Cell, Vol. 65, 859–873, May 31, 1991, Copyright © 1991 by Cell Press

Leukocytes Roll on a Selectin at Physiologic Flow Rates: Distinction from and Prerequisite for Adhesion through Integrins

Michael B. Lawrence and Timothy A. Springer
Center for Blood Research
and Harvard Medical School
800 Huntington Avenue
Boston, Massachusetts 02115

Summary

Rolling of leukocytes on vascular endothelial cells, an early event in inflammation, can be reproduced in vitro on artificial lipid bilayers containing purified CD62, a selectin also named PADGEM and GMP-140 that is inducible on endothelial cells. Neutrophils roll on this selectin under flow conditions similar to those found in postcapillary venules. Adhesion of resting or activated neutrophils through the integrins LFA-1 and Mac-1 to ICAM-1 in a lipid bilayer does not occur at physiologic shear stresses; however, static incubation of activated neutrophils allows development of adhesion that is greater than 100-fold more shear resistant than found on CD62. Addition of a chemoattractant to activate LFA-1 and Mac-1 results in the arrest of neutrophils rolling on bilayers containing both CD62 and ICAM-1. Thus, at physiologic shear stress, rolling on a selectin is a prerequisite for activation-induced adhesion strengthening through integrins.

Introduction

The migration of leukocytes into tissues is the central event in the inflammatory response. Leukocyte emigration is responsible for the successful host response to tissue injury and infection, but is also potentially harmful and contributes to the pathology of many diseases and inflammatory disorders. The first step in this process is margination, when leukocytes leave the central stream of flowing blood cells in a postcapillary venule and roll along the endothelial lining of the vessel (Cohnheim, 1889). Leukocytic margination in postcapillary venules should be distinguished from the "marginating pool" of about 50% of leukocytes that may be in capillary beds in the lung or tissues and enter the circulation in response to exercise or epinephrine. Postcapillary venules are major sites of leukocyte emigration in inflammation, and there are few or no marginating leukocytes in these venules in the healthy state (Fiebig et al., 1991).

As observed more than 100 years ago using intravital microscopy (Cohnheim, 1889), leukocytes begin to interact with the vessel wall by rolling along the endothelium within minutes after injury to adjacent tissue. The rolling response is seen throughout Vertebrata, in cold-blooded animals such as amphibians, as well as in mammals (Cohnheim, 1889). The number of rolling cells increases dramatically during the course of an inflammatory reaction (Atherton and Born, 1972) and is important in the accumulation of cells at the site (Fiebig et al., 1991). As the inflammatory reaction progresses, the endothelium becomes paved with leukocytes, and their rolling decreases in velocity and is interrupted by halts until they come to a firm stop (Cohnheim, 1889). Throughout this process the cells remain round, but undergo a dramatic change in shape immediately upon initiation of emigration. A pseudopod is extended through the vessel at a junction between endothelial cells, and this often is accompanied by a flattening of the leukocyte against the vessel wall (Marchesi, 1961). Transmigration continues as the pseudopod grows in ramifications and size until the entire cell body has emerged through a narrow gap between endothelial cells (Cohnheim, 1889). Cells appear to reach the point at which they emigrate by rolling; no active migration along the vessel wall is evident by intravital microscopy.

Both the rheology of blood and specific adhesive interactions may regulate the rolling response. Hydrodynamic studies of particles in suspension show that in Poiseuille flow, the larger particles are forced to the center of the stream, and this effect is more pronounced as shear forces increase (Segre and Silberberg, 1962). This effect has been confirmed for blood cells both in vivo and in vitro; the larger leukocytes are forced to the center of the stream in normal flow (Goldsmith and Spain, 1984; Nobis et al., 1985). In inflammation, vessels dilate and flow is slowed. Vascular permeability is increased, leading to plasma leakage and an increased hematocrit, and together with slower flow leads to erythrocyte rouleaux formation. A combination of these factors causes leukocytes to be displaced to the marginal region of flow near the vessel wall (Chien, 1982). This makes contact of a circulating leukocyte with the vessel wall more probable, but shear forces acting on the leukocyte at the vessel wall oppose adhesion to the endothelium. The velocity profile of a vessel shows no flow at the vessel wall and a parabolic increase toward the centerline. Because fluid velocity increases with distance from the wall, cells near the wall have torque exerted on them and will tumble even if not in contact with the wall. However, the velocity at which cells tumble in a shear flow near to the vessel wall is much faster than observed for rolling cells in inflammatory reactions, suggesting that adhesive interactions occur between the leukocyte and vessel endothelium (Atherton and Born, 1973).

More than 100 years after Cohnheim (1889) postulated molecular changes in vessel endothelium in inflammation, the molecular basis of leukocyte rolling remains unknown. However, three families of adhesion receptors that participate in leukocyte interactions with endothelium have been defined: the integrin, immunoglobulin (Ig)-related, and selectin molecules (reviewed in Springer, 1990). The integrins LFA-1 and Mac-1 on the neutrophil bind to the Ig family member ICAM-1 on endothelium (Smith et al., 1988, 1989; Diamond et al., 1990). LFA-1 and not Mac-1 binds to ICAM-2 (de Fougerolles et al., 1991; Diamond et al., 1990), an endothelial cell molecule that is more closely related to ICAM-1 than these molecules are to other Ig superfamily members (Staunton et al., 1989). Stimulation

of neutrophils with chemoattractants is required to activate binding of these integrins to ICAM-1 (Smith et al., 1989; Diamond et al., 1990). Stimulation of neutrophil integrin avidity is a rapid response that occurs within minutes, does not require increased integrin surface expression (Buyon et al., 1988; Philips et al., 1988; Vedder and Harlan, 1988; Lo et al., 1989), and appears analogous to an increase in avidity described for LFA-1 on T lymphocytes in response to antigen receptor cross-linking (Dustin and Springer, 1989).

ICAM-1 induction is a second mechanism for regulating inflammatory cell interactions; it occurs on a time scale of hours and requires mRNA and protein synthesis (reviewed in Springer, 1990). ICAM-1 is expressed basally on endothelial cells but is greatly increased at inflammatory sites and by stimulation with lipopolysaccharide and cytokines such as IL-1 and TNF. By contrast to ICAM-1, ICAM-2 is expressed at higher surface density on resting endothelium but is not inducible (de Fougerolles et al., 1991).

LFA-1 and Mac-1 together with p150,95 comprise the leukocyte integrins, a subfamily of integrins that share a common β subunit (CD18) and have distinct αL, αM, and αX (CD11a, b, and c) α subunits (reviewed in Larson and Springer, 1990; Springer, 1990). They are required for leukocyte emigration as demonstrated by an absence of neutrophil extravasation in patients with mutations in the common β subunit (leukocyte adhesion deficiency), and after treatment of healthy neutrophils with a monoclonal antibody (MAb) to the common β subunit in vivo or in vitro (reviewed in Anderson and Springer, 1987; Larson and Springer, 1990). Patient neutrophils, and healthy neutrophils treated with MAb to the common β subunit or a combination of MAb to LFA-1 and Mac-1 α subunits, are deficient in binding to endothelial cells in static adhesion assays (Buchanan et al., 1982; Harlan et al., 1985). However, when binding of neutrophils in shear flow is measured, the leukocyte integrin-dependent component of binding is lost at a shear stress below the physiologic range (Lawrence et al., 1990). Nonetheless, patient and CD18-treated cells that bind to the endothelium through other adhesion mechanisms fail to undergo transendothelial migration, in agreement with the lack of neutrophil diapedesis in leukocyte adhesion deficiency (Smith et al., 1988).

The selectins are the most recently recognized class of leukocyte adhesion molecules (reviewed in Springer, 1990). They have an N-terminal lectin domain, one epidermal growth factor–like module, and from two to nine short consensus repeats. By contrast to integrins and Ig family members, selectins have been found to date only on circulating cells and the endothelium, suggesting that they may be specialized for interactions within the vasculature. CD62 (PADGEM or GMP-140) is expressed in α granules of platelets and Weibel–Palade bodies of endothelial cells, and is mobilized to the plasma membranes of these cells after activation by mediators of inflammation and hemostasis, allowing these cells to bind neutrophils and monocytes at the site of tissue injury (Larsen et al., 1989; Geng et al., 1990). ELAM-1 is synthesized by endothelial cells in response to inflammatory agents and promotes adhesion of neutrophils, monocytes, and a subpopulation of lymphocytes (Bevilacqua et al., 1989; Picker et al., 1991; Shimizu et al., 1991). The LAM-1 or LECAM-1 molecule is expressed on leukocytes and facilitates their binding to endothelium during lymphocyte recirculation through peripheral lymph nodes and neutrophil emigration at inflammatory sites (Jutila et al., 1989; Spertini et al., 1991; Watson et al., 1991). Carbohydrate ligands for selectins have recently been defined (reviewed in Springer and Lasky, 1991); that for CD62 has Lewis x as an important component (Larsen et al., 1990) and also appears to be sialylated (Moore et al., 1991). Neutrophils bear Lewis x both on glycolipids and at the termini of N- and O-linked oligosaccharides (Symington et al., 1985; Fukuda et al., 1984). Antibodies to selectins and integrins additively inhibit neutrophil adhesion to endothelium, suggesting that they mediate distinct adhesion mechanisms (Luscinskas et al., 1989; Dobrina et al., 1989; Smith et al., 1991; Hallmann et al., 1991).

Here we have tested the hypothesis that a selectin is a rolling receptor. We chose CD62 because its rapid up-regulation on the endothelial cell surface suggests it may be important early in inflammation (Hattori et al., 1989; Geng et al., 1990) and because its expression on platelets provides a convenient source for biochemical isolation (Larsen et al., 1989). We demonstrate that at physiologic shear stress, neutrophils bind to and roll on CD62 in artificial bilayers. Further studies demonstrate qualitative differences between selectin and integrin adhesion mechanisms, and cooperation between them. On bilayers containing both CD62 and ICAM-1, the rolling interaction through CD62 is a prerequisite for chemoattractant-stimulated interaction of integrins on neutrophils with ICAM-1 that arrests rolling and dramatically strengthens adhesion. This essentially reproduces in vitro the steps of leukocyte accumulation at an inflammatory site in vivo.

Results

Reconstitution of CD62 and ICAM-1 in Lipid Bilayers

Immunoaffinity-purified CD62 and ICAM-1 in octyl-β-D-glucopyranoside (OG), alone or together, were mixed with phosphatidylcholine, and liposomes were prepared by dialysis. Liposome suspensions were placed on clean glass slides to form planar lipid bilayers with incorporated proteins (Watts et al., 1986). The incorporation of both CD62 and ICAM-1 into the membranes was quantitated by saturation binding with ^{125}I-MAb. Throughout the experiments described below, neutrophil binding to planar bilayers containing CD62 and ICAM-1 was found to be highly specific. Neutrophils did not bind to planar membranes containing phosphatidylcholine alone. Neutrophil binding to planar membranes containing CD62 was 98% reversible by incubation with EDTA for 10 min. Binding of N-formyl methionyl leucyl phenylalanine (fMLP)-stimulated neutrophils to planar membranes containing ICAM-1 was inhibited 97% by a combination of anti-LFA-1 (TS1/22) and anti-Mac-1 (LPM19c) α subunit antibodies, in agreement with previous reports (Smith et al., 1989; Diamond et al., 1990). TS1/22 or LPM19c alone did not inhibit fMLP-stimulated neutrophil binding to ICAM-1; thus, the results reported

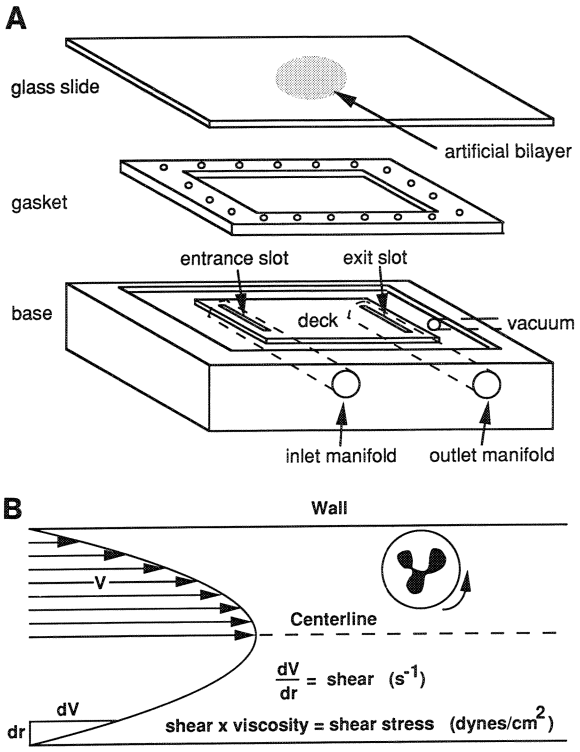

Figure 1. Experimental Apparatus to Measure Attachment of Neutrophils under Flow Conditions

(A) Parallel-plate flow chamber, after Lawrence et al. (1987). The chamber is shown upside down for illustration purposes; the glass slide with the artificial bilayer formed the lower parallel plate. It was mounted on the stage of an inverted microscope equipped with a video camera. The cell suspension was connected to the inlet manifold and a syringe pump to the outlet manifold.

(B) Schematic of parabolic flow profile in a blood vessel or parallel-plate flow chamber.

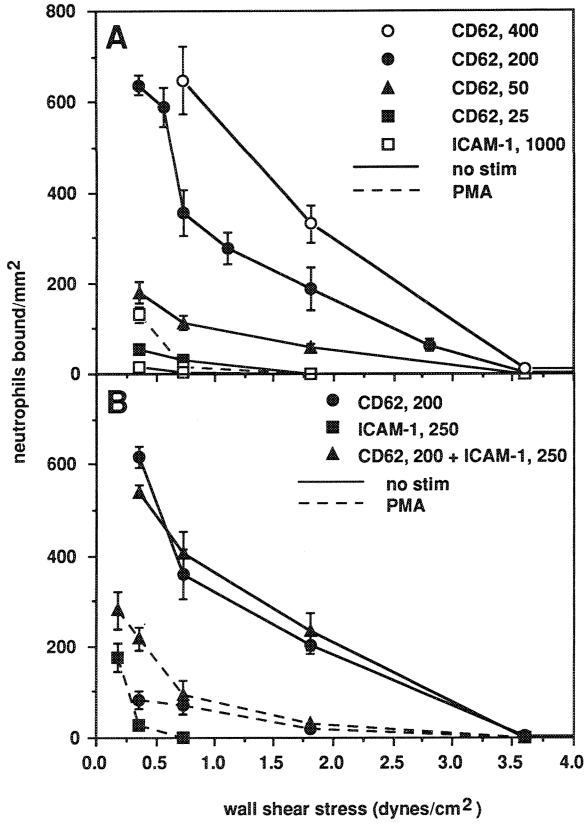

Figure 2. Attachment of Neutrophils to Artificial Planar Bilayers during Flow

Resting or PMA-stimulated neutrophils were infused at varying wall shear stresses through the parallel-plate flow chamber. A planar membrane containing CD62, ICAM-1, or both at the indicated density in sites per μm^2 was formed on one side of the chamber. After 3 min of continuous flow to equilibrate adherence and deadherence to the bilayer, adherent neutrophils were quantitated. (A) and (B) represent two different sets of experiments: day-to-day variation throughout this work was slight as exemplified by binding of unactivated neutrophils to CD62 at 200 sites per μm^2 in both panels. Data are averaged from four to six (A) and two (B) experiments. Bars show the SEM of the experiments. At the next higher experimental point, at 7.3 dyn/cm^2, binding was 0 in all cases.

below reflect interaction of both LFA-1 and Mac-1 on the neutrophil with ICAM-1 in the planar membrane.

The Selectin CD62 Is Distinctive in Support of Adhesion at Venular Levels of Shear Stress

To measure attachment of neutrophils under flow conditions, glass slides containing planar bilayers were incorporated into a parallel-plate flow chamber (Figure 1A). Neutrophils (10^6/ml) were infused at flow rates that were regulated to produce wall shear stresses bracketing the range estimated to exist in postcapillary venules. Wall shear rates and stresses in the flow chamber are calculated from chamber geometry and volumetric flow rate (Lawrence et al., 1990). Shear stresses in vivo can be calculated from centerline velocity and vessel diameter using the Hagen–Poiseuille equation. In both cylindrical and parallel-plate geometries, the velocity profile of a Newtonian fluid is parabolic (Figure 1B). The change in velocity per change in radial displacement is called shear and is highest at the wall. Shear stress, the product of shear and viscosity, better correlates with the forces acting on a cell under flow. Shear stresses of 1–10 dyn/cm^2 have been measured for postcapillary venules (Heisig, 1968), and in the classic studies of Atherton and Born (1972), leukocytic rolling was observed at shear stresses that we calculate to be 1.5–4.0 dyn/cm^2.

Flowing neutrophils readily bound to artificial bilayers containing CD62 at wall shear stresses within the physiologic range (Figure 2A). Attachment was dependent on the density of CD62, with efficient attachment at 1.8 to 2.8 dyn/cm^2 at 400 and 200 sites per μm^2, and significant attachment occurring at 1.8 dyn/cm^2 at a density of 50 sites per μm^2. By contrast, neutrophils did not adhere to artificial bilayers containing ICAM-1 under flow conditions, even at high ICAM-1 densities of 1000 sites per μm^2 (Figure 2A). Lower ICAM-1 densities than this support strong static binding of activated neutrophils (see below); however, neutrophils that were stimulated with PMA did not bind at physiologic shear stresses (>1 dyn/cm^2) and bound only at low levels in stagnant flow at a shear stress of 0.36

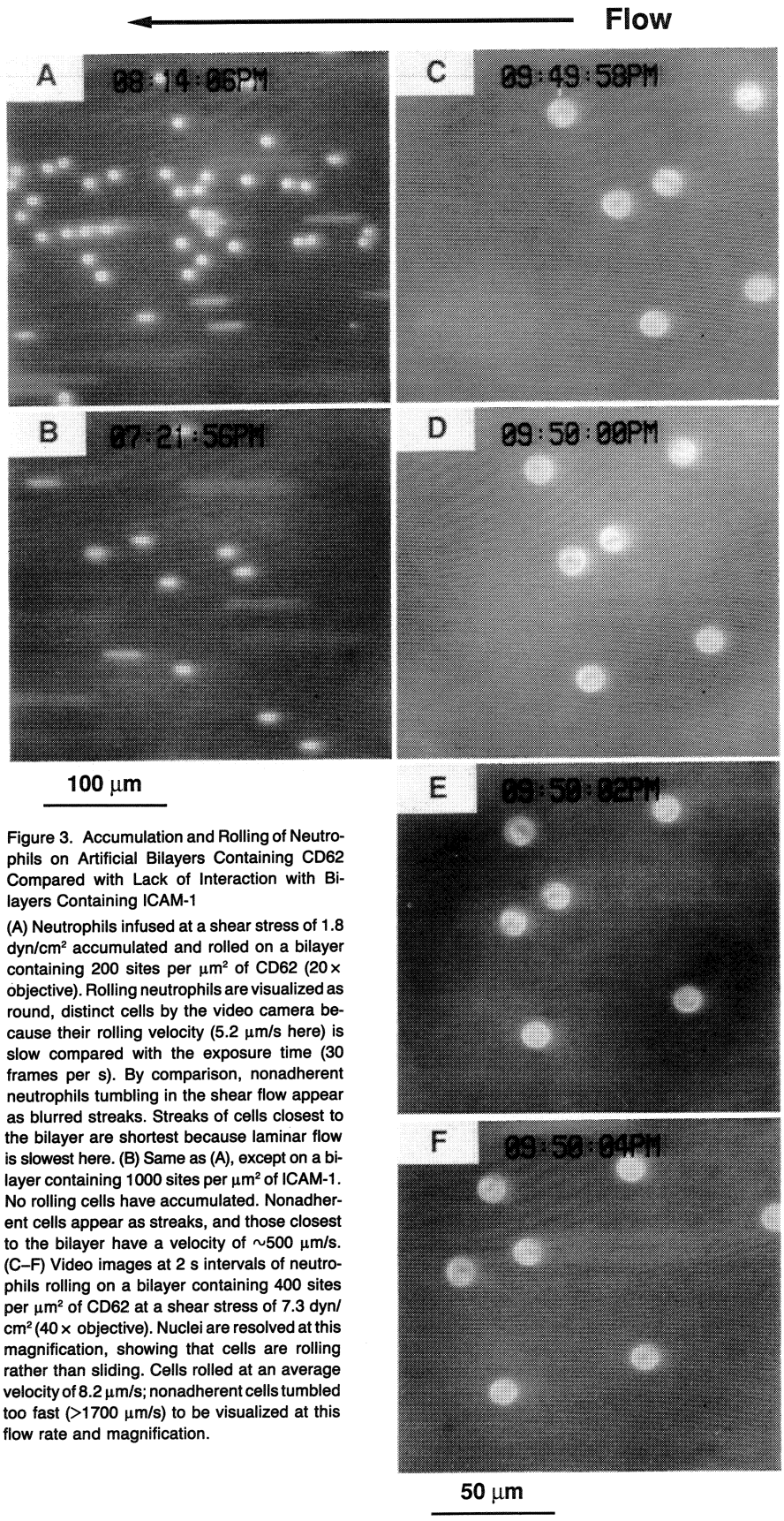

Figure 3. Accumulation and Rolling of Neutrophils on Artificial Bilayers Containing CD62 Compared with Lack of Interaction with Bilayers Containing ICAM-1

(A) Neutrophils infused at a shear stress of 1.8 dyn/cm^2 accumulated and rolled on a bilayer containing 200 sites per μm^2 of CD62 (20× objective). Rolling neutrophils are visualized as round, distinct cells by the video camera because their rolling velocity (5.2 μm/s here) is slow compared with the exposure time (30 frames per s). By comparison, nonadherent neutrophils tumbling in the shear flow appear as blurred streaks. Streaks of cells closest to the bilayer are shortest because laminar flow is slowest here. (B) Same as (A), except on a bilayer containing 1000 sites per μm^2 of ICAM-1. No rolling cells have accumulated. Nonadherent cells appear as streaks, and those closest to the bilayer have a velocity of ~500 μm/s. (C–F) Video images at 2 s intervals of neutrophils rolling on a bilayer containing 400 sites per μm^2 of CD62 at a shear stress of 7.3 dyn/cm^2 (40× objective). Nuclei are resolved at this magnification, showing that cells are rolling rather than sliding. Cells rolled at an average velocity of 8.2 μm/s; nonadherent cells tumbled too fast (>1700 μm/s) to be visualized at this flow rate and magnification.

dyn/cm² (Figure 2A). Flowing lymphocytes that have been activated by cross-linking the T cell antigen receptor with MAb also fail to bind to ICAM-1 under flow conditions (not shown) but bind avidly through LFA-1 to ICAM-1 under static conditions (Dustin and Springer, 1989). Lymphocytes also fail to bind through CD2 to LFA-3 (1000 sites per μm²) under flow conditions (not shown), but do so at stasis (Chan et al., submitted). The failure of several integrin–Ig superfamily interactions and Ig–Ig superfamily (CD2–LFA-3) interactions to occur under flow conditions is distinct from the efficiency of interaction through the selectin CD62.

The efficiency of adhesion of unactivated neutrophils to artificial bilayers containing a mixture of CD62 and ICAM-1 was indistinguishable from adhesion to CD62 alone (Figure 2B). Thus, no cooperative interactions between these adhesion mechanisms occur on resting neutrophils.

Activation with PMA prior to infusion greatly decreased the efficiency of adhesion to CD62 (Figure 2B). The amount of adhesion of PMA-stimulated neutrophils to the mixture of CD62 and ICAM-1 (Figure 2B) was additive for the adhesion to CD62 alone (Figure 2B) and ICAM-1 alone (Figure 2B).

Neutrophils Roll on CD62

Artificial bilayers containing CD62 were remarkable not only for their ability to support adhesion under physiologic flow conditions, but also in the rolling mode of this adhesion. Neutrophils rolled on the CD62 substrate, driven by fluid drag forces, as readily recorded using a video camera (Figure 3). Rolling neutrophils that accumulated on the CD62 substrate remained round and appeared as sharp images, whereas nonadherent cells that tumbled past in the shear flow were blurred streaks (Figure 3A). Only nonadherent, tumbling cells were visualized on ICAM-1 substrates (Figure 3B); the length of the blur is dependent on the distance from the wall because of the velocity profile (Figure 1B), with the slowest tumbling cells closest to the substrate.

Confirming that the cells roll rather than slide on the CD62 substrates, rotation of the polymorphic nucleus of the neutrophil was observed using a 40× objective and was clearly visible when videotapes were played back; the progress of rolling cells across a substrate at 2 s intervals is shown using a 40× objective in Figures 3C–3F. At lower shear stresses cells frequently wobbled as they rolled. Individual cells rolled with a relatively, but not completely, uniform velocity. Rolling was sometimes interrupted by brief pauses where the cells appeared to halt momentarily on the substrate, and also by brief increases in velocity during which the cells appeared to detach from the substrate and then reattach several cell diameters downstream. Rolling cells were in equilibrium with nonadherent cells, as reflected by detachment of some rolling cells with their loss from the field of view and attachment of other cells.

The overall similarity in rolling velocity among different cells led to the appearance that they rolled as a group over the substrate, as for the six cells in Figures 3C–3F, but there was some variation in velocity; comparison of Figures 3C and 3F shows that the cell in the upper left in Figure 3C is the slowest of the group and the one 5 o'clock to it in Figure 3C is the fastest and has overtaken it by Figure 3F.

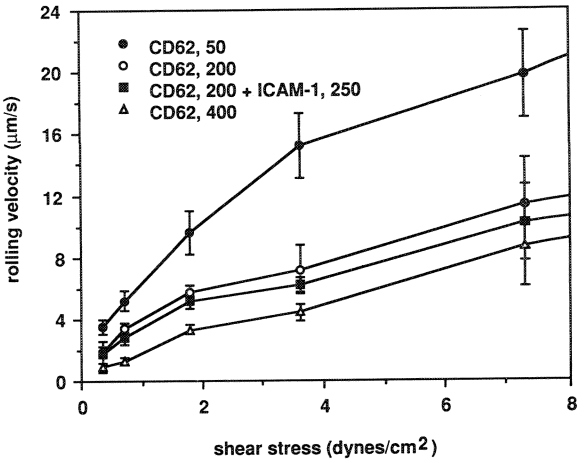

Figure 4. Rolling Velocity as a Function of CD62 Density and Shear Stress

CD62 and ICAM-1 were used at the indicated density in sites per μm². Error bars represent the SEM based on measurements from independent experiments. Experimental points at 14.6 dyn/cm² are not shown, but indicated by connecting lines.

Disturbance of the laminar flow profile by attached cells was evident from observation of cells attaching to artificial bilayers of CD62 at a shear stress of 1.8 dyn/cm² and above. Single cells bound in a random distribution over the substrate. However, once one cell bound, binding of further cells immediately downstream was much more likely than in areas of the substrate with no attached cells. It appeared that the neutrophils were following locally disturbed streamlines that increased the chance of collision with and subsequent attachment to the substrate. After several minutes the cells became more evenly distributed. At shear stress levels of 0.73 dyn/cm² and below, attachments were uniformly distributed even at the earliest times.

Rolling velocity increased with increasing shear force (Figure 4). The rolling velocity was proportional to the shear force at low shear stresses, and then began to plateau. This may reflect the effect of torque acting on a rolling but deformable object, leading to a greater contact area and less fluid drag. Rolling velocity was dependent on the site density of CD62 (Figure 4). The higher the CD62 density, the more slowly the neutrophils rolled at a given shear stress. This is as predicted, because a higher number of receptor–ligand interactions will lead to a greater resistance to the fluid drag force. The slower rolling velocities at higher site density correlated with the increased effectiveness of attachment. Inclusion of ICAM-1 in artificial bilayers containing CD62 did not alter rolling velocity (Figure 4), correlating with its lack of effect on attachment.

Rolling did not appear to result in any alteration in the cells or the substrate. The rolling velocity of groups of cells followed along the substrate did not appear to change with time. The rolling velocity of cells on CD62 substrates was

Table 1. Measured Neutrophil Rolling Velocities Compared with Calculated Velocities for a Sphere in Shear Flow

Shear Stress (dyn/cm²)	Measured Velocities (μm/s) on Various Substrates[a]			Calculated Velocities (μm/s) at Various Distances from Wall[b]		
	CD62	ICAM-1	ICAM-1 + PMA	500 nm	10 nm	1 nm
0.18	Not done	52.4 ± 11.2	0.0	54.9	28.5	21.4
0.36	1.9 ± 0.7 (89%)	138.6 ± 45.8	0.0	110.6	57.5	43
0.73	3.4 ± 0.4 (96%)	230.2 ± 20.9	0.0	221	114	86
1.8	5.7 ± 0.5 (99%)	541 ± 271	0.0	550	285	214
3.6	7.2 ± 1.6 (100%)	*	0.0	1104	573	429
7.3	11.4 ± 3.0 (99%)	*	0.0	2206	1145	858
14.6	15.9 ± 1.5 (100%)	*	0.0	4419	2296	1712

[a] Measured rolling velocities are from 2 or 4 s measurement periods on the indicated substrates (CD62 at 200 sites per μm²; ICAM-1 at 1000 sites per μm²); the percentage of cells that rolled in this time period on CD62 is shown in parentheses. Velocities of resting neutrophils on ICAM-1 at shear stresses of 0.73 dyn/cm² and above were measured from the leading edge of the streak on four consecutive frames. Asterisks indicate that cells moved too fast for velocity measurements. Measurements for PMA-stimulated neutrophils were made after cells were allowed to adhere to the substrate under static conditions for 6 min.

[b] Hydrodynamic calculations are for a 7 μm diameter hard sphere in a shear flow with no interaction with the wall, at the indicated distance from the wall.

unaltered for at least 15 min at a single observation condition, and the same substrate could be used for observations at many different shear stresses, without any alteration in rolling velocity measurements for the same shear stress replicated at the beginning and end of the set of observations. When the direction of flow was reversed, the direction of rolling was reversed and the cells rolled back "over their tracks" with the same velocity as in the forward direction.

We compared the velocities of unstimulated neutrophils rolling over a CD62 substrate and tumbling over an ICAM-1 substrate to the predicted velocity for a sphere of the same diameter, 7 μm (Table 1). The measurements on the ICAM-1 substrate are for the cells flowing closest to the substrate, as determined by the focal plane. The unstimulated neutrophils flowing over the ICAM-1 substrate moved at a velocity 100-fold greater than the cells rolling on the CD62 substrate. Because of the torque exerted by shear flow, a spherical object will rotate at an angular velocity of one-half the shear rate, so its motion, particularly if close to the wall, is not qualitatively different from rolling along a flat surface. Predicted velocities of a sphere 7 μm in diameter having no interaction with a smooth wall (Goldman et al., 1967) at distances of 1, 10, and 500 nm are more than an order of magnitude higher than the measured velocities for rolling on CD62 (Table 1). The rolling velocities observed on CD62 thus require an adhesive interaction. By contrast, the measured velocities on the ICAM-1 substrates are in excellent agreement with the predictions for a sphere tumbling in shear flow, particularly with a separation of 500 nm. Distances on the order of 1 and 10 nm are probably difficult to obtain without contact, since surface features such as ICAM-1 are on the order of this size; ICAM-1 is 18 nm long as measured in the electron microscope (Staunton et al., 1990).

Adhesion under Static Conditions

The ability of neutrophils to spread and develop adhesiveness on artificial bilayers under static conditions was examined for comparison to behavior under flow conditions. Neutrophils were injected through a port and examined at different time points; 2 min of the total time was required before all the neutrophils had settled onto the bilayer. Resting neutrophils remained round on ICAM-1 bilayers, with no spreading after prolonged incubation (Figure 5A). However, treatment with PMA or fMLP resulted in spreading of almost all neutrophils on ICAM-1 bilayers (Figures 5B–5F).

Neutrophils on CD62 bilayers remained round even after prolonged contact of up to 12 min (Figure 5G), in agreement with observations on rolling cells under flow conditions. Neutrophil activation with PMA induces a bipolar shape change even when cells are held in suspension; PMA-stimulated neutrophils had a bipolar appearance on CD62 bilayers, but no spreading was observed (Figure 5H).

To examine adherence under static conditions, neutrophils were allowed to settle onto the planar membrane, and after 6 min of contact, controlled flow was used to create a detachment force. Binding to CD62 and ICAM-1 was compared, with and without PMA present. This assay highlighted significant differences in patterns of adhesion strengthening between the CD18 and the CD62 pathways.

Unstimulated neutrophils did not adhere to artificial bilayers containing ICAM-1 (Figure 6A). By contrast, PMA-stimulated neutrophils developed strong adhesion to the bilayer during the 6 min contact period. These attachments were dramatically resistant to shear stress; almost all the cells remained attached at wall shear stresses as high as 36 dyn/cm². The same results were observed after stimulation with 10^{-7} M fMLP. These strongly adherent cells could not be induced to roll even at high shear stresses (Table 1). In the same assay, we found that binding was 97% inhibited by a combination of MAbs to the Mac-1 and LFA-1 α subunits, but not by either MAb alone. Thus, adhesion strengthening occurs through both Mac-1 and LFA-1. The differing abilities of ICAM-1 to mediate adhesion of PMA-stimulated neutrophils under flow as compared with static conditions were seen despite use of ICAM-1 at 1000 sites per μm² in flow assays and at 250 sites per μm² in static assays.

The formation of mechanically strong attachments

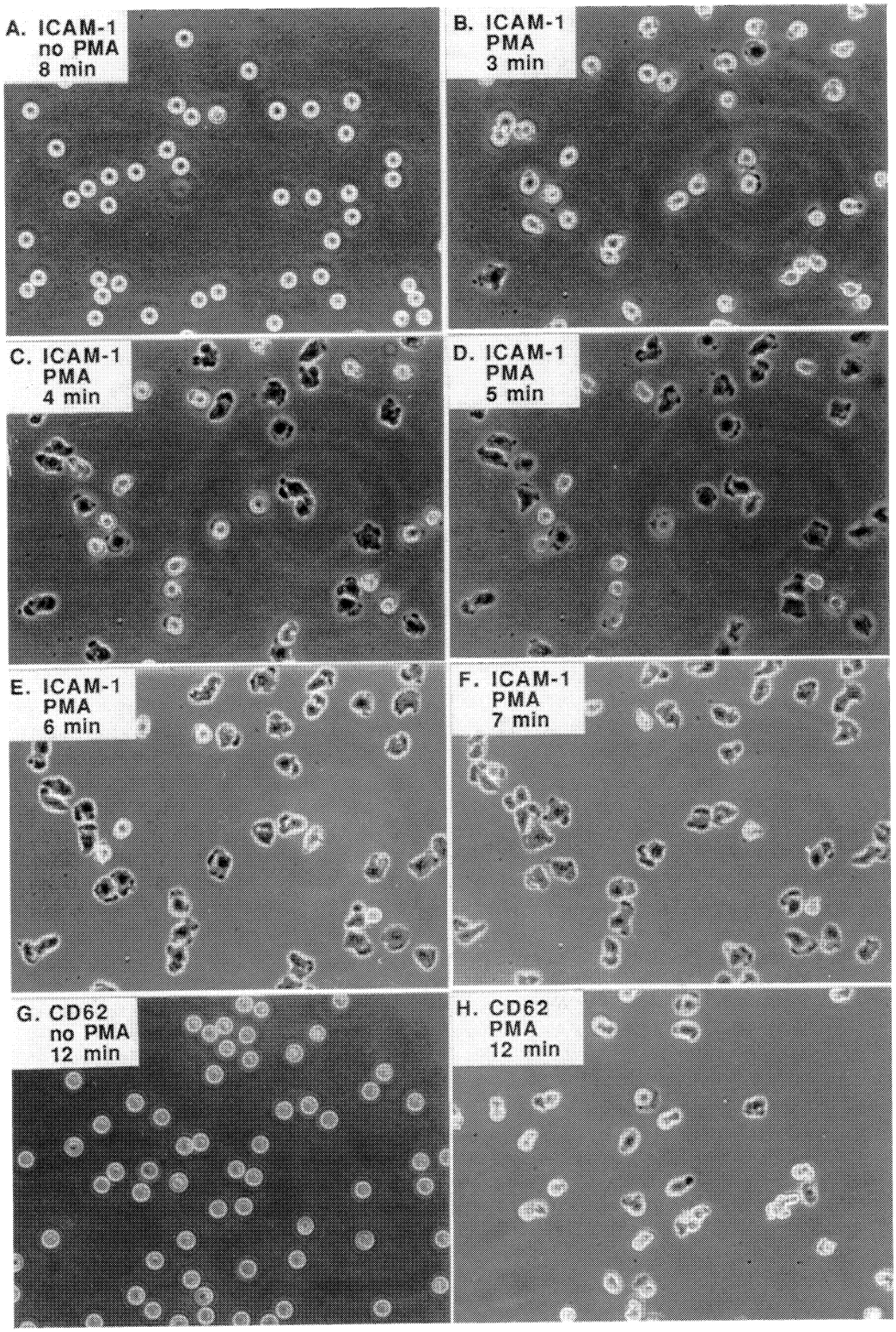

Figure 5. Neutrophils in Contact with Artificial Membranes under Static Conditions

Neutrophils, with or without stimulation with PMA, were allowed to adhere in the absence of flow to artificial bilayers containing 250 sites per μm^2 of ICAM-1 or 200 sites per μm^2 of CD62 for varying time periods, as indicated. (B)–(F) represent the same field of cells at 1 min intervals. Time points prior to 3 min are not shown because it takes 2 min for all cells to settle onto the bilayer.

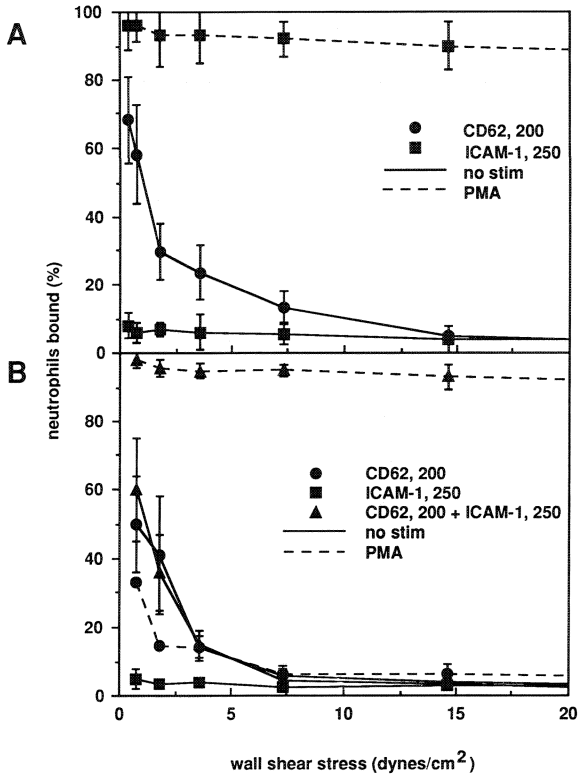

Figure 6. Detachment Assay following Static Incubation of Neutrophils on Artificial Membranes Containing either CD62, ICAM-1, or a Mixture of CD62 and ICAM-1

Neutrophils were injected through a port in the side of the flow chamber and allowed to settle onto artificial bilayers containing 200 sites per μm^2 of CD62, 250 sites per μm^2 of ICAM-1, or both. For some experiments, PMA was added to the neutrophil suspension before it was injected into the flow chamber (broken line). After 6 min of contact, shear stress was applied in staged increments. Neutrophils bound after 20 s at each shear stress point are expressed as the percentage of neutrophils that settled onto the bilayer in the initial contact period. Solid lines represent binding of unstimulated neutrophils to the membrane. Error bars represent the SEM of three to five independent experiments (A) and two experiments (B). Experimental points at 36 dyn/cm^2 are not shown but are indicated by connecting lines.

through integrins following PMA or fMLP stimulation contrasted with the pattern observed for CD62. With the same contact time neutrophils formed very reversible adhesions to CD62 (Figure 6A). The shear stresses required for detachment of neutrophils from CD62 were roughly comparable to the maximal shear stresses at which attachments could form (Figure 3, above). More importantly, all the neutrophils rolled along the planar membrane containing CD62, and at the same velocity as observed for neutrophils that adhered under flow conditions (data not shown). Thus, contact time did not result in adherence strengthening through the selectin CD62.

We used bilayers containing both CD62 and ICAM-1 to test for cooperativity in the static binding assay between the selectin and integrin/Ig gene family adhesion systems. No enhancement of resting neutrophil binding was observed on a bilayer containing both CD62 and ICAM-1 compared with CD62 alone (Figure 6B). The shear sensitivity of binding to both types of planar membranes was equivalent, and rolling occurred as on the CD62 membrane alone. Thus, even under conditions where contact with CD62 and ICAM-1 was maximized, CD62 did not stimulate adhesiveness of neutrophils for ICAM-1. The integrin/Ig and selectin pathways appear to function independently of each other; binding to the ligand for CD62 on neutrophils did not generate a signal that activated integrin avidity.

PMA activation of neutrophils resulted in strong attachments after 6 min of contact with the planar membranes containing both ICAM-1 and CD62 (Figure 6B). Adherent cells had a morphology indistinguishable from that of PMA-activated neutrophils binding to ICAM-1 alone, and were equally shear resistant. On CD62 alone, PMA did not stimulate development of shear-resistant attachments, but decreased shear resistance relative to unstimulated cells.

Cooperation between Selectin and Integrin Adhesion Mechanisms

We tested for cooperation between the CD62 rolling and leukocyte integrin adhesion-strengthening mechanisms under conditions that would approximate those at an inflammatory site by addition of fMLP to rolling neutrophils. Neutrophils were infused at two shear stresses in the physiologic range into a flow chamber with an artificial bilayer containing both CD62 and ICAM-1. After a 3 min equilibration period to allow accumulation of rolling neutrophils on the bilayer, 10^{-9} M fMLP was added to the infusion medium and reached the rolling neutrophils 12 or 30 s later (0 time, Figure 7A). Within 30 s of exposure, mean rolling velocities had begun to drop at both flow rates (Figure 7A) and continued to drop for 5 min. After 5 min essentially all the neutrophils became arrested on the bilayer, as seen both from the average rolling velocity (Figure 7A) and from the velocity distributions (Figures 7B and 7C). Arrest of round cells preceded spreading. The round, rolling neutrophils at 0 time had almost completely spread after 5 min (Figure 7D) and developed shear-resistant adhesion (Figure 7E). Cells did not become arrested on CD62 alone, because even after contact of activated neutrophils with CD62 bilayers under static conditions, rolling occurred after initiation of flow, and adhesion was not strengthened compared with unstimulated cells (Figure 6B).

Discussion

Following a localized tissue injury, nearby blood vessels undergo a rapid dilation. Blood flow slows and neutrophils begin to roll along the endothelial cell monolayer lining the vessel wall. Rolling appears to be the first step of the interaction of the leukocyte with the vessel wall during an inflammatory response and a prerequisite for later extravasation. The molecular basis of rolling does not appear to involve the leukocyte integrins, based on the inability of MAb to the leukocyte integrin common CD18 β subunit to inhibit rolling in vivo (Arfors et al., 1987).

In this report we demonstrate that leukocyte rolling, a hallmark of the early stages of an inflammatory response,

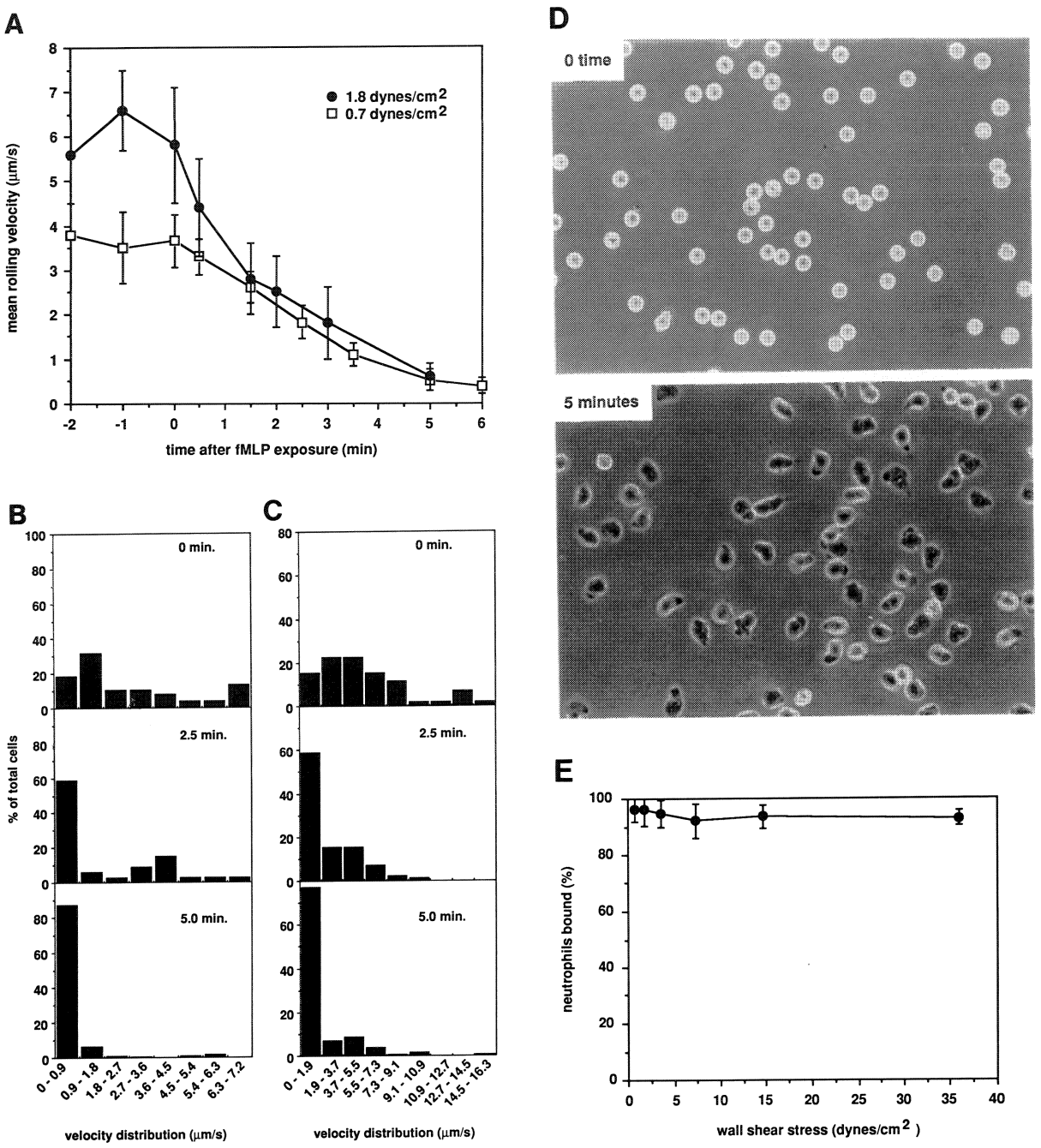

Figure 7. Stimulation with fMLP Arrests Neutrophil Rolling on Artificial Membranes Containing both CD62 and ICAM-1

Neutrophils were allowed to adhere to artificial bilayers containing CD62 (200 sites per μm^2) and ICAM-1 (250 sites per μm^2) during flow at a wall shear stress of 1.8 or 0.73 dyn/cm^2. Rolling velocity was constant for at least 5 min under these conditions. During the experiment, 10^{-9} M fMLP was added to the medium being infused into the chamber and reached the cells rolling in the field of view 30 or 12 s later (marked 0 time) as verified in another experiment with a dye solution.

(A) Mean rolling velocity as a function of time after fMLP addition. Bars show the SEM for two experiments.

(B and C) Distribution of rolling velocities of adherent neutrophils with time after fMLP exposure at 0.73 and 1.8 dyn/cm^2, respectively.

(D) Photomicrographs of neutrophils before the infusion of fMLP (0 time) and after exposure to 10^{-9} M fMLP (5 min) on the same area of the planar membrane. fMLP addition induced arrest and spreading of the neutrophils. Flow was stopped for approximately 30 s to take the photographs in a separate experiment from the two experiments averaged for data in (A)–(C).

(E) Shear resistance of neutrophil binding. Neutrophils were allowed to attach at 0.73 dyn/cm^2, and shear resistance was determined 5 min after fMLP exposure.

can be reconstituted in vitro on artificial lipid membranes containing an endothelial cell selectin, CD62. Neutrophils formed reversible rolling attachments to CD62 at physiologically relevant shear stresses. Another endothelial cell adhesion molecule, ICAM-1, did not support rolling adhesions, and was found to be significantly less effective than CD62 as a ligand for neutrophils under flow conditions. However, ICAM-1 was required for subsequent spreading and the development of a shear-resistant attachment.

The selectin CD62 was distinguishable from integrin and Ig family members both in its ability to mediate rolling and in its ability to mediate adhesion during flow at physiologic shear stresses. When the avidity of neutrophil integrins for ICAM-1 was stimulated with PMA or fMLP, flowing neutrophils did not bind to ICAM-1 significantly over a range of physiologic shear stresses at which CD62 was highly effective. This was despite the ability of activated neutrophils to form attachments in static assays to 4-fold lower densities of ICAM-1. These attachments to ICAM-1 were greater than 100-fold more shear resistant than attachments to CD62. Integrins on activated T cells also failed to bind to ICAM-1 and fibronectin at physiologic shear stresses. Furthermore, an Ig family–Ig family interaction between CD2 on T lymphocytes and LFA-3 in artificial bilayers failed to occur at physiologic shear stresses. Even when adhesion of neutrophils or lymphocytes to ICAM-1 or lymphocytes to LFA-3 was initiated under static conditions, firm adhesion was unable to be converted to a rolling adhesion when shear flow was applied; cells either remained adherent or were completely dislodged. The shear stress required to dislodge 50% of cells binding through CD2 to the transmembrane isoform of LFA-3 is 1.5 dyn/cm^2 (Chan et al., submitted); at a comparable ligand density a shear stress of 1 dyn/cm^2 dislodged 50% of neutrophils binding to CD62. The comparability of these shear stresses suggests that rolling is not directly related to binding strength.

We saw significant binding of neutrophils to CD62 at site densities as low as 50/μm^2, which is a physiologically relevant density. CD62 is stored in Weibel–Palade bodies of endothelial cells (Bonfanti et al., 1989; McEver et al., 1989), which in response to stimuli such as thrombin and histamine fuse with the plasma membrane and thereby up-regulate expression of CD62. An increase from 20 to 50 sites per μm^2 within 5 min after stimulation and a decline to baseline levels by 30 min is seen for endothelium cultured in vitro (Hattori et al., 1989). During passage of endothelial cells in vitro, there is a decline in CD62 and Weibel–Palade body content, and therefore densities of CD62 could be higher in vivo.

Our studies demonstrating that CD62 is a receptor that mediates rolling suggest that its up-regulation may be an important mechanism for regulating rolling in the inflammatory response. Thrombin stimulation mediates a transient binding of neutrophils that is CD18 independent and likely due to CD62 expression (Zimmerman and McIntyre, 1988; Geng et al., 1990). Neutrophil binding to primary-culture endothelial cells at 2.0 dyn/cm^2 wall shear stress is stimulated by thrombin and is characterized by the initiation of rolling detectable within less than 1 min of thrombin exposure, suggesting the involvement of CD62 (M. B. L. and L. V. McIntire, unpublished data). Thus CD62 is an excellent candidate for the initiation of neutrophil rolling during the early stages of an inflammatory response.

Rolling velocities on CD62 were comparable to in vivo rolling velocities. On bilayers containing CD62, velocities ranged from under 2 to over 30 μm/s, depending on site density and the shear stress. Mean rolling velocities in vivo were 10 μm/s in mouse mesentery venules and 20 μm/s in hamster cheek pouch venules (Atherton and Born, 1973). Rolling velocities on CD62 were proportional to the flow rate at low shear stresses, but increases were not proportional at higher shear stresses. Atherton and Born (1973) reported that rolling velocities in vivo were linearly dependent on shear stress (blood flow velocity) up to about 5 dyn/cm^2 and plateaued above this point. The in vivo velocities at a given shear stress (Atherton and Born, 1973) were about twice what we find on CD62 at 50 sites per μm^2; considering that other selectins may contribute to rolling in vivo and their concentration is unknown, the correspondence between these values is remarkable.

Neutrophils rolled on CD62 at shear stresses comparable to those found in postcapillary venules in vivo. Wall shear stresses at which rolling was observed in the measurements of Atherton and Born (1972, 1973) (assuming cylindrical geometry and a plasma viscosity of 2 cP) ranged from 1.5 to 4.0 dyn/cm^2. These values are similar to the shear stresses at which neutrophils form rolling attachments to bilayers containing CD62, and also to monolayers of endothelial cells stimulated with cytokines (Lawrence et al., 1990) or thrombin (M. B. L. and L. V. McIntire, unpublished data).

Other selectins have not been shown to mediate rolling; however, this family of molecules may be specialized to mediate rolling adhesions, and rolling observed in vivo may involve contributions from all three. LECAM-1 contributes to the interaction of unactivated neutrophils with cytokine-stimulated endothelial cells at physiologic shear stresses (Smith et al., 1991). On the basis of the ability of unactivated neutrophils to adhere under flow conditions, the selectins so far studied appear to be structures that are capable of mediating the initial attachment of neutrophils to the vessel wall.

The structure of selectins and their carbohydrate ligands appears ideally suited for their function in rolling and adhesion at high shear stresses. Kinetic considerations are very important here; a high on-rate for formation of the selectin–ligand complex is required for efficient interactions of rapidly flowing cells with a substrate, and both a rapid on-rate and rapid off-rate are required for rolling. We found no change in the cells or the substrate after prolonged rolling, suggesting that dissociation at the upstream edge of the cell is due to dissociation of the CD62–ligand complex rather than to loss of molecules from the cell or substrate.

Interactions between biological macromolecules are frequently diffusion limited; i.e., the rate at which they can form specific noncovalent interactions is limited by their rates of diffusion. Diffusion of the extracellular ligand-binding domain of an adhesion receptor has two components, one due to segmental flexibility of the tether by

which it is attached to the membrane and the other due to lateral diffusion in the membrane bilayer of the membrane anchor. With an appropriately high number of segments and high segmental flexibility, the former type of diffusion can occur more rapidly than the latter, since proteins of adhesion receptor size have diffusion coefficients on the order of 10^{-7} cm^2/s (Tanford, 1961), whereas bilayer diffusion coefficients are on the order of 10^{-9} cm^2/s (Jacobson et al., 1987). The volume of solvent above the cell surface in which diffusion can occur and the diffusion rate are related to the length of the membrane tether, its segmental flexibility, and the size of the segments. Molecules composed of short consensus repeats of the type found in selectins have random configurations as revealed by electron microscopy (Weisman et al., 1990), suggesting a high degree of segmental flexibility. The (sialylated) Lewis x ligand of CD62 is found at the termini of long carbohydrate structures that are predicted to confer flexibility (Fukuda et al., 1984). The location of the lectin-binding domain and the Lewis x determinant at the termini of their respective structures maximizes diffusiveness due to segmental flexibility. The diffusion coefficient is inversely related to size; therefore, sialylated Lewis x, by virtue of its small size (M_r 779) relative to protein adhesion ligands, allows a faster rate for diffusion-limited processes.

Adhesiveness through integrins and adhesiveness through the selectin CD62 differ drastically in the time scale required for their development. Binding of flowing cells to a substrate places stringent time constraints on adhesive bond formation that appear to prevent leukocyte integrin interactions with ICAM-1, even when neutrophils are activated. CD62 mediates adhesion on a time scale at which the leukocyte integrin interactions are ineffective. Bond formation through CD62 appears so rapid that little adhesion strengthening is apparent when binding under flow and static conditions is compared. Adhesion strengthening through integrins may take time to develop both because the globular putative ligand-binding regions are quite large and are attached by two stalks to the membrane (Nermut et al., 1988), limiting diffusiveness, and because cooperative interactions between multiple integrins or with other molecules such as cytoskeletal components may be required. The latter may be reflected in the spreading that occurs during adhesion strengthening on ICAM-1 but not on CD62.

Inhibition of neutrophil binding to CD62 under flow conditions by prior activation with PMA may reflect an effect of the change to a bipolar shape rather than of loss of ligand-binding sites. CD62 binds at saturation to 20,000 sites per cell with a similar affinity or avidity of 10^9 M^{-1} to both resting and PMA-stimulated neutrophils (Moore et al., 1991). When we bound PMA-stimulated and resting neutrophils to artificial bilayers containing CD62 at stasis and subjected them to detachment with shear, PMA-stimulated cells bound less efficiently, but the decrease in efficiency relative to untreated cells was much less marked than for binding under conditions of shear. Activated neutrophils with their bipolar, elongated shapes would experience higher transient torques than unstimulated, round cells in contact with a substrate. The shape change may therefore be an important factor that impedes adhesion in shear flow, and may help prevent activated leukocytes, if they fail to emigrate at an inflammatory site, from attaching and emigrating at an uninvolved site downstream.

Our studies demonstrate how a "weak," rolling interaction can develop into what is termed a "firm" adhesion to the vessel wall that neutrophils develop during an inflammatory response (Pober and Cotran, 1990). Since neutrophils do not migrate on the lumenal side of the vessel wall, rolling allows an unstimulated neutrophil to move to a site where it can undergo diapedesis. Transendothelial cell migration, however, first requires the arrest of the rolling neutrophil. While it has been shown that transendothelial migration requires CD18 (Smith et al., 1988), how a neutrophil comes to a stop at an endothelial cell junction has been unknown. We demonstrated here that following activation of leukocyte integrins with a chemoattractant, neutrophils rolling on a bilayer containing CD62 and ICAM-1 rapidly slowed and came to a stop. Activation of LFA-1 and Mac-1 permitted interactions with ICAM-1, which then led to arrest and spreading. The firm adhesion observed in the microcirculation is blocked by in vivo administration of MAb to CD18 (Arfors et al., 1987) and therefore appears equivalent to the shear-resistant attachment neutrophils form on ICAM-1 bilayers. At physiologic shear stress, rolling on a selectin was a prerequisite for neutrophil integrin interaction with ICAM-1. The rolling interaction may promote the integrin–ICAM-1 interaction both because it facilitates close physical interaction between integrins and ICAM-1 and because the neutrophil is rolling approximately two orders of magnitude more slowly than a tumbling neutrophil near the wall, which would raise the chance of enough bonds being formed for the rolling adhesion to be converted to a stationary one. Our findings suggest that activated integrins contribute to leukocyte arrest at sites of endothelial cell junctions and contribute to the mechanism for transendothelial migration. The neutrophil interaction with CD62 is highly reversible and should not impede subsequent migration mediated by leukocyte integrins, since interactions through the leukocyte integrins are much stronger.

The synergism between the selectin and leukocyte integrin/Ig pathways demonstrated in our flow system is qualitatively different from the additivity observed in static adhesion assays. Use of anti-ELAM-1 and anti-CD18 antibodies singly or in combination and anti-LECAM-1 and anti-CD18 antibodies singly or in combination had suggested that the selectin and integrin mechanisms, while distinct, functioned in an additive manner in neutrophil adhesion to cytokine-stimulated endothelial cells (Dobrina et al., 1989; Luscinskas et al., 1989; Smith et al., 1991; Hallmann et al., 1991). Similar studies on the relative contribution of CD62 and CD18 to binding of neutrophils to thrombin-stimulated endothelial cells have not been reported, but it can be inferred from the existence of both CD18-independent (presumably CD62) and CD18-dependent pathways in static assays (Zimmerman and McIntyre, 1988) that there would be additive effects of blocking both adhesion mechanisms. Under flow conditions, however, we observed no additivity between CD62- and CD18-

mediated mechanisms: no interaction was possible with ICAM-1 unless there was a rolling interaction with CD62. Our model of sequential interactions of rolling on CD62 followed by adhesion strengthening on ICAM-1 predicts that blocking interaction either through integrins or through selectins alone should completely block formation of firm adhesion in vivo, and is in agreement with observations that MAb to either integrins or a selectin (LECAM-1/LAM-1) can largely inhibit accumulation of leukocytes at inflammatory sites in vivo (Lewinsohn et al., 1987; Jutila et al., 1989; Arfors et al., 1987; Price et al., 1987).

The sequential steps of rolling on a selectin and adhesion strengthening through integrins elucidated here have important implications for the steps of leukocyte localization at inflammatory sites in vivo. Since rolling precedes firm sticking in vivo, and we have found that rolling is mediated by a selectin and is a prerequisite for subsequent adhesion strengthening through an integrin in vitro, we suggest that in vivo the initial accumulation of rolling leukocytes at an inflamed site must be regulated by selectins and by changes in vessel tone that lower fluid shear stresses to facilitate interactions through selectins. Small changes in vessel diameter and flow rate can result in significant reductions in wall shear stress. Stimulation of endothelial cells can induce expression of CD62 and later ELAM-1, and might also induce on these cells the expression of the ligand for LAM-1/LECAM-1. We found that rolling on CD62 by itself does not activate integrins on the neutrophil, but does permit adhesion strengthening through integrins if the neutrophil is activated. Chemoattractants that activate the avidity of integrins on neutrophils and monocytes are released at inflammatory sites. These include the complement component C5a, N-formylated bacterial peptides, platelet-derived growth factor, IL-8, and leukotriene B4. However, once these small molecules enter the bloodstream they are rapidly diluted and swept downstream, leading to the commonly held opinion that they cannot be responsible for regulating adhesion of leukocytes in postcapillary venules, and may only be effective once a neutrophil has undergone diapedesis. However, rolling in vivo will bring leukocytes into close proximity with chemoattractants that are diffusing from tissue through the junctions between endothelial cells, and to activating stimuli on the endothelial surface such as platelet-activating factor (Zimmerman et al., 1990), and rolling will greatly prolong the time period over which leukocytes are exposed to these stimuli. This promotes the activation of integrin avidity on the leukocyte that we have shown slows rolling; activation of LAM-1/LECAM-1 on the leukocyte (Spertini et al., 1991) might have a similar effect. The slowed rolling allows even more efficient exposure to chemostimulants and thereby sets in motion a positive feedback loop that results in arrest of the rolling leukocyte and finally spreading, integrin-mediated adhesion strengthening, and transendothelial migration.

In conclusion, we have shown that neutrophil rolling and subsequent arrest and adhesion strengthening at an inflammatory site in vivo can be accurately reproduced in vitro with a small number of purified components: a lipid bilayer, the selectin CD62, the Ig family member ICAM-1, and the chemostimulant fMLP. We predict that other types of selectins, integrin ligands, or chemostimulants will be found able to substitute for CD62, ICAM-1, or fMLP, respectively; nonetheless, the major point is that we have established the minimal molecular requirements for a complex physiological process. The small number of required components on the vessel wall demonstrates the simple elegance of this process and suggests how it may be refined by substitution or addition of other selectins or integrin ligands. Our studies illuminate the mechanisms by which adhesion molecule antibodies or analogs profoundly inhibit inflammatory responses in vivo, and suggest that antagonists of selectins or integrins may have similar biological effects despite distinct mechanisms of action.

Experimental Procedures

Monoclonal Antibodies

Monoclonal antibodies used in these studies as purified IgG were AC1.2 (anti-CD62, IgG1) (Larsen et al., 1989), R6.5 (anti-ICAM-1, IgG2a) (Smith et al., 1988), and TS1/22 (anti-CD11a, IgG1) (Sanchez-Madrid et al., 1982). They were used at 20 μg/ml for inhibition of neutrophil binding. LPM19c (anti-CD11b, IgG2a) (Uciechowski and Schmidt, 1989) was used as a 1:200 dilution of ascites fluid.

Purification of ICAM-1 and CD62

ICAM-1 was affinity purified from the Epstein–Barr virus-transformed B lymphoblastoid JY cell clone 33 (Hollander et al., 1988), as previously described (Marlin and Springer, 1987). Briefly, a Triton X-100 lysate was passed over a RR1/1 Sepharose column, and the column was eluted with a buffer containing 1% OG so that ICAM-1 could be incorporated into liposomes. CD62 was a generous gift of Drs. S. Sajer and B. Furie and was purified as previously described (Larsen et al., 1989).

Preparation of Liposomes

Liposomes were prepared by the method of OG dialysis (Mimms et al., 1981) with slight modification. Egg phosphatidylcholine (Avanti, AL) was diluted in chloroform and dried under an argon stream and then placed under a vacuum (30 μm Hg) for 2 hr to remove residual chloroform. The lipid film was redissolved at 0.4 mM in 250 μl of 25 mM Tris–HCl (pH 8.0), 150 mM NaCl (TS), 2% (w/v) OG, and was mixed with 250 μl of detergent solution containing approximately 1–18 μg/ml CD62, 6–30 μg/ml ICAM-1, or both in TS, 1% OG, followed by three changes of dialysis against TS at 4°C over 36 hr. After the removal of OG by dialysis, the liposome suspension was stored at 4°C under argon to minimize oxidation of lipids.

Preparation of Planar Bilayers

Planar bilayers were formed by incubating drops of liposome suspension on glass coverslips or slides at 22°C for 30 min. Prior to use, all glass surfaces were boiled in detergent (Linbro 7 × solution, Flow Lab, McLean, VA) for 15 min, rinsed extensively in deionized distilled water for at least 24 hr, and then stored in ethanol. For site-number determinations, glass coverslips (5 mm diameter, no. 1 thickness; Bellco, Vineland, NJ) were attached to the bottom of 96-well microtiter plates (Linbro Titertek, Flow Lab, McLean, VA) by silicone household glue (General Electric Co., Waterford, NY), and liposome drops (20 μl) were placed on top. For laminar flow assays, glass slides (45 × 60 mm, no. 2 thickness; Corning Glass Works, Corning, VA) were placed in 10 cm petri dishes. A liposome drop (20 μl) was placed in a demarcated area (1 cm diameter) and a glass coverslip (1 cm diameter; Bellco, Vineland, NJ) was then used to spread the liposome droplet. After the planar bilayers were formed, excess liposomes were removed by several changes of binding medium (DMEM, 10% FCS, 25 mM HEPES [pH 7.4]). Planar bilayers were never exposed to air.

Determination of Site Densities

Liposomes were reconstituted with different quantities of immunoaffinity-purified ICAM-1 or CD62, and planar membranes were formed

as described above. MAbs R6.5 to ICAM-1 (Smith et al., 1988) and AC1.2 to CD62 (Larsen et al., 1989) were iodinated to a known specific activity of about 70 μCi/μg, and site densities of ICAM-1 and CD62 were determined by saturation binding as previously described (Dustin and Springer, 1988). Site numbers assume binding of one IgG molecule per antigen molecule because saturation binding favors monomeric binding and because transmembrane proteins are immobile on glass-supported bilayers (McConnell et al., 1986) and at the highest density were on average too far apart (32 nm) for bivalent binding. After initial measurements of bilayer incorporation, protein concentrations were adjusted to give round numbers of sites per μm^2. The actual site densities were determined twice for each liposome preparation at each density, in triplicate. The round values of sites per μm^2 mentioned in Results and actual values ± SD compared are as follows: ICAM-1 1000: 950 ± 95; ICAM-1 250: 272 ± 16; CD62 400: 389 ± 62; CD62 200: 207 ± 41; CD62 50: 64 ± 12; CD62 25: 34 ± 7. Incorporation of both CD62 and ICAM-1 did not affect the efficiency of incorporation compared with either alone.

Isolation of Polymorphonuclear Leukocytes

Neutrophils were isolated from citrate-anticoagulated whole blood following dextran sedimentation and density separation over Ficoll-Hypaque (Miller et al., 1987). Following isolation, neutrophils were stored in Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY) supplemented with 10 mM HEPES at pH 7.3 and human serum albumin (0.1%) at room temperature for up to 6 hr. Before use in experiments, the neutrophils were washed into HBSS supplemented with 10 mM HEPES, 1.0 mM Mg^{2+}, and 1.2 mM Ca^{2+} at pH 7.3, since CD18 interactions with ICAM-1 require divalent cations (Marlin and Springer, 1987), as does the CD62 interaction with its counterstructure (Geng et al., 1990).

Laminar Flow Assays

A glass slide containing a planar bilayer was assembled in a parallel-plate laminar flow chamber (260 μm gap thickness) in which a uniform wall shear stress is generated. The flow chamber was mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD, Nikon Inc., Garden City, NY). For continuous flow assays, neutrophils were resuspended at a concentration of 10^6/ml in HBSS supplemented with 10 mM HEPES, 1.2 mM Ca^{2+}, and 1.0 mM Mg^{2+} and drawn through the chamber at controlled flow rates with a syringe pump attached to the outlet. The wall shear stress was calculated from a momentum balance on a Newtonian fluid, assuming a viscosity of 1.0 cP. The flow rate was stepped down to allow measurements of cell binding at different shear stresses. Three minutes was allowed for equilibration before the number of cells per unit area was measured. Measurements on different areas of the bilayer were averaged, and no evidence for nonuniformity was found. Attached neutrophils and their motion were observed with phase-contrast objectives and quantitated by analysis of videotaped images. For activation studies, neutrophils were treated with PMA (30 ng/ml, final concentration) for 5 min before perfusing the cell suspension through the flow chamber.

Detachment Assays

For detachment assays, neutrophils (4×10^6/ml) were injected into the chamber through a port and allowed to settle. To determine the effect of CD18 up-regulation, PMA was added to the neutrophils 1 min before injection into the flow chamber. All cells came in contact with the bilayer within 120 s, as indicated by their entry into the same focal plane. Controlled flow was applied following a 6 min incubation period. The initial shear force was 0.5 dyn/cm^2; this force was increased every 20 s to a maximum of 36 dyn/cm^2. All experiments were recorded on videotapes, and multiple fields of view were examined for each data point.

Analysis of Neutrophil Rolling

Rolling velocities were measured for all cells in two to five fields of view for each experiment at a given shear stress or ligand density. Results are presented as averages from experiments on different days. Velocities on CD62 were comparable whether cells were bound during shear flow or bound at stasis and then subjected to shear flow. Images were recorded on a time-lapse videocassette recorder at real time and played back at 6- or 9-fold slower speed. The tape was paused to mark the location of cells, and the displacement of the center of individual cells was measured 2 to 4 s later. In experiments in which the effect of chemotactic factors on neutrophil rolling was measured, cells were first allowed to form rolling attachments to the bilayer at the indicated shear stress. Flow at the same rate was continued with medium lacking cells for 3 min, followed by addition of fMLP (1×10^{-9} M, final concentration) to the perfusion medium without stopping flow.

Acknowledgments

We thank Drs. S. Sajer and B. Furie for the generous gift of 25 μg of purified PADGEM (CD62). This work was supported by National Institutes of Health grant CA 31799.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received April 15, 1991; revised May 1, 1991.

References

Anderson, D. C., and Springer, T. A. (1987). Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu. Rev. Med. *38*, 175–194.

Arfors, K.-E., Lundberg, C., Lindborm, L., Lundberg, K., Beatty, P. G., and Harlan, J. M. (1987). A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. Blood *69*, 338–340.

Atherton, A., and Born, G. V. R. (1972). Quantitative investigation of the adhesiveness of circulating polymorphonuclear leucocytes to blood vessel walls. J. Physiol. *222*, 447–474.

Atherton, A., and Born, G. V. R. (1973). Relationship between the velocity of rolling granulocytes and that of the blood flow in venules. J. Physiol. *233*, 157–165.

Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., and Seed, B. (1989). Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science *243*, 1160–1165.

Bonfanti, R., Furie, B. C., Furie, B., and Wagner, D. D. (1989). PADGEM (GMP140) is a component of Weibel–Palade bodies of human endothelial cells. Blood *73*, 1109–1112.

Buchanan, M. R., Crowley, C. A., Rosin, R. E., Gimbrone, M. A., and Babior, B. M. (1982). Studies on the interaction between GP-180-deficient neutrophils and vascular endothelium. Blood *60*, 160–165.

Buyon, J. P., Abramson, S. B., Philips, M. R., Slade, S. G., Ross, G. D., Weissman, G., and Winchester, R. J. (1988). Dissociation between increased surface expression of gp165/95 and homotypic neutrophil aggregation. J. Immunol. *140*, 3156–3160.

Chien, S. (1982). Rheology in the microcirculation in normal and low flow states. Adv. Shock Res. *8*, 71–80.

Cohnheim, J. (1889). Lectures on General Pathology: A Handbook for Practitioners and Students (London: The New Sydenham Society).

de Fougerolles, A. R., Stacker, S. A., Schwarting, R., and Springer, T. A. (1991). Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J. Exp. Med., in press.

Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacker, S.A., Garcia-Aguilar, J., Hibbs, M. L., and Springer, T. A. (1990). ICAM-1 (CD54)—a counter-receptor for Mac-1 (CD11b/CD18). J. Cell Biol. *111*, 3129–3139.

Dobrina, A., Schwartz, B. R., Carlos, T. M., Ochs, H. D., Beatty, P. G., and Harlan, J. M. (1989). CD11/CD18-independent neutrophil adherence to inducible endothelial-leucocyte adhesion molecules (E-LAM) in vitro. Immunology *67*, 502–508.

Dustin, M. L., and Springer, T. A. (1988). Lymphocyte function associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell Biol. *107*, 321–331.

Dustin, M. L., and Springer, T. A. (1989). T cell receptor cross-linking

transiently stimulates adhesiveness through LFA-1. Nature *341*, 619–624.

Fiebig, E., Ley, K., and Arfors, K.-E. (1991). Rapid leukocyte accumulation by "spontaneous" rolling and adhesion in the exteriorized rabbit mesentery. Int. J. Microcirc. Clin. Exp. *10*, 127–144.

Fukuda, M., Spooncer, E. S., Oates, J. E., Dell, A., and Klock, J. C. (1984). Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes. J. Biol. Chem. *259*, 10925–10935.

Geng, J. G., Bevilacqua, M. P., Moore, K. L., McIntyre, T. M., Prescott, S. M., Kim, J. M., Bliss, G. A., Zimmerman, G. A., and McEver, R. P. (1990). Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. Nature *343*, 757–760.

Goldman, A. J., Cox, R. G., and Brenner, H. (1967). Slow viscous motion of a sphere parallel to a plane wall—II. Couette flow. Chem. Eng. Sci. *22*, 653–660.

Goldsmith, H. L., and Spain, S. (1984). Margination of leukocytes in blood flow through small tubes. Microvasc. Res. *27*, 204–222.

Hallmann, R., Jutila, M. A., Smith, C. W., Anderson, D. C., Kishimoto, T. K., and Butcher, E. C. (1991). The peripheral lymph node homing receptor, LECAM-1, is involved in CD18-independent adhesion of human neutrophils to endothelium. Biochem. Biophys. Res. Commun. *174*, 236–243.

Harlan, J. M., Killen, P. D., Senecal, F. M., Schwartz, B. R., Yee, E. K., Taylor, R. F., Beatty, P. G., Price, T. H., and Ochs, H. D. (1985). The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium in vitro. Blood *66*, 167–178.

Hattori, R., Hamilton, K. K., Fugate, R. D., McEver, R. P., and Sims, P. J. (1989). Stimulation of secretion of endothelial cell von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. J. Biol. Chem. *264*, 7768–7771.

Heisig, N. (1968). Functional analysis of the microcirculation in the exocrine pancreas. Adv. Microcirc. *1*, 89–94.

Hollander, N., Selvaraj, P., and Springer, T. A. (1988). Biosynthesis and function of LFA-3 in human mutant cells deficient in phosphatidylinositol anchored proteins. J. Immunol. *141*, 4283–4290.

Jacobson, K., Ishihara, A., and Inman, R. (1987). Lateral diffusion of proteins in membranes. Annu. Rev. Physiol. *49*, 163–175.

Jutila, M. A., Rott, L., Berg, E. L., and Butcher, E. C. (1989). Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1. J. Immunol. *143*, 3318–3324.

Larsen, E., Celi, A., Gilbert, G. E., Furie, B. C., Erban, J. K., Bonfanti, R., Wagner, D. D., and Furie, B. (1989). PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell *59*, 305–312.

Larsen, E., Palabrica, T., Sajer, S., Gilbert, G. E., Wagner, D. D., Furie, B. C., and Furie, B. (1990). PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). Cell *63*, 467–474.

Larson, R. S., and Springer, T. A. (1990). Structure and function of leukocyte integrins. Immunol. Rev. *114*, 181–217.

Lawrence, M. B., McIntire, L. V., and Eskin, S. G. (1987). Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. Blood *70*, 1284–1290.

Lawrence, M. B., Smith, C. W., Eskin, S. G., and McIntire, L. V. (1990). Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. Blood *75*, 227–237.

Lewinsohn, D. M., Bargatze, R. F., and Butcher, E. C. (1987). Leukocyte–endothelial cell recognition: evidence of a common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. J. Immunol. *138*, 4313–4321.

Lo, S. K., Detmers, P. A., Levin, S. M., and Wright, S. D. (1989). Transient adhesion of neutrophils to endothelium. J. Exp. Med. *169*, 1779–1793.

Luscinskas, F. W., Brock, A. F., Arnaout, M. A., and Gimbrone, M.A. (1989). Endothelial–leukocyte adhesion molecule-1-dependent and leukocyte (CD11/CD18)-dependent mechanisms contribute to polymorphonuclear leukocyte adhesion to cytokine-activated human vascular endothelium. J. Immunol. *142*, 2257–2263.

Marchesi, V. T. (1961). The site of leucocyte emigration during inflammation. Q. J. Exp. Physiol. *46*, 115–133.

Marlin, S. D., and Springer, T. A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function–associated antigen 1 (LFA-1). Cell *51*, 813–819.

McConnell, H. M., Watts, T. H., Weis, R. M., and Brian, A. A. (1986). Supported planar membranes in studies of cell–cell recognition in the immune system. Biochim. Biophys. Acta *864*, 95–106.

McEver, R. P., Beckstead, J. H., Moore, K. L., Marshall-Carlson, L., and Bainton, D. F. (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel–Palade bodies. J. Clin. Invest. *84*, 92–99.

Miller, L. J., Bainton, D. F., Borregaard, N., and Springer, T. A. (1987). Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. J. Clin. Invest. *80*, 535–544.

Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981). Phosholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. Biochemistry *20*, 833–840.

Moore, K. L., Varki, A., and McEver, R. P. (1991). GMP-140 binds to a glycoprotein receptor on human neutrophils: evidence for a lectin-like interaction. J. Cell Biol. *112*, 491–499.

Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., and Yamada, K. M. (1988). Electron microscopy and structural model of human fibronectin receptor. EMBO J. *7*, 4093–4099.

Nobis, U., Pries, A. R., Cokelet, G. R., and Gaehtgens, P. (1985). Radial distribution of white cells during blood flow in small tubes. Microvasc. Res. *29*, 295–304.

Philips, M. R., Buyon, J. P., Winchester, R., Weissman, G., and Abramson, S. B. (1988). Up-regulation of the iC3b Receptor (CR3) is neither necessary nor sufficient to promote neutrophil aggregation. J. Clin. Invest. *82*, 495–501.

Picker, L. J., Kishimoto, T. K., Smith, C. W., Warnock, R. A., and Butcher, E. C. (1991). ELAM-1 is an adhesion molecule for skin-homing T cells. Nature *349*, 796–798.

Pober, J. S., and Cotran, R. S. (1990). The role of endothelial cells in inflammation. Transplantation *50*, 537–544.

Price, T. H., Beatty, P. G., and Corpuz, S. R. (1987). In vivo inhibition of neutrophil function in the rabbit using monoclonal antibody to CD18. J. Immunol. *139*, 4174–4177.

Sanchez-Madrid, F., Krensky, A. M., Ware, C. F., Robbins, E., Strominger, J. L., Burakoff, S. J., and Springer, T. A. (1982). Three distinct antigens associated with human T lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc. Natl. Acad. Sci. USA *79*, 7489–7493.

Segre, G., and Silberberg, A. (1962). Behavior of macroscopic rigid particles in Pouiseuille flow. II. Experimental results and interpretation. J. Fluid Mech. *14*, 136–157.

Shimizu, Y., Shaw, S., Graber, N., Gopal, T. V., Horgan, K. J., Van Seventer, G. A., and Newman, W. (1991). Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. Nature *349*, 799–802.

Smith, C. W., Rothlein, R., Hughes, B. J., Mariscalco, M. M., Schmalstieg, F. C., and Anderson, D. C. (1988). Recognition of an endothelial determinant for CD18-dependent neutrophil adherence and transendothelial migration. J. Clin. Invest. *82*, 1746–1756.

Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C., and Anderson, D. C. (1989). 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*, 2008–2017.

Smith, C. W., Kishimoto, T. K., Abbass, O., Hughes, B., Rothlein, R., McIntire, L. V., Butcher, E., and Anderson, D. C. (1991). Chemotactic factors regulate lectin adhesion molecule 1 (LECAM-1)-dependent neutrophil adhesion to cytokine-stimulated endothelial cells in vitro. J. Clin. Invest. *87*, 609–618.

Spertini, O., Kansas, G. S., Munro, J. M., Griffin, J. D., and Tedder, T. F. (1991). Regulation of leukocyte migration by activation of the leukocyte adhesion molecule (LAM-1) selectin. Nature *349*, 691–694.

Springer, T. A. (1990). Adhesion receptors of the immune system. Nature *346*, 425–433.

Springer, T. A., and Lasky, L. A. (1991). Sticky sugars for selectins. Nature *349*, 196–197.

Staunton, D. E., Dustin, M. L., and Springer, T. A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61–64.

Staunton, D. E., Dustin, M. L., Erickson, H. P., and Springer, T. A. (1990). The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. Cell *61*, 243–254.

Symington, F. W., Hedges, D. L., and Hakomori, S.-I. (1985). Glycolipid antigens of human polymorphonuclear neutrophils and the inducible HL-60 myeloid leukemia line. J. Immunol. *134*, 2498–2506.

Tanford, C. (1961). Physical Chemistry of Macromolecules (New York: John Wiley and Sons, Inc.).

Uciechowski, P., and Schmidt, R. E. (1989). NK and non-lineage antigens—cluster report: CD11. In Leucocyte Typing IV: White Cell Differentiation Antigens, W. Knapp, ed. (Oxford: Oxford University Press), pp. 543–551.

Vedder, N. B., and Harlan, J. M. (1988). Increased surface expression of CD11b/CD18 (Mac-1) is not required for stimulated neutrophil adherence to cultured endothelium. J. Clin. Invest. *81*, 676–682.

Watson, S. R., Fennie, C., and Lasky, L. A. (1991). Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor–IgG chimaera. Nature *349*, 164–167.

Watts, T. H., Gaub, H. E., and McConnell, H. M. (1986). T-cell-mediated association of peptide antigen and major histocompatibility complex protein detected by energy transfer in an evanescent wavefield. Nature *320*, 179–181.

Weisman, H. F., Bartow, T., Leppo, M. K., Marsh, H. C., Jr., Carson, G. R., Concino, M. F., Boyle, M. P., Roux, K. H., Weisfeldt, M. L., and Fearon, D. T. (1990). Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. Science *249*, 146–151.

Zimmerman, G. A., and McIntyre, T. M. (1988). Neutrophil adherence to human endothelium in vitro occurs by CDw18 (Mo1, Mac-1/LFA-1/GP 150,95) glycoprotein-dependent and -independent mechanisms. J. Clin. Invest. *81*, 531–537.

Zimmerman, G. A., McIntyre, T. M., Mehra, M., and Prescott, S. M. (1990). Endothelial cell-associated platelet-activating factor: a novel mechanism for signaling intercellular adhesion. J. Cell Biol. *110*, 529–540.