Effect of Flow on Polymorphonuclear Leukocyte/Endothelial Cell Adhesion

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The effect of flow on the adhesion of polymorphonuclear leukocytes (PMNL) to vascular endothelium was investigated using a parallel plate chamber with a well-defined flow field. Washed PMNL were perfused over a monolayer of primary human umbilical vein endothelial cells (HUVEC) pretreated with formyl-methionyl-leucyl-phenylalanine (FMLP, 1 x 10^-7 mol/L) for five minutes. In other experiments HUVEC were pretreated with interleukin 1 (IL1, 2 U/mL) for four hours. PMNL adhesion to stimulated and control HUVEC was measured over a physiologic range of shear stresses. PMNL adhesion to nylon-coated surface was also studied. At a wall shear stress of 0.98 dynes/cm^2, 283 ± 37.3 PMNL/mm^2 (mean ± SEM) adhered to FMLP-treated HUVEC while 195 ± 20.3 PMNL/mm^2 adhered to control HUVEC. At 1.96 dynes/cm^2, 68 ± 14.1 PMNL/mm^2 adhered to FMLP-treated HUVEC and 42 ± 6.0 PMNL/mm^2 adhered to control HUVEC. At 3.92 dynes/cm^2, virtually no PMNL adherence was noted on either control or FMLP-treated HUVEC. On IL1-treated HUVEC at 1.96 dynes/cm^2, 371 ± 25.8 PMNL/mm^2 adhered while 28 ± 2.9 PMNL/mm^2 adhered to control HUVEC. PMNL adhesion to IL1-treated and control HUVEC dropped to 10.2 ± 3.8 and 6.8 ± 3.5 PMNL/mm^2, respectively, at 3.01 dynes/cm^2. The effect of flow on PMNL adhesion appears to be an important factor in determining the outcome of the PMNL/HUVEC adhesive interaction under these experimental conditions.

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O NE OF THE FIRST responses of the immune system to injury or infection is the margination of leukocytes to the blood vessel wall. While the effects on leukocytes and endothelium of various inflammatory mediators have been extensively studied under quiescent conditions, little work has been done in characterizing the magnitude and significance of physical forces on the process of leukocyte attachment to the vessel wall. With the well-defined flow field that exists in a parallel-plate flow chamber, the importance of wall shear stress in the margination of polymorphonuclear leukocytes (PMNL) can be quantitated with respect to the effects of inflammatory mediators.

A number of recent investigations have pointed to an independent role for the endothelium in mediating PMNL adhesion. Chemotactic compounds such as leukotriene B4, complement fragment C5a, and formyl-methionyl-leucyl-phenylalanine (FMLP) in nanomolar concentrations have been shown in several laboratories to increase adhesion of PMNL to endothelium, apparently by action on the endothelial cell (EC). In one set of experiments we used the bacterial endotoxin analog, FMLP, to stimulate adhesion of PMNL to endothelium. Perhaps the most dramatic modulators of the adhesive interaction now being investigated are interleukin 1 (IL1), tumor necrosis factor, thrombin, and lipopolysaccharide. These agents induce large increases in

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MATERIALS AND METHODS

Endothelial cell cultures. Human umbilical vein endothelial cells (HUVEC) were harvested using collagenase digestion, pooled, seeded onto glass slides (34 × 74 mm, Fisher Scientific Co, Springfield, NJ), and cultured in M199 (GIBCO Laboratories, New York) supplemented with 20% fetal calf serum (FCS, HyClone Laboratories Inc, Logan, UT), penicillin, streptomycin, and neomycin (PSN, 1% by volume, GIBCO). Cultures were used in experiments two to four days after reaching confluence.

Preparation of human PMNL. Venous blood from normal, healthy male donors was drawn into heparin (10 U/mL, final concentration). PMNL were isolated from heparinized whole blood by dextran sedimentation followed by hypotonic lysis of erythrocytes and centrifugation over Ficoll-Hypaque (FH). The final pellet was re suspended in M199 at pH 7.4 to which 3.75 mg/mL of bovine...
serum albumin (BSA, Sigma Chemical Co, St. Louis) was added (M199 + BSA). PMNL were counted with a model ZBI Coulter Counter (Coulter Electronics Inc, Hialeah, FL) connected to a Coulter Channelyzer (Coulter Electronics) to determine the appropriate dilution ratio. Trypan blue exclusion showed greater than 96% viability.

Preparation of platelet-poor plasma. Venous blood was drawn into siliconized glass tubes containing heparin (10 U/mL, final concentration). After centrifugation at 400 × g for ten minutes, the supernatant was drawn off with a Pasteur pipet and centrifuged again at 1,000 × g for ten minutes. The resultant platelet-poor plasma (PPP) was transferred to a clean plastic tube.26

Experimental apparatus. To produce a well-defined wall shear stress, we used a parallel plate geometry for our flow chamber (Fig 1A).27-29 One side of the chamber was a slide on which HUVEC were cultured. The other side was machined from polycarbonate. Once assembled these two flat surfaces were held approximately 250 μm apart by a Silastic rubber gasket (Dow Corning, Midland, MI). Because of the small gap-to-width ratio, the channel could be approximated over most of its surface as two infinite parallel plates. Under laminar flow conditions such as used in our experiments (Reynolds number < 2), the velocity profile is parabolic and the wall shear stress can be calculated utilizing the momentum balance for a Newtonian fluid.30 As described in the following equation, the wall shear stress is equal to:

\[ \tau = \frac{3\mu Q}{2a b} \]

\[ \tau = \text{Wall shear stress} \]

\[ \mu = \text{Coefficient of viscosity} \]

\[ Q = \text{Volumetric flow rate} \]

\[ 2a = \text{Channel height} \]

\[ b = \text{Channel width} \]

A model 935 Harvard Apparatus syringe pump (South Natick, MA) was employed for flow control. Experiments were conducted at the following wall shear rates: 140 sec⁻¹, 280 sec⁻¹, 430 sec⁻¹, and 560 sec⁻¹. These shear rates, for a flowing tissue culture medium at 37°C, correspond respectively to wall shear stresses of 0.98, 1.96, 3.01, and 3.92 dynes/cm². This range of shear stresses is similar to those seen in venules and veins. PMNL/HUVEC interactions under these flow conditions were visualized directly using phase-contrast videomicroscopy and digital image processing (Fig 1).

Adhesion assay. The HUVEC covered slide was fitted onto the flow chamber and vacuum applied to hold it in place. The chamber was then rinsed with M199 + BSA at 37°C for 30 minutes. The PMNL suspension was diluted with M199 + BSA at 37°C to a cell concentration of 1,000 cells/μL and 30 minutes was allowed for the PMNL to stabilize. The PMNL suspension, the connecting tubing, and the microscope with flow chamber (Fig 2) were all maintained at 37°C by an air-curtain incubator (Laboratory Products, Boston; Model 279). Approximately 30 minutes after the PMNL were diluted, the HUVEC were exposed to 1 × 10⁻¹⁰ mol/L FMLP for five minutes and then rinsed for five minutes with M199 + BSA. The PMNL suspension was then perfused through the flow chamber. Using a Hamamatsu video camera (Waltham, MA; Model C1012) mounted on a Nikon Diaphot-TMD inverted phase-contrast microscope (Garden City, NY), the experiments were videotaped. The number of PMNL attached to the HUVEC monolayer were counted after ten minutes of flow. The control consisted of the same experimental procedure without the incubation of the HUVEC with FMLP. For experiments involving IL 1 (Genzyme Inc., Boston), 100 μL of IL 1 (200 U/mL) in 0.5% BSA was introduced into the petri dish containing the HUVEC monolayer and diluted in the tissue culture medium to 2 U/mL. The HUVEC were incubated with the IL 1 for four hours preceding the experiment.31 The control consisted of untreated HUVEC. The PMNL for each experiment were isolated from a single donor, and the HUVEC monolayers were from the same pooled seeding.

PMNL rolling velocities were measured by digital image processing (Perceptive Systems Inc., Houston; Model 327) of videotapes. The time interval used for these velocity measurements was 30 seconds to resolve PMNL velocities below 1 μm/sec. Accordingly, a stable adhesion was defined as one that lasted 30 seconds or longer. Normalized histograms of velocity distributions and the averages were determined from analyzing two to four frames of videotape separated by at least one minute in each experiment.

The same procedure for PMNL preparation was used with studies of PMNL adhesion to nylon. Glass slides were coated with Elvamide nylon (DuPont, Wilmington, DE) by solvent casting (MeOH, 1% solution). In some experiments the nylon surface was incubated with PPP for 20 minutes followed by a two-minute washout before the PMNL suspension was perfused through the chamber.

Statistical analysis. The variation in adhesion ratios was analyzed using a one-tailed t test for paired data since the control, FMLP-treated and IL 1-treated HUVEC, were from the same population of pooled cells, and the washed PMNL used in the experiment were from the same donor. Ratios of the means of PMNL rolling velocities on FMLP-treated HUVEC and controls as well as ratios of the means at two shear stresses were compared using a two-tailed t test. A two-tailed t test was also used to compare PMNL rolling velocity distributions. Error bars on all histograms represent the SEM.
RESULTS

In paired experiments at 0.98 dynes/cm² wall shear stress, the ratio of PMNL adhesion to FMLP-treated HUVEC vs control HUVEC was 1.45 (P < .01, n = 9, Fig 3). At this wall shear stress 283 ± 37.3 PMNL/mm² (mean ± SEM) adhered to FMLP-treated HUVEC and 195 ± 20.3 PMNL/mm² adhered to control HUVEC after ten minutes of flow. At 1.96 dynes/cm² wall shear stress, the ratio of PMNL adhesion to FMLP-treated HUVEC vs control HUVEC was 1.58 (P < .025, n = 8). At this wall stress 68 ± 14.1 PMNL/mm² adhered to FMLP-treated HUVEC and 42 ± 6.0 PMNL/mm² adhered to control HUVEC after ten minutes of flow. At 3.92 dynes/cm² wall shear stress, virtually no cell adherence was noted on either control or FMLP-treated HUVEC (<1 cell/mm², n = 3), although there were many transient white cell/endothelial cell interactions.

In paired experiments at 1.96 dynes/cm² wall shear stress, 371 ± 25.8 PMNL/mm² adhered to IL 1-treated HUVEC and 28 ± 2.9 PMNL/mm² adhered to control HUVEC, resulting in an adhesion ratio of 13.8 ± 1.2 (P < .01, n = 5, Fig 4). At 3.01 dynes/cm² wall shear stress, 10.2 ± 3.8 PMNL/mm² adhered to IL 1-treated HUVEC and 6.8 ± 3.5 PMNL/mm² adhered to control HUVEC, giving an adhesion ratio of 1.65 ± 0.25 (P < .05, n = 5).

At 0.98 dynes/cm² wall shear stress, 9.7 ± 3.8 PMNL/mm² adhered to the nylon surface while on nylon pretreated with PPP, 77 ± 21.7 PMNL/mm² adhered, giving an adhesion ratio of 7.9:1 (P < .01, n = 4, Fig 5). There was almost no adhesion (<1 cell/mm², n = 3) of PMNL to untreated nylon at 1.96 dynes/cm² (data not shown).
At a wall shear stress of 0.98 dynes/cm², PMNL that were attached to control HUVEC had an average rolling velocity of 0.166 ± 0.046 μm/sec; and PMNL attached to FMLP-treated HUVEC had an average rolling velocity of 0.134 ± 0.026 μm/sec (n = 3, Fig 6). The difference between these means due to FMLP treatment of the HUVEC monolayer was not significant (P > .4). At a wall shear stress of 1.96 dynes/cm², PMNL attached to control HUVEC had an average rolling velocity of 0.313 ± 0.054 μm/sec, and PMNL attached to FMLP-treated HUVEC had an average rolling velocity of 0.269 ± 0.047 μm/sec (n = 5). The difference between these means due to FMLP treatment of the HUVEC monolayer was also not significant (P > .4). The ratio of PMNL rolling velocities at 1.96 and 0.98 dynes/cm² wall shear stress was 1.94 (P < .02).

Between 20% and 90% of the PMNL with stable adhesions to the HUVEC monolayer rolled downstream. At 0.98 dynes/cm², less than 1% of the PMNL rolled faster than 1 μm/sec (Fig 7), while at 1.96 dynes/cm², approximately 5% of the PMNL rolled faster than 1 μm/sec (Fig 8). Differences in velocity distributions due to FMLP treatment of the HUVEC monolayer were also not significant (P > .4).

Fig 5. PMNL adhesion to nylon-coated surface under flow (0.98 dynes/cm², 140 sec⁻¹). PMNL were suspended in M199 + BSA (1,000 cells/μL) at 37°C. PMNL/mm² adhering to untreated nylon surface (A), PMNL/mm² adhering to nylon preincubated with PPP for 20 minutes (B), PMNL/mm² adherent to control HUVEC (C). Error bars represent SEM.

Fig 6. Effect of flow and FMLP treatment of HUVEC on the mean rolling velocities of PMNL. Experiments performed at wall shear stresses of 0.98 dynes/cm² (A, 140 sec⁻¹) and 1.96 dynes/cm² (B, 280 sec⁻¹). FMLP-treated HUVEC, cross-hatched bars; control HUVEC, open bars. Error bars represent SEM.

Fig 7. Distribution of PMNL rolling velocities at 0.98 dynes/cm². FMLP-treated HUVEC, cross-hatched bars; control HUVEC, open bars. Each pair of bars represents the fraction of adherent PMNL with rolling velocities ranging from 0.0 to 0.13 μm/sec (A), 0.13 to 0.26 μm/sec (B), 0.26 to 0.39 μm/sec (C), 0.39 to 0.52 μm/sec (D), 0.52 to 0.65 μm/sec (E), 0.65 to 0.78 μm/sec (F), 0.78 to 0.91 μm/sec (G), and 0.91 to 1.04 μm/sec (H). To generate a representative velocity distribution for each experiment, the normalized velocity distributions from two to four image frames of videotapes (separated in each case by at least one minute) were averaged. Histograms from experiments under the same conditions were again averaged to create a final normalized velocity distribution. Error bars represent SEM.
H U V E C monolayer were not significant at the two flow conditions examined, although a broader distribution of rolling velocities was observed at 1.96 dynes/cm².

In experiments with nylon surfaces, we observed no PMNL downstream rolling, although PMNL in the plane of focus next to the nylon surface had velocities that ranged from 300 to 600 μm/sec. Extensive spreading of PMNL attached to the PPP-incubated nylon surface was observed.

**DISCUSSION**

From our experiments fluid drag forces appear to have a major impact on the number of PMNL adhering to endothelium. Doubling the wall shear stress from approximately 1 to 2 dynes/cm² reduced the number of adherent PMNL by more than a factor of four on both control and FMLP-treated endothelium. Doubling the wall shear stress again from approximately 2 to 4 dynes/cm² reduced adhesion to virtually zero. On IL 1-treated endothelium, the level of adhesion was 13-fold more than on controls at 2 dynes/cm², as was expected from earlier results of static experiments on PMNL/EC adhesion.³ Increasing the wall shear stress to approximately 3 dynes/cm² on the IL 1-treated HUVEC dropped both the adhesion ratio and the absolute number of adherent PMNL dramatically. This large decrease in the level of PMNL adhesion, even on highly stimulated endothelium, over a small range of wall shear stresses is in accord with Lackie's proposal that the PMNL/EC adhesive force is delicately balanced with the shear force due to fluid drag.²⁵²⁶

The wall shear rates examined in these experiments are close to those estimated for the venules and large blood vessels but are lower than those estimated for the capillary bed where there is normally little PMNL adhesion.²³ In vivo, the abrupt drop in shear forces found in the transition from capillaries to venules coincides with the location of the bulk of PMNL adhesion in the microcirculation.²⁴ A qualitatively similar pattern can be seen in our model vessel data using dilute suspensions of PMNL. Our data suggest that PMNL adhesion to endothelium (even activated endothelium) can occur only after shear forces have dropped below a threshold level and may explain in part why PMNL normally adhere in venules and not in capillaries.

By eliminating complex geometry and the resultant complex fluid mechanics, the flow system described simplifies the type of forces a PMNL experiences and permits a direct correlation of wall shear stress with the level of PMNL/EC adhesion. In our experiments a dilute, washed PMNL suspension was used so that any influence of platelets or erythrocytes would be eliminated. However, there has been some recent evidence that in plasma PMNL adhesion to EC is reduced.²⁸ Pretreatment of the PMNL with FMLP was not done, as the purpose of this work was to evaluate the role of the endothelial cell substrate on PMNL adhesion. Preactivation of PMNL would probably lead to very different levels of leukocyte adhesion to HUVEC. It has been shown that FMLP causes a 20-minute aggregation response in PMNL, so the incubation times for endothelial cells were selected accordingly.²⁰ Tonneson et al²⁹ have reported that exposure of endothelial cell monolayers to FMLP at a comparable concentration for periods of time ranging from 15 minutes to 4 hours did not increase PMNL adhesion to the monolayer. Thus, the small increases in adhesion seen in our work (Fig 3) and that of others may be due to some PMNL activation by low levels of residual FMLP.¹³

The number of PMNL sticking to nylon was very low when compared with the number of PMNL that adhered to endothelium at a wall shear stress of 0.98 dynes/cm². When the wall stress was doubled, we observed no PMNL adhering to the untreated nylon surface. The contact of the nylon-coated flow chamber wall with PPP, as would occur if blood were in contact with a similar type of foreign surface, dramatically increased the adhesion of PMNL.³⁰ (Fig 5). PMNL have receptors for the complement fragments C3b and C3bi for effectively attaching to opsonized surfaces and facilitating the phagocytosis of bacteria.³¹,³² However, when nylon activates the C3 convertase enzyme, the C3b and C3bi fragments formed probably bind to nylon’s polysaccharide-like surface, possibly forming an “opsonized” surface for the PMNL to attach to.

An unattached PMNL near the wall of our flow chamber would be moving at a velocity of several hundred μm/sec if there were no interactions with the endothelial cell monolayer, as was the case of PMNL near the nylon surface. In
contrast, the mean rolling velocities of PMNL we report on endothelium are considerably less than 1 \( \mu m/sec \), which indicates that there is a strong adhesive interaction occurring. In addition, our data show a strong dependence of mean rolling velocity on wall shear stress. The mean rolling velocity of PMNL subjected to a wall shear stress of 1.96 dynes/cm\(^2\) was approximately double the mean rolling velocity of PMNL subjected to 0.98 dynes/cm\(^2\).

Atherton and Born\(^{10}\) have reported that in vivo granulocyte rolling velocities exhibit a strong flow dependence. Unexpectedly, there was no significant difference in average PMNL rolling velocities due to FMLP-pretreatment of the HUVEC at either flow rate examined. With both FMLP and control experiments, the effect of increased wall shear stress was to broaden the distribution of velocities of rolling PMNL at the higher flow rate as well as to raise the mean rolling velocity of attached cells. The velocity distributions in all the experiments analyzed were similar and show consistently that 20% to 90% of the attached PMNL were rolling downstream. Since a large fraction of PMNL, roll downstream due to flow, it is possible that this intermediate level of adhesion is a property of leukocytes that allows them to marginate from the bulk flow in the circulation yet still retain some mobility along the vessel wall. In our experiments with PMNL adhering to nylon, we observed what appeared to be cell spreading but no rolling PMNL, which suggests the interaction is of a different nature than the PMNL/HUVEC adhesive interaction.

Little is known about the type and strength of the bonds formed between PMNL and EC or the actual forces on the interface that determine whether or not the PMNL will adhere.\(^{8,11}\) Employing a centrifugation assay that determined the force needed to separate PMNL from an endothelial cell monolayer after coincubation of the two cell types, Charo et al\(^{19}\) have reported that FMLP and C5a increase the strength of PMNL adhesion to endothelium. A stronger adhesive interaction with the stimulated endothelium implies that rolling velocities would be lower, and PMNL flowing near the EC monolayer would be more readily arrested. However, only slight differences were observed between PMNL rolling velocities on FMLP-treated and control endothelium. Additionally, our data indicated the threshold shear stresses for PMNL adhesion to untreated, FMLP-treated, and IL 1-treated HUVEC were very close to each other. The pattern of PMNL adhesion to HUVEC at different shear stresses suggests that, while there were many more adhesive sites on IL 1 and FMLP-treated endothelium, the adhesive bonds formed were not necessarily stronger than on untreated HUVEC.

Even though static conditions in the blood rarely occur, most studies of leukocyte adhesion have been conducted in systems without controlled flow effects. These studies have revealed much about the biochemical interactions involved in leukocyte adhesion to endothelium but say little about the relative importance of different forms of activation under the wide range of physiologic conditions PMNL encounter in vivo. Our data, showing that slight changes in wall shear stresses can cause large changes in PMNL adhesion to both stimulated and unstimulated HUVEC, suggest that the local control of blood flow—perhaps by vessel dilation—may be as significant as the presence of inflammatory mediators in determining where the arrest of leukocytes occurs.

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