15 months after the beginning of the study). The number of leukocytes in the blood and their relative differentiation counts were determined using Coulter counter STKS (Coulter, England).

Oxidative burst measurement of blood phagocytes: Luminol-enhanced chemiluminescence of whole blood phagocytes was measured using BioOrbit 1251 Luminometer (BioOrbit, Finland). The principle of the method is based on luminol interaction with the phagocyte-derived free radicals, which results in large measurable amounts of light. Briefly: The reaction mixture consisted of whole blood (1 μl for zymosan opsonized in human serum (OZP) and 10 μl for FMLP), 1×10^{-3} mol/l luminol (stock solution of 0.01 mol/l luminol in 0.2 mol/l borate buffer) and one of the activators (FMLP – 2×10^{-6} mol/l or OZP – 0.1 g/l). The total reaction volume of 500 μl was adjusted with Hanks balanced salt solution, pH 7.4. The assays were run in duplicates. Spontaneous chemiluminescence measurements in samples containing 10 μl of whole blood and other substances except any activator were included in each assay. The chemiluminescence emission of each vial was followed up for 60 minutes at 37℃. The integral value of the chemiluminescence reaction, which represents the total ROS production by blood phagocytes, was corrected to the number of granulocytes.

Determination of the cell surface expression of adhesion molecules: The measurements were performed according to the manufacturer's protocol (Caltag Laboratories, USA) using unfixed whole blood. Briefly: 100 μl of blood were incubated in plastic tubes (Falcon, USA) with anti-CD11b and anti-CD62L monoclonal antibodies (7.5 μl of each) at room temperature for 15 minutes. 100 μl of blood incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Then the samples were fixed by Cal-lyse and the red blood cells were lysed by distilled water. The remaining cells were resuspended in phosphate buffered saline, placed on ice and analyzed within 2 h. Ten thousand blood granulocytes selected on the basis of their typical scattering characteristics were analyzed by flow cytometer FACSCalibur (Becton Dickinson, USA). The median of relative fluorescence (MRF) was determined and corrected to the background fluorescence of the isotype control. The results were expressed as the means of MRF and standard deviations.

In vitro determinations

Cell line: Promyelocytic HL-60 cell line was obtained from the American Type Culture Collection (USA). Cells were grown in RPMI-1640 medium supplemented with heat inactivated fetal calf serum (10%) and gentamicine (0.045 g/l) at 37℃ in a CO_2 incubator (Heraeus, Germany). Cells were seeded for 96 hours with dimethyl sulfoxide (1%) to differentiate them to cells with neutrophil like morphology. The correct stage of cell differentiation was verified by a flow cytometric analysis of CD11b expression and by the chemiluminescence measurement of ROS production.

Only cells with a viability higher a 95 % as assessed by the trypan blue exclusion test were used for experiments.

Co-incubation of cells with membranes: Differentiated HL-60 cell suspension (1×10^8 cells/l of RPMI-1640 with 10 % heat inactivated fetal calf serum) was prepared for tests. Hollow fibers of the membranes were cut in small pieces (1–3 mm) and 1 g of these was placed in a 50 ml plastic test tube. Then 40 ml of the cell suspension was added to each tube. The tubes were shaken gently in horizontal position for 3 hours and the HL-60 cells were then harvested for further analyses. Cells incubated without membrane were used as a negative control.

ROS production measurement by chemiluminescence: The reaction mixture consisted of 2×10^8 HL-60 cells, 1×10^{-3} mol/l luminol and one of the following activators PMA (5×10^{-6} mol/l) or OZP (0.1 g/l). The total reaction volume was adjusted to 250 μl with Hanks balanced salt solution. Spontaneous chemiluminescence was measured without the activators. The chemiluminescence emission expressed as relative light units was recorded continuously for 90 min at 37 ℃ by microplate luminometer LM-01T (Immunotech, Czech Republic).

Flow cytometric analyses: Determination of the cell surface expression of adhesion molecules: 40 μl of HL-60 cells (1×10^9 cells/l) were incubated in plastic tubes with anti-CD11b and anti-CD62L monoclonal antibodies (4 μl of each) at 4 ℃ for 30 minutes. Then the cells were washed twice in phosphate buffered saline and resuspended in 0.4 ml of phosphate buffered saline, placed on ice and analyzed by flow cytometer within 30 min. Negative isotype controls were also analyzed.

Oxidative burst measurement *in vitro*: 300 μl of HL-60 cells (1×10^8 cells/l) were incubated in sterile plastic tubes with DCFH-DA (2×10^{-5} mol/l) or HE (1×10^{-5} mol/l). DCFH-DA and HE were dissolved in dimethyl sulfoxide (less than 1 % in the final cell suspension). The tubes were shaken gently and incubated in dark at 37 ℃ for 20 min. Then 100 μl of either PMA (2×10^{-5}mol/l) or OZP (0.4 g/l), or just Hanks balanced salt solution were added and the cell suspensions were incubated in dark at 37 ℃ for additional 20 min. At the end of the incubation period, the tubes were placed on ice and analyzed.

Detection of mitochondrial membrane potential: fluorescent probe JC-1 (dissolved in dimethyl sulfoxide) was used for detection of mitochondrial membrane potential as described by Salvioli and co-workers (Salvioli et al. 1997). Briefly, 300 μl of HL-60 cells (1×10^8 cells/l) were incubated in plastic tubes with JC-1 (a final concentration of 2.5 mg/l) in dark at room temperature for 20 min. At the end of the incubation period, the cells were washed twice with Hanks balanced salt solution and analyzed by flow cytometer immediately.

Procedure of analysis of HL-60 cells by flow cytometer: ten thousand live cells selected on the basis of scattering characteristics were analyzed by flow cytometer FACSCalibur (Becton Dickinson, USA) and MRF was determined. The fluorescence of monoclonal antibodies was corrected for background fluorescence of isotype control. The results were expressed as the means of the MRF and the standard error of the mean, or as a mean ratio of the MRF of samples and the control cells plus standard error of the mean.

Statistical analysis:

Results obtained from *in vivo* experiments were analyzed by the Student's t-test for independent or dependent samples, and significances were verified by the non-parametric Mann-Whitney U-test or Wilcoxon test. These non-parametric tests were applied for the analysis of results obtained from *in vitro* experiments.

Results

Clinical study

No significant differences in the expression of CD11b and CD62L on blood granulocytes were found between healthy controls and patients before hemodialysis either with polysulfone or with hemophan membranes (data not shown). The expression of CD11b in patients after hemodialysis was significantly upregulated only in the case of hemophan hemodialysis when compared with the levels determined before the hemodialysis procedure (949 ± 113 MRF *vs.* 1457 ± 128 MRF, $p = 0.01$) whereas polysulfone membrane caused only a mild non-significant increase (890 ± 116 MRF *vs.* 1014 ± 168 MRF). No significant changes in CD62L expression were observed either in polysulfone or hemophan dialyzed patients (data not shown).

When compared with healthy controls a significant decrease in the spontaneous and in the OZP-activated production of ROS measured by chemiluminescence was observed in the patients before hemodialysis with polysulfone or with hemophan membranes (Table 1). No differences in spontaneous and in activated chemiluminescence were found in the patients before hemodialysis either with polysulfone or hemophan membranes. The spontaneous whole blood neutrophil-derived ROS production measured by chemiluminescence was not changed at the end of polysulfone hemodialysis compared with the pre-hemodialysis level, while it was decreased after the hemophan hemodialysis procedure (Table 2). However, hemodialysis reduced the activated chemiluminescence independently of the type of membrane used. This decrease was much more profound after the hemophan hemodialysis procedure, where both FMLP and OZP-activated chemiluminescence were significantly lower in comparison with the pre-hemodialysis levels and even in comparison with the chemiluminescence determined after polysulfone hemodialysis.

There were no significant differences in the total numbers of neutrophils and monocytes between healthy controls and patients before either polysulfone or hemophan hemodialysis. The total numbers of either neutrophils or monocytes did not change significantly after both the hemophan and the polysulfone hemodialysis procedures in comparison with the pre-hemodialysis levels (data not shown).

In vitro experiments

The spontaneous chemiluminescence of HL-60 cells at the end of their 3 hour incubation with polysulfone or hemophan hollow fibers did not changed significantly when compared with controls (Table 3). Activation of the cells with OZP or PMA

Table 1. ROS production in whole blood detected by chemiluminescence in healthy controls and patients dialyzed with hemophan or polysulfone membranes before hemodialysis procedure. Data are expressed as $V \cdot s \cdot 10^{-3}$ (mean ± S.E.M.). Asterisks indicate statistically significant differences ($p < 0.05$) compared with controls

	Spontaneous chemiluminescence (V·s)	Activated chemiluminescence (V·s)	
		FMLP	OZP
control	0.573 ± 0.035	8.649 ± 0.540	45.420 ± 1.826
polysulfone	0.450 ± 0.045*	8.614 ± 0.985	38.188 ± 1.722*
hemophan	0.467 ± 0.038*	8.248 ± 1.137	37.318 ± 2.144*

Table 2. ROS production in whole blood detected by chemiluminescence in patients after hemodialysis procedure with hemophan or polysulfone membranes. Data are expressed as percentages of predialysis levels (mean ± S.E.M.). Crosses indicate statistically significant differences ($p < 0.05$) comparing the effects of the two different membranes, and asterisks indicate statistically significant differences ($p < 0.05$) compared with predialysis levels

	Spontaneous chemiluminescence (%)	Activated chemiluminescence (%)	
		FMLP	OZP
polysulfone	98.4 ± 3.5	90.2 ± 4.8*	93.7 ± 5.2
hemophan	91.9 ± 4.6*	77.3 ± 10.5*$^{+}$	67.7 ± 13.6*$^{+}$

Table 3. Effect of 3 h incubation of HL-60 cells with hemophan or polysulfone membranes on ROS production measured by chemiluminescence. Data are expressed as a percentage of control cells (mean ± S.D. of four independent experiments). Cross indicates statistically significant difference ($p < 0.05$) comparing the effects of the two different membranes, and asterisks indicate statistically significant differences ($p < 0.05$) compared to control cells

	Spontaneous chemiluminescence (%)	Activated chemiluminescence (%)	
		FMLP	OZP
polysulfone	128 ± 24	67 ± 4*	66 ± 8*
hemophan	117 ± 12	58 ± 7*	45 ± 14*$^{+}$

measured by chemiluminescence was significantly reduced in comparison with untreated control cells. The reduction of chemiluminescence was most obvious in the hemophan-treated, PMA-activated cells, where the chemiluminescence was significantly lower not only in comparison with the controls but also with cells treated with polysulfone.

ROS production was also determined by flow-cytometry (Fig. 1). Incubation of HL-60 cells with polysulfone did not cause any significant changes in spontaneous or

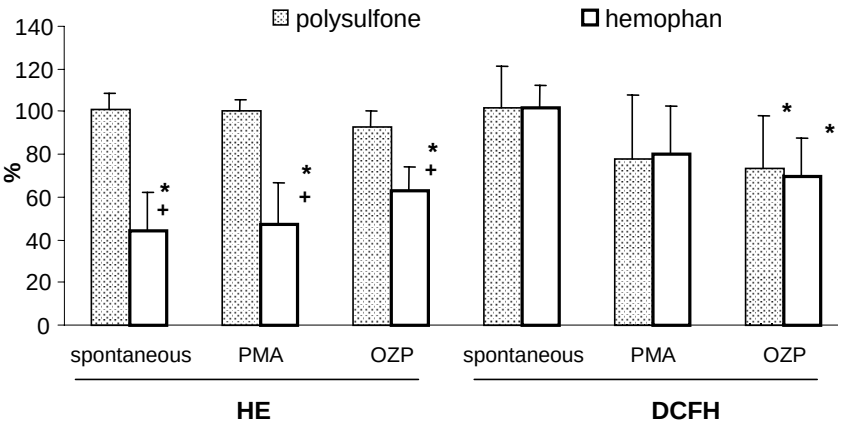

Figure 1. Effect of 3 h incubation of HL-60 cells with hemophan or polysulfone membranes on ROS production measured by HE and DCFH-DA. Data are expressed as percentages of controls (mean ± S.D. of four independent experiments). Crosses indicate statistically significant differences ($p < 0.05$) comparing the effect of the two different membranes, and asterics indicate statistically significant differences ($p < 0.05$) compared with control cells.

activated superoxide production measured with HE. On the other hand, pretreatment with hemophan caused a significant decrease in the spontaneous and in both OZP and PMA-activated production of superoxide. Hydrogen peroxide production measured with DCFH-DA was not significantly changed in the non-activated and the PMA activated HL-60 cells in any cases. In contrast, DCFH-DA fluorescence in the OZP activated cells dropped down after pretreatment with both types of membranes.

As regards the surface molecules, the expression of CD11b was significantly upregulated after 3 hours of incubation with either polysulfone (173 ± 21 MRF *vs.* 219 ± 17 MRF, $p = 0.02$) or hemophan (173 ± 21 MRF *vs.* 227 ± 24 MRF, $p = 0.03$) membranes. Expression of CD62L was not influenced by the membranes (data not shown).

Mitochondrial membrane potential detected by fluorescent probe JC-1 did not change significantly ($113.8 \pm 11.3\,\%$) after a 3 h incubation of the HL-60 cells with polysulfone membrane fibers whereas hemophan caused a significant decrease ($85.2 \pm 4.5\,\%$, $p = 0.01$) compared with control cells.

Discussion

This study was designed to assess and to compare changes in the functional properties and surface molecule expression of neutrophils after their contact with cellulose (hemophan) or synthetic (polysulfone) membranes either in the extracorporeal circulation during hemodialysis or *in vitro*.

The membrane contact with blood elements during the hemodialysis procedure is known to induce neutrophil activation as measured by the release of proinflammatory mediators (Horl et al. 1985), a decrease in viscoelasticity (Iijima et al. 1999), and changes in the surface expression of the integrin protein CD11b/CD18 and the selectin CD62L (Himmelfarb et al. 1992, 1993, 1994, 1995; Hernandez et al. 1998; Rosenkranz et al. 1999). Impaired leukocyte function in response to subsequent stimuli has also been described (Nguyen et al. 1985; Haag-Weber et al. 1989; Descamps-Latscha et al. 1991; Vanholder et al. 1991; Hernandez et al. 1998). The rate of membrane-induced effects depends on the membrane biocompatibility. Numerous investigators have demonstrated that cellulose membranes cause marked complement activation. They proposed that it could be a dominant mechanism for granulocyte activation during hemodialysis (Nguyen et al. 1985; Descamps-Latscha et al. 1991; Himmelfarb et al. 1995; Iijima et al. 1999).

The increased expression of CD11b (CR3) found in our study at the end of the hemodialysis procedure with hemophan is in good agreement with the literature (Himmelfarb et al. 1994, 1995; Hernandez et al. 1998; Iijima et al. 1999; Rosenkranz et al. 1999). Results of Himmelfarb et al. (1995) strongly support the hypothesis that upregulation of CD11b is connected with complement activation. No major changes in the expression of CD11b were detected in patients dialyzed with polysulfone complement non-activating membrane in our study as well as in the study of Rosenkranz et al. (1999).

The main parameter observed in our study was the production of ROS by blood phagocytes. Their metabolic response is altered generally in patients with chronic renal failure who do not receive hemodialysis (Ritchey et al. 1981; Haag-Weber et al. 1989; Vanholder et al. 1991; Cendoroglo et al. 1999). The hemodialysis procedure could further worsen this pathological state.

We extend this knowledge by findings that spontaneous and OZP-activated chemiluminescence were lower before both the polysulfone and the hemophan hemodialysis procedure when compared with the chemiluminescence of healthy controls. Moreover, the hemodialysis procedure further reduced the neutrophil chemiluminescence independently of the type of membrane used, however, this decrease was much more profound after hemophan hemodialysis. The decreased spontaneous generation of ROS after hemophan membrane hemodialysis measured by chemiluminescence was confirmed by the detection of intracellular superoxide production measured by flow cytometry with HE and by the detection of intracellular hydrogen peroxide production measured with DCFH-DA in selected patients (unpublished data). Our results are in good agreement with findings that the metabolic response of blood phagocytes measured by chemiluminescence is adversely affected by hemodialysis (Ritchey et al. 1981; Cohen et al. 1982; Nguyen et al. 1985). The significant decrease in leukocyte response to phagocytic challenge and metabolic activity was caused mainly by cellulose membranes and was not seriously affected by complement non-activating membranes (Nguyen et al. 1985; Vanholder et al. 1991; Descamps-Latscha et al. 1991; Himmelfarb et al. 1993). These findings support a theory that the defect of phagocytes is dependent on complement activation.

On the other hand, respiratory burst induction, CD11b/CD18 upregulation or the shedding of CD62L can also occur partially independently of complement activation being induced by other mechanisms (Kormoczi et al. 1999; Rosenkranz et al. 1999). Experiments of Himmelfarb et al. with aprotinin, complement activation inhibitor and sCR1 suggest that not all the effects of cellulose membranes on granulocyte activation are due strictly to complement activation (Himmelfarb et al. 1994, 1995). The alteration of the metabolic activity of phagocytes caused by the polysulfone membrane, although weaker than that caused by the hemophan membrane, are in good agreement with this theory.

The reason for the decreased phagocyte ROS production measured by chemiluminescence is unclear. One of the possible explanations can be a degranulation of polymorphonuclear cells and consequent decreased amount of myeloperoxidase, which can play key role in luminol-induced chemiluminescence. Degranulation of polymorphonuclear cells during the hemodialysis procedure is relatively well described (Horl et al. 1985; Haag-Weber et al. 1989). In contrary, Cohen et al. (1982) failed to detect any differences between pre- and intra-dialytic myeloperoxidase concentrations in polymorphonuclear cells. These data together with our results where the superoxide and hydrogen peroxide production as well as luminol-induced chemiluminescence were depressed similarly support an idea that the decrease in blood phagocyte metabolic activity after hemodialysis is myeloperoxidase-independent and is caused rather by a true defect in oxidative metabolism.

We used two activators of the oxidative burst of phagocytes which stimulate cells through different receptors and mechanisms. OZP are recognized by complement and immunoglobuline receptors, and FMLP binds to a specific formyl peptide receptor. Regardless the upregulation of complement receptor, a decreased metabolic activity of blood phagocytes by OZP was found in our study after hemodialysis. That is why another explanation for the depressed metabolic activity of phagocytes after hemodialysis can be a pre-existing stimulation or "exhaustion" of blood phagocytes. The decreased spontaneous ROS production as well as the absence of significant differences in ROS production induced by the used activators also support this theory.

The decreased production of ROS at the end of hemodialysis procedure is in contrast to observed increased oxidative stress during hemodialysis (Galli and Ronco 2000) which was even suggested to be caused by increased production of ROS by blood phagocytes (Lojek et al. 1998). Several authors described activation of neutrophils and increased ROS production by neutrophils from the beginning of hemodialysis procedure up to 60 min (Nguyen et al. 1985; Rosenkranz et al. 1994; Iijima et al. 1999; Kormoczi et al. 1999) However, it returned to predialysis levels at later phases of hemodialysis procedure. At the end of hemodialysis the capability of neutrophils to produce ROS was even reduced as was described above. It could be speculated that ROS production by blood phagocytes is an important source of oxidants contributed to increased oxidative stress during hemodialysis.

Beside *in vivo* and *ex vivo* studies, few articles evaluating *in vitro* effects of hemodialysis membranes on isolated neutrophil metabolic activity and sur-

face molecule expression have appeared during last years (Kuwahara et al. 1988; Rosenkranz et al. 1994, 1999). Though, results of these studies could be influenced by the fact that the expression of immunoglobuline and complement receptors on cell membranes, and also the functional properties of neutrophils, are changed during the separation processes (Lojek et al. 1997). It was the reason why we decided to use the HL-60 cell line differentiated by dimethyl sulfoxide to obtain cells exhibiting a phenotype very similar to normal human blood neutrophil granulocytes (Yen 1990; Narayanan and Robinson 1998; Sedlák et al. 1999). This allowed us to obtain sufficient amount of a homogenous population of resting cells without any activation for *in vitro* experiments (Horáková 1999).

A three hour incubation of HL-60 cells with both types of membranes induced an increased expression of CD11b, the expression of CD62L was without significant changes. This effect was similar as for *in vivo* hemodialysis. The activated chemiluminescence was reduced by both membranes, which was most obvious in the hemophan treated cells. Incubation of cells with hemophan also caused a significant decrease in ROS production measured with HE. In contrast, DCFH-DA fluorescence in the OZP-activated cells dropped after pretreatment with both types of membranes. It was already demonstrated in *in vitro* experiments that incubation of polymorphonuclear cells with cellulose membranes had more profound effects on spontaneous ROS production while polysulfone membranes did not elicit radical formation (Rosenkranz et al. 1999). The partial differences in the production of ROS measured by chemiluminescence and flow cytometry in our study can be explained by different specificites of these methods. Luminol-enhanced chemiluminescence detects all types of produced reactive oxygen and nitrogen species and oxidants included in the myeloperoxidase system (Lojek et al. 1997). HE and DCFH-DA-based flow cytometric analyses are considered as specific probes for the intracellular production of superoxide and hydrogen peroxide, respectively (Bass et al. 1983; Rothe and Valet 1990).

It was reported earlier, that the expression of CD11b increased and CD62L decreased only in the presence of normal serum with complement compounds (Rosenkranz et al. 1999). No significant effect was found in the presence of complement component deficient serum and in the absence of serum. In our experiments we used heat inactivated fetal calf serum, therefore an influence of complement components was excluded. These *in vitro* data for CD11b expression and ROS production again suggest the involvement of a complement-independent pathway. Our results are in agreement with the results of Kuwahara et al. (1988). These authors suggested that neutrophil activation occurred by direct membrane-neutrophil interaction and the produced ROS might play an initial and/or additional role in the events occurring at the initiation of hemodialysis.

The mechanism of direct membrane activation of neutrophils in absence of complement is not fully clarified. One of the possible explanations is that neutrophils are activated due to their adhesion to cellulose-based hemodialysis membranes *via* L-fucose-inhibitable lectine-like binding (Kormoczi et al. 1999). We suggest that also other non covalent low force interactions may play important role in

neutrophil activation by hemodialysis membranes in absence of complement. This is supported by such significant increase of neutrophil adhesiveness during hemodialysis which is not fully correlated with increase of main adhesion molecules (Iijima et al. 1999).

The influence of polysulfone and hemophan membranes on neutrophil metabolic activity was detected in our study also on the basis of changes in mitochondrial membrane potential. Mitochondrial membrane potential, which is generated by mitochondrial electron transport chain, is in turn responsible for the formation of ATP molecules by ATP synthase. For this reason, mitochondrial membrane potential is an important parameter for mitochondrial functionality and an indirect evidence of the energy status of the cell. We failed to observe any sharp collapse of mitochondrial membrane potential which is known to be typical for apoptotic process (Cossarizza et al. 1993). On the other hand, the decreased mitochondrial membrane potential of cells after incubation with hemophan membrane could indicate impaired metabolic functions of these cells. These results are in good consent mainly with the decreased ROS production detected with HE.

It was shown that neutrophils from uremic patients either without or with regular hemodialysis undergo accelerated *in vitro* apoptosis (Jaber et al. 1998; Cendoroglo et al. 1999) This phenomenon was caused mainly by uremic toxins and the apoptosis-inducing activity of uremic plasma could be modulated by the material of dialysis membrane. Only a contact of neutrophils with hemodialysis membranes in the absence of uremic toxins does not induce apoptotic process as was shown in our *in vitro* experiments.

In conclusion, a better biocompatibility of polysulfone membranes in comparison with hemophan membranes was proved in our study. Adverse effects of hemophan could be associated with activation of complement cascade but we also proved complement independent effects in *in vitro* experiments with polysulfone membrane. Differentiated neutrophil-like HL-60 cells had been shown to be sensitive to co-incubation with membrane fragments. A combination of chemiluminescence methods for the detection of total ROS production, flow cytometric methods for the intracellular production of ROS using different fluorescent probes and the detection of changes in mitochondrial membrane potential can be a useful tool to obtain a complex view of the activation and metabolic status of blood phagocytes. Therefore, it can be employed for the analysis of membrane biocompatibility.

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