Effect of Intravascular Complement Activation on Granulocyte Adhesiveness and Distribution

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The effects of manipulations that activate complement (C) by the alternate pathway upon the concentration of circulating granulocytes and their adhesiveness were studied in rabbits and humans under several conditions. Whether C is preactivated in vitro and then infused intravenously, activated in vivo by administration of cobra venom factor, or activated during extracorporeal circulation in human hemodialysis, a close correlation between changing granulocyte adhesiveness and granulocyte levels is noted. Shortly after C activation, circulating granulocytes disappear, while their adhesiveness (measured by nylon fiber filtration) increases strikingly. Thereafter, when circulating granulocytes return and actually rebound to above-baseline levels, their adhesiveness declines in parallel. The results suggest that activated C components induce stickiness of granulocytes, which engenders their sequestration. When C-activated plasma is infused intravenously, granulocytes are sequestered selectively in the pulmonary circulation. When C is activated throughout the entire body by the intravenous infusion of cobra venom factor, granulocytes sequester nonselectively. In the former instance, animals develop hypoxemia; in the latter instance, hypoxemia is not observed. Thus granulocytes made hyperadhesive sequester in the first capillary network traversed, and selective organ dysfunction may occur. The results help to explain the recently reported pulmonary leukostasis and dysfunction accompanying hemodialysis.

Interaction of Granulocytes with various activated complement (C) components in vitro provokes several alterations in their function and metabolism. Thus such components may cause granulocytes to demonstrate chemotaxis, attach to and phagocytose particulate matter, and release lysosomal constituents. Even brief exposure of granulocytes to activated complement alters their surface charge and stimulates their degradation of glucose.

However, the importance in vivo of these complement-related effects is uncertain, although it seems likely that complement-granulocyte interactions are critical to host defense and inflammatory phenomena. For instance, animals depleted of complement capable of being activated, are inefficient in localizing granulocytes to diverse inflammatory sites. Patients who genetically lack certain complement components may suffer severe and chronic bacterial infections.

To study the interaction of complement with granulocytes in vivo, other investigators infused animals with activated plasma, purified components of activated plasma, or various complement activators such as cobra venom factor. In rabbits each technique has been shown to induce profound, albeit transient, granulocytopenia. Our laboratory confirmed and extended these results by showing that the granulocytopenia following intravenous infusion of plasma...
activated with inulin, zymosan, polycellulose, or nylon fibers resulted from sequestration of granulocytes, particularly in pulmonary capillaries. Of pathophysiologic interest is our finding that the polycellulose membrane utilized in hemodialysis coils is capable of potently activating complement predominantly through the alternate pathway (much as do other polysaccharides such as zymosan, endotoxin, and inulin). From these data we suggested that complement-induced granulocytopenia with its accompanying pulmonary leukostasis causes the acute and chronic pulmonary dysfunction that can occur during hemodialysis of uremic patients. We also suggested that dialysis-coil cellophane might be utilized as a convenient, sterilizable, and easily removable complement activator.

In the present studies we explored the mechanism whereby activated complement components produce neutropenia. A previous, serendipitous observation that exposure of granulocytes to complement-activated plasma rendered them excessively adherent to plastic Petri dishes suggested that activated complement components in the circulation might increase granulocyte adherence to endothelium, i.e., might promote their "margination." Hence the potential for localized organ dysfunction from capillary plugging by granulocytes would seem real.

Indeed, utilizing a newly described assay of granulocyte adhesiveness to nylon fibers, we demonstrated in the present experiments that granulocyte adhesiveness, measured in vitro, is altered rapidly and inversely to circulating granulocyte counts during complement activation. Adhesiveness strikingly increased as granulocytes disappeared from the circulation shortly after complement activation. Conversely, when circulating granulocytes reappeared and actually rebounded to supernormal levels, adhesiveness in vitro decreased markedly. That the correlate in vivo of this complement-induced excessive adhesiveness (i.e., vessel plugging) can cause organ dysfunction—particularly of the lungs—is also shown.

MATERIALS AND METHODS

Infusion of Preactivated Complement Into Rabbits

Under local anesthesia (1%, procaine) a femoral artery and vein were cannulated in each of a number of albino white rabbits weighing 2-4 kg. Arterial blood was immediately placed in ice and centrifuged at 10,000 g for 10 min at 0°C. The derived plasma, with heparin (1 U/ml) added, was placed in plastic containers, and its complement was activated as previously described by exposure to either polycellulose dialysis membrane (Gambro, Newport News, Va.) (20 sq cm/cc) or inulin (0.2 mg/cc) for 30 min with gentle agitation at 37°C. Thereafter, plasma was freed of dialysis membrane manually or of inulin by recentrifugation (at 10,000 g for 10 min). Documentation of predominant alternate pathway activation of C by these two methods was provided previously by us and by others.

The activated plasma was rapidly infused into the venous catheter of the donor animal. At various times thereafter, samples from the arterial catheter were assayed for granulocyte counts and adhesiveness and compared to samples obtained 30 sec prior to infusion.

Activation in Vivo of Complement in Rabbits

Cobra venom factor (CVF), prepared and purified by the technique of Cochrane et al., was stored at -70°C until rewarmed and infused (40 U/kg) into the venous catheter of rabbits prepared as above. To measure pulmonary clearance of granulocytes after infusion of activated plasma or after injection of CVF, the arterial and venous catheters were advanced into the thorax and granulocyte counts were performed on samples obtained simultaneously from both catheters.
Studies in Hemodialyzed Uremic Patients

Four chronically hemodialyzed patients (two male, two female) with diverse renal diseases were studied during the first 3 hr of 5-hr dialysis periods. Serial blood samples were removed from the arterial line of the dialysis apparatus, further anticoagulated with heparin to approximately 10 U/ml (the patients were endogenously heparinized to approximately 1 U/ml levels), and assayed for granulocyte counts and adhesiveness.

Granulocyte adhesiveness was measured by a slight modification of the method reported by MacGregor et al.20 Briefly, 40 mg sterile nylon fibers were packed into glass pipettes. The pipettes were suspended about 3 cm above the bottom of vacutainer tubes containing powdered ethylenediaminetetraacetate (EDTA), and the entire apparatus was placed in a 37°C incubator.

For each assay, 0.8 ml heparinized (10 U/ml) blood removed from the arterial catheter of the rabbit or patient was immediately placed onto the nylon fiber column. Each assay was run in duplicate in rabbit and in triplicate in human experiments. Granulocyte adherence (GA) was calculated with the following formula:

\[
GA(\%_c) = \left(1 - \frac{\text{PMN/cu mm after filtration}}{\text{PMN/cu mm before filtration}}\right) \times 100.
\]

Total leukocyte counts were obtained with a Coulter counter, and 300 cell differentials were performed on Wright-stained smears. Bands and more immature myelogenous forms were not considered polymorphonuclear (PMN) cells for the purpose of this calculation. When purified PMN suspensions were studied (see below), differential counts were not performed, since the total leukocyte number was assumed to represent only PMN.

The possibility that the concentration of leukocytes in assayed blood might systematically affect GA in this assay system has not previously been adequately explored. The effects of erythrocyte or platelet numbers have also not been rigorously examined. Therefore we prepared blood cell suspensions of varying composition by reconstituting samples from purified fractions of blood cell elements. Leukocyte-free erythrocytes, obtained by cellulose column filtration, were suspended at various hematocrits in autologous, heparinized (10 U/ml) plasma containing a fixed concentration (3000/cu mm) of autologous dextran-sedimented leucocytes. No effect of varying hematocrit on the GA assay was detected (Fig. 1A). In contrast, if granulocyte numbers were artificially raised by adding dextran-sedimented leukocytes to reconstituted blood suspensions of constant hematocrit (35%), marked increases in GA occurred, and a very significant direct relationship between GA and the logarithm of PMN concentration became evident (Fig. 1B). Finally, if PMN were purified (99% PMN, 1% mononuclear cells) by the Ficoll-Hypaque density-gradient technique22 and then suspended in Hank's buffered albumin (pH 7.4), the same direct relationship between GA and the logarithm of PMN count was verified (Fig. 1C).

Arterial blood gases were measured as previously described17 on samples obtained from arterial catheters of rabbits or hemodialyzed patients. Immunoelectrophoretic and hemolytic assays of C activation in rabbit and human blood were performed as previously described16,17 All results were analyzed for statistical significance using an unpaired or, where appropriate, paired Student's t tests.

![Fig. 1. Effect of varying blood cell concentrations on the granulocyte adherence (GA) assay system. (A) No effect upon GA of widely-varying RBC concentrations noted in reconstituted whole blood suspensions of constant (300/cu mm) granulocyte (PMN) content; each point, mean of six determinations. In contrast, highly significant direct correlation between GA and PMN concentration evident both in (B) reconstituted whole blood and (C) suspensions of purified PMN in Hank's buffered albumin; each point, mean of three determinations.](image-url)
A consistent, rapid decrease in circulating granulocytes occurred in rabbits when activated complement components were present in the circulation (Figs. 2 and 3, solid lines). The induced neutropenia was short-lived, and neutrophil counts approached normal or even rebounded above baseline within 1 hr or so, whether complement components were activated in vitro and then infused intravenously (Fig. 2) or endogenously activated by injection of purified cobra venom factor (Fig. 3). In the former case, dialysis coil cellophane was utilized to activate the alternate pathway* (Fig. 2); inulin activation produced identical results (not shown). In both situations GA underwent rapid and striking changes that were inversely correlated with the alterations in circulating PMN levels (Figs. 2 and 3, dashed lines). As PMN disappeared from the circulation, their adhesiveness in vitro markedly increased. Conversely, when granulocytes began to reappear in the circulation, GA fell and ultimately reached levels well below baseline. If cobra venom factor was boiled prior to injection, no C activation occurred; concomitantly, no consistent alteration in white count or GA was evident (Table 1).

It is acknowledged that unknown cobra venom factors, other than those responsible for C activation, might also be destroyed by boiling. Other experiments to corroborate the critical role of C activation in altering GA therefore were devised. In such studies (not shown) prevention of C activation by preheating (56°C for 30 min) or exposing plasma to hydrazine before reinfusion simultaneously ablated its leukopenia and GA-promoting effects. In contrast,
when the ability to activate C was reconstituted by mixing the two differently inhibited plasmas together, leukopenia and GA increase were again demonstrated. Similar results using nylon fiber leukapheresis filters to activate C have just been published.\textsuperscript{22a}

We previously demonstrated that the severe neutropenia occurring during the first 30 min of hemodialysis (Fig. 4, solid line) results from infusion of complement components activated by the cellophane in dialysis coils.\textsuperscript{16} As is the case in rabbits, granulocytes harvested from such dialyzed patients manifested alterations in adhesiveness closely reflecting (albeit inversely) the changing granulocyte counts (Fig. 4, dashed line).

When plasma in which complement has been activated by preincubation with dialysis coil cellophane (or inulin) is infused intravenously, the first capillaries to which excessively adhesive granulocytes might lodge are in the pulmonary bed. Indeed, in our rabbits clearance of PMN across this bed increased two- or threefold within seconds after intravenous infusion of activated plasma (Fig. 5A, interrupted line). That such entrapment may adversely affect pulmonary function is indicated by the decrease in arterial oxygen tension that accompanied this clearance (Fig. 5A, solid line). An increase in the alveolar/arterial O\textsubscript{2} gradient also was demonstrated at this time (not shown). However, if C was activated throughout the vascular system (systemic and pulmonary beds) by infusion of CVF, pulmonary clearance of PMN was no longer predominant, and in fact such clearance declined somewhat from baseline levels (Fig. 5B, interrupted line). Predictably, pulmonary dysfunction (decreased arterial pO\textsubscript{2}) was not seen under these conditions (Fig. 5B, solid line).

![Fig. 4. Inverse effects of hemodialysis on granulocyte adherence (---) and circulating PMN levels (-----); triplicate assays at each time interval after institution of hemodialysis in four uremic patients. *: values significantly different from baseline (p < 0.05). Activation of C in all patients validated by CH\textsubscript{50} decrements of 10%-20% and by immunoelectrophoretic appearance of C3 conversion products.](image)
DISCUSSION

These data demonstrate that during complement activation in animals or in hemodialyzed patients a close association exists between excessive adhesiveness of granulocytes, as measured in vitro, and their sequestration from the circulation. A reasonable explanation is that granulocytes induced to be “sticky” by interaction with activated complement components attach to endothelium and/or to other granulocytes and are thereby removed from the circulating pool of cells. We acknowledge that rigorous proof of a causal relationship between these two phenomena is not yet available; other more recent observations, however, also tend to support a possible causal relationship. When infused into rabbits, highly purified C5 induces neutropenia and simultaneous changes in GA similar to those found in the present studies. Moreover, pulmonary capillary plugging by granulocytes can be demonstrated in animals infused with activated C components during the same period of particularly excessive GA (e.g., within 5 min of C infusion). It is noteworthy that others have also suggested that the nylon fiber adherence assay may predict granulocyte stickiness to endothelium and have reported changes in GA identical to those presented here that occur during human extracorporeal hemodialysis. Moreover, these authors have demonstrated that two fluid-phase constituents, one of which is probably activated C, may augment GA. The existence of other possible GA augmenters remains to be determined, although coagulation, fibrinolytic, and kallikrein activation products seem worthy potential candidates. In addition, the possibility that bacterial endotoxins might alter GA, independent of C activation, should also be considered. Thus others have observed that neutropenia may follow endotoxin administration to C-depleted or genetically C6-deficient rabbits.

Further knowledge of possible variables in the GA assay was also gained in the present studies. We became particularly concerned that changing concentrations of the various filtered blood elements might systematically affect granulocyte entrapment by the nylon fibers. Since levels of granulocytes themselves changed so drastically during C activation (Figs. 2-4), analysis of the effect of varying leukocyte numbers on GA was required before an interpretation of our results became feasible. In fact, GA increased directly with the logarithm of granulocyte count (Fig. 1). Since in the present studies adhesiveness increased
during C activation at a time when granulocyte levels were decreasing, this potential artifact, if anything, strengthened the impression that C-induced neutropenia was associated with excessive granulocyte adhesiveness in vivo. Of equal importance, our results (Fig. 1) exclude the possibility that changing red cell or platelet numbers were significant variables in the GA assay system.

The inverse relation between GA and circulating granulocyte levels continues to hold during late phases after C activation, when leukocyte counts normalize and actually rebound to higher-than-baseline levels. GA declines to subnormal levels during this period. A possible explanation for these results is that young granulocytes are released from the bone marrow in response to neutropenia—a phenomenon shown by others to occur with infusion of cellophane-activated plasma— and that such young granulocytes may have diminished adhesiveness. Validation of this interpretation awaits comparative studies of GA in cells of differing age.

The effects of C-induced granulocyte adhesiveness have been shown to reflect the site of C activation. Thus infusion of activated C components into the venous circulation, as occurs during hemodialysis, produces pulmonary capillary leukostasis and pulmonary dysfunction (Fig. 5 A). More generalized C activation, as exemplified by CVF injection or which might be expected to occur with circulating immune complexes or endotoxin, may engender more random organ involvement. Pulmonary function may be unaffected under these conditions (Fig. 5 B).

We believe that these results, besides demonstrating coherent effects of C activation on granulocyte adhesiveness and distribution, may also provide insights into inflammation more generally. C capable of being activated is known to be required for granulocytes to localize at sites of inflammation. Moreover, it seems reasonable to propose that the circulating traffic of granulocytes must initially adhere to endothelium before leaving the vascular compartment and entering the inflamed neighboring tissue. We suggest that complement components, activated by an inflammatory provocative (for instance, a microbe), promote this granulocyte/endothelial interaction necessary for the first stage of inflammation. Although such a response is often beneficial, it should also be apparent from these studies that granulocytes may adhere and accumulate abnormally in situations in which C is activated in a more chaotic fashion. We consider it likely that some of the damage noted in pulmonary and mesenteric areas during gram-negative sepsis and endotoxemia may result from C-mediated increases in granulocyte stickiness. In fact, increased GA has been noted after endotoxin injection, and the alternate pathway of C has been shown to be regularly activated in patients during gram-negative shock. We suggest that granulocytes excessively and randomly adhere to endothelium in such situations and that diffuse tissue damage results from the accompanying capillary leukostasis that such adhesiveness engenders.

Still another mechanism by which C-mediated hyperadhesiveness might engender tissue destruction is suggested by more recent results from our laboratory. A specific activated C component, C5a, was shown to aggregate granulocytes, supporting the hypothesis that C-induced embolization of leukocyte aggregates may also be tissue damaging. In this regard, it seems likely that some of the increased GA noted during C activation in the present studies might re-
fect formation of C-induced granulocyte aggregates, since such aggregates might interfere mechanically with cell passage through the nylon fiber bed used in the GA assay system.

REFERENCES

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