Neutrophil Rolling, Arrest, and Transmigration Across Activated, Surface-Adherent Platelets Via Sequential Action of P-Selectin and the β_2-Integrin CD11b/CD18

By Thomas G. Diacovo, Stephen J. Roth, Janet M. Buccola, Dorothy F. Bainton, and Timothy A. Springer

Platelets bound to thrombogenic surfaces have been shown to support activation-dependent firm adhesion of neutrophils in flow following selectin-mediated tethering and rolling. The specific receptor(s) responsible for mediating adhesion-strengthening interactions between neutrophils and platelets has not previously been identified. Furthermore, the ability of adherent platelets to support the migration of bound neutrophils has not been tested. We studied neutrophil interactions with activated, surface-adherent platelets as a model for leukocyte binding in vascular shear flow and emigration at thrombogenic sites. Our results demonstrate that the β_2-integrin Mac-1 (CD11b/CD18) is required for both firm attachment to and transmigration of neutrophils across surface-adherent platelets. In flow assays, neutrophils from patients with leukocyte adhesion deficiency-1 (LAD-I), which lack β_2-integrin receptors, formed P-selectin–mediated rolling interactions, but were unable to develop firm adhesion 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 β_2-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.

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.[1,2] The importance of such interactions is demonstrated in leukocyte adhesion deficiency-1 (LAD-I) patients with a genetic absence of leukocyte integrins (β_2-integrins; CD18 antigens). These patients have recurrent bacterial infections and delayed wound healing.[3] The leukocyte or β_2-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.[4] This requires activation of integrin adhesiveness.[5] 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 due to lack of β_2-integrins.[6,7] The leukocyte integrins are also crucial for movement of neutrophils through endothelial monolayers; transmigration of healthy neutrophils in vitro is completely blocked by anti-CD18 monoclonal antibodies (mAbs), and is deficient in LAD-I neutrophils.[8-10] Thus, neutrophils that lack β_2-integrin receptors are unable to complete a multistep adhesion process responsible for their recruitment to sites of inflammation.

Although well characterized for endothelium, a similar multistep model has not been established for platelets, which are localized to the vessel wall in thrombosis and would occupy a position analogous to endothelium with respect to leukocyte accumulation and emigration. There are many associations between neutrophils and platelets in inflammation and thrombosis. Neutrophils and platelets colocalize at sites of hemorrhage, on vascular grafts, on artherosclerotic lesions, and in areas of myocardial infarction.[11-15] Furthermore, interactions between neutrophils and platelets are required in the production of chemoattractants, such as neutrophil-activating peptide-2 (NAP-2), and in transcellular synthesis of leukotrienes and arachidonic acid metabolites.[16,17] Thus, stable adhesive interactions may be a prerequisite for promoting neutrophil/platelet cross-communication.

When platelets are activated, P-selectin is mobilized from α-granules to the plasma membrane. Purified P-selectin and P-selectin on the surface of adherent, activated platelets support tethering and accumulation of neutrophils in flow, in an interaction characterized by reversible adhesion and rolling.[11,18-21] mAbs to P-selectin inhibit platelet and neutrophil accumulation on an artificial vessel surface in vivo.[11] Although P-selectin participation in transient neutrophil attachment to adherent platelets is well established, the formation of more permanent interactions mediated through the β_2-integrins has not been characterized. Firm adherence of neutrophils to platelets may be important in facilitating the events described here and in subsequent emigration of neutrophils into thrombi and wounded tissue. Firm neutrophil/platelet interactions no doubt contribute to the maintenance of vascular integrity, as well as to its impairment in pathologic states.

Here, we characterize multiple steps in neutrophil/platelet

From The Center for Blood Research, Division of Newborn Medicine, and Departments of Cardiology and Pathology, Harvard Medical School, Boston, MA; and the Department of Pathology, University of California, San Francisco, CA.

Submitted November 20, 1995; accepted February 19, 1996.

Supported by National Institutes of Health Grant No. CA 31799.

Address reprint requests to Timothy A. Springer, PhD, The Center for Blood Research, 200 Longwood Ave, Boston, MA 02115.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/96/8801-0107$3.00/0

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-I, 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 β_2-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.[23] Platelets were washed twice in HEPES buffer (145 mmol/L NaCl, 10 mmol/L HEPES, 0.5 mmol/L Na_2HPO_4, 5 mmol/L KCL, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 0.1% glucose, and 0.2% human serum albumin [HSA], pH 7.4) and resuspended at a concentration of 5×10^8/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-I 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 $MgCl_2$ plus 5 mmol/L EGTA, pH 7.4, or 2 mmol/L $CaCl_2$ plus 1 mmol/L $MgCl_2$ 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 2.5×10^6 cells/mL.[29]

mAbs. The following previously described murine mAbs to human antigens were used: CBR-IC2/2 (anti–ICAM-2, immunoglobulin [Ig]G2a),[30] TS1/22 (anti-CD11a, IgG1),[31] OKM1 (anti-CD11b, IgG2a),[32] CBRM1/29 (anti-CD11b, IgG1),[33] CBRp150/2E1 (anti-CD11c, IgG1),[34] TS1/18 (anti-CD18, IgG1),[31] and PECAM 1.3 (anti-CD31, IgG1).[35] WAPS 12.2 (anti-CD62P, IgG1)[36] and S12 (anti-CD62P, IgG1),[37] adhesion-blocking and nonblocking monoclonal IgG1 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.[29,38] For neutrophil transmigration through surface-adherent platelets, 100 μL of various concentrations of purified human platelets (0.5×10^8/mL to 5×10^8/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 (200*g* for 2 minutes), the 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 NH_4OH in phosphate-buffered saline (PBS) for 30 minutes at 25°C.[39] The presence and uniformity of HUVEC-derived ECM and fibronectin was documented by staining Transwells with an antifibronectin mAb (AMAC, Westbrook, ME), followed by secondary staining with fluorescein isothiocyanate (FITC)-labeled goat antimouse Ig (Zymed, South San Francisco, CA), and visualization under fluorescence microscopy.

For chemotaxis assays, recombinant human IL-8 (Genzyme, Cambridge, MA) 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 (2.5×10^5) 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% CO_2, the inserts were removed and the transmigrated cells were counted by fluorescent microscopy as previously described.[40] 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.[40]

Electron microscopy. Platelet-coated Transwell membranes were fixed in 1% glutaraldehyde for 1 hour at 22°C, washed twice in sodium cacodylate buffer, postfixed in 1% OsO4 in veronal-acetate buffer, stained with aqueous 1% uranyl acetate, dehydrated in ethyl alcohol, infiltrated with propylene oxide, and flat embedded in Epon (Ted Tella Inc, Redding, CA).[41]

Platelet substrate. Purified human platelets were bound to 3-aminopropyltriethoxysilane (APES)-treated glass slides and activated with thrombin as previously described.[23] For comparison, platelets were also bound to fibronectin-coated slides (400 μg/mL, 37°C for 1 hour).[42] 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 described[38] and stimulated with human thrombin (1.0 U/mL) for 2 minutes.

Detachment 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.[18] The flow chamber was mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY). Neutrophils (2×10^6 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/cm^2 for 3 minutes. Controlled flow at an initial shear force of 0.73 dyn/cm^2 was applied and increased every 20 seconds to a maximum of 35 dyn/cm^2. 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-

tration 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 (40× objective) for two experiments performed in duplicate. Velocities were measured for 5-second intervals beginning at the initial tether, t = 0.

RESULTS

β_2-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 β_2-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-

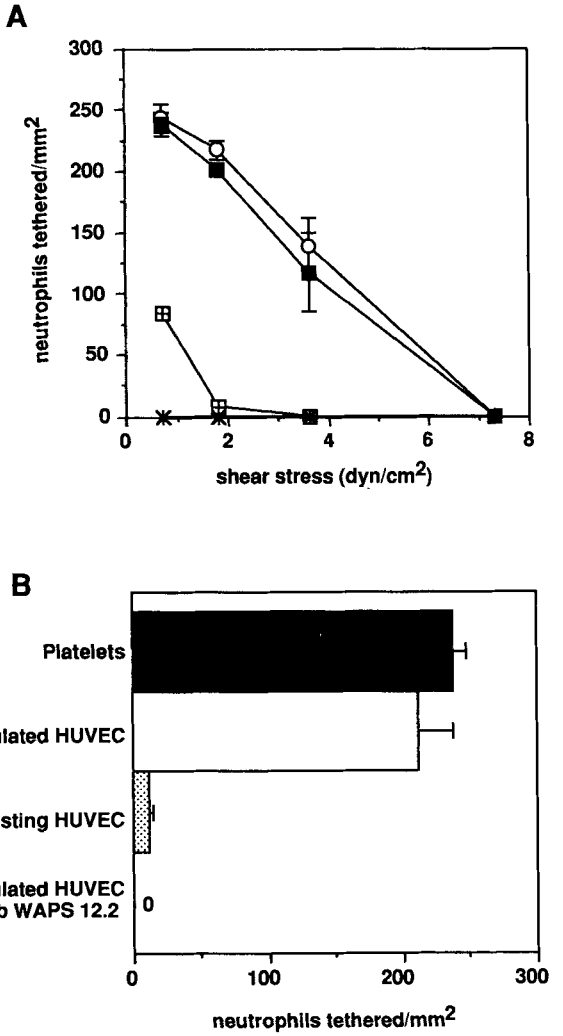

Fig 1. Tethering of healthy and LAD-I neutrophils to surface-adherent, activated platelets in physiologic shear flow. Neutrophils (10^6/mL in buffer with Ca^{2+} and Mg^{2+}) were infused at the indicated wall shear stresses through the parallel plate flow chamber. (A) Identical fields of view of immobilized platelets were used to score binding of different experimental groups of neutrophils during continuous flow for 3 minutes. Cells interacting with the platelet substrate for >5 seconds were considered stably tethered. ■, Healthy control; ○, LAD-I; ✱, healthy control, EDTA; ⊞, healthy control, WAPS 12.2. (B) Comparison of healthy neutrophil tethering to platelet and endothelial cell substrates. Neutrophils were infused over the resting endothelial substrate for 3 minutes at 0.73 dyn/cm^2 to determine binding in absence of stimulation. Subsequently, 1.0 U/mL of thrombin was infused for 2 minutes and neutrophil tethering evaluated using the identical field of view. Thrombin-stimulation for 5, 10, 15, and 20 minutes resulted in a similar number of bound neutrophils (data not shown). Treatments with the P-selectin mAb WAPS 12.2 (20 μg/mL) and EDTA (5 mmol/L) were as described in Materials and Methods. Data are the mean ± SD of 1 representative experiment performed in triplicate.

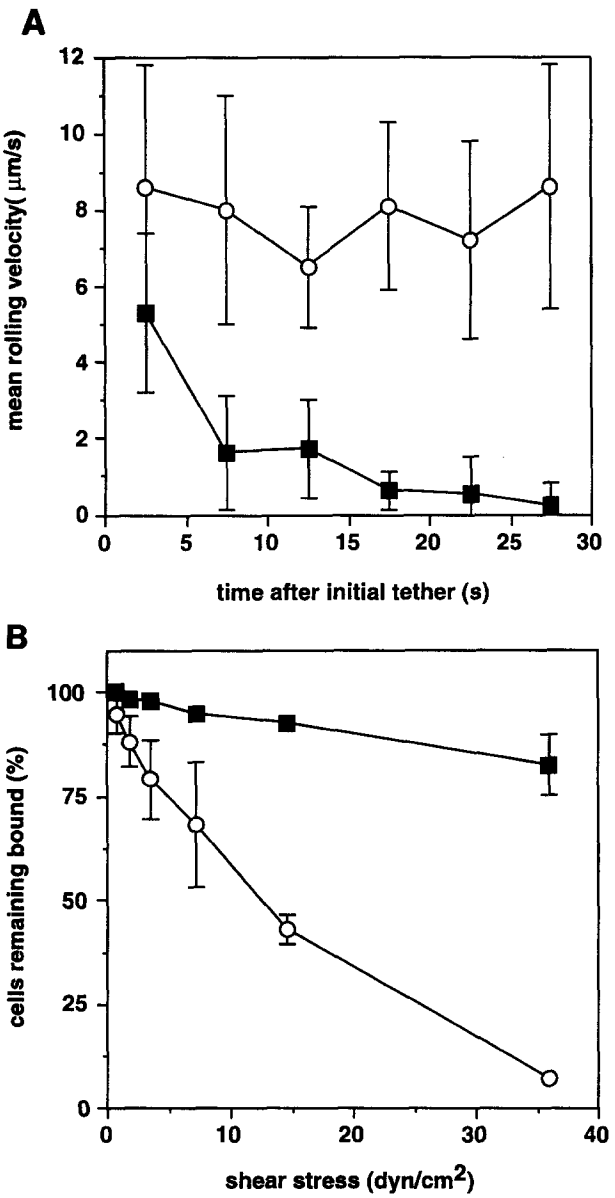

Fig 2. Unstimulated control neutrophils, but not LAD-I cells, arrest on surface-adherent platelets in flow. ■, Healthy control; ○, LAD-I. (A) Rolling velocity after tethering. Cells were allowed to tether to the platelet substrate at a shear stress of 1.8 dyn/cm² and mean rolling velocities calculated 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.

phil populations as determined by light microscopy, whereas when healthy neutrophils in shear flow were observed to develop integrin-dependent adhesion, shape change was minimal.

As shown by the ability to resist detachment in shear, healthy but not LAD-I neutrophils developed firm adhesion to activated platelets (Fig 3A). The strength of adhesion for healthy neutrophils did not significantly differ whether thrombin-stimulated platelets or HUVECs were used as the substrate (Fig 3C). At the highest shear stress tested, greater than 97% of β_2-integrin–deficient cells, but less than 10% of control cells, detached. The development of adhesion-strengthening interactions was confirmed by measurements of the fraction of rolling neutrophils (Fig 3B). Essentially all LAD-I neutrophils, but few of the healthy neutrophils, could be seen to roll for greater than two-cell diameter in 20 seconds at higher shear stresses. The rolling velocity for the LAD-I neutrophils was dependent on the hydrodynamic force acting on the cells and averaged 12.7 ± 3.8 μm/s at 3.6 dyn/cm². The same results were seen whether platelets had been bound to fibronectin or APES-coated slides, and were identical to results obtained on endothelial cell substrate (Fig 3D).

Antibodies to Mac-1 abolish Mg^{2+}-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.[9,43] To isolate firm adhesion from Ca^{2+}-dependent selectin interactions, adhesion was assayed in Mg^{2+} 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 (CBRM1/29) 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 β_2-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.[23] A blocking mAb to ICAM-2 that has been previously shown to inhibit LFA-1–dependent adhesion to platelet substrates[23] 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 trans-platelet 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 4.0×10^7 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 2×10^7 per membrane and confluent HUVEC monolayers retarded diffusion threefold and sixfold more, respectively, than fibronectin-coated or un-

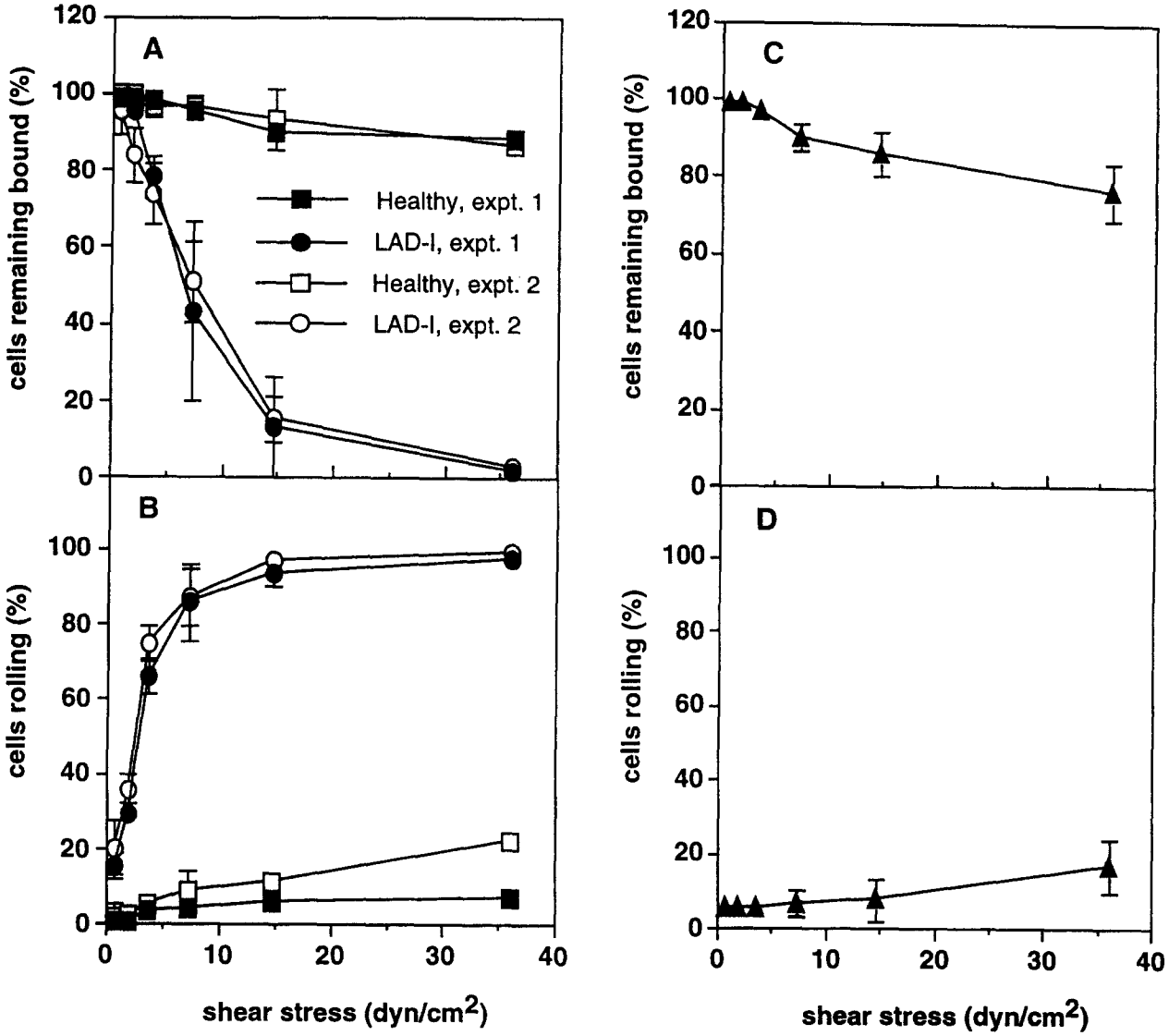

Fig 3. Detachment and rolling in shear flow of PMA-stimulated neutrophils incubated in stasis on activated, adherent platelets and thrombin-stimulated endothelial cells. (A) Neutrophils in Ca^{2+} and Mg^{2+} buffer exposed to 50 ng/mL PMA for 2 minutes were injected through a port in the side of the 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 ^{125}I–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 0.5×10^7 per membrane as compared with untreated filters, but was reduced as the density of platelets was increased to 2×10^7 and 4×10^7 per membrane. In comparison to a HUVEC monolayer, platelets at 2.0×10^7 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-

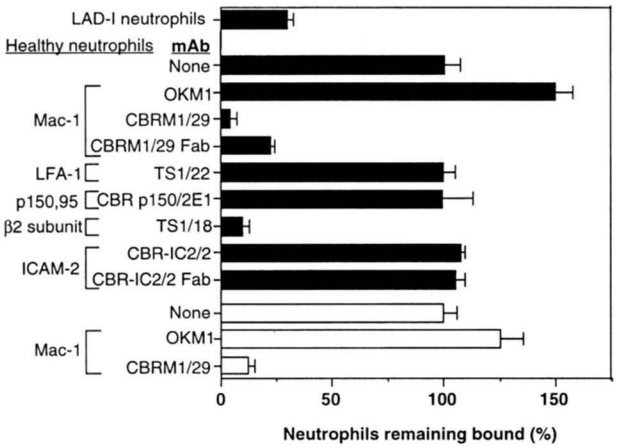

Fig 4. Inhibition by Mac-1 mAb of Mg^{2+}-dependent neutrophil adhesion to activated platelets. PMA-stimulated neutrophils, in buffer containing EGTA and $MgCl_2$, were injected into the flow chamber and allowed to settle on platelets that had been adhered to glass slides coated with APES (■) or fibronectin (□) 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.

tion, the plasma membrane of neutrophils was observed to be in close contact with the plasma membranes of activated platelets (Fig 7). Protrusion of a pseudopod into the platelet multilayer and then into a pore in the membrane preceded transmigration of the cell body, much as in transendothelial migration.

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.[44] 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 β_2-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

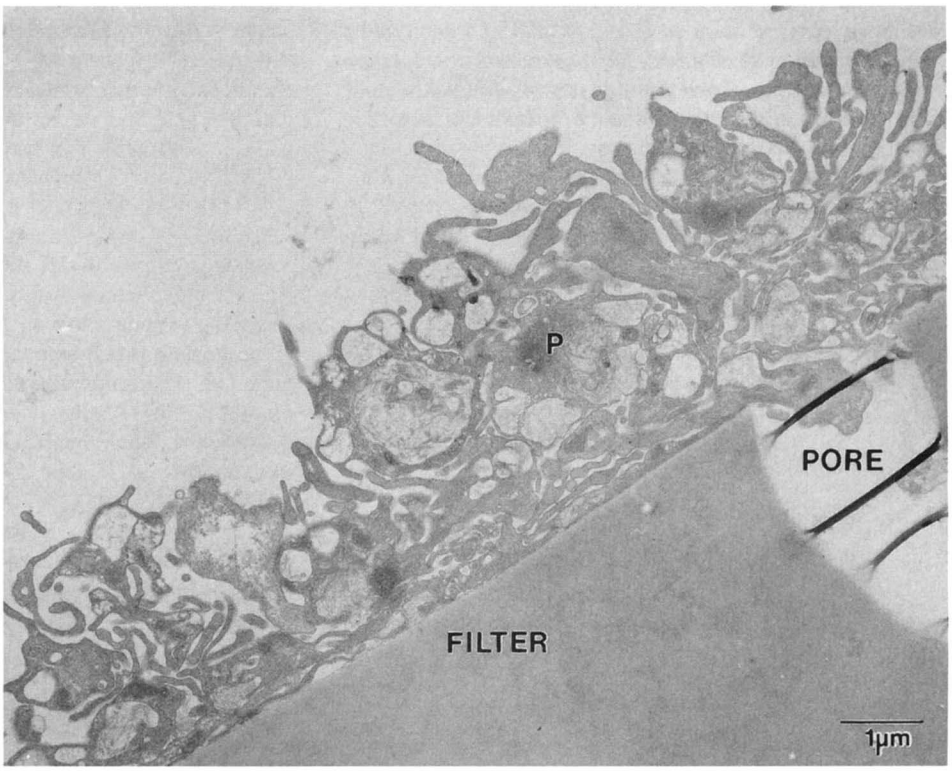

Fig 5. Transmission electron micrograph of thrombin-activated human platelets (P) (2×10^7 total) bound to a fibronectin-coated Transwell membrane. A confluent layer of degranulated platelets was also observed at a coating density of 1×10^7 total platelets (data not shown).

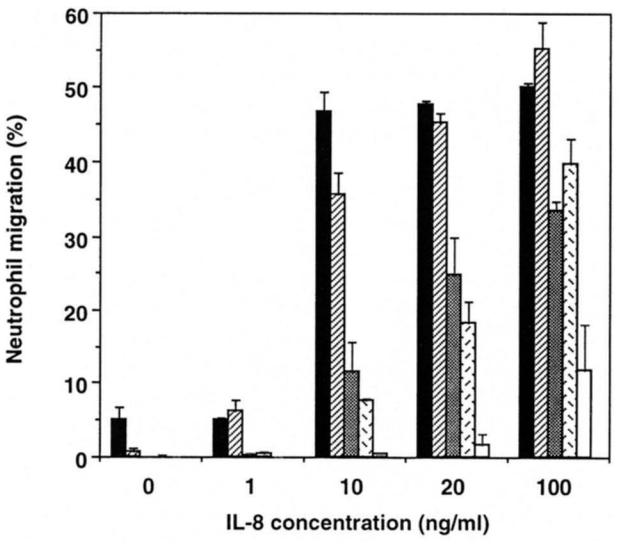

Fig 6. Neutrophil transmigration to IL-8 across platelet multilayers. Transwell insert membranes with 3-μm pores were coated with fibronectin alone (■) or fibronectin and then platelets at 0.5 (▨), 2.0 (▩), and 4.0 (□) × 10^7/insert followed by activation with thrombin. Alternatively, HUVECs (▧) were 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 × 10^7/insert; neutrophil transmigration across platelet-coating densities of 0.5 to 1.0 × 10^7/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 chemoattractant-induced activation of additional adhesion receptors (such as the β_2-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.[20] 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 β_2-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/cm^2 of LAD-I patient, but not healthy control, neutrophils that had bound to platelets in presence of Ca^{2+} and Mg^{2+}. Furthermore, greater than 80% of healthy control neutrophils bound in the presence of Mg^{2+} and absence of Ca^{2+}, 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)[45] and the chemokines NAP-2[16] and ENA-78.[46] Our results with platelets highlight the different functions of selectins and leukocyte integrins in adhesion in shear flow. Although neutrophils deficient in β_2-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-selectin[18,47] and E-selectin[48] substrates, and has been interpreted to be the result of neutrophil shape change[48] or counter-receptor redistribution.[47] Neutrophil activation has

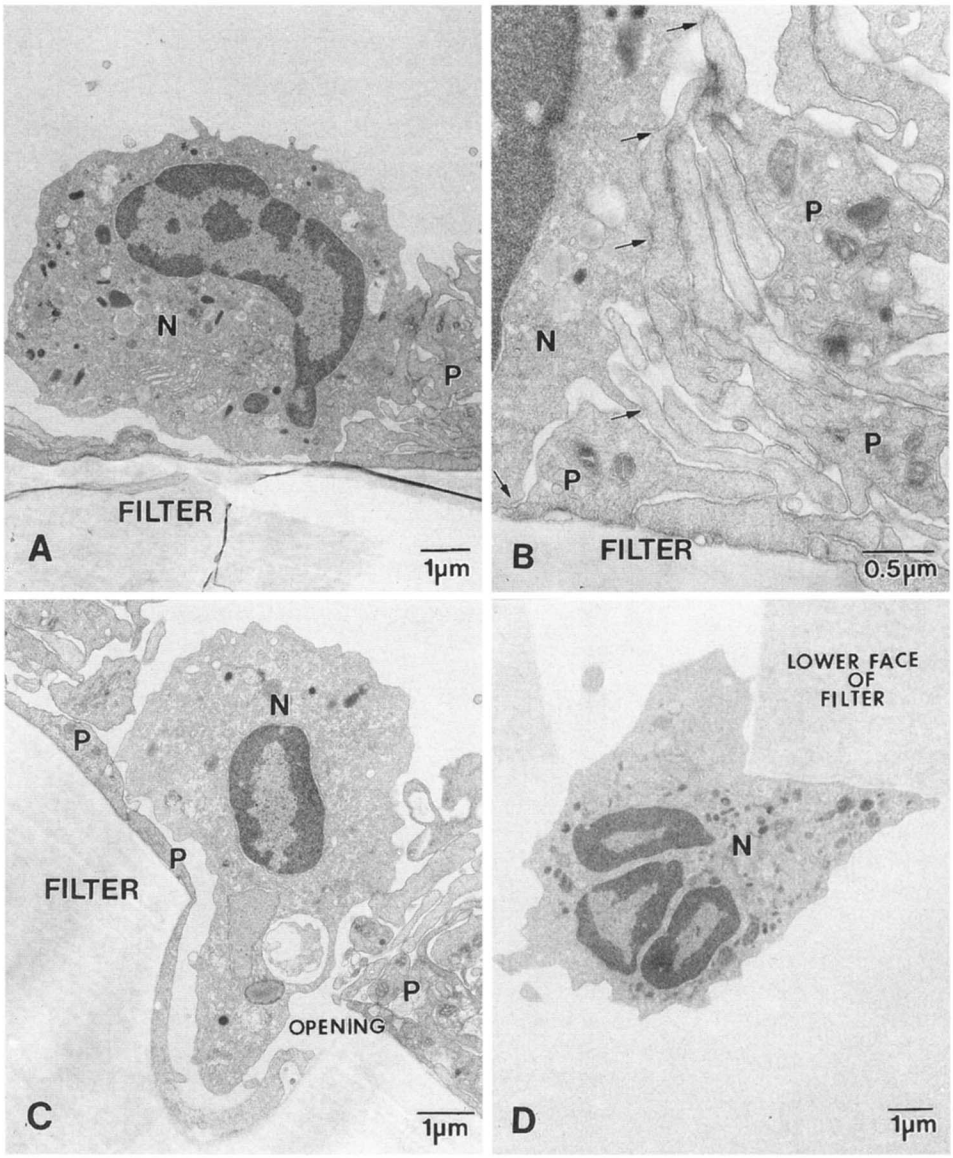

Fig 7. Electron micrographs of neutrophil/platelet interactions during transmigration through platelet multilayers to IL-8. Platelets (2.0×10^7) were bound to Transwell membranes and 30 minutes after initiation of neutrophil chemotaxis to 10 ng/mL IL-8, Transwells were fixed for electron microscopy. (A) A neutrophil (N) in close proximity to the platelet (P)-covered upper surface of a Transwell membrane (original magnification × 8,580). (B) Neutrophil/platelet interaction along the upper filter surface (original magnification × 9,254). (C) Neutrophil entering a platelet-coated pore on the upper filter surface (original magnification × 10,576). (D) Neutrophil exiting a pore at the lower filter surface (original magnification × 10,576).

also been reported to downregulate P-selectin–dependent binding to platelets in suspension assays.[49]

Using mAb to leukocyte integrin α- and β-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, β_2-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.[9] 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.[23] mAbs and Fabs to ICAM-2 that completely block binding

Table 1. Checkerboard Analysis of Neutrophil Transmigration Through Platelets to IL-8

IL-8 (ng/mL) in Bottom	% Neutrophil Migration (IL-8 [ng/mL] in top)			
	0	1	10	100
0	0.01 ± 0.01	0	0	0
1	0.2 ± 0.05	0.2 ± 0.025	0.03 ± 0.005	0
10	24.0 ± 2.0	12.0 ± 0.4	0.5 ± 0.15	0
100	18.3 ± 1.3	17.8 ± 2.3	9.8 ± 1.5	0.4 ± 0.2

Purified platelets (2×10^7) 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 2.5×10^5/insert and incubated for 1 hour at 37°C. Data are the mean ± half the range of duplicates.

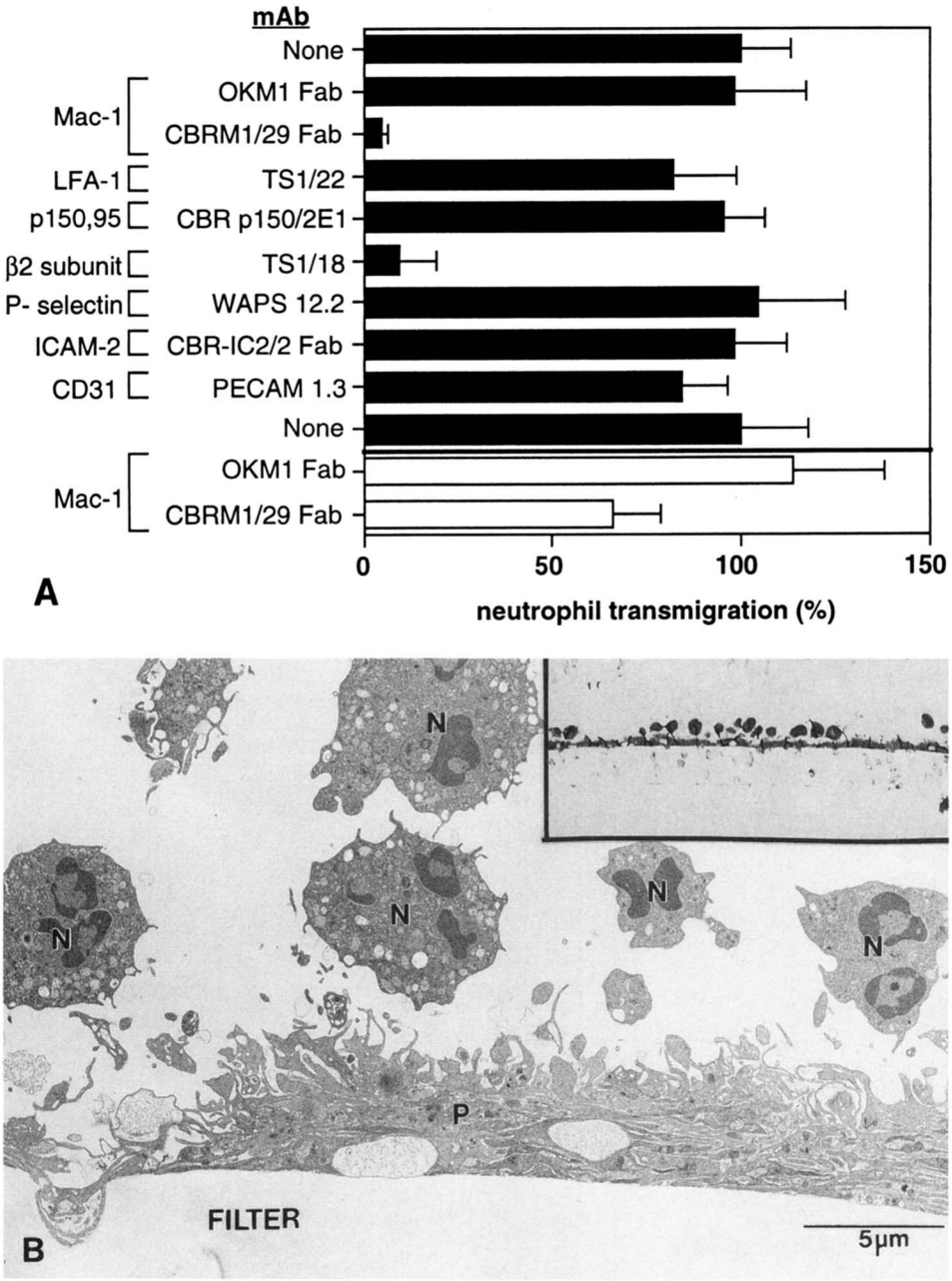

Fig 8. mAb to the Mac-1 α and β_2-subunits inhibit neutrophil chemotaxis to IL-8 through activated, adherent platelets. (A) Neutrophils (2.5×10^5/insert) were incubated at 37°C for 1 hour in the absence or presence of saturating concentrations of mAb (whole Ig or Fab) CBR-M1/29 (anti–Mac-1), mAb TS1/22 (anti–LFA-I), mAb TS1/18 (anti–β_2-subunit), mAb WAPS 12.2 (anti–P-selectin), or mAb CBR-IC2/2 (anti–ICAM-2). Human platelets (2×10^7 total) bound to fibronectin-coated membranes of 3-μm pore size and activated with thrombin (■); or fibronectin-coated membranes alone (□). Neutrophil transmigration was quantified by counting the number of cells that migrated into the bottom wells and expressed as percent input neutrophils migrated. Error bars represent the SD of the mean of experiments performed in triplicate. (B) Electron micrograph of neutrophil/platelet interactions during transmigration through platelet-covered Transwell membranes in the presence of anti–Mac-1 Fab CBRM1/29 (20 μg/mL). Platelets at 2.0×10^7/insert were bound to membranes, and IL-8 at 20 ng/mL used as chemoattractant (1-hour incubation). Essentially all neutrophils (N) (>95%) remained at the level of the platelet (P)-covered upper surface of the insert membrane (filter) (original magnification × 8,580). Insert depicts a photomicrograph of the same platelet-coated membrane (original magnification × 370).

to LFA-1,[30] and mAbs to LFA-1 that block binding to ICAM-1, ICAM-2, and ICAM-3,[50] 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,[51-54] 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[55]; however, we have been unable to find binding of Mac-1 to ICAM-2.[43,56] 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[55]; 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-I patient neutrophils are deficient in ability both to orient and migrate in chemoattractant gradients.[57] 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

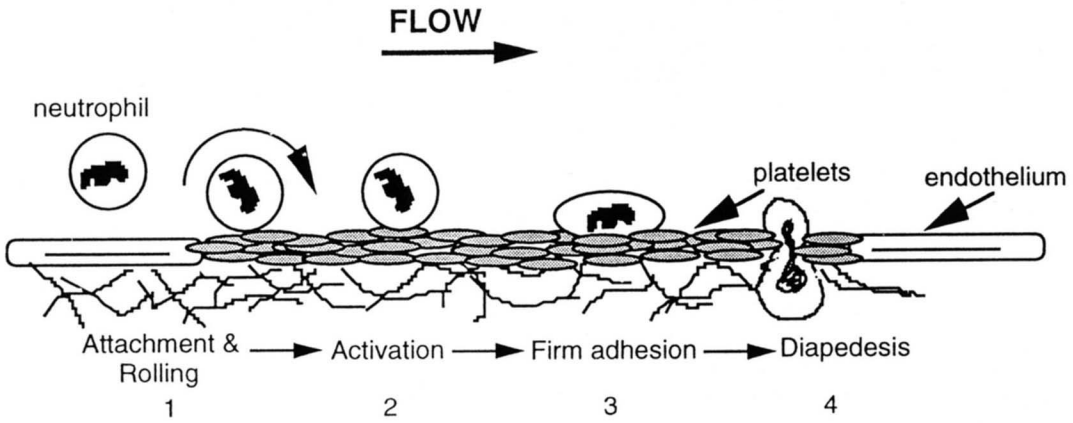

Fig 9. Proposed sequential adhesion model of neutrophil attachment to and transmigration across activated, surface-adherent platelets. P-selectin mediates the initial attachment and rolling in flow (1); surface receptor(s) or surface-bound cytokine(s) induce neutrophil activation (2); the β_2-integrin Mac-1 promotes firm adhesion (3), and also mediates chemoattractant-induced diapedesis (4).

manner, and that transplatelet migration occurs by chemotaxis, not random locomotion. The barrier to diffusion produced by platelets is intermediate to that produced by confluent HUVEC and fibronectin-coated Transwells. Background neutrophil migration through platelet multilayers was much lower than on Transwell membranes coated with fibronectin alone. The novel transplatelet chemotaxis assay system provided strong support for the central importance of the Mac-1 pathway in firm adhesion. Function-blocking mAb and Fab to Mac-1 inhibited greater than 95% of IL-8–induced neutrophil transplatelet migration. Furthermore, we demonstrated that inhibition of transmigration was the result of blocking neutrophil/platelet interactions and not neutrophil/fibronectin or neutrophil/membrane interactions, as greater than 95% of neutrophils remained bound on the platelet surface as shown by electron microscopy. Similar to adhesion assays, neither LFA-1 nor ICAM-2 appear to be involved in transmigration. P-selectin, which mediated the initial interactions between neutrophils and platelets in flow, also did not participate in transmigration, which illustrates further the distinct roles played by selectins and integrins in this proposed adhesion model. These results establish a parallel with neutrophil migration across endothelial monolayers in response to a chemoattractant, which has been shown to be mediated by Mac-1 and LFA-1 or the β_2-integrins in vitro and in vivo.[9,57,58] Although PECAM-1 (CD31), a member of the Ig superfamily, is important for spontaneous migration of neutrophils through endothelial cell monolayers grown on collagen gels,[59] we have failed to find an effect of multiple function-blocking mAb to CD31 on chemoattractant-stimulated transmigration of neutrophils either through platelets or endothelial monolayers (this report and unpublished data).

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.[12,15,60] 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.

ACKNOWLEDGMENT

We thank Dr Hans Ochs, University of Washington, Seattle, and Dr C. Wayne Smith, Baylor College of Medicine, Houston, TX, for LAD patient blood.

REFERENCES

1. Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: The multi-step paradigm. Cell 76:301, 1994

2. Butcher EC: Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. Cell 67:1033, 1991

3. Anderson DC, Schmalstieg FC, Shearer W, Becker-Freeman K, Kohl S, Smith CW, Tosi MF, Springer T: Leukocyte LFA-1, OKM1, p150,95 deficiency syndrome: Functional and biosynthetic studies in three kindreds. Fed Proc 44:2671, 1985

4. Kishimoto TK, Larson RS, Corbi AL, Dustin ML, Staunton DE, Springer TA: The leukocyte integrins: LFA-1, Mac-1, and p150,95. Adv Immunol 46:149, 1989

5. Diamond MS, Springer TA: The dynamic regulation of integrin adhesiveness. Curr Biol 4:506, 1994

6. Lawrence MB, Smith CW, Eskin SG, McIntire LV: Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. Blood 75:227, 1990

7. von Andrian UH, Berger EM, Ramezani L, Chambers JD, Ochs HD, Harlan JM, Paulson JC, Etzioni A, Arfors KE: In vivo behavior of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes. J Clin Invest 91:2893, 1993

8. Smith CW, Rothlein R, Hughes BJ, Mariscalco MM, Schmalstieg FC, Anderson DC: Recognition of an endothelial determinant for CD18-dependent neutrophil adherence and transendothelial migration. J Clin Invest 82:1746, 1988

9. Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC: Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. J Clin Invest 83:2008, 1989

10. Furie MB, Tancinco MCA, Smith CW: Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1 inhibit chemoattractant-stimulated neutrophil transendothelial migration in vitro. Blood 78:2089, 1991

11. Palabrica T, Lobb R, Furie BC, Aronivitz M, Benjamin C, Hsu Y-M, Sajer S, Furie B: Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. Nature 359:848, 1992

12. Ross R: The pathogenesis of atherosclerosis—An update. N Engl J Med 314:488, 1986

13. Laws KH, Clanton JA, Starnes VA: Kinetics and imaging of indium-111–labeled autologous platelets in experimental myocardial infarction. Circulation 67:110, 1983

14. Del Maschio A, Dejana E, Bazzoni G: Bidirectional modulation of platelet and polymorphonuclear leukocyte activities. Ann Hematol 67:23, 1993

15. Bednar M, Smith B, Pinto A, Mullane KM: Neutrophil depletion suppresses ^{111}In-labeled platelet accumulation in infarcted myocardium. J Cardiovasc Pharmacol 7:906, 1985

16. Cohen AB, Stevens MD, Miller EJ, Atkinson MAL, Mullenbach G: Generation of the neutrophil-activating peptide-2 by cathepsin G and cathepsin G-treated human platelets. Am J Physiol 263:L249, 1992

17. Marcus AJ, Safier LB, Ullman HL, Islam N, Broekman MJ, Falck JR, Fischer S, von Schacky C: Platelet-neutrophil interactions: (12*S*)-hydroxyeicosatetraen-1 20-dioic acid: A new eicosanoid synthesized by unstimulated neutrophils from (12-*S*)-20-dihydroxyeicosatetraenoic acid. J Biol Chem 263:2223, 1988

18. Lawrence MB, Springer TA: Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. Cell 65:859, 1991

19. Buttrum SM, Hatton R, Nash GB: Selectin-mediated rolling of neutrophils on immobilized platelets. Blood 82:1165, 1993

20. Yeo EL, Sheppard J-AI, Feuerstein IA: 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). Blood 83:2498, 1994

21. Lalor P, Nash GB: Adhesion of flowing leukocytes to immobilized platelets. Br J Haematol 89:725, 1995

22. Diacovo TG, Roth SJ, Bainton DF, Springer TA: Mac-1 (CD11b/CD18) mediates neutrophil adhesion to and transmigration across surface adherent platelets. FASEB 9:A796, 1995 (abstr)

23. Diacovo TG, de Fougerolles AR, Bainton DF, Springer TA: A functional integrin ligand on the surface of platelets: Intercellular adhesion molecule-2. J Clin Invest 94:1243, 1994

24. Miller LJ, Bainton DF, Borregaard N, Springer TA: Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. J Clin Invest 80:535, 1987

25. Bowen TJ, Ochs HD, Altman LC, Price TH, Van Epps DE, Brautigan DL, Rosin RE, Perkins WD, Babior BM, Klebanoff SJ, Wedgewood RJ: Severe recurrent bacterial infections associated with defective adherence and chemotaxis in two patients with neutrophils deficient in a cell-associated glycoprotein. J Pediatr 101:932, 1982

26. Beatty PG, Harlan JM, Rosen H, Hansen JA, Ochs HD, Price TD, Taylor RF, Klebanoff SJ: Absence of monoclonal-antibody–defined protein complex in boy with abnormal leucocyte function. Lancet 1:535, 1984

27. Diener AM, Beatty PG, Ochs HD, Harlan JM: The role of neutrophil membrane glycoprotein 150 (GP-150) in neutrophil-mediated endothelial cell injury in vitro. J Immunol 135:537, 1985

28. Anderson DC, Schmalstieg FC, Arnaout MA, Kohl S, Tosi MF, Dana N, Buffone GJ, Hughes BJ, Brinkley BR, Dickey WD, Abramson JS, Springer T, Boxer LA, Hollers JM, Smith CW: Abnormalities of polymorphonuclear leukocyte function associated with a heritable deficiency of high molecular weight surface glycoproteins (GP138): Common relationship to diminished cell adherence. J Clin Invest 74:536, 1984

29. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA: Monocyte chemoattractant protein-1 is a major T lymphocyte chemoattractant. Proc Natl Acad Sci USA 91:3652, 1994

30. de Fougerolles AR, Stacker SA, Schwarting R, Springer TA: Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J Exp Med 174:253, 1991

31. Sanchez-Madrid F, Krensky AM, Ware CF, Robbins E, Strominger JL, Burakoff SJ, Springer TA: Three distinct antigens associated with human T lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc Natl Acad Sci USA 79:7489, 1982

32. Wright SD, Rao PE, Van Voorhis WC, Craigmyle LS, Iida K, Talle MA, Westberg EF, Goldstein G, Silverstein SC: Identification of the C3bi receptor of human monocytes and macrophages with monoclonal antibodies. Proc Natl Acad Sci USA 80:5699, 1983

33. Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL, Springer TA: The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J Cell Biol 120:1031, 1993

34. Stacker SA, Springer TA: Leukocyte integrin p150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. J Immunol 146:648, 1991

35. Liao F, Huynh HK, Eiroa A, Greene T, Polizzi E, Muller WA: Migration of monocytes across endothelium and passage through extracellular matrix involve separate molecular domains of PECAM-1. J Exp Med 182:1337, 1995

36. Jutila MA, Bargatze RF, Kurk S, Warnock RA, Ehsani N, Watson SR, Walcheck B: Cell surface P- and E-selectin support shear-dependent rolling of bovine γ/δ T cells. J Immunol 153:3917, 1994

37. Hamburger S, McEver R: GMP-140 mediates adhesion of stimulated platelets to neutrophils. Blood 75:550, 1990

38. Gimbrone MA, Jr.: Culture of vascular endothelium. Prog Hemost Thromb 3:1, 1976

39. Eldor A, Fuks Z, Levine RF, Vlodavsky I: Measurement of platelet and megakaryocyte interaction with the subendothelial extracellular matrix. Methods Enzymol 169:76, 1989

40. Roth SJ, Carr MW, Rose SS, Springer TA: Characterization of transendothelial chemotaxis of T lymphocytes. J Immunol Methods 100:97, 1995

41. Bainton DF, Takemura R, Stenburg PE, Werb Z: Rapid fragmentation and reorganization of Golgi membranes during frustrated phagocytosis of immobile immune complexes by macrophages. Am J Pathol 134:15, 1989

42. Beumer S, IJsseldijk MJW, de Groot PG, Sixma JJ: Platelet adhesion to fibronectin in flow: Dependence on surface concentration and shear rate, role of platelet membrane glycoproteins GP IIb/IIIa and VLA-5, and inhibition by heparin. Blood 84:3724, 1994

43. Diamond MS, Staunton DE, de Fougerolles AR, Stacker SA, Garcia-Aguilar J, Hibbs ML, Springer TA: ICAM-1 (CD54): A counter-receptor for Mac-1 (CD11b/CD18). J Cell Biol 111:3129, 1990

44. Zigmond S, Hirsch JG: Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. J Exp Med 137:387, 1973

45. Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA: Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: A juxtacrine system for adhesion and activation of neutrophils. J Cell Biol 115:223, 1991

46. Power CA, Furness RB, Brawand C, Wells TNC: Cloning of a full-length cDNA encoding the neutrophil-activating peptide ENA-78 from human platelets. Gene 151:333, 1994

47. Lorant DE, McEver RP, McIntyre TM, Moore KL, Prescott SM, Zimmerman GA: Activation of polymorphonuclear leukocytes reduces their adhesion to P-selectin and causes redistribution of ligands for P-selectin on their surfaces. J Clin Invest 96:171, 1995

48. Lawrence MB, Bainton DF, Springer TA: Neutrophil tethering to and rolling on E-selectin are separable by requirement for L-selectin. Immunity 1:137, 1994

49. Rinder HM, Tracey JL, Rinder CS, Leitenberg D, Smith BR: Neutrophil but not monocyte activation inhibits P-selectin–mediated platelet adhesion. Thromb Haemost 71:750, 1994

50. de Fougerolles AR, Qin X, Springer TA: Characterization of the function of ICAM-3 and comparison to ICAM-1 and ICAM-2 in immune responses. J Exp Med 179:619, 1994

51. Wright SD, Weitz JI, Huang AD, Levin SM, Silverstein SC, Loike JD: Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. Proc Natl Acad Sci USA 85:7734, 1988

52. Wachtfogel YT, DeLa Cadena RA, Kunapuli SP, Rick L, Miller M, Schultz RL, Altieri DC, Edgington TS, Colman RW: High molecular weight kininogen binds to Mac-1 on neutrophils by its heavy chain (domain 3) and its light chain (domain 5). J Biol Chem 269:19307, 1994

53. Coombe DR, Watt SM, Parish CR: Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitor cells to stromal cell elements via recognition of stromal heparan sulfate. Blood 84:739, 1994

54. Diamond MS, Alon R, Parkos CA, Quinn MT, Springer TA: Heparin is an adhesive ligand for the leukocyte integrin Mac-1 (CD11b/CD18). J Cell Biol 130:1473, 1995

55. Xie J, Li R, Kotovuori P, Vermot-Desroches C, Wijdenes J, Arnaout MA, Nortamo P, Gahmberg CG: Intercellular adhesion molecule-2 (CD102) binds to the leukocyte integrin CD11b/CD18 through the A domain. J Immunol 155:3619, 1995

56. de Fougerolles AR, Diamond MS, Springer TA: Heterogenous glycosylation of ICAM-3 and lack of interaction with Mac-1 and p150,95. Eur J Immunol 25:1008, 1995

57. Anderson DC, Springer TA: Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Ann Rev Med 38:175, 1987

58. Anderson DC, Schmalsteig FC, Finegold MJ, Hughes BJ, Rothlein R, Miller LJ, Kohl S, Tosi MF, Jacobs RL, Waldrop TC, Goldman AS, Shearer WT, Springer TA: The severe and moderate phenotypes of heritable Mac-1, LFA-1 deficiency: Their quantitative definition and relation to leukocyte dysfunction and clinical features. J Infect Dis 152:668, 1985

59. Muller WA, Weigl SA, Deng X, Phillips DM: PECAM-1 is required for transendothelial migration of leukocytes. J Exp Med 178:449, 1993

60. Collins CE, Cahill MR, Newland AC, Rampton DS: Platelets circulate in an activated state in inflammatory bowel disease. Gastroenterology 106:840, 1994