Platelet-Dependent Primary Hemostasis Promotes Selectin- and Integrin-Mediated Neutrophil Adhesion to Damaged Endothelium Under Flow Conditions

By P.H.M. Kuijper, H.I. Gallardo Torres, J.A.M. van der Linden, J.-W.J. Lammers, J.J. Sixma, L. Koenderman, and J.J. Zwaginga

Co-localization of blood platelets and granulocytes at sites of hemostasis and inflammation has triggered an intense interest in possible interactions between these cellular processes and induction of vessel wall injury. Leukocyte adhesion to endothelial cells decreases with increasing shear and is dependent on an initial rolling phase mediated by selectins. We hypothesized that flow-dependent platelet adhesion at an injured vessel wall will lead to P-selectin expression by platelets, thus mediating leukocyte co-localization. A perfusion chamber was used in which flowing whole blood induced platelet adhesion to a subendothelial matrix (ECM) of cultured human umbilical vein endothelial cells (HUVEC). We compared neutrophil (polymorphonuclear leukocyte [PMN]) interactions with HUVEC and their ECM with and without adhered platelets. PMNs adhered predominantly to ECM-adhered platelets and not to endothelial cells. ECM alone did not support PMN adhesion under flow conditions.

PMN adhesion to unstimulated HUVEC was only substantial at low shear (up to 200 cells/mm² at shear stress 80 mPa). In marked contrast, PMN adhesion to ECM-adhered platelets was dramatically increased, and adhesion was demonstrated at much higher shear stress (up to 640 mPa). Studies with specific antibodies showed that the platelet-dependent neutrophil adhesion was selectin-mediated. Inhibition of P-selectin caused a marked inhibition of adhesion at high shear stress, whereas the role of leukocyte L-selectin was less pronounced. β2-Integrin-blocking antibodies inhibited static neutrophil adhesion. fMLP induced L-selectin shedding from leukocytes, resulting in decreased leukocyte adhesion. In conclusion, platelet-dependent hemostasis at the ECM appears to be a powerful intermediate in neutrophil–vessel wall interactions at shear stresses that normally do not allow neutrophil adhesion to intact endothelium.

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THE PHYSIOLOGY OF hemostasis and inflammation, as well as the pathophysiologist of thrombosis, vasculitis, and metastatic seeding, involves adhesion of cells suspended in flowing blood to the vessel wall. Extensive research has shown the importance of flow and of exposed vessel wall structures in these processes. In this respect, platelet-dependent hemostasis at injured vessel walls is more extensive at higher shear. However, adhesion of leukocytes to endothelium decreases at higher shear.1 Multiple receptor-ligand interactions are usually needed for firm attachment.2 Membrane-associated selectins mediate the initial margination and rolling, but activated β2-integrins (CD18 complexes) are necessary for subsequent firm attachment (or static adhesion) and spreading.3 P- and E-selectin are expressed on endothelial cells upon stimulation, and activated platelets express P-selectin as well. Leukocytes constitutively express L-selectin, which can be quickly shed from the membrane upon activation. Selectins recognize oligosaccharide-based ligands such as sialyl-Lewis X in a calcium-dependent way.4-5 This interaction is sensitive to inhibition by heparin oligosaccharides.6,7 The influence of β2-integrins is clear from the deficient inflammation-induced extravasation of neutrophils seen in leukocyte adhesion deficient (LAD) patients.8 Transgenic mice, deficient in P- and L-selectin, illustrate the importance of selectins for leukocyte extravasation in vivo.9

So far, in vitro experiments suggest that high or arterial shear virtually eliminates binding of granulocytes to endothelium—even after appropriate stimulation.10 However, observations in vivo show that vascular inflammation is certainly not limited to vessels in which a low shear is found.11 Together with the presence of platelet deposition at high shear inflammatory sites,1,2,11 these observations prompted us to study the role of platelets in leukocyte adhesion to a damaged vessel wall under high-shear conditions. Here, extracellular matrix (ECM) exposure to flowing blood leads to rapid platelet adhesion, activation, and immediate expression of P-selectin.12 In vivo observations in atherosclerosis, vasculitis, acute cardiovascular accidents, and thrombosis have shown that both local platelet adhesion and large numbers of leukocytes are found at these sites.12,13 Moreover, it has been shown that activated platelets in suspension or bound to artificial structures support leukocyte adhesion in a selectin-independent way.14-17 In line with these reports, we hypothesized that exposure of a subendothelial surface to blood leads to acute platelet adhesion and P-selectin expression. This platelet-covered vessel wall surface may then allow leukocyte adhesion that can be highly shear-resistant.

In this study, a perfusion system was used in which flowing whole blood was exposed to endothelium or ECM. In the latter, the mimicking of physiologic conditions leads to a homogeneous platelet adhesion. We subsequently examined the interaction of isolated neutrophils (polymorphonuclear leukocytes [PMNs]) under flow conditions with (damaged) vessel wall components, such as endothelial cells, ECM, and ECM-adhered platelets. The influence of shear on the rolling and adhesion of isolated PMNs to these sur-

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faces was evaluated with real-time video-assisted image analysis. We show that ECM-adhered platelets are a better adhesive substrate for neutrophils, compared with cultured endothelial cells. We hypothesize that in vivo, the primary hemostatic reaction involving platelet deposition provides an intriguing mechanism for leukocyte recruitment at the damaged vessel wall.

MATERIALS AND METHODS

Monoclonal antibodies. The monoclonal antibody (MoAb) CLB/Thromb-6 (anti-β1-selectin, CD62p) was kindly provided by Dr A. Von dem Borne (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The MoAb LAM1-3 (anti-L-selectin, CD62L, 5 µg/mL for blocking studies) was a kind gift from T. Tedder (Harvard Medical School, Boston, MA). The MoAb WASP12.2 (anti-β-selectin, CD62p, 1 µg/mL for blocking studies) was purchased from Endogen (Boston, MA). MoAbs 44a (CD11b, 10 µg/mL for blocking studies) and IB4 (CD18, 10 µg/mL for blocking studies) were purchased from the American Type Culture Collection (Rockville, MD). The MoAb Leu-8 (anti-L-selectin, CD62L) and CSELI (anti-sialylLea) were from Becton Dickinson (San Jose, CA). The MoAb 80H3 (CD66b) was purchased from Immunotech (Marseille, France).

Reagents. Percoll was obtained from Pharmacia (Uppsala, Sweden). DMLP, thrombin, and 4% polyborohydride, 12-myristate-acetate (PMA) were purchased from Sigma (St Louis, MO). Experiments were performed in HEPES buffer (20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1.2 mmol/L KH2PO4, 1 mmol/L MgSO4·7H2O, 5 mmol/L glucose, and 1 mmol/L CaCl2, pH 7.4). HEPES buffer was made from neutral calcium concentrations were made according to the method described by Marks and Maxfield. The heparin Thromboliquine was obtained from Organon Teknika (Boxtel, The Netherlands). Tissue culture supplies (media, antibiotics, and trypsin) were purchased from Gibco Biocult (Paisly, UK). All other reagents were of reagent grade.

Perfusion chamber and set-up. Perfusion experiments were performed in a transparent rectangular perfusion chamber with a slit height of 0.3 mm and width of 6 mm. It is a modification from the method of Jaffe et al., with some minor modifications. The perfusion chamber was mounted on a microscope stage (Boxtel, The Netherlands). Tissue culture supplies (media, antibiotics, and trypsin) were purchased from Gibco Biocult (Paisly, UK).

Materials and methods.

Monoclonal antibodies.

Methods.

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Endothelial cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins according to the method of Jaffe et al., with some minor modifications. The cells were cultured in RPMI 1640 containing penicillin, streptomycin, and amphotericin B, with 20% (vol/vol) heat-inactivated human serum. Endothelial cells of the second passage were harvested by trypsin digestion and subcultured on glass cover slips. Endothelial monolayers used in perfusion experiments were either cultured on gelatin-coated cover slips or on cover slips upon which the isolated matrix of smooth muscle cells was present. In the latter case, HUVEC were more firmly attached and more resistant to high shear stress compared with cells grown on gelatin-coated cover slips, as described by Reutelingsperger et al. HUVEC confluency and morphology were checked by phase-contrast microscopy before and after experiments. Confluent HUVEC were used in perfusion experiments with whole blood and neutrophils. In some cases, HUVEC were stimulated with tumor necrosis factor alpha (TNF-α) before the experiment. Therefore TNF-α (100 U/mL) was diluted in fresh culture medium and incubated for 6 hours at 37°C.

Surface ECM coating. Cover slips with ECM were obtained as described previously. In short, HUVEC were cultured on 1% gelatin-coated cover slips. Cell monolayers were grown to confluence in 5 to 7 days. The matrix was obtained after removal of endothelial cells by exposure to 1 mol/L NaOH for 5 minutes at room temperature. Isolated matrices were washed and kept in phosphate-buffered saline (PBS) 1:10 vol/vol 0.1-mol/L sodium phosphate buffer in 150 mmol/L NaCl) at 4°C for a maximum of 3 weeks.

Surface platelet-covered HUVEC/ECM. Platelet adherence to different surfaces was accomplished by perfusing whole blood from healthy volunteer donors (Blood Bank, Utrecht, The Netherlands) as described previously. The blood was anticoagulated with 92.5 vol 110 mmol/L trisodium citrate. When a monolayer of HUVEC were used as a surface, whole blood was perfused at 37°C for 5 minutes at a shear rate of 400 s⁻¹, which corresponds with a shear stress of approximately 2 Pa with a blood viscosity of 5 mPa·s. At this shear rate, the HUVEC monolayer stayed largely intact, while at higher shear rates HUVEC are progressively shedded from the matrix. To induce platelet adhesion to ECM-coated cover slips, 15 mL whole blood was perfused at 37°C for 7 minutes at a shear rate of 1,600 s⁻¹ (ie, a shear stress of ~7 Pa) in a recirculating system. After completion of the whole-blood perfusion, cover slips were rinsed with HEPES buffer to remove blood remnants. To determine the morphology and percentage of ECM surface covered with platelets, some cover slips were fixed (1% paraformaldehyde) and stained with May-Grünewald/Giemsa. Under given conditions, homogenous platelet coverage of 85% ± 4% (mean ± SE, n = 24) of the surface was accomplished. The coverage was evaluated by light microscopy using a Quantimet 570C image analysis system (Leica/ Cambridge, Cambridge, UK).

Neutrophil isolation. Blood was obtained from healthy volunteers from the Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were purified from theuffy coat of 500 mL blood anticoagulated with 0.34% (wt/vol) trisodium citrate (pH 7.4) as described previously. In short, mononuclear cells were removed by centrifugation over Ficoll-Paque (density, 1.077/mL; Pharmacia, Uppsala, Sweden). Remaining erythrocytes were lysed by incubation in isotonic 115 mmol/L ammonium chloride solution (pH 7.4) at 0°C for 20 minutes. After centrifugation, the remaining granulocytes were regenerared in RPMI 1640 (containing 0.5% human serum albumin) for 30 minutes at 37°C, diluted in HEPES buffer (2 × 10⁵ cells/mL), and kept at room temperature until the start of the perfusion. Neutrophil purity was more than 95%, and viability measured with Trypan blue exclusion was more than 98%; morphology was controlled by light microscopy. For blocking experiments, neutrophils were preincubated with monoclonal antibodies for 30 minutes at room temperature. Before perfusion, neutrophils were always prewarmed for 5 minutes at 37°C.

Perfusion and evaluation. Neutrophil perfusions were performed as individual runs under specific shear conditions. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE; Leica, Wetzlar, Germany) equipped with a B/W CCD-video camera (Sanyo, Osaka, Japan) coupled to a VHS video recorder. Perfusion experiments were recorded on video tape. Video images were evaluated for the number of adhered cells and the rolling velocity per cell with a Quantimet 570C image-analysis system.
system (Leica). The neutrophils, which were in contact with the surface, appeared as bright white-centered cells, after proper adjustment of the microscope during recording. Single adhering cells on the endothelium- or platelet-covered ECM were detected by the image analyzer. The number of surface-adhered neutrophils was measured after 5 minutes of perfusion, unless stated otherwise, at a minimal of 20 randomized high-power fields (total surface, \( \approx 1 \) mm\(^2\)). The software used was designed to automatically determine the (rolling) velocity of cells in focus. To accomplish this, a sequence of a given number of images was digitally captured with a certain time interval (\( \delta t \)). At each image, the position of every cell in focus was detected as a solid of the total area of the cell and as a central dot. For all subsequent images, overlapping solids were added together, resulting in a stretched total detected area for moving cells and a round total solid area for static cells. The length and width of all total outlines were measured, together with the total amount of dots within each outline. Cell velocities (\( v \)) in millimeters per second were calculated from the equation, 

\[
v = \frac{(C \cdot (1 - w))(\Delta t \cdot (x - l))}{(A + t(x - l))}
\]

in which \( C \) is the calibration value of the microscope image-analysis system in micrometers per pixel, \( l \) is the length and \( w \) the width of the total outline in pixels, \( \delta t \) is the time interval between images in milliseconds, and \( x \) the number of dots within a total outline. The cutoff value to distinguish between rolling and static adherent cells was set at 1 \( \mu \)m/s. With this method, static adherent, rolling, and freely flowing cells (which were not in focus) could be clearly distinguished.

**Immunoﬂuorescence flow cytometry.** Surface marker expression by activated neutrophils was determined on a FACSvantage flowcytometer (Becton Dickinson Co, Mountain View, CA). Neutrophils (2 \( \times 10^6\) /mL HEPES buffer) were incubated with different concentrations of fMLP for 5 minutes at 37\(^\circ\)C and immediately ﬁxed in ice-cold paraformaldehyde (1% /wt/vol). The cells were stained using anti-CD18 antibody IB4, anti-CD11b antibody 44a, anti-L-selectin antibody LAM1-3, or anti-CD67 antibody, together with FITC-labeled goat antimouse Ig serum. The samples were kept on ice until analysis.

**Statistical analysis.** Results are expressed as the mean ± SE. Statistical analysis of the data was performed using a paired Student’s \( t \) test for single measurements or repeated-mesures analysis of variance (MANOVA) for series of measurements. \( P \) values less than .05 were considered significant.

**RESULTS**

**Neutrophil adhesion to a damaged vessel wall model.** First, we studied the rolling and adhesion behavior of neutrophils on a model of a damaged vessel wall. A monolayer of HUVEC cultured on a gelatin surface were perfused with citrated whole blood at a shear rate of 400 s\(^{-1}\) for 5 minutes. This shear induced microlesions in the HUVEC monolayer due to partial cell retraction or HUVEC shedding from their ECM. The exposed ECM induced acute platelet adhesion, finally covering 20% ± 5% (mean ± SE, \( n = 16 \)) of the total surface (Fig 1A). The HUVEC surface with platelet deposition at the ECM was subsequently used to study rolling and adhesive behavior of puriﬁed unstimulated neutrophils under flow conditions. Interestingly, neutrophils adhered predominantly to blood platelets and not to endothelial cells (Fig 1B). At a shear stress of 80 mPa, an eightfold greater adhesion to platelet-covered surfaces as compared with HUVEC was observed (≈570 and 70 PMN/mm\(^2\), respectively; Table 1). At 320 mPa, this predominance of neutrophil adhesion to platelets was even more pronounced. Here, only 1% of adherent neutrophils interacted with HUVEC. Approximately half the adhering neutrophils showed a rolling interaction with the platelet-covered spots. Rolling neutrophils that reached the end of a platelet-rich spot often detached from the surface.

The effect of additional stimulation of the HUVEC/platelet surface was examined in similar experiments in which the surface was incubated with thrombin (1 U/mL for 5 minutes at 37\(^\circ\)C) or TNF-\( \alpha \) (100 U/mL for 6 hours at 37\(^\circ\)C). After thrombin stimulation, which induces maximal P-selectin expression, the prevalence of the neutrophils adhering to platelets did not change at shear stress 80 mPa, showing a small nonsignificant decrease at 320 mPa from 97% to 95% (Table 1). TNF-\( \alpha \), which induces E-selectin and ICAM-1 expression on the endothelium, did show an increased neutrophil adhesion to HUVEC, but still more neutrophils adhered to platelets. To further characterize the shear-dependent mechanisms of neutrophil adhesion to vessel wall structures, we performed subsequent perfusion experiments in which the reactivity of PMNs with HUVEC, ECM, or ECM-adhered platelets was separately tested.

**Neutrophil adhesion to HUVEC.** Neutrophil adhesion to a HUVEC monolayer cultured on a matrix of smooth muscle cells was examined at varying shear stresses. The observed neutrophil adhesion was both time- and shear-dependent, as determined by repeated-measures ANOVA (\( P < .01 \)). Neutrophil adhesion decreased with increasing shear up to 80 mPa (Fig 2); at less than 80 mPa, adhesion showed a linear nonsaturating increase in time of up to 1,100 cells/mm\(^2\) after 20 minutes. At a shear stress of 200 mPa (2 dynes/mm\(^2\)) and higher, no significant PMN rolling or adhesion to HUVEC could be detected. Incubation of HUVEC with TNF-\( \alpha \) (100 U/mL for 6 hours at 37\(^\circ\)C) increased neutrophil adhesion at all shear stresses, allowing adhesion up to a shear of 320 mPa (Fig 2).

To examine the effect of stimulus-induced maximal P-selectin expression, HUVEC were stimulated with thrombin (1 U/mL for 5 minutes) before PMN perfusion.\(^{25,26}\) Table 2 shows the increased neutrophil adhesion at shear stress 40 mPa to stimulated HUVEC versus unstimulated HUVEC. After the experiments, HUVEC did not show shear-induced changes in morphology, as examined with a phase-contrast microscope. Thrombin-stimulated HUVEC were slightly contracted after perfusion, but still covered 80% to 90% of the surface. Higher thrombin concentrations showed no additional effect on PMN adhesion but increased the change in HUVEC morphology (data not shown). Pretreatment of neutrophils with the chemotaxin fMLP at a concentration of 10\(^{-7}\) mol/L, which is known to induce integrin activation,\(^{27}\) resulted in increased PMN adhesion (Table 2). Stimulation of neutrophils with fMLP caused pseudopodia formation in 40% to 50% of the PMNs. Total adhesion was increased more after simultaneous stimulation of both neutrophils and HUVEC. These results are largely in agreement with known characteristics of neutrophil-EC interactions,\(^{26,28,29}\) thus validating the perfusion system.

**Neutrophil adhesion to ECM.** HUVEC ECM alone did not support PMN adhesion under flow conditions (shear...
Fig 1. (A) A monolayer of endothelial cells was perfused for 2 minutes with citrated whole blood at a shear rate of 400 s\(^{-2}\). The surface is covered with spread, slightly contracted HUVEC and platelets adhering to ECM (arrows). (B) Unstimulated neutrophils adhere predominantly to the platelet-covered loci under flow conditions (arrows). (D) Immunofluorescent staining of ECM-bound platelets using a P-selectin antibody. (C) Unstimulated neutrophils adhered to the platelets bound to ECM (arrow) and not to ECM alone (*). Under flow conditions.

Neutrophil adhesion to ECM-bound platelets. Since we have observed that activated platelets were more potent in supporting neutrophil adhesion in flow as compared with (activated) endothelium, we examined the platelet-PMN interaction in more detail. Platelets were allowed to adhere to ECM during a perfusion with anticoagulated whole blood (7 minutes at shear rate 1,600 s\(^{-1}\)). The platelets covered 85% ± 3% (mean ± SE, n = 24) of the ECM surface and were spread or dendritic with a few small aggregates. Immunofluorescent staining of ECM-adhered platelets with a...
monoclonal antibody to P-selectin showed the clear uniform presence of P-selectin on the platelet surface, indicating the highly activated state of the adhered platelets (Fig 1D). This expression was not enhanced after thrombin stimulation, and P-selectin expression was not decreased after periods of up to 3 hours. Subsequent perfusion of isolated PMNs showed that neutrophils adhered in large numbers to ECM-adherent platelets and that this adhesion decreased with increasing shear stress (Fig 3A). At a shear stress of 80 mPa, which permitted little neutrophil adhesion to HUVEC, 1,250 ± 90 cells/mm² adhered to platelets. Even at much higher shear stress (320 and 640 mPa), an appreciable amount of neutrophils still adhered to the platelet surface. All neutrophils were localized at platelet-covered parts of the cover slip, as far as could be determined by phase-contrast microscopy and after May-Grunwald-Giemsa staining (Fig 1C). Figure 3B shows the percentage of neutrophils that show a rolling interaction with the surface and the mean rolling velocity of these cells at different shear stresses. Both the percentage and rolling velocity increased with shear stress, reaching a maximum at a shear stress of 320 mPa.

Effect of Ca²⁺ depletion and heparin on neutrophil adhesion to platelets. Both integrins and selectins bind their ligands in a Ca²⁺-dependent way, whereas heparin treatment prevents the selectin-ligand binding. Therefore, the effect of Ca²⁺ depletion and heparin on neutrophil adhesion to platelet-covered surfaces was studied. Using HEPES buffer (1 mmol/L free Ca²⁺) as a control and buffers with different free Ca²⁺ concentrations, we observed a Ca²⁺-dependent inhibition of PMN adhesion (Fig 4A). At shear stress 80 mPa, the inhibitory effect was seen only at concentrations less than 1 µmol/L free Ca²⁺. The effect of Ca²⁺ depletion was significantly more pronounced at a shear stress of 320 mPa, resulting in a decrease of 40% at a Ca²⁺ concentration of 10 µmol/L. Depletion of free Ca²⁺ from the HEPES medium showed greater than 95% inhibition of adhesion at both shear stresses (Fig 4A). Addition of heparin to the

### Table 1. Neutrophil Adhesion to Endothelial Cells and ECM-Bound Platelets Under Flow: Effects of Shear Stress and Stimulation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (80 mPa)</th>
<th>+ Thrombin (80 mPa)</th>
<th>+ TNF (80 mPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells/mm²</td>
<td>640 ± 98</td>
<td>310 ± 66</td>
<td>960 ± 92</td>
</tr>
<tr>
<td>PMNs/mm² adhered to platelets: HUVEC</td>
<td>570:70</td>
<td>280:30</td>
<td>570:410</td>
</tr>
<tr>
<td>% Neutrophil adhesion to platelets</td>
<td>97</td>
<td>97</td>
<td>85</td>
</tr>
</tbody>
</table>

A monolayer of HUVEC cultured on a gelatin surface were perfused with citrated whole blood at a shear rate of 400 s⁻¹ for 5 minutes. A second perfusion with neutrophils (10⁶ cells/mL) was performed at a shear stress of 80 or 320 mPa for 5 minutes (see Fig 1A and B). Thrombin (1 U/mL) was used to stimulate HUVEC and blood platelets on a coverslip for 5 minutes before the neutrophil perfusion. HUVEC were incubated with TNF (100 U/mL) for 6 hours at 37°C and washed before the whole blood perfusion. Platelet coverage and neutrophil adhesion (to either platelets or HUVEC) were determined using an image analyzer system.

### Table 2. Effect of Stimulation on Neutrophil Adhesion to a HUVEC Monolayer Under Flow Conditions

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>HUVEC + Thrombin 1 U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>60 ± 20*</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>350 ± 60*</td>
</tr>
<tr>
<td>FMLP 10⁻⁷ mol/L</td>
<td>572 ± 60</td>
</tr>
</tbody>
</table>

Neutrophils were perfused over a HUVEC monolayer at shear stress 40 mPa with or without stimulation. Neutrophils (2 × 10⁶/mL HEPES buffer) were stimulated with chemotaxin FMLP (10⁻⁷ mol/L) 5 minutes before perfusion at 37°C. HUVEC were activated 5 minutes before perfusion with thrombin (1 U/mL HEPES), and thrombin was washed away before perfusion. The number of surface-interacting neutrophils per mm² after 5 minutes perfusion is represented as the mean ± SE of 3 experiments.

*Statistically significant difference between two perfusions with unstimulated and stimulated cells determined by Student's two-tailed t-test (P < .05).
Fig 3. Neutrophil rolling and adhesion to ECM-bound platelets: effect of shear stress. (A) Number of neutrophils adhering under flow conditions to ECM-coated cover slips with or without preadhered platelets. After a first perfusion with anticoagulated whole blood (○), platelet poor plasma (□), or filtered plasma (△) over an ECM-coated cover slip, the surface was covered with 86%, <2%, or 0% spread platelets, respectively. Subsequently, the total number of adhering and rolling neutrophils/mm² were measured after 5 minutes of perfusion. Mean ± SE of 6 experiments shown. (B) Rolling velocity and percentage of rolling neutrophils during perfusion at different shear stresses. An ECM-coated cover slip preperfused with anticoagulated whole blood and therefore covered with platelets (85% of the surface) was perfused with resting neutrophils (2 × 10⁶/mL HEPES buffer) at a shear stress varying from 20 to 680 mPa. (○) Number of rolling neutrophils as a percentage of total adhered cells; (□) Rolling velocity (µm/s). Mean ± SE of 6 experiments shown.

Fig 4. Effect of calcium depletion and heparin on neutrophil adhesion to ECM-bound platelets. Neutrophils were perfused over a surface of ECM-bound platelets as described earlier. After 5 minutes, the total number of adhering neutrophils was calculated as neutrophils/mm². Numbers are given as % of control values. (A) Neutrophils were preincubated in HEPES buffer with different concentrations of free Ca²⁺ in the presence of 1 mmol/L Mg²⁺ for 5 minutes at 37°C. Subsequently, perfusions were performed at shear stress 80 mPa (○) and 320 mPa (□). Mean ± SE of 3 experiments shown. Significant effects of Ca²⁺ depletion and different shear stresses were determined by repeated-measures ANOVA (P < .05). (B) Neutrophils were incubated with different concentrations of heparin (trombolidine) for 5 minutes at 37°C; perfusions were performed at shear stress 80 mPa (○) and shear stress 320 mPa (□). Mean ± SE of 4 to 6 experiments shown. Significant effects of heparin incubation and different shear stresses are determined by repeated-measures ANOVA (P < .05).
neutrophils before perfusion caused a decrease in adhesion in a concentration- and shear-dependent fashion. At shear stress 320 mPa, addition of 0.1 U/mL heparin already showed an inhibition of 35% (Fig 4B). Under these conditions, absolute inhibition of 97% was seen at a concentration of 250 U/mL heparin, whereas at 80 mPa, inhibition was significantly less effective (63%).

Inhibition of neutrophil-platelet interaction by antibodies. The role of P- and L-selectin and β₂-integrins in neutrophil adhesion to platelets was investigated using monoclonal antibodies. We determined the role of P-selectin expressed by platelets by incubating the platelet surface with monoclonal antibody WASP12.2 against P-selectin (15 minutes at RT). Subsequent perfusion with PMNs over the P-selectin–blocked platelet surface showed a 60% decrease at shear stress 80 mPa, whereas at 320 mPa, approximately 90% of neutrophil adhesion was inhibited (Table 3). Further studies were performed by blocking L-selectin by preincubation of the neutrophils with the antibody LAM1-3. This resulted in a significant inhibition of 70% of neutrophil adhesion at shear stress 320 mPa (P < .05), whereas no significant inhibition was seen at lower shear (Table 3). Blocking of β₂-integrins with the CD11b (clone 44a) and CD18 antibodies (clone IB4) did show a small but nonsignificant inhibition of the total number of PMNs adhered to the surface at shear stresses of 80 and 320 mPa (Table 3). However, at 80 mPa, the number of rolling cells increased significantly compared with the control level (183 rolling PMNs/mm² with control monoclonal antibody, compared with 360 and 408 rolling PMNs/mm² using CD18 and CD11b, respectively). Functional blocking capacity of the integrin antibodies was confirmed in a neutrophil aggregation assay. Here, the antibodies effectively inhibited PMA-stimulated aggregation, which is integrin-mediated (results not shown). Functional inhibition of both P- and L-selectins resulted in a decrease in the number of rolling cells. Interestingly, at the shear stress of 80 mPa, total inhibition (>95%) of neutrophil adhesion was only observed when P-selectin, L-selectin, and CD18 antibodies were used simultaneously. The results indicate that the integrin antibodies can only block static adhesion, whereas the selectin antibodies are more effective in blocking the rolling interaction of PMNs to platelets. In all experiments, similar incubations with isotype-matched control antibodies showed no significant effects.

**Neutrophil adhesion to platelets after fMLP stimulation.** To characterize the effect of neutrophil stimulation on PMN interaction with ECM-associated platelets, we incubated PMNs with the bacterial formyl peptide and chemoattractant, fMLP. Subsequent perfusions were performed at different shear stresses. In contrast to the increased adhesion by activation of neutrophils to HUVEC (Table 2), fMLP stimulation of PMNs caused a shear-dependent inhibition of neutrophil adhesion to platelets (Fig 5A). At shear stress 20 and 40 mPa, no significant inhibition was observed. At shear stress 80 and 320 mPa, activation with fMLP (10⁻⁷ mol/L) resulted in a maximal inhibition of 50%. The absolute number of rolling neutrophils per square millimeter after activation with fMLP was also determined (Fig 5B).

Neutrophil rolling was decreased dose-dependently after stimulation with fMLP at both shear stresses, with a maximal inhibition of 70% after stimulation with 10⁻⁷ mol/L fMLP. A more pronounced effect of fMLP treatment was observed at a shear stress of 80 mPa versus 320 mPa. We measured the expression of L-selectin, CD11b, CD18, and a neutrophil degranulation/activation marker (CD67) to determine the effect of fMLP treatment on the expression of adhesion molecules by neutrophils. L-selectin expression at the neutrophil surface was decreased by 60% upon stimulation with fMLP at concentrations of 10⁻⁸ mol/L and higher. Under the same conditions, CD11b and CD67 expression increased by 90% and 65%, respectively, while CD18 expression was unaffected (results not shown).

**DISCUSSION**

**Neutrophil adhesion to HUVEC.** In our experiments, we used a well-defined perfusion system to study neutrophil interaction with ECM-bound platelets, and to compare this interaction with adhesion of PMNs to HUVEC. The initial attachment of leukocytes to endothelial cells under flow conditions is known to be mediated by a selectin-dependent rolling interaction. Subsequent integrin binding is supposed to be necessary for static adhesion, spreading, and transmigration. In accordance with these studies, we observed diminished neutrophil adhesion to unstimulated HUVEC with increasing shear and virtually no adhesion at a shear stress of 200 mPa or higher (Fig 2). We studied the effect of induction of adhesive properties of both endothe-

<table>
<thead>
<tr>
<th>Table 3. Neutrophil Adhesion to ECM-Bound Platelets Under Flow: Effects of Monoclonal Antibodies</th>
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<tbody>
<tr>
<td>Shear Stress (mPa)</td>
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<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>D6</td>
</tr>
<tr>
<td>CD18</td>
</tr>
<tr>
<td>CD11b</td>
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<td>L-sel</td>
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<td>P-sel/L-sel</td>
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<td>P-sel/CD18</td>
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<td>P-sel/L-sel/CD18</td>
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Cover slips with ECM-bound platelets were incubated with control HEPES buffer or with anti-P-selectin (Waps 12.2); neutrophils were incubated with control HEPES buffer, anti-L-selectin (LAM1-3), anti-CD18 (IB4), and/or anti-CD11b (44a) for 30 minutes at room temperature. Neutrophils (2 × 10⁶/mL HEPES buffer) were perfused in the presence of the monoclonal antibodies at 37°C at shear stresses of 80 and 320 mPa. Data represent the total number of adhering and rolling neutrophils per mm², or the number of rolling cells per mm² after 5 minutes of perfusion (mean ± SE, n = 4-8).

Abbreviation: ND, not determined.

* Statistically significant effect of antibody treatment vs control cells (P < .05).
† Statistically significant decrease vs P-selectin–treated cells (P < .05).
The platelet surface we used was formed on the matrix of endothelium in the presence of whole blood and under physiologic flow conditions. Subsequent perfusions showed that high numbers of neutrophils (up to 1,600/mm²) adhered to these platelet surfaces (Fig 3A). The absence of PMN adhesion to bare or plasma-perfused ECM clearly indicated the platelet-dependency. Both the mean rolling velocity and the percentage of rolling neutrophils increased with higher shear (Fig 3B). Interestingly, the maximal percentage of rolling cells does not exceed 60%, indicating that even at a high shear stress, part of the neutrophils are able to adhere firmly to the platelet-covered surface. Moreover, a maximal rolling velocity was observed, probably related to the number of available rolling receptors.⁵ ECM-adherent platelets seem more PMN adhesion at higher shear stresses as compared with HUVEC (compare Figs 3A and 2). This was nicely illustrated in perfusions in which both HUVEC and ECM-adherent platelets were present (Fig 1 and Table 1).

Selectins and ligands. Receptors and ligands involved in the adhesion of neutrophils to platelets under flow were further characterized. Clearly, neutrophil adhesion to ECM-bound and activated platelets under flow was critically dependent on selectins, particularly at higher shear stresses (Table 3 and Fig 4B). Earlier reports have shown that P-selectin supported adhesion of thrombin-activated platelets in suspension to neutrophils.¹⁵ Moreover, in vivo studies have shown that antibodies against P- and L-selectin have a protective effect against an experimentally induced inflammatory reaction and pulmonary vascular damage in animal models.⁴⁰,⁴¹ The fact that heparin treatment, known to pre-
vent selectin-ligand binding in vitro and during acute inflammation in vivo.\textsuperscript{7,42} induced shear-dependent inhibition was in agreement with the role for selectin/mucin-ligand interactions. At high shear, adhesion was blocked more than 90% by anti-P-selectin. Also, anti-L-selectin was partially able to inhibit PMN adhesion at high shear stress (Table 3). This could be an indication for a possible interaction between P- and L-selectin. L-selectin does indeed possess the sLeX carbohydrates that may serve as a ligand for P-selectin.\textsuperscript{43} Nevertheless, P-selectin recognizes other ligands, such as PMN-associated P-selectin glycoprotein ligand-1 (PSGL-1), as well.\textsuperscript{\textsuperscript{44}} An alternative (mucin-like) ligand for L-selectin that could be present on the platelet surface remains to be defined; neuraminidase treatment of the platelet surface decreased neutrophil rolling at shear 80 mPa by approximately 50% and the total number of adhered neutrophils by approximately 30% (data not shown). Additional evidence for the role of L-selectin may be deduced from the fact that fMLP-treated neutrophils adhered less to the platelet surface at shear stresses of 80 and 320 mPa (Fig 5A). The concentration of fMLP that induced the inhibitory effect also caused β2-integrin activation and L-selectin shedding from the neutrophil surface (as confirmed by FACS experiments). Under high-shear conditions, which favor selectin-mediated adhesion, shedding of L-selectin\textsuperscript{27} obviously overshadows a simultaneous increase of integrin-dependent adhesion. In contrast, leukocyte adhesion to unstimulated HUVEC, which is integrin-dependent and only occurs at low shear, is enhanced after stimulation with fMLP\textsuperscript{28,29} (Fig 2). In the experiments with HUVEC, the presence of L-selectin seems less critical for leukocyte adhesion.

Role of integrins. Indirect evidence for the role of integrin-dependent adhesion of PMNs to ECM-bound platelets could be derived from the fMLP experiments. Preactivated neutrophils exhibited 70% less rolling and more static adhesion as compared with unstimulated neutrophils (Fig 5B). At a low shear stress (25 mPa), when integrin-mediated adhesion is less dependent on rolling, fMLP prestimulation tends to increase PMN adhesion. In agreement, at shear 320 mPa, addition of fMLP (10^{-8} \text{ mol/L}) during the perfusion resulted in an immediate arrest of all rolling cells and subsequent spreading (results not shown). Our results indicate that rolling facilitated integrin-mediated adhesion. In agreement with the findings obtained by Buttrum et al,\textsuperscript{16} both CD18 and CD11b antibodies failed to significantly decrease the total amount of neutrophils adhered to ECM-bound platelets. However, we observed that these monoclonal antibodies caused a clear shift toward rolling adhesion at shear stress 80 mPa (Table 3). When both P- and L-selectins were blocked with monoclonal antibodies, some neutrophil adhesion could still be observed, which was abolished when CD18 was added. The data on fMLP-induced and integrin monoclonal antibody–blocked static adhesion (Table 3) indicated a clear role for integrins in these processes.

Integrins and ligands. The finding that calcium depletion during perfusion completely inhibits PMN adhesion to platelets (Fig 4A) demonstrated the calcium dependency of the process, and is in agreement with the described calcium-dependent binding of selectins and integrins to their ligands.\textsuperscript{45,46} In studies by others, in which isolated platelets adhered to lysine-coated glass under static conditions, the characteristics of PMN adhesion were different.\textsuperscript{6,37} In contrast to our results, PMNs adhered only at shear stresses less than 200 mPa, and no integrin-dependent adhesion was reported. Additionally, in these studies, 60% of the cells show rolling adhesion at 80 mPa, as compared with 15% in our experiments. The described differences could be explained by the presence of more or more potent integrin ligands in our system. The use of extracellular matrix and flowing whole blood to prepare the platelet surface not only may change the platelet activation during adhesion but could also influence the presence of plasma proteins on the surface. A possible ligand for the β2-integrins in our system is ICAM-2, which has been reported to be expressed on platelets.\textsuperscript{47} However, preliminary experiments show that incubation of the platelet surface with ICAM-2 antibodies only slightly inhibited neutrophil adhesion (15% ± 7%, n = 3). Fibrogen, which is present in high concentrations during the whole-blood perfusion, is a second candidate for β2-integrin–mediated adhesion. It binds activated platelets and may become the ligand for β2-integrins on neutrophils.\textsuperscript{48,49} However, the precise role of integrin binding and activation in PMN-platelet interactions under flow conditions remains to be characterized.

In conclusion, our model for endothelial damage provides evidence that activated platelets are more potent in supporting neutrophil adhesion under flow as compared with (activated) endothelium, particularly at higher shear stresses. An explanation for our findings may be that matrix-activated platelets express P-selectin in much higher numbers per surface area and for a longer period as compared with thrombin-activated HUVEC.\textsuperscript{42,43} Long-term activation of the endothelium (eg, by TNF) can further increase PMN adhesion by upregulation of ICAM-1 and E-selectin, but adhesion to activated platelets remains more potent. PMN adhesion to the platelet surface can be characterized by a rolling interaction, which is dependent on selectins, and a static adhesion. The described mechanisms are likely of key importance in the pathogenesis of thrombosis, atherosclerosis, or vasculitis, in which the inflammatory response goes hand in hand with platelet-dependent hemostasis.

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Platelet-dependent primary hemostasis promotes selectin- and integrin-mediated neutrophil adhesion to damaged endothelium under flow conditions

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