Cuprophan membranes during haemodialysis significantly increase the plasma levels of C5a desArg (maximal 55 μg C5a desArg/l blood after 30 min) whereas Hemophane or Polysulphonemembranes induce only low plasma levels of C5a desArg. C5a desArg generated in vitro by yeast incubation of autologous plasma stimulates PMN chemotaxis and oxidative metabolism but has no effect on enzyme release. Preincubation of whole blood with C5a desArg causes aggregation and changed oxidative burst activity of the isolated PMN. These changes are similar to those found in cells from patients after haemodialysis with cuprophan membranes. So the elevated plasma levels of C5a desArg after haemodialysis explain some of the changes in PMN functions, but additional mechanisms have to be assumed.

Key words: C5a desArg, Cuprophan, Haemodialysis, PMN functions

Stimulation of neutrophil functions by C5a desArg: an in vitro model of haemodialysis

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Introduction

During haemodialysis (HD) with various membranes complement components are activated, which results in elevated levels of C5a and C3a. Additionally neutrophil granulocytes (PMN) are activated, which results in the release of lysosomal enzymes and an altered oxidative metabolism. To explain the change in the PMN functions two effects have to be evaluated: a direct effect of the membrane material on PMN and an indirect effect of membrane activated plasma components on the cells. If the two effects are exerted, they may differ in their extent, in their kinetic, and in their dependence on the membrane material. They can be studied in two ways: by using membrane activated cells without the addition of plasma and by using normal cells with activated plasma.

In this study we set out to investigate the incubation of normal PMN with yeast activated plasma as an in vitro model of the plasma effects during haemodialysis.

Material and Methods

Isolation of human PMN: Blood was drawn by venipuncture after obtaining written consent from normal, healthy donors. PMN were isolated using Ficoll (Pharmacia-LKB, Bromma, Sweden) separation and sedimentation of erythrocytes. The cells represented more than 98% granulocytes with a viability of about 98% by Trypan exclusion.

Generation of C5a desArg: Autologous plasma was activated by incubation with baker’s yeast (1 mg ml⁻¹) for 60 min at 37°C. Plasma was inactivated by incubation for 30 min at 56°C.

Concentration of C5a and C5a desArg was measured with an ELISA (Behringwerke, Marburg, Germany).

Chemotaxis assay: Chemotactic assays were performed in modified Boyden chambers. Activated plasma (2.5–20%) in HBSS (Gibco, Germany) was put in the lower compartment and the PMN (10⁶) in the upper compartment of the Boyden chambers. Cellulose ester filters (ME 29, Schleicher and Schüll, Dassel, Germany) were used. The chambers were incubated for 90 min at 37°C, then the filters were removed, stained with hematoxylin, and mounted on slides. The PMN were counted in the filters at every 10 μm interval by a computerized imaging method (Bausch and Lomb, Frankfurt, Germany) and the chemotactic index (CI) calculated, which reflects the mean distance travelled by the activated cells.

Cytochrome c test: Superoxide anion generation was measured by cytochrome c reduction. PMN (10⁶) were incubated with 75 μM cytochrome c and stimuli (Phorbol 12-myristate 13-acetate, PMN, 1 μg ml⁻¹ or activated plasma 0.3–20% as indicated in results) in Hanks’ balanced salt solution (HBSS) for 10 min at 37°C. The reaction was stopped by cooling in ice and centrifugation (10 min, 200 × g). The absorption of the supernatants was determined at 550 nm, and the superoxide generation was calculated.

Elastase release: PMN (4 × 10⁶) were incubated with activated plasma (2.5–20% as indicated in results) in HBSS for 15 min at 37°C. Released elastase (HLE) was complexed with α1-proteinase inhibitor (α1PI) by addition of plasma (25%) and further
incubated for 2 min. The samples were centrifuged (10 min, 200 × g) and the HLE-a1PI-complex was measured by ELISA (Merck, Darmstadt, Germany). Total HLE content of the PMN was measured after lysis with cetyltrimethylammonium bromide. 10

Preincubation experiments: Sixty ml of anticoagulated blood were centrifuged and the plasma separated. Ten ml of the plasma were activated and 10 ml inactivated as described above. The blood was then divided into three parts and each was treated in one of the following ways: (1) PMN were directly isolated from one part, (2) one part of the blood was mixed with activated plasma (final concentration 10%) and incubated for 10 min at 37°C, then the PMN were isolated, (3) one part of the blood was mixed with inactivated plasma (final concentration 10%) and treated as part 2.

Patient studies: Twenty-seven patients gave their informed consent to participate in the study. Haemodialysis (HD) was performed by an A 2008 C (Fresenius, Bad Homburg, Germany) or an AK-10 (Gambro, Hechingen, Germany) volume controlled equipment. As our dialyser we used cuprohane: SMAD 125/140 (SMAD, Lyon, France), heombohane: MO 450 (SMAD) and GFS 120/140 MCH (Gambro, Lund, Sweden), and polysulfone: F6 (Fresenius).

EDTA blood was taken from the arterial line before systemic heparinization prior to HD and 10, 30, 60, 120 and 180 min after the beginning of HD. The blood was immediately centrifuged and the plasma stored deep frozen until assessed for C5a by ELISA.

Chemicals were if not otherwise stated by Sigma Chemie, Deisenhofen, Germany.

Statistical analysis: Data are represented as the mean ± 1 standard deviation (SD). Statistical comparisons were performed by the paired Student’s t-test.

Results

C5adesArg generation during dialysis: Figure 1 shows the kinetics of C5adesArg generation in the venous blood samples during haemodialysis (HD). HD with Cuprophane gives maximal levels of 55 μg C5adesArg/l after 30 min. HD with Hemophane Mo 450 or GF 120 results in lower values of maximal 8 μg C5adesArg/l after 10 min of dialysis. Similar results are obtained for HD with Polysulfone (maximal 5 μg l⁻¹).
Neutrophil functions and C5a\textsubscript{desArg}

In vitro generation of C5a\textsubscript{desArg}: Generation of C5a\textsubscript{desArg} by yeast activation of autologous plasma samples results in a mean value of 1040 ± 450 µg C5a\textsubscript{desArg}/l (n = 8). In parallel inactivated plasma samples contain 1.7 ± 0.9 µg C5a\textsubscript{desArg}/l (n = 8).

Stimulation of chemotaxis by activated plasma: Figure 2 shows the concentration dependent stimulation of chemotaxis by the autologous plasma samples. Maximal stimulation is achieved by 10% activated plasma which corresponds to about 100 µg C5a\textsubscript{desArg}/l.

Stimulation of oxidative burst by activated plasma:

Direct effect of activated plasma. The superoxide generation of PMN is already stimulated by 1% of activated plasma corresponding to about 10 µg C5a\textsubscript{desArg}/l to 2 nmol O\textsubscript{2}−/10 min/10⁶ PMN (Fig. 3). Further addition of activated plasma up to 20% (about 200 µg C5a\textsubscript{desArg}/l) enhances the superoxide production to 13.7 nmol/10 min/10⁶ PMN.

Preincubation of PMN with activated plasma. To test the effect of priming, blood was mixed with activated or inactivated plasma (final concentration 10%) and incubated for 10 min at 37°C, and then the PMN were isolated. As a control, PMN from the same donor were directly isolated.

The yield of PMN is reduced to 18% of the control after incubation with activated plasma (Table 1, p < 0.002). Incubation of the blood with inactivated plasma also reduces the yield (55% of control, not significant = NS). If the superoxide generation of the isolated PMN is tested without the addition of a stimulus, the plasma preincubated cells produce higher amounts of superoxide than untreated cells (4.5 and 4.1 vs. 2.2 nmol O\textsubscript{2}−/10⁶ PMN/10 min, NS, Fig. 4). In contrast, generation of superoxide is significantly reduced after PMN stimulation of the C5a\textsubscript{desArg} preincubated cells: 9.5 nmol O\textsubscript{2}−/10 min/10⁶ PMN vs. 20.1 nmol of control cells (p < 0.01). After stimulation with activated plasma the C5a\textsubscript{desArg} pretreated cells produce more superoxide than the other groups: 9.3 nmol/10 min/10⁶ PMN vs. 5.5 nmol of control cells (NS) and 5.1 nmol of inactivated plasma incubated cells (p < 0.05).

<table>
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<tr>
<th>Table 1. Yield of PMN after preincubation of the blood with activated or inactivated plasma (final concentration 10%, 10 min, 37°C) compared to untreated control blood</th>
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<td><strong>Preincubation</strong></td>
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<td>10% act. plasma</td>
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<td>10% inact. plasma</td>
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Statistical significance by Student's t-test: act.plasma vs. control p < 0.002, act. vs. inact. plasma p < 0.05.
Preincubation of blood with lower concentrations of activated plasma (final concentration 5%) gives similar results as preincubation with inactivated plasma (data not shown).

**Stimulation of HLE release:** Incubation of isolated PMN with activated plasma results in no stimulation of HLE release in the range of 1–20% activated plasma.

**Discussion**

Human C5a is cleaved from the fifth component of complement during activation and then rapidly converted by a plasma enzyme to the C5a$_{desArg}$ derivative. The plasma levels of C5a$_{desArg}$ in healthy adults are normally below 0.2 µg l$^{-1}$. The measurement of C5a or C5a$_{desArg}$ is difficult because of its short half-life. Measurements during haemodialysis have to be made in venous blood samples which come directly from the dialyser, where C5a is supposed to be generated.

We found maximal plasma levels of 55 µg C5a$_{desArg}$ after 30 min of haemodialysis with cuprophan membranes (Fig. 1) in accordance with results of Knudsen, Bingel and Freyria (maximal 35–61 µg l$^{-1}$). The other membranes only produced about 8 µg C5a$_{desArg}$ (Fig. 1).

For our *in vitro* experiments we generated C5a$_{desArg}$ by yeast activation of the autologous plasma samples, which resulted in 1040 ± 450 µg C5a$_{desArg}$/l on average, while the inactivated samples only contained about 2 µg l$^{-1}$. To test the C5a$_{desArg}$ activity we performed chemotaxis experiments (Fig. 2). Maximal stimulation was achieved by 10% activated plasma corresponding to 60 nM C5a$_{desArg}$, the same value as described by Yancey *et al.* and Chenoweth and Hugli. The native C5a fragment was about 20-fold more potent (EC$_{50}$ C5a 0.5 nM).

The stimulation of the oxidative burst with activated plasma (Fig. 3) gave no maximum but was increased in the whole range tested (final concentration 1–20% activated plasma) as already described by Goldstein.

So we found stimulation of chemotaxis and oxidative burst by about 10% activated plasma, which corresponded to 100 µg C5a$_{desArg}$/l, the same values found after haemodialysis with cuprophan membranes. To simulate the complement exposure during dialysis, we preincubated blood with 10% activated plasma (final concentration 100 µg C5a$_{desArg}$/l blood) and as the control with 10% inactivated plasma (final concentration 0.2 µg C5a$_{desArg}$/l), and tested the subsequently isolated PMN.

C5a$_{desArg}$ aggregates PMN and the aggregated
cells were separated by Ficoll centrifugation. So the yield of PMN was rather reduced after incubation with activated plasma (Table 1). The prestimulated PMN produced less superoxide after further stimulation with PMA than the control cells or the cells that were preincubated with inactivated plasma (Fig. 4). The extent of these effects differed from one blood donor to the other, reflected by the large standard deviations, but the ratio of the values was always the same.

So the preincubation experiments imitate the in vivo results, which also show leukopenia after cuprophane dialysis and diminished superoxide generation of the isolated PMN.

It seems probable that after stimulation a subpopulation of PMN with lower activity was isolated, because priming of PMN with a stimulus normally resulted in enhanced activity towards a second stimulus. However, if the prestimulated PMN are further stimulated with activated plasma, the superoxide generation is enhanced in comparison to untreated cells (Fig. 4). So the activated plasma seems to contain a factor which compensates the cellular effect. These apparently contradictory results are also produced during dialysis, where the superoxide generation of PMN in autologous plasma is enhanced at the same time as the superoxide generation of isolated PMN is reduced.

Our preincubation experiments were performed with high C5a desArg concentrations which correspond to the values after cuprophane dialysis. And they show similar results for PMN function as are produced during cuprophane dialysis. Preincubation with lower C5a desArg concentrations (5% activated plasma corresponding to a final concentration of about 50 μg C5a desArg/l) shows no effect. But other membrane materials such as hemophane also induce leukopenia and changes of PMN oxidative burst although they only generate very small amounts of C5a desArg. So C5a desArg may be responsible for the complement induced effects after cuprophane dialysis but not for the effects after HD with the other membranes. This is confirmed by several other authors who find no correlation between the extent of complement activation and leukopenia. Moreover complement activation by cuprophane also depends on additional serum factors which differ greatly between individuals.

The second cellular effect during dialysis, the enzyme release, is not mimicked by activated plasma. Incubation of isolated PMN with up to 20% activated plasma results in no release of HLE (Fig. 5) Release of azurophil granules is only stimulated after cytochalasin B preincubation while only specific granules are released by soluble stimuli. So the HLE release of PMN during dialysis can not be explained by the activated plasma components but additional stimulating effects of the dialysis membrane have to be assumed.

C5a desArg generated during haemodialysis may influence some of PMN functions e.g. the oxidative burst. But for other functions e.g. the enzyme release an additional influence of the membrane material has to take effect.

References

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