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Original Article

Neutrophil Oxygen Radical Production by Dialysis Membranes

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Abstract. The ability of different dialysis membranes to activate polymorphonuclear neutrophil oxygen radical production was investigated with chemiluminescence. All the six membranes, namely cuprophan, cellulose acetate, polycarbonate, polysulphone, polyacrilonitrile and polymethylmethacrylate were able to interact with neutrophils and stimulate their oxygen radical production, the highest responses being seen with polyacrilonitrile, polymethylmethacrylate and polycarbonate. To analyse the role of complement in this interaction, fresh plasma, heat-inactivated and zymosan-activated plasma were added: with fresh plasma oxygen radical production was stimulated on cuprophan, cellulose acetate and polysulphone, not modified on polycarbonate, and decreased on polyacrilonitrile and polymethylmethacrylate. With heat-inactivated plasma, the responses were decreased or abrogated on all the membranes except polycarbonate and polymethylmethacrylate, whereas with zymosan-activated plasma similar responses to fresh plasma were observed. In addition, when plasma was used to precoat the membrane, cuprophan, cellulose acetate and polysulphone disclosed an enhanced neutrophil oxidative burst, while precoated polyacrilonitrile and polymethylmethacrylate were less stimulatory than uncoated membranes. In contrast the precoating of polycarbonate did not modify oxygen radical production. These data suggest that neutrophil activation occurs by direct membrane-neutrophil interaction. Plasmatic factors modulate this interaction but complement seems involved on cellulosic and polysulphone membranes only. Therefore, it appears that oxygen radicals produced from contact of neutrophils with the dialysis membrane might play an initial and/or additional role in the events occurring at the initiation of haemodialysis.

Key words: Biocompatibility; Complement; Haemodialysis; Neutrophils; Oxygen radicals

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Introduction

The neutropenia occurring at the initiation of haemodialysis is generally thought to result from activation of complement generated by the contact of plasma with the dialyser membrane, which subsequently promotes aggregation and sequestration of granulocytes in the pulmonary bed [1]. However, other mechanisms have been postulated, such as polymorphonuclear degranulation [2], up-regulation of the C_{3b} receptor [3] and the increased expression of an adhesion-promoting protein [4]. Moreover, it was shown that the degree of neutropenia was not always linked to the degree of complement activation [5] nor to the oxidative metabolism of dialysed cells [6]. The electrical charges of dialysis membranes also were shown to play a role in triggering neutrophil degranulation [7]. These observations point to the fact that the membrane itself could modulate neutrophil functions.

When neutrophils are activated by bacteria or other stimuli, they take up oxygen from the surrounding medium (a phenomenon called the respiratory burst) and generate a group of lethal oxidants including superoxide (O_2^-), hydroxyl radicals (OH°) and hydrogen peroxide (H_2O_2), which produce electronically excited states and emit light on relaxation to ground state [8,9]. These oxygen

radicals have been shown to be injurious to cells and tissues [10,11]. In addition O_2^- is able to react with plasma and generate a chemotactic factor for neutrophils, thus amplifying the inflammatory response [12]. Since oxygen radicals reflect neutrophil activation, their measurement may not only represent an index to assess the effect of dialyser membranes on this function, but also help to understand whether oxygen radicals play a role in haemodialysis neutropenia.

We investigated in vitro the oxidative metabolism of neutrophils in contact with different dialyser membranes using luminol- and lucigenin-enhanced chemiluminescence. These two techniques were chosen for their distinctive sensitivity and specificity towards different oxygen radical species. This study demonstrates that neutrophil activation occurs by direct membrane–neutrophil interaction and that plasma factors modulate this activation on some membranes only.

Materials and Methods

Neutrophil Isolation

Venous blood from healthy adult volunteers was collected into plastic tubes containing sodium heparin (10 $U \cdot ml^{-1}$ blood). Purified preparations of polymorphonuclear leukocytes were obtained in a single centrifugation step on discontinuous density Percoll Gradient (Pharmacia) as previously described [13]. Plasma was removed and kept on ice until use. Finally, the cells were suspended in Hank's balanced salt-solution containing glucose (5 $mmol \cdot l^{-1}$) (HBSS) at a concentration of 2×10^7 $cells \cdot ml^{-1}$.

Reagents

Heat-inactivated plasma was prepared by incubating autologous plasma for 30 min at 56°C. Zymosan-activated plasma was prepared as already described [13]. Zymosan-activated plasma was frozen in aliquots and thawed on the day of use. Luminol (6-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma, St Louis, Missouri, USA), was stored as a stock solution in dimethylsulphoxide (DMSO) (Sigma) ($5.6 \cdot 10^{-2}$ $mmol \cdot l^{-1}$) at −20°C and diluted to a concentration of $5.6 \cdot 10^{-4}$ $mmol \cdot l^{-1}$ in HBSS for use. Lucigenin (bis-N-methylacridinium nitrate) (Sigma) was dissolved in HBSS to a concentration of 10^{-3} $mmol \cdot l^{-1}$.

Membranes

Six different types of membranes were studied. Cuprophan, cellulose acetate, polysulphone and polymethylmethacrylate fibers were removed respectively from CF 1511 (Travenol, USA), CA 170 (Travenol, USA), Hemoflow F60 (Fresenius, West Germany) and B2-150 (Toray, Japan) hollow-fiber dialysers, and cut into small fragments (~1 cm). Polyacrilonitrile and polycarbonate membranes were obtained from Biospal 2400S (Hospal, France) and Lundia Pro 5 (Gambro Lundia, Sweden) plate membrane dialysers, respectively, and cut into small pieces. The membrane fragments were washed three times with saline prior to use. In some experiments, these saline-washed membranes were treated with fresh or heat-inactivated plasmas. Ten-milligram fragments were incubated in 1 ml plasma for 10 min at 37°C, then washed 3–4 times with saline.

Chemiluminescence

Luminol- and lucigenin-enhanced chemiluminescence were performed in a LKB 1250 luminometer as previously described [14]. The reaction was initiated by the addition of 50 µl cells to 3-ml plastic tubes containing the prewarmed buffer (HBSS) together with 10 mg of the dialyser membrane fragments, 50 µl luminol with or without autologous plasma, in a final volume of 0.5 ml. With lucigenin as a chemiluminescence amplifier, 25 µl cells were used. In some experiments, heat-inactivated plasma or zymosan-activated plasma were substituted for fresh plasma.

Statistics

Statistical analysis was done by the Wilcoxon's signed-rank test (≥ 6 experiments) or by the paired and unpaired Student's t test (< 6 experiments).

Results

Oxygen-radical production from neutrophils in contact with the different dialyser membranes and the influence of plasma on this interaction is shown in Figs 1, 2.

Luminol-Enhanced Chemiluminescence

The luminol-enhanced chemiluminescence has been shown to depend primarily on H_2O_2 and myeloperoxidase [15]. Modulation of chemiluminescence by each of the membranes is depicted in Fig. 1. In contact with neutrophils only (open bars), saline washed polycarbonate, polymethylmethacrylate and polyacrilonitrile were able to induce a high chemiluminescence response, cuprophan stimulated the chemiluminescence to a lower degree, whereas cellulose acetate and polysulphone failed to enhance the chemiluminescence. The addition of fresh

plasma (Fig. 1a) slightly potentiated the responses on polysulphone, but did not significantly modify those on cuprophan, cellulose acetate and polycarbonate. In contrast it decreased the chemiluminescence on polymethylmethacrylate and polyacrilonitrile.

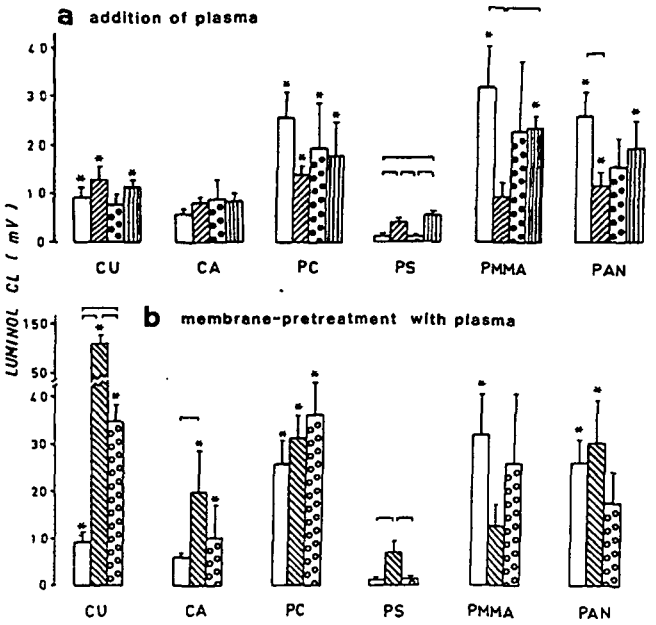

Fig. 1. Luminol-amplified chemiluminescence of neutrophils in contact with dialyser membranes and its modulation by plasma. Values are the mean ± SEM of three experiments for polycarbonate and polymethylmethacrylate and at least six experiments for the other membranes.

Asterisks indicate the level of significance in comparison to control (absence of membranes). Brackets indicate the level of significance between the groups indicated under the brackets. * and ⌐ = $P < 0.05$. Symbols are: ☐ saline-washed membranes; ▨ + fresh autologous plasma; •° + heat-inactivated plasma; ▥ + zymosan-activated plasma; ▩ fresh autologous plasma-treated membranes, °° heat-inactivated plasma-treated membranes.

To further analyse the role of complement in neutrophil activation by the membrane, heat-inactivated plasma or zymosan-activated plasma were substituted for fresh plasma. With heat-inactivated plasma, a significant chemiluminescence was maintained on polycarbonate only. With zymosan-activated plasma, the chemiluminescence responses were similar to these with fresh plasma (except for polymethylmethacrylate).

Since the contact between neutrophils and dialyser membrane can in vivo be preceded by plasma protein adsorption on the membrane, the membranes were incubated for 10 min with plasma and washed afterwards (Fig. 1b). Compared to the saline-washed membranes, only fresh-plasma-treated cuprophan, cellulose acetate and polysulphone were able to further stimulate the chemiluminescence, cuprophan stimulating to the highest degree. The already high chemiluminescence seen with polycarbonate, polymethylmethacrylate and polyacrilonitrile was not modified upon pretreating these membranes with fresh plasma, indicating that plasma did not play a role in the neutrophil activation on those membranes. After heat-inactivated plasma treatment, cuprophan and polysulphone only produced a decreased chemiluminescence. These results suggest that neutrophil activation is modulated by adsorbed plasma factors (related to complement or not) only on cuprophan, cellulose acetate and polysulphone.

Lucigenin-Enhanced Chemiluminescence

Production of oxygen radicals by interaction of neutrophils with dialyser membranes was further analysed by measuring the lucigenin-enhanced chemiluminescence, which has been shown to mostly depend on superoxide [16] (Fig. 2). It can be observed that polycarbonate and polymethylmethacrylate induced a fairly high chemiluminescence response, whereas cuprophan and polysulphone were unable to significantly stimulate the neutrophils. Upon adding plasma (Fig. 2a), chemiluminescence of polymorphonuclear in contact with cuprophan, cellulose acetate and polysulphone was significantly increased above control values. The already elevated chemiluminescence observed with polycarbonate was not modified by plasma as well as that obtained with polymethylmethacrylate. The addition of heat-inactivated plasma did not modify the polycarbonate- or polymethylmethacrylate-induced chemiluminescence compared to fresh plasma, whereas it virtually abolished the other membrane-induced chemiluminescence. In comparison to fresh plasma, zymosan-activated plasma did not modify the chemiluminescence observed with all the membranes. It is worth noting that the chemiluminescence induced by polysulphone was the greatest, while it has been shown that in vivo this membrane activated complement minimally [13]. The results indicate that as for luminol chemiluminescence, cuprophan, cellulose acetate and polysulphone depend on plasma factors for neutrophil activation, whereas polycarbonate and polymethylmethacrylate do not.

When pre-treated with plasma (Fig. 2b), all the membranes except polymethylmethacrylate were able to stimulate the chemiluminescence above control values. However, only coated cuprophan and polysulphone were significantly greater than uncoated membranes. Furthermore, the high chemiluminescence observed on polysulphone with plasma present in the medium (Fig. 2a) was greatly decreased upon pretreating the membrane. Further addition of plasma to the medium containing this plasma-treated polysulphone membrane did not restore a high chemiluminescence response (data not shown). It suggests that polysulphone became coated with a plasma

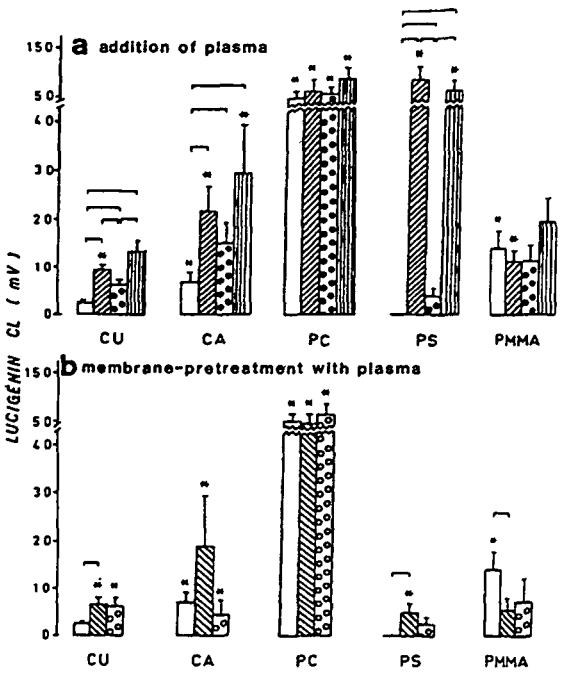

Fig. 2. Lucigenin-amplified chemiluminescence of neutrophils in contact with dialyser membranes and its modulation by plasma. Values are the mean ± SEM of four experiments for polycarbonate and polymethylmethacrylate and at least six experiments for the other membranes. Symbols are as in Fig. 1.

factor that prevented activation of neutrophils. Treating polycarbonate with plasma again did not modify the neutrophil response. When treated with heat-inactivated plasma, all membranes except polycarbonate induced a very low level of chemiluminescence.

Discussion

During haemodialysis a direct contact between neutrophils and the dialyser membrane might lead to activation [17,18]. The present in vitro study demonstrated the effect of six dialyser membranes on neutrophil oxidative metabolism and its modulation by different plasma components. All the membranes were able to stimulate neutrophil oxygen-radical production, albeit to different degrees. Activation occurred in some cases independently of complement activation, and the ability of the different membranes to stimulate neutrophils was not linked to their in vivo degree of neutropenia [13]. Indeed, polyacrilonitrile, polymethylmethacrylate and polycarbonate were the most potent activating membranes. Similarly it was reported that polyacrilonitrile activated Hageman factor more intensively than polymethylmethacrylate, cuprophan and cellulose acetate, when these membranes were incubated with purified Hageman factor in vitro [19]. In addition, a comparable increase of plasma neutrophil elastase was observed in patients dialysed either with cuprophan or polymethylmethacrylate [20].

A special recognition by leukocytes of certain determinants on the dialysis membrane could probably be responsible for this different oxygen-radical production. These stimulating properties might be mediated with the membrane capacity to interact by electrostatic forces with the corresponding negatively charged sites on the polymorphonuclear membrane. Indeed it was observed that only cationic, but not anionic or neutral polymethylmethacrylate induced a marked release of lysozyme or platelet activating factor [7]. It was also shown that bacteria which had been preopsonised by cationic polyelectrolytes generated intense luminol-dependent chemiluminescence [21]. However, other membrane properties must also play a role, since the anionic polyacrilonitrile was able to trigger a large neutrophil activation. It has also recently been reported that polyacrilonitrile was able to induce interleukin-1 production in monocytes, whereas regenerated cellulose had much less effect [22].

The addition of fresh plasma further modulated the oxygen-radical production, depending on the type of membrane. The increased stimulation observed upon addition of plasma on cuprophan, cellulose acetate and polysulphone could be mediated by complement activation. Indeed cuprophan but not polyacrilonitrile incubated in vitro with normal plasma was able to activate complement components [23]. In addition cuprophan-treated plasma had the capacity of triggering the release of oxygen radicals from their autologous neutrophils [24]. Our experiments show that heat-inactivated plasma decreased or abrogated the responses on all membranes tested except polycarbonate and polymethylmethacrylate. These two membranes showed identical oxygen-radical production whether fresh or heat-inactivated plasma was added, suggesting a complement-independent pathway for activation. If the differences observed upon addition of plasma were due to the ability of the membranes to activate complement differently, similar responses should be expected when plasma already containing activated complement was used. However, zymosan-activated plasma induced almost similar responses as fresh plasma. Therefore, it must be hypothesised that either complement was not completely activated by zymosan or that the responses depended more on the nature of the membrane than on activated complement.

Contact of biopolymers with blood is followed within minutes by surface adsorption of proteins [25]. It was shown that polysaccharide-containing membranes (i.e. cuprophan, cellulose acetate) activate complement through the alternative pathway [26] and that cuprophan binds C_{3b} to its surface [27]. Our study showed that membranes precoated or in other words preopsonized by plasma disclosed different responses, namely an enhanced activation on cuprophan, cellulose acetate and polysulphone and an inhibition on polyacrilonitrile or polymethylmethacrylate. On polycarbonate, precoating did

not modify the stimulation, suggesting that this procedure did not play a role in neutrophil activation. Polyacrilonitrile and polymethylmethacrylate must adsorb more or other proteins than cuprophan, cellulose acetate and polysulphone, and therefore prevent neutrophils from interacting with the membrane surface. It is worth noting that this plasma coating considerably decreased the high stimulation observed on polysulphone when fresh plasma (but not heat-inactivated plasma) was present in the medium. This indicates that the activating capacity of polysulphone required the presence of plasma and that the factor responsible for activation (in this case complement) was either not fixed by this membrane or became superseded by other proteins such as albumin.

That plasma stimulates, inhibits or has no effect on the capacity of the dialysis membranes to induce the oxidative burst is suggestive of a complex modulation. The relative role of different mediators may be closely interwoven and therefore difficult to discriminate. Activated neutrophils could at the same time become desensitised due to their stimulation by the dialyser (complement-related or not), but could also generate additional molecules with stimulatory, chemotactic or adherence-promoting properties, thereby providing a self-amplifying mechanism.

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