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P-Selectin Mediates Neutrophil Rolling on Histamine-Stimulated Endothelial Cells

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ABSTRACT In postcapillary venules, marginating neutrophils (PMNs) are often seen rolling along the vessel wall prior to stopping and emigrating. There is substantial evidence in vitro and in vivo that the adhesion receptors E- and L-selectin participate in this phenomenon on cytokine-stimulated endothelium, and recent evidence has shown that a closely related adhesion receptor, P-selectin, is capable of mediating neutrophil rolling on an artificial membrane. Here we demonstrate and characterize PMN rolling on monolayers of human umbilical vein endothelial cells (HUVECs) stimulated with histamine to induce surface expression of P-selectin. Peak association of PMNs with the HUVECs occurs 10 min after histamine stimulation, and at a postcapillary venular wall shear stress of 2.0 dyn/cm^2 the rolling velocity is 14 μm/s. Approximately 95% of the PMNs roll on the endothelial cells, 5% adhere firmly, and none migrate beneath the endothelial monolayer. Monoclonal antibody (MAb) G1, which binds P-selectin and blocks its adhesive function, completely prevents association of the PMNs with histamine-stimulated HUVEC, whereas the nonblocking anti-P-selectin MAb S12 does not. Treatment of PMNs with the anti-L-selectin MAb DREG56 reduces PMN adherence by approximately 50%. Anti-CD54 MAb R6.5 and anti-CD18 MAb R15.7 have little effect on the number of PMNs rolling on the HUVECs but completely prevent PMNs from stopping and significantly increase rolling velocity. Nonblocking control MAbs for R6.5 (CL203) and R15.7 (CL18/1D1) lack these effects. Rolling adhesion of PMNs on histamine-stimulated HUVECs therefore appears to be completely dependent on endothelial cell P-selectin, with a minor adhesion-stabilizing contribution from intercellular adhesion molecule 1 and β_2 integrins. The partial inhibition of rolling with DREG56 suggests that L-selectin may also play a role in neutrophil interactions with histamine-stimulated endothelium. We further characterize these interactions by determining the effects of the various MAbs and wall shear stresses on adhesion patterns, rolling velocities, and distributions of rolling velocities.

INTRODUCTION

The inflammatory response is a multistep process involving changes in vascular permeability, changes in expression of endothelial cell adhesion receptors, and the triggering of adherent leukocytes to emigrate into the tissues at the site of inflammation. It is becoming clear that several types of adhesion receptors are involved in binding and transmigration and that their expression is tightly regulated to produce the sequence of events that leads to leukocyte extravasation. These events are coordinated by a wide variety of inflammatory mediators, including histamine, thrombin, bradykinin, interleukin 1 (IL-1), tumor necrosis factor α, and lipopolysaccharide. These mediators signal the endothelium to alter its extensive control over vascular permeability, hemostasis, and leukocyte recruitment. The process in general begins with an increase in vascular permeability, which alters the characteristics of the flow profile through the vessel (1). There are also changes in the expression of endothelial adhesion receptors and in the production of endothelial-derived inflammatory regulating substances (2). The first adhesive interaction observed is the rolling of neutrophils along the endothelium (3, 4) with a much slower velocity than the tumbling of neutrophils in the fluid stream (5, 6). Rolling neutrophils may then either adhere firmly or dissociate from the endothelium and re-enter the fluid stream. Finally, if appropriate activating and chemoattracting substances are present, leukocytes that adhere firmly can flatten against the vessel wall, migrate to interendothelial junctions, and diapedese into the extravascular space.

The details of these adhesive interactions at the molecular level are currently of great research interest, both in terms of the importance to pathophysiology and implications for therapeutic development. Previous work has led to the elucidation of a multistep pathway for neutrophil binding and emigration through cytokine-stimulated endothelial monolayers. Although this pathway is different from the case of histamine-stimulated endothelial monolayers, we will describe it here to introduce the various receptors involved. In response to IL-1, tumor necrosis factor α, or lipopolysaccharide stimulation, endothelial cells express E-selectin (ELAM-1) with maximum expression occurring around 3–4 h (7). E-selectin, along with L-selectin (LAM-1, LECCAM-1, the human homologue of the murine MEL-14 antigen gp90^{Mel14}) and P-selectin (CD62, GMP-140, PADGEM) form the selectin family of adhesion receptors (reviewed in Refs. 8 and 9). This classification is based on the common structural motifs of an NH_2-terminal lectinlike domain followed by an epidermal growth factor-like domain and a series of consensus repeats related to those in

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complement-binding proteins, coupled to a transmembrane domain and a cytoplasmic tail. E-selectin is known to bind directly to sialyl Lewis X (sLe^X, CD15) moieties on neutrophil L-selectin, and it is likely that both molecules bind additional undetermined counter-receptors (10, 11). This selectin-mediated binding appears to underly the neutrophil rolling interaction (12, 13), as seen with flow studies in vitro (11, 14, 15) and experiments in vivo (16–18). Expression of intercellular adhesion molecule 1 (ICAM-1) is also induced on cytokine-stimulated endothelium (19, 20), and the interaction of this receptor with β_2 integrins (CD11a/CD18, LFA-1 and CD11b/CD18, Mac-1) (21, 22) on the neutrophil produces a much more shear-resistant, firm adhesion (14, 23). Integrin binding alone is not effective in the initial arrest of neutrophils from the bloodstream, however (14, 18, 23–26). Slowing of the neutrophils by selectin-mediated rolling is necessary for efficient recruitment, and it appears that the reason for this is that at some point during their initial interaction with the vessel wall, neutrophils become activated by a substance or substances produced by the endothelial cells. Evidence suggests that IL-8 is a major factor in this step (27–29) and that endothelial cell surface platelet activating factor (PAF) may also play a role (29, 30). Activation concurrently causes rapid proteolytic shedding of L-selectin from the neutrophil surface (31, 32), mobilization of Mac-1 from intracellular stores (33), and modulation of leukocyte integrins to a high-avidity state (34). This combination of events apparently facilitates subsequent integrin-dependent migration of neutrophils to interendothelial junctions and transmigration.

The present study provides in vitro evidence for another pathway of neutrophil adhesion—in this case to endothelium activated with the rapid-acting inflammatory mediator histamine. Rapid-acting inflammatory mediators such as histamine, thrombin, and bradykinin produce a variety of effects related to hemostasis, and in the case of histamine, vasodilation and an increase in vascular permeability are especially prominent. Histamine also causes the rapid mobilization of P-selectin from Weibel-Palade bodies to the surface of endothelial cells. This mobilization begins in seconds, there is a maximum in expression occurring around 6–10 min, and endocytosis depletes most surface receptors by 30 min (35). Thrombin and bradykinin also induce the mobilization of P-selectin, but these also cause considerable endothelial cell retraction, which complicates the interpretation of in vitro leukocyte adhesion. Earlier studies of P-selectin reconstituted in artificial lipid bilayers have shown that neutrophils will roll on the bilayers at physiological wall shear stresses (23), and we here demonstrate that histamine-stimulated human umbilical vein endothelial cells (HUVECs) support neutrophil rolling. We also characterize the contributions of various adhesion receptors to this interaction with monoclonal antibody blocking studies and characterize the effects of the antibodies and various wall shear stresses on adhesion patterns, rolling velocities, and distributions of rolling velocities.

MATERIALS AND METHODS

Isolation of polymorphonuclear leukocytes

Neutrophils are obtained from citrate-anticoagulated (citrate phosphate dextrose solution, 1.4 ml/10 ml blood; Abbott Laboratories, North Chicago, IL) venous blood samples by centrifugation through a PolyMorphPrep (Nycomed Pharma AS, Oslo, Norway) one-step Ficoll-Hypaque gradient. Approximately 20 normal healthy adults contributed blood samples to the study. The neutrophils are suspended in modified Hanks' buffered saline solution (Sigma Chemical Co., St. Louis, MO) lacking calcium and magnesium and stored with occasional stirring at room temperature, at a concentration of 10^7 cells/ml for up to 3 h until used in the flow experiments. The isolated cells are >99% viable as determined by trypan blue exclusion assay but slowly become activated, necessitating use within 3 h. Polymorphonuclear neutrophils (PMNs) are clearly visible with phase-contrast microscopy, so that no fluorescent labeling was necessary.

Tissue culture of HUVECs

Cells are isolated by collagenase treatment according to established techniques (36). Cells from five to eight umbilical cords are pooled and plated on fibronectin-coated (1 ml of 5 μg/ml human plasma fibronectin for 30 min; Gibco Laboratories, Grand Island, NY) 35-mm-diameter tissue culture dishes (Corning Glass Works, Corning, NY) at sufficiently high density to form a confluent monolayer without cell division. Monolayers are cultured in M199 (Gibco Laboratories) supplemented with 15% fetal bovine serum (Gibco defined fetal bovine serum), hydrocortisone (1 μg/ml; Sigma), low-molecular-weight heparin (1 μg/ml; Sigma), gentamicin (25 μg/ml; Sigma), and amphotericin B (1.25 μg/ml as Fungizone; Gibco). No growth factors are used. Cultures are maintained for 3–5 days at 37°C in a humidified atmosphere with 5% CO_2.

Adherence assay under flow conditions

Physiological flow conditions are produced in vitro using a flow chamber with parallel-plate geometry as described previously (26). Briefly, the chamber produces a well-defined laminar flow over monolayers grown in 35-mm tissue culture dishes. Shear rates and the corresponding wall shear stresses can be calculated from the chamber geometry and volumetric flow rate as described previously (26). The isolated neutrophils are diluted to 10^6 cells/ml in Hanks' buffered saline solution (with calcium and magnesium) and perfused through the chamber at a rate that produces the desired wall shear stress. Stimuli such as histamine (10^{-5} M final concentration; Sigma) or thrombin (2 units/ml final concentration; Calbiochem, La Jolla, CA) are normally added directly to this feed solution so that HUVECs are stimulated for the duration of the 20-min experiments. For a small number of experiments, endothelial cells were exposed to a 2-min pulse of histamine, which was washed out before the neutrophils were added. These experiments serve as one control for the continuous exposure of neutrophils to histamine. Interactions between neutrophils and the endothelial monolayer are observed by phase-contrast video microscopy (Diaphot-TMD microscope, Nikon, Inc., Garden City, NY; Panasonic WV-BL200 video camera, Yokohama, Japan) and quantified with a digital image processing system (Sun Microsystems, Mountainview, CA; Inovision, Inc., Durham, NC). The flow system is maintained at 37°C in a warm air box surrounding the microscope.

Quantitation of adherence

A computer-controlled stage drive is used in conjunction with the image-processing system to cycle through a set of fields of view in the flow channels, typically acquiring images for three fields of view each minute. Rolling and firmly adherent neutrophils are counted for each of the three fields and averaged to give the values at that time point. Rolling and firmly adherent neutrophils are differentiated by acquiring two images for each field (see Fig. 1). The first image is a "snapshot" that shows both rolling and firmly

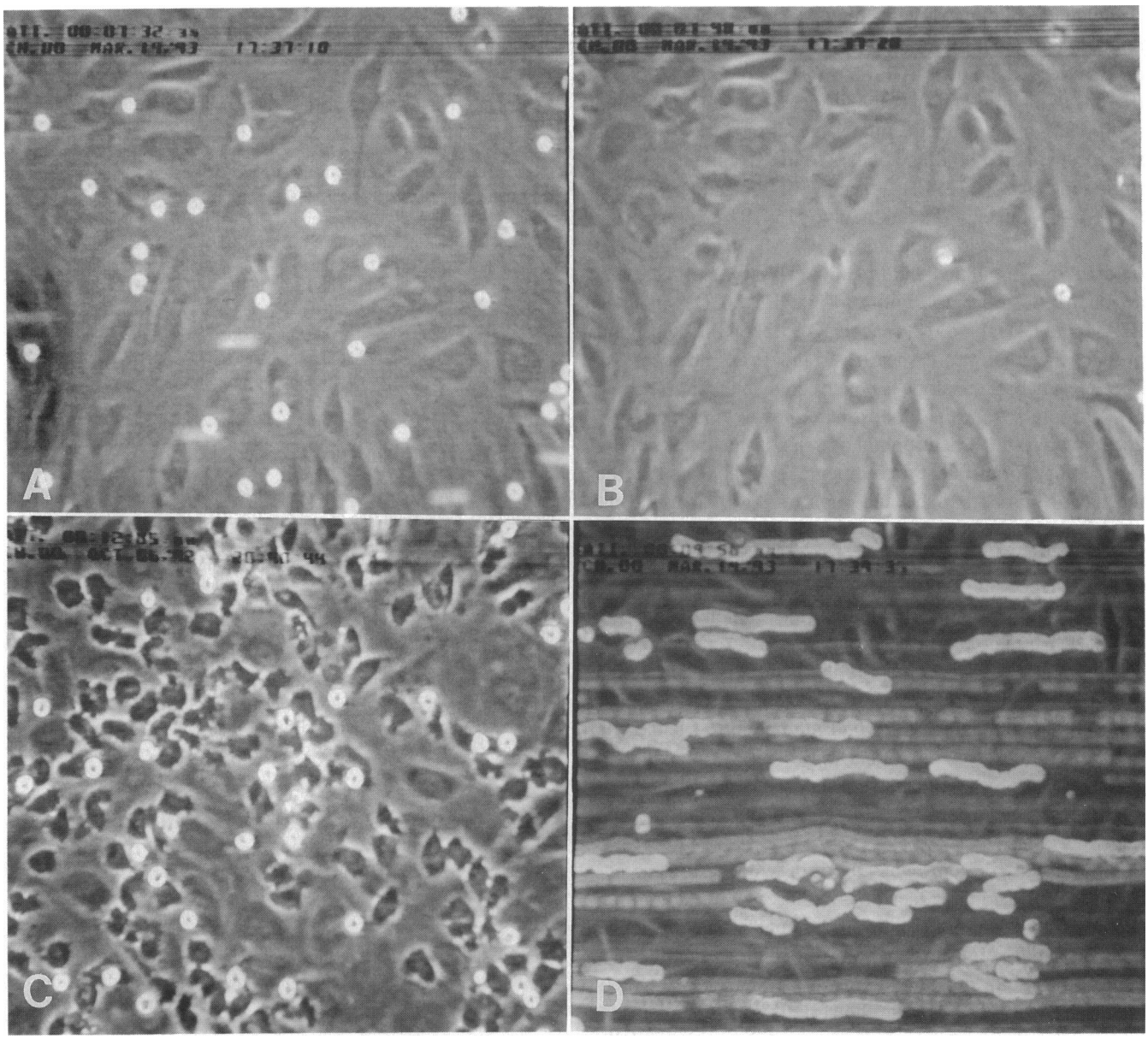

FIGURE 1 Example images acquired using our flow system. (*A* and *C*) "Snapshots" of the adhesion of neutrophils to HUVECs stimulated with histamine (10 min) or IL-1 (4 h), respectively. Both images were acquired approximately 10 min after introduction of neutrophils into the flow (wall shear stress of 2 dyn/cm^2). With histamine stimulation, most neutrophils roll along the endothelium and do not adhere firmly or transmigrate, whereas with IL-1 stimulation many diapedesed neutrophils (*phase-dark patches*) are seen. (*B*) 3-s minimization image showing that very few of the neutrophils shown in (*A*) are firmly adherent. (*D*) 4-s maximization image used to quantitate the rolling velocity of the neutrophils.

adherent neutrophils. Neutrophils flowing by without contacting the endothelium are out of focus and move quickly enough that they do not appear in this image. The second image is a 3-s minimization image. In this image, frames are acquired at the rate of 32 frames/s, but pixel intensities from each frame are stored in the final image only if the intensity at a particular pixel is lower than the previously stored intensity. Since neutrophils appear as phase-bright objects on a darker endothelial cell background, if a neutrophil moves, the pixel intensities at its previous location are replaced by the endothelial cell background of lower intensity, while pixels at its new location retain the lower background intensity. The result is that only neutrophils that do not move during the 3-s acquisition appear in this image, giving the number of firmly adherent neutrophils. Firm adhesion is therefore defined for this system as remaining stationary for at least 3 s. Subtraction of the number of firmly adherent neutrophils from the number of neutrophils appearing in the snapshot image then gives the number of rolling neutrophils.

Rolling velocities are quantified by acquiring 4-s maximization images (see Fig. 1). In these images, frames are acquired at the rate of 32 frames/s, and the maximum intensity which appears at each pixel over the 4-s acquisition is stored to form the final image. The result of this procedure is that rolling phase-bright neutrophils produce long blurs in these images. The length of each blur is measured and divided by the time of acquisition to give the rolling velocity for each neutrophil in the field of view. Rolling velocities are determined using fields of view at or near the time of maximum adhesion. Data from $n = 6$ experiments for each condition are pooled to give measurements for approximately 100–300 neutrophils. Those neutrophils that remain stationary for the 4 s of acquisition (typically approximately 5%) are excluded, since this population of neutrophils may be activated and may be utilizing different binding mechanisms. Also, neutrophils that either begin rolling on the endothelium or re-enter the free stream during the acquisition period are excluded. The blurs made by these neutrophils are readily distinguished by their long "tails" of diminishing intensity. Rolling

velocity distributions for each data set were calculated using SAS statistical analysis software (SAS Institute, Inc., Cary, NC). The frequencies for each rolling velocity range were determined and expressed as percentages to normalize for differences in actual numbers of neutrophils measured for each condition. Summary statistics for the distributions were also calculated and are given in the tables.

Monoclonal antibodies

All monoclonal antibodies were used as purified IgG. Monoclonal antibody (MAb) G1 binds to P-selectin and is known to block receptor-binding function. MAb S12 also binds P-selectin but does not block binding function (37, 38). MAb R6.5 was provided by Dr. Robert Rothlein of Boeringher-Ingleheim and binds ICAM-1 at an epitope that blocks integrin binding (20). CL203 binds the fifth immunoglobulin domain of ICAM-1 and does not block integrin binding. MAb R15.7 binds CD18 and is known to block the binding function of β_2 integrins in diapedesis assays (39). MAb DREG56 binds to L-selectin and inhibits this molecule's binding fuction (40). We attempted to use the nonblocking anti-L-selectin MAb LAM1.14 provided by Dr. Thomas Tedder as a control MAb but found that this activated the neutrophils extensively. CL18/1D8 is a nonbinding MAb used as a control (41).

MAbs are added to the neutrophil suspension and perfused over the endothelial cell monolayer at a concentration of 20 μg/ml for the duration of the experiment. G1 and S12 had been shown previously to completely saturate P-selectin at a concentration of 2 μg/ml, so this concentration was used for G1 and S12. Preincubation of cells with MAbs for 30 min gave the same results in all cases as simply adding the MAbs directly to the feed solution, so for most experiments cells were not preincubated with MAbs.

RESULTS

Neutrophils roll on histamine-stimulated HUVECs

Fig. 1 shows some example images acquired using our system. Fig. 1 (*A*, *B*, and *D*) shows that in general, many neutrophils roll on histamine-stimulated HUVECs while very few adhere firmly. Morphologically, rolling PMNs are spherical, whereas many of those that are firmly adherent are less regular in shape, having spread out somewhat on the endothelial cells. Fig. 2 shows the time course of neutrophil adhesion to histamine-stimulated HUVECs at a wall shear stress of 2.0 dyn/cm^2. As discussed in Materials and Methods, firmly adherent neutrophils are defined as those neutrophils that remain stationary for a period of at least 3 s. Thrombin stimulation produced very similar results (data not shown). An important feature of this neutrophil adhesion is that none of the firmly adherent neutrophils migrated through the monolayer. This is in sharp contrast to experiments using IL-1β-stimulated HUVECs (2 units/ml, 4 h), where most of the firmly adherent neutrophils eventually migrate beneath the monolayer (Fig. 1 *C*) (14, 15, 42, 43).

To control for the fact that neutrophils are normally exposed to histamine for the duration of the 20-min experiments, several experiments were performed using only a 2-min pulse of histamine to stimulate the endothelial cells. Results of these experiments were qualitatively very similar, although peak adhesion was slightly lower, and adhesion peaked and began to decrease somewhat sooner with the pulse stimulation. In other experiments to test the effects of histamine on neutrophils, we examined the activation of neutrophils in the presence and absence of histamine using previously published flow cytometry techniques (33). As indicators of activation, we measured the up-regulation of surface CD18 expression and the down-regulation of surface L-selectin. These experiments showed that 10^{-5} M histamine does not itself activate neutrophils and only slightly inhibits the ability of neutrophils to be stimulated with 10^{-8} M *N*-formyl methionyl leucyl phenylalanine (fMLP). Histamine reduced the ability of neutrophils to modulate these surface antigens by approximately 10–15%.

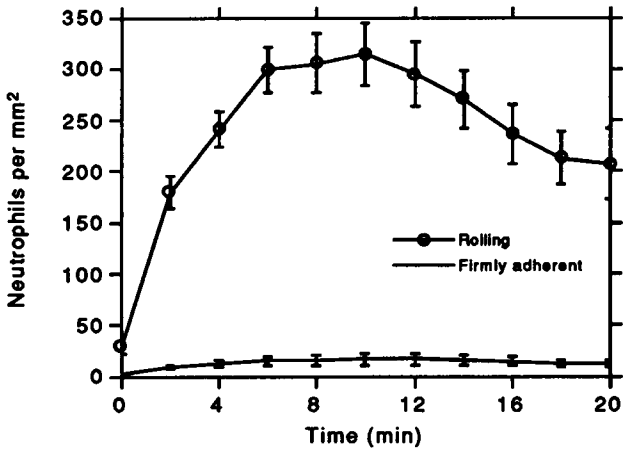

FIGURE 2 Time course of neutrophil adhesion to histamine-stimulated HUVECs under flow conditions. HUVECs are exposed to a suspension of 10^6 neutrophils/ml with 10^{-5} M histamine at a wall shear stress of 2.0 dyn/cm^2. Firmly adherent neutrophils are defined as those that remain stationary for at least 3 s. Error bars represent mean ± SEM for $n = 6$ experiments.

Monoclonal antibody blocking experiments

Fig. 3 shows the effects of various MAbs on neutrophil adhesion to histamine-stimulated HUVECs at a wall shear stress of 2.0 dyn/cm^2. The adherence plotted is the maximum adherence during the 20-min experiments, which was normally observed at approximately 10 min.

Fig. 4 shows the effect of monoclonal antibodies on firm adhesion. These data are the same as those shown in Fig. 3, plotted with an expanded scale. The decreases in firmly adherent neutrophils with R6.5 anti-ICAM-1 MAb and R15.7 anti-CD18 MAb are statistically significant ($p < 0.03$) decreases relative to histamine-stimulated HUVECs without MAbs.

Fig. 5 shows the effects of the MAbs on average neutrophil rolling velocities. All rolling velocity measurements were made at or near the time of peak adherence. Fig. 6 shows the effect of the various MAb treatments on the distribution of neutrophil rolling velocities, and Table 1 presents some summary statistics for these distributions.

Shear stress effects

Fig. 7 shows the variation with wall shear stress of neutrophil adhesion and rolling velocity on histamine-stimulated

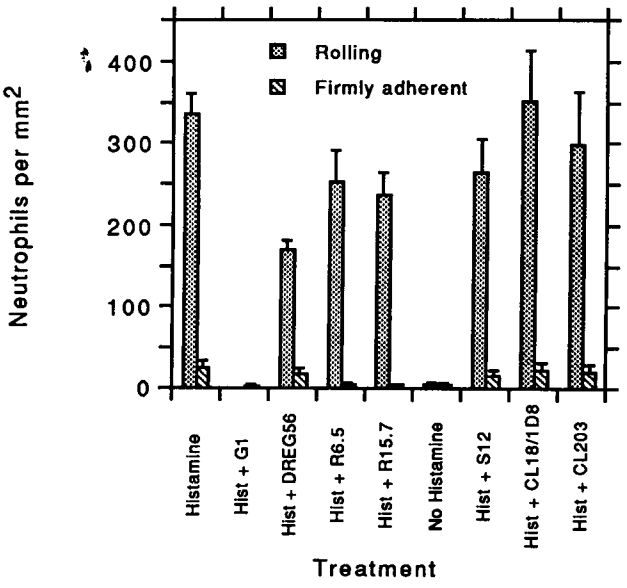

FIGURE 3 Effects of MAbs on neutrophil adhesion to histamine-stimulated HUVECs under flow conditions (wall shear stress = 2.0 dyn/cm^2). MAb specificities are as follows: G1 binds P-selectin and blocks receptor binding function; S12 binds P-selectin but does not block binding function; DREG56 binds L-selectin and blocks ligand binding function; R6.5 binds ICAM-1 and blocks receptor binding; R15.7 binds CD18 and blocks ligand binding. CL18/1D8 is a nonbinding control MAb. Error bars represent mean ± SEM for n = 6 experiments.

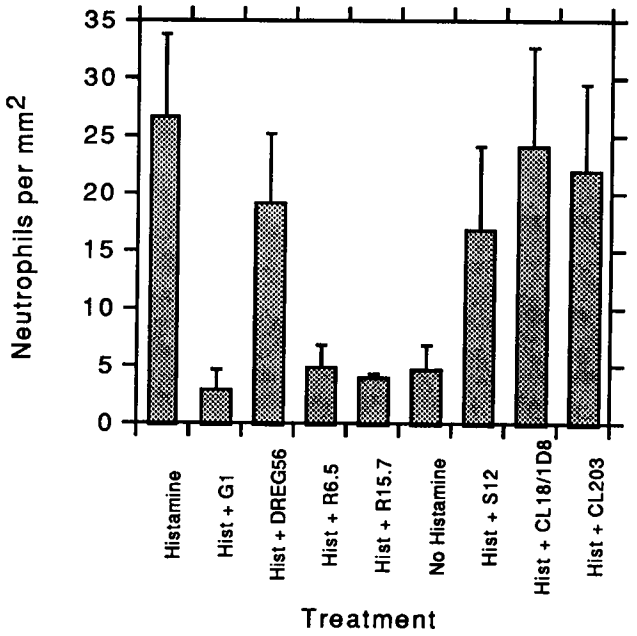

FIGURE 4 Effects of MAbs on firm adhesion of neutrophils to histamine-stimulated HUVECs. Data is an expanded-scale plot of the firm adhesion data in Fig. 3.

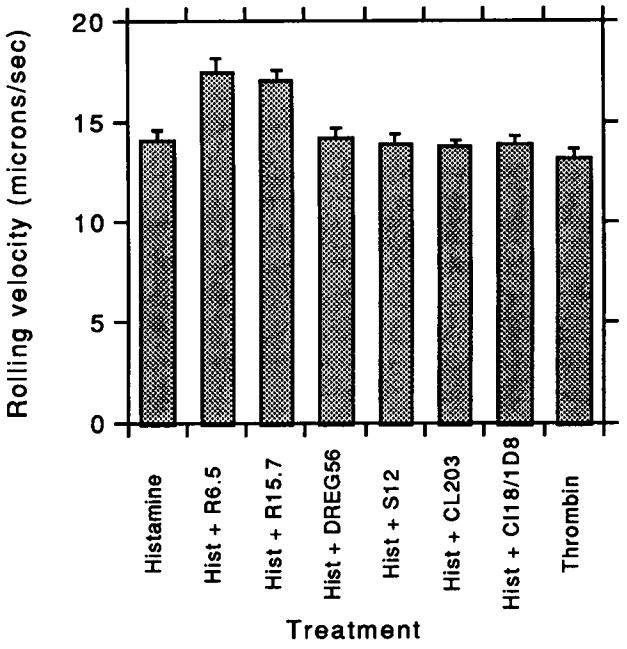

FIGURE 5 Effects of MAbs on average neutrophil rolling velocity over histamine-stimulated HUVECs at a wall shear stress of 2.0 dyn/cm^2.

HUVECs. Fig. 8 shows the effect of varying wall shear stress on the distribution of neutrophil rolling velocities and Table 2 presents some summary statistics for these distributions.

DISCUSSION

This work demonstrates the contributions of P-selectin, β_2 integrins, ICAM-1, and L-selectin to neutrophil adhesion to histamine-stimulated HUVECs under flow conditions. At 2.0 dyn/cm^2 wall shear stress, neutrophils roll on histamine-stimulated HUVECs with a speed of 14 μm/s. This rolling interaction is dependent on endothelial cell expression of P-selectin, as suggested by the time course of neutrophil rolling and as demonstrated by MAb blocking experiments. The rapid peak and short time course of neutrophil binding closely correlate with the time course of P-selectin expression (35), whereas the time courses of expression of the adhesion molecules E-selectin (7), ICAM-1 (19, 44), and vascular cell adhesion molecule 1 (VCAM-1) (45) do not correlate. More convincingly, the P-selectin-specific MAb G1 blocks essentially 100% of neutrophil adhesion, whereas the P-selectin-specific but nonblocking control MAb S12 does not. MAb S12 does reduce adhesion slightly; this is presumably due to minor steric interference even though the MAb does not bind directly to the receptor binding site. Surface expression of PAF by endothelial cells has been shown to occur rapidly upon histamine stimulation (46, 47), although we did not measure PAF levels under our own tissue culture conditions. If PAF is expressed in our system, it does not appear to activate the rolling neutrophils extensively, and since adhesion can be completely blocked by anti-P-selectin MAb, it is also not likely that PAF is contributing as an adhesive receptor. Under conditions where extensive firm adherence does occur, however, PAF might carry out a signaling function between endothelial cells and neutrophils.

The contribution of constitutively expressed ICAM-1 appears to be a small adhesion-stabilizing effect. Our results show that neutrophil rolling velocities are significantly

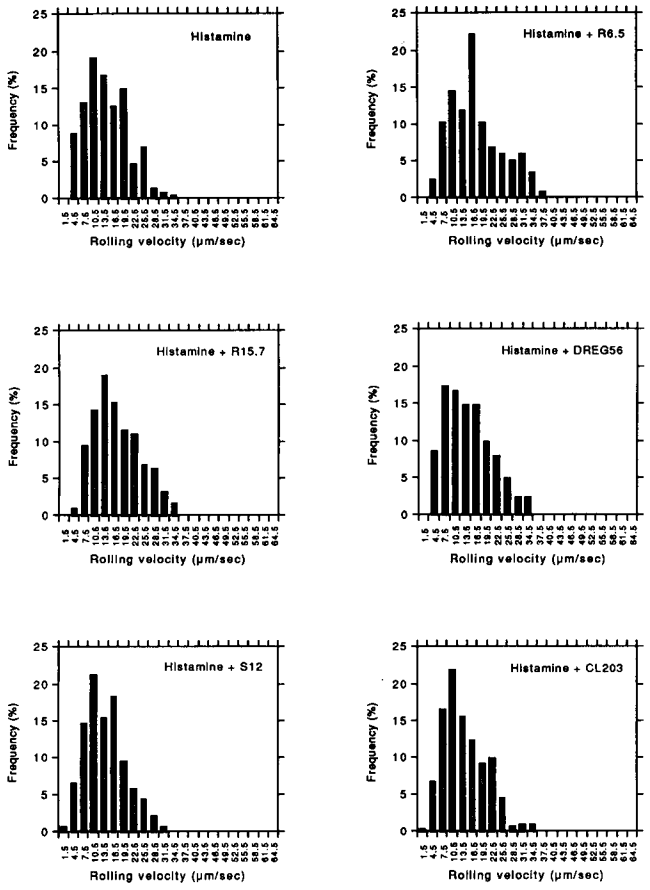

FIGURE 6 Distributions of neutrophil rolling velocities over histamine-stimulated HUVECs at 2.0 dyn/cm^2. Frequencies are expressed as percentages of the total number of neutrophils observed. Each plot corresponds to the same MAb treatments shown in Figs. 2–5; treatments are indicated on each plot. The plots for thrombin treatment and MAb CL18/1D1 are not shown but were similar.

higher in the presence of R6.5 anti-ICAM-1 antibody or R15.7 anti-CD18 antibody (Fig. 5) and that firm adhesion (defined as remaining stationary for at least 3 s) can be inhibited to control levels by R6.5 or R15.7 (Fig. 4). One possible explanation for the latter finding is that the combination of selectin-mediated binding and binding mediated by basal levels of integrins on unactivated neutrophils is capable of firmly arresting some neutrophils. Another possible explanation is that the small population of firmly adherent neutrophils is normally activated (48) or has been activated during isolation, by exposure to the flow system, or by endothelial-derived factors. This subpopulation of neutrophils could then adhere firmly via up-regulated integrins. The increases in rolling velocity caused by R6.5 and R15.7, on the other hand, cannot be explained on the basis of a small subpopulation of activated neutrophils since firmly adherent neutrophils were excluded from all rolling velocity measurements. It is also unlikely that steric effects of binding by the MAbs account for the increase in rolling velocity, since the use of MAb CL203 (which binds ICAM-1 at a nonfunctional site) did not increase rolling velocity. The observed increases in rolling velocity therefore give evidence for an adhesion-stabilizing contribution of basal levels of leukocyte integrins. Note, however, that it does not rule out the possibility that the firmly adherent neutrophils have indeed been activated.

A similar increase in rolling velocity was found in vivo using an anti-CD18 antibody (24). In contrast, Lawrence and Springer (23) showed that on artificial lipid bilayers, the addition of ICAM-1 (250 sites/μm^2) to P-selectin at 200 sites/μm^2 did not significantly decrease neutrophil rolling velocity relative to P-selectin alone. Our rolling velocity data more closely match their results at 50 sites/μm^2, however, and they do not report data for the combination of P-selectin at this density and ICAM-1. They also mention that a density of 50 sites/μm^2 is closer to physiological values, based on the reported density of P-selectin on histamine-stimulated HUVECs in tissue culture. The actual density of P-selectin on endothelial cells in vivo is not known.

Our results show that the anti-L-selectin MAb DREG56 inhibits approximately 50 ± 5% of neutrophil binding to histamine-stimulated HUVEC. There are several possible explanations for this observation. First, neutrophil L-selectin might present carbohydrate ligands that are bound by P-selectin, as suggested by Picker et al. (10). They found that approximately 70% of neutrophil binding to P-selectin transfected cells is inhibited by DREG56. Our results show a similar magnitude of inhibition. The fact that anti-L-selectin MAb does not completely inhibit adhesion suggests that this particular antibody might block incompletely in the flow assay or that additional neutrophil ligands for P-selectin may exist. One candidate is a recently described high-affinity glycoprotein ligand for P-selectin that contains sialylated, O-linked oligosaccharides (49, 50). We attempted experiments in which neutrophil L-selectin was removed from the surface by fMLP activation to see if binding under flow was

TABLE 1 Summary statistics for the distributions of neutrophil rolling velocities with various MAb treatments

MAb treatment (dyn/cm^2)	No. of neutrophils	Mean rolling velocity (μm/s)	SD	SE	Skewness	Kurtosis
No MAbs	214	14.20	6.48	0.44	0.53	−0.20
R6.5	117	17.51	7.74	0.71	0.59	−0.35
R15.7	189	17.18	6.96	0.51	0.52	−0.37
DREG56	246	14.26	7.07	0.55	0.70	0.14
S12	136	13.92	6.13	0.52	0.48	−0.12
CL203	283	14.00	6.48	0.38	0.64	−0.04
CL18/1D1	145	14.12	6.62	0.47	0.54	−0.11
Thrombin	148	13.30	5.95	0.49	0.45	−0.09

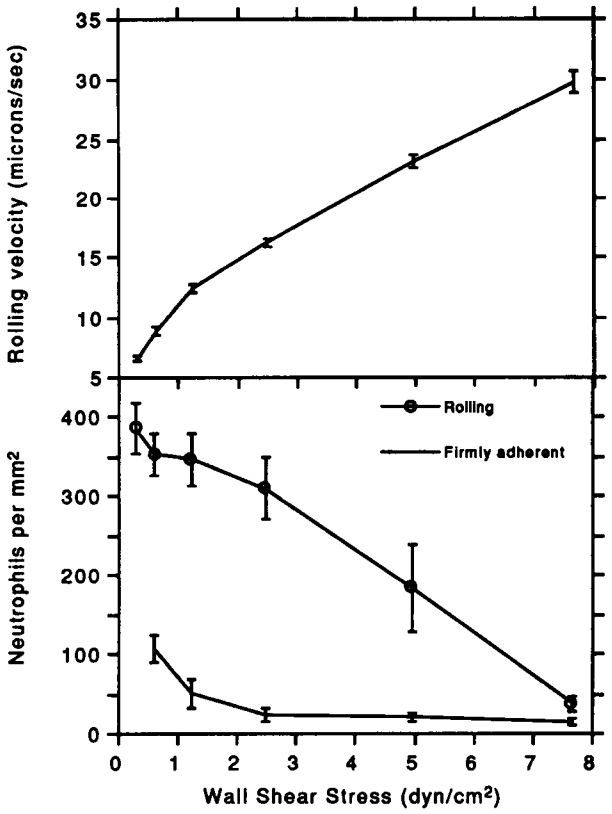

FIGURE 7 Variation with wall shear stress of neutrophil adhesion and rolling velocity on histamine-stimulated HUVECs. Error bars for rolling and firmly adherent neutrophil adhesion represent mean ± SEM for $n = 6$ experiments.

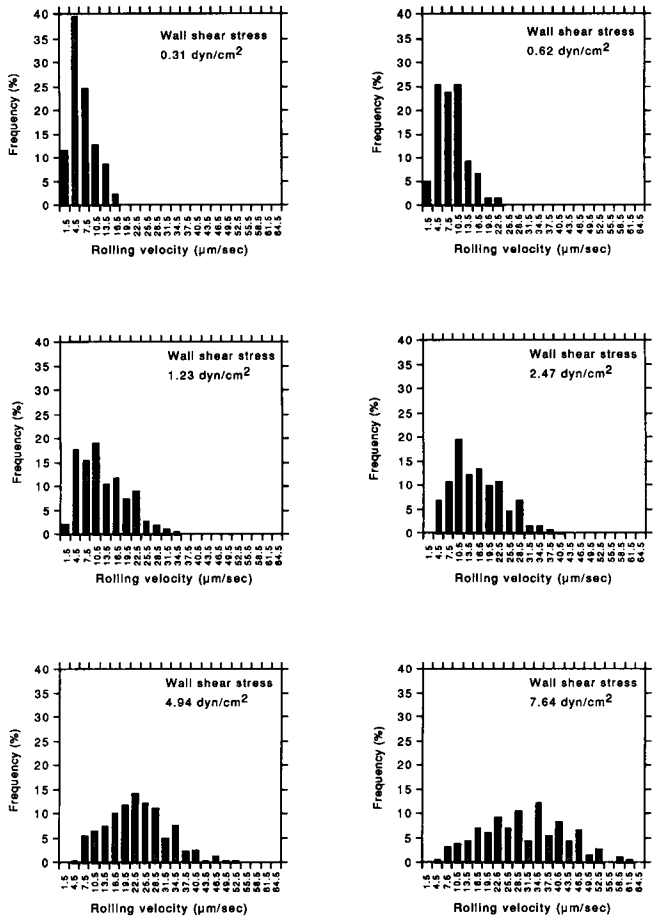

FIGURE 8 Variation with wall shear stress of the distribution of individual neutrophil rolling velocities over histamine-stimulated HUVECs. Frequencies are expressed as a percentage of the total number of neutrophils observed.

still possible, but these proved difficult to interpret. The activation causes drastic shape changes in the neutrophils that hinder rolling and modulate additional binding mechanisms. Although we saw significant binding (mostly firm adhesion, very little rolling) with L-selectin largely shed, we decided that these data should not be interpreted in terms of the relative roles of L-selectin versus other ligands for P-selectin.

Second, the anti-L-selectin antibody might indirectly affect neutrophil function such that the cells no longer adhere to P-selectin. This possibility is particularly relevant, since other anti-L-selectin MAbs are known to activate neutrophils. Unlike several other anti-L-selectin MAbs, DREG56 does not appear to activate neutrophils extensively, as measured by shape change (Jones, unpublished observations), Ca^{2+} mobilization, and L-selectin shedding (Smith, unpublished observations). We were unable to identify a nonblocking anti-L-selectin antibody that does not activate PMNs extensively to serve as a control for DREG56. Therefore, we cannot exclude the possibility that DREG56 indirectly affects neutrophil adhesion rather than specifically blocks binding of L-selectin to P-selectin. A third possible explanation for the partial blocking found with DREG56 is that histamine might mobilize a second carbohydrate ligand recognized by L-selectin. In this case, binding of L-selectin to its endothelial carbohydrate ligand would combine with binding of P-selectin to the neutrophil surface to produce adhesion. Both receptors would be necessary to produce adhesion, but neither would be sufficient by itself. The 50% inhibition with DREG56 we observe is also close to the 60–65% inhibition of adhesion of neutrophils to cytokine-stimulated endothelial cells at 1.85 dyn/cm^2 (15, 42, 43), indicating a similarity in the level of interaction of L-selectin with endothelium activated by histamine (which up-regulates P-selectin) or cytokines (which up-regulate E-selectin).

The variation of adhesion and rolling velocity with wall shear stress indicates that P-selectin-mediated rolling adhesion is optimal at postcapillary venular wall shear stresses (1–4 dyn/cm^2) but not at arteriolar or arterial wall shear stresses (>8 dyn/cm^2). The number of rolling cells decreases with increasing wall shear stress. This may be because contact with the endothelium in rapid flow is too short for bonds to form or because increased fluid forces on the neutrophils break bonds more easily. Both effects probably contribute. In either case, since fewer bonds provide adhesion for those neutrophils that do roll, the rolling velocity is greater at higher shear stresses as well. This observation also supports the findings of two groups that have recently developed mathematical models of P-selectin/L-selectin-mediated adhesion (12, 13). These models consist of mathematical descriptions of neutrophil rolling based on available data for

TABLE 2 Summary statistics for the distributions of neutrophil rolling velocities at various wall shear stresses

Wall shear stress (dyn/cm^2)	No. of neutrophils	Mean rolling velocity (μm/s)	SD	SE	Skewness	Kurtosis
0.3	240	6.63	3.55	0.23	0.83	−0.06
0.6	180	8.81	4.34	0.32	0.71	0.16
1.2	361	12.80	7.02	0.37	0.69	−0.19
2.5	377	16.32	7.63	0.39	0.61	−0.21
4.9	294	23.21	9.17	0.53	0.37	−0.04
7.6	181	29.87	12.1	0.90	0.12	−0.64

rate and equilibrium constants, receptor densities, etc. Both groups concluded that in general the unique response of P-selectin–L-selectin bonds to strain is largely responsible for their ability to arrest flowing neutrophils and allow rolling. These models are based on data for P-selectin reconstituted in artificial membranes at 50 sites/μm^2 (23), and since our data are very similar, it is likely that these conclusions can still be drawn for neutrophil interactions with intact endothelial cells. This modeling may apply equally well to interactions of P-selectin or L-selectin with distinct ligands rather than with each other. Models such as these can always be improved by making use of additional experimental data for parameter estimation and comparison with model predictions. Our data showing variations with wall shear stress of neutrophil adhesion, average rolling velocity, and distribution or rolling velocities should prove useful in these respects. Our data for a wall shear stress of 0.31 dyn/cm^2 are in good agreement with a previously reported in vivo velocity distribution (5), and the trend toward a broader distribution at higher shear stresses also agrees with their results, although the exact wall shear stresses in vivo are difficult to quantitate.

The physiological significance of neutrophil binding to histamine-stimulated endothelium remains an unresolved question. Our studies show that this P-selectin-mediated rolling acts optimally on a time scale of less than 30 min, rarely leads to firm adhesion, and does not lead to neutrophil diapedesis. This is in dramatic contrast to observations of neutrophil adhesion to cytokine-stimulated endothelial monolayers which show that a large number of neutrophils bind firmly and transmigrate (14, 15, 42, 43). The difference is most likely due to lack of endothelial ICAM-1 up-regulation or to lack of endothelial-derived IL-8 with histamine stimulation. Experiments using cytokine-stimulated endothelial cells are generally carried out at the point of maximum E-selectin expression (approximately 4 to 6 h), and significant up-regulation of ICAM-1 occurs within this time frame. Our experiments, on the other hand, are carried out only to 20 min, at which point ICAM-1 levels are still low. Recent experiments using thrombin stimulation (44) have demonstrated that ICAM-1 may be up-regulated from intracellular stores after 30 min of thrombin stimulation. However, since our experiments did not examine these later times, it remains unknown whether this ICAM-1 up-regulation can function to allow firm adhesion of neutrophils following P-selectin-mediated rolling.

The lack of firm adhesion and diapedesis with histamine stimulation relative to cytokine stimulation can be explained by the hypothesis that cytokine stimulation but not histamine stimulation results in endothelial cell production of a factor or factors that activate neutrophils to undergo integrin-dependent firm adhesion and diapedesis. As mentioned in the introduction, several factors have been implicated in this activation, including soluble IL-8 and endothelial-bound PAF. PAF up-regulation, unlike other known candidate factors, was recently shown to be rapidly induced by histamine stimulation (47), and it was demonstrated in static assays that PAF activates significant numbers of stationary neutrophils leading to activation-dependent adhesion. Our results using a flow assay, however, lead to the conclusion that at most a small number of rolling neutrophils are activated by PAF. This result might reflect the inability of PAF to stimulate neutrophils under shear conditions or it might be due to differences in PAF mobilization using our endothelial cell culture conditions. In addition to soluble and cell-associated activating factors, the possibility exists that receptor binding can itself transduce activating signals to the neutrophil (51). Our results indicate that this mechanism does not function to activate large numbers of neutrophils to bind firmly to histamine-stimulated endothelial cells. Other investigators (52, 53) have given evidence for a direct inhibitory effect of both membrane-bound and soluble P-selectin on neutrophil diapedesis. This might explain the lack of diapedesis, but it should be noted that these findings have proved difficult to reproduce (54). Oxygen radical stimulation of the endothelial cells produces longer-lived expression of P-selectin (55, 56). This could be a situation where P-selectin-mediated adhesion leads to more transmigration. A final possible function for this rapid adhesion pathway is that the neutrophils play a role in thrombosis by binding activated platelets via platelet P-selectin. The enhanced accumulation of activated platelets and neutrophils could be a mechanism for the modulation of clot formation in response to injury.

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