Modulation of granulocyte LAM-1 and MAC-1 during dialysis—A prospective, randomized controlled trial

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Modulation of granulocyte LAM-1 and MAC-1 during dialysis—A prospective, randomized controlled trial. Hemodialysis with first-use cellulosic dialysis membranes results in activation of the alternative pathway of complement and profound neutropenia followed by rebound leukocytosis. The neutropenia has been shown to be associated with increased expression of adhesion receptors and pulmonary sequestration of granulocytes. However, the mechanism underlying the return of the granulocytes has not been elucidated. We determined simultaneously the changes in the granulocyte adhesion receptor MAC-1 (CD11b-CD18) and the selectin LAM-1 receptor during dialysis using a complement activating and a non-complement activating membrane, in a randomized, cross-over study. With initiation of dialysis with cellulosic membranes, there was a rapid and prominent increase in the expression of MAC-1 receptors. At the nadir of granulocyte count, 15 minutes after initiation of dialysis with the complement activating membrane, there was a four-fold increase in the MAC-1 receptor expression. At the same time, there was a two-fold decrease in LAM-1 expression. There were no changes in the expression of two other granulocyte receptors CD11a and CD15 which are known not to be modulated during granulocyte activation. Granulocytes harvested during dialysis and which had high MAC-1 and low LAM-1 expression had a significantly decreased adherence to endothelial cell monolayers. Dialysis of the same patients with non-complement activating membranes resulted in no significant change in the expression of these receptors on granulocytes nor in their adherence to endothelial cells. These results shed new light on the mechanism of the cyclical granulocytopenia and rebound granulocytosis during dialysis with new cellulosic membranes.

Hemodialysis with first-use cellulosic dialysis membranes results in profound activation of the alternative pathway of complement [1, 2]. This is associated with a transient granulocytopenia followed by a rebound granulocytosis [3]. In this setting, granulocytes have been demonstrated to undergo activation, as demonstrated by degranulation and production of reactive oxygen species [4–6]. In addition, up-regulation of specific cell surface receptor proteins, including CR1 [7] and MAC-1 (CD11b-CD18) [8], the so-called integrin receptors, have been demonstrated.

Several studies have clearly documented the importance of the MAC-1 receptor in adhesion of granulocytes to endothelium and to other granulocytes. During dialysis with first-use cellu-

losic membranes, Arnaout et al have previously demonstrated a rapid up-regulation of granulocyte cell surface MAC-1 (CD11b-CD18) receptors [8]. The time course of increased cell surface expression of this receptor coincided with the development of granulocytopenia and their pulmonary sequestration, consistent with the functional importance of increased cell surface MAC-1 expression in the increased adhesion of these activated granulocytes to the pulmonary endothelium. However, surprisingly, granulocyte cell surface MAC-1 remained elevated through most of the course of dialysis, despite the egress of these neutrophils from the pulmonary circulation and the reversal of granulocytopenia beginning at 15 minutes after the initiation of dialysis. Thus, while increased cell surface MAC-1 expression may be necessary in the development of dialysis granulocytopenia, it was not sufficient to maintain a granulocytopenic state. The exact cause of the reversal of granulocytopenia has not been demonstrated.

Recently a new family of cell adhesion molecules known as selectins or LEC-CAMS have been described [9]. All members of the selectin family appear to be involved in the adhesion of circulating blood cells to endothelium. Kishimoto et al have recently described shedding or down-regulation of the LAM-1 from human granulocytes in vitro upon activation with chemotactic factors and phorbol esters, and proposed a potential role for this shedding in regulating cell adhesion and transendothelial migration [10]. To our knowledge, there are no studies to date of human in vivo shedding of granulocyte LAM-1.

We postulated that granulocyte activation in vivo by first-use cellulosic dialysis membranes may cause simultaneous up-regulation of granulocyte cell surface MAC-1 and down-regulation of cell surface LAM-1, and that the inverse regulation of integrin and selectin granulocyte cell adhesion molecules during hemodialysis may be involved in the sequence of initial development and subsequent reversal of granulocytopenia in the hemodialysis procedure. Furthermore, we postulated that these reciprocal changes are initiated by complement activation and therefore are attenuated either by the reuse of these cellulosic membranes or during dialysis with synthetic membranes such as PAN or PMMA, which do not activate complement.

Methods

Patient characteristics

Ten patients on chronic maintenance hemodialysis were selected for study. Informed consent was obtained from the

subjects and the project was reviewed by the Institutional Review Board of the institution. The mean age of the patients was 55 (range 27 to 75 years), with a mean time on dialysis of 1.5 years (range 6 months to 4 years). Seven were male and three were female. None of these patients had clinical evidence of infection at the time of the study. Prior to the study, all patients were on a hemodialysis reuse program using 1% formaldehyde incubated at 40°C for at least 24 hours as a sterilant. Bleach was not used in the processing of these dialyzers.

Study design

All patients were studied in a cross-over fashion using first-use cellulosic (Focus 90, National Medical Care, Rockleigh, New Jersey, USA) hemodialysis membranes—high complement activating membranes—and fifth or greater reused cellulosic dialysis membranes, which have minimal complement activating potential.

The sequence of sampling was random. Thus, in six studies, patients were initially studied on first-use cellulosic membrane, followed by ≥fifth reuse sampling, whereas in four other studies, the initial sampling was obtained during dialysis with a reused (≥ fifth reuse) dialyzer, and patients were then placed on a first-use dialyzer and sampled again. The interval between these two studies for any one patient ranged from two weeks to four months.

A bicarbonate based dialysate (Na 140 mEq/liter, K 2 mEq/liter, Ca 3.5 mEq/liter, HCO_3 30 mEq/liter, acetate 3 mEq/liter) was used in all cases. Blood flow in all studies was 300 ml/min. Blood samples were drawn pre-dialysis from the afferent line and then subsequently from efferent lines at 5, 10, 15, 30, and 90 minutes after the initiation of dialysis and at the end of the dialysis procedure. Anticoagulation was maintained using heparin at an initial bolus of 100 U/kg, with additional doses if activated clotting times were less than 1.5 times pre-dialysis values.

Methods

Preparation of whole blood samples for measuring MAC-1 and LAM-1. Duplicate 100 μl aliquots of blood were removed from the dialysis line at specified times. Ten microliters of phycoerythrin conjugated anti-LEU-15 (anti-PE-MAC-1, Becton Dickinson, Mountainview, California, USA) was added to one tube and 20 ul of fluorescein isothyocyanate conjugated (FITC) anti-LEU-8 (FITC-anti-LAM-1, Becton Dickinson) was added to the other tube. These were incubated for 20 minutes at 4°C, washed ×1 with PBS (Difco Laboratories, Detroit, Michigan, USA) and then resuspended in 850 μl of PBS. Thirty microliters of 1.67 mg/ml LDS-751 (Exciton, Dayton, Ohio, USA) was added. This method allows the determination of fluorescence in whole blood specimens and avoids the artifacts that can potentially be introduced during granulocyte separation (see below) [11]. A 1:1 volume of 2% paraformaldehyde as a fixative was then added to each tube. In subsequent experiments, FITC conjugated antibody to CD11a (LFA-1; AMAC, Westbrook, Maine, USA) and murine antibody to CD15 (Becton Dickinson) were added to whole blood and analyzed as described above in three patients on first-use cellulosic dialyzers. Goat anti-mouse (GAM-FITC) was used as the second antibody for the CD15 antibody (Coulter, Hileah, Florida, USA). Control samples consisting of FITC-mouse IgG$_1$ and

PE-mouse IgG$_{2\alpha}$ (Becton Dickinson) were also processed simultaneously. All samples were then analyzed within two hours by flow cytometry.

Flow cytometric analysis of granulocyte MAC-1 and LAM-1. All samples were analyzed on a FACScan flow cytometer (Becton Dickinson). The instrument was set to measure linear forward light scatter (FSC) which is a measure of cell size, linear side scatter (SSC), which is a measure of granularity, 530 nM fluorescein fluorescence (FL1), 580 nM phycoerythrin fluorescence (FL2), and >620 nM LDS-751 fluorescence (FL3). Fluorescence parameters were collected using four decade logarithmic amplification.

The instrument was triggered on FL3 so that only nucleated cells staining with LDS-751 were detected. This effectively eliminates erythrocytes from the analysis and permits analysis of the major leukocyte populations in whole blood samples [11]. Data on at least 10,000 cells was collected in list mode files. For in vitro experiments, 2000 events were collected.

Data analysis was performed by first displaying FSC versus SSC and gating on the lymphocytes and granulocyte populations. Then histograms of FL1 or FL2 were generated for each population, and the mean fluorescence level calculated. In some studies two parameter correlated histograms of FL1 versus FL2 were also generated.

For each leukocyte population, control FL1 and FL2 fluorescence levels were determined from cells labeled with control reagents consisting of FITC and PE conjugated mouse immunoglobulin (Becton Dickinson). The results are expressed as mean log fluorescence channel (MLFC) of the test cells minus the mean log fluorescence channel of the control. To facilitate comparisons of fluorescence at different times, relative fluorescence is used to define the relative changes in receptor number. Since a four decade, 1024 channel logarithmic scale was used, the mean fluorescence channel could be converted to relative fluorescence by the following formula:

Relative fluorescence =

$$10^{(\text{Test mean channel} - \text{Control mean channel})/256}$$

HUVEC cultures. Endothelial cells from human umbilical cords were isolated by collagenase treatment and seeded on 100 mm petri dishes as described previously [12]. Morphology and anti-factor VIII antibody staining confirmed the endothelial origin of these cells. After confluence, cells were expanded by successive passages on new petri dishes coated with 1% gelatin. HUVEC were transferred to 24-well plates at passages 3 to 12 and used for adhesion experiments following confluence. PMN from patients and normal donors were tested simultaneously on HUVEC cultures from the same donor. In this series of experiments, the PMN were obtained from patients on new celluloscic membranes or PMMA (B2-150) membranes (Toray Industries, New York, USA), which is known to cause only minimal complement activation.

PMN isolation and Cr^{51} labelling. Ten milliliters of blood were drawn in a chilled vacutainer tube containing 50 U heparin from the efferent line of the dialyzer at time 0, 5, 15, 30, 60, 120 and 240 minutes (end of the session). All isolation procedures were maintained at 4°C until the adhesion assay or unless otherwise stated. PMN were separated by dextran sedimentation (vol/vol) for 30 minutes and red cells were lysed by

hypotonic shock with distilled water. This procedure obtained a high recovery of PMN with a contamination of less than 10 ± 5% monocytes as assessed by size and granularity on cytofluorometry analysis. Approximately 10^7 PMN were incubated in 500 μl Hanks' balanced salt solution (HBSS) without Ca^{++} and Mg^{++} (Gibco Lab., Grand Island, New York, USA), with 100 μCi Cr^{51} (1 mCi/ml) (Du Pont NEN, Boston, Massachusetts, USA) for one hour with frequent gentle shaking in order to obtain a total labelling of 20,000 cpm/10^6 PMN. PMN were washed twice and resuspended at 10^6/ml in HBSS containing Ca^{++} and Mg^{++} and immediately assayed for adhesion to endothelial cells as shown below.

Adhesion assay. A total of 5.10^5 PMN resuspended in 500 μl HBSS + 1% HEPES (pH 7.4) were allowed to adhere in duplicates on HUVEC wells at 37°C in a 5% CO_2 incubator for various amounts of time (5, 10, 15, 20, 30, 45 min). At the end of each time point, supernatants were harvested and wells washed with cold HBSS. HUVEC monolayers and adherent PMN were lysed with 500 μl 1 N NH_4OH for one hour. The amount of Cr^{51} in the supernatants, washes and lysis suspension was determined in a scintillation counter. Interassay variation was 4.2 ± 1.8%. Percentage of adherent PMN was determined as follows:

% adherence = mean of duplicates of

$$\frac{cpm\ lysis}{cpm\ (lysis\ +\ supernatant)} \times 100$$

In vitro stimulation of granulocytes with C5a and PMA. In this series of experiments C5a (1×10^{-8} M) (Sigma) or PMA (100 nM) (Sigma) was added separately to 10 ml of whole blood obtained from normals ($N = 3$) and dialysis patients ($N = 3$) and maintained at 37°C. Aliquots were removed at selected time points and analyzed for granulocyte cell surface MAC-1, LAM-1, CD15 and CD11a/CD18 density as described above.

White blood cell measurement. White blood cell (WBC) counts were measured using a Coulter Counter.

Statistical methods

Statistical analysis was performed using multivariate analysis packages available in SAS (Statistical Analysis Institute, Cary, North Carolina, USA). Initial analysis comparing changes in receptor fluorescence intradialytically and between new and reused dialyzers was done using the ANOVA procedure and the DUNCAN multiple range test, with significance determined at the $P = 0.05$ level. Specific values of the statistical differences (P values) between pre-dialysis values and a specific time point as well as for each time point between new and reused receptor data was determined using post-hoc t-tests of adjusted treatment means (least-square means).

Results

In vivo granulocyte LAM-1 and MAC-1 expression during first-use cellulosic (complement activating) and non-complement activating dialysis membranes

With first-use cellulosic dialysis membranes, granulocyte LAM-1 surface expression began to drop within five minutes after the initiation of dialysis from a mean log channel fluores-
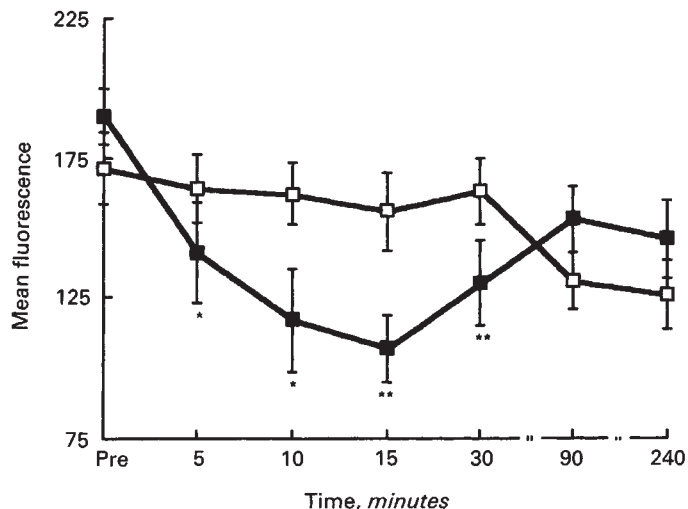

Fig. 1. *Granulocyte LAM-1 modulation during dialysis with cellulosic membranes.* Whole blood was obtained at specified time points with either first-use [■] or greater than fifth reuse cellulosic membranes [□]. Results are expressed as log mean fluorescence of granulocyte cell surface LAM-1 ± SEM ($N = 10$ experiments). * = $P < 0.05$, ** = $P < 0.001$ compared to pre-dialysis values.

cence of 190 ± 11 to 141 ± 17 MLFC ($P < 0.01$; Fig. 1). Subsequently, LAM-1 levels decreased further and reached a nadir 15 minutes after the initiation of dialysis, at which point mean channel fluorescence was 107 ± 12 channels ($P < 0.004$ compared to pre-dialysis levels). This represents a 2.1-fold decrease, to 47% of pre-dialysis levels. At the 30-minute sampling time, there was a trend toward increasing granulocyte LAM-1 levels to 130 ± 15 MLFC, though still significantly less compared to pre-dialysis values ($P < 0.002$). By the end of dialysis, granulocyte LAM-1 levels had increased to 146 ± 14 MLFC, not significantly different than pre-dialysis values.

Intradialytic changes of LAM-1 receptors during dialysis with non-complement activating membranes were markedly attenuated. Thus, after 15 minutes, granulocyte LAM-1 expression had declined from 171 ± 13 pre-dialysis to 164 ± 18 MLFC (P = NS; Fig. 1). By the end of dialysis, granulocyte LAM-1 had declined to 136 ± 12 MLFC, which is not statistically different from pre-dialysis values.

Changes in granulocyte cell surface MAC-1 (CD11b-CD18) were also analyzed intradialytically (Fig. 2). As has previously been demonstrated, there was a dramatic up-regulation of granulocyte cell surface MAC-1 beginning within five minutes after the initiation of new cellulosic hemodialysis, at a time when there was no significant decrease in WBC values (vide infra). By 15 minutes, fluorescence for cell surface MAC-1 increased to 308 ± 25 MLFC compared to 161 ± 22 channels pre-dialysis ($P < 0.003$). This represents a four-fold increase compared to pre-dialysis values. Cell surface granulocyte MAC-1 remained elevated compared to pre-dialysis, even at the end of the dialysis session with new cellulosic dialysis membranes. Thus at the end of dialysis, MLFC of MAC-1 was 246 ± 24 channels versus 161 ± 22 channels pre-dialysis, representing a 2.3-fold increase compared to pre-dialysis. Similar to the LAM-1 receptor, there were no statistical changes in MAC-1 expression during dialysis with reused membranes (Fig. 2).
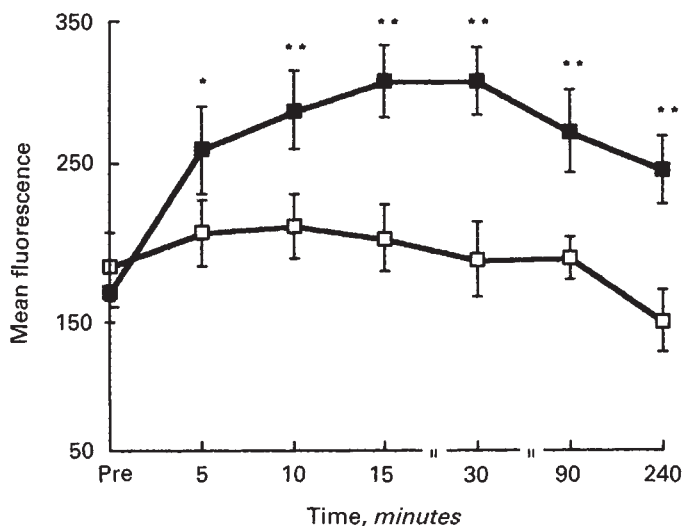
Fig. 2. *Granulocyte MAC-1 (CD11b-CD18) modulation during hemodialysis with cellulosic membranes.* Whole blood was obtained at selected time points with either first-use [■] or greater than 5th reuse cellulosic membranes [□]. Results are analyzed as log mean fluorescence of granulocyte MAC-1 ± SEM (N = 10 experiments). *P < 0.05, **P = 0.001 compared to pre-dialysis values.

Taken together, these studies clearly demonstrate that complement activation of granulocytes is associated with a rapid and dramatic shedding of granulocyte cell surface LAM-1 and up-regulation of MAC-1 receptors that does not occur with non-complement activating membranes.

Two-parameter correlated plots of granulocyte LAM-1 and MAC-1 expression during first-use cellulosic hemodialysis

Two-color immunofluorescence correlated plots of granulocyte LAM-1 and MAC-1 cell surface expression during first-use cellulosic hemodialysis were obtained. Figure 3 is a representative bicontour plot obtained over the course of a single hemodialysis session. Analysis of granulocytes obtained pre-dialysis showed a relatively uniform population in expression of both MAC-1 and LAM-1. Five minutes after the initiation of dialysis, MAC-1 cell surface expression had increased in a uniform fashion in all granulocytes; however, the decrease at five minutes in LAM-1 expression was more heterogeneous, indicating that not all cells had shed their LAM-1 receptors. The heterogeneity of LAM-1 expression continued through the first 15 minutes of dialysis. Thereafter, as LAM-1 cell surface expression decreased the population again became more uniform for all cells. By the end of the hemodialysis session, there again was a single population of granulocytes in terms of both MAC-1 and LAM-1 expression.

Specificity of intradialytic granulocyte receptor changes

To establish the specificity of intradialytic granulocyte LAM-1 and MAC-1 modulation during first-use cellulosic hemodialysis, we used a combination of forward-angle light scatter (FSC) and 90° side scatter (SSC) to flow cytometrically isolate the lymphocyte population for analysis in five patients. There were minimal changes in lymphocyte cell surface LAM-1 expression (Fig. 4) over the course of hemodialysis with either first-use or multiply reused cellulosic membranes.

To determine the role of complement activation, we assessed in in vitro experiments the changes of granulocyte receptors in response to C5a. As can be seen for the LAM-1 receptor in Figure 5 for blood obtained from normal volunteers, incubation of whole blood with C5a led to a decline of MLFC from 184 ± 9 to 131 ± 17 MLFC after five minutes of incubation. Thirty minutes after incubation with C5a, there was a further decline in LAM-1 granulocyte expression to 92 ± 20 MLFC. This represents a 2.1-fold decline, to 48% of control values.

Addition of PMA led to similar but more pronounced results (Fig. 5). Thus, by five minutes after addition of PMA, mean log channel fluorescence (MLFC) had declined from 184 ± 9 to 39 ± 22. This represents a 3.7-fold decline in the expression of these receptors, to 27% of control values. There was no further decline in these PMA stimulated cells after five minutes, and by 30 minutes, LAM-1 expression had increased to only 75 ± 18. The addition of buffer as a control showed no effect on cell surface LAM-1 expression (Fig. 5).

The addition of either C5a or PMA to granulocytes in whole blood obtained from normals also caused a rapid up-regulation of granulocyte surface MAC-1 expression (Fig. 6). Using PMA, MLFC increased from 164 ± 19 channels to 497 ± 39 units over 30 minutes. This represents a 20-fold increase compared to control values. With C5a, up-regulation of cell surface MAC-1 was equally prompt, but to a lower cell density than with PMA (Fig. 6). Control values using buffer remained unchanged until the 15 minute time point of incubation, after which there was a slight increase in granulocyte MAC-1 expression, from 164 ± 19 to 260 ± 49 MLFC. This most likely represents granulocyte stimulation during in vitro incubation at this temperature [13]. Identical results were obtained when the experiments were repeated on blood obtained pre-dialysis from dialysis-dependent patients (data not shown). Thus the addition of either C5a or PMA on whole blood resulted in increased granulocyte MAC-1 cell surface expression and decreased granulocyte LAM-1 expression.

In other in vivo experiments, we investigated the intradialytic changes of granulocyte receptors CD11a and CD15 in response to dialysis with new cellulosic membranes (Focus 90, National Medical Care, Rockleigh, New Jersey). As can be seen in Table 1, these receptors had little or no change in their expression intradialytically. The changes in the expression of either of these receptors were not statistically significant.

White blood cell counts during first-use cellulosic dialysis membranes

White blood cell (WBC) counts began to decline by 10 minutes after the initiation of dialysis (Table 1). Note, however, that at five minutes after initiation of dialysis, the number of WBC was not significantly different from the pre-dialysis value. By fifteen minutes after the initiation of new cellulosic hemodialysis, blood cell counts had declined to 37% of pre-dialysis (P < 0.001). White blood cell counts began to increase after 15 minutes, and by 90 minutes after the initiation of dialysis white blood cell counts were not significantly different than pre-dialysis. When cellulosic dialysis membranes were reused, the changes in WBC were markedly attenuated (Table 2).
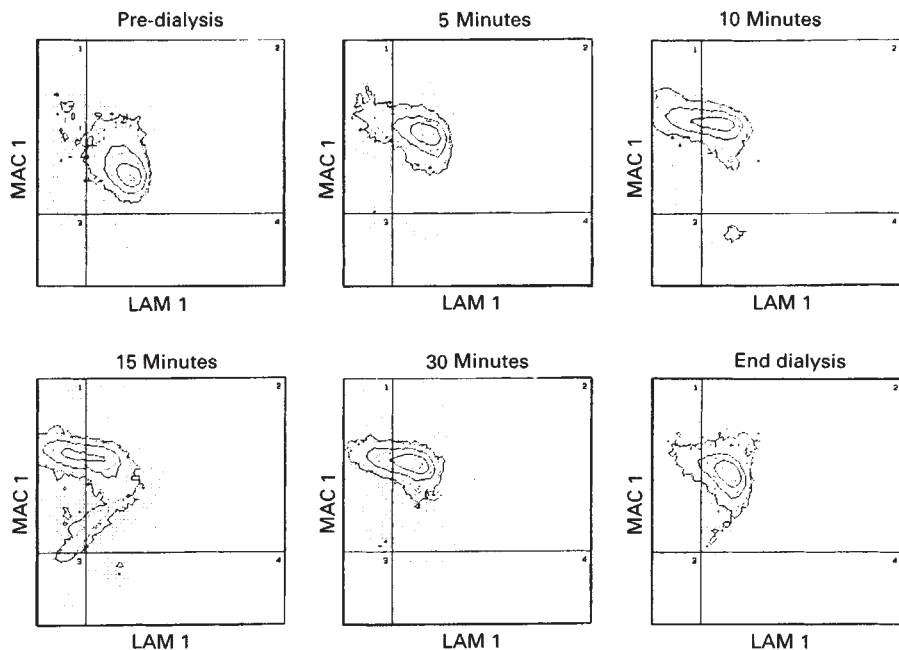
Fig. 3. *Two color immunofluorescence of granulocyte LAM-1 and MAC-1 expression during a representative first-use cellulosic hemodialysis.* Whole blood was obtained at selected time points over the course of first-use cellulosic hemodialysis. Simultaneous two color immunofluorescence was used to analyze log mean fluorescence of granulocyte LAM-1 and MAC-1, respectively. Analysis reveals a relatively uniform population pre-dialysis. MAC-1 cell surface expression is uniform at all time points. The corresponding decrease in LAM-1 expression is more heterogeneous over the first 30 minutes of dialysis.
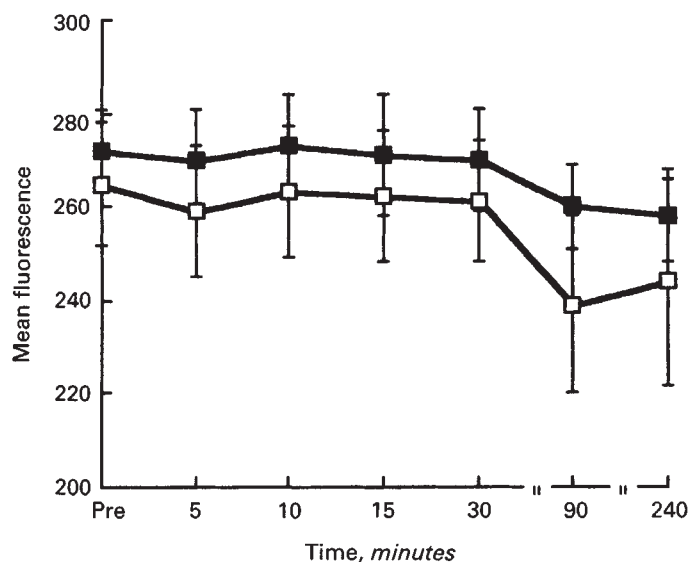
Fig. 4. *Lymphocyte LAM-1 expression during dialysis with cellulosic membranes.* Whole blood was obtained at selected time points with either first-use [■] or greater than fifth reuse cellulosic membranes [□]. A lymphocyte population was gated using flow cytometric criteria of forward angle light scatter and 90° side scatter. Lymphocyte LAM-1 cell surface expression was expressed as log mean fluorescence ± SEM (*N* = 5 experiments). *$P < 0.05$.

Fig. 5. *In vitro modulation of granulocyte LAM-1 in response to exogenous C5a and PMA.* Whole blood was incubated with either C5a (1×10^{-8} M) [●], PMA (100 nM) [■], or buffer [□], at 37°C. Results are expressed as log mean fluorescence ± SEM (*N* = 3 experiments).

Adherence of intradialytic granulocytes to HUVEC

Percent of adherent granulocytes obtained from normal controls, assessed in parallel with hemodialyzed subjects, increased with time of exposure to HUVEC, reaching a plateau of 14.1 ± 3.2% at 30 minutes of adhesion time to HUVEC and remained at the same level at 45 minutes. Consequently, to facilitate the comparison of adherence, we compared all adherence values 45 minutes after incubating these granulocytes with
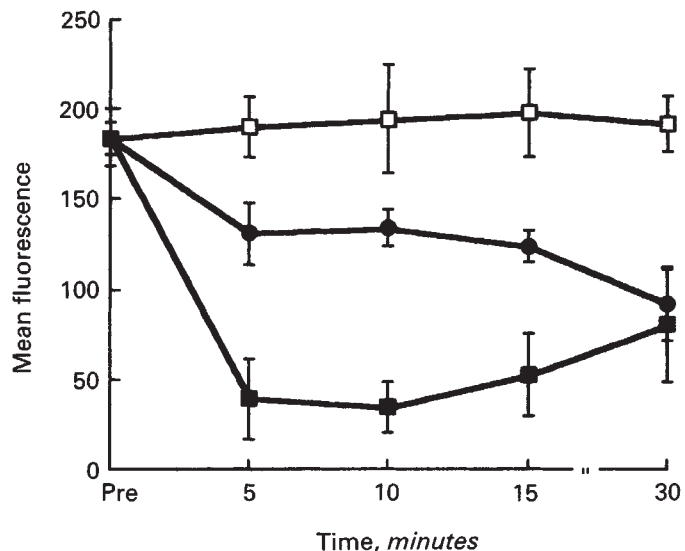
HUVEC. Day-to-day and inter-subject variability was less than 4%. Percent adherence of granulocytes harvested pre-dialysis was in the same range (12.3 ± 3.7% at 45 minutes) as controls (*P* = NS), confirming that baseline adherence of these cells from hemodialysis subjects was not significantly affected by the uremic state. Adhesion of granulocytes harvested as soon as five minutes after the initiation of first-use cellulosic membranes was found to be markedly decreased at all HUVEC incubation time points, compared to pre-dialysis levels (Fig. 7). Granulocytes harvested at 15 minutes after initiation of dialysis with new cellulosic membranes had percent adhesion to HUVEC as

Table 1. Intradialytic changes in CD11a and CD15 granulocyte receptors ($N = 3$)

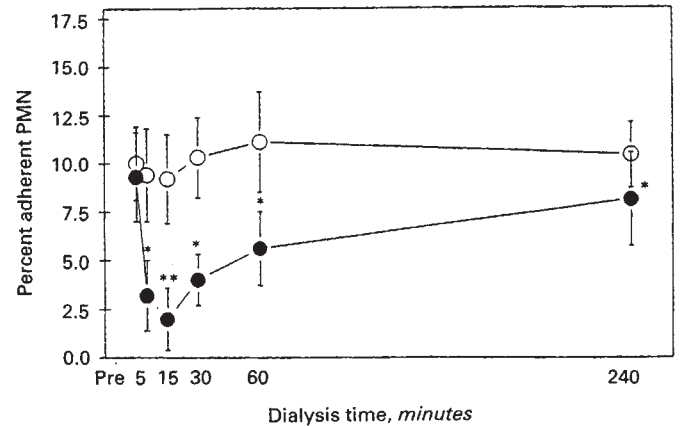| Time	Pre	5	10	15	30	90
				minutes		
CD11a (LFA-1)	183 ± 9.7	180 ± 11.2	200 ± 22.9	186 ± 31	179 ± 12.9	165 ± 4.9
CD15	545 ± 29.1	521 ± 26.3	464 ± 27.4	514 ± 41.3	559 ± 6.4	543 ± 7.7
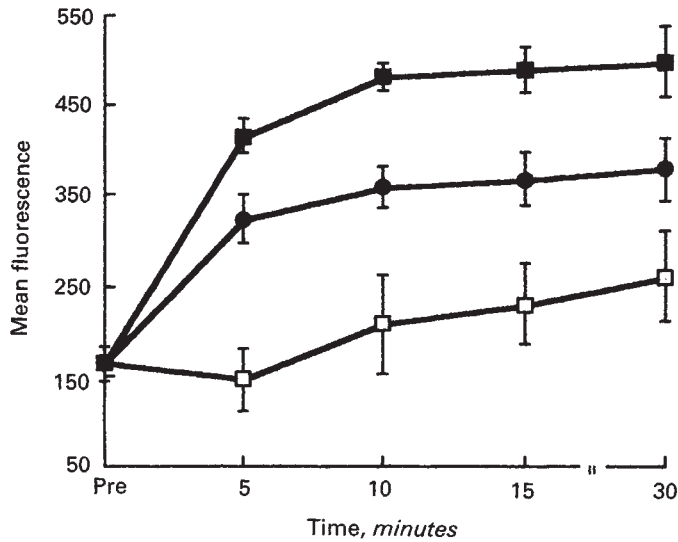
Fig. 6. *In vitro modulation of granulocyte MAC-1 after incubation with exogenous C5a and PMA.* Whole blood was incubated with either C5a (1×10^{-8} M) [●], PMA (100 nM) [■], or buffer [□], at 37°C. Granulocyte MAC-1 fluorescence was analyzed as log mean fluorescence ± SEM (N = 3 experiments).

Fig. 7. *Percent adherence of granulocytes to endothelial cell monolayers.* Granulocytes were harvested from blood drawn at different times after initiation of dialysis with complement activating membrane (●) and a non-complement activating membrane (□).

Table 2. White blood cell counts ($\times 10^3$) during new and reuse (>5) cuprophane dialysis

Time	New	Reuse
Pre	7.3 ± 0.6	7.6 ± 0.4
5 min	7.1 ± 0.9	7.6 ± 0.4
10 min	3.9 ± 1.2[a]	7.3 ± 0.5
15 min	2.7 ± 0.7[b]	7.2 ± 0.6
30 min	4.9 ± 0.6[b]	7.5 ± 0.4
90 min	7.6 ± 0.8	7.7 ± 0.3
240 min	6.9 ± 0.8	6.7 ± 0.4[a]

[a] $P < 0.05$
[b] $P < 0.001$

low as 2.42 ± 2.1% ($P < 0.001$ compared to pre-dialysis samples). This was comparable to adherence on uncoated plastic dishes (data not shown). Adhesion of granulocytes harvested at 60 minutes of dialysis with this complement activating membrane remained persistently low (<6%) and recovered slightly toward the end of the dialysis session (7.9 ± 2.8%). When granulocytes from the same patients were harvested during dialysis with non-complement activating membranes, no intradialytic changes in adherence occurred (Fig. 7).

Discussion

The mechanisms that cause granulocytopenia, and in particular its subsequent reversal during dialysis with new cellulosic membranes, have not been entirely elucidated. Previously, investigators have postulated that the development of granulocytopenia is related to C5a-induced granulocyte leukoaggregation and adhesion to endothelial cells. On the other hand, the reversal of granulocytopenia was attributed to down-regulation of the number and/or functional activity of granulocyte C5a receptors [14–16]. However, this hypothesis has recently been disputed by the demonstration that there is little if any down-regulation of granulocyte C5a receptors during first-use cellulosic dialysis [17, 18].

In this study, we have confirmed the time course of increased granulocyte cell surface MAC-1 expression and its association with the development of granulocytopenia, suggesting that increased cell surface MAC-1 expression may indeed be involved in increased granulocyte adhesiveness to endothelium, resulting in granulocytopenia. Of interest is that the increased expression of MAC-1 appears to precede the development of granulocytopenia. These results confirm those of Arnaout et al [8]. However, in the present study as well as in Arnaout et al's study, granulocyte cell surface MAC-1 receptors remain elevated throughout most of the course of hemodialysis, despite the reversal of granulocytopenia starting as early as 15 minutes after initiation of dialysis and the return of WBC count to pre-dialysis levels 60 minutes after the initiation of dialysis. Thus, while increased cell surface MAC-1 expression may be necessary in the development of dialysis granulocytopenia, it is not sufficient to maintain a granulocytopenic state.

Recently, a new family of cell adhesion molecules, known as selectins or LEC-CAMs, have been described. All three members of this family appear to have carbohydrate moieties as their ligands, and appear to be involved in the adhesion of circulating

blood cells to endothelium. The LEC-CAM GMP-140 (CD 62), found on activated platelet and endothelial cell plasma membranes has been demonstrated to bind granulocytes and monocytes [19–22]. A second member of this family, known as ELAM-1, is found solely on activated endothelial cells and is involved in leukocyte adhesion [23]. The most recent member of this family to have its structure elucidated is LAM-1 (also previously known as LEU-8, TQ-1, LEC-CAM-1 and its murine equivalent as MEL-14) [24]. LAM-1 is found on granulocytes, monocytes, lymphocytes and their precursor cells, and has an unknown ligand found on endothelial cells [25, 26]. Kishimoto et al have recently described shedding of LAM-1 from human granulocytes in vitro upon activation with chemotactic factors or phorbol esters [10]. Shedding of LAM-1 from activated granulocytes has been postulated to prevent extravasation into and damage of normal tissues by activated granulocytes [27]. In an animal model, Jutila et al demonstrated that "high MAC-1, low LAM-1" granulocytes are not able to transmigrate endothelium at a site of inflammation into a focus of infection [27].

In this study, we have demonstrated that coincident with the reversal of granulocytopenia, granulocyte LAM-1 surface expression reaches a nadir at 15 minutes and only returns towards pre-dialysis values toward the end of dialysis. The shedding of the LAM-1 receptor is likely a result of complement activation since it occurred with complement activating surfaces in vivo and were reproduced in vitro in response to C5a. These changes are specific for the granulocytes, as shown by the absence of similar changes in lymphocytes.

We were also able to demonstrate that granulocytes harvested between 5 and 60 minutes after initiation of first-use cellulosic membrane hemodialysis have dramatically decreased binding to endothelial monolayers. Granulocytes harvested intradialytically using PMMA, a non-complement activating dialysis membrane, did not change significantly compared to pre-dialysis values. The temporal relationship between the development of "high MAC-1, low LAM-1" granulocytes in circulation and decreased binding of granulocytes to endothelial cells, strongly support the contention that changes in granulocyte cell surface adhesion expression govern the initial development of granulocytopenia and its subsequent reversal and cause the complement mediated changes in white blood cells during cellulosic dialysis.

We postulate that during the early (first 5 min) stages of first-use cellulosic hemodialysis, complement activation leads to rapid and prominent up-regulation of MAC-1 on granulocyte cell surfaces. The increased density of MAC-1 on these granulocytes leads to increased adhesiveness both to other granulocytes and to endothelium resulting in granulocytopenia. In the absence of transendothelial migration (and possibly endothelial cell activation), activated granulocytes shed their LAM-1 receptors and detach from the endothelium since these "high MAC-1, low LAM-1" granulocytes have decreased endothelial cell adherence. This leads to the reversal of granulocytopenia. It is only by the end of dialysis that the expression of LAM-1 on granulocytes has returned to near basal levels and the binding of granulocytes to endothelial cell monolayers is partially restored. Note, however, that at the end of dialysis with the complement activating membrane, the level of MAC-1 is still substantially above baseline level.

It is possible to speculate that these observations may have

clinical implications. Infections remain one of the leading causes of morbidity and mortality in patients on chronic maintenance hemodialysis. Many of these infections are vascular access-related and occur during or around the time of the dialysis procedure [28, 29]. Thus, the "high MAC-1, low LAM-1" granulocyte state that occurs during first-use cellulosic hemodialysis could prevent granulocyte transmigration into sites of infection. Furthermore, granulocytes stimulated by cellulosic membranes will not respond to additional stimuli such as FMLP or C5a in elaborating reactive oxygen species during the dialysis procedure [5, 6]. Vanholder et al have demonstrated, in a prospective study, higher rates of infections and hospitalization in patients chronically dialyzed with complement activating membranes compared to polysulfone dialyzed patients [30]. However, whether changes in granulocyte function during first-use cellulosic hemodialysis contributes to morbidity from infections in hemodialysis patients will require further study in the future.

In summary, we have demonstrated that during first-use cellulosic hemodialysis, there is a rapid and pronounced up-regulation of the granulocyte integrin cell adhesion molecule MAC-1, but in addition, down-regulation of granulocyte LEC-CAM molecule LAM-1 occurs. Furthermore, the reciprocal changes in granulocyte cell adhesion molecules coincide with decreased adhesion to endothelial cell monolayers. Changes in granulocyte cell adhesion molecules may explain development of neutropenia and subsequent reversal in first-use cellulosic membrane hemodialysis.

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