

## Neutrophil–Neutrophil Interactions under Hydrodynamic Shear Stress Involve L-Selectin and PSGL-1

### A Mechanism That Amplifies Initial Leukocyte Accumulation on P-Selectin In Vitro

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#### Abstract

Leukocytes attach to and roll on inflamed endothelium and on leukocyte monolayers that form on the endothelial cells. Leukocyte–leukocyte interactions occurring under hydrodynamic shear stress are mediated by binding of L-selectin to unknown sialomucin-like glycoproteins. We show that purified neutrophil PSGL-1, a sialomucin glycoprotein that serves as a ligand for both P- and E-selectin, can also support the attachment and rolling of free flowing neutrophils in vitro. Neutrophil rolling on PSGL-1 was abolished by the anti-L-selectin mAb DREG200 and by the anti-PSGL-1 mAb PL1, indicating that L-selectin can interact directly with PSGL-1. Neutrophil rolling on neutrophil monolayers was also blocked by PL1 ( $60 \pm 9\%$  SEM inhibition); however, DREG200 blocked more efficiently ( $93 \pm 7\%$  SEM inhibition), suggesting that other L-selectin ligands may exist on the neutrophil surface. These studies demonstrate that PSGL-1 on the neutrophil surface is a major functional ligand for L-selectin. The avidity of this L-selectin–dependent adhesion event was sufficient to allow individual neutrophils rolling on P-selectin to capture free flowing neutrophils, which progressed to form linear strings and discrete foci of rolling neutrophils. Neutrophil accumulation on P-selectin accelerated with time as a result of neutrophil-assisted capture of free flowing neutrophils. When neutrophil–neutrophil interactions were blocked by DREG200, neutrophils accumulated on P-selectin in a random pattern and at a uniform rate. Thus, leukocyte-assisted capture of flowing leukocytes may play an important role in amplifying the rate of initial leukocyte recruitment at sites of inflammation. (*J. Clin. Invest.* 1996; 98:1081–1087.) Key words: myeloid cells • adhesion • inflammation • mucin • glycoprotein • rolling

#### Introduction

Leukocyte accumulation on activated endothelium is initiated, augmented, and prolonged by adhesion events that mediate leukocyte–endothelial cell, leukocyte–leukocyte, and leukocyte–platelet interactions. The selectin family of adhesion proteins (1–3), which are expressed on activated endothelial cells (E-selectin [CD62E] and P-selectin [CD62P]), activated platelets (P-selectin) and peripheral blood leukocytes (L-selectin [CD62L]), promote the attachment and rolling of leukocytes on activated endothelium. This process reduces the velocity of leukocytes in the blood flow by approximately two orders of magnitude. Jutila and colleagues (4, 5) have demonstrated that leukocytes not only roll directly on activated endothelium but also roll on a monolayer of accumulated leukocytes bound to the activated endothelial cells. Leukocyte monolayers may promote continued and augmented leukocyte recruitment even after access to the endothelial surface is obstructed by attached leukocytes. Adhesion receptors expressed on the leukocyte monolayer could add to the repertoire of endothelial cell adhesion receptors (E- and/or P-selectin) and allow for the attachment of leukocytes that previously were unable to bind directly to the activated endothelium (4, 5).

Leukocyte–leukocyte interactions occurring under shear stress are exclusively L-selectin–dependent (4–6). The leukocyte ligands recognized by L-selectin appear to be sialomucin-like, demonstrated by the ability of O-glycoprotease (5) and neuraminidase (4) to abrogate leukocyte rolling on leukocyte monolayers. Activation–induced neutrophil aggregation has a L-selectin–dependent component that involves sialomucin-like ligands (7), and previously characterized L-selectin ligands on endothelial cells (GlyCAM-1 and CD34) are also sialomucin glycoproteins (8, 9). Human neutrophils express P-selectin glycoprotein ligand-1 (PSGL-1),<sup>1</sup> a heavily glycosylated sialomucin that can serve as a ligand for both P- and E-selectin (10–15). PL1, a mAb that binds near the NH<sub>2</sub>-terminus of PSGL-1 (16), inhibits neutrophil rolling on P-selectin surfaces both in vitro (17, 18) and in vivo (19). PL1, however, blocks neutrophil accumulation on E-selectin in vitro less efficiently, suggesting that E-selectin may bind to other regions of PSGL-1 and other ligands (18). Sulfation of NH<sub>2</sub>-terminal tyrosine residues on PSGL-1 is critical for binding to P-selectin (15, 20–22), but not to E-selectin (15, 20, 22). The presentation of PSGL-1 on the

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1. Abbreviation used in this paper: PSGL-1, P-selectin glycoprotein ligand-1.

surface of neutrophils appears to occur preferentially at the tips of microvilli (17). As previously described for L-selectin (23, 24), this location may be advantageous for initiating the attachment of free flowing leukocytes to the endothelium (25).

In this study, we examined whether PSGL-1 could serve as a functional ligand for L-selectin. We show that free flowing neutrophils attached to and rolled on purified PSGL-1 and that this interaction was completely blocked by either an anti-PSGL-1 (PL1) or an anti-L-selectin (DREG200) mAb. The PL1 mAb also significantly reduced neutrophil rolling on neutrophil monolayers, demonstrating the functional importance of PSGL-1 as a L-selectin ligand. We show that individual neutrophils rolling on purified P-selectin can mediate the capture of free flowing neutrophils. Neutrophil-assisted capture of free flowing neutrophils dramatically amplified the rate of initial neutrophil accumulation on P-selectin.

## Methods

**Antibodies.** The following mouse mAb were used in this study: DREG200, which recognizes and blocks the function of human L-selectin (26); PL1 and PL2, which recognize function- and nonfunction-blocking epitopes, respectively, on human PSGL-1 (16, 17); WAPS12.2, which recognizes and blocks the function of human P-selectin (27) (generously provided by Dr. E.C. Butcher, Stanford University, Stanford, CA); and R15.7, which recognizes and blocks the function of human CD18 (28) (generously provided by Dr. R. Rothlein, Boehringer Ingelheim).

**Purification of adhesion proteins.** PSGL-1 was isolated from human neutrophils as previously described (13), with the exception that the Brij-58 detergent (Pierce Chemical Co., Rockford, IL) was exchanged with *n*-octyl- $\beta$ -glucopyranoside (Sigma Chemical Co., St. Louis, MO). P-selectin was isolated from human platelets. Approximately  $3.6 \times 10^{10}$  platelets were lysed in 10 ml 1% Triton X-100 (Sigma Chemical Co.), 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 1 mM PMSF, and aprotinin (1/100 dilution). The lysate was precleared on a Sepharose-4B (Sigma Chemical Co.) column and then applied to a WAPS12.2-conjugated Sepharose-4B column. After extensive washing with 50 mM *n*-octyl- $\beta$ -glucopyranoside, 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 1 mM PMSF, and aprotinin (1/100 dilution), 1 ml elution fractions were collected using 50 mM *n*-octyl- $\beta$ -glucopyranoside, 0.2 M acetic acid, 0.5 M NaCl, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 1 mM PMSF, and neutralized with 100  $\mu$ l 1 M Tris-HCl (pH 8).

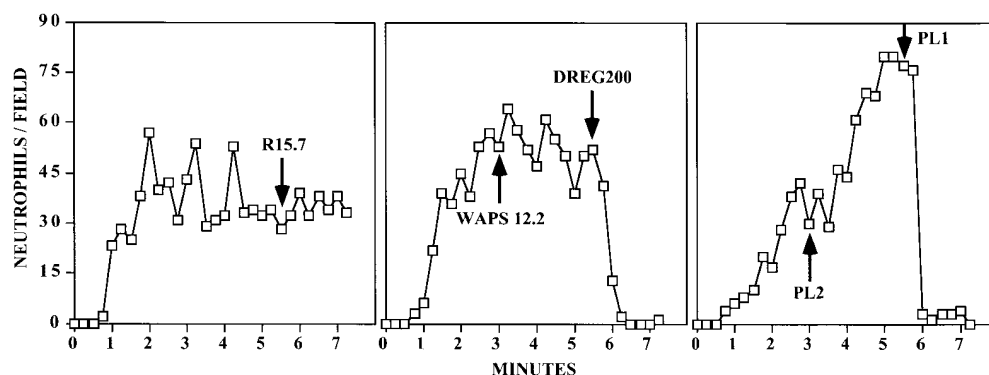
**Preparation of adhesive substrates.** Adhesive substrates used in the flow assays consisted of adsorbed PSGL-1, P-selectin, or neutrophil

monolayers. Purified PSGL-1 and P-selectin in 50 mM *n*-octyl- $\beta$ -glucopyranoside were diluted below the detergent's critical micelle concentration and adsorbed to 35 mm Falcon petri dishes (No. 1008) obtained from Becton Dickinson Labware (Franklin Lakes, NJ) for 2 h at room temperature. The plates were then blocked with 100% de-complemented FBS (GIBCO BRL, Gaithersburg, MD) or 1% HSA (Calbiochem-Behring Corp., La Jolla, CA), 25 mM Hepes, and 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> at 4°C for at least 1 h. For preparation of neutrophil monolayers, neutrophils were suspended at  $2.5 \times 10^6$  in 100  $\mu$ l flow media (1 $\times$  HBSS, 2 mM CaCl<sub>2</sub>, and 25 mM Hepes), infused into the flow chamber containing an untreated 35 mm Falcon tissue culture dish (No. 3001) (Becton Dickinson Labware), and allowed to attach to the surface of the dish for 1.5 min. After this brief incubation a uniform monolayer of round neutrophils was formed ( $> 2,500$  neutrophils/mm<sup>2</sup>), which was then fixed with 0.25% paraformaldehyde in 1 $\times$  HBSS without CaCl<sub>2</sub> and MgCl<sub>2</sub> for 10 min and extensively washed with flow media. Neutrophil monolayers were used immediately.

**Hydrodynamic flow assays.** Peripheral blood neutrophils were isolated by dextran sedimentation and by Ficoll centrifugation, as previously described (29). Neutrophils were stored in 1 $\times$  HBSS without CaCl<sub>2</sub> and MgCl<sub>2</sub>, 0.5 mM EDTA, and 25 mM Hepes at 4°C until their use. Immediately before their use in the flow assay the neutrophils were suspended in flow media. Neutrophil rolling under hydrodynamic shear stress was performed with a parallel plate flow chamber obtained from Glycotech (Rockville, MD) as previously described (29, 30). Plates coated with an adhesive substrate were attached to the flow chamber containing a 5-mm-wide gasket. Leukocytes suspended in flow media were drawn from a cell reservoir into the flow chamber by a syringe pump (Harvard Apparatus, South Natick, MA) at a constant shear stress calculated per manufacturer's instructions (Glycotech, viscosity = 1 centiPoise). Neutrophil rolling was visualized with an inverted microscope equipped with a Newvicon camera (NC-70) (Dage-MTI, Michigan City, IN), and recorded to VHS video tape for later analysis. Image analysis was performed with the public domain National Institutes of Health image software (version 1.57) on a Macintosh Quadra 950 equipped with a Scion LG-3 board obtained from Scion (Frederick, MD).

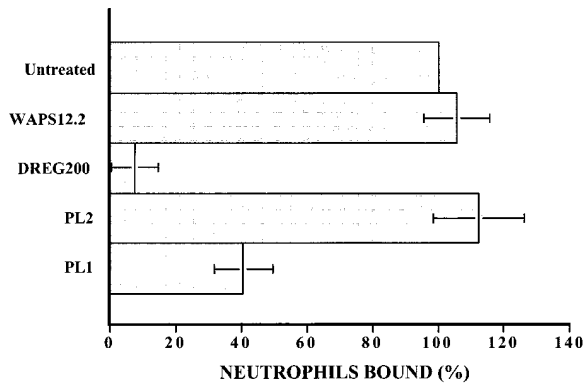
## Results

**PSGL-1 supports L-selectin-dependent neutrophil rolling in vitro under hydrodynamic shear stress.** Neutrophil rolling assays were performed using a parallel plate flow chamber containing a plastic base to which PSGL-1 was adsorbed. When a suspension of neutrophils from a cell reservoir was drawn into the flow chamber at a constant shear stress, free flowing neu-



**Figure 1.** Anti-L-selectin and anti-PSGL-1 mAb block neutrophil rolling on purified PSGL-1. PSGL-1 was adsorbed to the base of the flow chamber as described in Methods. Neutrophils suspended at a concentration of  $6 \times 10^5$ /ml in flow media were drawn through the flow chamber at a shear stress of 2 dynes/cm<sup>2</sup>. In all three panels the same PSGL-1 surface and the same microscopic field (10 $\times$ , area = 1.2 mm<sup>2</sup>) were observed.

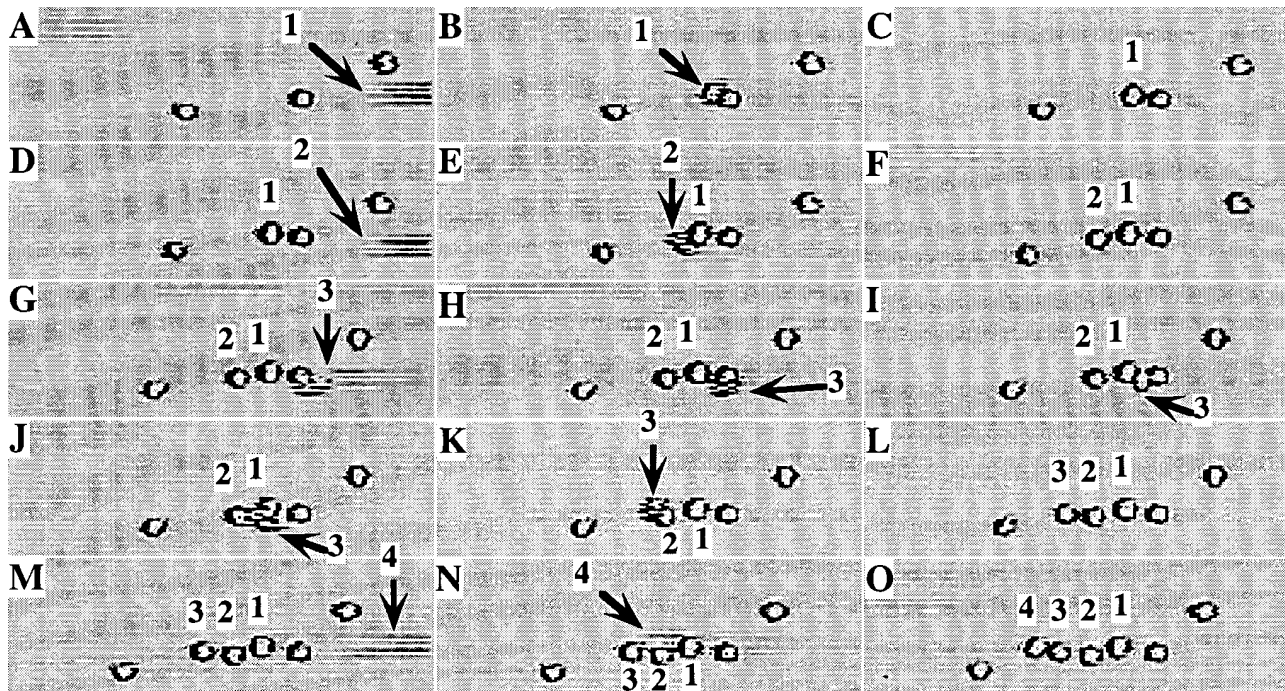
In each panel, rolling interactions of fresh neutrophils were established on the PSGL-1 surface. Previously infused neutrophils were removed from the flow chamber with 5 mM EDTA followed by extensive washing with flow media. The indicated mAb were added (20  $\mu$ g/ml final concentration) to the cell reservoir at 3 or 5.5 min, as denoted by the arrows. Data are representative of three experiments.



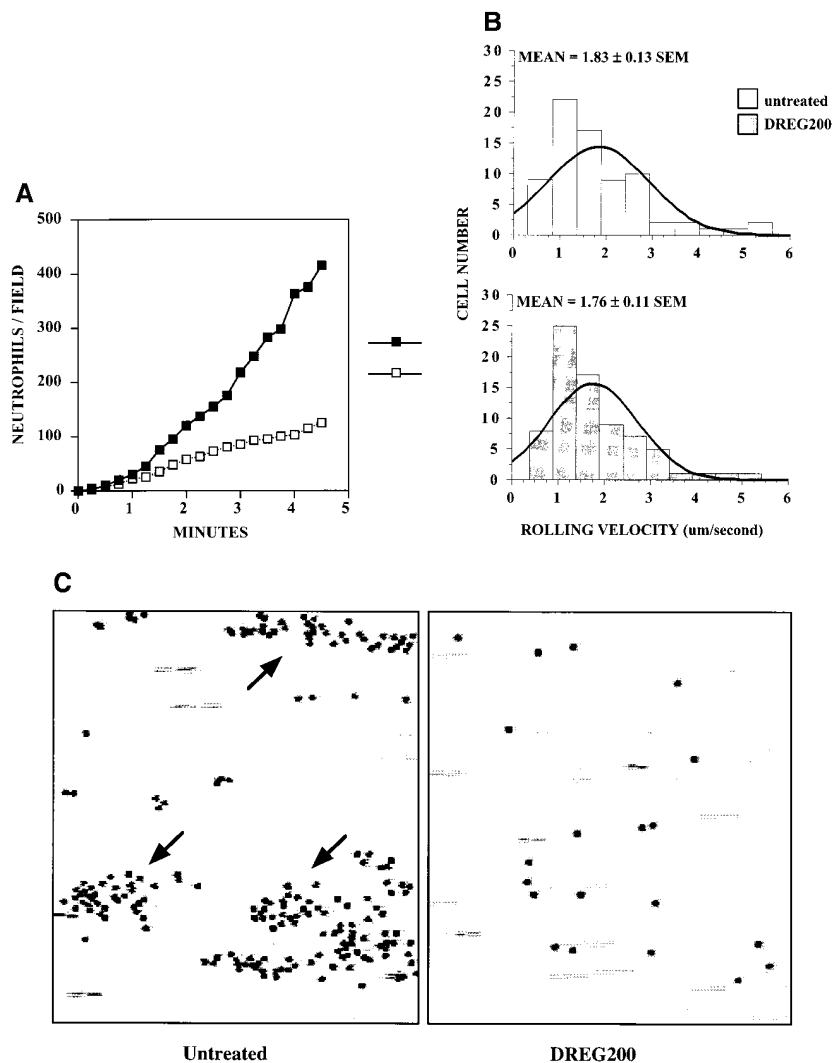
**Figure 2.** Anti-L-selectin and anti-PSGL-1 mAb block neutrophil rolling on preformed neutrophil monolayers. Neutrophil monolayers were formed on plastic dishes as described in Methods. Neutrophils suspended at a concentration of  $2 \times 10^6$ /ml in flow media were drawn through the flow chamber at a shear stress of 2.5 dynes/cm<sup>2</sup>. For each experiment, neutrophil rolling on the neutrophil monolayer was established and allowed to equilibrate, and then a mAb (20  $\mu$ g/ml final concentration) was injected into the cell reservoir, as indicated. A fresh neutrophil monolayer was used for each experiment. The number of neutrophils rolling three minutes after the addition of mAb was compared to the initial number of rolling neutrophils (*untreated*) and data presented as percent of untreated. The number of neutrophils rolling on a neutrophil monolayer was determined by digitally subtracting the neutrophil monolayer with image analysis software. Each mAb treatment represents the mean  $\pm$  SEM of three independent experiments using neutrophils from three separate donors.

trophils attached to and rolled on PSGL-1. Fig. 1 shows data from a representative experiment where we examined the effects of various mAb on neutrophil rolling. R15.7, a function-blocking anti-CD18 mAb, had no effect on the number of neutrophils rolling on PSGL-1 (Fig. 1, *left*). Similarly, WAPS12.2, a function-blocking anti-P-selectin mAb, did not affect the number of rolling neutrophils (Fig. 1, *middle*). This demonstrates that contaminating, activated platelets did not contribute to neutrophil rolling on PSGL-1 through the use of P-selectin. Addition of the anti-L-selectin mAb DREG200 to the cell reservoir abolished neutrophil rolling in less than one minute (Fig. 1, *middle*). Addition of the anti-PSGL-1 mAb PL1 to the cell reservoir also abolished neutrophil rolling on PSGL-1 (Fig. 1, *right*). The anti-PSGL-1 mAb PL2 appeared to increase the number of rolling neutrophils in this experiment (Fig. 1, *right*); however, this was not a consistent finding in other experiments or in a different neutrophil rolling assay (data not shown and Fig. 2). These data demonstrate that purified PSGL-1 supports L-selectin-dependent neutrophil rolling.

*MAb to PSGL-1 and L-selectin block neutrophil rolling on neutrophil monolayers.* We next examined the functional importance of PSGL-1 as a L-selectin ligand during neutrophil rolling on a preformed monolayer of neutrophils. Neutrophil monolayers were formed on the plastic base of a parallel plate flow chamber and fixed with 0.25% paraformaldehyde to prevent morphologic change and redistribution of cell surface proteins. Freshly infused neutrophils rolled on the neutrophil monolayer, as previously described (4, 6). The anti-P-selectin mAb WAPS12.2 had no significant affect on the number of rolling neutrophils (Fig. 2), again indicating that P-selectin on activated platelets did



**Figure 3.** An individual neutrophil rolling on P-selectin facilitates the capture of free flowing neutrophils. P-selectin was adsorbed to the base of the flow chamber as described in Methods. Neutrophils from a cell reservoir ( $5 \times 10^5$ /ml) were drawn over the P-selectin surface at a shear stress of 3 dynes/cm<sup>2</sup>. The capture of free flowing neutrophils by P-selectin-bound neutrophils was examined by computer-assisted frame by frame video analysis (30 frames per s). 15 of 165 frames over a time window of 5.5 s are shown in chronological order (A–O). Free flowing neutrophils, which appear as horizontal stripes, and neutrophils tumbling over P-selectin-attached neutrophils are indicated by arrows. The four newly recruited neutrophils are represented by the numbers 1–4. Approximate  $\times 200$ .



**Figure 4.** Comparison of the accumulation efficiency and rolling velocity of DREG200-treated and -untreated neutrophils on plastic-adsorbed P-selectin. (A) Treated or untreated neutrophils ( $5 \times 10^5/\text{ml}$ ) were drawn through the flow chamber at a shear stress of 3 dynes/cm<sup>2</sup> and their accumulation on the P-selectin surface was determined over time. DREG200-treated neutrophil rolling was initially established on the P-selectin surface. Before the infusion of the untreated neutrophils, any treated leukocytes remaining in the flow chamber were removed with 5 mM EDTA followed by extensive washing with flow media. The order of neutrophil infusion had no effect on the results. ■, untreated; □, DREG200. (B) The distributions of rolling velocities of 75 DREG200-treated and untreated neutrophils on P-selectin were determined by measuring the distance rolled in a time window of 20 s. (C) The accumulation patterns of DREG200-treated and untreated neutrophils on P-selectin are compared at 3 min after their infusion into the flow chamber. Neutrophil foci are indicated by the arrows. Approximate  $\times 100$ . The same P-selectin surface and microscopic field were observed for both DREG200-treated and untreated neutrophils. Inclusion of the anti-CD18 mAb R15.7 had no effect on the results (data not shown). All data are representative of three experiments.

not contribute to rolling. The anti-L-selectin mAb DREG200 blocked neutrophil rolling on the neutrophil monolayer by  $93 \pm 7\%$  (Fig. 2), as previously shown with different anti-L-selectin mAb (4). The anti-PSGL-1 mAb PL1, but not PL2, blocked neutrophil rolling by  $60 \pm 9\%$  (Fig. 2), suggesting that PSGL-1 may serve as a major ligand for L-selectin. PL1 did not block neutrophil-neutrophil rolling as effectively as DREG200. Extended pre-incubation of neutrophils with PL1 at concentrations up to 50  $\mu\text{g}/\text{ml}$  had no additional blocking affect (data not shown). It is unlikely that PL1 did not efficiently access its epitope on the neutrophil, because it abolishes neutrophil rolling on various P-selectin surfaces (data not shown and 17, 18). These data suggest the possibility of other L-selectin ligands on the neutrophil surface.

*Neutrophil-neutrophil interactions accelerate the rate of neutrophil accumulation on P-selectin.* Leukocytes functioning collectively as a monolayer support rolling of other leukocytes, a potential mechanism for continuing leukocyte accumulation on endothelial surfaces obstructed by the attached leukocytes (4, 5). We examined whether individual neutrophils rolling on an endothelial selectin could capture free flowing neutrophils. P-selectin, adsorbed to the base of the flow chamber, was used as a substrate to support neutrophil rolling. Free flowing neutrophils attached to and rolled on the P-selectin surface. As

previously described, this interaction was completely blocked by either an anti-PSGL-1 mAb (17) or anti-P-selectin mAb (17, 30) (data not shown). Upon close examination, using high magnification and computer-assisted frame by frame video analysis, it was observed that free flowing neutrophils frequently hit and momentarily attached to individual P-selectin-bound neutrophils, before advancing forward and adhering to the P-selectin surface. Fig. 3 shows a sequence of selected video frames from 5.5 s of video illustrating a single P-selectin-bound neutrophil initiating the capture of four additional neutrophils. In Fig. 3 A, three neutrophils are shown rolling on the P-selectin surface. A free flowing neutrophil entered the field (Fig. 3 A, arrow), hit and attached to the middle P-selectin-rolling neutrophil (Fig. 3 B), then bound to the P-selectin surface (Fig. 3 C). Fig. 3, D–O show three additional free flowing neutrophils that were captured by the P-selectin-bound neutrophils to form a string of rolling neutrophils.

Next we examined the contribution of L-selectin-dependent neutrophil-neutrophil capture to the rate of neutrophil accumulation on P-selectin. To address this, the accumulation over time of DREG200-treated and untreated neutrophils on P-selectin was compared. At early time points, both the untreated and DREG200-treated neutrophils accumulated on the P-selectin surface at nearly equivalent levels (30 versus 22

neutrophils per field, respectively, by 1 min, Fig. 4*a*). The regression over the first minute was not statistically different between untreated and treated cells (Fig. 4*a*). However, after this initial seeding of neutrophils, the efficiency of accumulation by untreated neutrophils on P-selectin increased dramatically compared to DREG200-treated neutrophils (414 versus 125 neutrophils per field, respectively, by 4.5 min, Fig. 4*a*). The accumulation of DREG200-treated neutrophils was fitted to a linear equation ( $a = [27.1 \pm 0.8]t$ , where  $a$  = accumulation and  $t$  = time min; R-square = 0.996,  $P = 0.0001$ ), whereas the accumulation of untreated cells was fitted to a quadratic equation ( $a = [23.5 \pm 2.9]t + [15.6 \pm 0.8]t^2$ ; R-square = 0.998,  $P = 0.0001$ ). Interestingly, the linear components of both equations are similar, reflecting direct neutrophil capture on P-selectin. The accelerated accumulation with time due to L-selectin-dependent neutrophil–neutrophil interactions is reflected in the quadratic element of the equation for untreated cells. At later time points, the rate of accumulation was physically limited by the available P-selectin surface area. The mean rolling velocity and variance of DREG200-treated and untreated neutrophils on P-selectin were equivalent (Fig. 4*b*). The accumulation pattern of untreated neutrophils was nonrandom, initially occurring as short strings of cells on the P-selectin surface (Fig. 3) that typically increased in size over time to form discrete foci of rolling neutrophils (Fig. 4*c*, *arrows*). The accumulation pattern of DREG200-treated neutrophils, however, was random (Fig. 4*c*). The contribution of neutrophil–neutrophil interactions to neutrophil accumulation on P-selectin was evident under low shear stress (1 dyne/cm<sup>2</sup>, data not shown) and became more prominent as shear stress increased (Fig. 4). These data demonstrate that neutrophil-assisted capture of free flowing neutrophils amplifies the rate at which neutrophils accumulate on the substratum.

## Discussion

Previous studies have demonstrated that free flowing leukocytes can attach to and roll on monolayers of adherent leukocytes under hydrodynamic shear stress (4–6). This interaction was shown to be inhibited by either pretreatment of the flowing leukocytes with anti-L-selectin mAb (4, 5) or of the leukocyte monolayer with neuraminidase (4) or O-glycoprotease (5), suggesting that L-selectin mediates rolling by interacting with sialomucin-like glycoproteins. We extend these studies by providing evidence that PSGL-1 serves as a functional ligand for L-selectin during neutrophil–neutrophil interactions under physiologic shear stress. Purified PSGL-1 adsorbed to plastic was sufficient to support the attachment and subsequent rolling of neutrophils. This interaction was abolished by the anti-PSGL-1 mAb PL1, but not PL2, and by the anti-L-selectin mAb DREG200. The complete inhibition of neutrophil rolling by either DREG200 or PL1 suggests a direct interaction of L-selectin with PSGL-1. Since PL1 also abolishes neutrophil rolling on P-selectin (17, 18), L- and P-selectin may recognize a similar region of PSGL-1. The rolling behavior of neutrophils on PSGL-1 was similar to that on other L-selectin ligands. L-selectin-dependent neutrophil rolling on MECA-79 antigen (29, 31) or neutrophil monolayers (6) occurs at a faster velocity than rolling on E- and P-selectin (30, 32, 18) and requires a threshold shear stress in order for attachment and rolling to occur (6). We observed that L-selectin-dependent neutrophil rolling behavior on PSGL-1 was phenotypically similar to rolling on MECA-79 antigen, and at

a very low shear stress neutrophils detached from the PSGL-1 surface (data not shown).

The functional relevance of PSGL-1 as a L-selectin ligand was demonstrated by the ability of PL1 to block neutrophil rolling on neutrophil monolayers. Interestingly, though anti-L-selectin mAb essentially blocked all neutrophil–neutrophil rolling (~93% inhibition), as previously shown (4), PL1 did not block as effectively (~60% inhibition). The complete blocking by PL1 of neutrophil rolling on purified PSGL-1 but incomplete inhibition of neutrophil rolling on neutrophil monolayers suggests that ligands for L-selectin in addition to PSGL-1 may exist on the neutrophil surface. However, we cannot exclude the possibility that L-selectin may bind to other regions of cell surface PSGL-1, as described for E-selectin (18). Sulfated tyrosine residues on the NH<sub>2</sub>-terminus of PSGL-1 contribute to binding to P-selectin but not to E-selectin (15, 20–22). Further studies are required to determine whether these sulfated tyrosine residues are necessary for L-selectin to bind PSGL-1.

We demonstrate that individual neutrophils rolling on the P-selectin substrate can assist in the capture of free flowing neutrophils, indicating that the adhesive avidity between a single neutrophil bound to P-selectin and a free flowing neutrophil is sufficient to counter physiological shear stress and facilitate attachment on to the P-selectin surface. Certain physical attributes of the rolling neutrophil probably enhance its ability to interact with free flowing neutrophils. Rolling neutrophils project further into the lumen of the flow chamber than adsorbed P-selectin, and rolling leukocytes could present oligomeric clusters of L-selectin (23, 24) and PSGL-1 (17) on the tips of their microvilli. We observed that a free flowing neutrophil would hit and momentarily attach to a P-selectin-bound neutrophil, then tumble over the bound neutrophil and adhere to the P-selectin surface. The forward advancement of the captured neutrophil may be due, in part, to the faster detachment rate of L-selectin bonds (6, 33) compared to the P-selectin bonds (34) supporting the bound neutrophil. Certain hydrodynamic factors may contribute to the forward displacement of the captured neutrophil as well. A neutrophil attached on top of a P-selectin-bound neutrophil would be at a greater radial distance from the P-selectin surface and thus exposed to increasing laminar flow velocities due to decreased shear. In addition, the P-selectin-bound neutrophil may disrupt the immediate downstream flow profile allowing the captured neutrophil to more efficiently attach to the P-selectin surface. Continued neutrophil capture typically progressed to linear strings and then small foci of accumulated cells. These nonrandom patterns of accumulation became more evident with increasing shear stress (Fig. 4 and data not shown), suggesting that rolling neutrophils support the capture of free flowing neutrophils more efficiently than the P-selectin substrate. The effect of neutrophil–neutrophil interactions on the rate of neutrophil accumulation on P-selectin was dramatic. When neutrophil–neutrophil interactions were blocked by DREG200, neutrophils accumulated on P-selectin at a uniform rate. However, untreated neutrophils accumulated on P-selectin at a rate that accelerated over time. Neutrophil–neutrophil interactions accounted for approximately 70% of the bound neutrophils after 4.5 min of accumulation (Fig. 4*a*). These results suggest that PSGL-1 molecules on the same neutrophil serve a dual role by facilitating both rolling on P-selectin and the capture of free flowing neutrophils. Leukocyte–leukocyte capture may be an

important mechanism to accelerate the accumulation of these cells.

The reduced accumulation of DREG200-treated neutrophils on P-selectin could be due to inhibition of sLex-modified, neutrophil L-selectin binding to P-selectin, as proposed by others (23, 35). Although some anti-L-selectin mAb reduce neutrophil attachment to P-selectin surfaces under shear stress (35, 36), a direct interaction of L-selectin with P-selectin has not been observed (37, 38). Three lines of evidence presented here suggest that P-selectin does not bind directly to L-selectin. (a) We show that the mean rolling velocity and variance of DREG200-treated and untreated neutrophils on P-selectin were equivalent. Consistent with these findings, Jones et al. (35) showed that the anti-L-selectin mAb DREG56 reduced P-selectin-mediated neutrophil rolling on histamine-stimulated HUVEC by approximately 50%, but did not affect neutrophil rolling velocity. It has been established that under constant shear stress, leukocyte rolling velocity will increase when the frequency of selectin-ligand bond formations are decreased (30–32). Taken together these results suggest that P-selectin ligand density was not reduced on DREG200-treated neutrophils. (b) DREG200 did not cause a constant reduction in neutrophil accumulation on P-selectin. At early time points ( $\leq 1$  min, Fig 4 a) similar numbers of untreated and DREG200-treated neutrophils attached directly to the P-selectin substrate. After this initial seeding the accumulation rate accelerated with time for untreated neutrophils while remaining uniform for the DREG200-treated neutrophils. (c) DREG200-treated neutrophils accumulated on the P-selectin surface in a random manner, whereas untreated neutrophils accumulated in a nonrandom manner forming strings and foci of rolling neutrophils. This suggests that P-selectin-bound neutrophils contributed to leukocyte accumulation. Together, these data indicate that L-selectin did not serve as an additional ligand for immobilized P-selectin but cooperated as an additional receptor mediating leukocyte–leukocyte interactions. Leukocyte-assisted capture of free flowing leukocytes may also account, in part, for the previously described L-selectin component involved in leukocyte accumulation on E-selectin surfaces (18, 23, 39, 40).

Jutila and colleagues proposed that leukocyte monolayers support leukocyte rolling and augment leukocyte recruitment at sites of inflammation (4, 5). We show that individual neutrophils rolling on an adhesive substrate can capture free flowing neutrophils and propose that this is also an important mechanism to amplify neutrophil recruitment early in the inflammatory response. Leukocyte-assisted capture of flowing leukocytes may explain in part the broad role L-selectin appears to play in a variety of inflammatory settings, including cobra venom factor and IgG immune complex-induced lung injury (41), burn injury (42), induced acute and chronic peritonitis (43–46), and ischemia-reperfusion injury (47, 48). The direct interaction of L-selectin with PSGL-1 indicates that this sialomucin glycoprotein can be recognized by all three selectin members, and participates in leukocyte–leukocyte, leukocyte–endothelial cell, and leukocyte–platelet interactions. Sklar and colleagues have recently shown that PSGL-1 serves as a ligand for L-selectin during activation-induced neutrophil aggregation (49), a process that also involves  $\beta_2$ -integrins (7, 50). Thus, PSGL-1 may be involved in a broad range of inflammatory settings. In respect to the multi-step model of leukocyte recruitment (attachment, rolling, activation, firm adhesion, and ex-

travasation), leukocyte–leukocyte interactions may significantly amplify leukocyte attachment and thus affect later steps. The contribution of leukocyte–leukocyte capture may hasten the kinetics of leukocyte recruitment as well as exacerbate certain inflammatory conditions.

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## References

1. McEver, R.P., K.L. Moore, and R.D. Cummings. 1995. Leukocyte trafficking mediated by selectin-carbohydrate interactions. [Review]. *J. Biol. Chem.* 270:11025–11028.
2. Varki, A. 1994. Selectin ligands. *Proc. Natl. Acad. Sci. USA.* 91:7390–7397.
3. Bevilacqua, M.P., and R.M. Nelson. 1993. Selectins. *J. Clin. Invest.* 91: 379–387.
4. Bargatze, R.F., S. Kurk, E.C. Butcher, and M.A. Jutila. 1994. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. *J. Exp. Med.* 180:1785–1792.
5. Jutila, M.A., and S. Kurk. 1996. Analysis of bovine  $\gamma/8$  T cell interactions with E-, P-, and L-selectin. Characterization of lymphocyte on lymphocyte rolling and the effects of O-glycoprotease. *J. Immunol.* 156:289–296.
6. Finger, E.B., K.D. Puri, R. Alon, M.B. Lawrence, U.H. Von Andrian, and T.A. Springer. 1996. Adhesion through L-selectin requires a threshold hydrodynamic shear. *Nature (Lond.)* 379:266–269.
7. Bennett, T.A., C.M. Schammel, E.B. Lynam, D.A. Guyer, A. Mellors, B. Edwards, S. Rogelj, and L.A. Sklar. 1995. Evidence for a third component in neutrophil aggregation: potential roles of O-linked glycoproteins as L-selectin counter-structures. *J. Leukocyte Biol.* 58:510–518.
8. Lasky, L.A., M.S. Singer, D. Dowbenko, Y. Imai, W.J. Henzel, C. Grimley, C. Fennie, N. Gillett, S.R. Watson, and S.D. Rosen. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell.* 69:927–938.
9. Baumhueter, S., M.S. Singer, W. Henzel, S. Hemmerich, M. Renz, S.D. Rosen, and L.A. Lasky. 1993. Binding of L-selectin to the vascular sialomucin CD34. *Science (Wash. DC)* 262:436–438.
10. Moore, K.L., N.L. Stults, S. Diaz, D.F. Smith, R.D. Cummings, A. Varki, and R.P. McEver. 1992. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J. Cell Biol.* 118:445–456.
11. Norgard, K.E., K.L. Moore, S. Diaz, N.L. Stults, S. Ushiyama, R.P. McEver, R.D. Cummings, and A. Varki. 1993. Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides. *J. Biol. Chem.* 268:12764–12774.
12. Sako, D., X.-J. Chang, K.M. Barone, G. Vachino, H.M. White, G. Shaw, G.M. Veldman, K.M. Bean, T.J. Ahern, B. Furie, D.A. Cumming, and G.R. Larsen. 1993. Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell.* 75:1179–1186.
13. Moore, K.L., S.F. Eaton, D.E. Lyons, H.S. Lichenstein, R.D. Cummings, and R.P. McEver. 1994. The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetylglucosamine. *J. Biol. Chem.* 269:23318–23327.
14. Asa, D., L. Raycroft, L. Ma, P.A. Aeed, P.S. Kaytes, A.P. Elhammer, and J. Geng. 1995. The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. *J. Biol. Chem.* 270:11662–11670.
15. Li, F., P.P. Wilkins, S. Crawley, J. Weinstein, R.D. Cummings, and R.P. McEver. 1996. Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. *J. Biol. Chem.* 271: 3255–3264.
16. Li, F., H.P. Erickson, J.A. James, K.L. Moore, R.D. Cummings, and R.P. McEver. 1996. Visualization of P-selectin glycoprotein ligand-1 as a highly extended molecule and mapping of protein epitopes for monoclonal antibodies. *J. Biol. Chem.* 271:6342–6348.
17. Moore, K.L., K.D. Patel, R.E. Bruehl, F. Li, D.A. Johnson, H.S. Lichenstein, R.D. Cummings, D.F. Bainton, and R.P. McEver. 1995. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J. Cell Biol.* 128:661–671.
18. Patel, K.D., K.L. Moore, M.U. Nollert, and R.P. McEver. 1995. Neutrophils use both shared and distinct mechanisms to adhere to selectins under static and flow conditions. *J. Clin. Invest.* 96:1887–1896.
19. Norman, K.E., K.L. Moore, R.P. McEver, and K. Ley. 1995. Leukocyte rolling in vivo is mediated by P-selectin glycoprotein ligand-1. *Blood.* 86:4417–

4421.

20. Pouyani, T., and B. Seed. 1995. PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell* 83:333-343.
21. Wilkins, P.P., K.L. Moore, R.P. McEver, and R.D. Cummings. 1995. Tyrosine sulfation of P-selectin glycoprotein ligand-1 is required for high affinity binding to P-selectin. *J. Biol. Chem.* 270:22677-22680.
22. Sako, D., K.M. Comess, K.M. Barone, R.T. Camphausen, D.A. Cumming, and G.D. Shaw. 1995. A sulphated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. *Cell* 83:323-331.
23. Picker, L.J., R.A. Warnock, A.R. Burns, C.M. Doerschuk, E.L. Berg, and E.C. Butcher. 1991. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* 66:921-933.
24. Erlandsen, S.L., S.R. Hasslen, and R.D. Nelson. 1993. Detection and spatial distribution of the  $\beta_2$  integrin (Mac-1) and L-selectin (LECAM-1) adherence receptors on human neutrophils by high-resolution field emission SEM. *J. Histochem. Cytochem.* 41:327-333.
25. Von Andrian, U.H., S.R. Hasslen, R.D. Nelson, S.L. Erlandsen, and E.C. Butcher. 1995. A central role for microvillus receptor presentation in leukocyte adhesion under flow. *Cell* 82:989-999.
26. Kishimoto, T.K., M.A. Jutila, and E.C. Butcher. 1990. Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. *Proc. Natl. Acad. Sci. USA* 87:2244-2248.
27. Jutila, M.A., R.F. Bargatze, S. Kurk, R.A. Warnock, N. Ehsani, S.R. Watson, and B. Walcheck. 1994. Cell surface P- and E-selectin support shear-dependent rolling of bovine  $\gamma\delta$  T cells. *J. Immunol.* 153:3917-3928.
28. Smith, C.W., R. Rothlein, B.J. Hughes, M.M. Mariscalco, F.C. Schmalstieg, and D.C. Anderson. 1988. Recognition of an endothelial determinant for CD18-dependent neutrophil adherence and transendothelial migration. *J. Clin. Invest.* 82:1746-1756.
29. Walcheck, B., J. Kahn, J.M. Fisher, B.B. Wang, R.S. Fisk, D.G. Payan, C. Feehan, R. Betageri, K. Darlak, A.F. Spatola, and T.K. Kishimoto. 1996. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. *Nature (Lond.)* 380:720-723.
30. Lawrence, M.B., and T.A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 65:1-20.
31. Lawrence, M.B., E.L. Berg, E.C. Butcher, and T.A. Springer. 1995. Rolling of lymphocytes and neutrophils on peripheral node addressin and subsequent arrest on ICAM-1 in shear flow. *Eur. J. Immunol.* 25:1025-1031.
32. Lawrence, M.B., and T.A. Springer. 1993. Neutrophils roll on E-selectin. *J. Immunol.* 151:6338-6346.
33. Alon, R., K.D. Puri, R.C. Fuhlbrigge, E.B. Finger, S. Chen, and T.A. Springer. 1995. The lifetime of the L-selectin:carbohydrate bond is considerably shorter than P- and E-selectin:ligand bonds and correlates with faster rolling adhesions. Abstract #4777 presented at the 9th International Congress of Immunology, San Francisco, CA, July 23-29, 1995. 805(Abstr.)
34. Alon, R., D.A. Hammer, and T.A. Springer. 1995. Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. *Nature (Lond.)* 374:539-542.
35. Jones, D.A., O. Abbassi, L.V. McIntire, R.P. McEver, and C.W. Smith. 1993. P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. *Biophys. J.* 65:1560-1569.
36. Buttrum, S.M., R. Hatton, and G.B. Nash. 1993. Selectin-mediated rolling of neutrophils on immobilized platelets. *Blood* 82:1165-1174.
37. Moore, K.L., A. Varki, and R.P. McEver. 1991. GMP-140 binds to a glycoprotein receptor on human neutrophils: evidence for a lectin-like interaction. *J. Cell Biol.* 112:491-499.
38. Levinovitz, A., J. Mühlhoff, S. Isenmann, and D. Vestweber. 1993. Identification of a glycoprotein ligand for E-selectin on mouse myeloid cells. *J. Cell Biol.* 121:449-459.
39. Kishimoto, T.K., R.A. Warnock, M.A. Jutila, E.C. Butcher, C. Lane, D.C. Anderson, and C.W. Smith. 1991. Antibodies against human neutrophil LECAM-1 (LAM-1/Leu-8/DREG-56 antigen) and endothelial cell ELAM-1 inhibit a common CD18-independent adhesion pathway in vitro. *Blood* 78:805-811.
40. Lawrence, M.B., D.F. Bainton, and T.A. Springer. 1994. Neutrophil tethering to and rolling on E-selectin are separable by requirement for L-selectin. *Immunity* 1:137-145.
41. Mulligan, M.S., M. Miyasaka, T. Tamatani, M.L. Jones, and P.A. Ward. 1994. Requirements for L-selectin in neutrophil-mediated lung injury in rats. *J. Immunol.* 152:832-840.
42. Mulligan, M.S., G.O. Till, C.W. Smith, D.C. Anderson, M. Miyasaka, T. Tamatani, R.F. Todd III, T.B. Issekutz, and P.A. Ward. 1994. Role of leukocyte adhesion molecules in lung and dermal vascular injury after thermal trauma of skin. *Am. J. Pathol.* 144:1008-1015.
43. Jutila, M.A., L. Rott, E.L. Berg, and E.C. Butcher. 1989. Function and regulation of the neutrophil MEL-14 antigen in vivo: Comparison with LFA-1 and Mac-1. *J. Immunol.* 143:3318-3324.
44. Bosse, R., and D. Vestweber. 1994. Only simultaneous blocking of the L- and P-selectin completely inhibits neutrophil migration into mouse peritoneum. *Eur. J. Immunol.* 24:3019-3024.
45. Pizcueta, P., and F.W. Luscinskas. 1994. Monoclonal antibody blockade of L-selectin inhibits mononuclear leukocyte recruitment to inflammatory sites in vivo. *Am. J. Pathol.* 145:461-469.
46. Tedder, T.F., D.A. Steeber, and P. Pizcueta. 1995. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *J. Exp. Med.* 181:2259-2264.
47. Ma, X., A.S. Weyrich, D.J. Lefer, M. Buerke, K.H. Albertine, T.K. Kishimoto, and A.M. Lefer. 1993. Monoclonal antibody to L-selectin attenuates neutrophil accumulation and protects ischemic reperfused cat myocardium. *Circulation* 88:649-658.
48. Seekamp, A., G.O. Till, M.S. Mulligan, J.C. Paulson, D.C. Anderson, M. Miyasaka, and P.A. Ward. 1994. Role of selectins in local and remote tissue injury following ischemia and reperfusion. *Am. J. Pathol.* 144:592-598.
49. Guyer, D.A., K.L. Moore, E. Lynam, C.M.G. Schammel, S. Rogelj, R.P. McEver, and L.A. Sklar. 1996. P-selectin glycoprotein ligand (PSGL-1) is a ligand for L-selectin in neutrophil aggregation. *Blood*. In press.
50. Simon, S.I., J.D. Chambers, E. Butcher, and L.A. Sklar. 1992. Neutrophil aggregation is  $\beta_2$ -integrin- and L-selectin-dependent in blood and isolated cells. *J. Immunol.* 149:2765-2771.