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EXPERIMENTAL

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The effect of CVVHD and endotoxin on the oxidative burst, adhesion molecules and distribution in tissues of granulocytes

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Abstract *Objective*: Extracorporeal circulation, such as cardiopulmonary bypass and haemodialysis, has been associated with an activation of the immune system, especially the granulocytes. Continuous veno-venous haemodiafiltration (CVVHD) is used in critically ill septic patients. During CVVHD cytokines are excreted in the ultrafiltrate. But when the membranes used in CVVHD are cultured with granulocytes, the granulocytes are slightly activated. This effect is potentiated by endotoxin. We therefore, in vivo, compared the effect on granulocyte activation of CVVHD with an endotoxin group and a control group. *Methods*: Thirty-one pigs were anaesthetized and mechanically ventilated. In ten pigs CVVHD was performed. Eleven pigs received an infusion of *Escherichia coli* endotoxin 30 μ/kg^{-1} and ten pigs served as a control group. The adhesion molecules CD18 and CD62L were measured using monoclonal antibodies. The oxidative burst activity was assayed as superoxide dismutase-inhibitory reduction of cytochrome c. The number of granulocytes in peripheral blood and in the lungs and liver were counted. *Results*: The infusion of endotoxin was followed by granulocytopenia, reduced oxidative burst activity, increased expression of CD18 and decreased expression of CD62L on granulocytes. Accumulation of granulocytes in liver and lung tissue was also noted in this group. CVVHD was only associated with a non-significant decrease in CD62L expression on granulocytes. It did not affect any of the other measured immunological parameters. *Conclusion*: In contrast to endotoxin-induced sepsis, the granulocytes were not activated during CVVHD.

Key words Adhesion molecules · CVVHD · Endotoxin · Granulocytes · Oxidative burst

Introduction

Several forms of extracorporeal circulation have been associated with an activation of the granulocytes. During cardiopulmonary bypass (CPB) granulocytopenia was observed in peripheral blood followed by granulocytosis hours later [1]. CPB was also associated with increased secretion of cytokines, especially IL-1, IL-6,

IL-8 and TNF [2]. The cytokines activated the endothelium as well as the granulocytes. Following CPB, activation of granulocytes with increased levels of elastase in the blood and increased expression of adhesion molecules on the surface of granulocytes have been observed [3]. The adhesion molecules are essential for the granulocytes' ability to transmigrate through endothelium to underlying tissues. The blood surface contact-induced activation of granulocytes, especially, has even been described as a systemic inflammatory reaction [4]. However CPB using a membrane oxygenator differs from dialysis.

Haemodialysis (HD), another form of extracorporeal circulation, has also been associated with increased expression of adhesion molecules on granulocytes and increased secretion of cytokines, especially TNF [5, 6]. During HD granulocytopenia has been observed in peripheral blood while granulocytes accumulated in the lungs [7]. At the nadir of granulocytopenia, the granulocytes that remained in the circulation had a decreased oxidative burst activity [8]. These effects of HD are largely due to the use of cuprophane membranes.

Continuous veno-venous haemodiafiltration (CVVHD) is a rather new form of combined dialysis and filtration, which can be used in critically ill patients with unstable circulation [9]. In CVVHD very biocompatible high-flux membranes are always used. Especially the polyacrylonitrile (PAN) membrane has been associated with adsorption of complement to the filter. There has been great interest in the immune-modulating potential of CVVHD, as it was discovered that cytokines were removed by filtration as well as adsorption during CVVHD [10, 11]. However, when granulocytes were cultured in vitro in the presence of biocompatible membranes used in CVVHD modest quantities of cytokines were produced [12]. Thus CVVHD might not only attenuate the inflammatory reaction in sepsis, it also has the potential to activate granulocytes. Toft et al. [13] were not able to demonstrate an effect of CVVHD on the number of granulocytes in peripheral blood, the cytokines and the expression of adhesion molecules on granulocytes in septic patients. However, they observed a large interindividual difference between the critically ill patients with sepsis [13]. They concluded that the heterogeneity of the study population and the "noise" from the underlying sepsis made it difficult to separate any effect of CVVHD on granulocyte activation from the activation caused by sepsis.

The purpose of the present study was to assess the number of granulocytes in blood and tissues, the expression of adhesion molecules on granulocytes and the oxidative burst activity of granulocytes during CVVHD compared to a control group and to endotoxin-induced sepsis in otherwise healthy pigs.

Material and methods

Thirty-one healthy male pigs, bred in pathogen-free environment and with body weights between 28 and 31 kg, were used in this study. The investigation was approved by the Animal Ethics Committee of Denmark.

The animals were premedicated with azaperon (Stresnil, Jansen Pharmaceutica, Beerse, Belgium) 6 mg/kg^{-1}. After induction of anaesthesia with ketamine (Ketalar, Parke Davis) 5 mg/kg^{-1} i.v. and midazolam (Dormicum, Roche, Basel, Schwitzerland) 0.25 mg/kg^{-1} i.v., the animals were intubated orotracheally and connected to a ventilator (Siemens-Elema 900 B, Solna, Sweden). The animals were mechanically ventilated with 70 % N_2O in O_2, the respiratory frequency was 12 min^{-1} and the tidal volumes were adjusted to achieve an arterial $PaCO_2$ of 4.5–5 kPa. Blood gases were measured on an OSM3 oximeter (Radiometer, Copenhagen, Denmark). Anaesthesia was maintained by a continuous infusion of fentanyl (Haldid, Jansen Pharmaceutica, Beerse, Belgium) 50 μg/kg^{-1} · h^{-1}, pancuronium (Pavulon, Organon Teknika, Boxtel, Holland) 100 μg/kg^{-1} · h^{-1} and propofol (Diprivan, Zeneca, Moodsfield, U.K.) 8 mg/kg^{-1} · h^{-1}.

A double lumen central venous line was introduced through the right external jugular vein for infusion of drugs and fluids. An arterial catheter was introduced via the right carotid artery. Mean arterial blood pressure (MAP) and mean central venous pressure (CVP) were measured by a Hewlett Packard monitor (Hewlett Packard, Boeblingen, Germany). All animals received 7.5 ml/kg^{-1} · h^{-1} of isotonic sodium chloride throughout the observation period.

Ten pigs were anaesthetized and ventilated as described and served as a control group. Eleven pigs received an intravenous infusion of *E. coli* endotoxin (OS5:B5, Difco Laboratorium, Detroit, USA) 30 μg/kg^{-1} over a period of 20 min.

In ten pigs a double lumen dialysis catheter was introduced into the right external jugular vein and CVVHD was performed using a BSM22 haemofiltration system (Hospal, Lyon, France). Blood was driven by a peristaltic pump at a rate of 100 ml/min^{-1} through the polyacrylonitrile parallel plate haemofilter (AN 69S, Hospal, Lyon, France) and back into the venous lumen of the dialysis catheter. Dialysate (Gambrosol 1.5 %, Gambro, Lund, Sweden) was delivered by a pump at a rate of 1 l/h^{-1} countercurrent to the blood flow. The ultrafiltrate was collected in a bag and measured hourly. The ultrafiltrate was replaced with isotonic saline to keep the animals normohydrated. Heparin 600 IU/h^{-1} was infused directly into the extracorporeal circuit pre-filter to achieve an activated clotting time of 150–220 s.

Blood samples were collected as soon as the central venous catheter was introduced and half an hour (except for measurements of oxidative burst and chemotaxis), 2 h and 5 h following the end of the endotoxin infusion or the start of CVVHD. The samples were stored in EDTA tubes. The total leukocyte counts were made with a Coulter Counter S (Coulter Elektronics, UK) and differential counts were performed on Wright-stained blood smears. The oxidative burst was assayed as superoxide dismutase (SOD)-inhibitory reduction of cytochrome c. The neutrophils were diluted to 5×10^5 ml^{-1} in Krebs-Ringer phosphate buffer containing 5 mM glucose and 1 mg/ml^{-1} cytochrome c (Sigma). The cells were activated by 1.6 μM phorbol-12-myristate-13-acetate (PMA) and incubated for 10 min in the presence or absence of 30 μg/ml^{-1} SOD (Sigma). The basal level of superoxide generation was determined in the absence of stimulators. Reduction in cytochrome c was terminated by adding 10 μl of 3 μg/ml^{-1} SOD to samples without this enzyme. Reduction in cytochrome c was measured spectro-photometrically as the change in absorbance at 550 nm [14].

For measurements of adhesion and activation molecules on granulocytes we used flow cytometry and monoclonal antibodies, Anticoagulated peripheral blood, 100 µl, was incubated with 20 µl of monoclonal antibodies for 15 min in darkness at room temperature. The following antibodies were used: anti-CD18-PE (MαP, IgG_1) and anti-CD 62L-PE (MαP, IgG_1), all from Pharmingen. Cells were fixed in 1% formalin in PBS before analysis with flow cytometry (Coulter Elite, Luton, UK) after gating the granulocytes on forward and side scatter. Cells positive for the specific antigens were scored on the basis of Simultest Control (M, IgG_1-PE).

Lungs and livers from control, surgical or endotoxin-treated pigs were frozen in Hexan (–70 °C). From each lung and liver 8 µm frozen sections were cut at 10–15 different levels. The sections were stained with chloroacetate esterase (NAFDCL).

The number of granulocytes in lung and liver sections from each animal was counted in 10 randomly selected high power fields (ocular X10, objective X40) and expressed as mean number of granulocytes per high power field. The results were grouped in four subgroups: less than 10 granulocytes per high power field, 10–25 granulocytes per high power field, 26–50 granulocytes per high power field and more than 50 granulocytes per high power field, for each experiment.

Friedman's analysis of variance was used to test for changes within the groups. Kruskal-Wallis one-way analysis of variance was used to test for differences between the groups. Rank sum test was used to test for differences between the number of granulocytes in tissues. A probability value of less than 0.05 was considered statistically significant. The results are expressed as mean ± standard error of the mean.

Results

In the endotoxin group we observed a pronounced decrease in the number of granulocytes in peripheral blood from $7.3 \pm 1.8\,10^9l^{-1}$ before the infusion of endotoxin to $0.8 \pm 0.1\,10^9l^{-1}$ half an hour after the end of endotoxin infusion ($p < 0.05$) (Fig. 1). During CVVHD treatment the number of granulocytes increased from an initial value of $6.7 \pm 0.7\,10^9l^{-1}$ to $11.3 \pm 0.7\,10^9l^{-1}$ after 5 h of CVVHD ($p < 0.05$) (Fig. 1). The granulocytosis in the control group was not significant (NS) (Fig. 1).

Following infusion of endotoxin the ability of the granulocytes from peripheral blood to respond with an oxidative burst decreased from 48.4 ± 3.3 to 23.5 ± 5.3 2 h later ($p < 0.05$). In the CVVHD group as well as in the control group no significant decrease in the oxidative burst was observed (NS) (Fig. 1).

In the endotoxin group the expression of the adhesion molecule CD18 increased from a mean fluorescence channel value (CV) of 963 ± 224 before the infusion to 1511 ± 328 and 1453 ± 271 ,respectively, half an hour and 2 h later ($p < 0.05$). However CVVHD did not affect the expression of CD18 on granulocytes. Before CVVHD the expression of CD18 was 782 ± 109 CV compared to 771 ± 74 after 5 h of CVVHD (NS) (Fig. 2). The expression of the adhesion molecule CD62L decreased non-significantly from 84.7 ± 4.7% to 69.0 ± 11.5% after 5 h of CVVHD (Fig. 2). Following infusion of endotoxin the expression of CD62L decreased significantly. Before infusion of endotoxin, CD62L was detected on 78.8 ± 5.8% of the granulocytes. However, the percentage of CD62L positive granulocytes declined steadily to 36.9 ± 4.1% 5 h later ($p < 0.05$) (Fig. 2).

The number of granulocytes in both liver and lung was significantly higher following infusion of endotoxin than in the other groups ($p < 0.05$). No significant difference between the control group and CVVHD-treated animals was found. (NS) (Table 1).

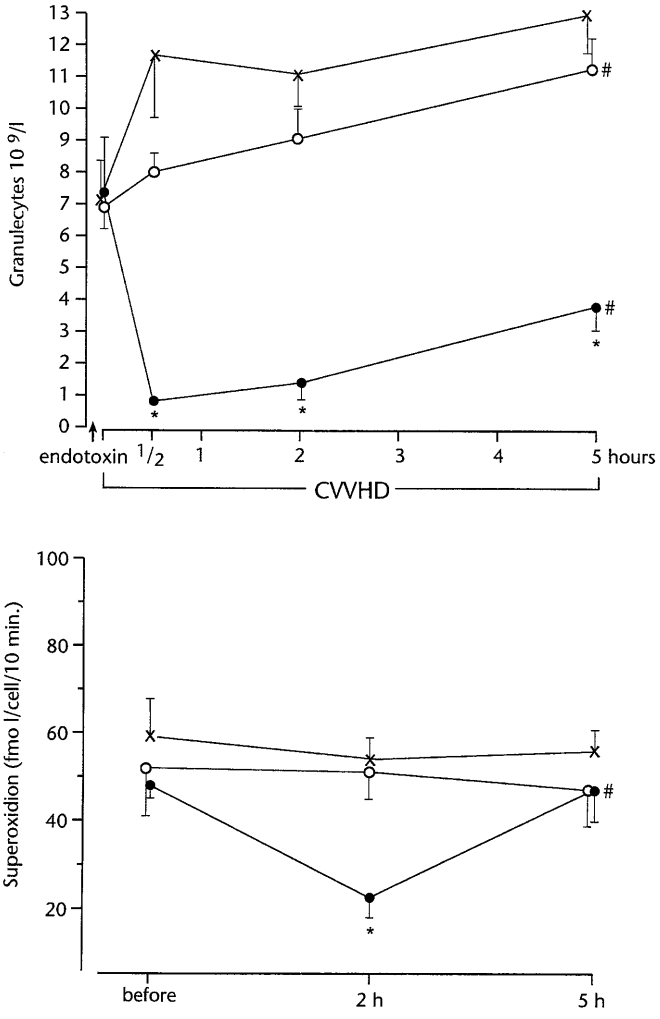

Fig. 1 The number of granulocytes in peripheral blood and the oxidative burst activity of granulocytes following infusion of endotoxin (●—●), CVVHD (○—○) and in the control group (x – x). * Represents a significant difference ($p < 0.05$) between the groups; # Denotes a significant difference within the group. The values are expressed as mean ± SE

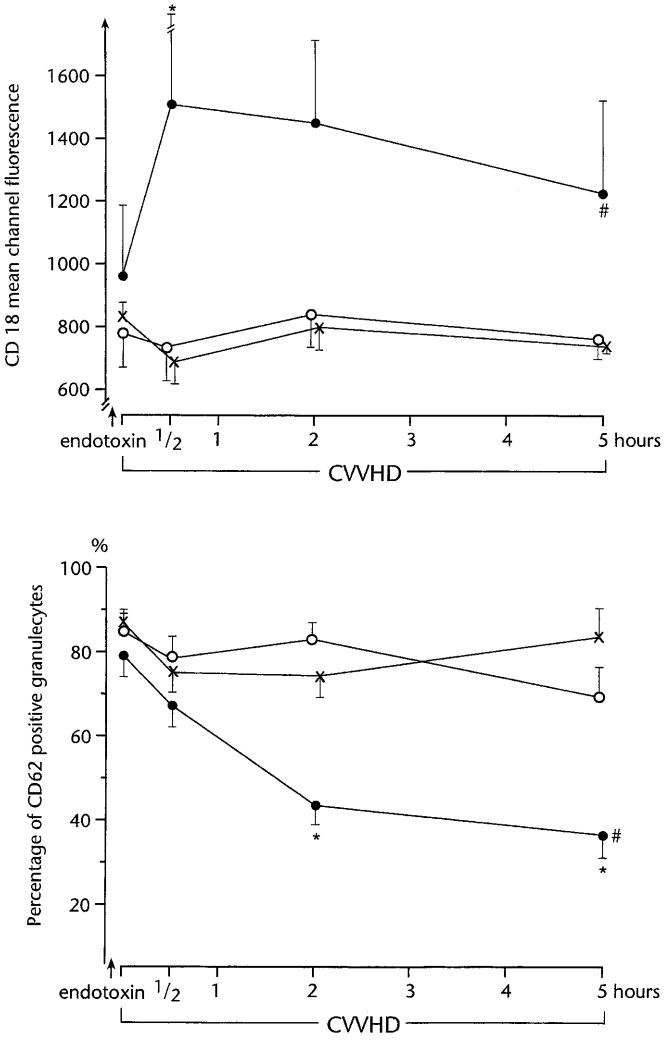

Fig. 2 The expression of the adhesion molecules CD18 and CD62L on granulocytes following infusion of endotoxin (● – ●), CVVHD (o – o) and in the control group. * Represents a significant ($p < 0.05$) difference between the groups. # Denotes a significant difference within the group. Mean values ± SE

Table 1 Number of granulocytes per high power field in liver and lung

	< 10	10–25	26–50	> 50	Total
Liver					
K	5	5			10
CVVHD	7	4			11
LPS			7	3	10
Lung					
K	4	6			10
CVVHD	1	10			11
LPS			8	2	10

Discussion

The granulocytopenia following endotoxin infusion is in accordance with other investigations [15, 16]. The lack of granulocytopenia during CVVHD is in contrast to the well documented granulocytopenia during CPB and HD [1, 8]. This indicates that the granulocytes are not activated to a larger extent during CVVHD.

The decreased oxidative burst activity of the granulocytes from peripheral blood upon PMA stimulation in vitro in the endotoxin group corresponds in time with the granulocytopenia. The same refractoriness of granulocytes during and after the granulocytopenia has been observed by Cohen et al. [8] during HD and by Toft et al. [1] during CPB. The decreased oxidative burst upon stimulation in vitro can be explained with the hypothesis that an exhausted granulocyte subpopulation remains in the circulation during the granulocytopenia induced by some forms of extracorporeal circulation. The unaffected oxidative burst activity in the CVVHD group indicates that the granulocytes are not activated to a larger extent during CVVHD.

The decreased oxidative burst of the granulocytes following endotoxin infusion is in accordance with some studies of septic patients. Vespasiano et al. [17] observed a reduced oxidative burst activity in septic patients when blood samples were taken within the first 12 h after admission to the ICU. In patients with gram-negative septicaemia, Wenisch et al. [18] also observed a decreased oxidative burst activity of granulocytes prior to treatment. The oxidative burst activity normalized within 7 days of treatment. In contrast, Trautinger et al. [19], who took blood samples immediately at the time of admittance to the ICU, found an increased oxidative burst activity in critically ill patients. The oxidative burst activity was, however, lower in those patients who did not survive. Thus granulocytes might become exhausted upon continuous stimulation in critically ill septic patients.

The adhesion molecules can be divided into several families. The two most important families of adhesion molecules on granulocytes are the integrin and the selectin families. CD11b/CD18 is probably the most important adhesion molecule within the integrin family, while CD62L constitutes the most important adhesion molecule on granulocytes within the selectin family.

Kawabata et al. [20] and Carreno et al. [21] have demonstrated an increased expression of CD11b/CD18 (Mac-1) on granulocytes after 15–30 min of HD. An increased expression of CD11b has also been observed following CPB [1]. This is in contrast to the present study where the expression of CD18 was almost unaffected by CVVHD. Thus CVVHD does not induce any major upregulation of the integrin adhesion molecules on granulocytes. Though Nakae et al. [22] observed a normal expression of CD11b/CD18 on granulocytes in septic patients, most studies have reported an increased

expression of this adhesion molecule in septic patients [23, 24]. This is in accordance with the present study where infusion of endotoxin induced a significantly increased expression of CD18 on granulocytes.

Several studies have demonstrated a decreased expression of CD62L on granulocytes after 15–30 min of HD [18, 19]. The non-significant decrease in the expression of CD62L during CVVHD is in accordance with other studies [13, 25]. Toft et al. [10] observed a non-significant decrease in CD62L expression on granulocytes in septic patients during CVVHD. Kellum et al. [25] registered a non-significant decrease in soluble L-selectin levels in the blood during CVVHD as well as during continuous veno-venous haemofiltration (CVVH) in septic patients. There was no difference in soluble L-selectin levels between patients treated with CVVHD compared to CVVH.

The effect of endotoxin infusion on the expression of CD62L on granulocytes has, to our knowledge, not been investigated before. In a preliminary report Rosenbloom et al. [26] showed, however, that circulating granulocytes from septic patients had a low L-selectin expression. This is in accordance with the present study.

The lack of any accumulation of granulocytes in the lungs and liver during CVVHD is in contrast to haemodialysis using cuprophane membranes. During haemodialysis Dodd et al. [7] observed a significant accumulation of labelled granulocytes in the lungs. The accumulation of granulocytes in the lungs and in the liver following endotoxin infusion is in accordance with other studies [15]. The accumulation of activated granulocytes in the lungs might play a role in the development of ARDS.

The hepatic failure in sepsis is probably multifactorial. A modest infiltration of granulocytes in the liver might protect against translocation, whereas a larger accumulation of activated granulocytes probably participates in the development of hepatic failure. Jaeschke et al. [27], by using monoclonal antibodies against granulocytes, showed that that injury after hepatic ischaemia was partly mediated by granulocytes.

We conclude that, in contrast to the pronounced activation of granulocytes following endotoxin-induced sepsis, granulocytes were not activated during CVVHD.

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