Intradialytic Modulation of Granulocyte C5a Receptors

Jonathan Himmelfarb, Norma P. Gerard, and Raymond M. Hakim

J. Himmelfarb, Division of Nephrology, Maine Medical Center, Portland, ME
N.P. Gerard, Harvard Medical School, Brookline, MA
R.M. Hakim, Division of Nephrology, Vanderbilt University Medical Center, Nashville, TN

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ABSTRACT
Hemodialysis with new cellulosic membranes is associated with profound granulocytopenia, with a nadir 15 min after initiation, followed by a rebound leukocytosis seen 1 h after initiation and persisting up to the termination of dialysis. The rapid reversal of granulocytopenia during hemodialysis has previously been ascribed to down-regulation of granulocyte C5a receptors. In this report, a method of characterizing C5a receptors by using a novel probe consisting of C5a attached to biotin via a six-carbon spacer chain is described. Cellulose acetate electrophoresis and cation exchange HPLC demonstrated a biotin-to-C5a ratio of 1:1. Analysis of granulocyte cell surface C5a receptors were performed with the probe with a fluorescein-avidin conjugate and by using fluorescence flow cytometry. The maximum decrease in C5a receptors was measured at the 15-min sampling time, when the number of C5a receptor decreased from 189,240 ± 24,500 predialysis to 160,740 ± 19,380 receptors (P was not significant) at the nadir of granulocytopenia. However, during recovery from neutropenia, granulocyte cell surface C5a receptors increased to 172,140 ± 19,380 at 30 min and 193,800 ± 24,510 at the end of dialysis. Concentrations of C3a and C5a peaked at 15 min and declined rapidly thereafter, but both remained significantly above baseline at all times. These studies suggest that down-regulation of C5a receptors, which is seen maximally at 15 min after initiation of dialysis, does not sufficiently account for the reversal of granulocytopenia during hemodialysis.

Key Words: C5a receptors, granulocytopenia, hemodialysis, biocompatibility, cytometry

Cellulosic membranes, the most widely used dialysis membranes in clinical practice, are made up of a repeating polysaccharide structure and activate the alternative pathway of complement. In contact with blood, C5a, a potent anaphylatoxin and neutrophil chemotactic agent, is generated and results in granulocyte activation (1). This is associated with pulmonary leukostasis and profound leukopenia with a nadir 15 min after the initiation of hemodialysis (2,3). However, this leukopenia is transient, and, within 1 h after the initiation of dialysis, granulocyte counts have returned to predialysis levels. By the termination of dialysis, usually 3 to 4 h after inititation, a rebound leukocytosis, to levels 120 to 140% of predialysis values, is commonly observed.

The mechanism for the reversal of the granulocytopenia during dialysis remains unclear. Skubitz and Craddock, by using granulocyte aggregation assays, proposed that this was due to selective down-regulation of granulocyte responsiveness to C5a (4). It was further postulated that this may be due to internalization of C5a receptors on these granulocytes (5). Gardinali et al., also employing granulocyte aggre-gometry, recently offered a different hypothesis (6). They postulated that the transience of granulocytopenia during dialysis is caused by the decline of plasma C5a levels below a critical threshold needed for granulocyte-endothelial cell adhesion.

To investigate this issue further, we employed a probe for C5a receptors consisting of recombinant human C5a bound to biotin via a six-carbon spacer chain. By using a fluorescein-avidin conjugate, we were able to directly characterize modulation of granulocyte C5a receptors during hemodialysis. The results indicate that, after a transient decrease at 15 min, the number of C5a receptors increases during the period of rebound leukocytosis and suggest that down-regulation of C5a receptors does not sufficiently account for the transience of granulocytopenia during dialysis.

MATERIALS AND METHODS
Patient Characteristics

Six patients on chronic maintenance hemodialysis were selected for study. Informed consent was obtained from the subjects, and the protocol was ap-
proved by the Institutional Review Board. The mean age of the patients was 57.5 yr (range, 35 to 73), with a mean time on dialysis of 55 months (range, 15 to 156 months). Three were male and three were female. None of these patients had clinical evidence of infection at the time of the study.

Preparation of Recombinant Human C5a

C5a was synthesized in Escherichia coli with a synthetic C5a gene constructed from the amino acid sequence of the peptide isolated from complement-activated human serum. The peptide was renatured and purified as described previously (7). Material obtained from E. coli cultures was solubilized in 100 mM potassium phosphate (pH 7.4) containing 100 mM 2-mercaptoethanol and 6 M guanidine hydrochloride. This was dialyzed against 20 mM potassium phosphate (pH 7.5) followed by 20 mM potassium phosphate (pH 6.6). The C5a was then purified by ion exchange chromatography on SP-Sephadex C-25, followed by gel filtration on Bio-Gel P-6 (Bio-Rad Laboratories, Richmond, CA) as described previously (7). The material obtained by these procedures was >90% pure and was further purified by HPLC-ion exchange chromatography on Mono-S (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 300 mM NaOAc (pH 6.3). The column was eluted at 1 mL/min with a linear gradient to 400 mM NaOAc (pH 6.3) over 40 min. The effluent was monitored by absorbance at 280 nm. Recombinant C5a elutes at 26 to 27 min under these conditions and is well separated from contaminating peptides. Homogeneity of this material was evaluated by amino acid composition and amino-terminal sequence analysis. Biological activity was assessed by the spasmodogenic activity of guinea pig lung parenchymal strips compared with natural human C5a and was fully functional (7).

Preparation of Biotinylated C5a

Recombinant human C5a was reacted with caproic acid-biotin-N-hydroxysuccinimide (CAB-NHS; ICN Biochemicals, Costa Mesa, CA) under conditions that optimized biotinylation and maintained biological activity of the molecule. Incubation of a twofold molar excess of CAB-NHS with C5a at pH 8.0 for 10 min at 25°C results in the formation of several products separated by cation-exchange HPLC (Figure 1). Peaks 1, 2, and 3 appear after reaction of CAB-NHS with C5a; peak 4 eluted in the position of the derivatized molecule. Material recovered in peaks 2, 3, and 4 was further analyzed by cellulose acetate electrophoresis and staining for protein with amido black and for biotin (data not shown). Peak 4 comigrated with unmodified C5a and did not stain for biotin. Material in peaks 2 and 3 both reacted with streptavidin-biotinhorseradish peroxidase followed by 3,3′ diaminobenzidine, indicating the presence of biotin. Cellulose acetate electrophoresis also indicated a single protein staining band migrating with a single net charge less than unreacted C5a, consistent with a 1:1 biotin to C5a ratio.

Bioactivity of Biotinylated C5a

The biological activity of the biotinylated recombinant human C5a was assessed by the release of peroxidase from normal human polymorphonuclear leukocytes (PMN), as described by Gerard et al. (8). Both derivatives (peaks 2 and 3) were tested for biological activity and were equipotent to derivatized C5a for releasing peroxidase from normal human PMN (Figure 2). Only material eluting in peak 3 was used in subsequent studies.

Cell Preparations

Whole blood was obtained from dialysis patients and was immediately placed in heparinized tubes, cooled to 4°C, and centrifuged at 1,000 × g for 10 min at 4°C in a refrigerated centrifuge. The platelet-rich plasma was discarded. Tris-buffered balanced salt solution (TBSS; 1 M) was then added to make up the original volume. This was then layered over 1% dextran and allowed to sediment for 60 min at 4°C. The nonagglutinated aspirate was collected in TBSS and then washed twice with TBSS. Hypotonic lysis was performed with 0.2 M TBSS at 1°C for 45 s. The
neutrophils incubated with the fluorescein-avidin conjugate alone. Control fluorescence values were subtracted from values obtained with both biotinylated C5a and fluorescein-avidin conjugates. Standardization of fluorescein fluorescence was achieved by using standardized beads (Flow Cytometry Standards Corporation, Research Triangle Park, NC). One hundred fluorescent units was equal to approximately 57,000 molecules of fluorescein or receptors/cell. In each experiment, at least 4,000 cells were counted. All analyses were done as triplicate samples with a coefficient of variation of less than 12%.

Hemodialysis Studies

A bicarbonate dialysate (Na 140 mEq/L; K 2 mEq/L; Ca, 3 mEq/L; HCO3, 30 mEq/L; and acetate, 3 mEq/L) was used in all cases. Blood samples were drawn predialysis and then from the efferent line of the dialyzer at 15, 30, 60, and 240 min after the initiation of dialysis. Anticoagulation was maintained by using heparin at an initial rate of 50 U/kg, with additional doses given if activated clotting times were less than 1.5 times predialysis values.

All patients were studied by using first-use cellulose hollow fiber dialyzers (PCS series; National Medical Center, Rockleigh, NJ). Four patients used dialyzers with a surface area of 0.8 m² and two patients used dialyzers with a surface area of 1.1 m². Before the study, all patients were on a hemodialyzer reuse program that used 1% formaldehyde. Blood flow rates were 250 ml/min, and dialysate flow rates were 500 mL/min during this study.

RIA Analysis of C3a-desArg and C5a-desArg Levels

Blood for measurement of complement products was drawn in tubes containing EDTA, centrifuged, and separated immediately; plasma was stored in duplicate polypropylene tubes at −70°C until processing. Samples were thawed only once for processing. C3a-desArg and C5a-desArg RIA were performed according to previously published methods by using commercial kits (Amersham Corp., Arlington Heights, IL) [9].

Statistics

Results are expressed as mean ± SD. Statistical analysis was performed by using analysis of variance, with Duncan's multiple range test performed on all variables (WBC, C3a-desArg, C5a-desArg, and C5a receptor number). The actual P value was calculated by using a two-tailed paired Student's t test after the initial analysis of variance test.
RESULTS

Dose Response of Biotinylated C5a Binding to Neutrophil C5a Receptors

Optimal concentrations of biotinylated C5a for binding to C5a receptors was determined via dose-response curves in normal neutrophils (Figure 3). Saturation of C5a receptor sites was achieved at doses of biotinylated C5a greater than 20 to 30 nM, corresponding to approximately 190,000 receptors per cell. In all further experiments, 60 nM biotinylated C5a was used to saturate binding to C5a receptors.

Specificity of C5a Receptor Binding with a Biotinylated C5a Probe

The specificity of binding of biotinylated C5a to C5a receptors was demonstrated by inhibition experiments with unlabeled C5a (Figure 4). The binding of 20 nM biotinylated C5a to neutrophils was inhibited in a dose-dependent manner by excess unlabeled C5a. Using a 50-fold excess of unlabeled C5a resulted in inhibition of more than 75% binding of biotinylated C5a to neutrophils. The inability to achieve 100% inhibition of biotinylated C5a bindings suggests that there may be some nonspecific binding of the biotin-C5a preparation or fluorescein-avidin to neutrophils.

Generation of Anaphylatoxins

Plasma levels of C5a-desArg increased from a baseline level of 3.1 ± 0.3 to 47.1 ± 11.9 ng/mL 15 min after the initiation of dialysis (P < 0.01) (Figure 5). For the remainder of dialysis, C5a-desArg levels remained significantly elevated compared with predialysis levels. Thus, at 30 min, it averaged 28.7 ± 8.6 ng/mL (P < 0.01); at 60 min, C5a-desArg levels were 16.7 ± 2.1 ng/mL (P < 0.01); and at 240 min (the end of dialysis), C5a-desArg levels were 12.1 ± 1.2 (P < 0.05).

Change in WBC

While on dialysis, patients on cellulosic membrane developed a profound leukopenia 15 min after the initiation of hemodialysis with mean WBC counts of 1.9 ± 0.3 x 10^9/mm^3 compared with predialysis WBC of 7.0 ± 0.1 x 10^9/mm^3 (P < 0.01). By 30 min after the initiation of hemodialysis, WBC had increased to 4.4 ± 0.3 x 10^9/mm^3, though this was still significantly less than predialysis WBC (P < 0.01). Sixty minutes after the initiation of dialysis, mean WBC
was 7.3 ± 0.4 × 10^9/mm³, which was not statistically different compared with predialysis (P was not significant [NS]). At the termination of dialysis, mean WBC was 9.5 ± 0.9 × 10^9/mm³, significantly increased compared with predialysis WBC (P < 0.05).

Quantitation of C5a Binding Sites on Granulocytes During Hemodialysis

Granulocytes harvested from patients before dialysis had a mean of 332 ± 42 fluorescence channels, representing C5a receptors (Table 1). With fluorescently labeled beads as a standard, this corresponded to approximately 189,240 ± 24,500 C5a receptors per granulocyte. By 15 min after the initiation of hemodialysis, C5a receptor sites had declined to 282 ± 34 fluorescence channels, corresponding to 160,740 ± 19,380 C5a receptors. This was not statistically significantly different from baseline. Thirty minutes after the initiation of dialysis, C5a receptors were approximately 172,140 ± 19,380 and remained constant at 60 min after dialysis at 174,420 ± 20,520 (P = NS). By the termination of dialysis at 240 min, C5a receptor levels had returned to baseline levels of 193,800 ± 24,510 receptor sites (P = NS).

DISCUSSION

This study represents the first use of a novel probe consisting of C5a attached to biotin via a six-carbon spacer chain. This probe proved to have a 1:1 molar ratio of biotin to C5a and migrated on cellulose acetate electrophoresis as a single protein staining band, thus demonstrating a one-to-one ratio of biotin to C5a. Studies of bioactivity demonstrated that this probe has equivalent bioactivity with natural C5a. We also demonstrated that binding to C5a receptors was saturable and inhibitable by unlabeled C5a, thus confirming the specificity of binding to C5a receptors.

The C5a receptor on normal human polymorphonuclear leukocytes has previously been characterized (10–13). Studies by several groups have described variations in the number of C5a receptors on normal granulocytes between 50,000 and 300,000 C5a receptors. The most recent Scatchard analysis has demonstrated a single receptor population with a Kd of 4.8 ± 0.1 × 10⁻¹⁰ M on normal human granulocytes (9). Whether the reported wide variation in normal numbers of C5a receptors represents heterogeneity of C5a receptors on leukocytes or represents variations related to the methods used to isolate neutrophils is presently not clear. In this study, hemodialysis patients had an average of 189,240 ± 24,500 receptors per cell predialysis. This corresponded to levels seen on granulocytes from normal human volunteers.

Previous studies by Skubitz, Craddock, and co-workers have demonstrated that granulocytes obtained after reversal of granulocytopenia in hemodialysis patients are refractory to C5a-desArg stimulation in granulocyte aggregation assays and proposed that this was due to down-regulation (and internalization) of granulocyte C5a receptors (3–5). Further support for this hypothesis came from in vitro studies by O’Flaherty et al. (16) and Damereau et al. (17), who have demonstrated that, after aggregation with C5a, granulocytes could not be reaggregated with the same agent.

By using the biotinylated C5a probe, we have been able to analyze the modulation of C5a receptor binding sites on circulating granulocytes during hemodialysis. The results of our study suggest that down-regulation of the C5a receptor may not be the explanation for the transience of granulocytopenia because the maximum decrease in C5a receptor number, which is only 15% and which is not statistically different from baseline, occurs at the 15-min sampling time rather than at the time that granulocytopenia is resolved. Thus, at 60 min after initiation of dialysis, when the WBC count returns to normal, the C5a receptor cell surface density is only decreased by 9% less than the predialysis value (P = NS) and, importantly, is increasing to baseline values. Thus, the reversal of granulocytopenia does not coincide with maximal C5a receptor down-regulation and occurs at a time when these receptors are increasing; therefore, it is doubtful that C5a receptor down-reg-
ulation accounts for the reversal of granulocytopenia.

It is also important to recall that the samples analyzed were drawn from the efferent line of the dialyzer, when the neutrophils have been exposed to high levels of C5a in the dialyzer. Thus, the decrease in the C5a receptor number represents the maximal decrease of these receptors and, although it may represent some internalization or shedding of C5a receptor-C5a complexes or nonreversible occupancy of these receptors with C5a, it remains modest and unlikely to account for the subsequent reversal of granulocytopenia.

Lewis et al., by using a fluoresceinated C5a probe, found no change in the percentage of circulating C5a-receptor-positive cells during in vitro dialysis but found a decrease in the percentage of C5a binding granulocytes during in vitro dialysis with new cellulosic membranes. They also noted wide variation in individual responses (15). There are a number of differences in study design and techniques that may account for the somewhat differing results; perhaps the most important is that they did not study the 15-min time point after the initiation of dialysis, the point at which we found maximal C5a receptor density decrease. By 30-min postinitiation of dialysis (the first time point studied by them), C5a receptors were increasing in our study.

Secondly, Lewis et al. did not indicate whether the patients in their study were part of a dialyzer reuse program. If their patients were chronically and repetitively exposed to new cellulosic dialysis membranes, predialysis C5a receptors may already have been down-regulated. Indeed, Lewis et al., in an earlier study, demonstrated down-regulation of C5a receptors in predialysis samples compared with normal controls (14). Finally, their C5a was purified from plasma by chromatographic techniques and may have contained a large fraction of C5a-desArg with different receptor binding affinities. In our study, we used a purified cloned gene product. In addition, we measured the number of receptors/cell, whereas Lewis et al. reported on the percentage of fluorescein-positive WBC. Nevertheless, their conclusion that there is no correlation between the in vitro rebound leukocytosis and changes in C5a receptors agrees with the results of our studies.

Gardinall et al., by using RIA measurement of C5a levels concurrent with granulocyte aggregometry, recently demonstrated a persistent ability of granulocytes to aggregate in the presence of C5a or zymosan (6). They postulated that the recovery from C5a-induced neutropenia is due to a decrease in the level of complement activation after the first 30 min of dialysis below a threshold required for continued adhesion to endothelium. Further support for this hypothesis comes from three published reports demonstrating that neutropenia can reoccur if complement-activating hemodialyzer or leukopheresis filters are changed during the procedure (18–20). In our studies, although C5a-desArg levels remained significantly higher than baseline at all intradialytic time points, they decreased rapidly after 15 min and, by 1 h, were one third of the peak level. Thus, it is possible that the decrease of C5a below a threshold level may account for the return of neutrophils from the pulmonary to the systemic circulation.

Finally, it is possible that adhesion molecules on neutrophils and/or endothelial cells are also modulated during dialysis and account for the cyclical changes in systemic neutrophil count. Recent in vitro and in vivo studies have demonstrated that activated granulocytes adhere to endothelial cells via cooperative interactions between leukocyte adhesion molecules and endothelial cell adhesion molecules (21–29). Modulation of these adhesion molecules either on granulocytes or on endothelium could alternately account for the reversal of granulocytopenia during hemodialysis. Recently, Lo et al. demonstrated that the adhesion of C5a-stimulated neutrophils to endothelial cells is transient over time, with a dramatic reduction in the adhesion of neutrophils to endothelial cells by 15 min even in the continued presence of C5a (26). Kishimoto et al. also demonstrated that gp100 mem-14 is a granulocyte adhesion protein shed from neutrophil cell surfaces within 4 min after activation with C5a, which may also contribute to the transience of in vitro granulocyte/endothelial cell interactions (29).

In summary, we measured modulation of C5a receptors on granulocytes during hemodialysis with cellulosic membranes and have shown that significantly intradialytic down-regulation of C5a receptor numbers does not occur on granulocytes and is not likely to account for the reversal of granulocytopenia during hemodialysis. Further studies with the newly described cloned C5a receptor (30) may help to elucidate the specific mechanisms that reverse the granulocyte/endothelial cell interactions that occur during hemodialysis.

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REFERENCES


