FIRM ADHERENCE (termed ‘‘sticking’’) of circulating neutrophils to inflamed vascular endothelium is an essential component of a multistep adhesion cascade that results in the accumulation and eventual migration of neutrophils through the vessel wall. The importance of such interactions is demonstrated in leukocyte adhesion deficiency-1 (LAD-I) patients with a genetic absence of leukocyte integrins (β2-integrins; CD18 antigens). These patients have recurrent bacterial infections and delayed wound healing. The leukocyte or β2-integrins, lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18) on polymorphonuclear leukocytes (PMN) mediate adhesion-strengthening interactions. This requires activation of integrin adhesiveness. Multiple stimuli can increase the avidity of integrins on neutrophils for their counter-receptors, but chemoattractants are probably the most important. Neutrophils from LAD-I patients are capable of selectin-dependent tethering and rolling on endothelial cells in flow, similar to healthy neutrophils, but are unable to arrest or firmly adhere to activated platelets, in contrast to healthy neutrophils, which developed firm adhesion within 5 to 30 seconds after initiation of rolling. Furthermore, the adhesion-strengthening interaction observed for healthy neutrophils could be specifically inhibited by monoclonal antibodies (mAbs) to Mac-1, but not to lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) or intercellular adhesion molecule-2 (ICAM-2; CD102). Further evidence for a β2-integrin-dependent neutrophil/platelet interaction is demonstrated by the complete inhibition of interleukin (IL)-8-induced neutrophil transmigration across platelets bound to fibronectin-coated polycarbonate filters by mAbs to Mac-1. Thus, Mac-1 is required for firm adhesion of neutrophils to activated, adherent platelets and may play an important role in promoting neutrophil accumulation on and migration across platelets deposited at sites of vascular injury.

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interactions in hydrodynamic shear flow and in chemotaxis. A preliminary report of these observations has been reported.22 We find that healthy, but not LAD-1, neutrophils that tether and roll on adherent, activated platelets in shear flow arrest after several seconds and develop firm adhesion that is dependent on the β₂-integrin Mac-1. Furthermore, we have established a model of neutrophil transplatelet chemotaxis, and find that Mac-1-dependent adhesive interactions are required. Based on our observations and those of others and the parallels to neutrophil/endothelial cell adhesive interactions, we propose an analogous multistep adhesion model for neutrophil interactions with surface-adherent platelets.

**MATERIALS AND METHODS**

**Preparation of platelets and neutrophils.** Platelet-rich plasma was prepared by centrifugation of whole blood. Platelets were washed twice in HEPES buffer (145 mmol/L NaCl, 10 mmol/L HEPES, 0.5 mmol/L Na₂HPO₄, 5 mmol/L KCl, 2 mmol/L CaCl₂, 2 mmol/L MgCl₂, 0.1% glucose, and 0.2% human serum albumin [HSA], pH 7.4) and resuspended at a concentration of 5 × 10⁹/mL. Platelets were counted on a Technicon H3 (Technicon, Tarrytown, NY) cell counter and showed less than 0.1% contamination with leukocytes or erythrocytes. All samples were used within 2 hours of purification.

Peripheral blood neutrophils from healthy volunteers or patients with LAD-1 were isolated from whole venous blood by dextran sedimentation followed by density separation over Ficoll-Hypaque and hypotonic lysis.7,24-28 Purified cells were stored in Hanks' balanced Salt Solution (HBSS) supplemented with 10 mmol/L HEPES and 0.2% HSA, pH 7.4 (HBSS buffer) at room temperature for a maximum of 4 hours. For in vitro flow chamber assays, neutrophils were resuspended in HBSS buffer supplemented with either 2 mmol/L MgCl₂ plus 5 mmol/L EGTA, pH 7.4, or 2 mmol/L CaCl₂ plus 1 mmol/L MgCl₂ and 0.2% HSA, pH 7.4. For transmigration assays, neutrophils were fluorescently labeled with 2',7'-bis(2-carboxyethyl)-5- and 6-carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR) at 0.5 μg/mL for 30 minutes in assay medium (1:1 mixture of RPMI 1640 and medium 199 plus 0.25% HSA), washed, and resuspended at 2.5 × 10⁶ cells/mL.

mAbs. The following previously described murine mAbs to human antigens were used: CBR-IC2/2 (anti-ICAM-2, immunoglobulin γ [IgG2a]),29 TS1/22 (anti-CD11a, IgG1),30 OKM1 (anti-CD11b, IgG2a),31 CBRM1/29 (anti-CD11b, IgG1),32 CBRp130/2E1 (anti-CD11c, IgG1),33 CBRM1/3 (anti-CD11a, IgG1), TS1/18 (anti-CD18, IgG1),34 and PECAM 1.3 (anti-CD31, IgG1).35 WAPS 12.2 (anti-CD62P, IgG1)36 and SI2 (anti-IgGl antibodies to P-selectin, were obtained from Dr Eugene Butcher (Stanford, CA) and Dr Rodger McEver (University of Oklahoma College of Medicine, respectively). Fab fragments were prepared using the ImmunoPure Fab Preparation Kit (Pierce Chemical, Rockford, IL). The purity and binding capacity of the intact mAb and Fab fragments were ascertained by Coomassie staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) run under nonreducing conditions and flow cytometry (Becton Dickinson, San Jose, CA), respectively. All mAbs, either purified IgG (20 μg/mL) or Fab (20 μg/mL), were used at saturating conditions in both flow and transmigration assays. In addition, all experiments included isotype-matched IgG mAbs as negative controls.

**Transendothelial and transplatelet chemotaxis assays.** Isolation and culture of human umbilical vein endothelial cells (HUVEC), as well as the transendothelial chemotaxis assay, were as previously described.29-31 For neutrophil transmigration through surface-adherent platelets, 100 μL of various concentrations of purified human platelets (0.5 × 10⁹/mL to 5 × 10⁹/mL) were bound to fibronectin-coated (Becton Dickinson, 6.5-mm diameter polycarbonate membranes [Costar, Cambridge, MA]) of 3 μm pore size. After a 15-minute incubation period, the Transwells were briefly centrifuged (200g for 2 minutes), the plate bound platelets activated with 0.2 U/mL of human thrombin (Sigma, St Louis, MO) for 2 minutes, and then washed twice in HBSS buffer. Platelets were also coated on extracellular matrix (ECM) derived from HUVECs grown on Transwells for a period of 7 days. The confluent cell layer was removed by treatment with 0.5% Triton X-100 and 20 mmol/L NH₄OH in phosphate-buffered saline (PBS) for 30 minutes at 25°C.37 The presence and uniformity of HUVEC-derived ECM and fibronectin were documented by staining Transwells with an antifibronectin mAb (AMAC, Rockford, IL) or assay medium was added to 24-well tissue culture plates in a final volume of 600 μL.

Platelet or HUVEC-covered Transwells were placed into wells and BCECF-labeled neutrophils (2.5 × 10⁶) were added to each insert in a final volume of 100 μL. A 1:20 dilution of input cells added to a well that contained medium alone, but without a Transwell insert, served as a measure of the number of input cells. After incubation at 37°C in 5% CO₂, the inserts were removed and the transmigrated cells were counted by fluorescent microscopy as previously described.38 All samples were tested in either duplicate or triplicate, and each experiment repeated a minimum of three times. In each experiment, the ability of either untreated filters, fibronectin-coated filters, ECM-coated, platelet-covered, or endothelial-covered filters to act as a barrier to the upward diffusion of FITC-dextran (Sigma) of average molecular weight (mol wt) 4,400 was assessed for up to 4 hours.

Electron microscopy. Platelet-covered Transwell membranes were fixed in 1% glutaraldehyde for 1 hour at 22°C, washed twice in sodium cacodylate buffer, postfixed in 1% OsO₄ in phosphate-buffered saline, and embedded in Epon (Ted Pella Inc, Redding, CA).

Platelet substrate. Purified human platelets were bound to 3-aminopropyltriethoxysilane (APES)-treated glass slides and activated with thrombin as previously described.24 For comparison, platelets were also bound to fibronectin-coated slides (400 μg/mL, 37°C for 1 hour).39 A confluent layer of spread platelets was formed and confirmed by light microscopy for each experiment.

Endothelial cell substrate. Primary HUVECs were harvested and cultured as previously described38 and stimulated with human thrombin (1.0 U/mL) for 2 minutes.

**Detection assays.** A glass slide that contained adherent platelets or a 150-mm tissue culture dish on which endothelial cells were cultured was assembled in a parallel-plate laminar flow chamber (260 μm gap thickness) in which a uniform wall shear stress is generated.40 The flow chamber was mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY). Neutrophils (2 × 10⁵ cells) were injected through a side port and allowed to settle for 5 minutes in stasis. Alternatively, neutrophils were allowed to accumulate on the platelet substrate at a shear stress of 0.73 dyn/cm² for 3 minutes. Controlled flow at an initial shear force of 0.73 dyn/cm² was applied and increased every 20 seconds to a maximum of 35 dyn/cm². The number of adherent cells was counted after 20 seconds of flow at each shear stress. All experiments were recorded with a TEC-470 CCD video camera (Optronics, Goleta, CA) and Hi 8 Sony CVD-1000 recorder. For activation studies, neutrophils were treated with phorbol myristate acetate (PMA; Calbiochem, La Jolla, CA) at 50 ng/mL final concen-
tion for 2 minutes before injection into the flow chamber. For antibody-inhibition studies, neutrophils, surface-adherent platelets, or cultured endothelial cells were incubated with mAb (20 μg/mL) or Fab (20 μg/mL) for 15 minutes at room temperature before applying shear. HBSS containing 5 mmol/L EDTA was infused between platelet data sets, resulting in the release of all cells, and confirming specific interactions.

For tethering assays, unstimulated neutrophils (1 × 10^6/mL) were perfused over the substrate for 3 minutes at the desired shear stress and the number of cells that tethered in the identical field of view was measured. A tether was defined as a cell bound to the substrate for greater than 5 seconds.

Neutrophil rolling. Rolling velocities were measured for all cells (>20 cells) using the identical field of view (40X objective) for two experiments performed in duplicate. Velocities were measured for 5-second intervals beginning at the initial tether, t = 0.

RESULTS

β2-integrin–deficient neutrophils tether as efficiently as control neutrophils to surface-adherent platelets, but fail to develop firm adhesion. Stable tethering to thrombin-stimulated platelets adherent to a glass slide that formed the lower wall of a flow chamber was compared between LAD-I and healthy control neutrophils. LAD-I neutrophils tethered as efficiently as control neutrophils to the platelet substrate over a wide range of shear stresses (Fig 1A). Adhesion of both populations of cells was divalent cation-dependent as shown by abrogation with 5 mmol/L EDTA. Treatment of platelets with a blocking P-selectin mAb WAPS 12.2, but not with a noninhibitory P-selectin mAb S12, significantly reduced binding of both LAD-I and healthy neutrophils (Fig 1A and data not shown). For comparison, neutrophil tethering to thrombin-stimulated primary HUVECs was evaluated at 0.73 dyn/cm^2. Thrombin-stimulated, but not resting, endothelium supported neutrophil adhesion at levels comparable to the platelet substrate (Fig 1B). As observed for surface-adherent platelets, treatment of activated endothelial cells with a blocking P-selectin mAb abrogated the binding of healthy neutrophils. Healthy control, but not LAD-I, neutrophils were observed to arrest on the platelet substrate. LAD-I neutrophils continued to roll at virtually identical velocity throughout the observation period. In the first 5-second interval after tethering to the platelet substrate, mean rolling velocities of control cells were already diminished relative to the LAD-I cells, which suggests the involvement of additional adhesion receptors (Fig 2A). After approximately 30 seconds, essentially all of the control neutrophils became arrested on the platelet substrate.

Neutrophils tethered to the adherent, activated platelets in flow for 3 minutes at 0.73 dyn/cm^2 were assessed for their ability to develop adhesion-strengthening interactions. Healthy control, but not LAD-I, neutrophils were resistant to shear-induced detachment forces (Fig 2B). At a shear stress of 36 dyn/cm^2, more than 85% of healthy control, as compared with less than 10% of LAD-I, cells remained bound.

To compare more directly the ability of healthy and LAD-I neutrophils to develop firm adhesion on activated platelets, neutrophils were stimulated with PMA (50 ng/mL) before injection into the flow chamber to ensure maximal β2-integrin activation, and allowed to bind in stasis to the platelets to permit firm adhesion to develop under identical conditions. PMA stimulation resulted in a marked polarization, pseudopod formation, and refractile changes in both neutro-
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A

149

B

100

75

50

25

cells remaining bound (%)

0 10 20 30 shear stress (dyn/cm²)

mean rolling velocity (μm/s)

0 5 10 15 20 25 30 time after initial tether (s)

Fig 2. Unstimulated control neutrophils, but not LAD-I cells, arrest on surface-adherent platelets in flow. (A) Healthy control; ○, LAD-I. (A) Rolling velocity after tethering. Cells were allowed to tether to the platelet substrate at a shear stress of 1.8 dyn/cm² and mean rolling velocities calculated at 5-second intervals. Mean ± SD of velocities of 15 to 20 cells from 2 independent experiments performed in duplicate. (B) Shear-induced detachment of neutrophils bound to adherent, activated platelets under flow conditions. Cells tethered in flow for 3 minutes at 0.73 dyn/cm² were subjected to incremental increases in shear stress. The percentage of neutrophils that remained bound to the platelet substrate was determined after 20 seconds at each shear stress.

Antibodies to Mac-1 abolish Mg²⁺-dependent firm adhesion of control neutrophils to activated, adherent platelets. Previous studies have shown that both the integrins Mac-1 and LFA-1 are important for neutrophil firm adherence to endothelial cells, and that ICAM-1 is the most important ligand on endothelium. To isolate firm adhesion from Ca²⁺-dependent selectin interactions, adhesion was assayed in Mg²⁺ and EGTA. Neutrophils were stimulated with PMA and allowed to bind in stasis to activated, adherent platelets, and then subjected to shear flow at 0.73 dyn/cm². Pretreatment of neutrophils with either whole mAb or Fab fragment to Mac-1 diminished binding by greater than 95% (Fig 4). Inhibition by either mAb to the α-subunit (C9M1/29) or β-subunit of Mac-1 (TS1/18) was equally effective, whereas mAb to LFA-1 or p150,95 had no observable effect, which suggests that Mac-1 is the major or only β₂-integrin responsible for firm adhesion. An mAb to a different epitope on Mac-1, OKM1, had no effect and acted as a control. Similar results with Mac-1 mAbs were obtained whether platelets had been adhered to APES- or fibronectin-coated slides. Of the three ICAMs, only ICAM-2 is expressed on platelets. A blocking mAb to ICAM-2 that has been previously shown to inhibit LFA-1-dependent adhesion to platelet substrates had no effect on neutrophil firm adhesion to platelets.

Surface-adherent platelets support IL-8-induced neutrophil transmigration. The ability of surface-adherent platelets to support both P-selectin–mediated tethering and Mac-1–dependent firm adherence of neutrophils suggested that platelets, like endothelium, may be capable of supporting leukocyte transmigration. To test this and determine what adhesion molecules would be required, we developed a transplatelet chemotaxis assay. Purified human platelets were adhered to fibronectin-coated Transwell membranes and activated with thrombin. The application of either 1.0, 2.0, or 4.0 × 10⁷ platelets per membrane resulted in a confluent platelet multilayer as determined by electron microscopy (Fig 5). The platelet multilayer acted as barrier to the diffusion of FITC-dextran (4,400 mol wt) from the lower to the upper chamber. Platelets at 2 × 10⁷ per membrane and confluent HUVEC monolayers retarded diffusion threefold and sixfold more, respectively, than fibronectin-coated or un-
Fig 3. Detachment and rolling in shear flow of PMA-stimulated neutrophils incubated in stasis on activated, adherent platelets and thrombin-stimulated endothelial cells. (A) Neutrophils in Ca²⁺ and Mg²⁺ buffer exposed to 50 ng/mL PMA for 2 minutes were injected through a port in the side of the flow chamber. After 5 minutes of contact with the platelet substrate under quiescent conditions, flow was started and increased in staged increments. The percentage of neutrophils that remained bound (rolling or firmly adherent) on immobilized platelets was quantified after 20 seconds at each shear stress tested. (B) The number of rolling cells was quantified after 20 seconds at each shear stress tested in the experiment described above. Rolling cells were defined as those that moved >2-cell diameters in 20 seconds. Experiments 1 and 2 were with cells from two different LAD patients. (C) The resistance to shear-reduced attachment of PMA-activated healthy neutrophils bound to thrombin-stimulated endothelial cells was determined as described in (A). (D) The percentage of PMA-stimulated neutrophils that rolled in increasing shear stress on thrombin-stimulated HUVECs was quantitated as in (B). Mean ± SD for triplicate determinations.

treated membranes. Similar results were obtained in experiments assessing the diffusion of 125I-IL-8 (results not shown). Neutrophils transmigrated across platelets to the chemotactic agent IL-8 in a dose-dependent manner (Fig 6). Transmigration of neutrophils to IL-8 was unimpeded by fibronectin alone or platelets at 0.5 × 10⁷ per membrane as compared with untreated filters, but was reduced as the density of platelets was increased to 2 × 10⁷ and 4 × 10⁷ per membrane. In comparison to a HUVEC monolayer, platelets at 2.0 × 10⁷ per membrane yielded similar neutrophil transmigration at all IL-8 concentrations tested. Platelets greatly reduced background neutrophil transmigration in the absence of IL-8, yielding migration indices greater than 1,000 to IL-8. The majority of neutrophils (~65% of the total transmigrating population) migrated across platelets within 1 hour, with the remainder migrating in the second hour (data not shown). Transmigration across platelets bound to HUVEC-derived ECM, a substrate more physiologically relevant to vessels denuded of endothelium, gave essentially identical results (data not shown). During the process of transmigra-
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Inhibition by Mac-1 mAb of Mg²⁺-dependent neutrophil adhesion to activated platelets. PMA-stimulated neutrophils, in buffer containing EGTA and MgCl₂, were injected into the flow chamber and allowed to settle on platelets that had been adhered to glass slides coated with APES (■) or fibronectin (□) and activated with thrombin. After 5 minutes of contact, a shear of 0.73 dyn/cm² was applied. Neutrophils that remained bound after 20 seconds of flow are expressed as the percentage of cells that settled in the initial contact period. Neutrophils or adherent platelets were incubated with the indicated mAb (20 μg/mL) for 15 minutes. Data are the mean ± SD of 3 independent experiments, each performed in triplicate.

To test whether IL-8-induced neutrophil migration across adherent platelets was due to chemotaxis (directed locomotion) or chemokinesis (stimulation of random locomotion), a checkerboard analysis was performed with different concentrations of IL-8 in the top and bottom chamber. Significant neutrophil migration occurred only when a gradient existed, with the higher concentration of IL-8 present in the bottom compartment (Table 1). Thus, neutrophil migration across platelets to IL-8 is the result of chemotaxis, with little or no random locomotion.

Mac-1 is required for neutrophil migration across platelets. The function of adhesion molecules in neutrophil transplatelet migration was evaluated by antibody-inhibition assays. mAb to both the α-subunit of Mac-1 and the common β₂-subunit of the leukocyte integrins reduced transmigration by greater than 90% (Fig 8A). Furthermore, this inhibition was specific as the mAb OKM1, an mAb of the same isotype to a different epitope on Mac-1, had no effect on the ability of neutrophils to migrate across fibronectin-bound platelets. Function-blocking mAb to the leukocyte integrins p150,95 and LFA-1 had no and little effect, respectively. Furthermore, function-blocking mAb to P-selectin and ICAM-2 had
fibronectin alone (HI or fibronectin and then platelets at 0.5
ence. Neutrophil migration was for 1 hours at 37°C. Values are the
mean and range of duplicates. Transwell insert membranes with 3-pm pores were coated with
fibronectin and then platelets at 0.5 (b), 2.0 (b), and 4.0 (b) × 10⁷/insert followed by activation with thrombin. Alternatively, HUVECs (b) were cultured on membranes to confluence. Neutrophil migration was for 1 hours at 37°C. Values are the mean and range of duplicates.

no effect. Incubation of the platelet substrate with function-blocking mAb to CD31 also had little effect on neutrophil transmigration. By contrast to results on transplatelet chemotaxis, migration across filters coated with fibronectin alone was much less dependent on Mac-1, with 35% inhibition by the CBRM1/29 mAb (Fig 8A). Identical results on inhibition of migration across platelet multilayers or fibronectin alone were obtained when the incubation period was extended from 1 to 4 hours (data not shown).

To examine the step in transmigration blocked by Mac-1, after 1 hour of chemotaxis in the presence of CBRM1/29 Fab to Mac-1, Transwell membranes were fixed and examined by electron microscopy (Fig 8B). Greater than 95% of neutrophils remained at the platelet surface, which shows that platelet transmigration was blocked; no neutrophils were observed at the platelet/fibronectin interface. These results were at a platelet-coating density of 2 × 10⁷/insert; neutrophil transmigration across platelet-coating densities of 0.5 to 1.0 × 10⁷/insert was similarly inhibited by CBRM1/29 Fab (data not shown).

DISCUSSION
The observation that neutrophils tether, roll, and subsequently arrest on activated platelet monolayers suggests that platelets, like endothelial cells,¹ may recruit neutrophils through a multi-step, sequential process of adhesive interactions (Fig 9). In accordance with this model, we and others have clearly demonstrated that the initial interaction is mediated by P-selectin, an event that results in rolling adhesions (step 1).¹⁹,²⁰ P-selectin has been shown to be important in neutrophil recruitment to sites of platelet deposition in vivo.¹¹ P-selectin–dependent attachment was confirmed by the ability of a function-blocking mAb to significantly inhibit binding of neutrophils in flow. The subsequent arrest of rolling neutrophils on surface-adherent platelets is reminiscent of chemotactant-induced activation of additional adhesion receptors (such as the β₂-integrins) (step 2) and the binding of these molecules to counter-receptors on endothelium (step 3). Platelet-induced activation of rolling neutrophils has been established in a previous study by the requirement for metabolically intact neutrophils for the arrest of rolling cells in flow.²⁶ We have shown that unstimulated healthy control neutrophils, but not LAD-I cells, undergo a rapid reduction in mean rolling velocities, with greater than 50% of cells arrested within the first 5 seconds of attachment and the remainder within 30 seconds of contact with platelets. Similar observations have been reported with fMLP-stimulated arrest of neutrophils on supported phospholipid bilayers that contain purified P-selectin and ICAM-1.¹⁸ However, the mechanism(s) of neutrophil activation by platelets remains unknown, and may be the result of interactions with a surface receptor or surface-bound chemoattractant(s). In the present study, we have extended this multistep model of neutrophil/platelet adhesive interactions by identifying Mac-1 as the β₂-integrin required for neutrophil firm adhesion (step 3) to surface-adherent platelets. We also report for the first time that surface-bound platelets can support Mac-1–dependent, chemoattractant-induced neutrophil transmigration, in parallel with transendothelial migration (step 4).

Development of firm adhesion of neutrophils to platelets and its dependence on the integrin Mac-1 was verified in several ways. The formation of mechanically strong attachments was confirmed by demonstrating that the majority of adherent healthy neutrophils remained bound to the platelet substrate at high shear stresses. P-selectin did not contribute significantly to these interactions, as shown by the complete detachment at 36 dyn/cm² of LAD-I patient, but not healthy control, neutrophils that had bound to platelets in presence of Ca²⁺ and Mg²⁺. Furthermore, greater than 80% of healthy control neutrophils that had bound to platelets in presence of Ca²⁺ and Mg²⁺ and absence of Ca²⁺, thus negating selectin-dependent interactions, remained adherent at this shear stress (data not shown). Adhesion strengthening was not accompanied by significant spreading of neutrophils on the platelet substrate, by contrast to results with fMLP-stimulated arrest of neutrophils on supported phospholipid bilayers that contained purified P-selectin and ICAM-1.¹⁸ Elucidating the mechanism of this rapid activation requires much further work; possible candidates for the stimulus include platelet-activating factor (PAF)¹⁸ and the chemokines NAP-2⁴ and ENA-78.⁴⁰ Our results with platelets highlight the different functions of selectins and leukocyte integrins in adhesion in shear flow. Although neutrophils deficient in β₂-integrins exhibited P-selectin–dependent tethering in flow that was as efficient as control cells, they did not develop firm adherence to the platelet substrate even when stimulated with PMA. In fact, PMA stimulation resulted in decreased resistance to shear stresses. This is consistent with previous reports studying interactions with P-selectin¹⁸,⁴⁷ and E-selectin⁴⁸ substrates, and has been interpreted to be the result of neutrophil shape change⁴⁸ or counter-receptor redistribution.⁴⁷ Neutrophil activation has

![Fig 6. Neutrophil transmigration to IL-8 across platelet multilayers. Transwell insert membranes with 3-μm pores were coated with fibronectin alone (b) or fibronectin and then platelets at 0.5 (b), 2.0 (b), and 4.0 (b) × 10⁷/insert followed by activation with thrombin. Alternatively, HUVECs (b) were cultured on membranes to confluence. Neutrophil migration was for 1 hours at 37°C. Values are the mean and range of duplicates.](image-url)
Fig 7. Electron micrographs of neutrophil/platelet interactions during transmigration through platelet multilayers to IL-8. Platelets (2.0 \times 10^7) were bound to Transwell membranes and 30 minutes after initiation of neutrophil chemotaxis to 10 ng/mL IL-8, Transwells were fixed for electron microscopy. (A) A neutrophil (N) in close proximity to the platelet (P)-covered upper surface of a Transwell membrane (original magnification \times 8,580). (B) Neutrophil/platelet interaction along the upper filter surface (original magnification \times 9,254). (C) Neutrophil entering a platelet-coated pore on the upper filter surface (original magnification \times 10,576). (D) Neutrophil exiting a pore at the lower filter surface (original magnification \times 10,576).

Also been reported to downregulate P-selectin-dependent binding to platelets in suspension assays.

Using mAb to leukocyte integrin \( \alpha \) and \( \beta \)-subunits, we showed that Mac-1 mediates greater than 90% of the adhesion-strengthening interaction between neutrophils and surface-immobilized platelets. Neither LFA-1 nor p150,95 played any detectable role. Thus, \( \beta \)-integrin-dependent firm adhesion via Mac-1 is the principal mechanism responsible for the arrest of activated neutrophils on adherent, activated platelets. By contrast, a combination of mAb to Mac-1 and LFA-1 is required for significant inhibition of neutrophil adhesion to stimulated endothelial monolayers. The identity of the surface molecule(s) on platelets that support Mac-1 binding remains unknown. We have shown that ICAM-2, a ligand for LFA-1, is constitutively present on platelets and mediates binding of PMA-stimulated T lymphocytes. MAbs and Fabs to ICAM-2 that completely block binding

<table>
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<th>IL-8 (ng/mL) in Bottom</th>
<th>% Neutrophil Migration (IL-8 [ng/mL] in top)</th>
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<tr>
<td>0</td>
<td>0.01 ± 0.01</td>
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<tr>
<td>1</td>
<td>0.2 ± 0.05</td>
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<tr>
<td>10</td>
<td>24.0 ± 2.0</td>
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<tr>
<td>100</td>
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Purified platelets (2 \times 10^7) were adhered to fibronectin-coated Transwell inserts and activated with thrombin. IL-8 or control media was placed into plate wells (bottom) and inserts (top) to achieve the concentrations indicated. Purified neutrophils were added at 2.5 \times 10^6/insert and incubated for 1 hour at 37°C. Data are the mean ± half the range of duplicates.
to LFA-1, and mAbs to LFA-1 that block binding to ICAM-1, ICAM-2, and ICAM-3, had no effect on firm adhesion of neutrophils to platelets. This clearly illustrates the predominance of a Mac-1 adhesion pathway over the LFA-1/ICAM-2 pathway, and implicates the presence of a Mac-1 ligand on activated platelets. Potential Mac-1 ligands include fibrinogen, high molecular weight kininogen (HK), and heparan sulfate, all of which may be present on the surface of activated platelets. ICAM-2 has also been found to be a ligand for Mac-1; however, we have been unable to find binding of Mac-1 to ICAM-2. The group that found binding of Mac-1 to ICAM-2 found partial inhibition of this interaction with CBR-IC2/2 mAb to ICAM-2; however, we consistently found no inhibition by this mAb of neutrophil interactions with platelets. Further work is required to identify the Mac-1 ligand(s) on platelets responsible for firm adhesion.

Adhesion to a substrate is thought to be prerequisite to provide traction for chemotactic migration; LAD-1 patient neutrophils are deficient in ability both to orient and migrate in chemoattractant gradients. The observation that neutrophils form firm attachments to adherent, activated platelets led us to investigate the possibility that adherent platelets could also support chemoattractant-induced neutrophil transmigration. We demonstrated that IL-8 attracts neutrophils across a confluent multilayer of platelets in a dose-dependent...
MAC-1 AND FIRM ADHESION OF NEUTROPHILS TO PLATELETS

**FLOW**

neutrophil

platelets

endothelium

Attachment & Rolling → Activation → Firm adhesion → Diapedesis

- **Fig 9.** Proposed sequential adhesion model of neutrophil attachment to and transmigration across activated, surface-adherent platelets. P-selectin mediates the initial attachment and rolling in flow (1); surface receptor(s) or surface-bound cytokine(s) induce neutrophil activation (2); the β₂-integrin Mac-1 promotes firm adhesion (3), and also mediates chemoattractant-induced diapedesis (4).

In conclusion, activated, adherent platelets support all steps thought to be required for accumulation and emigration of neutrophils at sites of vascular injury. Both development of firm adhesion after initial tethering and rolling on P-selectin, and transplatelet emigration to chemoattractants, are entirely dependent on the leukocyte integrin Mac-1. Except for the lack of participation of LFA-1, there is a complete parallel with the proposed adhesion cascade responsible for neutrophil/endothelial cell interactions, and with subsequent transendothelial migration, which suggests that platelets deposited at sites of vascular damage may serve as surrogates for endothelial cells in their ability to recruit circulating neutrophils through selectin- and integrin-dependent adhesion. Such an adhesive mechanism may play a vital role in the colocalization of neutrophils and platelets in various pathologic conditions in humans, such as myocardial infarction, inflammatory bowel disease, and atherosclerosis. Furthermore, the interaction of platelets with leukocytes may facilitate the functions of both cell types in thrombosis and inflammation. Understanding the adhesive basis of these events may enable development of new therapeutic strategies aimed at altering platelet/neutrophil interactions.

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Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18

TG Diacovo, SJ Roth, JM Buccola, DF Bainton and TA Springer