Exogenous eosinophil activation converts PSGL-1-dependent binding to CD18-dependent stable adhesion to platelets in shear flow

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LEUKOCYTE ADHESION to activated platelets represents a key event in the sequence of thrombus formation, as demonstrated in vitro (19) and observed in vivo after arterial injury (30) and during propagation of venous thrombosis (42). Several lines of evidence also suggest that enhanced leukocyte-platelet adhesion occurs in the circulation of patients with acute myocardial infarction (35) or stroke (23) or after coronary angioplasty (31). These heterotypic adhesive interactions are thought to promote thrombosis and vascular occlusion, thereby impairing blood flow and exacerbating ischemia (13).

To date, most work has focused on delineating the molecular mechanisms by which neutrophils interact with activated platelets, because neutrophils represent the largest leukocyte subpopulation in blood (10, 14, 25, 26, 36, 39, 50). As a result, very little is known about the molecular constituents mediating attachment of other leukocyte subpopulations to platelets. For instance, eosinophils, although they normally comprise $<4\%$ of circulating leukocytes in blood, are dramatically increased in certain disease states and can account for $\geq20\%$ of the total leukocyte population. Several reports have noted the occurrence of thrombotic disorders in hypereosinophilic patients that, in certain cases, was accompanied by occlusion of arteries and small blood vessels (33, 38). In particular, 58% of patients with idiopathic hypereosinophilic syndrome (HES), characterized by persistent eosinophilia and organ damage, develop cardiovascular disease, often with associated mural thrombi (51). In addition, neurological complications caused by thromboemboli either of cardiac origin or locally produced within cerebral vessels are frequently detected in HES (51). The pathogenesis of eosinophil-mediated organ (e.g., cardiac) damage is currently unknown but is thought to involve both the presence of increased number of eosinophils and other as yet ill-defined stimuli for recruitment and/or activation of these leukocytes. It has been suggested that eosinophils may undergo a respiratory burst to generate oxidative products that, alone or in concert with eosinophil peroxidase, may cause oxidant-mediated damage (51). Earlier work demonstrated that activated platelets induce superoxide anion release by monocytes and neutrophils (34). It is therefore likely that enhanced eosinophil-platelet adhesive interactions in the microcirculation of eosinophilic patients result in eosinophil activation and release of oxidants that may precipitate and/or exacerbate thrombotic disorders in these patients. Consequently, elucidation of the detailed molecular basis underlying eosinophil attachment to platelets...
may provide insights for the rational development of novel therapeutic agents, based on the blockade of these adhesive interactions, to effectively combat thrombotic disorders in eosinophilic patients.

Prior work has shown that thrombin-activated platelets interact with isolated eosinophils in a Ca\textsuperscript{2+}-dependent manner under stationary conditions (9). Antibody blocking experiments have revealed a role for platelet CD62P (P-selectin) in this process. However, the molecular determinants (other than P-selectin) and the detailed sequence of events involved in this heterotypic interaction remain unknown. In contrast, a multistep, detailed sequence of events involved in this heterotypic leukocyte interactions in shear flow (6, 24), we wanted to compare the pattern and extent of eosinophil binding to immobilized platelets with those of neutrophils.

### MATERIALS AND METHODS

#### Reagents.

The IgG murine monoclonal antibodies (MAbs) 7E4 (blocking anti-CD18), HP2/1 (blocking anti-CD49d), and D1G10VL2 (blocking anti-fibrinogen; Ref. 5) were purchased from Immunotech (Westbrook, ME). The blocking anti-CD29 MAb 4B4 was from Coulter (Miami, FL). The blocking MAbs KPL-1 [anti-CD162 (anti-FSGL-1)], HI111 [anti-CD11a], ICRF44(44) (anti-CD11b), and HIP1 [anti-CD42b (anti-GPIb)] were obtained from BD-Pharmagen (San Diego, CA). A blocking anti-CD62P/E (anti-P-/E-selectin) MAb (EP5C7), which does not affect CD62L function (47), was generously provided by Dr. Nicholas F. Landolfi (Protein Design Labs, Fremont, CA). The function-blocking anti-CD62L (anti-L-selectin) MAb LAM1–116 and anti-CD11d (anti-CD11d) MAb 240I were generously provided by Dr. Thomas F. Tedder (Duke University Medical Center, Durham, NC) and Dr. Pat Hoffman (ICOS, Bothell, WA), respectively. The nonpeptide small-molecule platelet-\(\alpha_{I\beta3}\) antagonist XV454 (1) was a kind gift of Dr. Shaker A. Mousa (DuPont Pharmaceuticals, Wilmington, DE). The Fab anti-\(\alpha_{I\beta3}\) MAb c7E3 was from Centocor (Malvern, PA). Human eotaxin-2 was kindly provided by Dr. John White (GlaxoSmithKline, King of Prussia, PA). Formalin was purchased from Richard Allan Scientific, Fremont, CA.

Human eosinophils were kindly provided by Dr. Shaker A. Mousa (DuPont Pharmaceuticals, Bothell, WA). Eosinophil purity (based on the examination of Diff-Quik-stained cytocentrifugation preparations) was 96%, and viability (by erythrosin B dye exclusion) was nearly 100%

#### Isolation of human granulocytes.

Protocols involving human subjects were performed in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society. Human eosinophils were isolated from EDTA-anticoagulated venous blood of donors with mild allergic rhinitis or asthma by 1,090 g/min Percoll density-gradient centrifugation at room temperature (RT) and removal of CD16-positive cells (neutrophils) with immunomagnetic beads (46). Eosinophil purity (based on the examination of Diff-Quik-stained cytocentrifugation preparations) was >96%, and viability (by erythrosin B dye exclusion) was nearly 100%. Eosinophils (5 × 10\textsuperscript{6} per ml) were kept in assay buffer (RPMI 1640 medium containing 1 mM sodium pyruvate, 10 mM HEPES, 4.5 g/l glucose, and 0.1% BSA) at 4°C for no longer than 4 h before use in adhesion assays. In selected experiments, human neutrophils were isolated from CPD-anticoagulated venous blood of healthy volunteers by centrifugation through PMN isolation medium (16) and held (10\textsuperscript{7} neutrophils/ml) at 4°C for up to 4 h before being used in flow-based adhesion assays. Before flow experiments, eosinophils (or neutrophils) were allowed to equilibrate at 37°C for 2 min and then were diluted to a cell concentration of 0.5 × 10\textsuperscript{6} for assay buffer at 37°C and perfused over platelet-coated surfaces at prescribed wall shear stresses.
Immobilization of platelet layers on glass slides. Platelet-rich plasma (PRP) was prepared by centrifugation (160 g for 15 min) of sodium citrate (0.38% wt/vol)-anticoagulated human blood of healthy volunteers (28). PRP specimens were subjected to a further centrifugation (1,100 g for 15 min) in the presence of 2 μM PGE₁, and the platelet pellet was resuspended in HEPES-Tyrode buffer (in mM: 129 NaCl, 8.9 NaHCO₃, 2.8 KCl, 0.8 K₂PO₄, 0.8 MgCl₂, 10 HEPES, 5.6 dextrose) containing 5 mM EGTA and 2 μM PGE₁ (14). Thereafter, platelets were washed once via centrifugation (1,100 g for 10 min), resuspended at 2 × 10⁶/ml in HEPES-Tyrode buffer (14), and kept at RT for no longer than 2 h before use in flow assays. Before the perfusion experiments, purified platelets were allowed to bind to 4% APES-treated coverslips (24 × 50 mm; Corning) for 30 min at 37°C in a humid environment (28). Under these conditions, a confluent layer of platelets was formed, as evaluated by light microscopy for each experiment (Fig. 1). The density and confluence of platelet layers were not affected during the flow experiment.

Hydrodynamic flow assays. Leukocyte adhesion to immobilized platelets was quantitated under dynamic flow conditions with a parallel-plate flow chamber (1, 7, 28, 32). A platelet-coated coverslip was assembled with a flow chamber and mounted on the stage of an inverted microscope (Nikon, Melville, NY), a 0.55× phase objective (Nikon, and a CCD100 camera (Dage-MTI, Michigan City, IN)) connected to a VCR and TV monitor. Surface-adherent platelets were then incubated with 1 U/ml thrombin (unless otherwise stated) in the presence of 0.1% BSA for 10 min at 37°C. After the platelet layer was washed with D-PBS-0.1% BSA for ~2 min, leukocytes were perfused through the chamber for 3 min at appropriate flow rates to obtain wall shear stresses of 0.5–3 dyn/cm², thereby mimicking the fluid mechanical environment of the microcirculation and postcapillary venules. Leukocyte binding to surface-anchored platelets was visualized in real-time by phase-contrast videomicroscopy. A single field of view (0.55 mm²) was monitored during the 3 min of the attachment assay, and at the end five fields of view (each 0.55 mm²) were monitored for 10 s each. During all experiments, the entire flow system was maintained at 37°C in a warm air box surrounding the microscope.

Data analysis of attachment assays in flow. Five parameters were quantified in the analysis: 1) the number of primary interacting cells per square millimeter during the entire 3-min perfusion experiment, 2) the number of secondary interacting cells per square millimeter during that period, 3) the number of stationary interacting cells per square millimeter after 3 min of shear flow, 4) the percentage of total (primary + secondary) interacting cells that were rolling after 3 min of shear flow, and 5) the average rolling velocity (μm/s) of interacting leukocytes (1, 28). Cells that tethered directly to the platelet layer in the absence of any interaction with previously bound leukocytes were defined as primary interacting cells (3). Primary interacting leukocytes that tethered upstream of and continued to translate into the field of view were distinguished from those that initially tethered directly to the platelets within the field of view. Cells that attached to the substrate after first forming homotypic tethers with leukocytes already bound to the platelet surface were defined as secondary interacting cells (3). The number of primary and secondary interacting cells was determined manually by reviewing the videotapes. Stationary interacting leukocytes were considered as the interactions as determined within 10 s at the end of the 3-min attachment assay. To quantify their number, images were digitized from the videotape recorder with a Scion frame grabber and a personal computer and processed with OPTIMAS 6.5 software package (Argis-Schoen Vision Systems, Alexandria, VA; Ref. 28). Rolling velocities were computed as the distance traveled by the centroid of the translating cell divided by the time interval (1, 28) with OPTIMAS 6.5 software. Only cells that rolled without stopping during the entire acquisition period were included in the analysis.

Cell treatment with MAbS, enzymes, and eotaxin-2. For some inhibition studies, leukocytes were pretreated for 30 min at 4°C with saturating concentrations of function-blocking MAbs that were kept present during the perfusion assays. For other studies, surface-adherent platelets were preincubated with MAbs (50 μg/ml, unless otherwise stated), the small-molecule α₁bβ₃ antagonist XV454 (150 nM), or the RGD-containing peptide GRGDSP (4 mg/ml) for 10 min at 37°C during the thrombin-BSA incubation. Saturating concentrations of EP5C7 MAb, XV454, and GRGDSP were also maintained in the flow buffer during the flow assays. The extent of leukocyte binding to platelet layers, as well as the strength of these adhesions, was measured in controlled detachment assays, were unaltered by the presence or absence of the appropriate isotype-matched control MAbs (data not shown).

In some experiments, leukocytes (5 × 10⁶/ml) were incubated with 0.1 U/ml Vibrio cholerae neuraminidase (Roche...
Molecular Biochemicals, Indianapolis, IN) for 30 min at RT to remove terminal cell surface sialic acid residues (28). In others, leukocytes were treated for 20 min at RT with the proteolytic enzyme chymotrypsin (1 U/10^6 cells), which has been shown to cleave L-selectin and PSGL-1 from the cell surface (3, 16). After enzyme treatment, leukocytes were washed once, resuspended in flow buffer, and infused into the flow chamber for adhesion assays. In selected experiments, eosinophils were perfused over immobilized platelets in the presence of the sialyl Lewisx (sLex) mimic glycyrrhizin (10 mM) (37). In others, the actin polymerization inhibitors cytochalasin B (10 μg/ml) or latrunculin A (1 μM) were added to the flow buffer, which was superfused over leukocytes that had been allowed to tether to the platelet surface for 3 min at a stress level of 2 dyn/cm^2. Control experiments verified that superfusion of 0.1% ethanol alone, a concentration level used as a diluent for cytochalasin B, did not alter the pattern and extent of leukocyte binding to platelets. In some experiments, the platelet layer was exposed to 1% formalin for 15 min, followed by a washing step before use in adhesion assays. To assess the role of exogenous eosinophil activation in eosinophil binding to platelet layers under flow, eotaxin-2 (3 nM) was added to the flow buffer 1 s before the initiation of the perfusion assay (46).

**Statistics.** Data are expressed as means ± SE. Statistical significance of differences between means was determined by one-way ANOVA. If means were shown to be significantly different, multiple comparisons by pairs were performed by the Tukey test. Probability values of P < 0.05 were selected to be statistically significant.

**RESULTS**

Surface-anchored platelets support eosinophil adhesion under flow. To study the pattern and extent of eosinophil adhesive interactions with platelets in shear flow, eosinophils were perfused through a parallel-plate flow chamber, whose lower plate was coated with platelets, at prescribed wall shear stresses ranging from 0.5 to 3 dyn/cm^2. Our data indicate that immobilized platelets formed a highly efficient surface for eosinophil capture (Fig. 1). The majority of eosinophils that tethered from the fluid stream were observed to roll ~1–3 cell diameters before becoming stationary. Treatment of platelet layers with thrombin increased the number of stationary eosinophils and concomitantly decreased the percentage of rolling cells (240 ± 9 vs. 148 ± 10 stationary cells/mm^2; 9.5 ± 2.4 vs. 37.0 ± 0.5% of tethered cells rolling in the presence and absence of thrombin, respectively, after 3 min of perfusion at 2 dyn/cm^2). Therefore, to investigate the mechanisms by which platelets recruit and efficiently stabilize free-flowing eosinophils, platelets were pre-treated with thrombin in all experiments reported hereafter. A progressive decrease in the number of stationary eosinophils was detected between 0.5 and 3.0 dyn/cm^2 (Fig. 2A). Concomitantly, an increasing proportion of tethered eosinophils were observed to translocate slowly along the platelet layer. The percentage of rolling eosinophils increased from <4% at a wall shear stress of 0.5 dyn/cm^2 to ~35% at 3 dyn/cm^2 (Table 1).

Hydrodynamic shear also regulates the pattern of recruitment of free-flowing eosinophils to the platelet surface. In particular, in the low-shear regime (0.5 dyn/cm^2), eosinophils primarily tethered directly to the platelet layer in the absence of any interaction with

<table>
<thead>
<tr>
<th>Shear Stress, dyn/cm^2</th>
<th>Percentage of Interacting Cells Rolling</th>
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<tbody>
<tr>
<td>0.5</td>
<td>3.7 ± 1.6</td>
</tr>
<tr>
<td>1</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>1.5</td>
<td>8.6 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>9.5 ± 2.4</td>
</tr>
<tr>
<td>2.5</td>
<td>13.7 ± 4.0</td>
</tr>
<tr>
<td>3</td>
<td>34.7 ± 0.9</td>
</tr>
</tbody>
</table>

Data are means ± SE for n = 3–8 experiments and represent the percentage of total interacting cells that continuously rolled rather than developing stationary adhesion. Platelet layers were incubated with thrombin (1 U/ml) for 10 min. Eosinophils (5 × 10^6/ml) were perfused over the platelet layer at indicated shear stress levels. The instantaneous number of both rolling and stationary eosinophils present in the field of view at the end of the 3-min perfusion experiment was recorded.

Fig. 2. Effect of wall shear stress on leukocyte interactions with surface-anchored platelets. Platelet layers were incubated with thrombin (1 U/ml) for 10 min. Eosinophils (5 × 10^6/ml) were then perfused over the platelet layer for 3 min at the indicated wall shear stress levels. Data represent the number of stationary interacting cells (A) and primary interacting cells that tethered directly to platelet layer within the field of view and secondary interacting cells (B). Values are means ± SE of 3–6 experiments.
previously captured eosinophils and were therefore termed primary interacting cells (Fig. 2B). In contrast, at high shear (3 dyn/cm²), the majority of interacting eosinophils were observed to result from the formation of homotypic tethers with eosinophils already bound to the platelet surface and were termed secondary interacting cells (Fig. 2B). These eosinophil-eosinophil interactions led to the formation of strings of bound cells (Fig. 1), as previously reported with neutrophils and monocytes (3, 27, 48), and accounted for up to 65% of total eosinophil accumulation on platelet layers at higher shear stresses.

At a wall shear stress level of 2 dyn/cm², the relative percentage of tethered eosinophils rolling along the platelet layer was lower than that of rolling neutrophils (9.5 ± 2.4 vs. 20.3 ± 3.7% of interacting cells rolling in the case of eosinophils and neutrophils, respectively; n = 4–8; means ± SE). Furthermore, as shown in Fig. 3, the number of primary interacting eosinophils was consistently larger than that of neutrophils at 2 dyn/cm². Concomitantly, a decreased number of secondary interacting eosinophils was detected compared with that of neutrophils. It is noteworthy that homotypic leukocyte interactions account for ~70% of the total neutrophil accumulation on platelet layers compared with ~50% for eosinophils at 2 dyn/cm². Together, these data indicate that the pattern of leukocyte recruitment to the platelet surface is qualitatively, yet not quantitatively, similar between eosinophils and neutrophils.

Relative contribution of selectins and selectin ligands to eosinophil binding to surface-adherent platelets under flow. Ensuing experiments focused on the elucidation of the molecular pathways involved in the adhesive interactions between eosinophils and immobilized, thrombin-treated platelets at a wall shear stress of 2 dyn/cm². Treatment of eosinophils with neuraminidase, an enzyme that cleaves sialic acid residues from the cell surface (16), dramatically reduced the number of stationary eosinophils on the platelet layer as well as secondary homotypic eosinophil interactions (Fig. 4A). Similar results were obtained when eosinophils were incubated with chymotrypsin, a protease that cleaves both L-selectin and the highly sialylated PSGL-1 (10 mM). B: eosinophils pretreated with KPL-1 (blocking-anti-PSGL-1; 30 μg/ml) or LAM1–116 (blocking-anti-L-selectin MAb; 15 μg/ml) for 30 min at 4°C before infusion to the flow chamber. In selected experiments, eosinophils were perfused over immobilized platelets in the presence of stialyl Lewis vaccines mimic glycyrrhizin (10 mM).

Relative contribution of selectins and selectin ligands to eosinophil binding to surface-adherent platelets under flow. Ensuing experiments focused on the elucidation of the molecular pathways involved in the adhesive interactions between eosinophils and immobilized, thrombin-treated platelets at a wall shear stress of 2 dyn/cm². Treatment of eosinophils with neuraminidase, an enzyme that cleaves sialic acid residues from the cell surface (16), dramatically reduced the number of stationary eosinophils on the platelet layer as well as secondary homotypic eosinophil interactions (Fig. 4A). Similar results were obtained when eosinophils were incubated with chymotrypsin, a protease that cleaves both L-selectin and the highly sialylated PSGL-1 (Fig. 4A; Ref. 16). Nevertheless, eosinophils treated with either neuraminidase (0.1 U/ml) or chymotrypsin (1 U/10⁶ cells) alone were observed to tether directly and roll on the platelet surface (Fig. 4A), albeit significantly faster than untreated cells (Table 2). It is noteworthy that, whereas 275 ± 26 primary interacting neuramin-
Table 2. Effects of enzymes and function-blocking antibodies on the pattern of eosinophil interactions with surface-bound platelets

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Interacting Cells Rolling</th>
<th>Rolling Velocity, μm/s</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>9.5 ± 2.4</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>71.5 ± 1.8*</td>
<td>21.1 ± 4.6*</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>73.5 ± 3.8*</td>
<td>9.6 ± 3.2*</td>
</tr>
<tr>
<td>KPL-1 (anti-PSGL-1)</td>
<td>87.2 ± 1.2*</td>
<td>68.3 ± 9.8*</td>
</tr>
<tr>
<td>EPS57 (anti-P-selectin)</td>
<td>75.8 ± 5.9*</td>
<td>16.9 ± 1.2*</td>
</tr>
<tr>
<td>LAM1-116 (anti-L-selectin)</td>
<td>9.9 ± 1.1</td>
<td>2.6 ± 0.3</td>
</tr>
</tbody>
</table>

Data are means ± SE and represent the percentage of total interacting cells that continuously rolled rather than developing stationary adhesion (n = 3–21 experiments) and the average rolling velocity of the corresponding rolling cells (n = 25–85 cells). Platelet layers were incubated with thrombin (1 U/ml) for 10 min. Eosinophils (5 × 10⁶/ml) were perfused over the platelet layer for 3 min at a wall shear stress level of 2 dyn/cm². Eosinophils were treated with neuraminidase (0.1 U/ml for 30 min at room temperature (RT)), chymotrypsin (1 U/ml for 30 min at RT), KPL-1 (blocking-anti-PSGL-1 monoclonal antibody (MAb); 30 μg/ml) or LAM1–116 (anti-L-selectin MAb; 15 μg/ml) before infusion into the flow chamber. Immobilized platelets were treated with EPS57 (blocking-anti-P-selectin MAb; 50 μg/ml) during the 10-min thrombin incubation, which was maintained in the flow buffer during the perfusion assay as well. The instantaneous number of both rolling and stationary eosinophils present in the field of view at the end of the 3-min perfusion experiment was recorded. *P < 0.05 with respect to untreated eosinophils.

idase-treated eosinophils/mm² were observed to tether in the field of view (Fig. 4A), another 357 ± 46 cells/mm² were observed to roll into the field of view after having previously tethered upstream. Similarly, another 58 ± 15 chymotrypsin-treated eosinophils/mm² rolled into the field of view from upstream. In contrast, in matched control samples all eosinophils tethered directly to platelets within the field of view, and no eosinophils were observed to enter the field of observation by rolling from upstream. Flow cytometric analysis of the eosinophil cell surface revealed that treatment with neuraminidase reduced the sLeα expression level by 94%, whereas treatment with chymotrypsin cleaved L-selectin and reduced the PSGL-1 expression level by 35% (data not shown). It is noteworthy that the combination of these enzymes completely blocked eosinophil interactions with immobilized platelets under dynamic flow conditions (Fig. 4A). The inability of neuraminidase or chymotrypsin alone to effectively abrogate the primary heterotypic adhesive interactions may be due to the presence of residual sLeα and PSGL-1 expression levels on the cell surface after enzyme treatment. However, the sLeα mimic glycyrrhizin (18, 37), which has been shown to block selectin binding to sLeα both in vitro and in vivo, essentially abolished eosinophil attachment to platelets at 2 dyn/cm² (Fig. 4A). Together, these data are suggestive of the potential involvement of sialylated PSGL-1 and L-selectin in eosinophil accumulation on platelet layers in shear flow.

To verify the potential role of PSGL-1 in this process, eosinophils were pretreated with a function-blocking anti-PSGL-1 MAb, KPL-1, before being perfused over immobilized platelets. This intervention abrogated both stationary interacting cells and secondary homotypic eosinophil interactions (Fig. 4B). Similar to either neuraminidase or chymotrypsin treatment, eosinophils that did tether to the platelet surface via primary interactions rolled at markedly higher velocities than nontreated cells (Table 2).

Prior work has shown that PSGL-1 is a ligand for both platelet P-selectin and leukocyte L-selectin (29). Pretreatment of platelets as well as the presence in the flow buffer of a function-blocking P-selectin MAb, EPS57, significantly reduced the number of interacting cells relative to control (Fig. 4B) and increased both the percentage of tethered eosinophils rolling on the platelet surface and their average rolling velocity (Table 2). It is noteworthy that whereas 52 ± 13 primary interacting eosinophils/mm² were observed to tether to EPS57-treated platelets in the field of view (Fig. 4B), another 234 ± 47 primary interacting cells/mm² were detected to roll into the field of view after having previously tethered upstream. Incubation of eosinophils with a function-blocking anti-L-selectin MAb, LAM1–116, nearly eliminated secondary homotypic eosinophil interactions, which resulted in ~90% decrease in the relative number of stationary interacting eosinophils with respect to nontreated controls (Fig. 4B). However, this molecular intervention had no effect on either the percentage of rolling eosinophils or their corresponding average rolling velocities compared with nontreated eosinophils (Table 2). Cumulatively, these data indicate that eosinophil PSGL-1 binding to platelet P-selectin mediates primary interactions whereas homotypic eosinophil PSGL-1-L-selectin binding is required for secondary interactions.

Role of CD18 integrins in eosinophil adhesion to immobilized platelets in absence and presence of exogenous stimulation by eotaxin-2. Several lines of evidence suggest that initial tethering and rolling of neutrophils on activated platelet layers is mediated by platelet P-selectin and that subsequent involvement of neutrophil CD18 integrins converts these transient adhesive interactions into firm adhesion (10, 26, 50). We therefore wished to examine whether CD18 integrins mediate stationary adhesion of eosinophils to immobilized platelets under dynamic flow conditions. Our data indicate that the treatment of eosinophils with the function-blocking anti-CD18 integrin MAb 7E4 failed to reduce the number of stationary interacting eosinophils at a wall shear stress of 2 dyn/cm² (Fig. 5A). Moreover, treatment of eosinophils with function-blocking MAbs specific for eosinophil CD29 (β1 integrins), CD49d (α4 integrins), or CD11d (α6 integrins) had no effect on the extent of stationary adhesion (data not shown).

Controlled detachment assays were performed to probe the adhesive strength of eosinophils tethered to immobilized platelets at 2 dyn/cm² by incrementally increasing the flow rate every 30 s to achieve wall shear stress levels of 4, 8, 16, and 32 dyn/cm². Our data indicate that ~75% of stationary eosinophils detached...
from the platelet layer by the time that shear exposure of 32 dyn/cm² was achieved (Fig. 5B). It is noteworthy that the extent of shear-induced eosinophil detachment was further augmented by the presence of the anti-CD18 integrin MAb 7E4 (Fig. 5B). For example, 41.5 ± 4.1 vs. 18.1 ± 0.3% of tethered eosinophils remained adherent after exposure to a stress level of 8 dyn/cm² in the absence and presence of 7E4, respectively. Furthermore, the average rolling velocity of eosinophils increased with increasing shear stress, as shown in Table 3.

We next wanted to evaluate the effects of eosinophil activation induced by the CCR3-active chemokine eotaxin-2, which rapidly upregulates CD18 integrin function (21, 46) while decreasing CD49d integrin avidity (46), on eosinophil interactions with surface-anchored platelets in shear flow. Neither the number of primary interacting eosinophils nor the extent of stationary adhesion to immobilized platelets was significantly affected by the presence or absence of eotaxin-2 in the perfusion buffer at a wall shear stress level of 2 dyn/cm² (Fig. 5A). However, there was a small but statistically significant reduction in the number of secondary interacting eosinophils (Fig. 5A), which may be ascribed to eotaxin-2-induced partial L-selectin shedding (45).

In distinct contrast to untreated (control) cells, eosinophils became resistant to shear-induced detachment forces when exposed to eotaxin-2, with <25% of the stationary eosinophils detaching from the platelet layer even with a shear stress level of 32 dyn/cm². The conversion from stationary to firmly adherent cells was dependent on eosinophil CD18 integrin binding to the platelet surface, as evidenced by the marked reduction of the percentage of eosinophils remaining bound in the presence of the anti-CD18 MAb 7E4 (Fig. 5B). In an attempt to determine the potential contributions of CD11a/CD18, CD11b/CD18, and CD11d/CD18 to this process, eosinophils were pretreated with function-blocking anti-CD11a, CD11b, or CD11d MAbs, respectively. However, none of these antibodies when used either alone or in combination with the other two in the presence of eotaxin-2 affected the extent of eosinophil firm adhesion in the controlled detachment assays (data not shown). A possible explanation for the inability of the aforementioned MAbs to interfere with eosinophil stable adhesion to platelet layers may be that these MAbs block epitopes different from those involved in eosinophil binding to platelets, as previously demonstrated for a panel of similar MAbs (50).

Some previous studies suggested that platelet αⅡbβ3- and/or αMβ2-bound fibrinogen mediates neutrophil adhesion to immobilized platelets under dynamic flow conditions (26, 50), but others have failed to confirm

Table 3. Effects of shear level on the rolling velocities of eosinophils and neutrophils over platelets

<table>
<thead>
<tr>
<th>Shear Stress, dyn/cm²</th>
<th>Eosinophil Rolling Velocity, μm/s</th>
<th>Neutrophil Rolling Velocity, μm/s</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>2.3 ± 0.2</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>3.8 ± 0.5</td>
<td>7.0 ± 0.7</td>
</tr>
<tr>
<td>8</td>
<td>7.1 ± 0.7</td>
<td>13.4 ± 1.1</td>
</tr>
<tr>
<td>16</td>
<td>12.1 ± 1.1</td>
<td>19.0 ± 2.0</td>
</tr>
<tr>
<td>32</td>
<td>19.0 ± 1.8</td>
<td>30.4 ± 2.8</td>
</tr>
</tbody>
</table>

Data represent the average ± SE rolling velocity of the corresponding rolling cells (μm/s) for n = 25–85 cells. Platelet layers were incubated with thrombin (1 U/ml) for 10 min. Eosinophils (5 × 10⁶/ml) or neutrophils (5 × 10⁶/ml) were perfused for 4 min at a shear stress of 2 dyn/cm², after which the flow rate was doubled every 30 s to achieve shear stress levels of 4, 8, 16, and 32 dyn/cm².
this finding (36, 39). We therefore wanted to explore the potential role of platelet αIIbβ3 in eosinophil attachment to surface-anchored platelets in shear flow. Treatment of the platelet layer with the highly specific αIIbβ3 antagonist XV454 (1) or the function-blocking anti-αIIbβ3/α,β3 MAb c7E3, which were maintained in the perfusion buffer during the experiment, had no effect on the extent of firm or stationary adhesion in the presence or absence of eotaxin-2, respectively, compared with matched control specimens (data not shown). Furthermore, superfusion of the RGD-containing peptide GRGDSP during the controlled detachment assays failed to reduce the percentage of eotaxin-2-treated eosinophils remaining bound to the platelet surface (data not shown). These findings are in agreement with previous studies by Ostrovsky et al. (36), which indicate that neutrophil binding to platelets via CD18 integrins is RGD- and platelet αIIbβ3 integrin independent. Moreover, treatment of platelets with the blocking anti-fibrinogen MAb D1G10VL2 (5), which was maintained in the perfusion buffer during the flow experiment, failed to affect the extent and strength of firm adhesion of eotaxin-2-treated eosinophils, compared with matched controls (data not shown). Recent studies have identified platelet GPIb as a potential counter-ligand for the CD18 integrin receptor Mac-1 (41). However, pretreatment of the platelet layer with an anti-GPIb MAb had no effect on the extent and strength of eosinophil adhesion to platelets (data not shown). Altogether, these data suggest that treatment of eosinophils with eotaxin-2 rapidly upregulates the avidity of CD18 integrins, which mediate eosinophil shear-resistant firm adhesion to immobilized platelets by binding to a platelet counterreceptor(s) in a RGD-independent manner.

Role of eosinophil cytoskeleton in eosinophil-platelet interactions under flow. Our data show that PSGL-1 binding to platelet P-selectin not only initiates the majority of eosinophil tethering and rolling but also plays a predominant role in mediating stationary adhesion to surface-bound platelets in shear flow in the absence of any exogenously added stimulus. The ability of PSGL-1 to support eosinophil stationary adhesion to platelet P-selectin may be regulated by its interaction with the leukocyte actin cytoskeleton (40). We therefore wanted to investigate how disruption of the eosinophil actin cytoskeleton affects the pattern of eosinophil-platelet interactions. To rule out any possible effects of the actin polymerization inhibitors cytochalasin B or latrunculin A on the platelet cytoskeleton, experiments were performed with thrombin-treated, formalin-fixed immobilized platelets. Treatment of platelets with formalin for 15 min before the perfusion of eosinophils reduced, but did not eliminate, the ability of eosinophils to arrest on the platelet surface, which is in accord with previous studies on neutrophil-platelet interactions (40). At a shear stress level of 2 dyn/cm², >60% of eosinophils were observed to roll on the fixed platelet surface. As shown in Fig. 6, eosinophils that developed stationary interactions with fixed platelets at 2 dyn/cm² detached on exposure to increasing shear forces in a similar yet enhanced manner compared with unfixed platelets.

Superfusion of cytochalasin B, a compound that caps the growing end of actin filaments, after 3 min of flow at 2 dyn/cm² caused rolling eosinophils to arrest on formalin-treated platelets. This is reflected by the marked increase in the percentage of eosinophils remaining stationary at 2 dyn/cm² when reevaluated after the superfusion of cytochalasin B (Fig. 6). Moreover, these cells exhibited increased deformability (data not shown) and resistance to shear-induced detachment forces at all shear levels examined in this work (Fig. 6). In distinct contrast, when latrunculin A, a compound that prevents actin polymerization by irreversibly binding to actin monomers (52), was superfused after 3 min of flow, stationary cells became shear sensitive and detached rapidly from the formalin-treated platelet surface at 2 dyn/cm². Accordingly, this is reflected by a pronounced decrease in the percentage of eosinophils remaining stationary at 2 dyn/cm² when reevaluated after the superfusion of latrunculin A (Fig. 6). Similar trends were observed when cytochalasin B and latrunculin A were superfused over eosinophils interacting with non-formalin-treated platelets (data not shown). Together, our data demonstrate the absolute requirement of an intact cytoskeleton for the efficient recruitment and interaction of eosinophils with surface-bound platelets under hydrodynamic shear.

**DISCUSSION**

The major findings of this work are as follows. 1) Eosinophils, tethered either directly to surface-anchored platelets via PSGL-1 binding to platelet P-selectin or through homotypic secondary interactions mediated by L-selectin-PSGL-1 tethering, become rap-
idly immobilized on the platelet layer at low shear. The majority of these stationary interactions are dependent on the high degree of eosinophil PSGL-1 binding to platelet P-selectin and have an absolute requirement for intact eosinophil cytoskeleton. Only a small fraction of these stationary eosinophils develop shear-resistant attachments mediated by CD18 integrins. 2) Exogenous stimulation of eosinophils with the CCR3-active chemokine eotaxin-2 converts PSGL-1-P-selectin-dependent stationary adhesion to CD18-mediated stable attachment.

**Eosinophil tethering, rolling, and stationary adhesion to immobilized platelets in shear flow are mediated by PSGL-1-P-selectin interactions.** In concert with previous studies using neutrophils (10, 36, 39), PSGL-1 binding supports eosinophil primary tethering and rolling along surface-bound platelets under flow. However, significant differences are observed in the adhesive interactions of these two leukocyte subpopulations with immobilized platelets in shear flow. More specifically, the number of eosinophils tethered directly to the platelet surface is higher than that of neutrophils. Furthermore, the relative percentage of tethered cells rolling along the platelet layer and their respective average rolling velocity are markedly diminished for eosinophils compared with neutrophils. Direct comparison of these interactions with respect to cell surface ligand expression must be avoided because eosinophils and neutrophils were purified via different isolation methods from different donors. However, our observations are in accord with previously published data in purified P-selectin, P-selectin-expressing Chinese hamster ovary cells, or fixed platelets (12, 18, 20). The aforementioned discrepancies may be attributed to qualitative differences in the molecular structure of PSGL-1 on eosinophils from that on neutrophils (29, 44) and/or the higher PSGL-1 expression levels detected on the eosinophil relative to the neutrophil surface (8, 12, 44). Interestingly, as shown in Table 3, the average rolling velocity of neutrophils at a given shear stress level (e.g., $7.0 \pm 0.7 \mu m/s$ at 4 dyn/cm$^2$) is essentially equal to that of eosinophils expressing nearly twice as much PSGL-1 (8) at twice the level of shear (e.g., $7.1 \pm 0.7 \mu m/s$ at 8 dyn/cm$^2$). This finding further supports the concept that the biomechanics of cell rolling depends on both the ligand density and the fluid shear at a given receptor-site density.

Previous studies suggested that nearly all tethered neutrophils become stably arrested via CD18 integrin involvement within seconds of the initial PSGL-1-P-selectin-mediated binding (10, 50). This finding is in clear contrast to other reports (36, 43) showing that nearly 50% of the tethered neutrophils roll continuously along immobilized platelets in shear flow and the remaining ~50% become firmly adherent via CD18-dependent binding. Our data indicate that the majority of eosinophils and neutrophils tethered to the platelet surface become rapidly stationary at a wall shear stress level of 2 dyn/cm$^2$ in a P-selectin-dependent manner. However, the percentage of eosinophils (or neutrophils) remaining stationary decreases dramatically with increasing shear, with <25% of cells forming shear-resistant attachments mediated by CD18 integrins at a shear level of 32 dyn/cm$^2$. It is noteworthy that increasing shear caused eosinophils (and neutrophils) to begin to roll on the platelet surface rather than abruptly detaching. A subsequent reduction in the shear stress level to 2 dyn/cm$^2$ caused these “rolling” cells to become stationary again (data not shown). Cumulatively, our data suggest that immobilization of free-flowing, resting eosinophils (or neutrophils) to platelet layers at low shear results predominantly from the eosinophil (or neutrophil) PSGL-1 binding to high site densities of platelet P-selectin. Along these lines, eosinophils (and neutrophils) were observed to tether, roll and develop stationary adhesive interactions when perfused at 2 dyn/cm$^2$ over high levels of immobilized, purified P-selectin (0.5 $\mu g/ml$), in contrast to rolling interactions that were exclusively observed at lower P-selectin concentrations (<0.25 $\mu g/ml$; data not shown).

Prior work suggests that the energy provided by the fluid flow to the rolling cell is dissipated principally into two parts: energy dissipation due to adhesion bond separation and energy loss due to cytoplasm viscous dissipation (11). Cytochalasins inhibit actin polymerization, thereby increasing the fluidity of the cell (49) and thus increasing the relative contribution of cytoplasmic viscous dissipation. Partial disruption of the actin cytoskeleton by cytochalasin B converts rolling of otherwise resting eosinophils instantly to stationary adhesion to platelet P-selectin, as previously reported for neutrophils (15, 40). Moreover, the pattern of detachment of cytochalasin B-treated eosinophils was markedly different from that of untreated cells. Application of high shear caused cytochalasin-treated eosinophils to elongate in the direction of shear before abruptly detaching from the platelet surface. Treatment of leukocytes with cytochalasin B has been reported to partially decrease the number of microvilli and sum of microvillus tip widths present on the cell surface (15). Because microvilli are points of attachment of actin bundles to the plasma membrane and because of the higher affinity of cytochalasin B for cytoplasmic rather than cortical actin filaments, this actin-capping agent primarily interferes with cytoplasmic actin filaments while having little effect on localized polymerization of cortical actin. Consequently, the possible association of PSGL-1 with cortical actin coupled with biomechanical factors such as increased intercellular contact area and decreased shear forces on adherent leukocytes due to enhanced cell deformation may support greater resistance to detachment of cytochalasin B-treated leukocytes over control cells. In distinct contrast, exposure of interacting eosinophils to latrunculin A, which abrogates both cortical and cytoplasmic actin polymerization by binding to G-actin monomers, completely eliminated their ability to maintain stationary adhesion to surface-anchored platelets in shear flow. To this end, previously stationary eosinophils immediately began to roll and their resultant average rolling velocity was substantially elevated.
compared with that of nontreated cells (data not shown). Consequently, we speculate that latrunculin A eliminates PSGL-1 association with cortical actin filaments, thereby drastically diminishing the magnitude of strain that eosinophils bound to platelets via PSGL-1-P-selectin bond(s) can withstand before detaching under shear stress.

Tethered leukocytes are capable of recruiting free-flowing cells via homotypic cell interactions predominantly mediated by L-selectin binding to PSGL-1. However, the contribution of this pathway relative to PSGL-1-P-selectin-mediated recruitment increases with increasing shear, which is in accord with the relative binding kinetics of the respective receptor-ligand pairs (2). The enhanced neutrophil recruitment relative to eosinophil accumulation to immobilized platelets via secondary tethering may be ascribed to the twofold higher L-selectin expression levels on the neutrophil surface compared with those on eosinophils (12). Taking into account the relative L-selectin and PSGL-1 expression levels on the eosinophil and neutrophil surfaces, our data suggest that homotypic leukocyte interactions are primarily dependent on the surface expression level of L-selectin and are less sensitive to the differences in PSGL-1 expression levels.

**Eotaxin-2-induced eosinophil activation is required for shear-resistant CD18 integrin-mediated adhesion to immobilized platelets.** The activation of leukocytes represents a key component in their adhesion cascade to vascular endothelium, platelets, or other leukocytes and upregulates the binding affinity of integrins via both conformational changes and altered interactions with the cytoskeleton (6, 24). Previous studies showed that activation of tethered neutrophils via platelet-derived activating agents such as PAF converts neutrophil rolling to shear-resistant, CD18-mediated firm adhesion to immobilized platelets (36, 50). The percentage of tethered neutrophils activated by agents generated by or through platelets varies from 25–50% (36) up to nearly 100% (50). In the current study, only ~25% of stationary eosinophils remained adherent to the platelet surface on exposure to a wall shear stress of 32 dyn/cm² and ~60% of these adhesion events were eliminated by the use of the function-blocking anti-CD18 MAb 7E4. These data suggest that a small fraction of stationary eosinophils may have been activated by locally platelet-secreted agents such as PAF or RANTES, which was previously shown to be released by thrombin-stimulated platelets (17).

Selective leukocyte recruitment is the result of the orchestrated events involving cell surface receptors, their respective ligands, and chemokines. The CCR3-active chemokines such as eotaxin-2 selectively regulate eosinophil adhesion in a mitogen-activated protein kinase-dependent manner while having no chemotactic effect on neutrophils (4). Along these lines, addition of eotaxin-2 in the perfusion buffer induced rapid shear-resistant eosinophil firm adhesion to platelet layers via eosinophil CD18-integrins, as evidenced by the dramatic reduction of adhesion by the use of an anti-CD18 MAb.

Previous studies suggest that neutrophil firm adhesion to immobilized platelets is predominantly mediated via CD11b/CD18 integrin binding to platelet-associated fibrinogen presented by αIIbβ3 integrins (26, 50). However, our data indicate that blockade of platelet αIIbβ3 or platelet-bound fibrinogen or the incorporation of an RGD peptide had no effect on the extent of eosinophil binding to platelets in the presence or absence of eotaxin-2 in both attachment and detachment assays. These findings are in accord with other previously published reports (36, 39) that failed to demonstrate any potential platelet αIIbβ3 or RGD peptide involvement in these leukocyte-platelet interactions. Therefore, we have yet to demonstrate which platelet ligand(s) is responsible for mediating CD18-dependent firm adhesion of activated eosinophils.

In summary, eosinophil recruitment to surface-bound platelets in shear flow follows a cascade of events that shares common features with that outlined for neutrophils. In particular, PSGL-1 predominantly binds to platelet P-selectin to initiate primary tethering and rolling of free-flowing eosinophils, which assist in the secondary eosinophil recruitment mediated by L-selectin-PSGL-1 interactions. In the absence of any exogenous eosinophil activation, cortical actin-associated PSGL-1 binding is sufficient to mediate stationary interactions at low shear levels, presumably because of the high density of the receptor-ligand pairs on the apposing cell surfaces. Disruption of cortical actin polymerization eliminates these heterotypic cell adhesive interactions in shear flow. In the absence of exogenously added chemokines, only a small proportion of stationary cells (<25%) may develop shear-resistant CD18-integrin-dependent attachments to platelet layers. However, eotaxin-2-induced activation of eosinophils converts PSGL-1-dependent binding to shear-resistant firm adhesion mediated by CD18-integrin binding to adhesive proteins attached to the platelet surface. Together, these findings enhance our understanding of the molecular mechanisms of eosinophil-platelet adhesion and may provide insights for the rational development of novel therapeutic strategies aimed at altering these adhesive interactions.

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