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HYDRODYNAMIC AND MOLECULAR DEPENDENCE OF NEUTROPHIL-NEUTROPHIL ADHESION

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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ABSTRACT

Hydrodynamic and Molecular Dependence of Neutrophil-Neutrophil Adhesion

by

Andrew Taylor

Homotypic adhesion of neutrophils stimulated with chemoattractant is analogous to capture on vascular endothelium in that both processes depend on L-selectin and $\beta_2$-integrin adhesion receptors. Under hydrodynamic shear, cell adhesion requires that receptors bind sufficient ligand over the duration of intercellular contact to withstand hydrodynamic stresses. A detailed examination of the effect of shear rate and shear stress on neutrophil-neutrophil adhesion was performed using cone plate viscometry. A collisional analysis based on Smoluchowski's theory was employed to fit the kinetics of aggregation with an adhesion efficiency. Adhesion efficiency increased with shear rate from $\sim$20% at 100 s$^{-1}$ to $\sim$80% at 400 s$^{-1}$. The increase in adhesion efficiency with shear was dependent on L-selectin and peak efficiency was maintained over a relatively narrow range of shear rates (400-800 s$^{-1}$) and shear stresses (4-7 dyn/cm$^2$). When L-selectin was blocked with antibody, $\beta_2$-integrin supported adhesion at low shear rates which decreased with increasing shear $\geq$100 s$^{-1}$. At low shear, neutrophil-neutrophil adhesion was dependent on LFA-1 (CD11a/CD18) binding to ICAM-3 (CD50) and Mac-1 (CD11b/CD18) binding an
unknown ligand. Adhesion became more dependent on Mac-1 with time after chemotactic stimulation and as shear rate was increased. The binding kinetics of selectin and integrin appear to be optimized to function within discrete ranges of shear rate and stress, providing an intrinsic mechanism for the transition from neutrophil tethering to stable adhesion.
To Melissa

and

My Family
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Chapter 1

Background and Introduction

1.1 Cells of the Immune System

The human immune system has evolved to protect the body against microbial infections and promote wound healing. The immune system must be capable of recognizing a wide variety of foreign organisms and responding appropriately. The cells of the human immune system include phagocytes and lymphocytes. Phagocytes are responsible for the engulfment and digestion, or "phagocytosis" of foreign agents. The lymphocytes detect and eliminate infectious agents and recruit additional cells of the immune system into the infected area. Together, the phagocytes and lymphocytes are termed white blood cells, or leukocytes.

Leukocytes originate from a pluripotent bone marrow stem cell, which differentiates to form all of the phagocytic and lymphocytic subclasses. The phagocytes consist of the monocytes and macrophages, and the polymorphonuclear leukocytes (PMN's). The lymphocytes consist primarily of the T lymphocytes and the B lymphocytes.

Lymphocytes are responsible for the specific recognition of foreign antigens,
and account for 20-40% of the circulating white blood cells. Both B and T lymphocytes exist as individual clones, each cell recognizing a specific antigen epitope. When a naive lymphocyte encounters its antigen, it undergoes rapid growth and division, producing effector (antibody secreting or cytotoxic lymphocytes) or memory cells. If the same epitope is encountered in the future, the relatively large numbers of memory cells in circulation help recruit a much faster immune response, and the antigen is destroyed before an infection develops. This lymphocyte “memory” enables immunization to protect against future contraction of a disease.

Monocytes account for about 7% of the total white blood cell count and are the largest circulating leukocytes. Circulating monocytes have a half life of a few days, but when localized into the tissue they can live for weeks or months. In the tissue monocytes differentiate into macrophages, developing functions specialized for the tissue into which they are recruited.

The PMN’s include the eosinophils, basophils, and neutrophils. They are termed polymorphonuclear leukocytes because of the morphology of their nucleus, which appears segmented and multi-lobed, hence “polymorphic”. PMN’s represent 60-70% of the total leukocyte count in human blood. The cytoplasm of these cells contains a large number of granules, which are membrane-bound organelles containing a wide variety of proteins and proteolytic enzymes. The PMN’s are divided into three classes based on the staining properties of their cytoplasms.
Eosinophils stain red with acid dyes such as eosin, basophils stain blue with basic dyes, and neutrophils are stained by both dyes, appearing purple. The granules of the three PMN classes contain distinct molecules and enzymes, and convey specialized roles on each sub-type.

Basophils normally constitute less than 1% of white blood cells in circulation and are the least well defined biochemically. Basophil granules contain histamine and other molecules that mediate allergic or inflammatory responses. Eosinophils account for 1-7% of the total white blood cell count and are 8-10 μm in diameter. The granules in eosinophils are larger than those in neutrophils and contain enzymes which are biochemically distinct from those in neutrophil granules. The main function of eosinophils is the destruction of parasites, and the number of eosinophils in circulation is elevated during parasitic infections.

Neutrophils are the most abundant PMN, comprising 40-65% of total white blood cells. They are found at a concentration of 3 - 5*10^6 cells/ml in human blood and this concentration can increase ten-fold in cases of infection. Neutrophils are short-lived cells, remaining in the blood stream 8-20 hours after release from the bone marrow. The lifetime is extended up to several days when neutrophils leave the circulation to enter the tissue. In the human body, tremendous numbers of neutrophils enter and leave the blood stream, approximately 5*10^10 neutrophils must be generated
daily in an adult human with 5 liters of blood (1). Unactivated neutrophils are spherical cells of approximately 7 μm in diameter. In response to chemical stimuli, neutrophils rapidly change shape. Cell polarization enables them to sense and move in the direction of the chemical gradient. The neutrophil is the first cell recruited to sites of infection, underscoring its importance as the body’s first line of defense against a bacterial invasion.

1.2 Cell Adhesion

All phagocytic function is dependent on cell-cell or cell-matrix adhesion. Recruitment from the blood stream requires adhesion to the endothelial cell lining of the blood vessel, extravasation between endothelial cells, and mobility into the tissue. This mobility is enabled by phagocytes adhering reversibly to surrounding cells and to extracellular matrix molecules. Once at the site of infection, the phagocyte may also need to bind its target for engulfment.

An example of adhesion dependent localization of an immune cell is neutrophils being recruited from flowing blood in the microcirculation. Neutrophils in the free stream first undergo a transient “tether” or initial adhesion. If the proper signals are present, this initial adhesion is followed by cell “rolling” along the endothelial cells lining the blood vessel. If the rolling cell encounters a chemical
attractant, which may be generated at the site of infection, or a bacterial protein, the neutrophil becomes activated. The activated neutrophil then binds firmly to the endothelium, squeezes between adjacent endothelial cells, and moves toward the invading microorganisms or necrotic tissue.

1.3 Adhesion Receptors

Neutrophil localization is dependent on specific cell surface receptors binding their counter-structures on the vascular endothelium. Research during the past 10-15 years has led to the characterization of many of the molecules involved in neutrophil-endothelial cell adhesion. Three groups of molecules have been identified in neutrophil-endothelial cell adhesion, the selectins, the integrins, and members of the immunoglobulin (Ig) superfamily. In the current paradigm, the initial adhesion and rolling are mediated by selectins expressed both on the neutrophil and the endothelial cell surfaces (2,3). Subsequent firm adhesion and diapedesis are mediated by integrins on the neutrophil binding to intercellular adhesion molecules (ICAM’s), members of the Ig superfamily expressed on the endothelium.
1.3.1 Selectins

Three members of the selectin family of been identified: L-selectin (CD62L), E-selectin (CD62E), and P-selectin (CD62P). Selectins are characterized based on structural similarities, each containing three extracellular domains: An NH₂ terminal lectin domain, an epidermal growth factor (EGF) domain, and a variable number of complement regulatory (CR) domains. The length of the selectins differs according to the number of complement regulatory domains. P-selectin is the longest with nine CR domains, E-selectin contains six, and L-selectin is the shortest with just two. The binding sites on selectins have been mapped to the lectin-like binding site, and like lectins most selectin ligands are carbohydrates. In addition to their structural homology, selectins also display functional similarities. Selectin binding is Ca²⁺ dependent, and selectin function is regulated by a variety of mechanisms.

L-selectin is expressed on the surface of most circulating T- and B-lymphocytes, and essentially all eosinophils, neutrophils, and monocytes. L-selectin was discovered through study of lymphocyte recirculation in the mouse. It was found that blocking L-selectin with the monoclonal antibody Mel 14 abrogated the interaction of lymphocytes with the murine high endothelial venules (4,5). On neutrophils, L-selectin is preferentially expressed on the microvillus (6,7). This distribution may in part explain L-selectins ability to mediate initial adhesion and
rolling along the endothelium. L-selectin may have a low and high affinity conformation. In one report, activation of L-selectin by lineage specific stimuli was reported to regulate leukocyte migration (8). Modulation of receptor number is another mechanism to regulate the avidity of adhesion through L-selectin. Neutrophils and lymphocytes proteolytically cleave L-selectin from their surfaces in response to chemotactic stimuli (9-11). Within 90 seconds of formyl Met-Leu-Phe (fMLP) stimulation, almost 50% of L-selectin is shed from the cell surface, and greater than 90% is cleaved after 7 minutes (12,13). This shedding may help prevent inappropriate adhesion of activated neutrophils to sites down stream of the inflammatory site. L-selectin has also been reported to act as a signaling molecule. Cross-linking of L-selectin increases $\beta_2$-integrin expression on neutrophils (14), and activates $\beta_2$-integrin dependent adhesion (15,16).

E-selectin expression is limited to the endothelium, and is only expressed in response to inflammatory stimuli (2). E-selectin surface expression requires de novo protein synthesis, and peaks between 3 and 6 hours after TNF-α stimulation, and returns to basal levels after 10-16 hours, even in the continuous presence of cytokine (2,17). Similar to the other selectins, E-selectin mediates rolling of neutrophils along the inflamed endothelium (18,19).

P-selectin expression is inducible on both platelets and the endothelium. In
contrast to E-selectin, P-selectin is stored pre-formed, and may be produced transcriptionally (2). In platelets, P-selectin is stored within α granules, and in endothelial cells it is found in the Weibel-Palade bodies (20-22). P-selectin is expressed rapidly in response to agonists such as thrombin, histamine, and adenine diphosphate. P-selectin is expressed via granule fusion with the cell membrane and is detectable on the cell surface within minutes of stimulation. This expression is transient, being internalized by 20-60 minutes after stimulation (20). Like the other two selectins, P-selectin has been demonstrated to mediate leukocyte rolling (23).

As mentioned previously, the selectin ligands are mostly carbohydrate, as expected from the lectin-like binding domain. Selectins recognize a diverse number of carbohydrate ligands, which can be sialylated, fucosylated, and/or sulfated (2). The type and extent glycosylation may contribute to regulation of endothelial interactions with a specific leukocyte (6). The role of the protein “core” of selectin ligands is an area of active research (24,25). The best characterized glycoprotein selectin ligand is P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is expressed on all major classes of leukocytes and is a counter structure for P-selectin (26), L-selectin (27,28), and E-selectin (29). Ligands for L-selectin on inflamed endothelium include the sialomucin glycoproteins GlyCam-1 (30) and CD34 (31), and more remain to be identified.
1.3.2 Integrins

Integrins are heterodimeric proteins consisting of noncovalently linked $\alpha$ and $\beta$ subunits. Currently, 16 $\alpha$ and 8 $\beta$ subunits have been characterized, and more are being discovered each year (32). Although different combinations of $\alpha$ and $\beta$ subunits could give rise to more than one hundred possible heterodimers, there are currently just 22 known heterodimeric integrin complexes (32,33). Many $\alpha$ and $\beta$ subunits associate with only one partner, while some have many partners. In particular, the $\beta_1$, $\beta_2$, and $\beta_3$ subunits have three or more $\alpha$ partners, and the $\alpha_4$, $\alpha_6$, and $\alpha_v$ subunits form complexes with multiple $\beta$ subunits (32). The ability to bind multiple ligands is another attribute of several integrin heterodimers. Integrins are similar in structure, with $\alpha$ subunits averaging ~1100 amino acids and $\beta$ subunits averaging ~780 amino acids (34). A common feature to many integrins is the expression of a constitutively inactive conformation. In response to the proper signals, integrins attain an “active” conformation, capable of ligand(s) binding. Integrins are expressed on every human cell type, and are involved in both cell-matrix and cell-cell adhesion (34).

The first leukocyte specific integrin characterized was the $\alpha_{M/0}/\beta_2$ integrin identified in the mouse (34). The human homolog was then characterized and found to be identical to complement receptor type 3 (CR3). Currently, four $\alpha$ subunits have
been discovered which associate with the $\beta_2$ subunit in leukocytes: $\alpha_M/\beta_2$ (Mac-1, CD11b/CD18), $\alpha_4/\beta_2$ (LFA-1, CD11a/CD18), $\alpha_x/\beta_2$ (p150,95, CD11c/CD18), and the recently characterized $\alpha_d/\beta_2$. The $\beta_2$-integrins are expressed solely on leukocytes, with different $\alpha/\beta_2$ pairs being expressed on different leukocytes. LFA-1 is expressed by virtually all white blood cells, while Mac-1 expression is limited to monocytes, macrophages, granulocytes, and some minor subpopulations of lymphocytes (35). The $\alpha_x/\beta_2$ integrin expression is similar to Mac-1, and $\alpha_d\beta_2$ is expressed in myelomonocytic cell lines in a pattern distinct from the other $\beta_2$-integrins (36).

The $\beta_2$-integrins are responsible for the firm adhesion of neutrophils to the vascular endothelium, and for the subsequent transmigration into the inflamed tissue. The two major integrins involved in this process are the Mac-1 and LFA-1 integrins. The avidity of Mac-1 and LFA-1 must be highly regulated in order to prevent inappropriate adhesion and ensure localization to the proper sites in the microvasculature. Both of these integrins have been shown to be inactive on an unstimulated cell, and to attain an active conformation in response to stimuli. In addition to affinity modulation, Mac-1 but not LFA-1 avidity may be increased by the upregulation of new receptors from intracellular pools. However, these newly upregulated receptors have been shown to be active only when a higher concentration of agonist or different stimulus is encountered (37).
1.3.3 Intercellular Adhesion Molecules (ICAMs)

The counter-receptors for $\beta_2$-integrins in the immune system are the Intercellular Adhesion Molecules (ICAMs), members of the immunoglobulin superfamily. This group of adhesion molecules is composed of three homologous proteins: ICAM-1, ICAM-2, and ICAM-3. ICAM-1 and ICAM-3 are the most structurally similar, each containing five Ig-like domains, while ICAM-2 contains just two. Despite their homology, the ICAMs have distinct binding characteristics and cellular distributions, suggesting unique roles for individual ICAMs.

ICAM-1 is expressed at low basal levels on endothelial cells, epithelial cells, fibroblasts, and many leukocyte populations. The expression of ICAM-1 is greatly increased when the tissue is exposed to pro-inflammatory molecules (38,39), or shear stress (40). ICAM-1 expression is transcriptionally regulated, with additional receptors appearing on endothelial cells within hours of cytokine stimulation, and maximum expression after 24-48 hours (41). Both Mac-1 and LFA-1 will bind to ICAM-1 on the inflamed endothelium.

ICAM-2 is constitutively expressed on endothelial cells and its expression is not modulated in response to cytokine stimulation. ICAM-2 is a ligand for LFA-1, and not Mac-1. The third ICAM, the newly characterized ICAM-3, is strongly expressed on all leukocyte populations, and is absent on the resting and inflamed
endothelium. However, ICAM-3 expression on endothelial cells has been detected in certain disease states, particularly in lymphomas (42,43). ICAM-3 has been shown to be a major LFA-1 ligand in the resting immune system (44). In addition to its adhesive function, ICAM-3 may also be a signaling molecule. Cross-linking of ICAM-3 has recently been reported to activate both the $\beta_1$, and $\beta_2$ integrins on T-lymphocytes (44).

1.4 Role of Hydrodynamics in regulating neutrophil adhesion

As discussed above, the recruitment of circulating neutrophils to sites of inflammation is a multistep process. The four basic steps are: initial capture or "tethering", rolling, stable adhesion, and finally migration of the neutrophil through the endothelium to the site of inflammation. The majority of neutrophil extravasation occurs in the postcapillary and high endothelial venules. Both the hydrodynamics and the adhesion receptors expressed on venules may promote adhesion to these sites (45). The size of these vessels (~20 - 60 $\mu$m) is small enough so that neutrophils in the free stream frequently contact the vessel wall, enabling cell capture under appropriate conditions. The presence of erythrocytes in flowing blood also increases the number of intercellular contacts between neutrophils and the vessel wall (46). In the resting immune system these frequent contacts are transient, and the neutrophil soon returns
to the free stream. However, during inflammation the blood vessel dilates and vascular permeability increases, which causes lower shear stress and even more frequent leukocyte-endothelium contact. In an inflammatory setting, the endothelium also releases cytokines and upregulates adhesion molecules as discussed above. These factors enable the initial contact of the neutrophil to evolve into a rolling interaction. Both rolling and capture have been shown to require adhesive interactions between adhesion molecules expressed on the endothelium and the neutrophil (47). Neutrophil rolling also enables further cell activation, which is required for stable adhesion. Finally, the activated and shape changed neutrophil moves between endothelial cells along the chemotactic gradient toward the site of infection.

The fluid dynamics within the vessel play a vital role in regulating the adhesion of neutrophils to the endothelium. At wall shear stresses above ~3 dynes/cm², neutrophils are unable to form rolling interactions on IL-1 stimulated endothelium in vitro (48). A central hypothesis of this thesis is that at these high shears, both the shorter intercellular contact duration and the increased shear stress prevent neutrophil-endothelium interactions. At shear stresses below ~3 dynes/cm² in vitro, the multi-step adhesion cascade is observed, with initial tethering and rolling leading to firm adhesion and transmigration (48). At shear stresses of 2 dynes/cm² the initial adhesion and rolling have been shown to be selectin dependent events, both in
vitro (49,50) and in vivo (51,52). At shear stresses below 0.5 dynes/cm² in vitro (48,53-55), and below ~2 dynes/cm² in vivo (45), there is some evidence that β₂-integrins can mediate both primary adhesion and firm adhesion. The average levels of shear stress in the venous circulation are on the order of 1-10 dynes/cm², but in chronic inflammation or in ischemic tissue, the shear may be much lower (48). In these cases activated neutrophils may localize to sites of inflammation entirely through an integrin-mediated mechanism.

Why are selectin-carbohydrate interactions more effective in mediating the initial interactions of neutrophils with the endothelium at higher shear stresses? The preferential localization of L-selectin to the microvilli of neutrophils may be a significant part of this process. In an elegant study, von Andrian et al. swapped the cytoplasmic domains of L-selectin and CD44, a molecule which is excluded from the microvillus (56). It was determined that when L-selectin was not expressed on the microvillus, much of its ability to mediate the primary adhesion of a lymphoid cell line at high shear was abolished. Also the length of selectins may promote initial adhesion. The vascular selectins E- and P-selectin in particular have relatively long lengths (30 and 40 nm respectively) relative to integrins. The longer lengths of selectins could enable them to avoid stearic hindrance from both the glycocalyx and glycoprotein surface decorations (57). Selectins are also predicted to have very fast bond-formation and dissociation rates (58,59). In a recent report Alon et al. found
that bonds between P-selectin and PSGL-1 had a fast off rate \( \sim 5/\text{sec} \) that was relatively insensitive to shear stress and a high tensile strength (60). In another recent report, adhesion through L-selectin was found to require hydrodynamic shear above a threshold level to mediate rolling both \textit{in vitro} and \textit{in vivo} (61). Thus, it appears that selectins function optimally within a discrete range of shear.

1.5 Thesis overview

In this work, I have focused on the transition from selectin-mediated to integrin-mediated adhesion, both as a function of time and shear rate/stress. A cone-plate viscometer was used to apply known, uniform shear fields to activated neutrophil suspensions. Neutrophil-neutrophil adhesion was quantitated using fluorescence flow cytometry.

In Chapter 2, the viscometric and flow cytometric methods used throughout the study are outlined, along with basic experimental techniques such as neutrophil isolation and monoclonal antibody preparation. In Chapter 3 neutrophil-neutrophil adhesion kinetics as a function of shear rate are presented. The results from Chapter 3 are utilized in the formulation of a mathematical model in Chapter 4. The dependence of L-selectin and \( \beta_2 \)-integrin function on shear rate and shear stress are presented in Chapter 5. In Chapter 6, the roles of Mac-1, LFA-1, and ICAM-3 at low and high shear are discussed.
Chapter 2

Methods

In this chapter, methods which are used throughout this work will be presented. Reagents and experimental techniques which are specific to a single chapter will be presented in their respective chapters.

2.1 Blood collection and neutrophil isolation

Human blood was collected from healthy volunteers by venipuncture into a sterile syringe containing 10 U heparin/ml of blood. Neutrophils were isolated using a one step Ficoll-Hypaque density gradient (Mono-Poly resolving medium, Flow laboratories Inc., McLean VA). In brief, whole blood was layered on resolving medium (+6% sterile deionized water) and centrifuged at 2100 revolutions/minute, 24°C, for 30 minutes. The neutrophil band was then collected, washed once in buffer, and maintained at 4°C in a Ca²⁺-free HEPES buffer (containing 110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM MgCl₂ and 30 mM HEPES, pH = 7.4). With this separation method, neutrophil viability was >95% as assessed by trypan blue exclusion. Neutrophil suspensions (10⁶ cells/ml) were incubated for 2-3 min at 37°C
in buffer containing 1.5 mM Ca$^{2+}$ prior to each experimental run. Samples were then stimulated with 1 μM fMLP and sheared in a cone-plate viscometer maintained at 37°C. Aliquots of 30 μl were taken at 10-30 second intervals for up to 10 minutes after stimulation and fixed in 200 μl of cold 2% glutaraldehyde.

2.2 Cone-Plate viscometry

Neutrophil suspensions were exposed to shear stress in a cone-plate viscometer (Ferranti Electric, Inc., Commack, NY), which consists of a stationary plate beneath a rotating cone (Figure 2.1). Samples are placed on the plate below and rotation of the cone above applies shear stress to the sample. The design of the viscometer enables a uniform shear rate to be applied to the entire sample volume(62). The shear rate $G$ (1/sec) is independent of distance from the cone center and is given by (63):

$$ G = \frac{\omega}{\tan \theta} \quad (2.1) $$

where $\omega$ (radians/sec) is the angular velocity of the cone, and $\theta$ (radians) is the cone angle. A cone 7 centimeters in diameter with an angle of 1° was used, and the gap between the cone and plate ranged from less than 10 μm in the center to 610 μm at the outside edge. The viscometer has a built in controller which keeps the gap
Figure 2.1. *Cone-Plate Viscometer*. Shown above is a schematic of a cone-plate viscometer. a) Rotating cone (cone angle of 1°) b) Plate c) Neutrophil suspension in linear shear field.
between the tip of the cone and the plate stable. To inhibit cell-surface interactions, the cone and plate surfaces were treated with 1% Prosil-28® (PCR Research Chemicals; Gainsville, Fl) one hour before each experiment. This organic silicon coating inhibits interactions between cells and the apparatus. At the angular velocities utilized in this study, the flow field was laminar and the shear stress \( \tau \) (dyne/cm\(^2\)) for a Newtonian fluid may be calculated as \( \tau = \mu G \), where \( \mu \) is the fluid viscosity (poise). The viscosity of the buffer was measured to be \( \sim 0.0075 \) poise at 37°C in a Brookfield cone plate viscometer (Stoughton MA.). An estimate of the maximum theoretical constant shear rate that can be applied with a 1° cone is \( \sim 4200 \) s\(^{-1}\), due to secondary flow (64). The maximum shear rate applied in these studies was 3000 s\(^{-1}\).

2.3 Flow cytometry

A FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to analyze the particle distributions of fixed cell suspensions and the fluorescence intensity of labeled cells (Figure 2.2). A flow cytometer is an instrument in which particles in a fluid stream pass in single file through a beam of illuminating light, usually a laser. The light transmitted consists of scattered light and fluorescent light. The flow cytometer used in these studies enabled measurement of 5 parameters (Forward scatter, Side scatter or 90 degree scatter, and 3 fluorescence wavelengths) on each cell or cell aggregate. The forward scatter detector on the
Figure 2.2 FACScan Flow Cytometer
FACScan flow cytometer is a silicon photo diode, and the other four are photo multiplier tubes. The light scattered at small angles (<10 degrees), termed forward scatter, is relatively independent of cell shape, and is proportional to the cell volume. Measurement of the light scattered at right angles, termed side scatter, provides a measure of the cell granularity or cell shape. The neutrophil population studied in this work was identified by its characteristic forward and side scatter (65).

Flow cytometry enables measurement of the properties of large numbers of individual cells in relatively short times. Hundreds to thousands of particles per second can be studied, depending on the particle concentration in suspension and flow rate of the sheath fluid. A typical data acquisition for these studies quantitated the binding properties of 3000 particles, and the data acquisition time ranged from 10 seconds to 2 minutes depending on the extent of cell aggregation.

2.4 Production of fab antibody fragments

Monoclonal antibodies (mAbs) were used to inhibit the function of adhesion receptors. The mAbs used in these studies were in the IgG class, consisting of two fab, or antigen binding regions, and one Fc region. We found that whole IgG antibody caused cell activation and cell-cell bridging via Fc receptors expressed on the neutrophil surface. To eliminate these phenomena from our adhesion assays, we
used fab and f(ab')₂ mAb fragments. A fab preparation kit from Pierce (Rockford, IL) was used to produce the fab fragments. Briefly, whole antibody was incubated at 37°C overnight with papain which was immobilized in a gel. Papain is a non-specific protease which cleaves one or more peptide bonds in the hinge region, producing two fab molecules and one Fc molecule per whole antibody molecule. The fab and Fc fragments were then separated from the papain gel using a separator tube, and the fragments were passed through a protein-A column which binds the Fc fragments and prevents their passage. To test for the presence of contaminating whole antibody, western gels were run and fabs were identified by their characteristic 50 kd molecular weight. F(ab')₂ fragments are produced via a similar protocol, but all f(ab')₂ mAbs used in these studies were obtained as such.

2.5 Fluorescently labeled mAbs

All of the fluorescently labeled mAbs used in these studies were obtained commercially, with the exception of ICR1.1 (anti-ICAM-3). ICR1.1 was fluorescently labeled using a CY3 labeling kit from Amersham Life Science (Arlington Heights, IL). Briefly, mAb is mixed with a coupling buffer and transferred to a vial containing the reactive dye. The mAb-dye solution is incubated at room temperature for 30 minutes. The labeled protein was then separated from the free dye
by passage through a gel filtration column. The labeled protein passes through faster than the free dye, and is collected at the bottom. CY3 is a water soluble and highly fluorescent dye which fluoresces in the orange (550 nm) wavelength. The dye to protein (D/P) molar ratio was estimated with a spectrophotometer, the ICR1.1 CY3 had a D/P ratio of ~5.
Chapter 3

Shear Dependence of Neutrophil Aggregation Kinetics

3.1 Background and Introduction

As discussed in chapter 1, the reversible adhesion of neutrophils is vitally important for many immunologic functions including intravascular margination (66), diapedesis (67), and phagocytosis (68). In addition to these heterotypic intercellular interactions, homotypic neutrophil adhesion may also have physiologic relevance. Vascular neutrophil aggregation may be important in increasing the number of phagocytes in the vicinity of tissue insult (69). In 1992, Rochon et al. postulated that neutrophils adherent to the vascular endothelium could recruit additional neutrophils from the circulation (70). The interaction of neutrophils in flow with adherent neutrophils was first demonstrated by Bargatze et al. in 1994, wherein they utilized a monolayer of neutrophils adherent on activated endothelium for the recruitment of additional neutrophils (71). Walcheck et al. extended this finding to include the recruitment of neutrophils from the flow stream by free rolling cells (72). This phenomena has also been demonstrated in vivo (61) and reproduced by other investigators (73,74).
Neutrophil aggregation is also important in many pathophysiological settings. Intravascular neutrophil aggregation can lead to the trapping of cells within capillaries and postcapillary venules (75,76). The trapped neutrophil aggregates may then release oxidants and proteolytic enzymes, damaging healthy tissue (77,78). Under conditions of ischemia, as in hypovolemic and endotoxic shock or myocardial infarction, microvascular obstruction can lead to organ dysfunction (76,79). It has been reported that high leukocyte counts are predictive of both stroke (80) and myocardial infarction (81,82). Neutrophil aggregation and embolization have also been implicated as pathogenic mechanisms in disease states associated with connective tissue damage in rheumatoid arthritis (83), and complement activation (84).

The mechanism by which neutrophils become activated in the free stream or while tethered to the endothelial wall is an area of active research. One possible mechanism for rapid and local activation of leukocytes is through ligation of chemokines such as IL8 and PAF which are synthesized by endothelial cells stimulated by IL1 or TNF. Cells which are rolling on the vessel wall proximal to these secreted chemokines are thought to become rapidly activated upon ligation. A variety of cytokines will activate neutrophils to adhere to endothelium including complement (C5a), PAF, or IL8. In addition to these cytokines, chemotactic peptide stimulation of neutrophils has been shown to induce transient neutrophil aggregation
both *in vivo* (84), and *in vitro* (65,85). In these studies, neutrophils were activated with the chemotactic peptide formyl-Met-Leu-Phe (fMLP), a peptide expressed on the surface of bacteria.

Neutrophil aggregation provides a sensitive assay for determining adhesion function and identifying adhesion deficiencies (86,87). Isolated neutrophils exposed to continuous shear mixing in suspension exhibit a rapid homotypic aggregation in response to formyl peptide stimulation which is reversible over several minutes (65,88,89). The extent of cell aggregation appears to depend on the concentration of stimulus, duration of agonist binding, cell density, and the level of degranulation (85,89,90). Cellular responses decay and neutrophils disaggregate as agonist binding approaches equilibrium. Neutrophil aggregation requires shear mixing to provide the collisions necessary to form aggregates. Chemotactic stimulation shifts the efficiency of adhesion such that cell-cell encounters result in aggregate formation. This may be analogous to the arrest of rolling neutrophils on cytokine-stimulated endothelial monolayers under conditions of shear flow.

Three methods have commonly been used to quantify neutrophil aggregation; 1) measurement of changes in light transmittance through a stirred cell suspension, typically termed aggregometry (88) 2) quantitation of cell count and particle size using electronic impedance of aggregates as they pass through an aperture (91) 3)
flow cytometric quantitation of fluorescently labeled cells or fixative induced autofluorescence. The first two techniques have significant limitations in that they are applicable only to purified suspensions of cells. Light transmittance assays only give a measure of the bulk or average responses of the cell suspensions, and both aggregometry and electronic particle counting are sensitive to changes in cell shape and volume (92). Flow cytometric analysis of either whole blood (93) or purified granulocytes (65,90) enables simultaneous measurements of several cellular parameters in real time. This analysis can be targeted to subpopulations of cells and provide statistical detail of the size distribution of aggregates as well as quantitate the binding of fluorescent antibody and peptide to specific cell surface receptors on individual cells.

We have been investigating neutrophil-neutrophil adhesion in an effort to define the molecular and physical requirements of aggregate formation and stable adhesion under defined hydrodynamic shear (12,65,94). In the present study, we investigated homotypic neutrophil adhesion using cone-plate viscometry and flow cytometry. Cone-plate viscometry was chosen because it enables the application of precise and uniform shear rates (62). Previous studies of homotypic neutrophil adhesion utilized mixing systems in which the shear field was too complex to allow anything other than estimates of average shear rates (65). This is the first study where the aggregation kinetics of neutrophils were quantitated as a function of shear rate.
The molecular components of neutrophil-neutrophil adhesion, and analogies to neutrophil-endothelial adhesion will be discussed in chapters 5 and 6.

3.2 Materials and Methods

3.2.1 Calculation of percentage aggregation.

The particle distributions of neutrophil aggregates were determined using histograms of fluorescence intensity as described in Results. The percentage of cells in aggregates was determined by dividing the number of single cells by the total number of neutrophils detected:

\[
\%_{\text{Agg}} = 100 \times \left(1 - \frac{S}{S + 2D + 3T + 4Q + 5P + 6Sx}\right)
\]  \hspace{1cm} (3.1)

where the neutrophil aggregate sizes are given by S=singlets, D=doublets, T=triplets, Q=quartets, P=pentuplets, and Sx=sextuplets and larger unresolved aggregates. Particles larger than sextuplets typically comprised <20% of the total aggregates formed.
3.3 Results

The adhesive properties of neutrophils interacting in shear flow were measured with a combination of cone-plate viscometry and flow cytometry. This strategy enabled the application of precise shear rates to cell suspensions (10^6 cells/mL). Samples were fixed *in situ* and aggregate distributions representative of the entire neutrophil population were quantitated via flow cytometry.

3.3.1 Neutrophil adherence determined by flow cytometry.

Autofluorescence due to glutaraldehyde fixation enabled the detection of neutrophil singlets and aggregates. Samples were removed by gentle pipette aspiration into fixative at defined time points prior to and following application of stimulation and shear. In previous studies it was confirmed that the process of sample removal and fixation did not cause aggregate breakup by demonstrating that aggregate distributions were equivalent between live cells injected over time into the cytometer and those fixed in the test tube (65,90). In the current studies it was confirmed that aggregate distributions were equivalent between samples removed by pipette into fixative, and those fixed directly on the plate during shear at comparable times. Fewer than 2% of neutrophils were detected in aggregates in response to shear stress alone (Figure 3.1). Homotypic neutrophil adherence was observed within seconds of
Figure 3.1. *Flow cytometric detection of unstimulated neutrophils.* Isolated neutrophils were incubated in 37°C buffer for 3 minutes, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. A two parameter dot-plot of side scatter and glutaraldehyde-induced autofluorescence is shown for neutrophils prior to stimulation.
Figure 3.2. *Flow cytometric detection of neutrophil-neutrophil adhesion at low shear*. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 μM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. A two parameter dot-plot of side scatter and glutaraldehyde-induced autofluorescence is shown for fMLP stimulated cells subjected to $G = 100 \text{s}^{-1}$ for 1 minute. Particle sizes ranging from singlets to sextuplets were resolved.
Figure 3.3. Flow cytometric detection of neutrophil-neutrophil adhesion at optimum shear. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 μM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. A two parameter dot-plot of side scatter versus glutaraldehyde-induced autofluorescence is shown for fMLP stimulated cells subjected to $G = 800 \text{ s}^{-1}$ for 1 minute. Particle sizes ranging from singlets to sextuplets were resolved.
stimulation with 1 μM fMLP. After stimulation and exposure to a shear rate of 100 s\(^{-1}\) for 30 seconds, aggregates were chiefly comprised of doublets and triplets (Figure 3.2), while at 800 s\(^{-1}\) neutrophils were recruited into aggregates up to sextuplets and larger (Figure 3.3). Formation of larger aggregates at the higher shear rates was consistent with the dependence of encounter frequency on shear rate as predicted by two body collision theory (see Chapter 4).

### 3.3.2 Neutrophil aggregate distributions

The kinetics of neutrophil aggregation following chemotactic stimulus for cells at a concentration of 1x10^6 cell/ml were determined. The distribution of singlets and aggregates was measured by gating on each particle size (singlets to sextuplets and larger aggregates) and normalizing by the total number of particles detected. At low shear, aggregates formed in response to 1 μM fMLP stimulation during the first 90 to 180 seconds, after which aggregates remained relatively stable (Figure 3.4). Small aggregates are formed initially, leading to larger aggregate formation. The aggregates formed remained stable up to 300 seconds after stimulation and shear. At higher shear rates, three distinct phases characterize the reversible kinetics of neutrophil aggregation (65). At a shear rate of 800 s\(^{-1}\) the initial phase of rapid aggregate formation lasts from 0 to ~30 seconds (Figure 3.5). Single cells were rapidly taken
Figure 3.4. *Kinetics of neutrophil aggregate distributions at low shear*. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 μM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. Kinetics of neutrophil aggregate distributions are shown at a shear rate of 100 s⁻¹. Aggregate distributions were calculated as percentage of total neutrophils, as described for singlets. The distributions of a) singlets and b) doublets through sextuplets⁺ are shown.
Figure 3.5. Kinetics of neutrophil aggregate distributions at optimum shear. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 µM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. Kinetics of neutrophil aggregate distributions are shown at a shear rate of 800 s⁻¹. Aggregate distributions were calculated as percentage of total neutrophils, as described for singlets. The distributions of a) singlets and b) doublets through sextuplets* are shown.
up into aggregates, depleting the total number of singlets in the cell suspension by 
~90%. Doublets were formed in the first 10 seconds, and in the subsequent time 
interval (10-30 seconds), aggregates grew in size leading to the formation of triplets, 
quadruplets and larger aggregates. Thus, there was a sequential rise of aggregates of 
increasing size after stimulus. The rapid decrease in the particle concentration with 
time lead to a decrease in the collision frequency and a consequent reduction in the 
rate of aggregation. In the second phase of the kinetics, which lasted from ~30 to 
~120 seconds, there was very little change in the aggregate size distribution. A rapid 
transition to disaggregation was consistently observed during the final phase which 
started 120-150 seconds after stimulation. The singlet population was almost totally 
recovered by 600 seconds. At high shear rates (>1600 s⁻¹) the shear was too high for 
large aggregates to form (Figure 3.6). The aggregates formed were almost all 
doublets, and disaggregation was complete by 300 seconds after stimulation. The 
three distinct phases of aggregation were consistently observed at high shear, and 
variation among donors caused less than a 5% change in the time of onset of each of 
these phases.
Figure 3.6. Kinetics of neutrophil aggregate distributions at high shear. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 μM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. Kinetics of neutrophil aggregate distributions are shown at a shear rate of 3000 s⁻¹. Aggregate distributions were calculated as percentage of total neutrophils, as described for singlets. The distributions of a) singlets and b) doublets through sextuplets are shown.
3.3.3 Kinetics of neutrophil aggregation over a range of shear rates.

Aggregation experiments were performed over a range of shear rates from 100 s\(^{-1}\) to 3000 s\(^{-1}\). Stimulation induced adhesion within 10 seconds, the earliest time point measured with this technique. The aggregation rate was highest during the first 30 seconds and the extent of aggregation was highest at a shear rate of 800 s\(^{-1}\), where more than 90% of neutrophils were recruited into aggregates. The aggregation rate increased from a minimum at 100 s\(^{-1}\) to a maximum at a shear of 800 s\(^{-1}\) (Figures 3.7 & 3.8). However, at shear rates above 800 s\(^{-1}\) the extent of aggregation decreased with increasing shear (Figure 3.8). At the time point of maximum aggregation, a steady state “plateau phase” in which formed aggregates remained stable was observed over a period of 1-2 minutes at all shear rates. This was confirmed by shearing stimulated cells and then diluting aggregates formed (1:10 in buffer containing 1 \(\mu\)M fMLP). Under these conditions, encounter frequency was decreased by a factor of \(~1000\), thereby limiting intercellular collisions and new aggregate formation. Aggregates displayed the same disaggregation rate in both the control and diluted samples (figure 3.9).
Figure 3.7. Kinetics of neutrophil adhesion at low shear. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 μM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. Kinetics of homotypic neutrophil adhesion at shear rates from 100 s⁻¹ to 400 s⁻¹. The percentage of neutrophils recruited into aggregates, mean ± SEM, n ≥10 is plotted.
Figure 3.8. Kinetics of neutrophil adhesion at high shear. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 μM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. Kinetics of homotypic neutrophil adhesion at shear rates from 800 s⁻¹ to 3000 s⁻¹. The percentage of neutrophils recruited into aggregates, mean ± SEM, n ≥5 is plotted.
Figure 3.9. *Dilution studies of disaggregation.* Neutrophils at a concentration of 1x10^6 cells/ml were stimulated and sheared at G = 1600 s\(^{-1}\). The suspension was diluted 10 times in excess buffer containing 1\(\mu\)M FMLP and 1.5\(\mu\)M Ca\(^{2+}\) at 30 seconds, 60 seconds, or 90 seconds. An undiluted control at 1600 s\(^{-1}\) is also displayed. A representative experiment is displayed.
3.4 Discussion

In the current study, cone-plate viscometry was employed in combination with flow cytometry to quantitate the kinetics of adhesion between neutrophils in response to chemotactic stimulation. Cone-plate viscometry enabled the application of precise shear rates and stresses to neutrophil suspensions. Discrete aggregate size distributions were quantitated using a fluorescence flow cytometer. The combination of cone-plate viscometry and flow cytometry has been applied successfully to the study of platelet aggregation (62,95), but this is the first study to utilize this technique for the study of neutrophil-neutrophil adhesion.

Neutrophils are rapidly recruited into aggregates when stimulated with 1 μM fMLP. Both the number of cells recruited into aggregates and the aggregate size distributions were shear-dependent. At low shear rates (100 - 200 s⁻¹) small aggregates are formed during the first 30 seconds, leading to larger aggregate formation by ~90 seconds after stimulation. The aggregates formed are then relatively stable up to 300 seconds after stimulation and shear. At moderate shear (400 - 800 s⁻¹) large aggregates are formed quickly, with up to 30% in aggregates of size pentuplet or larger after 30 seconds of shear and stimulation. At shear rates above ~200 s⁻¹, aggregates are stable until ~120 seconds after stimulation, after which shear stress caused disaggregation. The stability of aggregates in the plateau region was studied
by dilution experiments (Figure 3.9), and it was found that aggregates remained stable
during this time, rather than aggregating and dissaggregating cells being in a dynamic
equilibrium. At high shear rates (>1600 s\(^{-1}\)) large aggregates were unable to form
(Figure 3.6).

In this chapter, a viscometric-flow cytometric methodology for the study of
neutrophil adhesion under defined shear was presented. It was shown that neutrophil
adhesion is modulated by fluid dynamics. Neutrophil aggregation was demonstrated
to be a three phase process at shear rates greater than \(~200\) s\(^{-1}\). Analogies to
neutrophil-endothelial adhesion and distinct shear regimes for specific adhesion
receptors will be presented in further chapters.
Chapter 4

Modeling Neutrophil Aggregation Kinetics

4.1 Background and Introduction

Viscometry was first applied to the study of cell adhesion in suspension by Curtis in 1969 (96). Curtis recognized that the number of intercellular collisions is proportional to shear rate, making viscometry an ideal system for the quantification of parameters important for adhesion. In 1978, Evans et al. utilized the methodology developed by Curtis to analyze lymphoid cell aggregation (97). Evans measured the maximum aggregate diameter as a function of shear rate, and estimated the critical force required to dissociate an aggregate. A mathematical model was developed to study the evolution of lymphoid cell aggregates using a theory by von Smoluchowski for the interaction of uniform spheres in a linear shear field (98). To fit their data, Evans et al. modeled the lymphoid cells as distinct subpopulations with different adhesive characteristics. They found that the probability of an intercellular collision leading to lymphoid cell aggregation was on the order of .003, and that this probability decreased with shear rate.

Bell first applied the viscometric methodology to the study of cell adhesion
known to be mediated by specific molecular interactions (99). Bell also utilized von
Smoluchowski’s theory for the estimation of the intercellular collision frequency of
spherical cells. By averaging over all potential collision geometries in a linear shear
field, Bell estimated the collision duration and collision contact area as a function of
shear rate. Together with results from previous work (100), he also estimated the
number of molecular bonds required to hold two cells together, and the sticking
probability. Although no experimental data was shown, the sticking probability was
predicted to decrease with increasing shear rate due to decreased intercellular contact
duration and increased force on intermolecular bonds.

Another mathematical approach to analyze cell aggregation in a linear shear
field was performed by Belval and Hellums (101). Using von Smoluchowski’s
laminar shear coalescence kernel in a population balance approach, they modeled the
aggregation of platelets in the linear shear field of a cone and plate viscometer. A
more complex analysis was necessitated by platelet morphology and adhesion
characteristics. In particular, platelets are not spherical in shape or homogenous in
size, and platelet aggregates are irregularly shaped and contain significant void space
(101). Belval used a modified version of von Smoluchowski’s collision kernel to
account for particle void fraction. Fitting this model to experimental measurements,
he found that the maximum collision efficiency of shear induced platelet aggregation
was $\sim 3 \times 10^{-3}$. When platelets were stimulated exogenously with ADP and subjected
to lower shear rates, the efficiency rose to ~0.4. Belval's work was extended by Huang and Hellums to include disaggregation in the population balance equations (101).

Neutrophil aggregation was first modeled by Simon et al. in 1990 (65). Neutrophil aggregation was studied in a non-uniform shear field within a test tube mixed by a stir bar. A simple, linear model based on particle geometry was formulated to fit the kinetics of aggregation and disaggregation. Neutrophil aggregation was modeled as a three phase process, with distinct rate constants for each phase. The model was successful in fitting the data despite the complex hydrodynamics of the mixing system, but the dependence of neutrophil aggregation on shear rate was not determined.

Both the adhesive interactions between the neutrophil and the endothelium, and the homotypic aggregation of neutrophils are influenced by fluid mechanical forces. An increase in shear rate leads to a reduction in the interaction time during cell collision (13,48,99), which may in turn reduce the adhesion efficiency. A second consequence of an increase in shear rate is a concomitant increase in the shear and normal stresses acting between colliding cells. The resultant forces can increase both the extent of cell deformation and the tensile forces transmitted to the adhesive bonds between cells. The interplay of hydrodynamic factors and the time dependent changes
in receptor adhesivity following cell stimulation appear to determine the pattern of
neutrophil adhesion over the range of shear rates found in the circulation (60,102).

In previous reports a technique was presented to measures the kinetics of
aggregation of chemotactically stimulated neutrophils subjected to shear in a
magnetically stirred suspension within a test tube (12,65,94). The process was
mathematically modeled in three discrete phases which consisted of: a) an
aggregation phase where single neutrophils were recruited into aggregates with little
disaggregation, b) a plateau phase where the aggregate size distribution did not
change with time, and c) a disaggregation phase in which formed aggregates
spontaneously and rapidly broke up (65). This analysis did not account for the time
dependent changes in adhesion efficiency and disaggregation kinetics, and it was
limited to predicting the evolution of homotypic aggregates of up to five cells.
Moreover, as mentioned above, the previous model was applied to only a single
estimated shear rate (>30 s⁻¹) and a limited range of cell concentrations.

In the current study, cone-plate viscometry was used to apply a uniform shear
rate to neutrophil suspensions stimulated with formylated chemotactic agonist
(fMLP). At a given shear rate two-body collision theory enables the estimation of cell
collision frequency and the period of apparent contact between particles during
collision, as well as the hydrodynamic and other interaction forces between the
particles (98). Aggregate size distribution and geometry following stimulation were determined by flow cytometry as demonstrated in Chapter 3. These measurements were used as criteria in formulating a mathematical model for neutrophil aggregation. The model accounted for the time and shear dependent changes in aggregation kinetics. Disaggregation kinetics were studied in a subsequent manuscript by Neelamegham et al. (102). We succeeded in predicting the evolution of cell aggregates over a wide range of shear rates (100 s\(^{-1}\) - 3000 s\(^{-1}\)).

Previous studies of homotypic neutrophil adhesion utilized mixing systems in which the shear field was too complex to allow anything other than estimates of average shear rates (65). Accurate control of the shear rate enabled us to formulate a model of cell adhesion based on Smoluchowski's theory of particle flocculation in a linear shear field (102). This analysis was used to predict the collision frequency, adhesion efficiency, and average intercellular contact duration over a wide range of shear rates.
4.2 Mathematical Model For Neutrophil Aggregation

Neutrophils are cells which remain intrinsically unactivated and nonadherent until appropriately stimulated. As demonstrated in Chapter 3, less than 2% of the unstimulated neutrophils mixed in a cone-plate viscometer are incorporated into aggregates. However, upon stimulation with fMLP (formyl-met-leu-phe) and application of shear, neutrophil aggregation is a rapid and reversible process (12). Aggregates form most rapidly during the first 30 seconds after stimulation. A plateau phase of stable aggregates lasting ~2 minutes follows, and if the shear rate is above a critical level, the aggregates dissociate. We mathematically modeled the process in order to quantitate the absolute rate and extent of neutrophil aggregation and disaggregation over a range of shear rates and aggregate sizes. The efficiency of neutrophil-neutrophil adhesion was estimated from the aggregation kinetics over the first 30 seconds following chemotactic stimulation. In subsequent studies (102) it was determined that adhesion efficiency decreased with time following fMLP stimulation. However, this decrease is gradual over the initial 30 seconds (< 20% of the initial value) over which the data was modeled. The time interval over which adhesion efficiency was computed comprised of at least two sample points for all aggregate species measured. The model is based on a theory which describes the interaction of spherical particles mixed in a linear shear field as formulated by von Smoluchowski (65,98).
The concentration, $C_i$, of aggregates of size $i$ at any time, $t$, can be found by solving the following differential equation:

$$\frac{dC_i}{dt} = \frac{1}{2} \sum_{j=1}^{i-1} k_{i-j} C_i^j C_j^i - \sum_{j=1}^{N-i} k_{i-j} C_i^j C_j^i - \frac{1}{2} \sum_{j=1}^{i-1} b_{i-j} C_i^j + \sum_{j=i+1}^{N} b_{i-j} C_j^i \quad i=1,2,3...N$$

$$j=2,3,...,N \quad (4.1)$$

$k_{ij}$ ($\text{cells}^{-1}\text{s}^{-1}\text{cm}^3$) is the aggregation rate coefficient that describes the adhesion kinetics when two particles with $i$ and $j$ cells adhere. $b_{ij}$ ($\text{s}^{-1}$) is the disaggregation rate coefficient for the dissociation of an aggregate of size $j$ into particles of size $i$ and ($i-j$). $N$ is the maximum aggregate size used in the simulation and determines the number of simultaneous differential equations solved at each time point. Experimental observations revealed that at the highest cell concentration employed and under optimum shear conditions as many as $\sim 40\%$ of the aggregates contained six or more cells ($6x^+\text{s}$). However under most conditions, less than 20\% of the aggregates were found to be sextuplets or larger. For continuity, aggregates up to size 15 ($N=15$) were modeled, and the aggregation kinetics of singlets through sextuplets were fit to the experimental data. The first and second term on the right hand side of Eq. 4.1 quantify the aggregation process. The first term accounts for the rate of formation of large aggregates from two smaller aggregates (e.g. a triplet is formed from the adhesion of a singlet and a doublet). The second term accounts for particle depletion.
during aggregate formation (e.g. singlet and doublet populations are depleted during triplet formation). The last two terms describe the rate of disaggregation of large aggregates. The third term quantifies the rate at which aggregates break up (e.g. the rate at which a triplet breaks up into a singlet and a doublet). The fourth term accounts for the rate of formation of particles as a result of disaggregation (e.g. a singlet and a doublet are formed when a triplet disaggregates). The factor of $\frac{1}{2}$ in the first and third term are placed in order to avoid counting the contribution of these terms twice. The overall mass balance of the system was conserved and checked at each iteration. While modeling neutrophil aggregation following fMLP stimulation, we start with a population of single cells at an initial cell concentration $C_o$. Therefore, the initial conditions for solving Eq. 4.1 are:

$$C_i = \begin{cases} C_0 & \text{for } i=1 \\ 0 & \text{for } i>1 \end{cases}$$  \hspace{1cm} (4.2)

The model can also be applied for other initial conditions, for example, if the aggregate size distribution at any particular time is known and we only wish to model the aggregation kinetics beyond this point, then the initial conditions in Eq. 4.2 can
be appropriately modified. The model is based on two key assumptions: a) single cells and aggregates were assumed to have a spherical geometry, and b) only single collisions between two particles were considered at any time step in the iteration. The system of differential equations represented by Eq. 4.1 was solved simultaneously by the Runge-Kutta Fehlberg 4,5 algorithm (103) with a variable time step on a Pentium computer.

4.2.1 Rate of neutrophil aggregation

Neutrophil aggregation was modeled as a two-step process: 1) Cells exposed to the linear shear field of a cone and plate viscometer collide. The uniform gradient in the velocity streamlines of a linear shear field cause the cells closer to the rotating cone surface to move faster than the cells near the stationary plate, resulting in cell-cell collisions. The intercellular collision frequency per unit volume, \( f_{ij} \), was computed based on the physical parameters of the system: aggregate radius \( r_i \) and \( r_j \) (cm), shear rate \( G \) (s\(^{-1}\)), and concentration \( C_i \) and \( C_j \) (cells/ml) of aggregates of size \( i \) and \( j \) respectively (98):

\[
f_{ij} = \frac{2}{3} (r_i + r_j)^3 C_i C_j G
\]  

(4.3)
2) Collisions between the cells result in firm adhesion with a probability which may be expressed as the adhesion efficiency \( E \) given by:

\[
E = \frac{k_v C_v C_\ell}{f_v} \tag{4.4}
\]

The adhesion efficiency \( E \) was defined as the fraction of intercellular collisions which resulted in firm adhesion, and was assumed to be independent of aggregate size. While \( f_v \) accounts for the physical parameters of the system, efficiency is solely a function of the intrinsic properties of the cell which determine its adhesivity. These may include, but are not limited to, the number, affinity and distribution of adhesive receptors expressed on the cell surface, their response to applied shear and the time after stimulation. In this work, the adhesion efficiency is estimated from the first 30 seconds of aggregation, where disaggregation of stable aggregates is negligible and the neutrophils are still approximately spherical.
4.3 Results

The reversible kinetics of neutrophil aggregation following chemotactic stimulus were examined in cell suspensions sheared in a cone-plate viscometer. The distribution of aggregate sizes was measured using fluorescence flow cytometry as described in Chapter 3. Experimentally measured parameters were used in conjunction with a mathematical model to simulate the aggregation kinetics of neutrophils over a range of shear rates.

4.3.1 Modeling the formation of neutrophil aggregates

The simulation at 1x10^6 cells/ml and shear rate of 100 s\(^{-1}\) yielded <10% difference between the experimentally measured aggregate size distribution and the model predictions based on two-body collision theory (Figure 4.1 a,b). The adhesion efficiency at 100 s\(^{-1}\) was ~ 0.2, and there was no disaggregation during the 30 seconds of aggregate formation. Transient aggregates formed during the first 30 seconds break up in proportion to their size and the shear rate applied (102). Utilizing the breakage constant for transient aggregates at a shear rate of 800 s\(^{-1}\), an adhesion efficiency of 0.75 fit the aggregate distribution data within 10% (Figure 4.2 a,b). A wider comparison of the model’s predictions with the experimental data was performed over >10 fold range of shear rates (100 s\(^{-1}\) to 3000 s\(^{-1}\)), and the aggregate size distribution predicted by the model differed from the experimental data by less than 10%.
Figure 4.1. A model of the early kinetics of aggregation at low shear. Neutrophils were stimulated by 1μM FMLP and sheared at 100 s⁻¹. The aggregate distributions of singlets through sextuplets⁺ measured experimentally (denoted by symbols) are compared with simulation results (denoted by lines) from the mathematical model.
Figure 4.2. *A model of the early kinetics of aggregation at high shear.* Neutrophils were stimulated by 1μM FMLP and sheared at 800 s⁻¹. The aggregate distributions of singlets through sextuplets measured experimentally (denoted by symbols) are compared with simulation results (denoted by lines) from the mathematical model.
4.3.2 The efficiency of neutrophil adhesion as a function of shear rate

In chapter 3, the aggregation kinetics at various shear rates were demonstrated. It was evident that the rate of aggregation increased with shear rate up to an optimum level, before decreasing at shear rates >800 s\(^{-1}\). A useful measure of the rate at which neutrophils are recruited into aggregates is the aggregation efficiency (see Equation 4.4). The efficiency was defined as the probability that a cell-cell collision results in formation of a stable aggregate and may be used to quantitate the average adhesivity of cells over time. We computed the aggregation efficiency over a range of shear rates from 100 s\(^{-1}\) to 3000 s\(^{-1}\). Efficiency was calculated from the aggregation kinetics over the initial 30 seconds since the molecular recognition events that lead to adhesion occur on this time scale. At shear rates of 100-200 s\(^{-1}\), 20-30% of collisions resulted in aggregate formation (Figure 4.3). Adhesion efficiency more than doubled to ~80% at shear rates from 400-800 s\(^{-1}\), and decreased sharply to ~45% at 1600 s\(^{-1}\), and ~25% at 3000 s\(^{-1}\). The increase in neutrophil adhesion efficiency with shear rate is quite remarkable since neutrophils, unlike platelets, are not activated by shear. The dependence of neutrophil adhesion efficiency on specific adhesion receptors will be demonstrated and discussed in chapter 5.
Figure 4.3. Adhesion efficiency as a function of shear rate. Isolated neutrophils were incubated in 37°C buffer for 3 minutes, stimulated with 1 μM fMLP, and exposed to shear in a cone plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. Adhesion efficiency was determined at each shear rate from the aggregation kinetics over the first 30 s of fMLP stimulation. The adhesion efficiency was determined from the aggregate distributions of each experiment, and is plotted as mean ± SD for n ≥5 experiments.
4.4 Discussion

In this chapter a mathematical model was formulated to predict the formation of homotypic neutrophil aggregates over the initial 30 seconds following chemotactic stimulation in a linear shear field. Precise shearing of cell suspensions was achieved in a cone-plate viscometer over a range of shear rates from 100 s\(^{-1}\) to 3000 s\(^{-1}\). The efficiency of aggregate formation was estimated based on an appropriate choice of model parameters and the experimental characterization of the dependence on time and shear rate.

4.4.1 A balance between molecular bonding and hydrodynamic forces determines the kinetics of aggregation.

In this analysis, the entire population of neutrophils is assumed to be composed of equally sized rigid spheres. Collision between any two spheres in a linear shear field will result in a transient doublet, the geometry of which is assumed to be a prolate ellipsoid (rod-like aggregate) (104). When sheared in a cone-plate viscometer, the doublet tumbles with a period that is independent of its size, but is dependent on the aggregate geometry and the applied shear rate. The mean lifetime of the transient doublet has been estimated to be \(5\pi/6G\) (105). This ranges from \(\sim 1\) to 25 milliseconds for the shear rates applied in our experiments (\(G = 3000-100\) s\(^{-1}\)). Over
one complete rotational orbit, neutrophil doublets experience hydrodynamic normal and shear forces. These forces vary sinusoidally with the angle of orientation of the axis of revolution of the particle with respect to the direction of flow throughout each half orbit (63). Essentially, the rotating doublet experiences compressive forces during half the cycle of rotation and we presume that bond formation occurs during this membrane contact. In addition, twice in each orbit the neutrophil experiences maximal tensile forces which may act to rupture the bonds in the contact region between individual cells. Aggregates detected following fixation and flow cytometric analysis were only those which formed a sufficient number of bonds to overcome the tensile forces over successive cycles of rotation.

Adhesion efficiency was found to vary as a function of the shear rate applied in the cone plate viscometer. At both the highest (3000 s⁻¹, Figure 3.8) and lowest (100 s⁻¹, Figure 3.7) shear rates applied, ~50% of singlets were recruited into aggregates over the first 90 seconds of stimulation. Over a range of shear rates between 400 s⁻¹ and 1600 s⁻¹, greater than 80% of neutrophils were adherent. However, peak efficiency was observed only over a relatively narrow range of shear rates from 400 s⁻¹ to 800 s⁻¹. The molecular dependence of adhesion efficiency, and its modulation by shear will be discussed in subsequent chapters.
In this chapter a methodology is presented to analyze the aggregation kinetics of neutrophils. Homotypic neutrophil aggregation is a complex process in which receptor signaling leads within minutes to the adhesion and subsequent disaggregation of millions of cells. The combination of cone-plate viscometry, flow cytometry and mathematical analysis provides a means to delineate between the roles of hydrodynamic shear and molecular properties in modulating cell adhesion. The roles of specific adhesion receptors will be discussed in the following chapters.
Chapter 5

Effect of Hydrodynamics on L-selectin and β2-integrin Dependent Neutrophil-Neutrophil Adhesion.

5.1 Introduction

The functional significance of many leukocyte adhesive processes is best understood in the context of a cascade of events wherein an initial adhesive mechanism facilitates a subsequent one. An example of this phenomenon occurs with neutrophils forming stable adhesion under physiologic shear rates found in postcapillary venules (150 s⁻¹-1600 s⁻¹) (49,52,106). Members of the selectin family of adhesion molecules (CD62) exhibit sufficiently rapid binding kinetics to allow unactivated neutrophils in the flow stream to interact with endothelial cells and begin rolling at markedly reduced velocities (19,52,54,107). In contrast, members of the β₂ (CD18) integrin family expressed on the neutrophil surface cannot mediate tethering and rolling at these shear rates (49,55). Current data support at least two different explanations. The first is that CD18 integrins are in a low avidity conformation on unstimulated neutrophils (108,109). The second is that even in the presence of stimulation, integrin binding kinetics may preclude the formation of adhesive bonds at high shear rates and corresponding brief intercellular contact durations. Since the
adhesive bonds formed by selectins are insufficient to mediate sustained adhesion
(49,71), neither mechanism alone allows for localization of neutrophils to sites of
inflammation at physiologic shear rates. In the current paradigm, selectin-mediated
rolling reduces the velocity of the cell and may facilitate chemokine activation of β₂-
integrins (CD11a,b), that then bind to ICAM-1 and ICAM-2 on the endothelial
surface (110-113).

L-selectin (CD62L) has recently been shown to mediate neutrophil tethering
and rolling only above a threshold level of shear (≈90 s⁻¹) both in vitro on a CD34
substrate and in vivo (61). On the other hand, CD18 was found to mediate neutrophil
capture on endothelial monolayers at shear rates less than 70 s⁻¹ in vitro (48,49,55),
and below 200 s⁻¹ in mesenteric post capillary venules (114). These data support a
mechanism by which β₂-integrin binds to its counter-structure on the endothelium
below the threshold shear that promotes binding through L-selectin, provided the
duration of cell contact is sufficient. The mechanisms which underlie the cooperative
process of adhesion through both L-selectin and β₂-integrin over a range of
physiologic shears remain to be determined.

We have been investigating adhesion between neutrophils (homotypic) in an
effort to define the molecular and physical requirements of stable adhesion under
defined hydrodynamic shear (12,65,94). Past investigations examined homotypic
neutrophil adhesion following chemotactic stimulation in suspension mixed by a magnetic stir bar (12). These studies demonstrated that both L-selectin and Mac-1 were required, and it appeared that each of these adhesion molecules bound to distinct ligands on interacting cells (94). Much like the case of neutrophil/endothelial adhesion, in homotypic adhesion neither L-selectin nor β2-integrin alone is sufficient to allow stable adherence at physiologic shear (94). While we have found that cross-linking of L-selectin does enhance the adhesive function of Mac-1 (15), a potentially more important role for L-selectin in this experimental model is mediation of the primary adhesion in a two step cascade necessary at physiologic shear rates and stresses.

In the present study, we investigated homotypic neutrophil adhesion using cone-plate viscometry and flow cytometry. We chose cone-plate viscometry since it enables the application of precise and uniform shear rates (62). Previous studies of homotypic neutrophil adhesion utilized mixing systems in which the shear field was too complex to allow anything other than estimates of average shear rates (65). Accurate control of the shear rate enabled us to formulate a model of cell adhesion based on Smoluchowski’s theory of particle flocculation in a linear shear field (102). This analysis was used to predict the collision frequency, adhesion efficiency, and average intercellular contact duration over a wide range of shear rates. Neutrophil adhesion was found to be independent of L-selectin at relatively low shear rates (≤100
s⁻¹) and stresses (≤1 dyn/cm²). Remarkably, in cells expressing both selectin and integrin the adhesion efficiency increased up to a threshold level of shear rate and shear stress. This phenomena was not observed when L-selectin was blocked with mAb, or cleaved with chymotrypsin. It appears that L-selectin is critical for cells to achieve optimum adhesion efficiency at relatively high levels of shear stress and short intercellular encounter durations.

5.2 Materials and Methods

5.2.1 Reagents

Anti-L-selectin mAb DREG-200 (IgG1) was a gift of Dr. T. K. Kishimoto, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT. Anti-L-selectin mAbs Lam1-3 (IgG1) and Lam1-14 (IgG1) were obtained from Cell Genesis, Foster City, CA. Anti-CD18 mAb IB4 (IgG2a) was a gift of Dr. J.D. Chambers, Salk Institute, La Jolla, CA. Commercial mAb to L-selectin (Leu 8-FITC) was purchased from Becton Dickinson Immunocytometry, San Jose, CA. Anti-CD18 mAb MHM 23-PE was purchased from DakoPatts, Denmark, and anti-FcγRII mAb (IV.3 Fab) was purchased from Mederex, Inc., Annandale, NJ. Fab fragments were produced by digestion with papain and purified by passage over a protein A-Sepharose column using
ImmunoPure Fab preparation kits from Pierce, Rockford, IL, as described in Chapter 2. The proteolytic enzyme O-sialoglycoprotein endopeptidase (OSGE), which specifically cleaves proteins that are O-glycosylated on serine and threonine residues, was purchased from Accurate Chemical & Scientific Corporation, Westbury, NY. Formyl-methionyl-leucyl-phenylalanine (fMLP), chymotrypsin, and ficoll were purchased from Sigma Chemical Co., St. Louis, MO.

Buffer viscosity was increased by adding ficoll (m.w. 400,000., Sigma, St Louis MO.), a neutral hydrophilic polymer of sucrose which is commonly used in density gradients for cell separation. Ficoll dissolved easily in buffer and did not significantly increase the osmolarity, nor did it appear to penetrate the cell membrane as observed by flow cytometry and light microscopy. We suspended neutrophils in 6% (w/v) ficoll solutions and found no affect on L-selectin or β₂-integrin expression, or their modulation in response to fMLP stimulation. The viscosity of the ficoll enriched solutions was measured to be 1.7 cp at 37°C.

5.2.2 Inhibition of adhesion with mAb or protease pretreatment.

Monoclonal antibodies were preincubated for 15 minutes at room temperature. Whole antibody to L-selectin was found to cause sustained adherence of stimulated neutrophils, so Fab and F(ab’)_2 fragments were used for inhibition studies. Anti-L-
selectin DREG-200 Fab (40 μg/ml), anti-L-selectin Lam1-3 (30 μg/ml) and anti-CD18 IB4 (30 μg/ml) were used at concentrations determined to inhibit neutrophil aggregation. The mAb IV.3 Fab (20 μg/ml) to the FcγRII receptor on the neutrophil was used as a positive control. The proteolytic enzyme chymotrypsin was used to cleave L-selectin from the neutrophil surface. Chymotrypsin was preincubated (1 U/10^6 cells) at room temperature for 20 minutes in buffer without Ca^{2+}, washed and resuspended in buffer containing 1.5 mM Ca^{2+}. Flow cytometry was used to determine that L-selectin was shed to background levels using this procedure. The proteolytic enzyme OSGE was used to cleave O-linked glycosylated peptides from the neutrophil surface. OSGE was preincubated (30 μl reconstituted OSGE to 2.5x10^6 cells in 250 μl buffer) for 30 minutes at 37°C in Ca^{2+} free buffer. Cells were then washed and resuspended in buffer containing 1.5 mM Ca^{2+}. Protease-treated cells were equilibrated for 2 minutes at 37°C prior to each experiment.

5.2.3 Quantitation of receptor expression.

To measure receptor expression, sheared neutrophils were fixed in 0.25% paraformaldehyde and labeled with fluorescent mAbs. Samples were labeled for 25 minutes at 4°C with either Leu-8 FITC to L-selectin (10 μg/ml) or MHM-23 PE to CD18 (10 μg/ml). Excess label was removed by centrifugation and cells were
resuspended in 4°C buffer for analysis on the flow cytometer. Receptor expression
was quantitated on the green fluorescence channel (FL1) for L-selectin (Leu-8 FITC)
and on the red fluorescence channel (FL2) for β₂-integrin (MHM-23 PE). Analysis
of cellular events was performed using FACScan analysis software, Becton
Dickinson.

5.3 Results

5.3.1 Blocking of L-selectin and β₂-integrin with mAbs over the time-course of
aggregation.

In Chapters 3 and 4, it was demonstrated that there are three distinct phases of
aggregation following neutrophil activation; a rapid phase of singlet recruitment, a
plateau phase of aggregate stability, and a disaggregation phase (65,90). To
determine the relative requirements for L-selectin and β₂-integrin in the early phases
of singlet recruitment and aggregate stability, blocking mAbs were rapidly added at
10 and 30 seconds after stimulation. Based on the binding characteristics of anti-
CD62L (DREG-200 Fab) and anti-CD18 (IB4) (Kₐ ~16 nM and ~7.5 nM
respectively), we estimated that ~90% of the available receptors were bound with
blocking mAb within 30 s of addition of a saturating concentration of mAb (12). At
a shear rate of 1200 s⁻¹, addition of anti-CD18 10 seconds after stimulation prevented further aggregation and caused premature disaggregation of formed aggregates as observed previously in stir bar aggregometry (12). In contrast, addition of anti-CD62L after 10 seconds blocked further aggregation, but did not cause premature disaggregation (Figure 5.1). When anti-CD18 was added 30 seconds after stimulation, it again caused premature disaggregation, while addition of anti-CD62L at this time did not decrease the extent of aggregation. This lead us to hypothesize that L-selectin tethering lead to β₂-integrin mediated stable adhesion in the first seconds of aggregate formation, and β₂-integrin alone appeared to be required for sustaining formed aggregates.

5.3.2 Dependence of neutrophil adhesion on L-selectin and β₂-integrin at different shear rates.

It has been demonstrated that preincubation with mAbs to L-selectin or β₂-integrin will block homotypic neutrophil adherence (12,94). These studies of aggregation were performed in a test tube vigorously mixed with a small stir bar. While this technology served to determine the molecular requirements of homotypic aggregation at an undefined shear rate, it did not provide a quantitative measurement of avidity in a defined shear field. Although the average shear rate estimated for stir
Figure 5.1. Addition of anti-CD62L or anti-CD18 mAbs after fMLP stimulation. Neutrophils (10^6 cells/ml) were stimulated with 1 μM fMLP and exposed to G = 1200 s^-1. After 10 seconds, suprasaturating concentrations of DREG-200 Fab (50 μg/ml) to CD62L or IB4 (50 μg/ml) to CD18 were added. The kinetics of aggregate formation are plotted for control, anti-CD62L, and anti-CD18. Samples were taken at indicated time points and fixed with 2% glutaraldehyde. Shown is a plot representative of four separate experiments.
bar aggregometry was low (~30 s⁻¹) (65), the kinetics and extent of neutrophil aggregation and disaggregation were comparable to those observed at a shear rate of 3000 s⁻¹ in the cone-plate viscometer (Chapter 4). This large discrepancy between shear rate and aggregation behavior suggests that the hydrodynamics in the stir bar system are unpredictable, and uncharacteristic of laminar flow conditions. This prompted us to systematically examine the dependence of neutrophil adhesion on L-selectin and β₂-integrin at shear rates from 100 s⁻¹ to 3000 s⁻¹ in the cone-plate viscometer.

Neutrophils were preincubated with saturating concentrations of either anti-CD18 (IB4), or anti-CD62L (DREG-200 Fab, LAM1-3 Fab, or LAM1-14 Fab), then stimulated with 1 μM fMLP and exposed to a prescribed shear rate (Figures 5.2 a,b). At all shear rates examined, anti-CD18 inhibited adhesion completely (Figure 5.2). In contrast, blocking L-selectin did not change the rate of aggregation from the control level at shear rates ≤100 s⁻¹. Three well established blocking antibodies of L-selectin adhesion, DREG-200 Fab, DREG-56 Fab (data not shown), and LAM1-3 Fab (94, 115) preincubated alone or in combination did not inhibit aggregation. At a higher shear rate of 400 s⁻¹, almost complete inhibition was observed with mAb to L-selectin. As shown for L-selectin binding its ligand on the endothelium (115), the lectin like domain appears to be the binding site for L-selectin binding its ligand on an apposing neutrophil. Preincubation with mAbs which bind to the lectin like
Figure 5.2. *Inhibition of neutrophil adhesion by preincubation with anti-CD62L or anti-CD18 mAbs.* Neutrophil suspensions (10^6 cells/ml) were pretreated with DREG-200 Fab (30 μg/ml), LAM1-3 Fab (30 μg/ml), or LAM1-14 Fab (30 μg/ml) to CD62L, or IB4 to CD18 for 15 minutes at 25°C. Cells were then stimulated with 1 μM fMLP and exposed to prescribed shear rates in a cone-plate viscometer. Adhesion kinetics are plotted for untreated, and anti-CD62L treated neutrophils at shear rates of: a) G = 100 s^{-1} and b) G = 400 s^{-1}. The percentage of neutrophils recruited into aggregates is plotted as mean ± SD for n ≥3 experiments.
domain of L-selectin, (DREG-200 or LAM1-3), blocked 70% and 95% of adhesion respectively (Figure 5.2). Inhibition was dependent on specifically blocking the lectin-like domain since a high affinity mAb which maps to the short consensus repeats of L-selectin (LAM1-14), did not inhibit adhesion (Figure 5.2). A control mAb IV.3 Fab (20 µg/ml) which binds the FcγRII receptor on the neutrophil did not affect aggregation at any shear rate.

To confirm the results obtained with mAb blocking of L-selectin, neutrophils were treated with chymotrypsin to remove L-selectin, or OSGE to remove the O-linked carbohydrate ligand of L-selectin (27). Cells were treated with chymotrypsin at a concentration of 1 U/10^6 cells at room temperature for 15 minutes. Under these conditions, L-selectin was cleaved to undetectable levels, while CD18 function was retained as previously shown (94,116). The aggregation kinetics were assessed for protease treated samples at shear rates of 100 s⁻¹ and 400 s⁻¹ (Figure 5.3). At low shear rates there was no significant difference in the rate or extent of aggregate formation between control and enzyme treatment. At a shear rate of 400 s⁻¹ removing L-selectin with chymotrypsin or cleaving O-linked peptides with OSGE resulted in almost complete inhibition of aggregation. Inhibition due to protease pretreatment was equivalent to that observed with blocking mAbs to L-selectin. These results confirmed that β₂-integrin alone could support aggregate formation at low shear rates (<400 s⁻¹).
Figure 5.3. Inhibition of neutrophil adhesion by preincubation chymotrypsin. Neutrophil suspensions were incubated with chymotrypsin (1 U/10^6 cells) for 15 minutes at 25°C to shed L-selectin from the cell surface. Cells were then stimulated with 1 μM fMLP and exposed to prescribed shear rates in a cone-plate viscometer. Adhesion kinetics are plotted for untreated and enzyme treated cells at shear rates of: a) G = 100 s⁻¹ and b) G = 400 s⁻¹. The percentage of neutrophils recruited into aggregates is plotted as mean ± SD for n ≥3 experiments.
5.3.3 Receptor expression and shear rate.

It has been previously reported that homotypic neutrophil adherence is mediated by molecular recognition of L-selectin and $\beta_2$-integrin for distinct counter-structures (94). To determine whether shear rate could modulate receptor expression, and in turn adhesive function, molecular expression was measured at a low and a high shear rate. The affect of a relatively low (300 s$^{-1}$) and high (2000 s$^{-1}$) shear rate on the kinetics of CD62L and CD18 expression was examined by flow cytometric analysis of the binding of fluorescently conjugated mAb to sheared cell suspensions (Figure 5.4). On resting cells, CD62L is maximally expressed (~70,000 sites/cell), while CD18 is expressed at a low level (~40,000 sites/cell) (12). Within seconds of stimulation with 1 $\mu$M fMLP, CD62L was shed from the cell surface and CD18 was rapidly upregulated (Figure 5.4). During the first 2 min after stimulation ~70% of the initial CD62L was shed, and by 7 min expression was down to background levels. Shear had no significant effect on the rate or extent of L-selectin shedding. During the first 2 min after stimulation, when the aggregation rate was highest, CD18 was upregulated ~5 fold independent of the applied shear rate. After 7 min the expression of CD18 was significantly higher at 2000 s$^{-1}$ (~14 fold upregulation) than at 300 s$^{-1}$ (~10 fold upregulation) (p < .05). Incubating cells at 37°C with shear in the absence of stimulation caused <10% CD62L shedding and CD18 upregulation over the 7 min time course (data not shown). This level of adhesion receptor modulation with incubation alone indicated that the neutrophils remained unactivated in the absence of stimulation.
Figure 5.4. *Kinetics of the surface expression of CD62L and CD18 at low and high shear.* fMLP stimulated cell suspensions were exposed to $G = 300 \text{ s}^{-1}$ (circles) or $G = 2000 \text{ s}^{-1}$ (squares) and aliquots were taken at indicated time points. Cells were fixed with 0.5% paraformaldehyde and labeled with MHM 23-PE to CD18 or Leu-8-FITC to CD62L. The kinetics of anti-L-selectin (closed symbols) and anti-CD18 (open symbols) binding are plotted as mean fluorescence intensity ± SEM for two experiments.
5.3.4 Estimating the average intercellular contact duration.

The defined hydrodynamics of the cone-plate viscometer enabled estimation of the average intercellular contact duration ($t_{\text{contact}}$) required to mediate cell capture. The average contact duration was calculated on the basis of a collisional analysis for the interaction of two equally sized rigid spheres in a linear shear field (117). The contact duration depends on the collision angle, $\phi$, that the projection of the axis of revolution of the collision doublet makes with the median plane (see Figure 1, Goldsmith et al., 1995). Upon collision, the spheres move as a rigid dumbbell with angular velocity, $d\phi/dt$, which varies with the collision angle $\phi$ as previously described (118). Based on this analysis, the predicted contact duration of a doublet that collides with an initial angle $\phi$ has been shown to be (117):

$$t_{\text{contact}} = \frac{5}{G} \tan^{-1} \left( \frac{1}{2} \tan \phi \right)$$  \hspace{1cm} (5.1)

We calculated the average contact duration over all possible collision orientations, ranging from head-on collisions where $\phi$ tends to $\pi/2$, to glancing collisions where $\phi$ tends to 0. Neutrophils were assumed to be spherical in shape and their surface was discretized into a fine grid. For each point on the grid we calculated the angle of collision and the corresponding contact duration of interaction from Equation 5.1. We
also calculated the number of collisions at each grid point, since in a linear shear field the frequency of collision increases linearly as we move to higher streamlines. The average contact duration calculated, while taking into account the collision orientation at each point and weighing it by the frequency of collision at that point, was found to be \( \sim 2.6/G \). This agrees very well with 5\( \pi/6G \) as derived by Bartok and Mason for the mean lifetime of a transient doublet (105).

5.3.5 L-selectin and \( \beta_2 \)-integrin dependent adhesion efficiency.

It was apparent from the aggregation kinetics obtained at various shear rates (Chapter 3) that the rate of aggregation increased with shear rate up to an optimum level, before decreasing at shear rates >800 s\(^{-1}\). As described in Chapter 4, a useful measure of the rate at which neutrophils are recruited into aggregates is the aggregation efficiency. The efficiency of neutrophil-neutrophil adhesion was computed over a range of shear rates for untreated cells, and for cells preincubated with anti-CD62L, or anti-CD18. The efficiency was calculated from the aggregation kinetics over the initial 30 seconds since the molecular recognition events that lead to adhesion occur on this time scale. At shear rates of 100-200 s\(^{-1}\), only 20-30% of collisions resulted in aggregate formation (Figure 5.5). The adhesion efficiency increased with increasing shear rate, more than doubling to \( \sim 80\% \) at shear rates from
Figure 5.5. *The dependence of adhesion efficiency on L-selectin.* Isolated neutrophils (10^6 cells/ml) were incubated in 37°C buffer, exposed to shear in a cone-plate viscometer, and stimulated with 1 μM fMLP. Adhesion efficiency was determined at each shear rate from the aggregation kinetics over the first 30 seconds of fMLP stimulation, as described in Chapter 4. Adhesion efficiency is plotted for untreated and anti-CD62L treated neutrophils. The adhesion efficiency was determined from the aggregate distributions of each experiment, and is plotted as mean ± SD for n ≥ 3 experiments. Adhesion efficiency was significantly less (p < .05) for anti-CD62L treated cells only at shear rates ≥ 200 s⁻¹.
400 - 800 s\(^{-1}\). At shear rates above 800 s\(^{-1}\), efficiency decreased sharply to \(~45\%\) at 1600 s\(^{-1}\), and \(~25\%\) at 3000 s\(^{-1}\). At shear rates above 100 s\(^{-1}\) maximum adhesion efficiency required the expression of both L-selectin and \(\beta_2\)-integrin. Blocking with mAb to \(\beta_2\)-integrin inhibited aggregation completely at all the shear rates tested. L-selectin alone did not support the formation of aggregates which were sustained long enough to be measured by this method. At shear rates \(\geq 200\) s\(^{-1}\), mAb to L-selectin significantly decreased adhesion efficiency. However, Anti-CD62L reduced adhesion to baseline levels only at shear rates \(>400\) s\(^{-1}\) (corresponding to average contact durations \(<0.6\) ms), indicating an absolute requirement on selectin tethering at these contact intervals. The adhesion efficiency was unchanged by anti-CD62L at shear rates \(\leq 100\) s\(^{-1}\), corresponding to average contact durations \(\geq 25\) milliseconds.

5.3.6 The effects of shear stress on selectin and integrin mediated adhesion.

To differentiate the effects of shear rate and shear stress on neutrophil adhesion, buffer viscosity was manipulated. The effect of increased shear stress on neutrophil adhesion with and without L-selectin was examined (Figure 5.6 - 5.7). Ficoll was used to increase the viscosity of the suspension media, thereby increasing shear stress at a constant shear rate. At 37°C, the normal buffer had a viscosity of 0.75 cp and addition of 6% ficoll increased the buffer viscosity to 1.7 cp.
Figure 5.6. The effect of increased shear stress at a shear rate of 200 s\(^{-1}\) on neutrophil aggregation kinetics. Isolated neutrophils (10\(^6\) cells/ml) were incubated in 37\(^\circ\)C buffer at a low (0.75 cp) or high (1.7 cp) viscosity, exposed to shear in a cone-plate viscometer, and stimulated with 1 \(\mu\)M fMLP. Aggregation kinetics of untreated and anti-CD62L treated neutrophils are shown for G=200 s\(^{-1}\), at shear stresses of 1.5 dyn/cm\(^2\) and 3.4 dyn/cm\(^2\).
Figure 5.7. The effect of increased shear stress at a high shear rate on neutrophil aggregation kinetics. Isolated neutrophils (10^6 cells/ml) were incubated in 37°C buffer at a low (.75 cp) or high (1.7 cp) viscosity, exposed to shear in a cone-plate viscometer, and stimulated with 1 μM fMLP. Aggregation kinetics are shown for G=800 s⁻¹, at shear stresses of 6 dyn/cm² and 13.6 dyn/cm² for untreated and anti-CD62L treated cells.
The presence of ficoll had no apparent affect on L-selectin or β₂-integrin expression or modulation in response to fMLP stimulation (data not shown). Increasing the shear stress at a constant shear rate had a marked affect on neutrophil adhesion. At shear rates of 100 s⁻¹ (data not shown) or 200 s⁻¹ (Figure 5.6), doubling the viscosity from .75 cp to 1.7 cp increased the initial rate of aggregation by ~40%. Blocking L-selectin with LAM1-3 Fab inhibited the increase in adhesion observed when shear stress was doubled at 100 s⁻¹ and 200 s⁻¹ (Figure 5.6). At shear rates of 800 s⁻¹ (Figure 5.7) and higher (data not shown), increasing the shear stress caused a significant decrease in aggregation, and mAb to L-selectin completely inhibited adhesion.

A plot of adhesion efficiency versus shear rate for the two buffer viscosities provided a direct comparison of the effects of shear rate and shear stress on neutrophil adhesion (Figure 5.8). Adhesion efficiency increased ~2 fold at the higher viscosity for shear rates <400 s⁻¹. A peak efficiency of ~80% was observed at 400 s⁻¹ which was equivalent at both viscosities. At shear rates of 800 s⁻¹ or higher, increasing the viscosity at the same shear rate significantly reduced the adhesion efficiency. L-selectin-independent adhesion efficiency was unaffected by increased shear stress at a constant shear rate, as shown in Figure 5.9.

L-selectin dependent and independent adhesion efficiency versus shear stress is plotted in Figure 5.10. Adhesion efficiency was unaffected by mAb to L-selectin at a shear stress of 0.75 dyn/cm². In contrast, at shear stresses ≥1.5 dyn/cm² blocking mAb to L-selectin significantly inhibited adhesion efficiency. In untreated cells the adhesion efficiency increased from 20% at 0.75 dyn/cm² to 75% at ~7 dyn/cm². At shear stresses above 7 dyn/cm² the efficiency steadily decreased.
Figure 5.8. *The effect of shear rate and shear stress on adhesion efficiency.* Neutrophils were suspended in buffers with viscosities of either 0.75 cp or 1.7 cp, stimulated with 1 μM fMLP, and exposed to prescribed shear rates. Efficiency was plotted as a function of the shear rate for viscosities of 0.75 cp and 1.7 cp. The adhesion efficiency is plotted as mean ± SD for n ≥3 experiments.
Figure 5.9. *The effect of shear rate and shear stress on L-selectin independent adhesion efficiency.* Anti-L-selectin mAb (LAM1-3) treated neutrophils were suspended in buffers with viscosities of either .75 cp or 1.7 cp, stimulated with 1 μM fMLP, and exposed to prescribed shear rates. Efficiency was plotted as a function of the shear rate for viscosities of 0.75 cp and 1.7 cp. The adhesion efficiency is plotted as mean ± SD for n ≥3 experiments.
**Figure 5.10.** The dependence of L-selectin function on shear rate and shear stress. Isolated neutrophils (10^6 cells/ml) were incubated in 37°C buffer at a low (0.75 cp) or high (1.7 cp) viscosity, exposed to shear in a cone-plate viscometer, and stimulated with 1 μM fMLP. Adhesion efficiency was determined at each shear rate from the aggregation kinetics over the first 30 seconds of fMLP stimulation, as described in Analysis. Adhesion efficiency is plotted for untreated neutrophils in two buffer viscosities, with and without anti-CD62L. The adhesion efficiency was determined from the aggregate distributions of each experiment, and is plotted as mean ± SD for n ≥3 experiments.
5.4 Discussion

At sites of inflammation, neutrophils are recruited by a sequential process that involves initial cell contact and rolling on the vessel wall, followed by firm adhesion and transmigration (112,113). Neutrophils rolling on or firmly adherent to the vessel wall may also recruit additional neutrophils through capture from the free stream (71,72). Therefore, the key questions regarding neutrophil adhesion apply to both homotypic adhesion and margination to the vasculature: What are the relative contributions of the selectin and integrin molecules to the efficiency of cell adhesion under hydrodynamic shear? How does the rate of bond formation and dissociation compare to the duration over which cells are in contact and exposed to tensile and compressive stresses in a shear field?

In the current study, we employed cone-plate viscometry in combination with flow cytometry to quantitate the kinetics of adhesion between neutrophils in response to chemotactic stimulation. Cone-plate viscometry enabled the application of a linear shear field. It permitted the application of precise shear rates and stresses to neutrophil suspensions, and it allowed us to estimate the collision frequency and average intercellular contact duration. When coupled with a model of cell adhesion in a linear shear field, we were able to predict the adhesion efficiency over a range of physiologically relevant shear rates from 50 - 3000 s\(^{-1}\), and stresses from 1 - 30
dyn/cm². Adhesion efficiency was found to be invariant with respect to the cell concentrations used and was a function of the intrinsic properties of the receptors (102). Neutrophils were found to aggregate with an optimal adhesion efficiency of ~80% over a narrow range of shear rates between 400 s⁻¹ and 800 s⁻¹ where both selectin and integrin were necessary. The factors that mediate an increase in adhesion efficiency up to the peak value, and effect a decrease in efficiency at shear rates >800 s⁻¹, and stresses >7 dynes/cm² will be addressed.

5.4.1 Receptor expression and adhesion efficiency

Neutrophils adhere within seconds following chemotactic stimulation when sheared in suspension (12,65). This reversible adhesion requires the expression of L-selectin and β₂-integrin respectively on colliding cells (94). The L-selectin counterstructure appears to be an O-linked sialylated glycoprotein. Removal of these glycoproteins by an endopeptidase has been shown to inhibit neutrophil aggregation (27). L-selectin and β₂-integrin represent two parts in what appears to be a four-component recognition process. The O-linked ligand is proposed to be the third component, whereas the fourth component, the ligand bound by β₂-integrin, remains unknown. Several lines of evidence suggest that L-selectin functions to transiently tether neutrophils during aggregation. First, neutrophils which are fixed with
glutaraldehyde retain their ability to serve as adhesive counter targets for live cells, which bind via L-selectin and activated β₂-integrin (27,119). Secondly, data from at least two laboratories have demonstrated that neutrophils can be captured from the free stream and roll on a monolayer of adherent neutrophils in an L-selectin dependent manner (61,71).

We propose that the molecular dynamics of homotypic neutrophil adhesion are analogous to neutrophil adhesion on the vascular endothelium. We suggest that neutrophils interact transiently via binding of L-selectin to an O-linked glycoprotein ligand (107), thereby enabling firm adhesion through activated β₂-integrin. The initial expression of relatively high selectin and low integrin levels was sufficient for optimum adhesion efficiency during the first 30 seconds following stimulation. While L-selectin appears to be essential for transient adhesion, activated β₂-integrin plays a critical role in stabilizing the adhesive interaction. The experiments in which blocking mAbs were added to either L-selectin or β₂-integrin within 10 seconds of fMLP activation revealed a marked decrease in the extent of aggregation (Figure 5.1). In contrast, with the addition of anti-L-selectin blocking mAb at 30 seconds, a time point in which most aggregate formation is complete, the extent of aggregation was unaltered. This provided evidence that selectin was not critical to sustain formed aggregates. Maintenance of newly formed aggregates was attributed to CD18, since addition of anti-β₂-integrin mAb at 10 or 30 seconds caused the early breakup of
aggregates.

### 5.4.2 Effects of shear stress and shear rate on adhesion efficiency

The relative contributions of the selectin and integrin to adhesion efficiency were assessed by preblocking sites with mAbs to either molecule, or removing the L-selectin or the O-glycosylated ligand with proteases. We discovered that β₂-integrin alone could support adhesion at shear rates less than 400 s⁻¹. The direct dependence on shear rate was most evident in Figure 5.9 where the efficiency of integrin dependent adhesion decreased identically for the two viscosities, regardless of the shear stress.

Both L-selectin and its O-glycosylated counterstructure appeared to be critical to optimize efficiency at shear rates >100 s⁻¹, corresponding to average intercellular contact durations shorter than ~25 ms. Blocking with anti-L-selectin LAM1-3 or cleaving L-selectin with chymotrypsin significantly inhibited aggregation at shear rates >200 s⁻¹ (contact durations ≤~13 ms), and completely inhibited at shear rates >400 s⁻¹ (contact durations ≤~6 ms). In comparison, less effective inhibition was found with DREG-200 and LAM1-14. It appears that L-selectin binds rapidly via the lectin-like domain to O-sialylated counterstructures, thereby increasing the number of integrin bonds which may form and anchor cells together.
Neutrophils expressing both L-selectin and β₂-integrin exhibited an increase in adhesion efficiency as shear stress was increased. An increase in shear stress induced with the higher viscosity media boosted efficiency by ∼2 fold at shear rates of 100 and 200 s⁻¹ (Figure 5.6). Shear stress appeared to be the primary factor increasing efficiency up to the peak level. Interestingly, the peak efficiency was observed at the same level of shear rate (400 s⁻¹), but at two different levels of shear stress induced by the low and high viscosity media (Figure 5.8). Above the threshold shear rate, efficiency decreased to the same extent with an increase in either shear rate or stress as indicated in Figure 5.8 and 5.10.

5.4.3 The role of selectins in adhesion efficiency

The process of cell capture and rolling has been extensively studied in parallel plate flow chambers on substrates of human umbilical endothelial cells. Initial attachment and cell rolling is dependent on the selectin family of adhesion receptors, including E-selectin (19) and P-selectin (120) induced on vascular endothelial cells, and constitutively expressed L-selectin on leukocytes (8,121). Cell rolling mediated by selectins has also been demonstrated on substrates of reconstituted selectin ligands including peripheral node adressin (PNAd) (120) and glycolipids bearing s-\text{Le}^\alpha and s-\text{Le}^\beta (122). The molecular dynamics that support the transient tethering and rolling
of cells are thought to require the binding of relatively few selectin molecules to their carbohydrate ligands. These bonds apparently have high tensile strength and a rapid molecular on- and off-rate (59). The efficiency with which the selectins mediated the attachment and subsequent rolling of leukocytes appeared to be sensitive to the applied shear. Neutrophil capture and rolling on substrates of P- and E-selectin increased the efficiency of cell capture as shear flow was increased in a parallel plate flow chamber (120). This effect was also found for neutrophil rolling on L-selectin ligand (CD34), or on a monolayer of neutrophils (61). A minimum shear of ~0.5 dyn/cm² was required for cell capture, and the number of rolling cells steadily increased to a maximum at a shear of 1 dyn/cm² (61). In our studies, we also demonstrated that L-selectin mediated an increased adhesion efficiency up to an optimum level that was dependent on the applied shear stress. The underlying mechanism for the increase in efficiency with shear stress may be a function of the increased cell deformation and effective membrane contact area induced by a greater force of impact. For example, L-selectin is strategically positioned for binding to adjacent ligand as it is preferentially expressed on microvilli. The surface density of selectin may be many-fold higher than that of the integrin, which is not solely expressed on the microvilli (Dr. A. Burns, personal communication). Increased compression of the membranes would effectively increase the number of adhesive bonds, particularly integrins since they must make contact with ligand in the
membrane between microvilli. Another consequence of an increased intercellular compressive force would be to overcome the electrostatic repulsive forces due to negatively charged molecules in the membrane glycocalyx.

5.4.4 The interplay of intercellular contact duration and shear stress in modulating adhesion efficiency

The dynamics of cell interactions in a linear shear field are governed by the shear rate and the geometry of cell interaction (see Analysis). For the case of cell collisions in free suspension, the average contact duration is $\sim 2.6 \text{ G}^{-1}$, whereas it is $\sim 25$-fold less ($\sim 0.1 \text{ G}^{-1}$) (123) for interactions with a planar monolayer. During this interval, cells are exposed to hydrodynamic shear stresses that involve both compressive and tensile force components acting at the adhesive interface between cell membranes. These forces vary with time and are distributed in a complex manner at the interface as the cells rotate and change position in the shear field (124, 125). Nonetheless, a first approximation of the duration of contact between cells and the nature of the forces acting on the bonds may shed light on the relation between the kinetics of bond formation, bond lifetime, and the efficiency of adhesion.

In the case of homotypic neutrophil adhesion, a newly formed doublet tumbling in a shear field will have a period of rotation $\sim 5\pi \text{G}^{-1}$ (63). The adjacent cell
membranes will experience a cycle of compression over \( \sim \frac{1}{4} \) of the total rotation (63), and the number of selectin bonds would rapidly build up. Over a range of shear rates between 100-400 s\(^{-1}\) in which adhesion efficiency was observed to increase, the average contact duration is \( \sim 6 \) ms which is apparently sufficient for selectin bonds to form. Adhesion took place even at the highest shear rate applied (3000 s\(^{-1}\)), however, efficiency decreased to \( \sim 20\% \). At these high shear rates the tensile forces and the decreasing contact duration (<1.0 ms) may limit the number of integrin and selectin bonds which can form. Other investigators have found that the efficiency of cell capture via L-selectin decreased with shear rate (18,54,61). Finger et al. (1996) reported that the peak efficiency of L-selectin mediated adhesion occurred at a shear stress of 1 dyn/cm\(^2\). In the parallel plate flow chamber this corresponds to a shear rate of \( \sim 140 \) s\(^{-1}\) and a contact duration of \( \sim 1.0 \) ms. Interactions were eliminated at 3 dyn/cm\(^2\), corresponding to an estimated contact duration of <0.5 ms. In comparison, we found peak adhesion efficiency of homotypic adhesion at a shear rate of 400 s\(^{-1}\) corresponding to an average contact duration of \( \sim 6.0 \) ms. As noted, this peak efficiency was independent of the applied shear stress. Apparently, it is the brief contact duration that primarily limits the adhesion efficiency at shear rates above the threshold. It has been demonstrated that L-selectin dependent transient adhesion interactions such as capture and rolling is possible even under relatively high hydrodynamic shear stress. Neutrophils were observed rolling on high endothelial
venules within a range of shear stresses from ~2.5 to 40 dyn/cm² (61). In comparison, we found homotypic adhesion to occur over a range of shear stress from 1-30 dynes/cm².

Capture and rolling on endothelial cells is partially mediated by L-selectin (19,52,55). This event is both necessary and sufficient for β₂-integrin dependent firm adhesion at normal physiologic shear rates (51,126). β₂-integrin alone is sufficient to mediate cell capture only under conditions of low shear flow (48,55,114). We demonstrate here that β₂-integrin alone can mediate adhesion at shear rates less than 400 s⁻¹. β₂-integrins may require considerably more time for binding than L-selectin; an average intercellular contact duration of ~25 ms was necessary for engagement with its counter-structure on the neutrophil surface. L-selectin markedly enhanced the efficiency of homotypic adhesion at shear rates greater than 100 s⁻¹ and was absolutely required at shear rates greater than ~400 s⁻¹. Since L-selectin apparently acts over the initial seconds following activation, it may enable sufficient time and cell contact for β₂-integrin to engage in sufficient numbers. The presence of ligands for both L-selectin and β₂-integrin on the endothelium should markedly optimize adhesion efficiency over a wide range of shear rates. These results suggest that the interplay between adhesion molecule expression and the hydrodynamic conditions may provide a novel mechanism of selectivity for neutrophil localization at appropriate sites on vascular endothelium.
Chapter 6

Mac-1, LFA-1, and ICAM-3 Mediated Neutrophil Adhesion

6.1 Introduction

We have developed a model of neutrophil-neutrophil adhesion to study selectin and integrin function. Neutrophil adhesion was examined under uniform shear rates using cone-plate viscometry. In chapter 5, it was demonstrated that the molecular requirements for neutrophil-neutrophil adhesion are shear dependent. L-selectin is absolutely required for subsequent \( \beta_2 \)-integrin mediated stable adhesion at shear rates greater than 400 s\(^{-1}\). At shear rates between 100 and 400 s\(^{-1}\), \( \beta_2 \)-integrin can mediate primary adhesion independently of L-selectin, and at shear rates less than 100 s\(^{-1}\) adhesion efficiency is unaffected by the presence of L-selectin (13). In this chapter the adhesion mechanisms of the \( \beta_2 \)-integrins will be explored further.

The efficient recruitment of neutrophils to sites of inflammation in the microcirculation is a critical step in the immune response to infection or injury. At physiologic shear rates, this localization is mediated by two classes of adhesion molecules on the leukocyte, selectins and integrins (112). Selectins appear to mediate the initial capture and rolling of the neutrophil on the endothelium (2). Subsequent
firm adhesion and diapedesis into the inflamed tissue are mediated by integrins (33,127). The subset of integrins expressed on neutrophils, the $\beta_2$-integrins, are heterodimers consisting of a common $\beta_2$ chain noncovalently linked to distinct $\alpha$ subunits. The two major $\alpha$ subunits required for neutrophil adhesion are CD11a/CD18 (LFA-1), and CD11b/CD18 (Mac-1). Currently, all of the known counter-structures recognized by $\beta_2$-integrins on the vascular endothelium are intercellular adhesion molecules (ICAMs), members of the immunoglobulin (Ig) superfamily. To date, three members of the ICAM family of adhesion molecules have been characterized. ICAM-1, induced on activated endothelium, has been shown to be a ligand for both LFA-1 (39,128,129) and Mac-1 (109,130). Although both $\beta_2$-integrins adhere to ICAM-1, they do not bind in the same domain (131,132). Mac-1 binding has been mapped to the third Ig-like domain of ICAM-1, while LFA-1 binds an epitope in the first domain. ICAM-2, expressed on resting endothelium, is a ligand for LFA-1, but not Mac-1 (133). The most recently characterized $\beta_2$-integrin ligand, ICAM-3, is primarily expressed on circulating leukocytes and has only been shown to bind LFA-1 (134).

ICAM-3 is highly expressed on all populations of circulating leukocytes (135,136). It shares the greatest homology with ICAM-1, both having 5 Ig-like domains, an overall 47% amino-acid identity, and a maximum homology of 77% in the second domain (135). Like ICAM-1, the binding site on ICAM-3 for LFA-1 has
been mapped to the first Ig-like domain (137,138). Despite these similarities, LFA-1 shows specific binding sites for ICAM-1 and ICAM-3 (139). ICAM-3 displays more glycosylation than ICAM-1, and is glycosylated differently on neutrophils than on lymphoid cells (140). It was recently demonstrated that ICAM-3 is a major LFA-1 ligand in the resting immune system (141). ICAM-3 also appears to play a role in signaling, as activation of $\beta_1$- and $\beta_2$-integrins through cross-linking of ICAM-3 on T-cells has been reported (142). While ICAM-3 has not been found on resting or inflamed endothelium, it has been shown to be inducible in some disease states, particularly in lymphomas (42). The wide distribution of ICAM-3 on different leukocyte populations, its capacity to signal, and its distinct binding characteristics suggest a crucial role in modulating immune response.

The $\beta_2$-integrins are leukocyte-specific integrins. Mac-1 is found on neutrophils, monocytes, and some lymphocytes while LFA-1 is expressed on most white blood cells (35,127). A feature common to both $\beta_2$-integrin subunits is that on resting cells they are expressed in a low affinity conformation and require cellular activation to induce an adhesive conformation (112,127). The low affinity conformation of LFA-1 may mediate adhesion until chemotactic factors stimulate the active conformations of both integrin subunits. It has been reported that without exogenous chemotactic factors, LFA-1 but not Mac-1 on neutrophils can interact with ICAM-1 on IL-1 stimulated HUVEC, while both subunits are involved if the cells are
pre-stimulated with fMLP (143). In response to chemotactic stimulation, Mac-1 receptor numbers have been shown to increase dramatically, while LFA-1 expression remains constant (35). The function of the newly upregulated Mac-1 receptors in the immune response remains unclear. We postulate that LFA-1 and Mac-1 have distinct roles with time after stimulation, which will depend on the number, lifetime, mobility, and affinity of active receptors. It was recently demonstrated in vivo that at low hydrodynamic shear β2-integrin mediates transient adhesion and subsequent firm adhesion in the absence of selectins (114). The relative contributions of Mac-1 and LFA-1 to selectin independent neutrophil capture, rolling, and firm adhesion are unknown.

Recently, it was demonstrated that neutrophil adhesion efficiency decreases with time (102). It was determined that at shear rates >400 s⁻¹ this decrease is due both to L-selectin shedding from the cell surface and integrin deactivation or redistribution. It is unknown how Mac-1 and LFA-1 avidities change with time, and what ligands they bind in neutrophil-neutrophil adhesion. Also, modulation of Mac-1 and LFA-1 function by shear has not been demonstrated. In this study we demonstrate disparate functions for Mac-1 and LFA-1. We show for the first time that both LFA-1 and Mac-1 equally support neutrophil-neutrophil adhesion at low shear, while Mac-1 predominates at higher shear rates. The dependence of neutrophil-neutrophil adhesion on Mac-1 increases with time after chemotactic
stimulation. It appears that Mac-1 and LFA-1 mediate the formation of aggregates at low shear, but LFA-1 has a minor role in stabilizing aggregates. We also show for the first time that ICAM-3 is a ligand for LFA-1 in fMLP stimulated homotypic neutrophil aggregation.

The cascade from selectin mediated capture and rolling to integrin-ICAM mediated firm adhesion at physiologic shear rates has been studied extensively (49-52, 112, 144). However, less is known about the molecular requirements at shear rates below the shear regime of 100 s$^{-1}$ - 3000 s$^{-1}$ observed in the microcirculation. There is recent evidence which suggests that at low shear rates an initial selectin bond is unnecessary for recruitment of neutrophils to stimulated endothelium. In vivo, leukocytes were observed to roll independent of selectins at reduced shear (114). In fact, L-selectin (61) and more recently P- and E-selectin (145) have been shown to require shear above a threshold level to function. These findings suggest that integrin mediated capture may be important at shear rates below the threshold required for selectin function.
6.2 Materials and Methods

6.2.1 Reagents

Anti-CD11b mAb 60.1 (IgG1) was kindly provided as a F(ab')₂ fragment by Repligen Corporation, Cambridge, MA. Anti-CD11a mAb R3.1 (IgG1) was obtained from Dr. Robert Rothlein, Boehringer-Ingelheim Pharmaceuticals Inc., Ridgefield, CT. Anti-CD50 mAb ICR1.1 (IgG1) was obtained from Dr. Donald Staunton, ICOS Corporation, Bothell, WA. Commercial mAb to CD11a (CD11a FITC), CD11b (Leu15 FITC), and anti-CD45-FITC were purchased from Becton Dickinson Immunocytometry, San, Jose, CA. Fab fragments were produced by digestion with papain and purified by passage over a protein A-Sepharose column using ImmunoPure Fab preparation kits from Pierce, Rockford, IL. Formyl-methionyl-leucyl-phenylalanine (fMLP), was purchased from Sigma Chemical Co., St. Louis, MO. The fluorescent nuclear dye LDS-751 was purchased from Molecular Probes, OR. ICR1.1 was conjugated with the fluorescent dye CY3 using a FluoroLink-Ab™ Cy3™ labeling kit from Amersham Life Science, Inc., Arlington Heights, IL.

6.2.2 Flow cytometric detection of homotypic and heterotypic adherence
A FACScan flow cytometer (Becton Dickinson Immunocytopmetry Systems, San Jose, CA) was used to analyze the particle distributions of fixed cell suspensions. The neutrophil population was confirmed by gating on its characteristic forward scatter vs. side scatter. Adhesion was also quantitated between neutrophils and a mouse melanoma cell line transfected with full length human ICAM-1 (U11/E3 cells). The transfectants express abundant levels of surface ICAM-1 as determined by immunofluorescence (27). Neutrophils and E3-ICAM-1 cells were labeled with spectrally distinct fluorescent stains for heterotypic adhesion assays. Neutrophils were labeled with 5 µg/ml anti-CD45-FITC detected in the green (FL1) fluorescence channel, and E3-ICAM-1 cells were stained for 10 minutes at 25°C with the vital nucleic acid dye LDS-751 (0.5 µg/ml) for detection in the red (FL3) fluorescence channel. These labels did not alter cell function (94), and allowed the quantitation of homotypic neutrophil aggregates, as well as neutrophil E3-ICAM-1 heterotypic aggregates. Excess label was removed by a brief centrifugation (2-3 seconds at 3000xg) and the two cell populations (each at 3x10^6 cells/ml) were mixed and incubated for 2 minutes in buffer containing 1.5 mM Ca^{2+}. The combined sample was then stimulated and exposed to shear as described above. Aliquots of 50 µl were taken at desired time points and fixed in 50 µl of 0.5% cold paraformaldehyde. Use of paraformaldehyde avoids autofluorescence associated with glutaraldehyde, which would interfere with dual color fluorescence discrimination.
6.2.3 Two color homotypic aggregation assay

A two color assay was performed to quantitate adhesion between two populations of neutrophils labeled with spectrally distinct fluorescent markers. Neutrophils were labeled with either 5 μg/ml anti-CD45-FITC detected in the green (FL1) fluorescence channel, or were stained for 10 minutes at 25°C with the vital nucleic acid dye LDS-751 (0.5 μg/ml) for detection in the red (FL3) fluorescence channel. These labels did not alter cell function (94), and allowed the quantitation of one and two color homotypic neutrophil aggregates. Excess label was removed by a brief centrifugation (2-3 seconds at 3000xg) and the two cell populations (each at 3x10⁶ cells/ml) were mixed and incubated for 2 minutes in buffer containing 1.5 mM Ca²⁺. The combined sample was then stimulated and exposed to shear as described above. Aliquots of 50 μl were taken at desired time points and fixed in 50 μl of 0.5% cold paraformaldehyde.

6.2.4 Inhibition of adhesion with mAb pretreatment

Monoclonal antibodies were preincubated for 15 minutes at room temperature. Whole antibody was found to cause sustained adherence of stimulated neutrophils, so Fab and F(ab’)_2 fragments were used for inhibition studies. Anti-CD11b/CD18 60.1 F(ab’)_2 (30 μg/ml), anti-CD11a/CD18 R3.1 Fab (30 μg/ml), and anti-ICAM-3 ICR1.1
Fab (30 µg/ml) were used at concentrations determined to inhibit neutrophil aggregation.

6.3 Results

The kinetics of neutrophil aggregation were measured following stimulation with 1 µM fMLP as discussed in Chapters 2 and 3. Cell suspensions (10^6/ml) were exposed to a constant shear rate in a cone-plate viscometer. Samples were removed at prescribed time points and fixed with 2% glutaraldehyde (EM grade). Homotypic neutrophil aggregation was quantitated using flow cytometry to measure the fluorescence induced by glutaraldehyde fixation.

6.3.1 Heterotypic adhesion of neutrophils and E3-ICAM-1 transfectants

As demonstrated in chapter 5, β2-integrin was required for homotypic neutrophil aggregation over the entire range of shear rates. L-selectin was required for optimum adhesion at average contact durations <10 ms. To verify L-selectin-independent neutrophil adhesion at low shear rates, we explored β2-integrin dependent adhesion between human neutrophils and a mouse melanoma cell line expressing human ICAM-1 (E3-ICAM-1). These transfectants express relatively high levels of
ICAM-1 (350,000 sites/cell ± 50,000) and can be studied as single cells in suspension (27). Two-color fluorescence flow cytometry enabled the detection of neutrophils labeled green with CD45-FITC and E3-ICAM-1 cells labeled red with the nucleic dye LDS-751. In the absence of FMLP stimulation, shear alone did not cause heterotypic aggregation as shown in Figure 6.1a. At maximum aggregation, neutrophils formed both homotypic green-green aggregates and heterotypic red-green aggregates as shown in Figure 6.1b. The E3-ICAM-1 cells did not form homotypic aggregates, but aggregated with neutrophils in a manner dependent only on: 1) activation of CD11a/CD18 and CD11b/CD18 on the neutrophil; and 2) expression of ICAM-1 on the E3-ICAM-1 cells, as determined by mAb blocking of each receptor. It has been shown previously that the heterotypic adhesion of neutrophils to E3-ICAM-1 cells is independent of L-selectin (27).

Similar to homotypic neutrophil aggregation kinetics, heterotypic aggregation peaked by ~90 seconds after stimulation and disaggregation then ensued (Figure 6.2). The fraction of E3-ICAM-1 cells that captured neutrophils decreased as shear rate increased from 100 s⁻¹ to 800 s⁻¹, in contrast to homotypic neutrophil aggregation in the same suspensions. At shear rates >400 s⁻¹ heterotypic aggregates were markedly diminished.
Figure 6.1 CD18 dependent heterotypic adhesion between neutrophils and E3-ICAM-1 cells. Neutrophils (3x10^6 cells/ml) were fluorescently labeled green (CD45-FITC) and E3-ICAM-1 cells (3x10^6 cells/ml) were stained red (LDS-751) for 10 minutes at 25°C. Excess label was removed by centrifugation, and cell populations were equilibrated in 37°C buffer containing 1.5 mM Ca^{2+} for 3 minutes. Samples were fixed with 0.5% cold paraformaldehyde at the indicated time points and adhesion was quantitated on the flow cytometer. a) Detection of unstimulated neutrophils and E3-ICAM-1 cells after exposure to shear. Discrete neutrophil and E3-ICAM-1 populations are evident on the green (CD45-FITC) vs. red (LDS-751) dot plot. b) Heterotypic adhesion following 1.5 minutes of FMLP stimulation at G = 100 s^{-1}. Homotypic neutrophil (green-green) and heterotypic PMN-E3-ICAM-1 (red-green) aggregates are evident.
Figure 6.2 *CD18 dependent heterotypic adhesion between neutrophils and E3-ICAM-1 cells*. Neutrophils (3x10⁶ cells/ml) were fluorescently labeled green (CD45-FITC) and E3-ICAM-1 cells (3x10⁶ cells/ml) were stained red (LDS-751) for 10 minutes at 25°C. Excess label was removed by centrifugation, and cell populations were equilibrated in 37°C buffer containing 1.5 mM Ca²⁺ for 3 minutes. Samples were fixed with 0.5% cold paraformaldehyde at the indicated time points and adhesion was quantitated on the flow cytometer. Kinetics of neutrophil E3-ICAM-1 adhesion measured at the shear rates indicated. Data represent the percentage of total neutrophils recruited into heterotypic aggregates (mean ± SEM, n = 5).
Figure 6.3 The efficiency of homotypic neutrophil and neutrophil E3-ICAM-1 adhesion over a range of shear. Neutrophils and E3-ICAM-1 cells were stimulated with 1μM FMLP and exposed to prescribed shear rates. The adhesion efficiency of neutrophil-neutrophil and neutrophil E3-ICAM-1 cells was determined from the kinetics of the mean particle distributions for n = 5 experiments.
A plot of the homotypic and heterotypic aggregation efficiency over the range of shear rates tested provides a direct comparison of the integrin-selectin and integrin-ICAM-1 dependent processes (Figure 6.3). The efficiency of homotypic neutrophil adhesion was not inhibited in the presence of E3-ICAM-1 cells, increasing to a maximum at 800 s\(^{-1}\). Heterotypic aggregation demonstrated the highest efficiency at the lowest shear rate (100 s\(^{-1}\)), corresponding to an average contact duration of ~25 ms. The efficiency of heterotypic aggregation decreased monotonically with increased shear rate. This trend was virtually identical to that observed for homotypic adhesion of neutrophils treated with mAb to L-selectin. Moreover, the efficiency of heterotypic neutrophil E3-ICAM-1 aggregation was similar to that of integrin binding to its counterstructure in homotypic aggregation at 100 s\(^{-1}\).

6.3.2 Molecular specificity of neutrophil-neutrophil adhesion at low shear

Analogous to neutrophil margination on inflamed endothelium, neutrophil aggregation is a sequential process by which transient selectin bonds enable the formation of stable \(\beta_2\)-integrin dependent adhesion (13,94). Also analogous to neutrophil-endothelium adhesion (48,55), at low shear rates neutrophil-neutrophil adhesion is selectin independent (Figure 5.2). As demonstrated in Chapter 5, L-selectin function is absolutely required for the formation of neutrophil aggregates at
shear rates >400 s⁻¹. At shear rates less than 400 s⁻¹, neutrophils are capable of aggregating independently of L-selectin. At these low shear rates an initial selectin bond may not be required for the formation of neutrophil aggregates.

It has been reported that the integrin component of neutrophil aggregation is solely Mac-1 binding an unknown ligand (12,94,146,147). We hypothesized that at low shear, LFA-1 may also participate. When neutrophils were preincubated with either anti-LFA-1 or anti-Mac-1, aggregate formation was inhibited by 30-40% in the first 30 seconds following fMLP stimulation (Figure 6.4). There appears to be no significant contribution to aggregate formation by other adhesion receptors acting alone, as preincubation with a combination of anti-LFA-1 and anti-Mac-1 inhibited aggregation completely.

ICAM-3 is highly expressed on neutrophils (135), and is known to be a ligand for LFA-1 (134). After finding that LFA-1 partially mediates neutrophil aggregation, we examined the possibility that ICAM-3 may also play a role. Preincubation of neutrophils with anti-ICAM-3 also inhibited aggregate formation by 30-40% (Figure 6.5). This confirmed our hypothesis that ICAM-3 is an integrin ligand in fMLP stimulated neutrophil-neutrophil adhesion. In comparison, blocking ICAM-1 did not affect neutrophil aggregation alone or in combination with integrin antibodies. To determine which integrin subunit(s) bound ICAM-3, we incubated neutrophils with
Figure 6.4. Inhibition of neutrophil adhesion by preincubation with anti-CD11a,b/CD18 mAbs at $G = 100 \text{ s}^{-1}$. Neutrophil suspensions (10$^6$ cells/ml) were pretreated with 60.1 F(ab$'$$^2$) (30 $\mu$g/ml) to CD11b/CD18 or R3.1 Fab (30 $\mu$g/ml) to CD11a/CD18 for 15 minutes at 25°C and equilibrated for 3 minutes at 37°C. Cells were then stimulated with 1 $\mu$M fMLP and exposed to a shear rate of 100 s$^{-1}$ in a cone-plate viscometer. Adhesion kinetics are plotted for untreated, anti-CD11a, anti-CD11b, and both mAbs. The percentage of neutrophils recruited into aggregates is plotted as mean ± SD for n ≥6 experiments.
Figure 6.5. Inhibition of neutrophil adhesion by preincubation with anti-CD50 mAbs at $G = 100 \text{ s}^{-1}$. Neutrophil suspensions ($10^6$ cells/ml) were pretreated with 60.1 F(ab')$_2$ (30 $\mu$g/ml) to CD11b/CD18, R3.1 Fab (30 $\mu$g/ml) to CD11a/CD18, or lCR1.1 Fab (30 $\mu$g/ml) to ICAM-3 for 15 minutes at 25°C and equilibrated for 3 minutes at 37°C. Cells were then stimulated with 1 $\mu$M fMLP and exposed to a shear rate of 100 s$^{-1}$ in a cone-plate viscometer. Adhesion kinetics are plotted for untreated, anti-ICAM-3, and anti-ICAM-3/anti-CD11a,b combinations. The percentage of neutrophils recruited into aggregates is plotted as mean ± SD for $n \geq 6$ experiments.
mAbs to ICAM-3 in combination with anti-Mac-1 or anti-LFA-1 mAbs. Preincubation with anti-ICAM-3 and anti-Mac-1 together increased inhibition to ~70% (Figure 6.5). In contrast, co-incubation of anti-ICAM-3 and anti-LFA-1 did not increase inhibition (Figure 6.5). This data indicated that ICAM-3 was a ligand for LFA-1 and not Mac-1 on neutrophils.

The molecular requirements for the formation of neutrophil aggregates were estimated by computing the adhesion efficiency. Efficiency of aggregate formation was determined for untreated and mAb preincubated neutrophils (Figure 6.6). The efficiency of fMLP stimulated neutrophil-neutrophil adhesion was ~20% at a shear rate of 100 s⁻¹. Blocking both integrin subunits simultaneously with mAb inhibited adhesion completely and adhesion efficiency was reduced to zero. Blocking either LFA-1, Mac-1, or ICAM-3 alone with mAb resulted in ~30% decrease in adhesion efficiency. When both Mac-1 and ICAM-3 were blocked with mAb, a 70-75% decrease in efficiency was observed. Combinations of LFA-1 and ICAM-3 mAbs had no greater effect than either one added alone. The inability of anti-Mac-1 and anti-ICAM-3 combinations to completely inhibit aggregation raises the possibility that LFA-1 may bind other ligands on the neutrophil with low avidity.
Figure 6.6. *Integrin dependent adhesion efficiency at G = 100 s\(^{-1}\).* Neutrophil suspensions (10\(^6\) cells/ml) were pretreated with 60.1 F(ab')\(_2\) (30 \(\mu g/ml\)) to CD11b/CD18, R3.1 Fab (30 \(\mu g/ml\)) to CD11a/CD18, or ICR1.1 Fab (30 \(\mu g/ml\)) to ICAM-3 for 15 minutes at 25\(^\circ\)C and equilibrated for 3 minutes at 37\(^\circ\)C. Cells were then stimulated with 1 \(\mu M\) fMLP and exposed to a shear rate of 100 s\(^{-1}\) in a cone-plate viscometer. Adhesion efficiency at G=100 s\(^{-1}\) is plotted for untreated neutrophils and neutrophils pretreated with anti-CD11a/CD18, anti-CD11b/CD18, anti-ICAM-3, and mAb combinations.
6.3.3 Dual population analysis of adhesion

To compare the avidities of LFA-1 and Mac-1 for their ligands on adjacent neutrophils, we employed a two-color adhesion assay. One population of neutrophils was labeled green with anti-CD45 FITC, and the other red with the nucleic acid dye LDS-751, as described previously (94). It has been confirmed that LDS-751 and anti-CD45 FITC do not interfere with adhesive function (94). A two-color adhesion assay was used previously to demonstrate that the adhesive interactions of neutrophil receptors are heterophillic, and in a complex shear field the minimum requirements are L-selectin on one cell and β2-integrin on the other (94). Here, we examined the molecular requirements of neutrophil-neutrophil adhesion at a shear rate at which we have shown adhesion to be independent of L-selectin. In the absence of stimulation, neutrophils remained as discrete populations with no aggregate formation (Figure 6.7a). Following addition of chemotactic stimulus, red, green, and red-green aggregates were observed, as seen on the dot plots of cellular events (Figure 6.7b). The fraction of particles in each population was quantitated by drawing analysis gates on distinct aggregate species, as described previously (94). Two-color aggregation was determined by dividing the number of cells in dual-fluorescent aggregates by the total number of cells.
Figure 6.7. Detection of two color adhesion between fluorescently labeled neutrophil populations. Neutrophils (3×10^6 cells/ml) were fluorescently labeled green (CD45-FITC) or red (LDS-751) for 10 minutes at 25°C. Excess label was removed by centrifugation, and cell populations were equilibrated in 37°C buffer containing 1.5 mM Ca^{2+} for 3 minutes. Samples were fixed with 0.5% cold paraformaldehyde and adhesion was quantitated on the flow cytometer. a) Detection of unstimulated neutrophils. Discrete neutrophil populations are evident on the green (CD45-FITC) vs. red (LDS-751) dot plot. b) Red-Green aggregates formed following 30 seconds of fMLP stimulation at G = 100 s⁻¹.
Quantitation of one and two color aggregates enabled the determination of the receptor-ligand avidity between populations in which monoclonal antibodies were used to block specific adhesion receptors. Using the two-color adhesion assay we confirmed that ICAM-3 is a ligand for LFA-1 but not Mac-1 in neutrophil-neutrophil adhesion. In single population studies, when either Mac-1 or LFA-1 was blocked, we observed ~60% of control aggregation over the initial 30 seconds after fMLP stimulation (Figure 6.4). We demonstrate here that when Mac-1 was blocked on both populations, blocking ICAM-3 on one population decreased to ~30% of control two-color aggregation (Figure 6.8). In contrast, when anti-LFA-1 was added in excess, blocking ICAM-3 in one or both populations caused no additional inhibition (Figure 6.9). Additionally, when Mac-1 was blocked completely, blocking either ICAM-3 or LFA-1 on one population were equivalent in reducing two-color adhesion to ~30% of control. This supports the conclusion that ICAM-3 is the major ligand for LFA-1 on the neutrophil.

The two-color assay also enabled comparison of the avidities of Mac-1 and LFA-1 for their ligands on apposing neutrophils (Table 6.1). We found that either Mac-1 or LFA-1 expressed on one cell was sufficient to obtain ~30% of control adhesion. When ICAM-3 was blocked, LFA-1 bound an unknown counter-receptor on the neutrophil with a lower avidity, adhesion was only ~15% of control. If two receptor ligand pairs were available, the fraction of control was 60-70%, regardless of which pairs were available.
Figure 6.8. Two color adhesion between fluorescently labeled neutrophil populations, anti-CD11b in excess. Neutrophils (3x10^6 cells/ml) were fluorescently labeled green (CD45-FITC) or red (LDS-751) for 10 minutes at 25°C. Excess label was removed by centrifugation, and cell populations were equilibrated in 37°C buffer containing 1.5 mM Ca^{2+} for 3 minutes. Samples were fixed with 0.5% cold paraformaldehyde and adhesion was quantitated on the flow cytometer. Adhesion receptors on both populations were blocked with 60.1 fab (30 ug/ml). Adhesion receptors on one or both populations were blocked with R3.1 fab (30 ug/ml), or ICR-1.1 fab (30 ug/ml) as denoted on the abscissa. Cells were preincubated separately for 15 minutes at room temperature, washed of excess mAb, and equilibrated together for 2 minutes at 37 C. Two color aggregation is expressed as mean ± SEM relative to that of two-color untreated controls with data from at least 3 individual experiments at 10 and 30 seconds after stimulation. Cells depicted above represent available receptors.
Figure 6.9. **Two color adhesion between fluorescently labeled neutrophil populations, anti-CD11a in excess.** Neutrophils (3x10^6 cells/ml) were fluorescently labeled green (CD45-FITC) or red (LDS-751) for 10 minutes at 25°C. Excess label was removed by centrifugation, and cell populations were equilibrated in 37°C buffer containing 1.5 mM Ca^{2+} for 3 minutes. Samples were fixed with 0.5% cold paraformaldehyde and adhesion was quantitated on the flow cytometer. Adhesion receptors on both populations were blocked with R3.1 fab (30 ug/ml). Adhesion receptors on one or both populations were blocked with 60.1 fab₂ (30 ug/ml), or ICR-1.1 fab (30 ug/ml) as denoted on the abcissa. Cells were preincubated separately for 15 minutes at room temperature, washed of excess mAb, and equilibrated together for 2 minutes at 37°C. Two color aggregation is expressed as mean ± SEM relative to that of two-color untreated controls with data from at least 3 individual experiments at 10 and 30 seconds after stimulation.
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<td>(Two color)</td>
</tr>
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<td>ICAM-3 (50 or 100%)</td>
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</tr>
<tr>
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<td>(Two color)</td>
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<td>LFA-1 (50%)</td>
<td>1 LFA-1 - ?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac-1 (100%)</td>
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</tr>
<tr>
<td>ICAM-3 (100%)</td>
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Table 6.1
6.3.4 Roles of LFA-1 and Mac-1 with time after chemotactic stimulation

The efficiency of neutrophil-neutrophil adhesion in response to fMLP stimulation has been reported to decrease with time after stimulation (89,102). This decrease was hypothesized to be due to both L-selectin shedding and integrin deactivation. It has been shown that neutrophils can interact with ICAM-1 on IL-1 stimulated endothelium via LFA-1, but not Mac-1 (143). Mac-1 is not active on unstimulated neutrophils, so we hypothesized that its contribution to adhesive strength would increase with time after chemotactic stimulation. We chose to examine L-selectin independent aggregation by performing experiments at low shear to differentiate the contributions of Mac-1, LFA-1, and ICAM-3 to forming and maintaining aggregates after fMLP stimulation (Figure 6.10). The distinct functions of Mac-1 and LFA-1 in sustained adhesion were apparent after 60 seconds of stimulation at a shear rate of 100 s\(^{-1}\). Stimulation with fMLP induced aggregation which continued for 3 minutes, and aggregates remained stable for 5 minutes (Figure 6.10). The same aggregation pattern was observed when neutrophils were preincubated with mAb to LFA-1 or ICAM-3. Aggregation after 60 seconds was not significantly different for untreated compared to anti-LFA-1 or anti-ICAM-3 treated neutrophils. In contrast, when Mac-1 was preblocked with mAb, aggregation plateaued by 3 minutes and complete disaggregation was observed by 5 minutes after stimulation. Thus, it appears that the lifetime of LFA-1 mediated aggregates is much
Figure 6.10. The dependence of Mac-1, LFA-1, or ICAM-3 mediated adhesion on time at $G=100 \text{ s}^{-1}$. Neutrophil suspensions ($10^6$ cells/ml) were pretreated with 60.1 F(ab')$_2$ (30 $\mu$g/ml) to CD11b/CD18, R3.1 Fab (30 $\mu$g/ml) to CD11a/CD18, or ICR1.1 Fab (30 $\mu$g/ml) to ICAM-3 for 15 minutes at 25°C and equilibrated for 3 minutes at 37°C. Cells were then stimulated with 1 $\mu$M fMLP and exposed to a shear rate of 100 s$^{-1}$ in a cone-plate viscometer. Adhesion kinetics are plotted for untreated, anti-CD11a, anti-CD11b, and anti-ICAM-3. The percentage of neutrophils recruited into aggregates is plotted as mean ± SD for $n \geq 6$ experiments.
shorter than that of Mac-1 dependent aggregates. The distinct contributions of Mac-1 and LFA-1 to adhesion strength with time may be observed directly when plotted in terms of inhibition (Figure 6.11). Inhibition was determined compared to control (untreated cells) for individual experiments. Blocking either LFA-1 or ICAM-3 caused significant inhibition only over the first 60 seconds, and inhibition decreased with time following stimulation. Anti-ICAM-3 was slightly less effective at blocking adhesion over time as compared to anti-LFA-1, suggesting that LFA-1 may adhere to additional ligand on the neutrophil. Pre-incubation with anti-Mac-1 significantly inhibited adhesion initially, and blocking increased with time. Total inhibition of adhesion was observed by 5 minutes post stimulation.

Mac-1 and LFA-1 may regulate their adhesive avidity with time via several mechanisms. Changes in receptor expression, distribution, mobility, and conformation could all affect the overall contribution to bond strength. We next examined the expression of adhesion receptors on the neutrophil surface before and after chemotactic stimulation. We examined whether ICAM-3 shedding from the neutrophil surface in response to chemotactic stimulation contributed to the decreasing contribution of LFA-1 with time after fMLP stimulation. It has been shown previously that the surface expression of Mac-1 is up regulated in response to chemotactic stimulation, while LFA-1 expression remains constant (35). We confirmed previous observations that Mac-1 upregulated and LFA-1 receptor numbers
Figure 6.11. The inhibition of neutrophil aggregation with mAbs to Mac-1, LFA-1, or ICAM-3 at $G=100\text{ s}^{-1}$. Neutrophil suspensions (10^6 cells/ml) were pretreated with 60.1 F(ab')_2 (30 μg/ml) to CD11b/CD18, R3.1 Fab (30 μg/ml) to CD11a/CD18, or ICR1.1 Fab (30 μg/ml) to ICAM-3 for 15 minutes at 25°C and equilibrated for 3 minutes at 37°C. Cells were then stimulated with 1 μM fMLP and exposed to a shear rate of 100 s^{-1} in a cone-plate viscometer. Inhibition kinetics at $G=100\text{ s}^{-1}$ are plotted for untreated neutrophils and neutrophils pretreated with anti-CD11a/CD18, anti-CD11b/CD18, or anti-ICAM-3. Inhibition is defined as 1- fraction control at each time point for n ≥6 experiments.
Figure 6.12. Kinetics of the surface expression CD11a/CD18, CD11b/CD18, and ICAM-3. fMLP stimulated cell suspensions were exposed to G = 100 s⁻¹ and aliquots were taken at indicated time points. Cells were fixed with 0.5% paraformaldehyde and labeled with fluorescent mAb. The kinetics of anti-CD11a/CD18, anti-CD11b/CD18, and anti-ICAM-3 binding are plotted as mean fluorescence intensity. Shown is an experiment representative of four independent experiments.
were constant following fMLP stimulation (Figure 6.12). While it has been reported that ICAM-3 is shed from the cell surface in response to PMA or calcium ionophore stimulation (148), we did not observe a decrease in ICAM-3 expression in response to 1μM fMLP. To determine if shear rate modulates LFA-1 or ICAM-3 receptor numbers, we measured their expression in response to fMLP stimulation at a low (100 s⁻¹) and a high (800 s⁻¹) shear rate. The kinetics LFA-1 and ICAM-3 expression in response to fMLP stimulation were unaffected by shear rate (data not shown). Thus it appears that deactivation or redistribution of β₂-integrin and/or ICAM-3 contribute to disaggregation rather than receptor down-regulation.

6.3.5 Shear dependence of LFA-1 and Mac-1 dependent adhesion

As shear rate increases, the average intercellular contact duration of cells in suspension decreases proportionally, and the time available for receptor-ligand bonds to form is limited (63,102). When shear rate is increased, shear stress is increased in direct proportion, thereby increasing the tensile forces applied to intercellular bonds. We have demonstrated that L-selectin is required to mediate primary adhesion and enable subsequent β₂-integrin dependent stable adhesion at shear rates greater than 400 s⁻¹, while β₂-integrin can capture cells at lower shear rates (13). Selectins have been demonstrated to have “fast” molecular on- and off-rates, thereby enabling them
to mediate capture and rolling of cells at high shear rates (58,59). We postulated that without L-selectin, decreasing intercellular contact durations would limit integrin-ligand interactions as shear rate increases. The relative contributions of Mac-1 and LFA-1 to initial and stable adhesion as a function of shear rate have not been determined.

Here we further investigated the abilities of Mac-1 and LFA-1 to mediate adhesion as a function of shear rate (Figure 6.13). At a shear rate of 100 s\(^{-1}\), preincubating neutrophils with either anti-Mac-1 or anti-LFA-1 alone inhibited adhesion by 30-40\%. As shear rate increased, blocking mAb to LFA-1 was less effective at inhibiting aggregate formation. At a higher shear rate of 300 s\(^{-1}\), mAb to LFA-1 blocked aggregate formation by only ~15\%, and at 400 s\(^{-1}\) adhesion was decreased by less than 10\%. In contrast, anti-Mac-1 caused a constant inhibition of 40-45\% from 100 s\(^{-1}\) to 400 s\(^{-1}\). At shear rates above 400 s\(^{-1}\) mAb to Mac-1 became more efficient, inhibiting adhesion almost completely (>90\%) at a shear rate of 3000 s\(^{-1}\).
Figure 6.13. *The effect of shear rate on mAb inhibition.* Neutrophil suspensions (10^6 cells/ml) were pretreated with 60.1 F(ab')_2 (30 μg/ml) to CD11b/CD18 or R3.1 Fab (30 μg/ml) to CD11a/CD18 for 15 minutes at 25°C and equilibrated for 3 minutes at 37°C. Cells were then stimulated with 1 μM fMLP and exposed to a shear in a cone-plate viscometer. Inhibition due to mAb blocking during the first 30 seconds of aggregate formation was determined. Inhibition is plotted for untreated neutrophils and anti-CD11a/CD18 and anti-CD11b/CD18. Inhibition was determined as (1-fraction control) for each experiment, and is plotted as mean ± SEM for n≥3 experiments.
6.4 Discussion

In this chapter, the dynamics of adhesion via CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) in response to chemotactic factor at various shear rates was examined. Uniform shear rates were applied to activated neutrophil suspensions in a cone-plate viscometer. The kinetics of neutrophil-neutrophil adhesion were determined by removing samples over the time course of aggregation, and quantitating particle distributions with fluorescence flow cytometry. Using this technique, we assayed the average adhesive properties of large numbers of cells (3000 events/measurement) over the time course of stimulation. We demonstrate here that Mac-1 and LFA-1 function are modulated with time and shear rate after chemotactic stimulation. We also show for the first time that ICAM-3 contributes to fMLP stimulated neutrophil-neutrophil adhesion.

It has been demonstrated that neutrophils interacting with the endothelium or E-selectin substrates can recruit additional neutrophils from the free stream (71,74,149). At shear stresses of 2-3 dynes/cm² this process is dependent on L-selectin on the circulating neutrophil binding to PSGL-1 on the rolling or firmly adherent cell (72). Thus, neutrophil-neutrophil adhesion may play an important role in amplifying the immune response to injury or infection. Data indicates that at low shear rates, both neutrophil-neutrophil and neutrophil-endothelial cell interactions can
occur independently of an initial selectin bond. We have previously shown that homotypic neutrophil aggregation is L-selectin independent at low shear (13). In fact, there is evidence that L-selectin (61) and E- and P-selectin (145) require shear above a threshold level to mediate neutrophil adhesion a planar substrate. Slight increases in vessel diameter can have large effects on the shear rate in vivo (48). Vessel dilation in response to inflammatory mediators has been reported to decrease shear as much as 70% (114,150). It has been shown in vivo in the rat mesentery that reducing the shear rate by half from \( \sim 440 \text{ s}^{-1} \) to \( \sim 220 \text{ s}^{-1} \) supports significant integrin-independent, selectin-independent adhesion (114). Further, this transient adhesion was induced to firm adhesion when a chemotactic factor was added (114). The roles of specific \( \beta_2 \)-integrin subunits in this process have not been explored. In this study we examined the shear and time dependent function of Mac-1 and LFA-1 binding. We demonstrate here that ICAM-3 is a \( \beta_2 \)-integrin ligand in neutrophil-neutrophil adhesion. By systematically blocking with combinations of monoclonal antibodies (Figures 6.4 and 6.5), we have shown that ICAM-3 is a ligand for LFA-1, but not Mac-1 in homotypic neutrophil aggregation. The counter-receptor for Mac-1 on the neutrophil, as well as an additional LFA-1 ligand, remains to be determined. The LFA-1-ICAM-3 interactions between neutrophils may have physiologic significance in localizing cells. We demonstrate here that LFA-1 and ICAM-3 contribute mostly at low shear rates (Figure 6.13). Additionally, ligation of ICAM-3 has been shown
to activate $\beta_2$-integrin on the neutrophil (142,151). Therefore LFA-1 on an adherent cell binding transiently to ICAM-3 on circulating cells may activate $\beta_2$-integrin on the flowing cell, priming it for adhesion.

It was demonstrated that Mac-1 and LFA-1 contribute equally to neutrophil-neutrophil adhesion at low shear rates. This result adds to previous studies performed under non-uniform shear (12,94) or static adhesion (140,147,152), where Mac-1 was reported to be the only integrin component in neutrophil aggregation. We show here that when preincubated alone, mAb to LFA-1 inhibited neutrophil aggregation at shear rates $<400$ s$^{-1}$. Furthermore, the contribution of LFA-1 to adhesion decreased rapidly with time after chemotactic stimulation. A similar contribution to adhesion was observed for ICAM-3. The lessor extent of inhibition observed with mAbs to LFA-1 and ICAM-3 at high shear and with longer time after stimulation may explain why their participation in neutrophil aggregation has not been observed previously.

At low shear both the initial capture and firm adhesion appear to be integrin mediated events. Within the first minute of fMLP stimulation, Mac-1 and LFA-1 contributed equally to aggregate formation at low shear (Figure 6.10). After 60 seconds the contribution of LFA-1 to adhesion decreased markedly with time, while Mac-1 function appeared to dominate. By 3-5 minutes after stimulation, stable aggregates were completely Mac-1 dependent, and LFA-1 appeared to play little or
no role in maintaining adhesion. This decrease in LFA-1 avidity with time does not correlate with its expression on the cell surface. We confirmed (Figure 6.12) previous reports (35), that LFA-1 expression remains constant after chemotactic stimulation. ICAM-3, a major ligand for LFA-1 in neutrophil aggregation, also maintains relatively constant expression in response to chemotactic stimulation. It has been shown that LFA-1 receptor conformation is rapidly modulated to a high avidity state in response to chemotactic stimulation (153-155). This active conformation can also be induced using special monoclonal antibodies to the "I" domain of LFA-1 (156,157), or with certain cations such as Mn$^{2+}$ (158,159). There are also antibodies which bind to the active conformation of Mac-1 (109) or CD18 (160,161). A mAb which "reports" the level of active Mac-1 on the cell surface without affecting its level has been identified (109). With this antibody it was shown that only a subset of Mac-1 on the neutrophil surface becomes active with stimulation, and that active Mac-1 increases with time after fMLP stimulation for ~10 minutes after which it remains constant (109).

We show here LFA-1 avidity for its ligand on the neutrophil decreases rapidly with time after chemotactic stimulation. The reversibility of this high avidity state within ~1-2 minutes has at least two potential explanations. The most likely is that the high avidity conformation itself is transient. An antibody which recognizes an activation epitope of CD11a/CD18, without itself inducing it, would be useful to test
this hypothesis. Another possibility is that LFA-1 transiently clusters in focal adhesions, and that after ~60 seconds the receptors may diffuse away from high bond-density areas, or become clustered too tightly, reducing the surface area of bond formation (162-164). We have shown here that mAb to Mac-1 alone does not block aggregation completely until 5 minutes of chemotactic stimulus and shear, while the combination of anti-Mac-1 and anti-LFA-1 blocks aggregation at all time points (Figure 6.4). This data supports a complementary role for LFA-1 in neutrophil aggregation even after the time point when mAb to LFA-1 alone has no effect. It has been shown previously that interaction of LFA-1 with a ligand is required for full LFA-1 activation (160). Taken together, these data are consistent with a mechanism in which LFA-1 decreases in its ability to form new bonds rapidly with time after activation, but maintains those bonds formed within the first 1 minute of stimulation. Further support for this mechanism comes from the observation of a decrease in CD18 dependent neutrophil adhesion efficiency with time (102). The efficiency of LFA-1 mediated aggregate formation decreased much more rapidly than that of Mac-1, while both subunits were required for optimum aggregate stability (S. Neelamegham, manuscript in preparation).

Neutrophil recruitment from the circulation to sites of inflammation or injury occurs over a wide range of shear rates. However, most *in-vitro* flow experiments are performed at the so called “venous” shear stresses of 2 dynes/cm². This
corresponds to shear rates of \( \sim 290 \text{ s}^{-1} \) assuming a buffer viscosity of \( \sim 0.7 \text{ cP} \). At these shear rates, selectin mediated capture has been shown to be required for subsequent integrin dependent firm adhesion and diapedesis. In vessels with typically low shear, such as large vessels, the pulmonary bed (165), or in cases of acute inflammation (166), different mechanisms may act to recruit neutrophils to the inflamed tissue. In a parallel plate flow chamber, CD18 dependent attachment to IL-1 stimulated endothelial cells under flow was observed at shear stresses less than 0.5 dynes/cm\(^2\), corresponding to shear rates of \( \sim 70 \text{ s}^{-1} \) (48). \textit{In vivo}, neutrophils have been reported to roll independent of selectins in the rat mesentery when the shear rate was reduced by just half from \( \sim 440 \text{ s}^{-1} \) to \( \sim 220 \text{ s}^{-1} \) (114). The relative roles of Mac-1 and LFA-1 in this integrin dependent attachment and adhesion at low shear have not been thoroughly explored.

Our studies indicate that the molecular requirements for neutrophil aggregate formation change as shear rate increases. At low shear rates (\( \leq 100 \text{ s}^{-1} \)), aggregation is independent of L-selectin. As shear rate is increased, L-selectin plays a dominant role in the initial formation of neutrophil aggregates, becoming absolutely necessary for aggregate formation at shear rates greater than 400 s\(^{-1}\) (Figure 5.5). In contrast, we show here that mAb to LFA-1 inhibits aggregation significantly at low shear rates, but has no significant effect when added alone at shear rates above 400 s\(^{-1}\). When neutrophils were preincubated with mAb to Mac-1, a constant inhibition of 45-60\%
was observed at shear rates from 100 s\(^{-1}\) to 400 s\(^{-1}\). Although mAb to LFA-1 had no effect when added alone at shear rates \(\geq 400\) s\(^{-1}\), when mAbs to both integrin subunits were incubated together, they inhibited aggregation completely.

Several different mechanisms may account for the decrease of inhibition with shear rate observed with anti-LFA-1 and the increase observed with anti-Mac-1 mAbs: 1) The minimum duration of intercellular contact required for LFA-1 to mediate capture may be longer than that of Mac-1. In Chapter 5 it was demonstrated that \(\beta_2\)-integrin dependent aggregate formation required a contact duration of greater than 7 milliseconds. If contact duration limits LFA-1 mediated capture, we estimate that LFA-1 requires greater than 15 milliseconds to mediate primary adhesion. 2) The availability or number of "active" LFA-1 receptors may be less than Mac-1. At low shear LFA-1 may participate in the formation of a critical number of bonds required to form shear resistant stable adhesion. As shear rate is increased, L-selectin may fill this role, enabling sufficient \(\beta_2\)-integrin bonds to form a shear resistant aggregate. 3) Less "ligand induced activation" of LFA-1 may occur at high shear. In Chapter 5 it was demonstrated that L-selectin was required to mediate primary adhesion at shear rates greater than 400 s\(^{-1}\). Therefore it is possible that less LFA-1 molecules would interact with their ligands on the neutrophil, and less ligand induced activation would occur. 4) L-selectin may synergize preferentially to enhance Mac-1 binding. This could occur if Mac-1 and L-selectin were co-localized in the membrane to a greater
extent than LFA-1 and L-selectin, or L-selectin may activate or recruit Mac-1 through intercellular signaling. We have shown previously that L-selectin cross-linking activates Mac-1 function (15,16). Other labs have also reported that L-selectin ligation can upregulate Mac-1 expression (14).

In conclusion, the present study demonstrated for the first time that LFA-1 and ICAM-3 partially mediate neutrophil-neutrophil adhesion. Additionally, it was shown that Mac-1 and LFA-1 have distinct contributions to adhesion with time after chemotactic stimulation and shear rate. In vivo, the nature and severity of the inflammatory signal will vary depending on the type and location of injury or infection. The local hydrodynamics will be defined by the particular tissue and pathology involved. Mac-1 or LFA-1 may be selectively promoted to optimize the neutrophils response to a particular situation. Knowledge of the specific roles played by Mac-1, LFA-1, and ICAM-3 are crucial to mount a rationale for clinical interventions to enhance or inhibit neutrophil recruitment and adhesion.
References


