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# Reversal of Hemodialysis Granulocytopenia and Pulmonary Leukostasis

## A CLINICAL MANIFESTATION OF SELECTIVE DOWN-REGULATION OF GRANULOCYTE RESPONSES TO $C5a_{desarg}$

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ABSTRACT The transient granulocytopenia of hemodialysis results indirectly from plasma complement activation by dialyzer cellophane membranes. The $C5a_{desarg}$ 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 $C5a_{desarg}$ produced in the dialyzer. These studies were designed to measure $C5a_{desarg}$ generation during dialysis by granulocyte aggregometry and to determine the reason for the transience of the leukostasis. $C5a_{desarg}$ 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 $C5a_{desarg}$ 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 $C5a_{desarg}$, 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 $C5a_{desarg}$ in vitro. These studies suggest that $C5a_{desarg}$ 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 $C5a_{desarg}$—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 $C5a_{desarg}$ (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 $C5a_{desarg}$ 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; HBBSS buffer, Hepes-buffered balanced salt solution containing 0.5% (wt/vol) human serum albumin; HD, hemodialysis; *n*FMLP, *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 $C5a_{desarg}$ in the plasma of 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 $C5a_{desarg}$ during HD has not yet been directly investigated. If $C5a_{desarg}$ 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, $C5a_{desarg}$ 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 $C5a_{desarg}$ in vitro produces nonspecific inhibition of their responsiveness to all chemotactic stimuli, an effect attributed to depletion of a critical plasma membrane proesterase (11, 12). In contrast, human Gr exposed to $C5a_{desarg}$ in vitro lose only their responsiveness to $C5a_{desarg}$ itself 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 $C5a_{desarg}$ (17) and formylated peptides (18), which become selectively internalized after binding to the appropriate stimulus (19, 20).[2] 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 $C5a_{desarg}$ persists unabated throughout each HD, we explored the alternative possibility that the transience might instead result from down-regulation of Gr responsiveness to the $C5a_{desarg}$ 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 afferent (patient-to-dialyzer) and efferent (dialyzer-to-patient) lines. Pre-HD samples were drawn from the afferent 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 afferent 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 10HO-D, Payton Associates, Buffalo, N. Y.) (4). Maximum and minimum light transmission limits (full-scale deflection of 10 mV) were calibrated with suspensions containing $5 \times 10^6$ and $1 \times 10^7$ Gr/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 $1.11 \times 10^7$ 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 as 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-C5 and anti-C3 antisera (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.), and partially purified $C5a_{desarg}$ 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 $C5a_{desarg}$ and *n*-formyl-Met-Leu-Phe (*n*FMLP) (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 $1.25 \times 10^7$ Gr/ml) had been exposed to 0.05 ml of the alternative chemotaxin. Equipotent concentrations of zymosan-activated plasma C (undiluted) and *n*FMLP ($3.13 \times 10^{-7}$ 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 $\mu$m mean pore size and thickness of $158 \pm 16$ $\mu$m by the leading front technique

[2] Chenoweth, D. E., and T. E. Hugli. Binding, internalization and degradation of human C5a by human neutrophils. Submitted for publication.

for 1 h at 37°C (13, 23). The chemotactic stimuli, which included zymosan-activated plasma C (diluted 1:200 in buffer) and $10^{-8}$ M *n*FMLP were chosen because they are of approximately equal potency and normally induce intermediate rates of migration. All samples were assayed in triplicate, and the distance migrated by the leading front in each filter was measured in sextuplicate by a naive observer. Random movement was measured by estimating the distance migrated by Gr toward buffer alone.

*Statistical analysis.* Unless stated otherwise, all numerical data are expressed as the arithmetic mean±1 SD, and the significance of the difference between any two sets of data was assessed with Student's two-tailed unpaired *t* test (24). The difference between two sets of determinations was considered significant when the *P* value so derived was <0.05.

## 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

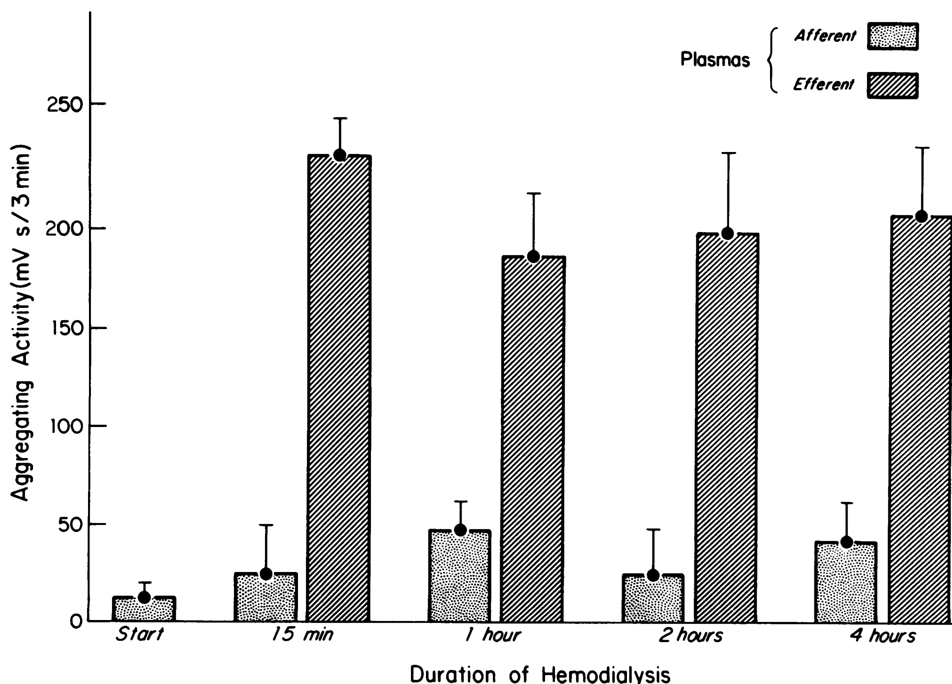


FIGURE 1 The aggregating activities in afferent (patient-to-dialyzer) and efferent (dialyzer-to-patient) plasma samples drawn from five consecutive patients during HD. Triplicate determinations of 3-min aggregation responses were made on each sample, and each point represents the arithmetic mean of the values for the whole group.

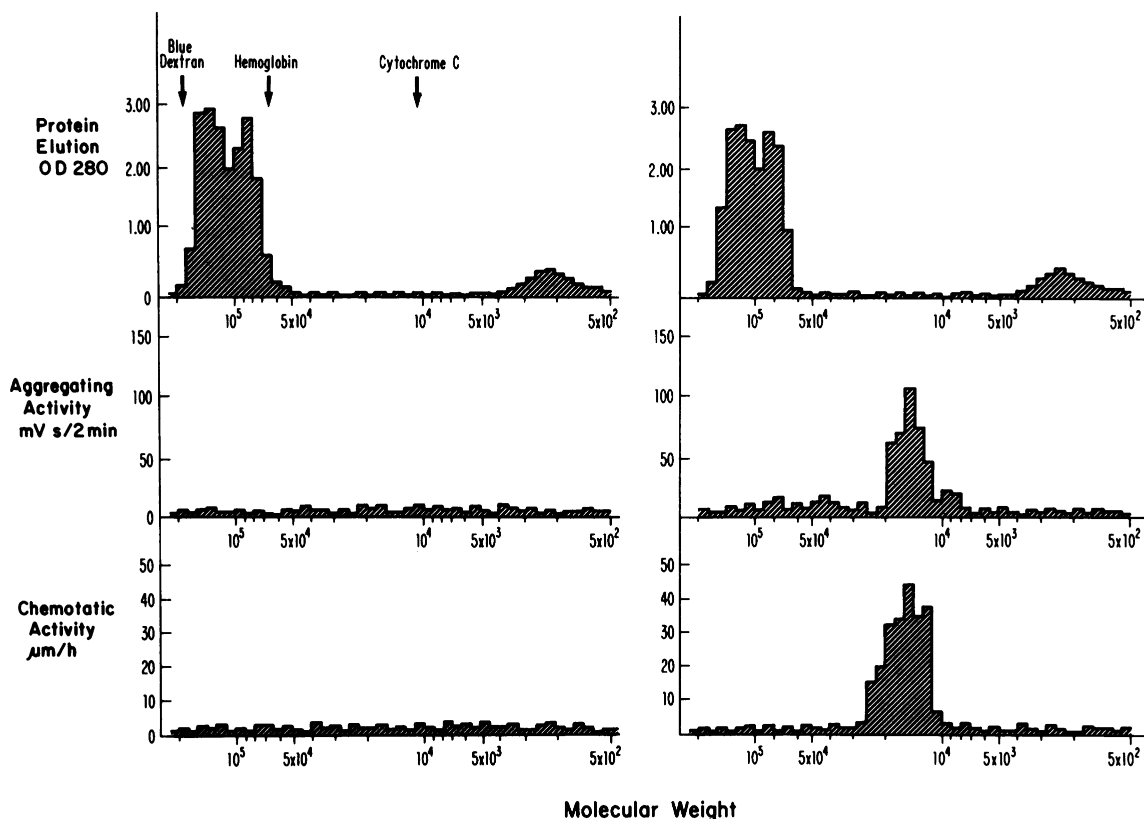


FIGURE 2 The aggregating and chemotactic activities in fractions of afferent (left) and efferent (right) plasma samples drawn simultaneously from a patient after 15 min of HD and prepared by filtration of 20 ml of each sample with Sephadex G-75. The aggregating activity of each fraction is expressed as raw data, and the chemotactic responses derived by subtracting the mean rate of migration of cells toward column buffer (43±13 µm/h) from the response produced by the fraction under test.

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 $C5a_{desarg}$ 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 I). Gr prepared from these patients just before HD responded to the $C5a_{desarg}$ 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 *n*FMLP and the higher concentrations of $C5a_{desarg}$ 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 $C5a_{desarg}$ in zymosan-activated plasma and *n*FMLP at near normal rates, but after 120 min of HD chemotaxis toward $C5a_{desarg}$ was reduced by ~67%, whereas their response to *n*FMLP 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 $C5a_{desarg}$ 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*

| Stimuli | Aggregation responses | | |
|---|---|---|---|
| | | Uremic granulocytes | |
| | Normal granulocytes | Predialysis | Dialyzed 120 min |
| | | *mV s/3 min* | |
| Afferent plasma | 22±30 | 19±8 | 12±9 |
| Efferent plasma | 230±31 | 98±16* | 16±8‡ |

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.

ary stimulation with partially purified $C5a_{desarg}$ 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 *n*FMLP ($3.13 \times 10^{-7}$ 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 $C5a_{desarg}$; 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.

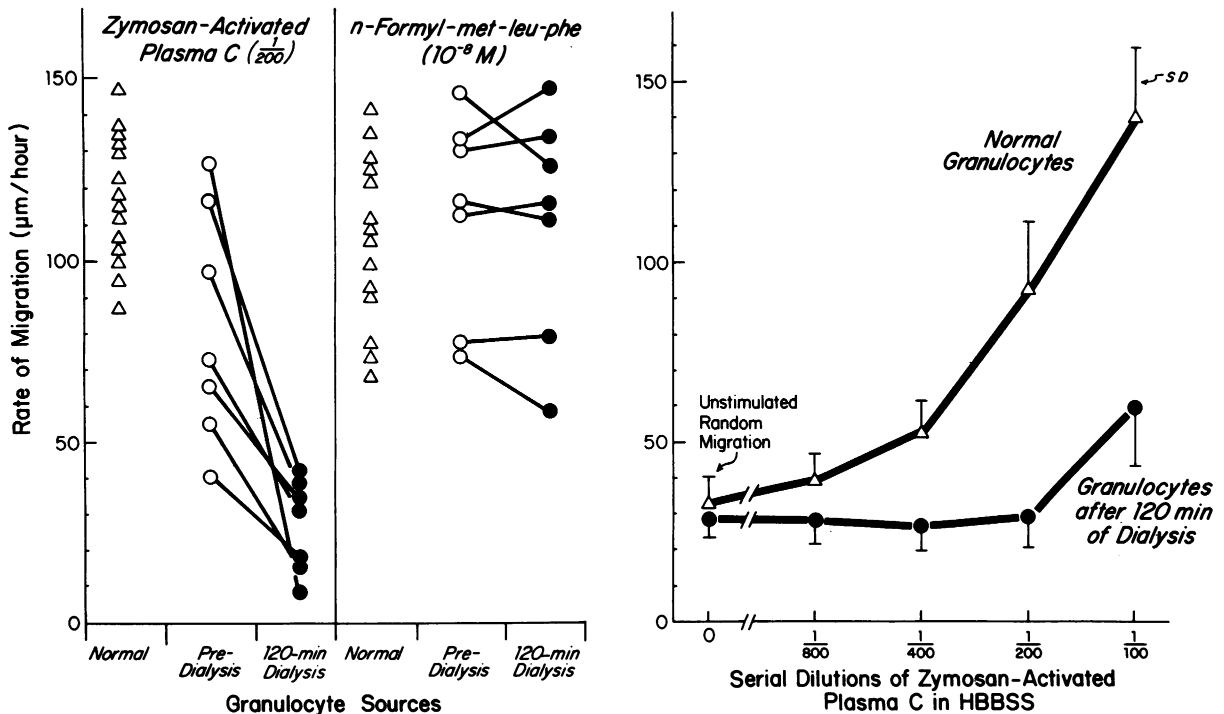


FIGURE 3 (Left) The differential chemotactic responses to zymosan-activated plasma and *n*FMLP of normal Gr (△) and Gr from seven consecutive uremic patients before (○) and after (●) 120 min of HD. Each point represents the arithmetic mean of at least three determinations. (Right) The chemotactic responses of normal (△) and dialyzed uremic (●) Gr produced by serial dilutions of zymosan-activated plasma in HBBSS buffer.

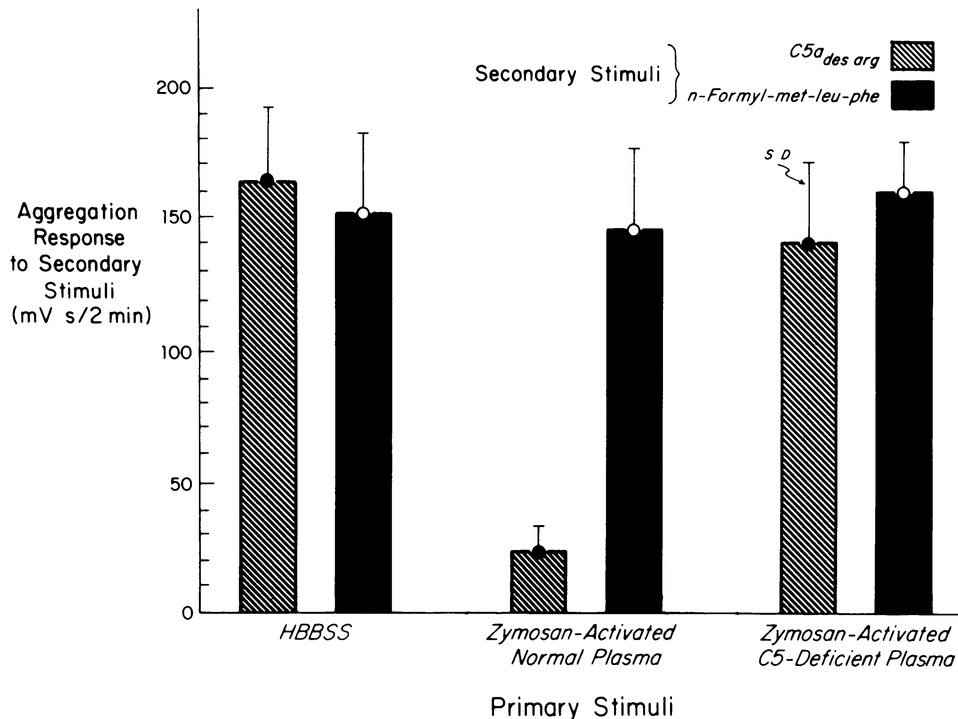


FIGURE 4 The aggregation responses exhibited by normal Gr stimulated with partially-purified $C5a_{desarg}$ or an equipotent concentration ($3 \times 10^{-7}$ M) of *n*FMLP 3 min after the cells had been primarily exposed to HBBSS buffer, zymosan-activated normal plasma, or zymosan-activated C5-deficient plasma. Each point represents the arithmetic mean of quadruplicate determinations.

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 *n*FMLP ($3.2 \times 10^{-8}$ 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 *n*FMLP 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

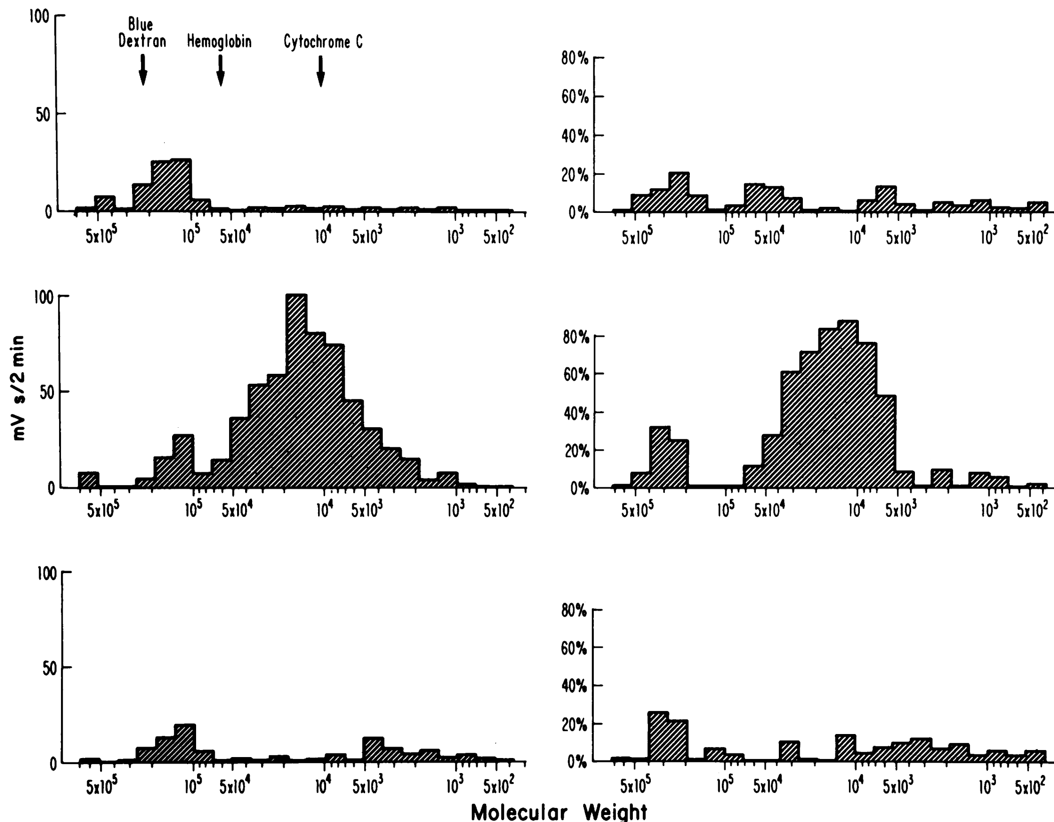


FIGURE 5 The aggregating (left) and inhibitory (right) activities of fractions of fresh normal (upper panel), zymosan-activated normal (center panel), and zymosan-activated C5-deficient (lower panel) plasma samples prepared by filtration with Sephadex G-75. Aggregating activities were determined directly by the addition of an aliquot of each fraction to normal Gr being stirred in an aggregometer. The inhibitory activity of each fraction was determined indirectly by measuring the aggregation response produced by the addition of 0.05 ml of partially purified $C5a_{desarg}$ 2 min after the Gr had been primarily stimulated with an equal volume of the fraction under test. This value was then expressed as a percent of that observed when Gr were stimulated wtih $C5a_{desarg}$ 2 min after exposure to column buffer, and the extent of inhibition was then calculated.

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 nonspecific cell damage—any of which would produce nonselective refractoriness to $C5a_{desarg}$ and to *n*FMLP as well. It is unlikely that our observations are an artefact of the increase in the proportion of the relatively unreactive, nonrosetting Gr that Klempner 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 $C5a_{desarg}$ 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 $C5a_{desarg}$ 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 re-emergence or regeneration of the internalized receptors for at least 2 h, but Sullivan and Zigmond (20) and Chenoweth and Hugli[2] 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 $C5a_{desarg}$ 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 $C5a_{desarg}$ and *n*FMLP will be necessary to resolve this critical issue.

There is clearly an increased risk of pyogenic in-

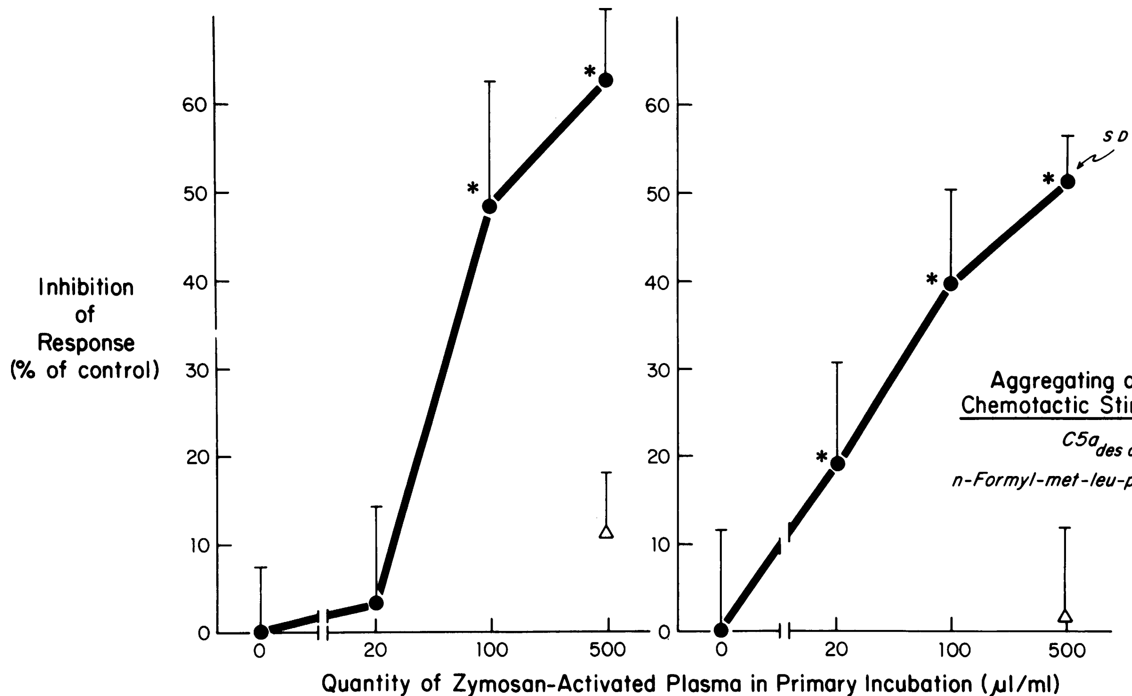


FIGURE 6 The dose-related and selective inhibition of $C5a_{desarg}$-induced aggregation (left) and chemotaxis (right) produced by longer exposure of normal Gr to zymosan-activated normal plasma. Gr were incubated for 15 min at 37°C in serial dilutions of plasma in buffer and washed twice before quantitation of their responses. Responses were expressed as a percent of that observed with Gr incubated in buffer alone (control); each point represents the mean inhibition produced by each plasma dilution and is denoted with an asterisk when significant inhibition was apparent.

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).

## ACKNOWLEDGMENTS

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