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Research Article

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The Induction of Augmented Granulocyte Adherence by Inflammation

MEDIATION BY A PLASMA FACTOR

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ABSTRACT The adherence of granulocytes to surfaces, measured in vitro in nylon fiber columns, is inhibited by in vivo administration of anti-inflammatory agents. Therefore, the effect of inflammation itself was assessed in blood from patients with acute inflammatory diseases. Mean adherence in these patients was twice normal ($56.4 \pm 5.6\%$ vs. $29.4 \pm 5.2\%$); their plasma contained a factor that augmented adherence of normal cells to $47.2 \pm 5.6\%$ whereas the patients' cells showed a normal level of adherence ($34.0 \pm 6.8\%$) when resuspended in normal plasma. Although exudate fluid from experimental inflammation also contained the augmenting factor, cells from the exudate maintained their high level of adherence after washing and suspension in normal plasma. The augmenting factor detected in plasma from patients with inflammation was not present in serum and was inactivated by heating plasma to 56°C for 30 min; restoration of augmenting activity was accomplished by addition of 20% guinea pig serum to the heat-treated plasma. Because the guinea pig serum itself did not increase adherence when added to normal plasma, it appears that the augmenting factor is heat-stable, but requires a heat-labile cofactor like complement. Sephadex G-200 fractionation of inflammatory plasma showed adherence-augmenting activity in the majority of fractions, with peak activity in the fractions corresponding to approximate molecular wts of 30,000, 160,000, and 400,000.

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INTRODUCTION

The delivery of granulocytes to the site of inflammation involves a series of steps including margination and adherence to the vascular endothelium, diapedesis through vascular walls, and directional movement of cells along chemotactic gradients. The mechanisms by which granulocytes marginate and adhere to vessel walls have been poorly characterized. A previous report from our laboratory (1) described a new, simple assay for measuring granulocyte adherence (GA)¹ in vitro with nylon fiber columns. Drugs with anti-inflammatory properties, including corticosteroids, aspirin, and ethanol, were found to inhibit GA, prompting us to investigate the effect of inflammation itself on GA. This report demonstrates that adherence is augmented in inflammatory states and that this augmentation is mediated by a plasma factor.

METHODS

Assay system. GA was measured with the assay described previously (1). Briefly, a specified weight of nylon fiber was packed into a Pasteur pipette and the column length carefully adjusted to 15 mm. 1 ml of heparinized venous whole blood is introduced at the top of the pipette and allowed to filter through the nylon fiber by gravity. The precolumn granulocyte count is compared with that in the effluent blood to determine the percentage of granulocytes adhering to the column. In normal conditions, this percentage is directly proportional to the weight of fiber packed into the column. "Low-adherence" columns, packed with 50 mg of fiber, were used for the present experiment; blood

¹ *Abbreviations used in this paper:* AGA, augmented granulocyte adherence; ESR, erythrocyte sedimentation rate; GA, granulocyte adherence; HBSS, Hanks' balanced salt solution; MHS, modified Hanks' solution.

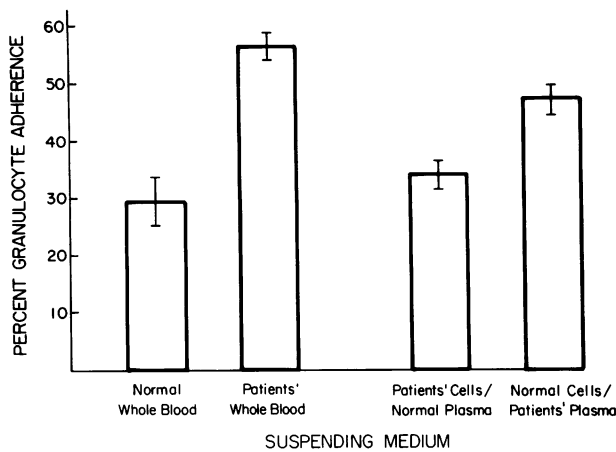


FIGURE 1 Mean granulocyte adherence in whole blood of patients with inflammatory diseases and in normal controls. Brackets represent \pm SE. The difference between the two groups (20 subjects each) is significant ($P < 0.01$, Student t test). Cells from patients with inflammation showed normal GA when suspended in normal plasma (third bar) and normal cells showed significantly increased adherence when suspended in plasma from patients with inflammation compared to their own plasma (fourth bar, $P < 0.01$, Student t test).

from normal controls has a mean GA of $29.2 \pm 4.3\%$ (SE) in columns of this weight.

Patients. Hospitalized patients with inflammation were selected by either of two criteria: the presence of an erythrocyte sedimentation rate (ESR) over 80 mm/h (Westergren) from any cause, or a rectal temperature of more than 38°C secondary to a bacterial infection. Nonbacterial inflammatory conditions with high sedimentation rates included vasculitis, systemic lupus erythematosus, and acute myocardial infarctions, while infections included pneumonia, osteomyelitis, and pyelonephritis. Patients treated with any anti-inflammatory agent (including aspirin) in the preceding week were excluded.

Blood separation. To determine whether inflammation directly affected the granulocyte's ability to adhere or worked indirectly through mediating plasma factors, cells were separated from plasma and GA was measured after the cells were resuspended in several different media. Heparinized venous whole blood (15 U heparin/ml) from normal controls and from patients with inflammations was centrifuged at 80 g for 10 min at 4°C and the decanted plasma kept on ice until used. The cells were washed three times in Hanks' buffered salt solution (HBSS) and resuspended in an equal volume of normal saline, HBSS, modified Hanks' solution (MHS) or ABO-compatible plasma. GA of the reconstituted blood was measured after incubation at 37°C for 30 min on a rotator.

Plasma factor evaluation. The adherence-augmenting factor found in plasma of patients with inflammation was evaluated as follows: heat-stability was assessed by incubating the plasma at 45, 50, and 56°C for 30 min. The heat-treated plasma was then used to suspend normal cells and the adherence was assayed. In some experiments, guinea pig serum (Grand Island Biological Co., Grand Island, N. Y.), in final concentration of 20% or 40% was added to the heparinized heat-treated plasma in an effort to restore complement activity. In other experiments, plasma from patients with inflammation was incubated with heat-killed yeast

particles for 30 min in an attempt to consume the late components of complement and, thereby, depress total hemolytic complement. This plasma was centrifuged at 1,300 g and passed through a Millipore filter (Millipore Corp., Bedford, Mass.) to remove the yeast. The effectiveness of complement inactivation was determined by measuring total hemolytic complement. Incubation of plasma with yeast resulted in a more than 95% decrease in CH_{50} . Because serum from patients with inflammation did not augment GA of normal cells (in contrast to plasma), human fibrinogen was added to the serum to produce a final concentration of 500 mg/100 ml. Thereafter, the GA of washed normal cells resuspended in the high-fibrinogen serum was measured. Finally, the adherence-augmenting activity of patients' plasma was evaluated after dialysis at 4°C for 24 h with 5 ml of plasma against 500 ml of MHS.

To characterize further and isolate the adherence-augmenting factors, plasma obtained from rabbits 4 h after the initiation of sterile peritonitis was subjected to euglobulin precipitation (precipitate resuspended in 3.5 mM phosphate buffer with 0.15 M normal saline) and Sephadex G-200 column fractionation (2). IgG, IgM, and albumin were identified by immunodiffusion (3). After each procedure, the effect of the plasma fractions on adherence of normal rabbit granulocytes was compared with that of similar fractions from normal rabbit plasma.

Rabbit peritonitis. To study the effects of inflammation on GA under controlled conditions, peritonitis was induced in 2.0–3.0-kg New Zealand white male rabbits (Skipack Farms, Skipack, Pa.) by the intraperitoneal injection of 120 ml of sterile normal saline. Clinical signs of peritonitis and a sterile exudate with 1,000–5,000 granulocytes/ mm^3 developed over the next 4–8 h, resolving completely by 24 h. Blood was drawn for GA determination before peritonitis and at intervals until it returned to normal. In addition, serum and plasma were assayed for augmented granulocyte adherence (AGA) factor activity by measuring adherence of normal granulocytes suspended in one or the other. The animal model also allowed measurement of the adherence of granulocytes that had migrated from the vascular space to the inflammatory site, with peritoneal exudate cells collected 4 h after induction of saline peritonitis. After centrifugation of the exudate at 80 g for 10 min, the cells were washed three times in HBSS (which reduced the cell number by 15–20%), and resuspended in isotonic saline or in plasma taken either before peritonitis or when exudate cells were harvested. The adherence of these exudate cells in the different media was compared to that of cells taken simultaneously from peripheral blood. In addition, the cell-free exudate supernate was used to suspend washed normal rabbit cells to determine whether or not AGA factor was present in the exudate itself.

RESULTS

All 20 patients with inflammatory disorders had an increased GA (Fig. 1), averaging $55.9\% \pm \text{SE } 2.6$ compared to $29.4\% \pm 4.3$ for 20 controls ($P < 0.01$, Student's t test). Representative values included 63.0% for five patients with osteomyelitis, 53.2% for three with pyelonephritis, 50.3% for two with pneumonia, 53.3% for two with abscess, 57.6% for two with vasculitis, 68.9% for one with systemic lupus erythematosus, and 60.0% for one with thrombophlebitis. Infectious and noninfectious inflammatory conditions caused a similar degree of

augmentation (average 56.2% vs. 55.2%). Although all patients had either a fever of more than 38°C and/or an ESR of more than 80 mm/h, the degree of augmented GA correlated poorly with extent which ESR or temperature were increased. The 13 patients with granulocyte counts below 10,000/mm³ had an average GA of 58.6%, compared to 50.9% for those with counts above 10,000/mm³. Adherence of granulocytes from patients with inflammation, washed and suspended in normal plasma, was 34.0%±2.4, not significantly different from normal ($P = 0.1$, Student's t test, Fig. 1). Conversely, normal cells suspended in plasma from patients with inflammatory disorders had mean adherence augmented from the normal value of 29.4% to 47.2%±2.8 ($P < 0.01$, Student's t test, Fig. 1). This augmentation was maximal within 1 min of mixing, without further increase with up to 60 min of incubation.

The process of separating, washing, and resuspending normal or patient granulocytes in autologous plasma did not change adherence from preseparation whole blood values. In seven studies with normal cells, resuspension in autologous plasma resulted in 104.6±3.5% of preseparation values; other results included 98.2±3.4 for heat-inactivated normal plasma, 95.2±4.0% for autologous serum, 94.2±3.1 for normal saline, 88.1±3.3 for MHS, and 108.7±3.0 for HBSS. Only ABO-compatible normal plasma produced a significant increase in GA to 111.1±4.4% ($P < 0.05$, paired sample t test).

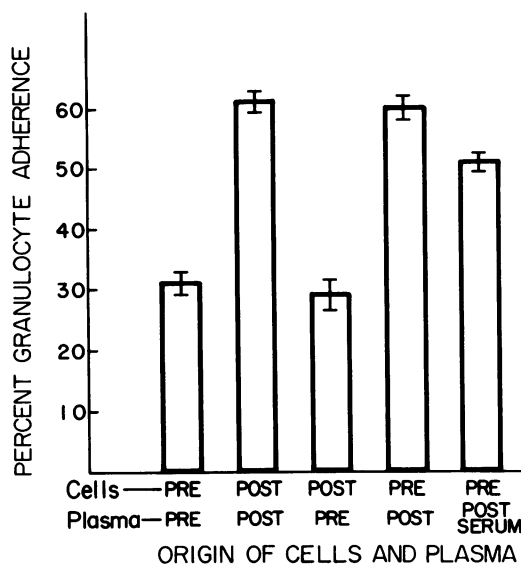


FIGURE 2 Adherence in rabbits before and 4 h after initiation of peritonitis is shown in the first two bars. Brackets represent \pm SE. Cells from the 4-h postperitonitis period suspended in preperitonitis plasma have normal adherence (bar 3) while preperitonitis cells suspended in postperitonitis plasma show adherence similar to that in postperitonitis whole blood (bar 4). Postperitonitis serum also augments the adherence of normal preperitonitis cells (bar 5).

Dose-Response of AGA Factor on Adherence of Normal Granulocytes

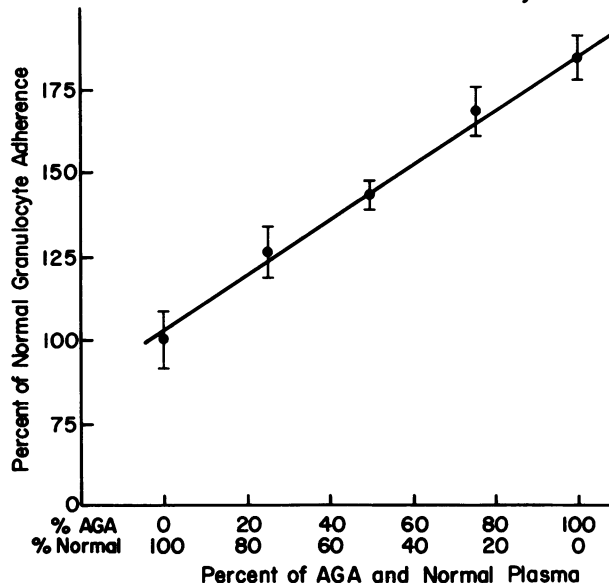


FIGURE 3 Dose-response of AGA factor on adherence of normal granulocytes. Normal GA is that of normal cells suspended in 100% normal plasma. The brackets represent ± 1 SD for the mean of three experiments. AGA plasma was obtained from rabbits with augmented granulocyte adherence induced by sterile peritonitis.

In rabbits given sterile peritonitis, mean preperitonitis GA was 31.7%±SE 1.9 (Fig. 2). It increased to 44.0±3.0 1 h after induction of peritonitis, 59.6±2.7 at 2 h, and reached a maximum of 61.8%±1.5 at 4 h ($P < 0.01$ from normal, Student t test). Thereafter it fell to 49.5±2.9 at 6 h, 28.1±3.1 at 8 h, and was normal by 24 h in all animals. As with humans, rabbit cells were unaffected by washing and resuspension in autologous plasma. Moreover, suspension of cells from rabbits with peritonitis in normal plasma reduced their adherence to 29.3%±2.4, while normal cells suspended in plasma from rabbits with peritonitis had a mean adherence of 60.5%±2.0 (Fig. 2). This augmentation was dose-dependent in three studies (Fig. 3). Unlike the case in human inflammation, the clotting of rabbit blood to yield serum rather than plasma only partially destroyed AGA factor activity: for both AGA serum and plasma, the augmentation of normal cells' adherence was significant at the 0.001 level by Student's t test. However, augmentation with AGA serum was below that caused by AGA plasma: 51.3%±1.4 vs. 60.5%±2.0 ($P < 0.02$, Student's t test). In four experiments, intravenous infusion of 50 ml of plasma taken from rabbits 4 h after initiation of peritonitis failed to alter adherence in normal rabbits, measured 1, 2, 3, 5, 8, and 24 h after infusion.

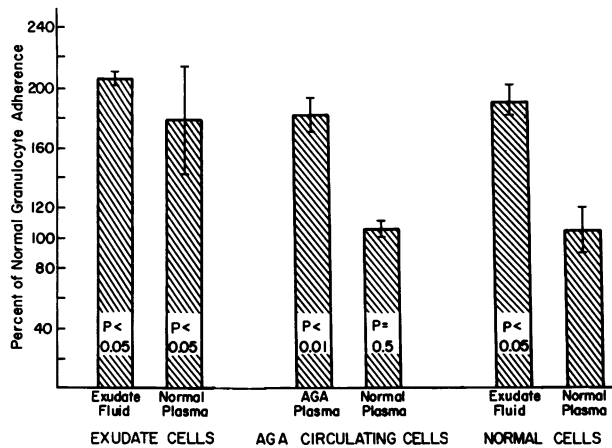


FIGURE 4 Adherence of rabbit granulocytes taken from different compartments in five experiments, suspended in plasma and peritoneal exudate fluid. Normal adherence is defined as that of normal cells in normal plasma, taken before induction of sterile peritonitis. 4 h later, peritoneal exudate cells, exudate fluid, circulating cells, and AGA plasma were obtained. Exudate fluid was cell-free, obtained by low-speed centrifugation of the exudate. Each animal served as his own control. *P* values represent significance compared to normal adherence (paired sample *t* test).

Granulocytes that had migrated from the vascular compartment into the rabbit peritoneal exudate had a high degree of adherence whether suspended in exudate fluid or normal preinflammation plasma (Fig. 4). In contrast, granulocytes taken from the intravascular compartment during inflammation showed increased adherence in their own plasma, but returned to normal adherence when resuspended in normal plasma. The cell-free peritoneal exudate fluid increased adherence of normal peripheral blood granulocytes to $190.7\% \pm 9.3$ of their adherence in normal plasma, similar to the augmentation induced by plasma taken at the time of peritonitis. Thus, equal AGA factor activity is present in the exudate fluid and in peripheral blood during inflammation.

Attempts to characterize the AGA factor found in humans with inflammation are shown on Fig. 5. Dialysis of AGA plasma against MHS did not alter its effect on normal granulocytes. Its adherence-augmenting effect was destroyed by heating to 56°C for 30 min, although heating to 45°C only reduced its effect by 7.7%, and 50°C by 12.4%. 20% guinea pig serum added to the heat-inactivated AGA plasma completely restored its adherence-augmenting property, but a 10% concentration restored it to only 87.6% of its preheated augmenting power. In a control study, 20% guinea pig serum added to normal plasma did not affect GA. Yeast-absorbed AGA plasma and AGA serum did not augment GA. Moreover, addition of fibrinogen to AGA serum

to reach a final concentration of 500 mg/100 ml caused no increase in GA.

Euglobulin precipitation of plasma from rabbits with peritonitis, performed on two occasions, demonstrated that augmenting factors were present in both soluble ($179.3 \pm 32.0\%$ of control) and precipitable ($197.0 \pm 34.6\%$ of control) fractions, with the untreated plasma augmenting adherence by $187.8 \pm 12.9\%$ over control values. Sephadex fractionation of rabbit AGA plasma, performed once, showed augmenting activity in the majority of fractions, with peaks in the fractions containing molecular wts of approximately 30,000, 160,000, and $\geq 400,000$ as extrapolated from the identification of IgM, IgG, and albumin peaks by immunodiffusion (Fig. 6).

DISCUSSION

These experiments demonstrate that inflammatory diseases are associated with augmented adherence of circulating granulocytes, occurring within 1 h of the onset of inflammation, and returning to normal with its resolution. The augmentation is mediated through a plasma factor rather than a direct effect of inflammation on the

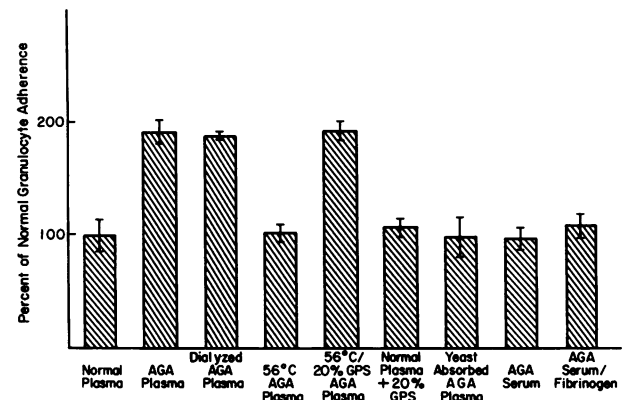


FIGURE 5 Effect of various manipulations on AGA factor activity. Normal ABO-compatible human granulocytes were used in all experiments and each experiment was performed three times. AGA plasma was taken from patients with augmented granulocyte adherence induced by various inflammatory diseases. The 56°C AGA plasma was from the same donors but incubated at 56°C for 30 min before mixing with the normal cells. The $56^\circ\text{C}/20\%$ GPS AGA plasma had 20% guinea pig serum added after the heat incubation. Normal plasma 20% GPS represents plasma from normal donors to which 20% guinea pig serum had been added. Yeast-absorbed AGA plasma was incubated with heat-killed yeast for 30 min before mixing. AGA serum represents serum from the same patients who donated AGA plasma. AGA serum/fibrinogen had fibrinogen added to a final concentration of 500 mg/100 ml before mixing with normal cells. The brackets represent ± 1 SE of the mean for each group. Adherence of normal cells in AGA plasma, dialyzed AGA plasma, and $56^\circ\text{C}/20\%$ GPS AGA plasma was significantly greater than in normal plasma ($P < 0.01$, paired sample *t* test).

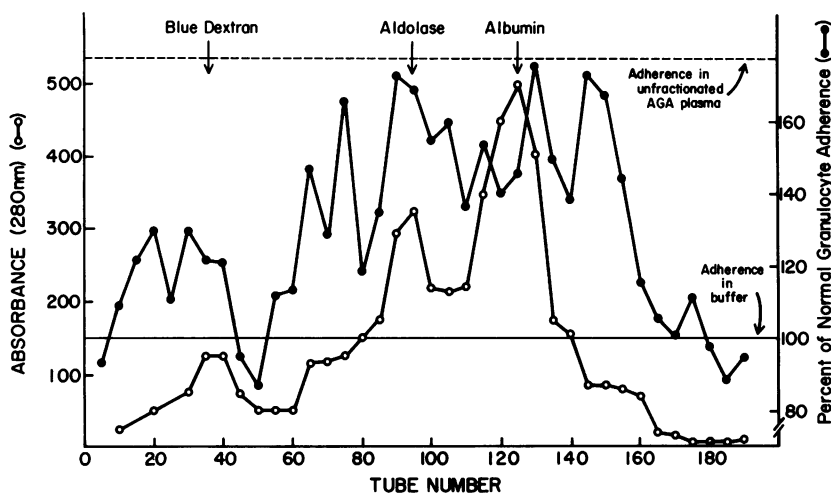


FIGURE 6 Adherence of normal granulocytes suspended in Sephadex G-200 fractions of AGA plasma from a rabbit with peritonitis. Normal adherence is defined as that of normal cells suspended in pure buffer. The dotted line at 177.5% of normal adherence represents the adherence of normal cells suspended in unfractionated AGA plasma. The arrows for the blue dextran, aldolase, and albumin identify the fractions in which these markers appear.

granulocyte. The factor acts rapidly (maximal increase in GA seen within 1 min of suspending normal granulocytes in AGA plasma) and reversibly ("hyperadherent" cells from individuals with inflammation show normal adherence after washing and suspension in normal plasma). In contrast, cells that have left the intravascular compartment and entered the peritoneal exudate show augmented adherence even after suspension in fluid not containing the augmenting factors. This suggests either that an irreversible change in adherence results from the granulocytes' extravascular migration, or that the cells that migrate successfully to the site of inflammation are those with the highest degree of adherence.

That adherence of normal granulocytes is the same whether suspended in normal plasma, normal plasma heated to 56°C for 30 min, or normal saline demonstrated that plasma factors are not required to maintain normal GA. Thus, it appears that the augmentation seen with inflammation involves production of a factor not present normally, rather than the increased concentration of a factor required in low concentration for a normal degree of GA.

The functional and physical-chemical studies of the adherence-augmenting humoral factor show it to be complex. Destruction of its activity by heating plasma to 56°C or by yeast absorption suggests that the factor is related to the complement system, as does reconstitution of activity to heated AGA plasma by addition of 20% guinea pig serum. Further, because guinea pig serum itself does not alter GA when added to normal plasma, it appears that inflammation may produce a

heat-stable factor that in turn requires a heat-labile complement protein(s) as a cofactor to increase GA. Thus, heat-inactivation of AGA plasma activity could be explained by removal of the heat-labile cofactor, and the heat-stability of the AGA factor itself demonstrated by return of its effect when guinea pig serum is added to the heated plasma. Removal of AGA activity from human plasma by clotting suggests that either the factor or a cofactor is related to the coagulation system. The factor does not appear to be fibrinogen because both heat-inactivated plasma and serum from individuals with inflammation fail to augment GA despite addition of 500 mg/100 ml fibrinogen. The fact that both pseudoglobulin and euglobulin fractions of AGA plasma show adherence-augmenting activity, as do most fractions from the plasma's Sephadex column separation, suggests that there may be a number of AGA factors and cofactors; alternatively, the factors may polymerize or split into subunits, and thus appear in multiple fractions. The failure of dialysis to decrease AGA plasma activity indicates that the factor(s) has a molecular weight greater than 10,000, substantiated by the Sephadex column separation results.

The significance of the increased GA found in inflammation is unclear. The induction of an AGA factor by inflammation may increase adherence of granulocytes *in vivo*, thereby delivering increased numbers of cells from the central blood flow to the endothelial surface, for potential diapedesis if attracted locally by chemotactic factor(s). This increased adherence could explain the margination of granulocytes that occurs early in inflammation and with endotoxin administration (4, 5).

We have shown that kinetic shifts between circulating and marginal granulocyte pools are associated with changes in GA: epinephrine and glucocorticoid therapy decrease GA, and shift cells from the marginal to the circulating pool, while endotoxin increases GA and causes granulocyte margination (6). Moreover, anti-inflammatory drugs inhibit GA in normal individuals, and return the augmented adherence associated with inflammation toward normal if successful in controlling the inflammation (7). Thus, there is evidence that granulocyte adherence may play an important role in the inflammatory process and in kinetic shifts of granulocytes among the various body compartments. Further studies of the relationship between GA and inflammation, including characterization of the mediating plasma factors, are in progress.

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