Selectin-Mediated Rolling of Neutrophils on Immobilized Platelets

By Stephen M. Buttrum, Raymond Hatton, and Gerard B. Nash

Interaction between neutrophils and platelets at the site of vascular damage or in ischaemic tissue may promote thrombosis and/or vascular occlusion. To study this interaction, we have developed a novel technique that allows visualization of adhesion of flowing neutrophils to immobilized, activated platelets. The total number of adherent neutrophils decreased with increasing wall shear stress in the range 0.05 to 0.4 Pa. Although a proportion of the adherent neutrophils were stationary, most were rolling with a velocity greater than 0.4 μm/s. The percentage of rolling cells increased with increasing wall shear stress, but the mean rolling cell velocity was nearly independent of shear stress. Adhesion of neutrophils was nearly abolished by treatment of the platelets with antibody to P-selectin, or by treatment of neutrophils with either neuraminidase, desulfate, or EDTA. Studies with a series of antibodies to L-selectin (TQ-1, Dreg-56, LAM1-3, and LAM1-10) suggested that this molecule was one neutrophil ligand for rolling adhesion. Thus, sialylated carbohydrate on neutrophils appears essential for P-selectin-mediated adhesion, and a proportion of this ligand may be presented by L-selectin. Treatment of the neutrophils with N-formyl-methionyl-leucyl-phenylalanine decreased the number of rolling cells, and increased the rolling velocity, possibly due to shedding of neutrophil ligand(s) and/or cell shape change. In vivo, immobilized platelets could play an important role in promoting attachment of neutrophils to vessel walls, e.g., by slowing neutrophils so that integrin-mediated immobilization could occur.

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PLATELETS AND NEUTROPHILS must adhere to the wall of blood vessels to perform their roles in hemo-
stasis and in the inflammatory and immune responses. On the other hand, these adhesive interactions may contribute to atherosclerosis, thrombosis, and ischemia. Platelets and neutrophils can adhere to each other, so that each might recruit the other to the surface of stimulated or damaged vascular endothelium. In addition, both cell types release factors that can directly activate or enhance the other’s response to agonists. Therefore, interaction between platelets and neutrophils may modulate their hemostatic and inflammatory functions, but could also augment vascular occlusive and thrombotic events.

The molecules that mediate the adhesive properties of platelets and neutrophils have been studied extensively, although fewer studies have examined the mechanisms underlying the adhesive interaction between the two cell types. P-selectin (GMP140/PADGEM/CD62) is a membrane glycoprotein found in α-granules of human platelets and also in Weibel-Palade bodies of endothelial cells. It is rapidly expressed on the surface of platelets and endothelium after thrombin stimulation, and is a mediator of adhesion of neutrophils to both of these cell types in static assays. Flowing neutrophils can form rolling attachment to surfaces coated with purified, platelet P-selectin at wall shear stresses up to 0.35 Pa, but no flow-based studies on neutrophil adhesion to cell-borne P-selectin have been reported to date. The rapid appearance of P-selectin after platelet and endothelial cell activation suggests that it could recruit neutrophils to platelet thrombi or stimulated endothelium, or bind platelets at sites of neutrophil extravasation in acute inflammatory or thrombotic conditions. Rolling adhesion on P-selectin may be a means of promoting immobilization of neutrophils by the more shear-sensitive, but stationary, interaction between neutrophil integrins and intercellular adhesion molecules (ICAM-1 and/or ICAM-2) expressed by endothelium.

The neutrophil ligand for P-selectin has not been fully characterized, but one or more sialylated, fucosylated oligo-
saccharides appear to be essential for binding. Lewis X antigen (CD15) and its sialylated form (sLe^X) are fucosyl-
lated oligosaccharides widely distributed on the surface of neutrophils, and are candidates as ligands or parts of ligands for P-selectin as well as for the endothelial adhesion molecule E-selectin (ELAM-1). The neutrophil form of L-selectin (peripheral lymph node lymphocyte homing receptor/human Leu 8 antigen/LECAM-1/TQ1) bears sLex and is involved in leukocyte rolling on endothelium both in vivo and in vitro. It has thus been suggested that L-selectin acts as (or bears) a ligand for P-selectin as well as E-selectin.

We report here that P-selectin on the surface of immobilized, activated platelets acts as a shear-dependent, rolling receptor for neutrophils. Sialylated carbohydrate(s), partly borne by L-selectin, appears to act as the neutrophil ligand. Rolling velocity was dependent on the density of the ligands or of P-selectin, and fMLP activation of neutrophils caused an overall decrease in adhesion and increase in rolling velocity.

MATERIALS AND METHODS

Isolation of neutrophils. Human venous blood was collected from healthy volunteers into buffered citrate anticoagulant (CPDA, 8:1; Blood Transfusion Service, Birmingham, UK) or into preservative-free sodium heparin (5 U/mL; Leo Laboratories Ltd, Princes Risborough, UK). All subsequent procedures were performed at room temperature. A portion of the heparinized blood was centrifuged at 1,500g for 10 minutes, and the plasma was harvested and filtered through a 0.2 μm pore sterile filter.

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Whole citrated blood was layered over a two-step density gradient of equal volumes of Histopaque-1077 and Histopaque-1119 (Sigma Chemical Co, Ltd, Poole, UK) and centrifuged at 700g for 25 minutes. The middle neutrophil fraction was harvested and washed in 5% autologous heparinized plasma in phosphate-buffered saline (PBS; without added calcium or magnesium; ICN Flow Labs, High Wycombe, UK). Contaminating red blood cells were removed by 30-second hypotonic lysis with distilled water (6:1), followed by the return of the osmolality to 290 mOsm/kg with 4X concentrated PBS. The lysed red blood cells were removed by centrifugation at 100g for 5 minutes and the neutrophils washed once with 5% autologous heparinized plasma in PBS. The neutrophils were counted by Coulter Counter (model ZF; Coulter Electronics, Luton, UK) and resuspended to a concentration of 1 x 10^6 cells/mL in 5% autologous heparinized plasma in PBS. Neutrophils prepared in this manner were greater than 95% viable, as judged by trypan blue exclusion.

In some experiments, the neutrophils were preincubated with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma) at a final concentration of 10^-7 mol/L. On other occasions, neutrophils were treated with either Clostridium perfringens neuraminidase (Type X, affinity purified; Sigma) at a range of concentrations from 0.1 to 1.0 U/mL for 90 minutes at room temperature, or a-chymotrypsin (type 1-S from bovine pancreas; Sigma) at 1 U/10^6 cells for 5 minutes at 37°C, followed by addition of excess ice-cold buffer plus 50% autologous plasma and washing at 4°C (as described by von Andrian et al22 for selective removal of L-selectin). The morphology of the neutrophils was unaltered by this treatment; as assessed by light microscopy, the cells maintained the passive, spherical shape characteristic of control samples (treated identically but without the enzyme). In contrast, fMLP-treated neutrophils showed marked distortion of shape with pseudopod formation. All assays were complete within 3 hours of neutrophil isolation.

Platelet coating of microslides. Microslides (glass tubes with a rectangular cross-section of 300 μm by 3 mm, and a length of 50 mm; Camlab Ltd, Cambridge, UK) were soaked overnight in 70% nitric acid and then washed with copious amounts of tap water. The microslides were dried with anhydrous acetone and immersed twice in a 4% solution of 3-aminopropyltriethoxysilane (APES; Sigma) in acetone for 2 minutes. The microslides were then rinsed once with acetone, washed four times with distilled water, and left overnight at 37°C to dry. Heparinized blood from the same donor as used in neutrophil isolation was centrifuged at 290g for 5 minutes and the platelet-rich plasma collected. Platelets were counted by Coulter Counter and diluted to 2 x 10^6 cells/mL with 5% autologous heparinized plasma in PBS. The platelets were loaded into an APES-treated microslide by capillary action, and 30 minutes was allowed for the platelets to settle, adhere, and spread onto the lower surface of the microslide. An essentially confluent layer of spread platelets was formed (Fig 1A).

Na-heparin was used as the anticoagulant for the platelets, because the adhesion and spreading of platelets on the microslides was totally inhibited if the blood was anticoagulated with EDTA. Some alternatives to APES were tested for pretreating the glass microslides. With untreated, washed microslides, very few platelets adhered to the surface of the microslide. If the microslides were pretreated with poly-D-lysine (0.2 mg/mL, Sigma), this produced a mean platelet coating density of 12,500 platelets/mm², compared with 16,900 cells/mm² (28% efficiency of platelet adhesion) with APES. Platelet coating density was counted directly by light microscopy, and efficiency of adhesion calculated from the known number of platelets originally loaded onto the microslide.

Adhesion assay. The adhesion assay was similar to that recently described by Cooke et al.23 All manipulations were performed at room temperature (22°C ± 1°C). The platelet-coated microslide was glued to a glass microscope slide using two small spots of Loc-tite super-glue gel (Loc-tite Holdings Ltd, Welwyn Garden City, UK) and mounted on a microscope stage. One end of the microslide was attached by silicon rubber tubing to an electronic valve, which allowed smooth switching between the neutrophil suspension and wash buffer (5% autologous heparinized plasma in PBS) held in vertical syringe barrels. The other end of the microslide was attached by silicon rubber tubing to a Harvard syringe pump (Harvard Apparatus, South Natic, MA). An air-tight seal between the microslide and the silicon tubing was established by first coating the ends of the microslide with double-sided adhesive tape. The pump controlled flow through the microslide at a desired flow rate (Q); the wall shear stress (t) exerted on the platelet surface was calculated from the equation24: \[ t = \left(6Q \cdot \eta \right) / \left(w \cdot h \right) \] where \( \eta \) is the suspending medium viscosity, w is the microslide internal width (3 mm), and h is the microslide internal depth (300 μm).

After insertion of the platelet-coated microslide into the flow system, any free platelets were washed out with 5% autologous plasma in PBS. Neutrophils were then perfused over the platelets at the selected wall shear stress (between 0.05 and 0.4 Pa; equivalent to 0.5 to 4 dyn/cm²). At chosen time points, the number of adherent neutrophils was counted by direct microscopic observation of a number of complete microscope fields (eg, Fig 1B) and expressed as cells adhered per square millimeter.

Videomicroscopic recordings were made and analyzed off-line using a computerized image analysis system. The images were digitized using an Archimedes computer (Watford Electronics, Watford, UK). In an initial frame each adherent cell was labeled by a pointing device. A subsequent video frame was digitized after a known time (typically 10 seconds), the distance travelled by each cell was measured after the cells had been relabeled, and the cell velocity was calculated. Adherent cells were easily distinguished from nonadherent cells, because the cells passing through with the bulk flow were seen as faint streaks on the video playback. The mean velocity of these nonadherent cells was determined using APES-treated microslides coated with platelet-free plasma instead of platelet-rich plasma. No adhesion (< 1 cell/mm²) was seen under these conditions. The mean velocity of the neutrophils passing adjacent to the lower surface of the microslide was directly proportional to the wall shear stress and varied between 311 and 1253 μm/s for stresses between 0.05 and 0.2 Pa (70 and 30 cells, respectively).

For categorization of adhesion, stationary cells were defined as those with velocity less than 0.4 μm/s, which represents the minimum velocity resolved by the analysis system. High power videomicroscopic images showed that the neutrophils moving on the platelet surface were rolling, as shown by the rotation of the intercellular granules. Rolling velocity varied between 0.4 and 41.5 μm/s for the cells analyzed in this study. The percentage of cells that were rolling (rather than stationary) was calculated from the digitized video recordings.

Antibodies. Antibodies were incubated with the neutrophil suspension for 30 minutes at room temperature before the flow adhesion assay. Monoclonal IgG1 antibodies to L-selectin were TQI (Coulter Immunology, Luton, UK), LAM-1 and LAM-10 (adhesion blocking and nonblocking antibodies, respectively24, gift of Dr Thomas Tedder, Diana Farber Cancer Institute), and Dreg-56 (gift of Dr Ulrich von Andrian, La Jolla Institute for Experimental Medicine). CLB 139 (monoclonal IgG1 antibody to CD66) was obtained from Janssen Biochimica (Geel, Belgium). MHM23 (monoclonal IgG1 antibody to CD18) and the nonspecific control antibody DAK-GO1 (IgG1 antibody to Asperillus niger glucose oxidase) were obtained from Dako Ltd (High Wycombe, UK).

To treat platelets, coated microslides were washed with 5% autologous plasma in PBS and then filled with the antibody diluted in 5% autologous plasma in PBS. The microslides were incubated at room temperature (22°C ± 1°C) for 30 minutes. After the platelet-coated microslide was glued to a glass microscope slide using two small spots of Loc-tite super-glue gel (Loc-tite Holdings Ltd, Welwyn Garden City, UK) and mounted on a microscope stage. One end of the microslide was attached by silicon rubber tubing to an electronic valve, which allowed smooth switching between the neutrophil suspension and wash buffer (5% autologous heparinized plasma in PBS) held in vertical syringe barrels. The other end of the microslide was attached by silicon rubber tubing to a Harvard syringe pump (Harvard Apparatus, South Natic, MA). An air-tight seal between the microslide and the silicon tubing was established by first coating the ends of the microslide with double-sided adhesive tape. The pump controlled flow through the microslide at a desired flow rate (Q); the wall shear stress (t) exerted on the platelet surface was calculated from the equation24: \[ t = \left(6Q \cdot \eta \right) / \left(w \cdot h \right) \] where \( \eta \) is the suspending medium viscosity, w is the microslide internal width (3 mm), and h is the microslide internal depth (300 μm).
temperature for 20 minutes before connection to the flow system. G1 and S12, adhesion-blocking and nonblocking monoclonal IgG1 antibodies to P-selectin were a gift from Dr Rodger McEver (University of Oklahoma College of Medicine). Y2/51 (IgG1 antibody to the platelet glycoprotein IIIa, CD61) was obtained from Dako Ltd.

RESULTS

Adhesion of unstimulated neutrophils to platelets. To study the time course of the accumulation of neutrophils on immobilized platelets, neutrophils were perfused continuously over the platelet monolayer at a range of wall shear stresses (0.05 to 0.2 Pa) for up to 10 minutes (Fig 2). Adhesion increased linearly with time at each of the shear stresses. At a shear stress of 0.1 Pa, the adhesion continued to increase linearly to a level of more than 5,000 adherent cells/mm² after 20 minutes of continuous perfusion (data not shown). For all subsequent experiments, a maximum of 5 minutes perfusion was chosen for any wall shear stress; thus, the adhesion was never saturated. Due to the linear
characteristic of the adhesion over time, the results from each experiment could be normalized as cells adhered per square millimeter per 10⁶ cells perfused, so that results from experiments with different flow rates and different total numbers of cells perfused could be compared.

The effect of increasing shear stress on the adhesion of unstimulated neutrophils is more clearly shown in Fig 3. Data are shown for rolling and static adhesion, as well as the total number of cells adhering. Adhesion was greatest at 0.05 Pa and decreased with increasing wall shear stress, so that at a wall shear stress of 0.4 Pa, very few neutrophils adhered to the platelets (<1 cell/mm²/10⁶ cells perfused). Rolling adhesion was less sensitive to stress than was static adhesion. There was an increase in the number of rolling neutrophils from 0.05 to 0.1 Pa, but the number decreased at 0.2 Pa. The percentage of the adhered neutrophils that were rolling increased continuously with increasing wall shear stress from 31% at 0.05 Pa to 95% at 0.2 Pa, whereas the number and percentage of static adherent cells decreased monotonically with stress.

After neutrophils had adhered to the platelets at a wall shear stress of 0.1 Pa, the stress could be increased to levels well above 0.4 Pa, with little removal of cells. Even at a shear stress of 1.0 Pa, 79% of the cells remained attached after 2 minutes, but the percentage of rolling cells increased to more than 90% from 63%. Hence, once adhesion had occurred, the cells could withstand stresses at which they could not initially adhere.

Because the platelets had not been specifically stimulated by any agonist, the effect of thrombin was tested. After the platelets had been allowed to coat the microslides, 0.5 U/mL thrombin (Leo Pharmaceutical Products, Ballerup, Denmark) in PBS was perfused into the microslide and left to incubate for 3 minutes. The thrombin was washed out and neutrophils were perfused over the activated platelets for 4 minutes. The level of adhesion at a wall shear stress of 0.1 Pa was unchanged by the addition of thrombin (700 cells/mm² for thrombin-stimulated platelets, and 707 cells/mm² for control), whereas at 0.2 Pa, the adhesion after thrombin stimulation was slightly lower than the control (725 cells/mm² vs 823 cells/mm², respectively).

Effect of fMLP on adhesion of neutrophils. The effect of shear stress on adhesion was also studied after treatment of the neutrophils with 10⁻⁷ mol/L fMLP. The neutrophils were incubated with fMLP for 1 minute and then perfused over the platelet monolayer for a further 5 minutes, after which the number of adherent cells was counted. The results are presented in Fig 4. After fMLP treatment, the level...
ADHESION OF FLOWING NEUTROPHILS TO PLATELETS

ADHESION OF FLOWING NEUTROPHILS TO PLATELETS

Fig 4. Effect of wall shear stress on adhesion of fMLP-treated neutrophils to immobilized platelets. Each point represents the mean ± SEM from three experiments. Data are shown for total adhesion (■), rolling adhesion (□), and stationary adhesion (Δ).

Fig 5. Effect of MoAbs on the adhesion of unstimulated neutrophils to immobilized platelets at a shear stress of 0.1 Pa. Data are expressed as percentage of adhesion compared with untreated control (mean ± SEM of 3 to 6 experiments). (■) Total; (□) stationary; (△) rolling.

of total adhesion was lower than for the unstimulated neutrophils, and the decrease in adhesion with increasing shear stress was more marked. FMLP caused a slight increase in the number of stationary neutrophils at each of the shear stresses studied, but a marked reduction in the number of rolling cells. Thus, the variation in stationary adhesion with shear stress closely paralleled that of the total adhesion (Fig 4). The number of rolling stimulated cells showed a small increase at 0.1 Pa compared with 0.05 Pa, as was the case for the unstimulated neutrophils.

Effects of monoclonal antibodies (MoAbs) on adhesion of neutrophils. The effects of various MoAbs to platelets and neutrophils were studied. Each antibody was titrated and the concentration that gave the maximal effect on adhesion was used in subsequent adhesion assays. Figures 5, 6, and 7 show the effects of antibodies to P-selectin and L-selectin on adhesion of unstimulated neutrophils at wall shear stresses of 0.1 and 0.2 Pa, and on adhesion of fMLP-treated cells at 0.1 Pa, respectively. At 0.2 Pa, rolling was essentially absent for fMLP-treated neutrophils and the antibodies were not tested.

Antibody G1 against P-selectin almost totally inhibited the adhesion of unstimulated and fMLP-treated neutrophils to platelets (>94% in all cases). Antibody S12 (nonblocking anti-P-selectin) had no significant effect on adhesion (adhesion, 99% ± 7% and 101% ± 4% of untreated control at 0.1 and 0.2 Pa, respectively; mean ± SD for 3 experiments on unstimulated neutrophils). Nor did antibody to platelet glycoprotein IIIa significantly influence adhesion (data not shown).

Antibodies TQ1, Dreg56, and LAM1-3 against L-selectin had similar effects on adhesion at 0.1 Pa (Fig 5). Each had an inconsistent effect on the low level of stationary adhesion, but decreased rolling adhesion by about 60% to 70%, so that total adhesion was reduced by about 40% to 50%. However, antibody LAM1-10, did not consistently alter stationary or rolling adhesion, and its overall effect was a 13% reduction in total adhesion. The antibody TQ1 also reduced rolling, but not stationary adhesion, of unstimulated neutrophils at 0.2 Pa (Fig 6). TQ1 inhibited the adhesion of fMLP-treated neutrophils to a lesser degree than unactivated neutrophils (Fig 7).

The role of L-selectin was also investigated by treating neutrophils with chymotrypsin to selectively remove this ligand.22 For unstimulated neutrophils tested at a wall shear stress of 0.1 Pa, both static and rolling adhesion were inhibited (by 97% ± 5% and 79% ± 9%, respectively; mean ± SD for comparisons to untreated controls on 3 occasions), whereas total adhesion was reduced by 81% ± 6%.

Neither antibody to the β subunit of the β2 integrin (CD18) nor antibody to CD66 had a consistent effect on adhesion of unstimulated or fMLP-treated neutrophils at 0.1 Pa (3 experiments of each type for each antibody; data not shown). Treatment of neutrophils with sodium azide at up to 10 mmol/L did not reduce adhesion of unstimulated or fMLP-stimulated neutrophils to platelets (3 experiments; data not shown).

Role of neutrophil carbohydrate ligands. Adhesion of
lectin molecules to carbohydrates is typically Ca-dependent and, more specifically, ligands for P-selectin are likely to be sulfated and sialylated, fucosylated glycans. Here, paired samples of neutrophils were incubated with or without 1 mmol/L calcium or 1 mmol/L EDTA added to the standard suspension medium (which itself had a free calcium level of approximately 100 mEq/L according to measurement with a calcium-selective electrode). When perfused over the platelets at a wall shear stress of 0.1 Pa for 4 minutes, the level of adhesion was not affected by additional calcium (805 cells/mm² without v 802 cells/mm² with added calcium), although the percentage of rolling cells was slightly decreased (from 76% to 66% with added calcium; mean of 3 experiments). However, EDTA abolished the adhesion of neutrophils to platelets (<1 cell/mm²). In addition, if 1 mmol/L EDTA was added to the wash medium and perfused through a microslide containing neutrophils already adherent to the platelets, within seconds more than 98% of the neutrophils became detached from the platelets (mean of 2 experiments).

The binding of neutrophils and HL60 cells to isolated platelet P-selectin, the rosetting of HL60 cells by thrombin-activated platelets, and rolling of leukocytes in vivo have all been shown to be inhibited by sulfated glycans, including dextran sulphate. We found that if unstimulated neutrophils were incubated with 400 μg/mL dextran sulphate (molecular weight of 500 Kd; Sigma) in PBS for 30 minutes, adhesion to platelets was nearly totally abolished at wall shear stresses of 0.1 and 0.2 Pa (96.1% ± 1.5% inhibition at 0.1 Pa and 99.3% ± 0.3% inhibition at 0.2 Pa, mean ± SD, n = 3).

We used neuraminidase to cleave sialic acid residues from the neutrophil surface, a procedure previously shown to inhibit binding between neutrophils and purified P-selectin or activated platelets. Treatment of the neutrophils with neuraminidase inhibited adhesion to platelets in a dose-dependent manner (Fig 8); at 1.0 U/mL neuraminidase, the adhesion was inhibited by more than 93%.

**Rolling velocity and receptor and ligand density.** The rolling velocities of unstimulated and fMLP-treated neutrophils are shown in Fig 9. The mean rolling velocity of the unstimulated neutrophils showed little dependence on wall shear stress. Over a large number of unpaired experiments, the rolling velocities of unstimulated neutrophils were identical at 0.1 and 0.2 Pa (mean velocity, 1.6 μm/s in both
cases; 829 cells from 22 experiments measured at 0.1 Pa; 522 cells from 11 experiments at 0.2 Pa). The fMLP-treated neutrophils rolled more rapidly than unstimulated neutrophils at all stresses, and their rolling velocities increased approximately in proportion to the wall shear stress from 1.6 μm/s at 0.05 Pa to 5.2 μm/s at 0.2 Pa.

The effects of treatment with antibodies on rolling velocities of neutrophils are shown in Table 1. After treatment with suboptimal levels of antibody to P-selectin (G1), there was a significant increase in rolling velocity in each of 2 paired experiments at 0.1 Pa, and in 1 paired experiment at 0.2 Pa (P < .02 by Student’s t-test). There was no consistent change in rolling velocity in 6 paired experiments at 0.1 Pa after treatment with TQ1, but a significant change in 2 of 3 paired experiments at 0.2 Pa (P < .05). At 0.1 Pa, Dreg-56 increased rolling velocity in 1 of 3 experiments (P < .05), whereas LAM1-3 and LAM1-10 did not alter velocity in any of 3 experiments. The mean cell rolling velocity was increased dose-dependently by treatment with neuraminidase, from 1.8 ± 1.4 μm/s for untreated neutrophils to 3.8 ± 2.1 μm/s at 0.1 U/mL, and 7.3 ± 4.9 μm/s at 1.0 U/ml (pooled data from 2 or 3 experiments; mean ± SD for 20 to 111 cells).

We have shown that flowing unstimulated neutrophils adhere to P-selectin (GMP140, PADGEM, CD62) on immobilized, activated platelets. The efficiency of adhesion was highly dependent on the wall shear stress, which was varied between 0.05 and 0.4 Pa (ie, from just below to within the range of stresses found in venules). Rolling rather than stationary adhesion predominated when the

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Data represent the pooled cell velocities (μm/s) (mean ± SD for 17 to 270 cells, in 1 to 6 experiments). Suboptimal concentrations of G1 were used, so that adhesion was present, but at a low level.
stress was increased to 0.2 Pa, but formation of adhesive interactions ceased at 0.4 Pa. Nevertheless, neutrophils that had adhered at a lower stress could withstand a much higher stress (at least 1.0 Pa) without becoming detached. Perhaps, surprisingly, the mean rolling velocity of the neutrophils was essentially independent of the shear stress. However, cells stationary at a low shear stress (eg, 0.05 Pa) did start to roll when the shear stress was increased.

Previous studies using whole blood showed that platelets and neutrophils adhered together to the surface of a rotating glass flask, and that platelet “satellites” could form around granulocytes. Isolated platelets and neutrophils have also been shown to form aggregates. The binding of neutrophils to activated platelets was shown to be mediated by P-selectin on the platelets. These studies did not address the question of whether immobilized platelets could act as an adhesive substrate for circulating neutrophils, or of which neutrophil ligands interacted with P-selectin.

A recent study showed that artificial bilayers incorporating purified, platelet P-selectin supported rolling adhesion of neutrophils at wall shear stresses nearly identical to those tested here. The rolling velocities measured were generally faster than those observed in the present study and were dependent on shear stress, a finding not reproduced in our experiments. Rolling velocity was dependent on the density of P-selectin incorporated in the bilayer. We found a similar result; if suboptimal concentrations of antibody to P-selectin were used to treat platelets, rolling velocity was increased. Also, if suboptimal concentrations of neuraminidase were used to partially inhibit adhesion, velocity increased. When neutrophils were treated with antibody to L-selectin (TQ1), the rolling velocity increased slightly at the higher stress (0.2 Pa). Thus, it seems that rolling velocity is a function of receptor and ligand density.

Adhesion to P-selectin does not require neutrophil activation and, indeed, treatment with fMLP reduced the number of neutrophils rolling on platelets and increased their velocity, but slightly increased stationary adhesion. The former results agree with the findings of Lawrence and Springer using purified immobilized P-selectin. It is possible that the stationary adhesion to platelets, which we observed even for unactivated neutrophils at low shear stress, was promoted by an additional, unidentified receptor unable to support adhesion on its own. However, adhesion was not affected by antibody to the common β subunit (CD18) of the neutrophil β2 integrins (which undergoes a conformational change and increase in adhesive potential after neutrophil activation) or antibody to ICAM-1 (data not shown), which is weakly expressed on platelets. Nor was adhesion altered by antibody to CD66, which recognizes neutrophil antigens that bear sialylated Lewis X upregulated during activation and that can mediate adhesion of resting or activated neutrophils to cytokine-stimulated endothelial cells presenting E-selectin. Therefore, upregulation of this ligand would not seem to be the basis for altered adhesion to platelets, which was predominantly decreased upon activation.

Adhesion was nearly abolished by Ca-chelation or treatment of neutrophils with neuraminidase or dextran sulfate. These findings agree with previous studies of the binding of neutrophils to selectins, and are consistent with lectin-mediated adhesion via a sialylated, sulfated carbohydrate neutrophil ligand. It has been suggested that both Lewis X (CD15) and sialylated Lewis X may fill the role of neutrophil ligand for P-selectin. We have found that antibodies to either of these carbohydrates inhibit neutrophil adhesion to platelets (data not shown), but because both were IgM antibodies and the antigens are widely distributed on the neutrophil surface, inhibition could have been by nonspecific steric hindrance.

Antibodies to L-selectin inhibit rolling adhesion on endothelium in vivo. We have found that a series of antibodies recognizing L-selectin (Dreg-56, TQ1, LAM1-3) preferentially inhibit rolling rather than stationary adhesion of neutrophils on P-selectin. Dreg-56 has previously been shown to inhibit rolling adhesion of neutrophils on cytokine-stimulated endothelium in vitro and in vivo, as well as adhesion of neutrophils to COS cells transfected with either E-selectin or P-selectin. TQ1 inhibits lymphocyte binding of the soluble L-selectin ligand phosphomannan monoester (PPME), whereas LAM1-3 blocks lymphocyte binding of PPME and high endothelial venules, but LAM1-10 does not block either binding function of L-selectin.

Here, the control antibody LAM1-10 had a minimal effect on neutrophil adhesion to platelets. Brief treatment of neutrophils with chymotrypsin, which has been shown to remove L-selectin, but not, for example, CD18 or sialylated Lewis X, also reduced adhesion. These data strongly suggest that L-selectin is a ligand for P-selectin.

Reduced rolling adhesion and increased rolling velocity after fMLP treatment may have been due to distortion of neutrophil shape, as well as the shedding of L-selectin that follows activation. TQ1 had relatively little effect on adhesion after fMLP treatment. HL60 cells do not bear L-selectin, but do carry sialylated Lewis X and bind to activated platelets. In preliminary experiments, we have found that HL60 cells do roll on immobilized platelets, but at lower levels than neutrophils. However, because of uncertainty about the relative density of ligands other than L-selectin on the HL60 cells, and their relatively large size (which could affect the shear forces exerted on them at the microslide wall), these results cannot be taken as proof of a role for L-selectin in mediating adhesion.

We also examined the possibility that treatment with antibody to L-selectin itself induced activation, and so altered adhesion indirectly rather than directly. The antibodies tested did not cause shape change characteristic of activation, although slight alteration in morphology was noted with the nonblocker, LAM1-10. Judged by immunofluorescence measurements, pretreatment with TQ1 did not increase labeling of neutrophils with antibody to CD11b; nor did labeling with TQ1 itself decrease with time, as would be expected for activated neutrophils (2 and 4 experiments, respectively; data not shown). Antibodies to L-selectin had slight or no effect on rolling velocity (depending on shear stress), in contradistinction to the effects of fMLP. These findings lead us to conclude that the antibodies did not inhibit adhesion by activating the neutrophils.
Overall, the data presented here indicate that sialylated carbohydrate ligands are essential for adhesion of neutrophils to P-selectin, and that L-selectin mediates a part of this interaction. Thus, L-selectin may present a carbohydrate moiety to P-selectin, while the same or other ligands are presumably also borne by other membrane glycoproteins. Others have shown that L-selectin bears a small proportion of neutrophil sLex, and it was suggested that this portion was preferentially presented as a ligand for P-selectin as well as E-selectin. However, definitive evidence of the nature of the carbohydrate ligand for P-selectin is currently lacking.

This study suggests that platelets adhered to vessel walls would act as receptors for adhesion of flowing neutrophils. In ischemic tissue, or where flow rates were low, the neutrophils might be immobilized by this interaction, but otherwise P-selectin could act as a first step in delaying neutrophils, so that they become locally activated and adherent via integrin-mediated binding to endothelium. Recent intravital observations of the microcirculation of rabbit mesentery have shown that under circumstances in which platelets line the walls of microvessels, this lining appears to support rolling adhesion of neutrophils (S. Nourshargh and S. Larkin, unpublished observation). Neutrophil adhesion to platelets could therefore be an important promoter of thrombotic or inflammatory responses. Because P-selectin is also rapidly expressed on endothelium after stimulation, neutrophil rolling on acutely insulted endothelium may be mediated by this receptor. It has been shown in animal models that antibody to L-selectin, or sulphated glyco- canes (eg, dextran sulphate, as used here), greatly reduce rolling observed intravitaly. Thus, it is likely that P-selectin exposed on endothelium or on immobilized platelets, and the same neutrophil ligands as described here, mediate rolling adhesion in vivo, and are important mediators of acute inflammatory and immune responses.

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Selectin-mediated rolling of neutrophils on immobilized platelets

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