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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CHARACTERIZATION OF THE BIOCHEMICAL AND BIOPHYSICAL MECHANISMS FUNDAMENTAL TO RECEPTOR-MEDIATED WHITE BLOOD CELL ACCUMULATION

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

Stephen Bradley Forlow

Norman, Oklahoma
1998

CHARACTERIZATION OF THE BIOCHEMICAL AND BIOPHYSICAL MECHANISMS FUNDAMENTAL TO RECEPTOR-MEDIATED WHITE BLOOD CELL ACCUMULATION

A Dissertation APPROVED FOR THE
SCHOOL OF CHEMICAL ENGINEERING AND MATERIALS SCIENCE

BY

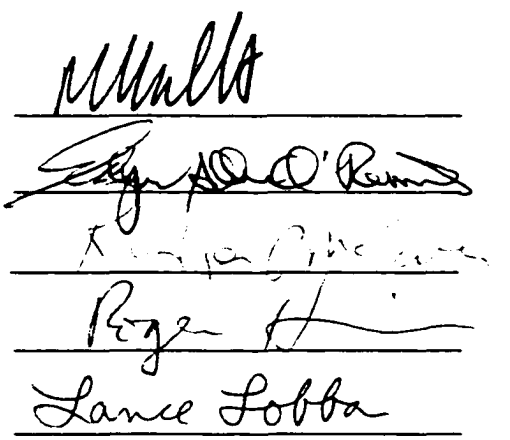


Table of Contents

List of Figures

List of Tables

Abstract

The recruitment of circulating leukocytes to areas of infection and injury is an important aspect of the immune response process. Leukocytes initially attach to and roll on the activated endothelial cells lining the blood vessel wall. The initial attachment and rolling of leukocytes on the vessel wall is largely mediated by P-selectin and E-selectin of the selectin family of adhesion molecules. It has recently been shown in vitro that leukocyte-leukocyte interactions mediated by L-selectin and PSGL-1 enhance accumulation of cells. We have recreated these initial cell recruitment events in vitro using simplified systems. The cell recruitment assays were done in a parallel plate flow chamber that mimics the body's fluid mechanical environment. We have identified a novel mechanism that enables cell-cell interactions to occur which may contribute in the recruitment of cells. Platelet microparticle concentrations have been shown to be elevated in many diseases and disorders; however, little work has been done to show if platelet microparticles play any role in clotting, cell aggregation, or cell accumulation. We investigated the possibility that platelet microparticles could mediate cell aggregation, cell-cell interactions, and enhanced cell recruitment through cell-cell interactions. It was predicted that P-selectin-expressing platelet microparticles could act as a bridge between PSGL-1-expressing cells allowing them to interact. HL-60 cells or neutrophils in the presence of increasing concentration levels of platelet microparticles were withdrawn over HL-60 cells prebound to an anti-human CD43 antibody coated glass slide to determine if platelet microparticles could mediate cell-cell interactions, cell aggregation, and possibly cell accumulation through cell-cell interactions. Increasing the concentration of platelet microparticles in the system resulted in an increase in the

number of cell-cell interactions, an increase in the number and size of cell aggregates, an increase in the number of cell-cell interactions at higher shear stresses, and an increase in cell accumulation on the surface. Therefore, platelet microparticles may play an important role in clotting, cell aggregation, and cell accumulation through cell-cell interactions. We also analyzed the kinetic off-rate of the selectin adhesion molecules. We have measured the off-rate of P-, E-, and L-selectin in various systems including using lipid bilayers and CHO cells as the surface and using neutrophils, HL-60 cells, and pre-B cells as the circulating cell. The off-rates were calculated over a range of shear stresses and compared between systems by calculating the force on the bond. The off-rates for P- and E-selectin were approximately the same; however, the off-rate for L-selectin was found to be much faster. The calculated off-rates were not dependent on how either the selectin or PSGL-1 was presented. This shows the off-rate is strongly dependent on the interactions between the molecules, but not strongly dependent on how the molecules are displayed on the surface. This also suggests that the kinetics are a result of the dissociation of the receptor-ligand bond and not the extraction of the receptor from the membrane.

Chapter 1: Introduction and Background

One of the most important functions of the immune response system is the recruitment of leukocytes to areas of infection or tissue damage (Figure 1). Leukocytes first leave the central stream of flowing blood and roll along activated endothelial cells lining the blood vessel wall. This initial rolling interaction is largely mediated by the selectin family of adhesion molecules (1-30), although, some integrins have been shown capable of mediating cell rolling on the endothelium (31-37). Leukocytes are able to roll under shear stress as a result of the high on-rates and off-rates of the selectin bonds (38). Therefore, bonds rapidly form at the leading edge of the cell as bonds are broken at the trailing edge. The rolling leukocytes subsequently adhere tightly to the vessel wall allowing them to emigrate between endothelial cells into areas of infection or injury. The arrest and emigration of leukocytes are mediated by integrins (6, 11, 18, 19, 21-25, 39-44) and adhesion molecules that are members of the immunoglobulin (Ig) superfamily.

The inflammatory response begins with infected or damaged tissue releasing various mediators that activate endothelial cells and leukocytes or act as chemoattractants for leukocytes. Endothelial cells activated by cytokines, thrombin, histamine, complement fragments, or oxygen-derived free radicals (45-59) are able to support selectin-mediated attachment and rolling of leukocytes on the surface. Once leukocytes roll on the endothelial surface many receptors are involved in the firm adhesion, activation, and emigration of the leukocyte into the subendothelial tissue. Most of these receptors are integrins or belong to the immunoglobulin superfamily of adhesion molecules. Figure 2 shows some of the many receptors and ligands involved in the complex cascade of events

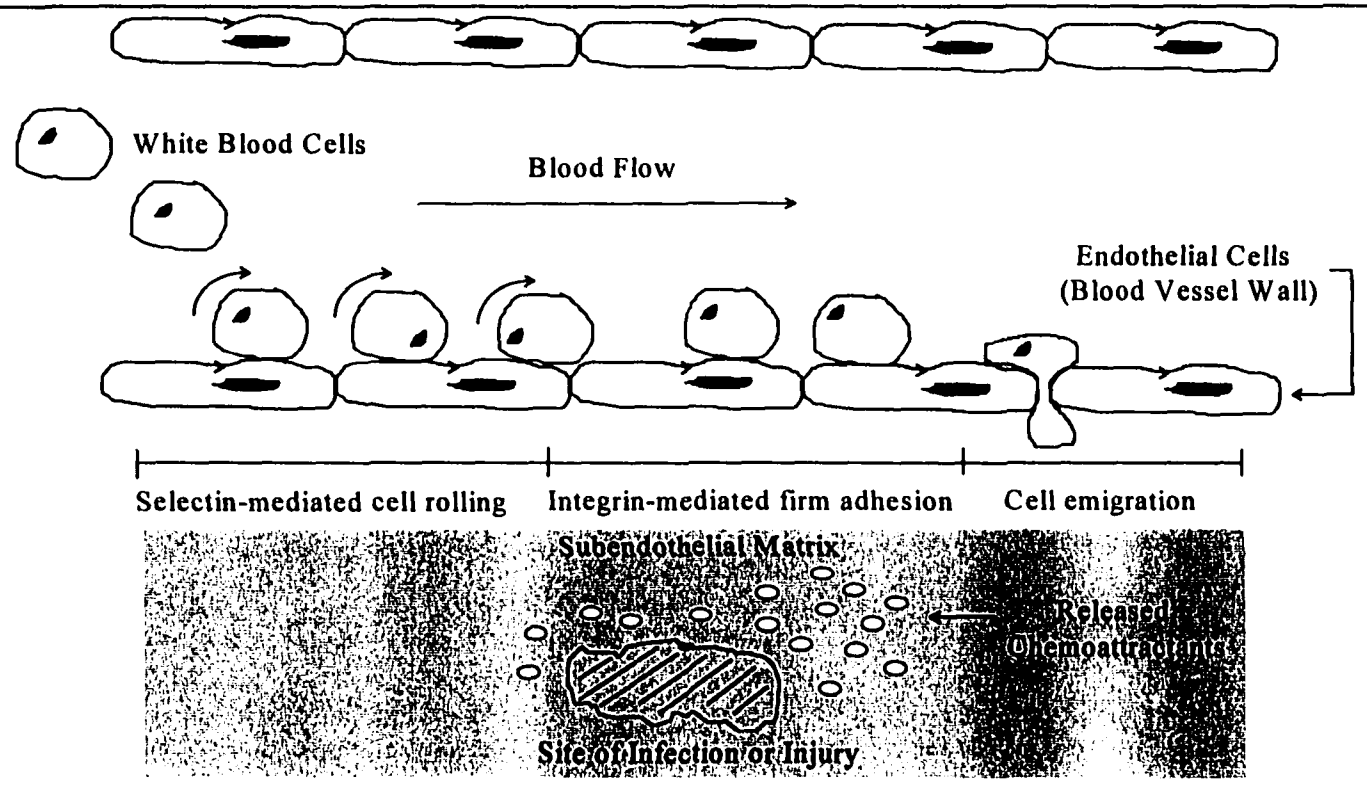


Figure 1: Immune response process. Normally white blood cells of the inflammatory response circulate throughout the body as nonadherent cells which do not interact with the blood vessel wall. However, tissue damage or infection stimulates the blood vessel walls to express various adhesion molecules. The selectin adhesion molecules initiate the immune response by binding to receptors on the white blood cells allowing them to begin rolling on the vessel wall. The rolling cells become firmly attached through the interactions of the integrin adhesion molecules. Once a cell is attached firmly to the wall, it is capable of emigrating through the vessel wall to the site of injury or infection.

that enables a leukocyte to roll on the endothelium, become firmly attached, and emigrate to a site of infection or injury.

ROLLING

Selectins

The selectin family of adhesion molecules is largely responsible for the recruitment of leukocytes to the blood vessel wall. Selectin receptors mediate the attachment and rolling of leukocytes to the blood vessel wall and can mediate leukocyte-leukocyte interactions. The selectin family consists of three molecules (Figure 3): E-selectin (ELAM-1, CD62E), L-selectin (LECAM-1, LAM-1, CD62L), and P-selectin (PADGEM, GMP-140, CD62P).

Each selectin consists of a signal peptide, a lectin domain, an epidermal growth factor (EGF)-like domain, a series of short consensus repeats (SCR) each consisting of approximately 62 residues, a hydrophobic transmembrane region, and a short cytoplasmic tail. The difference between E-, L-, and P-selectin is in the number of consensus repeats that are present. L-selectin contains only 2 repeats, E-selectin contains 6 repeats, and P-selectin contains 9 of the short consensus repeats (60-65). The three selectin molecules are very similar. They are approximately 40% identical in the SCR domain and 65% identical in the lectin and EGF-like domains. The lectin domain is the region of the receptor that supports Ca^{2+}-dependent ligand binding (66). However, recent data has shown that the EGF-like domain supports optimal ligand recognition since deletion of the EGF-like or SCR domains reduces the receptors ability to mediate cell binding (15, 67-69). Therefore, the EGF-like domain and one or more of the SCR seem to greatly

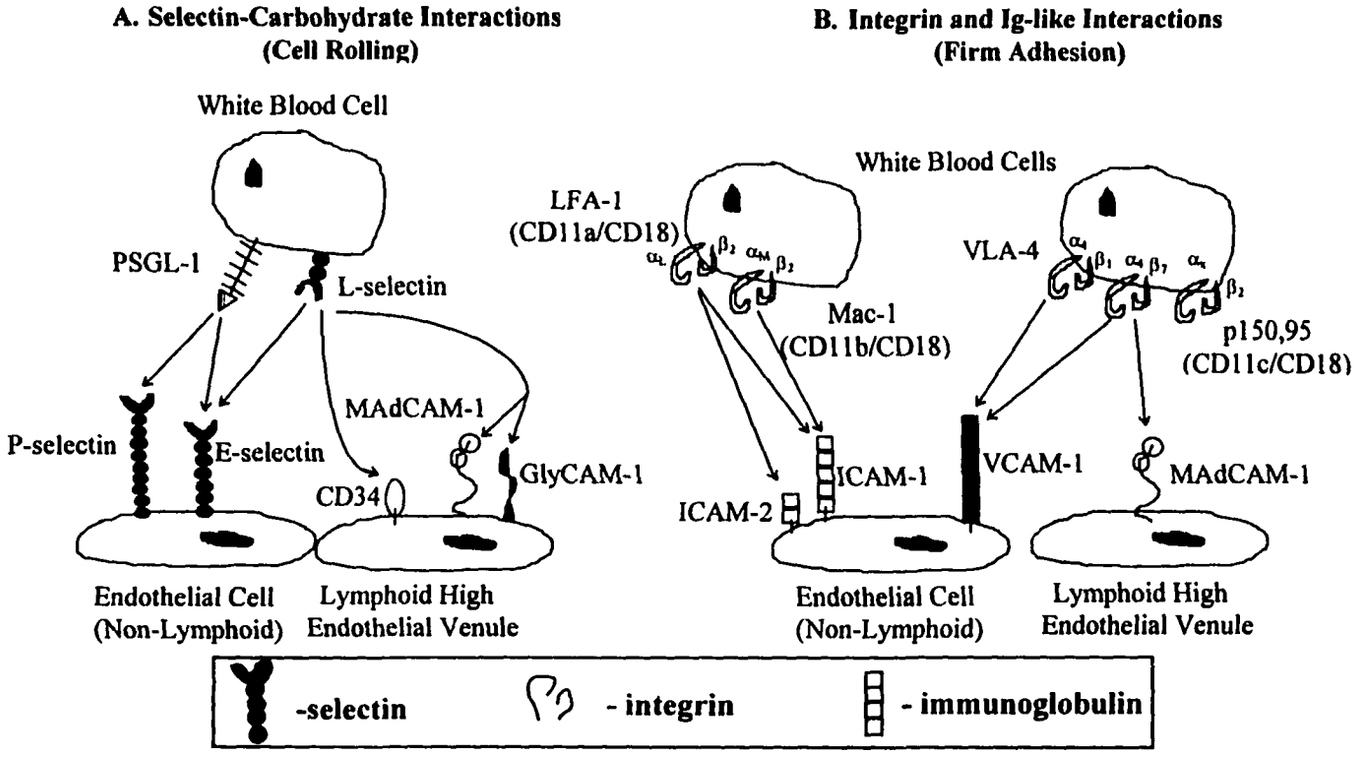


Figure 2. Adhesion molecules of the inflammatory response. The ligands of selectin adhesion molecules consist of various carbohydrate presenting molecules. These interactions are characterized by fast on- and off-rates which enable cells to come out of circulation and roll on the blood vessel wall. The integrin family of adhesion molecules bind to immunoglobulin-like molecules which provide firm adhesion of the white blood cells to the blood vessel wall.

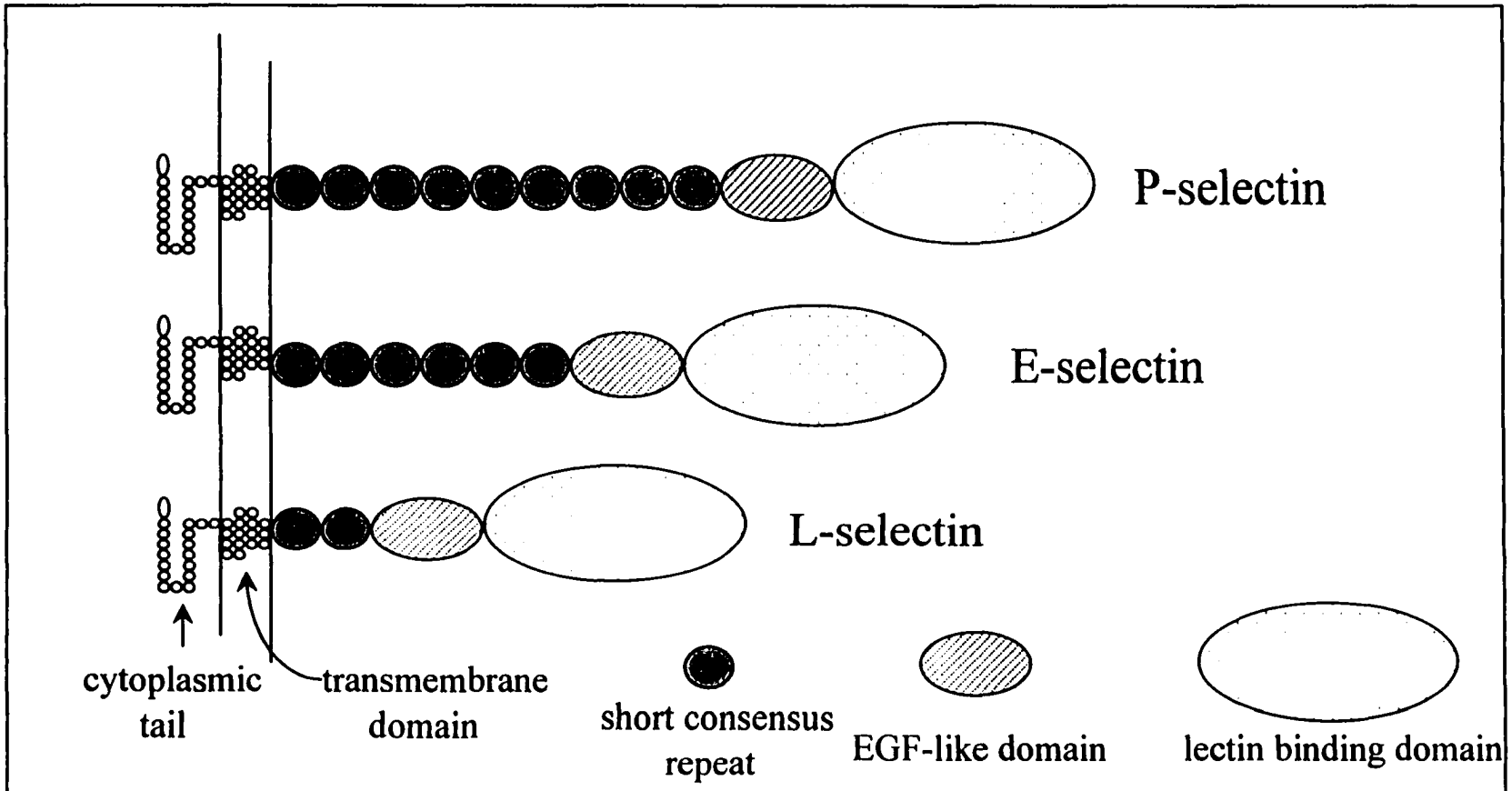


Figure 3. Selectin adhesion molecules. The three members of the selectin family of adhesion molecules are very similar in structure. Each selectin contains a signal peptide, a lectin domain, an epidermal growth factor (EGF)-like domain, a series of short consensus repeats (SCR), a transmembrane region, and a cytoplasmic tail. All the selectins bind to their ligands by a Ca^{2+}-dependent interaction that occurs in the lectin domain. The selectins bind glycoproteins that are generally sialylated, fucosylated, and sulfated.

influence the affinity and specificity of ligand interactions with the lectin domain under flow conditions (70).

Selectins recognize many types of carbohydrates (4, 5, 71-75). Generally the selectins bind to sialylated, sulfated, and/or fucosylated sequences found at nonreducing termini of N-linked or O-linked oligosaccharides or on glycospingolipids. The common feature of most of these is a lactosamine backbone of either type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc). The selectins bind with low affinity to these small sialylated and fucosylated oligosaccharides such as the tetrasaccharide sialyl Lewis x (76-94). The selectins bind more avidly to carbohydrates presented on a small number of specific glycoprotein ligands. Appropriate presentation of specific carbohydrate structures to lectin domains by membrane protein components may contribute to higher affinity binding of selectins to cellular ligands (5). This suggests that these serve as the physiologically relevant cell surface ligands for the selectins under shear force (70). All the selectins are able to bind to sialylated, fucosylated, and occasionally sulfated carbohydrates. However, the identification of high affinity glycoprotein ligands suggests that each selectin preferentially interacts with very specific ligand structures (17), but all may not be important for adhesion.

P-selectin

P-selectin is a rigid, highly extended structure that projects the lectin domain approximately 38 nm from the cell surface (70). P-selectin is contained in Weibel-Palade bodies of endothelial cells and in alpha granules of platelets (15, 95-97). P-selectin is

mobilized to the cell surface during fusion of the granule membrane with the plasma membrane within minutes after cell activation. Cell activation occurs by various thrombogenic and inflammatory mediators including thrombin, histamine, complement fragments, phorbol esters, calcium ionophore, oxygen-derived free radicals, and cytokines (45-49, 56, 58, 59). The surface concentration of P-selectin returns to basal levels after 20-60 minutes as a result of endocytosis (45, 98, 99). Endocytosis of P-selectin occurs through clathrin-coated pits and requires signals in the cytoplasmic domain (17). P-selectin is then recycled into newly formed secretory granules or degraded in lysosomes (17). It was recently shown that the interaction of the cytoplasmic domain of P-selectin with clathrin-coated pits enhanced the adhesion of leukocytes under flow. Neutrophils rolled on cells expressing internalization competent P-selectin better than on cells expressing internalization incompetent P-selectin. The interactions of the cytoplasmic domain with clathrin-coated pits may delay the dissociation of the bonds or prevent the molecule from being extracted from the cell (100).

P-selectin mediates the initial attachment and rolling of leukocytes to the blood vessel wall. The major ligand for P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1) which is constitutively expressed on leukocytes (76, 93, 101-104). P-selectin has also been shown to bind to CD24 on human carcinoma cells (105, 106) and certain glycolipids (107-110). P-selectin also mediates the adhesion of platelets to monocytes and neutrophils and the binding of activated B cells, a subset of T cells, and eosinophils to stimulated endothelium (111-117).

E-selectin

E-selectin is expressed on endothelial cells exposed to many inflammatory mediators including IL-1β, TNF-α, interferon-γ, substance P, thrombin, phorbol esters, oxygen radicals, and lipopolysaccharide (LPS) (15, 50-55, 59). E-selectin is expressed within the vasculature at sites of inflammation with maximum expression after 4-6 hours and requires de novo protein synthesis. E-selectin levels return to basal levels after 24-48 hours by degradation in lysosomes. It has been shown that E-selectin can bind to a single 150kDa sialoglycoprotein in lysates of murine myeloid cells (ESL-1) (118, 119) and to a 280kDa sialoglycoprotein in lysates of bovine γδ T cells (17). Other ligands for E-selectin are CD66 (120), β2 integrins (121), and L-selectin (122-124). E-selectin mediates attachment of neutrophils to activated vascular endothelium through interactions with unidentified ligands or possibly PSGL-1 (9, 101, 103, 125). Attachment of E-selectin to PSGL-1 occurs differently than P-selectin. E-selectin binding to PSGL-1 is only partially inhibited by an anti-PSGL-1 blocking antibody that blocks P-selectin binding, suggesting P-selectin and E-selectin bind to a related site on PSGL-1. E-selectin possibly binds to additional sites on PSGL-1. Neutrophils also attach more avidly and roll at lower velocities on P-selectin than on E-selectin. This implies that E-selectin possibly has a slower on-rate and a faster off-rate than P-selectin. Neutrophil attachment to P-selectin only requires PSGL-1; however, optimal attachment of neutrophils to E-selectin requires both PSGL-1 and L-selectin (126). E-selectin may also function as a tissue specific homing receptor for T cell subsets (15, 114-117).

L-selectin

L-selectin is constitutively expressed on many types of cells including thymocytes, B cells, T cells, neutrophils, monocytes, and eosinophils (127). L-selectin is expressed on the tips of microvilli in neutrophils. However, L-selectin is shed by proteolytic cleavage from the surface of neutrophils once they have been activated (128-132). L-selectin also binds to PSGL-1 (133, 134). This interaction mediates leukocyte-leukocyte interactions which amplifies the rate of cell accumulation in vitro (135-139). Other ligands for L-selectin include GlyCAM-1, which must be sulfated to bind L-selectin, CD34, MAdCAM, certain glycolipids, PNAd, and E-selectin (124, 140-144). GlyCAM-1 and CD34 are synthesized by peripheral lymph node high endothelial venules (HEV) and MAdCAM is synthesized by mucosal HEV (145-147). CD34 is constitutively expressed on most endothelial cells and hematopoietic stem cells. They all contain O-linked carbohydrates (3). GlyCAM-1 and CD34 both contain sulfated, sialylated, and fucosylated O-linked oligosaccharides and many serine and threonine residues which are potential sites for attachment of O-linked oligosaccharides.

P-selectin Glycoprotein Ligand-1 (PSGL-1)

P-selectin glycoprotein ligand-1 (PSGL-1) is a sialomucin consisting of repeating peptide motifs containing multiple serine/threonine-linked oligosaccharides (O-glycans) including poly-N-acetylactosamine terminating in the sialyl Lewis x structure (Figure 4) (76, 93, 148-151). Selectins bind with higher affinity to these glycoproteins than to oligosaccharides alone. PSGL-1 is a type 1 membrane protein with two identical 120kDa disulfide-linked subunits consisting of a NH_2-terminal signal peptide, an extracellular

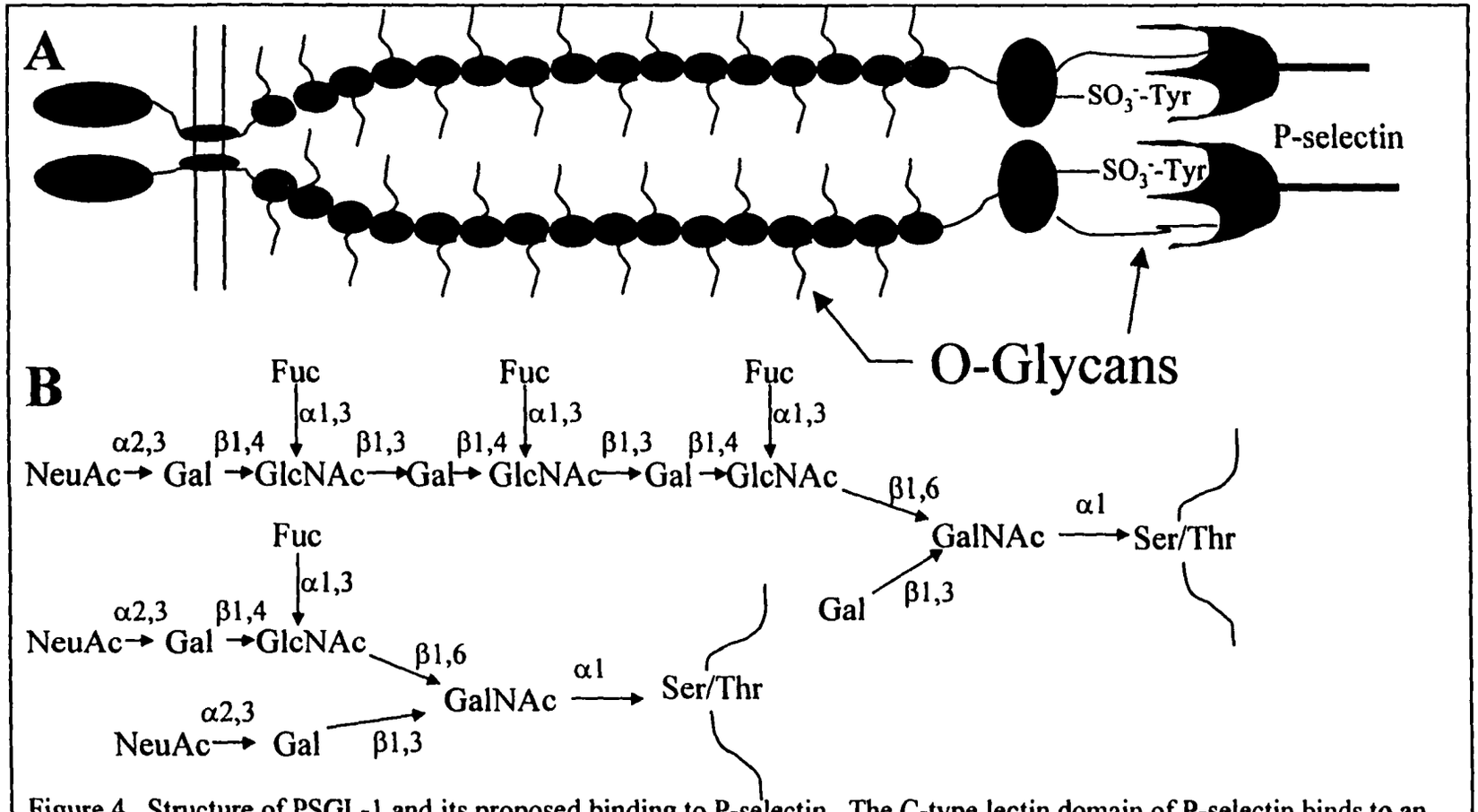


Figure 4. Structure of PSGL-1 and its proposed binding to P-selectin. The C-type lectin domain of P-selectin binds to an NH_2-terminal sLex-containing core-2 O-glycan and a tyrosine sulfate residue of PSGL-1. The 16 decameric repeats, rich in serines and threonines, are represented by the shaded ovals (●). The serine/threonine-linked oligosaccharide structures (O-glycans) are shown in B. The structures of the two fucosylated, core-2 O-glycans on PSGL-1 were derived from HL-60 cells. (Figure adapted from McEver and Cummings, 1997).

domain made of 16 decameric repeats that are rich in serines, threonines, and prolines. PSGL-1 extends approximately 60 nm above the cell surface (152). Tyrosine sulfation possibly occurs on three NH_2-terminal tyrosines located in an anionic consensus sequence (20).

The expression of PSGL-1 is widely distributed (153). It is expressed primarily on hematopoietic cells (104, 153) on the tips of microvilli (76, 93, 104, 154). PSGL-1 is found on myeloid cells (104) and almost all leukocytes. However, it is found at lower levels on B cells and only a subset of T cells (104, 114, 155, 156). PSGL-1 is expressed on circulating dendritic cells, on tissue monocyte-derived dendritic cells, and on some dendritic cells in lymphoid organs. CD43+ stem cells and some epithelial cells also express PSGL-1 (153, 157).

The binding of PSGL-1 to the different selectins is very specific. The binding of PSGL-1 to P-selectin requires that PSGL-1 is modified with α2,3-linked sialic acid and α1,3-linked fucose (6, 76, 91) on core-2 O-glycans as illustrated in Figure 4 (158, 159). The tyrosine residues in human PSGL-1 can also be sulfated (148, 160). The sulfation of one or more of these residues is required for P-selectin binding (160-162). The reason PSGL-1 binding to P-selectin is dependent on specific O-glycosylation and tyrosine sulfation is not known. PSGL-1 has been shown to bind to L-selectin (133, 134, 137). It appears that L-selectin and P-selectin bind similar NH_2-regions of PSGL-1. L-selectin also requires tyrosine sulfation and O-glycans (133, 134, 137, 163). The binding sites on PSGL-1 for E-selectin are uncharacterized (101, 126).

PSGL-1 is the major selectin ligand in the immune response since it can bind to L-selectin, P-selectin, and possibly E-selectin (101, 103, 104, 133, 134). The three selectin molecules and PSGL-1 contribute greatly to the inflammatory response due to their ability to bind under shear stress, their expression on endothelial cells, and their expression on various circulating cells (leukocytes and platelets). Figure 5 shows the many interactions mediated by PSGL-1 in cell recruitment.

PSGL-1 is the only high affinity ligand for P-selectin on leukocytes and is responsible for the initiation of leukocyte tethering and rolling on the endothelium (134). Blocking PSGL-1 with an anti-PSGL-1 blocking antibody eliminates neutrophil, eosinophil, and mononuclear cell tethering and rolling on P-selectin (104). Since platelets express P-selectin upon activation, leukocytes can also bind and roll on platelets or use platelets as a 'bridge' for cell-cell interactions providing another mechanism for leukocytes to be brought to the endothelium. After rolling neutrophils become activated, PSGL-1 undergoes a cytoskeletal-dependent redistribution to the uropods of polarized cells (154, 164, 165). This allows the P-selectin interaction to be weakened giving the integrins control of the adhesion (164, 165). The interaction of PSGL-1 with L-selectin has been shown to mediate leukocyte-leukocyte interactions which may amplify the rate of cell accumulation (135-139).

CELL-CELL INTERACTIONS

As shown in Figure 5, leukocytes also have the ability to interact with other leukocytes. Recent data has suggested leukocyte-leukocyte interactions could possibly be another

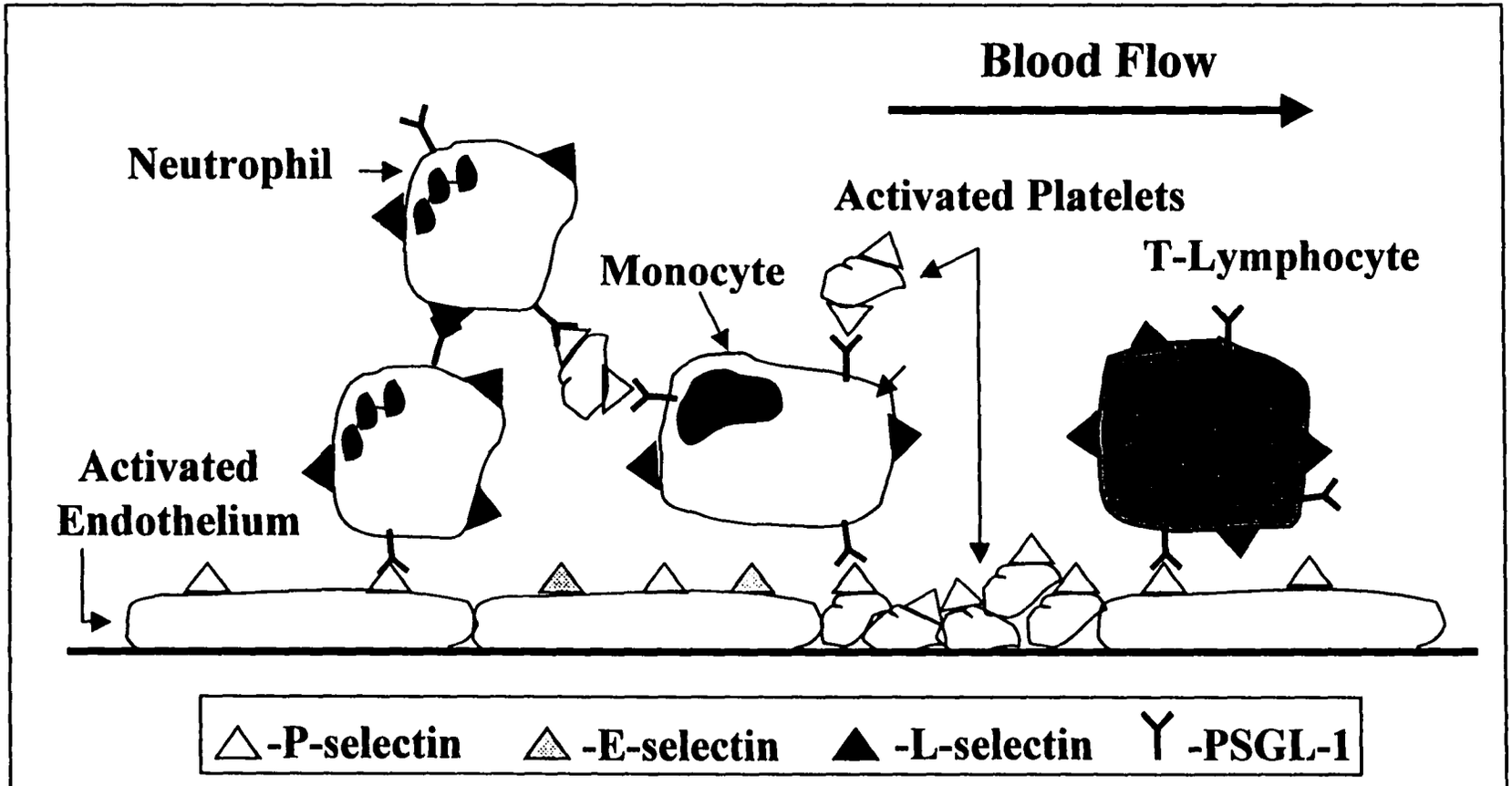


Figure 5. Cell-cell interactions mediated by PSGL-1 binding to P-selectin and L-selectin. Leukocytes expressing PSGL-1 initially attach and roll on activated endothelial cells and platelets by binding to P-selectin. Leukocyte-leukocyte interactions mediated by PSGL-1 and L-selectin may enhance cell recruitment at a site of injury or infection on the blood vessel wall. Activated platelets expressing P-selectin may also act as a bridge between PSGL-1-expressing cells to aid in the recruitment of cells to a site of inflammation.

important mechanism in the recruitment of cells to a site of inflammation (137-139, 166). The ability of bound leukocytes to bind to and bring flowing leukocytes to the surface enhanced or accelerated the accumulation of cells. Accumulation of leukocytes through these cell-cell interactions in vitro results in the formation of lines or strings of cells on the surface. This occurs because a flowing cell attaches to the upstream end of a bound cell and is pushed to the downstream end of the cell where it comes in contact with the surface. The line or string is extended by flowing cells attaching to bound cells at the upstream end of the line followed by the cells rolling on the surface of the bound cells before depositing on the surface at the end of the string. Once enough cells are bound to the surface, the resulting monolayers can support rolling of leukocytes on the surface of the bound cells (166, 167). It has been established that L-selectin and β2 integrins are the receptors that mediate neutrophil aggregation (135, 136, 163, 168), and the major ligand for leukocyte-leukocyte interactions is PSGL-1 (137, 163). Neutrophil aggregations or interactions can occur under flow conditions by the binding of L-selectin with its ligand PSGL-1. However, once neutrophils are activated and the L-selectin is shed from the surface, neutrophil aggregation occurs through β2 integrins that are expressed after activation. The role of cell-cell interactions is most likely physiologically relevant in enhancing accumulation of cells at a site once the selectin receptors on the endothelium are blocked by rolling or firmly attached cells. When the endothelium has become covered with cells the ability of leukocytes to roll on adherent leukocytes would allow the recruitment of cells to continue if this phenomenon occurs in vivo as it does in vitro.

LEUKOCYTE ACTIVATION

Circulating leukocytes express integrins in an inactive or low avidity state that do not bind or bind minimally to their ligands (24). Rolling leukocytes become activated by many mechanisms that produce changes in leukocyte integrin avidity. A conformational change in the integrin heterodimer results in greater affinity for ligands (169-172). β1 and β2 integrin-dependent adhesion is increased as a result of signals transduced by the binding of chemokines (IL-8, IL-3, TNF-α, MCP-1, MCP-1β), cytokines (GM-CSF, IL-5), or chemoattractants (C5a, FMLP) to receptors expressed on leukocytes (24, 173-181). These activating substances are produced by local tissue cells, infiltrating leukocytes, microorganisms, or the endothelium. Surface-expressed endothelial platelet activating factor (PAF) also stimulates neutrophil adhesion by activating β2 integrins (46, 47, 182, 183). β2 integrins on lymphocytes are activated by GlyCAM-1 binding to L-selectin (184). Activation can result in certain integrins contained in intercellular stores being released and expressed on the cell surface. This increases the binding capability of the cell due to a higher site density of receptors present on the surface. Leukocyte integrin activation can also occur after receptor binding due to crosslinking of certain surface proteins. Crosslinking results in some integrins binding more avidly or produces an increase in the surface expression of other integrins. Increased integrin avidity due to crosslinking is referred to as inside-out signaling (185-188).

FIRM ADHESION

Integrins

The receptors involved in firm adhesion of an activated leukocyte to the vessel wall are integrins and members of the immunoglobulin (Ig) superfamily (Figure 2B). Integrins consist of noncovalently linked, heterodimeric α and β chains. They are transmembrane cell surface proteins that bind to cytoskeletal proteins and communicate extracellular signals (39-43). Binding of the various α chains differs with respect to the necessity of divalent cations (42). The leukocyte β2 integrins share the common β chain CD18. Expression of β_2 integrins occurs only on leukocytes; however, the distribution of CD11/CD18 differs among the subtypes of leukocytes. Peripheral blood lymphocytes express primarily CD11a/CD18 (LFA-1) while neutrophils, monocytes, and natural killer (NK) cells express all three of the β2 integrins. CD11b/CD18 (Mac-1) and CD11c/CD18 (p150,95) are contained in intracellular storage pools in neutrophils and monocytes. Upon cell activation by calcium ionophore, phorbol esters, FMLP, GM-CSF, C5a, TNF-α, and LTB4, the surface expression level of CD11b/CD18 and CD11c/CD18 is increased (130, 189). The binding of neutrophils to E-selectin has also been shown to increase the expression of CD11b/CD18 (190). The ligands for the leukocyte β2 integrins that promote firm adhesion are proteins expressed by cells in the Ig-superfamily (ICAM-1, ICAM-2, ICAM-3) (191). Soluble proteins (fibrinogen, factor X) and complement fragments also bind β2 integrins (192, 193). The binding of neutrophils and monocytes to endothelial cells is mostly controlled by CD11a/CD18 and CD11b/CD18 while CD11a/CD18 is more involved in the adhesion of lymphocytes to the endothelium (192, 194-203).

The β1 integrins share CD29 as their common β subunit. The β1 integrins are widely distributed and are receptors for extracellular matrix proteins which include fibronectin, collagen, laminin, and vitronectin (24, 204). The integrin α4β1 (VLA-4, CD49d/CD29) is involved in lymphocyte, monocyte, eosinophil, basophil, and NK cell adhesion to cytokine-activated endothelial cells (205-213). VLA-4 has recently been shown to mediate initial cell tethering and rolling on endothelial cells (31-34). The expression of α4β1 differs among leukocytes. It is the major β1 integrin expressed on resting B and T lymphocytes. Resting B cells also express small amounts of α2β1 and α3β1 on the surface. T lymphocytes also express α4β1 and α5β1 and only α1β1 and α2β1 when the cells become activated (204). It has been shown that binding of T cells to endothelial PECAM-1 increases the function of β1 integrins (214). Monocytes express α2β1, α4β1, α5β1, and α6β1 (204, 215). Ligands for α4β1 include VCAM-1, extracellular matrix proteins fibronectin and thrombospondin, and the bacterial outer membrane protein invasin (205, 216-222). The β7 subunit is also expressed on T cells and B cells (223-225). VCAM-1, fibronectin, and MAdCAM-1 are all ligands for α4β7 (223, 226-231).

Immunoglobulin-like Receptors

The immunoglobulin (Ig) superfamily of adhesion receptors are important integrin ligands involved in the firm adhesion and emigration of leukocytes (232). ICAM-1 (CD54), ICAM-2 (CD102), and vascular cell adhesion molecule-1 (VCAM-1, CD106) are all integrin ligands expressed on the endothelium. ICAM-1 is a ligand for CD11a/CD18 with the binding site located in the NH_2- terminal first domain of ICAM-1 (233). ICAM-1 is expressed on leukocytes, fibroblasts, epithelial cells, and endothelial

cells. ICAM-2 is constitutively expressed on the endothelium and is a ligand for CD11a/CD18. ICAM-2 is not subject to upregulation by cytokines (TNF-α, IL-1, IFN-γ) or lipopolysaccharide (LPS) (234-237). The other ligand expressed on the endothelium, VCAM-1, binds α4β1 and α4β7. Both ICAM-1 and VCAM-1 are transcriptionally regulated by cytokines (TNF-α, IL-1), LPS, thrombin, polyinosinic acid, and other mediators of inflammation (51, 54, 60, 238-245). Therefore, the induced expression of VCAM-1 and ICAM-1 is largely dependent on synthesis of new mRNA and protein (246, 247). ICAM-1 upregulation and expression is also induced by phorbol esters (52, 245), hypoxia/reoxygenation, the generation of oxygen radicals (55, 248, 249), TNF-β (54), lysophosphatidylcholine (250), and INF-γ (54). VCAM-1 expression is also induced by lysophosphatidylcholine (250) and IL-4 (243, 251). ICAM-1 expression peaks at 12 hours and VCAM-1 expression peaks at 6 hours on TNF-γ stimulated endothelial cells. The expression of both persists for 72 hours after induction (50, 239, 243-244, 252). MAdCAM-1 is also a member of the Ig-superfamily. It is expressed on lymphoid high endothelial venules and is involved in lymphocyte emigration by binding L-selectin and α4β7 (228, 230, 253-256).

CELL EMIGRATION

The integrins interacting with their Ig-superfamily ligands mediate the firm adhesion of activated, rolling leukocytes to the vessel wall. This allows the leukocytes to emigrate or transmigrate between the endothelial cells to the extracellular matrix (257). A member of the endothelial Ig-superfamily, platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31), is a major mediator in leukocyte emigration (214, 258, 259). PECAM-1 is also

involved in homotypic adhesion of endothelial cells (260), binding of platelets to myeloid cells (261), and may transduce activating signals to leukocytes. PECAM-1 is found on endothelial cells, platelets, and some leukocytes (258, 260, 262, 263). On the endothelium, it is located at intercellular junctions. The emigration of neutrophils and monocytes is believed to occur by binding of leukocyte PECAM-1 to endothelial PECAM-1 (264). Emigration of neutrophils is promoted by endothelial-associated platelet activating factor (PAF) (46, 47, 182, 183) and endothelium-derived IL-8 (183, 265, 266).

CD11a/CD18 and CD11b/CD18 are also involved in the emigration of cells in response to a chemotactic gradient (267). Once a cell has emigrated between endothelial cells to the subendothelial matrix, the cell continues to migrate through the extracellular matrix in response to various chemoattractants that are released by the tissue. Chemoattractants include peptides like C5a and formyl peptides that activate phagocytosis and the chemokine family of chemoattractant proteins (268) that can attract leukocyte subtypes preferentially. The presence of IL-8 recruits neutrophils and lymphocytes while the chemokine RANTES recruits monocytes, eosinophils, and memory T cells. Monocytes also migrate in response to MCP-1 (24, 268).

SELECTIVE LEUKOCYTE RECRUITMENT

In many cases subtypes of leukocytes are predominantly found at a site of infection or injury. For example, eosinophils are found in extravascular sites of allergic reactions and memory T cells are found in synovial tissue in rheumatoid arthritis and in skin involved

with inflammatory dermatoses (24, 269, 270). This type of selective recruitment of leukocytes is largely due to the many activating molecules in the system. Upregulation of certain adhesion proteins is affected by several agonists while other agents are specific for one protein. Also, combinations of cytokines can modulate induction of different endothelial proteins. The expression of certain adhesion proteins or combinations of adhesion proteins can recruit specific leukocytes by preferentially binding to those subtypes (7, 8, 24).

Leukocyte subtypes also respond differently to the many chemoattractants released by the tissue. Selectivity can further occur at the chemoattractant level (174, 175, 271-274). Neutrophils and monocytes bind to PSGL-1, P-selectin, and E-selectin; however, if IL-8 is the major local chemoattractant, neutrophil accumulation should predominate. Monocytes will predominate in a situation where MCP-1 is the major chemoattractant. (24, 268).

SIGNALING THROUGH SELECTINS, PSGL-1, AND INTEGRINS

Recently a lot of effort has been put into determining the extent to which the selectins, PSGL-1, and the integrins act as signaling molecules (1, 2, 28, 39-41, 43, 275-286). It has been shown that crosslinking of L-selectin transmits signals into both myeloid and lymphoid cells (184, 287-294). Binding of monoclonal antibodies to L-selectin expressed on neutrophils induced an increase in intracellular Ca^{2+}, augmentation of the respiratory burst, production of oxygen radicals, and an increase in Mac-1 surface expression (292, 295-297). Sulfatides binding to L-selectin can also induce increases in

mRNA for TNF-α and IL-8 (296). L-selectin binding also signals the activation of MAP kinase and tyrosine phosphorylation (291). Signaling through L-selectin is possibly regulated through cytoskeletal associations. Crosslinking of L-selectin also directly upregulates the adhesive activity of Mac-1 integrins on neutrophils (294). E-selectin has been shown to function as a signaling molecule. Mac-1 adhesive activity is upregulated by the binding of neutrophils to E-selectin (190).

PSGL-1 also has signaling capabilities. Binding of PSGL-1 on monocytes to P-selectin on activated platelets induces superoxide anion release, enhances PAF synthesis and phagocytosis, and induces the expression of tissue factor (298-300). This interaction also costimulates secretion of MCP-1, TNF-α, IL-8 (282), and is associated with the nuclear translocation of NF-κB (301, 302) which is a widely expressed transcription factor involved in the expression of inducible genes involved in inflammation (303, 304). This translocation of NF-κB causes monocytes to respond to the chemokine RANTES (283, 302).

Integrins are capable of regulating gene expression, cell growth, differentiation, and survival (285, 305-307). Outside-in signaling results from integrin-ligand binding which controls cell shape, the organization of the cytoskeleton, and generates a variety of biochemical signals. The signaling through integrins is mediated by the integrin cytoplasmic domain (308-315). Integrin signaling may also involve the association of integrins with other transmembrane proteins (316, 317).

PLATELETS and PLATELET MICROPARTICLES

Platelets are approximately 2 μm in diameter and are an important cellular component of blood that helps regulate blood clot formation. Platelets play a vital role in hemostasis and inflammation. Figure 6 shows some of the many roles of platelets in inflammation and hemostasis. After a blood vessel is injured, circulating platelets bind to the damaged area and produce a 'plug' in the vessel wall to control the bleeding (318-320). Platelets quickly (within seconds) become activated and secrete the contents of their granules. Once activated, the platelets spread out over the damaged area and recruit more platelets to form a thrombus. This platelet 'plug' provides an activated surface which accelerates coagulation. The platelets are stabilized in the clot by fibrin (321). These initial coagulation events are mediated by surface receptors. The glycoprotein (GP) Ib-IX-V complex mediates the initial attachment of platelets to the exposed subendothelial matrix that contains its receptor, von Willebrand Factor (vWF) (322). GP Ib-IX-V is also involved in platelet activation by providing a binding site for the agonist α-thrombin (323, 324). vWF is a multimeric adhesive glycoprotein made by endothelial cells and megakaryocytes. vWF is stored in Weibel-Palade bodies of endothelial cells and in α-granules of platelets and is secreted into the extracellular matrix (321). Binding of platelets to vWF initiates signal transduction leading to platelet activation. This binding initiates intracellular signaling pathways, calcium influx, and the calcium-dependent activation of the platelet membrane aggregation receptor for fibrinogen (αIIbβ3 integrin, GP IIb-IIIa) (323, 325-328). Platelets are also able to adhere to the subendothelial matrix under shear by binding to exposed collagen and fibrinogen (329).

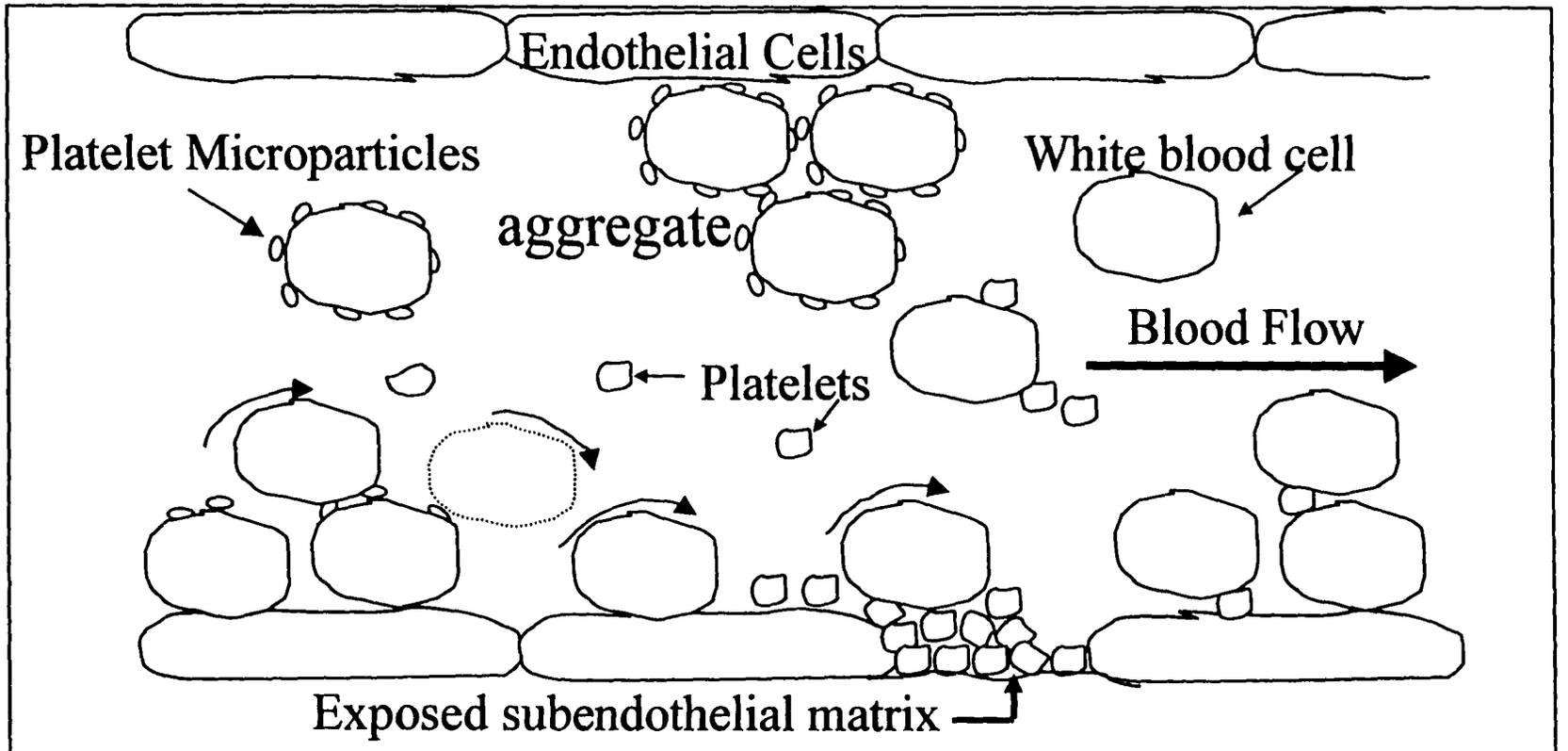


Figure 6. Roles of platelets and platelet microparticles in inflammation and hemostasis. Platelets primary function is clot formation to control bleeding. Platelets plug the area of the vessel exposed to the subendothelial matrix by binding to various exposed molecules. Platelets are also capable of aiding in cell recruitment by producing a receptor bridge between a circulating cell and the blood vessel wall. Platelet microparticles released from activated platelets are also possibly involved in cell recruitment by producing cell-cell interactions. The microparticles express P-selectin which binds to PSGL-1 on various inflammatory cells. Therefore, cells coated with microparticles can attach to cells already bound to the surface providing an additional cell accumulation mechanism.

Platelets stimulated by inflammatory mediators (vWF, collagen, α-thrombin) experience shape changes, adherence, liberation of arachidonate metabolites, release of granule contents (activation), and platelet aggregation (330, 331). Platelets are activated by a variety of substances including ADP, adrenaline, 5-hydroxytryptamine, thrombozane A_2, PGG_2, thrombin, and collagen. The release of granular material from the platelet storage pools is calcium-dependent and involves the transport of granules by actin and myosin microfilaments, and the fusion of the granular membrane with the plasmalemmus membrane (331, 332). Activation and/or aggregation of platelets saturates the endothelial lining with the substances stored in the organelles of the cell such as spasmogens, chemotactic factors, proteolytic enzymes, and superoxide anion (333-335) and are also capable of signaling chemokine synthesis by human monocytes (302).

Platelets participate in inflammation mainly through the release and expression of regulatory molecules. Platelets contain many active substances that participate in the inflammatory process (333). The substances released by platelets upon activation include spasmogens (5-hydroxytryptamine, platelet activating factor, thromboxane A_2, cationic proteins), chemotactic factors (platelet factor 4, growth factors, lipoxygenase products), and proteolytic enzymes. More recently, the role and importance of platelet-derived growth factor (PDGF), selectins, and platelet histamine has been reviewed (331). The release of PDGF and histamine and the expression of P-selectin during activation leads to many events involved in inflammation (96, 97). Activated platelets induce superoxide anion release by monocytes and neutrophils through P-selectin (299). It has also been

shown that P-selectin regulates platelet activating factor synthesis and phagocytosis by monocytes (300).

PDGF is stored in alpha granules of platelets and is released upon exposure to platelet-activating substances such as thrombin, collagen, and adenosine diphosphate during blood clotting and in inflammatory vascular lesions (336). PDGF is a chemotactic factor for fibroblasts, vascular smooth muscle cells (VSMC), and monocytes. PDGF also induces the proliferation of these cells. PDGF makes eosinophils produce superoxide anion (O_2) and inhibits platelet aggregation by acting on platelet PDGF-α-receptor.

Platelets are also involved in the inflammatory process through cell-cell interactions and cell recruitment (Figure 6). Platelets are able to roll on activated endothelial cells aiding in the recruitment of platelets to the site (337). Once a layer of activated platelets (which express P-selectin) is formed in the vessel, they can support rolling and extravasion of leukocytes (338-343). Platelets are also capable of bringing cells to the vessel surface by acting as a P-selectin 'bridge' between the cell and the vessel wall (84, 344-346). The ability of activated platelets expressing P-selectin to bind to leukocytes might be important in enhanced cell accumulation including cell aggregation and thrombus formation (347-349). Substances released by leukocytes are able to induce platelet activation (350). This interaction may play a role in thrombosis and inflammation.

Activated platelets release membrane fragments approximately 0.1 μm to 0.8 μm in size from the plasma membrane by a budding process. These platelet microparticles express

functional receptors including GP Ib, IIb, IIIa, and P-selectin (351, 352). The platelet microparticle surface also expresses negatively charged phospholipids, platelet factor 3 and receptors for coagulation factors Va and Xa, and provides a catalytic surface for the prothrombinase reaction (353-359). This high procoagulant activity on their surface suggests that platelet microparticles aid in the local activation of coagulation (353, 360-368) and accelerates clotting near a site of activation (365, 369-370). Platelet microparticle generation is induced by mediators such as thrombin, collagen, calcium ionophores, and complement proteins (351, 362, 371, 372). High shear stress has also been shown to produce platelet microparticles (353, 373). The concentration of platelet microparticles in normal circulation is reported to be anywhere from $5x10^5$-$5x10^8$/ml (362, 374). However, it is found that platelet microparticle concentrations are elevated in many diseases and disorders (375-380). High platelet microparticle concentrations are found in patients undergoing a cardiopulmonary bypass (362, 366, 381), in acute respiratory distress syndrome (366), in patients with idiopathic thrombocytopenic purpura (352, 382-384), unstable angina (385), transient ischemic attacks (386), atherosclerosis in diabetes mellitus (356, 387), activated coagulation fibrinolysis (353, 388), autoimmune thrombocytopenias (353, 389-391), small vessel cerebrovascular accidents (CVA) (370, 386), and acute coronary syndromes (ACS) (392). The function of platelet microparticles in the body has not been clearly defined. One possible mechanism is shown in Figure 6. Platelet microparticles that bind to leukocytes could enhance cell accumulation through cell-cell interactions by producing a P-selectin 'bridge'. Platelet microparticles might also cause increased cell aggregation when levels of platelet microparticles are such that cells become nearly saturated with the microparticles (393).

SOLUBLE ADHESION MOLECULES

Adhesion molecules have also been shown to exist in circulation in soluble forms which are biologically active (394-403). P-selectin, L-selectin, E-selectin, ICAM-1, and VCAM-1 are among the soluble adhesion molecules that possibly play a physiological role in adhesion events. These molecules become soluble by being released following cellular events which leads to proteolytic cleavage or membrane vesiculation (397). For example, soluble P-selectin results from platelets shedding membrane P-selectin from the surface (404). The concentration of these soluble molecules in normal circulation are as follows: ICAM-1 (271 +/- 27 ng/ml), VCAM-1 (615.8 +/- 215.8 ng/ml), P-selectin (168.8 +/- 116.1 ng/ml), E-selectin (35.3 +/- 15.2 ng/ml), and L-selectin (1658.8 +/- 335.3 ng/ml) (399, 400). It has been suggested that these soluble molecules can regulate adhesion. They possibly could act as competitive inhibitors which would down-regulate the response or they could be involved as co-signaling factors which would up-regulate the response (405, 406).

ROLE OF ADHESION MOLECULES IN DISEASES

The interactions between leukocytes and the endothelium mediated by various adhesion molecules are necessary events in host defense. However, the recruitment of cells at abnormal times leads to various autoimmune diseases and pathological inflammation disorders (394, 407-412). Also, the lack of cell recruitment greatly impairs the body's host defense system. The absence or reduced expression of β2 integrins results in the absence of neutrophils at a site of infection. This is referred to as leukocyte adhesion deficiency (LAD) (413-416). Without the β2 integrins, neutrophils are unable to form

firm adhesions with the blood vessel wall and emigrate into the tissue in response to an infection (407, 417). LAD is characterized by severe life threatening infections, chronic neutrophilia, and impaired wound healing (407). Another type of LAD prevents leukocyte accumulation due to the absence of fucose on the sialyl Lewis x residues. The neutrophils, therefore, cannot bind to E- and P-selectin (408, 418, 419). This is also characterized by recurrent infections, failure to form pus, gingivitis, and pronounced neutrophilia (407, 420).

Abnormal or excessive recruitment of cells from leukocyte-endothelial interactions can generate pathologic inflammation in various conditions including reperfusion injury after ischemia, asthma, allergic responses, and graft rejections (407, 415, 421-426). Diseases characterized by acute inflammation with infiltration of neutrophils are often associated with increased expression of E- and P-selectin (407, 415). The increased expression of ICAM-1 and VCAM-1 characterize chronic conditions (415, 427). The over expression of E-selectin, P-selectin, ICAM-1, and VCAM-1 are responsible for or involved in acute appendicitis (415), rheumatoid arthritis (428-431), acute lung injury (4, 432-434), extrinsic asthma (4, 435, 436), the spreading of cancer (4, 105, 437-440), reperfusion injury (98, 441-446), vasculitis (407, 412, 447-450), atherosclerosis (399, 407, 412, 451-454), renal and hepatic diseases (407, 412, 455-464), acute respiratory distress syndrome (98, 465, 466), and thrombotic disorders (98, 467-474). L-selectin has also been reported to be associated with insulin-dependent diabetes and kidney allograft rejection (15, 456, 475). Chronic lymphocytic infiltration seen in sarcoidosis and graft rejection results from the over expression of VCAM-1 (401, 415, 476, 477). The serum levels of the soluble

forms of these adhesion receptors are also increased in several inflammatory and autoimmune diseases (394, 399, 400, 478-493). Detecting these increased soluble receptor levels in circulation may provide information regarding the prognosis and inflammatory process of certain diseases (24, 394, 399, 400). The plasma levels of these soluble receptors are also increased in thrombostic or coagulation disorders (397, 399, 494). Diseases or disorders that have been reported to show an increase in the concentration of soluble E-selectin include sepsis, Kawasaki disease, scleroderma, systemic lupus erythematosus (394, 400, 495-501). Circulating soluble P-selectin concentrations are elevated in haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura (394, 397, 400). These increased levels may reflect the state of inflammation in various inflammatory disorders (400). Soluble P-selectin has been shown to prevent activated neutrophil adhesion to endothelial cells (400, 405).

The recent discovery of the involvement of selectins in various diseases and disorders has led to the investigation of selectin and immunoglobulin related therapies to control the abnormal inflammatory response. Various strategies are used in the attempt to control or prevent abnormal leukocyte-endothelial interactions. Monoclonal antibodies against different adhesion molecules, soluble adhesion receptor proteins that compete for ligands, small peptides that are specific for the binding site of adhesion receptors, and saccharides that block selectin-mediated rolling are all agents that have been used to diminish the effect of over expressed adhesion molecules (492, 502-512). Efforts have also been made to block the signaling pathway that leads to the over expression of the adhesion molecules (407).

Animal models have shown that these therapeutic agents can greatly inhibit the progression of the inflammatory response (15, 513-522). Ischemia and reperfusion produce tissue and organ damage. This occurs in a variety of clinical disorders including stroke, myocardial infarction, organ transplantation, and organ hyperfusion. Antibodies to β2 integrin and P-selectin or soluble carbohydrates to prevent selectin function decreased edema and necrosis in rabbit models (15, 407, 523). It has been reported that using anti-L-selectin antibodies diminished ischemia and reperfusion injury in rabbit models and decreased neutrophil accumulation in ischemic reperfused cat myocardium (15, 517, 523). The use of P-selectin antibodies protected feline heart and endothelium during myocardial ischemia and reperfusion injury (15). Myocardial reperfusion injury in cats was prevented by the use of an oligosaccharide containing sialyl Lewis x (15, 524). Sialylated oligosaccharides have also been effective in reducing immune complex-induced and P-selectin-dependent acute lung injury in rats (15, 525, 526). Studies done with primates showed that E-selectin antibodies inhibited neutrophil accumulation in delayed-type hypersensitivity reactions in skin (15).

Many other disorders have been treated using adhesion molecule antibody or oligosaccharide therapy. Antibodies to ICAM-1 reduced tissue infiltration and damage caused by neutrophils (415, 527) and helped prevent graft rejection and rheumatoid arthritis (415, 528). Human renal, cardiac, and liver transplant rejections show an increased expression of endothelial adhesion molecules (407, 529). Corticosteroid therapy of acute liver allograft rejection has been used to reduce ICAM-1 expression (415, 530). Infusions of sialyl Lewis x prevented neutrophil-mediated lung injury which

is similar to adult respiratory distress syndrome (415, 526). Anti-VLA-4 antibodies have been shown to reduce T-cell infiltration to the brain thus slowing autoimmune encephalitis (415, 531). Asthma is partially due to the accumulation of neutrophils and eosinophils. E-selectin, VLA-4, and ICAM-1 antibodies help reduce airway hyperreactivity and bronchoalveolar eosinophilia (407, 415, 518, 532).

During the past few years much has been learned about leukocyte-endothelial adhesion and the cell recruitment process. Identifying some of the major components of this system has provided many therapeutic strategies which have proved beneficial in many of the diseases and disorders associated with the abnormal accumulation of cells in tissue. It is still crucial to develop a more complete understanding of the complex cascade of events of the inflammatory response in order to continue developing new therapies to prevent or reduce abnormal responses which lead to the various diseases and disorders.

Chapter 2: Primary and Secondary Accumulation of HL-60 Cells on P-selectin Bilayers

Introduction

The ability of circulating cells to attach and roll on the blood vessel wall is a necessary process in the recruitment of cells to a site of injury or infection. There are many parameters that affect the accumulation of cells at a site of inflammation. Variations in the shear stress or the site density of the adhesion receptor could reduce or enhance the recruitment of cells. Cell attachment assays were studied at various shear stresses and adhesion receptor site densities to determine the effect of shear stress and site density on cell attachment, cell accumulation, cell rolling, and cell accumulation through cell-cell interactions. The initial rolling event was recreated in a simplified system using a parallel plate flow chamber that mimics the body's fluid mechanical environment. P-selectin, expressed by activated endothelial cells on the blood vessel wall, was incorporated into a lipid bilayer to provide a surface in which the P-selectin site density could be controlled. HL-60 cells (a human promyelocytic leukemia cell line) which express PSGL-1 (the P-selectin ligand found on white blood cells) and human neutrophils were used for the attachment assays. The attachment of HL-60 cells and neutrophils to the P-selectin surface was studied at various shear stresses and P-selectin site densities to determine the effect of shear stress and site density on cell accumulation, cell rolling, and cell-cell interactions.

Materials and Methods

Materials

L-α phosphatidylcholine Type X-E from dried egg yolk, TRIZMA® Hydrochloride (Tris-HCl), n-octyl β-D-glucopyranoside, heparin sodium salt from porcine intestinal mucosa, Dextran70, penicillin G sodium, human serum albumin without sodium azide (HSA), Histopaque1077®, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), Ca^{2+} and Mg^{2+} free HBSS (CMF HBSS), RPMI 1640, streptomycin sulfate, and sodium pyruvate were obtained from Gibco Life Technologies (Grand Island, NY). The 24-well tissue culture plates, Spectra/Por® cellulose ester dialysis membrane tubing (3500 MWCO), and the microscope glass cover slides were obtained from Fisher Scientific (Dallas, TX). P-selectin purified from platelets (92) was a generous gift from Dr. R. McEver (University of Oklahoma, Oklahoma City, OK).

Antibodies

Function blocking anti-PSGL-1 antibody PL1 and nonblocking anti-PSGL-1 antibody PL2 were provided by Dr. R. McEver. The anti-human P-selectin blocking antibody G1 and nonblocking antibody S12 were also provided by Dr. R. McEver.

Flow Chamber and Attachment of Glass Coverslips

All experiments done under flow were performed in a parallel plate flow chamber similar to the one in Figure 7. A glass coverslip coated with an antibody, a receptor molecule, or a lipid bilayer containing a receptor was attached to the flow chamber and held in place

by an applied vacuum. Attachment of the glass cover slide to the flow chamber was done underwater to prevent air from contacting the lipid bilayer, antibody, or the receptor coated slide. This was done by placing the cover slide on supports at the bottom of a large petri dish once it was filled with water. The flow chamber was submerged in the large petri dish and the cover slide was moved from the supports onto the flow chamber. The vacuum hose was connected to the flow chamber to secure the glass slide to the flow chamber while under water. The flow chamber was mounted on an inverted phase contrast microscope (Nikon). The cell suspension was withdrawn through the flow chamber at room temperature at a controlled shear stress by a syringe pump (Cole-Parmer 74900). The shear stress (dynes/cm^2) for the flow chamber was calculated using $\tau = (6Q\mu/B^2W)(1\ min/60\ sec)$, where Q is the flow rate (ml/min), μ is the viscosity (0.01 g/cm s), B is the height of the flow channel provided by the gasket (0.0254 cm), and W is the width of the flow channel (1.1 cm). One field of view per experiment was video taped with a video camera (Dage MTI 72SCCD) and VHS recorder (Panasonic AG-5700) allowing the experiment to be analyzed at a later time. A time bar was added to the recorded video tape using a Horita TG-50 time stamp generator. The complete experimental setup is shown in Figure 8.

Preparation of Lipid Vesicles

Egg phosphatidylcholine (5 mg) dissolved in chloroform was dried under nitrogen in a test tube to produce a thin lipid film on the test tube wall. Octyl-β-D-glucopyranoside (0.03 g) was added to the test tube containing the dried lipid. The lipid film and detergent were dissolved in an aqueous buffer containing 25 mM Tris-HCl and 150 mM

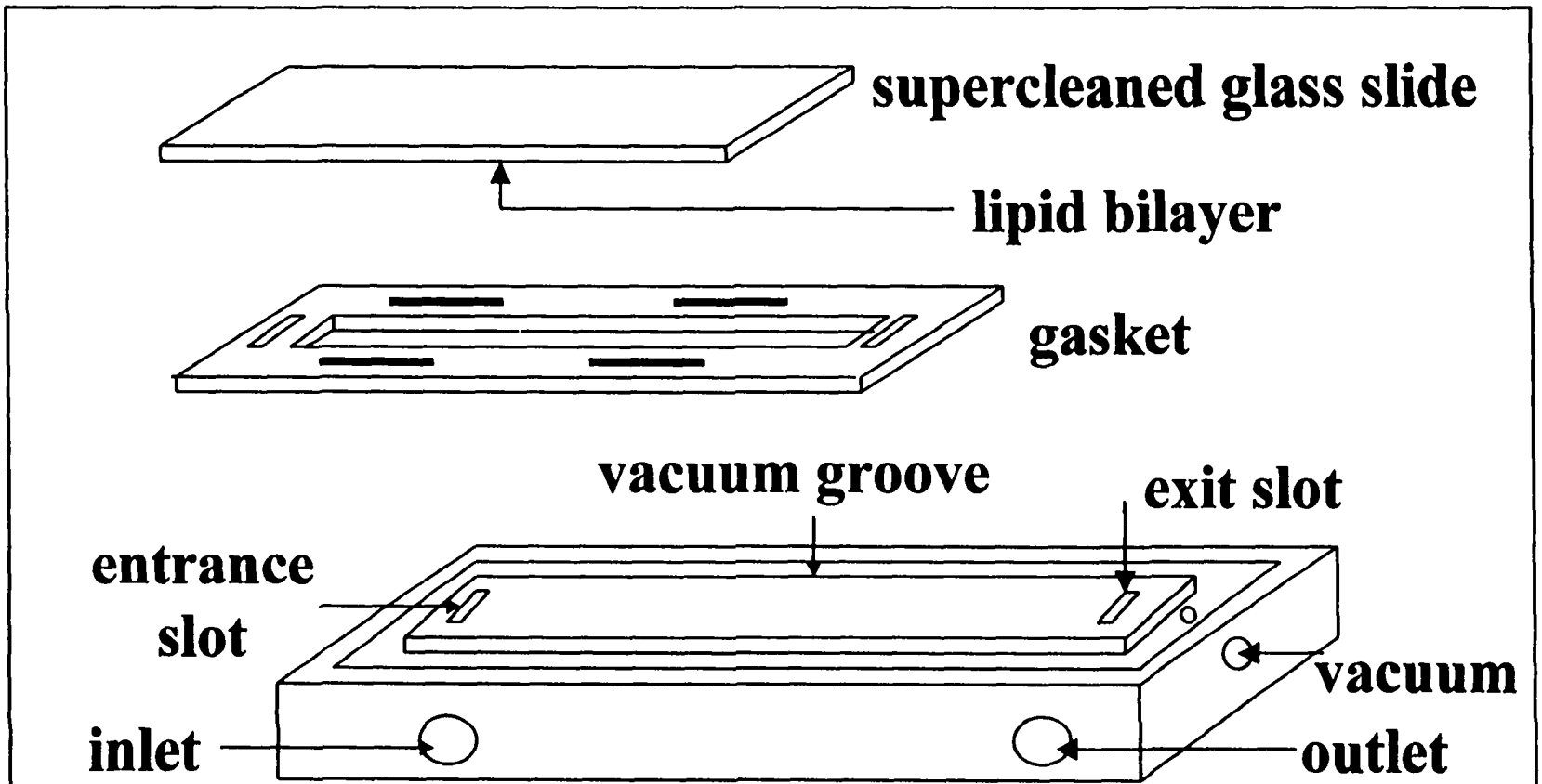


Figure 7. Flow chamber used for flow experiments. A supercleaned glass slide coated with an antibody, a receptor molecule, or a lipid bilayer containing an adhesion molecule is attached to the flow chamber by an applied vacuum. The height and width of the flow area is provided by a gasket. The shear stress (dynes/cm^2) at the wall is calculated using $\tau = 6Q\mu(1 \text{ min}/60 \text{ sec})/B^2W$ where Q is the flow rate (ml/min), μ is the viscosity (0.01 g/cm s), B is the height of the flow area (cm), and W is the width of the flow area (cm).

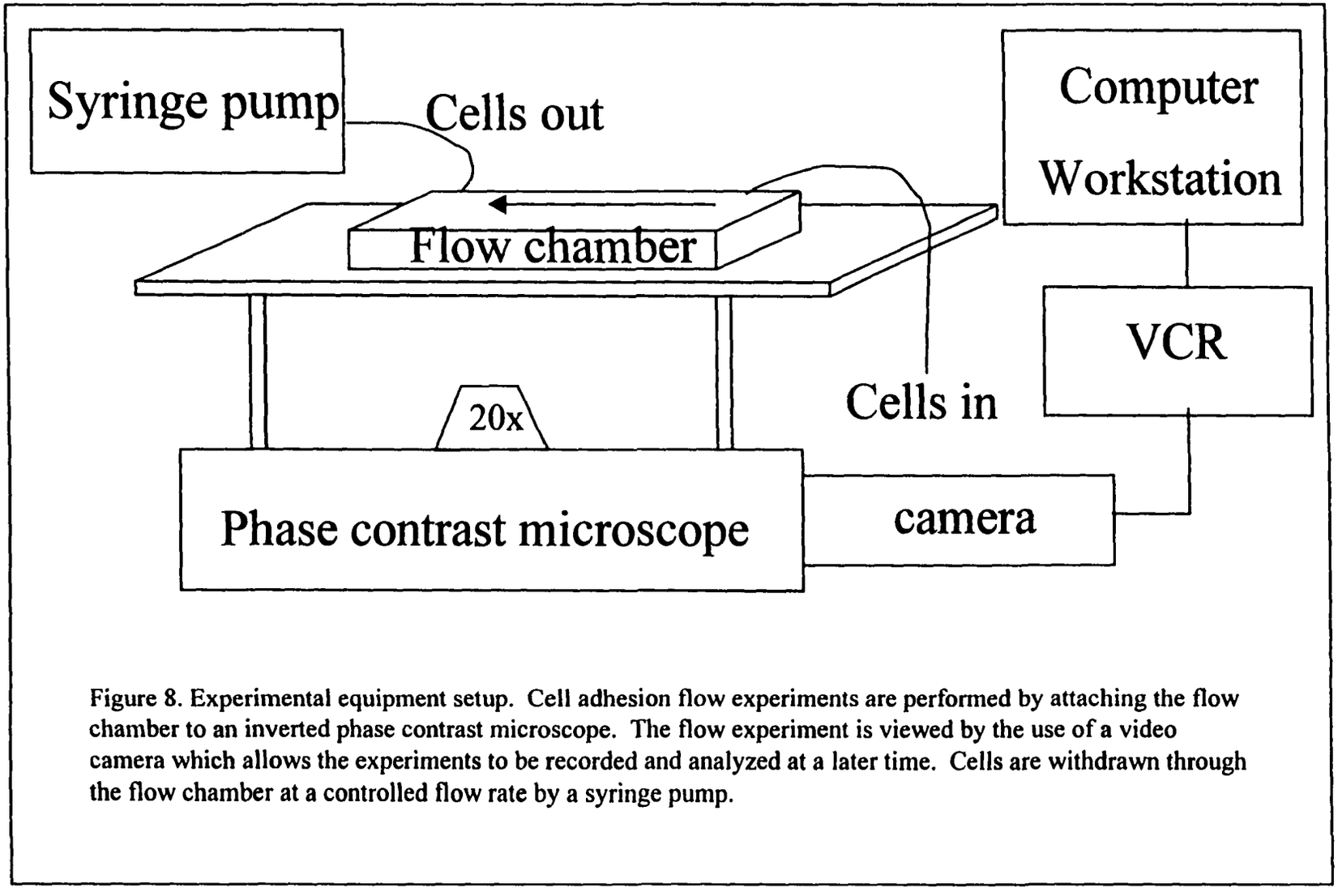


Figure 8. Experimental equipment setup. Cell adhesion flow experiments are performed by attaching the flow chamber to an inverted phase contrast microscope. The flow experiment is viewed by the use of a video camera which allows the experiments to be recorded and analyzed at a later time. Cells are withdrawn through the flow chamber at a controlled flow rate by a syringe pump.

NaCl (pH 7.4). The lipid was dissolved in 300 μl total volume of buffer which included the adhesion molecule to be incorporated into the vesicles. The solution was added to cellulose ester dialysis membrane tubing (Spectra/Por® 3500 MWCO) and the detergent was removed by dialysis against 25 mM Tris-HCl and 150 mM NaCl for 2 days with 2 changes of dialysis buffer.

Preparation of Lipid Bilayers

Lipid bilayers were formed on a supercleaned microscope glass cover slide (24 mm x 50 mm) that was prepared by boiling in detergent (Alconox) for 15 minutes, rinsed with ultrapure water, and stored in methanol at 4°C. A supercleaned cover slide was placed on a drop of the vesicle solution (35-50 μl) in a plastic petri dish. The bilayer was allowed to form for 5 minutes before being submerged in water in order to attach it to the flow chamber without being exposed to air.

Static Adhesion Assays

To demonstrate P-selectin was actively incorporated into the lipid vesicles, static adhesion assays were performed with HL-60 cells. 10 μl of a vesicle preparation made with 7 μg of P-selectin was incubated with 50 μl of cells ($2x10^6$/ml) in HBSS. Aggregation of cells indicated P-selectin was actively incorporated into the vesicles due to the vesicles producing a P-selectin 'bridge' between PSGL-1-expressing HL-60 cells. These assays were performed in the presence of 5 mM EDTA to show the interactions were calcium-dependent. To show the specificity of the aggregations, these assays were

also performed in the presence of the following antibodies, 10 μg/ml G1, S12, PL1, or PL2.

Iodination of Antibody

Glass tubes for iodination were coated with 10 μg iodogen in $CHCl_3$, dried, and stored at -20 ^{0}C. The antibody to be labeled was added to the tube (150 μg) followed by the addition of 500 μCi $Na^{125}I$ and incubated for 5 minutes at room temperature. This was added to a PD-10 (Pharmacia Biotech, Piscataway, NJ) column preequilibrated with 50 ml of elution buffer (25 mM HEPES, 116.25 mM NaCl, pH 7.4). Elution buffer was added to the PD-10 column in order to collect ten 500 μl fractions (1-5, 6, 7, 8, 9, 10). A 1 μl sample of each fraction was counted in a gamma counter and the two largest counts were pooled and added to a Centricon 30 tube (Millipore Corporation, Bedford, MA). The sample was run through the Centricon 30 tube for 15-25 minutes at 1900g and 4 ^{0}C. The labeled protein was resuspended in 1 ml elution buffer and ultracentrifuged for 30 minutes at 89,000g and 4 °C. A BCA protein assay (Pierce, Rockford, IL) was used to prepare a standard concentration curve. In a 15 ml centrifuge tube, reagent B (4.8 ml) and reagent C (200 μl) were mixed and incubated at room temperature for 10 minutes producing the B/C mix. 15 μl of a 2 mg/ml bovine serum albumin (BSA) stock solution was added to 1.5 ml of elution buffer (20 μg/ml). The standard concentration curve was prepared using 8 tubes and doing a serial dilution (20 μg/ml, 10 μg/ml, 5 μg/ml, 2.5 μg/ml, 1.25 μg/ml, 0.62 μg/ml, 0.31 μg/ml) in 500 μl volume. An antibody tube was made by diluting antibody 1:10 with elution buffer (50 μl in 450 μl). 5.0 ml of reagent A was added to the B/C mix and 500 μl of the A/B/C mix was added to each of the eight

standard curve tubes and the antibody tube. The tubes were incubated for 1 hour at 65 ^{0}C, allowed to cool, and read on a spectrophotometer at 562 nm. An equation giving optical density (OD) as a function of concentration was obtained from the standard curve. The unknown antibody concentration was determined based on its optical density reading. This provided the concentration of the antibody in ng/μl. The specific activity of the antibody was determined by diluting the antibody 1:10 (10 μl of antibody preparation into 90 μl elution buffer). 10 μl of the mixture was added to 8 test tubes and counted on a gamma counter to give the specific activity of the antibody in cpm/μl. The cpm/ng of the labeled antibody was calculated by dividing the specific activity of the antibody (cpm/μl) by the concentration of the antibody (ng/μl).

Site Density Determination for P-selectin Containing Lipid Bilayers

Lipid bilayers were formed on circular supercleaned glass slides that were placed in a well of a 24-well tissue culture plate by adding 75 μl of the liposome preparation to the top of the slide. The bilayer formed at room temperature over 5 minutes. HBSS (500 μl) was added to the well and the level of the solution was marked. The well was washed three times with HBSS. The buffer level was brought down to the 500 μl mark after the final wash. The bilayer was then incubated for 30 minutes with 200 μl of ^{125}I-S12 (2 μg/ml). The bilayer was washed 6 times to remove unbound labeled antibody. After the sixth wash the buffer level was again brought down to the 500 μl mark. The bilayer was removed from the slide by the addition of 500 μl NH_4OH (1 M) and scrubbed with a cotton swab. The cotton swab and solution in the well were put in a test tube. The well was washed with 500 μl NH_4OH and also added to the test tube. The test tube was

counted on a gamma counter to obtain the counts/minute which was converted to site density using:

$$\text{sites}/\mu m^2 = (\text{specific binding in cpm})(1 \times 10^{-9}\text{grams/specific activity in cpm/ng})(\text{MW of Ab})(\text{Avagadros Number})(\text{Surface Area})$$

where the specific binding equals the counts per minute of the labeled P-selectin bilayer minus the counts per minute of a plain bilayer (background) as determined on a gamma counter. The site density for each P-selectin concentration was determined using the average of three bilayers. CHO cells expressing P-selectin were grown to confluence in wells of a 24-well tissue culture plate. Six confluent monolayers were washed 3 times with HBSS and incubated with saturating concentrations of ^{125}I-S12 (1.0 μg/ml, 100 μl) in HBSS for 30 minutes at 4^0C. The monolayers were washed 5 times with HBSS and removed from the surface with 1 M NH_4OH (200 μl). The cell solutions were collected and counted in a gamma counter. The specific binding was defined as cpm bound to P-selectin CHO cells minus cpm bound to nontransfected CHO cells.

HL-60 Cell Culture

HL-60 cells maintained in RPMI 1640 supplemented with 100 U/ml penicillin G sodium, 100 U/ml streptomycin sulfate, 1 mM sodium pyruvate, and 15% FBS were split every 4-5 days by removing 75% of the media and replacing it with new warm media.

Cell Counts

Cell concentrations were determined using a hemocytometer. A 12-µl sample was added to the hemocytometer and allowed to settle. The number of cells counted in the outer squares were converted to concentration using:

$$\text{cells/ml} = (10^4)(\text{number of cells in (x) corners})/(\text{x})$$

Flow Experiments: Cell Accumulation Assays on P-selectin Bilayers

Initial experiments were done at various P-selectin site densities in lipid bilayers to determine the effect of site density and shear stress on the accumulation of HL-60 cells. Glass coverslips with a lipid bilayer containing less than 20 to approximately 350 sites/μm^2 of P-selectin were attached to the flow chamber as previously described (*see Flow Chamber and Attachment of Glass Coverslips* on page 33). HL-60 cells ($5x10^5$/ml) in HBSS/1% FBS were withdrawn over the bilayer and the interaction of cells with the surface and subsequent cell-cell interactions were observed. Flowing cells either attached directly to the surface and rolled (primary attachments) or interacted transiently with the surface. A rolling cell was defined as a cell remaining attached to the bilayer for more than one cell diameter. A transient interaction was defined as a cell which attached to the surface for at least one video frame (30 frames/sec) and released back into the hydrodynamic flow without moving one cell diameter on the surface. Flowing cells interacted with bound cells in three ways (Figure 9). First, flowing cells attached to bound cells followed by the cell becoming bound to the surface (secondary attachments) (Figure 9A). Secondly, cells briefly attached to bound cells before releasing back into the

hydrodynamic flow stream (Figure 9B). In some cases, flowing cells that attached to bound cells resulted in both cells releasing into the flowing stream (Figure 9C).

The various types of interactions directly occurring with the bilayer and the various types of cell-cell interactions were counted for a range of site densities and shear stresses to determine how the attachment rate (total events/min), percent of events that were transient, percent of cell-cell interactions that resulted in the cell attaching to the surface, accumulation of cells on the surface, and the percent of cells that accumulated on the surface by secondary interactions were affected by these parameters. The total number of events with the bilayer was calculated by summing the number of cells that attached directly to the bilayer and rolled (primary attachments), the number of cells that attached to the surface after attaching to a bound cell (secondary attachments), and the number of transient events.

Isolation of Neutrophils

Polymorphonuclear leukocytes (neutrophils) were isolated from whole blood anticoagulated with heparin (0.075 ml heparin/10 cc blood). Anticoagulated whole blood was mixed with half its volume of 6% Dextran70, 0.9% NaCl. Red blood cells sedimented for 60 minutes at room temperature. The supernatant (leukocyte-rich plasma) was carefully transferred to a centrifuge tube and centrifuged at 300g for 5 minutes at 4°C to obtain a pellet of leukocytes and red blood cells. The pellet was resuspended in a hypoosmotic buffer (25 ml of 0.2% NaCl for 20 seconds) to lyse the red blood cells followed by the addition of 25 ml 1.6% NaCl to restore isotonicity. Centrifugation at

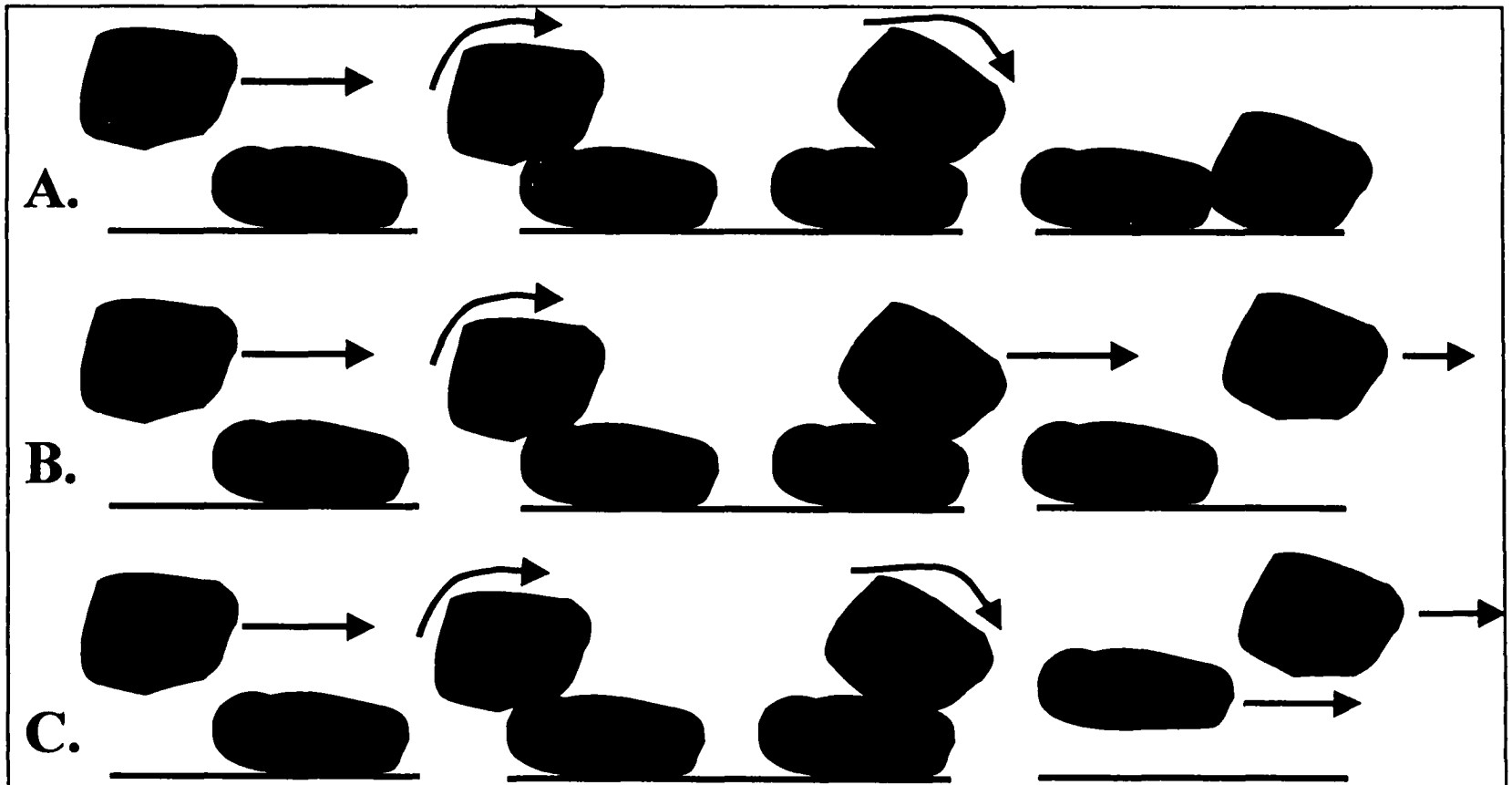


Figure 9. Types of cell-cell interactions. Once a cell is bound to the surface it can interact with flowing cells in three ways. A flowing cell can bind to a cell attached to the surface followed by it becoming attached to the surface (A). A flowing cell that attaches to a bound cell can also release back into the flowing stream (B). A flowing cell is also capable of bringing an attached cell back into the flowing stream (C).

300g and 4°C for 5 minutes produced a pellet containing mostly leukocytes which was resuspended in 5 ml of HBSS/0.6% HSA. The leukocytes were transferred to a round bottom centrifuge tube and 6 ml of a polysucrose solution (Histopaque1077®, density = 1.077 g/ml) was gently added to the bottom of the tube. Polymorphonuclear leukocytes were isolated from mononuclear leukocytes and red blood cells by a Histopaque1077® density gradient centrifugation at 300g for 30 minutes at 4°C. The polymorphonuclear leukocytes were pelleted at the bottom of the tube while the red blood cells and mononuclear leukocytes remained on top of the layer of Histopaque1077®.

Flow Experiments: Neutrophil Accumulation on P-selectin Bilayers through Cell-Cell Interactions

A glass coverslip coated with a lipid bilayer containing P-selectin (350 sites/μm^2) was attached to the flow chamber. Neutrophils ($5x10^5$/ml) in HBSS/1% FBS were withdrawn over the bilayer at 1.4 dynes/cm^2 to determine the role of cell-cell interactions in enhancing cell accumulation.

Cell Treatments

Flow Experiments for HL-60 Cell or Neutrophil Attachment Assays on P-selectin Bilayers

For flow experiments with PSGL-1 antibodies PL1 or PL2, HL-60 cells or neutrophils ($5 x10^5$ cells/ml) were preincubated at room temperature for 10 minutes in HBSS/1% FBS with 10 μg/ml mAb. For antibody inhibition studies with S12, G1, DREG56, or DREG200, HL-60 cells or neutrophils were incubated at room temperature in HBSS/1%

FBS for 10 minutes with 5 μg/ml mAb. For S12 and G1 inhibition studies, the surface was also incubated with the antibody (10 μg/ml) for 10 minutes.

Flow Cytometry Analysis

To determine if the HL-60 cells expressed L-selectin, 1 ml of $1x10^6$ HL-60 cells/ml were incubated with 4 μg/ml (20 μl) of a fluorescent L-selectin antibody, Leu-8-FITC, for 30 min on ice. The cells were then fixed with 1% paraformaldehyde. Control cells were either incubated with GAM-FITC, an irrelevant fluorescent antibody, or CD16-FITC, a fluorescent antibody that binds to the HL-60 cells. To determine if P-selectin was incorporated into the lipid vesicles, vesicles were incubated with a fluorescent P-selectin antibody for 30 minutes at room temperature. The fluorescence was compared with a vesicle sample incubated with a fluorescent antibody irrelevant for P-selectin and a vesicle sample with no fluorescence added.

Results

Activity of P-selectin in Lipid Vesicles

The site density of P-selectin incorporated into phosphatidylcholine bilayers was measured to determine if P-selectin was actively incorporated into the vesicles. The site density of P-selectin was directly proportional to the ratio of moles of P-selectin to the moles of phosphatidylcholine in the preparation. Vesicles prepared with and without membrane P-selectin were analyzed by flow cytometry to determine if P-selectin was incorporated into the vesicles. The fluorescence histogram showing the frequency of events as a function of fluorescence intensity is shown in Appendix I. The mean fluorescence intensity obtained for vesicles prepared with membrane P-selectin and incubated with a fluorescent anti-P-selectin antibody (curve C) is more than an order of magnitude higher than the mean fluorescence intensity of membrane P-selectin vesicles without the antibody (curve A) or membrane P-selectin vesicles incubated with an irrelevant antibody. This indicates that P-selectin was successfully incorporated into the vesicles in the proper orientation since the binding site for the antibody is located in the lectin domain.

The activity of P-selectin in lipid vesicles was also determined by static adhesion assays. HL-60 cells in suspension do not have a tendency to aggregate. However, due to the presence of PSGL-1 on the surface of HL-60 cells, P-selectin-containing vesicles could act as a bridge and cause large cell aggregates to form. This phenomenon was observed in 5 separate experiments. The specificity of the interaction was established by blocking the formation of aggregates with the calcium chelator EDTA or the anti-P-selectin

blocking antibody G1. The aggregates were not blocked by the presence of the nonblocking anti-P-selectin antibody S12 (data not shown).

Attachment Rate and Threshold Shear for Rolling for HL-60 Cells on P-selectin Bilayers

HL-60 cell flow experiments were done for a range of shear stresses over lipid bilayers containing P-selectin at site densities ranging from less than 20 sites/μm^2 to approximately 350 sites/μm^2. The attachment of cells to the surface (primary attachment) was calcium-dependent and mediated by P-selectin and PSGL-1. This was shown by performing experiments in the presence of various function blocking and nonblocking antibodies. The accumulation of cells on the surface was reduced by approximately 95% when the cells were incubated with either the anti-P-selectin antibody G1 or the function blocking anti-PSGL-1 antibody PL1 (data not shown). Accumulation of cells was not affected by the incubation of cells with the anti-P-selectin nonblocking antibody S12 or the nonblocking anti-PSGL-1 antibody PL2 (data not shown). Infusion of EDTA caused bound cells to rapidly release from the surface showing that their attachment was calcium-dependent.

The attachment rate (number of events per min) of HL-60 cells to the bilayer is shown in Figure 10. From 0.4-1.4 dynes/cm^2, the attachment rate was not very dependent on the shear stress. At lower site densities (<20), the majority of the interactions were transient. A cell could interact transiently with the surface more than one time in a field of view. Transient interactions also occurred more readily than established rolling interactions.

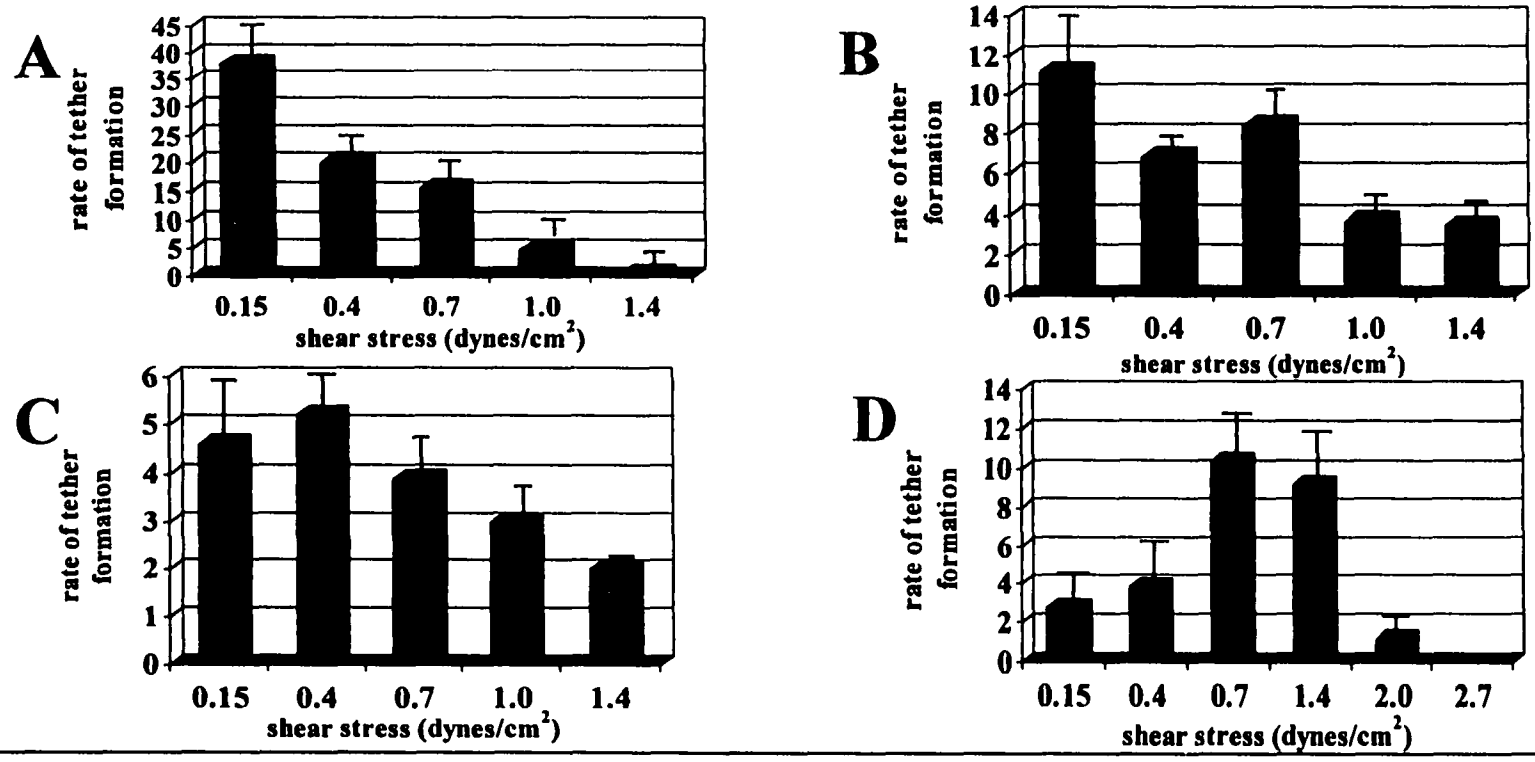


Figure 10. Rate of tether formation of HL-60 cells on P-selectin bilayers at various site densities. HL-60 cells were withdrawn over P-selectin bilayers at site densities of A) < 20 sites/μm^2 B) 25-50 sites/μm^2 C) 50-100 sites/μm^2 D) 350 sites/μm^2. The rate of tether formation was defined as the total number of events with the surface (primary, secondary, and transient attachments) per minute. At the low site densities (less than 100 sites/μm^2), the maximum attachment rate occurred at the lowest shear stresses tested (0.15-0.4 dynes/cm^2) (A-C). As the shear stress was increased, the attachment rate decreased since it becomes more difficult for bond formation. However, at sites densities above 250 sites/μm^2 (D), the attachment rate increased with an increase in shear stress due to unexpected accumulation through cell-cell interactions. Error bars represent +/- SEM. For < 20 sites, n=7; 25-50 sites, n=3; 50-100 sites, n=4; 250-350 sites, n=8-21.

Therefore, the attachment rate was increased under the conditions of low P-selectin site density and low shear stress.

A mixture of rolling and transient interactions occurred at site densities below 100 sites/μm^2. At these site densities, the ability of a cell to roll on the bilayer was dependent on the shear stress. This threshold shear stress for cell rolling is shown in Figure 11. This is consistent with the observations made by other groups using neutrophils and HL-60 cells on P-, L-, and E-selectin (533, 534). This phenomenon is most likely a hydrodynamic effect. A high enough shear stress is required to "roll" the cell over in order for the next ligand-receptor pair to interact. If the cell does not get "rolled" over enough for this interaction to occur before the current bond breaks, the cell detaches. Therefore, as the shear stress is increased, the majority of events are rolling interactions. At low shear stresses where the cells are not "pushed" over to the surface, the majority of the interactions are transient.

The attachment rate was defined as the number of freely suspended cells that attached to the surface (primary attachments, secondary attachments, and transient attachments) per 20x field of view (0.12 mm^2) per minute. At low site densities of P-selectin (less than 100 sites/μm^2), the maximum attachment rate occurred at the lowest shear stresses tested (0.15-0.4 dynes/cm^2, see Figure 10A-C). As the shear stress was increased, the attachment rate decreased and was essentially zero at 2.0 dynes/cm^2. In contrast, at site densities above 250 sites/μm^2 the attachment rate was found to increase with shear stress (Figure 10D). At intermediate shear stresses (0.7-1.4 dynes/cm^2), the attachment rate was

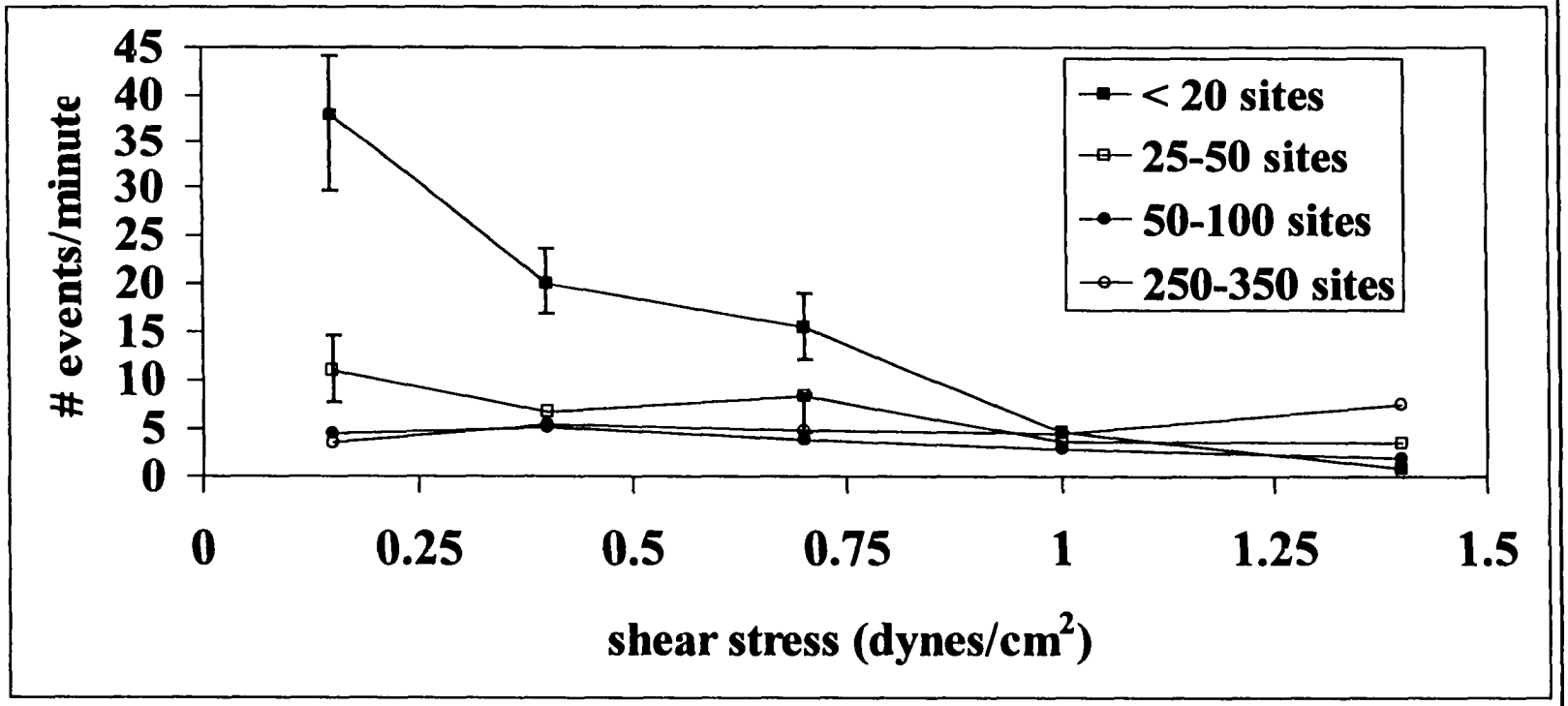


Figure 11. Attachment rate of HL-60 cells to P-selectin bilayers at various site densities. HL-60 cells were withdrawn over bilayers containing P-selectin and the total number of events (primary attachments, secondary attachments, and transient attachments) was counted. At < 20 sites/μm^2 the majority of the events were transient for 0.15-0.4 dynes/cm^2 which led to the elevated attachment rate. At sites densities under 100 sites/μm^2, a mixture of rolling and transient events was observed. The attachment rate decreased with increasing shear stress for site densities under 100 sites/μm^2. However, the attachment rate increased with an increase in shear stress due to unexpected attachments through cell-cell interactions at 250-350 sites/μm^2. Error bars represent +/- SEM. Symbols without error bars have SEM values smaller than the size of the symbol. For < 20 sites, n=7; 25-50 sites, n=3; 50-100 sites, n=4; 250-350 sites, n=8-21 depending on the shear stress.

higher due to accumulation through cell-cell interactions. At greater than 250 sites/μm^2, the cells all rolled very slowly (< 1.0 μm/min) or were firmly attached to the surface. Cell accumulation at these very high P-selectin site densities unexpectedly occurred through flowing cells attaching to bound cells followed by their deposition on the surface.

HL-60 Cell-Cell Interactions Occurring on P-selectin Bilayers (350 sites/μm^2)

Recently much research has been done concerning accelerated or enhanced neutrophil accumulation due to neutrophil-neutrophil interactions mediated by PSGL-1 and L-selectin (137-139, 166). Neutrophils isolated from whole blood were withdrawn over a bilayer containing P-selectin (350 sites/μm^2). Lines or strings of cells formed on the surface due to interactions between bound cells and flowing cells (Figure 12). Accumulation occurred primarily through cell-cell interactions which enhanced the accumulation at a site. Blocking the cell-cell interaction mechanism with an anti-L-selectin antibody (DREG200) reduced cell accumulation at the site by approximately 90% (data not shown). These types of cell-cell interactions also occurred with HL-60 cells on high P-selectin site density bilayers or P-selectin coated on glass slides. HL-60 cells express PSGL-1, but little or no L-selectin. The HL-60 cells used for these experiments expressed no detectable level of L-selectin by flow cytometry (Appendix II).

Additional flow experiments were done at 350 sites/μm^2 of P-selectin in lipid bilayers to further characterize the HL-60 cell-cell interactions. HL-60 cells bound directly to the surface under shear stresses of 0.15-2.0 dynes/cm^2. Cells that bound to this high P-selectin site density surface rolled very slowly (< 1.0 μm/min) on the surface or were

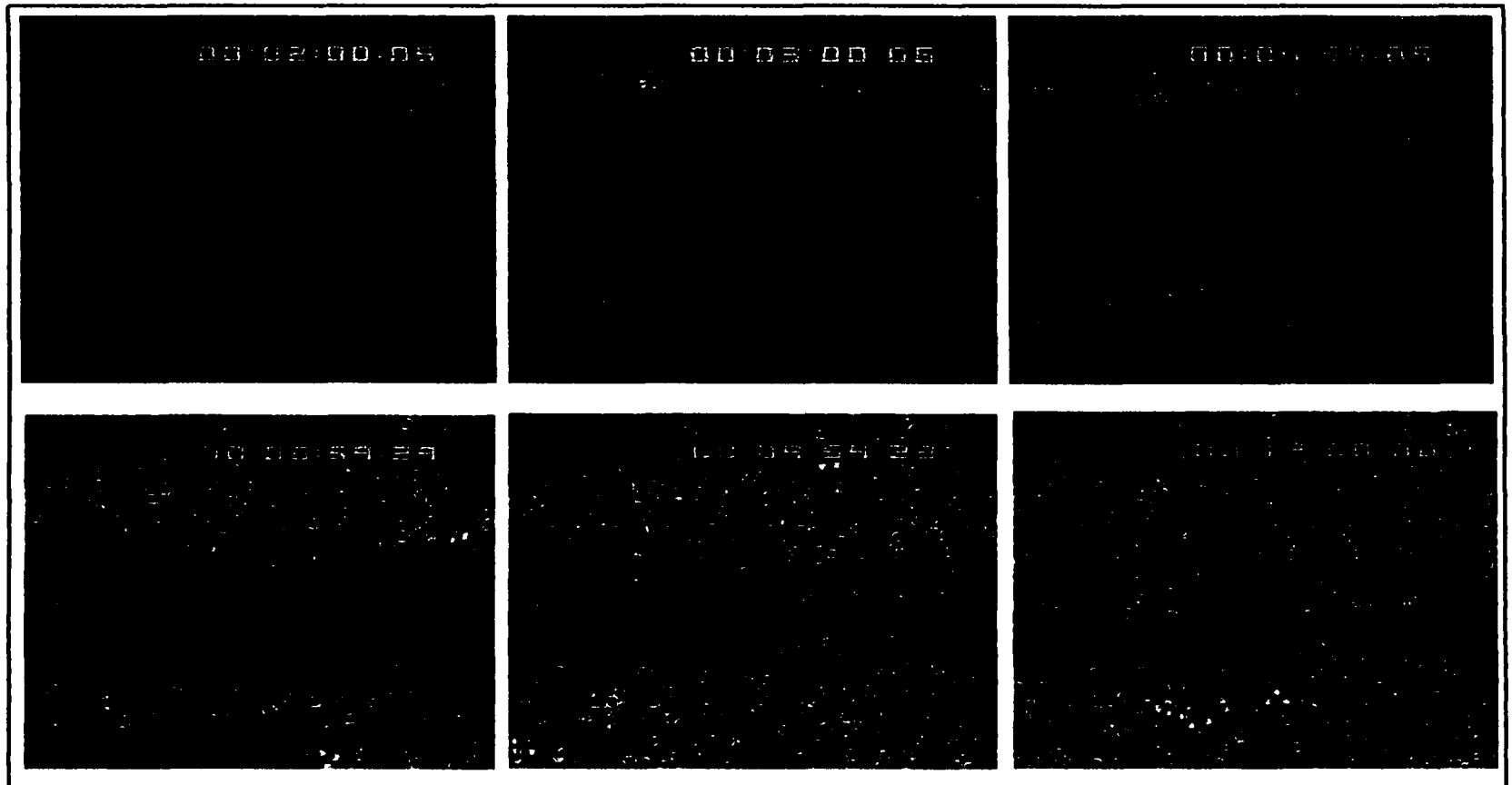


Figure 12. Neutrophil accumulation on a P-selectin bilayer at 1.4 dynes/cm^2. Neutrophils were withdrawn over a bilayer containing P-selectin (350 sites/μm^2) to show cell-cell interactions (secondary interactions) mediated by PSGL-1 and L-selectin greatly enhance accumulation. Experiments run in the presence of an anti-L-selectin antibody to block cell-cell interactions reduced accumulation by approximately 95%. Flow is from right to left.

firmly attached. Cell-cell interactions between flowing cells and cells bound to the surface occurred over the entire range of shear stresses studied (0.15-2.0 dynes/cm^2) (Figure 13). However, as the shear stress was increased a much larger percentage of the cell-cell interactions resulted in the flowing cell binding to the surface and remaining attached (Figure 14). At lower shear stresses (0.15-0.4 dynes/cm^2), the cell-cell interactions were mainly transient-- a flowing cell briefly attached to a bound cell then released back into the flowing stream. At higher shear stresses (0.7-2.0 dynes/cm^2), the flowing cells were more efficiently brought to the surface where they remained bound following a cell-cell interaction. At higher shear stresses (0.7-1.4 dynes/cm^2), cell recruitment was much more effective through cell-cell interactions than from cells directly attaching to the bilayer (Figure 15).

At lower shear stresses, where the cell-cell interactions did not result in the cell binding to the surface, accumulation of cells was in random fashion (Figure 16). Accumulation through cell-cell interactions at the higher shear stresses led to the formation of lines or strings of cells on the surface, as seen with neutrophils. Strings of cells were formed by a cell binding to an attached cell at the upstream end of the string and rolling on the string of cells before depositing on the surface at the end of the growing string of cells (Figure 17). The ability of cells to bind to the surface through cell-cell interactions greatly enhanced the accumulation of cells at a site. In experiments done at higher shear stresses, accumulation occurred throughout the entire experiment. However, at lower shear stresses when the cell-cell interactions were mainly transient, maximum accumulation on the surface occurred after only 3-4 minutes (Figure 18).

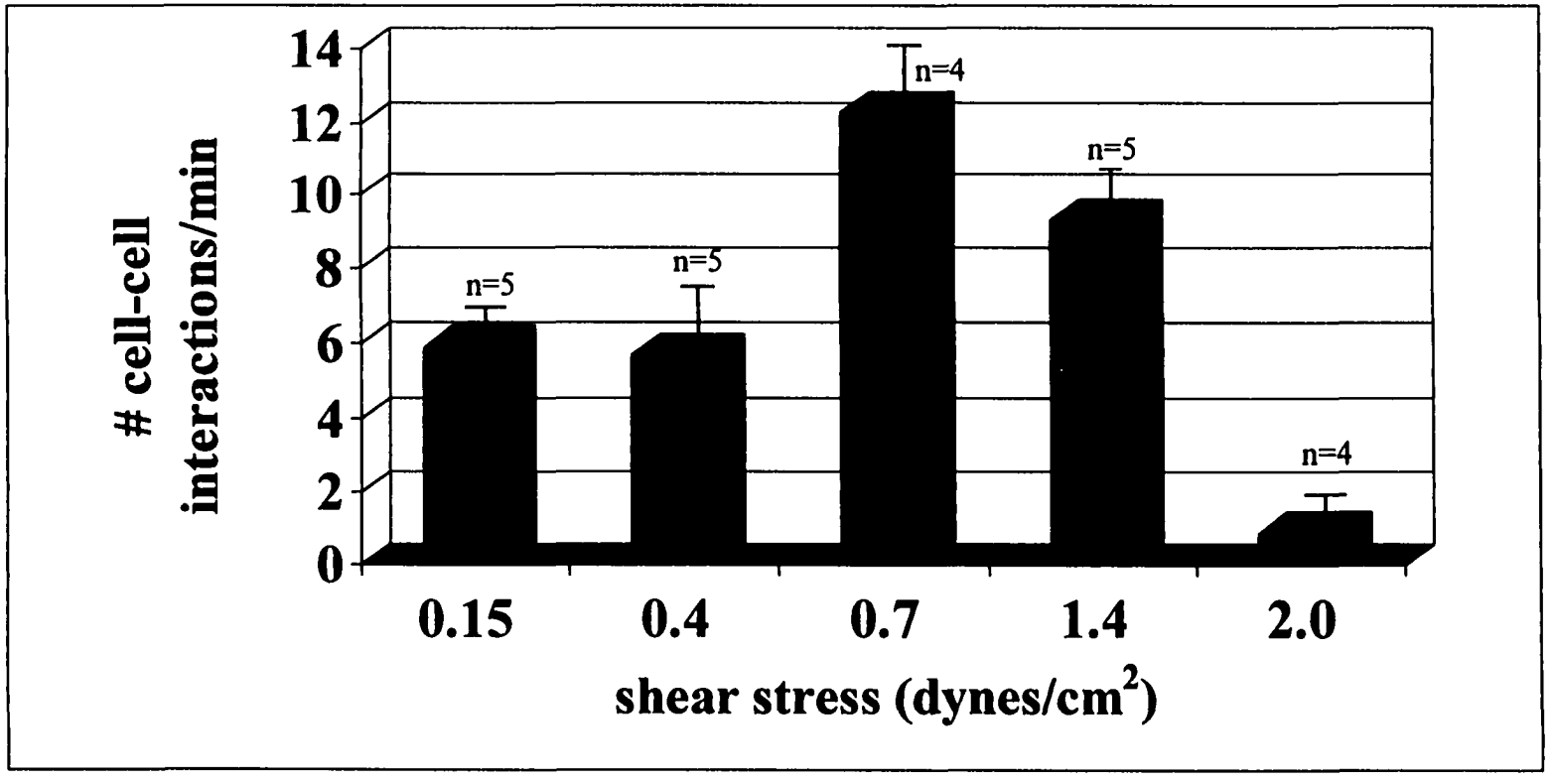


Figure 13. Rate of cell-cell interactions for HL-60 cells on a P-selectin bilayer (350 sites/μm^2). HL-60 cells were withdrawn over a bilayer containing P-selectin to determine the role of cell-cell interactions in cell accumulation. Cell-cell interactions between flowing cells and bound cells occurred over the entire range of shear stresses studied (0.15-2.0 dynes/cm^2). Error bars represent +/- SEM.

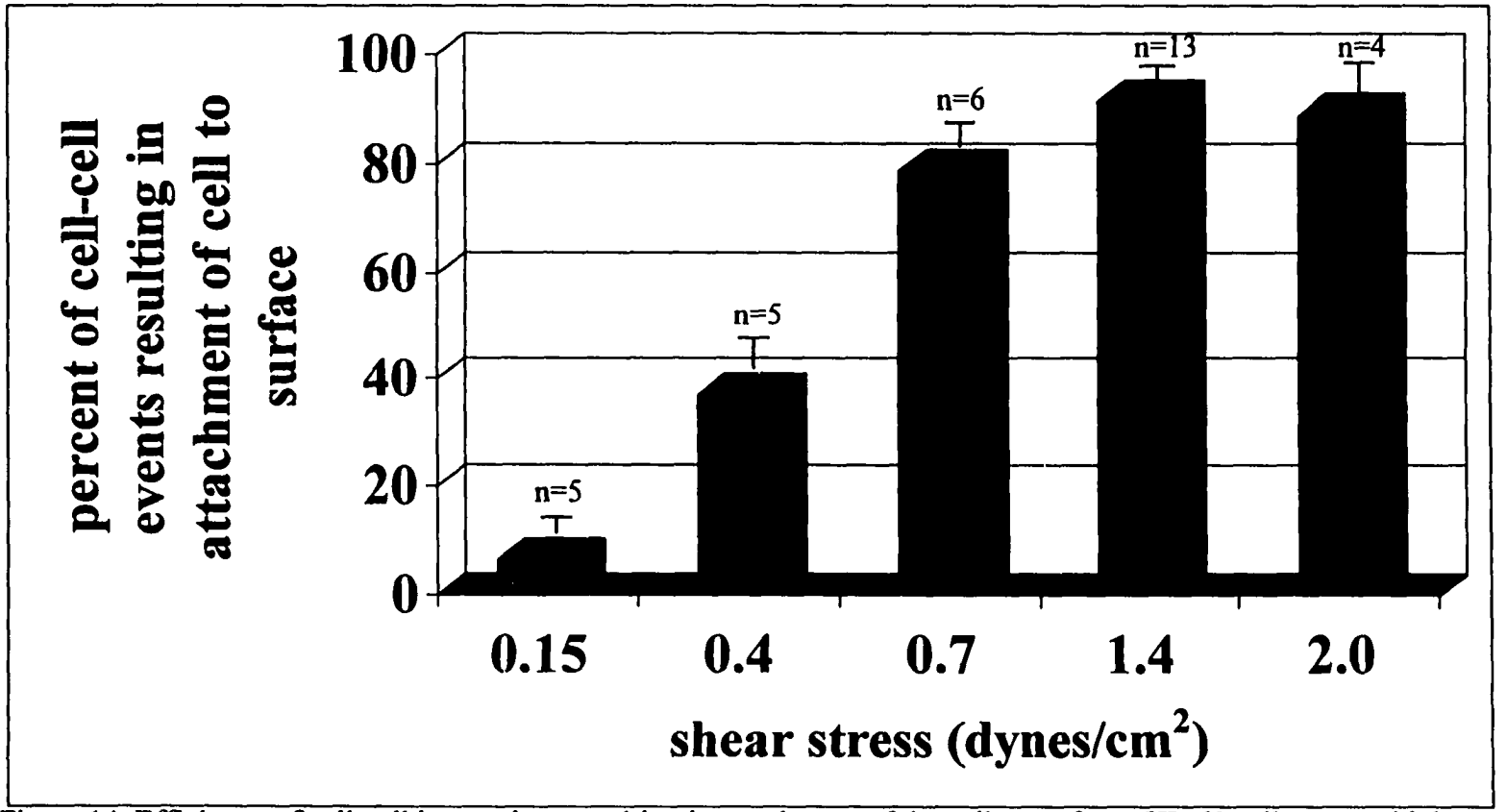


Figure 14. Efficiency of cell-cell interactions resulting in attachment of the cell to surface. HL-60 cells were withdrawn over a bilayer containing P-selectin (350 sites/μm^2) to determine the role of cell-cell interactions on cell accumulation on the surface. Cell-cell interactions occurred over the entire range of shear stresses studied. However, the ability of the cell to then attach to the surface was greatly enhanced as the shear stress was increased. At lower shear stresses (0.15-0.4 dynes/cm^2), the cell-cell interactions were mainly transient, while at higher shear stresses the flowing cells that attached to bound cells were more likely to be pushed onto the surface and remain bound. Error bars represent +/- SEM.

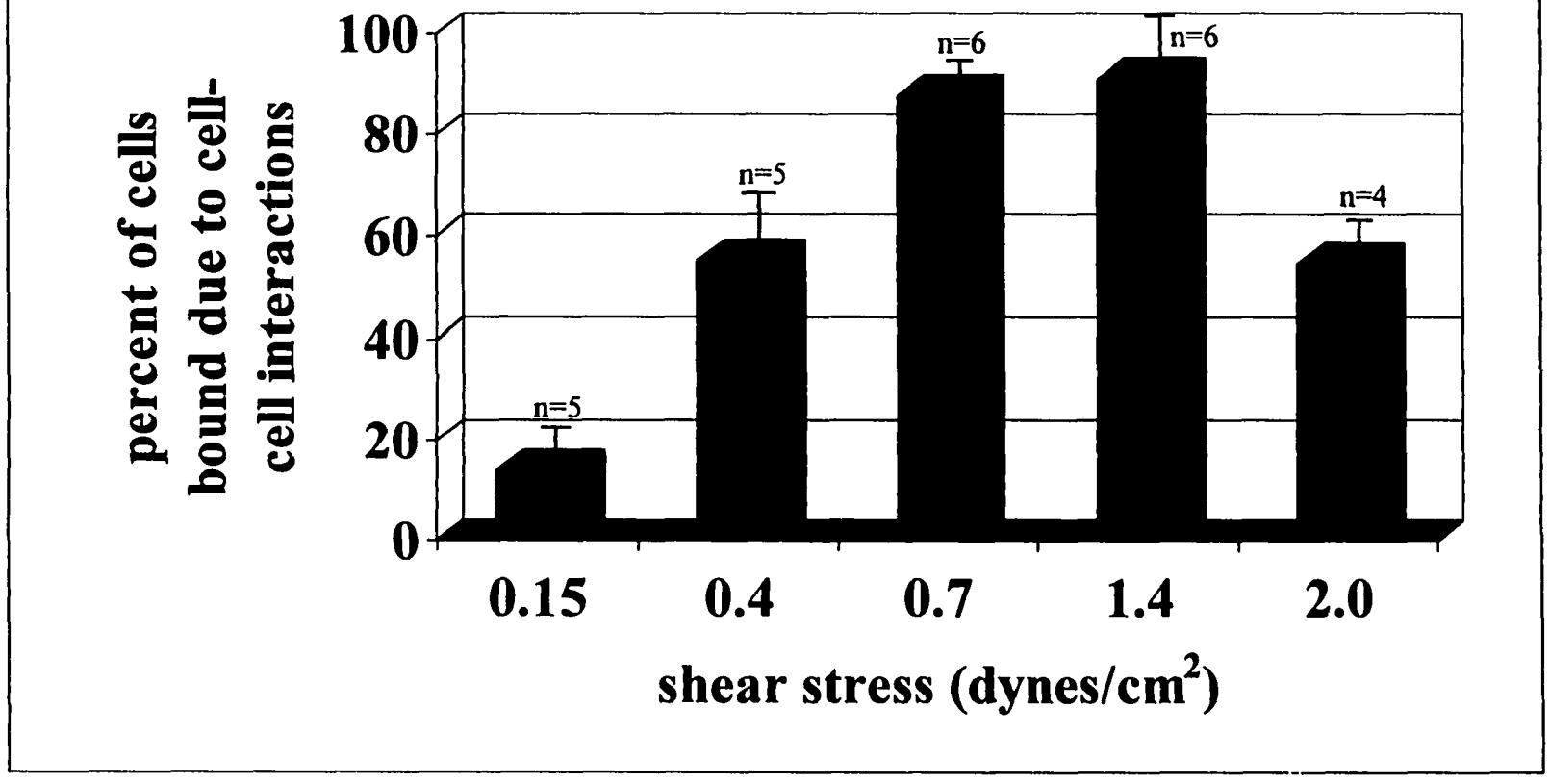


Figure 15. Effect of shear stress on cell accumulation by cell-cell interactions. HL-60 cells were withdrawn over a bilayer containing P-selectin (350 sites/μm^2) to determine the role of cell-cell interactions on cell accumulation. Cells accumulated more efficiently at higher shear stresses through cell-cell interactions rather than binding directly to the surface. At the higher shear stresses the cells were more likely to be pushed over to the surface where they remained bound compared to cell-cell interactions at lower shear stresses which were mainly transient. Error bars represent +/- SEM.

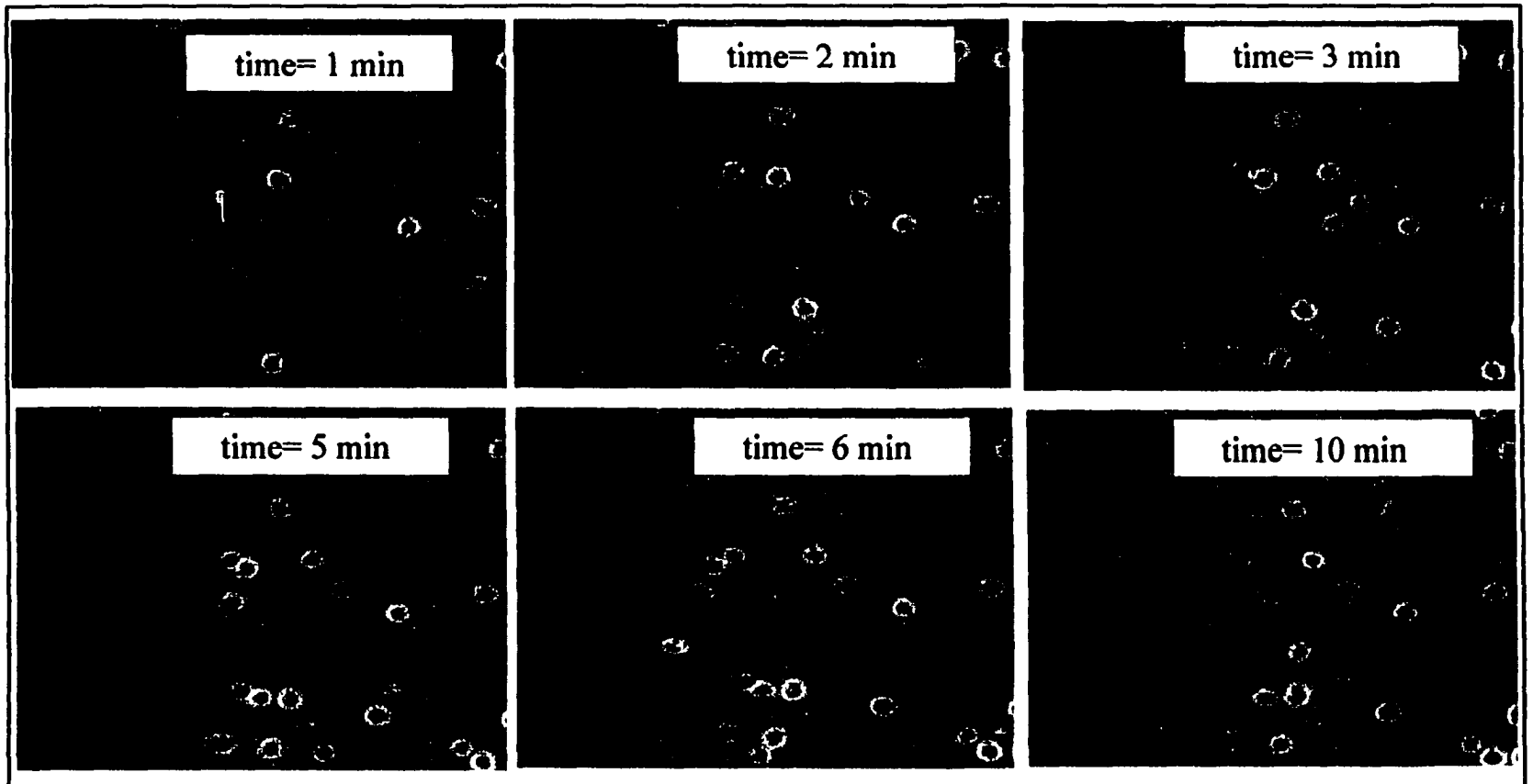


Figure 16. HL-60 cell accumulation on a P-selectin bilayer (350 sites/μm^2) at 0.15 dynes/cm^2. At lower shear stresses (0.15-0.4 dynes/cm^2), cell-cell interactions were mainly transient- a flowing cell attached to a bound cell briefly (less than 2 sec) before releasing back into the flowing stream. Therefore, accumulation did not occur through secondary interactions. Cells bound randomly throughout the field of view directly to the surface and maximum accumulation occurred after only 3-4 minutes. Cells are flowing from right to left.

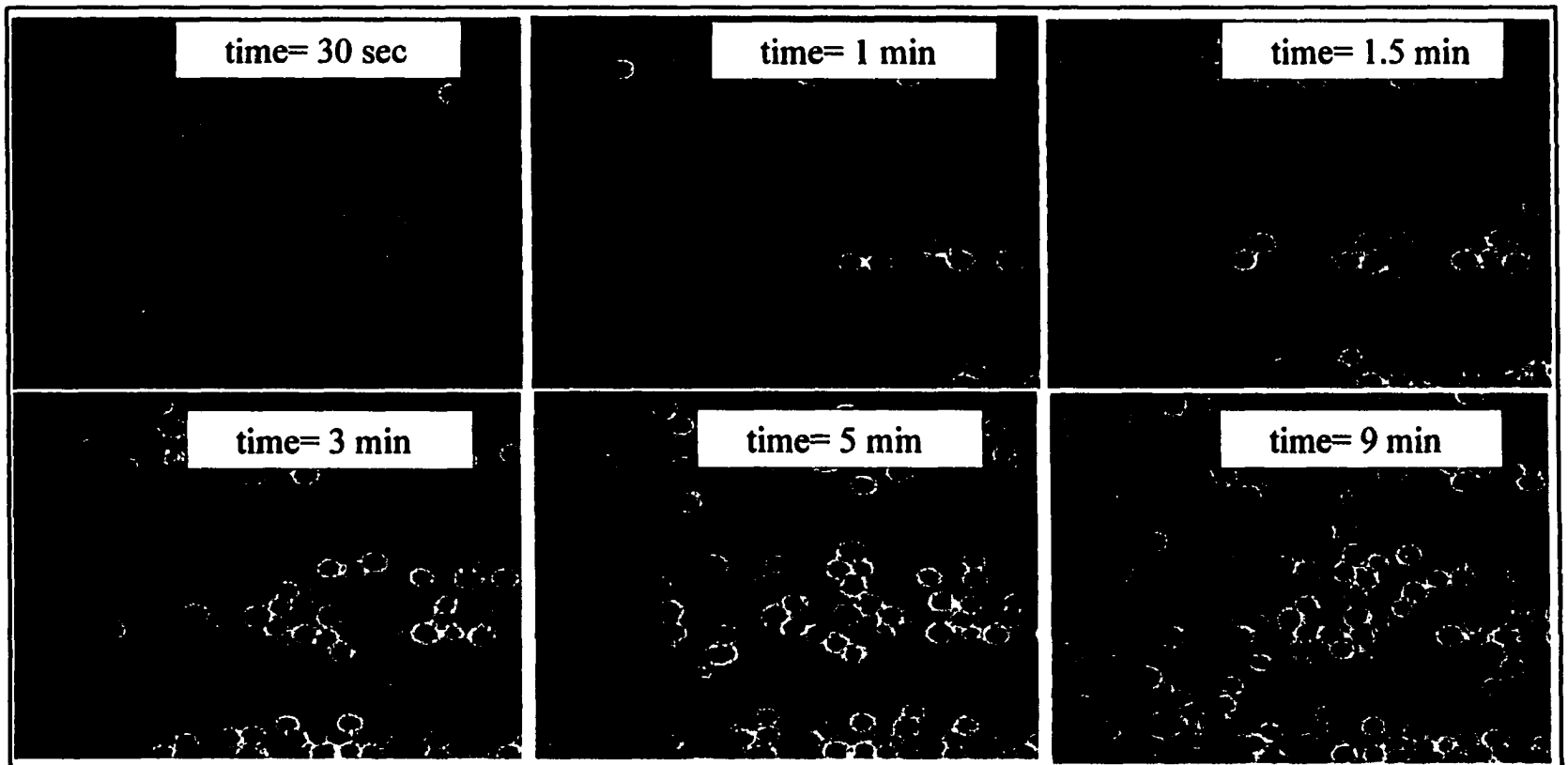


Figure 17. HL-60 cell accumulation on a P-selectin bilayer (350 sites/μm^2) at 1.4 dynes/cm^2. At higher shear stresses (0.7-2.0 dynes/cm^2), cell-cell interactions generally resulted in the flowing cell becoming attached to the surface (secondary attachment). A flowing cell attached to the upstream end of a bound cell and was pushed over the cell to the surface where it bound to P-selectin. These types of interactions lead to the formation of lines or strings of cells on the surface. Once enough cells were bound to the surface, flowing cells could bind to the upstream end of the string and roll on the tops of the bound cells before depositing on the surface at the end of the string of cells. The ability of cells to bind to the surface through cell-cell interactions greatly increased the number of cells accumulating at a site. Flow is from right to left.

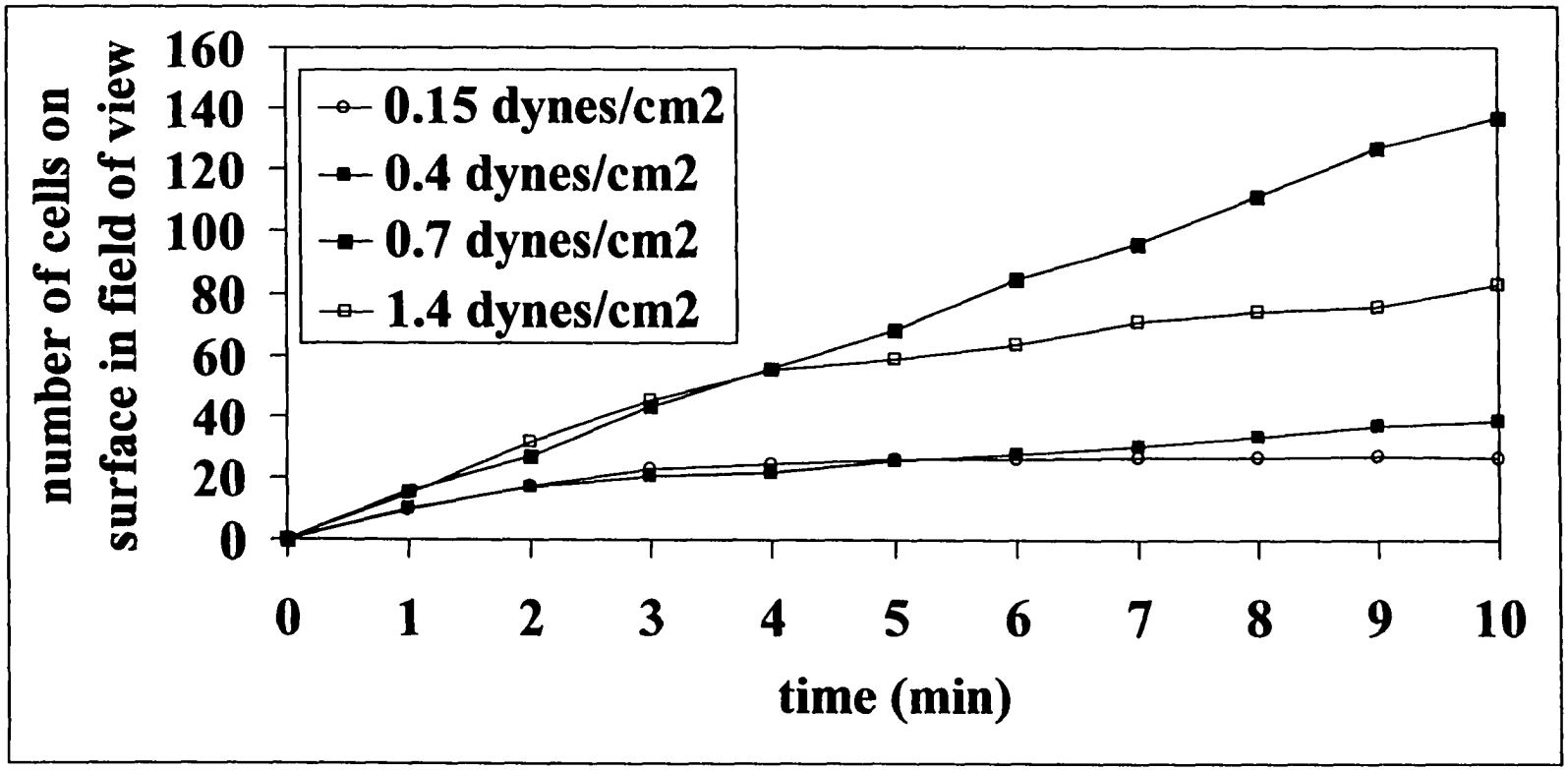


Figure 18. Accumulation of HL-60 cells on a P-selectin bilayer (350 sites/μm^2) as a function of time for a typical experiment. HL-60 cells were withdrawn over a bilayer containing P-selectin and the accumulation on the surface was measured every minute for 10 minutes. At lower shear stresses (0.15-0.4 dynes/cm^2) where cell-cell interactions were mainly transient and did not lead to the cell attaching to the surface, maximum accumulation occurred after only 3-4 minutes. However, at higher shear stresses (0.7-1.5 dynes/cm^2), cell-cell interactions resulted in the flowing cell attaching to the surface which led to cell accumulation over the entire experiment.

Discussion

The initial inflammatory event of cell rolling was recreated in vitro using a lipid bilayer containing P-selectin as an artificial blood vessel wall, and HL-60 cells expressing PSGL-1 as the flowing cells. The ability of cells to attach to the surface was studied at various shear stresses and P-selectin site densities to determine the effect shear stress and selectin site density had on cell attachment. The attachment rate defined as the total number of primary attachments, secondary attachments, and transient attachments per minute was measured for various shear stresses over a range of P-selectin site densities. At the lowest site densities, the majority of the interactions were transient. A mixture of transient and rolling interactions occurred up to approximately 100 sites/μm^2. At a given site density, as the shear stress decreased under approximately 0.4 dynes/cm^2 the events switched from rolling to transient interactions. This shear stress represents the hydrodynamic threshold shear stress required for cell rolling. At site densities which could support cell rolling (< 100 sites/μm^2), a high enough shear stress was necessary to produce cell rolling. The cell would roll at higher shear stresses due to the cell being pushed over to encounter another ligand receptor which allowed a new bond to form as trailing ones were broken. At the lower shear stresses, cell interactions were transient because the bonds at the trailing edge of the cell were broken which released the cell into the flowing stream before a new receptor was encountered to form a new bond.

Generally, the attachment rate decreased as the shear stress was increased since it becomes more difficult to form bonds when the cells are moving faster. However, above 250 sites/μm^2 the cells that attached to the surface remained firmly bound. Flowing cells

attached to bound cells followed by their attachment to the surface. This unexpected accumulation due to cell-cell interactions greatly enhanced cell accumulation. Accumulation of cells through cell-cell interactions also had a shear stress dependence. At higher shear stresses (0.7-1.4 dynes/cm^2), cells were more likely to attach to the surface after a cell-cell interaction rather than release back into the flowing stream. This was most likely a hydrodynamic effect. Higher flow rates enable flowing cells that attach to the top of a bound cell to be pushed over to the downstream end of the bound cell where it could contact and bind to the P-selectin surface. Higher flow rates enable the transfer of the flowing cell from the bound cell to the surface before the cell-cell interaction is broken and the cell is released into the flowing stream. At lower shear stresses, the cell-cell interactions were mainly transient. The cell would release back into the flowing stream because bonds formed with the bound cell were broken before the cell was pushed over to the P-selectin surface. Therefore, as the shear stress was increased, the majority of cells accumulated on the surface through cell-cell interactions. This indicated that recruitment through secondary attachments was more efficient than through primary attachments at the higher shear stresses. Accumulation of cells through cell-cell interactions possibly is an important physiological mechanism for cell recruitment when receptors on the surface are blocked by attached cells.

Chapter 3: HL-60 Cell-Cell Interactions can be Mediated by Platelet Microparticles in Fetal Bovine Serum

Introduction

Cell accumulation due to cell-cell interactions has been a recent area of investigation since accumulation by this mechanism leads to enhanced cell accumulation in vitro. Enhanced HL-60 cell accumulation through cell-cell interactions was unexpected since HL-60 cells do not express L-selectin which mediates neutrophil-neutrophil interactions. To determine the unknown mechanism of the HL-60 cell-cell interactions, additional simplified systems were used to characterize the HL-60 cell-cell interactions. HL-60 cells were prebound to anti-PSGL-1 antibody PL2, anti-human CD43 antibody, or E-selectin coated glass slides. These systems provided P-selectin free systems in which antibodies against PSGL-1 could be used to determine the role of PSGL-1 in the cell-cell interactions. HL-60 cell-cell interactions were studied by withdrawing HL-60 cells over the prebound cells in the presence of various antibodies and reagents to determine the specificity of the interactions. It was determined the cell-cell interactions were PSGL-1-dependent. Flow experiments using HL-60 cells were then performed over bilayers containing purified PSGL-1. HL-60 cells interacted transiently with these surfaces; therefore, the kinetic off-rate of the interaction was determined for the interaction and compared to off-rates of known interactions to provide additional information in the characterization of the unknown mechanism.

Materials and Methods

Materials

Paraformaldehyde, sodium azide, and 2-deoxyglucose were obtained from Sigma Chemical Co. (St. Louis, MO). E-selectin and PSGL-1 purified from leukocytes (151) were obtained from Dr. R. McEver (University of Oklahoma, Oklahoma City, OK).

Antibodies

The function blocking anti-L-selectin antibody DREG56 was obtained from ZYMED Laboratories Inc. (South San Francisco, CA). The function blocking anti-L-selectin antibody DREG200 was provided by Dr. T. K. Kishimoto (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). The anti-human CD43 antibody was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Treatments

Flow Experiments to Characterize Cell-Cell Interactions on P-selectin Bilayers

To determine if cell activation was involved in the cell-cell interactions, HL-60 cells were fixed with 1% paraformaldehyde at 4 °C for 20 minutes. For metabolic inhibition flow experiments, HL-60 cells ($5x10^5$/ml) in HBSS/1% FBS were incubated at room temperature with 0.06% NaN_3 and 50 mM 2-deoxyglucose for 30 minutes. To determine if L-selectin was mediating the cell-cell interactions, HL-60 cells ($5x10^5$/ml) were incubated with the anti-L-selectin antibody DREG200 (5 μg/ml).

Flow Experiments: Cell-Cell Interactions with Prebound Cells

HL-60 cell-cell interactions were studied using HL-60 cells preattached to an antibody coated or an adhesion molecule coated glass slide. A glass coverslide coated with either E-selectin, anti-PSGL-1 antibody PL2, or anti-human CD43 antibody was attached to the flow chamber. E-selectin and the different antibodies were coated with 2.0-2.5 μg (40-50 μl of a 0.05 μg/μl solution) by placing the glass slide on a 40-50 μl drop of the solution in a plastic petri dish. After 5 minutes the glass slide was attached to the flow chamber as previously described (Chapter 2: *Flow Chamber and Attachment of Glass Cover Slides, page 33*). HL-60 cells were infused into the flow chamber and allowed to settle to the surface for 10 minutes with no flow. Unbound cells were washed out of the flow chamber by withdrawing HBSS through at 1.4 dynes/cm^2 for 5 minutes before new HL-60 cells or neutrophils were withdrawn into the system. The prebound cells did not roll or detach under the levels of shear stress studied. Typically 20-40 cells remained randomly attached to the surface in a 20x field of view (approximately 0.12 mm^2). The number of cell-cell interactions occurring was counted and normalized by dividing the number of interactions by the number of cells bound to the surface and duration of the experiment. A cell-cell interaction was defined as a flowing cell binding to an attached cell for at least one video frame. Very few or no HL-60 cells or neutrophils attached to the antibody coated slide after interacting with a bound cell. HL-60 cells ($5x10^5$/ml) in HBSS/1% FBS were withdrawn at 0.4 dynes/cm^2. Neutrophils were withdrawn over the prebound cells at 1.4 dynes/cm^2. Additional experiments were performed using HL-60 cells that had been washed two times in 5 mM EDTA, once in HBSS, and resuspended in HBSS/0.6% HSA.

Cell Treatments

Flow Experiments for Analysis of Cell-Cell Interactions with Prebound Cells

HL-60 cells or neutrophils ($5x10^5$/ml) in HBSS/1% FBS were incubated with 10 μg/ml of S12, G1, PL1, or PL2 to determine the specificity of the interactions. Cells were incubated with the antibody for 10 minutes at room temperature before being withdrawn into the system. Cells were also withdrawn through the system in the presence of 5 mM EDTA to determine if the cell-cell interactions were calcium-dependent.

PSGL-1 Preparation

A leukopheresis product (200-250 ml) was obtained from the Oklahoma Blood Institute. The product was aliquoted into Falcon 250 ml conical centrifuge tubes and centrifuged at 200g for 20 minutes at room temperature. After the platelet-rich plasma (PRP) was aspirated, the tubes were swirled gently to resuspend the pellets. Red blood cells were lysed by the addition of 100 ml of 5 mM EDTA, pH 7.5 for 20 seconds followed by the addition of 100 ml 1.8% NaCl, 5 mM EDTA, pH 7.5 to restore isotonicity. The cell suspensions were centrifuged at 500g for 5 minutes and the supernatant aspirated. The pellets were resuspended with ice-cold Ca^{2+} and Mg^{2+} free HBSS, 5 mM EDTA, 10 mM MOPS, pH 7.5. The suspensions were combined into two 250 ml conical centrifuge tubes and the volume of each was brought up to 200 ml with ice-cold Ca^{2+} and Mg^{2+} free HBSS, 5 mM EDTA, 10 mM MOPS, pH 7.5. The two tubes were centrifuged at 500g for 5 minutes and the supernatant was aspirated. The pellets were resuspended with ice-cold Ca^{2+} and Mg^{2+} free HBSS, 5 mM EDTA, 10 mM MOPS, pH 7.5 and combined into one 250 ml conical centrifuge tube and the volume was brought up to 200 ml. 400 μl of 1 M

diisopropylfluorophosphate (DFP) was added to a final concentration of 2 mM and incubated on an ice bath for 20 minutes with occasional gentle stirring. This suspension was centrifuged at 500g for 5 minutes and the supernatant aspirated. The pellet was resuspended with ice-cold 1X relaxation buffer (10X: 1 M KCl, 30 mM NaCl, 10 mM Na_2ATP, 35 mM $MgCl_2$, 100 mM HEPES, pH 7.3) and the suspension was transferred to a flat-bottom container. The volume was brought up to 140 ml. 1.4 ml of 100X protease inhibitor cocktail (100X: 1 mg/ml leupeptin, 2 mg/ml antipain HCl, 10 mg/ml benzamidine HCl) and 0.28 ml of 1 M DFP was added to the container. The flat-bottom container was placed into a cell disruption bomb (Parr Instrument Company, Moline, IL) and pressurized with nitrogen at 350 psi for 5 minutes in a cold room (76, 535). Approximately 35 ml of cavitate was collected drop by drop into four 50 ml centrifuge tubes containing 0.35 ml of 0.2 M EGTA, mixed gently, and centrifuged at 500g for 10 minutes. The post-nuclear supernatant was layered (~20 ml) over 8 ml cold 40% sucrose in 1X relaxation buffer containing 1X protease inhibitor cocktail and centrifuged at 104,000g for 45 minutes at 4^0C. The cytosolic fraction was aspirated and the cloudy sucrose layer was collected. This fraction was diluted with four volumes of cold 0.1 M NaCl, 20 mM MOPS, pH 7.5 and centrifuged at 114,000g for 45 minutes at 4^0C. The supernatant was aspirated and the pellets were extracted with a total of 1 ml of extraction buffer (5% Triton-X-100, 0.1 M NaCl, 20 mM TAPS pH 8.5, 1X protease inhibitor cocktail, and 0.02% NaN_3) per 10^9 cells and incubated on a rocker overnight at 4^0C. The extract was centrifuged at 1800g for 10 minutes at 4^0C and the supernatant pH was adjusted to 7.5 by the addition of 1 M MOPS. PSGL-1 was purified by wheat germ agglutinin (WGA) affinity chromatography and a truncated P-selectin (tPS) column as

described in Moore et al (76). The WGA affinity column buffers used were an equilibration buffer and wash #1 (0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 1% Triton, 0.02% NaN_3), a wash #2 buffer (0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.2% Brij-58, 0.02% NaN_3), an elution buffer (wash #2 with 0.5 M N-acetylglucosamine), and a column regeneration buffer (1 nM NaCl, 50 mM sodium acetate, pH 4.0). The buffers used for the tPS column were an equilibration and wash buffer (0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% Brij-58, 0.02% NaN_3) and an elution buffer (0.1 M NaCl, 20 mM MOPS, pH 7.5, 5 mM EDTA, 0.02% Brij-58, 0.02% NaN_3).

Flow Experiments: HL-60 Cell and Neutrophil Interactions with Purified PSGL-1

HL-60 cells ($5x10^5$/ml) in HBSS/1% FBS were withdrawn over a PSGL-1 bilayer at 0.15 and 0.4 dynes/cm^2 to determine if HL-60 cells could bind purified PSGL-1 even though no L-selectin was detected on the cell surface. Neutrophils ($5x10^5$/ml) in HBSS/1% FBS were also withdrawn over the same surfaces to show PSGL-1 had been incorporated into the bilayers. The number of HL-60 cell transient events was counted for a 10 minute experiment. The number of neutrophil events with the surface (rolling + transient) was also counted for a 10 minute experiment.

Cell Treatments

Flow Experiments to Characterize HL-60 Cell and Neutrophil Interactions with Purified PSGL-1

HL-60 cells or neutrophils ($5x10^5$/ml) in HBSS/1% FBS were preincubated with 10 μg/ml of PL1, PL2, DREG56, or DREG200 for 10 minutes at room temperature to show the specificity of the interactions. Cells were also withdrawn through the system in 5 mM EDTA to show the interactions were calcium-dependent.

Results

Characterization of HL-60 Cell-Cell Interactions on P-selectin Bilayers

As described in Chapter 2, HL-60 cells were able to efficiently accumulate on P-selectin bilayers through cell-cell interactions. Accumulation of HL-60 cells through cell-cell interactions accounted for over 80% of HL-60 cells bound to the surface at 0.7-1.4 dynes/cm^2. The mechanism of the cell-cell interactions was unclear since HL-60 cells do not express L-selectin which mediates cell-cell interactions between neutrophils. Experiments run at a shear stress of 1.4 dynes/cm^2 using cells incubated with an anti-L-selectin antibody (DREG200), cells fixed with 1% paraformaldehyde, or cells treated with 50 mM 2-deoxyglucose and 0.06% NaN_3 produced no change in the ability of cells to attach to the P-selectin surface or in the accumulation of cells through cell-cell interactions. These results indicated the cell-cell interactions were not a result of L-selectin or cell activation and did not require metabolic energy (Figure 19).

Characterizing the HL-60 Cell-Cell Interactions Using Other Systems

A surface consisting of 20-40 HL-60 cells per 20x field of view (0.12 mm^2) bound to an anti-PSGL-1 antibody (PL2) coated onto a glass slide was used to further characterize the cell-cell interactions. HL-60 cells in HBSS/1% FBS withdrawn at a shear stress of 0.4 dynes/cm^2 interacted transiently with the preattached cells on the surface. No cells withdrawn through the chamber attached to the antibody coated glass slide and cell-cell interactions did not result in cell accumulation on the surface. A transient cell-cell interaction was defined as a flowing cell attaching to a bound cell for at least one video frame (30 frames/sec). The number of cell-cell interactions was counted and divided by

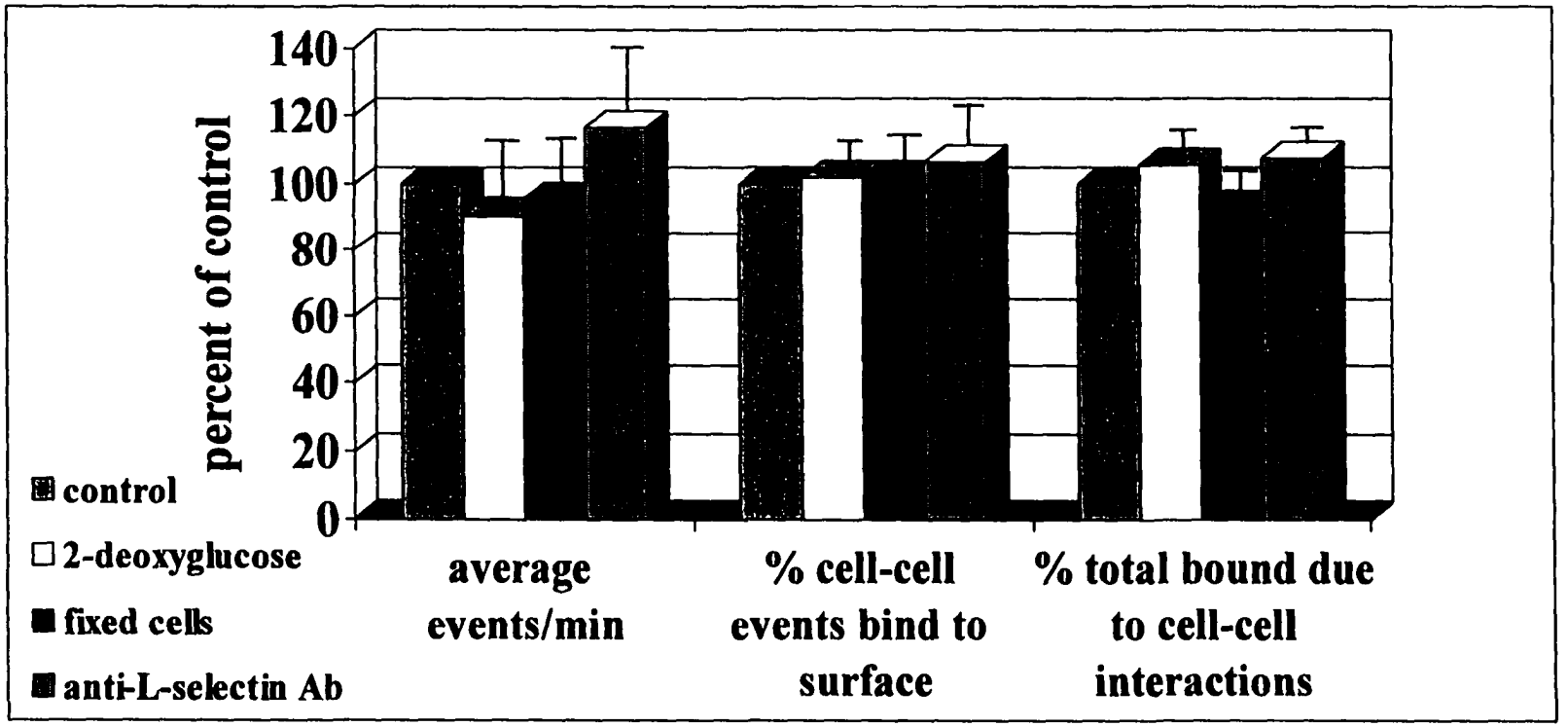


Figure 19. HL-60 cell-cell interactions did not depend on L-selectin or cell activation and did not require metabolic energy. HL-60 cells were withdrawn over bilayers containing P-selectin (350 sites/μm^2) to characterize cell accumulation due to cell-cell interactions. Incubating cells with 5 μg/ml of an anti-L-selectin antibody (DREG200), 50 mM 2-deoxyglucose/0.06% NaN_3, or 1% paraformaldehyde did not inhibit cells from binding to the P-selectin bilayer or accumulating through cell-cell interactions at 1.4 dynes/cm^2. Error bars represent +/- SEM. For 2-deoxyglucose and anti-L-selectin antibody, n=4. For fixed cells, n=3.

the number of cells attached to the surface and the duration of the experiment to calculate the rate of cell-cell interactions (Figure 20). Cell-cell interactions were eliminated when HL-60 cells being withdrawn over the surface were incubated with the anti-PSGL-1 blocking antibody (PL1) or resuspended in EDTA (Figure 20). Incubating the cells with a nonblocking anti-PSGL-1 antibody (PL2) or an anti-L-selectin antibody (DREG200) had no affect on the rate of cell-cell interactions (Figure 20).

A similar system was used to verify the results indicating the cell-cell interactions were mediated by PSGL-1, but not L-selectin. HL-60 cells in HBSS/1% FBS were withdrawn at a shear stress of 0.4 dynes/cm^2 over a surface of HL-60 cells bound (20-40 cells per 20x field of view, 0.12 mm^2) to an anti-human CD43 antibody coated glass slide. The cell-cell interactions were transient and there was no or little accumulation of cells on the surface. The rate of occurrence of cell-cell interactions was calculated as described above and is shown in Figure 21A. The cell-cell interactions were again blocked with an anti-PSGL-1 blocking antibody (PL1) and EDTA (Figure 21A). To verify L-selectin was not involved in the cell-cell interactions, HL-60 cells withdrawn through the system were incubated with a different anti-L-selectin antibody (DREG56). The anti-L-selectin antibody and the nonblocking anti-PSGL-1 antibody PL2 had no affect on the cell-cell interactions (Figure 21A).

Neutrophils were also withdrawn over bound HL-60 cells as a positive control for the anti-L-selectin antibody DREG56. Cell-cell interactions between neutrophils and HL-60 cells are a PSGL-1 and L-selectin-mediated process. The number of cell-cell interactions

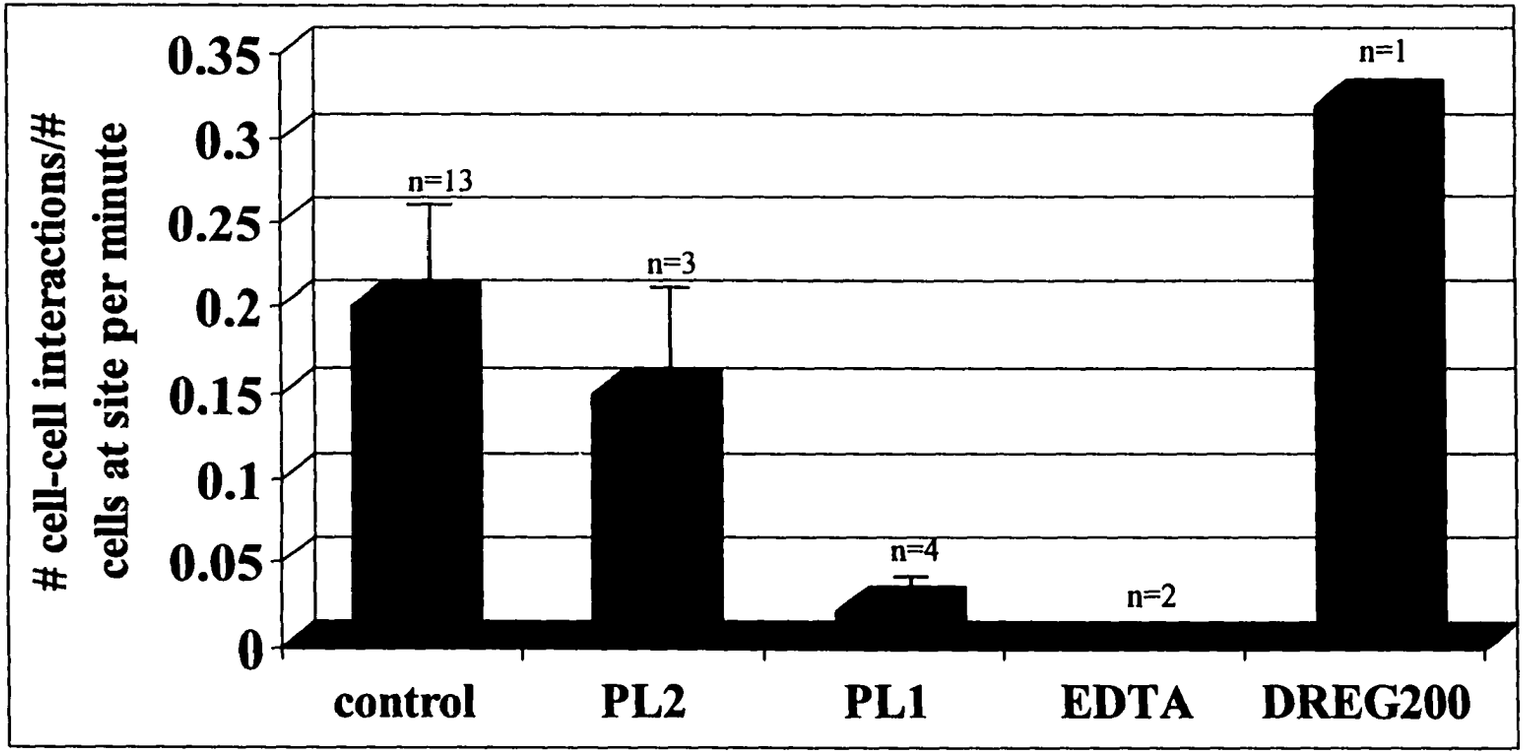


Figure 20. HL-60 cell-cell interactions were PSGL-1 and Ca^{2+}-dependent, but not L-selectin dependent. HL-60 were bound to a PSGL-1 antibody (PL2) coated glass slide. New HL-60 cells were withdrawn at 0.4 dynes/cm^2 over the bound cells and the number of transient cell-cell interactions was counted. The number of cell-cell interactions was normalized for the number of cells bound to the surface. Anti-L-selectin antibody (DREG200, 5 μg/ml) and a nonblocking PSGL-1 antibody (PL2, 10 μg/ml) did not block the cell-cell interactions. The cell-cell interactions were blocked by a PSGL-1 blocking antibody (PL1, 10 μg/ml) and also by 5 mM EDTA. Error bars represent +/- SEM.

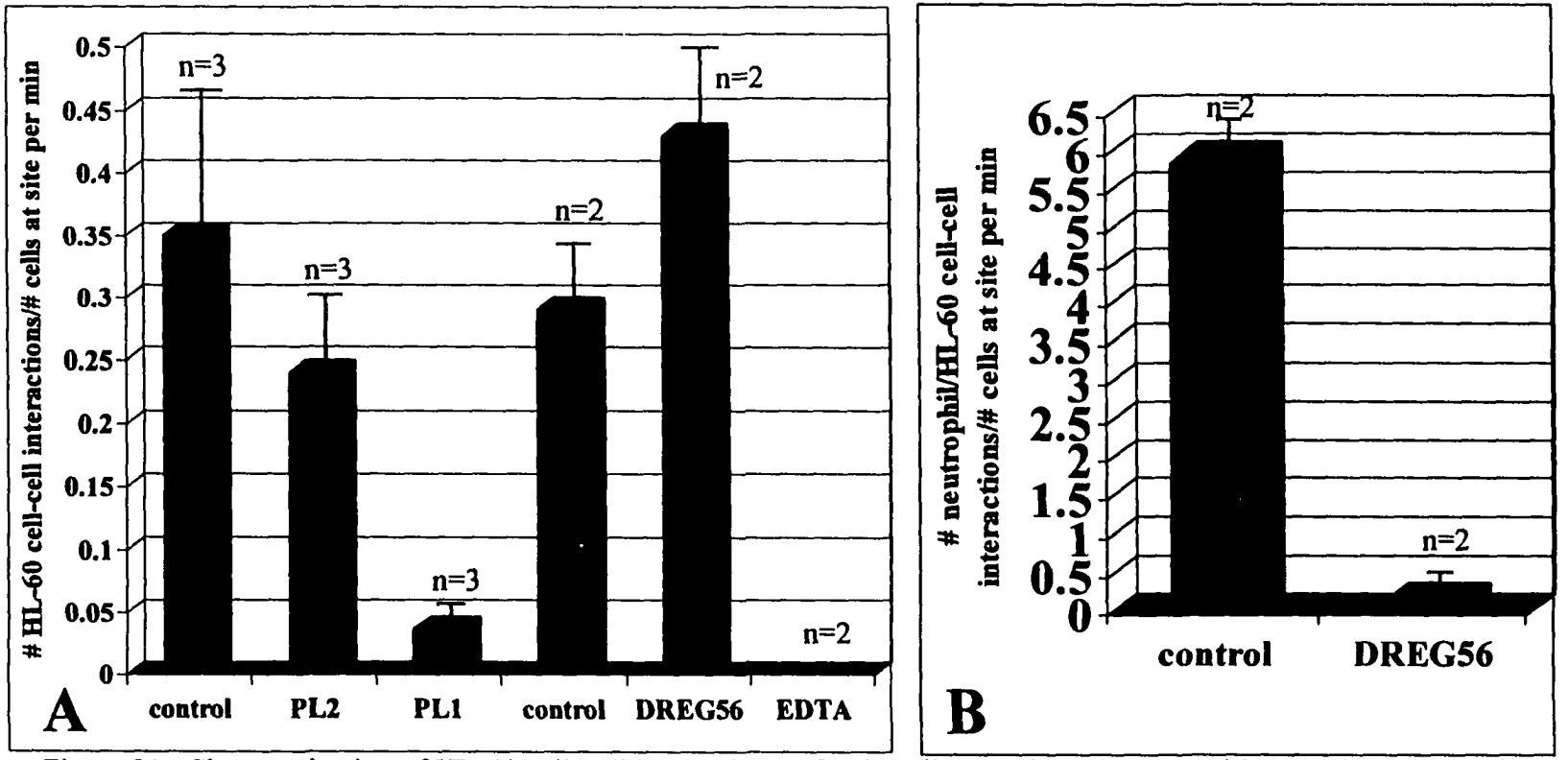


Figure 21. Characterization of HL-60 cell-cell interactions. HL-60 cells were bound to an anti-human CD43 antibody coated glass slide. New HL-60 cells were withdrawn at 0.4 dynes/cm^2 over the bound cells and the number of transient cell-cell interactions was counted (A). The anti-L-selectin antibody DREG56 (5 μg/ml) and the nonblocking PSGL-1 antibody PL2 (10 μg/ml) did not inhibit the cell-cell interactions. However, the cell-cell interactions were inhibited by the blocking PSGL-1 antibody PL1 (10 μg/ml) and the calcium chelator EDTA (5 mM). Neutrophils were also withdrawn over HL-60 cells at 1.4 dynes/cm^2 as a control to show the DREG56 was inhibiting L-selectin (B). Error bars represent +/- SEM.

at a shear stress of 1.4 dynes/cm^2 was counted for neutrophils with and without the presence of DREG56. As expected, the incubation of neutrophils with DREG56 inhibited interactions between neutrophils and HL-60 cells by approximately 95% (Figure 21B).

HL-60 Cell Interactions with Purified PSGL-1

Since the above experiments indicated the HL-60 cell-cell interactions were mediated by PSGL-1, the interaction of HL-60 cells with purified PSGL-1 was studied. HL-60 cells in HBSS/1% FBS withdrawn over a lipid bilayer containing purified PSGL-1 interacted transiently in experiments done at shear stresses of 0.15-0.7 dynes/cm^2. The transient interactions were significantly reduced by the presence of the anti-PSGL-1 blocking antibody PL1, but not affected by the nonblocking anti-PSGL-1 antibody PL2 (Figure 22). HL-60 cell interactions with PSGL-1 were also not affected by anti-L-selectin antibodies DREG56 and DREG200 (Figure 22 and data not shown). Removal of Ca^{2+} by EDTA completely blocked the transient interactions. Neutrophils were again used as a positive control for the various antibodies. Neutrophils rolled on the same PSGL-1 bilayers at 1.4-2.2 dynes/cm^2. At shear stresses of 0.15-0.7 dynes/cm^2, neutrophils interacted transiently with the PSGL-1 bilayer. The presence of the anti-L-selectin antibodies DREG56 or DREG200 (data not shown) or the blocking anti-PSGL-1 antibody PL1 eliminated all rolling at the higher shear stresses and greatly reduced the number of transient interactions occurring at the lower shear stresses. EDTA removed all types of interactions at all shear stresses (Figure 23). Neutrophils incubated with the

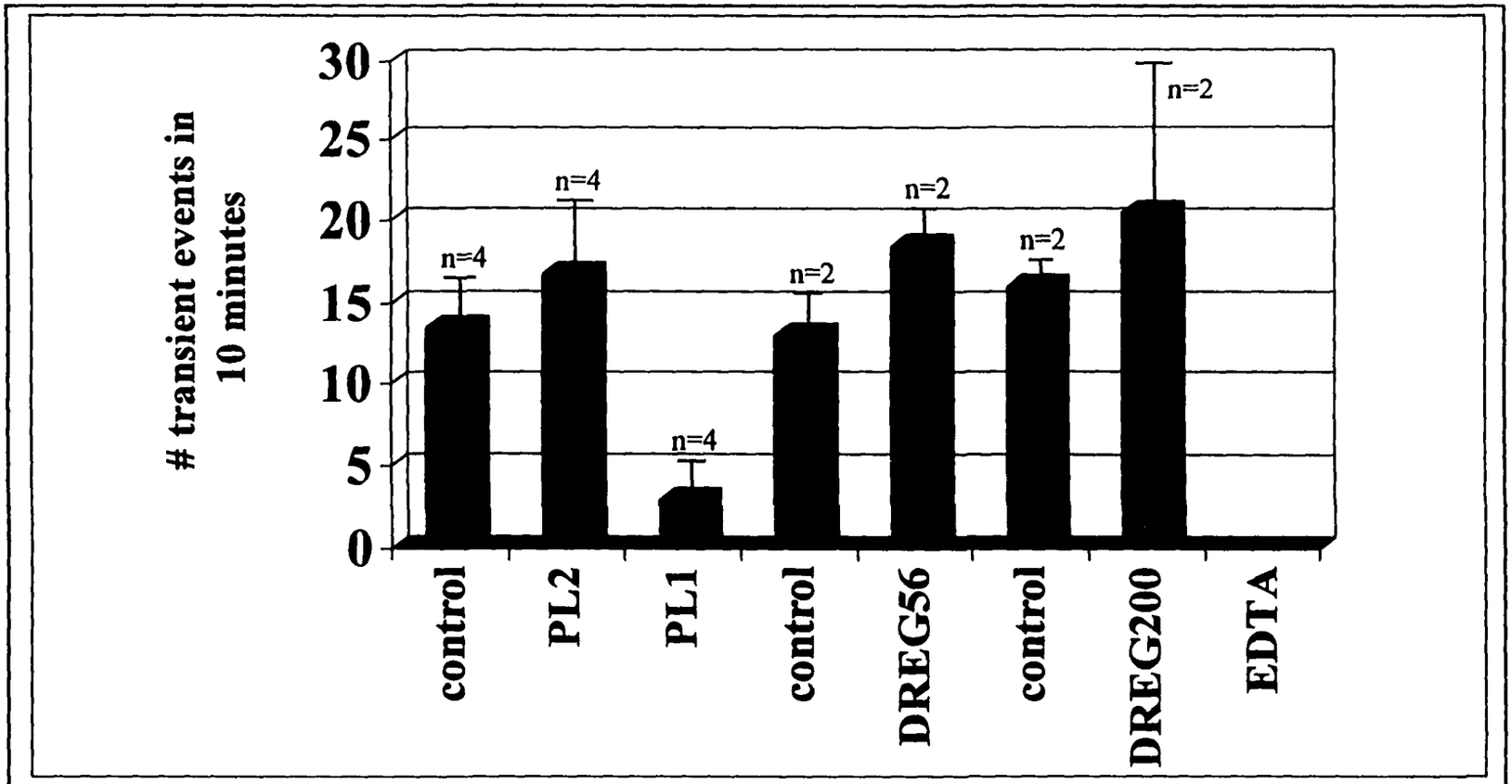


Figure 22. HL-60 cell interactions with PSGL-1 were not L-selectin dependent. HL-60 cells were withdrawn at 0.4 dynes/cm^2 over a bilayer containing purified PSGL-1. HL-60 cells only interacted transiently with the surface. The interactions were not blocked with anti-L-selectin antibodies DREG56 or DREG200 (5 μg/ml) or the nonblocking PSGL-1 antibody PL2 (10 μg/ml). The number of transient interactions was greatly reduced in the presence of the PSGL-1 blocking antibody PL1 (10 μg/ml) and the interactions were blocked in the presence of 5 mM EDTA. Error bars represent +/- SEM.

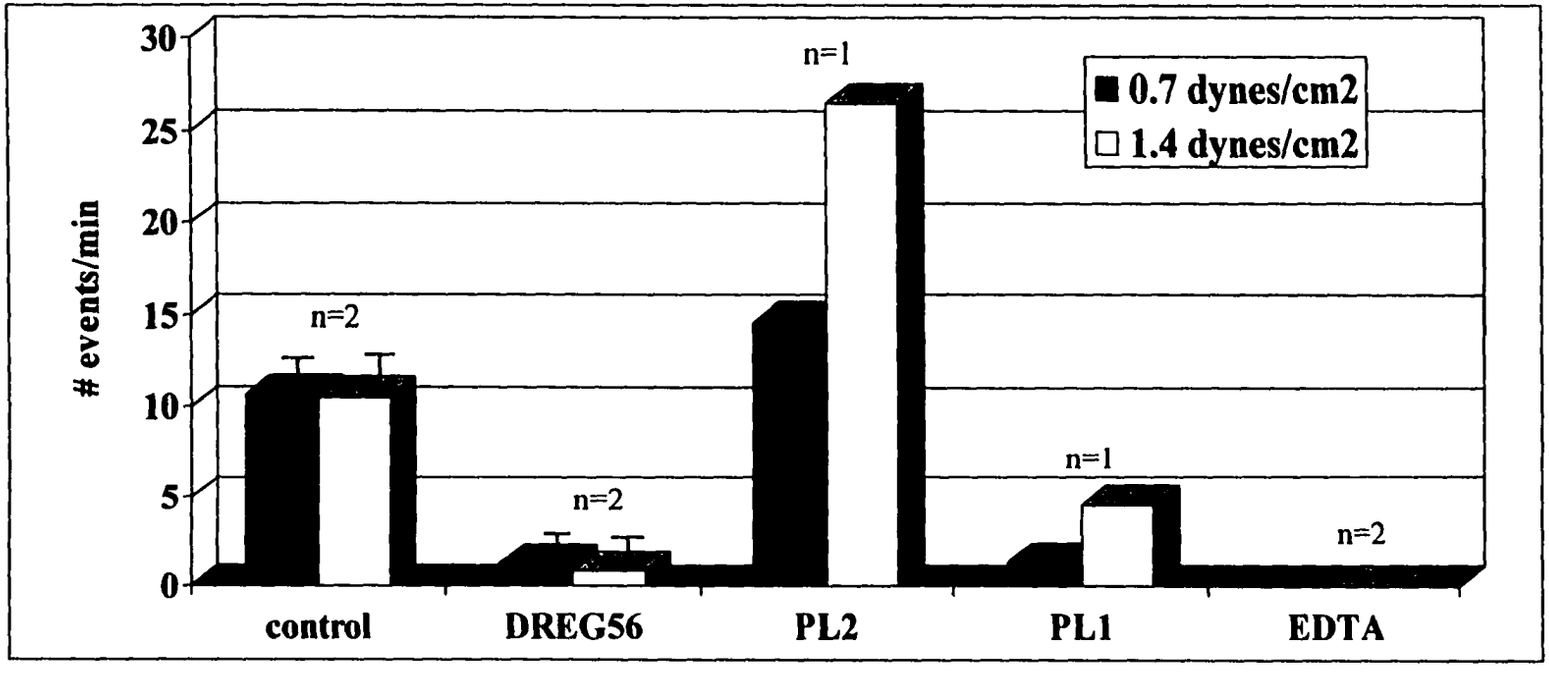


Figure 23. Neutrophil interactions with PSGL-1 were L-selectin dependent. Control experiments were run on the same PSGL-1 bilayers as the HL-60 cells to show the anti-L-selectin antibodies were functional. Neutrophils were withdrawn over the bilayers containing purified PSGL-1 at shear stresses of 0.15-2.2 dynes/cm^2. The number of neutrophil events with the surface (rolling + transient) was counted. At shear stresses of 0.14-0.7 dynes/cm^2 neutrophils mainly interacted transiently with the PSGL-1 surface. However, neutrophils rolled on the surfaces at shear stresses above 1.4 dynes/cm^2. The presence of the anti-L-selectin antibodies DREG56 or DREG200 (not shown) (5 μg/ml) and the PSGL-1 blocking antibody PL1 (10 μg/ml) eliminated all rolling at the higher shears stresses and greatly reduced the number of transient interactions at the lower shear stresses. EDTA inhibited all types of interactions at all shear stresses. The nonblocking PSGL-1 antibody PL2 had no affect on the neutrophil interactions with the surface. Error bars represent +/- SEM.

nonblocking anti-PSGL-1 antibody PL2 had no affect on the number of rolling cells or the number of transient events (Figure 23).

Kinetic Analysis of the HL-60 Cell Interactions with PSGL-1

Another method used to show that the interaction of HL-60 cells with purified PSGL-1 was not L-selectin-mediated was the comparison of the off-rate (k_{off}) to other selectin-mediated interactions that had been studied (see Chapter 5). The kinetics of the HL-60 cell interaction with PSGL-1 were nearly identical to the off-rates of neutrophils and HL-60 cells on P-selectin (Table 3: Off-rates of P-selectin/PSGL-1 Interactions, page 130). The interaction of neutrophils (L-selectin) with PSGL-1 had a much faster off-rate (Figure 38, page 127). This again showed the HL-60 cell-cell interactions mediated by PSGL-1 were not also a result of L-selectin, as seen with neutrophils. The kinetic data indicating the interaction was mediated by P-selectin led to the hypothesis that platelet microparticles expressing P-selectin in the fetal bovine serum were coating the cells enabling HL-60 cell-cell interactions by producing a P-selectin 'bridge' between PSGL-1-expressing HL-60 cells.

Analysis of Cell-Cell Interactions Using Washed Cells

Since the kinetic data indicated platelet microparticles could be mediating the cell-cell interactions, the cell-cell interaction assays were repeated after washing the cells in EDTA to remove platelet microparticle contamination. The cells were resuspended in HBSS/0.6% HSA instead of FBS for flow experiments. HL-60 cell-cell interaction assays were done on HL-60 cells prebound to an E-selectin or an anti-human CD43

antibody coated glass slide. Washed HL-60 cells did not display the cell-cell interactions (Figure 24) indicating that platelet microparticles in the fetal bovine serum were mediating the cell-cell interactions.

However, there was an interesting discrepancy between cell-cell interaction assays done on P-selectin bilayers compared with cell-cell interaction assays done on the other surfaces. HL-60 cell-cell interactions on P-selectin bilayers were always observed to be greatest at 0.7-1.4 dynes/cm^2 and using cells that had been washed did not remove cell-cell interactions in that system. When unwashed HL-60 cells were used in systems with HL-60 cells prebound to PL2 or the anti-human CD43 antibody coated slides, cell-cell interactions only occurred below 0.4 dynes/cm^2 and little or no cell-cell interactions occurred at shear stresses of 0.7-1.4 dynes/cm^2 (Table 1). We were able to reproduce the cell-cell interactions at high shear stresses (0.7-1.4 dynes/cm^2) with washed HL-60 cells by incubating the cells with 4.2 μg/ml membrane P-selectin (mPS). mPS is known to form an oligomer, micelle-like structure, in solution which could act as a bridge between cells.

Discussion

Accumulation of HL-60 cells on a bilayer containing P-selectin through cell-cell interactions was unexpected since HL-60 cells do not express L-selectin which mediates cell-cell interactions for neutrophils. In order to study the HL-60 cell-cell interactions in a calcium free system and in the presence of anti-PSGL-1 antibodies, HL-60 cells were prebound to an anti-PSGL-1 antibody or an anti-human CD43 antibody coated glass

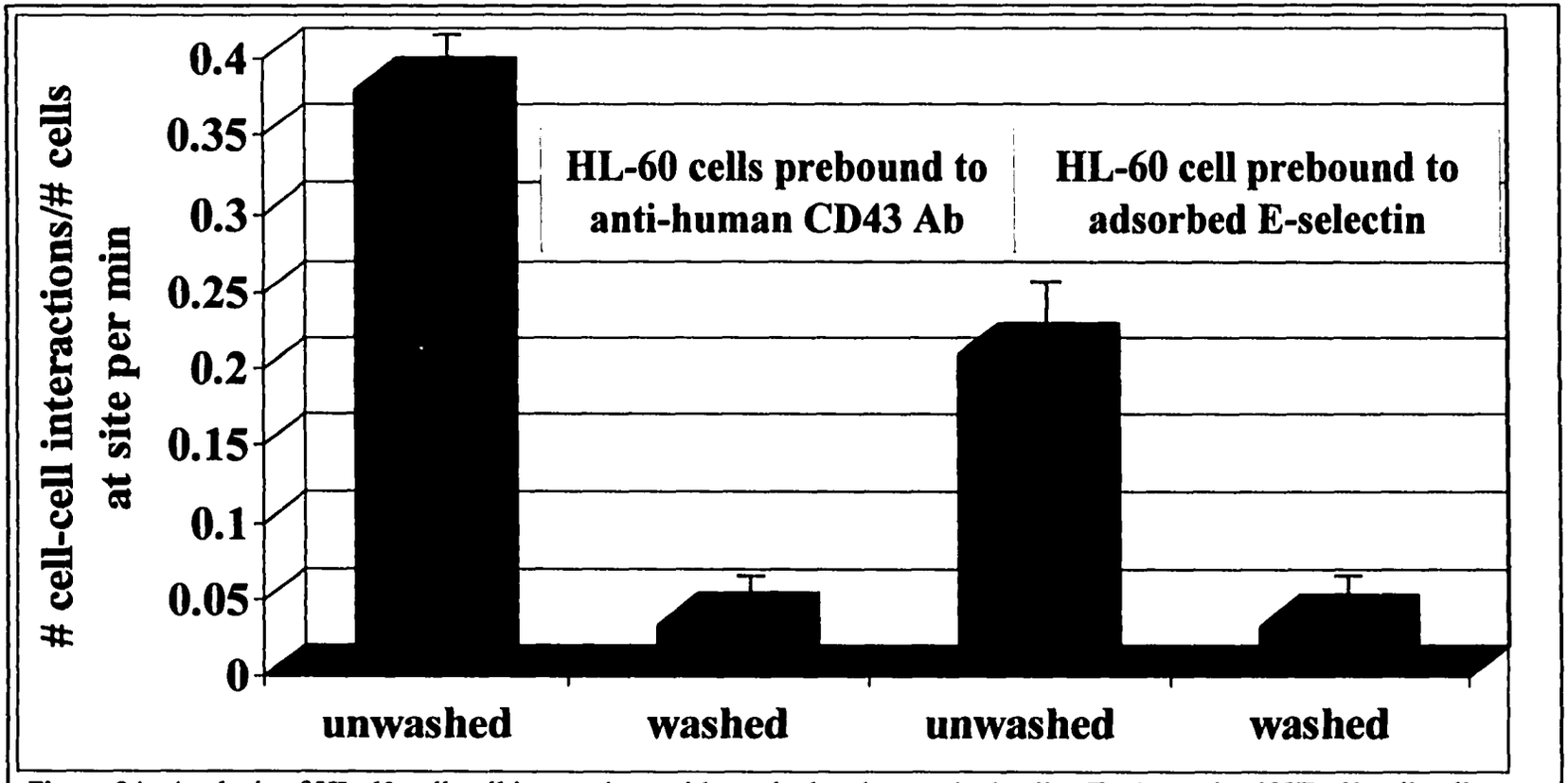


Figure 24. Analysis of HL-60 cell-cell interactions with washed and unwashed cells. To determine if HL-60 cell-cell interactions were the result of platelet microparticle contamination in the fetal bovine serum, cell-cell interaction assays were run with unwashed and washed cells. HL-60 cells were washed twice in 5 mM EDTA and once in HBSS. Unwashed cells in HBSS/1% FBS or washed HL-60 cells in HBSS/0.6% HSA were withdrawn over HL-60 cells prebound to an anti-human CD43 antibody or an E-selectin coated slide. The number of cell-cell interactions was greatly reduced using cells that had been washed indicating that cell-cell interactions were mediated by platelet microparticle contamination in the fetal bovine serum. Error bars represent +/- SEM. For anti-human CD43 Ab surface, n=3; for E-selectin surface, n=2.

# Cell-Cell Interactions/# Cells at Site per Minute										
	Surface:	HL-60 Prebound to PL2				HL-60 Prebound to anti-human CD43 Ab				
		control	PL2	PL1		control	PL2	PL1		
shear (dynes/cm2)		unwashed cells				unwashed cells				
1.4		0	0	0		-	-	-		
0.7		0.03	-	-		-	-	-		
0.4		0.2	0.15	0.021		0.35	0.24	0.037		
	Surface:	HL-60 Prebound to anti-human CD43 Ab			adsorbed E-selectin (sES)		P-selectin Bilayer (mPS)			
shear (dynes/cm2)		unwashed	washed cells		unwashed	washed cells	unwashed	washed cells		
1.4		0	0		0	0	**	**	** washed and unwashed HL-60 cells	
0.7		-	-		0.13	0.01	**	**	accumulated on mPS bilayers through cell-	
0.4		0.38	0.034		0.21	0.033	**	**	cell interactions	

Table 1. Comparison of HL-60 cell-cell interactions on different surfaces using unwashed and washed cells. There was a discrepancy in occurrence of cell-cell interactions when assays were done on P-selectin bilayers compared to assays done on prebound HL-60 cells or adsorbed E-selectin. On P-selectin bilayers, HL-60 cell-cell interactions were always observed to be greatest at 0.7-1.4 dynes/cm^2; however, on the other surfaces very few or no interactions occurred above 0.4 dynes/cm^2. Also, washing the HL-60 cells did not eliminate cell-cell interactions on P-selectin bilayers even though washing cells eliminated cell-cell interactions on the other surfaces. It was concluded that cell-cell interactions on prebound cells and on adsorbed E-selectin at 0.4 dynes/cm^2 resulted from platelet microparticle contamination since the interactions were removed with cell washing. It was concluded that cell-cell interactions on P-selectin bilayers were mediated from another unknown mechanism since washing did not remove the interactions and the interactions occurred at much higher shear stresses.

slide. These systems allowed HL-60 cells to be withdrawn over prebound HL-60 cells in the presence of a calcium chelator and various antibodies to determine the specificity of the cell-cell interactions. The cell-cell interactions in these systems were transient. A flowing cell would attach to a bound cell for less than 4 seconds and then release back into the flowing stream. Very few flowing cells attached directly to the antibody coated slide giving a system where accumulation of cells did not occur. Cell-cell interactions in these systems were not blocked with anti-L-selectin antibodies verifying that these interactions were not L-selectin-mediated as seen with neutrophils. The cell-cell interactions were inhibited by an anti-PSGL-1 blocking antibody showing that the cell-cell interactions were clearly PSGL-1-mediated. Elimination of the cell-cell interactions with the calcium chelator EDTA showed the cell-cell interactions were Ca^{2+}-dependent. Since cell-cell interaction assays in these systems indicated that the HL-60 cell-cell interactions were mediated by PSGL-1, additional flow experiments were performed on lipid bilayers containing purified PSGL-1. The HL-60 cells also interacted with these surfaces supporting the conclusion that the HL-60 cell-cell interactions were PSGL-1-mediated. HL-60 cell interactions with the purified PSGL-1 were transient which allowed the kinetic off-rate of the interaction to be measured. The off-rate calculated for this unknown interaction was nearly identical to the off-rate calculated for the P-selectin/PSGL-1 interaction. This kinetic analysis indicated that P-selectin contamination may be present. The hypothesis was that P-selectin-expressing platelet microparticles from the fetal bovine serum in the growth media were bound to the HL-60 cells and allowed the PSGL-1-expressing HL-60 cells to interact with each other. This allowed PSGL-1-expressing cells to display P-selectin as well. Platelet microparticles bound to a

cell then could act as a bridge to bind to another PSGL-1-expressing cell. To test this hypothesis, HL-60 cells were washed with EDTA to remove platelet microparticle contamination and the cells were resuspended in buffer with human serum albumin instead of fetal bovine serum. Elimination of cell-cell interactions using washed cells indicated the cell-cell interactions in the systems using prebound HL-60 cells were mediated by platelet microparticles.

However, as shown in Table 1 there was a discrepancy at which shear stresses the cell-cell interactions occurred in the P-selectin bilayer system versus the systems using prebound cells. Washing cells eliminated cell-cell interactions in the systems using prebound cells; however, accumulation through cell-cell interactions occurred using washed cells on P-selecting bilayers. Accumulation through cell-cell interactions on P-selectin bilayers occurred with washed or unwashed cells at 0.7-1.4 dynes/cm^2. Very few or no cell-cell interactions occurred in systems using prebound cells even when withdrawing unwashed HL-60 cells over the prebound cells above 0.7 dynes/cm^2. At this point it was concluded that cell-cell interactions at 0.4 dynes/cm^2 on prebound cells using unwashed cells were mediated by platelet microparticles since the cell-cell interactions were eliminated by washing the cells. It was also concluded that HL-60 cell accumulation on P-selectin bilayers at 0.7-1.4 dynes/cm^2 through cell-cell interactions was not mediated by platelet microparticles, but some unknown mechanism. It is believed that somehow the cell-cell interactions were being mediated by the P-selectin in the system. The P-selectin incorporated into bilayers or bound to a glass slide should not be in the system in such a way as to mediate cell binding. Monomeric P-selectin in the

system should in fact inhibit cell binding by binding to PSGL-1 and acting as a competitive inhibitor. However, if some of the P-selectin in the system was oligomeric and was not bound to the glass slide or was removed from the slide, the soluble oligomeric P-selectin micelles could bind to PSGL-1 on the HL-60 cells. Oligomeric P-selectin could then produce a P-selectin bridge between PSGL-1-expressing cells in the same manner as a platelet microparticle. This hypothesis was tested by withdrawing washed HL-60 cells in the presence of the membrane P-selectin (mPS) used in the lipid vesicle preparations over HL-60 cells prebound to anti-human CD43 antibody coated on a glass slide. The presence of the mPS produced HL-60 cell-cell interactions at shear stresses of 0.4-1.4 dynes/cm^2 (data not shown). This result indicates that possibly the P-selectin bilayer system contained P-selectin oligomers which were capable of mediating cell-cell interactions between HL-60 cells.

Chapter 4: Cell-Cell Interactions Mediated by Platelet Microparticles

Introduction

Platelet microparticles are released from activated platelets and express functional receptors, including P-selectin, on their surface. Platelet microparticle concentrations have been shown to be elevated in many disorders and diseases; however, little work has been done to show if platelet microparticles play any role in clotting, cell aggregation, or cell accumulation. We investigated the possibility that platelet microparticles could mediate cell aggregation, cell-cell interactions, and enhanced cell recruitment through cell-cell interactions. It was predicted that P-selectin-expressing platelet microparticles could act as a bridge between PSGL-1-expressing cells allowing them to interact. HL-60 cells or neutrophils in the presence of increasing concentration levels of platelet microparticles were withdrawn over HL-60 cells prebound to an anti-human CD43 antibody coated glass slide to determine if platelet microparticles could mediate cell-cell interactions, cell aggregation, and possibly cell accumulation through cell-cell interactions. We were also interested in studying the ability of platelet microparticles in mediating neutrophil/HL-60 cell interactions in the presence of an anti-L-selectin antibody. Platelet microparticles may be an important mediator of clotting, cell aggregation, and cell accumulation through cell-cell interactions, especially in disease states where platelet microparticle concentrations are elevated.

Materials and Methods

Materials

Acid citrate dextrose (ACD), calcium ionophore (A23187), dimethyl sulfoxide (DMSO), potassium chloride, magnesium chloride, sodium phosphate, glucose, HEPES, disodium salt EDTA, prostaglandin E_1 (PGE_1), potassium phosphate, sodium citrate, and sodium acetate were obtained from Sigma Chemical Co. (St. Louis, MO). D-Phe-Pro-Arg Chloromethyl Ketone, Dihydrochloride (PPAK) was obtained from Calbiochem-Novabiochem Corporation (San Diego, CA).

Antibodies

The anti-platelet integrin GPIIb antibody TAB was provided by Dr. R. McEver and labeled with FITC by Paul Friese (University of Oklahoma, Health Sciences Center, Oklahoma City, OK).

Isolation of Platelets and Platelet Microparticles

Whole blood was mixed with 1/9 its volume of acid citrate dextrose (ACD) anticoagulant and 0.2 μg/ml prostaglandin E_1 (PGE). The blood was centrifuged at 250g for 15 minutes to pellet red and white blood cells and leave a platelet-rich plasma (PRP) supernatant. The PRP was centrifuged at 1250g for 15 minutes to pellet the platelets and produce a platelet-poor plasma (PPP) supernatant. The PPP was spun down 2 more times at 1250g for 15 minutes to remove the remaining platelets. The final supernatant was PPP containing only platelet microparticles (particles less than approximately 1.0 μm) as shown by flow cytometry.

Generation of Platelet Microparticles by Calcium Ionophore

The platelet pellet was resuspended in 6 ml of wash buffer (9 mM Na_2EDTA, 26.4 mM $Na_2HPO_42H_2O$, 140 mM NaCl, pH 7.4) and centrifuged at 1250g for 15 minutes. This was repeated two more times to remove all plasma from the platelets. The final platelet pellet was resuspended in 6-7 ml of buffer A (137 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 5.5 mM glucose, 10 mM HEPES, pH 7.4) containing 2 mM $CaCl_2$. The calcium ionophore A23187 in DMSO was added to a final concentration of 10 μM and the platelet suspension was incubated at 37 °C for 20 minutes with occasional stirring. After the incubation, the platelets were pelleted by centrifugation at 1250g for 15 minutes. This was repeated to remove all the platelets. The resultant supernatant contained only platelet microparticles in buffer as shown by flow cytometry.

Generation of Platelet Microparticles by Aging Platelets

Platelet-rich plasma was obtained as described above and incubated at room temperature for 5 days. Platelet microparticles were generated spontaneously and detected by flow cytometry. Platelets were removed from the PRP by centrifugation at 1250g for 15 minutes after the 5 day incubation.

Detection of Platelet Microparticles by Flow Cytometry

Platelet microparticles were detected and distinguished from platelets by flow cytometry. The sample to be tested (10 μl) was added to 200 μl of buffered saline glucose citrate solution (BSGC: 1.6 mM KH_2PO_4, 8.6 mM Na_2HPO_4, 0.12 M NaCl, 13.6 mM $Na_3C_6H_5O_7$, 11.1 mM glucose, pH 7.3). A saturating concentration (2 μg/ml) of a

fluorescent anti-IIb antibody (2 μg of TAB-FITC) which binds to the platelet integrin GP IIb/IIIa was added and allowed to incubate at room temperature for 20 minutes. BSGC was added to bring the total volume up to 2.0 ml making the sample dilution 1:200. The sample was analyzed on a flow cytometer (FACScan, Becton Dickinson) and platelet microparticles were identified based on their fluorescence and forward scatter in relation to a platelet control. Platelet microparticles were defined as events with elevated levels of fluorescence that were less than 1 μm in size. The estimate of 1 μm on the flow cytometer was based on the center of the platelet population being approximately 2 μm which is the average diameter of platelets.

Flow Experiments: HL-60 Cell-Cell Interactions and Neutrophil/HL-60 Cell-Cell Interactions Mediated by Platelet Microparticles

HL-60 cells were attached to an anti-human CD43 antibody coated glass slide by infusing a high concentration of cells ($1x10^6$/ml) into the flow chamber and then allowing the cells to settle onto the surface for 10 minutes. The unbound cells were washed out of the flow chamber by withdrawing HBSS through the system at 1.4 dynes/cm^2 for 5 minutes. The HL-60 cells and neutrophils used in the cell-cell interaction assays were washed twice with 5 mM EDTA in HBSS, once with HBSS, and resuspended in HBSS/0.6% HSA. Platelet microparticles generated by calcium ionophore, platelet microparticles in new platelet-poor plasma, or platelet microparticles in 5 day old platelet-poor plasma were added at various concentrations to a 6 ml suspension of HL-60 cells or neutrophils and allowed to incubate at room temperature for 15 minutes. The HL-60 cells or neutrophils ($1x10^6$/ml in HBSS/0.6% HSA) were withdrawn over the prebound HL-60 cells to

determine the effect of platelet microparticles on cell-cell interactions. HL-60 cells were withdrawn over the prebound cells at 0.4 dynes/cm^2 and the neutrophils were withdrawn at 1.0 dynes/cm^2. The number of cell-cell interactions was counted and normalized for the number of cells on the surface and the duration of the experiment. Since HL-60 cells are larger than neutrophils, the shear stress was adjusted so that the force on the bond between cells would be approximately the same.

Cell Treatments

Flow Experiments for Platelet Microparticle Mediated Cell-Cell Interactions

In platelet microparticle cell-cell interaction assays, HL-60 cells or neutrophils ($1x10^6$/ml) in HBSS/0.6% HSA were incubated for 15 minutes at room temperature with 10 μg/ml PL1, PL2, G1, S12 or 5 μg/ml DREG56 in the presence of platelet microparticles. The cells used in the cell-cell interaction studies were washed two times in 5 mM EDTA and once in HBSS. For experiments performed with the addition of plasma (new or old platelet-poor plasma), heparin (7.5 U/ml) or PPAK (60 μmol/l) in 10 mM HCl was added to prevent fibrin clot formation.

Results

Generation and Isolation of Platelet Microparticles Verified by Flow Cytometry

Platelet microparticles (PMP) were isolated from freshly drawn blood by removing platelets from platelet-rich plasma leaving platelet microparticles in platelet-poor plasma. Platelet microparticles were generated by aging platelet-rich plasma for 5 days to allow platelets to spontaneously release microparticles. Platelets were removed by centrifugation and the platelet microparticles remained in the platelet-poor plasma. Platelet microparticles were also generated by activating platelets with the calcium ionophore A23187. Platelets were removed to leave only platelet microparticles in a buffer solution containing Ca^{2+}.

The generation of platelet microparticles was confirmed by flow cytometry in relation to a platelet control. Platelets and platelet microparticles were defined as events showing fluorescence from the TAB-FITC antibody (above horizontal line on plots). Events with no fluorescence obtained from a BSGC buffer sample were considered background noise (below the horizontal line) and were removed from the plots. Platelets were defined as fluorescent events in the upper right quadrant (above horizontal line and right of vertical line). Platelet microparticles were defined as fluorescent events in the upper left quadrant (above horizontal line and left of vertical line). Platelet microparticles were defined as particles less than 1 μm in size by forward scatter. The vertical line was set at approximately 1 μm based on the middle of the platelet population being 2 μm (average diameter of platelets). Platelet-rich plasma from freshly drawn blood contained very few platelet microparticles (3-5% of events) (Figure 25A). Removing the platelets from the

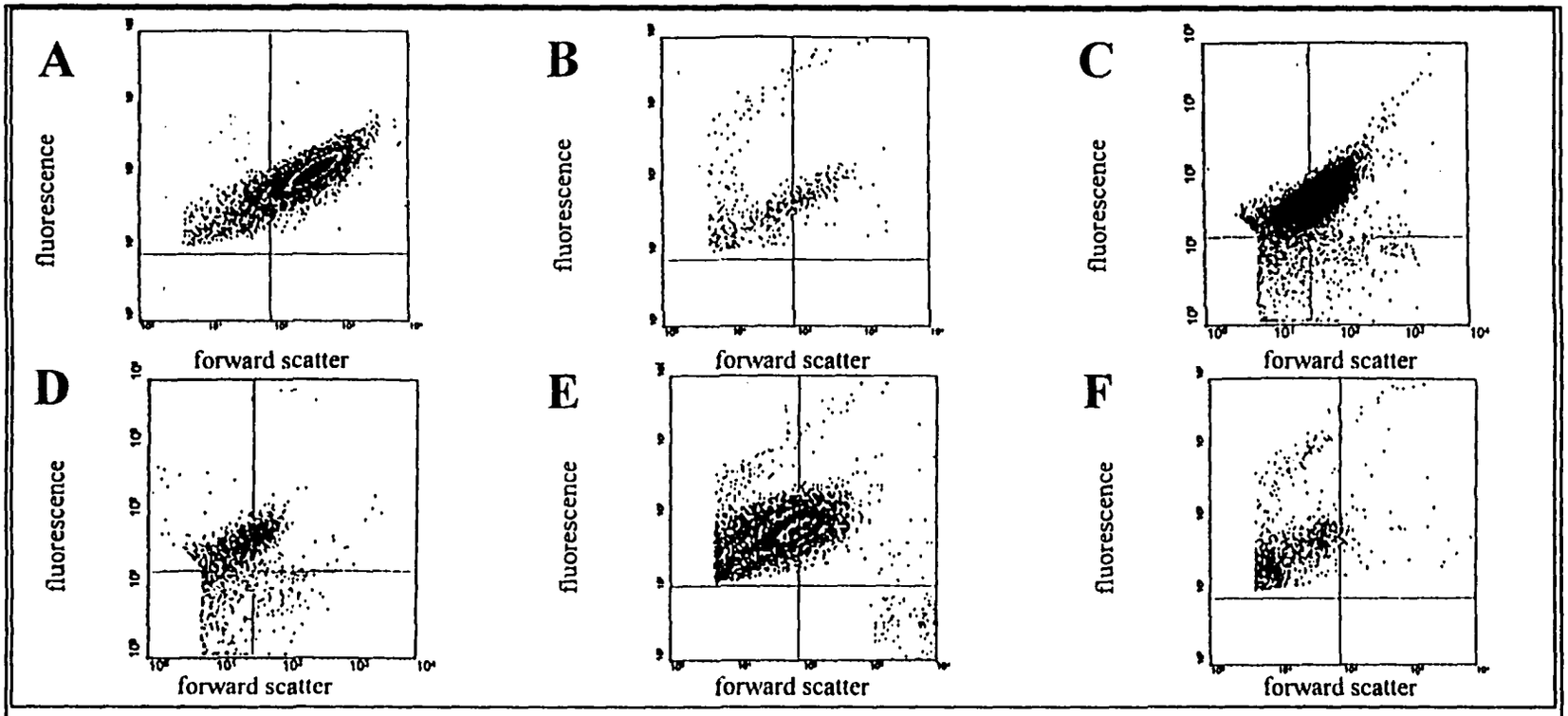


Figure 25. Generation and isolation of platelet microparticles (PMP) shown by flow cytometry. Freshly drawn blood contained very few PMP (3-5% of events) in platelet-rich plasma (A). Platelet-rich plasma that incubated for 5 days contained approximately 10 times the amount of PMP than new platelet-rich plasma (~30% of events) (C). Generation of platelet microparticles by activating platelets with calcium ionophore produced 10-30 times (~65% of events) more PMP than in new blood (E). Removing the platelets from the platelet-rich plasma yielded platelet-poor plasma containing mostly PMP (B, D). Removing activated platelets from the buffer yielded a plasma-free buffer containing mostly PMP (F). Platelets and platelet microparticles are events showing fluorescence (above horizontal line) for the GP IIb integrin. Platelet microparticles were defined as events less than 1 μm in size (left of vertical line).

platelet-rich plasma produced platelet-poor plasma containing mostly platelet microparticles (Figure 25B). Platelet-rich plasma that had been incubated at room temperature for 5 days contained approximately 10 times the amount of platelet microparticles than normal blood. (~30% of events) (Figure 25C). Removing platelets yielded a platelet-poor plasma containing mostly platelet microparticles (Figure 25D).

Platelets resuspended in a calcium-containing buffer were activated by calcium ionophore. Approximately 65-70% of the events of this platelet-rich solution were platelet microparticles (Figure 25E). Removing the platelets from the buffer solution gave a very clean suspension of platelet microparticles in a plasma-free buffer (Figure 25F).

Quantification of Platelet Microparticles by Flow Cytometry

The concentrations of platelet microparticles in new platelet-poor plasma, old platelet-poor plasma, and in the platelet microparticle plasma-free buffer solution generated by calcium ionophore were quantified by flow cytometry. The concentration of platelets in platelet-rich plasma was determined by a cell counter. Analysis of the platelet-rich plasma provided an event rate for the known concentration of particles. The procedure shown below is the method used to determine the unknown platelet microparticle concentrations. Quantification of platelet microparticles using this method was attempted 8 times. The calculated concentrations shown are representative for the specific sample analyzed. The concentration of platelets in platelet-rich plasma was determined by a cell counter (0.76×10^6 platelets/ml). An event rate was obtained from this control as shown in

Figure 26A (46650 events in upper right quadrant). Platelets were defined as events showing fluorescence (above horizontal line) larger than approximately 1 μm (right of vertical line).

0.76×10^6 platelets/ml = 46650 events/65 sec = 717.7 events/sec

The unknown platelet microparticle concentrations in new platelet-poor plasma, old platelet-poor plasma, and in the platelet microparticle solution generated by calcium ionophore were determined using the platelet event rate for the known platelet concentration (Figure 26B, C). Platelet microparticles were defined as events (in the upper left quadrant) less than approximately 1 μm (left of the vertical line) showing fluorescence (above horizontal line).

0.76×10^6 platelets/ml = 717.7 events/sec

from Figure B: [PMP in new PPP] = 1633 events/61 sec

therefore, with a sample dilution of 1:200 [PMP in new PPP] = 5.7×10^6 PMP/ml

0.76×10^6 platelets/ml = 717.7 events/sec

from Figure C: [PMP in ionophore soln] = 17826 events/61 sec

therefore, with a sample dilution of 1:200 [PMP in ionophore soln] = 6.2×10^7 PMP/ml

To verify the concentration that was calculated using the ratio of a known platelet concentration, a known concentration of red blood cells was added to the sample of platelet microparticles generated by calcium ionophore in order to use a ratio off the same flow cytometry plot (Figure 26D). The concentration of red blood cells added was determined by a cell counter. The red blood cell events were in the lower right quadrant while the platelet microparticle events were in the upper left quadrant.

$0.92x10^6$ red blood cells/ml = 12466 events

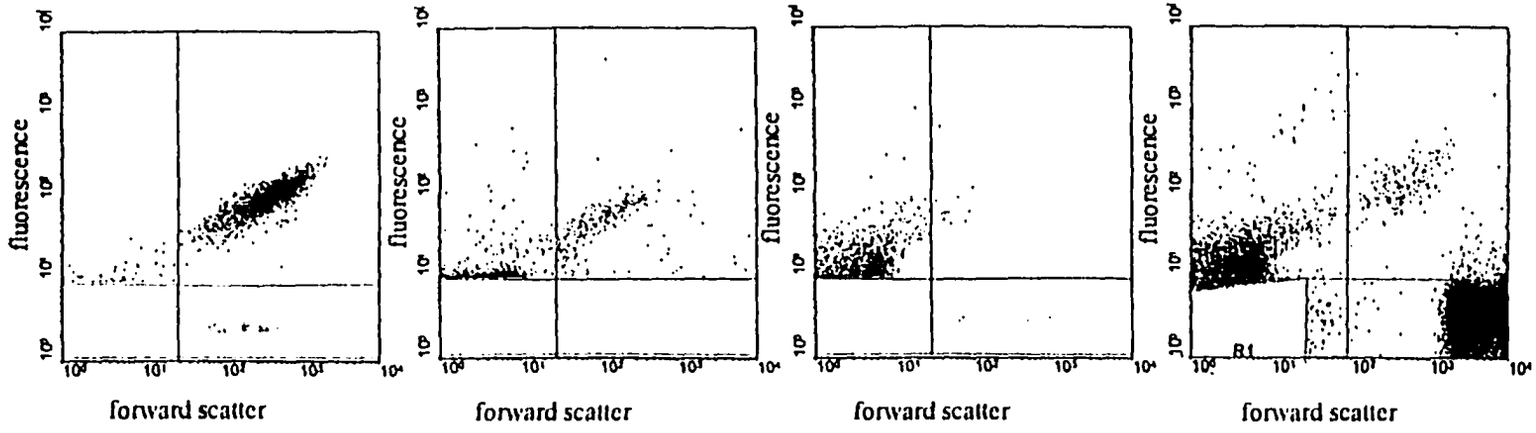
from Figure D: [PMP in ionophore soln] = 4377 events

therefore, with a sample dilution of 1:200 [PMP in ionophore soln] = $6.4x10^7$ PMP/ml

The concentration determined by this method was nearly identical to that obtained by using an event rate from a known platelet concentration. The quantification of platelet microparticles appeared accurate for samples analyzed on the same day since similar calculated concentrations were obtained based on using forward or side scatter and using platelets or red blood cells as the control. However, the results obtained in cell-cell interaction assays varied greatly with respect to cell aggregation and the number of cell-cell interactions using supposedly the same concentrations of platelet microparticles in experiments done on different days. Therefore, cell-cell interaction assays were done in the presence of various volumes of platelet microparticle solutions and the concentrations were not reported. It is likely that many platelet microparticles go undetected by flow cytometry due to the limited forward scatter resolution of the flow cytometer.

HL-60 Cell-Cell Interactions Mediated by Platelet Microparticles in New and Old Platelet-rich Plasma

Washed HL-60 cells ($1x10^6$/ml) in HBSS/0.6% HSA incubated with various concentrations of platelet microparticles were withdrawn over HL-60 cells prebound to an anti-human CD43 antibody coated glass slide. An anticoagulant (heparin or PPAK) was added to the plasma before the incubation of the cells to prevent fibrin clot formation. The number of cell-cell interactions was counted and normalized by the



Upper right quadrant:	Upper left quadrant:	Upper left quadrant:	Lower right quadrant:
46650 events	1633 events	17826 events	12466 events
time: 65 seconds	time: 61 seconds	time: 61 seconds	Upper left quadrant:
			4377 events
A	**B**	**C**	**D**

Figure 26. Quantification of platelet microparticle concentrations (PMP) by flow cytometry. The concentration of platelet microparticles was determined by calculating an event rate for a known number of particles. A known concentration of platelets (0.76×10^6/ml) was analyzed and an event rate was obtained (46650/65 sec) (A). An event rate was obtained for the unknown PMP concentrations in new platelet-poor plasma and the PMP solution obtained by activating platelets (B, C). The concentrations were determined by using the known ratio of concentration to event rate obtained for platelets. To verify the concentration calculated from the platelet ratio was accurate, a known concentration of red blood cells was added to the PMP solution obtained by activating platelets. Therefore, the unknown PMP concentration was obtained using the red blood cell concentration-event rate ratio obtained on the same flow cytometry plot (D).

number of cells on the surface and the duration of the experiment. Control experiments were performed to demonstrate that washed HL-60 cells interact minimally with bound cells. The addition of 1-4 ml of platelet microparticle suspension in new platelet-poor plasma had little affect on the rate of cell-cell interaction. The concentration of platelet microparticles in new platelet-poor plasma was approximately 3 times the concentration in whole blood since 20 ml of blood yielded approximately 6-7 ml of platelet-poor plasma. Therefore, the amount of platelet microparticles contained in 1-4 ml new platelet-poor plasma in a HL-60 cell suspension of 6 ml was a final concentration of approximately one-half to twice the normal platelet microparticle concentration in blood.

The platelet-poor plasma from aged platelet-rich plasma yielded approximately 10 times the amount of platelet microparticles than in new platelet-poor plasma. The number of cell-cell interactions was elevated in the presence of increasing concentrations of platelet microparticles in the system. Figure 27 shows a typical profile of the effect of increased platelet microparticle concentrations on the number of cell-cell interactions that occur at 0.4 dynes/cm^2. The results presented in Figure 27 are typical of 5 independent experiments.

As illustrated in Figure 27, at the highest concentration of platelet microparticles, the number of cell-cell interactions decreased. This decrease was the result of two separate phenomena that occurred from increasing platelet microparticle concentrations. The first phenomenon was cell aggregation. Small aggregates (2-5 cells) formed with the addition of 0.4 ml of old platelet-poor plasma. The size and number of aggregates increased as the

platelet microparticle concentration was increased. At high concentrations of platelet microparticles, the size of the aggregates became very large (10-30+ cells). Consequently, the concentration of single cells flowing through the system was reduced leading to a reduction in the number of cell-cell interactions. The second phenomenon was increased accumulation of cells on the surface. At the highest levels of platelet microparticles, cell-cell interactions sometimes led to accumulation of cells on the antibody coated slide. The microparticles mediated a long duration interaction with the surface allowing the cells to form a stable adhesion. With increased aggregation and accumulation, accurate quantification of the number of cell-cell interactions was not possible (Figure 27, unfilled column for 2.0+ ml old PPP). Both of these phenomena resulted in a decrease in the number of cell-cell interactions that occurred at the highest levels of platelet microparticle concentrations.

HL-60 Cell-Cell Interactions Mediated by Platelet Microparticles Generated by Calcium Ionophore

The same type of concentration dependent production of HL-60 cell-cell interactions was obtained in a plasma-free system using platelet microparticles generated by the calcium ionophore A23187 (Figure 28). As the concentration of platelet microparticles was increased in the system, the number of cell-cell interactions increased until significant cell accumulation and large cell aggregation occurred in the system. At 2.0+ ml in Figure 28, the unfilled column represents the situation in which accurate quantification could not be obtained due to the amount and size of cell aggregates and/or the amount of cell

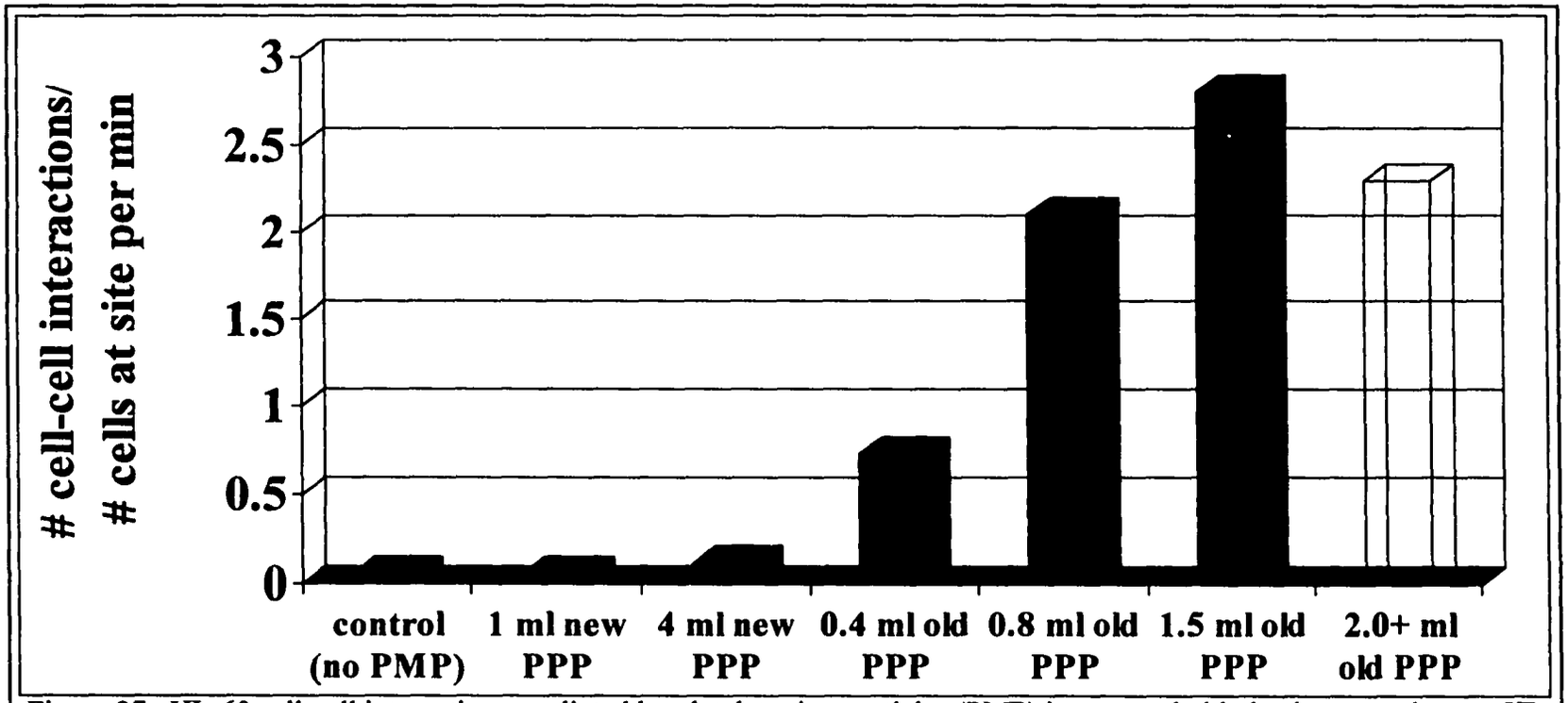


Figure 27. HL-60 cell-cell interactions mediated by platelet microparticles (PMP) in new and old platelet-poor plasma. HL-60 cells were incubated with platelet microparticles from freshly drawn blood (new PPP) or platelet microparticles generated by aging platelet-rich plasma for 5 days (old PPP). HL-60 cells (6 ml total volume) in the presence of platelet microparticles were withdrawn over HL-60 cells prebound to an anti-human CD43 antibody coated glass slide at 0.4 dynes/cm^2 and the number of cell-cell interactions was counted. Normal concentrations of PMP in blood (new PPP) had very little affect on the production of cell-cell interactions. Aging PRP for 5 days yielded ~10 times the normal PMP concentration. Increasing the concentration of platelet microparticles in the system greatly increased the number of cell-cell interactions. However, at the higher concentrations, cell accumulation on the surface and cell aggregation led to a decrease in the number of cell-cell interactions and limited accurate quantification (unfilled column). Graph is representative of 5 independent experiments.

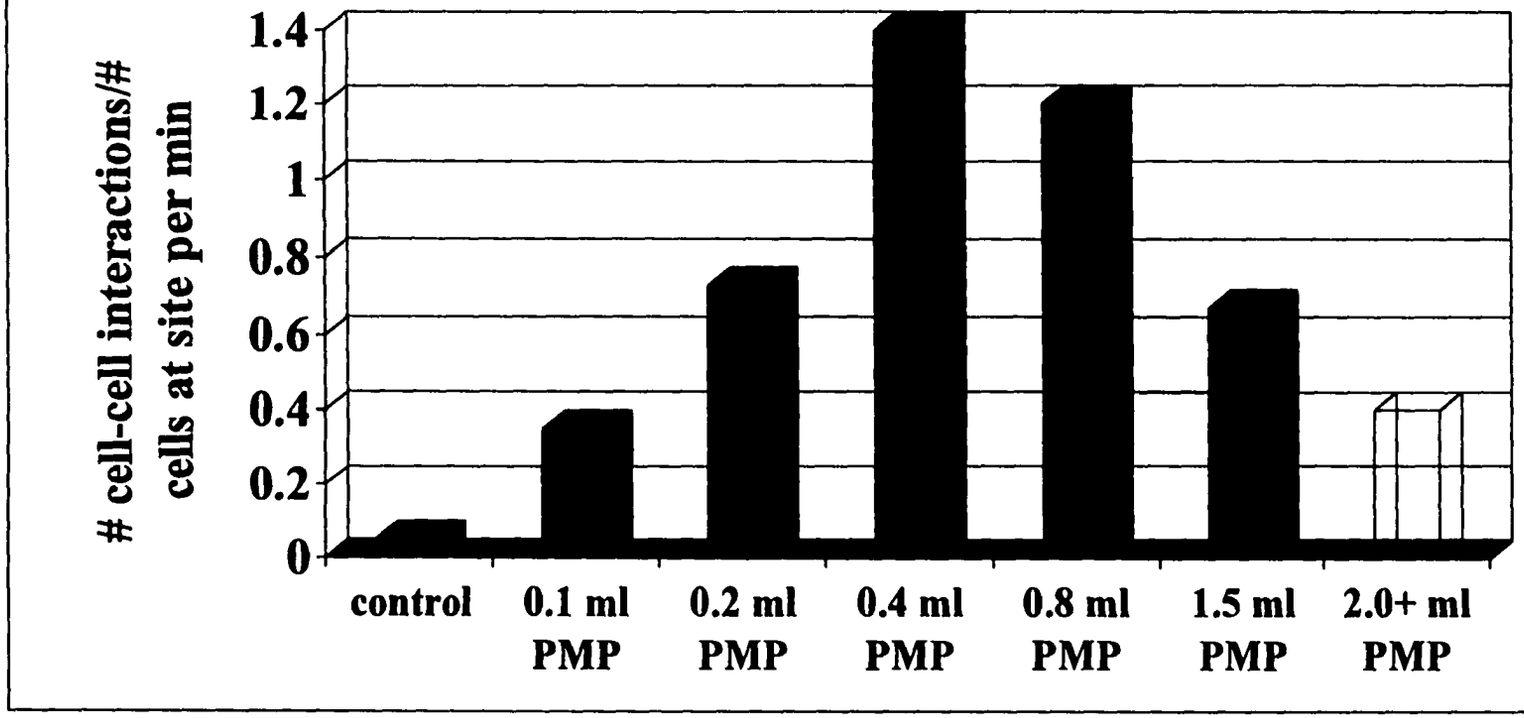


Figure 28. HL-60 cell-cell interactions mediated by platelet microparticles generated by calcium ionophore. HL-60 cells incubated with platelet microparticles (PMP) in a calcium containing buffer were withdrawn at 0.4 dynes/ cm^2 over HL-60 cells prebound to an anti-human CD43 antibody coated slide. The control was washed HL-60 cells with no PMP. As the concentration of PMP increased, the number of cell-cell interactions increased. A concentration was reached in which the PMP produced significant cell aggregates and also aided in cell accumulation on the surface. The number of cell-cell interactions decreased as a result of increased cell aggregation and a reduced number of single cells in the flowing stream. Significant cell accumulation and an increase in the number and size of cell aggregates limited accurate quantification of cell-cell interactions (unfilled column). Graph is representative of 5 independent experiments.

accumulation on the surface. Figure 28 is typical of the results obtained in 5 independent experiments.

HL-60 Cell-Cell Interactions Mediated by Platelet Microparticles were P-selectin and PSGL-1-Dependent

HL-60 cell-cell interaction assays with platelet microparticles generated by calcium ionophore were performed in the presence of various blocking and nonblocking antibodies to determine the specificity of the interactions. The nonblocking anti-P-selectin antibody S12 and the nonblocking anti-PSGL-1 antibody PL2 (10 μg/ml) had no affect on 0.4 ml of platelet microparticle solution producing cell-cell interactions (Figure 29). However, incubating the cells with the anti-P-selectin blocking antibody G1 or the anti-PSGL-1 blocking antibody PL1 eliminated the HL-60 cell-cell interactions (Figure 29). Therefore, HL-60 cell-cell interactions produced by the presence of platelet microparticles were mediated by P-selectin and PSGL-1. The antibody blocking data is representative of two independent experiments.

Neutrophil/HL-60 Cell-Cell Interactions Mediated by Platelet Microparticles

Neutrophils ($1x10^6$/ml) in HBSS/0.6% HSA were withdrawn over HL-60 cells prebound to an anti-human CD43 antibody coated glass slide. Neutrophils express both PSGL-1 and L-selectin (a PSGL-1 ligand); therefore, cell-cell interactions between neutrophils and HL-60 cells occurred readily without the addition of platelet microparticles (Figure 30). The neutrophil data is the average of three independent experiments. These cell-cell interactions were shown to be L-selectin-dependent by withdrawing neutrophils over the

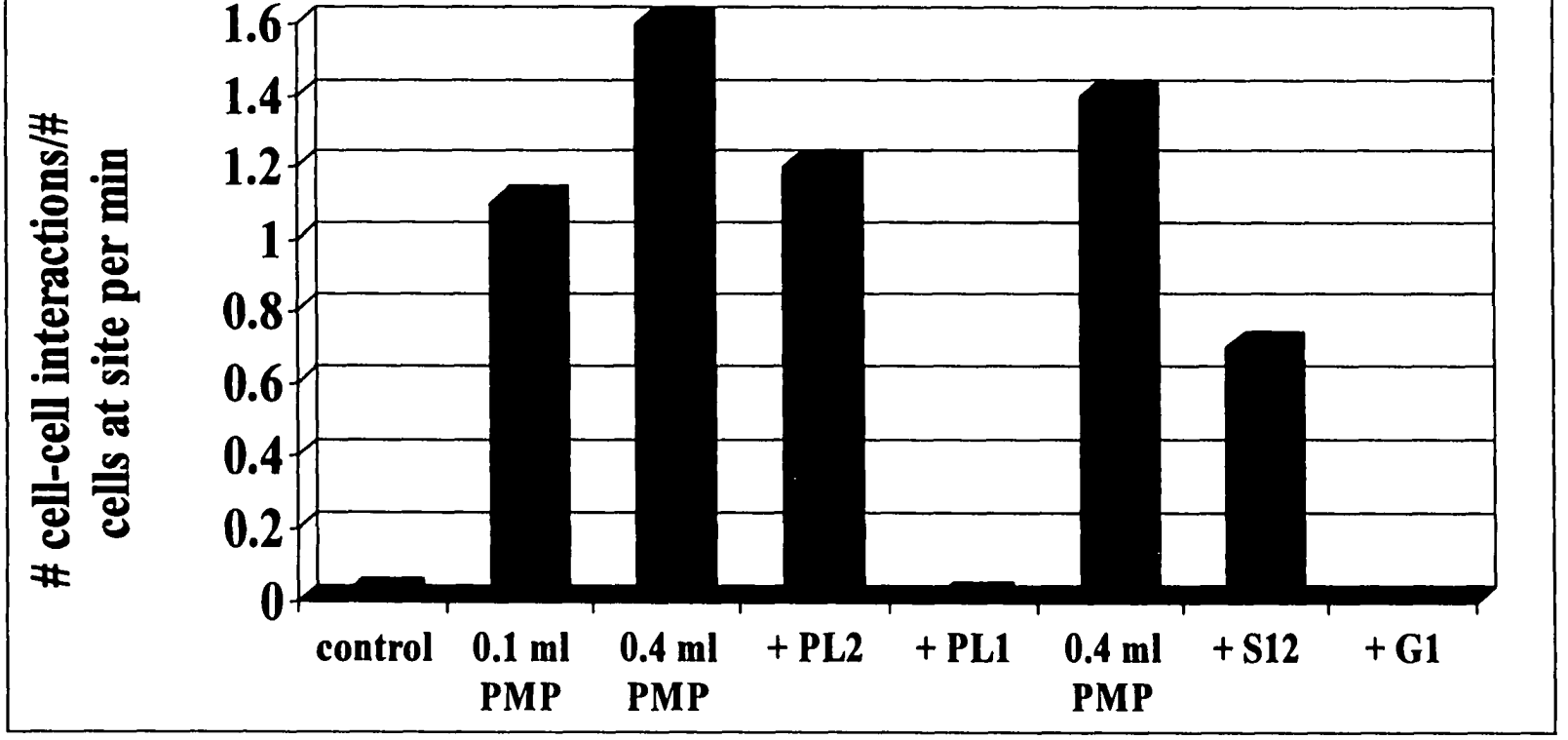


Figure 29. HL-60 cell-cell interactions mediated by platelet microparticles are P-selectin and PSGL-1 dependent. HL-60 cells incubated with platelet microparticles generated by calcium ionophore were withdrawn at 0.4 dynes/cm^2 over HL-60 cells prebound to an anti-human CD43 slide in the presence of various blocking and nonblocking antibodies. The cell-cell interactions were shown to be dependent on P-selectin-expressing microparticles and PSGL-1 on the HL-60 cells since the anti-P-selectin blocking antibody G1 and the anti-PSGL-1 blocking antibody PL1 inhibited cell-cell interactions. The nonblocking anti-P-selectin antibody S12 and the nonblocking anti-PSGL-1 antibody PL2 did not eliminate the cell-cell interactions. Graph is representative of 2 independent experiments.

HL-60 cells in the presence of an anti-L-selectin antibody (DREG56) (Figure 30). We were interested in determining if platelet microparticles could also mediate neutrophil interactions.

Even in the presence of DREG56, neutrophils incubated with platelet microparticles significantly interacted with bound HL-60 cells (Figure 30). The addition of an increased volume of platelet microparticles increased the number of cell-cell interactions (Figure 30). HL-60 cells were withdrawn through the system in the presence of 0.4 ml of platelet microparticle solution to show that microparticles had been generated. Neutrophil/HL-60 cell-cell interactions in the presence of platelet microparticles were shown to be P-selectin- and PSGL-1-mediated by the use of various blocking and nonblocking antibodies. Neutrophils were incubated with the anti-L-selectin antibody DREG56, platelet microparticles, and the nonblocking anti-P-selectin antibody S12 or the nonblocking anti-PSGL-1 antibody PL2. The presence of S12 or PL2 did not eliminate the neutrophil/HL-60 cell-cell interactions (Figure 31). Neutrophils incubated with platelet microparticles and either the blocking anti-P-selectin antibody G1 or the blocking anti-PSGL-1 antibody PL1 did not interact with bound HL-60 cells (Figure 31).

Neutrophil Accumulation on a P-selectin Bilayer Enhanced at Low Shear Stresses Due to Platelet Microparticle-Mediated Cell-Cell Interactions

Neutrophils ($1x10^6$/ml) in HBSS/0.6% HSA were withdrawn over a P-selectin bilayer and the number of cells accumulated on the surface was counted as a function of time (Figure 32). Neutrophils withdrawn over the P-selectin bilayer at 1.4 dynes/cm^2

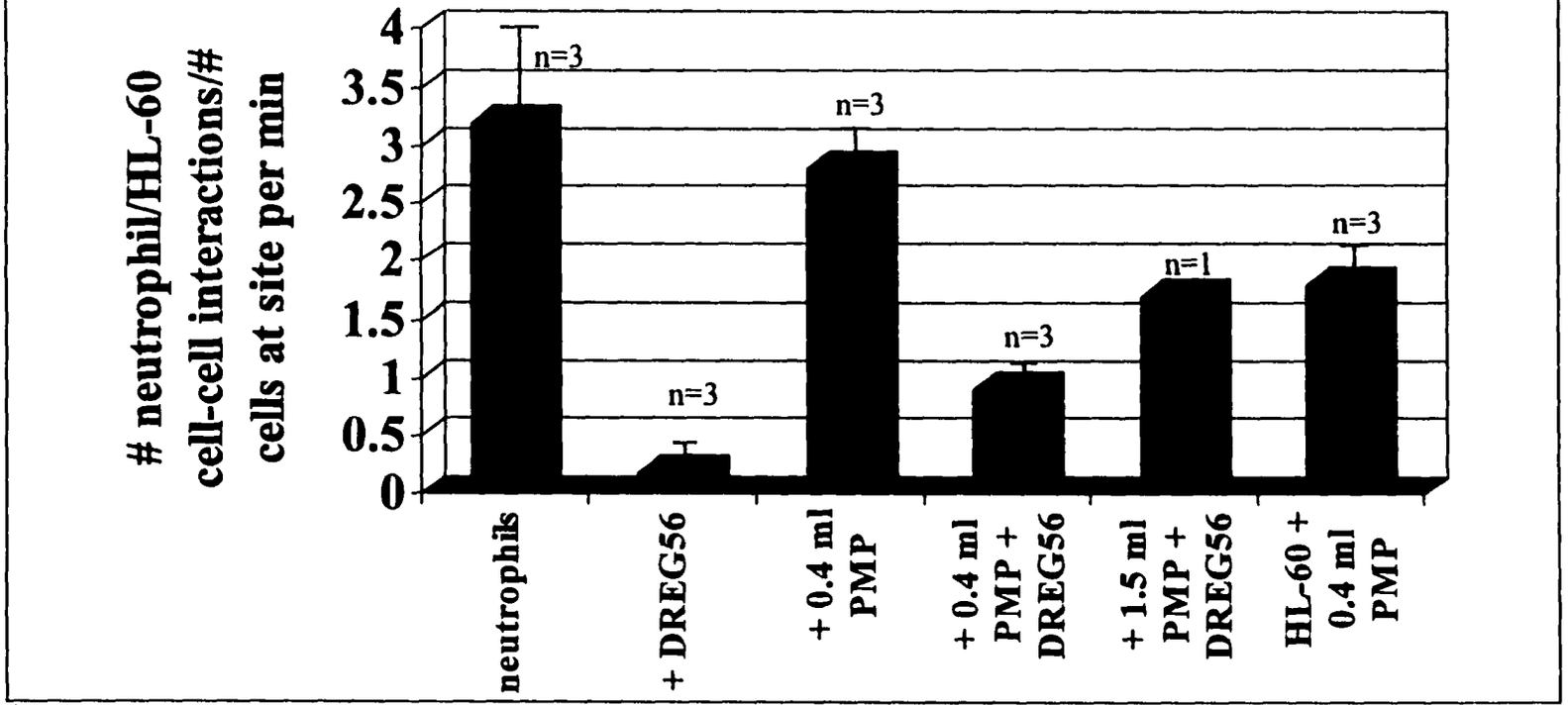


Figure 30. Interactions of neutrophils with HL-60 cells mediated by platelet microparticles (PMP) in the presence of an anti-L-selectin antibody. Neutrophils were withdrawn at 1.0 dynes/cm^2 over HL-60 cells prebound to an anti-human CD43 antibody coated glass slide and the number of cell-cell interactions was counted. Neutrophils express PSGL-1 and L-selectin (a PSGL-1 ligand) which allows neutrophils to transiently attach to HL-60 cells under flow. These interactions were removed with the anti-L-selectin antibody DREG56. However, the addition of platelet microparticles also aided in the production of cell-cell interactions. Neutrophils incubated with PMP interacted significantly with HL-60 cells in the presence of DREG56. Increasing the amount of PMP in the system increased the number of cell-cell interactions even though the L-selectin mechanism was blocked. HL-60 cells were also withdrawn in the presence of PMP to show that PMP were generated. Error bars represent +/- SEM.

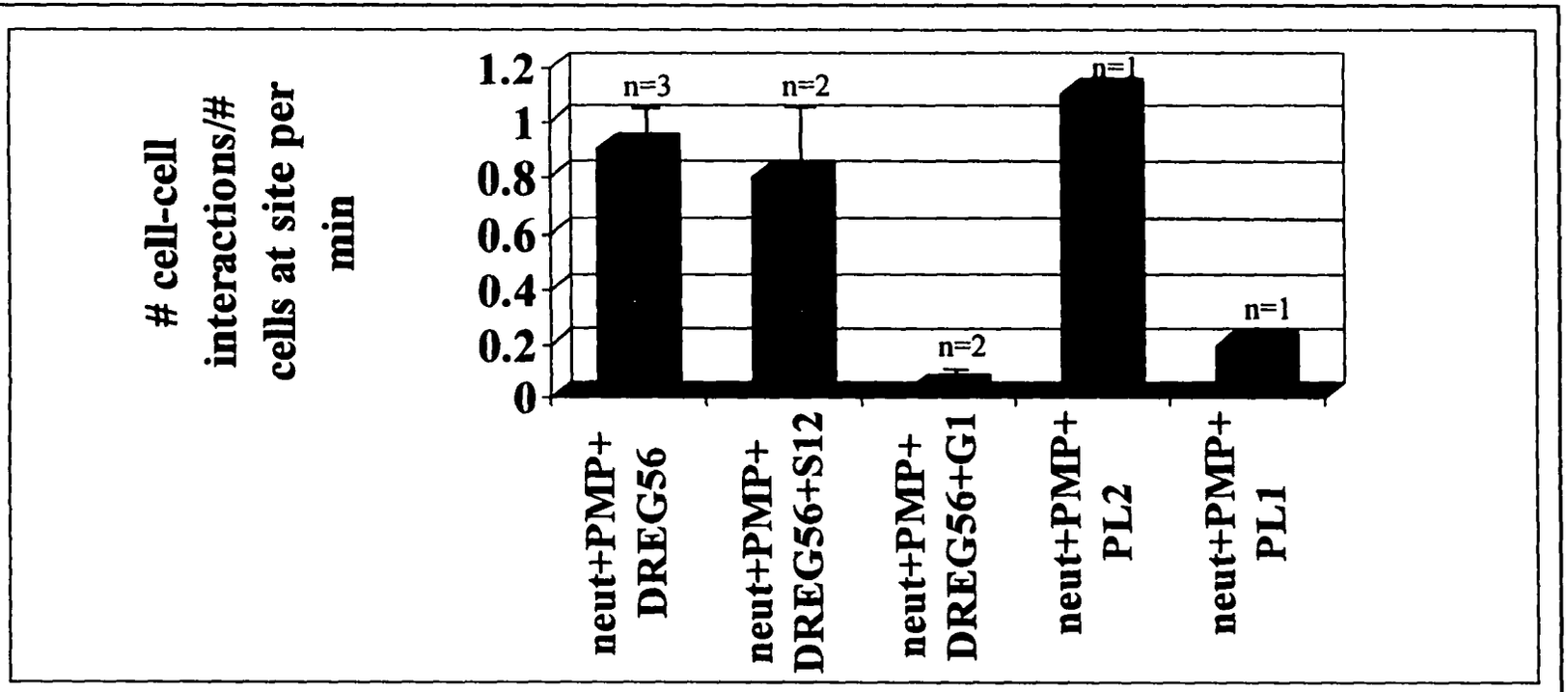


Figure 31. Interactions of neutrophils with HL-60 cells mediated by platelet microparticles (PMP) in the presence of an anti-L-selectin antibody are P-selectin and PSGL-1 dependent. Neutrophils incubated with 0.4 ml PMP generated by calcium ionophore were withdrawn at 1.0 dynes/cm^2 in the presence of an anti-L-selectin antibody DREG56 to eliminate L-selectin dependent binding to PSGL-1. Cells were withdrawn over HL-60 cells bound to an anti-human CD43 antibody coated slide and the number of cell-cell interactions was counted. These cell-cell interactions were shown to be dependent on P-selectin expressed by platelet microparticles since the interactions were eliminated in the presence of the anti-P-selectin blocking antibody G1. The nonblocking anti-P-selectin antibody S12 had no affect on cell-cell interactions. All neutrophil/HL-60 cell-cell interactions (L-selectin and P-selectin mediated) were PSGL-1 dependent as shown by the blocking (PL1) and nonblocking (PL2) anti-PSGL-1 antibodies. Error bars represent +/- SEM.

accumulated on the surface in lines or strings due to accumulation through cell-cell interactions mediated by L-selectin and PSGL-1 (as shown in Chapter 2, page 52). Accumulation of cells is enhanced by attachment of cells through cell-cell interactions (secondary attachments). Blocking the cell-cell interaction mechanism with an anti-L-selectin antibody reduced accumulation of neutrophils by approximately 60% (Figure 32). It has also been shown that a reduction in the shear stress from 1.4 dynes/cm^2 to 0.4 dynes/cm^2 greatly reduces the ability of L-selectin to form tethers with PSGL-1 (see Chapter 5, page 136). Accumulation of cells at 0.4 dynes/cm^2 occurred largely through attachment of cells directly to the surface (primary attachments) and very little through cell-cell interactions (secondary attachments). Primary attachments were increased at 0.4 dynes/cm^2 compared to experiments performed at 1.4 dynes/cm^2 where accumulation mostly occurred through cell-cell interactions. The increased ability of cells to bind to the surface under low flow did not off set the reduction in accumulation obtained through cell-cell interactions. Withdrawing neutrophils over a P-selectin bilayer at 0.4 dynes/cm^2 significantly reduced the accumulation of neutrophils on the surface since the cell-cell interaction mechanism was eliminated (Figure 32).

Neutrophils (6 ml total volume) were also withdrawn over a P-selectin bilayer at 0.4 dynes/cm^2 in the presence of platelet microparticles (0.4 ml) generated by calcium ionophore to determine if platelet microparticles could enhance accumulation through P-selectin-mediated cell-cell interactions. Platelet microparticles were able to mediate cell-cell interactions at 0.4 dynes/cm^2 which led to enhanced accumulation of neutrophils on the P-selectin surface (Figure 32).

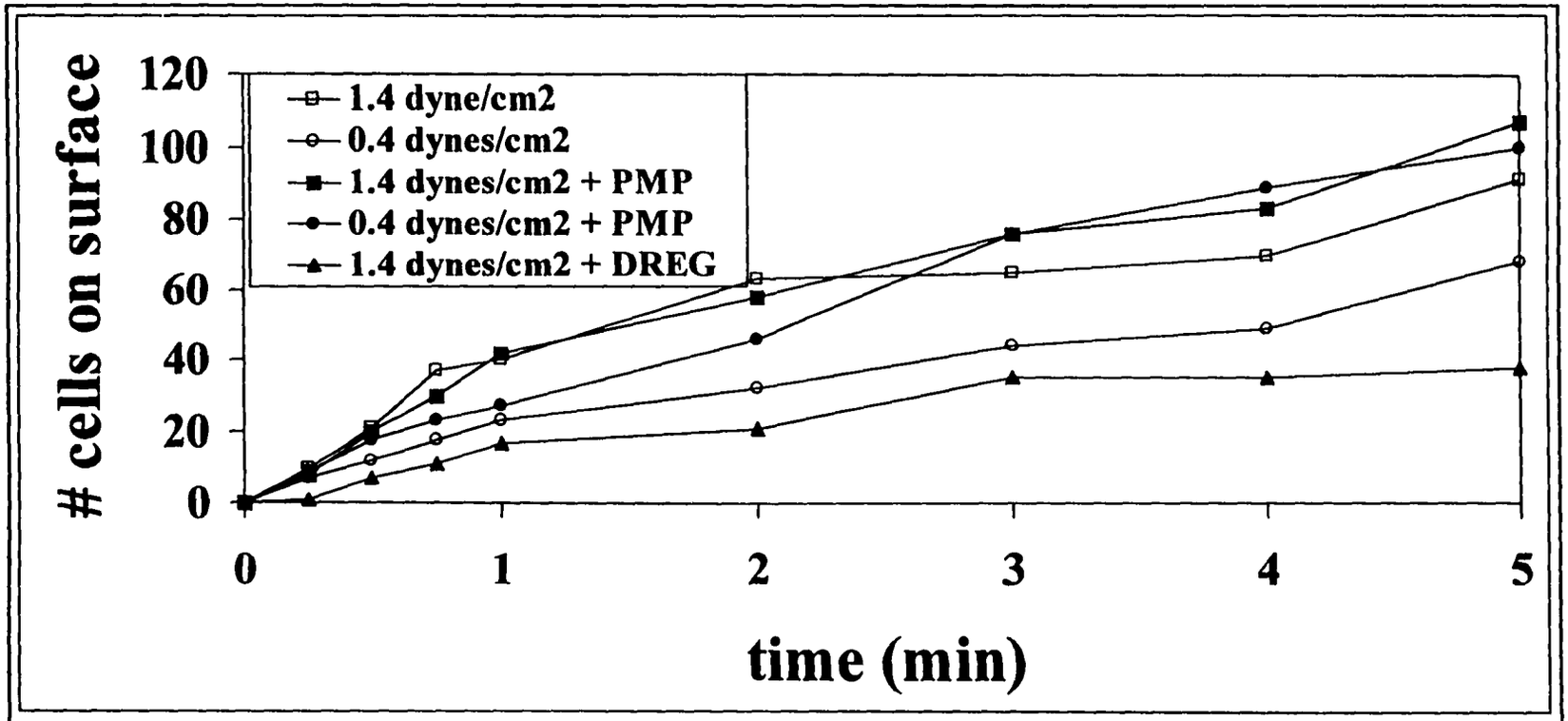


Figure 32. Neutrophil accumulation on a P-selectin bilayer enhanced by platelet microparticle-mediated cell-cell interactions. Neutrophils accumulate through cell-cell interactions at 1.4 dynes/cm^2. Accumulation through cell-cell interactions mediated by L-selectin and PSGL-1 enhance accumulation of cells at a site. Removing accumulation through cell-cell interactions with an anti-L-selectin antibody (DREG56) reduced accumulation by approximately 60%. Accumulation is also reduced with a reduction in shear stress from 1.4 dynes/cm^2 to 0.4 dynes/cm^2. The rate of L-selectin/PSGL-1 tethers is significantly decreased in low shear environments; therefore, accumulation at low shear stress does not occur through cell-cell interactions. The addition of platelet microparticles significantly enhanced the accumulation of neutrophils at 0.4 dynes/cm^2 through P-selectin-mediated cell-cell interactions. The data is representative of 4 independent experiments.

Discussion

Platelet microparticles are released from activated platelets and express functional receptors including P-selectin. It has been reported that platelet microparticle concentrations are elevated in various diseases and disorders; however, the role of platelet microparticles in clotting, cell aggregation, and cell accumulation remains uncertain. Platelet microparticles were isolated from fresh platelet-rich plasma and platelet-rich plasma that had incubated for 5 days. Platelet microparticles were also generated by activating platelets with the calcium ionophore A23187. Platelet microparticle isolation and generation was confirmed by flow cytometry. Consistent quantification of platelet microparticle concentrations by flow cytometry was not possible. The concentrations were determined using a suspension of platelets with a known concentration determined by a cell counter in order to obtain an event rate for a known number of particles. When this was repeated by adding a known concentration of red blood cells directly to the sample, the calculated concentration was nearly identical to that determined by the platelets. However, due to the small size of platelet microparticles, many platelet microparticles probably go undetected. Experiments done on separate days with supposedly the same concentration of platelet microparticles varied greatly in respect to the number of cell-cell interactions, the number and size of cell aggregates, and the ability of cells to accumulate on the surface. This indicated that the concentrations of platelet microparticles in the systems were not the same even though equal concentrations were added to the systems according to flow cytometry. Experiments were done by adding known volumes of platelet microparticles isolated or

generated in the same manner from day to day since quantification of the platelet microparticles was not accurate.

Platelet-poor plasma from freshly drawn blood contained platelet microparticles at 3 times the concentration of whole blood (20 ml of blood yielded ~7 ml of platelet-poor plasma). Therefore, adding platelet microparticles in new platelet-poor plasma to HL-60 cells only resulted in platelet microparticles at one-half to twice the normal concentration. However, elevated platelet microparticle concentrations in diseases can be as high as 100 times the normal concentration. Therefore, platelet microparticle generation was attempted by aging platelet-rich plasma and also by activating platelets with calcium ionophore to produce a much higher concentration of platelet microparticles. These procedures yielded platelet microparticle concentrations up to approximately 30 times normal concentrations.

Known volumes of platelet microparticles in new platelet-poor plasma, old platelet-poor plasma, or generated by activating platelets were added to HL-60 cells and withdrawn over prebound HL-60 cells. Experiments in which plasma was added to the system required the addition of an anticoagulant to prevent the formation of a fibrin clot. Also, platelets isolated from platelet-rich plasma required the plasma to be removed by washing to prevent the formation of a clot when the platelet pellet was resuspended in buffer. The addition of platelet microparticles in new platelet-poor plasma produced no cell aggregates, no cell accumulation, and very few cell-cell interactions suggesting that normal concentration levels of platelet microparticles might not be enough to affect

clotting or cell accumulation. As the concentration levels of platelet microparticles was increased by the addition of old platelet-poor plasma or platelet microparticles generated from activated platelets, cell aggregation, cell accumulation, and the number of cell-cell interactions was greatly increased. There was always a concentration of platelet microparticles that led to excessive cell aggregation and cell accumulation which made quantification of the number of cell-cell interactions inaccurate. Cell aggregation and cell accumulation in the system were not quantified in any way in the experiments; however, as the concentration of platelet microparticles was increased, the number and size of cell aggregates was increased as well as the number of cells which accumulated on the surface. The ability of platelet microparticles to coat the HL-60 cells allowed cell-cell interactions to occur through a platelet microparticle P-selectin bridge. This same mechanism resulted in cell aggregation as a greater number of cells were coated with platelet microparticles. Increased concentrations of platelet microparticles in the system led to increased cell accumulation most likely by two different mechanisms. First, HL-60 cells heavily coated with P-selectin platelet microparticles stayed attached longer to the bound HL-60 cells. This allowed the cell then to come in contact and bind to the surface. Also, platelets and platelet microparticles are adhesive particles which probably aided in HL-60 cells attaching to the surface under the low shear stress the experiments were performed. The cell-cell interactions were shown to be mediated by P-selectin and PSGL-1 by the use of various blocking antibodies verifying P-selectin-expressing platelet microparticles in the system were bridging the PSGL-1-expressing HL-60 cells.

The cell-cell interaction assays with platelet microparticles were repeated by withdrawing neutrophils over prebound HL-60 cells. Neutrophils express L-selectin and were able to interact efficiently with HL-60 cells over a wide range of shear stresses. These L-selectin-dependent cell-cell interactions could be blocked using an anti-L-selectin antibody. The addition of platelet microparticles to neutrophils that had the L-selectin adhesion mechanism blocked allowed neutrophils to interact significantly with the bound HL-60 cells. These cell-cell interactions were also shown to be completely P-selectin-dependent verifying the P-selectin-expressing platelet microparticles in fact could produce neutrophil/HL-60 cell-cell interactions in the L-selectin deficient system.

We were also interested in showing the presence of platelet microparticles could enhance accumulation of neutrophils on a P-selectin surface. Accumulation of neutrophils at 1.4 dynes/cm^2 occurred mostly through cell-cell interactions mediated by L-selectin and PSGL-1. Attachment of cells through cell-cell interactions enhances the accumulation of cells on the surface since removing the cell-cell interaction mechanism reduced the accumulation by approximately 60%. An interesting phenomenon occurs as the shear stress is lowered from 1.4 dynes/cm^2 to 0.4 dynes/cm^2. Chapter 5 discusses in detail the shear stress requirement for bond formation through L-selectin. A necessary shear stress is required to enable efficient bond formation between L-selectin and PSGL-1; therefore, at lower shear stresses, accumulation does not occur through cell-cell interactions. The lack of cell-cell interactions at low shear stresses is possibly a mechanism to control unwanted cell accumulation in low shear environments. We have shown that the presence of platelet microparticles mediates cell-cell interactions through P-selectin at

these low shear stresses. As a result of P-selectin-mediated cell-cell interactions, neutrophil accumulation on P-selectin bilayers was significantly enhanced at low shear stresses.

Therefore, the presence of elevated platelet microparticles in a system could override the attempt to control cell accumulation, cell aggregation, and possibly clotting by blocking the L-selectin/PSGL-1 cell-cell interaction. The presence of platelet microparticles might also enable significant accumulation through cell-cell interactions in low shear stress environments that do not promote cell-cell interactions through L-selectin and PSGL-1. Platelet microparticles might be an important aspect of clotting and cell recruitment in the immune response system. However, elevated concentrations could possibly lead to unwanted cell aggregation, clotting, and cell accumulation through cell-cell interactions.

Chapter 5: Kinetic Analysis of Selectin Adhesion Molecules

Introduction

The recruitment of leukocytes to areas of infection or injury is an important aspect of the immune response system. Leukocytes initially attach to and roll along the blood vessel wall before becoming firmly attached and emigrating into the subendothelial matrix. The arrest and emigration of leukocytes are mediated by integrins. The initial rolling event is largely mediated by the selectin family of adhesion molecules (14, 16, 20, 28), although some integrins are also capable of mediating cell rolling on the endothelium (33, 34). Leukocytes are able to attach to and roll on the blood vessel wall under shear stress as a result of the fast on-rates and off-rates of the selectin bonds (38). Bonds rapidly form at the leading edge of the cell, as bonds are broken at the trailing edge. Leukocyte tethering and rolling is mediated by P-selectin and E-selectin expressed on activated endothelial cells. P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) constitutively expressed on circulating leukocytes. E-selectin also binds to ligands on neutrophils. These interactions mediate cell recruitment directly to the endothelium. However, cell recruitment has also been shown to occur in vitro through leukocyte-leukocyte interactions (137, 138, 167). Circulating leukocytes are able to attach to and roll on bound leukocytes before depositing on the surface. Cell recruitment through these cell-cell interactions is mediated by L-selectin and PSGL-1 expressed on the leukocytes and may enhance recruitment of cells at a site of inflammation (137, 138, 167).

The process of cell rolling and cell recruitment has been studied in various systems; however, the kinetics of the selectin molecules involved in these processes have been

calculated in very few systems. The off-rates of P- and E-selectin have been calculated by measuring the dissociation of neutrophil transient tethers from P- and E-selectin bilayers (38, 536). The L-selectin interaction with PNAd coated on glass was also studied by measuring transient tethers of neutrophils (536). These results have shown that L-selectin has a faster off-rate than P-selectin and E-selectin. It has also been reported that L-selectin has a faster off-rate than E-selectin or P-selectin based on rolling velocities of cells on substrates of similar site densities (537, 538). We were interested in determining the off-rates of selectins in various systems that have been used to study the process of cell rolling and cell recruitment in which the selectin adhesion molecules were displayed on the surface (transfected cells or lipid bilayer) or expressed on the flowing cell. We have measured the off-rates for P-, E-, and L-selectin by measuring the dissociation of transient tethers in various systems including using lipid bilayers and CHO cells as the surface and using neutrophils, HL-60 cells, and pre-B cells as the circulating cell. By determining the off-rates in several systems we will be able to determine if the presentation of the selectin or PSGL-1 can affect the kinetics of the interactions.

An unresolved issue is whether rolling cells are able to extract adhesion molecules from the cell membrane of either the leukocyte or the endothelial cell. We would expect to see large variations in the calculated off-rates from system to system if the molecules were being extracted in the various presentations. Little variation in the calculated off-rates would suggest that the release of the cells from the surface was due to the dissociation of the receptor-ligand bond and not due to the extraction of one or both of the receptors.

It has been established that cell rolling through L-selectin requires a certain threshold hydrodynamic shear stress (533, 534). Previous studies have shown that leukocytes rolling uniformly on an L-selectin substrate at a shear stress above the shear threshold detach from the surface and enter the hydrodynamic flowing stream almost immediately after the shear stress is decreased below the threshold shear stress (533, 534). This phenomenon suggests that a certain shear stress is required not only for cell rolling, but also for bond formation to occur through L-selectin. Others have shown that the rate of L-selectin tethers on a PNAd substrate decreased as the shear stress was decreased, opposite of the trend seen on a P-selectin substrate (536). We investigated this shear dependence on efficient bond formation for the L-selectin/PSGL-1 interaction which mediates cell-cell interactions. We were also interested in determining if this phenomenon would occur with P-selectin and E-selectin at low enough shear stresses. The absence of bond formation in a low shear stress environment might be an important mechanism to prevent enhanced or inappropriate cell accumulation.

Materials and Methods

Materials

Versene, Alpha Modification of Eagle's media, HT Supplement (hypoxanthine and thymidine), heat-inactivated fetal bovine serum (FBS), L-glutamine, gentamicin, 2-mercaptoethanol, and GENETICIN® (G418) were obtained from Gibco Life Technologies (Grand Island, NY). Glutamine-Pen-Strep (GPS) was obtained from Irvine Scientific (Santa Ana, CA). Xanthine and mycophenolic acid were obtained from Sigma Chemical Co. (St. Louis, MO). CHO cells were supplied by Dr. R. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, OK). Pre-B cells expressing either P-, E-, or L-selectin were supplied by Dr. T. K. Kishimoto (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT).

CHO Cell Culture

Chinese hamster ovary (CHO) cells were cultured in Alpha Modification of Eagle's Medium 1X (α-MEM) supplemented with 1% Glutamine Pen-Strep (GPS) and 10% fetal bovine serum. Once the cells reached confluence on the flask they were split. All media was removed from the flask and 10-12 ml HBSS without Ca^{2+}, Mg^{2+} was added to wash the cells. After the removal of the HBSS, 6 ml Versene was added to the flask and incubated at room temperature for 5 minutes. Using a 5 ml pipet the cells were washed off the flask and the resulting suspension of cells was centrifuged at 500g for 5 minutes. The supernatant was removed and the pellet was resuspended in 10 ml α-MEM/GPS/FBS media. 2 ml of the cell suspension was added to a flask containing 10 ml of media. The same procedure was used for CHO cells expressing P-selectin or E-selectin except the

media contained 400 μg/ml active drug GENETICIN® (G418) to select for the selectin-expressing cells.

Pre-B Cell Culture

Pre-B cells expressing P-, E-, or L-selectin were cultured in gpt selection media. The bacterial gpt gene encodes xanthine-guanine phosphoribosyl transferase. Mycophenolic acid is used to inhibit the mammalian cell enzyme IMP dehydrogenase, which is necessary for the formation of guanosine monophosphate. Therefore, in the presence of xanthine, hypoxanthine and thymidine, cells expressing the gpt gene survive. The selection media was made with 93 ml of complete media, 1 ml hypoxanthine and thymidine, 1 ml xanthine, 1 ml mycophenolic acid, and 0.5 ml 1 N HCl. The complete media (~500 ml) was 500 ml RPMI 1640, 50 ml heat-inactivated FBS, 5 ml L-glutamine (100x stock = 200 mM), 2.5 ml gentamicin (200x stock = 10 mg/ml), and 0.5 ml 2-mercaptoethanol (100x stock = 55 mM). The hypoxanthine and thymidine stock solution (HT solution) was 10 mM sodium hypoxanthine and 1.6 mM thymidine. The xanthine (100x) stock solution was 12.5 mg/ml in 0.2 N NaOH (sterile filtered and stored at –20 °C). The mycophenolic acid (100x) stock solution was 250 μg/ml in 0.1 N NaOH (sterile filtered and stored at –20 °C). Every 4-5 days, 75% of the media was removed and replaced with new warm media.

Flow Experiments: Kinetic Analysis of Neutrophil, HL-60 cell, and Pre-B Cell Dissociation from P-selectin, E-selectin, and PSGL-1

Cells in HBSS/0.6% HSA were withdrawn over lipid bilayers displaying an adhesion molecule (selectin or PSGL-1) at low site densities so that only transient interactions occurred. The off-rates for HL-60 cells, neutrophils, and pre-B cells expressing P-, E-, or L-selectin were calculated for a range of shear stresses following the method described by Alon et al. (38). The number of video frames (0.033 sec/frame) a cell transiently interacted with the surface was counted and the duration of the transient interaction was calculated. A transient event was defined as a cell attaching to the surface for at least one video frame before releasing back into the flowing stream without moving more than one cell diameter on the surface. The off-rate of the interaction was determined from the duration of the transient events by assuming that the dissociation of a bound receptor follows first order kinetics:

$$N_{bound} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} N_{free} + P$$

where N_{bound} is a cell bound to a receptor, N_{free} is a free cell in solution, and P is a free unattached receptor. The rate at which cells detach from the receptors is directly proportional to the off-rate, k_{off}, and to the number of cells bound, N_{bound}.

$$r_{off} = k_{off} N_{bound}$$

Taking the total number of transient events as the initial number of cells attached to receptors, N_b^o, the number of cells that are bound, N_b, will diminish as time progresses following the above rate equation.

$$dN_b/dt = -k_{off}N_b = -r_{off}$$

Integration of the above equation gives:

$$\int_{N_b^o}^{N_b} dN_b/N_b = \int^{t} -k_{off}dt$$

$$\ln (N_b(t)) - \ln (N_b^o) = -k_{off}t$$

$$\ln (N_b(t)) = \ln (N_b^o) - k_{off}t$$

Therefore, a plot of the natural log of the number of cells that remained bound ($N_b(t)$) for some time (duration of transient interaction), t, versus t produced a straight line with the slope of $-k_{off}$. The off-rate (k_{off}) was determined using 95% of the data points (unless otherwise stated) where the data most closely demonstrated first order kinetics. Therefore, the 5% of transient events with longest duration were not used in the calculation of the off-rate. These points generally did not follow the slope of the fastest 95% of the transient events suggesting the cells formed more than one receptor-ligand bond making their dissociation much slower. An example of this is shown in Figure 33. The points on the right side of the vertical line were ignored in the off-rate calculations. The points (longest 5% of events) which did not follow first order kinetics most likely represent the formation of more than one receptor-ligand bond.

Calculating the Force on a Receptor-Ligand Bond

The off-rates of HL-60 cells, neutrophils, and the different pre-B cells were compared as a function of the force on the bond instead of the shear stress since differences in cell size result in different forces on the receptor-ligand bond. The force on the bond was

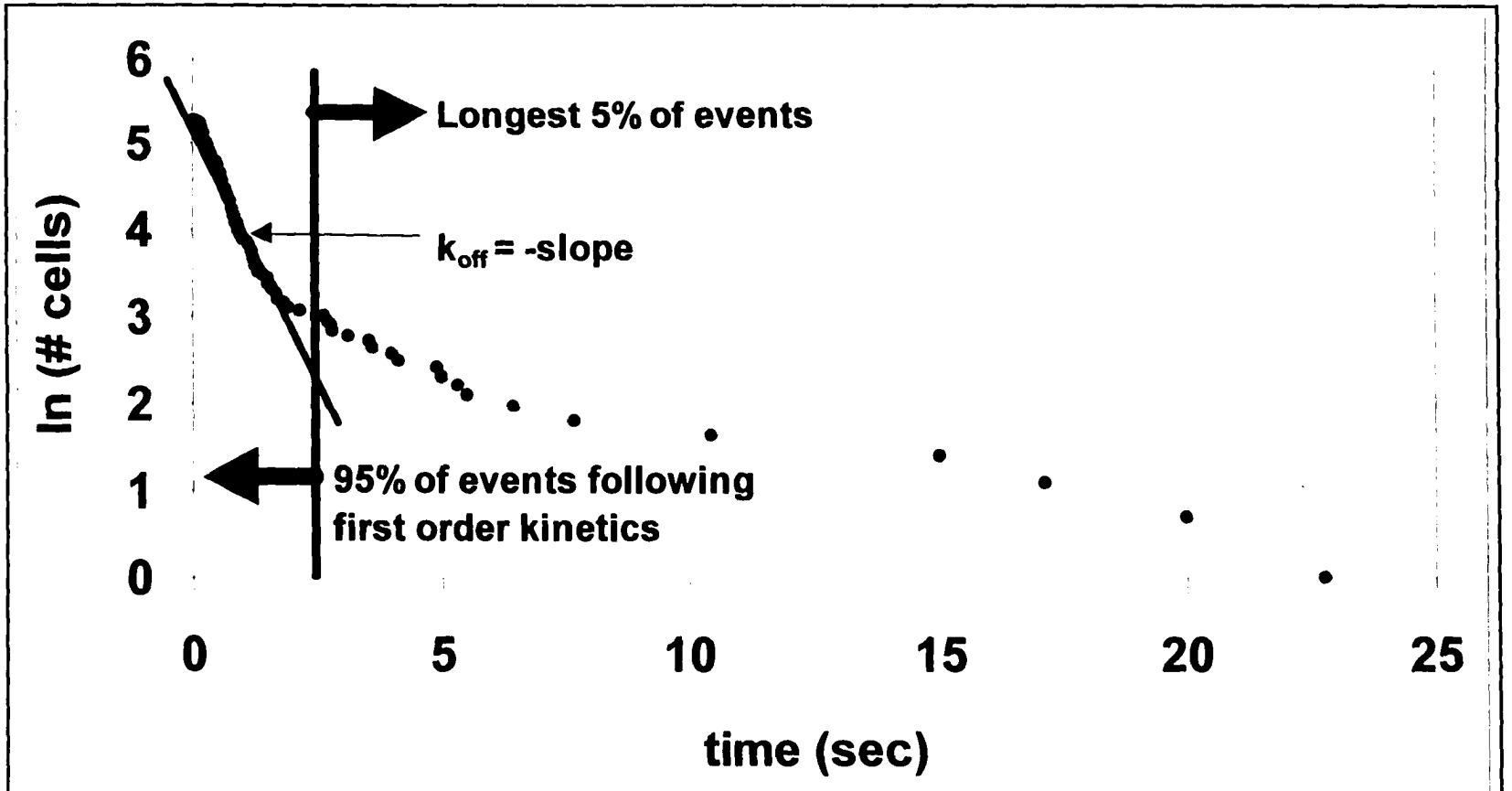


Figure 33. Calculation of the kinetic off-rate assuming first order kinetics. Assuming first order kinetics, the slope of the plot ln (# cells) versus duration of transient events (t) gives $-k_{off}$. The figure shows all the transient events observed for the experiment. However, it is evident only a portion of the events follow first order kinetics. Therefore, only the fastest 95% of events that follow first order kinetics were used in the calculation of the off-rate. The longest 5% of the events not following first order kinetics are most likely due to the formation of multiple receptor-ligand bonds.

calculated using the equations given by Alon et al. (38) and Goldman et al. (539) using 4.25 μm as the radius of a neutrophil, 6.5 μm as the radius of a HL-60 cell, and 4.75 μm as the radius of a pre-B cell. The force a receptor-ligand bond will experience depends on the shear stress, the size of the cell, and the angle at which the adhesion receptor attaches to the cell (Figure 34).

Torque and forces balances on the above system give the following equations:

$$\text{Torque balance: } l(F_b \sin\theta) = \tau_s + RF_s$$

$$\text{Force balance: } F_b \cos\theta = F_s$$

Goldman has shown that for a fixed sphere adjacent to a wall the torque, τ_s, is:

$$\tau_s = \tau^* 4\pi\mu R^3 S$$

where τ^* is the dimensionless torque (0.94399), l is the lever arm, R is the radius, μ is the viscosity, and S is the shear rate. The shear rate is calculated by $S=6Q/B^2W$, where Q is the flow rate in ml/min, B is the height of the flow area, and W is the width of the flow area. The force on the sphere is given by:

$$F_s = F^* 6\pi\mu R^2 S$$

where F* is the dimensionless force (1.7005).

Solving the torque and force balances simultaneously yields:

$$\sin\theta/\cos\theta = (\tau_s/F_s + R)/l$$

Using the above equations for τ_s and F_s, the bond angle θ can be solved for assuming a value for l. At the shear stresses studied for these assays, no cell deformation was observed. Therefore, for a neutrophil with a radius of 4.25 μm, l was assumed to be

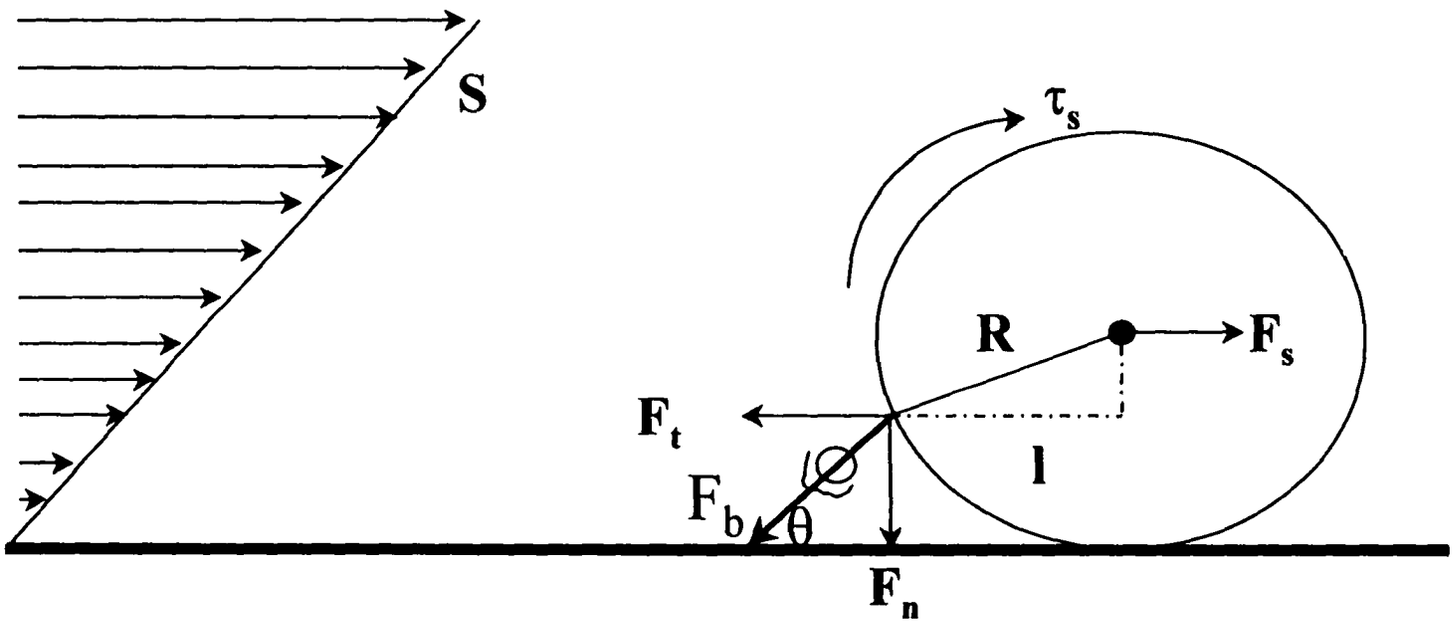


Figure 34. Force on a receptor-ligand bond. The force on a bond, F_b, can be calculated by doing a torque and force balance on the system following the procedure outlined by Alon et al. (38) and Goldman et al. (539). An assumption has to be made for the lever arm, l, in order to determine the bond angle θ. Assuming the cell does not deform greatly when it binds, l is approximately equal to the cell radius (for neutrophils R = 4.25 μm, l = 4.0 μm). The force on the bond can be calculated using $F_b = 1.7005(6)\pi\mu R^2 S/\cos\theta$, where μ is the viscosity, R is the cell radius, and S is the shear rate.

4.0 μm. However, the force on the bond, F_b, is not affected greatly by values of l as low as 60% of the cell radius (F_b reduced by 30%) (540).

$$\tan \theta = (2\tau^*R/3F^* + R)/l$$

$$\tan \theta = ((2(0.94399)4.25/3(1.7005) + 4.25)/4.0$$

$$\theta = 55.5^0$$

The force on the bond can be calculated using:

$$F_b \cos \theta = F_s$$

and $$F_s = F^*6\pi\mu R^2 S$$

to give $$F_b = 1.7005(6)\pi\mu R^2 S/\cos \theta$$

Flow Experiments: Analysis of the Selectin On-rates and Off-rates Through Cell-Cell Interactions

Pre-B cells expressing E-, P-, or L-selectin ($1x10^6$/ml) in HBSS/0.6% HSA were withdrawn over HL-60 cells prebound to an anti-human CD43 coated glass slide at various shear stresses. The pre-B cells interacted transiently with the prebound cells. A transient cell-cell interaction was defined as a flowing cell briefly attaching to a bound cell then releasing back into the hydrodynamic flow. The duration of the transient cell-cell interactions was always less than 4 seconds. The number of transient cell-cell interactions was counted and normalized for the number of cells on the surface, the duration of the experiment, and the flux of cells passing through the flow chamber.

Cell Treatments

Flow Experiments: Analysis of the Selectin On-rates and Off-rates Through Cell-Cell Interactions

Pre-B cells expressing E-, P-, or L-selectin ($1x10^6$/ml) in HBSS/0.6% HSA were withdrawn over prebound HL-60 cells in the presence of various blocking and nonblocking antibodies to show the specificity of the interactions. The cells were incubated for 10 minutes at room temperature with 10 μg/ml PL1, PL2, ES1, ES2, or G1 or 5 μg/ml DREG56. PL1 is an anti-PSGL-1 antibody that blocks the PSGL-1 binding site for P-selectin and L-selectin. PL2 is a nonblocking anti-PSGL-1 antibody. ES1 is an anti-E-selectin blocking antibody and ES2 is the nonblocking anti-E-selectin antibody. DREG56 is an anti-L-selectin antibody.

Results

Kinetic Off-rates of P-, E-, and L-Selectin Determined from the Dissociation of Transient Interactions

Other groups have shown that L-selectin has a faster off-rate than P-selectin and E-selectin by measuring the rolling velocities of cells (536-538) and by measuring the dissociation of neutrophil transient tethers (38, 536). We have calculated the off-rates of P-, E-, and L-selectin by directly measuring the duration of transient cell interactions. HL-60 cells expressing PSGL-1 were withdrawn over lipid bilayers containing P-selectin or adsorbed E-selectin. Neutrophils expressing L-selectin were withdrawn over lipid bilayers containing PSGL-1. The adhesion molecules were coated on the surface or incorporated into the lipid bilayers at low site densities so that there were few or no rolling interactions and transient cell interactions predominated. The calculated off-rates of P-selectin, E-selectin, and L-selectin over a range of shear stresses are shown in Figures 35, 36, and 37, respectively. HL-60 cells are larger than neutrophils; therefore, the off-rates for P-, E-, and L-selectin were compared as a function of the force on the receptor-ligand bond (Figure 38). The off-rates for the P-selectin and E-selectin interactions were very similar. However, the off-rates of P-selectin and E-selectin were much slower than the off-rate for L-selectin with PSGL-1 (Figure 38). The data shown in Figure 38 indicates the L-selectin/PSGL-1 interaction is more compliant than the P-selectin and E-selectin interactions. The slopes of the lines indicate the off-rate of the L-selectin interaction is affected more with an increase in the force on the bond than the P- and E-selectin interactions.

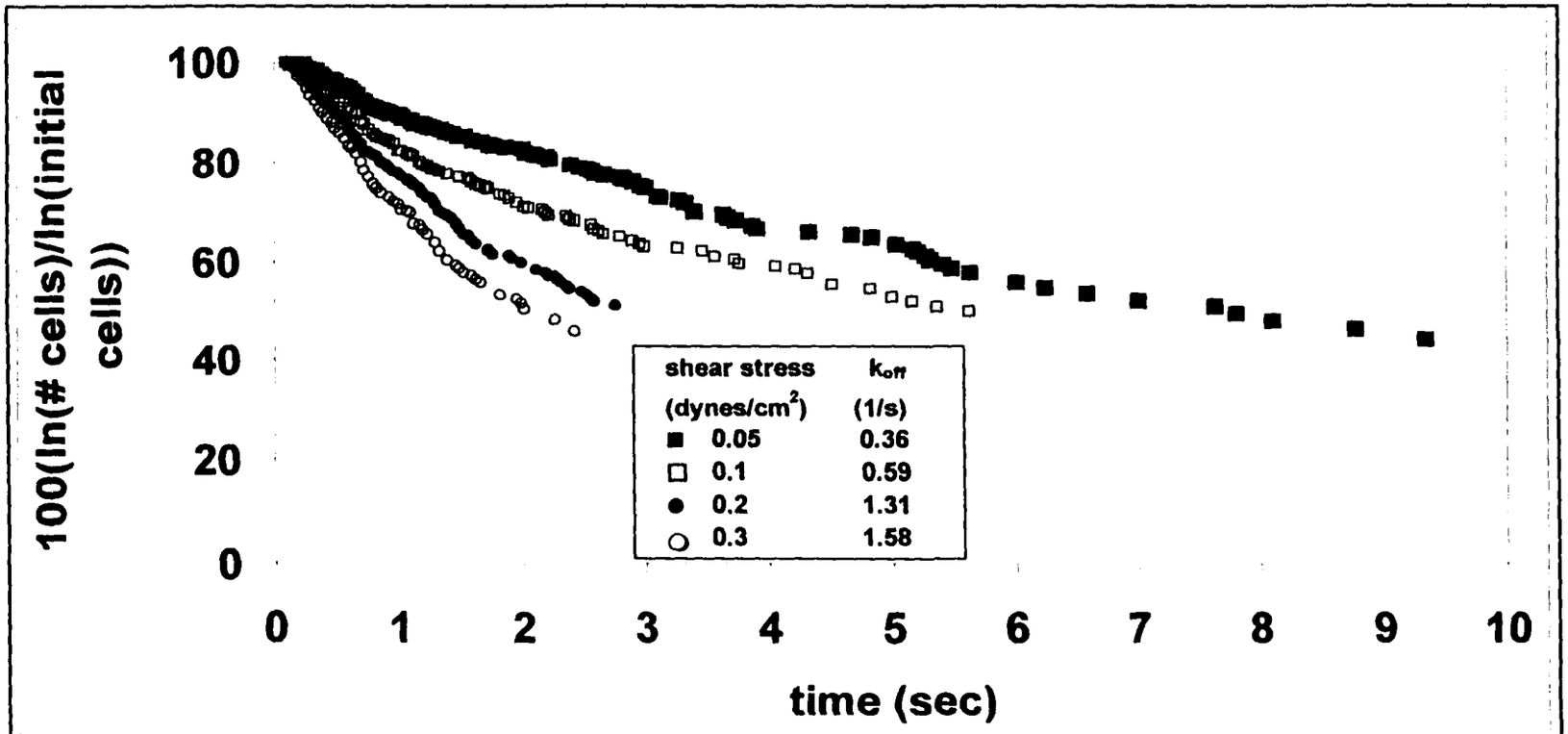


Figure 35. Kinetic off-rate of the P-selectin/PSGL-1 interaction calculated by withdrawing HL-60 cells over a P-selectin bilayer. HL-60 cells expressing PSGL-1 were withdrawn over a P-selectin bilayer at various shear stresses. P-selectin was incorporated into the bilayer at a low site density to produce transient interactions. The duration of the transient interactions were determined and the natural log of the number of cells remaining bound was plotted versus the duration of the transient events (t) to obtain the off-rate. The natural log of the number of cells was normalized for each shear stress by the natural log of the initial number of cells (ln (# cells)/ln (initial # cells)). The off-rate was calculated at 0.05 dynes/cm^2 from 183 cells; at 0.1 dynes/cm^2 from 273 cells; at 0.2 dynes/cm^2 from 407 cells, and at 0.3 dynes/cm^2 from 283 cells.

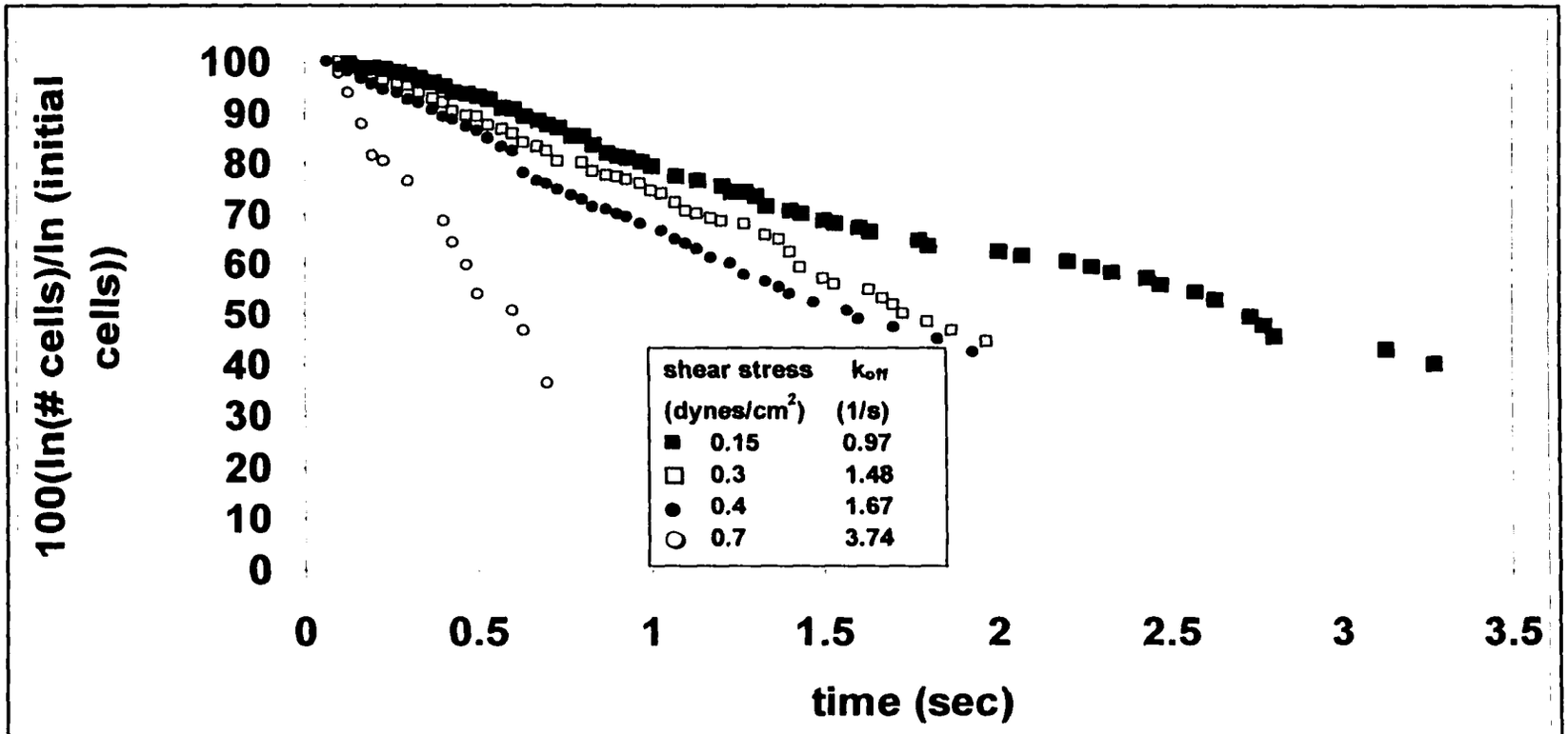


Figure 36. Kinetic off-rate of E-selectin calculated by withdrawing HL-60 cells over an adsorbed E-selectin coated glass slide. HL-60 cells expressing PSGL-1 were withdrawn over a surface of adsorbed E-selectin at various shear stresses. E-selectin was adsorbed on the surface at a low site density to produce transient interactions. The duration of the transient interactions were determined and the natural log of the number of cells remaining bound was plotted versus the duration of the transient events (t) to obtain the off-rate. The natural log of the number of cells was normalized for each shear stress by the natural log of the initial number of cells (ln (# cells)/ln (initial # cells)). The off-rate was calculated at 0.15 dynes/cm^2 from 127 cells, at 0.3 dynes/cm^2 from 142 cells, at 0.4 dynes/cm^2 from 134 cells, and at 0.7 dynes/cm^2 from 26 cells.

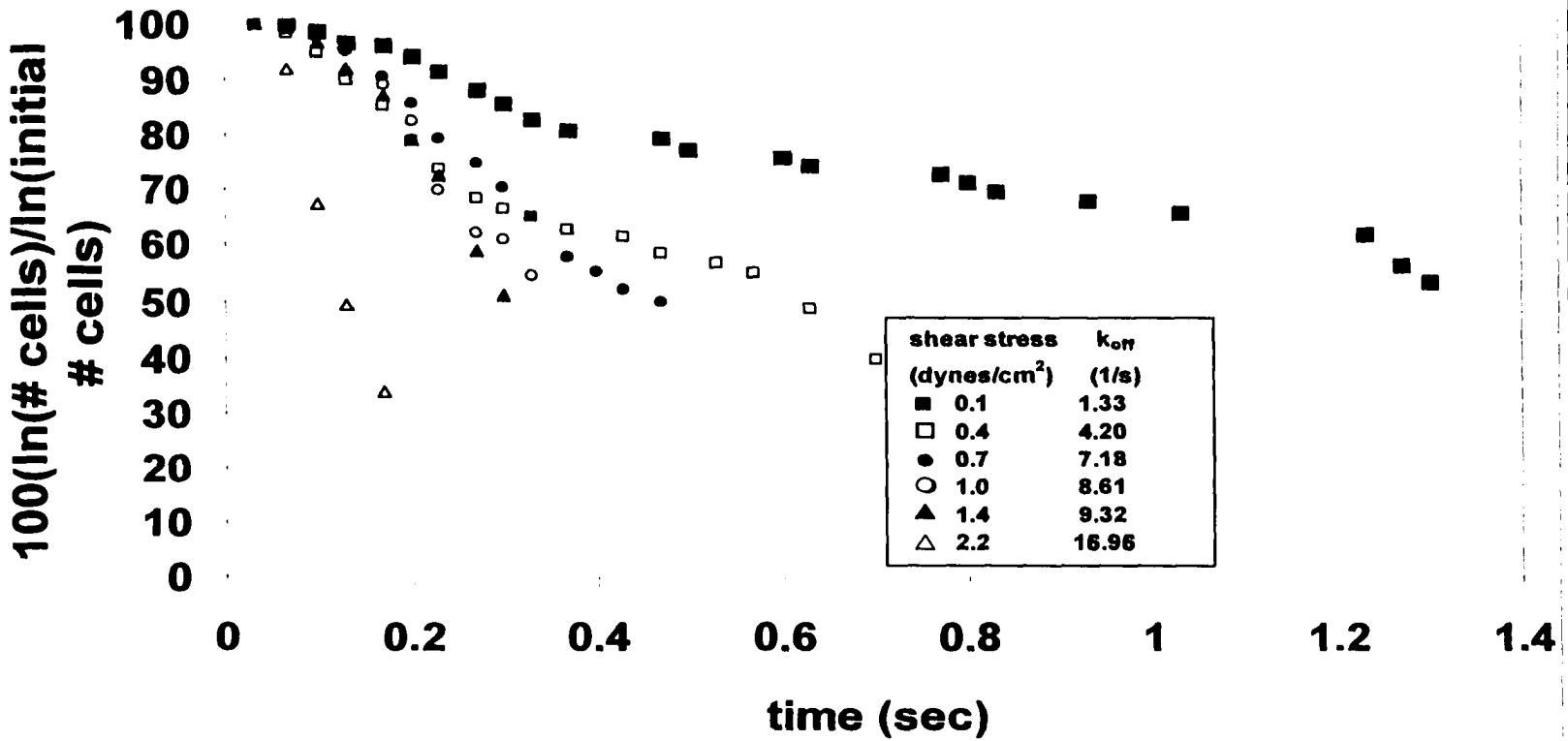


Figure 37. Kinetic off-rate of the L-selectin/PSGL-1 interaction calculated by withdrawing neutrophils over a PSGL-1 bilayer. Human neutrophils expressing L-selectin were withdrawn over a PSGL-1 bilayer that produced transient interactions. The duration of the transient interactions were determined and the natural log of the number of cells remaining bound was plotted versus the duration of the transient events (t) to obtain the off-rate. The natural log of the number of cells was normalized for each shear stress by the natural log of the initial number of cells (ln (# cells)/ln (initial # cells)). The off-rate was calculated at 0.1 dynes/cm^2 from 49 cells, at 0.4 dynes/cm^2 from 92 cells, at 0.7 dynes/cm^2 from 135 cells, at 1.0 dynes/cm^2 from 96 cells, at 1.4 dynes/cm^2 from 148 cells, and at 2.2 dynes/cm^2 from 26 cells.

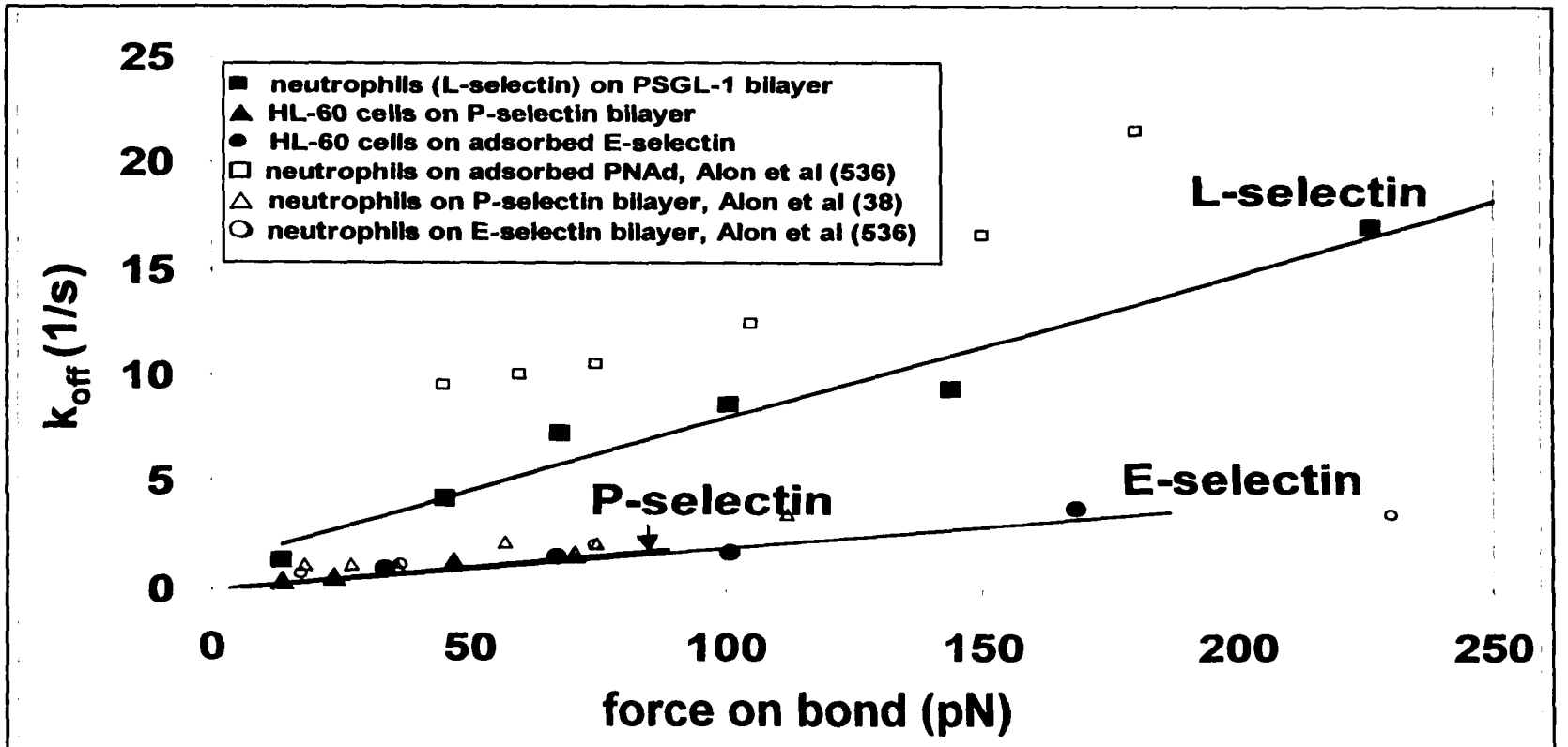


Figure 38. The off-rates of P-, E-, and L-selectin compared as a function of the force on the bond. The off-rates calculated for P-, E-, and L-selectin using HL-60 cells and neutrophils are shown as a function of the force on the bond to normalize for the different cell sizes. The off-rates for P- and E-selectin were nearly identical. The off-rate for the L-selectin/PSGL-1 interaction was much faster than the P-selectin or E-selectin interaction. The off-rates obtained by Alon et al (38, 536) for neutrophils on P- and E-selectin bilayers compare well with the off-rates calculated using HL-60 cells. The off-rates calculated for L-selectin using neutrophils on PSGL-1 bilayers are similar to the off-rates calculated by Alon (536) using neutrophils on adsorbed PNAd.

Dependence of Off-rate on the Presentation of the Molecule

We next wanted to determine if the presentation of the selectin or PSGL-1 would affect the calculated off-rate. The duration of transient tethers was measured for the interaction of each selectin in several systems as is outlined in Table 2. Selectins were presented either in a planar lipid bilayer, adsorbed on glass, or expressed in a transfected cell line (either CHO cells or pre-B cells). PSGL-1 was presented either in a planar lipid bilayer or in cells (neutrophils or HL-60 cells). The calculated values of the off-rates for P-, E-, and L-selectin are presented in Tables 3, 4, 5, respectively. These tables also include the kinetic data obtained by Matt Rose (540). The results are presented as a function of the force on the bond rather than shear stress since the different cell types have different sizes. Larger cells will experience greater force on the bond than smaller cells for the same shear stress. The calculated off-rates did not vary substantially for each selectin in different systems. This is also illustrated in Figure 39 where the off-rates are plotted as a function of the force on the bond for all of the systems except the selectin-expressing pre-B cells. The off-rates for E- and P-selectin were very similar, while the off-rate for L-selectin was much faster. It is also clear from Figure 39 that the presentation of the selectin did not affect the off-rate. Some of the off-rates calculated using the pre-B cells were consistently faster than the off-rates determined for other systems (Figure 40). This may have occurred because there was a substantial amount (up to 25% of the transient interactions) of nonspecific binding of the selectin-expressing pre-B cells to planar lipid bilayer surfaces containing PSGL-1. The binding was shown to be nonspecific since it occurred in the presence of a selectin blocking antibody and in the absence of calcium (data not shown). Substantial numbers of pre-B cells became permanently bound to the

Table 2: Systems Examined to Determine the Off-Rate of Selectin-mediated Interactions

P-selectin	E-selectin	L-selectin
HL-60/ selectin bilayer	HL-60/ adsorbed selectin	
PMN/ selectin bilayer		PMN/ PSGL-1 bilayer
HL-60/ selectin CHO	HL-60/ selectin CHO	
PMN/ selectin CHO	PMN/ selectin CHO	
HL-60 + PMP/PSGL-1 bilayer		
selectin pre-B/ PSGL-1 bilayer	selectin pre-B/ PSGL-1 bilayer	selectin pre-B/ PSGL-1 bilayer

Table 3: Off-rates Calculated for the P-selectin/PSGL-1 Interaction

Surface:	P-selectin bilayer		tP-selectin CHO		PSGL-1 bilayer	
Flowing Cell:	HL-60	neutrophils	HL-60	neutrophils	pre-B (P-selectin)	HL-60 + PMP
Force (pN)	k_{off} (1/s)	k_{off} (1/s)	k_{off} (1/s)	k_{off} (1/s)	k_{off} (1/s)^	k_{off} (1/s)^
13.5	0.36					
18				0.78*	1.19	
24-27	0.59			0.88*		
34-41			0.99*	0.79*	1.44	1.34#
44-47	1.31	1.15*		1.15*		
54-62			1.05*		2.38	
71	1.58					
84-90		1.64*	1.15*		3.42	
101-103	1.61*		1.21*			2.15#
126-133		2.17*			6.97	
208	2.43*					
225		2.51*				
311	3.39*					
526	8.55*					

only 90% of the transient events were used in the calculation of the off-rate
* off-rates calculated by Matt Rose (540)
^ off-rates shown as a function of the shear stress in Appendix III

Table 4: Off-rates Calculated for E-selectin-mediated Interactions

Surface:	adsorbed E-selectin	E-selectin CHO		PSGL-1 bilayer
Flowing Cell:	HL-60	HL-60	neutrophils	pre-B (E-selectin)
Force (pN)	k_{off} (1/s)	k_{off} (1/s)	k_{off} (1/s)	k_{off} (1/s)^
17			0.82*	
27			1.04*	
34-36	0.97		1.06*	
41-44		0.93*	1.31*	
54				1.73
62-67	1.48	0.98*		
84-90		1.01*		2.59
101-103	1.67	1.10*		
126				5.28
168	3.74			

* off-rates calculated by Matt Rose (540)
^ off-rates shown as a function of shear stress in Appendix III

Table 5: Off-rates of the L-selectin/PSGL-1 Interaction

Surface:	PSGL-1 bilayer	
Flowing Cell:	neutrophils	pre-B (L-selectin)
Force (pN)	k_{off} (1/s)	k_{off} (1/s)^
13.5	1.33+	
45	4.20	
54		4.55#
68	7.18	
90		7.19
101	8.61	
126		7.23
144	9.32	
226	16.95	

+ only the fastest 85% of the transient events used to calculate k_{off}
# only the fastest 90% of the transient events used to calculate k_{off}
^ off-rates shown as a function of shear stress in Appendix III

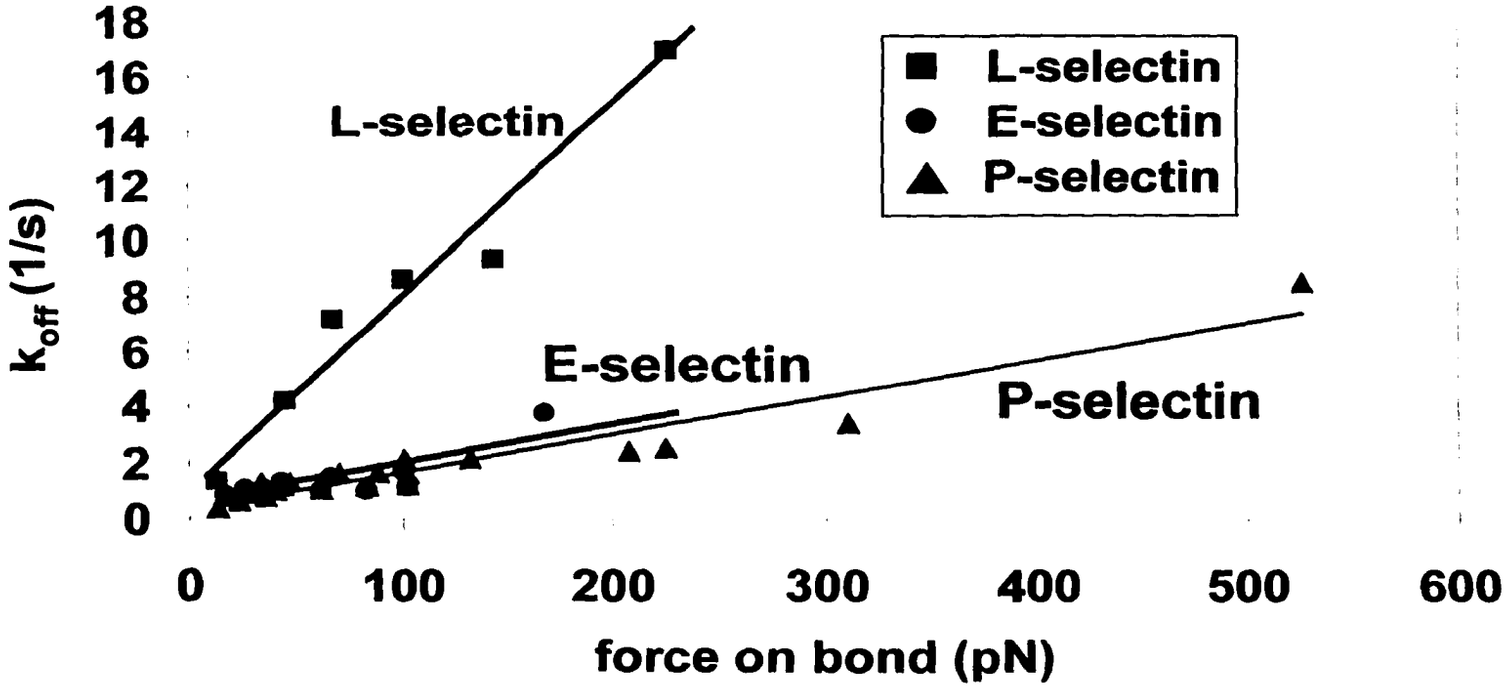


Figure 39. Comparison of calculated off-rates for P-, E-, and L-selectin in various systems to the force on the bond. The off-rates calculated in the various systems (shown in Tables 3, 4, and 5, pre-B systems not included) are plotted as a function of the force on the bond to normalize for the differences in cell size in the different systems. The data shows the off-rates for P-selectin and E-selectin are nearly identical, but much slower than the off-rate of the L-selectin interaction. Little variation was obtained in the off-rates for the P-selectin and E-selectin interactions in the different systems. This suggests the off-rates are dependent on the interactions of the molecules and not dependent on how the molecules are displayed in the system. This also suggests the kinetics are a function of the dissociation of the receptor-ligand bond and not the extraction of the receptor from the membrane.

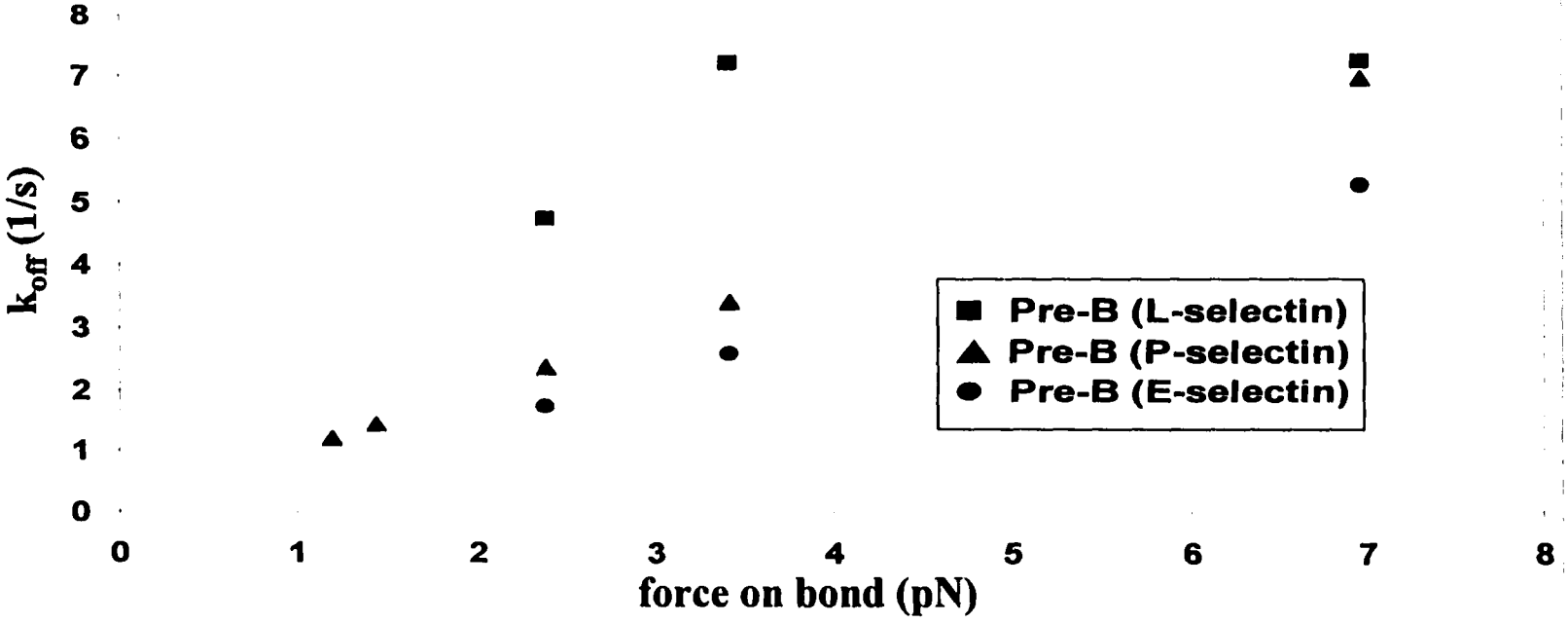


Figure 40. Off-rates of the selectins calculated using pre-B cells expressing P-, E-, or L-selectin. These transfected cells allowed the off-rates for the interaction of P-, E-, and L-selectin with PSGL-1 to be calculated in identical systems (all selectins expressed on the flowing cell). The off-rates calculated for P-, E-, and L-selectin followed the same trend as seen in the other systems. P-selectin and E-selectin had similar off-rates, but slower than L-selectin. However, the pre-B cells expressing a selectin gave faster off-rates than calculated in the rest of the systems. The faster off-rates calculated in the pre-B cell systems probably resulted from nonspecific binding of the pre-B cells to the surface. A portion of the faster events might not have been selectin-mediated. The pre-B cells that bound to the surface probably altered the off-rate due to longer transient events not being observed since the cell remained bound to the surface. Removing these longer transient events and adding fast transient interactions that were not selectin-mediated would lead to the calculation of a faster off-rate. Since the pre-B cells bound nonspecifically to the surface, the calculated off-rates are not likely very accurate.

PSGL-1 planar lipid bilayers. These cells were not included in computing the off-rates. Consequently, only the cells that interacted with the surface for short periods of time were included in the analysis making the calculated off-rates faster than expected.

Indirect Analysis of Selectin Kinetics Through Cell-Cell Interactions

Since the pre-B cells nonspecifically interacted with the PSGL-1 planar lipid bilayers, we examined the kinetics of these cells interacting with HL-60 cells that had been prebound to a surface using an antibody to CD43. The HL-60 cells constitutively express PSGL-1 on their surface. Studying the cell-cell interactions of P-, E-, and L-selectin-expressing pre-B cells with PSGL-1-expressing HL-60 cells gave an indirect comparison of the on-rates and off-rates of the different selectin adhesion molecules. Cell-cell interactions occurred with the pre-B cells expressing P-selectin and E-selectin up to about 4.0 dynes/cm^2 while the L-selectin-expressing pre-B cells interacted with HL-60 cells up to approximately 7.0 dynes/cm^2. This suggests the on-rate of L-selectin is faster than the on-rates of P-selectin and E-selectin. It also indicates that P-selectin and E-selectin have about the same on-rate. The transient cell-cell interactions obtained with pre-B cells expressing P-selectin or E-selectin were very long (0.5~4.0 seconds, data not shown) in comparison with the duration of the cell-cell interactions with L-selectin-expressing pre-B cells (less than 0.5 seconds, data not shown). This indirectly shows the off-rate for the L-selectin interaction is faster than the E-selectin and P-selectin interactions which is in agreement with the off-rates calculated from the dissociation of transient interactions.

The specificity of the cell-cell interactions was shown by various blocking and nonblocking antibodies. Incubating pre-B cells expressing P-selectin with the anti-P-selectin blocking antibody G1 or the anti-PSGL-1 blocking antibody PL1 reduced cell-cell interactions by approximately 95% (data not shown). The nonblocking anti-PSGL-1 antibody PL2 had no affect on the cell-cell interactions. Pre-B cells expressing E-selectin incubated with the anti-E-selectin blocking antibody ES1 reduced the number of cell-cell interactions by approximately 95% (data not shown). Incubating the E-selectin-expressing pre-B cells with the anti-E-selectin nonblocking antibody ES2, the anti-PSGL-1 blocking antibody PL1, or the anti-PSGL-1 nonblocking antibody PL2 had no affect on the occurrence of cell-cell interactions (data not shown). The inability of PL1 to block E-selectin interactions shows that E-selectin binds to a region on PSGL-1 distinct from P-selectin and L-selectin. Cell-cell interactions between HL-60 cells and pre-B cells expressing L-selectin were also reduced by approximately 95% by the incubation of the cells with the anti-L-selectin antibody DREG56 (data not shown).

Shear Stress Dependence on Binding Through the Selectins

The shear stress dependence of the cell-cell interactions obtained with the pre-B cells expressing L-selectin was counterintuitive (Figure 41). Cell-cell interactions occurred at shear stresses up to about 7.0 dynes/cm^2. Cell-cell interactions increased as the shear stress was decreased from 7.0 dynes/cm^2 to 1.4 dynes/cm^2. However, the number of cell-cell interactions was a maximum at 1.4 dynes/cm^2 and decreased with a decrease in shear stress from 1.4 dynes/cm^2 to 0.15 dynes/cm^2. This data indicates that L-selectin/PSGL-1 bond formation requires a threshold shear stress. This phenomenon was also obtained

with neutrophils interacting with prebound HL-60 cells (data not shown). The shear stress dependence on the rate of L-selectin/PSGL-1 interactions was seen on a PSGL-1 bilayer (Figure 42). The number of events (rolling + transient) decreased as the shear stress was decreased. Neutrophils rolled on the surface at 0.7-2.2 dynes/cm^2. A transition from rolling to transient events occurred at 1.4 dynes/cm^2 where there was a mixture of rolling and transient interactions. All events below 0.7 dynes/cm^2 were transient interactions.

The cell-cell interactions obtained with pre-B cells expressing P-selectin and E-selectin with prebound HL-60 cells followed the same shear dependence (Figures 43 and 44, respectively). No cell-cell interactions occurred at shear stresses above approximately 4.0 dynes/cm^2. As expected, the number of cell-cell interactions increased as the shear stress was decreased. However, at 0.15 dynes/cm^2 the number of cell-cell interactions decreased dramatically for the P-selectin- and E-selectin-expressing pre-B cells. This result may reflect a possible shear stress requirement for bond formation through all three selectin adhesion molecules. The same type of shear dependence on bond formation through P-selectin and E-selectin was seen using HL-60 cells and neutrophils on transfected CHO cells (540). At shear stresses over 0.35 dynes/cm^2, HL-60 cells and neutrophils rolled on the CHO cells expressing P- or E-selectin. As the shear was decreased, the interactions with the surface were transient. However, the rate of interactions decreased with a decrease in shear stress below 0.35 dyne/cm^2. No interactions were seen with the surface at 0.10 dynes/cm^2.

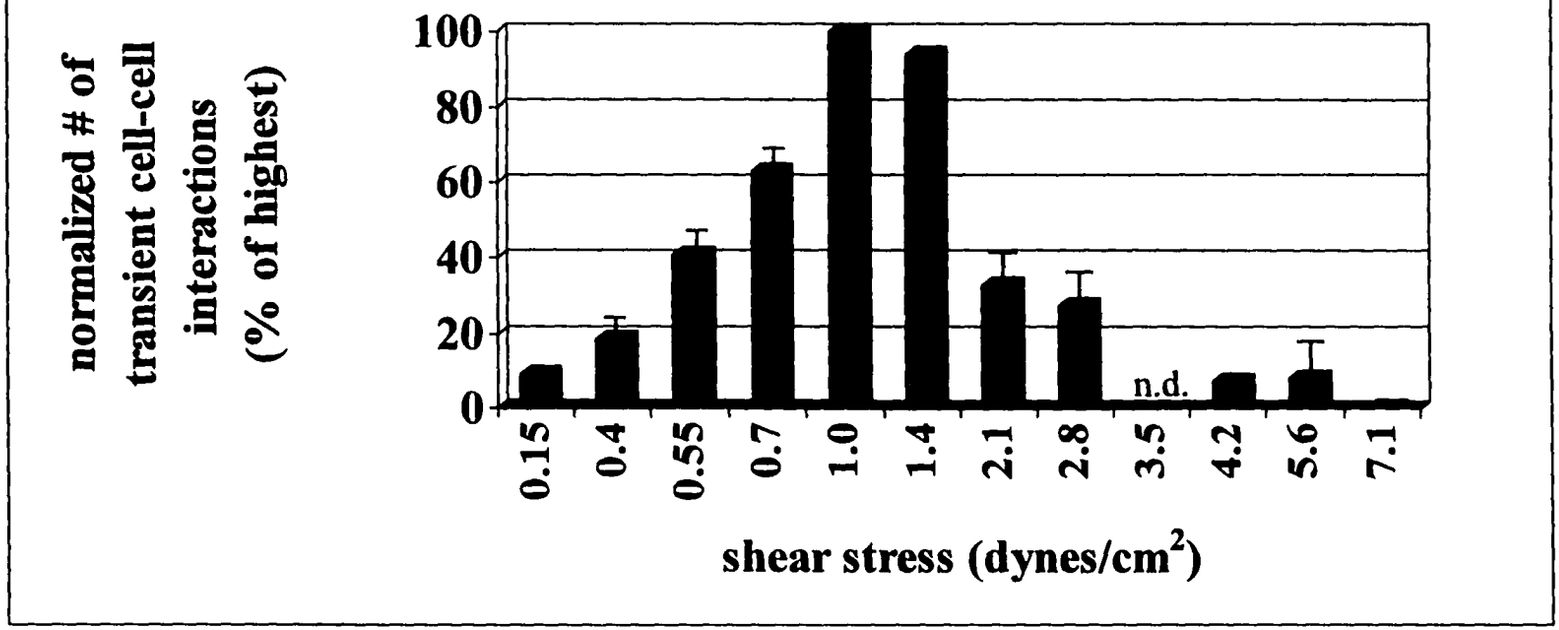


Figure 41. Analysis of the L-selectin/PSGL-1 interaction through cell-cell interactions. HL-60 cells expressing PSGL-1 were prebound to an anti-human CD43 antibody coated glass slide. Pre-B cells expressing L-selectin were withdrawn over the prebound HL-60 cells and the number of transient cell-cell interactions was counted and normalized for the number of cells on the surface, the duration of the experiment, and the flux of cells passing through the system. The number of interactions decreased dramatically as the shear stress was decreased from 1.4 dynes/cm^2. This phenomenon possibly indicates a threshold shear stress requirement for bond formation between PSGL-1 and L-selectin. Cell-cell interactions were observed up to approximately 7.0 dynes/cm^2. The occurrence of cell-cell interactions at these high shear stresses indicates the L-selectin/PSGL-1 interaction has a very fast on-rate. Cell-cell interactions occurring over the range of shear stresses were very fast transient interactions. The flowing cell remained attached to the bound cell less than 0.5 seconds. This supports the kinetic calculations that L-selectin has a very fast off-rate. Error bars represent +/- SEM, n=2.

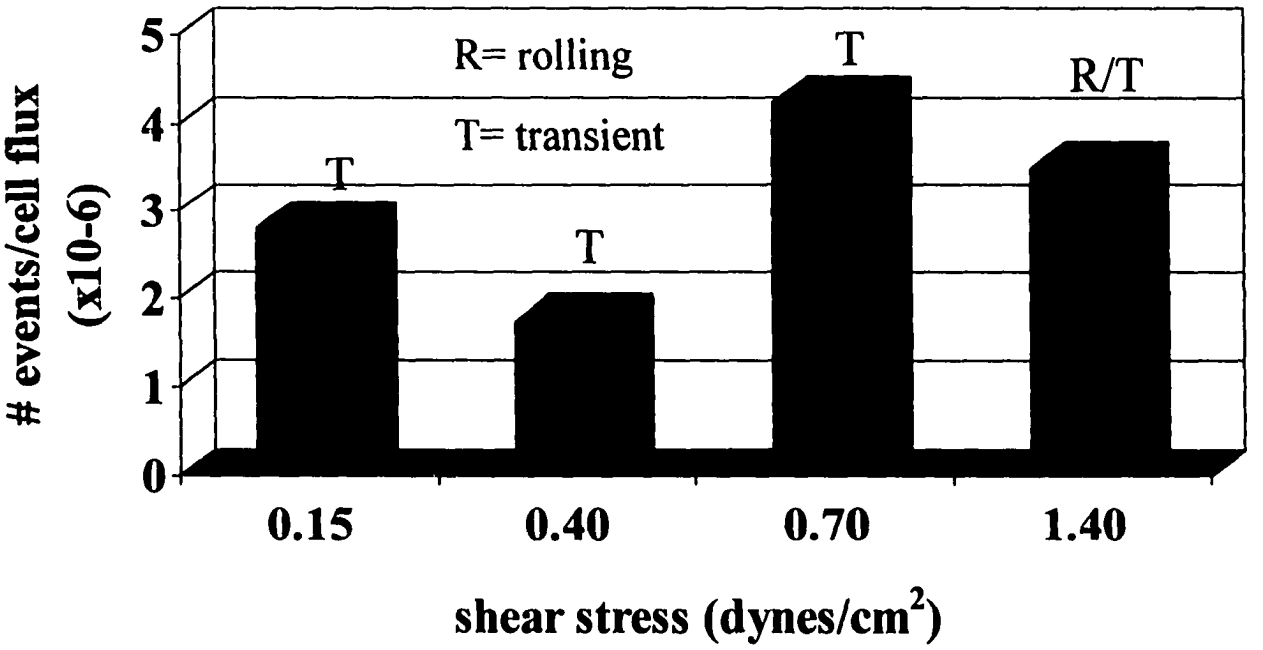


Figure 42. Neutrophil interactions with PSGL-1 incorporated into a lipid bilayer. Neutrophils were withdrawn over a lipid bilayer containing purified PSGL-1 and the number of events (rolling + transient) was counted and normalized for the flux of cells and the duration of the experiment. The neutrophils rolled on the surface at 0.7-2.2 dynes/cm^2. There was a transition to transient events at 1.4 dynes/cm^2 where a mixture of rolling and transient interactions occurred. All events below 0.7 dynes/cm^2 were transient interactions. The decrease in the number of events with a decrease in shear stress suggests bond formation becomes less efficient as the shear stress is lowered. This was consistent with the observations made using pre-B cells expressing L-selectin. Obtaining the same trend on a lipid bilayer indicates the phenomenon was not a hydrodynamic result of the cell-cell interactions. The data is representative of 8 independent experiments.

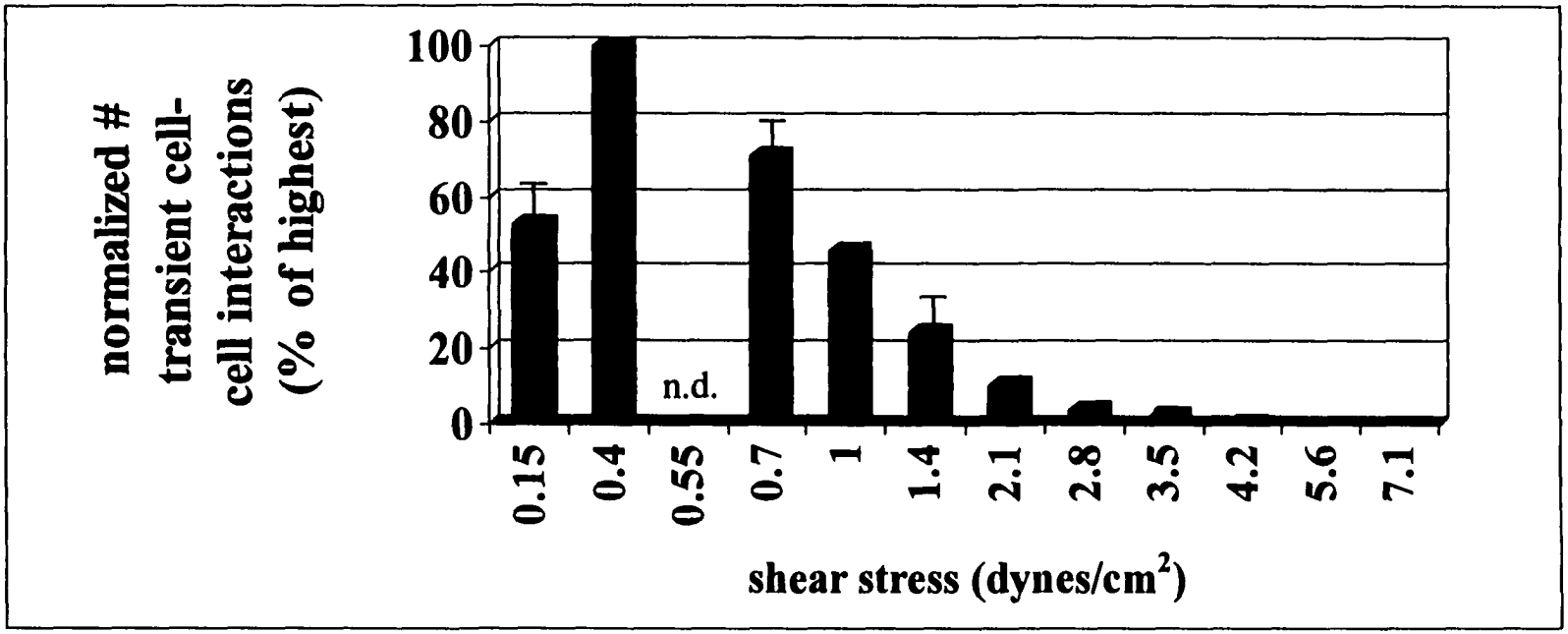


Figure 43. Analysis of the P-selectin/PSGL-1 interaction through cell-cell interactions. HL-60 cells expressing PSGL-1 were prebound to an anti-human CD43 antibody coated glass slide. Pre-B cells expressing P-selectin were withdrawn over the prebound HL-60 cells and the number of transient cell-cell interactions was counted and normalized for the number of cells on the surface, the duration of the experiment, and the flux of cells. The number of cell-cell interactions was maximum at approximately 0.4-0.7 dynes/cm^2 and decreased as the shear stress was increased. Cell-cell interactions occurred at shear stresses below 4.0 dynes/cm^2 suggesting the on-rate of P-selectin is slower than L-selectin (events up to 7.0 dynes/cm^2). The number of cell-cell interactions decreased significantly at 0.15 dynes/cm^2. This possibly indicates a certain shear stress is required to promote bond formation. Cell-cell interactions occurring over the range of shear stress were very slow transient interactions. The flowing cell remained attached to the bound cell for 0.5-2.0+ seconds. This supports the kinetic calculations that P-selectin has a slower off-rate than L-selectin.. Error bars represent +/- SEM, n=2.

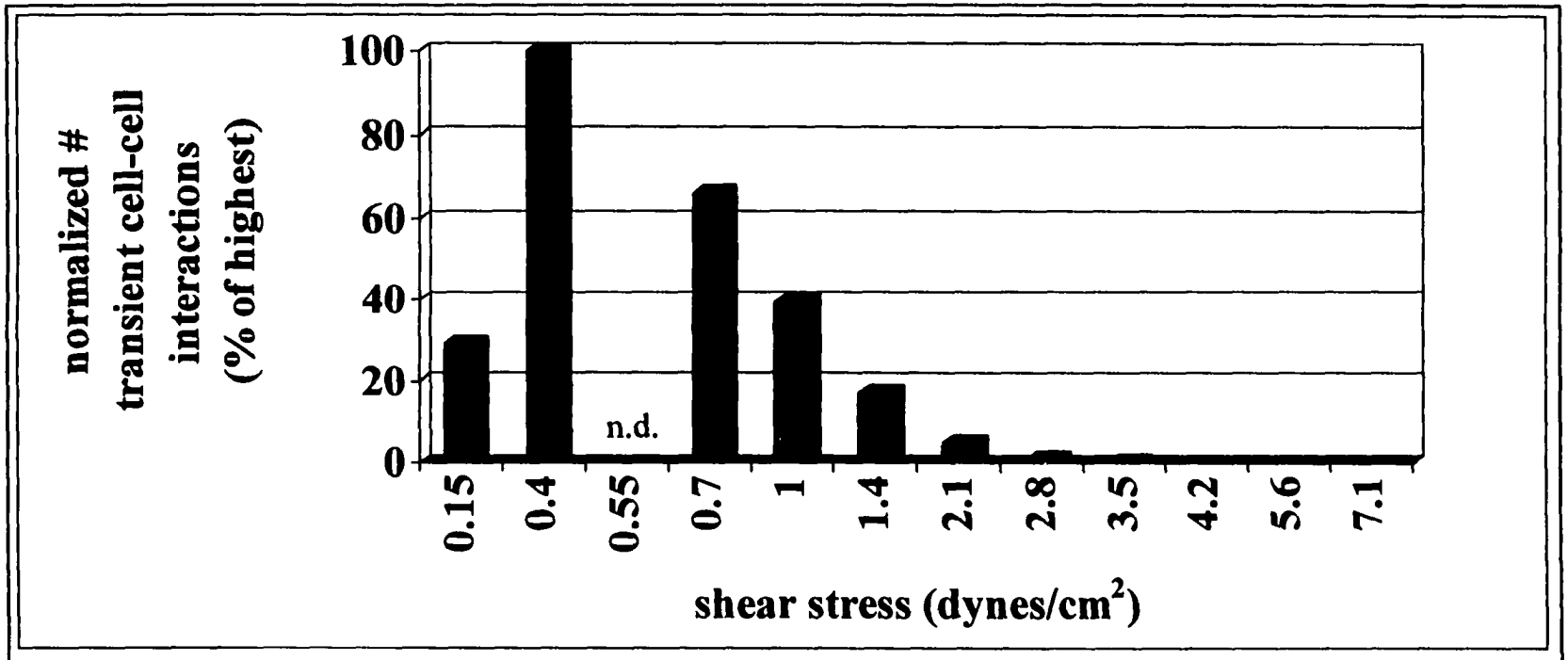


Figure 44. Analysis of E-selectin through cell-cell interactions. HL-60 cells expressing PSGL-1 were prebound to an anti-human CD43 antibody coated glass slide. Pre-B cells expressing E-selectin were withdrawn over the prebound HL-60 cells and the number of transient cell-cell interactions was counted and normalized for the number of cells on the surface, the duration of the experiment, and the flux of cells. The number of cell-cell interactions was maximum at approximately 0.4-0.7 dynes/cm^2 and decreased as the shear stress was increased. Cell-cell interactions occurred at shear stresses below 4.0 dynes/cm^2 suggesting the on-rate of E-selectin is slower than L-selectin (events up to 7.0 dynes/cm^2). The number of cell-cell interactions decreased significantly at 0.15 dynes/cm^2. This possibly indicates a certain shear stress is required to promote bond formation. Cell-cell interactions occurring over the range of shear stress were very slow transient interactions. The flowing cell remained attached to the bound cell for 0.5-2.0+ seconds. This supports the kinetic calculations that E-selectin has a slower off-rate than L-selectin.. Error bars representing +/- SEM (n=2) all less than 2%.

Discussion

The goal of measuring the off-rates of P-, E-, and L-selectin in various systems used to study cell rolling and cell recruitment was to determine if the presentation of the molecules in the systems affected the kinetics of the bond. The off-rates for P-, E-, and L-selectin were calculated by measuring the duration of transient events that are believed to result from the formation of a single receptor-ligand bond. The off-rates calculated for the three selectin adhesion molecules did not vary significantly in the various systems studied. This suggests the kinetics of the interactions are not affected by how the molecules are displayed in the system. It also indicates the kinetics are a result of receptor-ligand dissociation and not receptor extraction. We would expect to see a wide variation in the calculated off-rates from system to system if the kinetics were a result of the receptors being extracted out of the various cells and surfaces.

The off-rates of P-selectin and E-selectin were nearly identical over a range of shear stresses, although they were both slower than the L-selectin/PSGL-1 interaction. These findings are in agreement with others who have compared the off-rates of P-, E-, and L-selectin by measuring the dissociation of neutrophil transient tethers from P- and E-selectin bilayers (38, 536) and a PNAd coated surface (536). These results are also consistent with others who have compared the off-rates of selectins by measuring cell rolling velocities to show the L-selectin off-rate is faster than the off-rate of either P-selectin or E-selectin (537, 538). The off-rates we calculated for P- and E-selectin were very similar to those calculated by Alon et al on lipid bilayers (38, 536). An interesting finding is that the L-selectin/PSGL-1 interaction has a much faster off-rate than the P-

selectin/PSGL-1 interaction. Studies using blocking antibodies and site specific mutagenesis suggest that both selectins bind to a similar region on the N-terminal end of the PSGL-1 molecule (150). In contrast, E-selectin may have multiple binding sites on PSGL-1 or other ligands on leukocytes. In spite of this difference in the molecular mechanism of the interactions, P-selectin and E-selectin have very similar off-rates and the off-rate varies in the same way with increasing shear stress suggesting that the bond compliance of the two interactions is also similar. Our off-rate data for L-selectin with PSGL-1 is also similar to the off-rates calculated for L-selectin with PNAd (536). Our data shows that not only does L-selectin have a faster off-rate than the other selectins, the off-rate increases more rapidly with an increase in shear stress. This indicates that the L-selectin/PSGL-1 bond is more compliant, greater increase in k_{off} with an increase in applied force, than P- and E-selectin which is opposite of what was found with the L-selectin/PNAd interaction (536). Perhaps the mechanism by which L-selectin binds to PSGL-1 might be slightly different than to PNAd making the bond more susceptible to applied force.

The off-rates obtained using pre-B cells expressing P-, E-, or L-selectin were all faster than the off-rates calculated for the same interaction in the other systems. This is most likely the result of nonspecific binding of the pre-B cells to the surface. Transient interactions were only reduced about 75% with a blocking antibody. This indicates most of the events measured were selectin-mediated; however, some of the faster transient events may have been the result of nonspecific interactions. Also, cells that might have had long duration transient events might have remained permanently on the surface due

to nonspecific interactions. Removing some of the longer transient events and adding fast nonspecific transient events would lead to the calculation of a faster off-rate for the interaction using the pre-B cells.

The on- and off-rates for P-, E-, and L-selectin were also indirectly compared through cell-cell interactions. Pre-B cells expressing P-, E-, or L-selectin were withdrawn over bound PSGL-1-expressing HL-60 cells. The site density of the selectin and the size of the cells were approximately the same for each type of cell. This provided a system in which the expression of the selectins and PSGL-1 was consistent for all three selectin molecules. The flowing pre-B cells interacted transiently with the bound HL-60 cells. A flowing cell attached to a bound cell briefly (less than 4 seconds) then released back into the flowing stream. Studying the transient cell-cell interactions over a range of shear stresses gave an indirect comparison of the on-rates and the off-rates for the selectins. Pre-B cells expressing P-selectin and E-selectin interacted with bound HL-60 cells at shear stresses up to about 4.0 dynes/cm^2. However, L-selectin-expressing pre-B cells attached to bound HL-60 cells at shear stresses up to approximately 7.0 dynes/cm^2 indicating the on-rate for L-selectin is faster than the on-rate for P- and E-selectin. The transient cell-cell interactions resulted in pre-B cells expressing P-selectin and E-selectin remaining attached to HL-60 cells for 0.5~4.0 seconds. The duration of the L-selectin-expressing pre-B cells was always less than 0.5 seconds. This indirectly shows the off-rate for the L-selectin interaction is faster than the P-selectin and E-selectin interactions, which is in agreement with the calculated off-rates. The cell-cell interactions were shown

to be mediated by the selectin on the pre-B cell by performing the cell-cell interaction assays in the presence of various blocking and nonblocking antibodies (data not shown).

Physiologically a faster on- and off-rate is logical if L-selectin is involved in cell recruitment through cell-cell interactions. A faster on-rate would allow cells to be brought to a P-selectin or E-selectin surface under shear stress conditions that would not promote direct adhesion to the P- and E-selectin-expressing endothelium. A faster off-rate would allow neutrophils to roll on bound neutrophils faster than cells are rolling on the endothelium in order for the leukocytes attaching through cell-cell interactions to reach the endothelium instead of accumulating up into the vessel.

The threshold shear stress requirement for cell rolling is an interesting phenomenon that has been described for L-selectin (533, 534). It has been shown that cells will only roll above a certain critical shear stress and below this threshold shear stress the cell interactions are transient (533, 534, and data not shown). Also, cells rolling uniformly on the surface at shear stresses above the threshold shear stress detach and enter the hydrodynamic flow when the shear stress is decreased below the threshold shear stress (533, 534, and data not shown). These observations suggest a necessary shear stress is required for efficient bond formation as well as cell rolling. Alon et al showed that the rate of L-selectin tethers with PNAd decreased with a decrease in shear stress from 0.7 dynes/cm^2 to 0.3 dynes/cm^2, opposite of what was found on P-selectin (536). We were interested in studying this phenomenon of L-selectin with PSGL-1 which mediates cell-cell interactions. The shear stress dependence on the rate of cell-cell interactions for pre-

B cells expressing L-selectin was counterintuitive. The number of cell-cell interactions increased as shear was decreased from 7.0 dynes/cm^2 to 1.4 dynes/cm^2; however, as the shear was decreased from 1.4 dynes/cm^2 to 0.15 dynes/cm^2, the number of cell-cell interactions decreased dramatically. The same type of shear dependence on the rate of cell-cell interactions was seen with neutrophils on prebound HL-60 cells. The phenomenon was also studied with neutrophils on a purified PSGL-1 bilayer to show the decrease in the rate of interactions was not a result of the cell-cell interaction systems. Neutrophils rolled on the PSGL-1 bilayer above 0.7 dynes/cm^2 and interacted transiently with the surface below 0.7 dynes/cm^2. This observation is consistent with the threshold shear stress requirement for cell rolling. However, as the shear stress was decreased, there was a significant decrease in the rate of interactions with the surface. This data supports the idea that a certain shear stress is not only required for cell rolling, but also for transient tether formation between L-selectin and PSGL-1. Cell recruitment can occur from leukocyte-leukocyte interactions mediated by PSGL-1 and L-selectin. The lack of efficient bond formation below a critical shear stress might be a mechanism in which cell accumulation is controlled in low shear stress environments.

The shear stress dependence on cell-cell interactions for P-selectin and E-selectin-expressing pre-B cells was the same. The number of cell-cell interactions increased as the shear stress decreased. This is the expected result since bond formation occurs more readily at lower flow rates. A significant decrease in the rate of cell-cell interactions was seen at 0.15 dynes/cm^2. This phenomenon for P-selectin and E-selectin was also obtained with HL-60 cells and neutrophils on P- and E-selectin transfected CHO cells.

As the shear stress was decreased below the threshold shear stress required for cell rolling, the rate of interactions with the surface decreased significantly, reaching approximately zero at 0.1 dynes/cm^2. These results indicate a possible shear stress requirement for bond formation for all three selectin adhesion molecules. However, the shear stress at which it becomes evident for P- and E-selectin is much lower than through L-selectin. Alon et al did not see this trend with P-selectin possibly because the shear stress range studied was too high (536).

The determination of the off-rates for P-, E-, and L-selectin has shown that L-selectin has a much faster off-rate than P-selectin and E-selectin. The data suggests that the off-rates of the selectins are not affected by the presentation of the molecules in the systems and that the magnitude of the off-rate is a result of bond dissociation and not receptor extraction from the membrane. This study has also shown a shear stress requirement for L-selectin and PSGL-1 bond formation that might be an important cell accumulation control mechanism. It appears that P- and E-selectin bond formation is also controlled by shear stress in low shear stress environments.

Conclusion

We have identified a novel mechanism by which cell-cell interactions occur which may contribute in the recruitment of leukocytes to the vessel wall near sites of infection and injury. P-selectin-expressing platelet microparticles act as a bridge between PSGL-1-expressing cells allowing them to interact. HL-60 cells or neutrophils in the presence of platelet microparticles were withdrawn over HL-60 cells prebound to a glass slide to determine if platelet microparticles could mediate cell-cell interactions, cell aggregation, and possibly cell accumulation through cell-cell interactions. Increasing the concentration of platelet microparticles in the system resulted in an increase in the number of cell-cell interactions, an increase in the number and size of cell aggregates, an increase in the number of cell-cell interactions at higher shear stresses, and an increase in cell accumulation on the surface. Leukocyte-leukocyte interactions can occur through the interaction of L-selectin with PSGL-1. However, leukocytes incubated with platelet microparticles could significantly interact with HL-60 cells expressing PSGL-1 in the presence of an L-selectin antibody that blocked the L-selectin/PSGL-1 mechanism.

An interesting phenomenon was observed for cell-cell interactions occurring through the L-selectin/PSGL-1 mechanism. A decrease in the number of cell-cell interactions occurred with a decrease in shear stress from 1.4 dynes/cm^2 to 0.4 dynes/cm^2 when neutrophils or pre-B cells expressing L-selectin were withdrawn over prebound HL-60 cells. This phenomenon was also seen when neutrophils were withdrawn over a lipid bilayer containing purified PSGL-1. As the shear stress was decreased, the number of interactions with the surface greatly decreased. The number of cell-cell interactions was

increased when pre-B cells expressing P- or E-selectin were withdrawn over HL-60 cells as the shear stress was decreased from 1.4 dynes/cm^2 to 0.4 dynes/cm^2. A significant decrease in the number of cell-cell interactions was seen when the shear stress was lowered to 0.15 dynes/cm^2. These results indicate a shear stress requirement for efficient bond formation through the selectins. The shear stress requirement for efficient bond formation between L-selectin and PSGL-1 is possibly a control mechanism to regulate unwanted accumulation or aggregation through cell-cell interactions. However, in the presence of platelet microparticles, neutrophils were able to accumulate on a P-selectin bilayer through cell-cell interactions below this threshold shear stress requirement for the L-selectin/PSGL-1 interaction. Attachment of cells through P-selectin-mediated cell-cell interactions significantly enhanced the accumulation of neutrophils on the P-selectin bilayer.

Platelet microparticles may mediate significant cell aggregation and accumulation through cell-cell interactions in low shear stress environments which would override the possible control mechanism of limited cell-cell interactions through the L-selectin/PSGL-1 interaction in low shear environments. The ability of platelet microparticles to mediate leukocyte-leukocyte interactions when the L-selectin/PSGL-1 mechanism is blocked by an L-selectin antibody could also override attempts to control cell aggregation and accumulation through the L-selectin mechanism. Therefore, platelet microparticles may play an important role in clotting, cell aggregation, and cell accumulation through cell-cell interactions especially in disease states where platelet microparticle concentrations are elevated.

We also analyzed the kinetic off-rates of the selectin adhesion molecules. We have measured the off-rates of P-, E-, and L-selectin in various systems including using lipid bilayers and CHO cells as the surface and using neutrophils, HL-60 cells, and pre-B cells as the circulating cell. The off-rates were calculated in many systems to determine if how the molecules were displayed in the systems would affect the off-rate of the interaction and if the kinetics were a result of bond dissociation or receptor extraction. We would expect to obtain significant variations in the calculated off-rates in the different systems if the off-rates were dependent on how the molecules were displayed or if the off-rates were a result of receptor extraction. The off-rates were calculated over a range of shear stresses and compared between systems by calculating the force on the bond. The off-rates for P- and E-selectin were approximately the same; however, the off-rate for L-selectin was found to be much faster. The calculated off-rates were not dependent on how either the selectin or PSGL-1 was presented in the various systems. This shows the off-rate is strongly dependent on the interactions between the molecules, but not strongly dependent on how the molecules are displayed on the surface. This also suggests that the kinetics are a result of the dissociation of the receptor-ligand bond and not the extraction of the receptor from the membrane.

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Appendix I

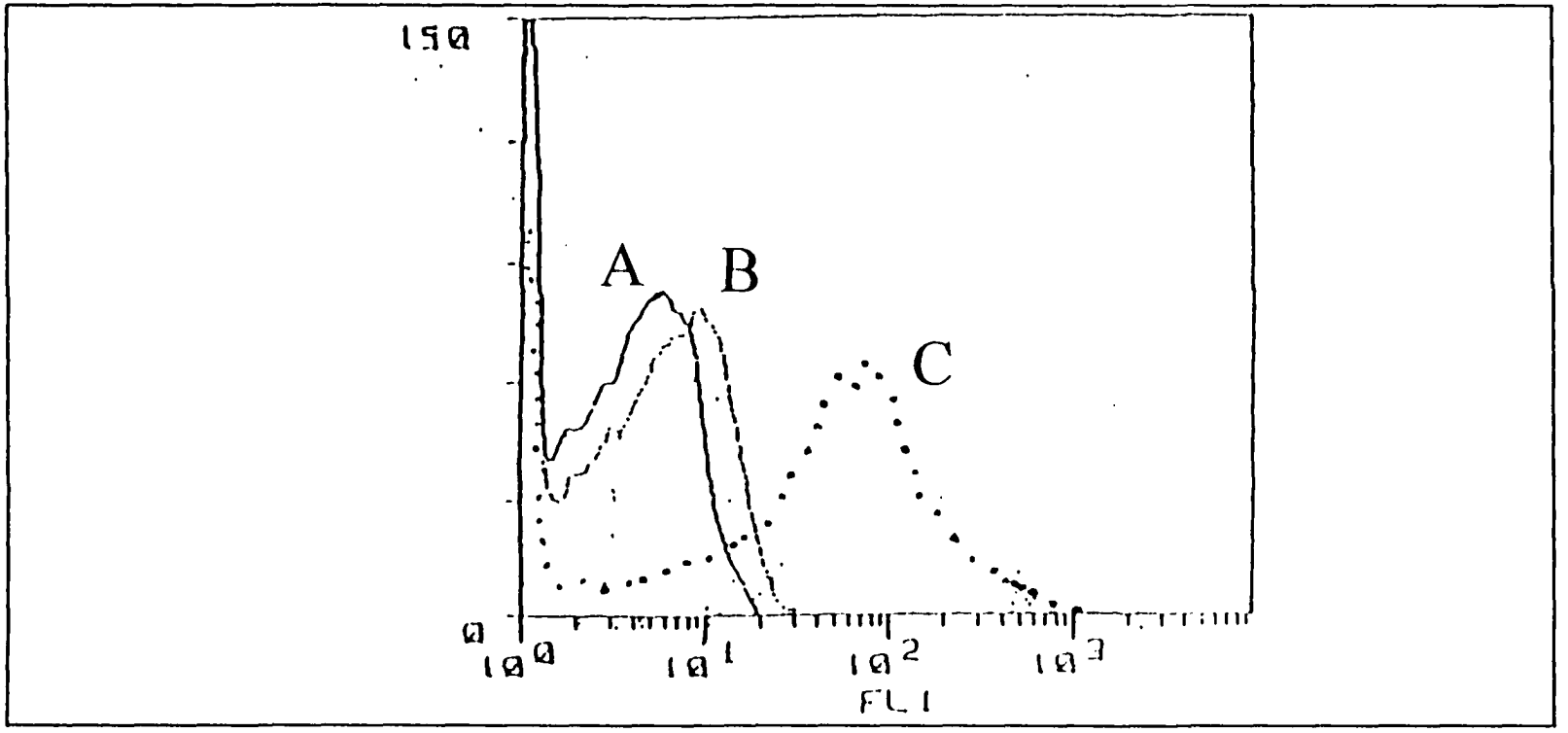


Figure A1. Fluorescence histogram to verify P-selectin was incorporated into lipid vesicles. Lipid vesicles made with P-selectin were analyzed by flow cytometry to determine if the P-selectin was incorporated into the vesicle correctly. Curve A is a sample of vesicles containing P-selectin without the addition of a fluorescent antibody. Curve B is a sample of P-selectin containing vesicles incubated with an irrelevant antibody for P-selectin. Curve C is a sample of vesicles containing P-selectin incubated with a fluorescent antibody for P-selectin. The higher fluorescence in Curve C is due to the binding of the fluorescent antibody to P-selectin indicating it was incorporated in the bilayer. It also shows that P-selectin was incorporated in the correct orientation since the binding site for the antibody is in the lectin domain of the molecule.

Appendix II

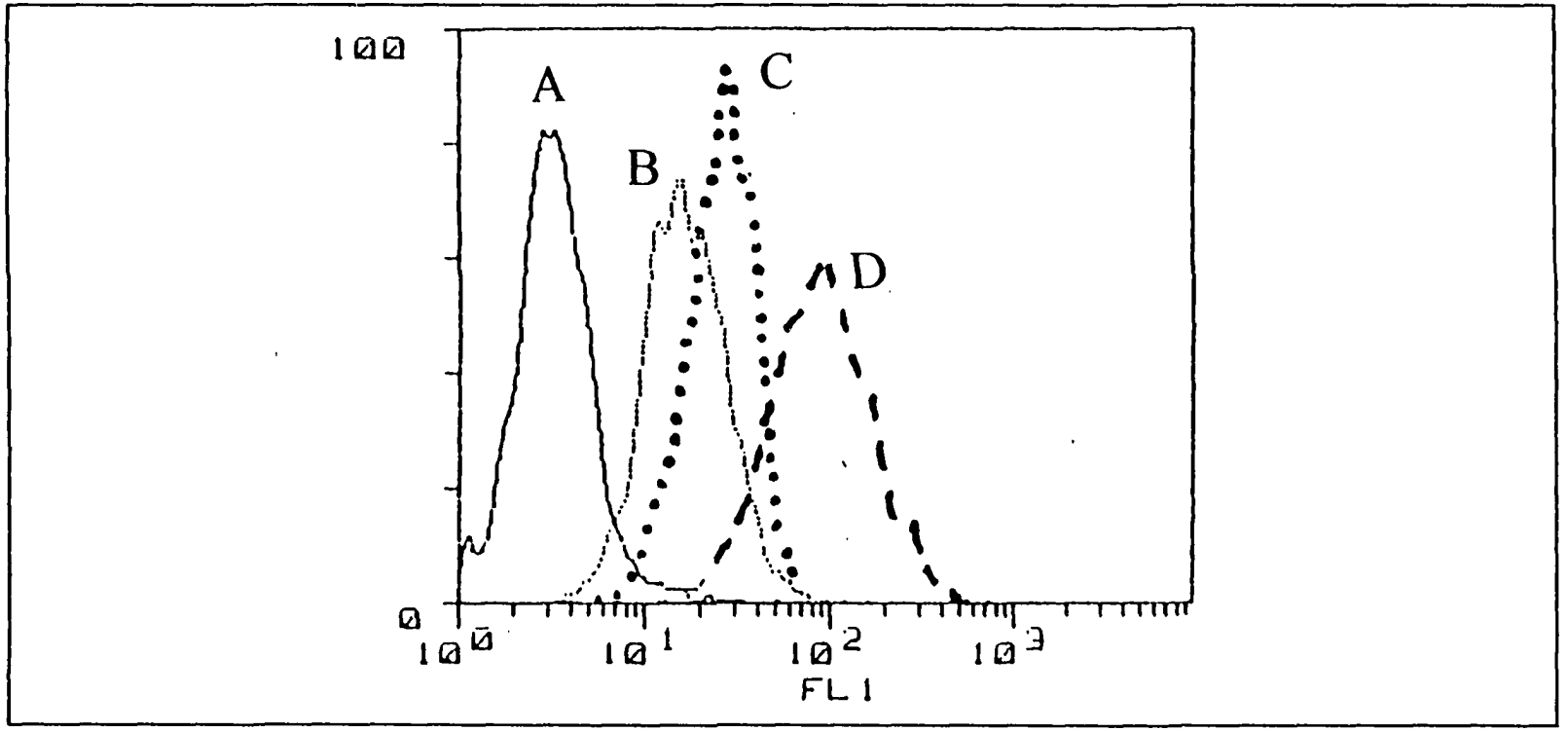


Figure A2. Fluorescence histogram to verify HL-60 cells do not express L-selectin. HL-60 cells were analyzed by flow cytometry to show that the HL-60 cells used in the flow experiments did not express L-selectin. HL-60 cells were incubated with either GAM-FITC, an irrelevant fluorescent antibody (C), Leu-8-FITC, an anti-L-selectin antibody (B), or CD16-FITC, a fluorescent antibody that binds to HL-60 cells (D). Incubation with the fluorescent L-selectin antibody did not show fluorescence compared with the fluorescence obtained by the incubation of cells with CD16-FITC indicating there was no L-selectin on the HL-60 cells. Curve A was a sample of HL-60 cells with no fluorescence added.

Appendix III

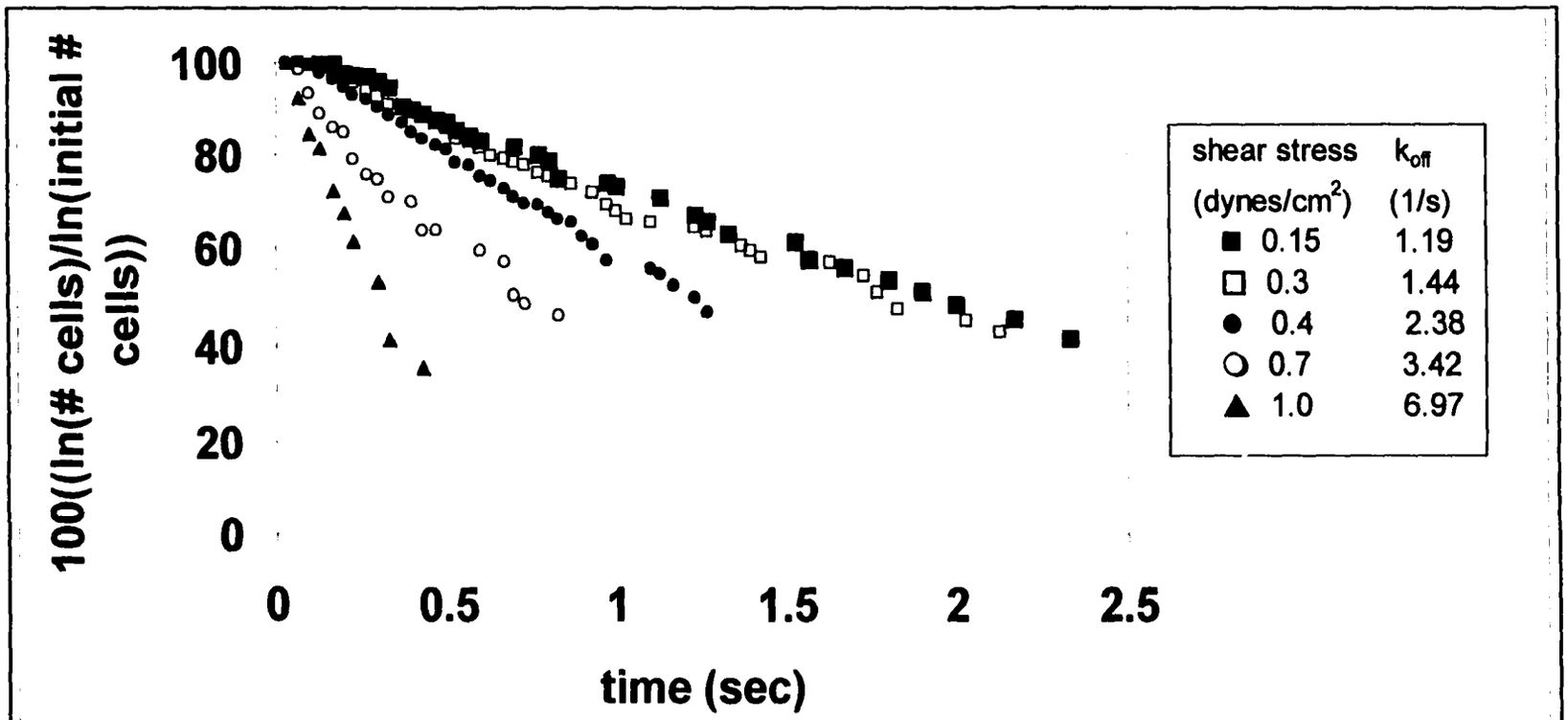


Figure A3. Kinetic off-rate of the P-selectin/PSGL-1 interaction calculated by withdrawing pre-B cells expressing P-selectin over a PSGL-1 bilayer. Pre-B cells expressing P-selectin were withdrawn over a PSGL-1 bilayer at various shear stresses. The duration of the transient interactions was determined and the natural log of the number of cells remaining bound was plotted versus the duration of the transient events (t) to obtain the off-rate. The natural log of the number of cells was normalized for each shear stress by the natural log of the initial number of cells (ln(#cells)/ln(initial # cells)). The off-rate was calculated at 0.15 dynes/cm^2 from 73 cells; at 0.3 dynes/cm^2 from 129 cells; at 0.4 dynes/cm^2 from 199 cells, at 0.7 dynes/cm^2 from 117 cells; and at 1.0 dynes/cm^2 from 50 cells.

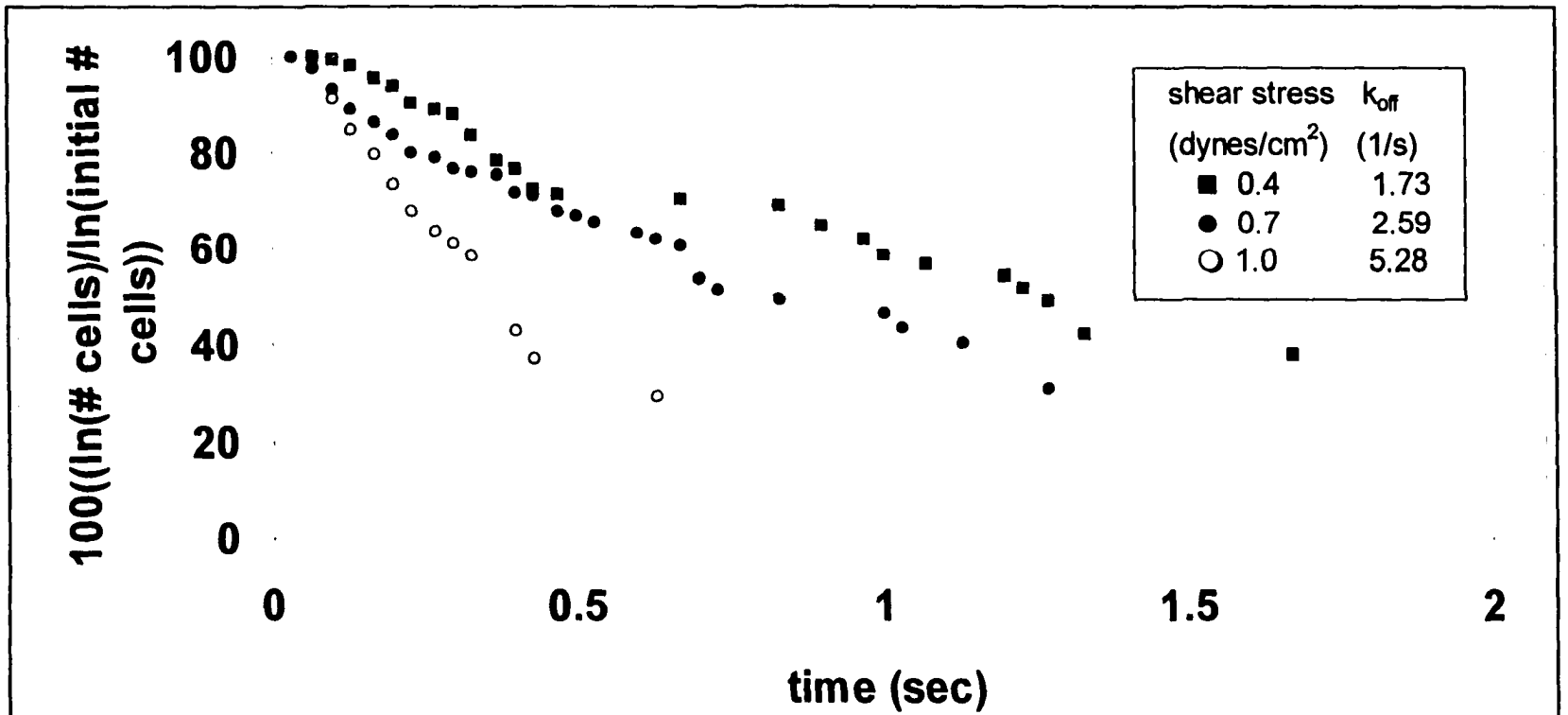


Figure A4. Kinetic off-rate of E-selectin calculated by withdrawing pre-B cells expressing E-selectin over a PSGL-1 bilayer. Pre-B cells expressing E-selectin were withdrawn over a PSGL-1 bilayer that produced transient interactions. The duration of the transient interactions was determined and the natural log of the number of cells remaining bound was plotted versus the duration of the transient events (t) to obtain the off-rate. The natural log of the number of cells was normalized for each shear stress by the natural log of the initial number of cells (ln(# cells)/ln(initial # cells)). The off-rate was calculated at 0.4 dynes/cm^2 from 70 cells, at 0.7 dynes/cm^2 from 87 cells, and at 1.0 dynes/cm^2 from 43 cells.

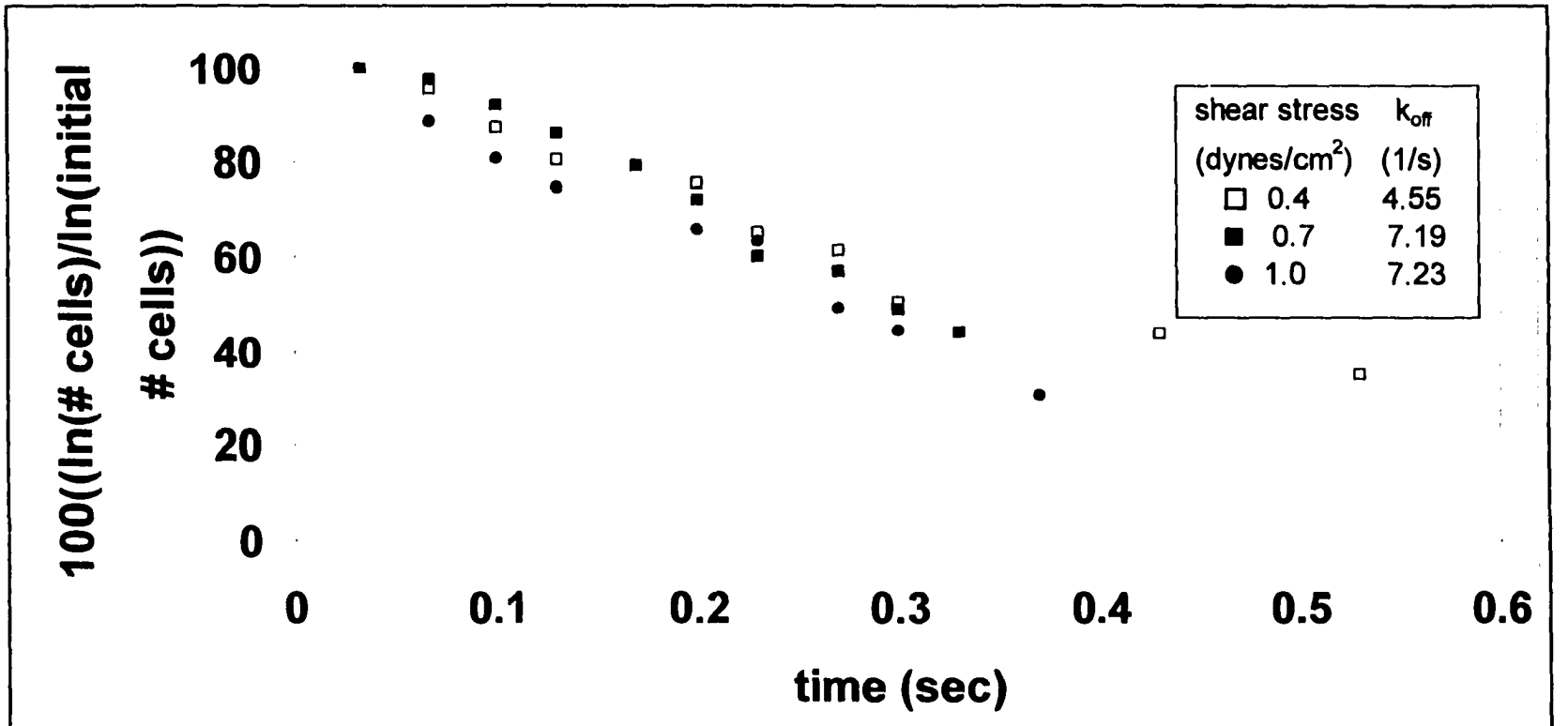


Figure A5. Kinetic off-rate of the L-selectin/PSGL-1 interaction calculated by withdrawing pre-B cells expressing L-selectin over a PSGL-1 bilayer. Pre-B cells expressing L-selectin were withdrawn over a PSGL-1 bilayer that produced transient interactions. The duration of the transient interactions was determined and the natural log of the number of cells remaining bound was plotted versus the duration of the transient events (t) to obtain the off-rate. The natural log of the number of cells was normalized for each shear stress by the natural log of the initial number of cells (ln(# cells)/ln(initial # cells)). The off-rate was calculated at 0.4 dynes/cm^2 from 24 cells, at 0.7 dynes/cm^2 from 39 cells, and at 1.0 dynes/cm^2 from 38 cells.

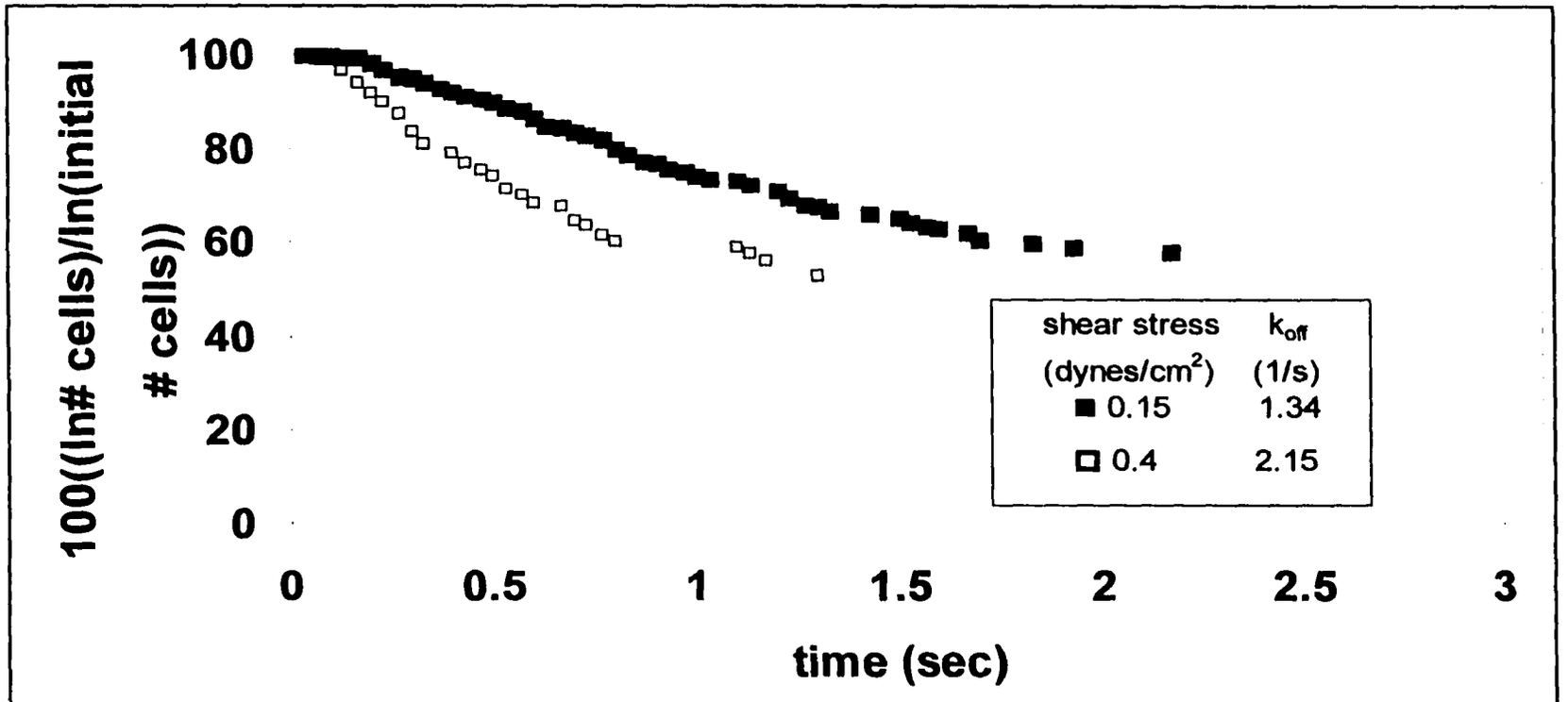


Figure A6. Kinetic off-rate of the P-selectin/PSGL-1 interaction calculated by withdrawing HL-60 cells contaminated with P-selectin platelet microparticles over a PSGL-1 bilayer. Platelet microparticles, expressing P-selectin, contained in fetal bovine serum bound to PSGL-1 expressed on the HL-60 cells. This enabled HL-60 cells to interact with PSGL-1 expressed in lipid bilayers. HL-60 cells (with platelet microparticles) were withdrawn over PSGL-1 bilayers at two shear stresses that produced transient interactions. The duration of the transient interactions was determined and the natural log of the number of cells remaining bound was plotted versus the duration of the transient events (t) to obtain the off-rate. The natural log of the number of cells was normalized for each shear stress by the natural log of the initial number of cells (ln(# cells)/ln(initial # cells)). The off-rate was calculated at 0.15 dynes/cm^2 from 194 cells and at 0.4 dynes/cm^2 from 112 cells.

Appendix IV

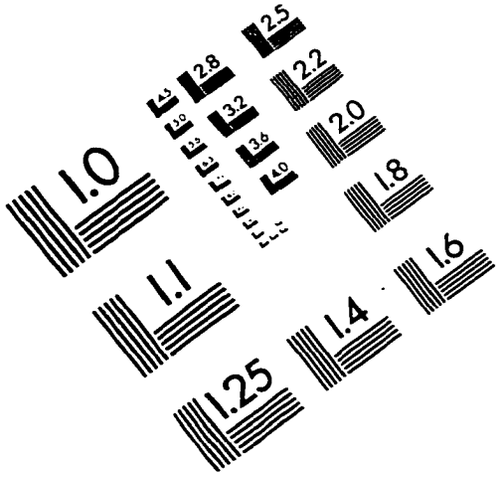
Acronym Key

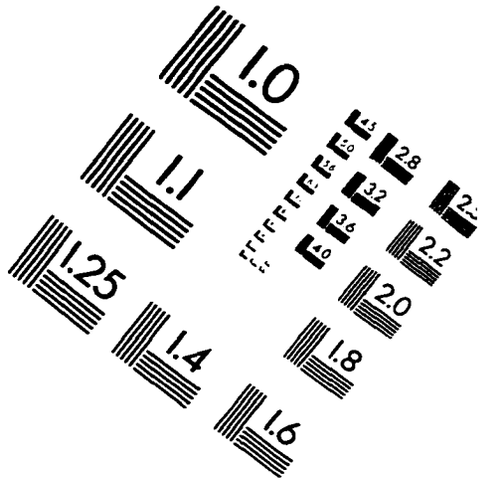
ACD	acid citrate dextrose
ACS	acute coronary syndromes
ADP	adenosine diphosphate
BSGC	buffered saline glucose citrate
CHO	chinese hamster ovary
CVA	cerebrovascular accidents
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
ELAM-1	endothelial leukocyte adhesion molecule
ESL-1	E-selectin ligand-1
FBS	fetal bovine serum
FMLP	formyl-methionyl-leucyl-phenylalanine
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP-140	granule membrane protein-140
GPS	glutamine-pen-strep
HBSS	Hank's balanced salt solution
HEV	high endothelial venule
HSA	human serum albumin
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
LAD	leukocyte adhesion deficiency
LAM-1	lymphocyte-associated molecule-1
LECAM-1	leukocyte-endothelial cell adhesion molecule-1
LFA-1	leukocyte function-associated antigen-1
LPS	lipopolysaccharide
LTB4	leukotriene B4
Mac-1	macrophage antigen-1
MAdCAM	mucosal addressin cell adhesion molecule
MAP	mitogen-activated protein
MCP	monocyte chemotactic peptide
αMEM	Alpha Modification of Eagle's Medium
NF-κB	nuclear factor-kappaB
NK	natural killer cell
PADGEM	platelet activation dependent granule-external membrane protein
PAF	platelet activating factor
PDGF	platelet-derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule-1
PGE_1	prostaglandin E_1
PGG_2	prostaglandin G_2

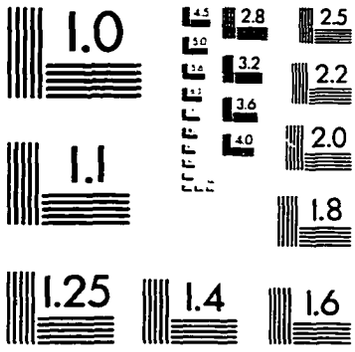
Acronym Key

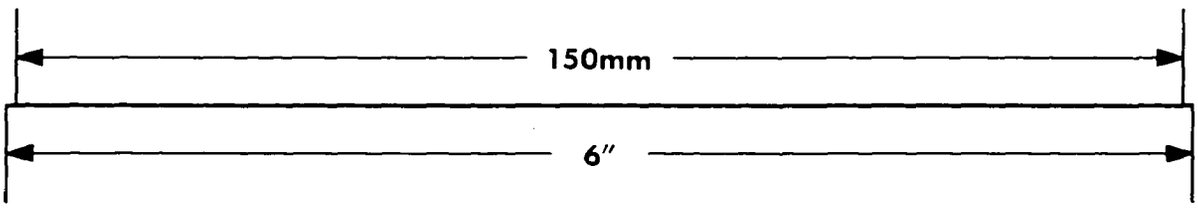
PMP	platelet microparticle
PNAd	peripheral node addressin
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	regulated upon activation, normal T cell expressed and presumably sectreted
SCR	short consensus repeat
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VSMC	vascular smooth muscle cells
vWF	von Willebrand Factor

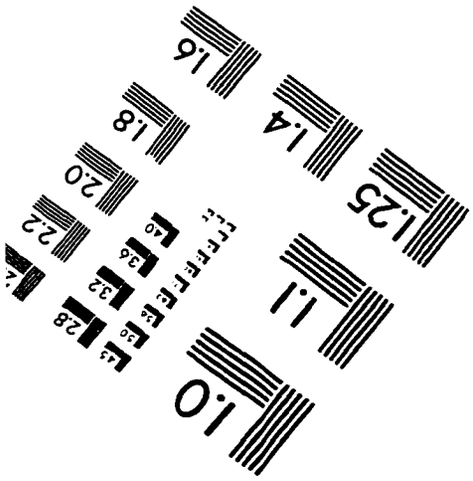
IMAGE EVALUATION
TEST TARGET (QA–3)

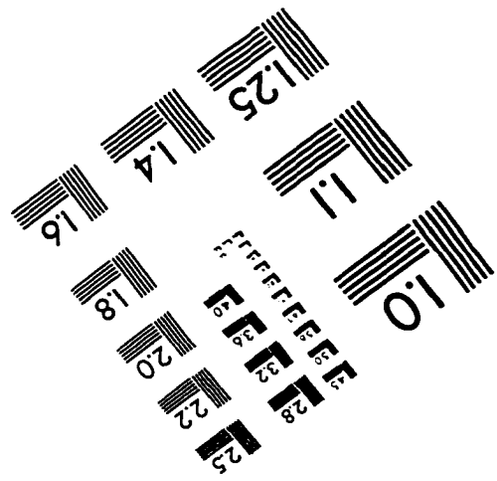












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