# HISTAMINE AS A POTENTIAL INITIATOR OF SICKLE PAIN CRISIS BY MEDIATION OF SICKLE ERYTHROCYTE ADHERENCE IN A SHEAR-DEPENDENT MANNER

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**HISTAMINE AS A POTENTIAL INITIATOR OF SICKLE PAIN CRISIS BY MEDIATION OF SICKLE ERYTHROCYTE ADHERENCE IN A SHEAR-DEPENDENT MANNER**

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To God, who gave me guidance,

To my colleagues, who taught me science,

To my parents, who taught me determination,

To my friends, who kept me sane.

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| | |
|---|---|
| ACS | acute chest syndrome |
| AMP | adenosine monophosphate |
| ANOVA | analysis of variance |
| CEC | circulating endothelial cells |
| DPBS | Delbecco's phosphate-buffered saline |
| DPI | diphenyleneiodonium chloride |
| dMECs | dermal microvascular endothelial cells |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| eNOS | endothelial nitric oxide synthase |
| FBS | fetal bovine serum |
| HbF | fetal hemoglobin |
| HbS | sickle hemoglobin |
| HBSS | Hank's Balanced Salts Solution |
| UHMWvWF | Ultra High Molecular Weight von Willebrand Factor |
| $H_1$ - $H_4$ | histamine receptor #1 - 4 |
| Hu | hydroxyurea |
| HUVECS | human umbilical vein endothelial cells |
| IgG | immunoglobulin |
| IL-1 | interleukin-1 |
| IL1$\beta$ | interleukin-1$\beta$ |
| IL-6 | interleukin-6 |
| IL-8 | interleukin-8 |
| ISC | irreversibly sickled cells |

| | |
|---|---|
| L-NAME | Nω-nitro-L-arginine methyl ester |
| MEC | microvascular endothelial cells |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| NS | not statistically significant |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PI | phosphatidylinositol |
| PS | phosphatidylserine |
| PSGL-1 | P-selectin glycoprotein-1 |
| RBC | red blood cells |
| REU | research experience for undergraduates |
| RNA | ribonucleic acid |
| SCA | sickle cell anemia |
| SEM | standard error of the mean |
| SFM | serum-free media |
| SSRBC | sickle red blood cells |
| STOP | Stroke Prevention Trial |
| TCD | transcranial Doppler ultrasound |
| TNF&TNF-α | tumor necrosis factor-α |
| TSP | thrombospondin |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VLA-4 | very late antigen-4 (also called $\alpha_4\beta_1$) |
| vWF | von Willebrand Factor |

## FORMULA NOMENCLATURE

| | |
|---|---|
| b | slope inflection (unitless) |
| b | gasket half-thickness (cm) |
| f | fraction of adherent cells |
| h | gap height (cm) |
| L | characteristic length in the plane of the plates (cm) |
| Q | perfusion solution flowrate (ml/min) |
| w | gasket cutout width (cm) |
| $w_1$ | entrance width (cm) |
| z | position coordinate measured from the channel entrance (cm) |

## GREEK NOMENCLATURE

| | |
|---|---|
| $\mu$ | viscosity of the media at 37° C (cp) |
| $\tau$ | shear stress (dynes/$cm^2$) |
| $\tau_w$ | shear stress at position "w" (dynes/$cm^2$) |
| $\tau_{50}$ | surface shear stress (dyne/$cm^2$) when f = 0.50 |

## SUMMARY

The genetic disorder sickle cell anemia causes hemolytic anemia and sickle pain crisis, episodes of microvascular occlusion resulting in painful ischemic tissue damage. Pain crisis is thought to occur when sickle erythrocytes adhere in the post-capillary venule, partially occluding the vessel. The resulting slowed blood flow causes more extensive cell adherence and entrapment of rigid, deoxygenated erythrocytes until the vessel is entirely occluded. It was hypothesized that the inflammatory mediators histamine and tumor necrosis factor-$\alpha$, factors known to cause endothelial expression of adhesive ligands, might significantly increase sickle erythrocyte adhesion, and thus be capable of initiating sickle pain crisis. It was also hypothesized that the perfusion shear stress environment of the endothelium, known to be oscillatory and reduced in sickle cell patients, was a significant mediating factor of sickle cell adhesion. An *in-vitro* flow chamber using cultured endothelial cells and erythrocytes from blood samples of sickle cell anemic patients was used to quantify sickle erythrocyte adherence to stimulated and unstimulated endothelial cells under shear stresses from 1.0 to 0.1 dyne/cm$^2$. Results showed that both endothelial stimulation and reduction of the perfusion shear stress increased sickle erythrocyte adherence. In combination, the use of inflammatory stimulation with reduced shear stress resulted in further increased adhesion, but only when above the range of 0.1 – 0.2 or 0.4 dyne/cm$^2$, depending on the inflammatory mediator. Adhesion below this level of shear is not significantly increased by endothelial stimulation. The mechanism by which histamine mediates adhesion was investigated, and found to involve the endothelial $H_2$ and $H_4$ receptors and expression of the P-selectin ligand. These data suggest that irregular flow, typical of sickle microvasculature, may act in conjunction with the pro-inflammatory state of sickle vasculature and the histaminergic nature of some pain treatments to initiate or propagate

sickle vaso-occlusion. Findings concerning histamine, tumor necrosis factor $\alpha$, and shear stress effects on adherence are discussed in relation to their possible applicability to patient health, future studies are outlined to confirm the relation of *in vitro* data to *in vivo* patient condition, and proposals are made for applying these methodologies to other potential mediators of sickle erythrocyte adhesion.

# CHAPTER I

## Introduction and Rationale

### 1.1 Introduction

The genetic disorder sickle cell anemia is characterized by homozygous inheritance of the gene for the production of hemoglobin S. The gelation of sickle hemoglobin when deoxygenated leads to the distortion or “sickling” of the sickle red blood cell (SSRBC) shape, increases cell rigidity, and damages the exterior membrane, altering blood flow dynamics and altering the red blood cell population. The clinical complications of sickle cell anemia are related to the sickling of red blood cells, either directly through erythrocyte changes that alter hemodynamics, or indirectly through its effect on blood chemistry, activation of thrombotic, inflammatory, and immune pathways. These complications include hemolytic anemia and pain crises that are self-limited episodes of diffuse, reversible pain in the extremities, back, abdomen, or chest. These are thought to be caused by microvascular occlusion, resulting in painful oxygen starvation and tissue damage. Although some of the effects of sickling on blood chemistry are understood, the detailed connection between genetics, external environmental effects, and clinical manifestations has not been fully defined.

Adhesion of sickle erythrocytes to endothelial monolayers likely contributes to the pathophysiology of sickle pain crises. Adhesion of erythrocytes in the microvasculature may cause a general slowing of blood flow by partly occluding vessels. Slower blood flow leads to a cascade of additional adhesion, sickling of red blood cells, entrapment of irreversibly sickled cells, and complete obstruction in the capillaries. The post-capillary venules are thought to be the site of the initial adhesion event.

The high levels of inflammatory cytokines, immune response mediators, and thrombotic mediators found in patient plasma may also induce endothelial activation,

causing expression of adhesive ligands that are known to elevate sickle erythrocyte adhesion. Known adhesive pathways include TNF-$\alpha$ or IL-8 up regulation of adhesive ligands, and von Willebrand factor or platelet thrombospondin bridging of endothelial sickle erythrocyte ligands. This may explain why infection, inflammation, and trauma are followed by a pain crisis in sickle patients.

Despite extensive investigation, initiation of sickle cell pain crisis is not yet fully understood. This thesis investigates histamine as a potentially powerful adhesion pathway in the microvasculature by examining capacity for promotion of sickle erythrocyte adhesion under conditions of flow, and determining the receptors and ligands involved in the promotion of sickle erythrocyte adhesion.

### 1.2 Rationale

It was hypothesized that histamine may initiate sickle complications by promoting the adhesion of SSRBC in the sickle microvasculature. Histamine is an inflammatory mediator known to cause the expression of adhesive ligands on endothelial monolayers. Highly specific histamine receptors $H_1$, $H_2$, $H_3$, or $H_4$ are involved in histamine's biological effects (Gantner et al., 2002; Morse et al., 2001; van der Werf & Timmerman, 1989; Heltianu et al., 1982). Histamine and TNF-$\alpha$ cause endothelial activation and promote adherence of sickle erythrocytes to vascular endothelium (Gee & Platt, 1995; Swerlick et al., 1993; Vordermeier et al., 1992). The dynamics of endothelial cell adhesion molecule expression induced by histamine are much more rapid, requiring minutes instead of the hours of stimulation characteristic of TNF-$\alpha$ activation (Sugama et al., 1992). Studies also show that histamine plasma levels are typically elevated during patient steady-state (asymptomatic), increase during pain crisis (Enwonwu & Lu, 1991), and increase after some typical treatments of sickle pain (Friedman & Dello Buono, 2001; Chaney, 1995;

Fuller et al., 1990; Muldoon et al., 1984). This thesis examines the adhesion of sickle erythrocytes to endothelium induced by histamine and characterizes the dynamics and mediators involved.

Blood flow in the microcirculation of sickle patients is known to be periodic and reduced over time (Kennedy et al., 1988; Lipowsky et al., 1987; Rodgers et al., 1984). Because sickle erythrocyte adhesion to the endothelium *in vivo* must take place under microvascular flow conditions, proposed adhesive interactions studied *in vitro* need to be evaluated over a range of flow conditions corresponding to that encountered in sickle patient microvasculature. Adhesive ligands incapable of promoting adhesion at normal vascular flow may have a profound effect at the reduced flow conditions typical of sickle patients.

Based on the known endothelial cell responses to histamine stimulation, the following hypothesis is explored: Histamine promotes sickle erythrocyte adherence to endothelium in a shear-dependent manner under physiologic flow. This is mediated by binding to endothelial histamine receptors inducing expression of adhesive ligands.

This hypothesis is explored through studies designed with the following specific aims:

(1) Use histamine receptor agonists and antagonists to characterize the endothelial histamine receptors involved in sickle adherence and demonstrate the dependence of sickle cell adherence on expression of specific ligands.

(2) Quantify sickle cell adherence levels in response to histamine stimulation under shear stress in the range of 1.0 to 0.1 dyne/cm$^2$.

These studies were designed to characterize important additional pathways that potentially act as triggering mechanisms of occlusive complications. Understanding the characteristics of sickle red blood cell adhesion promoted by histamine to endothelial monolayers will aid in evaluating the potential of this pathway causing vascular obstructions in sickle complications. Understanding the endothelial cell signaling pathways and ligand expression mechanisms may potentially be exploited to prevent sickle obstructive complications by blocking activation pathways or expressed ligands.

### 1.3 Chapter Guide

Chapter II reviews relevant literature and background information. Materials and methodology are presented in Chapter III. Chapter IV directly examines the mechanisms of histamine-mediated adhesion of sickle erythrocytes to cultured endothelial cells, addressing specific aim #1. In these experiments, cultured endothelial monolayers are exposed to a range of histamine concentrations both prior to and during perfusion with sickle erythrocyte suspensions while under a perfusion shear stress of 1.0 dyne/mm$^2$ in a parallel plate flow chamber. In addition, the mechanism promoting sickle erythrocyte adherence is examined through the use of blocking agents to determine the endothelial receptors and ligand expression involved in the histamine signal propagation.

The next section of this thesis (Chapter V) examines the effect of prevailing shear stress on the adhesion of sickle erythrocytes through the use of a linear shear chamber. Decreased flow rates affect the dynamics of sickle erythrocyte adhesion in the microvasculature, because the associated shear stress determines the contact time and frequency of endothelial cell / erythrocyte interactions. Shear also stresses any bonds formed between erythrocytes and endothelial cells. Thus the flow rate is an important variable to consider in properly modeling erythrocyte adhesion in the sickle microvasculature. In these studies, flow is examined over a shear rate range from 0.1 to

1.0 dyne/cm$^2$ to examine the role of hemodynamics on sickle cell adherence. Tumor necrosis factor $\alpha$ (TNF-$\alpha$) is used as an agonist in this section because the effect of TNF-$\alpha$ to promote sickle erythrocyte adherence is well characterized.

Specific aim #2 is addressed in Chapter VI, bringing the two previous chapters together by testing the shear stress dependence of the newly characterized histamine-mediated sickle erythrocyte adhesion.

The final results chapter of this thesis (Chapter VII) collects additional experiments which explored subjects related to the previous chapters, but proved inconclusive, methodologically unfeasible, or only partially completed as preliminary investigations for future work. These studies include an examination of the role of eNOS in sickle cell adherence promoted by histamine, investigation of alternate endothelial cell sources to attain a more physiologically-relevant endothelial model, and examination of bond strength through detachment studies. An overview of the entire thesis is provided in the final chapter (Chapter VIII).

# CHAPTER II

# BACKGROUND AND LITERATURE REVIEW

## 2.1 Sickle Cell Anemia

### 2.1.1 Disorder Etiology and Effect

The term “sickle cell anemia” designates a group of genetic disorders resulting from a mutation that produces an alternate form of hemoglobin known as “sickle hemoglobin.” (HbS) This mutation is the substitution of valine for glutamic acid at the sixth amino acid from the N-terminus end of the coding sequence for the β-hemoglobin chain (INGRAM, 1956; PAULING et al., 1949). Sickle cell anemia resulting from this mutation is characterized by vascular obstruction, causing pain, tissue damage, and organ failure throughout the patient's life.

Homozygous inheritance of the sickle cell gene leads to the complete sickle cell anemia disorder. Heterozygous inheritance leads to the benign sickle cell trait that is a genetic carrier state (Stark et al., 1980). The reason for the difference in severity of these disorders is the relative amount of sickle hemoglobin in patient's erythrocytes. Patients with sickle cell anemia produce erythrocytes that contain HbS with small amounts of fetal hemoglobin. Patients with sickle trait typically produce less than half hemoglobin S, the majority of their hemoglobin being of the normal "A" type (Hiruma et al., 1995). Additional hematological disorders are caused by the combination of the sickle gene and other independent hemoglobin mutations. For example, sickle β-thalassemia results from inheriting a gene for sickle cell anemia with a thalassemia gene mutation that greatly reduces the production of the β hemoglobin chains (Greenwalt & Zelenski, 1984). Other hemoglobin disorders compounded with sickle cell anemia

comprise the remainder of these sickle disorders, known collectively as “sickle cell disease.” These include HbSC, $HbSD_{Los}$, and HbSO, named after the mutant hemoglobin co-inherited with sickle cell anemia (Bunn, 1997; Bunn & Forget, 1986; Greenwalt et al., 1984).

The homozygous form of sickle hemoglobin related anemia (here referred to as SCA), is the central focus of the studies conducted in completion of this thesis. For simplicity, only homozygous SCA was studied and only blood samples from patients exhibiting homozygous sickle cell anemia were employed in these studies. Nevertheless, techniques or discoveries concerning sickle cell anemia in this thesis may provide useful direction for research to define causes of complication and methods of treatment of these related hemoglobinopathies.

### 2.1.2 Origin

Sickle cell anemia originated in at least four different mutational events in three different areas of Africa, those being Benin, Senegal, and the Central African Republic (Pagnier et al., 1984), and once in India (Trabuchet et al., 1991; Labie et al., 1989), as indicated by studies showing regional similarities in genetic haplotypes. A fourth, independent African origin has also been proposed (Lapoumeroulie et al., 1992) in Cameroon. Anthropological studies employing current migration theories suggest simultaneous appearance of the African mutations approximately 2,000 years ago. An alternate theory proposes a single originating mutation that acquired widely variant haplotypes via gene conversion and then were distinguished by genetic drift and selection (Flint et al., 1993). Other heritable hemoglobin mutations are known, but sickle cell anemia has remarkably high prevalence in world population for originating from these few mutations.

The multiple origins of the sickle hemoglobin mutation do not alone explain the number of individuals with the sickle gene. In the US, sickle cell anemia affects approximately 72,000 people. Sickle cell anemia occurs in nearly 1 in 500 African American births and 1 in 900 Hispanic American births. Approximately 2 million Americans, or 1 in 12 African Americans and 1 in 16 Hispanic Americans carry the sickle cell trait (American Sickle Cell Anemia website, 2006). The gene's proliferation in specific regions of the world with a tropical climate is believed to be an effect of sickle hemoglobin production which protects individuals with sickle trait against infection by the *Plasmodium falciparum* parasite responsible for the disease malaria (Williams et al., 2005a; Williams et al., 2005b; Williams et al., 2005c; ALLISON, 1954). Malaria is a frequent cause of death in these tropical regions, so an inherited protection against this deadly disease results in environmental selection for those with sickle trait. Although malaria treatment has improved in these regions, the malaria parasite still has an influence in maintaining the high frequency of HbS in patients (Williams et al., 2005c).

### 2.1.3 Sickle Hemoglobin

The principle way in which sickle hemoglobin differs from non-sickle hemoglobin involves its response to changes in oxygen tension. Sickle hemoglobin has a decreased oxygen affinity in comparison to non-sickle hemoglobin (Young, Jr. et al., 2000). More importantly, when sickle hemoglobin looses oxygen in the low-oxygen tension environment of the vasculature, sickle hemoglobin molecules aggregate reversibly into long, twisted, rods (Danish & Harris, 1983; Noguchi et al., 1983; Magdoff-Fairchild et al., 1976; ALLISON, 1957). Individual strands then associate into thick, rigid bundles of 14 strings (Dykes et al., 1979; Dykes et al., 1978). Literature on the subject frequently refers to this process as “polymerization” because of the ordered assembly of "monomer-like" sickle hemoglobin molecules into long chains. Although the formation of

these chains appears similar to the process of polymerization, deoxygenated sickle hemoglobin molecules do not form covalent bonds. Instead, the chains are held together via a strong hydrophobic interaction that arises from the changed charge distribution on sickle hemoglobin. Technically this is an aggregation or "gelation," not a polymerization.

The net result of this low-oxygen-tension response is that the "polymer" aggregates distort and deform the shape of the sickle erythrocyte. Typically, the cell distorts from the normal biconcave disk to an elongated sickle-like shape for which the disorder is named (Herrick, 1910). The presence of rigid “polymerized” sickle hemoglobin greatly decreases the cell’s flexibility (Itoh et al., 1995), and results in an increased viscosity of blood from sickle patients, which increases with increasing polymer fraction (Hiruma et al., 1995). Upon exposure to high oxygen tension, the sickle hemoglobin aggregation reverses (Hahn et al., 1976; Messer & Harris, 1970), and the sickle erythrocyte relaxes back to normal from its distorted form. As the cycle continuously repeats over the lifetime of the erythrocyte, this "sickling" distortion can become irreversible, leading to the formation of "permanently sickled" erythrocyte fractions (discussed in section 2.4.2), whose presence in sickle patient blood increases blood viscosity even when blood is oxygenated (Chien et al., 1970). This alteration of sickle hemoglobin results in diverse clino-pathological problems for patients that frequently leads to reduced life expectancy and organ damage.

## 2.2 Sickle Cell Pathology

Although sickle cell anemia has far-reaching consequences affecting every organ and system in the body either directly or indirectly, the primary effects of the disorder can be grouped into two distinct categories, namely hemolytic anemia and altered or reduced blood flow.

### 2.2.1 Hemolytic Anemia

Chronic hemolytic anemia in sickle cell patients is caused by sickle erythrocyte lysis. Lysis occurs through repeated gelation and un-gelation of hemoglobin as the blood passes through the pulmonary system (high oxygen tension) and the microvasculature (low oxygen tension). For the sickle erythrocyte, this is an endless cycle of "sickling" and "unsickling," repeatedly distorting and flexing the outer membrane. Sickling further damages the red cell membranes through complex internal interactions believed to result from the abnormal binding of spectrin to other membrane proteins such as ankyrin or protein 3 (Platt & Falcone, 1995; Liu et al., 1991; Lux et al., 1976). Also, denatured hemoglobin can associate with the cell membrane and free iron in the cell can cause specific membrane protein aggregation (Kannan et al., 1988; Low et al., 1985). Similar protein aggregation is seen during the standard aging process of erythrocytes approaching senescence, reflecting the severity of membrane damage on the sickle erythrocytes. Decreased deformability also means that sickle erythrocytes are more susceptible to mechanical stresses of flow through the microvasculature (Messmann et al., 1990). The narrow confines of the microvasculature contain capillary vessels only half the diameter of an erythrocyte, and require flowing erythrocytes to bend and distort in order to pass. This deformation of erythrocytes with altered membrane rigidity in combination with the sickling / unsickling cycle applies greater mechanical stress to the outer membrane (Weed, 1975), resulting in cell damage and a drastic decrease in longevity. Sickle erythrocytes have a lifespan of only 8-25 days, as compared to the lifespan of as long as 120 days for most non-sickle erythrocytes (Solanki et al., 1988). The spleen in sickle patients is often engorged as a result of accumulation of these short-lived erythrocytes, which is an especially dangerous condition in the first five years of life (Topley et al., 1981).

The resulting hemolysis in this chronic anemia is compensated for in patients by an increased erythropoesis (Ballas & Marcolina, 2000). As the demand for increased erythropesis is chronic during the life of the patient, there is an expansion of active marrow in the patient's bones (Mentzer, 2000; Mann et al., 1975). The population of very young erythrocytes, termed "reticulocytes" is increased to 5-16% of erythrocyte populations (Kaul et al., 1989b) as opposed to the more typical 1% in non-sickle patients. "Stress reticulocytes", even younger erythrocytes released prematurely from the bone marrow, and typically released in non-sickle patients only in the case of severe blood loss, are also present in increased numbers in the blood of sickle patients (Chang & Kass, 1997; Browne & Hebbel, 1996a). Unless properly accounted for in the patient's diet, this increased erythropesis may lead to the patient exhibiting nutritional deficiencies and slowed growth because of increased demand for calories, proteins, and folic acid (Serjeant, 1993; Enwonwu, 1988).

Constant hemolysis of the sickle erythrocyte population has effects that spread beyond the bone marrow and vasculature. The high turnover rate of erythrocytes leads to elevated levels of free hemoglobin and iron in the blood (Reiter et al., 2002), that may cause problems in the liver, kidneys, and heart, where excess free iron accumulates (Pippard, 1987). In patients where the iron overload condition is further exacerbated by the need for repeated transfusion, chelating agents are often required to clear excess iron from the blood and tissue (Kwiatkowski & Cohen, 2004; Pippard, 1987). Otherwise iron accumulates at toxic levels in the liver, heart, and endocrine organs.

### 2.2.2 Vaso-Occlusive Crisis

Vaso-occlusive crisis, a painful ischemic event caused by microvascular blockage, is a common pathology associated with sickle cell anemia. This effect occurs commonly in patients homozygous for sickle cell anemia, less severely in compound

heterozygotes, and very rarely in heterozygous patients under extreme conditions (Kerle & Nishimura, 1996). Vaso-occlusive events present as a self-limited episode of diffuse, reversible pain in the extremities, back, abdomen, or chest resulting from oxygen starvation of tissues with microvascular blockage (Platt et al., 2002; Platt et al., 1991; Platt & Eckman, 1989; Baum et al., 1987). These episodes typically lasts 4 to 6 days, but may last weeks. Vaso-occlusion is frequently associated with fever and the passage of dark or red urine. The localized tissue oxygen starvation resulting from the vaso-occlusion causes ischemic tissue damage and intense musculoskeletal pain. Recurrent ischemia over the lifetime of the patient leads to tissue dysfunction, organ failure, and death. Vaso-occlusion is the most serious pathological feature of sickle cell anemia, and is likely the primary cause of morbidity and mortality in older children and adults. Other clinical complications of vaso-occlusive events include recurrent infection, pulmonary infarction, stroke, splenic pathology, priapism, and retinopathy (Francis, 1991).

Sickle pain crises are thought to result from ischemia and infarction in the bone marrow (Milner & Brown, 1982; Lutzker & Alavi, 1976). The hyperplasic (over-cellularized) and expanded nature of blood-producing bone marrow in sickle patients, developed in response to the chronic anemia, results in higher bone marrow blood flow (Thrall & Rucknagel, 1978) making it especially susceptible to this ischemic damage and resulting pain-causing inflammation (Hammel et al., 1973). Increased intermedullary pressure from the inflammatory response following ischemic damage is believed to be the source of the pain.

A wide range of conditions are known to precipitate painful crisis episodes (Baum et al., 1987), including conditions arising from other pathologies; infection, fever, hypoxia, acidosis, and dehydration, patient physical or psychological condition; pregnancy, exhaustion, stress, anxiety and depression, or environmental conditions; extremes of cold, heat, or high altitude. Many pain crises occur without apparent

precipitant (Platt et al., 1989) highlighting the complexity of interacting factors leading to vaso-occlusive pain episodes.

### 2.2.3 Inflammation and Sickle Pain Crisis

Inflammation is one of the major precipitants of crisis, often arising directly from infection or injury. Pain episodes are often precipitated by or coincide with bacterial or viral infection (Wierenga et al., 2001; Goldstein et al., 1987; Winkelstein, 1977; Barrett-Connor, 1971). Cytokines associated with inflammation have also been found in elevated levels in patient plasma (Taylor et al., 1999; Taylor et al., 1995; Francis, Jr. & Haywood, 1992) as have other inflammatory mediators such as substance P (Michaels et al., 1998), especially during crisis (Duits et al., 1998). These elevated levels of inflammatory mediators lead many researchers to conclude that, even in the absence of infection, the sickle cell patient exists in a pro-inflammatory state capable of triggering inflammation with less stimulation than non-sickle patients (Wun, 2001). A similar pro-inflammatory state is found in transgenic sickle mouse models (Holtzclaw et al., 2004), and evident in sickle cell patients from examination of circulating endothelial cells (Solovey et al., 1998; Solovey et al., 1997). This delicate pro-inflammatory state may lead to initiation of pain crisis through activation of adhesive ligands on vascular endothelium. For example, the endothelial response to infection with the double-stranded RNA of many pathogenic viruses includes the expression of VCAM-1 (Offermann et al., 1995). This adhesive ligand may interact with sickle erythrocytes, causing adhesion that triggers pain crisis via a mechanism detailed below (2.4.1). Other adhesive ligands similar to VCAM-1 that may interact with sickle erythrocytes are expressed or activated upon initiation of an inflammatory response. The pro-inflammatory state of sickle patient vasculature may therefore be a key factor of vaso-

occlusion, with expressed adhesive ligands mediating sickle erythrocyte adhesion and initiating pain events.

### 2.2.4 Acute Chest Syndrome

Another severe pathology of sickle cell anemia arising as an indirect result of sickle hemoglobin production is a pulmonary complication referred to as “Acute Chest Syndrome” or ACS. This pathology is characterized by a new pulmonary infiltrate in combination with chest pain, a temperature of more than 38.5$^{o}$C, tachypnea, wheezing or cough, and/or hypoxia in a sickle patient (Haupt et al., 1982). ACS is the second most common complication of sickle cell anemia (Castro et al., 1994) and the most common condition at the time of death (Platt et al., 1991), being responsible for up to 25% of sickle-cell related deaths (Platt et al., 1994). The course of ACS may progress to neurological events (most likely involving sudden decreases in oxygenation in the vascular bed of the central nervous system (Vichinsky et al., 2000)) and respiratory failure. The specific causes or mechanism of ACS are incompletely understood, but are known to include pulmonary fat embolisms, pneumonia, pulmonary infarction, and acute pulmonary infection (Vichinsky et al., 2000). The mechanism driving ACS may be a more severe or organ-specific version of the mechanisms driving sickle pain crisis. The conditions listed that are suspected catalysts of ACS are also conditions known to precipitate sickling, regional hypoxia, and ischemic damage (Aldrich et al., 1996; Smolinski et al., 1995; Hebbel et al., 1987). Thus, while ACS is not fully understood, ACS and vaso-occlusive events may both arise from similar pathological conditions of the vasculature.

### 2.2.5 Sickle Cell Stroke

A further clinical complication of sickle cell anemia is the increased incidence of stroke. Strokes are a significant cause of morbidity and mortality in people with sickle cell disease, although the connection between the genetic disorder and increased incidence of stroke is not well understood. The dramatically increased incidence of stroke in very young children with sickle cell anemia is a source of great concern, and has been the subject of a recent large clinical study named the Stroke Prevention Trial on Sickle Cell Anemia (STOP) (Adams et al., 1998). The overall risk of stroke in sickle cell anemia is difficult to summarize due to age dependence and the loose definition of stroke (confused by the occurrence of "silent infarcts" detailed below) but incidence of cerebral infarction in sickle patients approaches 10% in North America, and varies between 1% and 18% in other regions around the world (Powars, 2000; Ohene-Frempong et al., 1998; Goncalves et al., 1994; Perrine et al., 1978). Of sickle patients who experience a cerebral infarction, 20-30% will have recurrent episodes (Adams et al., 2001), and nearly two-thirds of children who experience a stroke will have a recurrent clinical stroke (Powars et al., 1978). Neurological incidents involve lesions of an ischemic or hemorrhagic nature in specific cerebro-vascular territories resulting in effects ranging from the loss of tactile sensibility, to slight paralysis of one side of the body, visual field deficits, impaired communication skills, and/or cranial nerve paralysis (Ohene-Frempong, 1991).

Factors used to evaluate the risk of stroke in sickle patients include a high white blood cell count, high reticulocyte count, low hemoglobin, and low fetal hemoglobin level. Further, when measuring cerebral artery blood flow velocities via Transcranial Doppler (TCD) Ultrasound, high readings (> 200 cm/s) correlate with development of a stroke (Seibert et al., 1998; Adams et al., 1998; Adams et al., 1997; Adams et al., 1992). The correlation is theorized to apply because the high TCD velocity may represent flow past

partially stenotic arteries. Irregular TCD in addition to the presence of an abnormal magnetic resonance angiograph image are the best predictors of sickle stroke (Adams et al., 2001).

In contrast to the vaso-occlusive sickle pain crisis, the nature of stroke in sickle cell disease has been convincingly shown to be both a large-vessel and small-vessel cerebral arterial disease (Russell et al., 1976; Stockman et al., 1972). Histopathologic study of stenotic cerebral arteries show intimal proliferation, smooth muscle cell hyperplasia, mural thrombosis, and fragmentation / disruption of the internal elastic lamina (Rothman et al., 1986; Wilimas et al., 1980). Sickle-patient stroke is apparently initiated by the proliferation of cerebrovascular intima (hyperplasia) that narrows specific arteries; the internal carotid, anterior and middle cerebral arteries. Thrombosis at the site follows, resulting in distal thromboembolism and cerebral infarction (Rothman et al., 1986; Merkel et al., 1978). The cause of the initiating intimal thickening is not fully understood, but is thought to derive from endothelial injury and dysfunction, indicating that the genesis of the stroke risk may lie in some unique cranial vessel pathology developed as a result of sickle cell anemia. Supporting this theory, the STOP study found a 8-14% incidence of central nervous system abnormalities in children (Adams et al., 2001). Our lab has studied the related dysfunction of abnormal cerebral endothelial response to shear stress when exposed to sickle plasma. In flow chambers similar to those used in this thesis, cerebral endothelium did not align or extend in the direction of flow (Lola Brown, unpublished data).

A high incidence of thromboembolism and intimal hyperplasia at arterial bifurcations (where blood flow turbulence is common) (Rothman et al., 1986) supports the theory of stroke dependence on high blood flow turbulence. Stroke caused directly by sickle erythrocyte adhesion in the cerebral vesicles has been largely dismissed (Hebbel et al., 1980a), due to lack of correlation between sickle erythrocyte adhesivity

and stroke risk. In the long term, sickle stroke disease may advance to moyamoya disease (Jones et al., 2001; Kugler et al., 1993; Prohovnik et al., 1989), which causes the formation of abnormal netlike vessels and transdural anastomoses. Also, aneurysms are known to result from similar intimal and smooth muscle cell phenotypic changes, resulting in smooth muscle layer atrophy and fragmentation of the internal elastic lamina (Seeler et al., 1978; Merkel et al., 1978).

A further concern for sickle cell anemia patients is the possibility of “silent infarcts,” infarction of brain tissue found on imaging studies in the absence of a history of neurological symptoms (Wang et al., 1998; Armstrong et al., 1996). As the occurrence of cerebral vessel damage greatly increases the chances of stroke reoccurrence, presence of "silent infarcts" is an indicator of high risk for more severe stroke.

Fortunately, preventative treatment for sickle cell stroke has been found, as 90% of strokes in the high risk group (as defined by high TCD values) can be prevented by a chronic red blood cell transfusion program, as detailed through the STOP study (Adams et al., 1998). Unfortunately, chronic transfusion carries with it patient tolerance problems and severe risks of complication, as outlined in 2.7.2. Alternately, bone marrow transplantation has been found to partially reverse the abnormalities in cerebral vessels in addition to reversing other pathologies of sickle cell anemia. (Walters et al., 2000; Mehta & Marks, 1992). There is also some new evidence that the use of hydroxyurea prophylactically greatly reduces the chances of repeated stroke incidence in children (Ware et al., 2004).

### 2.2.6 Additional Clinical Pathologies

Sequestration of sickle erythrocytes in the spleen is another major clinical difficulty in sickle cell anemia. The high rate of erythrocyte turnover in patients results in acute splenetic enlargement, due to trapping of excessive red cell mass (Topley et al.,

1981). This results in a drop of hemoglobin level and peripheral circulatory failure. In turn, this may lead to further splenic enlargement and hypersplenism.

Priapism is another complication of sickle cell anemia, resulting from obstruction of venous drainage of the penis (Gillenwater et al., 1968). This complication presents in both "stuttering" attacks which are brief (<3 hours) and major attacks which persist longer than 24 hours and may be followed by impotence (Fowler, Jr. et al., 1991).

Microvascular occlusion in the arterioles of the eye by vaso-occlusion causes very specific ocular complications, resulting in ischemia of the retinal vasculature (Talbot et al., 1982). Extensive peripheral vascular remodeling results from this complication, causing abnormally developed vessels through a process known as the "proliferative sickle retinopathy." Vitreous hemorrhage with periodic visual impairment or retinal detachment and permanent blindness may result (Condon & Serjeant, 1980).

### 2.2.7 Clinical Pathology Summary

These widely varied organ and tissue complications of sickle cell anemia are all results of the substitution of a single amino acid in the synthesis of hemoglobin. Although the most direct effect of sickle hemoglobin suffered by patients, chronic hemolysis, may be well tolerated in most patients, the vascular occlusion pathology, an indirect effect of sickle hemoglobin synthesis, causes most of the mortality and morbidity in patients through organ and tissue damage (Serjeant, 1993). Vaso-occlusion is thus one of the most serious pathological results of the mutated sickle hemoglobin, prevention of which would have the greatest positive effect on patient lifespan and quality of life. Reviewing the known aspects, theorized mechanism, and potential determinants of vaso-occlusion as well as the variables that affect its frequency, duration, and initiation is thus necessary for any thorough treatment of the aspects of sickle cell pathology.

## 2.3 Adhesive Nature of Sickle Vasculature

### 2.3.1 Vaso-Occlusion and Sickle Erythrocyte Adherence

Sickle cell pathology is driven by an event at the molecular level during the deoxygenation of sickle hemoglobin. The “polymerization” of sickle hemoglobin into long, rigid strands gives rise to the cellular level of the disorder: morphologically distorted, rigid sickle erythrocytes. The low deformability of these cells, in addition to erythrocyte membrane damage and erythrocyte population redistribution resulting from hemolytic anemia, manifest pathologically at the tissue-level as vaso-occlusive complications, the most common of which is the sickle vaso-occlusive event. Changes in the characteristics or activation states of erythrocytes, platelets, leucocytes, endothelium, or vaso-muscular and rheological characteristics of patient vasculature have, both individually and in concordance, all been theorized to determine the frequency and duration of sickle cell pain crises. This speculation arises largely from observed changes in these factors and states during the onset of sickle pain crisis. Unfortunately, the precise mechanism driving vaso-occlusive events have yet to be isolated. Rather than any rigid set of initiating factors, vaso-occlusion may even be caused by a set of biological state variations changing from occurrence to occurrence, triggering pain crisis in a synergistic manner. Studies, however, have isolated several possible key determinants. It is unlikely that any single one of these mechanisms is solely responsible for all sickle cell pain crises, but it is likely that several of these factors, acting in combination, precipitate many incidents of vaso-occlusion.

### 2.3.2 Sickle Hemoglobin "Polymerization"

The pathology associated with sickle cell anemia derives from the deoxygenation and subsequent “polymerization” of sickle hemoglobin. This gelation is strongly

dependent on the cellular concentration of sickle hemoglobin in the sickle erythrocytes. Sickle hemoglobin gelation rate is dependent to the 30th power on intracellular sickle hemoglobin concentration (Ferrone et al., 1985; Hofrichter et al., 1974), explaining why there are few pathologies present in sickle trait individuals (Stark et al., 1980). Sickle cell pathologies, including hemolytic anemia severity and risk for acute chest syndrome, are more severe in patients with higher sickle hemoglobin fractions, whereas patients with sickle trait are usually entirely asymptomatic, as they have only have around 40% sickle hemoglobin in their blood (Montgomery et al., 1983).

Although sickle hemoglobin concentration is strongly correlated with patient clinical condition and frequency of vaso-occlusive pain crisis (Lande et al., 1988; Brittenham et al., 1985), the precise mechanism whereby hemoglobin sickling results in vaso-occlusion has yet to be determined. The rigid nature of sickle erythrocytes as a result of hemoglobin “polymerization” would suggest a simple "log-jam" mechanism. Cells incapable of bending to pass through the capillaries would become stuck, accumulate, and result in vaso-occlusion. This would suggest that patients with high numbers of the most inflexible (or readily sickled, thus becoming inflexible) erythrocytes would experience increased incidence of pain crisis. However, there is no positive correlation between pain crisis frequency and the rigid “irreversibly sickled cell” (ISC) fraction (Ballas et al., 1988; Lande et al., 1988; Billett et al., 1986). Instead it is evident that pain crisis involves alternate mechanisms that may or may not require the presence of inflexible erythrocytes, but are not solely dependent on them. Surprisingly, the most flexible erythrocyte fraction, referred to as “reticulocytes,” correlate strongly with incidents of sickle pain crisis (Ballas et al., 1988). The nature of these erythrocyte fractions is explained at length in section 2.4.2.

### 2.3.3 Thrombotic Activation

The occurrence of sickle pain crisis superficially resembles, in some aspects, the occurrence of thrombotic clot formation within the microvasculature. Both can result in ischemic tissue damage and the rate of thrombin generation and fibrin formation are increased in steady-state sickle patients (Hagger et al., 1995; Peters et al., 1994; Francis, Jr., 1989). Further, naturally occurring clotting inhibitors, such as antithrombin III, protein C, and free protein S, are decreased in sickle patients (Karayalcin & Lanzkowsky, 1989; Cacciola et al., 1989; Francis, Jr., 1988; Green & Scott, 1986). Direct evidence of platelet activation in sickle patients is shown through elevated β-thromboglobulin, (Green et al., 1986) thromboxane $B_2$, platelet factor-4 (Westwick et al., 1983), and depletion of platelet adenosine diphosphate during pain episodes (Beurling-Harbury & Schade, 1989). Additionally, there are increased platelet counts in older children and adults (Westwick et al., 1983; Haut et al., 1973), and reduced platelet survival during pain episodes (Alkjaersig et al., 1976; Haut et al., 1973). These factors point to a state of constant hypercoagulative potential in sickle patients (Francis, Jr., 1991), characterized by constant activation of the coagulation system, rather than an over-response to standard signals. Additional studies have drawn a correlation between disease severity and coagulation state (Westerman et al., 2002). This thrombotic state has the greatest potential for contributing to vaso-occlusion at sites where the intima of blood vessels have been damaged, exposing subendothelial surfaces and smooth muscle tissue. These sites are especially vulnerable to thrombus formation, and partial occlusion of the vessels by platelet accumulation at these sites may directly result in more favorable conditions for pain crisis initiation.

Alternately, thrombospondin (TSP), either derived from activated platelets or as an exposed matrix protein, is known to promote adhesion of sickle erythrocytes (Brittain et al., 1993). Adhesion occurs via CD36 on microvascular endothelial cells (Leung et al.,

1992) in an interaction not seen with non-sickle erythrocytes (Brittain et al., 1993) . It is theorized that increased sickle erythrocyte adhesion could result from the hypercoagulative state of sickle cell anemia by causing endothelial activation and retraction due to elevated thrombin levels, resulting in matrix thrombospondin exposure (Manodori, 2001). Adhesion of platelets or other cells at this site would then result in partial vascular occlusion. Thrombin, another procoagulant factor, also has increased expression in blood from sickle cell patients (Setty et al., 2001), possibly in response to increased expression of phosphatidylserine on sickle erythrocytes. Thrombin is a strong activator of endothelial cells, inducing expression of P-selectin, a ligand that also promotes platelet, leukocyte, and sickle erythrocyte adhesion (Matsui et al., 2002; Matsui et al., 2001).

During pain episodes, sickle cell patients also exhibit highly activated platelet subsets, shown to be degranulated platelets or platelet microparticles (Tomer et al., 2001; Wun et al., 1998). Microparticles are small, membrane-derived vesicles released from cell bodies upon activation or apoptosis. Platelet-derived microparticles are at slightly elevated levels in sickle patients both during patient steady-state (non-crisis) and crisis (Shet et al., 2003). The presence of platelet-derived microparticles during pain crisis further suggests a link between vaso-occlusive events and thrombosis. Other studies have found tissue-factor expression on some of these microparticles, indicating that some are also monocyte or endothelial-derived (Shet et al., 2003). Monocyte-derived microparticles in particular are significantly elevated in sickle patients at steady state, and further elevated during crisis. Presence of these tissue-factor expressing microparticles significantly shortened clotting time in plasma assays (Shet et al., 2003), indicating a possible interaction between the pro-inflammatory (monocyte microparticle expression) and pro-thrombogenic state of the vasculature in sickle patients.

### 2.3.4 Immune Response

Sickle cell patients exhibit elevated levels of leukocytes while in steady-state (not experiencing pain crisis) (Asakura et al., 1996; West et al., 1992), which become further increased during pain crisis (Serjeant, 1985). A correlation has been found between elevated leukocyte counts and frequency of pain crisis (Charache, 1997; Platt et al., 1994) as well as other sickle cell complications such as ACS (Miller et al., 2000) and hemorrhagic stroke (Ohene-Frempong et al., 1998). There is also some evidence linking a prolonged rate of recovery from sickle pain crisis to elevated leukocyte counts (Lipowsky & Chien, 1989; Lipowsky et al., 1987). In addition, decreases in neutrophil and monocyte counts in response to hydroxyurea treatment may be one reason for the improvement after treatment with this drug (Wun, 2001; Charache et al., 1996). These correlations, and the known aggregation of leukocytes in response to inflammation, suggest that leukocytes may play a role in the initiation of sickle vaso-occlusion.

The deformability of leukocytes is less than that of normal erythrocytes (Chien et al., 1984), so the previously mentioned concerns about decreased flexibility of erythrocytes also apply to the presence of elevated levels of leukocytes in the blood, and adhesion of leukocytes in the vasculature is a major cause for concern. A primary function of leukocytes during inflammation is adhesive interaction with ligands expressed on the endothelial surface to initiate trans-endothelial passage (Suffredini et al., 1999). Dysfunction of this interaction either through endothelial damage, excessive expression of inflammatory mediator, or leukocyte dysfunction could lead to excessive adhesion and partial vessel occlusion, initiating sickle pain crisis (see section 2.4.1). Leukocytes may also act as a mediating factor to increase other cell type adhesion (Frenette, 2002) as recently demonstrated in the sickle mouse model (Turhan et al., 2004; Turhan et al., 2002). Adherent leukocytes in the postcapillary venules of transgenic sickle mouse microvasculature were seen to adhere to circulating sickle erythrocytes. Sufficient

accumulation of sickle erythrocytes in this manner may occlude the microvessels and initiate pain crisis.

Leukocytes also reflect the general pro-inflammatory state of sickle vasculature, as adhesion of leukocytes from sickle patients to fibronectin correlates with plasma levels of cytokines in sickle patient blood (Kasschau et al., 1996). Neutrophil adhesion to endothelial cells cultured *in vitro* has also been found to increase significantly when taken from patients in painful crisis (Fadlon et al., 1998; Lachant & Oseas, 1987). The typically elevated levels of cytokines in sickle plasma both during pain crisis and steady-state, therefore, translate into a state of constant heightened activation for leukocytes and monocytes, with leukocytes and other immune cell fractions themselves being further sources of cytokines (Suffredini et al., 1999; Wathelet et al., 1992). Aspects of the proinflammatory state as exhibited by leukocytes and neutrophils in sickle cell anemia include enhanced respiratory burst (Hofstra et al., 1996), increased Mac-1, L-selectin, and CD64 expression (Okpala et al., 2002; Lard et al., 1999; Fadlon et al., 1998), as well as an overall greater potential for response to inflammatory stimulus (Lum et al., 2004) in the form of up regulated and activated CD18 ligand levels. Similar pro-inflammatory activation was detected in monocytes, with increased expression of IL-1$\beta$, TNF-$\alpha$, and CD11b (Belcher et al., 2000). Although monocytes are known to adhere to platelets in sickle cell patients (Wun et al., 2002), the danger of monocytes is more in the subsequent activation of the endothelium than vascular obstruction caused by them alone. A further monocyte contribution to vaso-occlusive events is the microparticles mentioned above in section 2.3.3.

These increases in polymorphonucleocyte fractions, numbers, and various activation states can affect the vasculature through either direct adhesion or through the

induced expression of adhesive ligands following cytokine release. Adhesion may then result in partial vessel occlusion and subsequent full occlusion, initiating pain crisis.

### 2.3.5 Vascular Condition / Tone

As the key determinant of hemodynamic flow through the microcapillary system, the vaso-muscular condition of sickle microvasculature is of interest in the examination of sickle pain crises. Capillary transit time, the amount of time it takes for individual erythrocytes to pass through capillaries, is a measure of microvascular blood flow rate, and an essential factor in the theorized mechanism of sickle vaso-occlusion detailed in section 2.4. The capillary transit time can be altered by the relaxation or contraction of pre-capillary arterioles. A loss of vasorelaxing ability or increase in constriction would thus be consistent with increased incidence of vaso-occlusion in SCA. In accord with this theory, the vasomotor response in sickle patients is distinctly different from that in non-sickle patients, exhibiting a distinct instability which may be key in the causation of sickle pain crises (Ballas & Mohandas, 2004). Overall microvascular tone is the result of a balance of interactions provoking contraction and relaxation. The signals are received and interpreted via the endothelial layer coating the vasculature interior, and acted upon by the underlying muscle cells compromising the structure of the vessels. Damage to the endothelial layer and exposure of the subendothelial matrix (discussed in 2.3.6) may result in dysfunctional response to these signals (Belhassen et al., 2001).

Several signaling factors related to vaso-constriction and vaso-relaxation are altered in patients with sickle cell anemia. The endothelial-derived relaxing factor prostacyclin (Weksler et al., 1978) is released from cultured endothelial cells in response to sickle erythrocyte exposure (Wautier et al., 1986), and, in patients, is found in levels both elevated (Buchanan & Holtkamp, 1985) and decreased (Longenecker et al., 1992) in relation to non-sickle controls. Irregular expression levels of a vaso-relaxing factor

may indicate irregular vasomotor tone, and thus badly controlled or uncontrolled variable microvascular flow.

The vasoactive agent of the greatest interest in sickle cell anemia is the endothelial-derived vasorelaxing factor nitric oxide (NO). Nitric oxide, constitutively expressed in endothelial cells by endothelial NOS (eNOS) (Alderton et al., 2001), is a contributor to basal coronary vasodilator tone and blood flow (Quyyumi et al., 1995). While eNOS is normally associated with vasodilation and various "vaso-protective" actions of the endothelium (Albrecht et al., 2003), it is also known that inactivation of eNOS prevents neutrophil adhesion (Schaefer et al., 1998) and that histamine, a vaso-constricting inflammatory mediator, activates NOS (Li et al., 2003; Yan et al., 1994). Thus NOS production of NO is strangely contradictory in that it seems largely anti-inflammatory, but is also involved in mechanisms that are part of inflammation, such as neutrophil adhesion and histamine response.

NO appears to be a regulating factor of importance in numerous vascular mechanisms and protective functions, including regulation of oxidative enzyme activity and intracellular oxidative stress (Niu et al., 1994; Clancy et al., 1992). NO also regulates flow-dependent expression of VCAM-1 (Tsao et al., 1996) and adhesion of leukocytes (Kubes et al., 1991) and both sickle and non-sickle erythrocytes (Space et al., 2000) to endothelial cells. However, sickle vasculature complicates NO signaling in that sickle vasodilation is blunted and requires much higher levels of NO to elicit a full response (Eberhardt et al., 2003; Belhassen et al., 2001; Aslan et al., 2001). Blood from sickle patients is in a state of constant oxidant stress (Steinberg & Brugnara, 2003; Osarogiagbon et al., 2000) caused in part by the overproduction of reactive oxygen species (Aslan et al., 2001; Dias-Da-Motta et al., 1996). In addition to oxidatively damaging the endothelium, these reactive oxygen species react with NO, quickly removing it from circulation, thus requiring further increased levels of NO before eliciting

the normal vasodilatory response. Also asymmetric dimethylarginine (ADMA), an endogenously produced inhibitor of nitric oxide synthase, has been detected in increased levels in sickle patient plasma, and may further distort NO signaling by blocking the means of its production (Schnog et al., 2005).

Reduced levels of NO metabolites (Morris et al., 2000; Stuart & Setty, 1999; Enwonwu et al., 1990), NO bio-availability, and NO vaso-relaxing effectiveness (Gladwin et al., 2003; Reiter et al., 2002) are all typical of sickle cell anemia. Treatments addressing this NO imbalance have had some success in improving sickle patient condition. *In vivo* dosing of patients with L-arginine is known to promote NO production (Morris et al., 2003; Romero et al., 2002; Vichinsky, 2002; Morris et al., 2000) and has been proposed as a potential treatment for pulmonary hypertension in sickle cell anemia (Morris, 2006). Also, directly inhaled doses of NO have been used to treat acute sickle vaso-occlusive crisis (Weiner et al., 2003), resulting in reduction of pain and reduced hospital stays, and treatment of the acute chest syndrome pathology (Sullivan et al., 1999; Atz & Wessel, 1997) has found reductions in pulmonary arterial pressure and pulmonary vascular resistance. Treatments designed to increase nitric oxide levels in sickle patients may thus reduce severity of sickle-related pathologies and improve clinical outcomes.

### 2.3.6 Endothelial Dysfunction.

The deleterious effect of the sickle mutation influences many organs and systems in the body. Of those affected, the vasculature itself is of particular interest. It is affected not only by ischemia following vaso-occlusion, but also by exposure of the endothelium to sickle erythrocytes and altered inflammatory, thrombotic, and immune factor expression. This contact leads to morphologic and functional changes in the

endothelial bed through the influence of elevated levels of cytokines, free sickle hemoglobin levels, and the sickle erythrocytes themselves.

Morphological differences in sickle patient endothelium have been directly observed in the arterioles and sinuses of sickle cell patient spleen (Klug et al., 1982). The morphologic changes take the form of microfilament formation, cellular edema, nuclear degeneration, and basal lamina thickening, bearing some resemblance to the endothelial dysfunction mentioned in 2.2.5 indicating risk of sickle stroke. Functional changes in the endothelium are also implied by the tenfold increase in circulating endothelial cells (CEC) in sickle patient blood (Solovey et al., 1997). Whereas the shed endothelial cells usually number around 2.0±0.8 cells/ml in blood from non-sickle patients, the circulating endothelial cells in asymptomatic sickle patients are an average of 13.2±11.8 cells/ml, and increase further to approximately 22.8±18.2 cells/ml observed when the patient is in crisis. Lack of apoptotic markers in these circulating cells indicates that the increase in cells was not due to an increased rate of apoptosis, but detachment of viable cells from the endothelial monolayer. This is a strong indicator of widespread endothelial damage (Mutin et al., 1999), and the primarily microvascular phenotype of the cells (Solovey et al., 1997) suggests the microvasculature as the site of chronic injury. Further investigations of these circulating endothelial cells has found them to be in a chronic state of activation (Solovey et al., 2001a; Solovey et al., 1999; Solovey et al., 1998; Solovey et al., 1997). Activation and lack of apoptosis indicates a damaged or perturbed endothelial monolayer as their source, endothelial cells detached from the endothelium mechanically or through some non-apoptotic endothelial pathology, possibly arising from their constantly activated state. Further, the presence of activation markers on the CECs may indicate a direct link between adhesive activation and endothelial injury. Unfortunately, direct examination of the potentially altered sickle microvascular endothelium has not been possible outside of the circulating endothelial

cell model until the recent development of an animal model in transgenic mice (detailed in 2.8).

Morphological alterations of endothelial cells may result from influences of sickle erythrocytes and sickle patient plasma. Chronically elevated cytokine presence, or cell fragments and free iron resulting from hemolysis may be acting on the endothelium and affecting endothelial processes beyond the vasodilatory suppression already mentioned. The proliferation of human vascular endothelial cells is known to be decreased when exposed in culture to sickle erythrocytes (Weinstein et al., 1990). The lack of proliferation stemming from the reduced endothelial DNA synthesis persists for at least six hours after a one-minute incubation. Similar phenotypic alteration was found in the suppression of proper cellular alignment, wherein a five-minute incubation of bovine brain endothelial cells with sickle erythrocytes prevented normal shear-induced elongation and alignment of those cells in the direction of flow for several days (Lola Brown, unpublished data). The lack of alignment suggests a cytoskeletal dysfunction in the endothelial cells brought about by contact with sickle erythrocytes.

These endothelial alterations in response to sickle erythrocytes could be contributing factors to vaso-occlusive events. Endothelial dysfunction in sickle vasculature could lead to reduced capacity for signal response, possible detachment, and a subsequent formation of adhesive junctions or thrombus formation on the exposed subendothelial matrix. In fact, the retraction of endothelium under conditions prevalent in sickle vasculature is a possible initiating factor of sickle erythrocyte adhesion which, in turn, would lead directly to sickle pain crisis (Manodori, 2001; Manodori et al., 2000).

### 2.4 Pain Episode Initiation by Adhesion

The process of vaso-occlusive initiation on a molecular or cellular level is not known (Embury et al., 1994), but certain tissue conditions or patient symptoms are

known to increase pain event likelihood. These conditions include infection, fever, hypoxia, acidosis, dehydration, pregnancy, exhaustion, high altitude, stress, anxiety, anger, and depression.

It is proposed that increased adherence of sickle erythrocytes to the endothelium in response to some of these conditions may initiate or propagate microvascular occlusion (Hebbel et al., 1980a; Hoover et al., 1979). Erythrocytes adherent in the post-capillary venules would partially obstruct the vessel, slow blood flow rate, and increase capillary transit time (Hebbel, 1997a; Wick & Eckman, 1996; Kaul et al., 1989b). This drop in blood flow rate, in addition to allowing for more sickle erythrocyte adhesion, would cause sickling of sickle erythrocytes within the inter-capillary space. The increasingly rigid erythrocytes would be incapable of traversing the capillary under the reduced shear conditions, and accumulate sickled erythrocytes in a "log-jam" like mechanism, the process propagating to full microvascular occlusion. This initiation via sickle erythrocyte adhesion is especially likely as sickle patients exhibit abnormalities of blood and endothelium, resulting in elevated levels of adhesion receptors and dysfunctional endothelium. In this mechanism, preventing erythrocyte adherence would be an obvious way to interrupt the cascade of events leading to microvascular occlusion. Thus, this thesis focuses on the phenomenon of abnormal sickle erythrocyte adherence to the endothelium, examining two inflammatory-mediator-driven mechanisms of adhesion in particular.

*In vitro* and *in vivo* studies have found that sickle erythrocytes are much more adherent to both stimulated and unstimulated endothelial cells than erythrocytes from non-sickle patients. This has been demonstrated through the use of cultured endothelial cells (Hebbel et al., 1981; Hebbel et al., 1980b; Hoover et al., 1979), perfused rat cremaster muscle endothelium (Kaul et al., 1989b), and examination of the newly

created sickle mouse model which found elevated adhesion of different blood cell fractions (Embury et al., 2004; Kalambur et al., 2004; Wood et al., 2004b).

### 2.4.1 Sickle Hemoglobin Sickling and Microvascular Entrapment

Red blood cells in the microvascular circulation need to be flexible to pass through the narrow capillary system. Hemoglobin sickling distorts sickle erythrocytes, increases their rigidity (Itoh et al., 1995), and impedes capillary transit. However, the dynamics of sickling following erythrocyte hemoglobin deoxygenation show there is a significant delay (Hofrichter et al., 1974; Malfa & Steinhardt, 1974) of approximately one second in hemoglobin aggregation, a delay which ensures the escape of erythrocytes into the venous circulation without sickling under normal conditions (Hebbel, 1997a; Mozzarelli et al., 1987). A kinetic model of sickle vaso-occlusion is based on the importance of this delay time (Mozzarelli et al., 1987; Hofrichter et al., 1974). Longer gelation delay times and shorter capillary transit times decrease the chances of erythrocytes being trapped in the capillary or among adherent cells in the post-capillary venules. Conditions which decrease gelation delay times (erythrocyte dehydration, high hemoglobin density, increased temperature, and lowered pH) or extend capillary transit times (reduced blood flow, increased blood viscosity, and partial obstruction of microvascular flow) allow inter-capillary sickling, increasing the possibility of entrapment and vaso-occlusion (Wick et al., 1996; Fabry et al., 1992; Kaul et al., 1989b).

The dependence of microvascular occlusion on capillary transit time means that sickling rate is not the sole trigger of vaso-occlusion caused by sickle cells. Clinically there is no correlation between frequency of patient pain events and the presence of the densest (most-quickly sickling) fraction of red blood cells in patient erythrocyte sub-populations (Billett et al., 1986). Also, microvascular perfusion experiments with the densest sickle cell fractions (Fabry et al., 1989) have not shown a high incidence of

vaso-occlusive events. However, the denser erythrocyte fractions do become trapped more readily, leading to depletion of the densest red cell fraction in patients during pain events, and indicating entrapment or sequestration of the fastest-sickling cells after initiation of vaso-occlusion (Fabry et al., 1984). Therefore, fast sickling erythrocyte fractions may not initiate pain events, but their rigid nature leading to obstruction of slowed post-capillary venules could propagate an event initiated by other means.

### 2.4.2 Sickle Erythrocyte Subpopulations

As this thesis is to be a study of sickle erythrocyte adherence to endothelial monolayers, attention must be directed to the characteristics of blood from sickle patients, particularly the certain sub-populations of sickle erythrocytes most noted for their adhesive properties. The cycle of sickling and unsickling resulting from the production of sickle hemoglobin has a marked effect not only on the lifespan of individual sickle erythrocytes, but also produces a heterogeneity in the shape and density of even viable sickle erythrocytes, creating a number of different subpopulations. These subpopulations are best categorized by their sickling characteristics, and include reticulocytes, discocytes, and irreversibly sickled cells.

Reticulocytes

Reticulocytes are very “young” red blood cells; erythrocytes that have been recently released from bone marrow. This subpopulation of erythrocytes are characterized as being slightly larger than the mature sickle erythrocyte (discocyte), and will still contain some ribosomal RNA (Gilmer, Jr. & Koepke, 1976). Individuals with sickle cell anemia compensate for the stress of hemolytic anemia by requiring higher production of red blood cells (Kaul et al., 1989a). This increased production, combined with a decreased red blood cell life span, results in a much higher proportion of this subpopulation than in non-sickle patients. Sickle patients can have from 5-16% of all

erythrocytes be reticulocytes, with this increase correlating strongly with pain crisis frequency (Baum et al., 1987) while non-sickle patients typically have approximately 1% reticulocytes.

The increased erythrocyte production brought on by anemia and pain crisis causes the production of even younger reticulocyte cells (Westerman & Bacus, 1983) called “stress” reticulocytes (Chang et al., 1997; Browne et al., 1996a; Coulombel et al., 1979). Stress reticulocytes are physiologically different from normal reticulocytes in that they exhibit a multilobulated outer cell membrane and contain dense aggregates of reticulum granules (Coulombel et al., 1979). They are less dense than normal reticulocytes (Browne et al., 1996a). Stress reticulocytes derive from emergency release of underdeveloped erythrocytes from the bone marrow in response to a sudden demand for red blood cells. These cells are in evidence in normal individuals only after rapid loss of large amounts of blood (Chang et al., 1997; Browne et al., 1996a).

There is significant evidence for direct participation of sickle reticulocytes in the propagation of pain crises. The presence of reticulocytes in sickle patient blood increases and then drops over the course of a pain crisis event (Ballas & Smith, 1992) indicating their involvement. Another characteristic of reticulocytes is that they express an excess of adhesive ligands on the surface of the cell, such as $\alpha_{IIb}\beta_3$, $\alpha_4\beta_1$, and transferrin (Swerlick et al., 1993; Joneckis et al., 1993; Leung et al., 1992; Okumura et al., 1992; Seligman et al., 1983). These receptors and ligands are shed as the cell matures (Stone et al., 1996), meaning that mature erythrocytes are less capable of interacting adhesively with other cells. If the mechanism of vaso-occlusion were as proposed in 2.4, with erythrocyte adhesion in the microvasculature partially occluding the capillary system, then selective adhesion of sickle reticulocytes to the endothelium via the excess of endothelial receptors and ligands during pain crisis would account for the sudden reduction in their numbers.

Discocytes

Discocytes are the subpopulation of erythrocytes with sickle hemoglobin that have reached full maturity. These cells are analogous to mature non-sickle erythrocytes (Mohandas & Evans, 1985), and are nearly identical in all biological and mechanical respects while oxygenated. Discocytes are very similar in all viscoelastic properties to mature non-sickle cells (Nash et al., 1984), except for deformability, which is slightly impaired (Itoh et al., 1995; Havell et al., 1978). This is likely due to either the presence of polymerized HbS (Noguchi et al., 1983) or initial damage to the structure of the cell membrane (detailed in section 2.2.1). Upon deoxygenation, the deformability of these cells decreases as sickle hemoglobin aggregates within the cell (Itoh et al., 1995). A transition between regular discocytes and irreversibly sickled cells, described below, are discocytes which maintain their regular shape, but have irregular, damaged surface contours (Mohandas et al., 1985). Repeated deformation of this erythrocyte type by the constant cycle of sickling will gradually transform this cell type into the irreversibly sickled cell type by increasing the polymerized HbS content of the cells (Noguchi et al., 1983) and further damaging the erythrocyte membrane. The rate at which this transformation will occur is highly variable, however, and is strongly dependent on the amount of fetal hemoglobin present in the cell (see below).

Irreversibly Sickled Cells

Irreversibly sickled cells (ISC) maintain the abnormal, distorted shape into which the constant sickling-unsickling cycle has forced them (Mohandas et al., 1985). These cells usually have an elongated, distorted shape even when the cells have been fully oxygenated, often taking the classic “sickle” shape for which the disorder has been named. While the formation of the elongated strands of "polymerized" sickle hemoglobin dictate the shape into which the cells are eventually forced, the cells hold this shape due to extensive damage wrought on the cell cytoskeleton through a spectrin-ankyrin

cytoskeleton rearrangement (Lux et al., 1976), and hemoglobin binding to the membrane (Evans & Mohandas, 1987; Fischer et al., 1975). This causes the cells to have a rigid membrane (Nash et al., 1984; Evans et al., 1984), and an elevated hemoglobin concentration through dehydration of the cells (Browne et al., 1996a; Bertles & Milner, 1968). These cells are unique to patients with sickle cell anemia. ISC levels vary widely from patient to patient, but a positive correlation of clinical condition with ISC concentration has not been found (Ballas et al., 1988; Billett et al., 1986), indicating that the mere presence of inflexible, elongated and distorted cells is insufficient to initiate pain crisis.

As mentioned above, the irreversibly sickled cell fraction of sickle cell blood has the lowest levels of adhesive ligand expression. In addition, the rigid membrane of ISCs may lead to a reduced cell surface area available for interaction with the endothelium (Mohandas et al., 1985). It is thus unlikely that standard adhesive interactions with ISCs drive the vaso-occlusive process. Further, there is a reduction in average blood cell flexibility in sickle patients during the advent of sickle pain crisis, suggesting that more flexible fractions of sickle blood cells are sequestered during initiation (Ballas et al., 1992), excluding ISCs from the early adhesion process.

Red Blood Cell Density

The changes wrought on erythrocytes by the presence of sickle hemoglobin and the constant sickling / unsickling process results in the formation of the erythrocyte subpopulations detailed above. One factor in which these cells strongly differ is in cellular density (Rodgers et al., 1985). Thus the formation of these erythrocyte subpopulations results in formation of a heterogeneous density distribution within a blood sample. Density of an erythrocyte is dependent upon ion and water content of individual cells. The sickling process is known to interfere with cation balances within the cells, resulting in ion (specifically potassium) loss and ion-gradient-driven

dehydration (Brugnara et al., 1985; Luthra & Sears, 1982; Clark et al., 1978). In simple centrifugation separations, discocytes and stress reticulocytes are found predominantly among the lightest fractions, whereas irreversibly sickled cells are found predominantly in the densest fractions (Mohandas et al., 1985; Nash et al., 1984). The separations are not perfect, however, as each cell subpopulation actually fills a distribution curve about the average density, complicating any strict density-based division. Generally speaking, density for sickle erythrocytes progresses from lowest to highest as "stress" reticulocytes, reticulocytes and discocytes, and irreversibly sickled cells (Browne et al., 1996a).

Other factors affect the rate of increase in sickle erythrocyte density by dehydration effects over the lifespan of the cell. Individual sickle erythrocytes may increase in density at drastically different rates, dependent upon the presence of fetal hemoglobin in the cell. Fetal hemoglobin levels are elevated in sickle reticulocytes due to an unbalanced synthesis of the $\gamma$-globin chain at birth (Rogers et al., 1981) which is used instead of the $\beta$ chain in the construction of hemoglobin. For non-sickle patients, fetal hemoglobin is replaced by adult hemoglobin a few months after birth, but sickle patients often retain some expression of this hemoglobin throughout their lives as a side effect of their hemolytic anemia. As fetal hemoglobin (HbF) does not posses the $\beta$–globin chain for sickle cell anemia, it does not "polymerize" under decreased oxygen tension and directly disrupts hemoglobin aggregation (Levasseur et al., 2004; Brittenham et al., 1985; Noguchi et al., 1983). Further, the very high dependence of sickle hemoglobin sickling rate on the concentration of sickle hemoglobin in the cell (Ferrone et al., 1985; Hofrichter et al., 1974) means that any HbF presence dilutes the HbS concentration and dramatically slows the sickling process.

Decreasing cell sickling decreases the rate of dehydration in individual cells (Evans et al., 1984; Bertles et al., 1968), enabling erythrocytes to maintain lower densities and experience longer viability. Therefore, sickle erythrocytes containing some HbF exhibit extended lifespan and decreased density, while dense cells form quickly if fetal hemoglobin levels are low. Improved viability dictates that HbF-containing cells will become over-represented in the sickle microvasculature as non-HbF-containing erythrocytes are removed from circulation. As a result of increasing HbF-expressing red blood cells, hemolytic anemia and all associated pathologies are reduced in severity. In fact, fetal hemoglobin levels have a strong inverse correlation with clinical severity and frequency of sickle pain events (Schechter & Bunn, 1982; Serjeant, 1975; Wrightstone & Huisman, 1974; Jackson et al., 1961). Many sickle cell anemia treatments attempt to ameliorate symptoms by increasing the fraction of fetal hemoglobin in the sickle patient vasculature, including treatments involving 5-azacytidine, myleran, hydroxyurea, erythropoietin, and butyrates (Steinberg, 2003; Yang & Pace, 2001) detailed in section 2.7.2.

## 2.5 Sickle Erythrocyte Adhesiveness

The initiation of sickle pain crisis is theorized to involve adhesion of blood-borne cells to the post-capillary venular endothelial layer. Adhesion of cells in that region would partially obstruct the post-capillary venules, and subsequently slow capillary blood flow. Propagation of the vaso-occlusion would then follow by additional adhesion under lowered shear stresses and simple entrapment of other flowing blood cells, especially the rigid irreversibly sickled erythrocytes in a process colloquially referred to as a "log-jam mechanism." (Hebbel, 1997a; Wick et al., 1996; Kaul et al., 1989b)

Involvement of erythrocyte adhesion in sickle pain crisis is supported by a correlation between adhesivity of sickle red blood cells and patient vaso-occlusive

severity (Ballas et al., 1988; Lande et al., 1988; Hebbel et al., 1980a). The reticulocyte fraction expresses the largest number of erythrocyte adhesion receptors (Swerlick et al., 1993; Joneckis et al., 1993) because these less mature cells express receptors (Hillery et al., 1996; Swerlick et al., 1993; Joneckis et al., 1993; Sugihara et al., 1992) lost or reduced during maturation (Hebbel, 1997b; Okumura et al., 1992; Patel et al., 1985). $\alpha_4\beta_1$ (Swerlick et al., 1993), $\alpha_{11}\beta_3$ (Okumura et al., 1992), CD36 (Wick et al., 1993), aggregated band 3 (Thevenin et al., 1997), phosphotidylserine (Manodori et al., 2000), sulfated glycolipids (Joneckis et al., 1996), and sialic acid (Montes et al., 2002) are all adhesive factors expressed on sickle erythrocytes which may promote adhesion to endothelial cells. Participation of reticulocytes in vaso-occlusive events is also indicated by a sharp drop in their circulating numbers during a pain episode (Ballas et al., 1988; Lande et al., 1988), suggesting that reticulocytes have been sequestered at the sites of occlusion. While this does not prove their participation in initiation, their selective removal from the system the positive correlation between their increased expression and vaso-occlusive frequency strongly implicates them in the propagation of pain crisis.

### 2.5.1 Sickle Erythrocyte Membranes

Although most studies theorize that the more adhesive nature of sickle erythrocytes adhesion is due largely to the adhesive receptors expressed on reticulocytes, as observed in flow assays (Wick et al., 1993; Barabino et al., 1987a; Barabino et al., 1987b) and animal perfusion studies (Kaul et al., 1993; Fabry et al., 1992) , other studies have shown significant adhesivity in the high density subpopulation (Smith & La Celle, 1987; Wautier et al., 1985; Hebbel et al., 1980b). Dense sickle erythrocytes that are more adherent under some conditions than adhesive-factor expressing reticulocytes would indicate adhesion derived from irregularities in sickle erythrocyte membrane surface instead of adhesive ligand expression. However, this

increase of dense erythrocyte adhesion (2.4.2) has been seen primarily under static conditions. This indicates that the adhesive mechanisms for receptor driven adhesion and the non-receptor driven adhesion are profoundly different.

In previous studies (Montes et al., 2002; Montes, 1999) the different types of observed *in vitro* cellular adhesion were categorized according to fluid flow conditions. Those mechanisms predominant under venular flow (at approximately 1.0 dyne/cm$^2$), acting through the stimulated endothelial monolayer interaction with erythrocyte membrane receptors, were termed "high affinity" adhesion mechanisms. Those mechanisms which drove adhesion independent of endothelial activation, but act only at greatly reduced "sludging" flow (approximately 0.1 dyne/cm$^2$) or under static conditions were termed "low affinity" adhesion. The determining factor between these mechanisms appears to be the duration of erythrocyte-endothelial cell contact. "High affinity" is capable of initiating adhesion during the brief contact driven under "high" shear, whereas "low affinity" requires significantly longer contact, implying either low-shear or entirely static conditions, in order to initiate adhesion.

The sites of the adhesive interaction of "low affinity" mechanisms likely derive from the afore-mentioned membrane abnormalities in sickle erythrocytes. These include changes in the orientation of membrane phospholipids (Manodori et al., 2000), autologous immunoglobulin, band 3 clustering (Thevenin et al., 1997), and changes in the charge of the surface membrane (Montes et al., 2002).

The normal erythrocyte outer membrane consists of a bilayer of phospholipids oriented so that the hydrophobic ends of each phospholipid are turned inward, to the center of the bilayer (Op den Kamp, 1979). Composition of the two leaflets of the bilayer are different (Verkleij et al., 1973), the outer one being rich in phosphatidylcholine (PC), sphingomyelin, and glycolipids, while the inner leaflet is high in phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). This asymmetry is

maintained by the action of the enzyme flipase which actively transports specific phospholipids (phosphatidylserine and phosphatidylethanolamine) from the outer to the inner monolayer if they become mixed during the life of the cell (de Jong et al., 2001; Kuypers et al., 1998; Kuypers, 1998; Devaux & Zachowski, 1994).

Sickle erythrocytes exhibit abnormalities in their membrane lipids. The act of sickling affects the phospholipid composition asymmetry, increasing the amount of PE and PS present in the outer leaflets, while increasing the amount of PC in the inner leaflet (Kuypers et al., 1996; Wood et al., 1996; Tait & Gibson, 1994; Chiu et al., 1981). In mature, deformable erythrocytes this change is reversed upon oxygenation (Lubin et al., 1981), but is not reversed in the more thoroughly damaged ISC, alluding that the asymmetry in ISCs results from cumulative damage to the erythrocyte membrane over many iterations of the sickling process, although PS exposing cells are found both in very young and mature erythrocytes (de Jong et al., 2001). Dysfunction of the "flipase" (aminophospholipid translocase) enzyme, possibly through interference of reactive oxygen species in sickle microvasculature (Hebbel, 1991), has been proposed as a reason for the PS asymmetry persisting on older erythrocytes in the sickle mouse model (Banerjee & Kuypers, 2004). Exposed erythrocyte PS correlates with increased blood cell adhesivity (Setty et al., 2002) although it is blocked with the phospholipid-"cloaking" protein Annexin V. Exposed phosphatidylserine is known to serve as a site of endothelial adhesion (Setty et al., 2002) and may act as a catalytic site for thrombotic response (Marcus, 1966), through interaction with thrombospondin (Manodori et al., 2000; Closse et al., 1999). It may also become targeted by the immune system through adherence to cultured human phagocytic monocytes (Schwartz et al., 1985). Targeting by the immune system suggests a mechanism designed to remove from circulation and break down senescent sickle cells, attesting to the hemolytic nature of SCA.

Increased levels of cell-bound immunoglobulin may also be important in the destruction of sickle erythrocytes. Erythrocyte aging studies have found that cell-bound autologous immunoglobulin, factors designed to remove old erythrocytes from circulation, are found on normal cells after aging 120 days, while they act on sickle erythrocytes within 10-40 days of their release from the bone marrow (Green et al., 1985). It is possible that the action of sickling and unsickling damages and artificially "ages" the sickle erythrocyte membrane by causing the exposure of signaling sites for the autologous immunoglobulin. Further, ISCs are known to be targeted by macrophages (Hebbel & Miller, 1984), and the presence of the autologous immunoglobulin may provide the signal necessary for such targeting.

The accumulation of cell-bound immunoglobulin may be partially driven by the presence of band 3 clustering in the sickle erythrocyte membrane. Transmembrane protein band 3 co-clusters with Heinz bodies; portions of denatured hemoglobin from the cytoplasm, which then copolymerize with the cytoplasmic domain of band 3, resulting in the formation of a band 3 cluster (Kannan et al., 1988; Schluter & Drenckhahn, 1986; Waugh et al., 1986). In addition to co-localization of these Hienz bodies with phosphatidylserine and phosphatidylcholine (Liu et al., 1996), the bodies are the signal of an aged erythrocyte to be removed from circulation (Lutz, 1992). Therefore, the unusual targeting of ISCs by macrophages noted above may alternately be in response to the formation of these Heinz bodies. Band 3 may also be directly involved in sickle erythrocyte-endothelial cell adhesion, though the mechanism is unclear (Thevenin et al., 1997).

Lastly, the sickle erythrocyte membranes experience atypical charge distribution. The charge distribution on erythrocytes is determined by the presence of surface glycoproteins containing derivatives of a neuraminic acid called sialic acid. These sialic acids carry a negative charge, imparting an overall negative charge to the surface of the

erythrocyte (EYLAR et al., 1962). Sickle erythrocytes, however, possess less sialic acid than non-sickle red blood cells, resulting in a more positive charge (Ekeke & Ibeh, 1988; Aminoff et al., 1980; Hebbel et al., 1980b), and what they do have is clustered atypically on the surface of the sickle erythrocyte membrane (Wise et al., 1987). As the negatively charged surface has insufficient charge to repel other close-approaching cells, this may provide opportunity for a closer approach to the endothelium, as low-charged portions of the membrane would not be repelled as strongly from the surface (Hebbel et al., 1980b). There is also some indication that the sialic acid aids in sickling recovery (Ekeke et al., 1988), although the mechanism is unknown.

### 2.5.2 Hemodynamic Differences of Sickle Vasculature

Thus far, the background has discussed primarily cellular aspects of sickle erythrocyte adhesion, concerning receptors and membrane factors mediating the cell-cell interaction of sickle erythrocytes with other cell types. However, the mechanism of sickle pain crisis is likely also strongly dependent upon the localized hemodynamics of the sickle microvasculature. As outlined in section 2.4, adherent cells in the post-venular microvasculature would create additional resistance to blood flow, slowing erythrocyte capillary transit time and providing sufficient opportunity for additional adhesion and inter-capillary “sickling” of the sickle erythrocytes as they deoxygenate. Thus the flow rate of blood through the microvasculature is of key importance in the propagation of sickle pain crisis. Of further importance is that, as the blood flow hemodynamics are responsible for the delivery of sickle erythrocytes to the endothelial cell monolayer, flow rate of the blood through the microvasculature also determines the cell-cell contact frequency and duration, effectively changing key factors in the mechanics of sickle erythrocyte adhesion.

Due to this involvement of blood flow rate in adhesive interactions, hemodynamics have to be carefully considered in the construction of any system modeling the processes of sickle cell anemia. For non-sickle vasculature, this presents a fairly straightforward problem. Microvascular blood flow rate is normally regulated and maintained by an elaborate system of signals to the vascular muscle cells. Blood flow is normalized by dilation and contraction of the blood vessels, resulting in an associated shear stress of approximately 1.0 dyne/cm$^2$ in post-capillary venules. Under these conditions blood, while being a non-Newtonian shear-thinning fluid, can be treated as a Newtonian fluid with constant viscosity, as the shear stress would not change throughout any test of blood-substitute flowing through at a constant steady state. Unfortunately, the same cannot be said of the flow rate and rheological properties of blood in sickle patient microvasculature.

Laser-Doppler measurements of blood flow in sickle dermal microvasculature show intermittent or periodic large, local oscillations in flow, and periods of general reduced flow (Rodgers et al., 1990; Kennedy et al., 1988; Rodgers et al., 1984). Studies visualizing blood flow in nail fold capillaries also detected a higher occurrence of slowed and stopped microcirculatory flow in sickle subjects compared to controls (Lipowsky et al., 1987). Even in patients with mild sickle cell disease, conjunctival blood flow velocity is approximately 20% lower than that measured in non-sickle patients (Cheung et al., 2002), while during painful crisis, a further decrease in vascularity (caused by flow stoppage in small vessels) was observed and a 36.7% +/- 5.2% decrease in large vessel (mostly venular) diameter resulted. In addition, the conjunctival red cell velocities either slowed significantly (6.6% +/- 13.1%; P <.01) or were reduced to a trickle (un-measurable) during crisis. These microvascular changes during crisis were transient and reverted to steady-state baseline after resolution of crisis. Flow variation between subjects is high, with some sickle cell anemic patients exhibiting a greater than 90%

reduction in microvascular blood flow (Cheung et al., 2002). Studies measuring the tissue flow rate of blood in sickle patients concluded that the blood flow rate regularly approaches 50% of typical flow during oscillations (Rodgers et al., 1984), giving an associated shear stress of approximately 0.5 dyne/cm$^2$.

Several theories exist to account for this variation in microvascular flowrate, including the generally dysfunctional nature of sickle endothelium as reflected in circulating endothelial cell samples (2.3.6), and the blunted effect of some vasodilators on sickle endothelium (2.3.5). Another possible cause is an unrelated loss of normal response to mechanical stimulation in the patient's muscular arteries (Belhassen et al., 2001). As the shear environment is an important hemodynamic endothelial signal, the altered hemodynamics of sickle microvasculature may exacerbate a chronic elevation of cytokines, and low microvascular blood flow may thus be a source of widespread endothelial damage (Croizat, 1994; Embury et al., 1994; Francis, Jr. et al., 1992). High levels of expressed cytokines in the blood of sickle cell patients may also have destroyed some vasodilator receptor sensitivity, and damaging of the endothelial monolayer (2.3.6), may adversely affect the system by exposing the vascular lumen, directly damaging the smooth muscle layer.

Further altering the hemodynamic properties of blood in sickle patients is the nature of the blood itself. The rheology of blood from sickle cell anemic patients is different from that of non-sickle patients. Plasma viscosity is increased, largely due to higher total protein content (Chien et al., 1982), but whole blood viscosity, when oxygenated, is decreased, due to the anemic aspect of the disorder (Chien et al., 1982; Chien et al., 1970). During deoxygenation, however, the increasing rigidity of the sickle erythrocytes eventually results in a 10-fold increase in viscosity at high shear rates (Danish et al., 1983). Simple extrapolation of this trend in an ischemic tissue site highlights the importance of this variable, increasing viscosity *in vivo*.

Thus the pain crises of sickle cell anemia are known to have a strong hemodynamic aspect. The blood flow through the microcapillaries dictates several of the key mechanisms driving pain crisis, but aspects of both the vascular condition and the blood itself alter the blood flow and the vascular response to that flow *in vivo*. This results in periodically variable, reduced flowrate that may be more disadvantageous, as it may drive the initial adhesion of sickle erythrocytes resulting in pain crisis initiation and propagation.

## 2.6 Signaling Mechanisms Responsible for the Expression of Adhesive Ligands

Adhesion as a possible initiating factor in the occurrence of sickle cell pain crisis and as a major difference between the behavior of erythrocytes in sickle and non-sickle microvasculature has been recognized by the scientific community since first reported in 1980 (Hebbel et al., 1980a). However, while the general inflammatory conditions known to often promote sickle cell adhesion and pain crisis are widely agreed upon, research has been unable to isolate a sole cause of vaso-occlusion, instead uncovering numerous mechanisms which may be responsible for *in vivo* pain-crisis initiating adhesion. As the inflammatory response triggers many cell adhesion mechanisms in the vasculature it is likely that multiple adhesion mechanisms work in concordance in the pathology of sickle pain crisis. This thesis proposes to study two mechanisms in particular, and a brief review of the known characteristics of these mechanisms is provided, in addition to a brief review of plasma factor-derived mechanisms and inflammation in general.

### 2.6.1 Plasma Factors

Plasma separated from sickle patients is known to induce adhesion when incubated with endothelial layers (Mohandas et al., 1985; Mohandas & Evans, 1984), promoting adhesion of subsequently introduced sickle erythrocytes or normal

erythrocytes above unstimulated levels. The precise factor responsible for this cytoadhesive effect in sickle plasma is unknown but likely derives either from signaling agents within the plasma typical of the sickle patients' chronically activated thrombogenic and inflammatory system, or some change in the long-chain proteins that make up the denser sickle plasma fraction. Interestingly, it has also been shown that the mere presence of sickle erythrocytes (washed of their plasma) is sufficient to cause expression of adhesogenic factors on endothelial layers (Shiu et al., 2000).

Studies have employed purified fractions of possible adhesion-promoting plasma factors in an attempt to discern which factors in sickle cell plasma are responsible for the increased adhesion. Thrombospondin (TSP), a large adhesive glycoprotein discussed in brief above (2.3.3) derived from either platelets (Lawler et al., 1978) or endothelial cells (Kramer et al., 1985), is known to interact with microvascular and umbilical vein endothelial cells via the integrin $\alpha_v\beta_3$ (Natarajan et al., 1996; Brittain et al., 1993; Charo et al., 1987), and interacts with sickle erythrocytes via cell receptor CD36 on reticulocyte fractions (Browne et al., 1996a; Sugihara et al., 1992). Due to the elevated levels of reticulocytes in blood from sickle patients, the elevated levels of TSP observed in sickle patients during crisis (Browne et al., 1996a), and the chronic thrombotic state discussed above, TSP may be a strong initiating factor in the occurrence of sickle vaso-occlusive crisis. Further, TSP is expressed in the subendothelial matrix, and treatments which cause cellular retraction, exposing TSP, may promote adhesion, although this adhesion is apparently dependent upon glycosaminoglycans, and not CD36 interaction (Joneckis et al., 1996; Hillery et al., 1996).

von Willebrand Factor is another potentially adhesive plasma factor found in elevated levels in sickle patients (Mackie et al., 1980). Stored in Weibel-Palade bodies of endothelial cells (Lynch et al., 1986), its release is triggered by histamine, thrombin (van Mourik et al., 2002), vasopressin (Mannucci et al., 1975), or fibrin (Ribes et al.,

1987), implicating its irregular expression in sickle cell anemia to both the result of altered vasomotor state and the chronically thrombogenic nature of the disorder. Assays *in vitro* have shown the ability of the high molecular weight fraction of this glycoprotein (Ruggeri & Zimmerman, 1987) to promote sickle erythrocyte adherence to HUVECs both under flow (Wick et al., 1993; Wick et al., 1987) and statically (Walmet et al., 2003) although adherence was relatively weak. The mechanism of adhesion is unknown but antibody blocking of platelet vWF receptor glycoprotein complexes GPIIb/IIIa (Weiss et al., 1993; Lombardo et al., 1985) or GPIb (Handa et al., 1986) in assay inhibits von Willebrand Factor-mediated adherence (Wick et al., 1993).

Interaction with GPIIb/IIIa receptors is also a characteristic of fibrinogen (Ikeda et al., 1991), which has also been shown to increase sickle erythrocyte adherence to endothelial cells (Wautier et al., 1983; Hebbel et al., 1981) under static conditions. Fibrinogen normally mediates platelet adhesion and aggregation, so the elevated levels of this factor in sickle patients during infection (Hebbel et al., 1981) or during acute pain crisis (Lawrence & Fabry, 1986) may be a further reflection of the pro-thrombotic state of sickle cell anemia, and its ability to promote erythrocyte adhesion may contribute to vaso-occlusion.

Fibronectin is another high molecular weight glycoprotein produced by human endothelial cells and released into the supernatants (Ruoslahti et al., 1981). Although levels of fibronectin are not significantly elevated in sickle patients, they are reduced during sickle pain crisis (Bolarin & Adenuga, 1986), suggesting sequestration of the factor and involvement in the mechanism of vascular occlusion. Similar to fibrinogen, fibronectin promotes sickle erythrocyte adhesion in static attachment studies either to cultured endothelial layers (Wautier et al., 1983) or surfaces pre-coated with fibrinogen (Kasschau et al., 1996; Patel et al., 1985). The adhesion, once formed under static conditions to endothelial layers, is sufficient to maintain adhesion under physiologic flow

for normal microvasculature (Wick et al., 1987), but is not maintained for immobilized fibronectin without the endothelial monolayer (Joneckis et al., 1996). Also, neutrophils from sickle patients, when activated by IL-6 or IL-8, exhibit increased adhesion to fibronectin (Assis et al., 2005).

### 2.6.2 Cytokine Agonist-Induced Receptor-Ligand Interactions

Adhesion of sickle erythrocytes to the endothelium is not necessarily accomplished via bridging factors in the plasma. Alternately, the vascular endothelium, when stimulated with specific cytokines, can express a variety of adhesion receptors that directly mediate cell adhesion. Presence of these cytokines drives the thrombogenic, inflammatory, or immune response mechanisms, initiating such actions as localization of flowing leukocytes for transport out of the vasculature into the tissue. The pro-inflammatory, pro-thrombogenic state of sickle microvasculature is reflected in increased levels of circulating cytokines both during pain crisis and at steady state. Chronic cytokine levels determine the chronically activated state of the vascular endothelium (Solovey et al., 1998; Solovey et al., 1997), expressing, or readily expressing, adhesive receptors in excess of normal functionality and likely initiating pain crisis through excessive cell adherence. Supporting this theory is the fact that infection, a trigger of inflammation and associated cytokine expression, has been identified as one of the precipitants of painful crisis, and is a major cause of morbidity and mortality in sickle cell patients (Barrett-Connor, 1971).

Inflammatory events in sickle cell anemia are closely associated with increased incidence of pain crisis. Increased levels of cytokines and inflammatory mediators such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin1 (IL-1), interleukin-6 (IL-6), and histamine found in increased levels in sickle plasma (Kasschau et al., 1996; Malave et al., 1993; Francis, Jr. et al., 1992) are believed to contribute to this association by driving

vaso-occlusion initiating cellular adhesion. As this thesis intends to investigate the specific intercellular interactions of VCAM-1/$\alpha_4\beta_1$, and P-selectin as mediated by TNF-$\alpha$ and histamine exposure respectively, these cellular interactions will be reviewed at length.

### 2.6.3 Tumor Necrosis Factor - $\alpha$ Mediated Adherence

TNF-$\alpha$ is known to cause expression of vascular adhesion molecule-1 (VCAM-1) on cultured endothelial cells (Osborn et al., 1989). TNF-$\alpha$ shares this ability with a number of other inflammatory cytokines including interleukin-1, interleukin-4 (IL-4)(Masinovsky et al., 1990), interleukin-1$\beta$ (Natarajan et al., 1996), bacterial endotoxin (Carlos et al., 1990), and viral-like double stranded RNA (Smolinski et al., 1995). Interestingly, long-term incubation with sickle erythrocytes (Brown et al., 2001) is also known to cause expression of VCAM-1. VCAM-1 expression is relatively slowly developed, as the adhesive ligand is expressed as a result of synthesis following cytokine stimulation. Synthesis reaches maximal levels at about 2.5 hours (Hession et al., 1991; Osborn et al., 1989) and binding mediated by VCAM-1 is typically maximal after 4-6 hours of stimulation (Carlos et al., 1990; Osborn et al., 1989).

The receptor for interacting with VCAM-1 is the integrin (cell-surface heterodimer adhesion receptor for cell ligands) $\alpha_4\beta_1$, also known as very late antigen-4 (VLA-4). Typically this receptor is expressed on leukocytes (Elices et al., 1990; Osborn et al., 1989) where VCAM-1/$\alpha_4\beta_1$ interactions mediate adhesion of leukocytes to the endothelium as part of standard long-term inflammation response. However, the $\alpha_4\beta_1$ receptor is also expressed on sickle reticulocytes (Swerlick et al., 1993; Joneckis et al., 1993), and thus sickle erythrocytes can adhere to endothelial cells expressing VCAM-1 after stimulation with TNF-$\alpha$ (Gee et al., 1995; Swerlick et al., 1993; Vordermeier et al.,

1992) or similar factors (Natarajan et al., 1996; Smolinski et al., 1995). Thus, the standard inflammatory response of the vasculature to events like inflammation inadvertently promotes the detrimental adhesion of sickle erythrocytes and possibly initiate sickle pain crisis.

Further, even at steady-state (non-pain crisis state) VCAM-1 is at increased levels in sickle patient plasma in comparison to non-sickle controls (Duits et al., 1996), and is further increased during pain crisis. Thus, even in the absence of inflammatory incidents, VCAM-1 is expressed in sickle vasculature.

### 2.6.4 Histamine Mediated Adherence

Histamine, an early inflammatory agent mechanistically and kinetically different from TNF-$\alpha$, is also elevated in sickle patients both during asymptomatic periods and further elevated during pain events (Enwonwu et al., 1991). Histamine mediated adherence is of clinical interest because opioid analgesics used for treatment of vaso-occlusive events are known to result in histamine expression, and histamine-related side effects such as itching (Friedman et al., 2001; Chaney, 1995; Fuller et al., 1990; Muldoon et al., 1984). Recent studies have highlighted an even more severe danger associated with the opioid derivative morphine, as orally administered morphine appears to increase the likelihood of the potentially lethal sickle complication ACS (Kopecky et al., 2004) (section 2.2.4), which may be related to the histaminergic effect of the drug.

Histamine is responsible for a number of systemic responses in addition to inflammatory mediation. It is know to control allergic response (Smit et al., 1999), control pepsin release in the digestive system (Ash & Schild, 1966), act as a feedback inhibition loop in neural pathways (Arrang et al., 1983), and is also involved in immune response (Gantner et al., 2002). Histamine-driven cellular signaling mechanisms are similarly varied, including an increase in the tight-junction permeability of endothelial

cells (Ratcliffe et al., 1999), rapid upregulation of IL8 (Utgaard et al., 1998), promotion of neutrophil adhesion, (Schaefer et al., 1998; Ley, 1994; Watanabe et al., 1991) and nitric oxide mediated relaxation in some arteries (Suzuki et al., 2000).

These varied responses to histamine in different tissues are caused by histamine interaction with one or more of the $H_1$, $H_2$, $H_3$, and $H_4$ receptors. Endothelial cells express different levels of histamine "H" receptors (Gantner et al., 2002; Morse et al., 2001; van der Werf et al., 1989; Heltianu et al., 1982) depending upon their phenotype, and binding to these receptors initiates a variety of intracellular signaling responses (Smit et al., 1999; Del Valle & Gantz, 1997; Leurs et al., 1995). Attempts to assign individual receptors to specific signal pathways have lead to ambiguous results. Evidence of interactions between receptor mechanisms at the level of second messengers allows for more elaborate mechanisms of activation. There may be independent methods of activating the same system, systems requiring simultaneous activation of multiple receptors for a single outcome, or negative feedback control of a system by activation of an alternate receptor. Known second messenger interactions are both tissue and species specific, including $H_1$ (Ayajiki et al., 1992) or $H_2$ (Kostic & Petronijevic, 1995) receptor-mediated synthesis of nitric oxide, allowing for the same system to be activated by alternate mechanisms. $H_2$ receptor mediated release of $Ca^{2+}$ (Mitsuhashi et al., 1989; Chew, 1986) or alteration of receptor mediated signal transduction (Mitsuhashi & Payan, 1989) are known examples of cross-effects of one histamine-mediated system on the relative activity of another histamine-mediated system. Understanding the mechanism that drives sickle erythrocyte adherence induced by histamine may prove similarly complicated.

Histamine promotes expression of P-selectin by translocation of the ligand from intercellular Weibel-Palade storage granules (Datta & Ewenstein, 2001; Utgaard et al., 1998) to the membrane surface (Barkalow et al., 1996; McEver et al., 1989; Stenberg et

al., 1985). In general, P-selectin acts to increase the specificity of endothelial cell interactions with platelets and leukocytes during inflammation, coagulation, and atherosclerosis (Varki et al., 1999; Varki, 1997; Rosen & Bertozzi, 1996; Furie & Furie, 1995; McEver et al., 1995; Springer, 1994; Rosen & Bertozzi, 1994). The formation of long P-selectin "tethers" to neutrophils and platelets has also been well established and their dynamics extensively studied (Schmidtke & Diamond, 2000; Utgaard et al., 1998). Expression of factors stored in the Weibel-Palade bodies requires only minutes, but expression of P-selectin peaks at 10 minutes of cytokine exposure and falls to non-stimulated levels by shedding or re-internalization of receptors between 45 and 60 minutes (Sugama et al., 1992). This is a relatively rapidly response of the endothelium to cytokine presence, in comparison to other inflammatory mediators, such as TNF-$\alpha$, which may require hours of stimulation. The difference is largely due to P-selectin being expressed from stores, whereas high VCAM-1 levels need to be synthesized on demand (Vordermeier et al., 1992). Note that the short lifetime of P-selectin expression compared to the long duration of pain events does not eliminate P-selectin as a key interaction. Histamine mediated adhesion could act as an initiator only, halting individual erythrocytes in the post-capillary venules. Once the erythrocyte was stationary, slower-acting adhesive pathways could independently immobilize the red blood cell (Solovey et al., 1998; Solovey et al., 1997; Duits et al., 1996).

Erythrocytes were once thought incapable of interacting with P-selectin, because they do not posses a complimentary ligand (Matsui et al., 2001; Varki et al., 1999). Specifically, the primary complimentary ligand P-selectin glycoprotine-1 (PSGL-1) has not been found on sickle erythrocytes. However, evidence for a sialic acid-containing ligand which interacts with P-selectin and is markedly enhanced on SSRBC has been discovered, although the exact nature of the ligand has not been defined (Matsui et al., 2001), and the involvement of P-selectin participating in erythrocyte adhesion *in vivo* in a

sickle mouse model has recently been observed (Embury et al., 2004; Wood et al., 2004b). Alternately, adhesive interactions between endothelial P-selectin and sickle erythrocytes might occur via exposed phosphatidylserine (PS) on the sickle erythrocytes (Setty et al., 2002; de Jong et al., 2001). PS expression is a result of damage from repeated sickling, as noted above in section 2.5.1, creating a subpopulation of PS-expressing cells (de Jong et al., 2001; Kuypers et al., 1996; Wood et al., 1996). Externally expressed membrane PS is normally corrected by the enzyme flipase (Kuypers et al., 1998; Kuypers, 1998). Sickle erythrocytes expressing PS in patient blood indicate a disabled or overwhelmed flipase function, implying severe red cell damage. Whichever erythrocyte factor interacts with P-selectin, thrombin has caused both non-sickle and, to a greater extent, sickle erythrocyte adherence to endothelium via this ligand (Matsui et al., 2001). As histamine is also known to cause P-selectin expression, it is likely that adherence induced by histamine may cause adhesion by a similar mechanism.

P-selectin is not the only adhesive factor released by histamine from storage in the Weibel-Palade bodies. von Willebrand Factor, which has also been shown to promote sickle erythrocyte adherence, as noted above in section 2.6.1, is also expressed (van Mourik et al., 2002). However, this mechanism does not promote adherence to certain phenotypes of endothelial cells (Brittain et al., 1992). Recent studies have also pointed out that ultra-large von Willebrand Factor multimers interact with P-selectin, P-selectin anchoring the long chain protein under shear (Padilla et al., 2004) to facilitate ultra-large vWF cleavage to less active forms (ULvWF). If the factors driving thrombogenic adherence of platelets to von Willebrand Factor are also the mechanisms promoting sickle erythrocyte adherence to vWF, then von Willebrand Factor-mediated sickle erythrocyte adherence is also highly dependent upon P-selectin

expression for anchoring, further emphasizing P-selectin's importance in sickle pain events.

## 2.7 Sickle Cell Anemia Therapy

Sickle cell anemia is a severe, debilitating disorder whose recurrent clinical complications require repeated hospitalization during the lifetime of patients. A study conducted in 1997 found that there were 75,000 annual hospitalizations between 1989 and 1993, while the average direct cost per hospitalization in 1996 was estimated at $6,300, for a total cost of $475 million per year (Davis et al., 1997). While much of this cost is covered by governmental programs, patients suffer further costs in the form of lost work or school time which can greatly impact their livelihood, and the impact of financial costs felt by patients and their families are further destructive to the overall reduction in quality of life and well being of patients with sickle cell anemia.

While deriving from a single point mutation in the genetic code for hemoglobin synthesis, the resulting complications spread throughout multiple organ systems of the sickle patients and have complex and interacting effects. Such a disorder requires not only study of treatments capable of addressing the source of the disorder, but treatments capable of alleviating or reversing damage and pain caused by the myriad clinical complications, and preventative treatments to blunt the damage caused by these complications. Therefore, research into long term treatments and cure, such as bone marrow transplants, research into prophylactic preventative treatments, such as anti-adhesion regimes, hydroxyurea, and transfusion therapy, and research into short-term treatments, such as pain management, are all needed to best improve the prospects of patients with sickle cell anemia. While research into a genetic treatment capable of completely curing this disorder is in progress and has met with some success, the small population of patients to whom this is a viable option indicates many hurdles that must

be passed before such treatments can be applied to the entire patient population, and thus demands development of more short-term treatments.

### 2.7.1 Genetic Treatments and Cure

Currently the only curative therapy for sickle cell anemia involves the use of a bone marrow transplant. Successful use of this therapy has resulted in replacement of sickle hemoglobin expression with non-sickle (AA-type), or sickle trait (AS-type) hemoglobin, followed by complete secession of vaso-occlusive episodes and hemolytic anemia (Vermylen, 2003; Walters et al., 2001; Ferster et al., 1995b; Johnson et al., 1994; Vermylen et al., 1988). However, the morbidity and mortality risks involved with the bone-marrow-ablative processes used in the standard bone marrow transplant procedure, as well as the possibility of sickle cell disease recurrence (Walters et al., 1997), and various other complications like post-transplant infection (Kalinyak et al., 1995; Johnson et al., 1994) and neurologic complications observed post-transplant (Ferster et al., 1995a) mean that it is considered only for those patients suffering the most severe sickle cell complications (Walters et al., 2000). Further, the current limitations of pharmacologic immunosuppression post-transplant to prevent the onset of graft-vs.-host disease (Ferster et al., 1995b; Johnson et al., 1994) mean that bone marrow transplant is available only to those patients with a full HLA-matched donor, requiring the donor be an identical sibling. Unfortunately, these limitations translate to only approximately 1% of the sickle cell population for whom bone marrow transplant is a viable option (Walters et al., 2000). Further developments in immunosuppression, allowing patient-donor matches with fewer HLA-factors in common, would considerably increase the number of candidates for bone marrow transplant, as would improvements in myeloablative treatments.

Because of these severe limitations on standard bone marrow transplant, alternate therapies have been sought. Hematopoietic stem cells derived from umbilical cord blood (Kelly et al., 1997) are proposed to address donor scarcity. A more complex form of bone marrow transplant may act as a final genetic "repair" to the point mutation at the root of sickle cell anemia. Patient bone marrow would be removed and retroviral vectors would be employed to "re-code" the genetic code for the sickle β-globin as the normal (AA-type) β-globin. Standard myeloablative treatments would follow, and the "re-coded" hematopoietic cells would be used to re-populate the patient's ablated marrow. While still employing the myeloablative treatments, this would completely eliminate HLA-matching limitations, as the patient allograft would already be a perfect match. Alternately, use of a non-myeloablative technique designed to induce mixed hematopoetic chimerism, expression of two compatible genotypes of hematopoietic stem cells in patient bone marrow, has been sought to avoid the dangers of myeloablation (Walters et al., 2001). Despite encouraging results observed in patients with acquired chimerism (Walters et al., 2001; Walters et al., 2000), and in murine models (Kean et al., 2003), bone marrow transplant is likely to remain an option only for pediatric patients due to treatment intolerance in the form of chronic end-organ diseases in adults (Vermylen, 2003).

### 2.7.2 Prophylactic Treatments and Preventative Care

One of the most direct forms of preventative care for sickle patients is the use of regular transfusions. Replacement of a substantial quantity of a patient's sickle-hemoglobin blood with transfusion blood temporarily dilutes the remaining blood of sickling cells, relieves anemia symptoms, drastically improves oxygenation and provides relief from pain crisis occurrence and hemolysis (McIntire et al., 1980). However, as

mentioned above (2.2.1) chronic transfusion therapy also carries a number of risks, including the possibility of iron overload (Davies et al., 1984; Sarnaik et al., 1979) requiring treatment with chelating agents (Kwiatkowski et al., 2004), alloimmunization (Wenz et al., 1982), and infection risk (Francis, 1991). As a result, transfusion therapy is typically used only as a preventative measure in patients exhibiting symptoms of severe sickle cell anemic complications such as stroke (Adams et al., 2004; Pegelow, 2001; Adams et al., 1998) and acute chest syndrome (Emre et al., 1995).

Alternate therapies for sickle cell anemia include the application of agents to prevent, minimize, and/or delay the sickling process when sickle hemoglobin is deoxygenated. One alternate form of hemoglobin from both normal “AA” and sickle “SS” hemoglobin that is present in sickle patients is "fetal" hemoglobin. So named because of its presence in fetuses, it is necessary for the transmission of oxygen from the mother’s bloodstream. In normal human development, the production of fetal hemoglobin drops to negligible levels within weeks following birth (Rogers et al., 1981). However, the pernicious nature of the hemolytic anemia in sickle cell patients usually results in elevated levels of fetal hemoglobin in patient blood. Because fetal hemoglobin inhibits gelation of sickle hemoglobin (Rogers et al., 1981; Nagel et al., 1979; Moffat, 1974), and because it thus improves erythrocyte longevity, there is a strong inverse correlation between fetal hemoglobin levels and the vaso-occlusive complications of sickle cell anemia (Powars et al., 1984; Stevens et al., 1981). Techniques that could further raise this proportion could be applied to improve patient condition by reducing the incidence of pain crisis and the severity of patient anemia.

Hydroxyurea (Hu), a ribonucleotide reductase inhibitor and a cancer treatment for use against leukemias, is a drug capable of reducing the incidence of sickle pain crisis and other severe sickle complications (stroke, ACS) in highly symptomatic patients (Vichinsky, 2002; Charache et al., 1995). Use in treating sickle cell anemia has resulted

in a 40% decrease in mortality within a high-risk subset of sickle patients (Steinberg, 2003). A large multi-center study of the drug (Charache, 1997; Bunn, 1997) found that regular oral administration of hydroxyurea resulted in 44% decrease in mean painful crisis incidence, as well as decreases in the number of acute chest occurrences and reduction in the number of transfusions required in patients. The action of hydroxyurea believed to be responsible for the long-term improvement of sickle patient condition is the increase in HbF production promoted by the drug (Ware et al., 2002), but the clinical improvements resulting from hydroxyurea treatment before HbF increase is observed (Ballas et al., 1989) is not fully understood. The improvement may occur through improved erythrocyte hydration, anti-sickling effects of the drug, rheologic improvements in blood flow, or reduction of neutrophil and leukocyte counts (Wun, 2001; Charache, 1997; Charache et al., 1992; Kaufman, 1992; Goldberg et al., 1990). Hu use is considered relatively safe and effective in preventing sickle crisis, although major complications during treatment may still occur. Further, it's efficacy is limited, in that a sizeable proportion of patients (~40-60%) do not respond at all to hydroxyurea treatment (Amrolia et al., 2003; Steinberg et al., 2003). Side effects of the drug can be serious as well, leading to myelosuppression and leg ulceration, in addition to concerns about the unknown risks of long-term exposure to the drug (Amrolia et al., 2003; Chaine et al., 2001; Hanft et al., 2000; Steinberg, 1999). Alternate treatments intended to induce production of fetal hemoglobin include short-chain fatty acids such as four-carbon-chain butyrate-derived compounds (Liakopoulou et al., 1995; Faller & Perrine, 1995), 5-aza-2'-deoxycytidine (Saunthararajah et al., 2003) and hydroxyurea analogs such as Zileuton (Haynes, Jr. et al., 2004), in attempts to avoid some of hydroxyurea's cytotoxic side effects (Atweh et al., 2003).

Hydration of sickle erythrocytes is also a potential sickling-prevention treatment. Hydration of the erythrocytes will act to dilute the sickle hemoglobin within the

erythrocytes and greatly reduce their sickling rate (Ferrone et al., 1985) and subsequent cellular damage. Several drugs designed to address erythrocyte dehydration do so through the blocking of potassium egress from the cells. Calcium-mediated potassium ion channels in the erythrocyte membrane called "Gardos" pathways (GARDOS, 1958) provide one potential route, and drugs known to block these channels include charybdotoxin (Ohnishi et al., 1989; Wolff et al., 1988), quinidine and nitrendipine (Ellory et al., 1992), clotrimazole, and similar imidazole antimycotics (Stuart et al., 1994; Alvarez et al., 1992), with numerous variations of these drugs under investigation.

Finally, rheologic effects serve as another target for the prevention of sickle pain crisis. Aside from simple observance of proper transfusion protocol to prevent excessive accumulation of red cell beyond 35 volume percent, where blood viscosity increases and worsens existing infarctions (Jan et al., 1982), a number of treatments have been used to alter the rheologic conditions present in sickle vasculature. It is hoped that by reversing the flow abnormalities (2.5.2) arising from complex interactions between sickle erythrocytes, endothelium, platelets, and mediators of inflammation and thrombogenesis, as well as improving flow conditions by improving erythrocyte flexibility and reducing blood viscosity, microvascular occlusion incidents can be reduced. The ability of corticotrophin and pentoxifylline to reduce blood viscosity and improve sickle cell deformability have been investigated (Keller & Leonhardt, 1979). Similar results have been seen with the use of a perfluorochemical (Fluosol-43) (Reindorf et al., 1985), a polyol nonionic surfactant (Pluronic F-68) which acts as a dispersing, wetting and defoaming agent (Smith et al., 1987) and also abolishes sickle erythrocyte adherence, and the nonionic block copolymer surfactant RheothRx (Adams-Graves et al., 1997). Unfortunately, several of these treatments have toxicity problems (Vercellotti et al., 1982; Endrich et al., 1979; Yokoyama et al., 1975) or operate via mechanisms only partially understood (Toth et al., 1997).

### 2.7.3 Short-Term Treatment and Care

The most common and widespread complication experienced by patients with sickle cell anemia is the pain crisis associated with vaso-occlusion. Unfortunately, there are currently no treatments available for administration during pain crisis which will quickly reverse the condition or shorten the duration of the vaso-occlusion, although there is promising research in the field of inhaled nitric oxide therapy recently that may significantly shorten vaso-occlusion duration and additionally aid in acute chest syndrome (Weiner et al., 2003).

Current patient care techniques during pain crisis are symptomatic treatments, and include antibiotic treatment for patients whose pain crises may have been initiated as a result of infection-related inflammation, hydration, administration of analgesics for the treatment of associated pain, and counseling on avoidance of conditions likely to initiate pain crisis (Benjamin, 1982). Studies (Chapter V & VI) conducted in this thesis, however, have highlighted the importance of proper analgesic selection, as associated inflammatory mediator release in response to certain opioid analgesics may initiate or further exacerbate patient pain crisis. This is further emphasized by the correlation drawn between increased incidence of severe sickle cell complication acute pain crisis and the use of morphine in treatment of sickle cell patients (Kopecky et al., 2004).

## 2.8 Animal Models

Special note needs to be made of a recent development in the study of sickle cell anemia. The development of an animal model for the study of sickle cell anemia has long been an objective of the field because of its immense potential for explaining and illustrating the many factors in action during pain crisis, and for examining and devising treatments for the resulting organ damage. With an animal model, invasive observation techniques impossible with human subjects could be conducted and the efficacy of anti-

vaso-occlusive event drugs could be observed directly, offering immense insight into the pathophysiology of sickle cell anemia and quickly advancing the development of sickle cell treatments. Unfortunately, no other animal naturally carries the gene for sickle hemoglobin, so an animal model had to be transgenically developed.

Before the current sickle mouse model, several other mouse models were developed and had some success in expressing different amounts of sickle-patient like blood. These models included the SAD-1 mouse (Trudel et al., 1994), the Constantini-Fabry-Nagel (NYC1) mouse (Roy et al., 1993), and the S+S-Antilles model (Fabry et al., 1995) all of which exhibited some pathologies useful for the study of sickle cell anemia. However, the recent advances in the field of transgenics have resulted in a transgenic sickle mouse model expressing exclusively human globin chains (Paszty et al., 1997; Ryan et al., 1997) and given scientists a model more useful than any previous attempt. Although there are some notable differences (Manci et al., 2006), such as low expression of HbF, evaluation of the transgenic model found remarkable similarities between the pathologies exhibited by the mice and those seen in sickle patients. Studies evaluating the vascular characteristics of these mice have found the similar loss of vaso-motor tone and the blunted response to vaso-relaxing agents like nitric oxide (Nath et al., 2000; Kaul et al., 2000b). Further similarities were the presence of pro-inflammatory state cytokines, increased leukocyte-endothelium interaction, endothelial oxidant generation, and significant flow abnormalities (Nath et al., 2000; Kaul & Hebbel, 2000a). TNF-$\alpha$ driven microvascular occlusion via blood cell adhesion (Turhan et al., 2002), increased expression of adhesive ligands like E -selectin and P-selectin (Wood et al., 2004a; Wood et al., 2004b), and well documented organ and blood vessel pathology (Manci et al., 2006; Chang et al., 1998; Paszty et al., 1997; Ryan et al., 1997) were observed. Current studies involving these transgenic models cover a wide range of sickle-related topics, from examination of brain vasculopathy (Wood et al., 2005; Wood

et al., 2004b), pain crisis initiation (Belcher et al., 2005), cure variants on bone marrow transplant and chimerism (Kean et al., 2003), or genetic treatments (Chang et al., 2006).

## 2.9 Summary

The inherited genetic disorder of sickle cell anemia is the most common heritable hematological disease. Although a proven cure has been found in bone marrow transplantation, this cure carries with it additional risks and an inherently limited population applicability, due to a lack of appropriate HLA-matched donors for sickle cell anemic recipients. Other current treatments, such as chronic transfusion or hydroxyurea, while temporarily alleviating the pathology associated with sickle cell anemia, also have long-term risk factors and complications that make their continuous use undesirable. Attempts to pharmacologically address the clinical complication of vaso-occlusive pain crisis, the painful, ischemically damaging microvascular occlusion believed to be initiated by sickle erythrocyte adhesion and leading to tissue damage, organ dysfunction, and death, should thus be continuously developed in parallel with those studies designed to address the disorder at the genetic level. The aim of pain crisis studies should be the development of a safe therapeutic agent administered during crisis for lessening the vaso-occlusive severity, or, alternately, administered prophylactically for the prevention of pain crisis.

The complex nature of vaso-occlusive crisis requires a thorough understanding of the altered nature of sickle microvasculature and the altered blood cells with which it is interacting. Studies have pointed to profoundly altered blood cell membranes, activated and chronically damaged endothelium, and irregular blood flow in sickle patients. Extensive *in vitro* and more recent *in vivo* studies have focused on the adhesive interaction between various blood fractions and the endothelial layer, characterizing many potential inflammatory, thrombogenic, or immune-response related

mechanisms now known to promote the adhesion of sickle erythrocytes to stimulated endothelial cells. Despite extensive investigation, a comprehensive, accurate model of sickle pain crisis has yet to emerge. This implies that there are yet factors unaccounted for in previous examinations of sickle pain crisis.

One potential missing piece in the understanding of sickle pain crisis is the inflammatory mediator histamine. Capable of causing rapid expression of adhesive ligands from endothelial monolayers, histamine is of special interest because of the histaminergic nature of several standard treatments for sickle pain crisis.

Further missing from the current model of sickle pain crisis is an accounting for the altered blood flow found in sickle patient microvasculature. Blood flow in sickle microvasculature is known to be oscillatory and decreased, which, as a primary factor in determining cell-cell interaction frequency and duration, is a key factor for understanding the progression of sickle erythrocyte adherence. However, few studies address this atypical blood flow, examining adhesion under non-sickle flow rates or statically.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Endothelial Cell Culture

### 3.1.1 Cell Sources

Human umbilical vein endothelial cells (HUVECs) was the primary endothelial cell type used in these studies. Human dermal microvascular endothelial cells (dMECs) was a second cell type used in a few controls. HUVEC cells were purchased as variable cultures at first or second passage from the Emory Skin Diseases Research Center (Atlanta, GA) in either confluent T-75 flasks or frozen ampules. In some experiments HUVECs frozen at first passage from Clonetics (East Rutherford, NJ) were used. No phenotypic difference was observed in cultures between the two cell sources during cell adhesion assays, but the cells from the Emory Skin Diseases Research Center were preferred as they displayed faster proliferation up to the target passage.

dMECs were purchased at 2nd or 3rd passage from the Emory Skin Diseases Research Center at Emory University School of Medicine. Cells were received as frozen cryovials of cell suspension or T-75 flasks with confluent endothelial monolayers of endothelial cells.

### 3.1.2 Cell Thawing and Culture Protocol

Adhesion assays used HUVECs at passage 6-7. To reach this passage, cells stored in frozen form were plated to T-75 flasks (Corning, Corning, NY) by the following protocol: A T-75 flask was pre-coated with 6 ml of a 0.1% porcine gelatin (Sigma-Aldrich, St. Louis MO) solution in Delbecco's Phosphate Buffered Saline Solution (DPBS, Sigma) and incubated overnight (minimum of 5 hours) at 37°C. The gelatin

solution was then aspirated from the flask and a cryovial of cells, thawed in a water bath, was added. Ten ml of warmed, complete HUVEC growth media (defined in detail below) was then added dropwise over ten minutes to reduce the effects of any sudden osmotic imbalance which might otherwise damage the endothelial cells. While adding the HUVEC media, the T-75 was rocked by hand to ensure even distribution of the cell suspension over the culture surface. A further 5 ml of complete media were added after ten minutes for a total suspension volume of 15 ml. A second technique for thawing of cells was later employed when a decline in quality of cell proliferation and confluency was noted in several newly-thawed cell cultures (which, as a result, were not employed for adhesion studies). Suspecting that the extended initial exposure of the cells to traces of dimethylsulfoxide (DMSO, Sigma-Aldrich) from the freezing solution was affecting growth, the thawed cryovial was instead transferred to a 15 ml centrifuge tube and centrifuged for 10 minutes at 100 g. The DMSO-containing supernatant was aspirated, the cell pellet resuspended in 15 ml HUVEC media, and the solution was placed directly in a pre-coated T-75 culture flask.

HUVECs were fed every 48 hours following thawing with 15 ml of fresh, warmed (37°C) complete HUVEC growth media, and grown to 90-95% confluency prior to passaging. The complete HUVEC growth media used to feed the cells consisted of the basal media M199 reconstituted from powder (Sigma-Aldrich), supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich or Atlanta Biological, Atlanta, GA), ~2 mmol/L L-glutamine, 90 U/ml penicillin, 90 μg/ml streptomycin, 17 U/ml cell-culture grade heparin (all from Sigma-Aldrich), and 25 μg/ml endothelial cell growth factor (Roche Biologicals, Indianapolis, IN). Media was made only in ~300 ml batches to avoid denaturing of growth factors that would follow repeated warming and cooling cycles during standard feeding protocols.

Cells were grown out to fifth passage by culture in a sequence of T-75 flasks. After reaching 90-95% confluency (which took from 3-5 days, depending on the cell passage), cells were split 1-3 into either new T-75 flasks and cultured or into 3 cryovials and frozen down for storage in liquid nitrogen. Passaging was accomplished by the following protocol: The endothelial monolayer was washed twice with 6 ml of warmed M199 basal media in order to remove long-chain proteins from the heat-inactivated FBS, which would otherwise interfere with the action of trypsin. 4 ml of trypsin/EDTA solution (0.05%, GIBCO Laboratories, Grand Island, NY) was then added to the T-75 flask and incubated for one minute. The flask was inspected under the microscope and agitated as necessary to remove endothelial cells from the surface. 6 ml of complete growth media (either HUVEC or dMEC growth media, depending on the culture) was added to the flask to neutralize the trypsin, the solution transferred to a 15 ml centrifuge tube, and then centrifuged at 100 g for 10 minutes. Following centrifugation, the supernatant solution was aspirated and discarded. If being prepared for transfer to culture flasks, the cell pellet was re-suspended in 3 ml of complete growth media, and divided equally into three gelatin pre-coated T-75s as detailed above. If the cells were to be frozen down for storage, the cell pellet was resuspended in 3 ml of an ice-chilled "freezing solution" consisting of 90% (by volume) heat-inactivated FBS and 10% DMSO (Sigma-Aldrich). This cell suspension is then divided into three sealed cryovials (Nalge-Nunc International, Rochester, NY), placed in an isopropanol-filled cell holder (Nalge-Nunc International), and cooled in a -70 freezer for 24 hours before being placed in long-term storage within a liquid nitrogen dewar. Cells were typically stored at 3$^{rd}$ and 5$^{th}$ passage for later use.

dMECS were similarly passaged, cultured, and stored with identical protocols and feeding schedules, but microvascular endothelial growth media was substituted for HUVEC media in all cases. Microvascular endothelial growth media (EGM-MV) was

purchased from Cambrex (East Rutherford, NJ). The precise makeup of the media is a proprietary formula maintained by Cambrex. Hydrocortisone supplements provided by Cambrex as part of the media formulation were not added to the media as they might have affected adhesion-based studies.

### 3.1.3 Endothelial Cell Culture for Adhesion Assays

HUVEC cells intended for adhesion assays were thawed and cultured in T-75 flasks as detailed in 3.1.2. Cells were trypsinized at fifth passage and resuspended in 3 ml of complete media, again as described in 3.1.2. The numbers of endothelial cells were determined by staining a sample of cell suspension with trypan blue (Sigma-Aldrich) for viability and counting unstained cells in a hematocytometer. Cells were plated on Labtek® permanox single-well chamber slides (Nalge Nunc International) that had been coated overnight with 2 ml of the 0.1% porcine gelatin solution in the same manner as preparation of the T-75 flasks detailed in 3.1.2. The number of plates and the total volume of media used in the cell suspension were adjusted such that the cells were plated at approximately 15,000 cells per single-well chamber. The number of cell chambers prepared was determined by the anticipated flow assay for that week, with 3-4 additional plates prepared in case of contamination or uneven confluency. To ensure even cell distribution on the rectangular slides, a custom protocol was developed. The single-well chambers were tilted (~10°) by positioning one end of the plates on a 1 ml pipette, placed lengthwise along the row of single-well chambers. The cell suspension (2 ml apiece) was then placed in each chamber slide and incubated for five minutes. Afterwards, the cell suspension was aspirated and pooled. The plates were tilted in the other direction, and the cell suspension added to each plate for another five-minute incubation. Finally, the plates were aspirated and re-filled with the plates lying flat. This technique ensured that cells populated the entire surface of the chamber slide, rather

than selectively populating one side or the center only. These cells were then grown to full confluency on the slides before use in adhesion assays. Confluency was typically reached within 2-4 days, and media was changed every 48 hours.

dMEC cells were treated identically as HUVEC cells, except that complete MEC growth media was used in place of complete HUVEC growth media, and at each stage of the single-well chamber slide culture process, the cell suspension was allowed to settle for 6, not 5 minutes within the slides. In addition, media for dMECs in chamber plates was first exchanged within 12 hours of plating. These changes were part a custom protocol developed in response to apparent differences in the two endothelial cell culture growth and attachment characteristics.

### 3.1.4 Alternate Endothelial Cell Culture for Adhesion Assays

In a limited number of experiments, the single well chamber slides were not available. As a substitution, plates of the appropriate dimensions were cut from the cell-culture surface of T-75 flasks by scoring it with a heated scalpel and fracturing the surface along the scored lines. The plates were carefully trimmed and filed to fit the flow chamber, and then sterilized using a UV sterilizer, 1 hour of exposure to each side. Plates were pre-coated with the gelatin solution by placing 4-5 cut plates in 75 mm Petri dishes, culture treated side up, and careful addition, without spilling into the Petri dish, of ~ 1.75 ml of the gelatin solution from 3.1.2 to the culture surface, for overnight incubation. Addition of the cell suspension was accomplished similarly, the gelatin solution being aspirated and replaced with the cell suspension, being sure not to spill the cell suspension from the cell culture surface. Subsequent feedings every other day were accomplished by filling the Petri dish until all the plates were submerged (~20 ml of complete media), and being careful to ensure that the plates did not rest on top of one another. All other protocols for these plates were identical.

## 3.2 Erythrocyte Suspension

### 3.2.1 Blood Sample Acquisition

Whole blood was collected from asymptomatic volunteers with homozygous sickle cell anemia by means of venipuncture during regular clinic visits to the Georgia Comprehensive Sickle Cell Center at Grady Memorial Hospital in Atlanta, Georgia. Study subjects were screened by the center staff to be sure they were not taking hydroxyurea, had not recently received transfusions, and were not pregnant, thromboembolic, nor exhibiting signs of infection or liver disease.

Blood was drawn into heparin anticoagulant-charged Vacutainer tubes (VWR, West Chester PA) provided to the center. Written informed consent was obtained from each subject before sample collection, in accordance with protocols approved by the investigational review boards of the Georgia Institute of Technology, Emory University School of Medicine, and the Research Oversight Committee of Grady Memorial Hospital, and also in accordance with the principles of the Declaration of Helsinki. Blood samples given to our lab through these protocols had all patient-specific identifiers removed, as per IRB requirements, thus "blinding" the samples and preventing the tracing of blood samples back to specific patients. As such, it is unknown the frequency with which blood was received from specific patients or the total number of different patients studied, only the total number of samples.

Blood samples collected at the hospital were retrieved for laboratory work within 4 hours of drawing, and kept refrigerated both at the hospital and within the lab before use in experiments. (Exception to this schedule is noted below.)

### 3.2.2 Erythrocyte Preparation for Adhesion Assays

Within 1 hour of beginning adhesion assays, whole blood was transferred to a 15 ml centrifuge tube and centrifuged at 100 g for 10 minutes at room temperature. (For blood sample volumes smaller than 1 ml, a microcentrifuge tube was substituted for the 15 ml centrifuge tube, and a microcentrifuge was employed at the same 100 g for the same period of time. This was necessitated by the difficulty in handling and separating very small volumes of blood, and the smaller diameter of the microcentrifuge tube aided in discerning the different layers of the blood sample.) The plasma supernatant was then removed by Pasteur pipette aspiration and discarded, taking care to also remove the buffy coat. The erythrocyte fraction was then washed twice with 3-4 ml of a DPBS solution (or 1 ml in the case of samples smaller than 1 ml), supplemented with 0.2% (w/v) human albumin, 5 μg/ml human transferrin and 5 μg/ml of bovine insulin (all from Sigma-Aldrich). Erythrocyte washing diluted the heparin anti-coagulant to prevent its effect on sickle cell adhesion (Embury et al., 2004; Matsui et al., 2002; Barabino et al., 1999). It also removed most leukocytes and essentially all platelets from the sample, but some minor leukocyte contamination likely remained (Brown et al., 2001).

Washed red cells were reconstituted to approximately 20% hematocrit by addition of a serum-free medium, consisting of MCDB-131 (GIBCO) supplemented with 87 U/ml penicillin and 87 μg/ml streptomycin (GIBCO), 0.01 μg/ml epidermal growth factor (Clonetics, San Diego, CA), 0.292 mg/ml L-glutamine, 0.277 mg/mL cyclic adenosine monophosphate (AMP), 0.2% wt/vol) human albumin, 5μg/mL human transferrin, and 5 μg/mL bovine insulin (all from Sigma-Aldrich). The ~20% hematocrit solution is reconstituted by resuspending the erythrocyte solution in SFM to its original volume + 1-2 ml. All erythrocyte solutions were warmed to 37°C for use in flow assays. The hematocrit of resuspended erythrocyte solutions was determined by using a

hematocrit microcapillary tube (GIBCO), hematocrit centrifuge, and microcapillary reader (both from Damon/IEC division). This measurement was used in determining mixing proportions for erythrocyte perfusion solutions.

A 0.22% hematocrit washed sickle erythrocyte suspension was reconstituted with serum-free media (SFM) from the 20% hematocrit stock solution immediately before use in adherence assays. The 20% solution was used as a "stock solution" to avoid degradation of the blood sample (Montes, 1999) over the duration of the experiments (as much as 14 hours).

### 3.2.3 "Day Old" Blood Samples

In a limited number of cases, blood samples were not available immediately after drawing them from the patient. In some cases, these blood samples were not available for use until 24 hours following phlebotomy. The samples belonging to this "day old" group were carefully distinguished during experimentation, but the data were compiled into the same set with "fresh" blood samples. A comparison of data between "day old" blood and "fresh" blood for the applicable experiments (sickle erythrocyte adherence induced by histamine stimulation, Chapter IV) found the response in all cases where "day old" blood was used to be qualitatively similar to that for "fresh" blood. However, total adhesion was quantitatively reduced for the baseline (unstimulated) adhesion for "day old" blood. (Figure 3.1) Upon analysis, this difference was not found to be statistically significant for either unstimulated ($P=0.193$), or histamine stimulated ($P=0.984$) adhesion.


**Figure 3.1: Sickle Erythrocyte Adhesion is not Significantly Altered by 24 hour Storage** Sickle erythrocyte adherence following 40 minutes of perfusion with or without 100 μM histamine stimulation after erythrocyte storage as whole blood for ~4 ("Fresh") or ~24 ("Day Old") hours. Data are mean ± standard deviation for n=14 ("Fresh) or n=29 ("Day Old") blood samples. No significant difference found between "Fresh" and "Day Old" samples for either "Histamine stimulated" or "Unstimulated" data.

## 3.3 Stimulation Protocols

### 3.3.1 Endothelial Cell Stimulation with TNF-α

Cytokine treatments applied to endothelial cells differed according to the desired effect and the cytokine employed. The cytokine tumor necrosis factor α (TNF-α) requires a relatively long incubation time with the endothelial cell monolayer to promote the desired adhesive ligand expression (Montes et al., 2002; Swerlick et al., 1993). The protocol used to promote the expression of vascular cell adhesion molecule -1 (VCAM-1) in a time-dependent manner is as follows: Cell cultures were grown to confluence at sixth passage in individual single-chamber Labtek® Permanox cell-culture plates. The plates were then rinsed twice with 2 ml warmed basal medium M-199, and incubated with 2 ml of TNF-α (Sigma-Aldrich) solution for a fixed duration. Concentrations of TNF-α used in these studies were 100, 200, and 500 U/ml in fresh HUVEC media for an incubation time of 6 hours. Alternately, the TNF-α solution was kept constant at 500 U/ml, and the exposure time was varied, used at 2, 4, 6, or 8 hours. In each case, the individual slides were stored in the incubator for the duration of the stimulation. Following stimulation, the slides were disassembled, rinsed with basal media, and placed in the appropriate flow chamber as detailed below. As the duration of TNF-α stimulation stretches over so many hours, the stimulation schedule is structured such that multiple plates are being stimulated simultaneously during the experiment.

### 3.3.2 Endothelial Cell Stimulation with Histamine

The histamine signaling cascade leading to the expression of adhesive ligands is active within minutes, and not hours, of initial exposure (Lorenzon et al., 1998). As a result, the time required to assemble the flow chamber, as well as the time required for

sickle erythrocyte adhesion to reach an equilibrium level, is a significant delay following histamine stimulation. During this delay, the expressed adhesive ligands might be shed or retracted by the endothelial cells, due to their relatively short expression time (Easton & Dorovini-Zis, 2001; Lorenzon et al., 1998; Sugama et al., 1992). As such, the protocol for histamine-mediated adhesion of sickle erythrocytes required stimulation and sickle erythrocyte perfusion through the flow chamber system to be simultaneous. The histamine (Sigma-Aldrich) solution (1 to 1000 μM in SFM) was included in the same perfusate solution as the 0.22% hematocrit for erythrocyte perfusion.

A limited number of control histamine experiments were conducted with a pre-stimulation schema, where endothelial cells were stimulated with histamine independent of erythrocyte perfusion. This required a more specialized protocol. Endothelial cells were rinsed twice with warmed basal media, and the appropriate level of histamine in 2 ml of complete media was added. Where appropriate, the endothelium was incubated in this solution until 20 minutes before the completion of scheduled histamine stimulation. The slide was then assembled into the appropriate flow chamber, which was also filled with the same histamine / complete media solution. This assembly took place during the 10 minutes designated as “assembly time” for standard parallel plate flow chamber assembly, thus constituting an additional 10 minutes of histamine stimulation. The normal 10 minute SFM rinse described below (section 3.4) is replaced by a 10 minute perfusion with histamine in SFM to maintain the pre-stimulation of the endothelium with histamine for a final 10 minutes. Finally, at the conclusion of histamine stimulation, the chamber is rinsed with SFM at the standard flowrate (given in section 3.4.1) for 1.5 minutes (to remove residual histamine from the chamber) and sickle erythrocytes suspended in SFM (without histamine) are then perfused. Thus, for the 40 minute pre-stimulation with histamine, cells are histamine incubated for 20 minutes before chamber assembly, for 20 minute pre-stimulation the cells are stimulated during chamber

assembly and “rinse” perfusion only, and for 10 minute pre-stimulation, the endothelium is stimulated during the “rinse” perfusion only.

### 3.3.3 Stimulation and Blockade of Specific Histamine Receptors

To further characterize the effect of histamine on sickle cell adherence, histamine receptor agonists and antagonists, chosen for their specificity towards individual histamine receptors, were used in the sickle erythrocyte suspension. Antagonists included mepyrimine (Sigma-Aldrich) (an $H_1$ receptor antagonist) (Hide et al., 1988; Vickers et al., 1982), famotidine ($H_2$) (Sigma-Aldrich) (Shepherd-Rose & Pendleton, 1984), and thioperamide ($H_3/H_4$) (Sigma-Aldrich) (Arrang et al., 1987). Agonists used were amthamine (Tocris, Ellisville, MO) (an $H_2$ receptor agonist) (van der Goot & Timmerman, 2000), and (R)-(-)-$\alpha$-methylhistamine (Sigma-Aldrich) ($H_3/H_4$) (Schaefer et al., 1998). Histamine $H_3$ and $H_4$ receptors are highly homologous and most reagents target both receptors (Hough, 2001). Clobenpropit (Sigma-Aldrich), an $H_3$ receptor antagonist and an $H_4$ receptor agonist (Gantner et al., 2002; Oda et al., 2000) was used to determine the involvement of $H_3$ and $H_4$ receptors in histamine-induced sickle cell adherence, because no other $H_4$ specific agonists are available. Stimulation with histamine receptor agonists was identical to histamine stimulation in all studies, except that different concentrations were required for efficacy. (See individual graphs for concentration details.) For histamine receptor antagonist studies, the endothelial cells were pre-stimulated with the antagonist in SFM for a total of 30 minutes, and then included in the erythrocyte perfusion to maintain the antagonist presence throughout the erythrocyte perfusion. Prestimulation with the antagonist was accomplished by the same technique detailed in 3.3.2, adjusted for 30 minute exposure instead of 40 minute.

### 3.3.4 Erythrocyte Stimulation

A series of controls were run in order to determine the effect, if any, of histamine on sickle erythrocyte adhesion during the inclusion of histamine in the sickle erythrocyte perfusate. Sickle erythrocytes were reconstituted from the stock solution to 0.22% hematocrit solution, and histamine was added at 100 μM. The solution was then incubated for the indicated time, minus fourteen minutes. The remaining fourteen minutes of exposure, carefully timed, occurred while the solution was centrifuged at 100 g for 10 minutes, and the time it took for supernatant aspiration and travel to and from the centrifuge. The sickle erythrocytes were then resuspended to 0.22% hematocrit for immediate perfusion through the flow chamber. As the additional centrifugation (as well as residual histamine presence) might have damaged or otherwise altered the surface chemistry of the sickle erythrocytes, these samples were compared against a "blank" sickle cell suspension: a suspension which had also been reconstituted to 0.22% hematocrit, incubated for the appropriate time, then centrifuged and resuspended, but without the presence of histamine.

### 3.3.5 Blockade of Adhesive Ligands

Adherence to VCAM-1 was inhibited by adding 10 μg/ml anti-VCAM-1 $IgG_1$ mouse antibody (Immunotech, Marseille, France) to the TNF-α solution during endothelial-cell activation. After 6 hours of exposure the endothelial monolayer was rinsed with Hank's balanced salt solution (HBSS) (Sigma-Aldrich) and added to the linear shear stress flow chamber as detailed in section 3.4 and 3.4.2. A mouse $IgG_{1\kappa}$ myeloma protein antibody from a mouse tumor line (MOPC-21) (Sigma Aldrich) used under identical conditions served as a negative control for the anti-VCAM-1 antibody.

Sickle erythrocyte binding mediated by the expression of P-selectin in response to endothelial histamine stimulation was inhibited by the inclusion of an anti-P-selectin antagonist (EWVDV) in the perfusate, a high affinity peptide previously shown to block P-selectin mediated adherence (Appeldoorn et al., 2003; Molenaar et al., 2002). Treatment with the peptide was accomplished by pre-stimulating the endothelium for 30 minutes with the peptide at 100 μM concentration (employing the same technique detailed in 3.3.2, and including it in the sickle erythrocyte suspension at the same concentration). Control for this peptide was an alternate peptide with no affinity for P-selectin (EWVKV), used in the same manner (Appeldoorn et al., 2003; Molenaar et al., 2002). Involvement of P-selectin in sickle cell binding was confirmed in experiments substituting blocking anti-P-selectin antibodies (Ancell, Bayport MN) at 5μg/ml concentration for the peptide. Anti-P-selectin antibodies could not be used for all P-selectin blockade experiments, as the requirement that the blocking agent be included in the sickle erythrocyte perfusate required unmanageably large quantities of antibody for each plate.

Inhibition resulting from these blockade tactics is calculated as follows:

**Percent Inhibition =**

$$\left[1-\frac{\left[RBC/mm^2\right]_{AbBlocking}-\left[RBC/mm^2\right]_{Baseline}}{\left[RBC/mm^2\right]_{Stimulated}-\left[RBC/mm^2\right]_{Baseline}}\right]\times 100 \quad \text{(Equation 3.1)}$$

In the case of shear-stress levels for which an increase in adherence was observed with the inflammatory stimulant, percent sickle cell adherence induced by the stimulation was calculated as: **Percent Inducible Adherence =**

$$\left[\frac{\left[RBC/mm^2\right]_{Stimulated}-\left[RBC/mm^2\right]_{Baseline}}{\left[RBC/mm^2\right]_{Stimulated}}\right]\times 100 \quad \text{(Equation 3.2)}$$

### 3.3.6 Blocking of Nitric Oxide Synthase Activity

To verify the involvement of nitric oxide synthase (NOS) in sickle cell adherence elevated by histamine, specific NOS-blocking agents diphenyleneiodonium chloride (DPI) at 30 nM (Sigma) (Wang et al., 1993; Stuehr et al., 1991) or Nω-nitro-L-arginine methyl ester at 100 μM (L-NAME, Sigma) (Albrecht et al., 2003) were added to endothelial cells for 30 minutes prior to assembly of the perfusion chamber. eNOS blocking agents were also maintained at the given concentration in the perfusate solution both during the rinse stage and during the sickle erythrocyte perfusion, in a manner similar to that used for the histamine receptor antagonists.

## 3.4 Flow Chamber Assays

Conducting flow chamber assays with different styles of flow chamber (Figure 3.3 and 3.4) and different stimulation regimes requires subtle alterations in the standard assay protocol. The general protocol is given here, with specific alterations to the system for each particular case following.

The chamber slide is prepared by assembling the polypropylene flow director with entrance and exit stopcocks / spigots and sealing any unused holes with plugs. The assembly is lubricated and sealed with vacuum grease at the assembly joints. The surface of the polypropylene flow director is then lightly coated with vacuum grease where the gasket will rest, while carefully avoiding any surface that will be exposed to flowing media or through which the microscope will focus. The gasket is pressed hard into place, eliminating any bubbles trapped beneath the gasket and removing any excess vacuum grease in the central flow chamber. Another thin layer of vacuum grease is applied to the top side of the gasket. The assembled chamber is then repeatedly rinsed with warmed basal media. The half-assembled chamber has the

stopcock adjusted to point its open port upwards and is filled at either end with warmed complete media (or a SFM + agent solution in those experiments involving prestimulation with an agonist or blocking agent), care being taken not to allow bubbles to form within the stopcock or flow director. Enough complete media is added to the empty space defined by the gasket to form a “bubble” of media.

A confluent endothelial cell monolayer on a Labtek® chamber slide is then rinsed twice with basal media solution (M199 or HBSS) and filled with 2 ml of complete media (or SFM solution with or without stimulation factors, depending on the protocol), at which point a timer is set for 10 minutes. The thumb-hold of the slide is removed to fit the flow chamber. The outer walls of the chamber slide and the silicon gasket that holds the chamber walls in place are removed. Media remaining on the slide is poured into the half-assembled flow chamber, and the slide is inverted and pressed into place atop the flow chamber gasket. Being sure to eliminate bubbles and to not shift the permanox slide, the chamber is inverted and placed into the aluminum brackets (Figures 3.3 & 3.4). The stopcock position must be rotated 180° to properly fit. Spacers are added to hold the chamber in place, and the upper bracket is secured with six screws. The chamber is held together with sufficient force to keep the slide from slipping or the chamber from leaking, but over-tightening results in bending of the permanox slide, bowing it and distorting the flow fields within the chamber.

The solution intended to flow through the chamber for 10 minutes prior to erythrocyte perfusion is next constituted in a 15 ml centrifuge tube, a solution of either SFM or SFM with some stimulant or blocking agent added. The chamber is positioned in a specially-built holder on the stage of a Nikon Diaphot-TMD inverted phase-contrast microscope (Southern Micro Instruments, Atlanta GA) to visualize adherence. An air-curtain incubator (Nicholson Precision Instruments, Bethesda MD) was used to direct warm air across the flow chamber and microscope stage, maintaining the chamber

temperature at 37°C. The centrifuge tube of rinse solution is placed in a water bath at 37°C adjacent to the flow chamber (water bath is elevated to stage level). Tubing with leuer-lock connectors attached to the stopcock on the flow chamber (the stopcock was used to easily change perfusion between the serum-free media and the erythrocyte suspension flows) are run from the rinse solution (tubing is taped in place), again being careful not to allow bubbles to form in the tubing or flow chamber. The exit spigot of the flow chamber is connected via pre-filled tubing to a 60 ml syringe locked in a syringe pump (Model 33; Harvard Apparatus, South Natic, Mass). After the 10 minute assembly time expires, the 10 minute "rinse" phase begins and the warmed rinse solution is

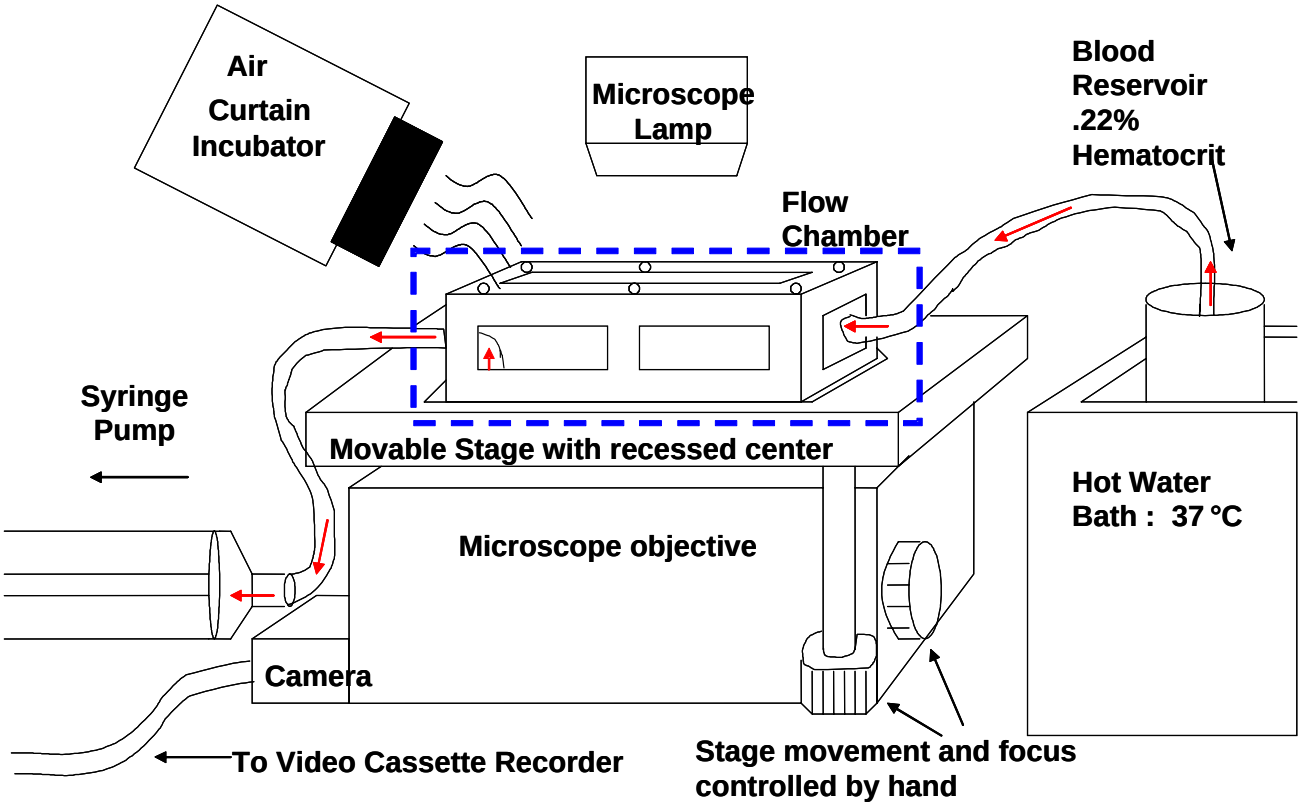


**Figure 3.2: Rough Schema of Flow Experiment During Perfusion.** Schema of experiment in progress. Arrows indicate direction of flowing hematocrit, dashed box indicates position of perfusion flow chamber.

perfused through the chamber by the syringe pump set at “withdraw” at a rate appropriate to the shear stress level desired. During the rinse stage, the chamber is positioned upside-down in the path of the air curtain incubator in order to prevent settling of the few sickle erythrocytes left from previous experiments. The purpose of the rinse stage is both to further rinse the chamber and to pre-condition the system, so that changes to endothelial monolayer in response to applied shear, such as detachment or peeling of sections, occur separately from those changes brought about by the introduction of cytokines or sickle erythrocytes. Endothelial monolayers that do peel or detach are discarded, and data from those monolayers is not recorded.

During the “rinse” stage, the sickle erythrocyte suspension is formulated. The formulation is timed to minimize the time the erythrocytes spend as a 0.22% solution in order to minimize the effects of dilution on the blood. The sickle erythrocyte suspension is attached to the flow chamber as was the rinse solution, using the other stopcock spigot. At the finish of the “rinse” stage, the flow chamber is righted and repositioned, the entrance spigot switched and erythrocyte perfusion begun. Flow is always begun within two minutes of attachment to the erythrocyte reservoir in order to minimize the effect of sickle erythrocyte settling in the tubing (Fig 3.2).

Each experiment is viewed under 400X total magnification through a CCD-72 series camera (Dage-MTI, Michigan City, IN) and recorded on VHS videocassette. Perfusion time for the experiment is kept with an inline VTG-33 video timer (FOR-A, Boston Mass). The “perfusion time” officially begins with the observation of an influx of erythrocytes at the furthest upstream field resolvable. Field sampling and cell counting protocols depended on the flow chamber employed.

For each field, the endothelial monolayer was inspected via the display monitor. In the case of uneven confluency or some localized cell detachment leading to uneven cellular monolayers (as might happen during a flow experiment), data was only collected

over confluent regions of the plate, to avoid confusing adhesion to the endothelial monolayer with adhesion of cells to exposed basal matrix or cell culture plastic.

Following the completion of a flow assay, the system is halted, and the flow chamber removed to the biological safety hood. There, the permanox plate is discarded, and all parts (excepting the exit tubing attached to the syringe pump) were rinsed twice with warmed SFM or basal media to remove residual cytokine or sickle erythrocyte contamination. A cotton swab soaked in SFM was used to scrub the exposed areas of the flow chamber to further remove sickle erythrocytes. The chamber was then refilled with complete media for the next run as previously detailed.

In a limited number of experiments, detachment assays followed sickle erythrocyte adherence assays to measure the adherence strength of attached cells (Walmet et al., 2003). For detachment assays, the additional "rinse" solution was formulated during flow chamber assembly. Following the 30 or 40-minute assay, the 3-way stopcock was turned to once again perfuse the chamber with "rinse" solution without sickle erythrocytes. Flow was allowed to continue for 5 minutes, at which point standard data collection resumed, counting 4 to 5 fields. Media flowrate was increased so that the applied shear increased by 0.5 dyne/cm$^2$, flow was allowed to continue for 5 minutes, and another 4 to 5 fields were examined. This process continued until no sickle erythrocytes remained adherent to the endothelial surface or 3.0 dynes/cm$^2$ was reached.

### 3.4.1 Parallel Plate Flow Chamber

The shear stress experienced by an endothelial monolayer placed in the parallel plate flow chamber (Figure 3.3) is determined by the following formula:

$$\tau = \frac{6\mu Q}{w(2b)^2} \quad \text{(Equation 3.3)}$$

Where $\tau$ is the shear stress in dynes/cm$^2$

$\mu$ = the viscosity of the media at the running temperature (37° C) (0.75 cp)

Q = the flowrate of the perfusing solution (ml/min)

w = gasket cut-out width (1.060 cm)

b = gasket half-thickness (0.0055 cm)

Flowrate for the thickness of the gasket and the width of the flow field defined in the standard parallel plate flow chamber is 1.71 mL/min. After flow chamber assembly, the endothelial monolayer is rinsed with serum free media for 10 minutes at 1.0 dyne/cm$^2$ shear stress and then perfused with erythrocytes for 30-40 minutes, depending on the assay. Erythrocyte adherence to endothelium is counted in 4-5 randomly selected microscopic fields (excluding those fields within 4 mm of the entrance and exit where entrance and exit effects might interfere) per minute continuously for the first 11 minutes, and then for three minutes at each ten minute interval following. Data were pooled in 1-10 minute intervals and reported as adherence after 1, 3, 5, 10, 20, 30 and/or 40 minutes erythrocyte perfusion. Cell counts for each time point were averaged and normalized to adherent cells per square millimeter.

In early experiments, data was collected from the videocassette recordings made of each plate, but in later experiments adherent cells were counted and recorded during the run itself. Videocassette recordings are still made of the experiments in case specific data fields needed to be reviewed.

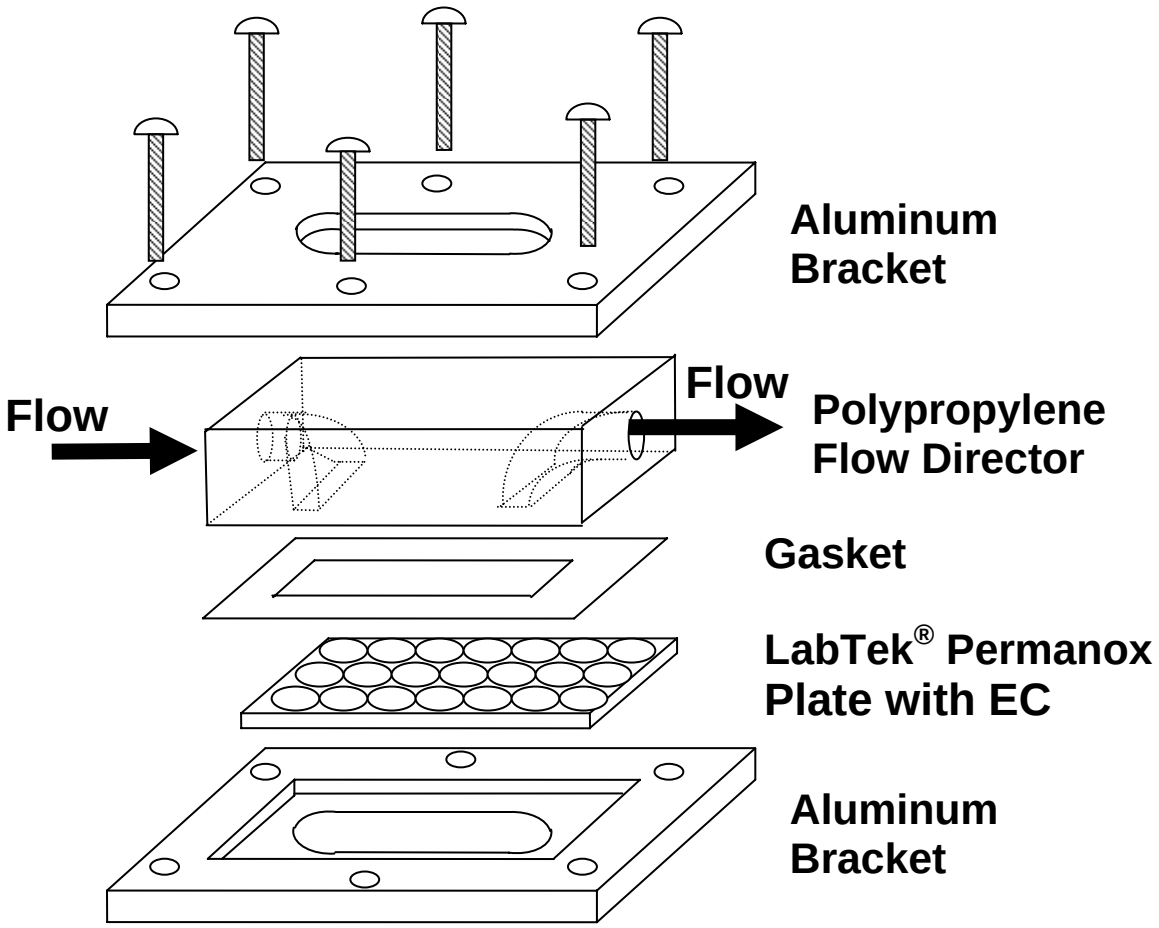


**Figure 3.3: Parallel-Plate Flow Chamber**. Schematic of parallel-plate flow chamber components. The arrow indicates flow direction from inlet to outlet.

### 3.4.2 Linear Shear Stress Flow Chamber

The shear stress experienced by an endothelial layer in the linear shear chamber (Figure 3.3) is determined, as derived by Usami et al (Usami et al., 1993) by the following formula

$$\tau_w = \frac{6\mu Q}{h^2 w_1}\left(1 - \frac{z}{L}\right) \quad \text{(Equation 3.4)}$$

Where $\tau_w$ = the shear stress (dyne/cm$^2$)

$\mu$ = the viscosity of the media at the running temperature (37° C) (0.75 cp)

Q = the flowrate of the perfusing solution (ml/min)

h = the gap height (0.024 cm)

$w_1$ = is the entrance width (0.195 cm)

z = the coordinate measured from the channel entrance (cm) (see Figure 3.4)

L = the characteristic length in the plane of the plates (4.20 cm)

A linear shear stress flow chamber (Usami et al., 1993) was used to quantify sickle cell adherence at different shear stresses simultaneously in a single experiment. Flow for most experiments were kept constant at a rate of 0.156 mL/min, which dictated a shear range of 1.0 dyne/cm$^2$ at 2 millimeters from the entrance point for the chamber. These few millimeters were necessary to avoid the entrance effects altering the flow dynamics driving sickle erythrocyte adherence. A few experiments were conducted at half this rate to examine a reduced range of shear. As this flow chamber is significantly narrower at the entrance than the parallel plate flow chamber, especial care was taken to remain at or near (within 1 microscope field) the center of the chamber, so that edge effects would not interfere with the flow dynamics and adhesion data.

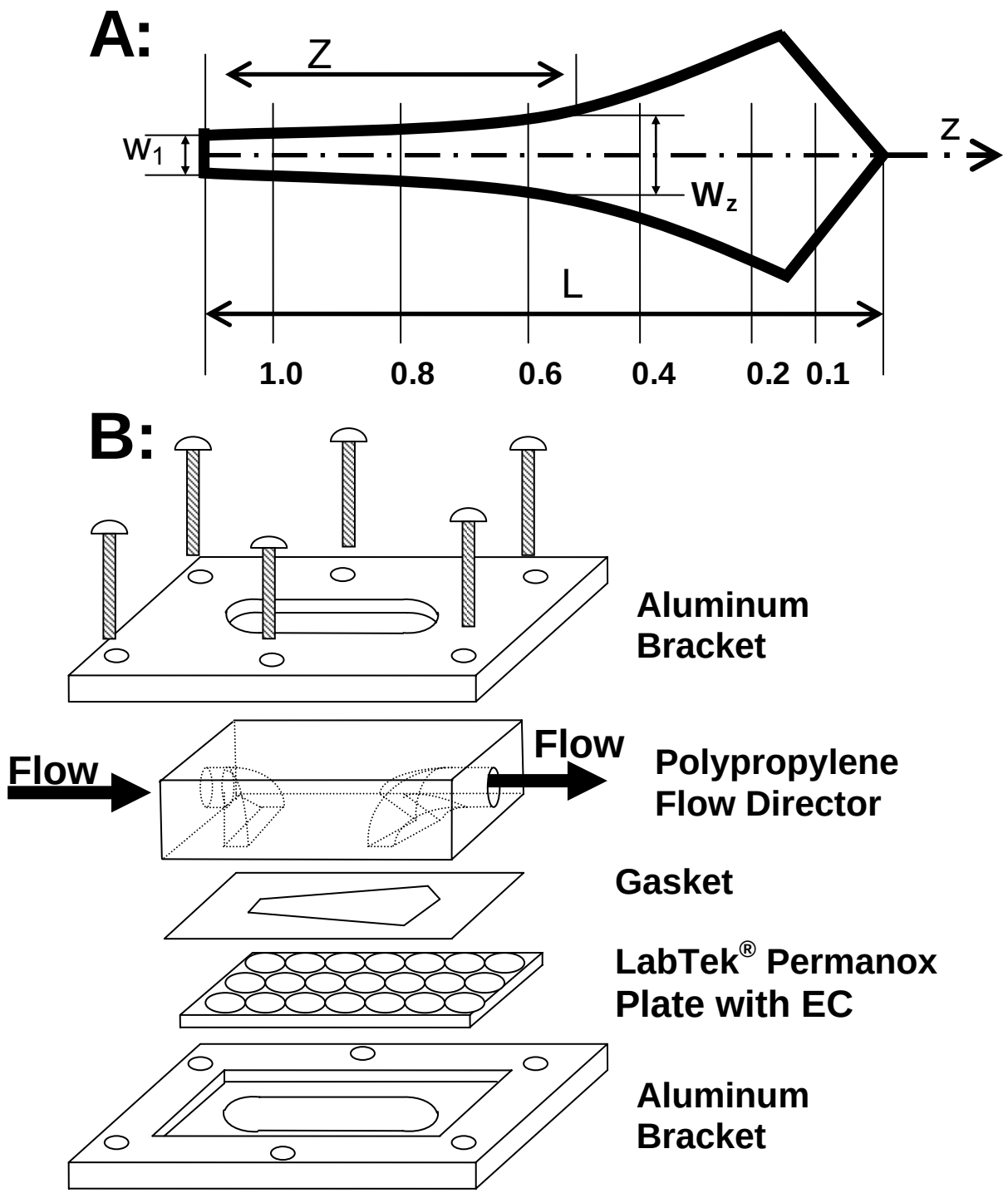


**Figure 3.4: Linear Shear Stress Flow Chamber** (A) Schematic diagram of flow-channel geometry used to achieve 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 dyne/cm$^2$ shear stress at the approximate locations indicated on the basis of Hele-Shaw flow (Usami et al., 1993) and an inlet flow rate of 0.154 mL/min. Flow chamber height is constant at 0.24 mm, defined by the gasket shown in (B). (B) Schematic of flow chamber components arranged for assembly. The arrow indicates flow direction from inlet to outlet.

To acquire data at specific shear rates, the microscope had to resolve fields at specific, pre-assigned positions down the length of the chamber. This was done by measuring the positions on the permanox plate of the fully assembled flow chamber with a microcaliper and scoring the position on the underside of the plate with a 12-gauge needle. This step was preformed during the rinse or assembly stage of each run. During the assay, these marks appear as a bold black line (due to diffraction of the microscope light) and were used to orient the position of each field. Initially, another line was scored down the length of the chamber to mark the center line, but this proved both difficult (as it needed to be perfectly straight) and unnecessary once the operator got used to the system.

Data for the linear shear system was collected continuously for the duration of the experiment, starting 30 seconds after erythrocyte perfusion initiation. Data was collected first at 4-6 fields at the 1.0 dyne/cm$^2$ position, then proceeded down the length of the chamber in the direction of flow to similarly collect data at each marked shear rate. Once data was collected at the 0.1 dyne/cm$^2$ position, the objective was returned to the 1.0 dyne/cm$^2$ mark to repeat the process. Data was not collected in fields where the endothelial cells had detached from the surface, and cells were not counted when obviously adhering to exposed sections of permanox plate. In those cases, other fields within the same range of shear rate were selected for data collection.

As the collection of data for the linear shear system covered six times the shear rates as that examined in the parallel plate flow chamber, fields were selected and sampled at a much faster rate. This additional speed made it impossible to count and mark the adherent cells visible in each resolved field by hand during the experiment. For each linear shear assay run, the entire experiment was recorded on videocassette, and it was from this recording that the count of adherent cells was tallied.

Erythrocyte adherence was recorded in 4-6 microscopic fields at 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 dyne/cm$^2$ shear stress (at the locations indicated in Figure 3.4) at approximately 1, 3, 8 ,13, 18, 23, and 30 minutes of erythrocyte perfusion for VCAM-1 studies and the same shear set at 3, 8, 13, 18, 23, 30, 34 and 39 minutes of erythrocyte perfusion for histamine studies. Erythrocyte adherence values were averaged and normalized to adherent red cells per square millimeter for each level of shear stress and pooled at the perfusion times listed above. Adherence was quantified visually to distinguish sickle erythrocytes from any contaminating white cells, although leukocyte contamination was infrequent (~1 adherent white cell in 10-15 microscopic fields).

### 3.5 Experimental Design

To minimize the effect of donor-to-donor variability on the level of red cell adherence, each donor sample served as its own control. Experiments were performed with blood samples from different donors and sickle cell adherence is reported as the average adherence level (adherent cells/mm$^2$) for all donors. These individual plates were tested serially in a set order for a single blood sample. For each set of experiments the order was reversed or mixed at least once to be certain that the sequence was not affecting results.

### 3.6 Statistical Analysis

Testing of significance through analysis of variance (ANOVA) with repeated measures (Minitab Inc., 2000) was accomplished with Minitab statistical software, version 13.31 (Minitab Inc., State College, PA). Pair-wise comparisons were made between data sets at the specific times or conditions indicated. Differences in adherence were considered statistically significant when $P \leq 0.05$. Formulas for percent inhibition and percent inducible adhesion given above under 3.3.5.

# CHAPTER IV:

# HISTAMINE INCREASES SICKLE ERYTHROYCTE ADHESION TO ENDOTHELIUM

## 4.1 Abstract

Complications of sickle cell anemia include vascular occlusion triggered by the adherence of sickle erythrocytes to endothelium in the post capillary venules. Inflammatory mediators that promote endothelial cell adhesion molecule expression and arrest flowing erythrocytes can induce adherence. This study characterized the effect of histamine stimulation on the kinetics of sickle cell adherence to large vessel and microvascular endothelium under venular flow. Increased sickle cell adherence was observed within minutes of endothelial activation by histamine and reached a maximum value within 30 min. At steady state, sickle cell adherence to histamine-stimulated endothelium was 47 ± 4 adherent cells/mm$^2$, 2.6-fold higher than sickle cell adherence to unstimulated endothelial cells. Histamine-induced sickle cell adherence occurred rapidly and transiently. Studies using histamine receptor agonists and antagonists suggest that histamine-induced sickle cell adhesion depends on simultaneous stimulation of the $H_2$ and $H_4$ histamine receptors and endothelial P-selectin expression. These data show that histamine release may promote sickle cell adherence and vaso-occlusion. *In vivo* histamine release should be studied to determine its role in sickle complications and whether blocking of specific histamine receptors may prevent clinical complications or adverse effects from histamine release stimulated by opiate analgesic treatment.

## 4.2 Introduction

The etiology of the microvascular occlusion and episodic pain typical of sickle cell anemia is multifactorial and complex. Literature reports suggest that understanding the cytokine-driven pro-inflammatory state evident in sickle cell anemia (Wun, 2001; Chies & Nardi, 2001; Platt, 2000) can contribute to understanding of mechanisms of pain episodes. Thrombosis (Brittain et al., 1993), inflammation (Walmet et al., 2003), endothelial activation (Solovey et al., 1998; Solovey et al., 1997; Duits et al., 1996) and sickle erythrocyte adherence to endothelium (Hebbel et al., 1980a) may contribute to these episodic vaso-occlusive pain episodes (Francis & Johnson, 1991). Unusually-large von Willebrand factor multimers (Wick et al., 1993; Wick et al., 1987), thrombospondin (Brittain et al., 1993), thrombin (Matsui et al., 2001), cytokines (Makis et al., 2000), and chemokines (Kumar et al., 1996) all increase sickle cell adherence to endothelium *in vitro*. Prothrombotic and proinflammatory factors (Brown et al., 2001; Makis et al., 2000; Duits et al., 1998; Duits et al., 1996; Vordermeier et al., 1992), activated monocytes and granulocytes (Belcher et al., 2000), and activated platelets (Tomer et al., 2001; Wun et al., 1998; Brittain et al., 1993) are typically elevated in sickle patients, and may contribute to endothelial cell activation and sickle erythrocyte adherence *in vivo*. Thus, chronic inflammation, immune system activation, and thrombogenicity evident in sickle cell patients would contribute to sickle cell anemia severity by increasing sickle erythrocyte adhesion to activated endothelial cells. Inflammation, therefore, is both causative in the vaso-occlusive damage of sickle pain crises, and a principle, chronic aspect of the patient's condition. Understanding different aspects of inflammation in sickle patients may provide techniques for better treatment of the disorder.

Histamine is a powerful inflammatory agent stored in mast cells and basophils that is rapidly released by degranulation in response to pro-inflammatory stimuli or some

drug treatments (Muldoon et al., 1984). Histamine causes rapid expression of P-selectin and von Willebrand factor on endothelial cells by translocation from intracellular Weibel-Palade bodies (Datta et al., 2001; Utgaard et al., 1998; Hattori et al., 1989). P-selectin expression increases the specificity of endothelial cell interactions with platelets and leukocytes during inflammation, coagulation, and atherosclerosis (Varki et al., 1999; Varki, 1997; Kansas, 1996; Furie et al., 1995; McEver et al., 1995; Springer, 1994) P-selectin expressed on endothelial cells also increases sickle erythrocyte adhesion, although the precise ligand interaction involved is not known (Matsui et al., 2001).

Endothelial cell P-selectin expression increases within minutes of histamine stimulation, peaks within 10-20 minutes, and returns to baseline within approximately 40 minutes; although endothelial cells show phenotypic variability in the expression rate (Easton et al., 2001; Lorenzon et al., 1998; Sugama et al., 1992). One or more of the histamine-specific $H_1$, $H_2$, $H_3$, and $H_4$ receptors may mediate histamine-induced P-selectin expression. Endothelial cells show differential expression of these receptors (Gantner et al., 2002; Morse et al., 2001; van der Werf et al., 1989; Heltianu et al., 1982), and histamine activation of these receptors initiates different intracellular responses (Smit et al., 1999; Del Valle et al., 1997; Leurs et al., 1995).

Plasma histamine levels are elevated in sickle patients both during pain episodes and during asymptomatic periods (Enwonwu et al., 1991). Clinically, opioid analgesics such as morphine, often used to treat sickle cell pain episodes, cause histamine release (Barke & Hough, 1993; Casale et al., 1984).

The current study quantifies sickle cell adherence kinetics under venular flow conditions in response to endothelial cell stimulation with histamine. The results demonstrate that histamine promotes sickle erythrocyte adherence, defining a novel adhesive mechanism that may respond to receptor pathway blockade, reducing *in vivo* sickle cell adherence. Determining the adhesion kinetics and biochemical factors

involved in histamine-mediated adhesion may provide insights into new therapeutic strategies for inhibiting or reversing sickle cell adherence that is thought to occur during vaso-occlusive pain episodes.

## 4.3 Materials and Methods

These experiments employed cultured HUVEC and dMEC on single-well Labtek® permanox plates in parallel-plate flow chambers. Blood samples used were both "fresh" blood samples and "day old" samples from discard blood. Data from both "fresh" and "day old" blood samples were pooled. Straightforward stimulation protocols were used, as well as the modified protocols necessary for blocking experiments. The detailed protocols for these experiments are covered in full in sections 3.3.1-3, 3.2.1-3, 3.3.2-5, 3.4, 3.4.1, 3.5, and 3.6.

## 4.4 Results

### 4.4.1 Histamine Mediated Adhesion to Endothelium at 1.0 dyne/cm$^2$

Adherence of sickle red blood cells to unstimulated endothelium was low during 40 minutes of erythrocyte perfusion at 1.0 dyne/cm$^2$ (Figure 4.1A). In the presence of 100 μM histamine, sickle cell adherence to endothelium increased within 1-3 minutes of stimulation and reached steady-state within 30-40 minutes of erythrocyte perfusion. At steady state, sickle red cell adherence to histamine-stimulated endothelium (47±4 adherent cells/mm$^2$, average ± SEM) was significantly higher than sickle cell adherence to unstimulated endothelium (18±3 adherent cells/mm$^2$, Figure 1A). Sickle cell adherence increased with histamine stimulation for all 37 different patient samples tested, with increases between 1.2 to 9.6-fold baseline (unstimulated) adherence. The maximum increase in sickle cell adherence was observed at a histamine concentration

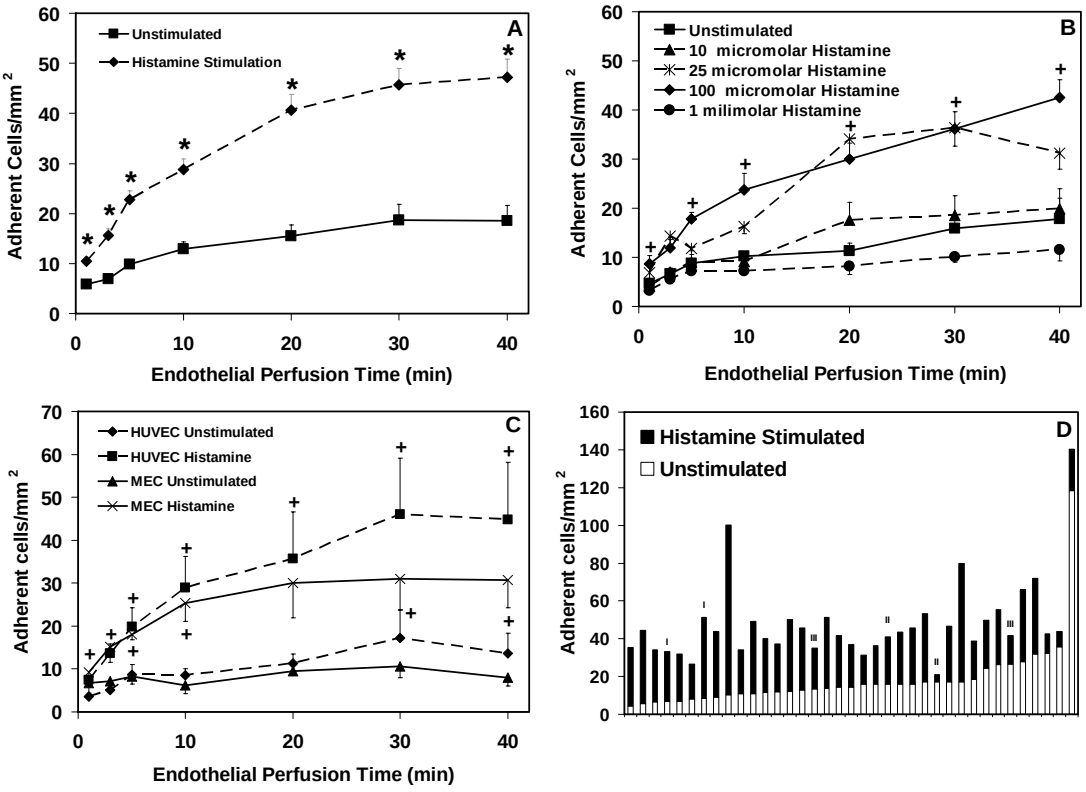


**Figure 4.1: Histamine Stimulation Promotes Sickle Erythrocyte Adherence** For continuous stimulation conditions, endothelial monolayers were exposed to (A, C, & D) 0 or 100 μM histamine or (B) 0, 10, 25, 100, and 1000 μM histamine stimulation starting at time 0, simultaneous with perfusion of washed sickle erythrocytes. Figure C represents direct comparison of sickle erythrocyte adhesion to histamine-stimulated HUVEC and dMEC monolayers. Figure D shows unstimulated (open) and histamine-stimulated (open + filled) red cell adhesion levels for individual experiments following 40 minutes perfusion, arranged in order of increasing unstimulated adherence. In three experiments, different blood samples were analyzed on the same day over endothelial cultures derived from the same 5$^{th}$ passage culture. These results are marked "I," "II," and "III" respectively. Unlabeled data sets were collected with endothelial cells derived from different 5$^{th}$ passage cultures. *P≤.001 and $^{+}$P≤.050 versus unstimulated (0 μM histamine) adherence at the same time point in all figures. Data are mean ± SEM for (A) & (D) n=37, (C) n=4, and (B) n=4, except n=3 at 1000 μM and n=2 at 25 μM histamine.

of 100μM (Figure 4.1B). Direct comparison of sickle cell adherence to umbilical vein (HUVEC) and microvascular (dMEC) endothelial cells demonstrate a similar effect of histamine stimulation on sickle erythrocyte adherence (Figure 4.1C). Steady-state (e.g. after 40 minutes perfusion) and histamine-stimulated adherence levels for individual blood samples are shown in Figure 4.1D.

### 4.4.2 Induced Adhesion Acts by Time-Dependent Stimulation of the Endothelium

Treatment of endothelial cells with histamine for 20 (but not 10 or 40) minutes prior to sickle erythrocyte perfusion (Figure 4.2) replicates the kinetics of sickle cell adherence induced by the continuous exposure of endothelial cells to histamine (Figure

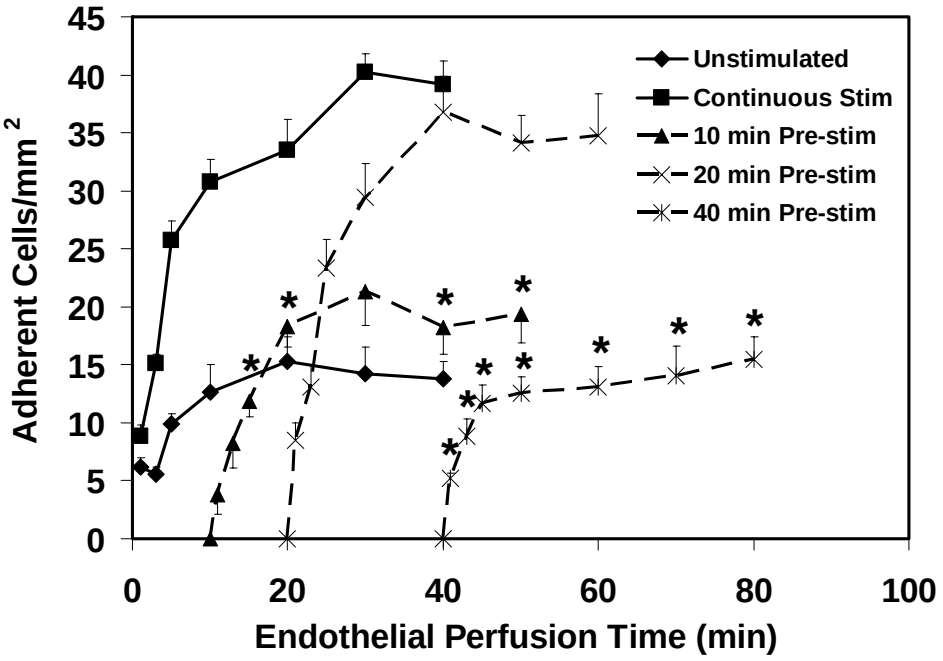


**Figure 4.2: Histamine Stimulated Adhesion is Time Dependent** Endothelial monolayers were prestimulated with 100 μM histamine for the indicated time, rinsed for 1 min with SFM alone, and then perfused with sickle erythrocytes for 40 minutes without histamine. "Continuous Stim" indicates continuous stimulation of endothelium with histamine solution beginning at time zero during erythrocyte perfusion as described in the Materials and Methods. * P≤.043 versus continuous histamine stimulation for prestimulated runs. Data are mean ± SEM for n=5 blood samples, except n=4 at 10 min pre-stim.

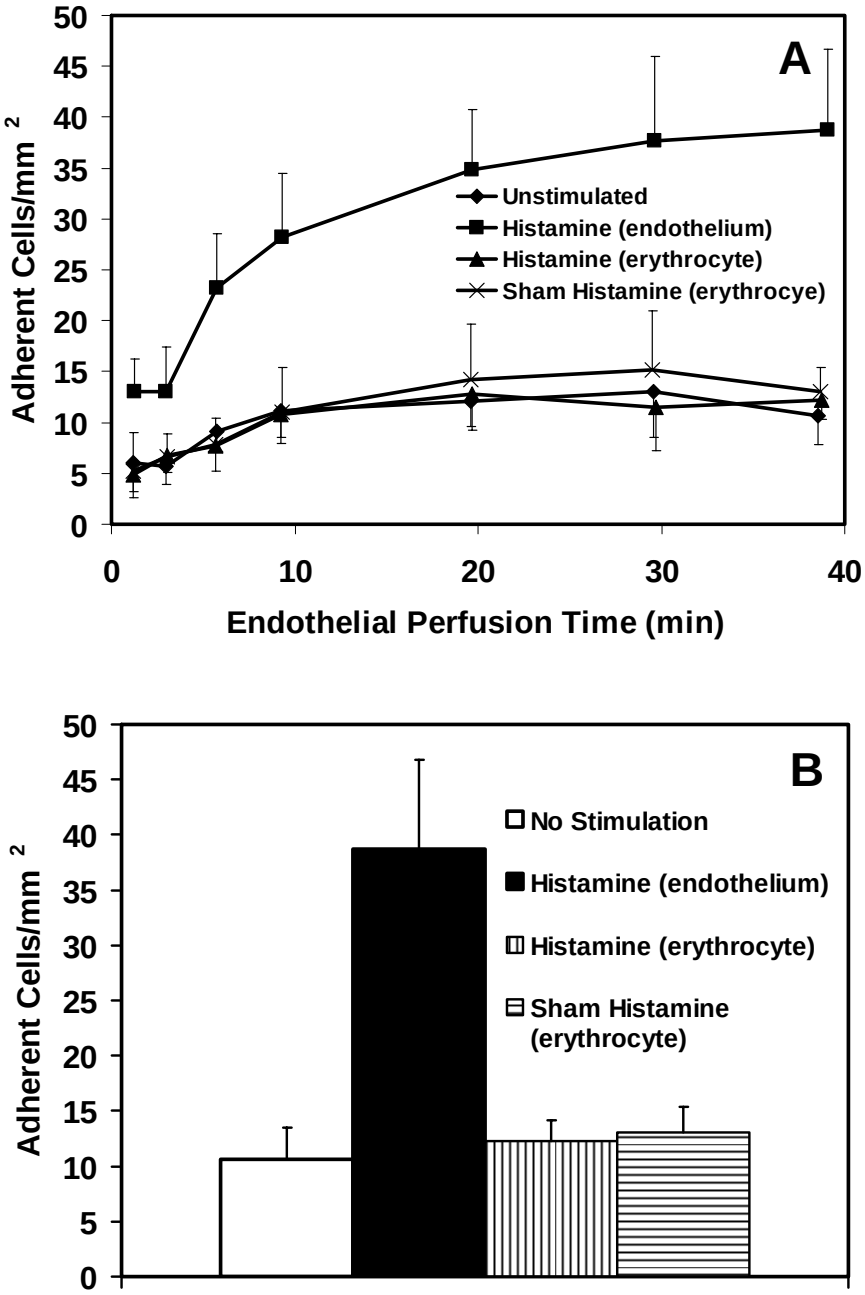


**Figure 4.3: Histamine Stimulated Adhesion is Endothelium Dependent** Sickle erythrocytes suspended in SFM were prestimulated with 100 μM histamine for 40 minutes. The cells were then centrifuged, resuspended without histamine and perfused over unstimulated endothelium. "Histamine (endothelium)" and "unstimulated" treatments have identical protocols to Figure 4.1 and 4.2. "Sham" indicates treatment identical to "Histamine (erythrocyte)," without the histamine, as described in 3.3.4. No significant difference was found between either erythrocyte stimulated and unstimulated data. Data are mean ± standard deviation for n=7 (except n=5 for "Sham") blood samples over (A) full perfusion time and (B) values after 40 min perfusion only.

4.1A). In control experiments, incubation of sickle erythrocytes with histamine without endothelial stimulation did not increase sickle cell adherence to endothelium (Figure 4.3). Additional controls observed no endothelial retraction during histamine stimulation of the endothelium (n=3 data not shown). Taken together, these results suggest that the effect of histamine stimulation is primarily localized to the endothelium.

### 4.4.3 Activation and Blockade of Endothelial Receptors

Additional experiments explored possible adherence mechanisms induced through histamine stimulation by investigating the roles of histamine $H_1$, $H_2$, $H_3$, and $H_4$ receptors and endothelial cell P-selectin expression. Mepyramine, an $H_1$ antagonist, did not significantly alter adherence of sickle erythrocytes to cultured endothelial monolayers induced by histamine (Figure 4.4A) at concentrations up to 25μM (data beyond 10 μM not shown). In contrast, endothelial treatment with famotidine, an $H_2$ antagonist, or thioperamide, an $H_3/H_4$ antagonist, inhibited sickle cell adherence induced by histamine essentially 100% (Figures 4.4B-C).

Experiments with histamine receptor agonists demonstrated that endothelial cell stimulation with either amthamine ($H_2$ agonist) or (R)-(-)-α-methylhistamine ($H_3/H_4$ agonist) alone does not promote sickle cell adherence above baseline, even at agonist concentrations up to 25 μM. However, simultaneous endothelial stimulation with amthamine (10 μM) and (R)-(-)-α-methylhistamine (10 μM) induced sickle cell adherence comparable to that induced by histamine (Figure 4.5). Distinguishing between activation of the highly homologous $H_3$ and $H_4$ receptors was accomplished by treating endothelial cells with clobenpropit, a reagent that simultaneously acts as a histamine $H_3$ receptor antagonist and an $H_4$ receptor agonist (Gantner et al., 2002; Oda et al., 2000). Endothelial cell stimulation with either 10 μM clobenpropit (Figure 4.6B) or 10 μM

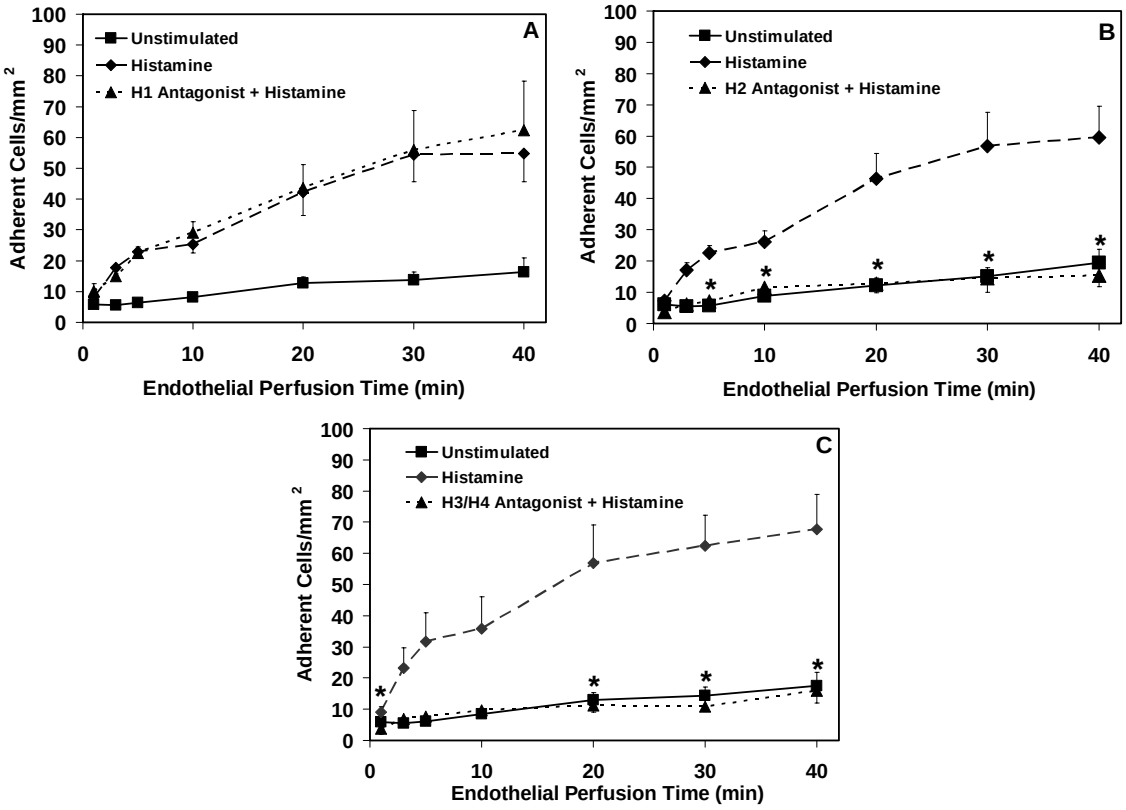


**Figure 4.4: Receptor Antagonism Inhibits Histamine-Induced Sickle Cell Adherence** Endothelial monolayers were pretreated with the indicated receptor antagonist for 30 minutes prior to sickle erythrocyte perfusion in the presence of 100 μM histamine and (A) 10 μM mepyramine ($H_1$ antagonist), (B) 10 μM famotidine ($H_2$), or (C) 10 μM thioperamide ($H_3/H_4$). Data are mean ± SEM for (A) n=5, (B) n=4, and (C) n=5 blood samples. * P≤.040 (B) or P≤.019 versus histamine-stimulated adherence at the same time point.

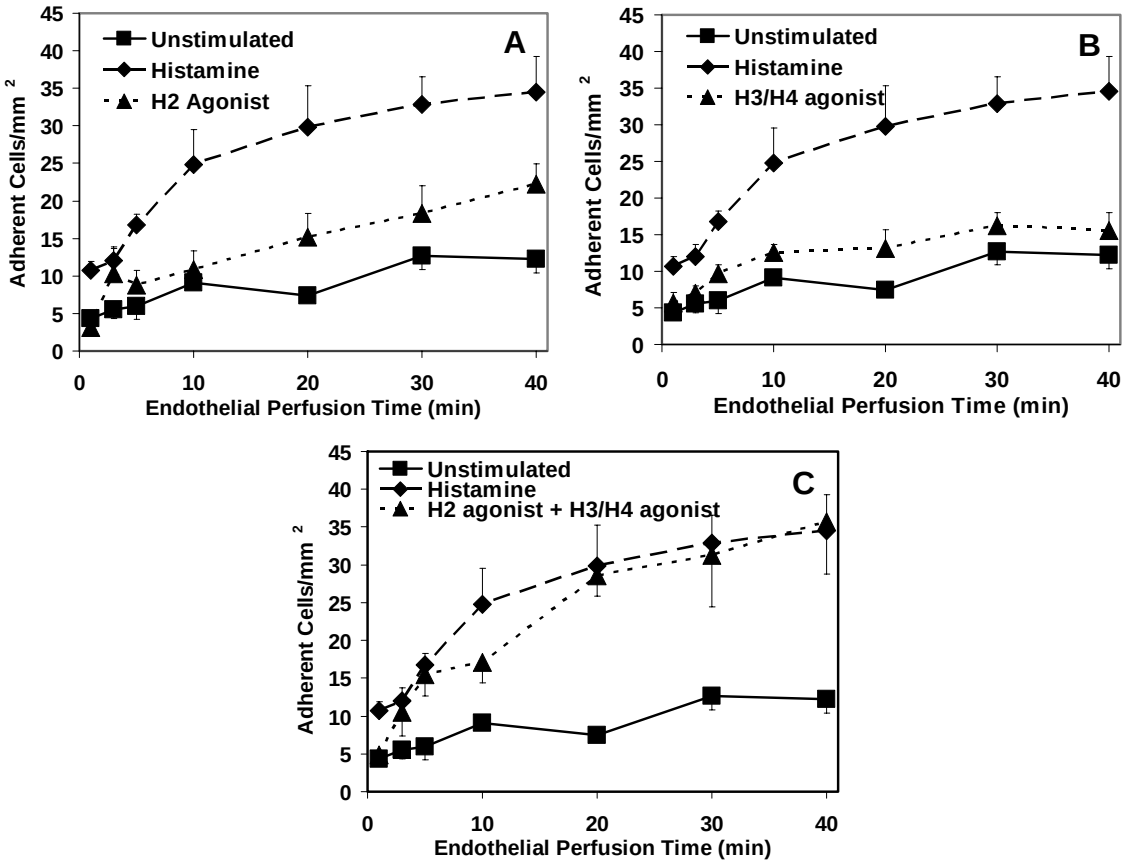


**Figure 4.5: Adhesion Induced by the Presence of Histamine Requires Binding to Histamine $H_2$ and $H_3$ / $H_4$ Receptors** Endothelial monolayers were continuously stimulated with (A) 10 μM amthamine ($H_2$ agonist), (B) 10 μM (R)-(-)-α-methylhistamine ($H_3/H_4$ agonist) or (C) 10 μM amthamine + 10 μM (R)-(-)-α-methylhistamine during erythrocyte perfusion. Data are mean ± SEM for n=3 blood samples. Significance not noted due to low n value.

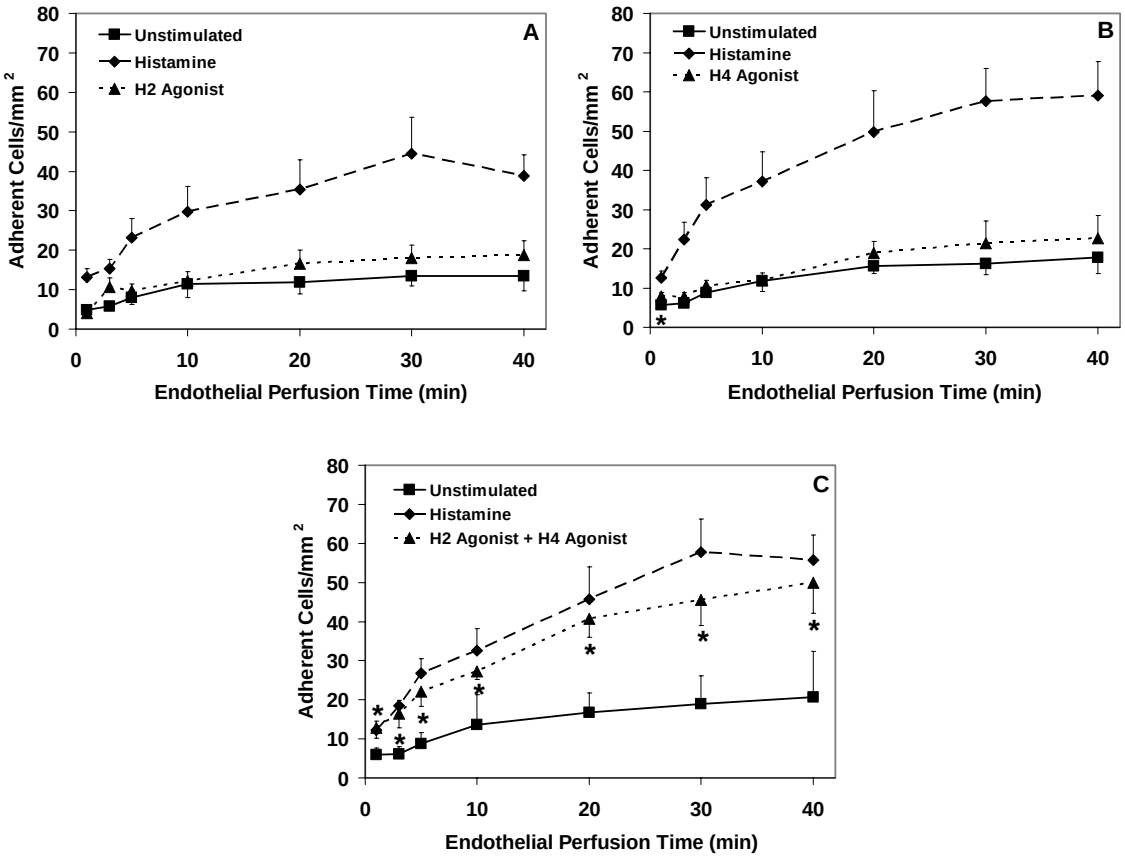


**Figure 4.6: Adhesion Induced by the Presence of Histamine Requires Binding to Histamine $H_2$ and $H_4$ Receptors** Endothelial monolayers were continuously stimulated with (A) 10 μM amthamine ($H_2$ agonist), (B) 10 μM clobenpropit ($H_4$ agonist) or (C) 10 μM amthamine + 10 μM clobenpropit during erythrocyte perfusion. Data are mean ± SEM for (A) n=5, (B) n=7, and (C) n=5 blood samples. * P≤.049 versus baseline (unstimulated) adherence at the same time point.

amthamine (Figure 4.6A) alone did not increase sickle erythrocyte adherence. In contrast, endothelial stimulation with both amthamine and clobenpropit together increased sickle cell adherence of 50±8 adherent cells/mm$^2$, a level similar to that observed with histamine stimulation (56±6 adherent cells/mm$^2$, Figure 4.6C).

### 4.4.4 Activation and Blockade of P-selectin Adhesive Ligand

Peptide blocking of P-selectin activity on histamine-stimulated endothelial cells reduced sickle cell adherence induced by histamine between 69-100% (Figure 4.7).

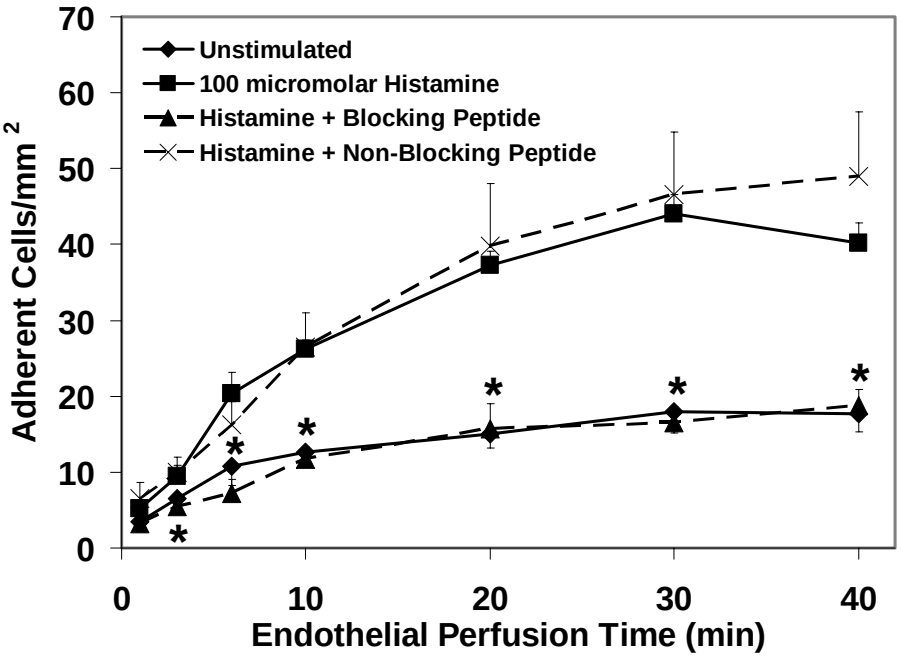


**Figure 4.7: P-selectin Peptide Antagonist Prevents Histamine-Induced Adhesion** The endothelium was pre-stimulated with 100 μM P-selectin blocking peptide (EWVDV) for 30 minutes prior to sickle erythrocyte perfusion and histamine stimulation (100μM). Blocking or non-blocking peptide (EWVKV) at 100 μM concentration was also included in the erythrocyte perfusion media during the adhesion assay. Data are mean ± SEM for n=6 blood samples, except for "Non-Blocking Peptide" (n=5). *P≤.004 versus histamine mediated adhesion at the same time point.

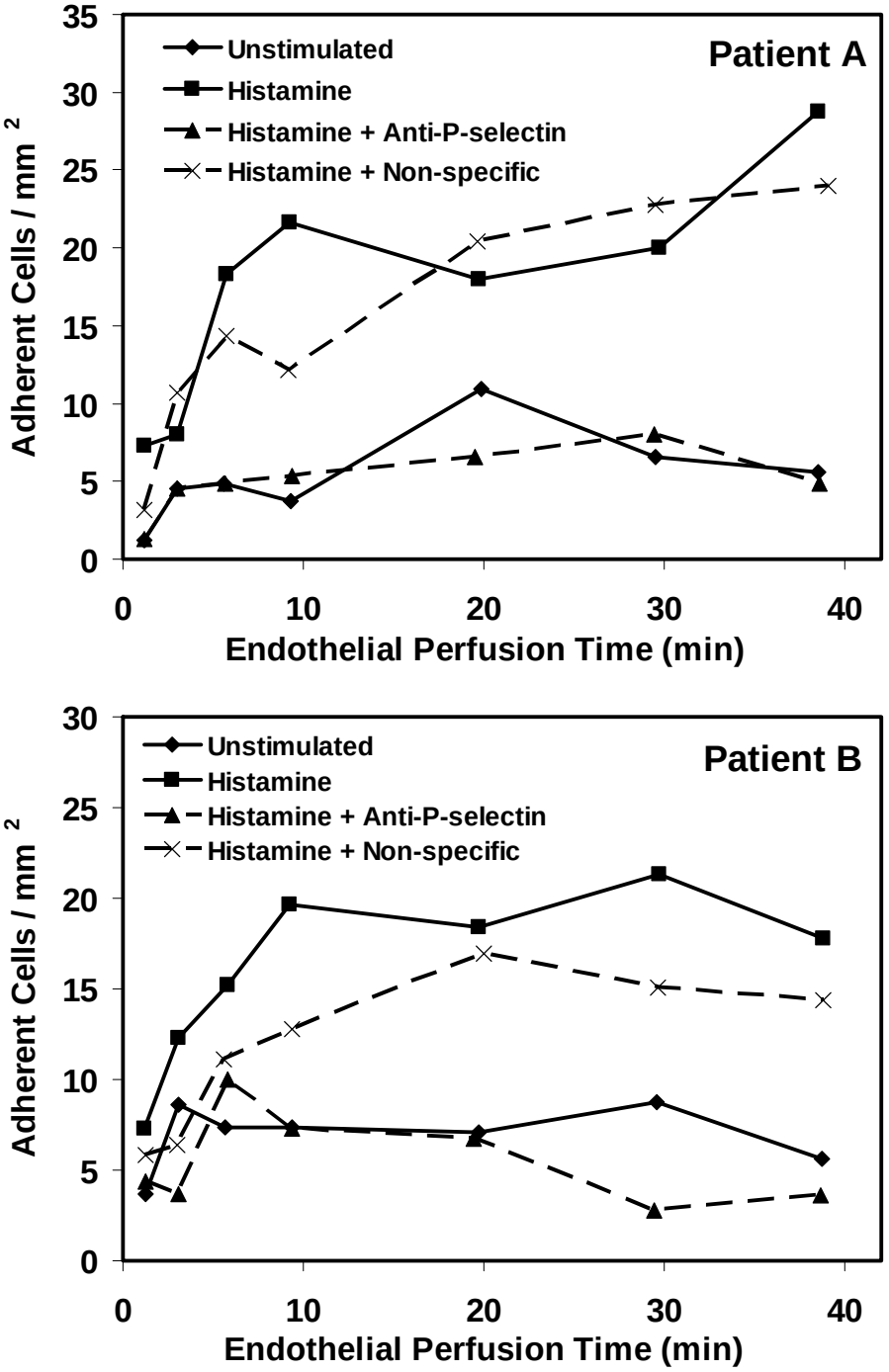


**Figure 4.8: P-selectin Antibody Blocking Prevents Histamine Mediated Adhesion to Microvascular Endothelium (dMEC)** The dMEC monolayer was pre-stimulated with 10 μg/ml blocking or nonspecific antibody for 30 minutes prior to sickle erythrocyte perfusion and histamine stimulation (100μM). Blocking or non-specific antibody at 10 μg/ml concentration was also included with red cell perfusion media during the adhesion assay. Data are from single experiments.

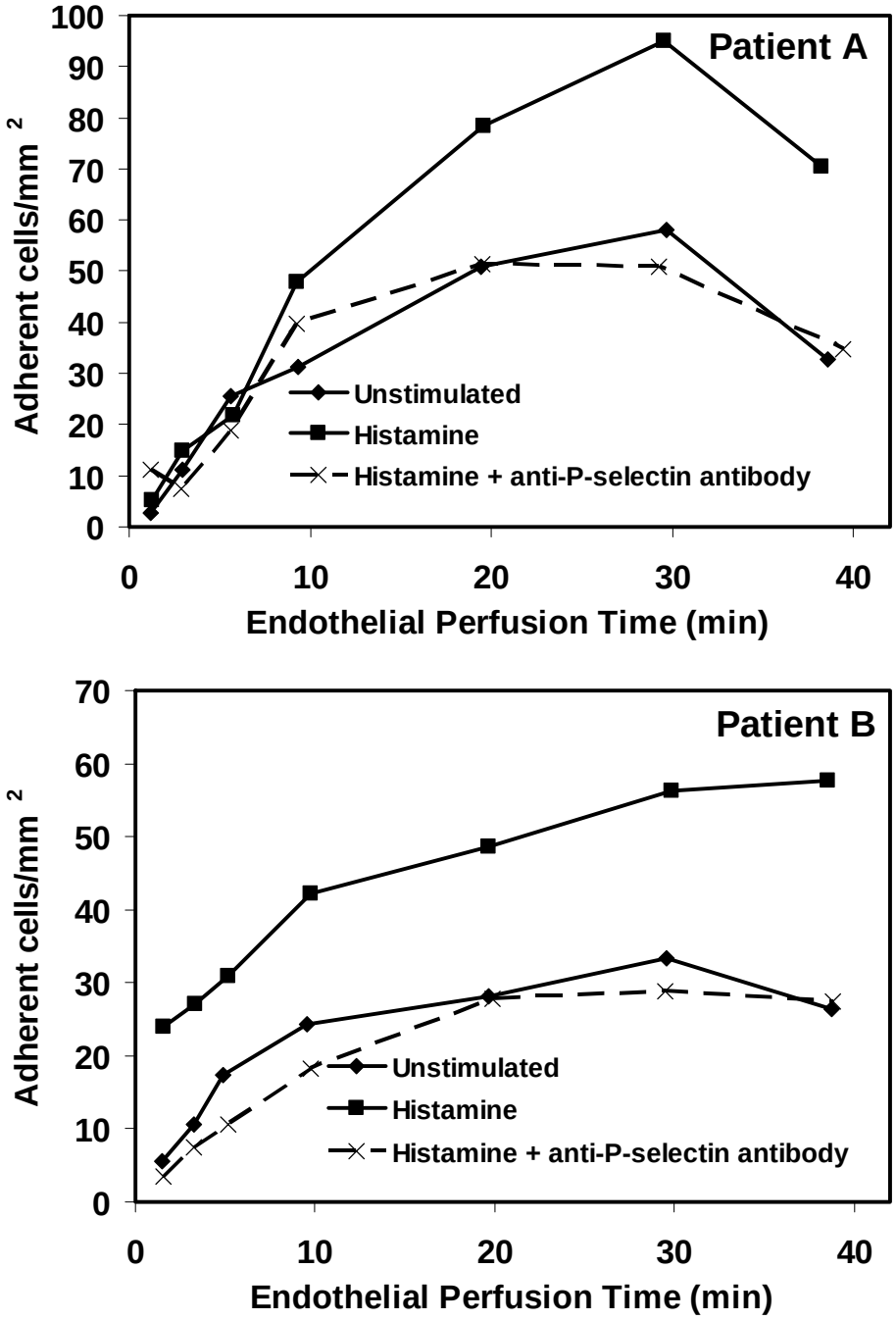


**Figure 4.9: P-selectin Antibody Blocking Prevents Histamine Mediated Adhesion to HUVEC** The HUVEC monolayer was pre-stimulated with 10 μg/ml blocking antibody for 30 minutes prior to sickle erythrocyte perfusion and histamine stimulation (100μM). Blocking antibody at 10 μg/ml concentration was also included in the erythrocyte perfusion media during the adhesion assay. Data are from single experiments. See Chapter III for similar antibody blocking.

The control peptide did not significantly affect sickle cell adherence to histamine stimulated endothelium. P-selectin involvement was confirmed in two experiments with monoclonal anti-P-selectin antibody, where sickle cell adherence induced by histamine was entirely inhibited, (Figure 4.9). Similarly, incubation of histamine-stimulated dMECs with anti-P-selectin antibody reduced sickle erythrocyte adherence essentially 100% in two separate experiments (Figure 4.8).

## 4.5 Discussion

Sickle erythrocyte adherence to endothelium likely contributes to sickle cell vascular complications. This study was designed to evaluate the possible contribution of histamine to sickle erythrocyte adherence and to examine the mechanisms of histamine signaling which promotes sickle cell adherence. The results demonstrate that histamine mediates sickle erythrocyte adhesion to venous (HUVEC) and microvascular (dMEC) endothelium *in vitro* under venular flow conditions. Sickle cell adhesion occurs rapidly following histamine exposure; reaching a plateau within 30-40 minutes of stimulation. Elevated sickle cell adhesion correlates with stimulation of histamine $H_2$ and $H_4$ receptors and with expression of P-selectin on the surface of the endothelium.

P-selectin as a mediator of sickle erythrocyte adhesion is particularly interesting, in part, because of its rapid expression kinetics. Endothelial expression of P-selectin is increased within minutes of histamine stimulation, reaches a maximum in 10-20 minutes, and returns to baseline levels within 30-40 minutes of histamine stimulation (Lorenzon et al., 1998). To capture the dynamics of sickle cell adherence to endothelium based on the known kinetics of P-selectin expression, experiments were designed to allow continuous histamine stimulation during erythrocyte perfusion through the flow chamber. Consistent with P-selectin expression kinetics, adhesion results demonstrate that sickle cell adherence increases within minutes of histamine stimulation and reaches steady-

state values within 30-40 minutes of erythrocyte perfusion (Figure 1A). After establishing the kinetics of sickle cell adherence under continuous histamine stimulation, subsequent experiments quantified adherence kinetics under conditions where endothelial cells were prestimulated with histamine and histamine was not present during erythrocyte perfusion. Under these conditions, increased sickle cell adherence occurs after 20 minutes of histamine prestimulation. Prestimulation 40 minutes before perfusion showed no increase in adhesion, possibly because 40 minutes of stimulation allows sufficient time for downregulation of P-selectin (Lorenzon et al., 1998). Taken together, these data demonstrate that sickle cell adherence induced by histamine stimulation is rapid.

The primary complimentary adhesive ligand for P-selectin is P-selectin glycoprotein-1 (PSGL-1). Previous studies have not found this ligand on the surface of sickle erythrocytes (Matsui et al., 2001) but evidence for a sialic acid-containing ligand which interacts with P-selectin and is markedly enhanced on SSRBC has been discovered, although the exact nature of the ligand has not been defined. It has further been observed both that platelets and platelet-derived microparticles (both capable of interaction with P-selectin) are elevated in sickle patient blood (Wun et al., 1997). Considering the known ability of platelets to adhere to sickle erythrocytes (Wun et al., 1997), we can speculate that P-selectin mediated erythrocyte adhesion might occur through adhesion of platelet-derived microparticles possessing PSGL-1 to the sickle erythrocyte. Similarly, PSGL-1-expressing monocyte-derived microparticles (detailed in section 2.3.3) may become associated with sickle erythrocytes. The elevated PS on sickle erythrocytes could cause this association, and PSGL-1 expressed on the microparticle could adhere to P-selectin.

The rapid increase in sickle cell adherence following histamine stimulation is in contrast to the kinetics of adherence reported for stimulation with cytokines like tumor

necrosis factor $\alpha$ or interlukin-1$\beta$ that upregulate cell adhesion molecule expression and sickle cell adherence after several hours (Natarajan et al., 1996; Swerlick et al., 1993). If adherence of sickle erythrocytes precipitates vascular complications, histamine could initiate complications by brief changes in tissue condition, compared to pathways requiring more time to express ligands. However, the subsequent rapid downregulation of P-selectin below levels necessary for erythrocyte adhesion would make these effects transient and, therefore, may be difficult targets for therapy. However, consideration of these rapidly-expressed ligands could be important in designing preventive strategies for reduction of sickle pain crisis.

Histamine interacts with cells through activation of one or more of four specific $G_{\alpha i/o}$ receptors, named $H_1$-$H_4$, whose effects are strongly tissue-dependent. Although the $H_2$ receptor is known to mediate gastric acid and mucus production in the stomach (Hill, 1990), it has also been shown to mediate leukocyte rolling and adhesion on endothelial cells (Yamaki et al., 1998) as well as endothelial cell adhesion molecule expression and tumor cell adhesion (Tang et al., 2004). The other three histamine receptors exhibit similarly diverse functionalities. $H_1$ receptors are commonly associated with allergic inflammatory response, for example, increasing vascular permeability, edema, and vasodilation in cutaneous response (Owen et al., 1980). It is not clear whether $H_1$, $H_2$, or the two working synergistically are responsible for driving cell adhesion (Torres et al., 2002; Tang et al., 1998; Yamaki et al., 1998). $H_3$ receptors are best known as neural receptors (Hill et al., 1997), that mediate histamine levels via feedback control. The recently discovered $H_4$ receptor appears to be responsible for aspects of immune response, as well as recruitment of effector cells to sites of inflammation (Ling et al., 2004; Buckland et al., 2003).

The present data (Figures 4.4-4.6) indicate that sickle cell adhesion induced by histamine occurs through simultaneous stimulation of both $H_2$ and $H_4$ receptors, but not through stimulation of $H_1$ receptors. $H_3$ receptors were shown to be unnecessary for initiation of histamine mediated adhesion, but complete lack of involvement could not be proven due to the cross-reactivity of most $H_3$ receptor agents with $H_4$ receptors. Lack of participation of the $H_3$ receptor, however, is likely because $H_3$ receptors do not appear in significant numbers on umbilical vein endothelial cells (Gantner et al., 2002). The effect of simultaneous $H_2$ and $H_4$ agonist stimulation is not a simple concentration effect, as H-receptor agonists were tested individually in increased concentrations (data not shown). A requirement for simultaneous $H_2$ and $H_4$ histamine receptor stimulation to promote cellular action has a precedent in the histamine-induced release of interleukin-16 from human $CD8^+$ T cells where activation of both receptors is required to elicit an effect (Gantner et al., 2002). Similarly, *in vivo*, activation of both $H_1$ and $H_2$ receptors is necessary for the accumulation of neutrophils following intraperitoneal injection of histamine or the neutrophil accumulation at the site of biomaterial implantation in mice (Tang et al., 1998). Further studies are necessary to elucidate the mechanism of signaling in $H_2$ and $H_4$ receptor mediated adhesion. Nevertheless, in contrast to the treatment suggested by the P-selectin studies above, these data identify H-receptors as targets for more effective prevention of histamine-induced sickle erythrocyte adhesion.

Histamine in sickle patient plasma is elevated even during asymptomatic periods. Histamine levels are further increased during morphine administration and have been reported to be within the nanomolar range in blood plasma (Withington et al., 1993; Enwonwu et al., 1991). This level may not be representative of tissue histamine concentrations due to localized histamine release from mast cells and basophils that accumulate at sites of inflammation (Bochner & Schleimer, 2001). Animal studies demonstrate that intraperitoneal sequestration of polymorphonuclear leukocytes, part of

a common inflammatory response, requires direct injection of histamine to the site in millimolar levels (Yamaki et al., 1998). Similarly, inflammatory response induced by biomaterial implantation in a mouse stimulates the release of nanomoles of histamine within the first two hours of implantation, presumably at the implantation site (Tang et al., 1998). The comparatively low increase in plasma histamine concentration despite high tissue-localized increases is consistent with histamine infusion experiments, showing that infusion of large quantities of histamine causes only small elevations in blood plasma histamine (Kaliner et al., 1982). This observation is likely due to a combination of dilution and the relatively short half-life of histamine in the circulation (Irman-Florjanc & Erjavec, 1994; Beaven et al., 1982; Ferreira et al., 1973). Therefore, histamine release occurring in tissues may lead to localized concentrations much higher than levels observed in blood plasma.

The present experiments found that 100 μM histamine promotes the highest level of sickle erythrocyte adherence. Concentrations of 10, 25, and 200 μM histamine show lower levels of adherence and 1mM shows little or no response. The present studies were designed with 100 μM histamine concentration, because this concentration elicits the largest and clearest effect of histamine stimulation on sickle erythrocyte adhesion. Reduced adherence at 200 or 1000 μM (compared to 100 μM) histamine concentration may occur because of receptor desensitization through internalization or other mechanisms attenuating adhesive ligand activity (Del Valle et al., 1997; Smit et al., 1995; Smit et al., 1994). A recent study found direct evidence for internalization and desensitization of the $H_4$ receptor (Ling et al., 2004). The *in vitro* endothelium of sickle patients, however, is unlikely to have histamine receptors remain in a desensitized state. The increased levels of histamine found in sickle plasma (Enwonwu et al., 1991) are on the order of nanomolar increases. Histamine is a short-term, localized inflammatory

mediator that quickly disappears from circulation (Irman-Florjanc et al., 1994; Beaven et al., 1982; Ferreira et al., 1973), and thus would attain receptor-desensitization levels only for a short time in a localized tissue region, and be incapable of widespread receptor desensitization. Increased plasma levels of histamine are significant only in that it is a generalized indication of frequent histamine release within the system. Thus the sickle patient endothelium likely remains responsive to the presence of histamine.

Exposure of endothelial cells to high concentrations of histamine (10,000 μM, a concentration 100-fold higher than that used in the present experiments) is known to induce endothelial cell retraction and sickle cell adhesion to newly exposed subendothelial thrombospondin (Manodori et al., 2000). Endothelial retraction of HUVECs in the presence of 100 μM histamine is also observed, but only when the endothelial cell monolayer is minimally confluent (confluent ≤ 18 hours) (Andriopoulou et al., 1999). Under these conditions endothelial cells exhibit gap formation, but endothelium confluent for longer (confluent ≥ 48 hours) shows no similar gap formation. Controls in the current studies observed endothelial cell cultures exposed to 100 μM histamine in the absence of sickle erythrocytes for up to 50 minutes with or without flow, but endothelial cell retraction was not observed (n=3, data not shown). Since endothelial monolayers in the current studies were cultured to full confluence (remaining confluent for over 1-2 days) before adhesion assays, it is not surprising that intercellular gap formation was not observed. In addition, adherent sickle erythrocytes were uniformly distributed across the endothelial cell surface and not localized to the cell edges, suggesting little if any involvement of exposed subendothelial matrix in adherence for the present study. Taken together, these results indicate that the effects of histamine on endothelial cell phenotype and sickle cell adherence are complex and

dependent upon histamine concentration, exposure time, and endothelial cell monolayer maturity.

Histamine was the focus of these studies because sickle patients have plasma histamine levels that are elevated both during pain crisis and at steady state (Enwonwu et al., 1991). Furthermore, some clinical treatments given to patients in crisis are known to stimulate acute histamine release. Opiates like morphine are analgesics of choice in treating severe pain episodes. Many opioids are highly histaminergic (Barke et al., 1993; Casale et al., 1984). Itching, vasodilation, airway constriction and urticaria from histamine release may occur in patients after administration of opiates (Muldoon et al., 1984; Moss & Rosow, 1983; Beaven, 1981). Also, a recent study suggested that excessive histamine blood levels after oral administration of morphine may lead to an increase in the incidence of acute chest syndrome (Kopecky et al., 2004).

Antihistamines that bind to the $H_1$ receptors are commonly administered as adjuvant pharmacological interventions to improve treatment of pain episodes and to reduce side effects of histamine release by opiates (Ballas, 2002; Platt et al., 2002). Further, pharmacologic blocking of histamine receptors is used as an over-the-counter treatment for prevention of the seasonal allergen response associated with $H_1$ activation or control of gastric acid secretion by blockade of the $H_2$ receptor. More recent work has suggested the use of multiple antihistamines in blocking synergistic effects of adhesion, including blocking $H_1$ and $H_4$ receptors for better treatment of allergic reactions (Daugherty, 2004), or $H_1$ and $H_2$ receptor antagonism to prevent neutrophil accumulation at sites of inflammation (Tang et al., 1998; Yamaki et al., 1998).

This demonstration that histamine stimulates adhesion of sickle erythrocytes to endothelial cells suggests that $H_2$ and/or $H_4$ antagonists in combination with those analgesics that promote histamine release may prevent some complications of treating sickle pain episodes. As full activation requires activity of both receptors (Figures 4.4-

4.6), blocking of either individually may be effective in reducing sickle cell adherence *in vivo* and the associated clinical complications. Further, use of either $H_2$ or $H_4$ antihistamines in combination with $H_1$ antihistamine may prevent adhesion via P-selectin expression and limit pain episodes by blunting other inflammatory processes. However, proof of P-selectin involvement and histamine-mediated adhesion *in vivo* requires further validation before clinical recommendations can be made. This proof may be forthcoming, as some very recent studies have underlined the importance of P-selectin in the vasculature of the sickle mouse model (Embury et al., 2004; Wood et al., 2004a) both as indicative of a pro-inflammatory and pro-thrombogenic vascular environment, and in direct observed in mediating sickle erythrocyte adhesion.

The present data demonstrate that histamine increases adherence of sickle erythrocytes to vascular (HUVEC) endothelium via simultaneous activation of $H_2$ and $H_4$ receptors leading to expression of P-selectin in a time and dose dependent manner. Further, the increase in adherence and involvement of P-selectin in histamine-stimulated sickle erythrocyte adhesion was also demonstrated on microvascular (dMEC) endothelium. Activation of sickle endothelium by histamine, occurring directly or from opioid administration to patients suffering from pain episodes, may play an important role in initiating or propagating microvasculature occlusion during pain episodes. This study shows that selective blockade of the associated receptors or expressed ligands prevents a histamine-mediated increase in sickle erythrocyte adhesion. These results suggest that selectively blocking specific histamine receptors may also prevent endothelial adherence occurring from inflammatory stimulation caused by opiate-induced histamine release.

These data, excepting those shown in Figures 4.3, 4.5, 4.8, and 4.9 have been previously published in the British Journal of Haematology, February 2006 (Wagner et al., 2006).

# CHAPTER V

# SICKLE CELL ADHESION DEPENDS ON HEMODYNAMICS AND ENDOTHELIAL ACTIVATION

## 5.1 Abstract

The venular microvascular circulation of patients with sickle cell anemia exhibits reduced and episodic blood flow. Sickle erythrocyte adhesion to post-capillary venular endothelium is postulated to initiate and propagate vaso-occlusive pain episodes. Hemodynamics likely mediate sickle cell adherence to the endothelium, controlling delivery of potentially adherent erythrocytes and removal of loosely adherent erythrocytes. This study found high shear dependence of sickle erythrocyte adhesion to vascular cell adhesion molecule-1 (VCAM-1) on endothelium stimulated by tumor necrosis factor -$\alpha$ (TNF$-\alpha$). Shear stress varied from 1.0 dyne/cm$^2$ (microvascular venular flow), where VCAM-1 ligand interactions induced by TNF-$\alpha$ primarily controlled adherence, to 0.1 dyne/cm$^2$ (low flow), where stimulation had little effect on adherence. At shear stresses analogous to *in vivo* velocities from laser Doppler studies (0.8 and 0.6 dyne/cm$^2$), TNF-$\alpha$ induced 1.9- and 2.7- fold increased adhesion compared to unstimulated (baseline) adherence. These findings suggest a dynamic vaso-occlusive process, dependent on both receptor expression and shear stress. These results indicate that in the microvasculature *in vivo* slightly reduced inflow rate and/or increased endothelial cell adhesion molecule expression may result in large increases in sickle erythrocyte adhesion.

## 5.2 Introduction

Sickle hemoglobin gelation and adherence of sickle erythrocytes to endothelium are thought to initiate sickle vaso-occlusive pain episodes (Bunn, 1997; Mozzarelli et al., 1987; Hebbel et al., 1980a; Hoover et al., 1979; Hofrichter et al., 1974). Adherent sickle erythrocytes alter microvascular hemodynamics (Hebbel, 1997a; Wick et al., 1996) which delays erythrocyte transit time through the microvasculature. This delayed transit allows the hemoglobin in erythrocytes sufficient time to polymerize after oxygen depletion while the red cells are still in the capillaries. The gelation then, in turn, enables rigid and morphologically sickled red cells to be trapped, causing complete occlusion (Fabry et al., 1992; Kaul et al., 1989b). Compensatory mechanisms, including an increased pressure drop in response to obstruction, tend to restore tissue blood flow (Lipowsky et al., 1987; Rodgers et al., 1984). Therefore, changes in local shearing forces may promote endothelial adherence and detachment of sickle erythrocytes. The effects are complex because increased shear stress determines delivery of potentially sticky erythrocytes but also exerts forces that detach loosely adherent cells.

Shear stresses studied in this work ranged from 1.0 dyne/cm$^2$ (venular microvascular venular flow) to 0.1 dyne/cm$^2$ (“low “ flow), where adhesion is “affinity controlled,” and “transport controlled,” respectively (Montes et al., 2002). Affinity controlled sickle cell adherence (Pries et al., 2001; Turitto, 1982) is adhesion requiring high-affinity receptor-ligand binding (Montes et al., 2002) strong enough to resist venular blood shearing forces and increased reperfusion pressures (Walmet et al., 2003). Transport-controlled adherence occurs under conditions of reduced flow (Rodgers et al., 1990; Kennedy et al., 1988; Rodgers et al., 1984), causing extensive, if relatively weak (Walmet et al., 2003; Lipowsky et al., 1987) adherence even in the absence of high-affinity interactions (Montes et al., 2002). Presumably, low shear stress enables adherence via low-affinity binding mechanisms (Walmet et al., 2003; Montes et al., 2002; Hebbel, 1997a; Embury et al., 1994) incapable of initiating or maintaining adhesion at higher shear rates. Lower shear

allows for longer cell-cell contact time between flowing sickle erythrocytes and the endothelium, and applies less pressure to established bonds, meaning that weaker, slower-initiating bonds are viable only under these reduced shear conditions.

Blood flow in sickle microcirculation is periodic (Kennedy et al., 1988; Rodgers et al., 1984), and reduced (Kennedy et al., 1988; Lipowsky et al., 1987; Rodgers et al., 1984), even during pain free periods. It is theorized that this variability is due to the damaged nature of the sickle vascular endothelium (Solovey et al., 1999; Solovey et al., 1998; Solovey et al., 1997; Hebbel & Vercellotti, 1997) or loss of microvascular tone most noticeable in the reduced responsiveness to NO as exhibited in transgenic sickle mice (Kaul et al., 2000b). Thus, studies modeling sickle erythrocyte adhesion *in vitro* over the range of shear stresses directly observed in post-capillary venules are likely relevant to understanding adherence and vaso-occlusion in the microvasculature of patients with sickle cell anemia. In the present study, sickle cell adherence via $\alpha_4\beta_1$/vascular cell adhesion molecule-1 (VCAM-1) interactions induced by stimulation of the endothelium with the inflammatory cytokine tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (Swerlick et al., 1993) was quantified under steady flow conditions at shear stresses between 0.1 and 1.0 dyne/cm$^2$. These studies demonstrate that both flow conditions and endothelial activation modulate sickle cell adherence over shear stresses physiologically relevant to sickle microvascular blood flow.

## 5.3 Materials and Methods

These experiments employed HUVEC cultures on single-well Labtek® chamber slides in the linear-shear flow chamber. Stimulation of the endothelium employed primarily TNF-$\alpha$, a cytokine known to promote VCAM-1 expression in endothelial cells over a period of hours. The long stimulation time required for TNF-$\alpha$ meant that the more elaborate perfusion protocols were unnecessary except during antibody blockade experiments, and that the cytokine did not need to be included in the perfusate solution. The effect of TNF-$\alpha$ on sickle erythrocytes is thus irrelevant to this study. The protocols for these experiments are covered in detail in sections 3.1.1-3, 3.2.1-2, 3.3.1, 3.3.5, 3.4, 3.4.2, 3.5, and 3.6.

## 5.4 Results

### 5.4.1 Sickle Erythrocyte Adhesion is Time and Shear Dependent

For all shear and TNF-$\alpha$ activation conditions studied, sickle cell adherence increased with time and reached a steady-state level after 30 minutes of red-cell perfusion. Maximal adherence values depend on both shear stress and TNF-$\alpha$ stimulation levels (Figure 5.3, 5.4, 5.1). Consistent with previous results (Montes et al., 2002), sickle cell adherence is highest at the lower shear stresses (0.1, 0.2, and 0.4 dyne/ $cm^2$) and not significantly increased by TNF-$\alpha$ stimulation (Figure 5.3, A, B, and C, Figure 5.4 A, B, and C, and Figure V1 A and B ). Similar results were seen when TNF-$\alpha$ concentration was kept constant at 500 U/ml and exposure time varied over 0-8 hours (Figure 5.3, 5.4), and when TNF-$\alpha$ concentration was varied for a constant stimulation time of 6 hours (Figure 5.1). Six hours of TNF-$\alpha$ treatment at 500 U/ml yielded the

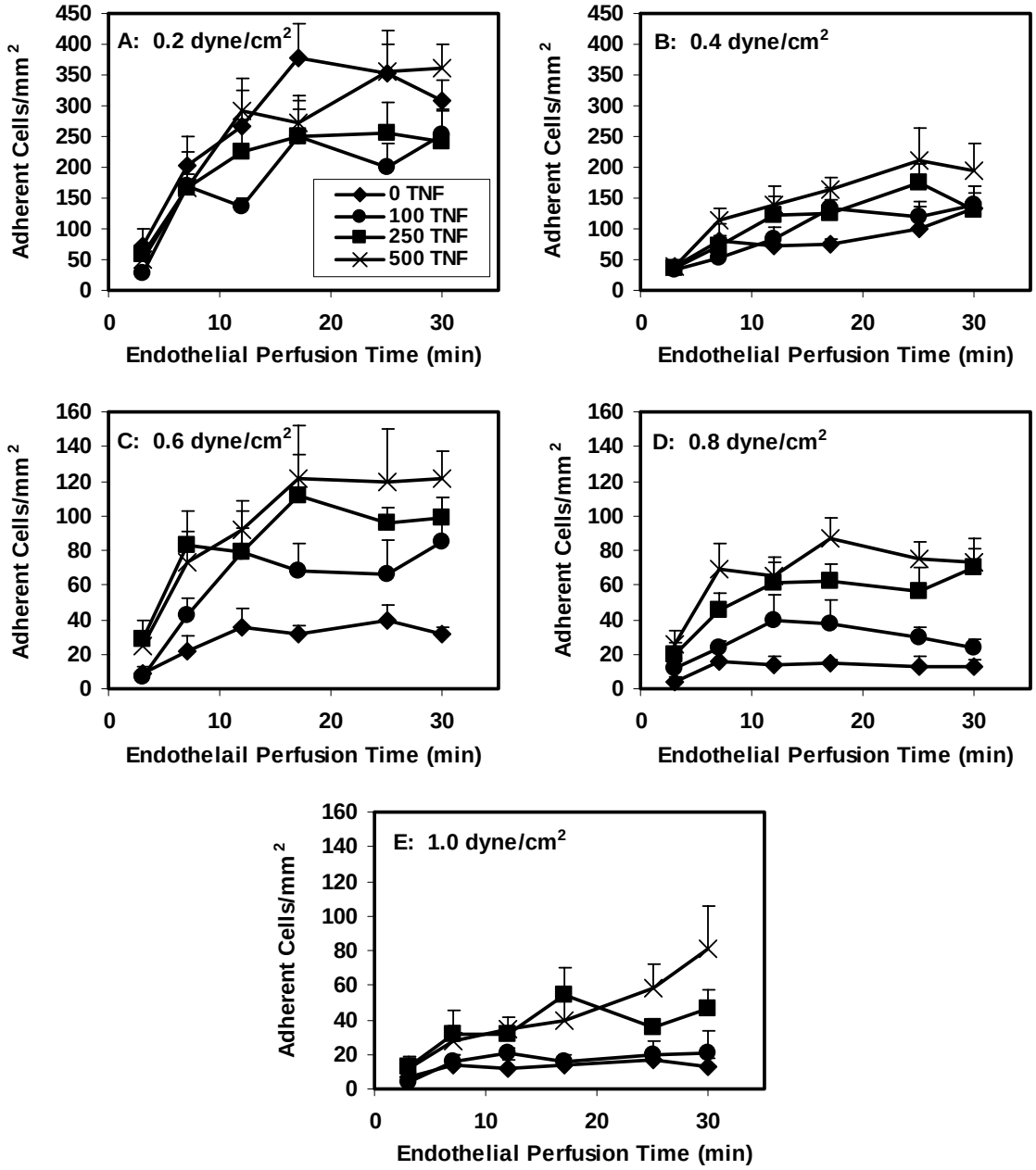


**Figure 5.1**: **TNF-α Concentration Stimulation Dependence of Sickle Red Cell Adherence Under a Range of Shear** Data are mean ± standard error of the mean (SEM) sickle erythrocyte adherence (A) 0.2, (B) 0.4, (C) 0.6, (D) 0.8, and (E) 1.0 dyne/cm$^2$ shear stress after endothelial stimulation for 6 hours with TNF-α at 0, 100, 250, or 500 U/ml concentration (n=6).

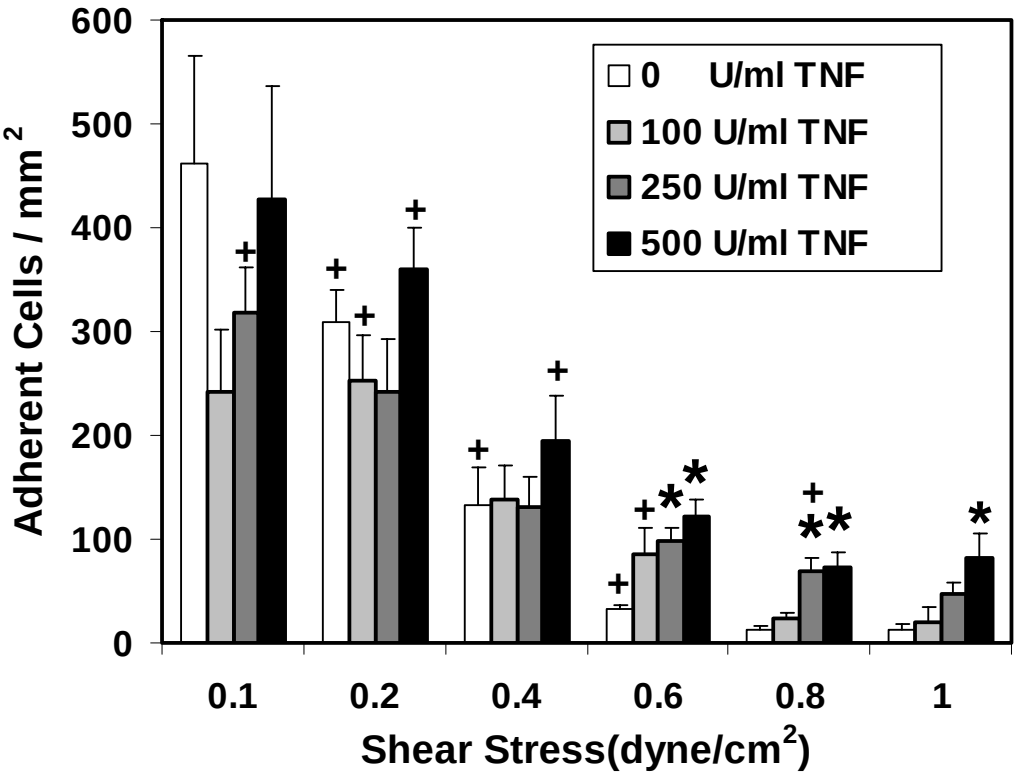


**Figure 5.2**: **Steady State Sickle Cell Adherence Across a Range of Shear is a Function of TNF-α Activation Level** Data are expressed as mean ± SEM sickle erythrocyte adherence at 30 minutes of perfusion at the indicated shear stress following endothelial cell stimulation for 6 hours with 0, 100, 250, or 500 U/ml of TNF-α (n=6). *Statistically significant increase in sickle cell adherence with TNF-α stimulation ($P \leq 0.05$) compared with adherence without TNF-α stimulation at the same shear stress. +Statistically significant increase in sickle cell adherence for the indicated TNF-α stimulation time compared with adherence for the same TNF-α stimulation at the next-lowest shear increment ($P \leq 0.05$).

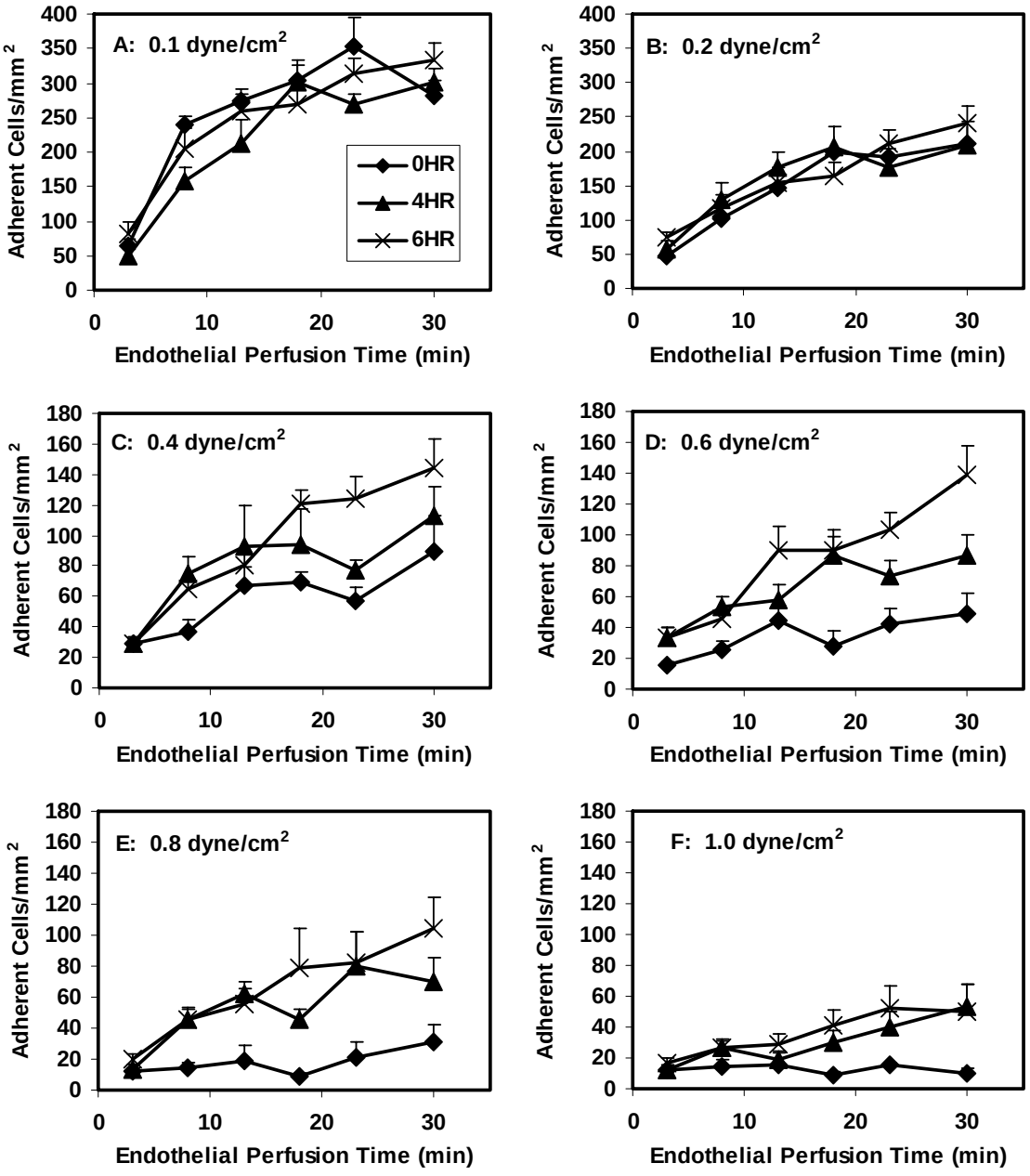


**Figure 5.3**: **Endothelial Stimulation and Shear Stress Regulate Sickle-Cell Adherence** Data are mean ± standard error of the mean (SEM) sickle erythrocyte adherence at flow locations corresponding to (a) 0.1, (B) 0.2, (C) 0.4, (D) 0.6, (E) 0.8, (F) 1.0 dyne/cm$^2$ shear stress after endothelial stimulation with TNF-$\alpha$ for 0, 4, or 6 hours (n=7). Adherence data for 2 and 8 hours of TNF-$\alpha$ stimulation omitted here and included separately in Figure 5.4 for clarity.

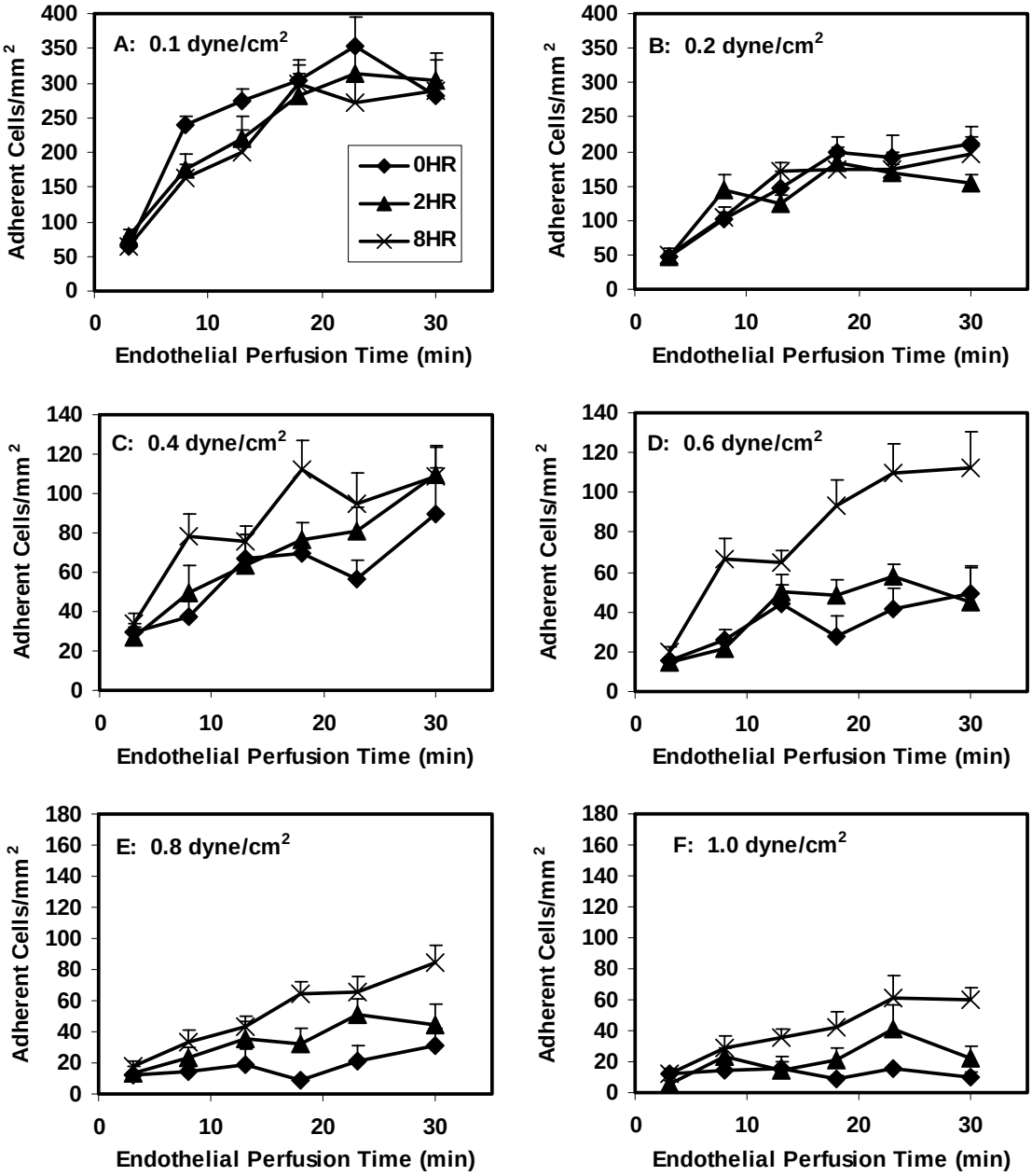


**Figure 5.4**: **Endothelial Stimulation and Shear Stress Regulate Sickle-cell Adherence** Data are mean ± standard error of the mean (SEM) sickle erythrocyte adherence at flow locations corresponding to (a) 0.1, (B) 0.2, (C) 0.4, (D) 0.6, (E) 0.8, (F) 1.0 dyne/cm$^2$ shear stress after endothelial stimulation with TNF-$\alpha$ for 0, 2, or 8 hours (n=7). Adherence data for 4 and 6 hours of TNF-$\alpha$ stimulation omitted here and included previously in Figure 5.1.

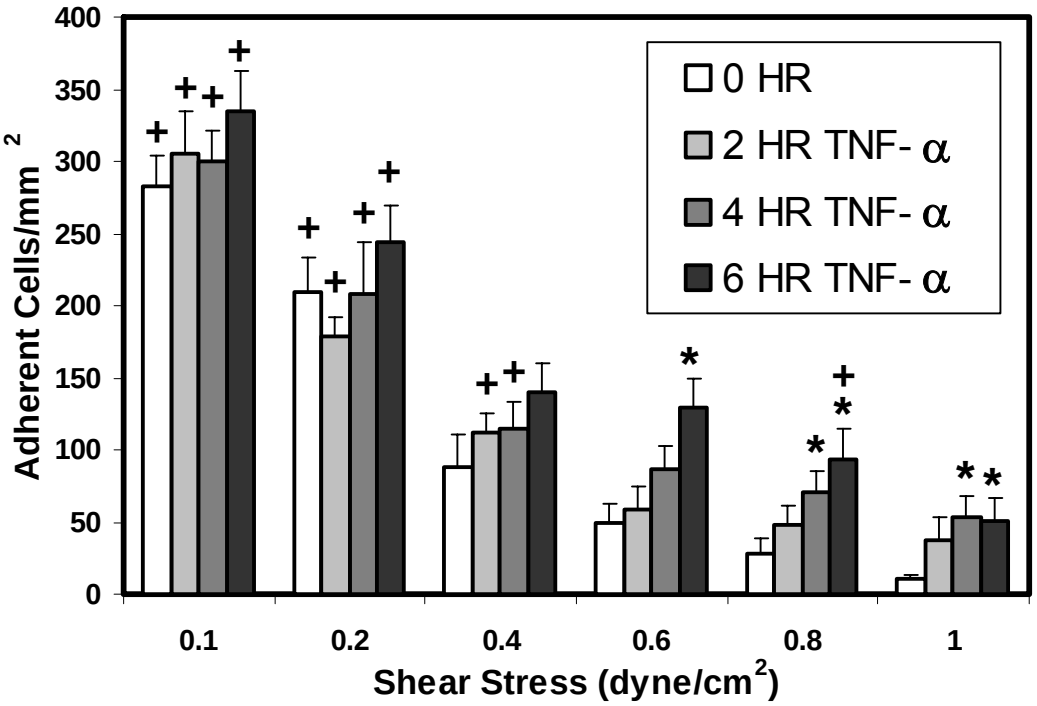


**Figure 5.5**: **Steady-State Sickle Cell Adherence is a Function of Shear Stress and Endothelial Cell Activation** Data expressed as mean ± SEM sickle erythrocyte adherence at 30 minutes of perfusion at the indicated shear stress following endothelial cell stimulation with 500 U/ml TNF-α for 0, 2, 4, or 6 hours (n=7). Data for 8 hours TNF-α stimulation presented separately *Statistically significant increase in sickle cell adherence with TNF-α stimulation ($P \leq 0.05$) compared to adherence without TNF-α stimulation at the same shear-stress rate. $^{+}$ Statistically significant increase in sickle cell adherence for the indicated TNF-α stimulation time compared with adherence for the same TNF-α stimulation at the next-higher shear increment ($P \leq 0.05$).

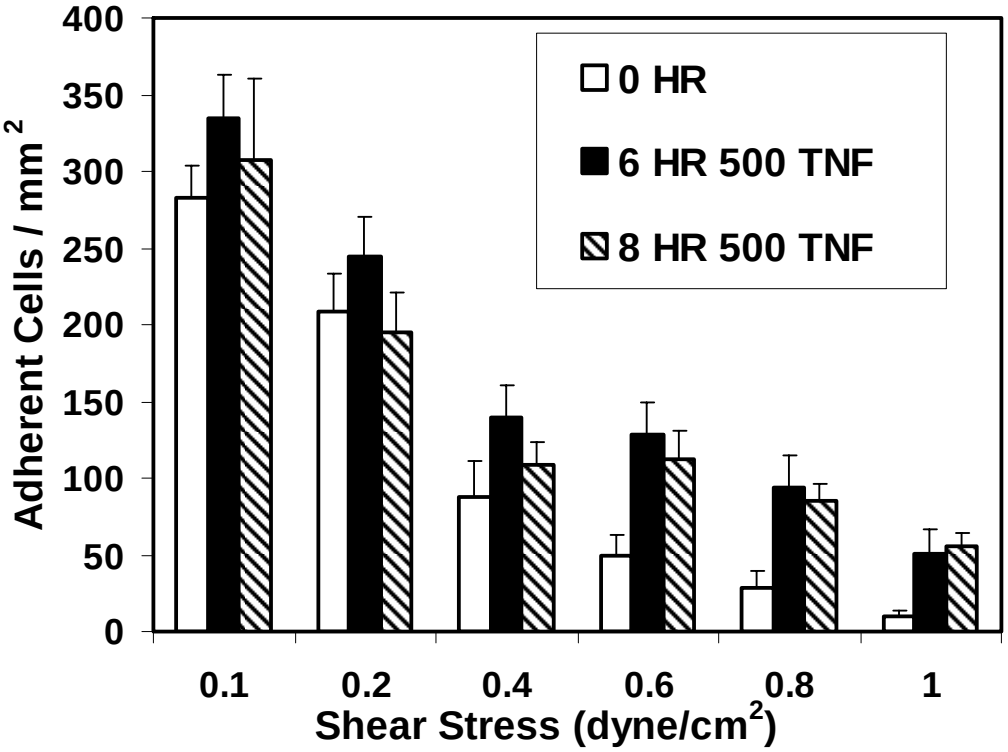


**Figure 5.6**: **TNF-α stimulation beyond 6 hours does not further increase adhesion** Data expressed as mean ± SEM sickle erythrocyte adherence at 30 minutes of perfusion at the indicated shear stress following endothelial cell stimulation with 500 U/ml TNF-α for 0, 6 or 8 hours (n=7). No significant difference was found between adhesion following 6 hours or 8 hours of stimulation at any shear rate. (Minimum P value = 0.118)

maximal endothelial adhesion in all data sets. No significant further increase was found when the TNF-$\alpha$ stimulation was extended to 8 hours, as shown in Figure 5.6. As shown in Figure 5.5 and 5.2, the effect of TNF-$\alpha$ stimulation on sickle cell adhesion is statistically significant after 30 minutes of perfusion for 0.6, 0.8, and 1.0 dyne/cm$^2$ shear stress. Statistically significant differences between 2 shear increments are mostly confined to shear rates between 0.1 and 0.4 dyne/cm$^2$.

### 5.4.2 Stimulation and Blockade of VCAM-1 Ligand

Adherence-inhibition studies with anti-VCAM-1 monoclonal antibody show that the increased sickle cell adherence induced by TNF-$\alpha$ stimulation is blocked between 90 to 100% in the range of 1.0 to 0.4 dyne/cm$^2$ shear stress (Fig 5.7 A inset). At 0.2 and 0.1 dyne/cm$^2$ shear stress, anti-VCAM-1 antibody did not significantly reduce sickle cell adherence, in part because TNF-$\alpha$ stimulation did not significantly increase sickle cell adherence beyond unstimulated adherence at these lower shear-stress levels (Fig 5.7). The high-affinity mechanism of VCAM-1/$\alpha_4\beta_1$ adhesion dominated sickle cell adherence at higher shear-stress levels (0.6, 0.8. 1.0 dyne/cm$^2$) and thus could be significantly suppressed by the anti-VCAM-1 antibody (Fig 5.7 B).

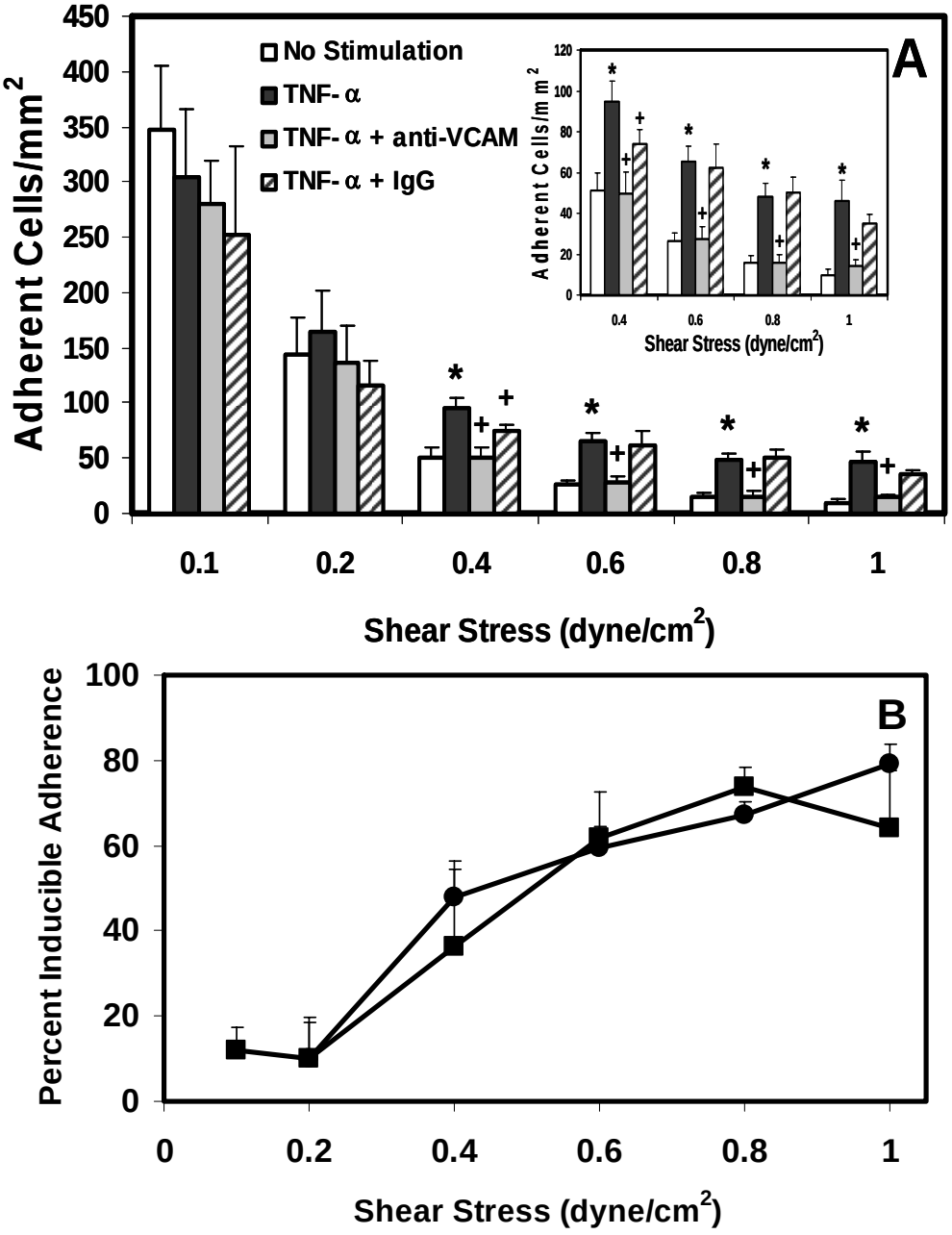


**Figure 5.7**: **Sickle cells bind to endothelial VCAM-1 receptors** (A) Data are expressed as mean ± SEM for steady-state sickle cell adherence after 30 minutes of erythrocyte perfusion at the indicated shear stress for unstimulated, TNF-α -stimulated (6 hours 500 U/ml), and TNF-α -stimulated endothelium incubated with anti-VCAM antibody or nonspecific control antibody (isotype matched IgG) (n=5). *Statistically significant ($P \leq 0.05$) increase after TNF-α stimulation with the listed antibody at the given shear rates. Data for shear stress values between 0.4 and 1.0 dyne/cm$^2$ are replotted in *inset* to clearly show the effect of anti-VCAM-1 antibody on adherence at higher shear stresses. (B) Data from Figure 5.3 (*black square*) or Figure 5.7 A (*black circle*), plotted in accordance with equation 3.2 to demonstrate the large contribution of endothelial VCAM-1 to adherence mechanisms at higher shear-stress levels.

## 5.5 Discussion

Abnormal adherence of sickle erythrocytes to vascular endothelium is a well established phenomenon and is considered an important factor regulating the microvascular occlusion that causes sickle pain episodes (Hebbel, 1997a; Wick et al., 1996). Adherence of sickle erythrocytes to the endothelium in post-capillary venules of sickle microvasculature is thought to initiate vaso-occlusive complications by two complimentary mechanisms. Erythrocytes adherent in the post-capillary venules partially obstruct blood flow and increase residence time of individual erythrocytes within the capillary. When the capillary clearance time exceeds the delay time for hemoglobin gelation following deoxygenation, sickling occurs within the capillary and the suddenly rigid cells become trapped (Wick et al., 1996). Alternatively, sickle erythrocytes adhering in the post-capillary venules may directly trap irreversibly sickled erythrocytes there (Kaul et al., 1989b). Under venular flow conditions (1.0 dyne/cm$^2$ shear stress) high levels of this initiating adherence do not occur without endothelial activation by inflammatory mediators or the presence of adhesive proteins (Walmet et al., 2003; Montes et al., 2002). High-affinity adherence is fast enough to initiate during brief cell-cell contact and strong enough to withstand elevated shear stresses (Walmet et al., 2003), thus maintaining formed bonds. In contrast, under lower flow conditions (static to 0.2 dyne/cm$^2$ shear stress) greater adhesion occurs even in the absence of activation or adhesive proteins (Montes et al., 2002; Hebbel et al., 1980b; Hoover et al., 1979). However, these low affinity interactions are weaker and unable to withstand increased perfusion pressure (Walmet et al., 2003). Thus, both shear conditions and endothelial activation regulate sickle cell adherence.

Previous studies have demonstrated the increased sickle erythrocyte adhesion under low shear (Montes et al., 2002) or static incubation conditions (Lipowsky et al., 1987). However, those studies represented only the most severe case of flow reduction.

The work presented here quantifies sickle cell adherence over a range of shear stresses with greater *in vivo* significance, representing progressively reduced microvascular flow (Lipowsky et al., 1987; Rodgers et al., 1984; Turitto, 1982) using a linear shear flow chamber. Here it was shown that, at shear stresses representing moderately depressed flow (0.6 & 0.8 dyne/cm$^2$), sickle cell adherence is significantly increased, both with and without TNF-$\alpha$ stimulation (Figure 5.1-5.7). With progressive reduction in shear stress from 1.0 to 0.6 dyne/cm$^2$, sickle cell adherence following TNF-$\alpha$ stimulation increases approximately 2-3 fold (Figure 5.5, 5.2, and 5.7, final column at each shear rate). Note that this is a relatively slow climb, and adhesion to TNF-$\alpha$ stimulated endothelium is almost stable for certain shear ranges (Figure 5.5: 0.4-0.6 dyne/cm$^2$; Figure 5.2 & B.7: 1.0-0.8 dyne/cm$^2$). This is in contrast to adherence without TNF-$\alpha$ stimulation, which progressively increases in response to reduced shear in all cases (Figures 5.5, 5.2, & 5.7 first column at each shear rate) within this range. These two trends acting together mean that, as shear decreases within this range, erythrocyte adhesion induced by TNF-$\alpha$ grows, but becomes a less significant portion of the overall adhesion, as seen in Figure 5.7 B. Despite this trend, moderate reduction in shear rate and introducing TNF-$\alpha$ stimulation results in a greater increase in adhesion than either factor individually.

When shear is depressed from 0.6 to 0.4 dyne/cm$^2$ (Figure 5.5, 5.2, & 5.7) adherence in the absence of TNF-$\alpha$ begins to rapidly rise in a trend that continues to 0.1 dyne/cm$^2$. This sudden increase may indicate a transition between different mechanisms of adherence, the increase being the contribution of some lower-affinity mechanism incapable of initiating or maintaining a bond at higher shear, or an interaction with different fractions of sickle erythrocytes as individual red cells experience longer endothelial contact at lower shear, thus increasing the likelihood of bond

formation. The increased adherence with TNF-$\alpha$ stimulation observed in the shear range of 6.0-1.0 dyne/cm$^2$ may indicate selective recruitment of a particular fraction of blood, such as the reticulocytes, which are known to express $\alpha_4\beta_1$ receptors and bind to endothelial cell VCAM-1 (Swerlick et al., 1993; Joneckis et al., 1993). Adhesion at high shear in these experiments was shown to be largely mediated via VCAM-1 activity, and thus TNF-$\alpha$ is likely selectively adhering young reticulocytes, due to their retention of $\alpha_4\beta_1$ ligand expression. Adhesion at low shear, however, depends largely on the nature of the bonds formed. If sickle erythrocytes adhere through interaction of specific ligands, then reticulocytes, as they retain more membrane ligand expression, would still be primary targets. However, the low-shear interactions are likely a combination of factors including abnormal membrane charge interactions due to altered sickle erythrocyte membrane sialic acid distribution (Montes, 1999; Hebbel et al., 1980b), erythrocyte interactions with endothelial fibronectin (Montes, 1999; Patel et al., 1985; Wautier et al., 1983) or interaction of sickle erythrocyte membrane phosphatidylserine and phosphatidylethanolamine (Choe et al., 1986; Lubin et al., 1981; Chiu et al., 1981) interacting with unknown endothelial ligands. If the adhesion at low shear arises through endothelial interaction with erythrocyte membrane characteristics typical of a dysfunctional membrane, then the denser, more damaged ISC fraction is likely becoming selectively adhered under low shear. Future studies characterizing the nature of adherent erythrocytes, could determine the contribution of specific red cell fractions responsible for the observed increases in adherence, possibly indicating them for targeted treatment.

At 0.2 dyne/cm$^2$ shear stress and above, TNF-$\alpha$ stimulation increases adherence of sickle erythrocytes to vascular endothelium, although this increase is only statistically significant for 0.6 to 1.0 dyne/cm$^2$ (Figure 5.5 & 5.2) or 0.4 to 1.0 dyne/cm$^2$ (Figure 5.7).

The effect of TNF-$\alpha$ below 0.6 or 0.4 dyne/cm$^2$ is no greater than the standard variance at these shears. Adhesion at 0.1 dyne/cm$^2$ is so little affected by TNF-$\alpha$ stimulation that standard variance in erythrocyte adhesion assays can make it appear to both increase (Figure 5.5) and decrease (Figure 5.2 & 5.7) adhesion, though never to a significant degree.

The increase in sickle cell adhesion due to endothelial stimulation is predominantly to endothelial cell VCAM-1 receptors, as adherence induced by TNF-$\alpha$ is blocked 90-100% by a VCAM-1 specific antibody in the range of 0.4 to 1.0 dyne/cm$^2$ (Figure 5.7). This is consistent with the results of previous work by our group which found 90 to 100% blocking of sickle cell adherence induced by TNF-$\alpha$ in the presence of anti-VCAM-1 antibody at 1.0 dyne/cm$^2$ shear stress (Walmet et al., 2003; Gee et al., 1995; Swerlick et al., 1993). Below 0.4 dyne/cm$^2$ shear stress, the increase in sickle cell adherence by endothelial cell stimulation is small and not statistically significant (Figure 5.5, 5.2, & 5.7), the suppression of this effect by the presence of an anti-VCAM-1 antibody is similarly not statistically significant (Figure 5.7), though decreased adhesion was seen for all VCAM-1 blocking experiments at the shear rates of 0.2 and 0.1 dyne/cm$^2$. Thus the relative contribution of endothelial cell VCAM-1 expression to sickle cell adherence decreases rapidly below approximately 0.6 dyne/cm$^2$ shear stress (Figure 5.7 A, inset & Figure B), and suggests that, at these lower shear stress values, sickle cell adherence is dominated not by the high-affinity VCAM-1/$\alpha_4\beta_1$ interaction induced by TNF-$\alpha$, but some weaker 'low affinity' interactions. Previous studies by our group suggest that at 0.1 dyne/cm$^2$ shear stress, these low affinity interactions depend partially on the presence of sialic acid residues on sickle erythrocytes and on the presence of fibronectin on the endothelium (Montes et al., 2002). These data demonstrate that the known effect of endothelial inflammatory activation on sickle erythrocyte adhesion is

strongly determined by the shear stress conditions. Both endothelial activation and microvascular hemodynamics have an important influence on sickle cell adherence and are consistent with the hypothesis that modest reduction of venular blood flow over activated endothelium could result in increased retention of highly adherent erythrocytes, possibly initiating vaso-occlusive episodes.

Laser-Doppler measurements of blood flow in sickle dermal microvasculature support the *in vivo* significance of these results. *In vivo* measurements show intermittent or periodic large, local oscillations in blood flow, and periods of general reduced flow (Rodgers et al., 1990; Kennedy et al., 1988; Rodgers et al., 1984). Studies visualizing blood flow in nail fold capillaries also detect a higher occurrence of slowed and stopped microcirculatory blood flow in sickle subjects compared to controls (Lipowsky et al., 1987). Even in patients with mild sickle cell disease, conjunctival blood flow velocity is approximately 20% lower than that measured in non-sickle patients (Cheung et al., 2002). Flow variation between subjects is high, with some sickle cell anemia patients exhibiting a greater than 90% reduction in microvascular blood flow (Cheung et al., 2002). Because those *in vivo* studies were designed to measure microvascular flow rate and not shear stress, it is unclear whether venular shear stresses reach the 0.1 dyne/cm$^2$ previously reported (Montes et al., 2002). However, those studies concluded that the blood flow rate regularly reach 50% of typical flow during oscillations (Rodgers et al., 1984), giving an associated shear stress of approximately 0.5 dyne/cm$^2$, which directly supports the *in vivo* significance of these studies. Indeed, the range of 0.4 to 0.6 dyne/cm$^2$ shows a sudden increase in unstimulated erythrocyte adhesion and a rapidly-reduced significance of TNF-$\alpha$ activated erythrocyte adhesion to the VCAM-1 ligand, which may be key to the adhesion-initiated pain crisis mechanism. Sickle erythrocyte adhesion under stimulation, therefore, needs to be evaluated within the range of shear

rates defined here in order to determine the *in vivo* significance of a particular adhesion mechanism.

The present data reinforce the importance of interactions between two factors; the availability of high affinity adherence pathways and reduced shear stress, rather than a single factor, in modulating sickle cell adherence in the microcirculation. This interaction may also account for the variability of some sickle cell pain events *in vivo*. Moderately elevated cytokine levels, typical of sickle patient plasma (Tomer et al., 2001; Duits et al., 1998; Duits et al., 1996; Browne et al., 1996b; Vordermeier et al., 1992), would be capable of promoting sufficient ligand expression for microvascular occlusion if the patient's blood flow were moderately depressed. Alternately, a sudden increase in expression of pro-adhesive cytokines might alone be sufficient in a patient who is not experiencing reduced microvascular flow. With a larger decrease in microvascular flow, sickle cell adherence may be sufficient to initiate or exacerbate microvascular occlusion and pain events independent of endothelial activation. Further studies should examine the influence of shear stress on the effectiveness of other adhesive mechanisms to promote sickle erythrocyte adherence. Correlation of these activation and shear stress effects to *in vivo* conditions should further aid in identifying dangerous vascular states for sickle patients, and help target treatments for pain crisis prevention.

This data (excluding figures 5.4, 5.6, 5.1, and 5.2) has been published as "Sickle cell adhesion depends on hemodynamics and endothelial activation" in the Journal of Laboratory and Clinical Medicine, November 2004 (Wagner et al., 2004).

# CHAPTER VI:

# SICKLE CELL ADHERENCE INDUCED BY HISTAMINE IS SHEAR DEPENDENT

## 6.1 Abstract

The episodic and reduced nature of venular microvascular circulation in patients with sickle cell anemia is thought to mediate the inflammation-driven adhesion of sickle erythrocytes to the microvascular endothelium by hemodynamically altering the contact conditions. This adhesion, in turn, is thought to initiate and propagate vaso-occlusive pain episodes. This study investigated the strong shear dependence of the *in vitro* interaction of sickle erythrocyte adhesion to endothelial monolayers when induced by the inflammatory mediator histamine. Adhesion was also strongly shear dependent in the absence of histamine. Shear stress was studied in a range from the standard post-venular microvascular flow of 1.0 dyne/cm$^2$, where P-selectin ligand interactions induced by histamine primarily controlled adherence, to the extreme case of 0.1 dyne/cm$^2$, where stimulation did not increase adhesion. Within this range, analogous to *in vivo* microvascular flow rates of sickle patients which are typically reduced and oscillatory, histamine was found to further increase adhesion (2.6, 2.0, and 1.9 fold for 0.8, 0.6, and 0.4 dyne/cm$^2$ respectively) compared to unstimulated conditions (baseline). These findings re-affirm the importance of considering shear rate as an active factor in the vaso-occlusive process, and the importance of shear in the adhesive interactions of inflammation-induced ligands. As reflected by our *in vitro* model, slight reductions of shear *in vivo* may be sufficient to increase adhesion rates and drive the vaso-occlusion mechanism forward.

## 6.2 Introduction

Adhesion of sickle erythrocytes to the post-capillary venules initiates or propagates vaso-occlusive pain episodes in sickle cell patients (Francis et al., 1991; Hebbel et al., 1980b). Activation of the endothelium with inflammatory mediators and expression of cell adhesion molecules increases sickle erythrocyte adherence to cultured endothelium (Makis et al., 2000; Wick et al., 1993). Under venular flow conditions (at a wall shear stress of 1.0 dyne/cm$^2$), histamine, a potent inflammatory mediator, increases sickle erythrocyte adherence to endothelium in a time and dose dependent manner that is strongly dependent on P-selectin expression (Wagner et al., 2006). This response is relatively rapid; endothelial activation and P-selectin expression occurring within minutes of histamine stimulation (Easton et al., 2001; Lorenzon et al., 1998), in accord with histamine's status as a localized, early-response inflammation mechanism. Histamine release *in vivo* may be relevant to clinical complications in sickle cell anemia since some analgesics, including morphine, which are used to treat vaso-occlusive events in sickle patients are known histaminergic agents (Barke et al., 1993; Casale et al., 1984) associated with other sickle cell complications (Kopecky et al., 2004).

Physiological blood flow in venules results in a shear stress of approximately 1.0 dyne/cm$^2$ (Montes et al., 2002). However, blood flow is periodic and reduced in sickle microcirculation even during pain-free periods (Kennedy et al., 1988; Lipowsky et al., 1987; Rodgers et al., 1984). Microvascular blood flow variation between sickle patients is high. Reductions in tissue perfusion frequently approach 50% (Rodgers et al., 1984) with some sickle cell patients exhibiting a microvascular flow reduction of greater than 90% (Cheung et al., 2002). Sickle erythrocyte adhesion is dependent on shear stress and endothelial cell activation (Wagner et al., 2004; Montes et al., 2002). Under venular flow conditions sickle cell adherence requires the presence of high-affinity receptor

ligand interactions (Wagner et al., 2004; Montes et al., 2002). Under low flow conditions (on the order of 0.1-0.4 dyne/cm$^2$ shear stress) sickle erythrocyte adherence is high and essentially independent of endothelial cell activation or the presence of adhesive proteins (Wagner et al., 2004; Montes et al., 2002). At shear stress levels below venular (i.e. 0.6-0.8 dyne/cm$^2$), sickle cell adherence is a function of both blood flow rate and cell adhesion molecule expression (Wagner et al., 2004). This transition likely arises due to complex interactions between receptor-ligand bond strength and hydrodynamics that regulate cell adherence. Transport of cells to the endothelial surface, and thus the frequency of cell-cell contact, is greater at venular shear stress, but higher shear reduces the duration of erythrocyte-endothelium contact times and applies greater stress on formed bonds, requiring higher-affinity, faster-forming, stronger cell-cell bonds to resist blood shearing forces (Walmet et al., 2003). At lower shear stresses, cell-cell contact time is longer and hemodynamic forces opposing adherence are weaker, allowing red cells to bind to endothelium via low-affinity non-specific interactions (Wagner et al., 2006; Montes et al., 2002). Therefore, at low shear stresses erythrocyte binding appears to depend primarily on cell transport to the endothelium. It is this interaction between cell transport and cell-cell affinity that locally controls the level of red cell binding to the endothelium (Montes et al., 2002). Thus, over a range of shear stresses in sickle microvasculature suggested by laser Doppler and intravital microscopic measurements (Kennedy et al., 1988; Lipowsky et al., 1987; Rodgers et al., 1984), sickle cell adherence, microvascular occlusion, and pain episodes *in vivo* may be regulated by both blood flow rate and availability of high-affinity receptor-ligand adherence interactions.

Since both blood flow rate and cell adhesion molecule expression contribute to sickle cell adherence, the adhesive potential of specific inflammatory mediators under a range of shear stress relevant to sickle microvascular blood flow should be evaluated to

better understand the mechanisms of vaso-occlusive events. The present study tested the hypothesis that at shear stresses typical of the sickle microcirculation, shear stress regulates the degree to which histamine stimulation increases sickle cell adherence. This hypothesis was tested in flow adherence experiments at shear stresses between 1.0-0.1 dyne/cm$^2$ (Kennedy et al., 1988; Lipowsky et al., 1987; Rodgers et al., 1984) to characterize the conditions under which shear stress and endothelial cell adhesion molecule expression regulate sickle cell adherence.

## 6.3 Materials and Methods

Experiments in this section employed HUVEC cultures on single-well Labtek® Permanox cell- culture plates in a linear-shear flow chamber. The expected short-term expression time of the ligands up-regulated in response to the presence of histamine requires the more elaborate flow chamber protocol use. These protocols are summarized in sections 3.1.1-4, 3.2.1-3, 3.3.2&5, 3.4, 3.4.2, 3.5, and 3.6.

## 6.4 Results

### 6.4.1 Histamine Mediated Adhesion is Shear Dependent

Under all conditions of shear stress studied, sickle cell adherence to endothelial cells increases from zero adherent cells/mm$^2$ to a steady-state adherence level within 30-40 minutes of the onset of sickle cell perfusion. This trend is observed for both unstimulated (Figure 6.1A) and histamine-stimulated (Figure 6.1B) conditions. At shear stresses in the range of 0.1-0.2 dyne/cm$^2$, sickle erythrocyte adherence is largely dependent upon shear stress, with histamine stimulation leading to little, if any, additional increase in sickle cell adherence (Figure 6.1D). In contrast, at shear stresses between 0.4 and 1.0 dyne/cm$^2$, sickle cell adherence was significantly increased when

stimulated with histamine (Figure 6.1C). Steady-state sickle cell adherence is a function of shear stress, with the highest adherence observed at the lowest shear stress (Figure 6.2). At shear stress levels of 0.2 dyne/cm$^2$ and 0.1 dyne/cm$^2$, steady state sickle cell adherence to endothelial cells is high even in the absence of endothelial stimulation with histamine (Figure 6.2), and is not significantly increased by histamine stimulation. In contrast, for shear stresses between 0.4 dyne/cm$^2$ to 1.0 dyne/cm$^2$, steady-state sickle cell adherence is a function of both histamine stimulation and media perfusion shear stress. At venous shear stress levels (0.8 and 1.0 dyne/cm$^2$) increased sickle cell adherence is observed only when endothelial cells are stimulated with histamine (Figure 6.2).

### 6.4.2 Stimulation and Blockade of P-Selectin Ligand

A subset (7 of 11) of patients studied in Figures 6.1 & 6.2 was further examined to characterize the role of P-selectin expressed by histamine stimulated endothelial cells in sickle cell adherence. For shear stresses where histamine significantly increased sickle cell adherence, blockade of P-selectin with a highly specific P-selectin antagonist peptide inhibited sickle cell adherence induced by histamine stimulation 69±15% - 95±5% (Figure 3) (Average ± SEM). Blockade at lower shear, where no effect of histamine stimulation was observed, produced no significant effect. Similarly, in two experiments using P-selectin blocking antibody, sickle cell adherence induced by histamine was inhibited 73-100% at those shear ranges where histamine produced a significant rise in erythrocyte adhesion. At all shear stresses where histamine increased adhesion, blockade of endothelial cell P-selectin with P-selectin antagonists reduced sickle cell adherence to the level measured on an unstimulated endothelial cell monolayer at the same shear stress.

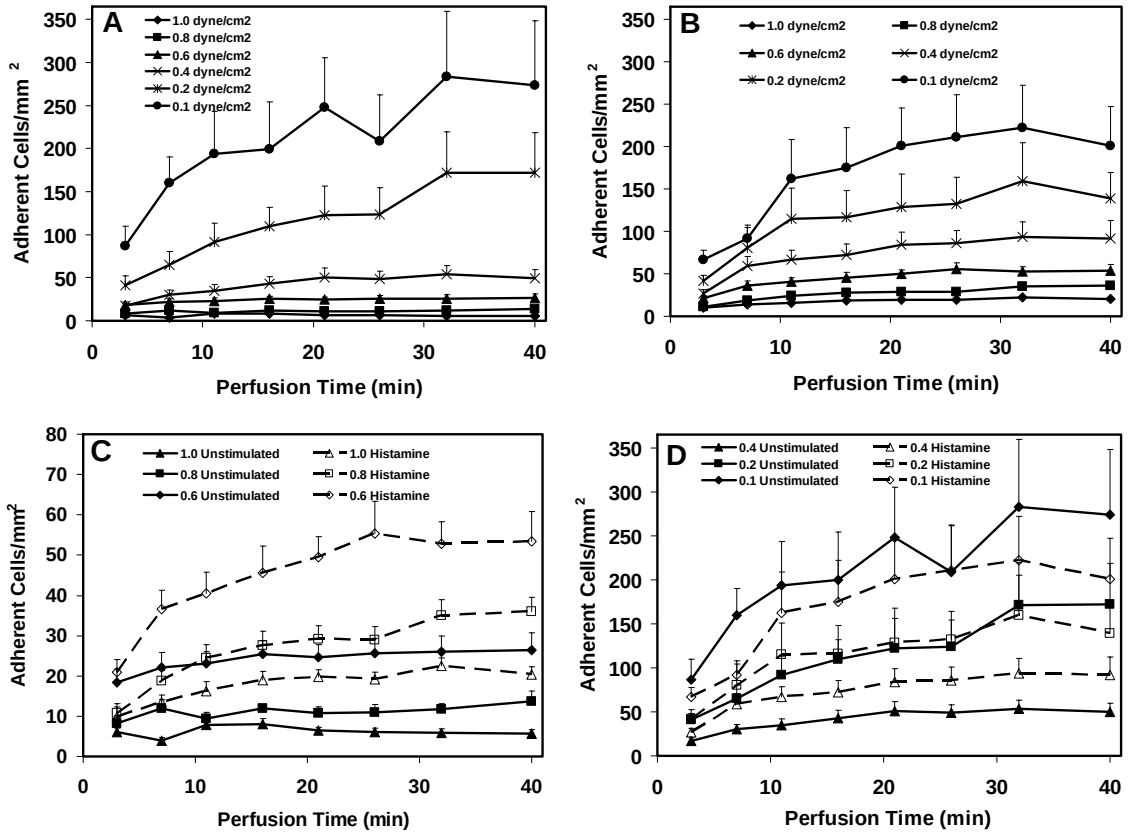


**Figure 6.1: Time-dependence of histamine-mediated sickle erythrocyte adhesion**
Data are mean ± SEM adherent red cells per $mm^2$ (n=11) for (A) unstimulated or (B) histamine stimulated endothelium (100 μM) at 3, 7, 11, 16, 21, 26, 32, and 40 minutes after the onset of erythrocyte perfusion. Unstimulated and histamine stimulated data are replotted for direct comparison in the (C) low (0.1 to 0.4 dyne/$cm^2$) and (D) high (0.6 to 1.0 dyne/$cm^2$) shear stress ranges.

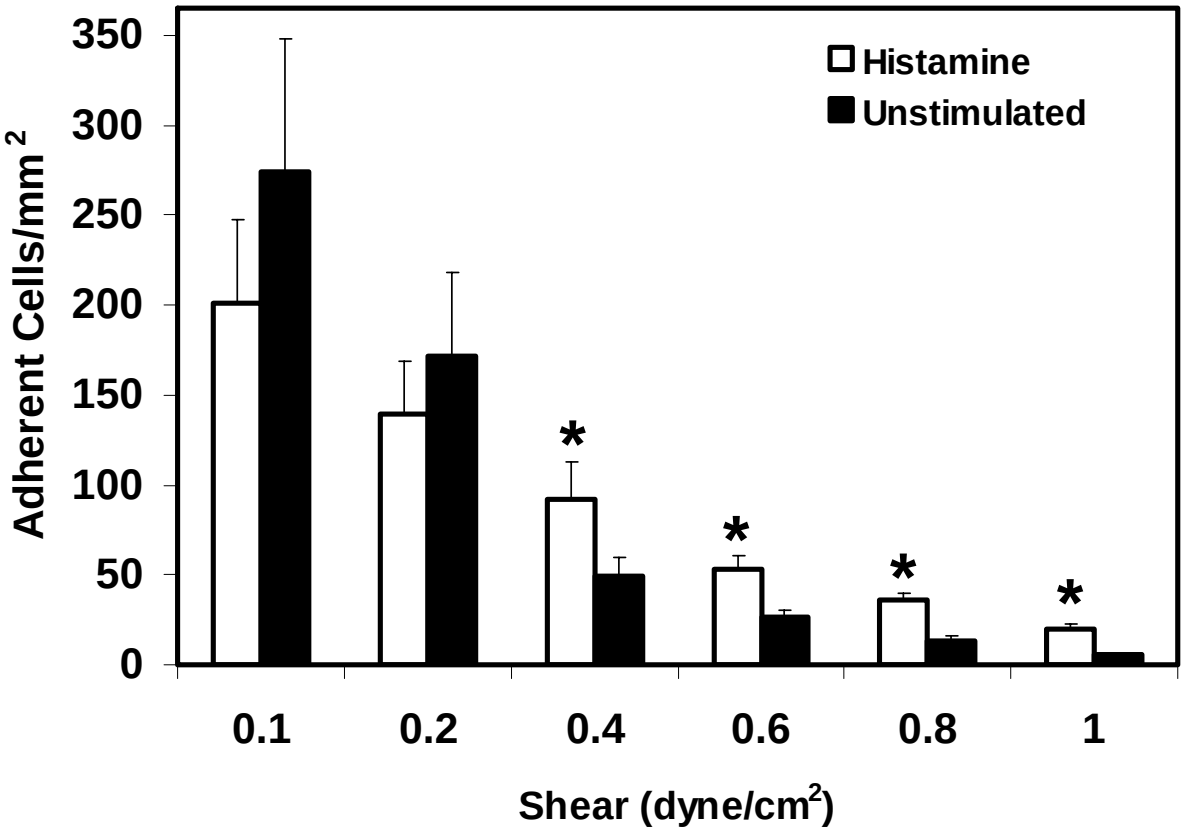


**Figure 6.2: Histamine-mediated equilibrium sickle erythrocyte adhesion is controlled by endothelial cell stimulation and shear stress** Data are mean ± SEM adherent red cells/mm$^2$ following 40 minutes of perfusion for n = 11 asymptomatic sickle patients at 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 dyne/cm$^2$ shear stress. *Statistically significant change in sickle erythrocyte adherence with histamine stimulation compared to unstimulated adherence at the same shear stress ($p \leq 0.05$).

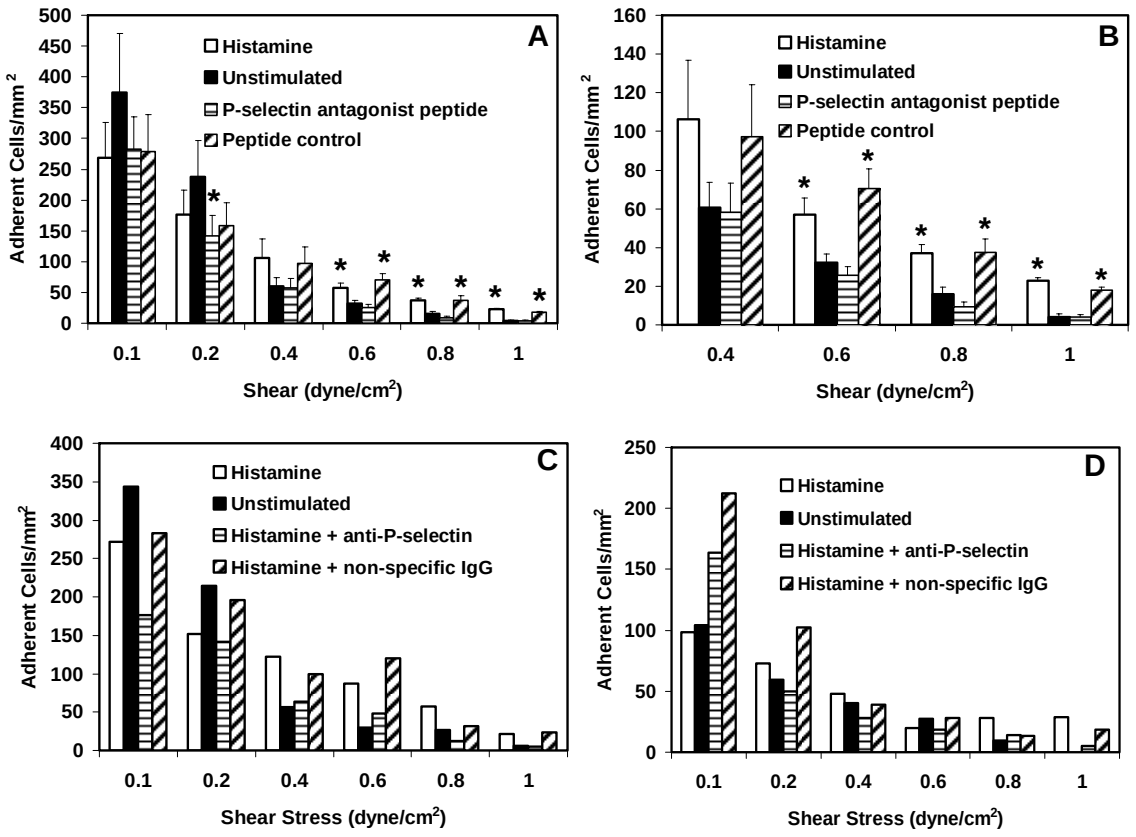


**Figure 6.3: Histamine Mediates Sickle Cell Adhesion to P-selectin** Data are mean ± SEM adherent red cells per $mm^2$ (A) following 40 minutes of erythrocyte perfusion at the indicated shear stress for unstimulated endothelial cells, endothelial cells stimulated with 100 μM of histamine during perfusion, and endothelial cells both prestimulated for 30 minutes before erythrocyte perfusion and continuously stimulated during perfusion with 100 μM anti P-selectin peptide or non-specific peptide control (n=7). These data represent a further analysis for 7 of the 11 samples studied in Figures 1 & 2. (B) Data for shear stress values between 0.4 to 1.0 dyne/$cm^2$ are replotted to more clearly show the effect of the anti-P-selectin peptide on sickle cell adherence at these higher shear stresses. *****Indicates a statistically significant ($p \leq 0.05$) change in adherence compared to unstimulated values for the same shear stress. (C&D) Two individual experiments using 5 μg/ml anti-P-selectin antibody or control non-specific antibody to block histamine-mediated sickle erythrocyte adherence.

## 6.5 Discussion

Adherence of sickle erythrocytes to endothelium in the microvasculature contributes to microvascular blockage and sickle cell vaso-occlusive pain episodes. Sickle erythrocyte adherence is a complex function of shear stress and endothelial cell activation. Adherence is increased by either an inflammatory challenge that promotes endothelial cell adhesion molecule expression to support high-affinity receptor-ligand interactions or by a reduced tissue blood flow rate that facilitates low-affinity interactions even in the absence of endothelial cell activation (Wagner et al., 2004; Montes et al., 2002). The data presented here demonstrate that histamine stimulation or reduced shear stress (even in the absence of histamine stimulation) can significantly increase sickle cell adherence. At the lowest and highest shear stress levels investigated, sickle cell adherence depends almost entirely on red cell-endothelial cell contact time and high affinity receptor-ligand interactions, respectively. Most interestingly, in the intermediate range of shear stresses (e.g. 0.4-0.8 dyne/cm$^2$), the combined effect of shear stress and endothelial cell activation is apparent. This observation is clinically relevant because sickle patients exhibit increased levels of plasma histamine (Enwonwu et al., 1991) and experience oscillatory and reduced microvascular flow conditions (Kennedy et al., 1988; Lipowsky et al., 1987; Rodgers et al., 1984). Confirmation of a role for endothelial cell P-selectin in sickle cell adherence increased by histamine under both venular and moderately reduced shear stresses further illustrates the relationship between vascular activation and local hemodynamics in regulating sickle cell adherence contributing to microvascular occlusion and pain episodes.

The presence of histamine affects sickle cell adhesion, but does so differently at venular (1.0 dyne/cm$^2$) and reduced (0.1 dyne/cm$^2$) shear, indicating that the mechanism of adhesion changes with shear stress. In the shear range between 0.4 and 1.0 dyne/cm$^2$, total sickle cell adhesion is a function of both shear stress and histamine

stimulation, with histamine stimulation accounting for ~50-80% of the observed adherence (Figure 6.2). In contrast, at 0.1 or 0.2 dyne/cm$^2$ shear stress, sickle cell adhesion is higher than that observed at 0.4 dyne/cm$^2$ and above, but is largely independent of histamine stimulation, suggesting that adhesion mechanisms other than the high-affinity ligands dominate at lower shear stresses. These low affinity adhesive interactions either require more time to form or have weaker bond strength when formed. The low-shear interactions, as detailed previously in Chapter V, are likely a combination of factors including abnormal membrane charge interactions due to altered membrane sialic acid distribution on sickle erythrocytes (Montes, 1999; Hebbel et al., 1980b), erythrocyte interactions with endothelial surface fibronectin (Montes, 1999; Patel et al., 1985; Wautier et al., 1983) or interactions between phosphatidylserine and phosphatidylethanolamine on the outer leaflet of the sickle erythrocyte membranes (Choe et al., 1986; Lubin et al., 1981; Chiu et al., 1981) interacting with unknown endothelial ligands. With low-affinity bonds dominating sickle cell- endothelial cell adherence at low shear, the addition of histamine causes no further increase in adhesion.

Sickle erythrocyte adhesion to histamine-stimulated endothelium under a range of physiologically relevant shear stresses is qualitatively similar to adhesion seen previously for TNF-$\alpha$ stimulated endothelium (Wagner et al., 2004). However, the sickle cell adherence upregulated by these inflammatory mediators is mechanistically and kinetically different. TNF-$\alpha$ stimulation increases endothelial cell VCAM-1 expression and $\alpha_4\beta_1$/VCAM-1 binding (Montes et al., 2002; Gee et al., 1995; Swerlick et al., 1993) whereas sickle erythrocytes bind to endothelial P-selectin induced by histamine stimulation (Figure 6.3). Furthermore, sickle cell adherence induced by TNF-$\alpha$ requires hours of endothelial stimulation, whereas sickle cell adherence occurs within 20 minutes

**Table 6.1: Comparison of Sickle Cell Adherence**
Data are fold increase in sickle cell adherence for agonist-stimulated endothelium compared to unstimulated endothelial cells at the indicated shear stress. NS=No statistical significance between unstimulated and agonist stimulated adherence at this shear stress. * Indicates statistical significance, defined as $p \leq 0.05$.

| | Shear Stress (dyne/cm$^2$) | | | | | |
|---|---|---|---|---|---|---|
| | 1.0 | 0.8 | 0.6 | 0.4 | 0.2 | 0.1 |
| Histamine | *3.6 | *2.6 | *2.0 | *1.9 | NS (0.8) | NS (0.7) |
| TNF-$\alpha$ (16) | *4.9 | *3.4 | *2.8 | NS (1.6) | NS (1.1) | NS (1.2) |

of histamine stimulation. A more subtle difference between the effect of TNF-$\alpha$ and histamine is related to the interaction between the shear stress and agonist stimulation on sickle cell adherence. Histamine stimulation significantly increases sickle cell adherence at 0.4 dyne/cm$^2$ shear stress and higher whereas TNF-$\alpha$ stimulation only increases sickle cell adherence at 0.6 dyne/cm$^2$ and above (Table 1). A smaller increase in adhesion at the highest shear stress may reflect lower bond strength for histamine stimulated adherence or that bond formation may require a longer cell-cell contact time unavailable at higher shear stresses. Significant increases in adhesion at lower shear rates both support the hypothesis of an adhesion mechanism able to take advantage of longer cell-cell contact times or lower bond strengths, and indicate recruitment of erythrocytes that are not otherwise recruited by the "low affinity" adhesion mechanisms significant at that shear.

This comparison between adhesive mechanisms encourages speculation as to whether histamine and TNF-$\alpha$ are even promoting the same subpopulations of sickle erythrocytes to adhere. TNF-$\alpha$, acting through promotion of the well-characterized adhesive ligand of VCAM-1, is likely selectively promoting the adherence of reticulocytes, as they retain many adhesive ligands lost upon later maturation. It is likely that many more matured erythrocytes, which have retained some $\alpha_4\beta_1$ ligand

expression, are also adhering, but reticulocytes would be overrepresented. Histamine, however, may act through very different mechanisms. It may also be primarily retaining the very young, highly adhesive reticulocytes if P-selectin is acting through the expression of some sialic acid-based ligand that, similarly to VCAM-1, is shed during erythrocyte maturation. However, if P-selectin adheres to erythrocytes through exposed PS, it is likely that histamine is promoting the adhesion of the more damaged ISC erythrocyte fraction, as those cells are more likely to have the dysfunctional membrane necessary for PS expression. In the event of microparticle-mediated sickle erythrocyte adhesion, as detailed in 2.3.3, adhesion would also likely be selective for ISCs, as the platelet or monocyte derived microparticles might be more readily bound to dysfunctional membrane than the fully functional membrane of very young erythrocytes. Finally, if adhesion is leukocyte mediated as outlined in section 2.3.4, adhesion would once again likely selectively target the damaged, PS expressing membrane of ISCs or similarly damaged erythrocytes, as this dysfunctional membrane may accumulate autologous immunoglobulin (as outlined in 2.5.1) which could serve as a targeting factor for adherent leukocytes.

As shown in the results, histamine stimulation requires a greater reduction in blood flow velocity than TNF-$\alpha$ before histamine stimulation and P-selectin expression can exert a strong effect on adhesion. Quantitative comparison is somewhat misleading, however, as the stimulation level for each factor was chosen not for direct *in vivo* relevance, but rather for maximal increase in sickle erythrocyte adhesion. Unfortunately, the localized and short-lived nature of histamine in the circulation (Irman-Florjanc et al., 1994; Beaven et al., 1982; Ferreira et al., 1973) has made estimation of *in vivo* stimulation levels very difficult. However, the results show that sickle cell adherence is dependent upon degree of endothelial activation, the specific mechanism activated, and local hemodynamics that dictate the interaction of sickle erythrocytes with the

endothelium. Relatively subtle changes in any of these factors can lead to large increases in sickle cell adherence. As a result of these complicated interactions, sickle patients may exhibit different time-dependent susceptibility to various inflammatory mediators depending on microvascular hemodynamic conditions.

The *in vitro* data presented here suggest that histamine stimulation combined with modest reduction in local blood flow rate leads to rapid and extensive adherence in sickle microvasculature following histamine release. If applied to *in vivo* conditions of sickle microvasculature, these data demonstrate that histamine release would have the greatest risk of initiating a pain crisis when release occurs in combination with a reduction of microvascular blood flow that reached 0.6 dyne/cm$^2$ or 0.4 dyne/cm$^2$. A pain crisis initiated by histamine-mediated adhesion would be dependant both on the intensity of histamine stimulation and a reduced prevailing shear stress.

The rapid and transient nature of histamine-mediated increase in sickle erythrocyte adhesion also suggests the possibility of involvement in alteration of microvascular flow in sickle patients. We can speculate that localized histamine tissue stimulation would cause upregulation of P-selectin expression on the endothelium. Resulting adhesion, both of sickle erythrocytes and leukocytes, would partially obstruct vessels of the microvasculature and slow the prevailing blood flow. Then, widespread weak (Walmet et al., 2003) adhesion of sickle erythrocytes via low-affinity adhesion mechanisms could take place, further reducing the prevailing shear stress. At this point, the process recalls the kinetic theory of vaso-occlusive events, initial adhesion leading to vaso-occlusion through reduced flow and increased capillary transit time. However, the transient expression of P-selectin means that it could be down-regulated in expression before vaso-occlusion occurs. Sickle erythrocytes and leukocytes would begin to detach and re-enter the bloodstream, enabling increased blood flow, and detachment of those cells adherent via low-affinity mechanisms. Thus the microvasculature could go through

a temporary cycle of reduced blood flow without vaso-occlusion in response to histamine stimulation. This is interesting because it resembles the oscillatory and reduced blood flow typical of sickle microvasculature. The rapid and transient expression of P-selectin in response to histamine stimulation may, therefore, cause, contribute to, or exacerbate the irregular microvascular flow known to occur in sickle cell patients.

Because histamine induces a rapid inflammatory response independent of longer-term inflammatory responses, it appears capable of contributing to sickle cell adherence and vaso-occlusion within minutes of exposure as a response to a localized inflammation, allergic response, or administration of a histaminergic drug. Other inflammatory mediators, such as TNF-$\alpha$ require hours to induce endothelial cell adhesion molecule expression. Given the potential for rapid increases in sickle cell adherence following histamine stimulation, use of antihistamines to prophylactically prevent endothelial cell activation and sickle erythrocyte adhesion may be a safe measure to manage histamine-related pain crisis. The data presented here have demonstrated the possible involvement of histamine in the mechanism of vaso-occlusive crisis when present under conditions of reduced microvascular flow. Further investigation of histamine-mediated sickle erythrocyte adhesion needs to be conducted with *in vitro* models to better evaluate the risk posed by histamine release in sickle patients.

# CHAPTER VII

# ADDITIONAL STUDIES

## 7.1 Nitric Oxide Synthase Study

### 7.1.1 Intent and background

Nitric Oxide and Sickle Erythrocyte Adhesion

During studies of histamine mediated sickle erythrocyte adhesion, an attempt was made to more thoroughly identify the progression of signal cascade leading from activation of endothelial histamine receptors to adhesive ligand expression. Our own studies and those of the literature showed the potential involvement of numerous inflammatory, thrombotic, and immune-response related pathways in promoting the adhesion of sickle erythrocytes, and the chronically activated nature of sickle patient vasculature hinted at simultaneous action of many adhesive mechanisms in the propagation, if not the initiation, of sickle pain crisis.

It was hypothesized that different intercellular processes leading from endothelial activation to adhesive ligand expression might have common mechanisms in the pathways for signal propagation. Identification of these common signaling mechanisms would offer good targets for treatment, enabling simple suppression of several adhesive mechanisms at once, and perhaps reducing pain crisis incidence by providing a blanket reduction in sickle erythrocyte adhesion. Such a reduction could act as a preventative measure in small doses, or, in large doses, a treatment for de-escalation of a patient experiencing inflammatory symptoms leading to pain crisis. Without signaling pathways in common, anti-adhesion treatments for patients with sickle cell anemia would involve use of multiple pharmacologic suppression treatments, or guessing at individual

adhesion pathways for treatment, while vaso-occlusive crisis might be triggered by still other unblocked pathways.

Assays were designed to determine the signaling pathway employed by the endothelium to propagate the histamine signal into the expression of adhesive ligand P-selectin. Even if common connections with other adhesive pathways weren't found, histamine itself is a fast-acting inflammatory mediator, and the expressed P-selectin ligands are known to be expressed and downregulated relatively quickly, making unfeasible any attempts *in vivo* to either remove released histamine from the blood plasma or block the adhesive ligand after expression. This left only systemic prevention of histamine expression (discussed in Chapter IV in relation to histaminergic analgesics) or prophylactic blockade of the intercellular signaling as viable targets for prevention of sickle pain crisis as mediated by histamine.

After examination of the endothelial histamine receptors (Chapter IV), intercellular signaling mechanisms were investigated, seeking a potentially controllable mediating factor for expression of P-selectin during histamine stimulation. The vascular signaling molecule nitric oxide (NO), and the enzyme responsible for its production, nitric oxide synthase (NOS), were found to be of particular interest, because histamine is known to increase the activity of endothelial nitric oxide synthase (eNOS) (Kostic et al., 1995; Yan et al., 1994; Ayajiki et al., 1992). It is also known that inactivation of eNOS prevents histamine mediated neutrophil adhesion (Schaefer et al., 1998). Processes similar to this may drive sickle red cell adhesion increased in the presence of histamine.

NO is a soluble gas constitutively synthesized in endothelial cells from the amino acid L-glutamine by endothelial NOS (eNOS), and is a critical endogenous vasodilator (Palmer et al., 1988; Ignarro et al., 1987; Palmer et al., 1987). While NO is normally associated with vasodilation and various "vaso-protective," anti-adhesive actions of the endothelium (Albrecht et al., 2003; Gewaltig & Kojda, 2002; Bolli, 2001), its involvement

in the mechanism of histamine and neutrophil adhesion indicate an involvement in inflammatory promotion. This apparently contradictory involvement makes NO control and mechanistic participation multifaceted and complex. Further complicating control of NO production, is the unusual response of sickle vasculature to nitric oxide. The vasodilation of sickle vasculature is blunted and requires much higher levels of NO to fully vasodilate (Belhassen et al., 2001; Aslan et al., 2001). This may occur because sickle plasma is a "sink" for NO, the plasma's increased levels of oxidative molecules can react with NO, removing it from the signaling system (Aslan et al., 2001; Dias-Da-Motta et al., 1996) while possibly oxidatively damaging the endothelium. These changes may alter the effect of NO signaling within the systems of ligand expression.

Nitric oxide is likely involved in mediating the previously examined (Chapter IV) interaction of histamine-mediated adhesion of sickle erythrocytes. Nitric oxide's clearly dysfunctional state in sickle microvasculature further implicates its involvement in microvascular occlusion. Control of this mechanism may enable prevention of sickle pain event-initiating erythrocyte adhesion as mediated by histamine and other adhesive mechanisms.

### 7.1.2 Materials and Methods

To verify the involvement of nitric oxide synthase (NOS) in sickle cell adherence elevated by histamine, specific NOS-blocking agents diphenyleneiodonium chloride (DPI) at 30 nM (Sigma) (Wang et al., 1993; Stuehr et al., 1991) or Nω-nitro-L-arginine methyl ester at 100 μM (L-NAME, Sigma) (Albrecht et al., 2003) were added to endothelial cells in a manner detailed in section 3.3.6 during parallel-plate adhesion assays. These concentrations have been reported previously to (DPI) inhibit the activity of nitric oxide synthase in endothelium-dependent vasodilation and partially inhibit the

relaxation elicited by acetylcholine in rabbit aortic rings (Stuehr et al., 1991), or (L-NAME) cause progressive and irreversible inhibition of NO synthase following initial competitive binding (Albrecht et al., 2003) and completely abolish endothelial-dependent relaxation of rat aorta induced by acetylcholine (Rand & Li, 1993).

### 7.1.3 Partial Results

Histamine-induced sickle erythrocyte adherence observed in 1.0 dyne/cm$^2$ parallel-plate assays is blocked by specific NOS-blocking agent diphenyleneiodonium chloride (DPI) or Nω-nitro-L-arginine methyl ester (L-NAME) antagonist treatment of endothelial cells (Figure 7.1). DPI, a NOS inhibitor, decreases sickle cell adherence induced by histamine an average of 80% from 41±6 red cells/mm$^2$ to 21±3 red cells/mm$^2$ (Figure 7.1A). Additional tests with DPI concentrations as high as 3 μM did not further reduce adherence. Direct examination of the endothelium following assays with these higher concentrations found signs of endothelial damage (data not shown), but no endothelial cell damage was seen at lower DPI concentrations or with 100μM histamine alone. Similarly, NOS inhibition by the eNOS-specific inhibitor L-NAME decreases sickle cell adherence induced by histamine an average of 98% from 46±4 to 14±3 (Figure 7.1B, $P < .01$ for all time points beyond 10 minutes).

### 7.1.4 Discussion

The participation of NO in histamine-stimulated adhesive ligand expression was supported by these studies in which endothelial nitric oxide synthase (NOS) activity was halted by two independent blocking mechanisms. This blocking resulted in sickle erythrocyte adhesion under histamine stimulation being reduced to unstimulated levels. Blocking NOS activity by flavoprotein inhibition (DPI) and, independently, by competitive

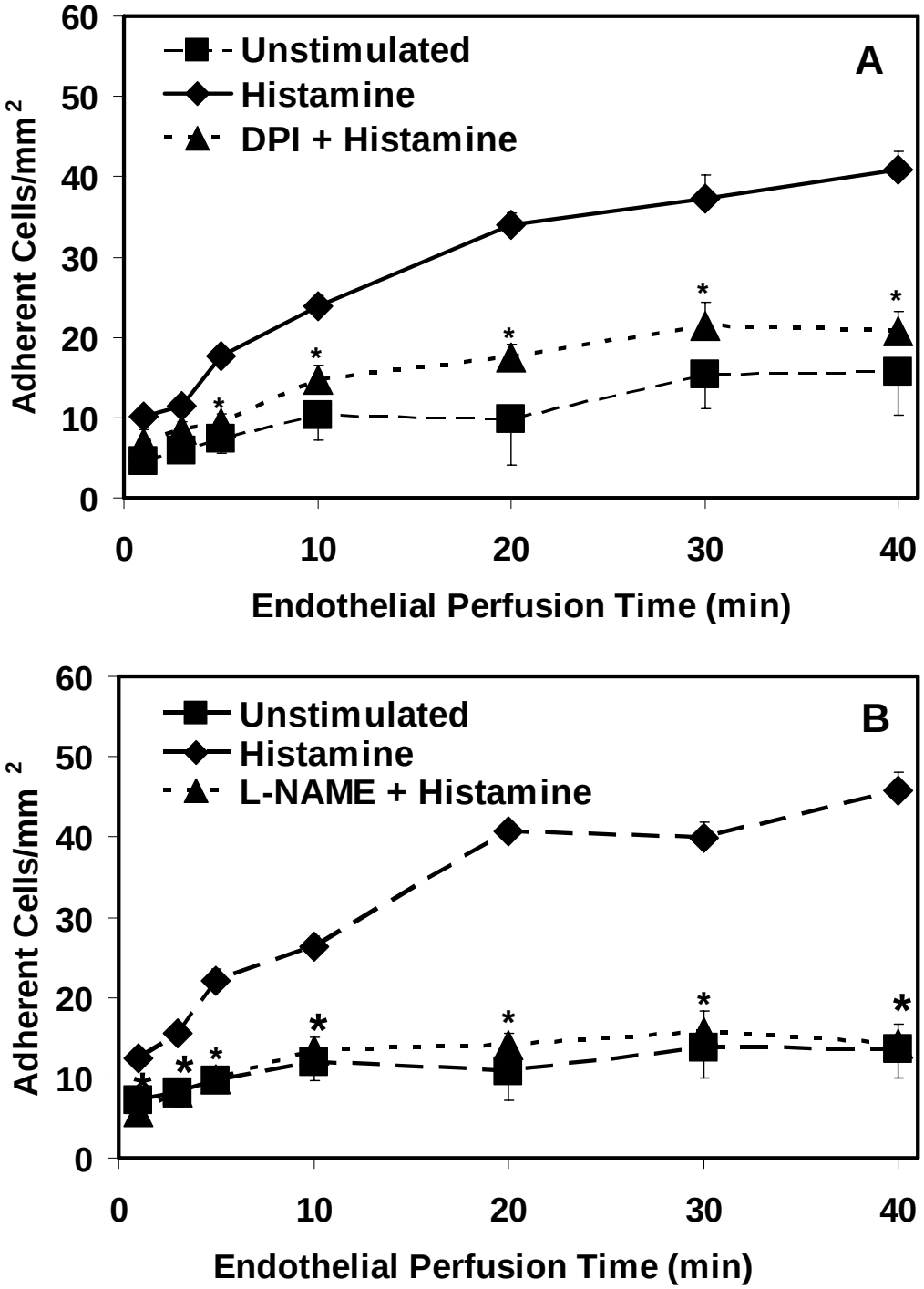


**Figure 7.1: Blocking of Nitric Oxide Synthase Activity Inhibits Sickle Cell Adherence Induced by Histamine** Endothelial monolayers were pretreated for 30 minutes with 30 μM diphenyleneiodonium chloride (DPI) (A) or 100 μM Nω-nitro-L-arginine methyl ester (L-NAME) (B) before histamine stimulation (100 μM) and sickle erythrocyte perfusion at 1.0 dyne/cm$^2$, maintaining this stimulation throughout the entire 40 minute erythrocyte perfusion. Data are mean ± SEM for n=7 (A) or n=9 (B). * Indicates statistically significant difference ($p<0.05$) from histamine stimulated adherence level at the same timepoint.

inhibition with an L-arginine analog (L-NAME) was necessary to be certain that NOS inactivation was the disabled mechanism reducing sickle erythrocyte adhesion, and not some secondary cellular process. Due to the broad action of the NOS blocking agents, it is conceivable that L-NAME might have reduced sickle erythrocyte adhesion through interaction with other cellular processes that are affected by L-arginine. Similarly, DPI, as a flavoprotein inhibitor, could conceivably have altered other cellular processes as well as interfering with the regulation of NOS. However, the intersection of these two inhibitor mechanisms in the eNOS system is unique. The fact that identical results are seen after inhibiting NOS activity by flavoprotein inhibition and after competitive inhibition with an L-arginine analog demonstrates that NOS inactivation is what causes the decrease in adherence, not secondary effects on another signaling pathway.

Nitric oxide, found here to be a key signaling mechanism in histamine mediated adhesion, is constitutively expressed in endothelial cells by endothelial NOS (eNOS) (Alderton et al., 2001). While normally associated with “vaso-protective” actions of the endothelium, as noted above, interpretation of the role of NO is greatly complicated by the prevention of neutrophil adhesion (Schaefer et al., 1998) by eNOS inactivation, and histamine's activation of NOS as seen in the literature (Li et al., 2003; Yan et al., 1994) and demonstrated in these results. Thus NOS production of NO is strangely contradictory in that it seems largely anti-inflammatory, but is also involved in mechanisms that are part of inflammation. The further complications offered by sickle vasculature in particular, the blunted vasodilatory response and sickle plasma acting as an NO "sink", only serve to make proposed NO-based treatments more problematic. And yet, some success has been had with *in vivo* administration of L-arginine to patients, a factor known to promote NO production (Morris et al., 2003; Romero et al., 2002; Vichinsky, 2002; Morris et al., 2000), or with directly inhaled doses of NO (Weiner

et al., 2003; Cannon, III et al., 2001; Gladwin & Schechter, 2001) which have shown potential for possibly reducing the severity and duration of acute sickle pain crisis.

This histamine and NO-dependent data suggest alternate or additional treatments for the decrease of pain crisis severity and frequency. Control of the signaling mechanism activated in response to adhesion-inducing reagents is promising, as this would preempt the signal before ligand expression, and halting the expression mechanism instead of trying to block each adhesive ligand as it is expressed. Further, inactivation of the intracellular signaling mechanism may inactivate multiple ligand expression mechanisms with a single treatment. Results in Chapter IV, elaborating the part played by the specific $H_2$ and $H_4$ receptors, suggest a simple pharmacologic blockade for prevention of initiating adhesion. Unfortunately, NO blocking is a more difficult prospect. The involvement of NO mechanisms in the control of numerous systems, including normal vascular tone, and the incomplete current understanding of these mechanisms, prevents considering pharmacologic blocking of NOS activity as a preventative therapy for pain crisis. However, the knowledge that histamine promotes adherence of sickle cell erythrocytes to stimulated endothelium via a NOS-dependent pathway may prove useful when more is known about NO vascular regulation.

### 7.1.5 Experimental Challenges

The data presented in this section proved to be highly problematic in several respects. Initial investigations of the nitric oxide literature led to the hypothesis that, as large doses of nitric oxide had led to an improvement in patient condition (Belhassen et al., 2001; Gladwin et al., 2001), the blockade of NOS and thus drastic reduction of NO (due to it's short in-vitro lifespan (Aslan et al., 2001; Dias-Da-Motta et al., 1996)) would lead to increased sickle erythrocyte adhesion. This increase might be even greater when in the presence of histamine, as NO was initially thought to be a mediating factor

to blunt the pro-adhesion histamine response. However, upon the discovery that our techniques for blocking the formation of NO in the cell led to a decrease in sickle erythrocyte adhesion, the literature was studied in much greater detail. A surprisingly complicated and apparently contradictory system of interactions and interrelations was found in relation to NOS, as detailed in 7.1.1 and 7.1.4.

From these limited results, the status of NOS and eNOS in relation to sickle erythrocyte adhesion increased in the presence of histamine would seem best interpreted as a regulatory factor. When the system is flooded with NO or made to enormously over-produce NO with the addition of L-arginine, the endothelium responds with drastic decreases in adhesive factors. However, when NO production is completely halted, sickle erythrocyte adhesion is similarly halted. When NO production is allowed to respond normally to the presence of histamine, adhesion is increased. Thus it seems that NOS acts as a messenger in the propagation of sickle erythrocyte adhesion via histamine stimulation, propagating the signal when active, incapable of propagating the signal when pharmacologically blocked, and when flooded with NO, alternate cellular systems respond to the high dose of the vaso-relaxer. Thus, small regulatory quantities of NO are required for normal cellular response, while large influxes of NO constitute an overwhelming signal that the endothelial cells deal with differently.

Further in-depth studies of NO and NOS to evaluate these points would require adhesion assays employing widely varied levels of nitroprusside, an NO donor, to test the level at which NO becomes severely anti-adhesion. ELISA studies could be employed for detection of intracellular NO production in the presence of histamine, as well as $H_2$ and $H_4$ agonists and antagonists to determine if the NOS activation is receptor-dependent. However, it is difficult to conceive of applying an NO blockade *in vivo* for potential patient treatment, especially in the face of other successful treatments acting in direct opposition to this proposed treatment. In light of the acknowledged

complexity of the NO system uncovered here, it seems unlikely that NO blocking treatments will soon acquire the degree of control necessary to fine-tune the NO signaling system and make prevention of sickle erythrocyte adhesion in patients via this method a viable alternative.

## 7.2 Alternate Endothelial Cell Sources

### 7.2.1 Intent and Background

The vasculature of sickle cell patients, specifically the endothelial monolayer of the microvasculature, is exposed to a hemodynamic and cell signaling environment very different from non-sickle patient vasculature. The shear environment is significantly reduced and oscillatory in comparison to non-sickle patient vasculature; likely as a result of blunted blood flow regulatory response (such as the blunted response to nitric oxide mentioned in section 7.1.1) or apparent loss of vaso motor tone. Further, sickle microvascular endothelial cells are under constant exposure to inflammatory, thrombotic, and immune-response mediating factors that are typically found at elevated levels in sickle patients (covered in detail in sections 2.2.3, 2.3.3, and 2.6). These conditions are supported by examination of circulating endothelial cells (CECs) from patients with sickle cell anemia. CECs are found in increased numbers and in a viable but activated state (Solovey et al., 1999; Solovey et al., 1997) implying both a mechanical trauma (Mutin et al., 1999) to the sickle endothelium and a response to a chronically activated vascular signaling system. Further, studies of the transgenic sickle mouse model have found similar vascular dysfunctionalities in this animal model as a result of its expression of sickle hemoglobin, including cytokine-mediated pro-inflammatory (Belcher et al., 2003) and pro-thrombotic (Wood et al., 2004b) states, and otherwise demonstrating a range of vascular instabilities (Nath et al., 2000; Kaul et al., 2000b). Thus it is believed that the

endothelial monolayers in sickle patient vasculature are chronically damaged and constantly exposed to a chronically pro-inflammatory, pro-thrombotic, and pro-immune responsive (and thus pro-adhesive) environment.

Importantly, this implies that sickle endothelium is phenotypically different from endothelial cells harvested from non-sickle patients. Endothelial cells constantly exposed to chronically elevated levels of activation factors, growing in a mechanically damaged environment, and perfused with altered shear dynamics, are likely to subsequently respond differently to further elevation of these factors or changes in shear when compared to standard endothelial cultures. As such, the altered endothelium of sickle microvasculature is likely a key factor in the adhesion that initiates and propagates incidents of sickle pain crisis. Yet this consideration is largely ignored in current *in vitro* modeling of sickle erythrocyte adhesion.

Two separate sets of studies were designed to acquire an endothelial culture for use in flow chamber assays that would be more physiologically relevant to sickle erythrocyte adhesion *in vivo*. These studies attempted to derive endothelial cell cultures from (7.2.2) circulating endothelial cells from sickle patient blood samples or (7.2.4) the lungs of the "sickle mice" animal model.

### 7.2.2 Circulating Endothelial Cells: Materials and Methods

The number of circulating endothelial cells found in blood samples from patients with sickle cell anemia are notably elevated (Solovey et al., 1997), with an average of tenfold more cells per milliliter of blood. Further, these cells show a much higher than normal degree of viability (Solovey et al., 1997), making them a target for both separation and culture. Several different techniques were employed by both an undergraduate REU assistant supervised by me, Chen Yang, and myself in an attempt to separate and culture these endothelial cells for use in perfusion chamber studies.

Two primary techniques were employed in attempting to culture circulating endothelial cells. The first involved collection of the ordinarily discarded buffy coat from patient blood samples. The buffy coat was then resuspended in complete MEC media, and plated in 10 cm Petri-dishes pre-coated overnight with 2 ml of 0.1% gelatin solution. (The number of dishes and volume of media used was varied experimentally.) This technique, derived from the experimental techniques of Dr. Hanson (protocol formulated from in-person conversation) of the Yerkes National Primate Research Center, was attempted both with 2-5 ml blood samples collected normally for adhesion studies (see section 3.2.1), and from large (~900 ml) discard blood samples. With the transfusion blood samples, large amounts of buffy coat were collected, and were repeatedly re-centrifuged to remove sickle erythrocyte fractions. After two hours, supernatant was gently aspirated from the Petri dishes, and fresh complete media was added, being sure to agitate the plated buffy coat into a suspension again. After a further eight hours, the Petri dishes were thoroughly rinsed to remove non-adherent cells and buffy coat, and fresh media was added. Dishes were fed regularly every other day following.

The second technique used an endothelial-targeted antibody coupled with magnetic beads used to specifically attack the CECs. The following protocol was derived from the literature (Solovey et al., 2001b; Lin et al., 2000; Solovey et al., 1997; George et al., 1992). A supply of Dynabeads® M-450 Goat anti-mouse IgG, a set of monosized, superparamagnetic, polystyrene beads with 4.5 μm diameter and affinity purified Goat anti-mouse IgG covalently bonded to their surface was acquired (Invitrogen, Carlsbad, CA.). Blood samples were then incubated with the mouse anti-human monoclonal antibody to P1H12 (Chemicon, Temecula, CA). In blood, this antibody reacts exclusively with CD146, a receptor only on endothelial cells. A concentration of 10 μg/ml of the monoclonal antibody was used. A Dynabead solution

was then added to and incubated with the blood sample. After incubation, the endothelial cells tethered to the paramagnetic polystyrene beads were drawn out of solution through the use of a strong magnet embedded in a centrifuge tube holder (Dynal A. S.), and the remainder of the blood solution was decanted away. Antibody and Dynabead® concentration as well as the incubation time for both were widely varied in an attempt to improve the technique's efficiency. After the supernatant blood was decanted away, the centrifuge tube was removed from the magnetic holder, the remaining cells were resuspended in complete MEC media, and the solution was plated in Petri dishes similarly prepared as in the first technique. The cells were allowed to attach overnight, then fed with fresh media 12 hours later, and every two days following.

### 7.2.3 Circulating Endothelial Cells: Partial Results

Unfortunately, despite exhaustive repetition (detailed below), none of these techniques resulted in viable cultures. On several occasions, dispersed individual cells or small clusters of endothelial cells were visually identified and attempts were made to culture them, but the cultures exhibited extremely slow growth, where growth was seen at all, and all eventually became contaminated or apoptotic and detached. The primary reason for the failure of these techniques was the scarcity of the endothelial cells sought. Although viable circulating endothelial cells are elevated in comparison to those found in blood from non-sickle patients, 12-22 cells per ml would give <100 cells per normal blood sample received. Even with 100% efficiency of cell harvesting, this is still an exceedingly small number of cells for culture. Further, endothelial cell proliferation is partially motivated by contact with other endothelial cells, naturally unavailable in such a small, dispersed culture. Although a culture was never attained, there is also some question as to whether CECs passaged the multiple times necessary to reach the

volume needed for repeated perfusion chamber assays would still retain the altered phenotypic nature of sickle endothelium.

Extensive alteration was made to the centrifugation technique outlined in section 7.2.2 in an attempt to increase the efficiency of endothelial cell separation and/or culture viability. Separation based on simple centrifugation was based on the work of Dr. Hanson, who had successfully separated circulating endothelial cells from baboon blood. Our concern with this technique focused on endothelial viability during the "plating" stage. The endothelial cells were plated along with concentrated portions of buffy coat, including leukocytes and platelets, whose response to centrifugation, separation, and incubation likely included large releases of cytokines and thrombotic factors adversely affecting endothelial cell adhesion and proliferation. Further, the small but significant fraction of erythrocytes captured with the buffy coat could lyse if retained in the media for a long time, releasing similarly detrimental heme, iron ions, and oxidative factors into solution. With this in mind, a range of incubation times and wash protocols were used in an attempt to allow endothelial cells maximum time to attach while balancing against the minimum exposure time to the other fractions of the buffy coat. These techniques included aspiration of excess media when the buffy coat had settled to the surface, followed by resuspension in fresh media to dilute released factors, as well as roughly washing-out buffy coat fractions once endothelial monolayers had been allowed to attach. Including all culture and plating variations, this technique was employed with a total of 21 blood samples, and produced only two potential cultures, one of which showed some growth (~250 cells) before becoming apoptotic and detached.

In an attempt to address the low quantity problem of circulating endothelial cells, much larger blood samples were employed on two different occasions in identical centrifugation separations. For each occasion, approximately 900 ml of discard blood from a single sickle-cell transfusion patient, acquired from the Georgia Comprehensive

Sickle Cell Center at Grady Memorial Hospital with the assistance of Dr. Hsu were employed in these studies. This, however, only compounds the difficulty of dealing with the excess buffy coat during the plating stage, as the total volume of buffy coat reached 30 to 40 ml, with considerable overlap with the lightest erythrocyte fractions. Unfortunately, this variation also did not result in viable sickle endothelial cell cultures, likely due to the high concentration of platelets and leukocytes activated by centrifugation.

Similar difficulties were encountered in the use of the paramagnetic polystyrene beads in imitation of the work of Dr. Lin (Lin et al., 2000) who had successfully separated, cultured, and characterized endothelial cells from human blood samples. These studies were conducted primarily by Chen Yang under my supervision. Despite extensive variation in the antibody and polystyrene bead concentration and the incubation time, both above and below that recommended by the manufacturer's protocol (Dynal, 2001), separation via the technique described in 7.2.2 was unsuccessful in isolating sufficient endothelial cells for a viable culture. The techniques upon which the magnetic separation protocols were based were designed for separations of many more cells than our 10~20 per milliliter of blood. Our protocol variations on this technique were thus a balance between sufficient paramagnetic beads and sufficient incubation time for the polystyrene beads to distribute and attach to the circulating endothelial cells, but keeping the number of beads and incubation time low enough that excessive numbers of paramagnetic beads don't attach to the cells and hinder their ability to attach to the cell culture surface and proliferate. This is an especially delicate manipulation considering that circulating endothelial cells have already been severely stressed by the mechanical damage that led to their circulating status. Further technique adjustments were also attempted. Approximately 19 attempts were made to separate viable circulating endothelial cells from individual blood samples. Of those

attempts, six yielded adherent endothelial cells. Culture attempts of up to six weeks were made with these cells, but were unsuccessful, as three were lost to bacterial contamination (a cumulative hazard of long-term culture), and only one showed significant growth, reaching approximately 500 cells (estimated by extrapolation of random microscopic fields) before becoming apoptotic and detaching. None of the protocol adjustments attempted with this technique resulted in viable cultures.

### 7.2.4 Sickle Mouse Derived Endothelial Cell Cultures: Materials and Methods

Recent advances in genetic engineering and improvements in the technique of breeding the notoriously delicate human-sickle-hemoglobin-expressing mice have provided researchers with a viable animal model for the study of sickle cell anemia *in vivo* (Paszty et al., 1997; Ryan et al., 1997). Studies evaluating the vascular characteristics of these mice have found many characteristics in common with sickle cell anemia in humans. These characteristics include loss of vaso-motor tone and blunted response to vaso-relaxing agents like nitric oxide (Nath et al., 2000; Kaul et al., 2000b), presence of pro-inflammatory state cytokines, increased leukocyte-endothelium interaction, endothelial oxidant generation, and significant flow abnormalities (Nath et al., 2000; Kaul et al., 2000a), TNF-$\alpha$ driven microvascular occlusion via blood cell adhesion (Turhan et al., 2002), expression of adhesive ligands like E -selectin and P-selectin (Wood et al., 2004a; Wood et al., 2004b), and well documented organ and blood vessel pathology (Paszty et al., 1997; Ryan et al., 1997). These symptoms all combine to make an excellent animal model of sickle cell anemia that is likely to be extremely helpful in modeling sickle pain crisis in humans. The endothelium from sickle mice is likely to be similarly to that in sickle patients, since, in addition to the endothelial-dependent dysfunctions mentioned above, it is also exposed to elevated inflammatory and thrombotic factors, and altered shear dynamics. As such, these sickle mice represent a

promising alternate cell source for the endothelial cell cultures used in *in vitro* flow adhesion assays. Use of sickle-mouse-derived cell cultures should give at least preliminary answers to the question of whether or not endothelium from sickle patients will interact with sickle erythrocytes differently than endothelial cells cultured from standard cell lines. However, standard MECs from the sickle mice are very difficult to harvest in significant quantities. Only the mouse lungs, with their heavily vascularized enormous surface area for oxygen / carbon dioxide exchange, provide sufficient endothelial cells to form a full culture.

Endothelial cells derived from the lungs of the sickle mouse model (Birmingham model) were generously provided after isolation and culture by the lab of Dr. Lou Ann Brown, Scientific Director of the Emory Alcohol and Lung Biology Center at Emory University, who maintains a dissection and cell culture lab for similar sickle-mouse model experiments at Emory University. For each attempt, four sickle mice were sacrificed by Dr. Brown's lab and their lungs excised under sterile conditions. The lungs were then cut into small pieces and immersed in a trypsin solution similar to that used during standard cell culture protocols as mentioned in 3.1.2. The trypsin was thus able to detach the endothelial cells from the underlying lung tissue with minimal cellular contamination. After gentle agitation to suspend the sickle mouse lung endothelial cells, the trypsin was neutralized by addition of complete lung endothelial cell media (also generously provided by Dr. Archer's lab), pooled, and plated into 4-5 Labtek® single-well chamber slides. Confluent plates were cultured by Dr. Archer's lab with this technique for our use in standard sickle cell adherence flow chamber assays. Plates seeded in this manner required only 2-3 days to reach full confluence.

### 7.2.5 Sickle Mouse Derived Endothelial Cell Cultures: Partial Results

Sufficient endothelial cells were readily derived from the harvested lung tissue. Four mice were sacrificed at each attempt, endothelial cells were harvested from their lungs, the cells were pooled, and then plated into chamber slides. Unfortunately, bacterial contamination struck the endothelial cell cultures on the three occasions this technique was attempted, causing mass detachment and cell culture death in all but one of the culture plates. The study was cancelled following this contamination in light of the number of sacrificed mice needed for the cultures. It was later found that the mouse subjects had contracted a mild respiratory infection prior to sacrifice, and it was hypothesized that, in the nutrient-rich culture media, the bacteria had blossomed into the observed contamination.

The single surviving plate salvaged from these cultures was used in a simple parallel-plate assay (as defined in 3.4 & 4.1) without stimulation. The results of this single experiment are presented in Figure 7.2. Of course, as the endothelial cells were pooled, this plate was likely also affected by the bacterial contamination, and, in any case, little can be derived from a single experimental run. However, taking those flaws into account, the dynamics of adhesion seen in Figure 7.2 seem to follow the same general rise to an equilibrium observed in our other experiments, indicating that sickle mouse-derived endothelial cultures employed in adhesion assays exhibit similar dynamics and thus are a viable subject for investigation.

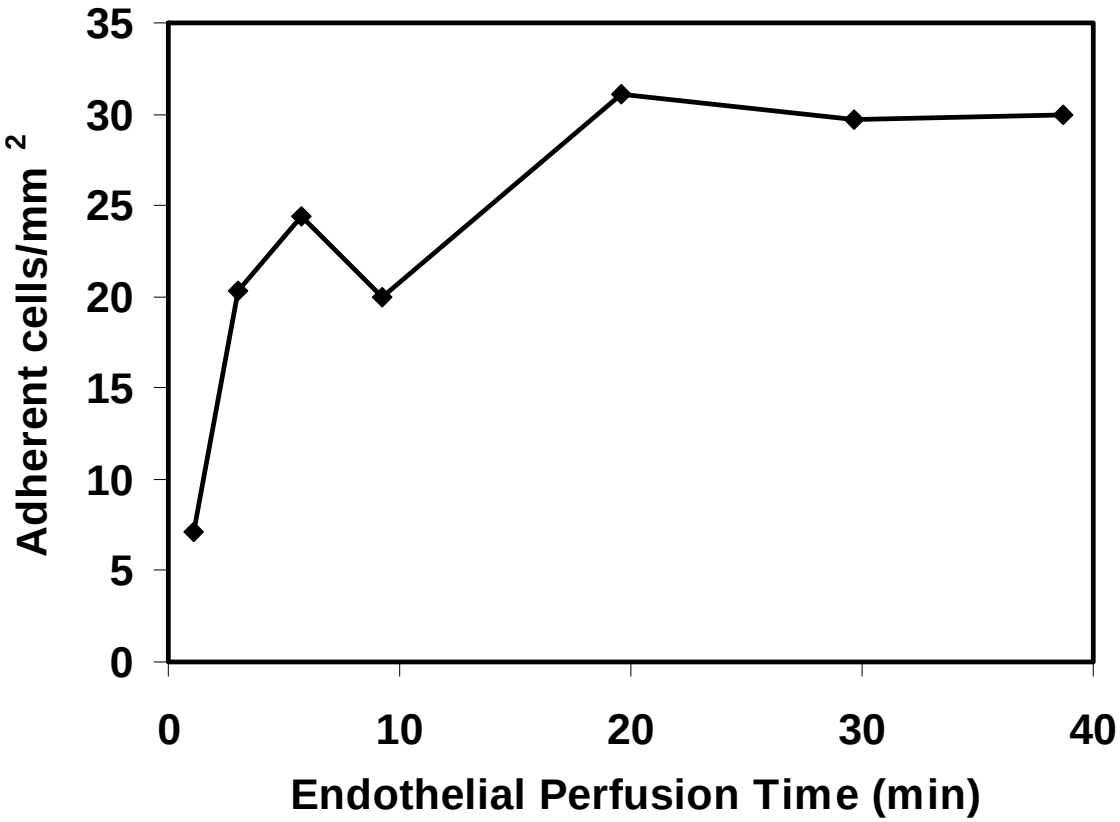


**Figure 7.2: Adhesion Assay With Sickle Mouse Derived Endothelial Cells** Transgenic sickle mouse lung-derived endothelial monolayer was used in a standard parallel-plate perfusion chamber adhesion assay with human sickle erythrocyte suspension. Endothelium was not pre-treated with inflammatory mediators. n=1

## 7.3 Sickle Erythrocyte Detachment Assays

### 7.3.1 Intent and Background

Detachment studies are a technique for judging the durability of the bonds holding adherent cells to a surface (Walmet et al., 2003). In these studies, adhesion is first induced via the selected bond either under low flow or static incubation of the target cell with ligands or other adherent structures secured to a stationary surface. After sufficient adhesion has developed, the perfusion rate is increased in a stepwise manner, perfusing cell-free media through the chamber at increasingly greater rates (Walmet et

al., 2003). After each stepwise increase, the number of adherent cells is allowed to reach a new equilibrium, whereupon cell adhesion is counted, and the flow rate is increased once more. The point at which particular fractions of adherent cells detach provides qualitative and quantitative information about the relative strength and nature of the bonds securing the adherent cells. Specific to the case of sickle cell anemia, detachment studies also have relevance in that the increased flowrate can be said to replicate the increased reperfusion pressure effect thought to eventually reverse the vaso-occlusive pain crisis in sickle microvasculature (Lipowsky et al., 1987; Rodgers et al., 1984). Consequently, adhesion of sickle erythrocytes by significantly stronger bonds is of special significance to the initiation of pain crisis. We hypothesized that those bonds capable of resisting higher reperfusion pressures would be the same bonds more capable of maintaining cell adhesion under increased shear and thus more capable of initiating pain crisis.

### 7.3.2 Materials and Methods

Detachment studies were performed in assays where adhesion had been up-regulated with standard histamine or TNF-$\alpha$ stimulation, as the methodology merely required further perfusion following the endpoint of any standard adhesion assay. The methods for these detachment assays are covered in section 3.4.

Assessment of results is done through the evaluation of the $\tau_{50}$, the shear rate at which half of the adherent cells become detached, which is a general measure of bond strength. This calculation is made by a line fit to a graph of shear (dyne/cm$^2$) vs. remaining adherent cells (% of total at 1.0 dyne/cm$^2$ -baseline). The line fit is begun with data collected at the 40 minute mark, and the data is fit to the following formula (Walmet et al., 2003; Garcia et al., 1998):

$$f = \frac{1.0}{1.0 + e^{[b(\tau - \tau_{50})]}}$$ (Equation 7.1)

Where:

f = fraction of adherent cells

$\tau$ = surface shear stress (dyne/cm$^2$)

$\tau_{50}$ = surface shear stress (dyne/cm$^2$) when f = 0.50

b = inflection of the slope

$\tau_{50}$ is then derived directly by setting f = 0.50 and solving for $\tau$.

### 7.3.3 Partial Results

A selection of data from these studies is presented in Figure 7.3. It includes (A) a summary of selected standard histamine mediated adhesion detachment assays comparing bond strength with or without histamine in the rinse media, (B) a comparison of bond strength formed by histamine and TNF-$\alpha$ both separately and in combination, and (C) a detachment assay following a standard amthamine / methylhistamine histamine receptor agonist experiment (see Chapter IV). These represent standard experimental trials involving detachment assays. Calculation of $\tau_{50}$ showed values of 1.637 dyne/cm$^2$ and 1.835 dyne/cm$^2$ for the detachment techniques in Figure 7.3 A. In Figure 7.3 B, the $\tau_{50}$ of adhesion for histamine is 1.809 dyne/cm$^2$, while the $\tau_{50}$ of adhesion for TNF-$\alpha$ is 2.873 dyne/cm$^2$. In combination, the $\tau_{50}$ is further increased to 3.460 dyne/cm$^2$. In Figure 7.3 C, the $\tau_{50}$ changed from 1.705 dyne/cm$^2$ to 2.239 dyne/cm$^2$ dependent upon whether adhesion is mediated via histamine or combined specific H-receptor agonists.

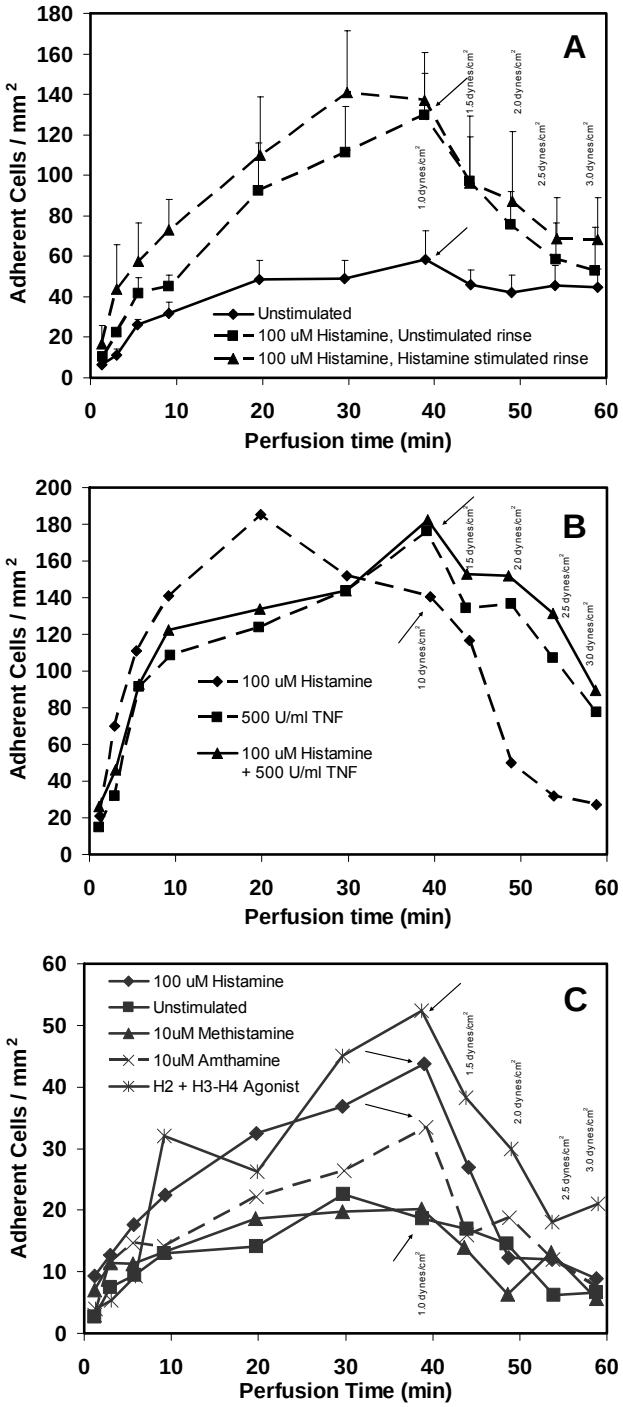


**Figure 7.3: Sample Detachment Assay Data Sets for Histamine** Data in (A) are mean ± SEM sickle erythrocyte adherence for n=4. Data in (B) and (C) are both n=1. Arrows in all panels indicate timepoint at which detachment phase of adhesion assay is begun, indicating an increase in media only perfusion rate. $\tau_{50}$ calculated for factors that increased adhesion. (A) $\tau_{50}$ = 1.637 for Unstimulated rinse, 1.835 for Histamine stimulated rinse, (B) $\tau_{50}$ = 1.809 for Histamine stimulated, 2.873 for TNF stimulated, 3.460 for Histamine + TNF, (C) $\tau_{50}$ = 1.705 for Histamine stimulated, 2.239 for H2 + H3/H4 Agonists. Shear stress at each point past 40 min. noted on the figures.

### 7.3.4 Discussion

Strong conclusions cannot be drawn from these studies due to their limited nature, but calculation of $\tau_{50}$ showed some interesting preliminary results. In Figure 7.3 A, it is evident that the presence or absence of histamine in the detachment perfusate is largely irrelevant. This would seem to support the conclusions reached in Figure 4.2 that histamine stimulation beyond a certain time no longer promotes new bond formation, nor reinforces bonds already formed. Surprisingly, the change in Figure 7.3 C from 1.705 dyne/cm$^2$ to 2.239 dyne/cm$^2$ dependent on histamine or combined specific H-receptor agonist adhesion promotion seems to indicate a difference in bond strength between the two different promotion techniques. This may indicate further strengthening of the bond would be achieved by further simultaneous activation of the $H_1$, or $H_3$ receptors with $H_2$ and $H_4$, but the change is more likely due to an inherent increase in inaccuracy of the line fit due to the decreased scale of adhesion (due to a less adherent blood sample). Further tests would be required to evaluate this point.

Most interesting is Figure 7.3 B, as it directly compares the strength of bonds formed with histamine and TNF-$\alpha$ mediated adhesion. The difference in $\tau_{50}$ indicates that bonds formed via the VCAM-1 pathway appear to be notably stronger than those formed via P-selectin. In combination, the two adhesion mechanisms result in a $\tau_{50}$ that is further increased to 3.460 dyne/cm$^2$, indicating an somewhat additive effect of the two bond formation mechanisms, which furthers the theory that multiple inflammatory effects could act in concordance to strengthen adhesive interactions in the initiation of vaso-occlusive events. This does not eliminate histamine as a potential initiator of pain events, however, as cells immobilized by P-selectin at lower shear by stimulation with histamine could then become adherent to the endothelial surface via the formation of other slower-forming bonds present on the chronically activated sickle endothelium.

Detachment assays, even from the preliminary studies completed here, allow a straightforward evaluation of the strength of bonds formed under shear during the course of flow assays. As the initiating event in sickle pain crisis, bond strength is exceedingly important in assessing the relative risk particular sickle erythrocyte adhesion mechanisms may present *in vivo*. Detachment assays, therefore, are simple techniques that allow the initial assessment, singly and in combination, of potential pain-crisis-initiating adhesion-causing inflammatory responses.

# CHAPTER VIII

# CONCLUSIONS AND FUTURE WORK

## 8.1 Conclusions

The adhesion of sickle erythrocytes to the endothelium of post capillary venules in the microvasculature of patients with sickle cell anemia is theorized to be the initiating factor in the vaso-occlusive event known as a sickle cell pain event. This vaso-occlusive event leads to severe pain and ischemic damage which, over time, can cause tissue and organ damage. In this thesis I showed, using flow assays, that the inflammatory mediator histamine promotes sickle erythrocyte adhesion to endothelium in a shear-dependent manner, suggesting histamine may promote sickle cell adhesion and pain crisis in patients. In the first part of this thesis, histamine was shown to promote increased sickle erythrocyte adhesion to cultured endothelial monolayers under steady shear stress of 1.0 dyne/cm$^2$. The signaling mechanisms induced by histamine to increase adhesion were investigated through the use of highly specific receptor agonists and antagonists as well as highly specific peptides for the blocking of ligands. It was shown that sickle erythrocyte adhesion was dependent upon the simultaneous activation of the $H_2$ and $H_4$ endothelial receptor, and the expression of P-selectin ligand on endothelial cells. Additional studies also suggest that the histamine signaling pathway requires active nitric oxide synthase. These studies both strongly suggest the importance of histamine in sickle cell pain event-initiating adhesion of sickle erythrocytes, and indicate possible targets for prevention of sickle cell adhesion by pharmacological interference with the histamine receptor, ligand expression, or histamine signal pathway.

In the second part of this thesis, the effect of reduced shear stress on the adhesion of sickle erythrocytes to stimulated endothelium was explored. Shear stress is especially significant to the mechanism of sickle erythrocyte adherence due to the chronically depressed and oscillatory nature of blood flow in the microvasculature of patients with sickle cell anemia. Tumor necrosis factor $\alpha$, a well-characterized inflammatory mediator capable of promoting strong sickle erythrocyte adhesion by the expression of vascular adhesion molecule 1, was used in this initial evaluation of shear effect on sickle erythrocyte adhesion. Results show that both lower shear stress and inflammatory stimulation increases sickle erythrocyte adhesion. When used in combination, TNF-$\alpha$ further increases adhesion elevated by depressed shear, so long as adhesion is not depressed below approximately 0.6 dyne/cm$^2$. Below this range, TNF-$\alpha$ stimulation quickly looses its efficacy, and in the low shear range, shear stress becomes the dominant controlling factor of sickle erythrocyte adhesion. These studies indicate the interaction of two different adhesion mechanisms dictating the adhesion of sickle erythrocytes. At high shear stress, adhesion is dominated by the expression of high-affinity bonds up regulated by stimulation with TNF-$\alpha$. At low shear stress, adhesion occurs independent of the expression of high-affinity bonds. These studies demonstrate that the chronically depressed and oscillatory nature of microvascular blood flow in sickle patients likely interacts with the chronically pro-adhesive nature of their blood chemistry to further increase the risk of pain-crisis-initiating sickle erythrocyte adhesion.

In the third part of this thesis, having established shear stress as an independent variable that controls sickle cell adhesion induced by inflammatory mediators, and having established the capacity of histamine to promote sickle cell adhesion, the two preceding parts were combined in a study of histamine stimulation on erythrocyte adhesion under a range of shear stresses. This study was conducted to evaluate the

effect of reduced shear stress on histamine's ability to promote adhesion. As in the study of TNF-$\alpha$, the intent was to evaluate adherence in a model of sickle vasculature, while accounting for the oscillatory and depressed nature of blood flow typical to sickle microcirculation. Sickle erythrocyte adherence was found to be increased both for depression of shear rate and for stimulation with histamine. In combination, histamine stimulation produced a further elevation in sickle erythrocyte adhesion under moderately depressed shear (0.8-0.4 dyne/cm$^2$). In the lowest shear range, however, histamine stimulation lost its efficacy, not contributing to adhesion in the shear stress range of 0.1 to 0.2 dyne/cm$^2$. In comparison with TNF-$\alpha$ results, histamine stimulation was less effective at promoting sickle erythrocyte adhesion at high shear stresses (1.0-0.8 dyne/cm$^2$), but roughly similar in promoting adhesion at 0.6 dyne/cm$^2$. Further, histamine was still effective at promoting adhesion at 0.4 dyne/cm$^2$, a range where TNF-$\alpha$ looses effect.

The difference in the degree of increased adhesion in the different ranges of shear stress is likely a result of differences in mechanisms of adhesion. Differences in bond type, strength, or cell-cell contact requirements for bond initiation may account for this difference, and the lower adhesion at high shear displayed by histamine stimulation could be due to lower bond strength or a requirement for longer cell-cell contact for bond formation. The faster rise in adhesion following a moderate reduction in shear further supports this evaluation. These results show that, similarly to TNF-$\alpha$ stimulation, histamine is more likely to initiate sickle cell adhesion, and thus sickle pain events, during periods of reduced flowrate in the microvasculature of patients with sickle cell anemia, but severe reduction to the range of 0.1 and 0.2 dyne/cm$^2$ results in high levels of adhesion independent of inflammatory activation. The potential risk of histamine to patient condition is further emphasized by the elevated levels of histamine known to

exist in sickle patient blood during both pain crisis and non-pain crisis periods (Kasschau et al., 1996; Malave et al., 1993; Francis, Jr. et al., 1992; Enwonwu et al., 1991), and due to the known histaminergic effects of some treatments for sickle pain (Chaney, 1995; Fuller et al., 1990; Muldoon et al., 1984).

The final part of this thesis presented preliminary studies in three fields tangential to the central aims of this thesis. Although the results are incomplete, they do raise interesting questions and highlight areas for further investigation. A study of NO and eNOS mediation of adhesion under histamine stimulation found an unexpected dependence of adhesion on having active NOS in the endothelial layer. This discovery implies a complex and problematic role for NO in regulating adhesion, implying that small regulatory amounts of NO are necessary for adhesion under histamine stimulation, but that large doses act as a blanket adhesion suppressant. In a second study, the likely phenotypic difference between the microvascular endothelium of sickle and non-sickle patients led to extensive attempts to acquire both human sickle and transgenic sickle mouse-derived endothelial cultures. Data from an adhesion assay employing a transgenic sickle mouse-derived culture are promising, and suggest that the mouse-model lung endothelial cells should be examined for phenotypic differences in mediating sickle erythrocyte adherence. Finally, preliminary detachment assays were run on sickle erythrocyte adherence formed under TNF-$\alpha$ and histamine. In a limited number of experiments, TNF-$\alpha$ mediated adherence was found to form stronger bonds than histamine, but the two factors used together caused cellular adherence stronger than either individually. Furthering these studies may reveal the basis of the differences observed between TNF-$\alpha$ and histamine-mediated adhesion under different shear stresses.

## 8.2 Recommended Future Studies

### 8.2.1 Sickle Mouse Model

The studies presented here could have their physiological relevance reinforced by demonstration of the illustrated adhesion mechanisms in the newly developed transgenic sickle-mouse models expressing human $\beta_s$ and $\alpha$ globin. As elaborated in section 7.2.1, our perfusion flow chambers lack a completely appropriate endothelial cell model, since the endothelium of sickle patients is likely to be phenotypically different from cell cultures due to the constant exposure to pro-inflammatory, pro-thrombotic, pro-immune active factors and exposure to the reduced and oscillatory blood flow rate typical of sickle endothelium. The endothelium of sickle mice, while not yet fully characterized, is likely to more directly mirror these conditions due to a similarly-affected vascular environment. Many of the details of the sickle mouse model are presented in section 2.8 and 7.2.4. The most directly relevant study using the mouse models to confirm the demonstrated histamine mechanism would be to show histamine promoting sickle erythrocyte adhesion and causing microvascular occlusion in the sickle mouse capillary system. Cremaster muscle visualization could be employed to visualize microvascular blood flow *in vivo*, and offers a simple protocol for direct addition of histamine or histaminergic treatments to the surface of the cremaster muscle in order to observe subsequent adhesion. This technique could be used to confirm that histamine promotes sickle erythrocyte adhesion to endothelium of sickle patients by microscopic observation during stimulation of the tissue with a histamine solution. Similarly, use of specific H-receptor agonists in solution could be used to confirm the receptors involved in histamine-mediated adhesion. Further, simple observation could show whether the histamine-mediated adhesion involves leukocytes or not, as *in vitro* P-selectin

expression will likely result in extensive leukocyte localization in the cremaster. Differences in histamine response between sickle and non-sickle mouse endothelium could be explored via similar assays with non-sickle controls. However, it would be necessary to characterize the transgenic sickle mouse microvascular endothelium in comparison to human endothelium. Phenotypically different human endothelial cells are known to have different levels of specific histamine receptor expression (Gantner et al., 2002; Morse et al., 2001; van der Werf et al., 1989; Heltianu et al., 1982). It is conceivable that mouse microvascular endothelium may be phenotypically different from human, and will require characterization, possibly via RT-PCR assay (Ling et al., 2004), to evaluate the suitability of the model.

The cremaster visualization technique is well established (Baez, 1973) and has been demonstrated in many other studies, including examination of sickling (Kaul et al., 1995) and cytokine-induced sickle erythrocyte adhesion (Liu et al., 1998) in earlier sickle mouse models. Fortunately, the relative speed with which histamine was shown to promote adhesion in this thesis (20-40 minute expression) simplifies many of the time-dependent difficulties involved with microvascular blood flow studies in animal models. Pharmacologically suppressing the animal's own histamine response would be necessary, as mast cell activation by the trauma of exposing the cremaster muscle would alter results and need to be controlled.

### 8.2.2 Comparative adhesion mechanisms studies

Tumor necrosis factor $\alpha$ and histamine are only two of the many mechanisms known to promote sickle erythrocyte adhesion *in vitro* which have been found to be upregulated in the vasculature of patients with sickle cell anemia. Many other potential adhesion pathways are detailed in Chapter II. *In vivo*, the mechanism of sickle pain

crisis propagation is likely dependent on multiple adhesion mechanisms acting in parallel. However, it is possible that the initiating mechanism, the adhesion of the first few sickle erythrocytes that begin the process, is acting through individual high-affinity adhesion pathways. Identifying those pathways most likely to initiate sickle pain crisis may allow for focused anti-adhesion therapies for the prevention of pain crisis in patients. As established in this thesis, the chronically depressed and oscillatory flow rate of blood in the sickle microvasculature may act to further amplify the effect of stimulated adhesion. Adhesion mechanisms that have little effect at a venular shear stress may be greatly amplified by a slightly decreased shear rate, and thus be more likely to initiate vaso-occlusive events than a factor that uniformly increases adhesion by a moderate amount over the same range. The linear shear chamber, as in the present studies, can be used to evaluate different adhesion mechanisms, highlighting those mechanisms most amplified by decreased shear. Those most amplified would present the greatest likelihood of initiating adhesion *in vivo*, and afford the most promising targets for treatment. Further comparison can be made between the strength of the bonds formed as a result of different stimulatory factors by employing a simple detachment assay as outlined in section 7.3.

The requirement of reduced shear for bond formation is no assurance that the bond is weaker than one formed under high shear. A particular adhesion mechanism may require the longer cell-cell contact time afforded by the reduced flowrate, but the bond formed may be strong enough to maintain integrity at shear rates far above those where more quickly formed bonds fail. In the sickle microvasculature, a transient reduction in blood flow might acquire sufficient adherent blood cells to initiate pain crisis even if blood velocity returns to normal before the event begins. Thus it becomes essential to evaluate not only the frequency of a particular bond formation, but the

frequency of that bond at reduced shear and the strength of that bond as well, marking any adhesion mechanisms with high bond strength that form readily under reduced flow as targets for potential treatment. Results would need to be confirmed via animal model or clinical study, but the use of the linear shear chamber as outlined in this thesis should provide an adequate first comparison of different adhesion mechanisms.

### 8.2.3 Signal Interruption of Histamine Mediated Sickle Erythrocyte Adhesion

As outlined in section 7.1, the rapid increase in sickle erythrocyte adhesion in response to histamine stimulation makes it a difficult process to interrupt once triggered, and expressed ligands are present for such a relatively short time that their blockade while expressed would be difficult and of limited utility. A promising alternative is to prophylactically interrupt the signaling cascade whereby histamine promotes the expression of the adhesive ligand P-selectin. If the signaling cascade is preemptively disabled, then the histamine-driven adhesion will not take place in response to histamine release. Although the inactivation of nitric oxide synthase in pursuit of this particular objective has proven to be a problematic for *in vivo* consideration, it is entirely possible that alternate approaches may be more specific to the adhesion mechanisms, making them more promising targets of regulation. Possible approaches include disabling the expression of the $H_4$ histamine receptor, as we have shown that sickle erythrocyte adhesion requires activation of both the $H_2$ and $H_4$ endothelial receptors. Alternately, expression of the P-selectin ligand might be hindered. As P-selectin evidently has a time-dependent expression, understanding the signaling control of the “shedding” or re-internalization of this ligand may provide possible regulatory approaches to shorten its expression time.

### 8.2.4 Summary

The data presented in this thesis suggest extension of current sickle cell studies in several specific directions. The first is in the use of a particular animal model, a transgenic sickle cell mouse, to confirm the *in vitro* observed potential of histamine to promote sickle erythrocyte adherence and to initiate sickle vaso-occlusive events. The second is to employ techniques outlined in this thesis to evaluate other sickle erythrocyte adhesion pathways under a range of shear stresses, in order to account for the unusual hemodynamics of sickle microvasculature. Doing so will give more accurate insight into the potential of any particular mechanism to initiate sickle vaso-occlusive events. The third is to investigate the feasibility of preemptively deactivating the ligand expression mechanism of histamine mediated sickle erythrocyte adhesion through blockade of histamine receptors, intercellular signaling mechanisms, or altering the expression dynamics of P-selectin. As a potential initiator of sickle pain crisis, application of these studies to patient care may lead to a decrease in sickle pain crisis incidence and associated ischemic damage.

# APPENDIX

## KEY DATA TABLES

Data presented in graphic form in the preceding chapters are included in expanded form here, in the order in which the referenced graphs appear in the main text. Refer to the referenced graphs and sections for details of data collection, agent concentration, and formulas. Data presented here are pooled data taken from adhesion assays, and not raw data, which are too extensive to include.

Frequently used notation:

Data sets are designated by the date of the assay. When more than one assay was conducted on one day, the data sets are distinguished by "A" and "B".

Histamine stimulation, unless otherwise stated, is at 100 μM, continuous for the duration of the assay.

TNF-α stimulation, unless otherwise stated, is a 6-hour stimulation at 500 U/ml preceding the assay.

In all cases, the normalized value "cells/$mm^2$" refers to sickle erythrocytes.

**Table A.1 Comparison of Adhesion for Fresh vs. "Day Old" Blood Samples**
Data for Figure 3.1

| Status | Date | Histamine | Unstimulated |
|---|---|---|---|
| Fresh | 1/8/2003 | 45.9 | 12.8 |
| Fresh | 1/16/2003 | 26.5 | 8.0 |
| Fresh | 1/23/2003 | 46.7 | 17.1 |
| Fresh | 2/5/2003 | 17.8 | 17.1 |
| Fresh | 2/12/2003 | 140.3 | 118.3 |
| Fresh | 2/20/2003 | 31.2 | 16.0 |
| Fresh | 3/12/2003 | 42.7 | 32.1 |
| Fresh | 3/19/2003 | 43.7 | 35.8 |
| Day Old | 5/15/2003 | 80.0 | 17.3 |
| Day Old | 5/29/2003 | 36.4 | 16.0 |
| Day Old | 6/12/2003 | 72.0 | 32.0 |
| Day Old | 6/19/2003 | 50.1 | 12.2 |
| Day Old | 7/18/2003 | 100.3 | 10.4 |
| Day Old | 7/31/2003 | 49.2 | 10.9 |
| Day Old | 8/13/2003 | 66.0 | 28.0 |
| Day Old | 8/21/2003 | 34.3 | 10.9 |
| Day Old | 9/5/2003 | 35.3 | 4.4 |
| Day Old | 9/11/2003 A | 33.1 | 7.0 |
| Day Old | 9/11/2003 B | 51.3 | 8.4 |
| Day Old | 9/18/2003 | 45.9 | 16.0 |
| Day Old | 10/16/2003 A | 21.1 | 17.1 |
| Day Old | 10/16/2003 B | 43.4 | 16.0 |
| Day Old | 10/23/2003 | 44.3 | 5.6 |
| Day Old | 10/30/2003 | 55.4 | 26.4 |
| Day Old | 11/6/2003 | 51.4 | 13.7 |
| Day Old | 1/29/2004 | 53.3 | 17.1 |
| Day Old | 2/19/2004 | 40.0 | 11.6 |
| Day Old | 3/05/04 | 49.9 | 24.4 |
| Day Old | 3/11/2004 | 37.1 | 14.5 |
| Day Old | 3/18/04A | 41.6 | 26.5 |
| Day Old | 3/18/04B | 35.0 | 13.6 |
| Day Old | 5/07/04 | 41.0 | 16.0 |
| Day Old | 5/13/2004 | 38.7 | 18.6 |
| Day Old | 5/27/04 | 34.0 | 6.7 |
| Day Old | 6/18/04 | 37.3 | 12.0 |
| Day Old | 8/27/2004 | 43.7 | 9.1 |
| Fresh | 9/20/04 | 32.0 | 7.0 |
| Fresh | 11/20/2003 A | 35.7 | 15.3 |
| Fresh | 11/20/2003 B | 35.7 | 8.3 |
| Fresh | 4/2/2004 | 54.4 | 15.3 |
| Fresh | 4/30/2004 | 30.4 | 11.1 |
| Fresh | 10/20/2004 | 76.0 | 32.8 |
| Fresh | 1/11/2005 | 41.7 | 14.3 |

**Table A.2 Histamine Promotes Sickle Erythrocyte Adhesion**
Data for Figure IV.1 A and IV.1 D.

| Stimulation | time | 1/8/2003 | 1/16/2003 | 1/23/2003 | 2/5/2003 | 2/12/2003 | 2/20/2003 | 3/12/2003 | 3/19/2003 | 5/15/2003 | 5/29/2003 | 6/12/2003 | 6/19/2003 | 7/18/2003 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (min) | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ |
| Histamine | 1.3 | 12.0 | 7.6 | 14.0 | 3.2 | 18.9 | 12.4 | 9.6 | 4.4 | 4.8 | 6.0 | 9.3 | 8.7 | 16.0 |
| | 2.9 | 15.8 | 11.7 | 13.8 | 5.6 | 34.9 | 8.6 | 17.6 | 4.9 | 19.7 | 11.1 | 16.0 | 21.7 | 48.0 |
| | 5.6 | 20.0 | 16.7 | 16.8 | 11.0 | 44.6 | 13.6 | 24.9 | 13.7 | 16.0 | 25.2 | 21.6 | 27.2 | 68.2 |
| | 9.4 | 34.0 | 14.3 | 22.3 | 11.0 | 69.0 | 26.2 | 32.0 | 17.5 | 32.0 | 19.0 | 20.4 | 32.8 | 75.3 |
| | 19.6 | 35.6 | 16.5 | 29.6 | 12.9 | 107.4 | 37.5 | 39.2 | 30.2 | 64.8 | 43.6 | 51.2 | 26.1 | 99.0 |
| | 29.6 | 36.7 | 24.0 | 41.7 | 22.2 | 128.0 | 38.0 | 56.7 | 32.0 | 78.2 | 43.4 | 72.0 | 33.8 | 84.3 |
| | 38.8 | 45.9 | 26.5 | 46.7 | 17.8 | 140.3 | 31.2 | 42.7 | 43.7 | 80.0 | 36.4 | 72.0 | 50.1 | 100.3 |
| | | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ |
| Unstimulated | 1.1 | 4.8 | 2.9 | 8.0 | 3.6 | 11.3 | 5.3 | 8.0 | 1.3 | 7.1 | 3.7 | 5.3 | 8.6 | 4.9 |
| | 2.9 | 8.0 | 2.8 | 7.5 | 7.3 | 17.9 | 5.8 | 10.5 | 6.2 | 4.6 | 6.2 | 3.0 | 8.0 | 6.2 |
| | 5.7 | 10.2 | 4.3 | 8.3 | 8.9 | 30.0 | 3.4 | 21.5 | 9.6 | 4.9 | 5.3 | 6.5 | 5.9 | 8.0 |
| | 9.2 | 10.9 | 8.3 | 10.4 | 5.1 | 49.6 | 8.0 | 24.8 | 9.6 | 5.3 | 9.7 | 11.6 | 8.8 | 6.9 |
| | 19.5 | 8.0 | 8.7 | 8.9 | 8.4 | 88.0 | 5.6 | 32.5 | 17.6 | 11.0 | 9.5 | 19.2 | 9.0 | 16.0 |
| | 29.6 | 12.2 | 9.0 | 26.7 | 8.0 | 123.1 | 16.8 | 41.8 | 16.0 | 11.6 | 10.4 | 22.6 | 16.0 | 11.6 |
| | 38.7 | 12.8 | 8.0 | 17.1 | 17.1 | 118.3 | 16.0 | 32.1 | 35.8 | 17.3 | 16.0 | 32.0 | 12.2 | 10.4 |
| Stimulation | time | 7/31/2003 | 5/7/2004 | 3/5/2004 | 3/11/2004 | 3/18/04B | 3/18/04A | 6/18/04 | 1/29/2004 | 2/19/2004 | 8/27/2004 | 9/20/2004 | 11/6/2003 | 10/30/2003 |
| | (min) | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ |
| Histamine | 1.3 | 11.6 | 5.8 | 4.4 | 6.0 | 1.5 | 5.3 | 16.0 | 7.1 | 5.3 | 12.0 | 7.1 | 10.2 | 21.3 |
| | 2.9 | 10.2 | 13.3 | 12.3 | 4.3 | 5.3 | 6.9 | 9.3 | 12.3 | 14.9 | 13.3 | 26.7 | 17.2 | 20.4 |
| | 5.6 | 26.7 | 22.1 | 18.9 | 10.9 | 16.8 | 16.0 | 22.9 | 29.0 | 21.6 | 21.1 | 17.9 | 22.1 | 41.3 |
| | 9.4 | 23.2 | 32.0 | 24.4 | 28.8 | 24.0 | 23.6 | 26.7 | 32.0 | 18.1 | 26.4 | 33.8 | 26.4 | 52.0 |
| | 19.6 | 40.5 | 25.3 | 41.6 | 36.8 | 37.3 | 35.2 | 28.2 | 45.9 | 35.4 | 37.7 | 42.0 | 48.0 | 61.7 |
| | 29.6 | 38.0 | 34.1 | 49.8 | 44.4 | 40.0 | 37.6 | 40.9 | 44.8 | 46.1 | 45.7 | 38.2 | 41.1 | 78.9 |
| | 38.8 | 49.2 | 41.0 | 49.9 | 37.1 | 35.0 | 41.6 | 37.3 | 53.3 | 40.0 | 43.7 | 32.0 | 51.4 | 55.4 |
| | | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ |
| Unstimulated | 1.1 | 2.7 | 4.0 | 6.9 | 5.3 | 1.2 | 1.1 | 10.7 | 5.8 | 2.9 | 7.3 | 6.4 | 10.7 | 6.7 |
| | 2.9 | 2.3 | 4.9 | 5.6 | 1.0 | 6.4 | 5.6 | 5.7 | 11.1 | 5.7 | 4.6 | 10.2 | 13.5 | 6.0 |
| | 5.7 | 7.3 | 9.9 | 17.8 | 5.5 | 5.3 | 7.7 | 8.8 | 13.8 | 10.4 | 10.4 | 11.4 | 11.1 | 13.2 |
| | 9.2 | 11.6 | 7.7 | 31.3 | 12.1 | 9.3 | 12.7 | 13.2 | 9.6 | 10.2 | 9.5 | 17.7 | 7.0 | 24.8 |
| | 19.5 | 10.9 | 14.5 | 33.8 | 18.2 | 9.7 | 15.3 | 13.5 | 16.9 | 9.6 | 9.9 | 13.6 | 10.7 | 22.2 |
| | 29.6 | 8.5 | 19.4 | 35.4 | 12.5 | 17.4 | 23.2 | 22.4 | 15.2 | 10.9 | 10.9 | 6.2 | 15.2 | 21.3 |
| | 38.7 | 10.9 | 16.0 | 24.4 | 14.5 | 13.6 | 26.5 | 12.0 | 17.1 | 11.6 | 9.1 | 7.0 | 13.7 | 26.4 |

Table A.2 Continued

| Stimulation | time | 10/23/2003 | 10/16/2003 B | 10/16/2004 A | 9/5/2004 | 8/21/2003 | 8/13/2003 | 9/18/2003 | 9/11/2003 A | 9/11/2003 B | 5/27/04 | 5/13/2004 | Average Adhesion | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (min) | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | SEM |
| Histamine | 1.3 | 9.6 | 14.2 | 19.6 | 12.0 | 10.7 | 9.8 | 14.5 | 14.4 | 12.8 | 17.8 | 10.7 | 10.5 | 0.8 |
| | 2.9 | 21.3 | 20.0 | 22.4 | 20.2 | 16.0 | 14.2 | 18.7 | 6.7 | 11.4 | 11.1 | 18.3 | 15.6 | 1.4 |
| | 5.6 | 19.8 | 21.3 | 20.8 | 24.1 | 16.8 | 19.4 | 25.8 | 26.4 | 17.5 | 18.5 | 26.4 | 22.8 | 1.7 |
| | 9.4 | 19.2 | 25.8 | 23.1 | 22.6 | 21.3 | 35.3 | 18.7 | 30.0 | 37.6 | 18.2 | 34.1 | 28.7 | 2.1 |
| | 19.6 | 32.0 | 46.1 | 29.0 | 25.6 | 20.8 | 63.6 | 35.8 | 28.8 | 39.3 | 32.9 | 40.4 | 40.6 | 3.1 |
| | 29.6 | 35.7 | 40.5 | 25.4 | 44.5 | 30.2 | 59.8 | 39.1 | 20.3 | 50.7 | 30.4 | 40.7 | 45.6 | 3.4 |
| | 38.8 | 44.3 | 43.4 | 21.1 | 35.3 | 34.3 | 66.0 | 45.9 | 33.1 | 51.3 | 34.0 | 38.7 | 47.2 | 3.6 |
| | | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | SEM |
| Unstimulated | 1.1 | 7.3 | 8.0 | 11.6 | 4.4 | 4.4 | 4.9 | 6.4 | 7.3 | 5.3 | 4.9 | 4.4 | 5.8 | 0.4 |
| | 2.9 | 5.3 | 6.9 | 12.6 | 6.2 | 6.7 | 7.4 | 9.3 | 8.0 | 8.0 | 3.4 | 6.4 | 6.9 | 0.5 |
| | 5.7 | 9.9 | 5.1 | 11.4 | 9.1 | 9.9 | 8.7 | 20.2 | 5.3 | 7.3 | 5.3 | 13.3 | 9.9 | 0.9 |
| | 9.2 | 18.3 | 12.4 | 9.7 | 5.1 | 7.7 | 17.5 | 15.3 | 7.0 | 8.3 | 6.2 | 23.0 | 12.9 | 1.4 |
| | 19.5 | 7.5 | 10.9 | 10.4 | 15.2 | 9.6 | 18.0 | 11.6 | 4.9 | 11.6 | 10.4 | 21.6 | 15.5 | 2.3 |
| | 29.6 | 8.9 | 16.8 | 11.3 | 8.0 | 7.7 | 26.7 | 17.8 | 7.7 | 10.4 | 10.9 | 21.1 | 18.7 | 3.2 |
| | 38.7 | 5.6 | 16.0 | 17.1 | 4.4 | 10.9 | 28.0 | 16.0 | 7.0 | 8.4 | 6.7 | 18.6 | 18.6 | 3.0 |

**Table A.3 Concentration Dependence of Histamine Stimulation** Data for Figure IV.1 B

| Histamine | 5/29/2003 | | 1/8/2003 | | 1/16/2003 | | 1/23/2003 | | 3/12/2003 | | 3/19/2003 | | AVERAGES | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stimulation | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| None | 1.2 | 6.2 | 1.1 | 4.8 | 1.2 | 2.9 | 1.2 | 8.0 | 1.2 | 8.0 | 1.2 | 1.3 | 1.2 | 4.6 | 1.0 |
| | 3.0 | 8.5 | 2.9 | 8.0 | 3.0 | 2.8 | 2.9 | 7.5 | 3.0 | 10.5 | 2.9 | 6.2 | 2.9 | 6.6 | 0.9 |
| | 5.8 | 11.3 | 5.7 | 10.2 | 5.8 | 4.3 | 5.8 | 8.3 | 5.6 | 21.5 | 5.6 | 9.6 | 5.7 | 8.7 | 1.1 |
| | 9.2 | 12.1 | 9.2 | 10.9 | 9.4 | 8.3 | 9.4 | 10.4 | 9.3 | 24.8 | 9.2 | 9.6 | 9.3 | 10.3 | 0.6 |
| | 19.6 | 13.2 | 19.5 | 8.0 | 19.6 | 8.7 | 19.7 | 8.9 | 19.7 | 32.5 | 19.7 | 17.6 | 19.6 | 11.3 | 1.6 |
| | 29.7 | 15.3 | 29.6 | 12.2 | 29.6 | 9.0 | 29.9 | 26.7 | 29.7 | 41.8 | 29.4 | 16.0 | 29.6 | 15.8 | 2.7 |
| | 38.2 | 15.3 | 38.7 | 12.8 | 38.5 | 8.0 | 39.1 | 17.1 | 38.8 | 32.1 | 38.9 | 35.8 | 38.8 | 17.8 | 4.2 |
| | 5/29/2003 | | 1/8/2003 | | 1/16/2003 | | 1/23/2003 | | 3/12/2003 | | | | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | | | time | cells/mm$^2$ | SEM |
| 10 micromol | 1.3 | 8.0 | 1.2 | 6.4 | 0.9 | 0.0 | 1.2 | 1.5 | 1.2 | 4.4 | | | 1.1 | 4.0 | 1.3 |
| | 2.9 | 9.6 | 2.9 | 6.4 | 2.6 | 5.1 | 2.9 | 9.1 | 2.9 | 4.6 | | | 2.8 | 7.0 | 0.9 |
| | 5.8 | 14.8 | 5.7 | 9.6 | 5.7 | 4.0 | 5.7 | 8.0 | 5.7 | 9.0 | | | 5.7 | 9.1 | 1.6 |
| | 9.2 | 11.7 | 9.5 | 9.9 | 9.3 | 6.0 | 9.3 | 8.8 | 9.4 | 9.5 | | | 9.4 | 9.2 | 0.8 |
| | 19.7 | 29.7 | 19.5 | 14.6 | 19.6 | 6.1 | 19.6 | 23.1 | 19.6 | 14.5 | | | 19.6 | 17.6 | 3.6 |
| | 29.8 | 33.7 | 29.9 | 20.7 | 29.6 | 6.5 | 30.0 | 14.5 | 29.5 | 17.5 | | | 29.7 | 18.6 | 4.0 |
| | 38.6 | 36.6 | 38.7 | 19.4 | 38.5 | 10.4 | 39.1 | 20.0 | 38.7 | 12.8 | | | 38.7 | 19.8 | 4.1 |
| | 5/29/2003 | | | | 1/16/2003 | | | | | | | | | | |
| | time | cells/mm$^2$ | | | time | cells/mm$^2$ | | | | | | | time | cells/mm$^2$ | SEM |
| 25 micromol | 1.3 | 4.9 | | | 1.2 | 8.7 | | | | | | | 1.2 | 6.8 | 1.3 |
| | 3.0 | 17.1 | | | 3.1 | 11.4 | | | | | | | 3.0 | 14.3 | 2.0 |
| | 5.7 | 11.1 | | | 5.8 | 12.6 | | | | | | | 5.7 | 11.9 | 0.5 |
| | 9.3 | 14.2 | | | 9.4 | 18.4 | | | | | | | 9.3 | 16.3 | 1.5 |
| | 19.5 | 40.7 | | | 19.6 | 27.6 | | | | | | | 19.6 | 34.1 | 4.7 |
| | 29.4 | 41.5 | | | 29.5 | 31.2 | | | | | | | 29.4 | 36.3 | 3.6 |
| | 39.0 | 35.7 | | | 38.9 | 26.7 | | | | | | | 39.0 | 31.2 | 3.2 |
| Histamine | 5/29/2003 | | 1/8/2003 | | 1/16/2003 | | 1/23/2003 | | 3/12/2003 | | 3/19/2003 | | | | |
| Stimulation | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| 100 micromol | 1.2 | 5.3 | 1.3 | 12.0 | 1.2 | 7.6 | 1.2 | 14.0 | 1.2 | 9.6 | 1.2 | 4.4 | 1.2 | 8.7 | 1.7 |
| | 2.9 | 13.3 | 2.9 | 15.8 | 3.0 | 11.7 | 3.0 | 13.8 | 3.1 | 17.6 | 3.0 | 4.9 | 3.0 | 11.9 | 1.7 |
| | 5.7 | 22.2 | 5.6 | 20.0 | 5.7 | 16.7 | 5.8 | 16.8 | 5.7 | 24.9 | 5.6 | 13.7 | 5.7 | 17.9 | 1.3 |
| | 9.3 | 30.7 | 9.4 | 34.0 | 9.2 | 14.3 | 9.3 | 22.3 | 9.3 | 32.0 | 9.4 | 17.5 | 9.3 | 23.7 | 3.4 |
| | 19.5 | 37.9 | 19.6 | 35.6 | 19.6 | 16.5 | 19.6 | 29.6 | 19.3 | 39.2 | 19.5 | 30.2 | 19.6 | 29.9 | 3.3 |
| | 29.5 | 46.3 | 29.6 | 36.7 | 29.7 | 24.0 | 29.4 | 41.7 | 29.9 | 56.7 | 29.6 | 32.0 | 29.6 | 36.1 | 3.5 |
| | 38.4 | 49.7 | 38.8 | 45.9 | 38.8 | 26.5 | 38.8 | 46.7 | 39.0 | 42.7 | 38.7 | 43.7 | 38.8 | 42.5 | 3.7 |
| | 1/8/2003 | | 1/16/2003 | | 1/23/2003 | | | | | | | | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | | | | | | | | | |
| 1 millimol | 1.2 | 2.9 | 1.3 | 2.9 | 1.2 | 4.0 | | | | | | | 1.2 | 3.3 | 0.3 |
| | 3.0 | 6.2 | 3.0 | 3.4 | 3.0 | 7.0 | | | | | | | 3.0 | 5.5 | 0.9 |
| | 5.7 | 7.3 | 5.7 | 9.0 | 5.7 | 5.5 | | | | | | | 5.7 | 7.3 | 0.8 |
| | 9.3 | 9.0 | 9.4 | 5.8 | 9.2 | 7.0 | | | | | | | 9.3 | 7.3 | 0.8 |
| | 19.7 | 12.0 | 19.5 | 5.1 | 19.4 | 7.3 | | | | | | | 19.5 | 8.2 | 1.7 |
| | 29.7 | 10.4 | 29.5 | 7.7 | 29.4 | 12.2 | | | | | | | 29.5 | 10.1 | 1.1 |
| | 38.7 | 12.2 | 38.6 | 6.4 | 38.6 | 16.0 | | | | | | | 38.6 | 11.5 | 2.3 |

**Table A.4 Test of Endothelial Type: HUVEC or dMEC**
Data for Figure IV.1 C and IV.8

10/12/2005

| HUVEC | | | | dMEC | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Unstimulated | | Histamine | | Unstimulated | | Histamine | | Non-Specific Antibody | | Anti-P-selectin Antibody | |
| time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ |
| 1.1 | 5.8 | 1.2 | 10.2 | 1.2 | 1.2 | 1.2 | 7.3 | 1.2 | 3.2 | 1.2 | 1.3 |
| 2.9 | 4.6 | 3.0 | 13.3 | 3.0 | 4.6 | 3.0 | 8.0 | 3.0 | 10.7 | 3.0 | 4.6 |
| 5.7 | 8.3 | 5.7 | 24.8 | 5.6 | 4.9 | 5.7 | 18.3 | 5.7 | 14.3 | 5.7 | 4.9 |
| 9.2 | 9.0 | 9.3 | 22.5 | 9.3 | 3.7 | 9.2 | 21.6 | 9.2 | 12.2 | 9.4 | 5.3 |
| 19.4 | 11.8 | 19.8 | 45.9 | 19.8 | 10.9 | 19.7 | 18.0 | 19.7 | 20.4 | 19.5 | 6.5 |
| 29.6 | 8.8 | 29.5 | 45.7 | 29.5 | 6.5 | 29.7 | 20.0 | 29.5 | 22.7 | 29.4 | 8.0 |
| 38.5 | 9.0 | 38.4 | 47.1 | 38.5 | 5.6 | 38.5 | 28.8 | 39.1 | 24.0 | 38.5 | 4.9 |

8/3/2005

| HUVEC | | | | dMEC | | | |
|---|---|---|---|---|---|---|---|
| Unstimulated | | Histamine | | Unstimulated | | Histamine | |
| time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ |
| 1.2 | 2.7 | 1.2 | 6.4 | 1.2 | 8.7 | 1.2 | 8.7 |
| 3.0 | 6.4 | 3.0 | 19.2 | 3.1 | 6.0 | 3.0 | 18.9 |
| 5.6 | 13.9 | 5.8 | 24.7 | 5.7 | 12.5 | 5.7 | 20.2 |
| 9.2 | 11.3 | 9.3 | 46.5 | 9.2 | 10.2 | 9.3 | 36.0 |
| 19.6 | 16.0 | 19.7 | 56.0 | 19.5 | 9.7 | 19.5 | 45.9 |
| 30.0 | 32.0 | 29.5 | 76.8 | 29.7 | 10.2 | 29.7 | 45.7 |
| 38.7 | 24.3 | 38.1 | 72.4 | 38.5 | 8.3 | 38.8 | 31.1 |

8/18/2005

| HUVEC | | | | dMEC | | | |
|---|---|---|---|---|---|---|---|
| Unstimulated | | Histamine | | Unstimulated | | Histamine | |
| time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ |
| 1.2 | 3.2 | 1.1 | 7.1 | 1.2 | 13.1 | 1.3 | 13.7 |
| 2.9 | 8.5 | 3.0 | 11.1 | 3.0 | 9.3 | 3.0 | 21.3 |
| 5.7 | 8.3 | 5.7 | 21.3 | 5.6 | 8.5 | 5.6 | 18.4 |
| 9.2 | 9.0 | 9.3 | 29.0 | 9.2 | 3.3 | 9.2 | 24.0 |
| 19.7 | 10.7 | 19.6 | 27.2 | 19.7 | 10.4 | 19.8 | 37.6 |
| 29.5 | 20.0 | 29.7 | 39.0 | 29.5 | 17.0 | 29.7 | 37.0 |
| 39.2 | 15.4 | 38.5 | 44.0 | 38.7 | 12.4 | 38.6 | 45.2 |

## Table A.4 Continued

| 9/7/2005 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HUVEC | | | | dMEC | | | | | | | |
| | Unstimulated | | Histamine | | Unstimulated | | Histamine | | Non-Specific Antibody | | Anti-P-selectin Antibody | |
| | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ |
| | 1.2 | 2.7 | 1.3 | 5.8 | 1.2 | 3.7 | 1.2 | 7.3 | 1.3 | 5.8 | 1.2 | 4.4 |
| | 3.0 | 0.9 | 3.0 | 11.1 | 3.1 | 8.6 | 3.1 | 12.3 | 3.0 | 6.4 | 3.0 | 3.7 |
| | 5.7 | 4.2 | 5.7 | 8.3 | 5.6 | 7.3 | 5.8 | 15.2 | 5.6 | 11.1 | 5.8 | 10.0 |
| | 9.3 | 4.9 | 9.2 | 18.0 | 9.4 | 7.3 | 9.2 | 19.6 | 9.4 | 12.8 | 9.4 | 7.3 |
| | 19.8 | 6.7 | 19.6 | 13.8 | 19.7 | 7.1 | 19.7 | 18.4 | 20.0 | 16.9 | 19.5 | 6.7 |
| | 29.4 | 8.0 | 29.6 | 22.7 | 29.6 | 8.7 | 29.7 | 21.3 | 29.7 | 15.1 | 29.4 | 2.8 |
| | 38.6 | 5.9 | 38.0 | 15.4 | 38.7 | 5.6 | 38.8 | 17.8 | 38.8 | 14.4 | 38.7 | 3.6 |
| AVERAGES | HUVEC | | | | dMEC | | | | | | | |
| | Unstimulated | | Histamine | | Unstimulated | | Histamine | | Non-Specific Antibody | | Anti-P-selectin Antibody | |
| | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ |
| | time | A/S/D | time | A/S/D | time | A/S/D | time | A/S/D | time | A/S/D | time | A/S/D |
| | 1.2 | 3.6 | 1.2 | 7.4 | 1.2 | 6.7 | 1.2 | 9.2 | 1.3 | 5.8 | 1.2 | 4.4 |
| | 3.0 | 5.1 | 3.0 | 13.7 | 3.0 | 7.1 | 3.0 | 15.1 | 3.0 | 6.4 | 3.0 | 3.7 |
| | 5.7 | 8.7 | 5.7 | 19.8 | 5.6 | 8.3 | 5.7 | 18.0 | 5.6 | 11.1 | 5.8 | 10.0 |
| | 9.3 | 8.6 | 9.3 | 29.0 | 9.3 | 6.1 | 9.2 | 25.3 | 9.4 | 12.8 | 9.4 | 7.3 |
| | 19.6 | 11.3 | 19.7 | 35.7 | 19.7 | 9.5 | 19.7 | 30.0 | 20.0 | 16.9 | 19.5 | 6.7 |
| | 29.6 | 17.2 | 29.6 | 46.1 | 29.6 | 10.6 | 29.7 | 31.0 | 29.7 | 15.1 | 29.4 | 2.8 |
| | 38.8 | 13.7 | 38.3 | 44.7 | 38.6 | 8.0 | 38.7 | 30.7 | 38.8 | 14.4 | 38.7 | 3.6 |
| SEM | HUVEC | | | | dMEC | | | | | | | |
| | Unstimulated | | Histamine | | Unstimulated | | Histamine | | | | | |
| | | SSRBC/mm$^2$ | | SSRBC/mm$^2$ | | SSRBC/mm$^2$ | | SSRBC/mm$^2$ | | | | |
| | | 0.9 | | 1.1 | | 3.1 | | 1.8 | | | | |
| | | 1.9 | | 2.2 | | 1.3 | | 3.5 | | | | |
| | | 2.3 | | 4.5 | | 1.8 | | 1.2 | | | | |
| | | 1.6 | | 7.2 | | 1.9 | | 4.2 | | | | |
| | | 2.2 | | 10.9 | | 1.0 | | 8.1 | | | | |
| | | 6.5 | | 13.1 | | 2.6 | | 7.2 | | | | |
| | | 4.7 | | 13.5 | | 1.9 | | 6.5 | | | | |

**Table A.5 Prestimulation Histamine Trials**

Data for Figure 4.2

| Time Delay Histamine Dataset | | | | | | | |
|---|---|---|---|---|---|---|---|
| time | No Histamine Stimulation | | | | | | |
| | 4/30/2004 | 5/7/2004 | 5/13/2004 | 8/27/2004 | 1/12/2005 | AVERAGE | SEM |
| | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | |
| 1 | 7.4 | 4.0 | 4.4 | 7.3 | 8.0 | 6.2 | 0.7 |
| 3 | 4.0 | 4.9 | 6.4 | 4.6 | 8.0 | 5.6 | 0.6 |
| 5 | 7.7 | 9.9 | 13.3 | 10.4 | 8.0 | 9.9 | 0.9 |
| 10 | 12.5 | 7.7 | 23.0 | 9.5 | 10.7 | 12.7 | 2.4 |
| 20 | 10.1 | 14.5 | 21.6 | 9.9 | 20.2 | 15.3 | 2.2 |
| 30 | 11.6 | 19.4 | 21.1 | 10.9 | 8.3 | 14.2 | 2.2 |
| 40 | 11.1 | 16.0 | 18.6 | 9.1 | 14.3 | 13.8 | 1.5 |
| Histamine Stimulation at t=0 | | | | | | | |
| 1 | 8.7 | 5.8 | 10.7 | 12.0 | 6.9 | 8.8 | 1.0 |
| 3 | 14.7 | 13.3 | 18.3 | 13.3 | 16.0 | 15.1 | 0.8 |
| 5 | 32.0 | 22.1 | 26.4 | 21.1 | 26.9 | 25.7 | 1.7 |
| 10 | 36.2 | 32.0 | 34.1 | 26.4 | 25.0 | 30.7 | 1.9 |
| 20 | 27.6 | 25.3 | 40.4 | 37.7 | 36.7 | 33.5 | 2.7 |
| 30 | 40.0 | 34.1 | 40.7 | 45.7 | 40.4 | 40.2 | 1.6 |
| 40 | 30.4 | 41.0 | 38.7 | 43.7 | 41.7 | 39.1 | 2.1 |
| Histamine Stimulation at t=10 min | | | | | | | |
| 0 | | | | | | | |
| 10 | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 11 | | 0.0 | 1.2 | 9.6 | 4.4 | 3.8 | 1.7 |
| 13 | | 5.3 | 3.7 | 16.0 | 8.0 | 8.3 | 2.1 |
| 15 | | 12.8 | 8.0 | 16.0 | 10.7 | 11.9 | 1.3 |
| 20 | | 22.5 | 14.0 | 21.9 | 14.8 | 18.3 | 1.8 |
| 30 | | 24.0 | 16.0 | 30.9 | 14.5 | 21.3 | 2.9 |
| 40 | | 21.6 | 16.0 | 24.0 | 11.1 | 18.2 | 2.2 |
| 50 | | 26.4 | 16.0 | 23.2 | 12.0 | 19.4 | 2.5 |
| Histamine Stimulation at t=20 min | | | | | | | |
| 0 | | | | | | | |
| 20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 21 | 9.3 | 8.7 | 2.5 | 13.1 | 8.7 | 8.5 | 1.5 |
| 23 | 16.0 | 6.2 | 12.3 | 18.5 | 12.3 | 13.0 | 1.9 |
| 25 | 31.1 | 15.4 | 20.6 | 21.9 | 27.8 | 23.4 | 2.5 |
| 30 | 22.9 | 22.7 | 39.7 | 33.6 | 28.4 | 29.4 | 2.9 |
| 40 | 29.0 | 33.7 | 46.3 | 30.9 | 43.8 | 36.7 | 3.1 |
| 50 | 30.4 | 32.8 | 44.4 | 34.1 | 29.0 | 34.1 | 2.4 |
| 60 | 26.5 | 35.4 | 46.2 | 24.7 | 40.8 | 34.7 | 3.7 |
| Histamine Stimulation at t=40 min | | | | | | | |
| 0 | | | | | | | |
| 40 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 41 | 5.8 | 3.7 | 4.4 | 5.3 | 6.7 | 5.2 | 0.5 |
| 43 | 5.3 | 8.0 | 5.7 | 10.2 | 14.9 | 8.8 | 1.6 |
| 45 | 10.2 | 7.7 | 14.0 | 9.3 | 17.3 | 11.7 | 1.6 |
| 50 | 17.7 | 9.7 | 10.9 | 14.3 | 10.2 | 12.6 | 1.3 |
| 60 | 17.5 | 10.4 | 18.1 | 8.0 | 11.3 | 13.1 | 1.8 |
| 70 | 13.7 | 10.1 | 25.4 | 9.4 | 11.5 | 14.0 | 2.6 |
| 80 | 19.2 | 9.5 | 18.2 | 10.9 | 19.5 | 15.5 | 1.9 |

**Table A.6 Erythrocyte Stimulation Trials**

Data for Figure 4.3

| Erythrocyte Stimulation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8/27/2004 | Hist (endo) | | none | | Hist (SS) | | Sham | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.2 | 12.0 | 1.2 | 7.3 | 1.2 | 8.0 | 1.2 | 9.6 |
| | 2.9 | 13.3 | 3.1 | 4.6 | 3.0 | 4.3 | 3.1 | 3.4 |
| | 5.8 | 21.1 | 5.7 | 10.4 | 5.6 | 10.1 | 5.7 | 6.3 |
| | 9.4 | 26.4 | 9.3 | 9.5 | 9.2 | 12.2 | 9.3 | 9.0 |
| | 19.7 | 37.7 | 19.7 | 9.9 | 19.7 | 10.4 | 19.7 | 12.8 |
| | 29.5 | 45.7 | 29.5 | 10.9 | 29.6 | 8.0 | 29.6 | 10.9 |
| | 38.6 | 43.7 | 38.7 | 9.1 | 38.7 | 10.4 | 39.0 | 13.2 |
| 5/27/2004 | Hist (endo) | | none | | Hist (SS) | | Sham | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.3 | 17.8 | 1.2 | 4.9 | 1.2 | 2.5 | 1.3 | 0.0 |
| | 3.0 | 11.1 | 3.0 | 3.4 | 3.1 | 6.2 | 3.0 | 5.6 |
| | 5.8 | 18.5 | 5.7 | 5.3 | 5.7 | 7.3 | 5.7 | 4.7 |
| | 9.3 | 18.2 | 9.2 | 6.2 | 9.3 | 5.7 | 9.2 | 6.4 |
| | 19.5 | 32.9 | 19.6 | 10.4 | 19.7 | 11.4 | 19.6 | 8.7 |
| | 29.5 | 30.4 | 29.7 | 10.9 | 29.6 | 7.0 | 29.4 | 10.7 |
| | 38.5 | 34.0 | 38.5 | 6.7 | 38.2 | 10.3 | 38.5 | 9.5 |
| 6/18/2004 | Hist (endo) | | none | | Hist (SS) | | Sham | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.2 | 16.0 | 1.2 | 10.7 | 1.2 | 2.5 | 1.2 | 7.4 |
| | 2.9 | 9.3 | 2.9 | 5.7 | 3.0 | 6.0 | 3.0 | 6.4 |
| | 5.8 | 22.9 | 5.7 | 8.8 | 5.7 | 9.0 | 5.7 | 8.0 |
| | 9.4 | 26.7 | 9.4 | 13.2 | 9.3 | 9.2 | 9.4 | 11.8 |
| | 19.7 | 28.2 | 19.5 | 13.5 | 19.7 | 10.7 | 19.6 | 14.0 |
| | 29.7 | 40.9 | 29.5 | 22.4 | 29.6 | 19.6 | 29.6 | 19.2 |
| | 38.6 | 37.3 | 38.0 | 12.0 | 38.7 | 11.6 | 38.7 | 16.0 |
| 4/30/2004 | Hist (endo) | | none | | Hist (SS) | | Sham | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.2 | 8.7 | 1.2 | 7.4 | 1.3 | 6.4 | 1.3 | 2.9 |
| | 3.0 | 14.7 | 3.0 | 4.0 | 3.1 | 6.4 | 3.0 | 8.0 |
| | 5.6 | 32.0 | 5.8 | 7.7 | 5.7 | 6.1 | 5.7 | 11.5 |
| | 9.4 | 36.2 | 9.2 | 12.5 | 9.2 | 10.0 | 9.2 | 10.1 |
| | 19.7 | 27.6 | 19.4 | 10.1 | 19.5 | 9.5 | 19.7 | 12.2 |
| | 29.4 | 40.0 | 29.5 | 11.6 | 29.7 | 10.4 | 29.5 | 11.4 |
| | 40.0 | 30.4 | 38.7 | 11.1 | 39.0 | 12.0 | 38.8 | 13.6 |
| 4/2/2004 | Hist (endo) | | none | | Hist (SS) | | Sham | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.3 | 11.2 | 1.2 | 6.2 | 1.2 | 4.0 | 1.2 | 6.2 |
| | 3.0 | 20.9 | 3.0 | 8.5 | 3.0 | 7.5 | 3.0 | 9.4 |
| | 5.8 | 28.4 | 5.8 | 11.3 | 5.6 | 10.4 | 5.7 | 8.5 |
| | 9.2 | 28.0 | 9.2 | 12.1 | 9.2 | 13.0 | 9.4 | 17.9 |
| | 19.7 | 33.8 | 19.6 | 13.2 | 19.6 | 18.2 | 19.4 | 23.3 |
| | 29.8 | 49.0 | 29.7 | 15.3 | 29.6 | 10.7 | 29.4 | 23.5 |
| | 38.6 | 54.4 | 38.2 | 15.3 | 38.6 | 11.8 | 38.6 | 12.9 |

**Table A.6 Continued**

| Erythrocyte Stimulation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 11/20/2004 A | Hist (endo) | | none | | Hist (SS) | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | | |
| | 1.2 | 14.4 | 1.2 | 4.6 | 1.2 | 3.7 | | |
| | 2.9 | 7.3 | 3.0 | 6.4 | 3.0 | 9.6 | | |
| | 5.6 | 22.9 | 5.8 | 10.0 | 5.8 | 7.7 | | |
| | 9.3 | 26.1 | 9.2 | 13.2 | 9.3 | 14.5 | | |
| | 19.6 | 41.8 | 19.7 | 16.9 | 19.7 | 17.7 | | |
| | 29.8 | 28.8 | 29.7 | 11.6 | 29.9 | 14.4 | | |
| | 39.7 | 35.7 | 38.6 | 12.0 | 38.9 | 14.9 | | |
| 11/20/2004 B | Hist (endo) | | none | | Hist (SS) | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | | |
| | 1.2 | 11.2 | 1.2 | 1.3 | 1.2 | 6.7 | | |
| | 2.8 | 14.4 | 3.0 | 6.9 | 3.0 | 7.5 | | |
| | 5.7 | 16.8 | 5.7 | 10.4 | 5.8 | 3.3 | | |
| | 9.4 | 36.0 | 9.3 | 11.2 | 9.2 | 11.1 | | |
| | 19.7 | 42.0 | 19.6 | 10.9 | 19.6 | 12.0 | | |
| | 29.8 | 28.6 | 29.6 | 8.8 | 29.6 | 10.1 | | |
| | 39.5 | 35.7 | 39.1 | 8.3 | 39.1 | 14.8 | | |
| AVERAGES | Hist (endo) | | none | | Hist (SS) | | Sham | |
| | | 13.0 | | 6.0 | 1.2 | 4.8 | 1.2 | 5.2 |
| | | 13.0 | | 5.6 | 3.0 | 6.8 | 3.0 | 6.6 |
| | | 23.2 | | 9.1 | 5.7 | 7.7 | 5.7 | 7.8 |
| | | 28.2 | | 11.1 | 9.3 | 10.8 | 9.3 | 11.1 |
| | | 34.9 | | 12.1 | 19.6 | 12.8 | 19.6 | 14.2 |
| | | 37.6 | | 13.1 | 29.7 | 11.5 | 29.5 | 15.1 |
| | | 38.8 | | 10.6 | 38.8 | 12.2 | 38.7 | 13.0 |
| SEM | Hist (endo) | | none | | Hist (SS) | | Sham | |
| | | 3.1 | | 2.9 | | 2.2 | | 3.8 |
| | | 4.4 | | 1.8 | | 1.7 | | 2.3 |
| | | 5.3 | | 2.1 | | 2.5 | | 2.6 |
| | | 6.3 | | 2.6 | | 2.9 | | 4.3 |
| | | 5.9 | | 2.6 | | 3.6 | | 5.4 |
| | | 8.4 | | 4.5 | | 4.3 | | 5.9 |
| | | 8.0 | | 2.9 | | 1.9 | | 2.3 |

**Table A.7 Thioperamide, Famotidine, Pyrilamine H-Receptor Antagonists**

Data for Figure 4.4

Famotidine and Thioperamide

| | 5/15/2003 | | 5/29/2003 | | 6/12/2003 | | 6/19/2003 | | 7/18/2003 | | 9/4/2003 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Histamine 100 uM | | | | | | | | | | | |
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm2 | time | cells/mm2 |
| 1.2 | 4.8 | 1.1 | 6.0 | 1.3 | 9.3 | 1.2 | 8.7 | 1.1 | 16.0 | 1.1 | 12.0 |
| 3.0 | 19.7 | 2.9 | 11.1 | 3.1 | 16.0 | 3.0 | 21.7 | 2.9 | 48.0 | 3.0 | 20.2 |
| 5.1 | 16.0 | 5.7 | 25.2 | 5.6 | 21.6 | 5.8 | 27.2 | 5.7 | 68.2 | 5.8 | 24.1 |
| 9.5 | 32.0 | 9.4 | 19.0 | 9.2 | 20.4 | 9.2 | 32.8 | 9.1 | 75.3 | 9.4 | 22.6 |
| 19.4 | 64.8 | 19.4 | 43.6 | 19.7 | 51.2 | 19.6 | 26.1 | 19.8 | 99.0 | 19.5 | 25.6 |
| 29.6 | 78.2 | 29.6 | 43.4 | 29.8 | 72.0 | 29.5 | 33.8 | 29.7 | 84.3 | 29.6 | 44.5 |
| 38.6 | 80.0 | 38.3 | 36.4 | 38.9 | 72.0 | 38.5 | 50.1 | 38.8 | 100.3 | 39.0 | 35.3 |
| Unstimulated | | | | | | | | | | | |
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm2 | time | cells/mm2 |
| 1.2 | 7.1 | 1.2 | 3.7 | 1.2 | 5.3 | 1.2 | 8.6 | 1.2 | 4.9 | 1.2 | 4.4 |
| 3.0 | 4.6 | 3.2 | 6.2 | 3.0 | 3.0 | 3.0 | 8.0 | 3.0 | 6.2 | 3.0 | 6.2 |
| 5.0 | 4.9 | 5.7 | 5.3 | 5.6 | 6.5 | 5.6 | 5.9 | 5.6 | 8.0 | 5.8 | 9.1 |
| 10.1 | 5.3 | 9.2 | 9.7 | 9.4 | 11.6 | 9.3 | 8.8 | 9.1 | 6.9 | 9.3 | 5.1 |
| 20.0 | 11.0 | 19.6 | 9.5 | 19.6 | 19.2 | 19.6 | 9.0 | 19.7 | 16.0 | 19.6 | 15.2 |
| 29.9 | 11.6 | 29.7 | 10.4 | 29.7 | 22.6 | 29.7 | 16.0 | 29.8 | 11.6 | 29.6 | 8.0 |
| 37.8 | 17.3 | 38.7 | 16.0 | 39.1 | 32.0 | 38.8 | 12.2 | 38.7 | 10.4 | 38.6 | 4.4 |
| Hist 100 uM + 3 uM Thioperamide | | | | | | | | | | | |
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm2 | | |
| 1.3 | 1.8 | 1.2 | 1.5 | 1.2 | 0.0 | 1.3 | 6.9 | 1.1 | 8.7 | | |
| 2.9 | 6.2 | 3.0 | 7.4 | 3.0 | 5.3 | 3.1 | 10.0 | 3.1 | 5.3 | | |
| 5.3 | 10.3 | 5.7 | 8.0 | 5.6 | 4.7 | 5.8 | 9.8 | 5.7 | 6.0 | | |
| 9.4 | 12.0 | 9.3 | 8.0 | 9.3 | 7.6 | 9.2 | 11.0 | 9.4 | 9.6 | | |
| 19.6 | 16.0 | 19.6 | 15.1 | 19.7 | 5.6 | 19.8 | 6.5 | 19.8 | 13.3 | | |
| 29.5 | 16.0 | 29.7 | 8.0 | 29.7 | 8.4 | 29.7 | 8.4 | 29.7 | 13.5 | | |
| 38.6 | 30.1 | 38.6 | 12.2 | 38.7 | 16.8 | 38.6 | 13.7 | 38.7 | 7.0 | | |
| Hist 100 uM + 3 uM Famotidine | | | | | | | | | | | |
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | | | | |
| 1.2 | 7.3 | 1.3 | 0.0 | 1.1 | 1.2 | 1.2 | 6.2 | | | | |
| 3.0 | 4.6 | 3.6 | 6.9 | 2.9 | 3.6 | 3.0 | 9.4 | | | | |
| 5.0 | 9.3 | 5.7 | 7.7 | 5.6 | 4.7 | 5.7 | 7.0 | | | | |
| 9.6 | 16.8 | 9.2 | 13.1 | 9.2 | 8.7 | 9.3 | 6.7 | | | | |
| 19.8 | 20.9 | 19.6 | 12.6 | 19.6 | 8.8 | 19.8 | 8.4 | | | | |
| 29.6 | 28.2 | 29.6 | 11.3 | 29.8 | 8.8 | 29.7 | 10.1 | | | | |
| 38.5 | 25.3 | 38.6 | 11.4 | 38.7 | 16.0 | 38.9 | 8.9 | | | | |
| 10 ⁄M Pyrilamine, 100 uM Hist | | | | | | | | | | | |
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | | | time | cells/mm2 |
| 1.2 | 3.2 | 1.2 | 8.0 | 1.2 | 9.6 | 1.2 | 16.0 | | | 1.2 | 14.4 |
| 3.0 | 5.7 | 3.0 | 13.7 | 3.0 | 26.7 | 2.9 | 17.1 | | | 3.0 | 11.2 |
| 5.0 | 27.4 | 5.7 | 16.9 | 5.7 | 25.5 | 5.7 | 17.9 | | | 5.8 | 24.5 |
| 10.2 | 39.5 | 9.3 | 26.4 | 9.3 | 34.9 | 9.2 | 18.7 | | | 9.4 | 25.6 |
| 19.7 | 50.1 | 20.0 | 30.5 | 19.8 | 70.9 | 19.8 | 32.0 | | | 19.6 | 34.7 |
| 29.5 | 84.4 | 29.6 | 32.0 | 29.8 | 89.8 | 29.8 | 36.3 | | | 29.5 | 37.0 |
| 38.9 | 94.1 | 38.7 | 32.0 | 38.7 | 107.1 | 38.7 | 41.4 | | | 38.8 | 37.3 |

**Table A.7 Continued**

| AVERAGES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AVERAGE Thiop Histamine 100 uM | | | AVERAGE Famotidine Histamine 100 uM | | | Average Pyril Histamine 100 uM | | |
| time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM |
| 1.2 | 9.0 | 1.9 | 1.2 | 7.2 | 1.1 | 1.2 | 8.2 | 1.3 |
| 3.0 | 23.3 | 6.4 | 3.0 | 17.1 | 2.3 | 3.0 | 17.7 | 1.9 |
| 5.6 | 31.6 | 9.3 | 5.6 | 22.5 | 2.5 | 5.6 | 22.8 | 1.9 |
| 9.3 | 35.9 | 10.3 | 9.3 | 26.1 | 3.7 | 9.3 | 25.4 | 2.9 |
| 19.6 | 56.9 | 12.2 | 19.6 | 46.4 | 8.1 | 19.5 | 42.3 | 7.5 |
| 29.6 | 62.3 | 10.0 | 29.6 | 56.9 | 10.8 | 29.6 | 54.4 | 8.7 |
| 38.6 | 67.8 | 11.2 | 38.6 | 59.6 | 10.0 | 38.7 | 54.8 | 9.1 |
| Unstimulated | | | Unstimulated | | | Unstimulated | | |
| time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM |
| 1.2 | 5.9 | 0.9 | 1.2 | 6.2 | 1.1 | 1.2 | 5.8 | 0.9 |
| 3.0 | 5.6 | 0.8 | 3.0 | 5.4 | 1.1 | 3.0 | 5.6 | 0.8 |
| 5.5 | 6.1 | 0.5 | 5.5 | 5.7 | 0.4 | 5.6 | 6.4 | 0.7 |
| 9.4 | 8.5 | 1.1 | 9.5 | 8.9 | 1.3 | 9.5 | 8.1 | 1.3 |
| 19.7 | 12.9 | 2.0 | 19.7 | 12.2 | 2.4 | 19.7 | 12.8 | 1.9 |
| 29.8 | 14.4 | 2.3 | 29.8 | 15.1 | 2.8 | 29.7 | 13.7 | 2.6 |
| 38.6 | 17.6 | 3.8 | 38.6 | 19.4 | 4.3 | 38.6 | 16.4 | 4.5 |
| 10 mM Thioperamide no hist | | | | | | | | |
| time | cells/mm$^2$ | SEM | | | | | | |
| 1.2 | 3.8 | 1.7 | | | | | | |
| 3.0 | 6.8 | 0.9 | | | | | | |
| 5.6 | 7.8 | 1.1 | | | | | | |
| 9.3 | 9.7 | 0.8 | | | | | | |
| 19.7 | 11.3 | 2.2 | | | | | | |
| 29.7 | 10.8 | 1.6 | | | | | | |
| 38.6 | 16.0 | 3.9 | | | | | | |
| | | | 10mM Famotidine, + Hist | | | | | |
| | | | time | cells/mm$^2$ | SEM | | | |
| | | | 1.2 | 3.7 | 1.8 | | | |
| | | | 3.1 | 6.1 | 1.3 | | | |
| | | | 5.5 | 7.2 | 1.0 | | | |
| | | | 9.3 | 11.3 | 2.3 | | | |
| | | | 19.7 | 12.7 | 2.9 | | | |
| | | | 29.7 | 14.6 | 4.6 | | | |
| | | | 38.7 | 15.4 | 3.6 | | | |
| | | | | | | 10 mM Pyrilamine, Hist | | |
| | | | | | | time | cells/mm$^2$ | SEM |
| | | | | | | 1.2 | 10.2 | 2.3 |
| | | | | | | 3.0 | 14.9 | 3.5 |
| | | | | | | 5.6 | 22.4 | 2.1 |
| | | | | | | 9.5 | 29.0 | 3.7 |
| | | | | | | 19.8 | 43.6 | 7.7 |
| | | | | | | 29.6 | 55.9 | 12.8 |
| | | | | | | 38.7 | 62.4 | 15.8 |

**Table A.8 Amthamine and Methylhistamine, H-Receptor Agonists**

Data for Figure 4.5

| | 1/8/2003 | | 1/16/2003 | | 2/20/2003 | | AVERAGES | | |
|---|---|---|---|---|---|---|---|---|---|
| Histamine | | | | | | | 100 mM Hist | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| | 1.3 | 12.0 | 1.2 | 7.6 | 1.2 | 12.4 | 1.2 | 10.7 | 1.3 |
| | 2.9 | 15.8 | 3.0 | 11.7 | 3.0 | 8.6 | 3.0 | 12.0 | 1.7 |
| | 5.6 | 20.0 | 5.7 | 16.7 | 5.7 | 13.6 | 5.7 | 16.8 | 1.5 |
| | 9.4 | 34.0 | 9.2 | 14.3 | 9.2 | 26.2 | 9.3 | 24.8 | 4.7 |
| | 19.6 | 35.6 | 19.6 | 16.5 | 19.5 | 37.5 | 19.6 | 29.8 | 5.5 |
| | 29.6 | 36.7 | 29.7 | 24.0 | 29.5 | 38.0 | 29.6 | 32.9 | 3.6 |
| | 38.8 | 45.9 | 38.8 | 26.5 | 38.8 | 31.2 | 38.8 | 34.5 | 4.8 |
| Unstimulated | | | | | | | No Stim | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| | 1.1 | 4.8 | 1.2 | 2.9 | 1.2 | 5.3 | 1.2 | 4.3 | 0.6 |
| | 2.9 | 8.0 | 3.0 | 2.8 | 2.9 | 5.8 | 2.9 | 5.5 | 1.2 |
| | 5.7 | 10.2 | 5.8 | 4.3 | 5.8 | 3.4 | 5.8 | 6.0 | 1.7 |
| | 9.2 | 10.9 | 9.4 | 8.3 | 9.4 | 8.0 | 9.3 | 9.1 | 0.8 |
| | 19.5 | 8.0 | 19.6 | 8.7 | 19.4 | 5.6 | 19.5 | 7.4 | 0.8 |
| | 29.6 | 12.2 | 29.6 | 9.0 | 29.7 | 16.8 | 29.6 | 12.7 | 1.8 |
| | 38.7 | 12.8 | 38.5 | 8.0 | 38.8 | 16.0 | 38.7 | 12.3 | 1.9 |
| 10 mM Methistamine | | | | | | | 10 mM Methistamine | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| | 1.2 | 6.7 | 1.2 | 2.5 | 1.1 | 8.0 | 1.2 | 5.7 | 1.4 |
| | 3.0 | 6.2 | 2.9 | 5.6 | 3.0 | 9.3 | 3.0 | 7.0 | 0.9 |
| | 5.7 | 8.0 | 5.7 | 8.3 | 5.7 | 12.6 | 5.7 | 9.6 | 1.2 |
| | 9.3 | 12.0 | 9.3 | 10.5 | 9.3 | 15.2 | 9.3 | 12.6 | 1.1 |
| | 19.5 | 10.7 | 19.6 | 9.3 | 19.4 | 19.4 | 19.5 | 13.1 | 2.6 |
| | 29.6 | 14.2 | 29.3 | 13.6 | 29.6 | 20.7 | 29.5 | 16.2 | 1.9 |
| | 38.7 | 16.0 | 38.6 | 10.2 | 38.8 | 20.6 | 38.7 | 15.6 | 2.4 |
| 10 mM Amthamine | | | | 0.1 | | | 10 mM Amthamine | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| | 1.4 | 3.6 | 1.2 | 2.5 | 1.3 | 3.6 | 1.3 | 3.2 | 0.3 |
| | 3.1 | 18.7 | 3.1 | 4.3 | 3.0 | 8.0 | 3.1 | 10.3 | 3.5 |
| | 5.8 | 12.8 | 5.7 | 9.0 | 5.6 | 4.7 | 5.7 | 8.8 | 1.9 |
| | 9.3 | 16.8 | 9.5 | 8.7 | 9.2 | 7.2 | 9.3 | 10.9 | 2.4 |
| | 19.5 | 22.4 | 19.6 | 9.3 | 19.4 | 13.8 | 19.5 | 15.2 | 3.1 |
| | 29.5 | 25.6 | 29.6 | 19.2 | 29.5 | 10.1 | 29.5 | 18.3 | 3.7 |
| | 38.6 | 29.0 | 38.6 | 19.6 | 39.0 | 18.1 | 38.8 | 22.2 | 2.8 |
| 10 mM Amthamine+10 mM Methistamine | | | | | | | Amthamine+Methistamine | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| | 1.3 | 8.0 | 1.2 | 2.9 | 1.2 | 3.6 | 1.2 | 4.8 | 1.3 |
| | 3.0 | 17.1 | 3.0 | 4.0 | 3.0 | 10.2 | 3.0 | 10.4 | 3.1 |
| | 5.8 | 22.1 | 5.7 | 10.7 | 5.8 | 13.6 | 5.8 | 15.5 | 2.8 |
| | 9.3 | 23.3 | 9.3 | 12.0 | 9.3 | 16.0 | 9.3 | 17.1 | 2.7 |
| | 19.5 | 34.8 | 19.4 | 23.6 | 19.4 | 27.3 | 19.5 | 28.6 | 2.7 |
| | 30.3 | 43.6 | 29.6 | 15.3 | 29.4 | 34.8 | 29.7 | 31.2 | 6.8 |
| | 38.5 | 44.2 | 38.7 | 18.8 | 38.6 | 44.0 | 38.6 | 35.7 | 6.9 |

## Table A.9 Amthamine and Clobenpropit, H-Receptor Agonists Data for Figure IV.6

| | 10/30/2003 | | 9/5/2003 | | 8/21/2003 | | 8/13/2003 | | 1/8/2003 | | 1/16/2003 | | 2/20/2003 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Histamine | | | | | | | | | | | | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.2 | 21.3 | 1.1 | 12.0 | 1.2 | 10.7 | 1.2 | 9.8 | 1.3 | 12.0 | 1.2 | 7.6 | 1.2 | 12.4 |
| | 3.0 | 20.4 | 3.0 | 20.2 | 3.0 | 16.0 | 3.0 | 14.2 | 2.9 | 15.8 | 3.0 | 11.7 | 3.0 | 8.6 |
| | 5.7 | 41.3 | 5.8 | 24.1 | 5.8 | 16.8 | 5.8 | 19.4 | 5.6 | 20.0 | 5.7 | 16.7 | 5.7 | 13.6 |
| | 9.2 | 52.0 | 9.4 | 22.6 | 9.4 | 21.3 | 9.3 | 35.3 | 9.4 | 34.0 | 9.2 | 14.3 | 9.2 | 26.2 |
| | 19.7 | 61.7 | 19.5 | 25.6 | 19.6 | 20.8 | 19.5 | 63.6 | 19.6 | 35.6 | 19.6 | 16.5 | 19.5 | 37.5 |
| | 29.6 | 78.9 | 29.6 | 44.5 | 29.5 | 30.2 | 29.6 | 59.8 | 29.6 | 36.7 | 29.7 | 24.0 | 29.5 | 38.0 |
| | 38.7 | 55.4 | 39.0 | 35.3 | 38.9 | 34.3 | 38.8 | 66.0 | 38.8 | 45.9 | 38.8 | 26.5 | 38.8 | 31.2 |
| Unstimulated | | | | | | | | | | | | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.1 | 6.7 | 1.2 | 4.4 | 1.2 | 4.4 | 1.2 | 4.9 | 1.1 | 4.8 | 1.2 | 2.9 | 1.2 | 5.3 |
| | 3.0 | 6.0 | 3.0 | 6.2 | 3.1 | 6.7 | 3.0 | 7.4 | 2.9 | 8.0 | 3.0 | 2.8 | 2.9 | 5.8 |
| | 5.7 | 13.2 | 5.8 | 9.1 | 5.8 | 9.9 | 5.7 | 8.7 | 5.7 | 10.2 | 5.8 | 4.3 | 5.8 | 3.4 |
| | 9.2 | 24.8 | 9.3 | 5.1 | 9.3 | 7.7 | 9.5 | 17.5 | 9.2 | 10.9 | 9.4 | 8.3 | 9.4 | 8.0 |
| | 19.5 | 22.2 | 19.6 | 15.2 | 19.4 | 9.6 | 19.7 | 18.0 | 19.5 | 8.0 | 19.6 | 8.7 | 19.4 | 5.6 |
| | 29.5 | 21.3 | 29.6 | 8.0 | 29.5 | 7.7 | 29.7 | 26.7 | 29.6 | 12.2 | 29.6 | 9.0 | 29.7 | 16.8 |
| | 38.6 | 26.4 | 38.6 | 4.4 | 38.5 | 10.9 | 38.7 | 28.0 | 38.7 | 12.8 | 38.5 | 8.0 | 38.8 | 16.0 |
| Clobenpropit | | | | | | | | | | | | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | | | | | | |
| | 1.2 | 10.2 | 1.2 | 5.8 | 1.1 | 5.8 | 1.2 | 5.8 | | | | | | |
| | 3.1 | 10.3 | 3.0 | 8.6 | 3.0 | 3.2 | 3.0 | 9.8 | | | | | | |
| | 5.8 | 12.5 | 5.8 | 10.9 | 5.6 | 3.6 | 5.8 | 16.0 | | | | | | |
| | 9.2 | 12.8 | 9.3 | 14.1 | 9.3 | 5.1 | 9.3 | 16.7 | | | | | | |
| | 19.7 | 20.8 | 19.7 | 14.4 | 19.6 | 7.6 | 19.6 | 28.6 | | | | | | |
| | 29.5 | 16.8 | 29.5 | 12.8 | 29.4 | 6.1 | 29.8 | 46.0 | | | | | | |
| | 38.7 | 23.0 | 38.7 | 13.5 | 38.5 | 6.5 | 38.7 | 52.9 | | | | | | |
| Amthamine | | | | | | | | | | | | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | | | | | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| | 1.2 | 6.4 | 1.2 | 4.4 | | | | | 1.4 | 3.6 | 1.2 | 2.5 | 1.3 | 3.6 |
| | 3.0 | 12.0 | 3.0 | 10.2 | | | | | 3.1 | 18.7 | 3.1 | 4.3 | 3.0 | 8.0 |
| | 5.7 | 14.6 | 5.7 | 7.2 | | | | | 5.8 | 12.8 | 5.7 | 9.0 | 5.6 | 4.7 |
| | 9.3 | 18.7 | 9.4 | 9.9 | | | | | 9.3 | 16.8 | 9.5 | 8.7 | 9.2 | 7.2 |
| | 19.6 | 27.0 | 19.7 | 10.7 | | | | | 19.5 | 22.4 | 19.6 | 9.3 | 19.4 | 13.8 |
| | 29.7 | 24.5 | 29.5 | 10.7 | | | | | 29.5 | 25.6 | 29.6 | 19.2 | 29.5 | 10.1 |
| | 38.8 | 20.4 | 39.4 | 7.1 | | | | | 38.6 | 29.0 | 38.6 | 19.6 | 39.0 | 18.1 |
| Amthamine + Clobenpropit | | | | | | | | | | | | | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | | | time | cells/mm$^2$ | | | | | | |
| | 1.2 | 22.4 | 1.2 | 12.4 | | | 1.1 | 8.0 | | | | | | |
| | 2.9 | 27.2 | 3.0 | 21.3 | | | 2.9 | 10.3 | | | | | | |
| | 5.7 | 26.7 | 5.6 | 21.6 | | | 5.8 | 12.5 | | | | | | |
| | 9.3 | 27.2 | 9.4 | 24.0 | | | 9.3 | 27.8 | | | | | | |
| | 19.6 | 38.4 | 19.5 | 33.0 | | | 20.0 | 47.1 | | | | | | |
| | 29.7 | 44.8 | 29.4 | 30.0 | | | 29.5 | 50.8 | | | | | | |
| | 38.9 | 55.5 | 38.9 | 45.1 | | | 39.7 | 50.0 | | | | | | |

## Table A.9 Continued

| | 6/12/2003 | | 6/19/2003 | | 7/18/2003 | | AVERAGE, AMTHAMINE | | | AVERAGE. CLOBENPROPIT | | | Amthamine + Clobinpropit | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Histamine | | | | | | | Histamine N=5 | | | Histamine N=7 | | | Histamine N=5 | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM |
| | 1.3 | 9.3 | 1.2 | 8.7 | 1.1 | 16.0 | 1.2 | 13.1 | 2.2 | 1.2 | 12.5 | 1.7 | 1.2 | 12.2 | 2.3 |
| | 3.1 | 16.0 | 3.0 | 21.7 | 2.9 | 48.0 | 3.0 | 15.3 | 2.3 | 3.0 | 22.4 | 4.4 | 3.0 | 18.5 | 1.4 |
| | 5.6 | 21.6 | 5.8 | 27.2 | 5.7 | 68.2 | 5.7 | 23.1 | 4.9 | 5.7 | 31.2 | 6.9 | 5.7 | 26.7 | 3.9 |
| | 9.2 | 20.4 | 9.2 | 32.8 | 9.1 | 75.3 | 9.3 | 29.8 | 6.4 | 9.3 | 37.1 | 7.6 | 9.3 | 32.6 | 5.6 |
| | 19.7 | 51.2 | 19.6 | 26.1 | 19.8 | 99.0 | 19.6 | 35.4 | 7.6 | 19.6 | 49.7 | 10.6 | 19.6 | 45.6 | 8.3 |
| | 29.8 | 72.0 | 29.5 | 33.8 | 29.7 | 84.3 | 29.6 | 44.4 | 9.2 | 29.6 | 57.7 | 8.3 | 29.6 | 57.8 | 8.4 |
| | 38.9 | 72.0 | 38.5 | 50.1 | 38.8 | 100.3 | 38.8 | 38.8 | 5.2 | 38.8 | 59.0 | 8.7 | 38.8 | 55.8 | 6.4 |
| Unstimulated | | | | | | | Unstimulated | | | Unstimulated | | | Unstimulated | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM |
| | 1.2 | 5.3 | 1.2 | 8.6 | 1.2 | 4.9 | 1.2 | 4.8 | 0.6 | 1.2 | 5.6 | 0.6 | 1.2 | 6.0 | 1.7 |
| | 3.0 | 3.0 | 3.0 | 8.0 | 3.0 | 6.2 | 3.0 | 5.8 | 0.8 | 3.0 | 6.2 | 0.6 | 3.0 | 6.1 | 1.9 |
| | 5.6 | 6.5 | 5.6 | 5.9 | 5.6 | 8.0 | 5.8 | 8.0 | 1.8 | 5.7 | 8.8 | 0.9 | 5.7 | 8.7 | 2.9 |
| | 9.4 | 11.6 | 9.3 | 8.8 | 9.1 | 6.9 | 9.3 | 11.4 | 3.5 | 9.3 | 11.8 | 2.7 | 9.3 | 13.6 | 7.7 |
| | 19.6 | 19.2 | 19.6 | 9.0 | 19.7 | 16.0 | 19.5 | 11.9 | 3.0 | 19.6 | 15.6 | 1.8 | 19.6 | 16.7 | 5.0 |
| | 29.7 | 22.6 | 29.7 | 16.0 | 29.8 | 11.6 | 29.6 | 13.5 | 2.5 | 29.6 | 16.3 | 2.8 | 29.6 | 18.9 | 7.2 |
| | 39.1 | 32.0 | 38.8 | 12.2 | 38.7 | 10.4 | 38.6 | 13.5 | 3.8 | 38.7 | 17.8 | 4.1 | 38.8 | 20.6 | 11.7 |
| Clobenpropit | | | | | | | Agonist Treatment | | | Agonist Treatment | | | Agonist Treatment | | |
| | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM | time | cells/mm$^2$ | SEM |
| | 1.2 | 5.8 | 1.2 | 10.2 | 1.2 | 11.6 | 1.3 | 4.1 | 0.7 | 1.2 | 7.9 | 1.0 | 1.2 | 12.8 | 2.5 |
| | 2.9 | 4.3 | 2.9 | 11.1 | 3.0 | 6.9 | 3.0 | 10.6 | 2.4 | 3.0 | 7.7 | 1.2 | 2.9 | 16.5 | 3.6 |
| | 5.8 | 10.9 | 5.7 | 9.1 | 5.8 | 10.9 | 5.7 | 9.7 | 1.8 | 5.7 | 10.6 | 1.4 | 5.7 | 22.1 | 3.7 |
| | 9.2 | 17.3 | 9.3 | 11.1 | 9.3 | 7.0 | 9.3 | 12.2 | 2.3 | 9.3 | 12.0 | 1.8 | 9.3 | 27.2 | 1.9 |
| | 19.7 | 28.6 | 19.6 | 14.2 | 19.8 | 18.5 | 19.6 | 16.6 | 3.5 | 19.7 | 19.0 | 2.9 | 19.7 | 40.6 | 4.6 |
| | 29.9 | 39.0 | 29.7 | 13.6 | 29.9 | 16.0 | 29.5 | 18.0 | 3.3 | 29.7 | 21.5 | 5.6 | 29.6 | 45.5 | 6.5 |
| | 39.2 | 29.4 | 38.7 | 17.6 | 38.7 | 16.0 | 38.9 | 18.8 | 3.5 | 38.7 | 22.7 | 5.7 | 39.2 | 49.9 | 7.9 |

Amthamine + Clobenpropit

| time | cells/mm$^2$ | time | cells/mm$^2$ |
|---|---|---|---|
| 1.3 | 9.3 | 1.2 | 11.6 |
| 3.0 | 7.5 | 3.0 | 16.0 |
| 5.7 | 33.5 | 5.8 | 16.0 |
| 9.4 | 34.1 | 9.2 | 23.0 |
| 19.7 | 55.0 | 19.9 | 29.7 |
| 29.6 | 66.8 | 29.8 | 35.0 |
| 38.7 | 73.8 | 40.0 | 25.2 |

**Table A.10 Peptide Blocking of Histamine-Mediated Adhesion**

Data for Figure 4.7

| time | Unstimulated | | | | | | Unstimulated | |
|---|---|---|---|---|---|---|---|---|
| | 3/4/2004 | 3/11/2004 | 3/18/2004 | 3/18/2004 I | 1/29/2004 | 2/19/2004 | AVERAGE | |
| | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | SEM |
| 1.2 | 4.4 | 5.3 | 1.1 | 1.2 | 5.8 | 2.9 | 3.5 | 0.8 |
| 3.0 | 9.3 | 1.0 | 5.6 | 6.4 | 11.1 | 5.7 | 6.5 | 1.4 |
| 5.9 | 22.2 | 5.5 | 7.7 | 5.3 | 13.8 | 10.4 | 10.8 | 2.6 |
| 9.3 | 21.6 | 12.1 | 12.7 | 9.3 | 9.6 | 10.2 | 12.6 | 1.9 |
| 19.7 | 20.2 | 18.2 | 15.3 | 9.7 | 16.9 | 9.6 | 15.0 | 1.8 |
| 29.7 | 28.8 | 12.5 | 23.2 | 17.4 | 15.2 | 10.9 | 18.0 | 2.8 |
| 39.1 | 23.2 | 14.5 | 26.5 | 13.6 | 17.1 | 11.6 | 17.8 | 2.4 |
| | Histamine | | | | | | Histamine | |
| | 5/4/2004 | 5/11/2004 | 5/18/2004 | 3/18/2004 I | 1/29/2004 | 2/19/2004 | AVERAGE | |
| | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | SEM |
| | 5.8 | 6.0 | 5.3 | 1.5 | 7.1 | 5.3 | 5.2 | 0.7 |
| | 13.3 | 4.3 | 6.9 | 5.3 | 12.3 | 14.9 | 9.5 | 1.7 |
| | 28.0 | 10.9 | 16.0 | 16.8 | 29.0 | 21.6 | 20.4 | 2.7 |
| | 30.6 | 28.8 | 23.6 | 24.0 | 32.0 | 18.1 | 26.2 | 2.0 |
| | 32.9 | 36.8 | 35.2 | 37.3 | 45.9 | 35.4 | 37.3 | 1.7 |
| | 51.0 | 44.4 | 37.6 | 40.0 | 44.8 | 46.1 | 44.0 | 1.8 |
| | 34.3 | 37.1 | 41.6 | 35.0 | 53.3 | 40.0 | 40.2 | 2.6 |
| | Blocking Peptide | | | | | | Blocking Peptide | |
| | 5/4/2004 | 5/11/2004 | 3/18/2004 | 3/18/2004 I | 1/29/2004 | 2/19/2004 | AVERAGE | |
| | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | SEM |
| | 5.3 | 2.5 | 0.0 | 0.0 | 5.3 | 5.8 | 3.2 | 1.0 |
| | 11.4 | 3.8 | 5.0 | 0.0 | 5.7 | 6.9 | 5.5 | 1.4 |
| | 10.4 | 7.4 | 5.5 | 0.0 | 14.0 | 6.0 | 7.2 | 1.8 |
| | 11.9 | 9.7 | 9.5 | | 19.3 | 8.0 | 11.7 | 1.6 |
| | 13.2 | 17.4 | 15.3 | 5.8 | 32.0 | 10.2 | 15.6 | 3.4 |
| | 16.0 | 14.7 | 12.2 | 16.7 | 27.2 | 12.2 | 16.5 | 2.1 |
| | 10.7 | 16.0 | 18.9 | 18.5 | 28.4 | 20.0 | 18.8 | 2.2 |
| | Non-Blocking Peptide | | | | | | Non-Blocking Pep | |
| | 5/4/2004 | 5/11/2004 | 5/18/2004 | 3/18/2004 I | 1/29/2004 | 2/19/2004 | AVERAGE | |
| | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | cells/mm$^2$ | SEM |
| | 7.4 | 6.7 | 1.3 | 5.3 | 13.3 | 4.8 | 6.5 | 1.5 |
| | 18.1 | 6.2 | 5.7 | 5.3 | 14.9 | 9.3 | 9.9 | 2.0 |
| | 28.4 | 11.5 | 12.3 | 8.9 | 26.4 | 10.2 | 16.3 | 3.2 |
| | 49.6 | 23.3 | 17.8 | 19.0 | 32.7 | 16.0 | 26.4 | 4.8 |
| | 83.8 | 41.9 | 24.3 | 32.0 | 40.8 | 16.0 | 39.8 | 8.8 |
| | 91.0 | 44.6 | 33.5 | 34.8 | 51.2 | 24.5 | 46.6 | 8.8 |
| | 96.0 | 45.2 | 37.6 | 39.5 | 51.0 | 24.4 | 48.9 | 9.2 |

**Table A.11 Antibody Blocking of P-Selectin Mediated Adhesion**

Data for Figure 4.9

| 3/28/2002 | | | | | | |
|---|---|---|---|---|---|---|
| | Unstimulated | | Histamine | | Anti P-selectin Antibody | |
| | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ |
| | 1.5 | 5.5 | 1.5 | 24.0 | 1.6 | 3.6 |
| | 3.3 | 10.7 | 3.3 | 27.1 | 3.2 | 7.4 |
| | 4.9 | 17.3 | 5.2 | 31.0 | 5.1 | 10.7 |
| | 9.5 | 24.3 | 9.8 | 42.2 | 9.7 | 18.2 |
| | 19.7 | 28.2 | 19.6 | 48.6 | 19.9 | 27.8 |
| | 29.6 | 33.3 | 29.8 | 56.3 | 29.5 | 28.8 |
| | 38.7 | 26.5 | 38.5 | 57.6 | 38.8 | 27.4 |
| 4/3/2002 | | | | | | |
| | Unstimulated | | Histamine | | Anti P-selectin Antibody | |
| | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ | time | SSRBC/mm$^2$ |
| | 1.4 | 6.7 | 1.4 | 7.1 | 1.4 | 13.3 |
| | 3.3 | 18.9 | 3.4 | 14.7 | 3.3 | 34.3 |
| | 5.1 | 20.6 | 5.0 | 32.0 | 5.1 | 48.3 |
| | 9.9 | 42.9 | 9.5 | 66.1 | 9.7 | 85.8 |
| | 19.7 | 63.3 | 19.8 | 101.6 | 19.7 | 118.2 |
| | 29.6 | 82.2 | 29.7 | 87.3 | 29.7 | 152.4 |
| | 38.7 | 92.6 | 38.9 | 104.8 | 38.9 | 129.1 |

**Table A.12 TNF Mediated Adhesion Under Shear Concentration Dependence**
Data for Figure V.1 and V.2

| Shear | 1/26/2000 | | | | 2/16/2000 | | | | 3/23/2000 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.1 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 32.0 | 16.0 | 40.0 | 32.0 | 96.0 | 16.0 | 24.0 | 80.0 | 48.0 | 88.0 | 64.0 | 48.0 |
| 7 | 192.0 | 128.0 | 116.0 | 144.0 | 160.0 | 128.0 | 72.0 | 144.0 | 440.0 | 240.0 | 245.3 | 296.0 |
| 12 | | 176.0 | 224.0 | 176.0 | 192.0 | 184.0 | 176.0 | 112.0 | 400.0 | 408.0 | 288.0 | 592.0 |
| 17 | | 112.0 | | | 304.0 | 96.0 | 197.3 | 192.0 | 496.0 | 416.0 | 312.0 | 664.0 |
| 25 | | | | | 304.0 | | 320.0 | | 528.0 | 496.0 | 416.0 | 672.0 |
| 30 | | | | | | | 200.0 | | 536.0 | 432.0 | 360.0 | 904.0 |
| 0.2 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 16.0 | 0.0 | 56.0 | 64.0 | 64.0 | 16.0 | 0.0 | 48.0 | 48.0 | 64.0 | 48.0 | 32.0 |
| 7 | 232.0 | 80.0 | 116.0 | 176.0 | 96.0 | 72.0 | 72.0 | 112.0 | 176.0 | 256.0 | 224.0 | 240.0 |
| 12 | 288.0 | 176.0 | 204.0 | 160.0 | 296.0 | 112.0 | 84.0 | 192.0 | 240.0 | 112.0 | 224.0 | 472.0 |
| 17 | 352.0 | 320.0 | 208.0 | 264.0 | 480.0 | 64.0 | 128.0 | 184.0 | 296.0 | 208.0 | 200.0 | 432.0 |
| 25 | 248.0 | 128.0 | 220.0 | 256.0 | 272.0 | 88.0 | 96.0 | 256.0 | 352.0 | 272.0 | 224.0 | 656.0 |
| 30 | 272.0 | 264.0 | 172.0 | 400.0 | 288.0 | 96.0 | 92.0 | 264.0 | 368.0 | 400.0 | 272.0 | 528.0 |
| 0.4 time(forc | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 16.0 | 56.0 | 36.0 | 48.0 | 48.0 | 0.0 | 40.0 | 48.0 | 40.0 | 48.0 | 16.0 | 32.0 |
| 7 | 56.0 | 32.0 | 92.0 | 80.0 | 96.0 | 64.0 | 36.0 | 56.0 | 0.0 | 56.0 | 64.0 | 160.0 |
| 12 | 112.0 | 48.0 | 60.0 | 72.0 | 48.0 | 56.0 | 44.0 | 64.0 | 64.0 | 160.0 | 72.0 | 224.0 |
| 17 | 80.0 | 152.0 | 152.0 | 168.0 | 64.0 | 32.0 | 56.0 | 88.0 | 48.0 | 192.0 | 64.0 | 224.0 |
| 25 | 248.0 | 208.0 | 136.0 | 224.0 | 40.0 | 48.0 | 80.0 | 48.0 | 16.0 | 176.0 | 128.0 | 424.0 |
| 30 | 304.0 | 96.0 | 184.0 | 88.0 | 128.0 | 80.0 | 90.7 | 104.0 | 56.0 | 128.0 | 80.0 | 352.0 |

## Table A.12 Continued

| Shear | 1/26/2000 | | | | 2/16/2000 | | | | 3/23/2000 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.6 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 0.0 | 8.0 | 8.0 | 16.0 | 8.0 | 0.0 | 4.0 | 16.0 | 8.0 | 0.0 | 32.0 | 16.0 |
| 7 | 24.0 | 16.0 | 24.0 | 16.0 | 0.0 | 16.0 | 52.0 | 80.0 | 48.0 | 56.0 | 80.0 | 112.0 |
| 12 | 69.3 | 32.0 | 112.0 | 56.0 | 0.0 | 32.0 | 44.0 | 64.0 | 48.0 | 80.0 | 88.0 | 120.0 |
| 17 | 32.0 | 32.0 | 76.0 | 32.0 | 40.0 | 16.0 | 36.0 | 72.0 | 32.0 | 104.0 | 112.0 | 256.0 |
| 25 | 24.0 | 8.0 | 88.0 | 56.0 | 48.0 | 32.0 | 68.0 | 96.0 | 48.0 | 64.0 | 136.0 | 192.0 |
| 30 | 26.7 | 32.0 | 140.0 | 96.0 | 40.0 | 32.0 | 52.0 | 72.0 | 16.0 | 176.0 | 112.0 | 176.0 |
| 0.8 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 0.0 | 0.0 | 4.0 | 0.0 | 16.0 | 24.0 | 12.0 | 0.0 | 0.0 | 0.0 | 16.0 | 40.0 |
| 7 | 16.0 | 16.0 | 16.0 | 32.0 | 16.0 | 16.0 | 32.0 | 48.0 | 16.0 | 24.0 | 80.0 | 48.0 |
| 12 | 10.7 | 40.0 | 40.0 | 40.0 | 8.0 | 24.0 | 48.0 | 72.0 | 0.0 | 72.0 | 72.0 | 56.0 |
| 17 | 16.0 | 10.7 | 56.0 | 64.0 | 16.0 | 16.0 | 20.0 | 80.0 | 16.0 | 48.0 | 80.0 | 80.0 |
| 25 | 0.0 | 32.0 | 52.0 | 56.0 | 32.0 | 16.0 | 8.0 | 72.0 | 16.0 | 16.0 | 96.0 | 112.0 |
| 30 | 16.0 | 16.0 | 88.0 | 16.0 | 24.0 | 8.0 | 52.0 | 80.0 | 0.0 | 37.3 | 80.0 | 112.0 |
| 1 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 0.0 | 0.0 | 4.0 | 0.0 | 8.0 | 0.0 | 4.0 | 8.0 | 8.0 | 16.0 | 0.0 | 5.3 |
| 7 | 8.0 | 16.0 | 24.0 | 8.0 | 32.0 | 32.0 | 8.0 | 32.0 | 32.0 | 16.0 | 32.0 | 64.0 |
| 12 | 0.0 | 16.0 | 36.0 | 16.0 | 24.0 | 16.0 | 24.0 | 48.0 | 24.0 | 21.3 | 48.0 | 32.0 |
| 17 | 16.0 | 8.0 | 28.0 | 0.0 | 16.0 | 16.0 | 28.0 | 16.0 | 0.0 | 32.0 | 128.0 | 80.0 |
| 25 | 16.0 | 24.0 | 48.0 | 8.0 | 32.0 | 16.0 | 32.0 | 56.0 | 8.0 | 56.0 | 40.0 | 112.0 |
| 30 | 0.0 | 0.0 | 56.0 | 16.0 | 24.0 | 8.0 | 16.0 | 184.0 | 0.0 | 32.0 | 64.0 | 112.0 |

## Table A.12 Continued

| Shear | 3/22/2000 | | | | 3/9/2000 | | | | 4/26/2000 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.1 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 96.0 | 104.0 | 64.0 | 32.0 | 160.0 | 48.0 | 224.0 | 56.0 | 184.0 | 72.0 | 32.0 | 112.0 |
| 7 | 480.0 | 336.0 | 336.0 | 144.0 | 292.0 | 208.0 | 240.0 | 320.0 | 336.0 | 80.0 | 186.7 | 384.0 |
| 12 | 864.0 | 352.0 | 464.0 | 328.0 | 409.3 | 248.0 | 328.0 | 272.0 | 208.0 | 112.0 | 352.0 | 368.0 |
| 17 | | 496.0 | | 480.0 | 512.0 | 192.0 | 320.0 | 464.0 | 448.0 | 168.0 | 256.0 | 304.0 |
| 25 | | 416.0 | | | 476.0 | 320.0 | 368.0 | 432.0 | 272.0 | 304.0 | 272.0 | 368.0 |
| 30 | | 384.0 | | | 608.0 | 144.0 | 264.0 | | | 288.0 | 400.0 | 384.0 |
| 0.2 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 192.0 | 64.0 | 112.0 | 16.0 | 40.0 | 0.0 | 48.0 | 64.0 | 80.0 | 24.0 | 85.3 | 80.0 |
| 7 | 400.0 | 416.0 | 344.0 | 160.0 | 216.0 | 104.0 | 106.7 | 208.0 | 96.0 | 88.0 | 136.0 | 112.0 |
| 12 | 496.0 | 152.0 | 456.0 | 400.0 | 236.0 | 160.0 | 144.0 | 320.0 | 51.5 | 112.0 | 240.0 | 208.0 |
| 17 | 597.3 | 528.0 | 448.0 | 240.0 | 288.0 | 176.0 | 232.0 | 272.0 | 256.0 | 208.0 | 288.0 | 248.0 |
| 25 | 576.0 | 336.0 | 416.0 | 448.0 | 344.0 | 184.0 | 384.0 | 272.0 | 320.0 | 200.0 | 200.0 | 240.0 |
| 30 | 416.0 | 272.0 | 464.0 | 352.0 | 314.0 | 184.0 | 208.0 | 360.0 | 200.0 | 304.0 | 240.0 | 256.0 |
| 0.4 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 64.0 | 24.0 | 88.0 | 16.0 | 16.0 | 16.0 | 16.0 | 64.0 | 48.0 | 48.0 | 24.0 | 24.0 |
| 7 | 208.0 | 32.0 | 144.0 | 144.0 | 60.0 | 48.0 | 56.0 | 160.0 | 64.0 | 88.0 | 48.0 | 80.0 |
| 12 | 96.0 | 104.0 | 176.0 | 224.0 | 56.0 | 80.0 | 128.0 | 160.0 | 49.7 | 58.7 | 245.3 | 96.0 |
| 17 | 96.0 | 80.0 | 176.0 | 176.0 | 88.0 | 80.0 | 106.7 | 184.0 | 80.0 | 256.0 | 192.0 | 144.0 |
| 25 | 112.0 | 96.0 | 272.0 | 176.0 | 144.0 | 56.0 | 192.0 | 272.0 | 32.0 | 128.0 | 240.0 | 128.0 |
| 30 | 80.0 | 80.0 | 184.0 | 128.0 | 124.0 | 168.0 | 208.0 | 280.0 | 104.0 | 280.0 | 42.7 | 216.0 |

## Table A.12 Continued

| Shear | 3/22/2000 | | | | 3/9/2000 | | | | 4/26/2000 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.6 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 16.0 | 8.0 | 80.0 | 26.7 | 0.0 | 0.0 | 32.0 | 32.0 | 24.0 | 24.0 | 16.0 | 40.0 |
| 7 | 0.0 | 72.0 | 160.0 | 48.0 | 8.0 | 56.0 | 80.0 | 128.0 | 48.0 | 40.0 | 104.0 | 56.0 |
| 12 | 8.0 | 128.0 | 58.7 | 80.0 | 40.0 | 168.0 | 122.7 | 72.0 | 48.2 | 32.0 | 48.0 | 160.0 |
| 17 | 16.0 | 64.0 | 160.0 | 112.0 | 48.0 | 112.0 | 192.0 | 144.0 | 22.4 | 80.0 | 96.0 | 112.0 |
| 25 | 32.0 | 144.0 | 96.0 | 40.0 | 72.0 | 56.0 | 96.0 | 224.0 | 16.0 | 96.0 | 88.0 | 112.0 |
| 30 | 32.0 | 128.0 | 96.0 | 112.0 | 40.0 | 112.0 | 112.0 | 160.0 | 37.3 | 32.0 | 80.0 | 112.0 |
| 0.8 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 0.0 | 8.0 | 40.0 | 24.0 | 0.0 | 32.0 | 40.0 | 48.0 | 8.0 | 8.0 | 8.0 | 40.0 |
| 7 | 16.0 | 16.0 | 64.0 | 128.0 | 16.0 | 40.0 | 48.0 | 96.0 | 16.0 | 32.0 | 32.0 | 64.0 |
| 12 | 16.0 | 0.0 | 32.0 | 64.0 | 37.3 | 88.0 | 128.0 | 96.0 | 10.7 | 16.0 | 48.0 | 64.0 |
| 17 | 24.0 | 44.0 | 56.0 | 80.0 | 4.0 | 96.0 | 80.0 | 144.0 | 10.7 | 10.7 | 80.0 | 72.0 |
| 25 | 8.0 | 16.0 | 48.0 | 64.0 | 24.0 | 48.0 | 88.0 | 96.0 | 0.0 | 48.0 | 48.0 | 48.0 |
| 30 | 8.0 | 32.0 | 40.0 | 80.0 | 16.0 | 32.0 | 48.0 | 64.0 | 16.0 | 16.0 | 112.0 | 88.0 |
| 1 time forced | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | None cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 3 | 8.0 | 0.0 | 24.0 | 0.0 | 4.0 | 0.0 | 24.0 | 48.0 | 16.0 | 8.0 | 24.0 | 8.0 |
| 7 | 0.0 | 16.0 | 32.3 | 8.0 | 4.0 | 16.0 | 0.0 | 32.0 | 8.0 | 0.0 | 96.0 | 24.0 |
| 12 | 16.0 | 32.0 | 24.0 | 40.0 | 8.0 | 24.0 | 40.0 | 56.0 | 0.0 | 16.0 | 16.0 | 16.0 |
| 17 | 24.0 | 8.0 | 37.3 | 32.0 | 20.0 | 16.0 | 64.0 | 80.0 | 8.0 | 16.0 | 42.7 | 32.0 |
| 25 | 32.0 | 16.0 | 48.0 | 64.0 | 4.0 | 0.0 | 32.0 | 56.0 | 8.0 | 8.0 | 16.0 | 56.0 |
| 30 | 8.0 | 0.0 | 48.0 | 48.0 | 28.0 | 80.0 | 16.0 | 80.0 | 16.0 | 5.3 | 80.0 | 48.0 |

**Table A.12 Continued**

| Shear | Averages | | | | SEM | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 0.1 | 102.7 | 57.3 | 74.7 | 60.0 | 24.5 | 15.1 | 30.6 | 12.7 |
| | 316.7 | 186.7 | 199.3 | 238.7 | 52.6 | 38.2 | 39.1 | 43.9 |
| | 414.7 | 246.7 | 305.3 | 308.0 | 110.8 | 46.3 | 41.4 | 68.7 |
| | 440.0 | 246.7 | 271.3 | 420.8 | 38.6 | 68.5 | 23.3 | 73.7 |
| | 395.0 | 384.0 | 344.0 | 490.7 | 51.5 | 36.6 | 25.3 | 65.4 |
| | 462.7 | 242.7 | 318.7 | 428.0 | 103.7 | 59.5 | 43.0 | 108.1 |
| | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 0.2 | 73.3 | 28.0 | 58.2 | 50.7 | 25.3 | 12.0 | 15.5 | 9.6 |
| | 202.7 | 169.3 | 166.4 | 168.0 | 46.0 | 56.8 | 41.2 | 21.0 |
| | 267.9 | 137.3 | 225.3 | 292.0 | 58.3 | 11.8 | 51.8 | 51.4 |
| | 378.2 | 250.7 | 250.7 | 273.3 | 54.4 | 64.8 | 44.8 | 34.2 |
| | 352.0 | 201.3 | 256.7 | 354.7 | 47.8 | 37.3 | 49.3 | 68.1 |
| | 309.7 | 253.3 | 241.3 | 360.0 | 30.9 | 42.5 | 51.2 | 40.8 |
| | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 0.4 | 38.7 | 32.0 | 36.7 | 38.7 | 7.8 | 9.0 | 11.0 | 7.3 |
| | 80.7 | 53.3 | 73.3 | 113.3 | 28.4 | 8.7 | 16.1 | 19.0 |
| | 70.9 | 84.4 | 120.9 | 140.0 | 10.9 | 17.2 | 31.9 | 29.9 |
| | 76.0 | 132.0 | 124.4 | 164.0 | 7.1 | 34.1 | 23.5 | 18.6 |
| | 98.7 | 118.7 | 174.7 | 212.0 | 36.2 | 26.3 | 29.8 | 52.9 |
| | 132.7 | 138.7 | 131.6 | 194.7 | 36.0 | 31.4 | 28.0 | 43.4 |
| | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 0.6 | 9.3 | 6.7 | 28.7 | 24.4 | 3.8 | 3.8 | 11.3 | 4.2 |
| | 21.3 | 42.7 | 83.3 | 73.3 | 9.2 | 9.4 | 19.0 | 17.1 |
| | 35.6 | 78.7 | 78.9 | 92.0 | 10.8 | 23.8 | 13.8 | 16.4 |
| | 31.7 | 68.0 | 112.0 | 121.3 | 4.7 | 15.7 | 23.1 | 31.2 |
| | 40.0 | 66.7 | 95.3 | 120.0 | 8.3 | 19.7 | 9.1 | 30.1 |
| | 32.0 | 85.3 | 98.7 | 121.3 | 3.8 | 25.4 | 12.4 | 16.1 |
| | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 0.8 | 4.0 | 12.0 | 20.0 | 25.3 | 2.7 | 5.4 | 6.5 | 8.6 |
| | 16.0 | 24.0 | 45.3 | 69.3 | 0.0 | 4.1 | 9.6 | 14.7 |
| | 13.8 | 40.0 | 61.3 | 65.3 | 5.2 | 13.9 | 14.4 | 7.6 |
| | 14.4 | 37.6 | 62.0 | 86.7 | 2.7 | 13.5 | 9.7 | 11.8 |
| | 13.3 | 29.3 | 56.7 | 74.7 | 5.3 | 6.4 | 13.0 | 10.0 |
| | 13.3 | 23.6 | 70.0 | 73.3 | 3.4 | 4.8 | 11.4 | 13.1 |
| | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ | 0 TNF cells/mm$^2$ | 100 TNF cells/mm$^2$ | 250 TNF cells/mm$^2$ | 500 TNF cells/mm$^2$ |
| 1 | 7.3 | 4.0 | 13.3 | 11.6 | 2.2 | 2.7 | 4.8 | 7.4 |
| | 14.0 | 16.0 | 32.1 | 28.0 | 5.8 | 4.1 | 13.9 | 8.5 |
| | 12.0 | 20.9 | 31.3 | 34.7 | 4.5 | 2.6 | 4.9 | 6.7 |
| | 14.0 | 16.0 | 54.7 | 40.0 | 3.5 | 3.6 | 15.6 | 13.5 |
| | 16.7 | 20.0 | 36.0 | 58.7 | 5.1 | 7.9 | 5.0 | 13.5 |
| | 12.7 | 20.9 | 46.7 | 81.3 | 4.9 | 12.8 | 10.6 | 24.5 |

**Table A.13 TNF Mediated Adherence Under Shear** Pooled data Data for Figures V.3, V.4, V.5, & V.6. (n=7).

| TNF | AVERAGES 1.0 | | | AVERAGES 0.8 | | | AVERAGES 0.6 | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm$^2$ | SEM | time | SSRBC/mm$^2$ | SEM |
| | 2.0 | 12.0 | 3.7 | 2.1 | 12.2 | 2.1 | 2.3 | 15.4 | 2.0 |
| | 7.4 | 14.3 | 5.1 | 7.5 | 14.9 | 2.6 | 7.4 | 25.9 | 5.2 |
| | 12.5 | 15.0 | 8.0 | 12.7 | 19.2 | 9.8 | 12.5 | 44.0 | 10.0 |
| | 17.3 | 8.6 | 2.6 | 17.0 | 8.6 | 2.3 | 17.5 | 27.9 | 9.8 |
| | 22.5 | 15.2 | 1.6 | 22.0 | 21.2 | 10.1 | 22.4 | 41.7 | 10.3 |
| | 27.4 | 10.0 | 2.9 | 27.3 | 30.9 | 11.2 | 27.8 | 49.1 | 13.0 |
| 2 HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM |
| | 1.8 | 5.7 | 2.2 | 2.1 | 13.7 | 3.9 | 2.4 | 14.4 | 2.7 |
| | 7.7 | 23.7 | 5.4 | 7.3 | 23.8 | 6.5 | 7.4 | 21.3 | 5.8 |
| | 12.7 | 14.9 | 5.0 | 12.3 | 35.8 | 10.7 | 12.5 | 49.9 | 9.0 |
| | 17.2 | 21.4 | 7.8 | 17.3 | 32.8 | 9.9 | 17.3 | 48.6 | 7.6 |
| | 22.7 | 41.1 | 15.7 | 22.3 | 50.7 | 10.4 | 22.0 | 58.1 | 5.7 |
| | 27.5 | 22.2 | 7.3 | 27.1 | 44.7 | 12.8 | 27.4 | 45.3 | 18.0 |
| 4 HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM |
| | 2.0 | 11.8 | 2.6 | 2.2 | 13.3 | 3.8 | 2.3 | 32.9 | 6.6 |
| | 7.4 | 26.7 | 5.0 | 7.2 | 45.9 | 7.0 | 7.6 | 53.8 | 6.7 |
| | 12.8 | 18.8 | 5.2 | 12.6 | 62.4 | 8.1 | 12.4 | 58.0 | 10.0 |
| | 17.7 | 30.4 | 7.8 | 17.3 | 46.0 | 6.4 | 17.5 | 86.4 | 16.4 |
| | 22.9 | 39.5 | 10.4 | 22.0 | 79.7 | 22.0 | 22.8 | 73.1 | 10.7 |
| | 27.5 | 53.8 | 14.1 | 27.6 | 70.2 | 15.2 | 27.8 | 87.1 | 12.8 |
| 6HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM |
| | 2.0 | 16.6 | 3.4 | 2.1 | 19.6 | 4.1 | 2.4 | 32.9 | 7.5 |
| | 7.5 | 26.3 | 5.4 | 7.2 | 45.7 | 6.3 | 7.5 | 45.9 | 6.4 |
| | 12.8 | 28.6 | 7.2 | 12.3 | 56.0 | 9.9 | 12.4 | 90.1 | 15.8 |
| | 17.7 | 41.0 | 10.6 | 17.4 | 79.4 | 24.8 | 17.5 | 90.1 | 8.5 |
| | 22.8 | 52.0 | 14.4 | 22.5 | 82.5 | 20.1 | 22.7 | 102.8 | 11.8 |
| | 27.8 | 49.7 | 17.5 | 27.5 | 104.0 | 20.6 | 27.7 | 138.9 | 18.9 |
| 8 HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM |
| | 2.0 | 12.7 | 2.6 | 2.2 | 17.5 | 3.6 | 2.5 | 19.7 | 2.7 |
| | 7.1 | 28.6 | 7.6 | 7.4 | 33.3 | 7.5 | 7.5 | 66.3 | 10.5 |
| | 12.6 | 35.5 | 5.7 | 12.4 | 42.9 | 7.5 | 12.1 | 64.6 | 5.9 |
| | 17.9 | 42.3 | 9.7 | 17.8 | 64.0 | 8.8 | 17.5 | 93.1 | 13.3 |
| | 22.7 | 61.0 | 14.4 | 22.6 | 65.1 | 10.2 | 22.5 | 109.3 | 15.2 |
| | 27.8 | 60.0 | 8.3 | 27.2 | 84.6 | 11.2 | 27.8 | 112.4 | 18.2 |

| TNF | AVERAGES 0.4 | | | AVERAGES 0.2 | | | AVERAGES 0.1 | | |
|---|---|---|---|---|---|---|---|---|---|
| 0HR | time | SSRBC/mm$^2$ | SEM | time | SSRBC/mm$^2$ | SEM | time | SSRBC/mm$^2$ | SEM |
| | 2.3 | 29.6 | 4.1 | 2.7 | 46.9 | 5.8 | 3.1 | 65.3 | 9.3 |
| | 7.5 | 37.3 | 7.6 | 7.6 | 101.3 | 9.6 | 8.0 | 239.6 | 13.3 |
| | 12.6 | 67.0 | 12.2 | 12.7 | 146.6 | 24.2 | 12.4 | 273.3 | 18.8 |
| | 17.8 | 69.7 | 6.4 | 17.7 | 199.9 | 20.7 | 16.8 | 304.2 | 22.5 |
| | 22.6 | 56.6 | 9.3 | 22.2 | 191.4 | 32.7 | 22.5 | 352.7 | 42.8 |
| | 27.4 | 89.7 | 23.1 | 27.2 | 210.5 | 25.7 | 27.4 | 280.4 | 23.0 |
| 2HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM |
| | 2.8 | 27.2 | 5.1 | 3.0 | 46.9 | 8.5 | 2.7 | 78.2 | 11.5 |
| | 7.5 | 49.5 | 14.0 | 7.6 | 144.0 | 22.3 | 7.5 | 176.0 | 20.8 |
| | 12.6 | 63.8 | 15.3 | 12.4 | 124.2 | 12.3 | 12.5 | 220.6 | 31.0 |
| | 17.8 | 76.4 | 8.8 | 17.2 | 184.8 | 22.2 | 17.8 | 281.1 | 31.4 |
| | 22.2 | 80.8 | 12.6 | 22.5 | 168.0 | 14.1 | 22.7 | 314.3 | 37.0 |
| | 27.4 | 109.4 | 14.7 | 27.3 | 153.7 | 13.6 | 27.8 | 302.7 | 31.6 |
| 4HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mr | 0 SEM |
| | 2.8 | 29.3 | 2.7 | 2.6 | 56.9 | 13.8 | 2.9 | 49.8 | 10.2 |
| | 8.1 | 75.1 | 11.2 | 7.4 | 128.9 | 25.4 | 7.1 | 158.2 | 18.4 |
| | 12.6 | 92.8 | 26.8 | 12.6 | 176.4 | 23.5 | 12.4 | 211.4 | 36.1 |
| | 17.5 | 94.1 | 23.7 | 17.4 | 206.7 | 29.8 | 17.4 | 302.2 | 30.2 |
| | 22.4 | 77.1 | 6.4 | 22.0 | 176.9 | 28.0 | 22.7 | 269.3 | 15.7 |
| | 27.5 | 112.9 | 19.5 | 27.4 | 208.7 | 35.6 | 27.5 | 300.4 | 20.8 |
| 6HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM |
| | 2.6 | 28.8 | 2.3 | 2.7 | 73.3 | 9.0 | 3.0 | 81.1 | 17.0 |
| | 7.5 | 65.4 | 6.5 | 7.7 | 117.5 | 19.1 | 7.8 | 204.6 | 31.0 |
| | 12.3 | 80.0 | 9.3 | 12.4 | 155.0 | 21.5 | 12.9 | 258.9 | 26.0 |
| | 17.3 | 120.6 | 9.6 | 17.6 | 163.2 | 20.2 | 17.6 | 268.0 | 28.9 |
| | 22.9 | 124.6 | 13.6 | 22.4 | 210.3 | 19.8 | 22.0 | 313.8 | 22.0 |
| | 27.5 | 144.2 | 19.3 | 27.0 | 241.0 | 24.8 | 27.3 | 332.4 | 24.7 |
| 8HR | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM | time | SSRBC/mm^2 | SEM |
| | 2.8 | 34.1 | 5.0 | 2.8 | 49.5 | 10.6 | 3.0 | 65.1 | 13.4 |
| | 7.8 | 78.4 | 11.1 | 7.6 | 103.6 | 15.0 | 7.5 | 162.5 | 19.7 |
| | 12.2 | 75.4 | 8.2 | 12.5 | 170.3 | 14.2 | 12.5 | 200.1 | 31.3 |
| | 17.4 | 112.0 | 14.8 | 17.6 | 172.8 | 20.4 | 17.3 | 298.7 | 33.7 |
| | 22.3 | 95.2 | 15.6 | 22.9 | 173.5 | 24.4 | 22.4 | 272.8 | 33.0 |
| | 27.4 | 108.8 | 14.6 | 27.8 | 195.0 | 25.7 | 27.7 | 289.1 | 54.3 |

**Table A.14 Antibody Blocking of VCAM-1**

Data for Figure 5.7

| Shear | 0.1 | | | | |
|---|---|---|---|---|---|
| | 7/22/2004 | 6/21/2004 | 6/18/2004 | 7/22/2004 B | 7/29/2004 |
| Unstimulated | 308.6 | 245.3 | 346.7 | 568.0 | 272.0 |
| TNF | 352.0 | 505.6 | 213.3 | 308.0 | 147.2 |
| TNF, anti-VCAM-1 | 304.0 | 412.8 | 250.7 | 256.0 | 176.0 |
| TNF non-Specific Antibody | 259.2 | 544.0 | 163.2 | 229.3 | 66.7 |
| Shear | 0.2 | | | | |
| | 7/22/2004 | 6/21/2004 | 6/18/2004 | 7/22/2004 B | 7/29/2004 |
| Unstimulated | 252.8 | 106.7 | 117.3 | 183.1 | 58.7 |
| TNF | 282.7 | 197.3 | 140.8 | 144.0 | 60.8 |
| TNF, anti-VCAM-1 | 244.0 | 122.7 | 136.0 | 140.8 | 38.9 |
| TNF non-Specific Antibody | 163.2 | 153.6 | 102.4 | 117.3 | 44.0 |
| Shear | 0.4 | | | | |
| | 7/22/2004 | 6/21/2004 | 6/18/2004 | 7/22/2004 B | 7/29/2004 |
| Unstimulated | 64.0 | 33.5 | 60.8 | 70.7 | 25.6 |
| TNF | 117.3 | 83.2 | 104.0 | 108.8 | 61.3 |
| TNF, anti-VCAM-1 | 50.7 | 80.0 | 40.0 | 61.7 | 16.0 |
| TNF non-Specific Antibody | 92.0 | 82.3 | 64.0 | 78.0 | 53.3 |
| Shear | 0.6 | | | | |
| | 7/22/2004 | 6/21/2004 | 6/18/2004 | 7/22/2004 B | 7/29/2004 |
| Unstimulated | 19.2 | 36.0 | 26.0 | 35.2 | 16.0 |
| TNF | 58.7 | 80.0 | 78.0 | 70.9 | 38.9 |
| TNF, anti-VCAM-1 | 30.2 | 24.0 | 32.0 | 44.0 | 5.3 |
| TNF non-Specific Antibody | 64.0 | 80.0 | 80.0 | 69.3 | 17.8 |
| Shear | 0.8 | | | | |
| | 7/22/2004 | 6/21/2004 | 6/18/2004 | 7/22/2004 B | 7/29/2004 |
| Unstimulated | 8.0 | 22.7 | 21.3 | 19.2 | 8.0 |
| TNF | 50.3 | 60.0 | 50.3 | 57.6 | 22.9 |
| TNF, anti-VCAM-1 | 16.0 | 24.0 | 9.6 | 25.6 | 4.0 |
| TNF non-Specific Antibody | 51.2 | 51.2 | 73.1 | 48.0 | 28.0 |
| Shear | 1.0 | | | | |
| | 7/22/2004 | 6/21/2004 | 6/18/2004 | 7/22/2004 B | 7/29/2004 |
| Unstimulated | 16.0 | 10.7 | 12.0 | 10.7 | 0.0 |
| TNF | 41.6 | 34.3 | 77.3 | 57.1 | 20.8 |
| TNF, anti-VCAM-1 | 16.0 | 11.4 | 24.9 | 12.8 | 6.4 |
| TNF non-Specific Antibody | 38.0 | 41.6 | 40.0 | 38.4 | 16.0 |

| AVERAGE | | | | | | |
|---|---|---|---|---|---|---|
| Shear | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| Unstimulated | 348.1 | 143.7 | 50.9 | 26.5 | 15.8 | 9.9 |
| TNF | 305.2 | 165.1 | 94.9 | 65.3 | 48.2 | 46.2 |
| TNF, anti-VCAM-1 | 279.9 | 136.5 | 49.7 | 27.1 | 15.8 | 14.3 |
| TNF non-Specific Antibody | 252.5 | 116.1 | 73.9 | 62.2 | 50.3 | 34.8 |

| Standard Error of the Mean | | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| Unstimulated | 57.6 | 33.7 | 9.0 | 4.1 | 3.2 | 2.7 |
| TNF | 61.5 | 36.6 | 10.1 | 7.6 | 6.6 | 9.7 |
| TNF, anti-VCAM-1 | 39.0 | 32.6 | 10.7 | 6.3 | 4.1 | 3.1 |
| TNF non-Specific Antibody | 80.0 | 21.2 | 6.8 | 11.5 | 7.2 | 4.7 |

**Table A.15 Shear Dependence of Histamine Mediated Adhesion**

Data for figure 6.1 and 6.2

| Date | 11/16/2005 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.6 | 52.4 | 2.6 | 23.1 | 2.1 | 10.7 | 1.7 | 19.2 | 1.1 | 8.0 | 0.6 | 8.0 |
| | 8.6 | 85.3 | 8.0 | 82.7 | 7.1 | 49.6 | 6.1 | 22.9 | 5.5 | 13.3 | 4.8 | 16.0 |
| | 13.2 | 96.0 | 12.6 | 112.0 | 11.7 | 66.7 | 11.1 | 29.3 | 10.3 | 26.7 | 9.5 | 16.0 |
| | 18.0 | 112.0 | 17.2 | 99.2 | 16.4 | 56.0 | 15.7 | 26.7 | 14.8 | 18.3 | 14.0 | 16.0 |
| | 23.8 | 106.7 | 23.0 | 96.0 | 21.7 | 84.8 | 20.7 | 61.7 | 19.8 | 24.0 | 18.9 | 16.0 |
| | 29.1 | 131.2 | 28.2 | 88.0 | 27.3 | 73.6 | 26.2 | 61.7 | 25.3 | 19.2 | 24.6 | 12.8 |
| | 34.4 | 172.8 | 33.6 | 137.1 | 32.7 | 78.2 | 31.7 | 43.2 | 30.9 | 29.3 | 30.2 | 22.4 |
| | 40.1 | 189.3 | 39.2 | 126.5 | 38.1 | 80.0 | 37.3 | 48.0 | 36.4 | 32.0 | 35.4 | 16.0 |
| Date | 11/10/2005 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.3 | 67.2 | 3.5 | 55.3 | 2.6 | 28.0 | 1.9 | 38.9 | 1.2 | 18.0 | 0.7 | 37.3 |
| | 8.9 | 149.3 | 8.1 | 82.7 | 7.4 | 60.0 | 6.7 | 56.0 | 5.9 | 24.9 | 5.0 | 25.1 |
| | 14.5 | 154.7 | 13.8 | 88.0 | 12.7 | 50.0 | 11.9 | 62.0 | 11.0 | 32.0 | 9.9 | 28.3 |
| | 19.4 | 170.7 | 18.8 | 84.0 | 17.9 | 57.1 | 16.9 | 45.3 | 16.1 | 22.9 | 15.4 | 36.0 |
| | 24.4 | 296.0 | 23.7 | 108.4 | 22.6 | 68.0 | 21.7 | 64.0 | 20.9 | 29.3 | 20.3 | 26.0 |
| | 29.7 | 236.0 | 29.0 | 140.8 | 28.1 | 58.0 | 27.1 | 64.0 | 26.1 | 20.6 | 25.3 | 24.0 |
| | 34.9 | 197.3 | 34.3 | 101.3 | 33.2 | 58.0 | 32.2 | 66.7 | 31.4 | 34.3 | 30.6 | 28.8 |
| | 40.0 | 217.6 | 39.3 | 123.4 | 38.3 | 46.0 | 37.4 | 44.4 | 36.6 | 32.0 | 35.7 | 28.4 |
| Date | 10/26/2005 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.9 | 136.0 | 3.3 | 80.0 | 2.5 | 56.0 | 1.7 | 11.2 | 0.9 | 12.0 | 0.5 | 0.0 |
| | 8.9 | 148.0 | 7.6 | 344.0 | 7.2 | 152.0 | 6.3 | 41.6 | 5.5 | 27.4 | 4.7 | 20.6 |
| | 12.4 | 576.0 | 11.9 | 448.0 | 11.1 | 152.0 | 10.5 | 28.8 | 9.7 | 24.0 | 9.2 | 13.3 |
| | 16.4 | 576.0 | 18.0 | 390.0 | 15.1 | 165.3 | 16.5 | 48.9 | 15.8 | 45.9 | 15.2 | 21.0 |
| | 21.1 | 472.0 | 25.0 | 460.8 | 19.7 | 186.7 | 23.5 | 80.0 | 22.8 | 30.0 | 22.0 | 16.0 |
| | 28.1 | 480.0 | 29.9 | 410.7 | 26.7 | 216.2 | 28.4 | 56.0 | 27.7 | 30.2 | 26.7 | 24.9 |
| | 35.5 | 549.3 | 34.6 | 524.0 | 33.9 | 244.0 | 33.2 | 58.7 | 32.5 | 46.2 | 31.6 | 30.0 |
| | 39.9 | 490.7 | 39.3 | 384.0 | 38.7 | 276.0 | 38.0 | 80.0 | 37.1 | 48.0 | 36.4 | 18.3 |
| Date | 10/19/2005 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 2.9 | 88.0 | 2.6 | 60.8 | 1.9 | 56.0 | 1.3 | 38.4 | 0.8 | 16.0 | 0.4 | 16.0 |
| | 6.9 | 144.0 | 6.3 | 85.3 | 5.5 | 80.0 | 4.8 | 60.8 | 4.3 | 36.0 | 3.8 | 13.3 |
| | 11.3 | 234.7 | 12.8 | 184.0 | 9.8 | 112.0 | 11.1 | 68.8 | 10.5 | 44.8 | 9.9 | 20.0 |
| | 15.6 | 304.0 | 20.9 | 178.7 | 14.1 | 118.4 | 18.3 | 54.4 | 17.3 | 39.1 | 16.4 | 12.4 |
| | 21.7 | 448.0 | 25.6 | 172.0 | 19.5 | 126.2 | 24.0 | 57.6 | 23.5 | 25.6 | 22.7 | 24.9 |
| | 26.2 | 485.3 | 30.6 | 229.3 | 24.8 | 107.4 | 28.5 | 61.1 | 27.6 | 40.0 | 26.9 | 16.0 |
| | 33.6 | 460.0 | 35.2 | 212.0 | 32.0 | 131.0 | 33.3 | 72.0 | 32.8 | 64.0 | 32.1 | 24.0 |
| | 40.5 | 464.0 | 40.2 | 245.3 | 39.0 | 114.9 | 37.9 | 61.7 | 37.2 | 36.0 | 36.5 | 21.3 |
| Date | 6/1/2005 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.1 | 96.0 | 3.4 | 42.7 | 2.5 | 25.1 | 1.8 | 29.3 | 1.3 | 5.3 | 0.7 | 9.6 |
| | 8.0 | 101.3 | 7.2 | 73.1 | 6.4 | 51.2 | 5.8 | 44.8 | 5.4 | 16.0 | 4.8 | 13.3 |
| | 13.3 | 112.0 | 12.4 | 74.7 | 11.4 | 53.3 | 10.6 | 48.0 | 9.7 | 20.6 | 8.8 | 12.0 |
| | 19.0 | 106.7 | 18.0 | 84.0 | 17.1 | 56.0 | 16.2 | 57.1 | 15.3 | 17.6 | 14.1 | 13.7 |
| | 24.3 | 134.4 | 23.4 | 100.6 | 22.3 | 54.9 | 21.5 | 58.0 | 20.8 | 19.2 | 20.1 | 24.0 |
| | 30.2 | 144.0 | 29.4 | 94.0 | 28.4 | 64.0 | 27.6 | 50.7 | 26.8 | 24.0 | 26.0 | 13.3 |
| | 35.5 | 144.0 | 34.5 | 176.0 | 33.3 | 68.4 | 32.3 | 70.9 | 31.6 | 26.7 | 31.0 | 16.0 |
| | 40.3 | 148.0 | 39.8 | 128.0 | 39.0 | 57.1 | 38.3 | 58.0 | 37.5 | 25.6 | 36.7 | 24.0 |

## Table A.15 Continued

| Date | 3/25/2005 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.3 | 69.3 | 3.7 | 61.3 | 2.8 | 22.0 | 2.1 | 18.7 | 1.5 | 13.7 | 0.9 | 4.6 |
| | 8.9 | 122.7 | 8.1 | 74.7 | 7.2 | 66.3 | 6.4 | 50.0 | 5.7 | 10.7 | 5.0 | 16.0 |
| | 14.0 | 240.0 | 13.2 | 136.0 | 12.4 | 93.3 | 11.6 | 64.0 | 10.7 | 40.0 | 9.9 | 10.7 |
| | 19.4 | 268.0 | 18.5 | 192.0 | 17.7 | 106.7 | 16.6 | 72.0 | 15.8 | 50.3 | 15.0 | 18.7 |
| | 24.2 | 192.0 | 23.6 | 220.0 | 22.9 | 117.3 | 22.1 | 50.7 | 21.3 | 58.0 | 20.5 | 21.3 |
| | 29.8 | 256.0 | 28.8 | 130.3 | 27.6 | 122.0 | 26.5 | 76.0 | 25.5 | 56.0 | 24.9 | 28.0 |
| | 35.9 | 282.7 | 35.0 | 198.9 | 33.9 | 120.0 | 32.9 | 82.7 | 32.1 | 44.4 | 31.3 | 34.7 |
| | 40.2 | 272.0 | 39.9 | 152.0 | 39.3 | 121.6 | 38.5 | 86.9 | 37.7 | 57.1 | 36.9 | 22.0 |
| Date | 2/16/2005 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.6 | 52.0 | 3.1 | 36.0 | 2.5 | 22.0 | 1.8 | 19.2 | 1.2 | 2.3 | 0.7 | 8.0 |
| | 8.3 | 108.8 | 7.5 | 50.7 | 6.6 | 45.3 | 5.9 | 26.0 | 5.1 | 22.4 | 4.5 | 4.0 |
| | 13.2 | 136.0 | 12.5 | 68.6 | 11.4 | 66.3 | 10.6 | 41.6 | 9.9 | 12.8 | 9.2 | 16.0 |
| | 18.3 | 128.0 | 17.6 | 64.0 | 16.8 | 80.0 | 16.0 | 89.6 | 15.1 | 24.0 | 14.5 | 22.9 |
| | 23.4 | 217.6 | 22.5 | 77.3 | 21.7 | 82.3 | 20.8 | 42.7 | 20.1 | 41.1 | 19.3 | 25.6 |
| | 27.8 | 196.6 | 26.8 | 112.0 | 26.0 | 88.0 | 25.3 | 117.3 | 24.8 | 32.0 | 26.6 | 25.8 |
| | 33.2 | 204.0 | 32.4 | 120.0 | 31.4 | 98.3 | 30.4 | 52.6 | 32.1 | 37.7 | 33.9 | 21.3 |
| | 38.1 | 166.4 | 37.1 | 138.7 | 36.1 | 112.0 | 35.3 | 88.0 | 39.9 | 53.3 | 39.1 | 24.0 |
| Date | 3/9/2005 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.3 | 32.0 | 3.6 | 32.0 | 3.0 | 24.0 | 2.4 | 18.7 | 1.6 | 2.7 | 0.9 | 4.8 |
| | 9.2 | 42.7 | 8.4 | 26.7 | 7.6 | 24.9 | 6.8 | 22.9 | 6.0 | 9.1 | 5.2 | 9.6 |
| | 14.3 | 74.0 | 13.2 | 52.0 | 12.3 | 35.6 | 11.5 | 20.6 | 10.7 | 13.7 | 10.0 | 11.4 |
| | 19.6 | 73.1 | 18.7 | 57.1 | 17.9 | 53.3 | 17.2 | 19.2 | 16.2 | 16.0 | 15.4 | 12.8 |
| | 25.7 | 118.0 | 24.7 | 58.0 | 21.7 | 82.3 | 22.9 | 34.7 | 22.0 | 25.1 | 20.9 | 18.9 |
| | 27.8 | 196.6 | 30.9 | 94.0 | 23.8 | 57.1 | 28.7 | 22.9 | 27.7 | 26.0 | 26.8 | 21.3 |
| | 31.8 | 85.3 | 32.4 | 120.0 | 29.8 | 80.0 | 34.7 | 32.0 | 33.8 | 20.6 | 32.9 | 18.0 |
| | 37.1 | 98.7 | 36.3 | 73.1 | 35.5 | 48.0 | 39.9 | 20.0 | 39.2 | 28.4 | 38.2 | 28.8 |
| Date | 1/20/2005 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.6 | 32.0 | 3.0 | 26.7 | 2.4 | 13.7 | 1.7 | 9.6 | 1.2 | 12.8 | 0.7 | 19.2 |
| | 8.9 | 57.6 | 8.4 | 29.3 | 7.7 | 27.2 | 7.1 | 21.3 | 4.9 | 16.0 | 4.3 | 13.7 |
| | 14.8 | 69.3 | 14.0 | 50.3 | 13.2 | 32.0 | 12.5 | 25.1 | 10.0 | 14.9 | 9.5 | 16.0 |
| | 19.3 | 58.7 | 18.4 | 50.0 | 17.6 | 34.3 | 16.8 | 29.7 | 16.1 | 24.0 | 15.6 | 13.3 |
| | 23.6 | 128.0 | 22.8 | 54.9 | 22.1 | 41.6 | 21.4 | 28.4 | 20.7 | 20.6 | 20.2 | 24.0 |
| | 29.3 | 66.4 | 28.6 | 33.3 | 26.2 | 35.2 | 27.4 | 43.2 | 26.8 | 22.7 | 26.1 | 19.2 |
| | 35.7 | 52.0 | 35.1 | 32.0 | 32.0 | 48.0 | 33.4 | 32.0 | 32.6 | 24.0 | 32.0 | 13.7 |
| | 39.9 | 42.7 | 39.2 | 40.0 | 38.5 | 36.0 | 37.9 | 44.8 | 37.2 | 20.6 | 36.6 | 12.0 |
| Date | 10/13/2004 | | | | | | | | | | | |
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.5 | 16.0 | 2.9 | 26.7 | 2.3 | 16.0 | 1.7 | 10.7 | 1.2 | 12.8 | 0.7 | 0.0 |
| | 7.7 | 68.0 | 7.0 | 19.2 | 6.1 | 30.0 | 5.5 | 26.7 | 4.8 | 20.6 | 4.3 | 8.0 |
| | 12.0 | 56.0 | 11.2 | 18.7 | 10.4 | 28.0 | 9.7 | 28.0 | 9.0 | 24.0 | 8.5 | 32.0 |
| | 17.1 | 54.9 | 15.7 | 48.0 | 15.0 | 21.3 | 14.3 | 34.7 | 13.6 | 25.1 | 12.9 | 19.2 |
| | 22.4 | 40.0 | 21.7 | 28.8 | 21.0 | 34.7 | 22.6 | 38.2 | 21.9 | 24.0 | 21.1 | 11.2 |
| | 26.7 | 34.7 | 26.3 | 57.6 | 25.6 | 54.4 | 29.4 | 21.3 | 28.6 | 20.6 | 27.8 | 14.0 |
| | 31.6 | 61.7 | 30.9 | 41.1 | 30.1 | 54.9 | 34.6 | 38.4 | 33.6 | 32.0 | 32.8 | 20.0 |
| | 37.2 | 66.7 | 38.2 | 65.2 | 37.5 | 64.2 | 38.9 | 16.0 | 38.6 | 26.7 | 38.1 | 8.0 |

## Table A.15 Continued

| Date | 9/9/2004 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stim | Histamine Stimulation | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.7 | 96.0 | 3.2 | 16.0 | 2.5 | 17.8 | 1.9 | 16.0 | 1.4 | 13.7 | 0.9 | 0.0 |
| | 7.6 | 35.2 | 6.7 | 13.3 | 6.1 | 34.7 | 5.6 | 19.2 | 5.0 | 9.6 | 4.4 | 9.1 |
| | 12.0 | 37.3 | 11.5 | 29.7 | 10.7 | 43.4 | 9.8 | 28.4 | 9.1 | 16.0 | 8.5 | 2.0 |
| | 16.0 | 72.0 | 17.6 | 32.6 | 14.8 | 49.6 | 16.1 | 24.0 | 15.3 | 19.4 | 14.7 | 22.7 |
| | 22.4 | 56.0 | 23.9 | 35.2 | 21.1 | 44.6 | 22.6 | 29.3 | 21.8 | 24.0 | 21.0 | 9.6 |
| | 28.6 | 80.0 | 28.0 | 66.7 | 27.2 | 66.7 | 26.5 | 34.7 | 25.8 | 27.4 | 25.0 | 10.7 |
| | 33.0 | 69.3 | 34.3 | 53.3 | 31.6 | 52.6 | 32.8 | 30.9 | 32.1 | 26.8 | 31.5 | 17.5 |
| | 38.5 | 56.0 | 40.1 | 56.0 | 37.5 | 57.6 | 38.6 | 40.0 | 37.9 | 36.0 | 37.4 | 21.3 |

AVERAGES
Histamine Stimulation

| 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| 3.8 | 67.0 | 3.2 | 41.9 | 2.5 | 26.5 | 1.8 | 20.9 | 1.2 | 10.7 | 0.7 | 9.8 |
| 8.3 | 91.5 | 7.6 | 80.2 | 6.9 | 59.1 | 6.1 | 36.5 | 5.3 | 18.7 | 4.6 | 13.5 |
| 13.2 | 162.4 | 12.6 | 114.7 | 11.6 | 66.6 | 11.0 | 40.4 | 10.1 | 24.5 | 9.4 | 16.2 |
| 18.0 | 174.9 | 18.1 | 116.3 | 16.4 | 72.6 | 16.4 | 45.6 | 15.6 | 27.5 | 14.8 | 19.0 |
| 23.4 | 200.8 | 23.6 | 128.4 | 21.5 | 84.1 | 22.2 | 49.6 | 21.4 | 29.2 | 20.6 | 19.8 |
| 28.5 | 211.0 | 28.8 | 132.4 | 26.5 | 85.7 | 27.4 | 55.4 | 26.6 | 29.0 | 26.1 | 19.1 |
| 33.9 | 222.7 | 34.0 | 159.6 | 32.2 | 93.9 | 32.9 | 52.7 | 32.3 | 35.1 | 31.8 | 22.4 |
| 39.3 | 201.1 | 39.0 | 139.3 | 38.0 | 92.1 | 38.0 | 53.4 | 37.7 | 36.0 | 37.0 | 20.4 |

Standard Error of the Mean
Histamine Stimulation

| 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 10.6 | | 6.0 | | 4.7 | | 3.1 | | 1.6 | | 3.3 |
| | 12.8 | | 27.7 | | 11.1 | | 4.8 | | 2.5 | | 1.8 |
| | 46.1 | | 36.3 | | 11.6 | | 5.3 | | 3.2 | | 2.5 |
| | 47.3 | | 31.5 | | 12.7 | | 6.6 | | 3.6 | | 2.1 |
| | 44.3 | | 39.3 | | 15.0 | | 4.9 | | 3.4 | | 1.8 |
| | 50.6 | | 31.9 | | 15.1 | | 8.1 | | 3.3 | | 1.8 |
| | 49.7 | | 45.3 | | 17.1 | | 5.6 | | 3.8 | | 1.9 |
| | 46.4 | | 29.9 | | 20.5 | | 7.4 | | 3.6 | | 1.9 |

## Table A.15 Continued

| Date | 11/16/2005 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.9 | 68.0 | 3.2 | 23.1 | 2.5 | 13.7 | 1.9 | 8.0 | 1.2 | 2.0 | 0.7 | 10.0 |
| | 9.6 | 160.0 | 8.7 | 64.0 | 7.7 | 22.4 | 6.8 | 16.0 | 5.9 | 13.5 | 4.9 | 5.3 |
| | 14.7 | 264.0 | 14.1 | 138.0 | 13.3 | 33.8 | 12.4 | 19.2 | 11.4 | 11.1 | 10.4 | 10.0 |
| | 17.6 | 248.3 | 19.0 | 126.2 | 17.9 | 60.4 | 16.9 | 22.9 | 16.2 | 16.0 | 15.4 | 8.9 |
| | 20.0 | 261.3 | 24.8 | 137.6 | 23.8 | 44.8 | 22.7 | 17.3 | 21.8 | 9.6 | 21.0 | 8.0 |
| | 25.6 | 296.0 | 29.9 | 189.7 | 29.0 | 56.0 | 28.1 | 28.4 | 27.3 | 20.6 | 26.5 | 5.3 |
| | 30.6 | 368.0 | 34.8 | 224.0 | 33.8 | 81.5 | 32.8 | 20.8 | 31.9 | 14.2 | 31.3 | 4.8 |
| | 35.5 | 325.3 | 39.1 | 209.8 | 38.2 | 90.7 | 37.4 | 29.1 | 36.7 | 19.2 | 37.9 | 5.0 |
| Date | 11/10/2005 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.3 | 74.7 | 2.8 | 42.7 | 2.3 | 13.3 | 1.6 | 16.0 | 1.0 | 9.1 | 0.6 | 0.0 |
| | 7.7 | 244.0 | 6.9 | 46.0 | 6.2 | 22.9 | 5.3 | 26.7 | 4.4 | 6.0 | 3.8 | 6.9 |
| | 12.0 | 216.0 | 11.4 | 54.9 | 10.7 | 16.0 | 9.8 | 29.3 | 9.0 | 2.3 | 8.4 | 9.1 |
| | 16.2 | 228.0 | 15.3 | 101.3 | 14.5 | 22.0 | 13.8 | 18.7 | 13.2 | 10.7 | 12.6 | 3.2 |
| | 20.7 | 325.3 | 22.4 | 66.6 | 21.5 | 26.7 | 18.5 | 18.0 | 20.0 | 7.3 | 19.3 | 4.1 |
| | 27.6 | 242.7 | 29.2 | 84.6 | 28.4 | 30.7 | 25.2 | 28.6 | 26.8 | 9.1 | 26.2 | 0.0 |
| | 33.9 | 197.3 | 33.2 | 101.3 | 32.4 | 24.7 | 31.5 | 30.0 | 30.8 | 8.0 | 30.4 | 4.0 |
| | 38.7 | 288.0 | 38.0 | 205.3 | 37.0 | 16.0 | 36.2 | 16.0 | 37.6 | 8.9 | 37.0 | 6.2 |
| Date | 10/26/2005 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.4 | 288.0 | 3.9 | 128.0 | 3.0 | 48.0 | 2.1 | 12.0 | 1.3 | 4.0 | 0.7 | 4.0 |
| | 8.7 | 320.0 | 8.1 | 163.2 | 7.2 | 64.0 | 6.4 | 18.7 | 5.7 | 10.7 | 5.1 | 2.7 |
| | 13.3 | 424.0 | 12.6 | 212.0 | 11.6 | 92.4 | 10.6 | 25.1 | 10.0 | 10.0 | 9.3 | 6.0 |
| | 18.6 | 394.7 | 17.9 | 208.0 | 16.9 | 109.7 | 15.9 | 46.0 | 15.0 | 12.4 | 14.2 | 6.0 |
| | 24.5 | 488.0 | 23.7 | 294.4 | 22.5 | 144.0 | 21.1 | 33.6 | 20.2 | 4.8 | 19.4 | 6.0 |
| | 30.9 | 536.0 | 30.1 | 325.3 | 28.8 | 112.0 | 27.5 | 41.8 | 26.4 | 4.6 | 25.6 | 1.3 |
| | 39.3 | 524.0 | 35.2 | 298.7 | 33.9 | 118.9 | 33.0 | 30.0 | 32.3 | 13.7 | 31.7 | 5.3 |
| | 42.3 | 528.0 | 41.1 | 293.3 | 39.9 | 102.9 | 38.9 | 46.2 | 38.1 | 8.0 | 37.3 | 4.6 |
| Date | 10/19/2005 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.0 | 112.0 | 2.7 | 90.7 | 2.0 | 21.3 | 1.3 | 48.0 | 0.7 | 0.0 | 0.4 | 5.3 |
| | 7.0 | 282.7 | 6.3 | 149.3 | 5.6 | 59.4 | 4.9 | 42.7 | 4.3 | 24.0 | 3.8 | 5.3 |
| | 12.0 | 536.0 | 11.5 | 210.7 | 10.7 | 46.2 | 9.8 | 34.0 | 9.0 | 18.0 | 8.2 | 7.4 |
| | 16.7 | 454.4 | 15.7 | 265.6 | 14.9 | 57.6 | 14.2 | 36.6 | 13.6 | 10.7 | 13.0 | 12.8 |
| | 22.1 | 608.0 | 21.3 | 370.7 | 20.3 | 65.8 | 19.3 | 44.0 | 18.7 | 16.0 | 17.8 | 10.2 |
| | 26.8 | 650.7 | 26.2 | 292.0 | 25.4 | 90.7 | 24.6 | 43.4 | 23.9 | 16.0 | 23.2 | 6.4 |
| | 33.0 | 883.2 | 31.9 | 532.6 | 30.7 | 50.0 | 29.7 | 32.0 | 28.8 | 16.0 | 31.1 | 4.9 |
| | 39.0 | 864.0 | 37.9 | 546.7 | 36.8 | 91.2 | 35.7 | 49.6 | 34.7 | 29.3 | 39.8 | 9.6 |
| Date | 6/1/2005 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.0 | 80.0 | 3.3 | 32.0 | 2.5 | 18.7 | 1.9 | 16.0 | 1.3 | 8.0 | 0.7 | 6.4 |
| | 8.5 | 106.7 | 7.7 | 64.0 | 6.9 | 26.7 | 6.0 | 10.7 | 5.1 | 10.7 | 4.6 | 0.0 |
| | 13.2 | 144.0 | 12.6 | 82.7 | 11.7 | 18.7 | 10.9 | 10.0 | 10.2 | 12.0 | 9.4 | 4.0 |
| | 18.1 | 124.0 | 17.3 | 90.7 | 16.5 | 26.7 | 15.6 | 18.0 | 14.8 | 10.7 | 14.0 | 4.0 |
| | 23.2 | 120.0 | 22.4 | 84.8 | 21.3 | 36.6 | 20.6 | 26.7 | 19.7 | 9.1 | 19.0 | 4.6 |
| | 27.8 | 181.3 | 27.1 | 108.0 | 26.1 | 24.9 | 25.3 | 17.6 | 24.6 | 6.9 | 24.0 | 4.0 |
| | 33.0 | 213.3 | 32.2 | 108.0 | 33.6 | 34.4 | 32.7 | 29.3 | 32.0 | 11.5 | 31.2 | 3.0 |
| | 39.6 | 162.1 | 39.6 | 162.1 | 40.3 | 27.4 | 40.0 | 28.0 | 39.4 | 8.0 | 38.9 | 0.0 |

## Table A.15 Continued

| Date | 3/25/2005 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.3 | 128.0 | 3.6 | 57.6 | 2.9 | 13.7 | 2.2 | 22.0 | 1.4 | 6.9 | 0.8 | 6.9 |
| | 8.3 | 256.0 | 7.6 | 72.0 | 6.7 | 29.3 | 6.0 | 36.0 | 5.5 | 21.3 | 4.9 | 4.6 |
| | 12.9 | 256.0 | 12.3 | 120.0 | 11.6 | 27.4 | 10.7 | 39.1 | 9.7 | 9.1 | 9.1 | 6.9 |
| | 17.6 | 352.0 | 16.8 | 144.0 | 15.9 | 30.2 | 14.9 | 32.0 | 14.1 | 12.0 | 13.5 | 3.2 |
| | 22.3 | 261.3 | 21.5 | 121.1 | 20.6 | 68.6 | 19.8 | 36.6 | 21.4 | 12.8 | 18.5 | 9.1 |
| | 26.5 | 260.0 | 25.8 | 118.4 | 27.4 | 60.6 | 26.5 | 36.6 | 28.1 | 21.3 | 25.3 | 8.6 |
| | 31.6 | 384.0 | 33.3 | 177.7 | 35.0 | 80.0 | 34.2 | 59.4 | 33.3 | 22.0 | 32.5 | 4.6 |
| | 38.5 | 352.8 | 40.1 | 214.4 | 39.5 | 56.0 | 38.8 | 30.2 | 38.1 | 26.7 | 37.4 | 6.0 |
| Date | 2/16/2005 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.2 | 71.1 | 3.5 | 27.4 | 2.9 | 16.0 | 2.4 | 8.0 | 1.8 | 6.0 | 1.0 | 10.0 |
| | 8.8 | 128.0 | 8.2 | 72.0 | 7.5 | 48.0 | 6.7 | 37.3 | 6.0 | 13.7 | 5.2 | 2.3 |
| | 13.9 | 73.6 | 13.0 | 81.8 | 12.1 | 61.3 | 11.1 | 33.2 | 10.3 | 2.7 | 9.7 | 8.0 |
| | 18.3 | 92.0 | 17.6 | 105.1 | 16.8 | 66.3 | 16.0 | 35.6 | 15.2 | 8.0 | 14.7 | 16.0 |
| | 22.9 | 179.2 | 22.1 | 130.0 | 21.3 | 56.9 | 20.5 | 32.0 | 19.8 | 23.1 | 19.1 | 8.0 |
| | 27.8 | 160.0 | 27.1 | 88.0 | 26.4 | 61.3 | 25.4 | 20.6 | 24.5 | 12.4 | 23.8 | 8.0 |
| | 33.2 | 268.8 | 32.2 | 148.6 | 31.3 | 85.3 | 30.2 | 24.7 | 29.2 | 12.0 | 31.3 | 7.8 |
| | 37.9 | 240.0 | 37.1 | 157.7 | 36.2 | 66.0 | 37.6 | 33.0 | 37.0 | 17.3 | 38.6 | 4.6 |
| Date | 3/9/2005 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.8 | 48.0 | 4.2 | 13.3 | 3.5 | 11.4 | 2.7 | 19.2 | 2.0 | 10.0 | 1.1 | 3.2 |
| | 8.8 | 83.2 | 8.2 | 28.4 | 7.3 | 11.4 | 6.7 | 24.0 | 6.0 | 0.0 | 5.5 | 0.0 |
| | 13.6 | 80.0 | 12.7 | 38.0 | 11.8 | 17.5 | 10.8 | 24.0 | 10.1 | 8.0 | 9.5 | 5.3 |
| | 18.3 | 92.0 | 18.2 | 59.2 | 17.2 | 35.2 | 16.4 | 20.0 | 15.6 | 6.0 | 14.8 | 5.3 |
| | 19.5 | 94.4 | 24.0 | 70.9 | 23.1 | 45.7 | 22.2 | 18.0 | 21.4 | 4.6 | 20.7 | 0.0 |
| | 25.0 | 94.0 | 30.2 | 59.4 | 29.2 | 32.0 | 28.3 | 30.2 | 27.3 | 0.0 | 26.4 | 5.3 |
| | 31.0 | 93.7 | 32.2 | 88.0 | 35.0 | 49.8 | 33.9 | 13.7 | 33.0 | 8.0 | 32.2 | 2.3 |
| | 36.9 | 104.0 | 36.0 | 59.4 | 39.8 | 40.0 | 39.2 | 27.4 | 38.5 | 9.6 | 37.9 | 0.0 |
| Date | 1/20/2005 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.4 | 16.0 | 2.9 | 5.3 | 2.4 | 13.3 | 1.8 | 16.0 | 1.3 | 8.0 | 0.7 | 5.3 |
| | 6.9 | 44.8 | 6.4 | 18.7 | 5.8 | 18.7 | 5.2 | 13.7 | 4.6 | 8.0 | 4.0 | 4.0 |
| | 11.8 | 26.7 | 11.2 | 21.3 | 10.6 | 24.9 | 9.9 | 12.8 | 9.2 | 9.1 | 8.4 | 8.0 |
| | 15.4 | 40.0 | 16.8 | 24.0 | 16.2 | 20.3 | 13.7 | 24.0 | 15.1 | 18.7 | 12.5 | 8.0 |
| | 21.3 | 54.0 | 22.4 | 13.7 | 21.6 | 22.9 | 19.3 | 15.6 | 20.5 | 10.7 | 18.3 | 6.4 |
| | 27.2 | 51.2 | 26.7 | 32.0 | 26.0 | 34.3 | 25.3 | 18.0 | 24.5 | 11.4 | 23.9 | 10.0 |
| | 33.6 | 61.0 | 33.0 | 40.0 | 32.4 | 21.6 | 31.6 | 14.0 | 30.9 | 11.4 | 30.3 | 6.4 |
| | 39.1 | 40.0 | 38.4 | 28.0 | 39.2 | 24.5 | 38.8 | 17.7 | 38.6 | 10.7 | 38.0 | 7.0 |
| Date | 10/13/2004 | | | | | | | | | | | |
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 4.0 | 21.3 | 3.5 | 10.7 | 2.8 | 11.6 | 2.0 | 10.0 | 1.3 | 12.8 | 0.7 | 2.7 |
| | 8.5 | 18.3 | 7.6 | 20.0 | 6.9 | 11.6 | 6.2 | 3.2 | 5.7 | 12.8 | 5.2 | 1.8 |
| | 13.9 | 34.0 | 13.2 | 13.3 | 12.3 | 28.8 | 11.5 | 14.4 | 10.8 | 3.2 | 9.9 | 9.6 |
| | 18.9 | 35.8 | 17.6 | 27.4 | 16.6 | 16.0 | 15.6 | 6.4 | 15.2 | 16.0 | 14.8 | 8.0 |
| | 25.2 | 58.0 | 24.3 | 45.3 | 23.6 | 22.4 | 22.8 | 13.7 | 21.8 | 13.5 | 20.6 | 7.1 |
| | 30.0 | 41.3 | 28.8 | 28.4 | 28.1 | 24.9 | 27.3 | 5.3 | 26.7 | 10.7 | 26.2 | 10.0 |
| | 34.6 | 41.1 | 33.7 | 32.0 | 33.0 | 13.7 | 32.4 | 10.7 | 32.0 | 6.9 | 31.4 | 10.7 |
| | 39.6 | 42.2 | 38.9 | 38.9 | 38.0 | 19.2 | 36.8 | 2.7 | 36.3 | 6.4 | 35.8 | 10.7 |

## Table A.15 Continued

| Date | 9/9/2004 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stim | Unstimulated | | | | | | | | | | | |
| Shear | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.9 | 48.0 | 3.1 | 17.8 | 2.4 | 8.0 | 1.8 | 26.7 | 1.2 | 24.0 | 0.6 | 12.0 |
| | 7.6 | 117.3 | 6.9 | 18.0 | 6.3 | 19.2 | 5.8 | 14.0 | 5.0 | 11.4 | 4.4 | 9.6 |
| | 11.5 | 74.7 | 12.8 | 37.3 | 12.2 | 12.8 | 9.6 | 12.4 | 10.7 | 17.0 | 8.3 | 12.8 |
| | 17.1 | 76.0 | 18.4 | 51.2 | 17.7 | 26.7 | 15.1 | 19.4 | 16.5 | 9.1 | 14.0 | 12.8 |
| | 23.4 | 85.3 | 22.8 | 9.6 | 22.1 | 21.3 | 21.2 | 16.0 | 20.2 | 6.9 | 19.5 | 6.9 |
| | 26.8 | 100.0 | 27.7 | 35.8 | 27.1 | 8.0 | 26.6 | 11.9 | 26.0 | 6.7 | 25.7 | 8.0 |
| | 32.2 | 70.0 | 33.6 | 53.3 | 32.9 | 29.7 | 32.1 | 20.6 | 31.6 | 4.6 | 30.9 | 11.4 |
| | 38.1 | 68.0 | 37.4 | 19.2 | 36.8 | 12.0 | 37.9 | 11.5 | 37.4 | 6.4 | 36.9 | 8.8 |
| | AVERAGES | | | | | | | | | | | |
| | Unstimulated | | | | | | | | | | | |
| | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | 3.9 | 86.8 | 3.3 | 40.8 | 2.6 | 17.2 | 2.0 | 18.4 | 1.3 | 8.3 | 0.7 | 6.0 |
| | 8.2 | 160.1 | 7.5 | 65.1 | 6.7 | 30.3 | 6.0 | 22.1 | 5.3 | 12.0 | 4.7 | 3.9 |
| | 13.0 | 193.5 | 12.5 | 91.8 | 11.7 | 34.5 | 10.7 | 23.1 | 10.0 | 9.3 | 9.2 | 7.9 |
| | 17.4 | 199.6 | 17.3 | 109.3 | 16.5 | 42.8 | 15.3 | 25.4 | 14.9 | 11.8 | 14.0 | 8.0 |
| | 22.0 | 247.7 | 22.9 | 122.2 | 22.0 | 50.5 | 20.7 | 24.7 | 20.5 | 10.8 | 19.4 | 6.4 |
| | 26.9 | 208.6 | 28.1 | 123.8 | 27.4 | 48.7 | 26.4 | 25.7 | 26.0 | 10.9 | 25.2 | 6.1 |
| | 32.5 | 283.3 | 33.3 | 171.6 | 33.1 | 53.6 | 32.2 | 25.9 | 31.4 | 11.7 | 31.3 | 5.9 |
| | 38.4 | 273.7 | 38.5 | 171.8 | 38.3 | 49.6 | 37.9 | 26.5 | 37.5 | 13.7 | 37.8 | 5.7 |
| | Standard Error of the Mean | | | | | | | | | | | |
| | Unstimulated | | | | | | | | | | | |
| | 0.1 | | 0.2 | | 0.4 | | 0.6 | | 0.8 | | 1 | |
| | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere | time | adhere |
| | | 22.5 | | 11.4 | | 3.3 | | 3.4 | | 1.9 | | 1.1 |
| | | 30.4 | | 15.1 | | 5.6 | | 3.7 | | 2.0 | | 0.9 |
| | | 50.2 | | 21.3 | | 7.2 | | 3.0 | | 1.6 | | 0.7 |
| | | 54.2 | | 22.6 | | 8.5 | | 3.3 | | 1.1 | | 1.3 |
| | | 58.0 | | 34.3 | | 10.7 | | 3.1 | | 1.6 | | 0.8 |
| | | 53.6 | | 31.0 | | 9.4 | | 3.7 | | 2.0 | | 1.0 |
| | | 76.5 | | 48.3 | | 10.1 | | 4.0 | | 1.5 | | 0.9 |
| | | 74.7 | | 46.4 | | 10.1 | | 4.2 | | 2.5 | | 1.0 |

**Figure A.16 Blocking of Shear Dependent Histamine-Mediated Adherence**
Data for Figure 6.3

| 10/26/2005 | Shear | Unstimulated | Histamine | Peptide Blocking | Peptide, non-blocking |
|---|---|---|---|---|---|
| | 0.1 | 528.0 | 490.7 | 408.0 | 416.0 |
| | 0.2 | 293.3 | 384.0 | 224.0 | 357.3 |
| | 0.4 | 102.9 | 276.0 | 60.8 | 240.0 |
| | 0.6 | 46.2 | 80.0 | 36.8 | 92.0 |
| | 0.8 | 8.0 | 48.0 | 5.3 | 73.1 |
| | 1 | 4.6 | 18.3 | 0.0 | 21.3 |

| 10/19/2005 | Shear | Unstimulated | Histamine | Peptide Blocking | Peptide, non-blocking |
|---|---|---|---|---|---|
| | 0.1 | 864.0 | 464.0 | 522.7 | 560.0 |
| | 0.2 | 546.7 | 245.3 | 284.0 | 196.6 |
| | 0.4 | 91.2 | 114.9 | 140.8 | 128.0 |
| | 0.6 | 49.6 | 61.7 | 36.6 | 96.0 |
| | 0.8 | 29.3 | 36.0 | 8.0 | 28.4 |
| | 1 | 9.6 | 21.3 | 5.3 | 18.3 |

| 11/16/2005 | Shear | Unstim | Histamine | P-block | P-non-block |
|---|---|---|---|---|---|
| | 0.1 | 325.3 | 189.3 | 256.0 | 149.3 |
| | 0.2 | 209.8 | 126.5 | 84.0 | 128.0 |
| | 0.4 | 90.7 | 80.0 | 38.9 | 102.4 |
| | 0.6 | 29.1 | 48.0 | 13.3 | 72.0 |
| | 0.8 | 19.2 | 32.0 | 5.3 | 39.1 |
| | 1 | 3.2 | 16.0 | 6.7 | 17.5 |

| 11/10/2005 | Shear | no stim | contin. | P-block | P-non-block |
|---|---|---|---|---|---|
| | 0.1 | 288.0 | 217.6 | 180.0 | 208.0 |
| | 0.2 | 205.3 | 123.4 | 104.0 | 91.4 |
| | 0.4 | 16.0 | 46.0 | 32.0 | 43.4 |
| | 0.6 | 16.0 | 44.4 | 18.0 | 57.6 |
| | 0.8 | 10.7 | 32.0 | 5.3 | 18.3 |
| | 1 | 6.4 | 28.4 | 2.3 | 16.0 |

| 6/1/2005 | Shear | Unstimulated | Histamine | Peptide Blocking | Peptide, non-blocking |
|---|---|---|---|---|---|
| | 0.1 | 170.7 | 148.0 | 182.4 | 200.0 |
| | 0.2 | 134.4 | 128.0 | 85.3 | 133.3 |
| | 0.4 | 27.4 | 57.1 | 28.4 | 72.0 |
| | 0.6 | 28.0 | 58.0 | 20.6 | 66.9 |
| | 0.8 | 8.0 | 25.6 | 14.0 | 33.6 |
| | 1 | 0.0 | 24.0 | 8.0 | 24.0 |

**Table A.16 Continued**

| 3/25/2005 | Shear | Unstimulated | Histamine | Peptide Blocking | Peptide, non-blocking | Antibody Block | Non-specific |
|---|---|---|---|---|---|---|---|
| | 0.1 | 344.0 | 272.0 | 281.6 | 297.6 | 176.0 | 282.7 |
| | 0.2 | 214.4 | 152.0 | 166.9 | 152.0 | 141.3 | 196.0 |
| | 0.4 | 56.0 | 121.6 | 74.0 | 66.7 | 64.0 | 100.0 |
| | 0.6 | 30.2 | 86.9 | 41.6 | 90.7 | 48.0 | 120.0 |
| | 0.8 | 26.7 | 57.1 | 21.3 | 45.7 | 12.0 | 32.0 |
| | 1.0 | 6.0 | 22.0 | 6.0 | 18.3 | 5.3 | 24.0 |

| 3/9/2005 | Shear | Unstimulated | Histamine | Peptide Blocking | Peptide, non-blocking | Antibody Block | Non-specific |
|---|---|---|---|---|---|---|---|
| | 0.1 | 104.0 | 98.7 | 149.3 | 121.6 | 164.0 | 212.0 |
| | 0.2 | 59.4 | 73.1 | 44.8 | 50.3 | 50.0 | 102.4 |
| | 0.4 | 40.0 | 48.0 | 32.0 | 29.3 | 28.4 | 38.9 |
| | 0.6 | 27.4 | 20.0 | 12.0 | 17.8 | 18.3 | 28.0 |
| | 0.8 | 9.6 | 28.4 | 6.0 | 24.0 | 14.0 | 13.7 |
| | 1.0 | 0.0 | 28.8 | 1.6 | 11.4 | 5.3 | 18.7 |

AVERAGES

| | Shear | Unstimulated | Histamine | Peptide Blocking | Peptide, non-blocking |
|---|---|---|---|---|---|
| | 0.1 | 374.9 | 268.6 | 282.9 | 278.9 |
| | 0.2 | 237.6 | 176.1 | 141.9 | 158.4 |
| | 0.4 | 60.6 | 106.2 | 58.1 | 97.4 |
| | 0.6 | 32.4 | 57.0 | 25.6 | 70.4 |
| | 0.8 | 15.9 | 37.0 | 9.3 | 37.5 |
| | 1.0 | 4.3 | 22.7 | 4.3 | 18.1 |

SEM

| | | Unstimulated | Histamine | Peptide Blocking | Peptide, non-blocking |
|---|---|---|---|---|---|
| | 0.1 | 96.2 | 57.7 | 51.7 | 59.9 |
| | 0.2 | 58.4 | 39.9 | 32.7 | 37.4 |
| | 0.4 | 13.1 | 30.6 | 15.2 | 26.9 |
| | 0.6 | 4.4 | 8.5 | 4.7 | 10.3 |
| | 0.8 | 3.4 | 4.3 | 2.3 | 6.9 |
| | 1.0 | 1.3 | 1.8 | 1.1 | 1.5 |

**Table A.17 NOS Blocking with DPI**
Data for Figure VII.1 A

| Stimulation | time AVG | 1/8/2003 | 1/16/2003 | 1/23/2003 | 2/5/2003 | 2/20/2003 | 7/31/2003 | 8/13/2003 | AVERAGE Adhesion | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| Histamine | 1.2 | 12.0 | 7.6 | 14.0 | 2.9 | 12.4 | 11.6 | 9.8 | 10.1 | 1.3 |
| | 3.0 | 15.8 | 11.7 | 13.8 | 5.3 | 8.6 | 10.2 | 14.2 | 11.4 | 1.3 |
| | 5.6 | 20.0 | 16.7 | 16.8 | 10.9 | 13.6 | 26.7 | 19.4 | 17.7 | 1.8 |
| | 9.3 | 34.0 | 14.3 | 22.3 | 11.6 | 26.2 | 23.2 | 35.3 | 23.8 | 3.1 |
| | 19.5 | 35.6 | 16.5 | 29.6 | 15.2 | 37.5 | 40.5 | 63.6 | 34.0 | 5.7 |
| | 29.6 | 36.7 | 24.0 | 41.7 | 23.0 | 38.0 | 38.0 | 59.8 | 37.3 | 4.3 |
| | 38.7 | 45.9 | 26.5 | 46.7 | 20.6 | 31.2 | 49.2 | 66.0 | 40.9 | 5.5 |
| Unstimulated | 1.2 | 4.8 | 2.9 | 8.0 | 3.6 | 5.3 | 2.7 | 4.9 | 4.6 | 0.6 |
| | 3.0 | 8.0 | 2.8 | 7.5 | 7.3 | 5.8 | 2.3 | 7.4 | 5.9 | 0.8 |
| | 5.6 | 10.2 | 4.3 | 8.3 | 8.9 | 3.4 | 7.3 | 8.7 | 7.3 | 0.9 |
| | 9.3 | 10.9 | 8.3 | 10.4 | 5.1 | 8.0 | 11.6 | 17.5 | 10.3 | 1.4 |
| | 19.5 | 8.0 | 8.7 | 8.9 | 8.4 | 5.6 | 10.9 | 18.0 | 9.8 | 1.4 |
| | 29.6 | 12.2 | 9.0 | 26.7 | 8.0 | 16.8 | 8.5 | 26.7 | 15.4 | 2.9 |
| | 38.7 | 12.8 | 8.0 | 17.1 | 17.1 | 16.0 | 10.9 | 28.0 | 15.7 | 2.2 |
| DPI | 1.2 | 9.6 | 3.2 | 3.2 | 4.0 | 8.0 | 16.0 | 3.7 | 6.8 | 1.7 |
| | 3.0 | 9.8 | 10.0 | 9.3 | 5.3 | 13.3 | 6.7 | 5.0 | 8.5 | 1.0 |
| | 5.6 | 11.2 | 5.8 | 14.3 | 9.8 | 11.0 | 7.3 | 6.3 | 9.4 | 1.1 |
| | 9.3 | 16.8 | 11.7 | 15.2 | 9.8 | 23.5 | 8.3 | 17.5 | 14.7 | 1.8 |
| | 19.5 | 14.3 | 18.8 | 16.0 | 16.9 | 23.5 | 9.8 | 23.2 | 17.5 | 1.7 |
| | 29.6 | 15.1 | 12.5 | 33.0 | 17.9 | 30.9 | 13.5 | 26.7 | 21.4 | 3.0 |
| | 38.7 | 21.6 | 13.0 | 22.4 | 17.1 | 33.5 | 13.3 | 24.0 | 20.7 | 2.5 |

**Table A.18 NOS Blocking with L-NAME**
Data for Figure VII.1 B

| Stimulation | time AVG | 7/31/2003 | 8/13/2003 | 9/11/2003 | 9/11/2003 | 9/18/2003 | 10/16/2003 | 10/16/2003 | 10/23/2003 | 11/6/2003 | AVERAGE | SEM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Histamine | 1.2 | 11.6 | 9.8 | 14.4 | 12.8 | 14.5 | 15.1 | 14.2 | 9.6 | 10.2 | 12.5 | 0.7 |
| | 3.0 | 10.2 | 14.2 | 6.7 | 11.4 | 18.7 | 19.9 | 20.0 | 21.3 | 17.2 | 15.5 | 1.7 |
| | 5.7 | 26.7 | 19.4 | 26.4 | 17.5 | 25.8 | 19.5 | 21.3 | 19.8 | 22.1 | 22.1 | 1.1 |
| | 9.3 | 23.2 | 35.3 | 30.0 | 37.6 | 18.7 | 20.3 | 25.8 | 19.2 | 26.4 | 26.3 | 2.3 |
| | 19.7 | 40.5 | 63.6 | 28.8 | 39.3 | 35.8 | 32.3 | 46.1 | 32.0 | 48.0 | 40.7 | 3.6 |
| | 29.8 | 38.0 | 59.8 | 20.3 | 50.7 | 39.1 | 33.4 | 40.5 | 35.7 | 41.1 | 39.8 | 3.7 |
| | 38.7 | 49.2 | 66.0 | 33.1 | 51.3 | 45.9 | 27.1 | 43.4 | 44.3 | 51.4 | 45.8 | 3.7 |
| Unstimulated | 1.2 | 2.7 | 4.9 | 7.3 | 5.3 | 6.4 | 11.6 | 8.0 | 7.3 | 10.7 | 7.1 | 0.9 |
| | 3.0 | 2.3 | 7.4 | 8.0 | 8.0 | 9.3 | 12.6 | 6.9 | 5.3 | 13.5 | 8.1 | 1.1 |
| | 5.7 | 7.3 | 8.7 | 5.3 | 7.3 | 20.2 | 11.4 | 5.1 | 9.9 | 11.1 | 9.6 | 1.5 |
| | 9.2 | 11.6 | 17.5 | 7.0 | 8.3 | 15.3 | 9.7 | 12.4 | 18.3 | 7.0 | 11.9 | 1.4 |
| | 19.7 | 10.9 | 18.0 | 4.9 | 11.6 | 11.6 | 10.4 | 10.9 | 7.5 | 10.7 | 10.7 | 1.2 |
| | 29.6 | 8.5 | 26.7 | 7.7 | 10.4 | 17.8 | 11.3 | 16.8 | 8.9 | 15.2 | 13.7 | 2.0 |
| | 38.7 | 10.9 | 28.0 | 7.0 | 8.4 | 16.0 | 17.1 | 16.0 | 5.6 | 13.7 | 13.6 | 2.3 |
| L-NAME | 1.2 | 8.0 | 1.8 | 4.0 | 5.8 | 2.7 | 2.7 | 7.3 | 5.8 | 13.1 | 5.7 | 1.2 |
| | 3.0 | 12.1 | 6.2 | 5.3 | 6.9 | 4.9 | 8.6 | 10.3 | 9.3 | 10.3 | 8.2 | 0.8 |
| | 5.7 | 10.6 | 10.9 | 5.3 | 8.4 | 11.8 | 10.9 | 9.5 | 12.0 | 9.0 | 9.8 | 0.7 |
| | 9.3 | 9.1 | 20.0 | 7.7 | 11.3 | 20.6 | 16.7 | 12.7 | 13.6 | 8.9 | 13.4 | 1.6 |
| | 19.7 | 12.3 | 22.2 | 8.4 | 13.0 | 13.7 | 8.9 | 15.1 | 16.9 | 16.8 | 14.1 | 1.4 |
| | 29.9 | 14.2 | 35.0 | 7.0 | 10.2 | 15.2 | 12.8 | 14.1 | 14.1 | 18.8 | 15.7 | 2.6 |
| | 38.9 | 13.5 | 31.2 | 7.6 | 12.2 | 13.5 | 8.4 | 10.4 | 9.5 | 21.3 | 14.2 | 2.5 |

**Table A.19 Sickle Mouse Cells Perfusion Assay** Data for Figure 7.2 (n=1)

| Unstimulated | |
|---|---|
| time | cells/mm$^2$ |
| 1.1 | 7.1 |
| 3.0 | 20.4 |
| 5.8 | 24.4 |
| 9.2 | 20.0 |
| 19.6 | 31.1 |
| 29.7 | 29.7 |
| 38.7 | 30.0 |

**Table A.20 Preliminary Detachment Assay Data** Data for Figure 7.3 A

| No Stimulation | | | | | | | | AVERAGE | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Detachment 1A | | Detachment 1B | | Detachment 2A | | Detachment 2B | | No Stimulation | | |
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | SEM |
| 1.2 | 2.9 | 1.2 | 11.1 | 1.3 | 6.7 | 1.6 | 4.4 | 1.3 | 6.3 | 1.8 |
| 3.0 | 9.6 | 3.0 | 19.7 | 2.9 | 9.1 | 3.1 | 6.4 | 3.0 | 11.2 | 2.9 |
| 5.4 | 27.1 | 5.5 | 28.8 | 5.5 | 18.8 | 5.4 | 29.9 | 5.5 | 26.1 | 2.5 |
| 9.1 | 31.3 | 8.9 | 31.4 | 9.2 | 18.0 | 9.0 | 46.2 | 9.1 | 31.7 | 5.8 |
| 19.5 | 40.0 | 19.8 | 44.6 | 19.7 | 32.9 | 19.5 | 76.3 | 19.6 | 48.5 | 9.6 |
| 29.4 | 32.0 | 29.5 | 55.6 | 29.7 | 38.7 | 29.5 | 70.4 | 29.5 | 49.2 | 8.6 |
| 38.7 | 37.3 | 39.0 | 64.0 | 39.1 | 35.7 | 38.9 | 96.0 | 38.9 | 58.3 | 14.2 |
| 43.9 | 29.9 | 44.4 | 58.3 | 43.8 | 38.2 | 44.6 | 57.6 | 44.2 | 46.0 | 7.1 |
| 48.8 | 27.1 | 48.9 | 43.7 | 49.0 | 32.0 | 49.1 | 65.8 | 49.0 | 42.1 | 8.6 |
| 53.9 | 32.0 | 54.1 | 52.0 | 54.1 | 27.1 | 54.1 | 70.4 | 54.1 | 45.4 | 9.9 |
| 58.7 | 23.5 | 58.8 | 65.3 | 59.2 | 38.7 | 59.1 | 50.9 | 58.9 | 44.6 | 8.9 |
| | | | | | | | | | | |
| Histamine Stimulation, none in detachment Phase | | | | | | | | Hist Stim & not in Detach | | |
| time | cells/mm2 | time | cells/mm2 | time | cells/mm2 | time | cells/mm2 | time | cells/mm2 | SEM |
| 1.3 | 14.2 | 1.3 | 9.6 | 1.4 | 6.0 | 1.5 | 10.7 | 1.4 | 10.1 | 1.7 |
| 3.0 | 23.1 | 3.0 | 20.4 | 2.9 | 18.7 | 3.2 | 27.4 | 3.0 | 22.4 | 1.9 |
| 5.6 | 55.3 | 5.4 | 22.0 | 5.6 | 51.2 | 5.7 | 38.9 | 5.6 | 41.8 | 7.5 |
| 9.0 | 48.8 | 8.9 | 34.8 | 9.3 | 36.7 | 9.1 | 59.7 | 9.1 | 45.0 | 5.8 |
| 19.5 | 76.3 | 19.7 | 58.7 | 19.7 | 73.8 | 19.3 | 161.3 | 19.6 | 92.5 | 23.3 |
| 29.2 | 82.3 | 30.0 | 86.7 | 29.8 | 97.3 | 29.6 | 179.2 | 29.6 | 111.4 | 22.8 |
| 38.7 | 119.1 | 38.9 | 104.0 | 39.1 | 106.7 | 38.9 | 190.0 | 38.9 | 129.9 | 20.3 |
| 44.1 | 83.6 | 44.0 | 72.9 | 44.6 | 70.0 | 43.8 | 162.0 | 44.1 | 97.1 | 21.8 |
| 48.7 | 59.1 | 48.9 | 47.0 | 49.0 | 76.0 | 48.9 | 120.9 | 48.9 | 75.7 | 16.2 |
| 54.0 | 33.1 | 54.3 | 45.5 | 54.1 | 42.7 | 54.2 | 112.0 | 54.1 | 58.3 | 18.1 |
| 58.8 | 16.0 | 58.8 | 38.0 | 58.4 | 43.4 | 58.8 | 114.5 | 58.7 | 53.0 | 21.3 |
| | | | | | | 64.0 | 118.0 | | | |
| Histamine Stimulation, Standard Histamine in Detachment Phase | | | | | | | | Hist Stim & in Detach | | |
| time | cells/mm2 | time | cells/mm2 | time | cells/mm2 | time | cells/mm2 | time | cells/mm2 | SEM |
| 1.3 | 13.3 | 1.2 | 6.7 | 1.3 | 5.3 | 1.4 | 41.3 | 1.3 | 16.7 | 9.2 |
| 3.0 | 27.4 | 3.0 | 22.4 | 3.0 | 24.0 | 3.2 | 101.3 | 3.1 | 43.8 | 22.1 |
| 5.4 | 56.0 | 5.5 | 35.8 | 5.7 | 28.8 | 5.5 | 109.1 | 5.5 | 57.4 | 18.9 |
| 8.9 | 91.3 | 9.4 | 46.9 | 9.3 | 50.0 | 8.9 | 104.0 | 9.1 | 73.1 | 15.0 |
| 19.6 | 80.0 | 19.6 | 97.3 | 19.9 | 76.8 | 19.6 | 186.2 | 19.7 | 110.1 | 28.5 |
| 29.6 | 139.1 | 30.2 | 100.9 | 29.7 | 102.7 | 29.8 | 220.8 | 29.8 | 140.9 | 30.6 |
| 38.5 | 130.5 | 39.0 | 110.0 | 39.3 | 106.0 | 38.8 | 200.9 | 38.9 | 136.8 | 23.8 |
| 44.0 | 62.5 | 44.2 | 105.6 | 44.0 | 24.5 | 44.1 | 192.0 | 44.1 | 96.2 | 33.0 |
| 49.1 | 59.6 | 48.9 | 77.3 | 48.9 | 26.7 | 49.3 | 186.0 | 49.0 | 87.4 | 34.2 |
| 54.0 | 49.5 | 54.0 | 81.3 | 54.0 | 14.9 | 54.8 | 129.5 | 54.2 | 68.8 | 20.1 |
| 58.7 | 62.8 | 59.0 | 60.3 | 59.0 | 18.1 | 59.2 | 132.8 | 59.0 | 68.5 | 20.6 |

**Table A.21 Mepyramine and Amthamine, TNF and Histamine Sample Detachment** Data for Figure 7.3 B&C

Figure B

| Histamine | | TNF | | Histamine + TNF | |
|---|---|---|---|---|---|
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| 1.2 | 20.9 | 1.1 | 14.5 | 1.1 | 26.2 |
| 3.0 | 69.8 | 2.9 | 32.0 | 3.0 | 46.4 |
| 5.4 | 110.8 | 5.8 | 91.4 | 5.7 | 92.8 |
| 9.1 | 141.0 | 9.4 | 108.6 | 9.2 | 122.2 |
| 19.9 | 185.1 | 19.6 | 123.6 | 19.8 | 133.8 |
| 29.9 | 152.0 | 29.8 | 144.0 | 29.8 | 144.0 |
| 39.5 | 140.4 | 39.1 | 176.0 | 39.3 | 182.4 |
| 44.0 | 116.8 | 43.7 | 134.4 | 43.7 | 152.7 |
| 48.9 | 50.1 | 48.9 | 136.9 | 48.6 | 152.0 |
| 53.8 | 32.0 | 53.8 | 107.2 | 53.8 | 131.2 |
| 58.8 | 26.9 | 58.8 | 77.5 | 58.7 | 89.3 |

Figure C

| Unstimulated | | Methistamine | | Amthamine | | Amthamine + Methistamine | | Histamine | |
|---|---|---|---|---|---|---|---|---|---|
| time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ | time | cells/mm$^2$ |
| 1.2 | 2.7 | 1.2 | 7.1 | 1.2 | 2.9 | 1.3 | 4.0 | 1.2 | 9.3 |
| 3.0 | 7.5 | 3.0 | 11.4 | 3.0 | 11.7 | 3.1 | 5.3 | 3.0 | 12.7 |
| 5.8 | 9.5 | 5.6 | 11.3 | 5.6 | 14.7 | 5.9 | 9.3 | 5.7 | 17.6 |
| 9.2 | 13.0 | 9.3 | 13.3 | 9.2 | 14.2 | 9.2 | 32.0 | 9.2 | 22.4 |
| 19.8 | 14.1 | 19.7 | 18.7 | 19.8 | 22.1 | 19.9 | 26.3 | 19.8 | 32.5 |
| 29.8 | 22.6 | 29.7 | 19.8 | 29.8 | 26.4 | 29.7 | 45.1 | 29.7 | 36.8 |
| 38.8 | 18.7 | 38.7 | 20.2 | 39.2 | 33.5 | 38.8 | 52.4 | 39.1 | 43.8 |
| 44.0 | 17.0 | 43.7 | 14.0 | 43.9 | 16.0 | 43.8 | 38.2 | 44.1 | 27.0 |
| 48.5 | 14.5 | 48.7 | 6.4 | 48.8 | 18.8 | 49.0 | 29.9 | 48.7 | 12.2 |
| 53.9 | 6.2 | 53.6 | 13.2 | 53.8 | 12.0 | 53.7 | 18.1 | 53.8 | 12.0 |
| 58.8 | 6.6 | 58.8 | 5.6 | 58.7 | 7.6 | 59.0 | 21.0 | 58.8 | 8.9 |

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## VITA

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