Role of P-Selectin and Leukocyte Activation in Polymorphonuclear Cell Adhesion to Surface Adherent Activated Platelets Under Physiologic Shear Conditions (An Injury Vessel Wall Model)

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Carbohydrate moieties on leukocytes adhere to activated platelets via P-selectin under static binding condition studies. We characterize polymorphonuclear cell (PMN) surface interactions with surface adherent platelets and the PMNs response, under physiologic flow conditions corresponding to a shear of 100 s⁻¹, in an in vitro flow chamber. Fluorescent labeled PMNs with red blood cells were drawn through a transparent flow channel and visually quantitated over 30 minutes, interacting with a confluent monolayer of activated, shear-spread platelets expressing P-selectin. PMN adhesion was saturable (2,250 ± 350/mm²), and time and cation (Ca²⁺, Mg²⁺) dependent, and PMNs did not bind to the experimental surface in the absence of a platelet monolayer. P-selectin antibodies completely abolished PMN adhesion in a concentration-dependent manner with half inhibition at 70 µg/mL. Antibodies to a putative P-selectin receptor CD15 (80H5 and MMA) maximally inhibited PMN adhesion by 73% and 10%, respectively. Adherent PMNs appeared morphologically activated and flow cytometric analysis of adherent PMNs confirmed activation because CD11b and CD18 surface expression was upregulated (100% and 27%, respectively), whereas L-selectin was downregulated (85%) compared with control nonadherent PMNs. In the presence of the metabolic inhibitor sodium azide (0.02% and 0.1%) there was a 23% ± 9% and 51% ± 3% decrease, respectively, in PMN adhesion at 100 s⁻¹. Thus, P-selectin is required for PMN adhesion to a pathophysiologic surface of activated adherent platelets at physiologic shear rates. Furthermore, a secondary step involving PMN activation after platelet binding appears necessary for complete (irreversible) adhesion to occur. This unique flow cell provides a model to explore, under controlled conditions, biologic mechanisms and ligands involved in leukocyte-platelet binding that play important roles in PMN localization at sites of thrombosis and vascular injury.

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sion to the platelet surface, a phenomena similar to that seen on endothelial cells. We hypothesize that, besides P-selectin, additional receptors or ligands on the platelet surface such as GPIIb/IIIa or additional receptors or ligands on the platelet surface are involved in adhesion under shear conditions.

MATERIALS AND METHODS

Reagents. Bovine albumin (fraction V; Sigma Chemical Co, St Louis, MO), dextran (D-7265; Sigma), phorbol myristate acetate (PMA), fluorescein isothiocyanate (FITC) isomer 1 on cell (Calbiochem, La Jolla, CA), pyridodisulphide R-phycoerythrin (R-PE; Molecular Probes Inc, Eugene, OR), mepacrine (quinacrine dihydrochloride; Sigma), fibrinogen (Grade L; Kabi, Stockholm, Sweden), rhodamine phalloidin (R415; Molecular Probes), and Ficol-Paque (Pharmacia [Canada], Dorval, Quebec, Canada) were used as reagents.

Antibodies. The monoclonal antibodies (MoAbs) to GPIIb/IIIa (A2A9; gift of J. Bennett, University of Pennsylvania, Philadelphia), to GPIb (AP1; gift of T. Kunicki, Scripps Clinic, La Jolla, CA), to CD11b (MY904; from American Type Culture Collection [ATCC], Rockville, MD), to CD18 (R15.7; from R. Rothlein, Boehringer Mannheim Co, Indianapolis, IN), to CD45 (gift of R. Sutherland, University of Toronto, Canada), and to L-selectin (DREG56, AMAC Inc, Westbrook, ME) were used. CD15 MoAbs (MMA; Becton Dickinson Canada Inc, Mississauga, Ontario, Canada) and 80H5 (AMAC), where necessary, were concentrated and salt exchanged over a PD-10 column (Pierce Chemical Co, Rockford, IL) before use.

The anti-F-selectin MoAb KC4.1 (IgG1) was developed against thrombin-activated whole platelets as previously described.24 KC4.1 was purified by protein G affinity chromatography and judged pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyclonal anti-P-selectin antibodies were developed as previously described.24 In brief, KC4.1 immobilized on protein A was used to chromatographically immunoaffinity purify P-selectin, followed by resolution by SDS-PAGE. The gel was excised, macerated, and injected intramuscularly into rabbits before antisera collection. Polyclonal anti-P-selectin antibodies were purified on a P-selectin affinity chromatography column as previously described.22 The anti-P-selectin antibodies immunoprecipitate and immunoblot P-selectin and do not recognize any leukocyte antigen.23 The MoAbs to P-selectin, GPIb/IIIa, GPIb, CD11b, CD18, and L-selectin were FITC-labeled as previously described.24 CD45 MoAb and KC4.1 were R-phycoerythrin-labeled following the methods of Molecular Probes. Appropriate control antibodies of the same species and isotype were used.

Platelet preparation. Venous blood of healthy donors was anticoagulated in ACD 6:1 (vol/vol), and the platelets were isolated by differential centrifugation as previously described by Mustard et al.24 In brief, platelet-rich plasma (PRP) was centrifuged and the platelet pellet resuspended in Tyrode's solution containing 0.35% (wt/vol) albumin (TA), buffer (Hepes 7.5), and asparagine at 37°C and were then allowed to stand for 45 minutes. The resultant washed platelets were further centrifuged and resuspended in TA with asparagine at 37°C and were allowed to stand for a further 45 minutes. This latter step was repeated again and the platelets resuspended at a final suspension concentration of 3 × 10^8 cells/mL with red blood cells (RBCs) at a hematocrit of 40% in TA with asparagine, pH 7.35. Platelet suspensions, in the absence of RBCs, were responsive to low concentrations of ADP, thrombin, and collagen when tested for aggregation.

PMN preparation. The PMNs were separated from the RBC concentrate according to methods previously described.25 After removal of the PRP, the remaining leukocyte/RBC concentrate was mixed (vol/vol) with 6% dextran in Dulbecco's phosphate-buffered saline (DPBS) and allowed to separate (1g) for 60 minutes at 37°C. The leukocyte/Dextran mixture was centrifuged and the pellet resuspended in DPBS containing 0.35% albumin, pH 7.35. The leukocyte preparation was layered over ice-cold Ficol-Paque 5:3 (vol/vol) and then centrifuged. The PMN-rich pellet was resuspended in DPBS containing 0.1% (wt/vol) D-glucose and 0.5% (wt/vol) albumin. The suspension was labeled, when necessary, to a final concentration of 25 μmol/L mepacrine, for 20 minutes at 20°C. After three further washes, the PMNs were resuspended in TA and kept in the dark at 4°C until needed. A cell count was taken using a Coulter Counter S-5TR (Coulter Electronics, Burlington, Canada). The PMN suspensions were greater than 99% viable by trypan blue dye exclusion throughout the course of the experiment and consisted of greater than 98% neutrophils based on Giemsa staining and Coulter Counter results. The RBCs isolated from the dextran sedimentation were washed thrice with Tyrode's 1:1 (vol/vol) and the final concentrate was kept at room temperature until needed. RBCs were added to the PMNs to achieve a hematocrit (Hct) of 40% and a final PMN concentration of 5.0 × 10^6 cells/mL. For the sodium azide (NaN3) experiments, the PMN suspension was incubated with 0.02% or 0.1% NaN3 for 20 minutes at 37°C. These NaN3-treated PMNs were either directly reconstituted with RBCs having a constant NaN3 concentration or washed free of NaN3 before reconstitution in an azide-free medium and then used directly in a flow experiment.

Flow cell design and experimental apparatus. The flow cell and experimental design have been previously described.33 The flow chamber consisted of a glass coverslip (Alkali-silicate; Fisher Scientific Ltd, Whitby, Ontario, Canada) separated from a Perspex, poly (methylmethacrylate), block by a thin sheet of Silastic (Dow Corning, Midland, MI; 254 μm thick). Into this sheet a channel 40 mm × 3.0 mm was cut. The coverslip and Silastic sheet were held in place on the Perspex block with a clamped metal plate. The Perspex block contained 1-mm diameter inlet and outlet ports directing the cell suspension to and from the channel. The configuration of the flow cell, when mounted on the microscope stage, is such that cell transport to the experimental surface was against the force of gravity.

The flow chamber was mounted on a Leitz Laborlux S microscope (Leica Canada Inc, Willowdale, Canada) equipped with Hoffman Modulation Contrast and epifluorescent attachments. The cell suspension was drawn from a reservoir at 37°C, unless otherwise stated, through Tygon tubing (polyethylene, 1.59-mm internal diameter) into the channel using a Harvard Apparatus Infusion/Withdrawal pump (Harvard Apparatus Co, Millis, MA) at a flow rate (0.2 mL/min) corresponding to a shear rate of 100 s⁻¹.

Surface preparation and monolayer assessment. The glass coverslip, cleaned with 1% Liqui-nox (Alconox Inc, New York, NY), was mounted in the flow cell and primed with Tyrode's solution. Fibrinogen (400 μg/mL), purified by the method of Lawrie et al.26 was drawn into the chamber and allowed to absorb into the surface for 1 hour at 37°C, followed by a Tyrode's solution rinse for 15 minutes at 0.2 mL/min (100 s⁻¹). To coat the fibrinogen-coated glass surface a shear spread platelets, the platelets/RBC preparation was drawn through the chamber at 1.0 mL/min (500 s⁻¹) for 4 minutes and then rinsed with TA for 5 and 25 minutes at 1.0 mL/min and 0.1 mL/min (50 s⁻¹), respectively, all at 37°C. Integrity of the platelet monolayer was assessed by Hoffman Modulation Contrast microscopy and permanently recorded with photomicrographs, before and after all PMN adhesion assays.

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In some experiments, the platelet-coated surface was further assessed for monolayer integrity by epifluorescent microscopy of platelet-F-actin stained with rhodamine phalloidin, both before and after the adhesion assay, and for platelet activation by staining of the platelet monolayer with FITC-labeled KC4.1.

The shear spread platelet-coated surfaces, before and after PMN exposure, were prepared for cytoskeletal staining. The cell-coated coverslips were quickly dip-rinsed in Tyrode's buffer at 37°C and then fixed with 0.2% glutaraldehyde for a minimum of 30 minutes at 21°C. This was followed by 15 minutes of incubation in 0.05 mol/L glycine at 4°C. Staining with 2.5 μg/mL rhodamine phalloidin took place for 10 minutes at 21°C. Two quick dips rinses in Tyrode's buffer were performed and the samples were glycerol-mounted onto glass slides for photographic analysis.

Cell adhesion assay. The PMN/RBC mixture was drawn through the flow chamber at 0.2 mL/min (100 s⁻¹) for 30 minutes at 37°C. For visual quantification of stationary PMNs adherent to the experimental surface, photomicrographs using epifluorescent optics were taken, at 6-minute time intervals, of six different sites on the platelet surface monolayer along the center of the flow channel (approximately 0.5 to 1.0 cm from the inlet port). Kodak TMZ 3200 ASA film (Eastman Kodak, Rochester, NY) was used for photography. At the conclusion of an experiment, the flow chamber was rinsed with TA and then with Tyrode’s solution for 2 minutes, respectively, and then fixed with fresh 1% paraformaldehyde in Tyrode’s solution for a further 5 minutes, all at 0.2 mL/min (100 s⁻¹). All experiments were simultaneously controlled in separate flow chambers with identical PMN/RBC suspensions.

For antibody binding inhibition assays, MoAbs or polyclonal antibodies, or appropriate controls, were preincubated with the PMN/RBC suspension or platelets adherent to the flow channel for 20 minutes before the initiation of a flow study. Antibodies were used at the concentrations stated and all flow experiments were performed a minimum of three times.

Preparation of samples for flow cytometry. For flow cytometric analysis of surface-adherent platelets and PMNs, coverslips were removed from the chamber after washing as above and incubated with HTB, pH 7.4, 5 mmol/L EDTA for 20 minutes at room temperature to lift off adherent cells. The removed platelets and PMNs were immediately fixed with fresh 1% paraformaldehyde in Tyrode’s solution. Cell recovery from the coverslips was greater than 95% complete, as judged by phase contrast microscopy. The cells were counted and PMNs were washed and concentrated (5 X 10⁷/mL) by centrifugation at 200g for 5 minutes in TA. For two-color platelet flow cytometric studies, 10⁷ platelets were incubated with saturating concentrations of PE-KC4.1 and FITC-API or FITC-A2A9 for 20 minutes, fixed with 1% paraformaldehyde in Tyrode’s solution, and then analyzed.²³ Control platelets from the nonadherent cells after movement through the flow chamber and washed presamples in the presence of the agonists 1 μmol/L PMA, 0.25 U/mL thrombin, or buffer were analyzed in parallel. For two-color PMN flow cytometric studies, 2.5 X 10⁶ cells were incubated with saturating concentrations of PE-CD45 and FITC–L-selectin, FITC-CD11b, and FITC-CD18 for 20 minutes, were fixed, and were then analyzed.²³ Control PMN samples taken from nonadherent cells after passage through the flow chamber and from washed preflow PMNs were incubated with 1 μmol/L PMA or buffer, fixed, and then labeled and analyzed as above. All studies were performed a minimum of three times.

Flow cytometric analysis. Samples were analyzed on a Becton Dickinson FACScan flow cytometer with LYSIS II software (Becton Dickinson, Mountain View, CA). The light scatter and fluorescence channels were set on logarithmic scale (platelet analysis) or linear scale (PMN analysis). For two-color analysis, the fluorescence threshold was set to analyze only those events that bound either the platelet-specific (FITC GPIb or GPIIb/IIIa) or leukocyte-specific (R-PE-CD45) labeled MoAbs.²² For platelet studies, 5,000 platelets (based on size characteristics and FITC) were analyzed for P-selectin, GPIb, and GPIIb/IIIa content.²³ Bound antibody for platelet studies is expressed in mean arbitrary fluorescence units (FLU) or as a percentage of positive cells. For PMN studies, 5,000 PMNs (based on size characteristics and CD45) were analyzed for CD11b, CD18, and L-selectin content. PMN surface antibody content is expressed as a ratio of the mean fluorescence intensity per cell of CD11b, CD18, or L-selectin to CD45.

RESULTS

Neutrophil-spread adherent platelet adhesion under flow.

The flow apparatus contains a well-defined lucite flow cell with a volume of 30.5 μL that has an experimental surface area of 120 mm² available to monitor cell-surface interactions.³⁰ The integrity, organization, and activation of the shear (500 s⁻¹) -induced confluent monolayer of spread platelets was maintained throughout all flow experiments, at the physiologic shear rate of 100 s⁻¹, as determined by Hoffman Modulation Contrast (HMC) photomicroscopy (Fig 1 A and C) and cytoskeletal and surface immunofluorescence. Studies using the cytoskeletal F-actin binding dye rhodamine phalloidin showed well-organized activated platelet cytoskeletons, each in immediate proximity to its neighboring spread adherent platelet (Fig 1 B and D), confirming the HMC microscopy observation that the platelets formed a confluent monolayer. In addition, immunofluorescence studies with anti-P-selectin antibodies showed homogeneous staining of the platelet monolayer, indicating that α-granule secretion and activation had occurred (data not shown). Full activation of spread adherent platelets was confirmed by flow cytometric platelet analysis that showed P-selectin surface expression (92%; 77 ± 4.6 FLU) compared with resting (4%; 8 ± 1.4 FLU) and thrombin-stimulated controls (98%; 85 ± 4.1 FLU) with downregulation of GPIb (68%; 47 ± 2.4 to 15 ± 1.3 FLU) and upregulation of GPIIb/IIIa (183%; 120 ± 5.5 to 220 ± 12 FLU) (Table 1). For a relative comparison of cell surface density of shear spread platelets versus monolayers of confluent endothelial cells, there were 2.75 ± 0.18 X 10⁵ spread platelets/cm² of our experimental surface compared with 1.5 ± 0.5 X 10⁵ cultured human umbilical vein endothelial cells (HUVECs)/cm².³⁴ Modeling of comparable P-selectin density between activated monolayers of platelets and cultured endothelial cells shows 358 ± 23 versus 35 ± 15 P-selectin molecules/μm², respectively.³⁵ The differences between P-selectin expression on platelets and endothelial cells is further magnified because P-selectin is transiently expressed on activated endothelial cells and is gone within 30 minutes, in contrast to sustained stable expression on activated platelets for up to 6 hours.³⁶,³⁷,³⁸,³⁹ All adhesion flow experiments were performed in the presence of a physiologic RBC Hct because RBCs play a significant role in shear wall forces and facilitate the radial movement of leukocytes to surfaces caused by RBC rotational and translational effects and, furthermore, to more closely approximate a biologic system.³⁶,³⁷ PMNs were mepracrine loaded to allow the dynamic visualization and quantification, by epifluorescent microscopy, of PMNs attaching and slowly moving on the surface during an experiment while under
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Fig 1. Washed human platelets (3 x 10^8 cells/mL) reconstituted in RBCs (Hct 40%) in TA buffer, pH 7.35, were drawn through the flow chamber (500 s⁻¹), in which they adhered and spread on a fibrinogen-coated glass surface. Mepacrine-loaded human resting PMNs (5 x 10^6 cells/mL) and washed RBCs (Hct 40%) in TA were drawn through the flow cell chamber over the experimental platelet surface at a shear rate of 100 s⁻¹ at 37°C for 30 minutes. After washing and gluteraldehyde fixation, the cell-adherent surfaces were stained with F-actin staining dye Rhodamine Phalloidin. HMC photomicrographs (A and C) and their corresponding epifluorescent images (B and D) of the confluent platelet monolayers before (A and B) and after (C and D) PMN adhesion are shown.

constant flow conditions. Nonadherent PMNs in the RBC bulk flow were not visualized because of the rapidity of their movement through the visual field. Surface adhesion studies performed in the presence or absence of PMN mepacrine loading demonstrated no significant difference (P = .558, N = 7) between the number of adherent PMNs, indicating that the fluorescent dye used for dynamic visualization did not interfere with the PMN adhesion capabilities (see Fig 5). Experiments were performed at a physiologic shear rate of 100 s⁻¹, which is greater than venous flow, because previous studies under the current suspension system conditions have shown this shear rate to give the greatest PMN binding to the experimental platelet surface. PMN binding to the platelet monolayer approached saturation (2,235 ± 105 PMN/mm²) at 30 minutes (Fig 2). PMN adhesion was divalent cation-dependent because 1 mmol/L EDTA (Fig 2) completely abolished adhesion to 0.07% ± 0.09% of control PMNs bound, whereas the integrity of the platelet monolayer remained intact. At higher concentrations of EDTA (5 mmol/L), the integrity of the platelet monolayer began to break down. In the absence of spread adherent platelets, PMN binding to the fibrinogen-coated glass experimental surface at 100 s⁻¹ was negligible (0.16% ± 0.13% of control bound PMNs on platelet monolayer), as we have previously described (Fig 2). Dynamic visualization of the experimental surface showed PMNs that rolled or jumped before final
Table 1. Platelet and Leukocyte Antigen Expression of Experimental Surface-Bound Cells

<table>
<thead>
<tr>
<th>Flow Conditions</th>
<th>Leukocyte Antigens</th>
<th>Leukocyte Antigens</th>
<th>Leukocyte Antigens</th>
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<tbody>
<tr>
<td></td>
<td>L-Selectin</td>
<td>CD11b</td>
<td>CD18</td>
</tr>
<tr>
<td>Control (N = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adherent PMNs</td>
<td>1.10 ± 0.05</td>
<td>4.50 ± 0.25</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Nonadherent PMNs</td>
<td>2.30 ± 0.15</td>
<td>2.25 ± 0.16</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>With azide (0.1%)</td>
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<tr>
<td>Adherent PMNs</td>
<td>1.41 ± 0.06</td>
<td>3.86 ± 0.21</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>Nonadherent PMNs</td>
<td>2.25 ± 0.1</td>
<td>2.26 ± 0.15</td>
<td>0.49 ± 0.04</td>
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<tr>
<td>Non-flow</td>
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<tr>
<td>PMA (1 μmol/L)</td>
<td>1.02 ± 0.07</td>
<td>4.11 ± 0.33</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>Buffer</td>
<td>2.30 ± 0.17</td>
<td>2.87 ± 0.19</td>
<td>0.51 ± 0.03</td>
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Platelet Antigens (arbitrary fluorescence units)

<table>
<thead>
<tr>
<th></th>
<th>GPIb</th>
<th>GPIb/III</th>
<th>P-Selectin</th>
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<tbody>
<tr>
<td>Control platelets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>47 ± 2.4</td>
<td>120 ± 5.5</td>
<td>8 ± 1.4 (4%)</td>
</tr>
<tr>
<td>Thrombin (0.25 U/mL)</td>
<td>25 ± 1.2</td>
<td>180 ± 7.0</td>
<td>95 ± 4.1 (98%)</td>
</tr>
<tr>
<td>Adherent platelets</td>
<td>15 ± 1.3</td>
<td>220 ± 12</td>
<td>77 ± 4.6 (92%)</td>
</tr>
</tbody>
</table>

Detached adherent PMNs and platelets and nonadherent PMNs from the 30-minute time point compared with control cells were analyzed by flow cytometry for leukocyte and platelet surface antigens. PMN data are expressed as the ratio of leukocyte activation antigen to CD45 arbitrary fluorescence units. Platelet data are mean arbitrary fluorescence units per platelet and for P-selectin include the percentage of positive cells above control. Results represent the mean ± SD of four experiments.

arrest as described on other selectin-expressing surfaces.\textsuperscript{24,26} Studies are ongoing in the quantitation of this phenomena.

Effects of anti-P-selectin antibodies on PMN-surface platelet adhesion. Lawrence and Springer\textsuperscript{24} have suggested that P-selectin is an initial primary adhesion molecule for leukocyte vessel wall interaction in an artificial phospholipid surface flow system. We evaluated the role of platelet P-selectin in flow-mediated PMN adhesion to our experimental surface of confluent activated platelets with P-selectin antibodies over a 30-minute time period. Anti-P-selectin IgG completely inhibited PMN binding to the spread platelets in a saturable and time-dependent fashion (Fig 3). Half-maximal antibody inhibition was observed at 62.5 μg/mL and maximal inhibition approached at 150 μg/mL (Fig 3). The time binding course of PMN-platelet surface adhesion demonstrated rapid surface PMN saturation at higher antibody inhibiting concentrations of anti-P-selectin antibody (half-maximal binding in 2.5 minutes at 100 μg/mL), with the number of adherent cells remaining constant throughout the remainder of the flow experiment (Fig 3, insert). This is consistent with an initial high on rate followed by a constant on-off rate for PMN adhesion. Dynamic visualization showed very few adherent PMNs leaving the surface after final arrest, suggesting a very low off rate once the surface was saturated with PMNs. Because PMN-P-selectin platelet surface adhesion approached a steady state at different antibody concentrations, adhesion was a direct function of the density of available P-selectin binding sites. The MoAb KC4.1, directed against the complement repeat region of P-selectin, weakly inhibited PMN binding (16.9% ± 4.6%) at saturating concentrations (100 μg/mL), whereas control antibodies failed to inhibit PMN-platelet surface binding (see Fig 5). The effects of KC4.1 are similar to its inhibition on platelet leukocyte adhesion under conditions of non-flow, suggesting either steric hindrance or an antibody binding epitope near but not at the critical lectin-binding site.\textsuperscript{32}

Effects of anti-Lewis X antibodies on PMN-surface platelet adhesion. All studies to define the platelet \textsuperscript{29}P-selectin counterreceptor component have been performed under non-flow conditions, the results of which may vary under shear. Initial observations by Larsen et al suggested that the LNFIII (Le\textsuperscript{a}) was a receptor, whereas others have been unable to confirm these observations.\textsuperscript{14,19} We tested two anti-Le\textsuperscript{a} MoAbs (80H5 and MMA) to assess this carbohydrate's role in PMN adhesion to platelets under flow conditions. The 80H5 antibody demonstrated half-maximal inhibition at 14 μg/mL (Fig 4). Maximal inhibition of 72% of total PMN binding was approached at 25 μg/mL. At the fully saturating concentration of 100 μg/mL of 80H5, PMN adhesion as a percentage of inhibition for each control time point was constant at 73% ± 2% throughout the 30-minute binding time course of the experiment. These results are in close agreement with the action of 80H5 described by Larsen et al\textsuperscript{19} under non-flow conditions. Inhibition of PMN binding with another anti-CD15 MoAb MMA showed little inhibi-
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Irreversible PMN adhesion on endothelial cells and artificial surfaces under shear requires leukocyte activation either by cell contact or stimulating cell factors, such as platelet-activating factor. This results in alterations of leukocyte adhesion receptors with upregulation of MAC-1 (CD11b/CD18), downregulation of L-selectin, and shear sensitive organization of constitutive receptors LFA-1 (CD11a/CD18). Whether PMN activation is involved in PMN adhesion to the P-selectin-expressing platelets is unknown, but activation is not required in static binding conditions. In this flow cell, the majority (>90%) of surface-bound PMNs, after 30 minutes, appeared morphologically to have undergone some degree of cellular activation with either flattened shapes or pseudopodal extensions (Fig 1C), as opposed to the rounded and raised contours noted on adherent PMNs at earlier time points. To further analyze whether bound PMNs had become activated, they were detached from the surface and their surface membranes analyzed by flow cytometry for leukocyte activation antigen content (CD18, CD11b, and L-selectin). Compared with unattached PMNs from the flow cell and resting controls similarly treated, CD18 and CD11b were upregulated (0.51 to 0.65 and 2.25 to 4.5, respectively), whereas L-selectin was downregulated (2.3 to 1.1), indicating that the bound PMNs had undergone activation consistent with the changes seen morphologically (Table 1). The unattached PMNs, after passage through the flow chamber, remained inactivated (Table 1). This indicated that, under shear conditions, adherent PMNs on a surface of P-selectin expressing activated platelets became activated.

Because the surface adherent PMNs were activated, we attempted to globally interfere with PMN activation during PMN-platelet surface contact using the nonspecific cellular inhibitor sodium azide (NaN₃) to determine whether cell activation was required for complete adhesion. In the pres-

Fig 3. Mepacrine-loaded human resting PMNs (5 × 10⁶ cells/mL) and washed RBCs (Hct 40%) in TA buffer, pH 7.35, were drawn through the flow cell chamber over the experimental platelet surface at a shear rate of 100 s⁻¹ at 37°C. PMNs binding to the experimental surface were determined from multiple (N = 6) epifluorescent photomicrographs taken at 6-minute time intervals. PMN binding data is shown as a percentage of total control PMNs bound at 30 minutes for increasing concentrations of polyclonal anti-P-selectin IgG and are controlled with normal rabbit IgG (N = 3). Insert shows the time binding profile at each concentration of polyclonal anti-P-selectin IgG, 20 pg/mL (•), 50 pg/mL ( ●), 100 pg/mL (△), and 200 pg/mL (▲) (N = 3), in a representative graph.

Fig 4. Mepacrine-loaded human resting PMNs (5 × 10⁶ cells/mL) and washed RBCs (Hct 40%) in TA buffer, pH 7.35, were drawn through the flow cell chamber over the experimental platelet surface at a shear rate of 100 s⁻¹ at 37°C. PMNs binding to the experimental surface were determined from multiple (N = 6) epifluorescent photomicrographs taken at 6-minute time intervals. PMN binding data is shown as a percentage of total control PMNs bound at 30 minutes, for increasing concentrations of monoclonal anti-CD15 antibody IgM (80H5) or control IgM (N = 4).
ence of 0.02% and 0.1% NaN₃, there was a 23% and 51% inhibition, respectively, in PMN adhesion to the activated platelet experimental surface (Table 2). There was no morphologic change in the platelet surface during any of these experiments and platelets were not lost from the surface as visualized by HMC optics. In parallel flow experiments, adhesion of sodium azide pretreated PMN was unaffected by the presence or absence of NaN₃ during flow (P = .122, N = 7; data not shown), thus confirming that the metabolic inhibitory effect was confined to the PMNs and not related to azide effects on the platelets. Sodium azide treatment did not affect PMN viability as determined by trypan blue exclusion. Furthermore, although fewer PMNs bound, those that did adhere were morphologically indistinguishable from the surface bound PMNs in the control flow cells, indicating incomplete inhibition of PMN activation at these concentrations of NaN₃. Flow cytometric leukocyte activation antigen analysis of azide-treated bound PMNs versus untreated bound controls confirmed PMN activation, although not to the degree seen in non-azide-treated cells, CD18 (0.57 v 0.65), CD11b (3.85 v 4.5), and L-selectin (1.41 v 1.1) (Table 1). These results suggest that PMN activation is required for complete adhesion to activated surface-bound platelets under conditions of shear, as has been described and modeled by others in leukocyte adhesion to artificial and endothelial cell surfaces. This extends the multistep leukocyte adhesion process (of reversible adhesion then leukocyte activation followed by irreversible leukocyte adhesion) to include the important physiologic cell, the platelet. We demonstrate that unstimulated PMNs bound to the experimental surface under differing conditions. Mepacrine-loaded human resting PMNs (5 x 10⁶ cells/ml) and washed RBCs (Hct 40%) in TA buffer, pH 7.35, were drawn through the flow cell chamber over the experimental platelet surface at a shear rate of 100 s⁻¹ at 37°C. PMNs binding to the experimental surface were determined from multiple (N = 6) epifluorescent photomicrographs taken at the 30-minute time point. PMN binding data is shown as a percentage of total control PMNs bound at 30 minutes. Where antibodies were assessed all MoAbs and polyclonal antibodies are at saturating concentrations and controlled with antibodies of the same species isotype. All binding experiments are performed on a platelet-covered experimental surface, except for the fibrinogen-coated glass surface alone (N = 3 to 6).

**Discussion**

Because flow is both an important physiologic state and known to affect cellular adhesion, we sought to characterize PMN adhesion to the previously undescribed physiologic cell surface of an activated platelet monolayer under flow conditions. To more closely approximate a biologic system, we reconstituted PMNs with RBCs, because RBCs play a major role in shear-induced cell-wall interactions. The results of this study show that in vitro flow chamber is a useful technique for quantifying and characterizing, in a dynamic manner, the adhesion of neutrophils to an experimental surface of activated adherent platelets. Specifically, the receptors/counter receptors involved in leukocyte-platelet adherent surface interactions can be studied with a physiologically reconstituted population of flowing cells under biologically relevant flow conditions. This flow system represents a model of an injured vessel wall covered with platelets.

We demonstrate that unstimulated PMNs bound to the confluent monolayer of adherent activated platelets in a saturable and time-dependent manner at a physiologic flow rate corresponding to a shear rate of 100 s⁻¹ but not to the experimental surface in the absence of adherent platelets. We have previously shown that lowering or raising the shear rate around 100 s⁻¹, which is within the physiologic range, caused a reduction in PMN surface adhesion. This is because cell-surface interactions under shear conditions are a summation of a series of flow related factors that include (1) increased lateral movement of leukocytes to the vessel wall due to translational and rotational effects from RBCs and (2) the effects of RBCs and fluid shear forces in the direction of flow that remove adherent PMNs and decrease surface contact time.

**Table 2. Effects of Sodium Azide on PMN Adhesion to Experimental Platelet Surface**

<table>
<thead>
<tr>
<th>Sodium Azide</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02%</td>
<td>77% ± 9%</td>
<td>100% ± 10%</td>
</tr>
<tr>
<td>0.1%</td>
<td>49% ± 3%</td>
<td>100% ± 14%</td>
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</tbody>
</table>

Calculated at the 30-minute time point from six spatially separated photomicrographs in a line between the inlet and outlet ports of the flow cell experimental surface, shown as the percent of control PMNs per square millimeter bound (mean ± SD) in the presence or absence of sodium azide (N = 3).
PMN ADHESION TO ACTIVATED PLATELETS

The receptor on the activated platelet responsible for PMN adhesion to surface-bound platelets under flow conditions is P-selectin. In the absence of flow, P-selectin antibodies, soluble P-selectin, the soluble lectin-like domain, and peptides of the lectin domain inhibit activated platelet adhesion to neutrophils, whereas resting platelets that do not express P-selectin do not adhere to leukocytes. We show that P-selectin antibody, in a concentration-dependent manner, completely inhibits PMN-activated platelet adhesion on an experimental surface under physiologic shear. Half-maximal inhibition was similar to that seen in adhesion assays in non-flowing conditions. Binding was stable at each concentration of P-selectin antibody, indicating that PMN adhesion was directly related to P-selectin surface density, consistent with the observations of Lawrence and Springer on an artificial P-selectin phospholipid surface. Furthermore, PMN adhesion was divalent cation-dependent, a condition consistent with P-selectin as the PMN adhesion ligand under non-shear states. Calcium is required for neutrophils to bind purified P-selectin by causing a conformational change in its lectin-like terminal end. This extends the observations in non-shear conditions to include P-selectin as a major ligand in PMN adhesion to activated platelets under conditions of shear. In vivo, this will occur at pathophysiologic situations of vascular injury and thrombosis.

Initial reports suggested that P-selectin recognizes the Leα (CD15) antigen because LNFIII and MoAbs to CD15 blocked platelet adhesion to neutrophils. Subsequently, multiple structures with different affinities for P-selectin have been identified that include sialylated Leα, sulfated glycoconjugates, sulphated glycans, and a leukocyte glycoprotein. Most recent evidence suggests that the P-selectin receptor is a sialylated glycoprotein with O-linked glycans. These studies were all performed in the absence of physiologically relevant shear forces. Because flow-related transport of PMNs to a surface will increase the availability of PMNs for adhesion, and shear stress will remove marginally adherent PMNs under shear as opposed to static adhesion conditions, shear will amplify the effects of inhibitory factors. We have demonstrated that the Leα (CD15) antigen, under conditions of shear, is involved as a P-selectin ligand in PMN-platelet adhesion because 80H5 inhibited adhesion to the same extent as that seen in non-flow experiments. Not all Leα MoAbs tested inhibited PMN adhesion, suggesting antigenic epitope specificity for the role of Leα in adhesion to P-selectin. This may explain, in part, the discrepant results with different Leα MoAbs assessed by different investigators. That sLeα is a component of adhesion of PMNs to activated platelets under conditions of shear is currently under investigation in conjunction with the development of real time, dynamic, video microscopy as an adjunct in our flow system.

Neutrophil adhesion to P-selectin expressing experimental surfaces has been modeled, in vitro, on activated endothelium and synthetic endothelial-like phospholipid monolayers as a multistep process involving initial slowing and rolling through P-selectin, neutrophil activation, and then irreversible adhesion through CD18/11b receptors. Under static binding conditions, the function of the adhesive lectin domain of P-selectin for neutrophils is highly stable and does not require leukocyte activation because leukocyte-platelet adhesion was unaffected regardless of whether it was performed with formaldehyde fixed neutrophils or at 4°C or 37°C. We have shown that, under flow conditions, adherent PMNs became morphologically progressively more activated over time on the activated platelet monolayer with flattened shapes and pseudopodal extensions (Fig 1). Analysis of surface expression of the leukocyte activation structures CD11b, CD18, and L-selectin confirmed that the adherent PMNs had become activated, whereas those that had either transiently contacted the surface or passed through the flow cell remained inactivated. Not only do platelet-surface-adherent PMNs become activated, but, when PMN activation was blocked with the metabolic inhibitor, sodium azide adhesion was significantly decreased. Thus, under conditions of shear, platelet-bound PMNs become activated and activation appears necessary for complete PMN adhesion to the physiologically relevant P-selectin—expressing surface-adherent activated platelets. The mechanism(s) of leukocyte activation in this system is unknown, but we hypothesize that contact activation or surface bound cytokine may play a role because potential secreted cytokines from the activated adherent platelets will have been washed away in preparing our surface. That leukocytes become activated upon adhesion to platelets is supported by our (unpublished observation) and others’ observations in static binding conditions.

We have shown that the first two steps of the multistep leukocyte-endothelium adhesion process are required for PMN surface adhesion to activated platelets and under flow conditions: (1) reversible adhesion through P-selection and (2) surface leukocyte activation. This suggests the requirement of yet-unidentified, secondary (irreversible) adhesion receptors on leukocytes and platelets for shear-resistant adhesion. Such receptors may include the leukocyte B2 integrins CD11b/CD18 as described for neutrophil-endothelial interactions and platelet ICAM-2 or platelet integrins GPIIIa/IIIA or GPIIb/IIIa and involve platelet/leukocyte adhesive ligands such as thrombospondin, fibrinogen, or fibronectin.

The significance of leukocytes adhering to a surface of activated platelets at sites of vascular injury is underscored by the modeling of Lawrence and Springer, who showed that the quantitative neutrophil adhesion correlated with P-selectin density under conditions of flow and has been confirmed in vivo in a thrombosis animal model. Activated adherent platelets as opposed to endothelial cells have (1) a calculated sevenfold increase in P-selectin density, (2) stable but not transient functional P-selectin expression, and (3) at sites of thrombus a greater three-dimensional surface area and thus P-selectin surface density. These modeled observations indicate that, at sites of vascular injury involving platelet monolayers or thrombi, the adherent platelet surface provides, through P-selectin, a physiologically important and potentially more adhesive surface than endothelial cells for the localization and recruitment of effector leukocytes. These studies have underlined the need to do cell-cell/surface studies under physiologic conditions of flow in which biologic processes may differ from static conditions. We have shown that platelet P-selectin, interacting via carbohydrate moieties, is required for surface PMN adhesion to acti-
vated platelets under physiologic shear, which emphasizes the pivotal role this molecule plays in the earliest interactions of PMNs with altered vascular surfaces such as thrombi and adherent platelets on injured or denuded endothelium. In addition, surface-adherent PMNs became contact-activated and, further, PMN activation was necessary for complete PMN adhesion, a previously unrecognized response for platelet-adherent PMNs. This indicates, under flow but not static conditions, a multistep adhesion process for complete PMN-platelet adhesion and suggests the involvement of secondary platelet and leukocyte-adhesion receptors. In addition to offering insights into the role of P-selectin on platelet surfaces, flow models such as this will be necessary for the identification of shear-requiring secondary platelet and leukocyte ligands/receptors involved in this biologic cell-cell interaction critical in the genesis of vessel injury response.

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Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model)

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