Reversal of Hemodialysis Granulocytopenia and Pulmonary Leukostasis

A CLINICAL MANIFESTATION OF SELECTIVE DOWN-REGULATION OF GRANULOCYTE RESPONSES TO C5adesarg

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ABSTRACT The transient granulocytopenia of hemodialysis results indirectly from plasma complement activation by dialyzer cellophane membranes. The C5adesarg so produced can induce reversible granulocyte aggregation in vitro and in vivo, and we hypothesized that the pulmonary leukostasis responsible for the granulocytopenia results from embolization of aggregates formed under the influence of C5adesarg produced in the dialyzer. These studies were designed to measure C5adesarg generation during dialysis by granulocyte aggregometry and to determine the reason for the transience of the leukostasis. C5adesarg generation was equally evident throughout dialysis, persisting well after granulocytopenia had reversed, and dialyzer-induced complement activation was insufficient to produce significant depletion of plasma complement titers. That granulocyte deactivation might be responsible for the transience was suggested by the absence of the usual granulocytopenia in a patient with uniquely high levels of C5adesarg in his predialysis plasma. Granulocytes drawn from seven stable uremic patients after granulocytopenia had reversed exhibited a dose-related, selective and irreversible refractoriness to stimulation with C5adesarg, but their responses to n-formyl-Met-Leu-Phe remained normal. Identical deactivation was produced in normal cells by short- or long-term exposure of C5adesarg in vitro. These studies suggest that C5adesarg is indeed generated by the dialyzer throughout hemodialysis and that the transience of the leukostasis and granulocytopenia is due to selective down-regulation of cellular responses to C5adesarg—a phenomenon that hitherto has been described only in vitro and that may be important in limiting the deleterious effects of adherent granulocytes on the endothelium in patients with intravascular complement activation.

INTRODUCTION

Severe granulocytopenia, the result of reversible pulmonary leukostasis, occurs in all patients during induction of hemodialysis (HD)1 with cellophane membranes (1, 2). We have reported that this leukostasis most likely results from embolization of granulocyte (Gr) aggregates that have formed in the peripheral circulation under the influence of the complement (C) fragment C5adesarg (3, 4); infusion of autologous plasma in which the alternative pathway of the C cascade has been activated by dialyzer cellophane produces in animals the same augmentation of Gr adherence (5), Gr aggregation (6), and pulmonary leukostasis (3) seen in patients during HD (1, 7). Of equal importance were the findings that these adherent cells can disrupt endothelial cell integrity in vitro (8) and cause lung dysfunction, including mismatching of ventilation:perfusion ratios and pulmonary edema in animals and dialyzed patients in vivo (9). Studies of C-mediated Gr aggregation in vitro revealed that C5adesarg is the major component in activated plasma C capable of producing such effects (4)—which

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1 Abbreviations used in this paper: C, complement; Gr, granulocyte; HBSS buffer, Heps-buffered balanced salt solution containing 0.5% (w/vol) human serum albumin; HD, hemodialysis; nFMLP, n-formyl-Met-Leu-Phe.
is entirely consistent with our earlier observation that the leukostatic activity of cellophane-incubated plasma resides primarily in its 16,000-dalton fractions (3) and with McGregor's finding that the factor responsible for increased Gr adherence in patients during HD is a heat-stable (56°C, 30 min) plasma component generated in the dialyzer (7). More recently, we have reported the close correlation between the onset of the adult respiratory distress syndrome ("shock lung") and the appearance of C5a during HD in patients after serious trauma, sepsis, or acute blood loss (10). Although critical to a complete understanding of HD granulocytopenia and the reason for its reversal, the generation of C5a during HD has not yet been directly investigated. If C5a generation could be demonstrated only during the first hour of HD, the reason for the transience of pulmonary leukostasis would be readily evident. If, on the other hand, C5a production were detectable throughout HD after granulocytopenia has reversed, an additional explanation for the transience, such as chemotactic factor-induced desensitization (11, 12), would be necessary if the primary hypothesis concerning C-mediated pulmonary leukostasis were to remain tenable.

Two inherently different modes of chemotactic factor deactivation have been described. Exposure of rabbit Gr to C5a in vitro produces nonspecific inhibition of their responsiveness to all chemotactic stimuli, an effect attributed to depletion of a critical plasma membrane prostesterase (11, 12). In contrast, human Gr exposed to C5a in vitro lose only their responsiveness to C5a and react normally to bacterial chemotactic factor or the synthetic formylated peptides (13–16); this deactivation and its selectivity have been attributed to internalization of chemotactic factor receptors, since it is known that human Gr carry specific and saturable receptors for C5a (17) and formylated peptides (18), which become selectively internalized after binding to the appropriate stimulus (19, 20). From these observations the concept of down-regulation of chemotactic responsiveness has been developed. The present studies were designed primarily to determine the reason for the transience of HD granulocytopenia. When it became clear that the generation of C5a persists unabated throughout each HD, we explored the alternative possibility that the transience might instead result from down-regulation of Gr responsiveness to the C5a produced in the dialyzer.

METHODS

Patient population. Adults with chronic renal failure of nonimmunologic etiology undergoing routine HD with single-use, parallel-plate Cuprophane dialyzers (Lundia Optima, A. B. Gambro, Lund, Sweden) were studied after providing informed consent as ratified by the Human Subjects in Research Committee. Blood samples were drawn from the arterer (patient-to-dialyzer) and efferent (dialyzer-to-patient) lines. Pre-HD samples were drawn from the arterer line just before institution of dialyzer flow. Automated leukocyte counts (Coulter model S, Coulter Electronics, Inc., Hialeah, Fla.) and 200 cell-differential counts were performed on EDTA-anticoagulated blood drawn from the arterer line. Hemolytic complement titers (total, C1, and C3) were measured by minor modifications of established techniques that utilize hemolysis of antibody-coated sheep erythrocytes as their endpoint (3). C5-deficient plasma was acquired from an adult male with homozygous deficiency of C5 who is free of autoimmune disease but who presented with recurrent meningococcal sepsis complicated by the Waterhouse-Friderichsen Syndrome.

Granulocyte preparation. Suspensions containing 95–99% pure Gr were prepared by a three-stage (dextran sedimentation, hypotonic lysis, and Ficoll-Hypaque density gradient centrifugation) technique (4), and the cells were suspended in a Hepes (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.)-buffered balanced salt solution (21) containing 0.5% (wt/vol) human serum albumin (HBBSS buffer) (22).

Granulocyte aggregometry. Aggregation responses were quantitated by digital integration (22) of light transmission increments of Gr being stirred in a dual-channel aggregometer-recorder system (models 300 BD and PF 10-HD, Payton Associates, Buffalo, N. Y.) (4). Maximum and minimum light transmission limits (full-scale deflection of 10 mV) were calibrated with suspensions containing 5 × 10^4 and 1 × 10^5 Gr/1 ml, respectively. The aggregating activity generated during HD was assayed in plasma anticoagulated with 10 mM sodium EDTA (to prevent additional C activation and heparin (2 U/ml)); 0.05 ml of plasma was added to 0.45 ml of Gr suspension containing 1.11 × 10^6 cells/ml that was being stirred in the calibrated aggregometer. Integration was initiated after a 3-s delay to avoid the inevitable dilution artefact (4), and the responses are expressed in units of millivolt seconds for intervals of either 2 or 3 min. Plasma was fractionated with Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, N. J.) in a calibrated ascending column previously described (4); additional concentration was achieved by filtration (P-10 filter, Amicon Corp., Bedford, Mass.). The activity of the column fractions was tested for heat stability (56°C, 30 min) or incubated (37°C, 30 min) with anti-C3 and anti-C3 antisera (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.), and partially purified C5a was prepared from zymosan-activated plasma C by similar filtration techniques using Sephadex G-75 (4). In those experiments in which Gr were serially exposed to C5a and n-formyl-Met-Leu-Phe (nFMLP) (Peninsula Laboratories, Inc., San Carlos, Calif.), 0.05 ml of the secondary stimulus was added 3 min after the cells (0.4 ml of a suspension containing 1.25 × 10^7 Gr/ml) had been exposed to 0.05 ml of the alternative chemotaxin. Equipotent concentrations of zymosan-activated plasma C (undiluted) and nFMLP (3.13 × 10^-7 M) were chosen. In those studies in which Gr underwent a more prolonged exposure to activated C, normal cells were incubated (37°C, 15 min) in serial dilutions of zymosan-activated or fresh plasma; they were then washed twice in buffer and their responses were tested as usual.

Granulocyte chemotaxis. Chemotaxis was assayed in Boyden chambers using filters (mixed cellulose acetate and nitrate, Millipore Corp., Bedford, Mass.) of 3.0 μm mean pore size and thickness of 158 ± 16 μm by the leading front technique.

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RESULTS

In none of the 16 patients studied during HD could we detect significant depletion of plasma hemolytic C titers. Total hemolytic C fell by a maximum of only 13±5% (SEM) during 6 h of HD, and C3 levels rose marginally by a maximum of only 19±11%—findings entirely consistent with the relative weakness of dialyzer cellophane as an activator of the C cascade (3). In contrast, and most likely as the result of an acute phase reaction to selective activation of the alternative pathway (3), Cl levels rose dramatically by 48±15% within 20 min of the induction of HD. To determine whether C5a desarg is generated during HD, we measured the aggregating activity in simultaneously drawn afferent plasmas from five consecutive patients during HD by quantitative aggregometry (Fig. 1). In contrast to plasma samples drawn at the start of HD and from the afferent line 15 min later, which were free of appreciable activity, efferent plasma drawn after 15 min consistently contained high levels of activity (Fig. 1). Sephadex filtration of this plasma revealed that this activity resided primarily in a single peak of ~16,000 daltons, which was also chemotactic for Gr in Boyden chambers (Fig. 2). When these fractions were pooled, concentrated by filtration, and incubated with anti-C5 antiserum, their aggregating activity was reduced 84% from 145±26 mV s/2 min to 24±19 mV s/2 min. Incubation with anti-C3 antiserum or heating at 56°C for 30 min produced no inhibitory effect. Afferent plasma fractions were free of detectable aggregating or chemotactic activity (Fig. 2).

High levels of aggregating activity were consistently detected in efferent plasmas throughout HD, whereas afferent plasma remained essentially unreactive (Fig. 1). It thus became clear that C5a desarg generation persists well after pulmonary leukostasis has reversed. The explanation for this paradox was first suggested by serendipitous findings in a uremic patient with disseminated cytomegalovirus infection whose predialysis plasma contained extraordinarily large quantities of...
aggregating activity (480±50 mV s/3 min), most likely generated by the immune complexes associated with this disease (25, 26). In contrast to all other patients we have studied, he failed to develop HD granulocytopenia; during the first hour his leukocyte and neutrophil counts actually rose from their predialysis values of 4,000 cells/μl and 2,000 cells/μl to 10,200 cells/μl and 4,900 cells/μl, respectively. These findings raised the possibility that circulating Gr can become desensitized to C5a <sub>desarg</sub> in vitro and that the transience of HD granulocytopenia might result from chemotactic factor deactivation. To test this hypothesis, we studied the effect of HD on Gr aggregation responses in stable patients with chronic uremia (Table 1). Gr prepared from these patients just before HD responded to the C5a <sub>desarg</sub> in efferent plasma (98±16 mV s/3 min), but when they were retested after 120 min of HD (during which time they had undergone reversible pulmonary leukostasis), they were almost completely unresponsive to this stimulus. In contrast, they retained their aggregation responses to equipotent quantities of nFMLP and the higher concentrations of C5a <sub>desarg</sub> present in zymosan-activated plasma (not shown).

Gr chemotaxis was also selectively impaired after reversal of pulmonary leukostasis (Fig. 3). Before HD, Gr from these patients migrated toward the C5a <sub>desarg</sub> in zymosan-activated plasma and nFMLP at near normal rates, but after 120 min of HD chemotaxis toward C5a <sub>desarg</sub> was reduced by ~67%, whereas their response to nFMLP was essentially unaltered. The random movement of predialysis Gr (34±24 μm/h) did not differ from normal (39±20 μm/h), but after 2 h of HD, random movement was moderately reduced to 14±6 μm/h. When serial dilutions of zymosan-activated plasma were used as chemotactic stimuli, the dose-response curve exhibited by dialyzed Gr was shifted considerably to the right (Fig. 3), which suggested that their refractoriness is relative as well as specific.

Identical unresponsiveness to C5a <sub>desarg</sub> was produced by short-term exposure of normal Gr to activated plasma C in vitro (Fig. 4). After they had been primarily exposed to zymosan-activated normal plasma for 3 min, second-
TABLE I
Impairment of Granulocyte Aggregation Responses Observed after Reversal of Hemodialysis-induced Granulocytopenia

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Aggregation responses</th>
<th>Uremic granulocytes</th>
<th>Normal granulocytes</th>
<th>Predialysis</th>
<th>Dialyzed 120 min</th>
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<td>mV ±3 min</td>
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<td>19±8</td>
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<tr>
<td>Efferent plasma</td>
<td>230±31</td>
<td>98±16*</td>
<td>16±8†</td>
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Aggregation responses were measured for 3-min intervals, and the results are expressed as the arithmetic mean±1 SEM of triplicate determinations using cells from five normal volunteers and five patients on chronic HD. The aggregating stimuli included afferent and efferent plasmas drawn from a patient after 15 min of HD (see Figs. 1 and 2).

* Significantly less than the value of normal Gr.
† Less than the value observed with predialysis cells.

Zymosan-activated normal plasma (C) resulted in an aggregation response 85% less than that observed when cells that had been primarily exposed to buffer or fresh plasma (not shown) were similarly stimulated. In contrast, the aggregation response produced by a usually equipotent concentration of nFMLP (3.13 x 10^-7 M) was essentially unaltered by prior exposure to zymosan-activated normal plasma, and primary exposure to zymosan-activated C5-deficient plasma had no effect on the aggregation responses produced by either stimulus.

When zymosan-activated normal plasma was fractionated with Sephadex G-75 (Fig. 5), its inhibitory activity resided primarily in a large, well-defined peak of ~16,000 daltons that also exhibited several other characteristics of C5adesargin; it coincided closely with the single peak of aggregating and chemotactic (4) activity in this plasma, and its inhibitory effects were reduced 82±5% by incubation with anti-C5 antiserum, unaffected by incubation with anti-C3 antiserum, and heat stable (56°C, 30 min). No inhibitory activity was detected in the corresponding fractions of either zymosan-activated C5-deficient plasma or fresh normal plasma. An additional peak of weaker, nonspecific inhibitory activity eluting at void volume was present in zymosan-activated normal plasma, but it was also detected in fresh plasma or zymosan-activated C5-deficient plasma, and its activity was both heat labile (56°C, 30 min) and unaffected by incubation with anti-C5 antisera.

![Figure 3](image-url)

Figure 3 (Left) The differential chemotactic responses to zymosan-activated plasma and nFMLP of normal Gr (Δ) and Gr from seven consecutive uremic patients before (○) and after (●) 120 min of HD. Each point represents the arithmetic mean of at least three determinations. (Right) The chemotactic responses of normal (Δ) and dialyzed uremic (●) Gr produced by serial dilutions of zymosan-activated plasma in HBBSS buffer.
Selective refractoriness to stimulation by C5a_desarg could also be induced in normal Gr by longer incubations in zymosan-incubated normal plasma (Fig. 6). In contrast to Gr incubated in buffer, which developed an aggregation response of 134±10 mV s/2 min when stimulated by C5a_desarg, cells incubated in zymosan-activated plasma diluted to 500 μM/ml in buffer developed a response of only 50±12 mV s/2 min, 63% less than normal. Cells incubated in a similar dilution of fresh plasma developed a near-normal response of 128±11 mV s/2 min (not shown). Serial fivefold dilutions of zymosan-activated plasma produced intermediate degrees of inhibition, and the aggregation response produced by an equipotent concentration of nFMLP (3.2×10−8 M) was not significantly impaired by exposure to any dilution of activated plasma C. Very similar degrees of selective inhibition of chemotaxis were observed under the same experimental conditions; the migration of cells toward C5a_desarg was reduced from 100±16 μM/h by 53% to 47±5 μM/h after incubation with 500 μM/ml zymosan-activated plasma C but was unaffected by incubation in fresh plasma (100±14 μM/h), whereas chemotaxis toward nFMLP remained entirely unaffected.

DISCUSSION

The present studies confirm that a biologically active agent capable of inducing Gr aggregation and possessing several other characteristics of C5a_desarg is indeed generated by the dialyzer in the plasma of patients undergoing HD. This new finding is entirely consistent with our original hypothesis that the pulmonary leukostasis seen during the first hour of HD results from embolization of Gr that have aggregated under the influence of C5a_desarg (4). But it is equally apparent that the generation of C5a_desarg by the dialyzer continues long after pulmonary leukostasis has reversed (2), and an additional explanation for this discrepancy is necessary. Two critical observations gave us our initial insight into the reason for this discrepancy. The first was that a patient with high levels of C5a_desarg before HD failed to develop the usual granulocytopenia associated with HD. The second was that, like normal human Gr exposed to activated plasma C (13, 14) or purified C5a_desarg (16) in vitro, the Gr that return to the circulation after pulmonary leukostasis has reversed have become selectively unresponsive to C5a_desarg. These data form the basis for our new hypothesis that the transience of HD granulocytopenia results from chemotactic factor-induced deactivation.

After their initial exposure to C5a_desarg during induction of HD, circulating Gr undergo a single episode of reversible aggregation and pulmonary leukostasis, after which they return to the circulation unable to react to the C5a_desarg continuously generated throughout the HD. The selectivity of the unresponsiveness displayed
by these cells suggests that their deactivation results from receptor internalization (19, 20, and footnote 2), rather than from depletion of an activatable esterase (11, 12), from exhaustion of another metabolic step critical to the chemotactic response, or from non-specific cell damage—any of which would produce nonselective refractoriness to C5adesarg and to nFMLP as well. It is unlikely that our observations are an artefact of the increase in the proportion of the relatively unreactive, nonrosetting Gr that Klemperer et al. have described in HD (27), because this population change reverses well before 2 h, when we took our samples, and we were able to induce an identical effect in Gr in vitro, where no such redistribution could occur. Although we have not been able to definitely demonstrate that the C5adesarg generated by the dialyzer is responsible for this deactivation, we were able to induce similar dose-related, irreversible and selective down-regulation of aggregation and chemotaxis in normal Gr by exposing them to the C5adesarg in zymosan-activated plasma C in vitro.

The potential reversibility of chemotactic factor-induced deactivation remains a point of controversy. In their initial description of the Gr down-regulation produced by exposure of cells to chemotactic peptides in vitro, Niedel et al. (28) were unable to detect regeneration of the internalized receptors for at least 2 h, but Sullivan and Zigmond (20) and Chenoweth and Hugli (20) have more recently made observations entirely to the contrary. In the present studies, we were unable to reverse the unresponsiveness of dialyzed Gr or Gr exposed to C5adesarg in vitro by incubation or washing, but the bioassay systems we used depend on more than just receptor availability. Direct determination of receptor numbers with isotopically labeled C5adesarg and nFMLP will be necessary to resolve this critical issue.

There is clearly an increased risk of pyogenic in-
fection in patients with renal failure (29) and in those on chronic HD (30), and there have been numerous reports of impaired Gr chemotaxis in both groups. Indeed, in four of the seven patients whose cells we studied (Fig. 4), we detected selective impairment of Gr migration toward the biologically relevant stimulus C5a(desarg). Other than the study of Goldblum et al. (31), who described a specific inhibitor of C5a-stimulated chemotaxis in patients on HD, little attention has been paid to stimulus specificity, an oversight which may well be responsible for the discrepant observations of various investigators. The inhibitor described by Goldblum et al. (31) is, however, distinct from the effects we describe because it is factor rather than cell directed, of relatively high molecular weight (>30,000), and heat stable, and it appears only after 3 mo of chronic HD. Regardless of whether Gr down-regulation is an important cause of the infection propensity in patients on chronic HD, it is very likely that it is at least responsible for the transience of HD granulocytopenia, and it may also play a role in the “anti-inflammatory effects” of HD seen in patients with familial Mediterranean fever (32), psoriasis (33), and systemic lupus erythematosus (34). An even more interesting speculation concerns the possibility that down-regulation may have a more general effect in limiting the deleterious effects of C-stimulated Gr on the integrity of vascular endothelium (8) in patients with intravascular C activation in this and other clinical contexts. This novel concept is entirely consistent with the paradoxical and otherwise unexplainable propensity for autoimmune vasculitis seen in patients congenitally deficient in C5 (or the earlier C components) but not observed in patients lacking C6–C8 (35).

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