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A major role for CD62P/CD15s interaction in leukocyte margination during hemodialysis

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A major role of CD62P/CD15s interaction in leukocyte margination during hemodialysis. We investigated expression of several antigens on neutrophils and monocytes, involved in cell adhesion, from patients hemodialyzed with cellulosic and polyacrylonitrile membranes. Among the antigens tested only the expression of CD15s and CD11b was significantly increased on neutrophils and monocytes in patients dialyzed with cellulosic membranes. No changes occurred with polyacrylonitrile membranes. Leukocyte counts from patients dialyzed with cuprophane membranes decreased at the same time as expression of cellular CD15s increased, resulting in a significant negative correlation at all time points tested. No correlation was found between the drop of monocytes and their expression of CD11b. When CD15s expression increased on neutrophils and monocytes, we observed a concomitant increase of CD62P, a specific selectin of activated platelets. When whole blood cells were incubated with complement activated serum both antigens increased but not when cells were incubated with hrC5a. We also observed that CD61, a platelet phenotypic antigen, was present on leukocytes incubated with complement activated serum. At the time when platelet-leukocyte coaggregates decreased, CD62P expression remained stable on leukocytes, suggesting that both neutrophils and monocytes are able to trap either CD62P shed by activated platelets or soluble CD62P present in normal human serum. The present study documents a major role of P-selectin (CD62P)/sialyl-Lewis x (CD15s) interaction in the transient leukocyte margination during hemodialysis.

The contact of blood with artificial membranes may activate cell adhesion processes and initiate the leukocyte inflammatory response which itself plays an important role in the pathophysiological changes occurring during hemodialysis [1–3]. Cellular adhesion is a critical phenomenon which guides cell margination and their subsequent transendothelial migration [4, 5]. Adhesion molecules are also implied in cell-cell interactions leading to cellular activation and production of mediators of the inflammatory response [6–8]. Several studies have shown that neutropenia and monocytopenia take place during hemodialysis procedure using cellulosic membranes [9, 10]. The leukocyte margination has been associated with complement activation which occurs during hemodialysis on cuprophane membranes [1, 11, 12] and to the increased expression of Mo1/Mac-1 antigen (CR3 receptors, CD11b/CD18 heterodimer) on leukocytes [13, 14]. Recently several adhesion molecules belonging to the selectin, integrin, immunoglobulin, and as yet unidentified families have been implicated in tethering, activation, strong adhesion and motility of leukocytes on the endothelium [15]. During hemodialysis it is proposed that the transient margination of leukocytes mainly involves tethering phenomena implicating interactions of selectin with their natural ligands. L-selectin, E-selectin and P-selectin have been characterized and shown to mediate a degree of adhesion that allows rolling (tethering) of the leukocyte but not firm adhesion, explaining the transient nature of cellular binding through selectin interactions [16]. The transient nature of this adhesive interaction is strengthened by the fact that selectins are shed by activated cells [17]. The purpose of our study was to investigate the mechanisms underlying leukocyte margination and activation during hemodialysis. Therefore, we have studied the expression of adhesion molecules on leukocytes obtained from patients dialyzed with cuprophane, cellulose acetate and polyacrylonitrile (AN69) membranes by cytofluorometric analysis.

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Methods

Patient population

Eighteen end-stage renal disease patients who were between 25 and 77 years old (mean ± SD, 49 ± 21) from the E. Rist Hemodialysis Center, 6 end-stage renal disease patients who were between 26 and 68 years old (mean ± SD, 42 ± 18) from the Nephrology Department, Hôpital Broussais, and 4 end-stage renal disease patients who were between 74 and 78 years old (mean ± SD, 76 ± 2) from the AURA Hemodialysis Center, were hemodialyzed for more than six months.

Positive history for first use syndrome, clinical evidence of infection at the time of the study, cardiac and vascular instability, unstabilized erythropoetin dosage and single needle dialysis were exclusion criteria. None of the patients received any medications known to affect leukocyte or platelet function for at least two weeks prior the study. Informed consent was obtained from all subjects.

Study design

Ten patients were dialyzed using cuprophane membranes (GFE15 Gambro, Sweden; Sorin 1508, France); 12 patients were dialyzed using polyacrylonitrile membranes (AN69, Hospal,

France) and 6 patients were dialyzed using acetate cellulose membranes (CA150, Baxter, USA). Recruitment of patients was without bias concerning the type of membrane. A bicarbonate base dialysate was used in all cases. Blood flow rates were between 250 and 300 ml/min; dialysate flow rate was 500 ml/min. Ultrafiltration rates were between 500 and 1000 ml/hour. Anticoagulation was maintained using heparin as an initial bolus and then by continuous infusion during the course of dialysis. Venous blood from hemodialysed individuals was collected in K_3 EDTA vacutainers. In a first set of experiments blood samples from 20 patients were drawn pre-dialysis (T:0) from the afferent line and then subsequently from the efferent line at 15 minutes (T:15) and at the end of the dialysis session (T:240). In a second set of experiments blood samples were drawn from 8 patients pre-dialysis (T:0) from the afferent line and then subsequentely from the efferent line at 5 (T:5), 10 (T:10), and 15 (T:15) minutes, and at the end of the dialysis session (T:240). Samples were kept cold before use.

Blood samples were also collected from seven healthy individuals for *in vitro* experiments.

Reagents and buffers

Phosphate buffered saline (PBS) was from bioMérieux (Marcy l'Etoile, France). Serum albumin bovine (BSA) and sodium azide (NaN_3) were from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex® G25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Suspension of particles was prepared as previouly reported [18]. Mouse monoclonal antibodies (mAb) anti-CD15s, anti-CD14, anti-CD15, anti-CD11a, anti-CD11b, anti-CD11c, anti-CD18, anti-CD49d, anti-CD54, anti-CD61, anti-CD62P and anti-CD62L, FITC-conjugated anti-CD15, PE-conjugated anti-CD14 and isotypic Ab controls were purchased from Becton Dickinson (Mountain View, CA, USA) and Immunotech (Marseille, France). FITC-conjugated anti-mouse IgF(ab')$_2$ fragment was from Silenus Laboratories (Australia).

Flow cytometric analysis

Neutrophils and monocytes. Whole blood (300 μl) was incubated with decomplemented human AB serum (1.5 ml) for 10 minutes at room temperature in order to decrease nonspecific binding, and centrifuged at 4°C. The pellet was incubated with 1 μg of first mAb diluted in PBS-BSA 1%-sodium azide 0.1% (staining medium) or 10 μl of conjugated mAb for 30 minutes at 4°C. Samples were washed twice in PBS-BSA 1%. If an unlabeled first step antibody was to be used, samples were incubated with a second affinity isolated FITC-conjugated anti-mouse Ig F(ab')$_2$ fragment (50 μl of 1/100 dilution) for 30 minutes at 4°C. Cells were washed once in staining medium. Erythrocytes were lysed by adding 2 ml of lysing solution (FACS lysing solution, Becton Dickinson) for five minutes at room temperature. This last procedure was repeated until complete lysis of red blood cells occurred. Samples were washed twice with the staining medium and cells were fixed by adding 0.3 ml of cold paraformaldehyde (1%) and stored in the dark at 4°C until analysis. Stained cells were characterized by flow cytometric analysis (FACScan cytometer Becton Dickinson). The instrument was set to measure linear forward light scatter (FSC), which is a measure of cell size, linear side light scatter (SSC), which is a measure of granularity, 538 nM fluorescein fluorescence (FL1), 507 nM phycoerythrin fluorescence (FL2). Fluorescence parameters were collected using four decade logarithmic amplification. Neutrophils and monocytes were identified in the linear forward versus linear side scatter plot by use of subgroup specific anti-mAbs: CD15 and CD14 for neutrophils and monocytes, respectively. Mouse isotypic antibodies were used as negative controls.

Platelets. CD62P is a specific marker of activated platelets [19] whereas CD61 is a marker of resting and activated platelets [20]. A total of 100 μl EDTA anticoagulated whole blood was incubated for two hours at 4°C with 1 ml of 1% cold paraformaldehyde. The fixed cells were centrifugated at 1200 $\times$g for five minutes at room temperature; the pellets were washed twice in 1 ml of PBS with 0.1% azide. Fifty microliters of fixed blood suspension were incubated with 20 μl of mAb. After 20 minutes incubation at room temperature samples were washed once in 1 ml of PBS 0.1% azide. Samples were then incubated with the second anti-mouse FITC-IgF(ab')2 fragments (50 μl of 1/100 dilution) in the dark, at room temperature, for 20 minutes. Samples were washed once in 1 ml of PBS 0.1% azide. Platelets were fixed by adding 1 ml of cold 1% paraformaldehyde and stored in the dark at 4°C. FACScan analysis was performed within three days. The FACScan instrument was set to measure logarithmic FSC, logarithmic SSC and 611 nM fluorescein fluorescence (FL1). Fluorescence parameters were collected using four decade logarithmic amplification. Platelets were identified in the logarithmic FSC versus logarithmic SSC plot by use anti-mAb CD61: the gated platelet population was found to bind > 90% the anti-CD61. Mouse IgG1 was used as a negative control.

Platelet-leukocyte interaction. mAb-anti CD62P was used to evaluate platelet-leukocyte interaction during hemodialysis with cuprophane and polyacrylonitrile membranes. The mean intensity of fluorescence of anti-CD62P on neutrophils and monocytes was used to characterize platelet leukocyte interaction. Neutrophil and monocyte surface staining with mAb anti-CD62P, and their FACS analysis were performed as described above.

Six thousand events were collected in list mode files for neutrophils, monocytes and platelets and were analyzed on Hewlett Packard computer equipped with FACScan software program (LYSIS II version 1.1).

In vitro stimulation of whole blood cells by Sephadex or rC5a. Human blood (N = 4) was collected from healthy individuals in dry vacutainers and in K_3 EDTA vacutainers. Serum was obtained after centrifugation and then incubated with 150 mg/ml of crushed Sephadex for one hour at 37°C before adding a solution of EDTA 5 mM [18]. Complement activation was evaluated by quantification of C3a/C3adesArg. Whole blood cells from the donors were obtained after discarding only the plasma by centrifugation. The plasma was replaced by the same amount of complement activated autologous serum, and the cells incubated for 5, 10, 15 and 45 minutes at 37°C. Whole blood cells from healthy donors (N = 3) were also stimulated with recombinant C5a (rC5a). Whole blood samples were incubated with rC5a (250ng/ml) for 30 minutes at 37°C. Leukocyte samples were then kept cold. Cell surface staining with mAbs anti-CD15s, anti-CD11b, anti-CD62P and anti-CD61, and their FACScan analysis were performed as described above.

Analytical methods

Blood cell counts were performed using the Coulter STKS System (Coultronics, USA); C3a/C3adesArg was done using a radioimmunoassay (Amersham, France).

Table 1. Expression of surface molecules involved in cell adhesion on neutrophils and monocytes obtained from hemodialyzed patients

	Neutrophils			Monocytes			
Antigens	T:0 MFI (%)	T:15 MFI (%)	T:240 MFI (%)	T:0 MFI (%)	T:15 MFI (%)	T:240 MFI (%)	
CD 15s	281 ± 74 (96)	469 ± 109 (96)	219 ± 79 (98)	117 ± 38 (80)	285 ± 101 (91)	105 ± 40 (94)	1
	272 ± 75 (99)	592 ± 226 (100)	308 ± 52 (10)	153 ± 46 (87)	463 ± 249 (97)	153 ± 28 (86)	2
	328 ± 109 (100)	357 ± 85 (100)	303 ± 82 (98)	166 ± 43 (87)	176 ± 73 (85)	168 ± 60 (83)	3
CD 11a	33 ± 8 (98)	30 ± 6 (97)	30 ± 11 (98)	83 ± 19 (95)	75 ± 20 (94)	80 ± 18 (91)	1
	37 ± 3 (97)	38 ± 4 (98)	37 ± 4 (95)	77 ± 7 (97)	71 ± 6 (94)	79 ± 10 (91)	2
	37 ± 3 (96)	37 ± 3 (96)	35 ± 2 (94)	84 ± 14 (98)	81 ± 8 (97)	77 ± 7 (95)	3
CD 11b	53 ± 20 (99)	71 ± 23 (99)	44 ± 17 (99)	54 ± 26 (97)	58 ± 26 (93)	51 ± 22 (91)	1
	49 ± 14 (97)	63 ± 19 (99)	56 ± 17 (98)	46 ± 8 (80)	56 ± 16 (90)	61 ± 11 (88)	2
	55 ± 17 (88)	54 ± 8 (96)	50 ± 13 (93)	58 ± 5 (96)	52 ± 7 (93)	57 ± 13 (92)	3
CD 11c	16 ± 4 (52)	16 ± 4 (36)	16 ± 4 (50)	27 ± 7 (86)	20 ± 5 (65)	28 ± 9 (90)	1
	26 ± 7 (34)	28 ± 8 (58)	29 ± 7 (31)	41 ± 7 (59)	38 ± 4 (62)	45 ± 5 (74)	2
	27 ± 5 (38)	26 ± 4 (36)	26 ± 5 (38)	43 ± 8 (80)	42 ± 6 (69)	41 ± 6 (77)	3
CD 18	84 ± 31 (97)	90 ± 31 (99)	88 ± 36 (96)	122 ± 16 (91)	149 ± 38 (90)	126 ± 49 (88)	1
	82 ± 17 (100)	96 ± 19 (100)	81 ± 16 (100)	127 ± 23 (94)	118 ± 18 (97)	126 ± 14 (99)	2
	83 ± 20 (99)	88 ± 10 (100)	78 ± 20 (100)	145 ± 31 (99)	138 ± 18 (99)	137 ± 32 (97)	3
CD 49d	24 ± 3 (6)	25 ± 6 (4)	21 ± 6 (3)	26 ± 6 (77)	29 ± 14 (69)	25 ± 6 (71)	1
	32 ± 6 (10)	33 ± 12 (17)	34 ± 5 (9)	38 ± 5 (60)	36 ± 5 (65)	38 ± 5 (60)	2
	26 ± 12 (7)	25 ± 11 (6)	24 ± 11 (6)	39 ± 4 (60)	39 ± 2 (66)	38 ± 5 (58)	3
CD 54	17 ± 7 (6)	21 ± 10 (7)	18 ± 6 (5)	29 ± 8 (80)	37 ± 30 (75)	27 ± 6 (74)	1
	26 ± 8 (9)	27 ± 10 (11)	27 ± 4 (6)	34 ± 6 (40)	32 ± 5 (40)	33 ± 5 (51)	2
	29 ± 3 (4)	28 ± 3 (6)	26 ± 4 (3)	35 ± 7 (56)	35 ± 7 (53)	33 ± 6 (45)	3
CD 62L	69 ± 22 (97)	80 ± 15 (97)	68 ± 23 (97)	68 ± 12 (86)	87 ± 11 (90)	66 ± 20 (82)	1
	89 ± 19 (99)	92 ± 18 (98)	86 ± 7 (99)	88 ± 12 (75)	95 ± 16 (90)	79 ± 15 (92)	2
	86 ± 13 (98)	88 ± 12 (98)	90 ± 13 (99)	88 ± 12 (83)	95 ± 16 (84)	79 ± 15 (80)	3

Patients were dialyzed with cuprophane ($N = 6$) (1), cellulose acetate ($N = 6$) (2) and polyacrylonitrile (AN69) ($N = 8$) (3) membranes. Cells were collected before dialysis (T:0), 15 minutes after (T:15) and at the end of dialysis session (T:240). Results are expressed as mean fluorescence intensity (MFI) ± SEM; numbers in parentheses are percentage of positive cells.

Statistics

For CD15s, CD11b, CD61 molecules, results were expressed as mean ± SEM of fluorescence intensity. For CD62P the marker expressed after platelet activation, results are represented as the percentage of positive cells ± SEM for platelets and means ± SEM of fluorescence intensity for neutrophils and monocytes. Statistical analysis was performed using the Student's paired *t*-test. P values < 0.05 were considered to be statistically significant.

Results

Expression of adhesive molecules on leukocytes from hemodialyzed patients

We have previously reported that cytokines are produced during hemodialysis in patients dialysed with cuprophane membranes and 24 hours after dialysis in patients dialyzed with synthetic high permeability membranes [12, 21]. Since cytokines, such as IL-1 and TNFα, regulate the expression of adhesive molecules responsible for neutrophils and monocyte margination and extravasation [22, 23], we have investigated the expression of adhesive molecules on both cell types. Expression of integrins CD11a/CD18(LFA-1), CD11b/CD18(CR3), CD11c/CD18(CR4), CD49d/CD29(VLA-4), the selectin CD62L, and antigens related to adhesive phenomena CD15s (sialyl-Lewis x) and CD54 (ICAM-1) was studied. Blood from patients dialyzed with cuprophane, cellulose acetate and polyacrilonitrile (AN69) membranes, known for their varying capacity to activate the complement system, were collected at different time points before the dialysis session (T:0), 15 minutes after starting the hemodialysis procedure (T:15) and at the end of the session (T:240). Expression of these molecules was investigated by indirect cytofluorometric analysis using specific fluorescent monoclonal antibodies as described in the **Methods** section. The results indicate that among the different antigens tested, only the expression of CD15s and CD11b increased significantly on neutrophils after 15 minutes of hemodialysis with cuprophane membranes as compared with predialysis values (469 ± 109 vs. 281 ± 74, $P < 0.007$ for CD15s antigen; 71 ± 23 vs. 53 ± 20, $P < 0.05$ for CD11b antigen; Table 1). On monocytes only CD15s expression was up-regulated (285 ± 101 at T:15 vs. 117 ± 38 at T0, $P < 0.005$). Expression of CD49d, CD11a, CD11c and CD54, on both type of cells, was not affected by the dialysis procedure. Similar results were observed with the cells obtained from the patients dialyzed with cellulose acetate (Table 1). Hemodialysis with polyacrylonitrile dialyzers did not affect the expression of CD15s and CD11b on both neutrophils and monocytes.

Leukocyte counts

Figure 1 depicts the changes in neutrophil and monocyte counts that occurred during hemodialysis using cuprophane and polyacrilonitrile membranes. The number of monocytes and neutrophils dropped dramatically at 15 minutes. Monocyte numbers returned to predialytic levels, but the number of neutrophils was significantly higher at the end of dialysis session than as predialysis values ($P < 0.05$). No significant changes occurred when patients were dialyzed with polyacrylonitrile membranes (AN69).

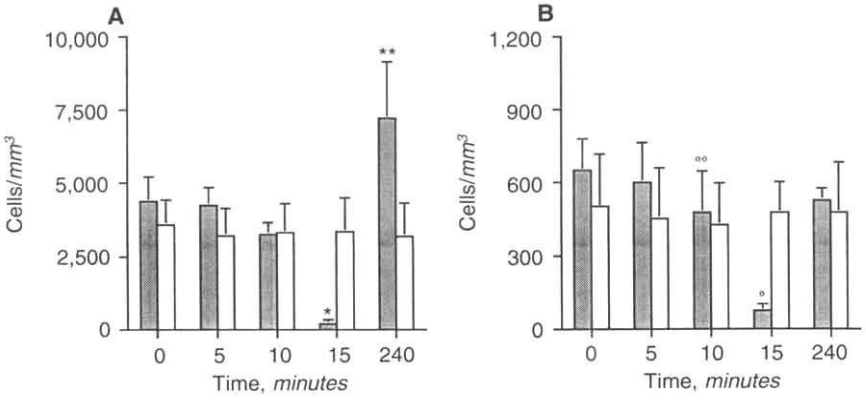

Fig. 1. *Time course of neutrophil (A) and monocyte (B) counts during dialysis with cuprophane (■) and polyacrylonitrile membranes (□).* Neutrophil and monocyte counts were determined for four patients dialyzed with cuprophane membranes and four patients dialyzed with polyacrylonitrile (AN69) at various time points of the dialysis session. Results are expressed as means ± SEM, compared to pre-dialysis values (T:0) by Student's *t*-test for paired data: $^{*}P < 0.005$, $^{**}P < 0.05$, $^{\circ}P < 0.005$, $^{\circ\circ}P < 0.05$.

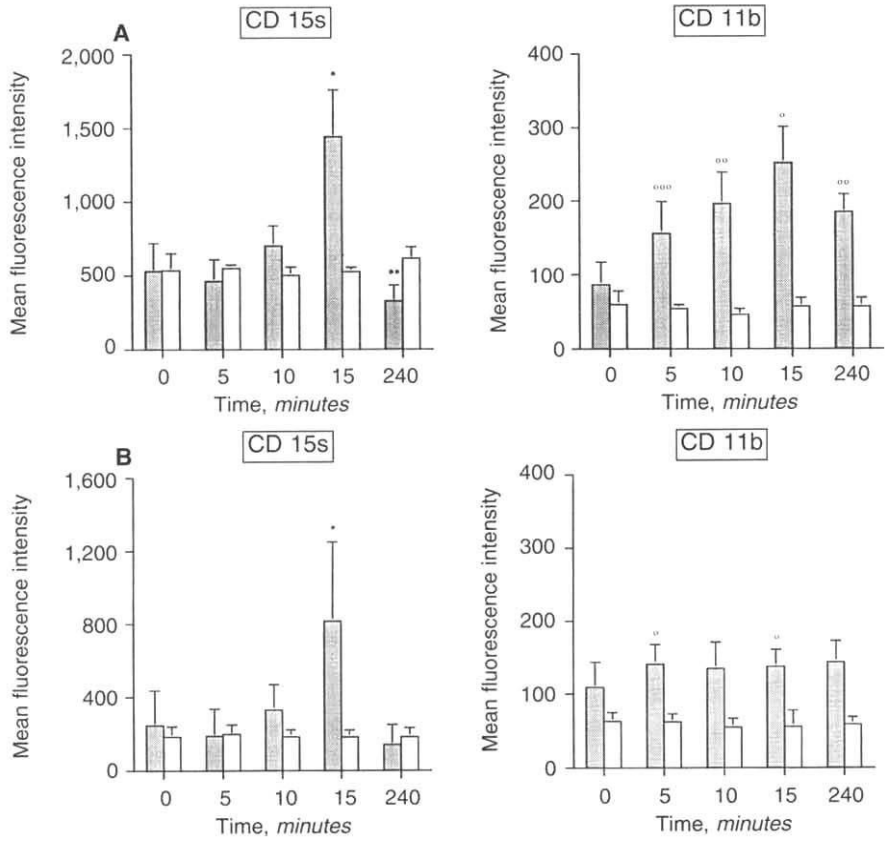

Fig. 2. *Modulation of neutrophil and monocyte CD15s and CD11b expression during dialysis with cuprophane (■) and polyacrylonitrile (AN69) membranes (□).* Expression of CD15s and CD11b was analyzed on neutrophils and monocytes obtained from the same patients at the same time points as those depicted in Figure 1. Results presented in **A** are expressed as mean fluorescence intensity ± SEM of surface CD15s and CD11b on neutrophils ($^{*}P < 0.002$, $^{**}P < 0.02$, $^{\circ}P < 0.005$, $^{\circ\circ}P < 0.008$, $^{\circ\circ\circ}P < 0.02$ compared to pre-dialysis values (T0) by Student's *t*-test for paired data); expression of the same surface antigens on monocytes is represented in **B** ($^{*}P < 0.04$, $^{\circ}P < 0.05$, compared to pre-dialysis values (T:0) by Student's *t*-test for paired data).

Expression of CD15s and CD11b on leukocytes during hemodialysis with cuprophane and polyacrylonitrile membranes

Expression of CD15s and CD11b was investigated on neutrophils and monocytes at the same time points depicted in Figure 1. Expression of CD15s on neutrophils increased significantly during hemodialysis with cuprophane membranes to reach maximal values at 15 minutes (1441 ± 319 T:15 vs. 529 ± 190 T:0; $P < 0.002$) and decreased to values below those obtained prior to dialysis at the end of the session (325 ± 110 T:240 vs. 529 ± 190 T:0, $P < 0.02$; Fig. 2A). Expression of CD15s increased similarly on monocytes obtained from the same patients (Fig. 2B). CD15s antigen expression did not change when patients were dialyzed with polyacrylonitrile membranes (AN69) (Fig. 2 A, B). Expression of CD11b on neutrophils from patients dialyzed with cuprophane membranes increased significantly at five minutes to reach maximal values at 15 minutes (87 ± 30 T:0 vs. 156 ± 43, $P < 0.02$; 87 ± 30 T:0 vs. 252 ± 49 T:15, $P < 0.005$). At the end of the dialysis session, expression of CD11b did not reach predialysis values (185 ± 24 T:240 vs. 87 ± 30 T:0, $P < 0.008$). CD11b expression was only slightly affected on monocytes during the dialysis procedure. No changes in CD11b expression occurred

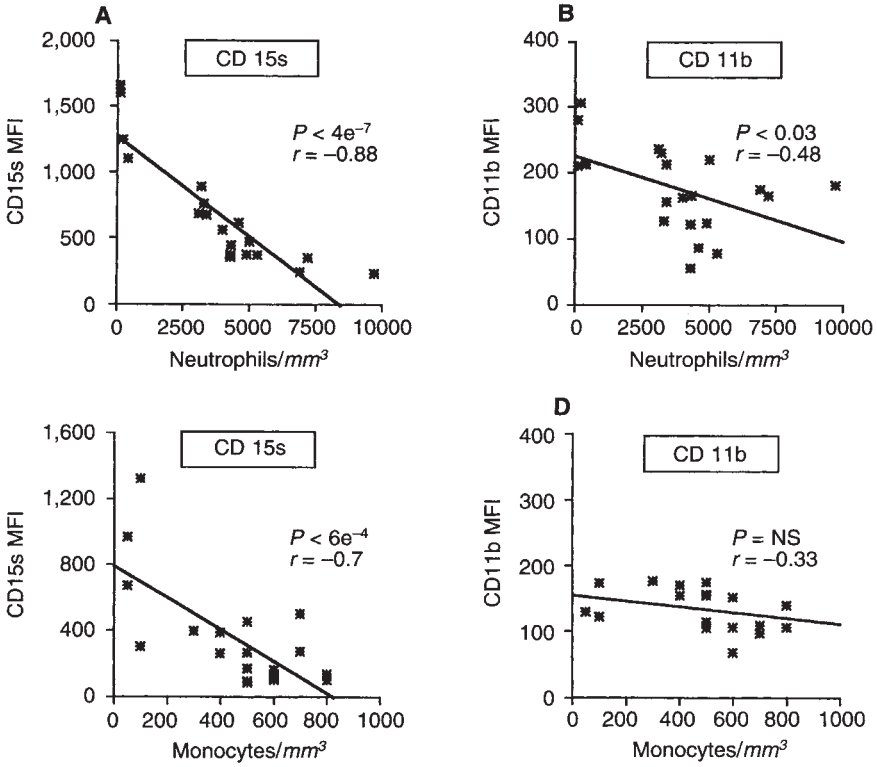

Fig. 3. *Correlation curves between neutrophils counts (x axis) and mean fluorescence intensity of CD15s and CD11b (y axis) on neutrophils (A, B) and monocytes (C, D) during cuprophane dialysis session.* A significantly negative correlation was found between the expression of CD11b molecules ($P < 0.03$) and neutrophil counts, as well as between CD15s molecules and cell counts, but to a higher magnitude ($P < 4e^{-7}$). A significantly negative correlation was also found between CD15s ($P < 6e^{-4}$) and monocyte counts during dialysis.

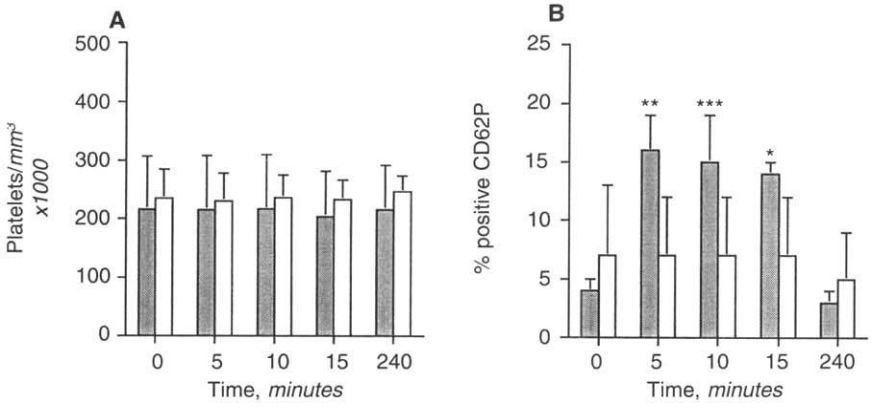

Fig. 4. *Total (A) and activated (B) platelet counts during dialysis with cuprophane (■) and polyacrylonitrile (AN69) membranes (□).* Activation of platelets was assessed by their binding to anti-CD62P. $*P < 0.005$, $**P < 0.009$, $***P < 0.03$ compared to pre-dialysis values (T:0) by Student's *t*-test for paired data.

when monocytes were obtained from patients dialyzed with polyacrylonitrile membranes (AN69).

Figure 3 depicts negative correlation between CD15s expression and neutrophil or monocyte numbers, indicating that CD15s is directly involved in the margination of circulating neutrophils and monocytes during hemodialysis using cuprophane devices.

Activation of platelets during hemodialysis session

We followed the expression of CD62P on platelets obtained from patients dialyzed with cuprophane and polyacrylonitrile membranes at the different time points during the hemodialysis session mentioned above. The results presented in Figure 4 indicate that platelets are highly activated at five minutes of dialysis on cuprophane and that the expression of CD62P decreases slowly at 10 minutes to reach minimal values at the end of dialysis. No significant changes were observed on platelets obtained from patients dialyzed with polyacrylonitrile (AN69) (Fig. 4).

Binding of platelets to leukocytes during hemodialysis

Since CD62P (P-selectin) molecules expressed on activated platelets constitue one of the ligands of CD15s expressed on both neutrophils and monocytes [19, 24, 25], we decided to investigate the binding of activated platelets on both types of cells. The results depicted in Figure 5 indicate that activated platelets bind to neutrophils and monocytes within five minutes of the dialysis session on cuprophane membranes. The relative numbers of CD62P positive neutrophils reached maximal values of positive CD62P cells (T:5, 73 ± 3 $CD62P^+$) within five minutes and decreased at the end of the dialysis session (T:240, 59 ± 3%

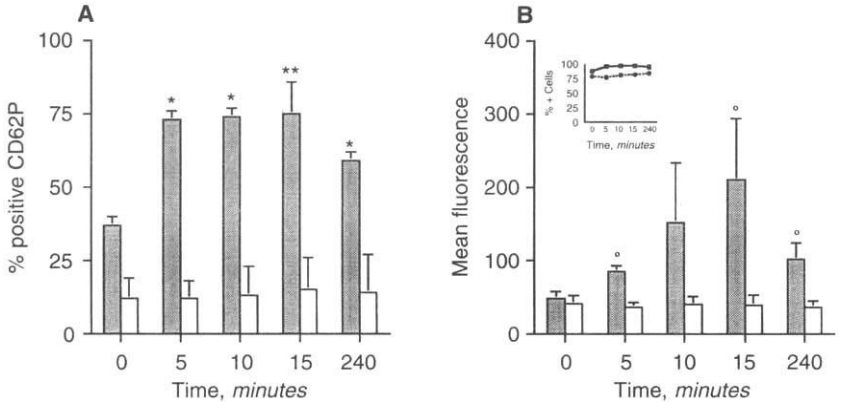

Fig. 5. *Effect of dialysis with cuprophane (■) and polyacrylonitrile (AN69) membranes (□) on platelet-neutrophil and platelet-monocyte aggregation.* Activated platelets induced by cuprophane dialysis bound significantly to neutrophils (**A**) and monocytes (**B**) during the dialysis session: $*P < 0.001$, $**P < 0.003$, $° P < 0.04$ compared to pre-dialysis values (T:0) by Student's *t*-test for paired data. Insert represent percentage of monocytes expressing CD62P during cuprophane (squares) and polyacrylonitrile (AN69) (circles) hemodialysis.

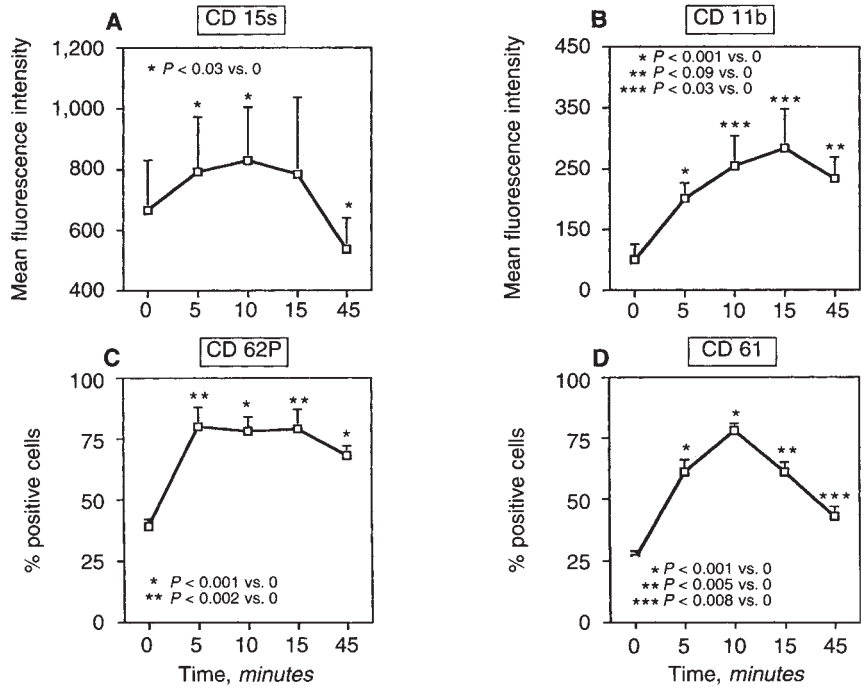

Fig. 6. *Stimulation of whole blood from healthy individuals with autologous normal human serum activated with Sephadex.* Expression of CD15s and CD11b antigens on neutrophils, obtained from four healthy individuals, at different time points of incubation (**A, B**) and evaluation of platelet-neutrophil aggregates as assessed by CD61 and CD62P expression on the neutrophils (**C, D**).

$CD62P^+$). One hundred percent of the circulating monocytes in patients dialyzed with cuprophane membranes expressed low levels of CD62P (T:0, MFI = 48 ± 10). CD62P expression increased during dialysis to reach maximal values at 15 minutes (T:15, MFI = 210 ± 84). The expression of CD62P antigen on both neutrophils and monocytes did not return to predialysis values. No changes occurred with cells from patients dialyzed with polyacrylonitrile (AN69) membranes.

In vitro studies

To further investigate the mechanisms implicated in the increase in the expression of CD15s and CD11b on circulating leukocytes and the induction of platelet-leukocyte aggregate formation, autologous activated human serum was added to whole blood from four healthy individuals. Normal human serum was activated by Sephadex, which has a closely related structure to cuprophane, and C3a/C3adesArg formation was assessed by RIA (Sephadex activated normal human serum = 72 140 ng/ml; unactivated normal human serum = 325 ng/ml). Figures 6, 7 and 8 show the modulation of the expression of CD15s, CD11b, CD62P and CD61 on neutrophils and monocytes. At the same time points expression of CD15s on neutrophils increased significantly at 5 minutes to reach maximal values at 10 minutes of incubation (666 ± 164 T:0 vs. 792 ± 180 T:5, $P < 0.03$; 666 ± 164 T:0 vs. 829 ± 175 T:10, $P < 0.02$). Expression of CD15s increased similarly and more significantly on monocytes (201 ± 10 T:0 vs. 321 ± 29 T:5, $P < 0.003$; 201 ± 10 T:0 vs. 353 ± 27 T:10, $P < 0.002$). The expression of CD11b antigen on both neutrophils and monocytes significantly increased at 5 minutes to reach maximal values at 15 minutes of incubation (neutrophils: 100 ± 25 T:0 vs. 201 ± 26 T:5, $P < 0.001$; 100 ± 25 T:0 vs. 284 ± 64 T:15, $P < 0.03$; monocytes: 109 ± 37 T:0 vs. 190 ± 24 T:5, $P < 0.02$; 109 ± 37 T:0 vs. 230 ± 24 T:15, $P < 0.02$). Despite the increased expression of CD15s and CD11b on neutrophils and monocytes incubated with complement-activated autologous serum, whole blood cells incubated in the presence of recombinant hC5a did not exhibit an

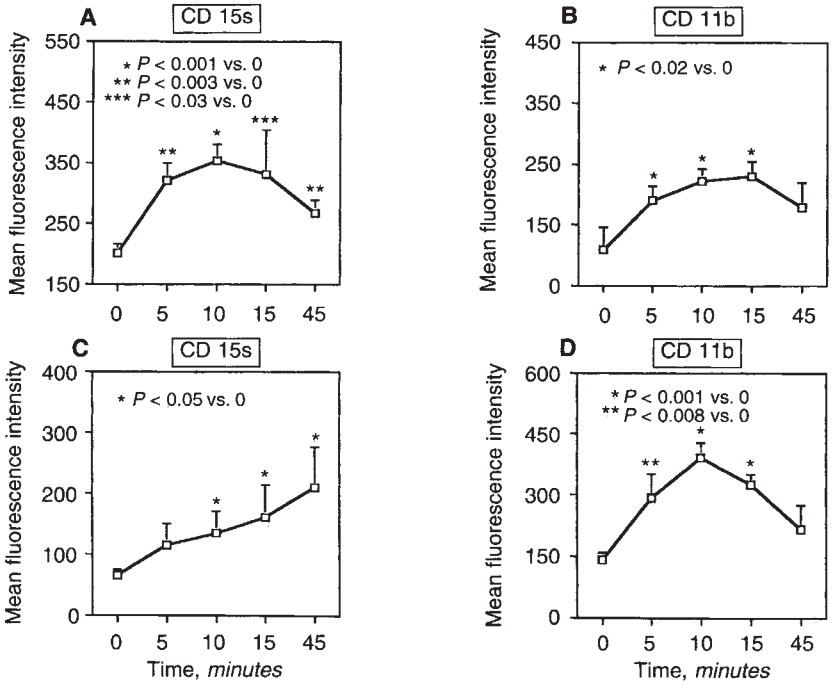

Fig. 7. *Stimulation of whole blood from healthy individuals with autologous normal human serum activated with Sephadex.* Expression of CD15s and CD11b antigens on monocytes obtained from the same donors as those depicted in Figure 6, at different time points of incubation (upper panels) and evaluation of platelet-monocytes aggregates as assessed by CD61 and CD62P expression on the monocytes (lower panels).

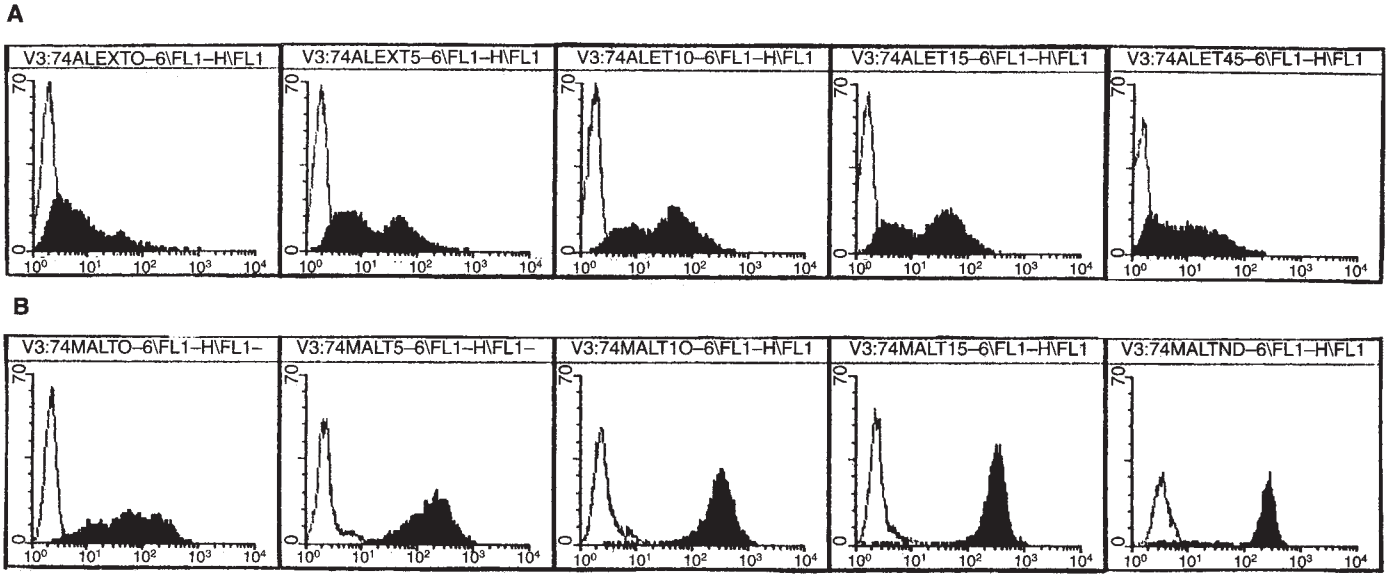

Fig. 8. *Flow cytometry analysis of CD61 expression on neutrophils* (**A**) *and monocytes* (**B**) *stimulated in vitro by autologous serum activated by Sephadex.* The results are those from one representative experiment out of four. The clear profiles show negative controls and darkened profile show CD61 expression on cells cultured for 0, 5, 10, 15 and 45 minutes in the presence of activated serum.

altered expression of CD15s on both cell types; expression of CD11b was only increased on monocytes (Table 2.). Figure 8 showed the kinetics of CD61 expression on neutrophils and monocytes as assessed by flow cytometry. The results are those from one representative experiment out of four. No significant change in surface antigen expression on leukocytes occurred in the presence of autologous serum that has not been incubated with Sephadex (data not shown).

To evaluate the formation of platelet-leukocytes aggregates at different time points during incubation of whole blood with autologous activated human serum, we examined the appearance of CD61 and CD62P on the surface of neutrophils and monocytes. CD62P is expressed on activated platelets, CD61 is expressed on resting and activated platelets. The results depicted in Figure 6 indicate that maximal values of positive CD61 neutrophils occurred at 10 minutes of incubation (T:10 78 ± 2 CD61+). The

Table 2. Expression of CD15s and CD11b (MFI) on neutrophils and monocytes cultured in the presence of recombinant C5a

	Neutrophils			Monocytes		
	4°C	37°C	C5a (37°C)	4°C	37°C	C5a (37°C)
CD15s	888	1081	1070	362	441	430
CD11b	102	146	143	102	107	123

The results are of a representative experiment out of three.

relative numbers of positive CD62 neutrophils reached maximal values at five minutes of incubation (T:5 80 ± 8 CD62+). In Figure 7 the mean fluorescence intensity of a 100% pure positive CD61 monocytes reached maximal values at 10 minutes of incubation (T:10 MFI = 390 ± 37). At 45 minutes of incubation the anti-CD62P binding to 100% of monocytes reached the maximal values (T:45 MFI = 210 ± 66).

Discussion

Hemodialysis on cellulosic membranes is associated with transient leukocyte margination [1, 9–11]. Leukocyte margination is dependent on the expression of selectins and integrins which is modulated by several mediators of the inflammatory response [5]. The present study demonstrates that dialysis with cellulosic membranes is associated with an increase of CD15s, CD11b, CD62P and CD62L expression on neutrophils and monocytes. Expression of CD11a, CD11c, CD18, CD49d and CD54 was not affected by the dialysis procedure. No changes in selectin or integrin expression occurred on leukocytes obtained from patients dialyzed with polyacrylonitrile (AN69) membranes. Our results also indicate that hemodialysis with cuprophane induces platelet activation followed by their aggregation on both neutrophils and monocytes.

The selectins initiate many critical interactions among blood cells [26]. Evidence that they play a crucial role in leukocyte trafficking has emerged in the last past few years [15, 16, 27]. Recently ligands for the selectins have been characterized [28] and among them, the sialyl-Lewis x molecules, also termed CD15s. CD15s has been shown to bind to E-selectin expressed on cytokine activated endothelial cells [25, 29], P-selectin expressed on rapidly activated endothelial cells and on platelets activated by thrombin, histamine or PAF [19, 30, 31], and L-selectins constitutively expressed on neutrophils, monocytes and lymphocyte subsets [4]. In human a genetic disorder called leukocyte adhesion deficiency type II, has been shown to be associated with CD15s deficiency and abnormal leukocyte trafficking [32]. In the present study, we investigated the expression of CD15s on neutrophils and monocytes from patients undergoing hemodialysis in order to gain some insight into the hemodialysis induced margination of leukocytes. Our results clearly indicate that CD15s is up-regulated on neutrophils and monocytes obtained at 15 minutes of hemodialysis with cuprophane membranes. At the end of hemodialysis, expression of CD15s was lower than that observed in samples collected prior to the dialysis session. Cells obtained from patients dialyzed with cellulose acetate membranes also expressed higher amounts of CD15s. No significant changes occurred with cells obtained from patients dialyzed with polyacrylonitrile (AN69) membranes. Similar results were obtained with monocytes from the same patients. As reported by other investigators, hemodialysis with cellulosic membranes was associated with an increased expression of CD11b on neutrophils and to a much lower extent on monocytes. Levels of CD11b on neutrophils had increased by five minutes of dialysis. Expression of CD11b at the end of the dialysis procedure was significantly higher than that observed prior to dialysis. No changes in CD11b expression on neutrophils and monocytes occurred in patients dialyzed with polyacrylonitrile membranes. Our results are in agreement with previous studies [33–35] but conflicting with those reported by Tielmans et al [36]. However, it was suggested by the authors that dialysis with polyacrylonitrile (AN69) could be associated with transfer of bacterial products, a phenomenon which could not explain the results of our study as particular attention was taken to prevent the contamination with bacterial endotoxins. At the time of the study no intracellular IL-1 production (data not shown) was observed in monocytes from patients dialyzed with high permeability membranes, strongly indicating that the dialysate was probably free of any endotoxin contamination [21, 37].

At the same time points in dialysis we simultaneously studied CD15s and CD11b expression and the time course of neutrophil and monocyte counts during hemodialysis with cuprophane and polyacrylonitrile membranes. The results indicate that there was a strong correlation between CD15s expression and the transient margination of neutrophils and monocytes at all time points tested (Fig. 3; $P < 4 \times 10^{-7}$ for neutrophils; $P < 6 \times 10^{-4}$ for monocytes throughout the dialysis procedure), whereas the drop in monocyte counts was independent on CD11b expression. Taken together, these results indicate that the transient leukopenia induced in patients dialyzed with cellulosic membranes is due to a mechanism which involves selectin mediated events rather than to the integrin adhesive phenomenon as suggested in earlier observations [38]. However, it can also be suggested that conformational changes of CD11b could occur during hemodialysis [39], a phenomenon which will not affect CD11b/CD18 expression but will involve their participation in margination. To investigate the mechanisms involved in up-regulation of CD15s and CD11b on neutrophils and monocytes, during the hemodialysis we cultured blood cells in the presence of Sephadex-activated autologous serum. We have previously shown that Sephadex activates human complement and induces the generation of both C3a and C5a anaphylatoxins *in vitro* [18]. We observed that CD15s and CD11b expression on neutrophils and monocytes incubated in complement-activated autologous serum is increased in a time dependent fashion. However, if the cells were incubated in the presence of recombinant C5a, the expression of CD15s was not altered and expression of CD11b was increased only on monocytes (Table 2). This result does not totally rule out complement involvement since anaphylatoxins could act in a permissive or synergistic fashion with other mediators, or activation of the autologous serum by Sephadex could also generate C3b and C3bi fragments which may interact with CR1 (CD35) and CR3 (CD11b/CD18) on both neutrophils and monocytes. However, it can be proposed that incubation of Sephadex with serum may also generate mediators which in turn may induce the release of platelet activating factor (PAF). It is known that PAF and P-selectin act synergistically in promoting the early adhesion of neutrophils to endothelium [27, 38]; therefore, PAF may modulate CD15s expression. This hypothesis is currently under investigation.

We have observed that both neutrophils and monocytes expressed CD62P antigens in patients dialyzed with cuprophane

membranes but not on cells from patients dialyzed with polyacrylonitrile membranes (AN69). Since CD62P antigens are expressed only on activated endothelial cells and activated platelets [19, 40], we investigated CD62P expression on platelets during hemodialysis. Platelets were gated in the logarithmic FSC versus logarithmic SSC plots using the CD61 platelet-specific antibody. The results indicate that platelets from patients dialyzed on cuprophane membranes were maximally activated after 5 minutes of dialysis whereas only a slight activation of platelets occurred in patients dialyzed with polyacrylonitrile membranes. The latter findings were also reported by other investigators using different types of synthetic membranes [41, 42]. We also studied the kinetics of platelet counts during hemodialysis. The data indicate that there was no significant thrombocytopenia in patients hemodialyzed with cuprophane membranes. At the same time points of hemodialysis as those used to measure platelets counts and CD62P expression we measured CD62P antigen on both neutrophils and monocytes. We observed that almost all monocytes (80 to 95%) expressed low levels of CD62P but only 30% of the circulating neutrophils expressed this antigen. During hemodialysis, increased CD62P expression was detectable on monocytes after just 5 minutes of dialysis and rose continuously to reach maximal values at 15 minutes. In contrast the percentage of $CD62P^+$ neutrophils increased at 5 minutes, remained stable throughout the procedure and remained elevated at the end of the dialysis session as compared with predialysis values. The higher affinity of monocytes for activated platelets as compared to neutrophils cannot be attributed to a higher expression of CD15s on monocytes, since monocytes expressed lower levels of CD15s as compared to neutrophils prior to hemodialysis (MFI = 529 ± 190 for neutrophils and 246 ± 151 for monocytes). It should be pointed out that neutrophils from hemodialyzed patients expressed lower levels of CD15s than cells from healthy individuals, but monocytes from the patients expressed similar levels of CD15s to normal monocytes (MFI = 529 ± 190 vs. 666 ± 164 for neutrophils and 246 ± 151 vs. 201 ± 10 for monocytes). Despite the absence of fall in platelet counts at the time when monocytes and neutrophils expressed CD62P, we observed a small decrease in CD15s expression on both type of cells (MFI = 529 ± 190 vs. 462 ± 148 for neutrophils and 246 ± 151 vs. 188 ± 135 for monocytes) at 5 minutes into the session in all patients dialyzed with cuprophane membranes. These results could be due to the formation of platelet-leukocyte coaggregates which will not affect platelet counts because the number of platelets in the circulation is far higher than leukocytes numbers. When microscopic analysis was performed on leukocytes isolated from patients dialyzed with cuprophane membranes 15 minutes after the dialysis session, the results indicated that formation of intact coaggregates between platelets and leukocytes occurred (data not shown). Direct visualization of platelet-neutrophil adhesion within the vascular lumen has been recently reported to occur *in vivo* in an animal model [43]. The greater amounts of coaggregates formed with monocytes as compared to neutrophils may be due to a different conformation of the CD15s molecule on monocytes which allows a better interaction with activated platelets or involvement of another monocytic membrane molecule in the recognition of activated platelets. In order to investigate the mechanism of coaggregate formation we incubated whole blood cells from several donors with Sephadex-activated autologous serum. The results indicated that for both neutrophils and monocytes the kinetics of CD62P expression were similar to those observed *in vivo*. The percent of neutrophils expressing CD62P reached a plateau at 5 minutes of incubation and the mean fluorescence intensity of CD62P rose continously on monocytes. For both neutrophils and monocytes, CD61 expression reached a maximal value after 10 minutes of incubation in activated serum and declined thereafter, indicating that platelets are either ingested or detached from the cells. The apparent discrepancy between CD62P and CD61 expression on both types of cells can be due to the shedding of CD62P from activated platelets at the membrane of cells or to an absorption of soluble CD62P. We and other investigators [17] have found that soluble CD62P is present in the serum of normal individuals. We have also observed that levels of soluble CD62P increased in patients undergoing cardiopulmonary bypass in which bioincompatibility of extracorporeal circulation is closely related to hemodialysis [12]. We are currently investigating the levels of soluble CD62P in hemodialyzed patients.

It has been demonstrated by *in vitro* studies that leukocytes cultured in the presence of activated platelets induce superoxide anion by monocytes and neutrophils through P-selectin/CD15s interactions and that leukotrienes are generated during platelet-neutrophil interactions [44, 45]. P-selectin also interacts in a juxtacrine fashion with $\beta 2$-integrins to increase phagocytosis by neutrophils. It was also reported that neutrophils and platelets may cooperate to increase the formation of PAF which is known to be a potential mediator involved in the amplification of inflammatory reactions. Taken together, our results indicate that hemodialysis on cuprophane membranes is associated with an increased expression of CD15s on neutrophils and monocytes. Despite the fact that neutrophil margination has been attributed to result from complement activation and generation of C3a and C5a, our results indicate that modulation of CD15s is independent of anaphylatoxin formation. We simultaneously observed increased CD15s expression on leukocytes and a concomitant coaggregate formation of platelets with neutrophils and monocytes, the monocytes having a higher tendency to form coaggregates. The P-selectin dependent coaggregation may contribute to physiological effects resulting from hemodialysis with cuprophane membranes since the leukocyte-platelet aggregates may impede microcirculatory flow and may induce activation of leukocyte function.

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