THE UNIVERSITY OF CALGARY

The Function of VLA-4 on Neutrophils

by

Paul H. Reinhardt

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF GASTROINTESTINAL SCIENCES

CALGARY, ALBERTA

FEBRUARY, 1998

© Paul H. Reinhardt 1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-31370-0

Canada

ABSTRACT

The aim of this thesis was to examine the role of $\alpha_4\beta_1$-integrin in neutrophil recruitment. Kubes et al. have described the presence of $\alpha_4\beta_1$-integrin (also called VLA-4) on the surface of neutrophils[12]. Using *in vitro* flow chamber techniques to mimic the shear conditions found in blood vessels, isolated human neutrophils that had been stimulated with DHCB (to maximally activate them) could use $\alpha_4\beta_1$ to adhere to cytokine-activated endothelium or a VCAM-1 expressing cell line independent of selectins and β_2-integrins. Recruitment did not occur on an ICAM-1 expressing cell line or on fibronectin-coated coverslips. $\alpha_4\beta_1$-dependent neutrophil recruitment onto VCAM-1 was enhanced by the presence of red blood cells, which are known to promote leukocyte recruitment under shear conditions. Emigrated but not circulating rat neutrophils were found to adhere to fibronectin on isolated cardiac myocytes using both α_4- and β_2-integrins. Thus neutrophils can use $\alpha_4\beta_1$-integrin at multiple steps in the recruitment cascade.

Acknowledgments

Thank you to Sophie, my best friend and partner. You convinced me that I could do it, and I know that it wasn't always easy to keep me convinced. Thank you Mum for your support and encouragement, it helped keep me confident that I was doing the right thing.

I am sincerely grateful to Paul Kubes for his support in all aspects of this endeavor. I'm not sure if others would have given me the same opportunity, and for this, I am indebted.

Thank you to my ever-tolerant lab mates, especially Derrice, Samina, Brent, and Donna Marie. I think having friends that you can both work and play with is a rare thing, and I count myself lucky.

Finally, I would like to thank the Medical Research Council of Canada for their financial support.

TABLE OF CONTENTS

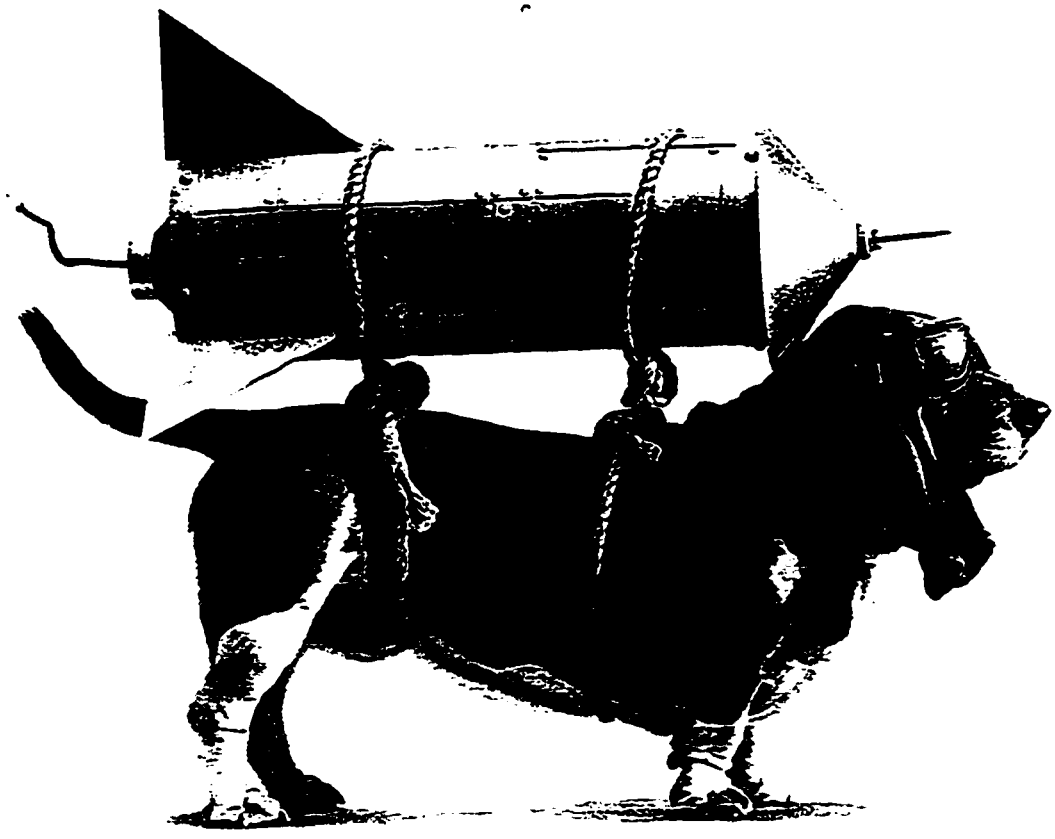
List of Tables

List of Figures

List of Abbreviations

BSA	bovine serum albumin
CLA	cutaneous lymphocyte antigen
DHCB	dihydrocytochalasin-B
ELISA	enzyme-linked immunosorbant assay
ESL-1	E-selectin ligand-1
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FN	fibronectin
HBSS	Hank's balanced salt solution
HUVEC	human umbilical vein endothelial cell
IL	interleukin
LAD	leukocyte adhesion deficiency
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
MAb	monoclonal antibody
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MSU	monosodium urate crystal
PAF	platelet-activating factor
PBS	Phosphate-buffered saline
PECAM-1	platelet/endothelial cell adhesion molecule-1
PMA	phorbol 12-myristate 13-acetate
PSGl-1	P-selectin glycoprotein ligand-1
TNFα	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4

Epigraph



A turtle proceeds only when he sticks his neck out of his shell.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Leukocytes are important during inflammatory and immune responses to foreign pathogens and injury, where they function in pathogen removal and tissue repair. While normal leukocyte function is essential, inappropriate leukocyte recruitment and activation is associated with pathology of several disease processes including rheumatoid arthritis and ischemia reperfusion injury of the heart [1-3]. The recruitment of leukocytes from the bloodstream is dependent on an organized cascade of events, starting with leukocyte tethering and rolling on activated endothelium. These requisite events are followed by firm leukocyte adhesion to the endothelium, and if appropriate signals are present, diapedesis across the vessel wall towards the inflammatory site. This cascade of events is mediated by various adhesion molecules that are expressed on the surface of the leukocyte. These molecules initially interact with adhesion molecules on activated endothelium and then with extravascular matrix proteins.

The mechanisms that mediate leukocyte recruitment have best been described for neutrophils. To leave the bloodstream, neutrophils initially contact (tether) then roll on activated endothelium. This primary adhesion is known to be mediated by the selectin family of adhesion molecules which are expressed on leukocytes and on activated endothelium. The velocity of rolling neutrophils is markedly reduced relative to the red blood cells, with some of the rolling cells becoming stationary and adherent to the endothelium (called secondary adhesion). Firm arrest is mediated by leukocyte adhesion molecules from the β_2-integrin family, which are activated by endothelial-derived factors such as IL-8 and platelet-activating factor or by signal transduction events initiated by

adhesion molecule engagement [4-6]. The ligands for β_2-integrins are varied, and include members of the immunoglobulin superfamily (such as ICAM-1, ICAM-2, and VCAM-1) and extracellular matrix proteins (such as fibronectin, laminin, and collagen [7]). Although this cascade of events has been proposed for neutrophils as well as other leukocytes, it is becoming clear that non-neutrophilic leukocytes can utilize a second integrin, VLA-4 ($\alpha_4\beta_1$) to firmly adhere and in some but not all instances, to tether and roll [8,9]. VLA-4 is a β_1-integrin whose ligands include the matrix protein, fibronectin and the adhesion molecule, VCAM-1 [8,10,11]. The importance of this observation is that VLA-4 could conceivably replace the selectin and β_2-integrin pathway and constitute an alternative recruitment pathway.

Based on the fact that neutrophils were generally thought not to express VLA-4, this pathway should be unimportant in neutrophil recruitment. However, recently Kubes et al. have reported that human neutrophils stimulated with chemotactic factors in the presence of dihydrocytochalasin B (DHCB) which optimizes neutrophil effector responses, expressed VLA-4 [12]. This expression occurred quickly (within 10 minutes), suggesting that VLA-4 was stored and available for rapid mobilization. Transmigration of neutrophils across endothelium also induced the expression of VLA-4 on neutrophils suggesting a role for this adhesion molecule once the neutrophil has extravasated.

Interestingly, a recent publication reported that circulating rat neutrophils express α_4-integrin, and that blockade of the α_4 improved the ability of a β_2-integrin antibody to inhibit neutrophil recruitment to arthritic joints [13]. Although intriguing, the inhibition of neutrophil infiltration by an antibody that immunoneutralizes α_4-integrin may be related to

indirect effects such as inhibition of mononuclear cell infiltration which could affect subsequent neutrophil recruitment. Therefore, one objective of these studies was to determine whether VLA-4 can directly contribute to neutrophil interactions with biologic substrata under shear conditions. This was accomplished by using an *in vitro* system that mimics the flow conditions found in blood vessels.

Studies have suggested that red cells can contribute to the forces that influence leukocyte interactions with vascular endothelium [14-16]. For example, red cells were found to enhance selectin- and integrin-mediated rolling and adhesion of T-cells on TNFα-activated endothelium [15]. To date, no one has demonstrated whether red blood cells can enhance neutrophil interactions with substrata under shear conditions. Therefore, a second objective was to determine if red blood cells could enhance $\alpha_4\beta_1$-dependent neutrophil recruitment.

The last step of leukocyte recruitment is emigration. The observation that emigrated neutrophils express $\alpha_4\beta_1$ suggests the possibility that, once out of the vasculature, neutrophils might use this molecule for adhesion to extravascular tissues. Myocardial inflammation associated ischemia/reperfusion, sepsis, and other pathologies has been shown to have a major neutrophil component inasmuch as prevention of neutrophil infiltration into myocardium decreases myocardial damage during the inflammatory process [2,3,17,18]. As with adhesion to endothelium, neutrophil adhesion to cardiac myocytes has been shown to involve CD18/ICAM-1 [19,20], however circulating (not emigrated) cells were used. Thus the third focus of this thesis was to determine if emigration will invoke $\alpha_4\beta_1$-dependent adhesion of neutrophils to cardiac myocytes.

1.2 Neutrophil Structure and Function.

Neutrophils (also called polymorphonuclear leukocytes) are members of the granulocyte family of leukocytes, and are the most numerous of all leukocytes in humans, comprising 50 to 70% of circulating white cells. They are the first leukocytes to infiltrate sites of inflammation, where they function in the killing and phagocytic removal of bacteria, and in the remodelling of damaged tissue. Neutrophils are derived from the self-renewing stem cells present in the bone marrow. Under the influence of cytokines produced by stromal cells and macrophages in the marrow, stem cells give rise to myeloid or lymphoid progenitor cells. Myeloid progenitors cells are committed to become neutrophils, monocytes, eosinophils, basophils, or megakaryocytes, while lymphoid progenitors become lymphocytes. Some myeloid progenitors will further differentiate towards neutrophils, passing through morphologically distinct stages (myeloblast, promyelocyte, myelocyte, metamyelocyte, and band cell) and eventually become mature neutrophils. The process of neutrophil development lasts about 5 days, at which point, mature neutrophils are released into the circulation. They circulate for about 10 hours, and eventually enter tissue sites, where they persist for about 2 days [22].

Neutrophils are morphologically characterized by their multilobed nuclei and numerous granules. Neutrophils have the ability to adhere to vascular endothelium and crawl from the vasculature following increasing gradients of chemotactic stimuli. On their surface, neutrophils have receptors that recognize ligands which are expressed on activated endothelium. Some of these receptors are adhesion molecules, and facilitate the

capture of neutrophils from the bloodstream. Others are G-linked proteins that detect various chemotactic agents expressed on the endothelial surface or in the extravascular tissue [23]. Once at an inflamed site, neutrophils perform their effector functions, which include phagocytosis, release of cytocidal agents (reactive oxygen metabolites and antimicrobial compounds), and the discharge of degradative enzymes [24]. Chemotactic factors for neutrophils include components of bacteria (for example, formylated peptides from the surface of bacteria), lipid products of cell membrane metabolism (eg, LTB_4 and PAF), and products of complement cascade activation (eg. C5a).

A primary cytocidal weapon used by neutrophils is the formation of reactive oxygen metabolites, which are generated by an enzyme called NADPH oxidase. This enzyme is found on the plasma membrane and is triggered when neutrophils are activated by a number of proinflammatory signals [25]. Once activated, NADPH oxidase shuttles electrons from cytosolic NADPH to oxygen, resulting in the formation of superoxide anion (a radical). Most of the superoxide anions are thought to spontaneously combine to form hydrogen peroxide (called the dismutation reaction). Hydrogen peroxide (a powerful antimicrobial agent) is used as a substrate by a neutrophil enzyme to form hypochlorous acid [25]. Hypochlorous acid is the principle active ingredient in household bleach, a commonly used disinfectant. Thus neutrophil activation results in the formation of several potent and potentially damaging molecules.

At the same time that NADPH oxidase is activated, the neutrophil granules fuse with the plasma membrane and release their contents into the extracellular space and into phagocytic vacuoles. Neutrophil granules are distinguishable morphologically and by their

contents. Primary (or azurophilic) granules contain myeloperoxidase. As well, primary granules contain other antimicrobial agents such as lysozymes and defensins, proteases such as cathepsin G and elastase, and acid hydrolases such as β glucuronidase. Secondary granules (called specific granules) contain metalloproteinases (collagenase and gelatinase) and antimicrobial agents such as lysozyme and lactoferrin. Thus neutrophil granules contain enzymes that can kill microbes as well as attack key components of the extracellular matrix.

1.3 Neutrophil Recruitment

1.3.1 The Multistep Paradigm of Neutrophil Recruitment.

To perform their effector functions, leukocytes normally leave the blood stream and enter nearby tissues. A complex set of mechanisms regulate this leukocyte trafficking and these mechanisms vary depending on the type of leukocyte involved as well as the tissue site affected. The process of leukocyte extravasation can be divided into 4 steps; 1) initial contact (tethering), 2) rolling, 3) firm adhesion, and 4) emigration. For neutrophils, these events occur primarily in post-capillary venules [26]. Neutrophils flowing through these venules marginate to the edge of the main stream of flow. This initial positioning of leukocytes near the venular endothelium may occur as a result of interactions with erythrocytes. Interactions of cells flowing in a blood vessel results in smaller cells concentrating towards the center of flow. In post-capillary venules, red cells tend to stick together to form small aggregates (called rouleau formation). These aggregates push the leukocytes toward the vessel wall [27]. As well, when leukocytes leave a capillary, they may

be pushed to the sides of the venule by the red cells that had lined up behind them in the capillary [14]. At points of blood vessel bifurcation red cells are observed to hang momentarily at the apex before re-entering the main flow. The relatively round and inflexible leukocytes do not do this but are swept downstream close to the wall of the daughter vessel they enter [14]. Thus local flow dynamics participate in the movement of leukocytes towards the blood vessel endothelium.

Once in proximity with the venular wall, leukocytes are observed to tether to and slowly roll along the surface of activated endothelium. These initial interactions are known to be mediated by the selectin family of adhesion molecules [28,29]. Selectins are transmembrane glycoproteins that bind to sialylated and fucosylated oligosaccharides in a calcium-dependent manner [28,30-32]. The three known members are P- and E-selectin (inducible to the surface of activated endothelium) and L-selectin (constitutively expressed on leukocytes). The selectins are structurally homologous, composed of a carbohydrate-binding lectin domain, an epidermal growth factor (EGF)-like domain, a series of consensus repeats, a transmembrane domain , and a short cytoplasmic tail. Selectins bind to their ligands primarily through the lectin domain, but can also use the EGF-like domain. The selectin types vary in length due to differences in the number of consensus repeats. The cytoplasmic tail is thought to function in signalling cell activation and in selectin reinternalization and storage [28].

P-selectin is found within membrane bound vesicles termed Weibel Palade bodies and is rapidly (within minutes) expressed to the endothelial surface in response to agonists such as histamine [33], thrombin [33,34], reactive oxygen metabolites [35], and terminal

complement proteins [36]. Most P-selectin expression on endothelium is transient, with only small amounts present after 30 to 60 minutes[36]. P-selectin has been shown to be important in the early stages of leukocyte recruitment both *in-vitro* [37] and *in-vivo* [38]. There is evidence that P-selectin can be transcriptionally regulated in response to inflammatory cytokines (IL-1, TNFα, and lipopolysachariде (LPS)) [39,40]. The significance of this may be to support leukocyte recruitment in ongoing (chronic) inflammation. The primary ligand for P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1), which is constitutively expressed on most leukocyte populations [41-43].

E-selectin expression requires *de novo* synthesis in response to inflammatory cytokines such as IL-1, TNFα, and LPS, with maximal amounts occurring about 4 hours after stimulation, and minimal expression by 24 hours [44,45]. E-selectin is also able to recognize PSGL-1, but other ligands (for example L-selectin, E-selectin ligand-1 (ESL-1), and cutaneous lymphocyte antigen (CLA)) may have significant roles [28,46,47]. E-selectin expression appears to be more tissue specific than P-selectin. For example, E-selectin is important in the dermal vasculature, where it functions as a homing receptor for memory T-cells [48].

L-selectin is located on the tips of microvillus projections of non-activated leukocytes [47,49]. This location allows L-selectin to interact with its endothelial ligands with minimal steric interference from other proteins. L-selectin is proteolytically shed from the surface upon cell activation, the purpose of which may be to expose adhesion molecules that are more proximal to the cell surface (i.e. reduce steric hindrance). This may facilitate the firm adhesion and subsequent emigration of neutrophils from the vasculature. L-

selectin has been shown to be important in lymphocyte homing to lymphoid tissue [50], as well as in neutrophil accumulation during inflammation [51,52]. P- and E-selectin have been implicated as potential ligands [47]. More likely ligands are glyoproteins that contain an epitope termed MECA-79 [28].

Leukocytes are also able to interact with adhesion molecules on other leukocytes. Bargatze et al. reported that neutrophils are able to roll on neutrophil monolayers via L-selectin [53]. Walcheck et al. further characterized this phenomenon by showing that L-selectin interacts with PSGL-1 during neutrophil on neutrophil rolling [54]. P-selectin is expressed on the surface of activated platelets, and can mediate the adhesion of platelets to neutrophils [55]. These data suggest that leukocyte-leukocyte interactions may enhance the rate and/or amount of leukocyte accumulation at inflammatory sites.

Some of the leukocytes rolling on activated endothelium will detach and rejoin the mainstream of blood flow, while others will become stationary and firmly adherent to the endothelium. This firm adhesion is known to be mediated by the integrin family of adhesion molecules. The integrins are a group of heterodimeric transmembrane glycoproteins that function in cell-cell and cell-substrate adhesion and communication. They are important in a wide range of processes including embryogenesis, wound healing, and tumour invasion [56]. The heterodimers are composed of α- and β-subunits, of which there are 14 known α and 8 known β subunits [11]. Most α-subunits non-covalently associate with only one type of β-subunit, but there are notable exceptions. For example, α_4 can associate with either β_1- or β_7-subunits [11]. Integrins are not constitutively active, and cells expressing them require a stimulatory signal before the integrins can function in

adhesion. Integrin activation appears to involve a change in molecular conformation, which allows it to interact with its ligand [56].

For neutrophils, the β_2 integrin subfamily (CD11/CD18 complex) are important for adhesive interactions with endothelium. The three members are LFA-1 (CD11a/CD18), MAC-1 (CD11b/CD18), and p150,95 (CD11c/CD18), each sharing a common β subunit. The importance of β_2 integrins to neutrophil function is demonstrated in leukocyte adhesion deficiency-1 (LAD-1), which is a condition resulting from a deficiency in the CD18 gene. In this disease, there is decreased or no β_2 integrin expression on leukocytes. People with LAD-1 have impaired neutrophil, monocyte, and lymphocyte recruitment, and as a result, suffer from severe and often fatal infections and delayed wound healing [57]. Ligands for the β_2 integrins are varied. The endothelial ligands are the intercellular adhesion molecules (ICAMs), which are members of the immunoglobulin supergene family. Of the three ICAM members, ICAM-1 is of primary importance to neutrophil adhesion, and is upregulated on endothelium in response to cytokines such as IL-1, TNFα, and LPS [58]. ICAM-1 is also expressed on a large variety of cells outside of the vasculature, including fibroblasts, dendritic cells and epithelial cells [59]. As well, there are other extravascular ligands for β_2 integrins, including matrix proteins and complement fragments [56]. This may be significant once neutrophils have left the vasculature and localize at points of inflammation.

As stated before, the integrins require activation before they can function in adhesion. For the β_2 integrins, the activation signal originates from the endothelium. Along with adhesion molecules, endothelium that has been stimulated will express

proadhesive signalling molecules that remain associated with the membrane. Rolling neutrophils have receptors for these molecules, and when engaged, the receptors set off a downstream signal that results in activation of the β_2 integrins [60]. This process has been termed juxtacrine activation and is best characterized for the proadhesive molecule platelet-activating factor (PAF) [33]. Interleukin-8 (IL-8), a cytokine from the C-X-C chemokine family, can also cause juxtracrine activation of neutrophils [60] This process adds a level of control to inflammatory process in that if rolling neutrophils do not receive the integrin activating signal, they will not firmly adhere to the endothelium, and will not enter a site of inflammation [61].

Once neutrophils have adhered to the endothelium they may or may not emigrate out of the vasculature. Whether they do is dependent on the presence of gradients of substances that are chemotactic for neutrophils. Besides being an activator of neutrophil adhesion, IL-8 is also a potent neutrophil chemokine, and is of primary importance in neutrophil emigration [62]. In response to IL-1, TNFα, and LPS, endothelium will produce IL-8, which will induce the transmigration of neutrophils [62]. Neutrophils induced to emigrate will undergo a shape change and spread across the endothelial surface. The cells extend pseudopodia and cross the endothelium via the endothelial cell junctions.

An adhesion molecule termed platelet-endothelial cell adhesion molecule-1 (PECAM-1) is highly expressed along the borders between endothelial cells and on the surface of neutrophils, monocytes, and lymphocytes [63], and has been implicated in leukocyte emigration. PECAM-1 has been shown to facilitate the transmigration of

neutrophils across endothelium *in vitro* [63] and to participate in neutrophil accumulation into the inflamed mouse peritoneal cavity [64].

After crossing the endothelium, neutrophils will continue towards the inflammatory site, following increasing gradients of chemotactic stimuli. Such inflammatory stimuli include bacterial products (formyl peptides), products of phospholipid metabolism (PAF and leukotriene B_4), complement cascade products (eg. C5a) and cytokines (IL-1 and IL-8). Thus through the sequential involvement of different adhesion molecules and receptors for chemotactic factors, neutrophils are able to leave the bloodstream and enter sites of inflammation.

1.3.2 Inappropriate Neutrophil Recruitment

While proper neutrophil recruitment and function are essential to the normal inflammatory response, inappropriate neutrophil activation is associated with the pathology of many diseases including respiratory distress syndrome, ulcerative colitis, and myocardial reperfusion injury [65]. As described in section 1.2, neutophils can release an arsenal of proteolytic and otherwise caustic agents, all of which can destroy cells and connective tissues. For the purpose of this introduction, the pathophysiologic action of neutrophils in myocardial dysfunction will be described.

Myocardial inflammation associated with ischemia/reperfusion, sepsis, and other pathologies has been shown to have a major neutrophil component. Engler et al. observed that neutrophils plug blood vessels in the post-ischemic dog heart [66] while Romson et al. reported reductions in reperfusion-associated myocardial necrosis in dogs that had been

treated with anti-neutrophil serum (reduces circulating neutrophil numbers) [3]. Studies have shown that antibodies against the β_2 integrins were effective at reducing myocardial necrosis [17,67]. The β_2 integrins are known to mediate neutrophil adhesion to vascular endothelium and to cardiac myocytes [19,20]. As well, immunoneutralization of P-selectin reduced myocardial necrosis in the cat[68]. Neutrophils can also induce cardiac dysfunction without causing myocardial cell death. Engler et al. demonstrated contractile disfunction in the absence of myocardial cell necrosis in dog hearts subjected to brief coronary occlusions [69]. Removal of neutrophils from the coronary circulation resulted in attenuation of contractile dysfunction in this model. These studies suggest that blood vessel blockade by adherent leukocytes may be important in cardiomyopathy. An alternate explanation may be that blockade of neutrophil adhesion to cardiac endothelium prevents neutrophil interactions with cardiac myocytes, which may be ultimately responsible for cardiac dysfunction.

Having emigrated out of the vasculature, neutrophils are able to interact directly with cardiac myocytes, however interactions of emigrated neutrophils with myocytes have not been characterized. Entman et al. observed that circulating canine neutrophils adhered to isolated cardiac myocytes, and that this event was mediated by β_2-integrins on the neutrophils [19]. Stimulation of neutrophils with platelet-activating factor (PAF) or zymosan-activated plasma and myocyte stimulation with the inflammatory cytokine, interleukin-1 (IL-1), resulted in increased neutrophil adhesion to myocytes. This adhesion was inhibited by anti-CD18 antibody (blocks β_2-integrins). Adhesion required that both the neutrophil and the myocyte were stimulated with inflammatory agonists, suggesting

that the neutrophil ligand on myocytes is increased during inflammation. Later work by Smith et al. showed that this ligand was ICAM-1 [20]. ICAM-1 expression on cardiac myocytes was increased by IL-1, TNFα, IL-6, or postischemic cardiac lymph [21].

Neutrophil adherence to cardiac myocytes was often associated with sustained contracture (an indicator of cell death) of the myocyte [19]. Entman et al. showed that myocyte death was adhesion- and oxidant-dependent [19]. Neutrophils that were adherent to myocytes were observed to undergo an oxidative burst (as measured by the oxidation of a fluorescent probe within the neutrophil). As well, at the position of neutrophil/myocyte adhesion, myocytes were observed to undergo intracellular oxidation followed shortly thereafter by myocyte contacture. Inhibition of neutrophil/myocyte adhesion (by anti-CD18 or anti-ICAM-1 antibodies) blocked oxidative burst and myocyte death. Intracellular but not extracellular oxygen radical scavengers were effective at blocking myocyte death but not adhesion of neutrophils to the myocytes. From this, the authors concluded in that the neutrophil oxidative burst affected the myocyte at the point of adhesion resulting in myocyte death.

Although these studies were seminal in demonstrating the importance of neutrophils and adhesion molecules in cardiomyopathy, they differ critically from the physiologic condition in that the neutrophils were isolated from whole blood (i.e. circulating neutrophils). Kubes et al. has shown that neutrophils that have emigrated in response to increasing concentrations of a chemotactic gradient express a novel adhesion molecule $\alpha_4\beta_1$ (VLA-4) [12]. Whether this can be extended to suggest that emigrated neutrophils will bind to myocytes via $\alpha_4\beta_1$ is highly speculative. Thus an objective of my

work was to determine if emigrated neutrophils are able to adhere to cardiac myocytes, and if so, to examine whether the adhesive profile that underlies the neutrophil-myocyte interactions changes as a result of emigration.

1.3.3 β_2-Integrin-Independent Neutrophil Recruitment.

While the β_2-integrins are a primary tool used by neutrophils to adhere to vascular and extravascular tissue, several lines of evidence suggest that neutrophils are able to adhere and accumulate by distinct, β_2-integrin (CD18)-independent mechanisms. Human neutrophils were observed to adhere to human umbilical vein endothelial cells (HUVEC) that had been treated with monosodium urate (MSU) crystals [70]. MSU crystals are found associated with tissue in several disease states, including diabetes, arthrosclerosis, and gout. Neutrophil adhesion could not be inhibited by antibodies against CD18, ICAM-1 or selectins, indicating that the activated endothelium was triggering neutrophils to adhere in a CD18- and selectin-independent manner[70]. As well, Issekutz et al. observed that C5a-induced human neutrophil emigration across IL-1-stimulated HUVEC monolayers was only 66% inhibitable by anti-CD18 antibody [71]. Several *in vivo* studies have shown CD18-independent neutrophil accumulation during inflammation. Doerschuk et al. found that rabbit neutrophil accumulation in response to intrabronchial but not intraperitoneal administration of *Streptococcus pneumoniae* or hydrochloric acid was not inhibited by intravascular anti-CD18 antibody [72]. Winn et al. showed that neutrophil emigration was inhibited by only 25% with intravascular anti-CD18 antibody 24 hours after intraperitoneal administration of live *Escherichia coli* into the rabbit peritoneum [73]. Issekutz et al.

observed that neutrophil accumulation in joints of arthritic rats was only partially CD18-dependent, with the remaining component undefined [74]. Thus, while β_2-integrins are important in neutrophil function, other adhesive mechanisms exist and require investigation.

1.4 The β_1-Integrins: VLA-4

1.4.1 Structure and Distribution.

The β_1 subfamily of integrins are called the "very late antigens" and are composed of at least eight different heterodimers (α_1 through α_8 / β_1). Most members of the VLA family mediate adhesion of cells to the extracellular matrix, but one member $\alpha_4\beta_1$ (VLA-4) is known to also be involved in cell-cell adhesion [11]. The α_4-subunit is a 999 amino acid (AA) protein that contains a short cytoplasmic tail (32 AA), a transmembrane domain, followed by seven homologous repeating domains. There are divalent cation binding sites in repeating domains V, VI, and VII [75]. In VLA-4, α_4 is non-covalently associated with a β_1- subunit, which is a 778 AA protein composed of a cytoplasmic tail, transmembrane domain, and an extracellular domain with 4 cystine-rich repeats. The cytoplasmic tail can be phosphorylated, which can affect integrin association with the cytoskeleton, and as a result, integrin location on the cell surface [76]. VLA-4 is expressed on the surface of lymphocytes (both B and T-cells), monocytes, basophils, eosinophils, and mast cells, and neutrophils [11-13,77,78].

Known ligands for VLA-4 include vascular cell adhesion molecule-1 (VCAM-1) and fibronectin [10]. VCAM-1 is a member of the immunoglobulin superfamily and was first

identified on the surface of cytokine-activated human umbilical vein endothelial cells (HUVEC) [10,79]. The predominant form of VCAM-1 found on endothelium contains seven immunoglobulin-like (Ig) domains, with domains I and IV being important for VLA-4 binding [80]. Fibronectin is a constituent of the extracellular matrix, and is also associated with extravascular cells. For example, fibronectin is found within the transverse tubules of cardiac myocytes, and is mobilized from these cells during inflammatory episodes [81].

1.4.1 Non-Neutrophilic Leukocytes and VLA-4.

VLA-4's ability to bind to endothelial ligands has implicated its role in the inflammatory process [9,79,82,83]. Using an *in vitro* system to mimic the flow conditions found within blood vessels, Alon et al. observed that T-lymphocytes and transfected cell lines that express VLA-4 were able to tether, roll, and adhere to VCAM-1-coated coverslips [8]. Rolling cells adhered firmly to VCAM-1 when stimulated with the integrin activating agents, Mn^{2+}, phorbol ester, or an activating antibody. Cells were not able to interact with fibronectin or ICAM-1 (a ligand for β_2-integrins) under shear conditions. Using antibodies against either domain I or IV of VCAM-1, Abe, et al have shown that domain I is important for the initial α_4-integrin-dependent capture of mono- and lymphoblastoid cell lines, while both domains can support adhesion under static conditions [84]. Fluorescently labelled human eosinophils were observed to roll in rabbit mesenteric venules by a mechanism involving both L-selectin and VLA-4 [85]. Thus VLA-4, like the β_2 -integrins, can support firm leukocyte adhesion to endothelium. Unlike the β_2 -integrins,

VLA-4 has the versatility to support leukocyte tethering and rolling, properties thought to be restricted to the selectins.

VLA-4 has been shown to play a role in several inflammatory diseases. In a rodent model of autoimmune disease (experimental allergic encephalomyelitis), Yednock et al. has shown that antibodies against α_4-integrin inhibited the extent of T-lymphocyte infiltration into the rat brainstem, and delayed the negative effects associated with the inflammatory process [86]. Issekutz has shown that both VLA-4 and LFA-1 (CD11a/CD18) contribute to lymphocyte accumulation in the skin during delayed type hypersensitivity responses in the rat, and that inhibition of both pathways is required to achieve maximal anti-inflammatory effect [87]. Baron et al has shown that antibodies to α_4 -integrin or to VCAM-1 delayed the onset of disease in a T-lymphocyte-dependent model of mouse diabetes [88]. Rats with adjuvant-induced arthritis have a systemic vasculitis, which is manifested as a large increase in leukocyte rolling and adherence observed in mesenteric post-capillary venules. Johnston et al. has shown that a significant number of these leukocytes are mononuclear cells (PMBCs), and that α_4 -integrin antibody eliminated PBMCs rolling and adherence [89].

The α_4-subunit can also associate with another β-subunit, namely β_7. $\alpha_4\beta_7$ (also called LPAM-1) has been shown to facilitate lymphocyte homing to lymphoid tissue (Peyer's patches) in the gut. $\alpha_4\beta_7$ binds to mucosal addressin cell adhesion molecule (MAdCAM) found on the high venular endothelium of Peyer's patches [90]. As well, $\alpha_4\beta_7$ will bind to VCAM-1 and fibronectin, but only when cells expressing $\alpha_4\beta_7$ have been activated with phorbol esters [91].

1.4.2 Neutrophils and VLA-4.

Neutrophils were the only leukocyte thought not to express VLA-4, but recently that view has changed. Kubes et al. have found that human neutrophils express both the α_4- and β_1- subunits of VLA-4 when stimulated appropriately[12]. Neutrophils expressed VLA-4 when treated with chemotactic factors in combination with dihydrocytochalasin B (DHCB), a compound that affects the cytoskeleton such that surface receptors are maximally expressed. Using a static adhesion assay, neutrophils stimulated with fMLP, PMA, IL-8, or TNFα adhered avidly to human endothelium (HUVEC), events that were inhibitable by an antibody that blocks CD18 (i.e. β_2-integrin). When DHCB was included with the neutrophil stimulus, neutrophil adhesion was not inhibited by the anti-CD18 antibody. If, in addition to an anti-CD18 antibody, antibodies that block either the α_4- or β_1- subunits of VLA-4 were included, adhesion of maximally activated neutrophils to endothelium was abrogated. Human neutrophils also expressed $\alpha_4\beta_1$ after transmigration across endothelial monolayers grown on semipermeable supports. These transmigrated neutrophils could effectively adhere to protein-coated plastic by a mechanism that involved both β_2- and β_1-integrins. This was the first study to show that human neutrophils can express the β_1-integrin, VLA-4, the function of which is revealed when β_2-integrins are blocked.

Subsequent to our study, Gao, et al has also demonstrated a role for VLA-4 in human neutrophil function [78]. Neutrophils expressed low levels of α_4-integrin when stimulated with the complement fragment, C5a. This occurred without the need for

costimulation with a cytoskeletal disrupting agent, such as DHCB. C5a-induced transmigration of neutrophils across monolayers of human synovial and dermal fibroblasts was inhibited (20-30% inhibition) by addition of an anti-α_4 antibody or a fibronectin fragment that contains the CS-1 binding domain of VLA-4. Interestingly, β_2-integrins did not need to be blocked to reveal the function of VLA-4.

Issekutz, et al, have shown that VLA-4 is constitutively expressed on rat neutrophils [13]. Treatment of rats with antibodies against α_4-integrin and CD11a/CD18 (LFA-1), but neither antibody alone, resulted in decreased neutrophil accumulation in arthritic joints and inflamed skin.

While implicating a role for VLA-4 in neutrophil recruitment, previous studies have not determined at which step(s) VLA-4 becomes involved in the recruitment cascade. To do this requires observation of VLA-4-expressing neutrophils interacting with relevant biologic substrata under shear conditions. As well, while emigrated neutrophils express functional VLA-4, whether this is sufficient to support adhesion to relevant extravascular tissue remains to be determined. The work proposed in this thesis was designed to further elucidate the function of VLA on neutrophils.

1.5 Statement of Hypothesis and Objectives.

Hypothesis 1: Neutrophils can employ $\alpha_4\beta_1$ (VLA-4) to interact with biologic substrata under shear conditions.

Objectives:

1) To determine if DHCB-primed neutrophils can utilize VLA-4 for tethering, rolling and adhesion to biologic substrates including TNFα-activated endothelium, a cell line expressing VCAM-1, or purified fibronectin protein.

2) To compare VLA-4-dependent neutrophil behaviour under shear conditions to that of a leukocyte cell line (Ramos) that constitutively expresses VLA-4.

Hypothesis 2: Erythrocytes will enhance neutrophil interactions with VCAM-1 under shear conditions.

Objectives:

1) To determine if unstimulated neutrophils in the presence of whole blood can be recruited onto VCAM-1.

2) To determine which shear forces are optimal for the recruitment of neutrophils to VCAM-1.

3) To determine if neutrophil recruitment is affected by changing the amount of VCAM-1 used as the experimental substrate (i.e. VCAM-1 density).

Hypothesis 3: Neutrophils can employ VLA-4 to adhere to inflamed parenchymal tissues.

Objectives:

1) To determine whether rat neutrophils use VLA-4 to adhere to freshly isolated rat ventricular myocytes, and if so, what is the ligand on the myocyte.

2) To determine if the process of emigration can alter the mechanisms by which neutrophils adhere to myocytes.

3) To determine whether the adhesion of neutrophils to myocytes can be affected by the inflammatory state of the myocyte.

CHAPTER 2

METHODS AND MATERIALS

2.1 Experimental Models

2.1.1 *In vitro* Flow Chamber Assay

To study neutrophil behaviour under shear conditions, a flow chamber assay was established as previously described [92] (Figure 2.1). This model allows for observations of neutrophil interactions with various biologic substrata at defined shear forces. Biologic substrata were coated onto glass coverslips, which were mounted into a polycarbonate chamber with parallel plate geometry. The flow chamber was placed onto the stage of an inverted microscope (Zeiss Canada) and monolayers were visualized at 100× magnification using phase contrast imagery. The stage area was enclosed in a warm air cabinet and maintained at 37°C. Leukocyte suspensions were warmed to 37°C using a water bath. A syringe pump (Harvard Apparatus, Canada) was used to draw the cell suspensions through the flow chamber at defined wall shear stresses. Experiments were video recorded for later analysis via a CCD camera (Hatachi Denshi, Ltd. Japan) and a video cassette recorder (Panasonic, Secuacus, NJ) that were attached to the microscope.

Neutrophils interacting with the coverslip surface experience shear force as a result of the fluid flowing past. Parameters that affect shear force include the flow rate and the viscosity of the perfusate as well as the dimensions of the chamber. Shear force within the flow chamber is calculated using the following equation.

$$R = \frac{6\mu Q}{B^2 W}$$

μ= Viscosity

Q= Flow Rate

B= Chamber Gap (see Figure 2.1)

W= Chamber Width (see Figure 2.1)

Various biologic substrates were used in the flow chamber apparatus, including human endothelium, transfected cell lines that express specific adhesion molecules, isolated adhesion molecules, and matrix proteins. Human umbilical vein endothelial cells (HUVEC) were harvested from freshly collected umbilical cords. Umbilical cord veins were rinsed of formed blood products with phosphate-buffered saline (PBS), after which, the vein was filled with a collagenase solution (320 units/ml in PBS). After a 20 minute incubation at 37°C, the cords were gently massaged to ensure detachment of endothelial cells from the vessel wall. The digest was collected into centrifuge tubes and the collagenase inactivated with fetal calf serum, after which, the tube was centrifuged (400g for 10 min at 37°C). The pellet was resuspended in medium 199 containing 10% fetal calf serum (FCS), endothelial mitogen (Biomedical Technologies, Inc., Stoughton, MA) and antibiotics. The endothelial cells were seeded onto 75 cm^2 culture flasks and maintained in 5% CO_2 at 37°C and 96% humidity. HUVEC were expanded by trypsinization and utilized for experimentation before the fifth passage.

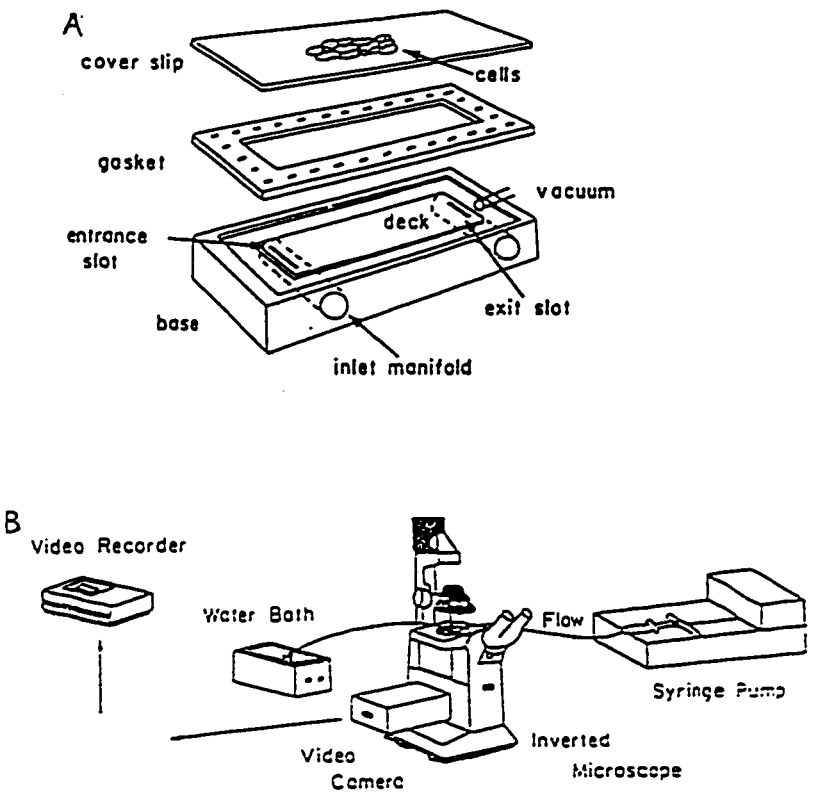


Figure 2.1: Diagram of the flow chamber apparatus used to examine leukocyte interactions with biologic substrata under shear conditions[92]. Biologic substrata were grown or coated onto a glass coverslip (A). Leukocyte suspensions were drawn through the chamber, and interactions were observed with phase-contrast microscopy (B).

VCAM-1 and ICAM-1 mouse L-celi transfectants were a gift from Dr. J.F. Elliot, Dept. of Medical Microbiology and Immunology, University of Alberta, Canada. The construction of mouse L-cell transfectants was accomplished as follows. cDNA's encoding VCAM-1 and ICAM-1 proteins were generated by polymerase chain reaction and ligated into the plasmid pJFE14 using the XbaI and NotI sites. pMSD4-HPRT, which expresses murine HPRT under the control of a Moloney murine leukemia virus promoter, was a gift from Dr. D. Denney (Department of Medical Microbiology and Immunology, University of Alberta, Canada). Mouse LA9 cells (ATCC, Rockville, Maryland) that permanently express VCAM-1 or ICAM-1 were constructed by co-transfecting Sal I cut pJFE14 containing the appropriate cDNA (200 μg/electrophoration) and SAL I cut pMSD4-HPRT DNA (10 μg/electrophoration). After electrophoration (BioRad Gene Pulser apparatus), cells were cultured in selection media (Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal calf serum (Hyclone) and hypoxanthine 100 μM/azaserine 5.8 μM)). After 10 to 14 days, single colonies of HPRT+ LA9 cells were picked using cloning cylinders and expanded. Clones were incubated for an additional 6 to 10 days in selection media, after which they were cultured in DMEM containing 10% FCS. VCAM-1 expression on TNFα-stimulated HUVEC was 60% of that found on VCAM-1 transfectants as determined by ELISA assay.

HUVEC, VCAM-1 transfectants, or ICAM-1 transfectants were seeded onto glass coverslips (Fisher Scientific, Ottawa, Ontario) at a density of 1×10^6 cells per coverslip. The cells were allowed to grow to form confluent monolayers. Coverslips were mounted into the flow chamber just prior to experimentation.

Human neutrophils were harvested from citrate anticoagulated venous blood collected from healthy donors as previously described [93] with minor modification. All isolation steps were performed at room temperature. Neutrophils were purified by dextran sedimentation (Dextran 250 000, Spectrum Chemicals) followed by centrifugation through a density gradient ((6.07% Ficoll Type 400 (Sigma Chemical Co., St. Louis) with 10% Hypaque Sodium (Winthrop-Breon, NY)). Isolated neutrophils were resuspended in HBSS at a density of 1×10^6 cells/ml. This yielded neutrophils that were 97% pure and 95% viable.

$\alpha_4\beta_1$-dependent interactions of neutrophils with various substrata was compared directly to Ramos cells (from American Type Culture Collection, Rockville, MD) which use this integrin to tether, roll and adhere. Ramos cells were cultured in RPMI medium 1640 with 10% FCS. For experimental procedures, Ramos cells were pelleted and resuspended in Hank's balanced salt solution (HBSS) at a density of 1×10^6 cells/ml.

Soluble recombinant human VCAM-1 was a gift from Dr. R. Lobb, Biogen Inc. Cambridge, MA. This VCAM-1 contains the seven extracellular domains and is truncated at the transmembrane domain. Coverslips were coated with VCAM-1 at various concentrations (1.0, 2.5, 5.0, and 10.0 μg/ml) and incubated at 4 °C for 18 hours. To inhibit non-specific interactions with the glass, coverslips were then incubated with 1% bovine serum albumin (BSA) at 37 °C for 2 hours. Whole blood for perfusion over VCAM-1-coated coverslips was collected from healthy donors and heparinized (30 units/ml) to prevent clotting.

2.1.2 Neutrophil/Myocyte Adhesion Assay. To assess rat neutrophil adhesion to isolated cardiac myocytes, an *in vitro* adhesion assay was employed (Figure 2.2). In this assay, two circular coverslips separated by an O-ring gasket (Bellco Glass Inc., Vineland, N.J.) are mounted into a small metal chamber. Neutrophil suspensions are injected through the gasket and into the chamber. The neutrophils will settle by gravity and interact with the bottom coverslip. Once the chamber is inverted, non-adherent neutrophils will fall, and the amount of neutrophils that remain adherent can be determined by microscopy. Smith et al. has used this system to successfully study neutrophil/endothelial cell adhesion [94]. For this study, freshly isolated rat cardiac myocytes were coated onto the coverslips,allowing for the assessment of neutrophil/myocyte adhesion.

Circulating rat neutrophils were isolated from citrate-anticoagulated whole blood collected by cardiac puncture. Red blood cells were removed by dextran sedimentation followed by hypotonic lysis. Neutrophils were further purified by centrifugation through a Histopaque gradient, and resuspended in Hanks balanced salt solution (HBSS) at $2x10^7$/ml. To obtain emigrated neutrophils, rats were injected intraperitoneally with 10 ml of 1% oyster glycogen in phosphate-buffered saline (PBS). After four hours, the rats were sacrificed and the peritoneal fluid collected. Following centrifugation, the emigrated neutrophils were resuspended in HBSS at $2x10^7$/ml. Neutrophils were

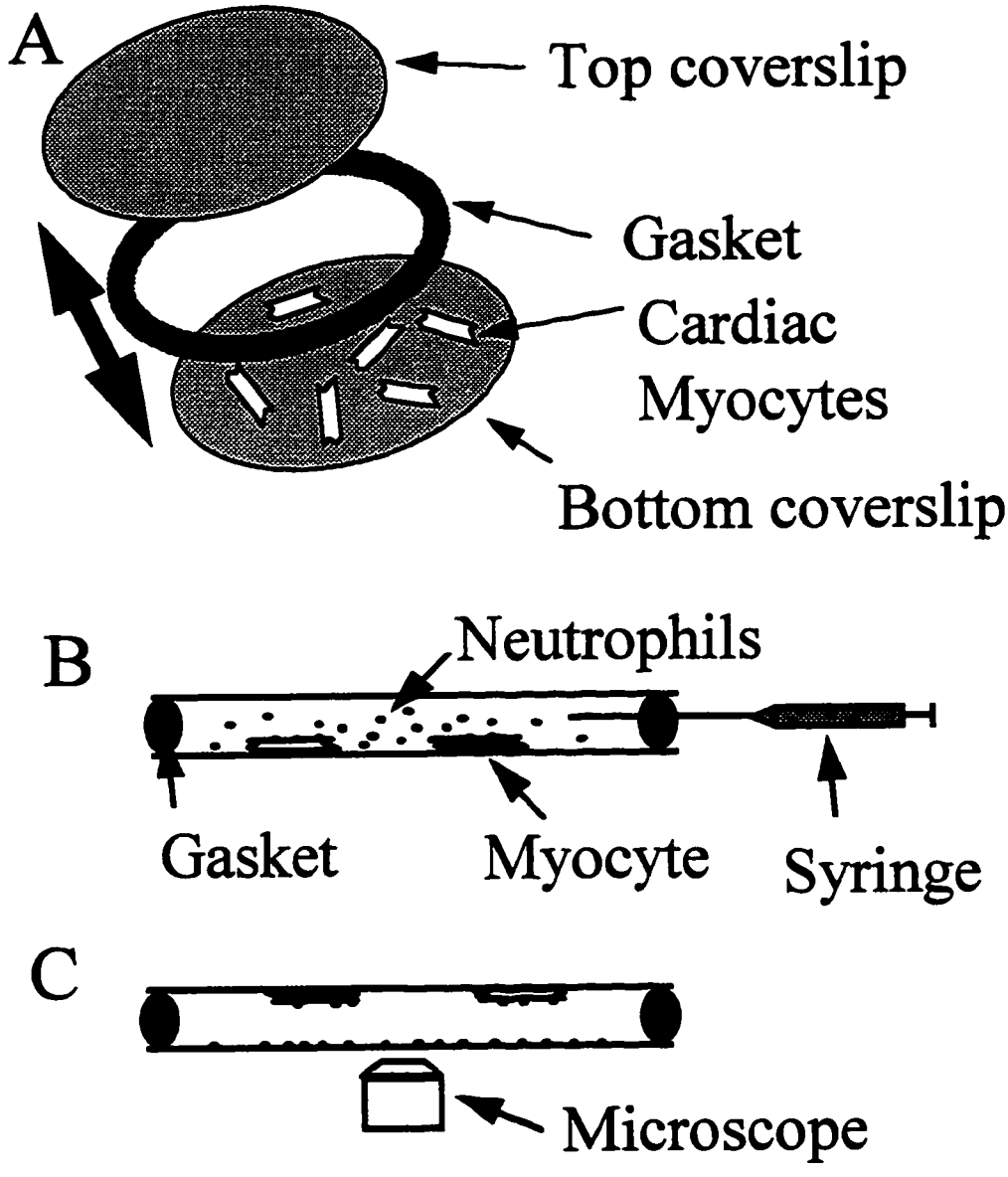


Figure 2.2: Diagram of the adhesion assay chamber used to examine neutrophil adhesion to cardiac myocytes (A). Neutrophils are injected into the chamber and allowed to settle onto the myocytes (B). When the chamber is inverted (C) the non-adherent neutrophils fall to the other side, allowing for assessment of neutrophil/myocyte adhesion.

activated by treating them with 20 μM N-formyl-Met-Leu-Phe (fMLP) or 10 nM CINC-gro just prior to injection into the adherence assay chambers. Antibodies were added at the time of activation.

To measure surface expression of α_4-integrin, flow cytometry techniques were employed. Circulating or emigrated rat neutrophils (1×10^6/tube) were fixed in 1% formalin (30 minutes at 4°C) then washed. Two μg of TA-2 antibody was added per tube to stain for α_4-integrin. After 30 minutes, the cells were washed and labelled with FITC-conjugated goat anti-mouse IgG (Serotec) and incubated at room temperature for a further 30 minutes. After washing, the level of α_4-integrin expression was measured on a FACScan® flow cytometer. (Becton Dickonson Immunocytochemistry Systems, Mountain View, CA).

Right ventricular myocytes from rats were isolated as previously described [95]. Briefly, the heart was rapidly removed from decapitated animals and Tyrode's solution was perfused retrograde (10 ml/minute for 5 minutes) via a canula attached to the aorta. The heart was then perfused with Tyrodes solution containing collagenase (0.02mg/ml), protease (0.004 mg/ml), and taurine (2.4 mg/ml) for 7 minutes, after which the right ventricle was separated from the heart and minced with 10 ml of Tyrode's containing collagenase (0.05 mg/ml), protease (0.1 mg/ml), taurine (2.5 mg/ml), bovine serum albumin (BSA) (5 mg/ml), and $CaCl_2$ (50 mM). Following agitation for 10 to 20 minutes at 34°C, the supernatant (containing separated myocytes), was collected and placed in

Tyrode's solution with BSA (5 mg/ml), taurine (2.5 mg/ml), and $CaCl_2$ (50 mM). Taurine was added to prolong the viability of the myocytes during storage.

To mimic an inflammatory state, tubes containing ventricular myocyte suspensions (approximately $1x10^4$ cells/ml) were dosed with TNFα (300 units/ml) and placed on a rotating rack for 4 hours at 37°C, after which time, the myocytes were layered onto coverslips and incubated for 1 more hour. Control myocytes were subjected to the same protocol without dosing with TNFα.

2.2 Experimental Protocols

2.2.1 Neutrophil Adhesion Via $\alpha_4\beta_1$-Integrin Under Flow Conditions.

$\alpha_4\beta_1$-dependent neutrophil interactions with TNFα-stimulated endothelium (300 units/ml) were performed in the presence of dihydrocytochalasin B (DHCB) at 2.5 μg/ml. This concentration of DHCB in the presence of an endothelial-derived chemotactic agent (as a result of TNFα stimulation) has previously been shown to induce significant $\alpha_4\beta_1$-integrin expression on neutrophils [12]. DHCB was present in the perfusion medium throughout the tethering, rolling and adhesion assays. Neutrophils were pre-treated with DHCB at 2.5 μg/ml for 10 minutes. Monoclonal antibody IB_4 (20 μg/ml) directed against the β_2-integrin was used alone or in combination with anti-α_4 MAbs [HP2/1 (2.0 μg/ml), HP1/2 (2.0 μg/ml)], anti-β1 integrin [MAbs LIA 1/2 (2.0μg/ml) or K20 (2.0 μg/ml)] to elucidate the adhesive mechanism of DHCB-pre-treated neutrophils with TNFα-stimulated HUVEC under shear conditions (2 dynes/cm^2). A role for selectins on $\alpha_4\beta_1$-expressing neutrophils

was determined using an anti-E/L-selectin antibody MAb EL-246 (50 μg/ml) or the selectin-binding carbohydrate fucoidan (75 μg/ml). In additional experiments, MAb EL-246 was added and neutrophils were perfused under conditions of decreasing shears (0.5 dynes/cm^2 at 2 minute intervals) in the presence and absence of HP2/1. This was done to establish whether $\alpha_4\beta_1$ could tether neutrophils to endothelium independent of selectins.

To determine whether $\alpha_4\beta_1$-expressing neutrophils could support interaction with VCAM-1 transfected L-cells, neutrophils expressing $\alpha_4\beta_1$ were perfused over either VCAM-1 or ICAM-1 transfectants at a starting shear of 2 dynes/cm^2. As neither Ramos cells nor $\alpha_4\beta_1$-expressing neutrophils would tether to VCAM-1 transfectants, shear was decreased by 0.5 dynes/cm^2 at 2 minute intervals. In this series of experiments TNFα could not be used (as endothelium was not used) so neutrophils were pre-treated with an exogenous chemotactic agent N-Formyl-Met-Lue-Phe (fMLP) (20 μM) + DHCB (2.5 μg/ml) for 10 minutes which also expresses $\alpha_4\beta_1$ [12]. In additional experiments Ramos cells were also pre-treated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml for 10 minutes) to increase $\alpha_4\beta_1$ avidity. Detachment assays were performed on $\alpha_4\beta_1$-expressing neutrophils or Ramos cells by allowing the leukocytes to settle for 2 minutes on VCAM-1 transfectants under static conditions and then re-establishing flow through the chamber to 2 dynes/cm^2.

In addition to $\alpha_4\beta_1$-integrin interacting with VCAM-1, $\alpha_4\beta_1$ also interacts with the CS-1 region of fibronectin [96]. $\alpha_4\beta_1$-expressing neutrophils were perfused over fibronectin-treated coverslips (25μg/ml for 30 minutes) under conditions of decreasing shear. Neither

Ramos nor $\alpha_4\beta_1$-expressing neutrophils interacted with this surface at any shear stress tested so static adhesion assays were performed.

Rolling and adhesive interactions of perfused cells with cell monolayers or fibronectin-coated coverslips were video-recorded and analyzed as follows. Cell counts were made for the field of microscopic view and represented as cells per millimetre squared of the monolayer surface. A rolling cell was defined as a cell that was in contact with the coverslip but was not stationary. These cells could be observed to roll end over end across the monolayer surface. An adherent cell was defined as one that was in contact with the monolayer and remained stationary for at least 10 seconds. All experiments were repeated between 3 and 8 times.

To test the ability of VLA-4 on neutrophils to support adhesion to fibronectin, experiments were performed under flow conditions (as described above) or under static conditions. For static adhesion assays, neutrophils were radiolabelled by incubating purified neutrophils with $Na^{51}Cr0_4$ (30μCi/ml) at 37°C for 30 minutes. The cells were washed 3 times and resuspended in HBSS at 2×10^7 cells/ml. Forty-eight well plates (Costar, Cambridge, MA.) were coated with human fibronectin (25μg/ml in PBS) and Cr^{51}-radiolabelled neutrophils were added to the wells (1×10^6/well in 500μL HBSS) and the plate was incubated for 30 min at 37°C. At the end of the incubation, the supernatant of each well was collected and the wells were gently washed with 500μL of HBSS. The cells that remained adherent were lysed by an overnight incubation with 500μL of NaOH (2 N). Supernatant, wash, and lysate were analyzed for ^{51}Cr activity and the neutrophil adherence was calculated as the ratio of radioactivity in the cell lysate versus that in

supernatant, wash, and lysate combined. $\alpha_4\beta_1$-expressing neutrophils were exposed to fibronectin in the presence of antibody IB_4 (20μg/ml) in combination with a fibronectin fragment (fragment 40K, 0.2 μg/ml) that contains the CS-1 binding region for $\alpha_4\beta_1$, or with antibody HP2/1 (2.0 μg/ml).

2.2.2 Neutrophil Recruitment From Whole Blood Via $\alpha_4\beta_1$-Integrin Under Flow Conditions. To determine if red blood cells could influence VLA-4-dependent neutrophil recruitment onto VCAM-1, whole blood was perfused over VCAM-1-coated coverslips at defined shear forces (15, 10, 5 or 2 dynes/cm^2) for 5 minutes. To examine if VCAM-1 density has an effect on neutrophil recruitment, coverslips were coated with VCAM-1 at various concentrations (1.0, 2.5, 5.0, and 10.0 μg/ml). To determine if leukocyte interactions were α_4 integrin-dependent, anti-α_4 integrin antibody (HP1/2 at 2 μg/ml) was added to the blood in some experiments. HBSS was perfused after the blood to remove red cells and non-interacting leukocytes. After approximately 20 seconds, the chamber became optically clear, allowing for videorecording of interacting (rolling and adhering) leukocytes. In the subsequent 2 minutes, a minimum of 4 fields of view were recorded, and the numbers of rolling and adhering leukocytes determined during later playback analysis. The coverslips were then gently removed from the chamber and allowed to air-dry before leukocyte differential staining (Wright-Giemsa). By this process, the morphology, and thus the type, of interacting leukocytes could be determined. Leukocytes were catagorized as either neutrophils eosinophils, lymphocytes, or monocytes.

2.2.3 Emigrated Rat Neutrophil Adhesion To Cardiac Myocytes Via α_4-Integrins. 25-mm round glass coverslips (Bellco Glass Inc., Vineland, N.J.) were pre-

treated with 1 ml of 1% gelatin in PBS for 60 minutes at 37 °C. The coverslips were washed once in PBS and 1 ml of myocyte suspension was gently layered onto the coverslip. The myocytes were allowed to settle and adhere to the coverslips for 60 minutes at 37 °C, after which the coverslips were inserted into adherence assay chamber. The chamber is designed to hold two coverslips separated by an O-ring gasket (Bellco Glass Inc., Vineland, N.J.). Neutrophil suspensions (diluted to 1×10^6/ml in HBSS) were injected into the chamber and allowed to settle onto the cardiac myocytes for 10 minutes, after which, the chambers were inverted. At this point, non-adherent neutrophils settled to the other side of the chamber. This design allowed for visualization of the cardiac myocytes (200× magnification) using phase contrast microscopy with an inverted microscope (Zeiss Canada).

The number of adherent neutrophils per myocyte was determined for a minimum of 16 myocytes on each coverslip with each condition repeated 3 to 8 times.

2.4 Statistics

All values are reported as mean ± standard error of the mean. The data were analyzed using standard statistical analysis (for example, students t test or analysis of variance). Statistical significance was set at $P < 0.05$.

CHAPTER 3:

NEUTROPHILS CAN ADHERE VIA $\alpha_4\beta_1$-INTEGRIN UNDER FLOW CONDITIONS

Hypothesis : Neutrophils can employ $\alpha_4\beta_1$ (VLA-4) to interact with biologic substrata under shear conditions.

Objectives:

1) To determine if DHCB-primed neutrophils can utilize VLA-4 for tethering, rolling and adhesion to biologic substrates including TNFα-activated endothelium, a cell line expressing VCAM-1, or purified fibronectin protein.

2) To compare VLA-4-dependent neutrophil behaviour under shear conditions to that of a leukocyte cell line (Ramos) that constitutively expresses VLA-4.

3.1 Results

Unstimulated neutrophils interact with cytokine-stimulated endothelium under shear conditions via selectins and β_2-integrins. Freshly isolated neutrophils were observed to tether and roll on TNF-α stimulated endothelium (HUVEC) at a wall shear stress of 2 dynes/cm^2 (Figure 3.1.A). As well, rolling cells were observed to stop and firmly adhere to the endothelium (Figure 3.1.B). Tethering, rolling and adhesive interactions increased during the time of perfusion. Addition of a monoclonal antibody (EL-246) that inhibits the actions of both E- and L-selectin completely blocked tethering and rolling (Figure 3.1.A), indicating that these interactions are selectin-dependent. Identical results were observed when fucoidan, a selectin binding polysaccharide, was administered. These regimens also completely inhibited neutrophil adhesion consistent with the view that tethering and rolling are a pre-requisite for firm adhesion (Figure 3.1.B). Addition of a monoclonal antibody (IB_4) that immunoneutralizes β_2-integrins also

blocked neutrophil adhesion to TNFα-stimulated endothelium (Figure 3.1.B). IB_4 did not inhibit this adhesion by affecting the ability of neutrophils to tether and roll on endothelium. In fact, IB_4-pre-treatment resulted in an increase in the number of rolling cells (untreated: 79.4 ±14.7 cells/mm^2 vs. IB_4-treated: 196.6 ±12.1 cells/mm^2; 5 minute values) most likely as a result of the reduced propensity of these cells to adhere.

$\alpha_4\beta_1$-expressing neutrophils adhere to TNFα-stimulated endothelium independent of β_2 -integrin. Kubes et al. have previously demonstrated that neutrophils can be induced to express $\alpha_4\beta_1$ when stimulated with DHCB in combination with an exogenous chemotactic stimulus or in the presence of an endogenous chemotactic stimulus (derived from TNFα-stimulated endothelium) [12]. Addition of DHCB did not increase the number of rolling cells (Figure 3.2.A) or affect rolling velocity (untreated neutrophils: 7.6 ± .52 μm /sec vs. DHCB-treated neutrophils: 6.75 ± .29 μm/sec) on TNFα-stimulated endothelium. DHCB-treatment did result in a greater propensity of rolling cells to adhere (a two fold increase) relative to their untreated neutrophil counterparts (Figure 3.2.B). The rolling of DHCB-treated neutrophils was still entirely selectin-mediated, as pre-treatment with EL-246 or fucoidan (Figure 3.3) completely blocked any rolling interaction. DHCB-treated neutrophil adhesion was reduced by the β_2-integrin antibody (IB_4) but a significant degree of CD18-independent adhesion remained (Figure 3.4). It should be noted that neither untreated nor DHCB-treated neutrophils interacted with unstimulated endothelium under shear conditions of 2 dynes/cm^2.

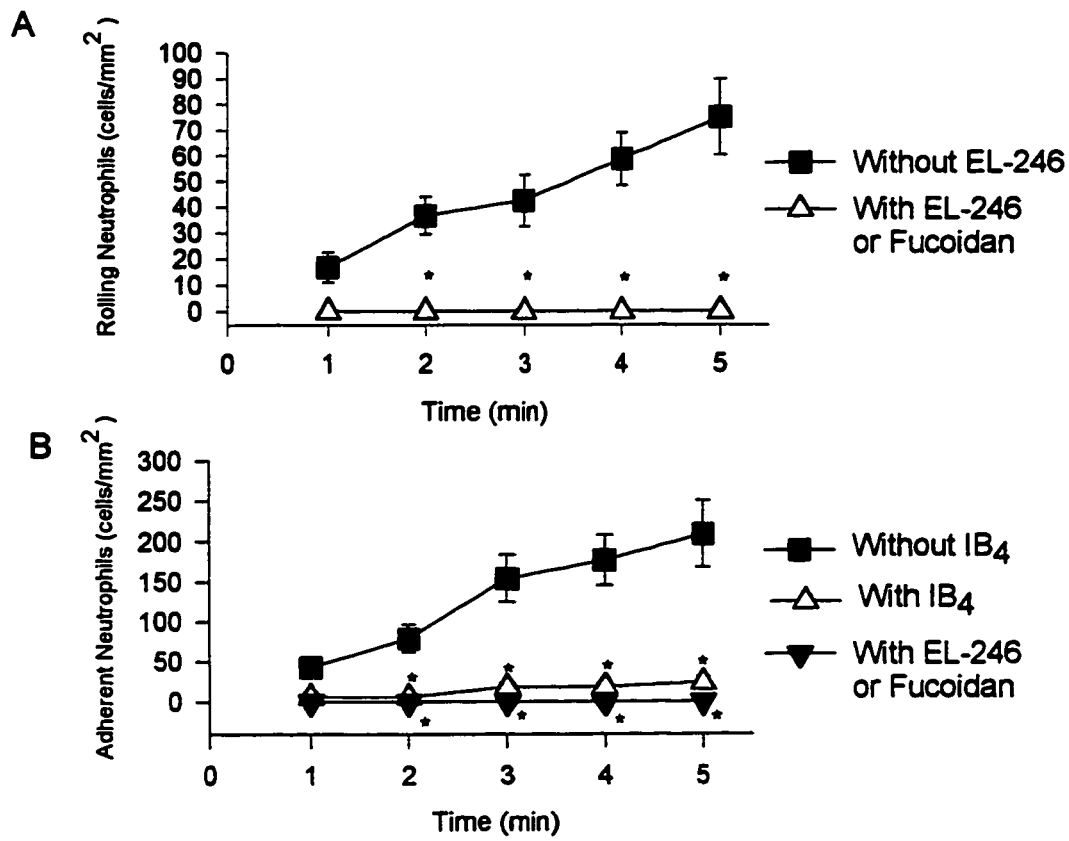


Figure 3.1: Neutrophils tether to TNFα-treated endothelium, roll (panel A) and adhere (panel B). The tethering and rolling was entirely inhibited by an E-selectin/L-selectin antibody (EL-246) or a selectin-binding carbohydrate (fucoidan) (panel A), and hence adhesion was eliminated (panel B). The anti-CD18 antibody (IB_4) also inhibited neutrophil adhesion (panel B).

* $p<0.05$ relative to untreated neutrophils.

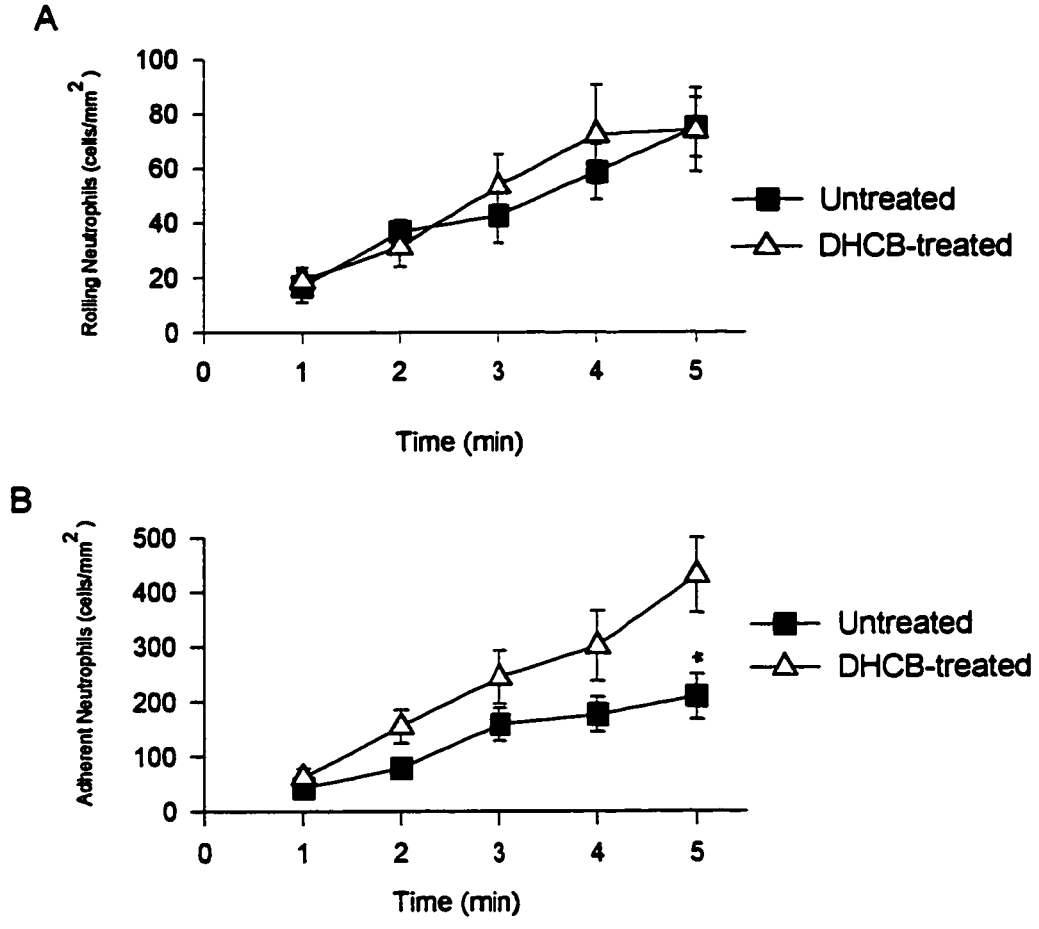


Figure 3.2: Illustrates that addition of DHCB (causes $\alpha_4\beta_1$ expression in the presence of chemotactic stimuli) had no further effect on the number of rolling neutrophils on TNFα-treated endothelium (panel A). Neutrophil adhesion (panel B) was further increased (doubled) relative to neutrophils not treated with DHCB.

* $p<0.05$ relative to untreated cells.

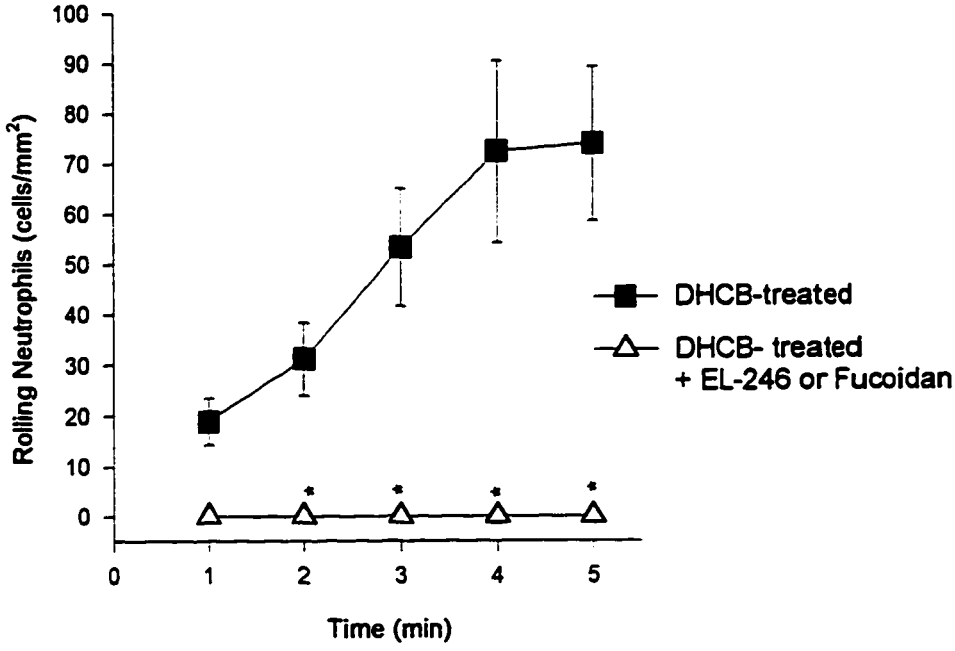


Figure 3.3: Addition of EL-246 or fucoidan eliminated rolling of DHCB-treated neutrophils on TNFα-stimulated endothelium.

* $p<0.05$ relative to DHCB-treated cells.

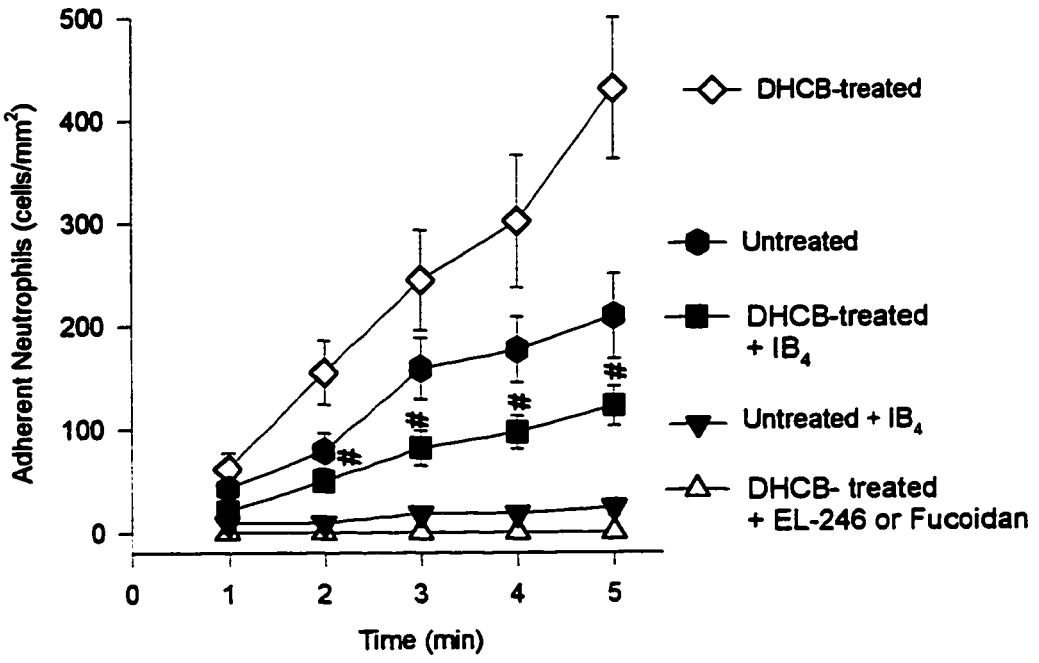


Figure 3.4: Whereas Anti-CD18 antibody (IB_4) completely inhibited adhesion of untreated neutrophils on endothelium, IB_4 was only 60% effective at reducing adhesion of DHCB-treated neutrophils. As with untreated neutrophils (Fig. 3.1.B), anti-selectin treatment (EL-246 or fucoidan) blocked adhesion of DHCB-treated neutrophils.

# $p<0.05$ relative to untreated neutrophils + IB_4.

DHCB-treated neutrophils adhere to cytokine stimulated endothelium via the integrin, $\alpha_4\beta_1$. Figure 3.5 demonstrates that the β_2-integrin-independent adhesion of DHCB pre-treated neutrophils was abrogated if, in addition to IB_4, the neutrophils were pre-treated with antibodies that inhibit the α_4 portion of the $\alpha_4\beta_1$ integrin (HP2/1 or HP1/2). To determine whether the β_1 subunit of $\alpha_4\beta_1$-integrin was involved, we pre-treated some cells with an inhibitory antibody that binds β_1-integrin (K20) [12,97]. This antibody was as effective as the α_4-integrin antibodies in preventing DHCB-treated neutrophil adhesion to TNFα-stimulated HUVEC. A second anti-β_1-integrin antibody (LIA1/2), also elicited identical inhibitory results. An isotype control antibody had no effect on the number of adherent cells. Addition of the anti-α_4 antibody, HP2/1, by itself (in the absence of an anti-β_2-integrin antibody) did not inhibit $\alpha_4\beta_1$-expressing neutrophil adhesion to TNFα-stimulated endothelium [DHCB-treated (430.8±68.8); DHCB-treated+HP2/1 (400.6±53.7), 5 minute values].

Table 3.1 summarizes the rolling data for $\alpha_4\beta_1$-expressing neutrophils. Each of the antibodies directed against either the α_4 or β_1 subunit did not decrease the number of rolling neutrophils. In fact, the inhibition of adhesion with α_4 or β_1-integrin antibodies resulted in increased numbers of rolling neutrophils, an observation previously reported by F.W. Luscinkas, et al, for monocytes [9]. Addition of the anti-α_4 antibody, HP2/1 by itself (in the absence of an anti-β_2-integrin antibody) did not effect neutrophil rolling. As shown in figure 3.3, selectins mediated all of the tethering and rolling at 2 dynes/cm^2.

Furthermore, when shear was reduced by 0.5 dyne/cm^2 at 2 minute intervals in the presence of EL-246, DHCB-treated neutrophils were observed to tether and immediately adhere to the endothelium (Figure 3.6), with adhesion increasing as shear was decreased. This interaction was inhibited by anti-α_4 antibody (HP2/1).

$\alpha_4\beta_1$-expressing neutrophils can tether to VCAM-1: To further characterize the ability of $\alpha_4\beta_1$ to support neutrophil interactions under shear conditions, we used L-cells that had been transfected with cDNA encoding for human VCAM-1, such that the only adhesion molecule that these cells express is VCAM-1. As with the human endothelium, transfected L-cells were grown onto coverslips before serving as a substrate in the flow chamber. For a control, L-cells transfected with human ICAM-1 were also employed. As shown above, the combination of DHCB and factors produced from cytokine stimulated endothelium was sufficient to induce $\alpha_4\beta_1$-dependent neutrophil behaviour. For experiments on L-cells expressing either VCAM-1 or ICAM-1, neutrophils were stimulated with DHCB and a chemoattractant (FMLP), a combination known to directly induce $\alpha_4\beta_1$ expression on neutrophils[12].

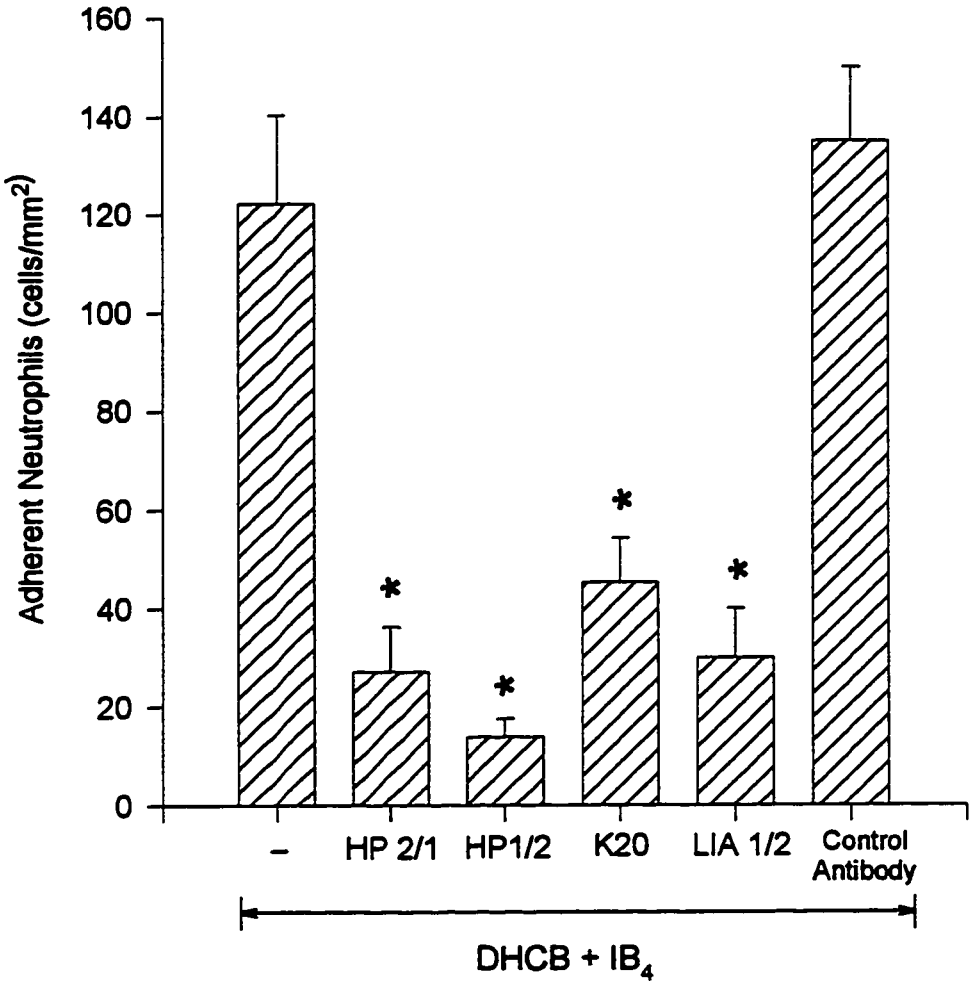


Figure 3.5: The adhesion of DHCB-treated neutrophils to TNFα–stimulated endothelium at 2 dynes/cm^2 (five minute values). Addition of the anti-α_4-integrin antibodies HP2/1 or HP1/2 inhibited the remaining firm adhesion in the presence of IB_4. Similar results were seen with the addition of the anti-β_1 antibodies K20 or LIA1/2. A control antibody was not inhibitory.

*$p<0.05$ relative to DHCB-treated neutrophils + IB_4.

Table 3.1: Rolling Data for $\alpha_4\beta_1$-Integrin-Expressing Neutrophils.

Neutrophil Treatment	Rolling Cells (#/mm^2)
DHCB + IB_4	84.5 ±12.3
DHCB + IB_4 + HP2/1 (anti-α_4)	149.9 ±22.7*
DHCB + IB_4 + HP1/2 (anti-α_4)	114.5 ±2.5*
DHCB + IB_4 + K20 (anti-β_1)	122.7 ±4.5*
DHCB + IB_4 + LIA1/2 (anti-β_1)	150.8 ±55.7*
DHCB + IB4 + Isotype Control	73.1 ±15.6
DHCB + HP2/1	96.2 ±9.2

Table 3.1 illustrates that $\alpha_4\beta_1$-integrin does not support rolling of DHCB-treated neutrophils on TNFα-stimulated endothelium. In each case, the addition of anti-α_4 (HP2/1 or HP1/2) or anti-β_1 (K20 or LIA1/2) antibodies blocked the adhesion, which translated into increased rolling (5 minute values).

*$P<0.05$ relative to DHCB+IB_4 condition.

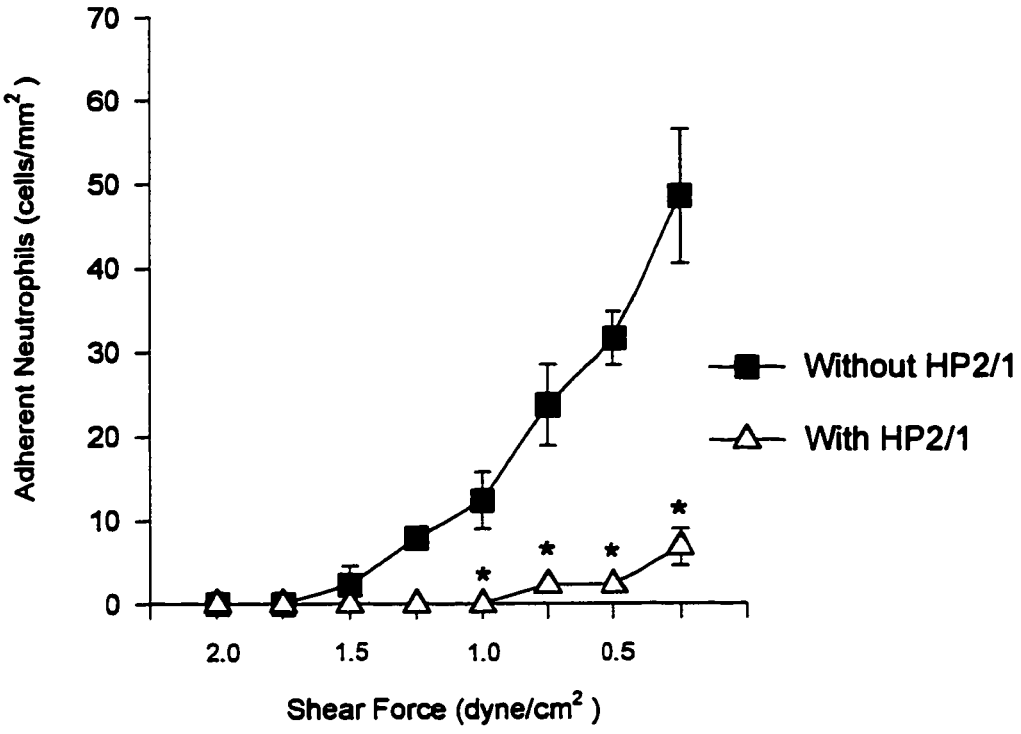


Figure 3.6: In the presence of anti-selectin antibody (EL-246), DHCB-treated neutrophils did not roll or adhere to TNFα-treated endothelium at 2 dynes/cm^2. Shear was decreased at 2 minute intervals and neutrophil tethering and adhesion was examined in the presence and absence of the anti-α_4-integrin antibody, HP2/1. It should be noted that rolling was not observed in this series of experiments.

*$p<0.05$ relative to the no antibody condition.

When these neutrophils were perfused at wall shear stress of 2 dynes/cm^2, they did not interact with monolayers of VCAM-1-transfected L-cells, consistent with the importance of selectins (absent on VCAM-1-transfected L-cells) in the initial adhesive interaction. When the shear stress was decreased by decrements of 0.5 dyne/cm^2 at 2 minute intervals, $\alpha_4\beta_1$-expressing neutrophils began to adhere at a shear stress of 1.5 dyne/cm^2, with adherence increasing as the shear stress was decreased (Figure 3.7.A). Unstimulated neutrophils, or neutrophils stimulated with either FMLP alone or DHCB alone did not roll on or adhere to VCAM-1 transfectants at any shear stress. Ramos cells (a cell line that constitutively expresses $\alpha_4\beta_1$) behaved similarly to the $\alpha_4\beta_1$-expressing neutrophils; Ramos cells also did not adhere at 2 dynes/cm^2, but adhered to monolayers of VCAM-1 transfectants at shears of 1.0 dyne/cm^2 or less (Figure 3.7.A). Interestingly, $\alpha_4\beta_1$-expressing neutrophils did not exhibit significant rolling behaviour (Figure 3.7.B) on VCAM-1 but rather tethered to the monolayer and adhered immediately at that position. In contrast to $\alpha_4\beta_1$-expressing neutrophils, Ramos cells exhibited significant rolling behaviour (Figure 3.7.B). However, when Ramos cells were activated with PMA, an agent shown to upregulate $\alpha_4\beta_1$ adhesivity [8], they also tethered and immediately adhered (27.0 $\pm$ 7.9 unstimulated vs. 52 $\pm$9.6 stimulated Ramos cells adherent at 5 minutes).

The interaction of $\alpha_4\beta_1$-expressing neutrophils with VCAM-1 could have occurred in a non-specific fashion in light of the fact that FMLP+DHCB is known to maximally activate neutrophils resulting in increased avidity and expression of CD18. However, when $\alpha_4\beta_1$-expressing neutrophils were perfused over monolayers of ICAM-1 transfected L-cells (ligand for CD18) under conditions of decreasing shear, tethering interactions did

not occur until shear stress was reduced to 0.5 dyne/cm^2, at which point, minimal tethering interactions were observed (Figure 3.8.A). Thus $\alpha_4\beta_1$-expressing neutrophils adhered preferentially to VCAM-1 when compared directly to the ICAM-1 substratum. The matrix protein, fibronectin, is also a ligand for $\alpha_4\beta_1$. When $\alpha_4\beta_1$-expressing neutrophils were perfused over coverslips coated with fibronectin, no interactions were observed at any shear force (Figure 3.8.A). The interaction with VCAM-1 was $\alpha_4\beta_1$-integrin-dependent as HP2/1 blocked adhesion of $\alpha_4\beta_1$-expressing neutrophils at various shear forces (Figure 3.8.B). Similar results were seen for the anti-β_1 antibody LIA1/2. In this series of experiments, it was not necessary to add an anti-CD18 antibody, suggesting that CD18 could not interact with VCAM-1 or other surface molecules on L-cells. Therefore, the neutrophil tethering and adhesion was entirely dependent on $\alpha_4\beta_1$-integrin and independent of the β_2-integrin. Thus, $\alpha_4\beta_1$ expressed on neutrophils can be used by these cells to tether and adhere under lower shear conditions, an event that is not generalizable to all integrins.

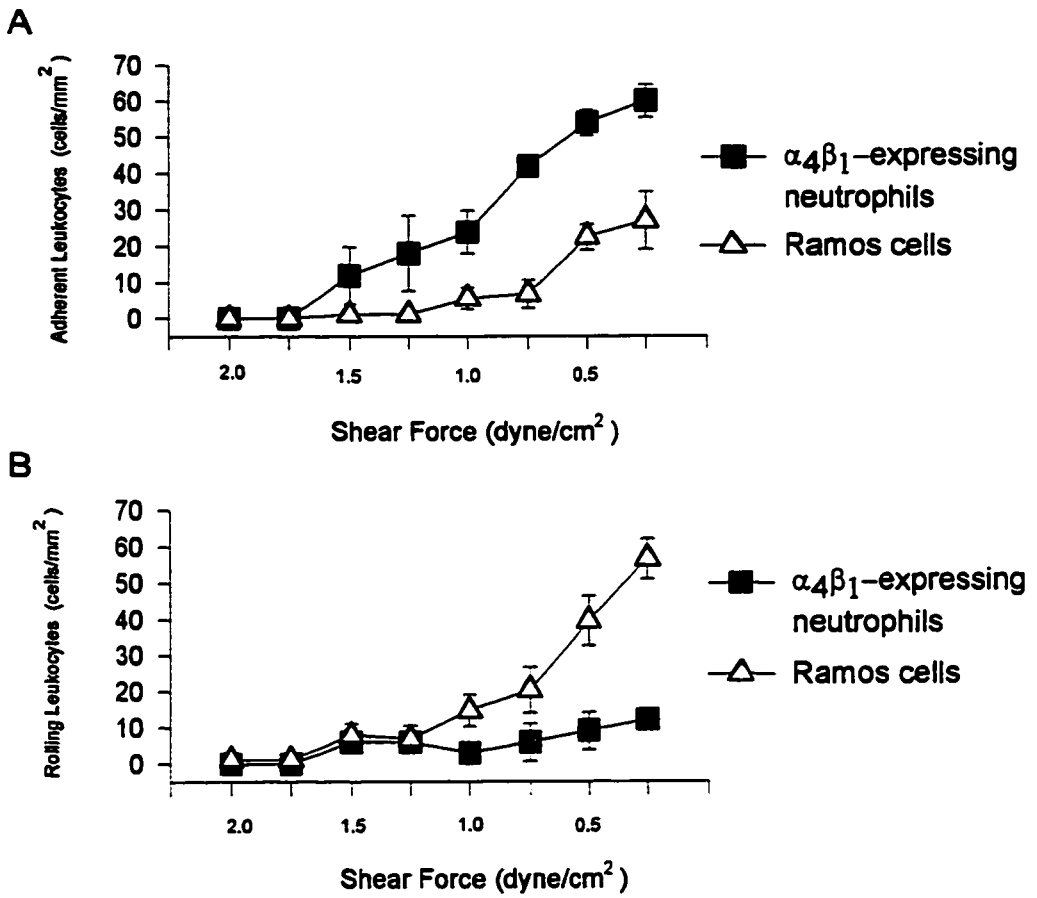


Figure 3.7: To induce $\alpha_4\beta_1$ expression on neutrophils, the cells were treated with DHCB and an exogenous chemotactic factor, FMLP. Perfusion of these cells or Ramos cells over VCAM-1 transfected L-cells revealed no interactions at 2 dynes/cm^2. Reducing shear below 2 dynes/cm^2 at 2 minute intervals caused neutrophils to tether to VCAM-1 transfectants (panel A) with very limited rolling (panel B). Untreated Ramos cells (known to express $\alpha_4\beta_1$) interacted with VCAM-1 transfectants at very similar shears as the neutrophils but displayed primarily rolling (panel B), not adhesion (panel A).

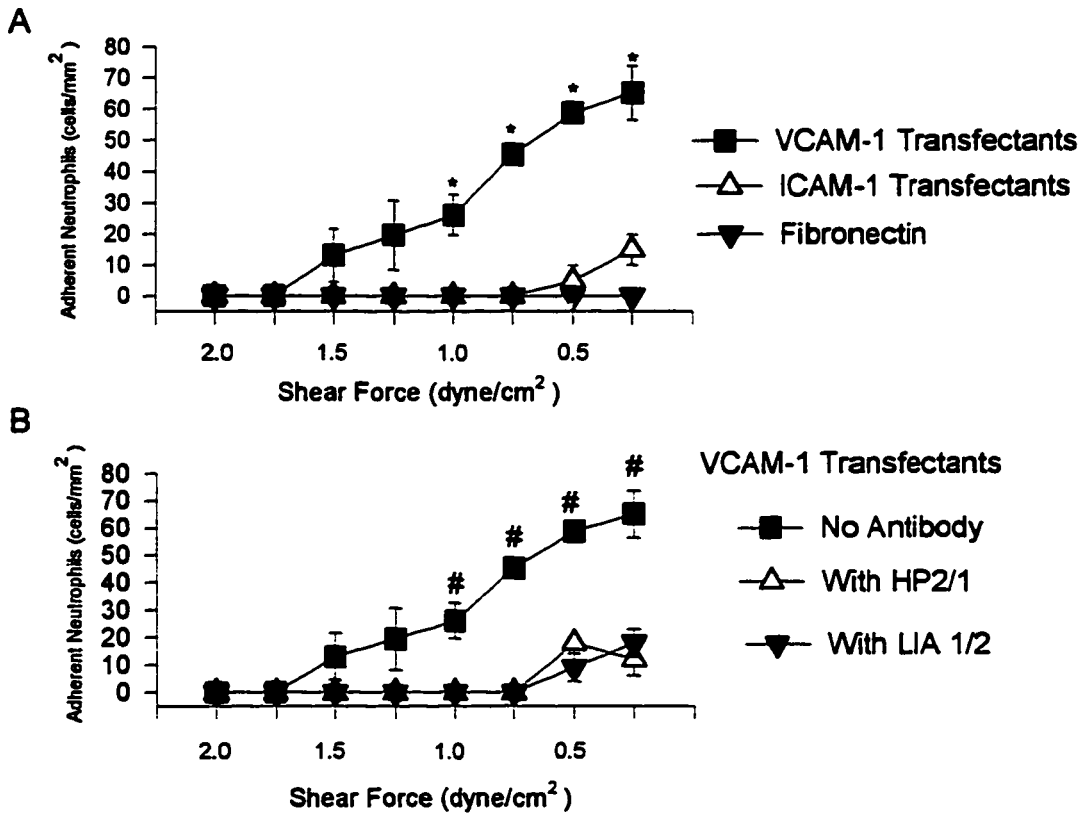


Figure 3.8: $\alpha_4\beta_1$-expressing neutrophils tethered to VCAM-1 transfected L-cells at shears of 1.5, 1.0, and 0.5 dyne/cm^2. Whereas these same cells did not interact with ICAM-1 transfected L-cells until very low shear stresses (panel A). When used alone, anti-α_4 (HP2/1) or anti-β_1 (LIA 1/2) antibodies blocked neutrophil adhesion to VCAM-1 (panel B)

*$p<0.05$ relative to ICAM-1 value. #$p<0.05$ relative to neutrophils + HP2/1.

To determine whether the neutrophils adherent to VCAM-1 transfectants could be detached, $\alpha_4\beta_1$-expressing neutrophils were allowed to settle on VCAM-1 transfectants for 2 minutes, after which, flow through the chamber was re-established to 2 dynes/cm^2. Stimulated neutrophils were observed to remain adherent to the VCAM-1 transfectants under shear, an event that was not inhibited by anti-CD18 antibody (IB_4) but was significantly inhibited by further addition of anti-β_1 antibody (K20) or anti-α_4 antibody (HP2/1) (Figure 3.9.A). Unstimulated Ramos cells also interacted with VCAM-1 transfectants and upon re-introduction of flow (2 dynes/cm^2) some cells detached, but the majority of the cells rolled along the VCAM-1 transfectants. The same concentrations of K20 or HP2/1 also inhibited Ramos cell rolling on VCAM-1 transfectants when flow conditions were re-established (Figure 3.9.B).

Neutrophils adhere to fibronectin under static but not flow conditions: Fibronectin is also a ligand for $\alpha_4\beta_1$. When $\alpha_4\beta_1$-expressing neutrophils were perfused over glass coverslips that were coated with human fibronectin, no interactions were observed between shears of 0.5 and 2.0 dyne/cm^2 (Figure 3.8.A). However, $\alpha_4\beta_1$-expressing neutrophils adhered to fibronectin in a static assay system. Moreover, the adhesion was almost entirely a CD18-independent mechanism (Figure 3.10). The adhesion could however be attenuated by addition of anti-CD18 antibody (IB_4) in combination with human fibronectin fragment 40K (FN40) or the anti-α_4 antibody (HP2/1). FN40 competes with $\alpha_4\beta_1$-integrin for the fibronectin binding region (CS-1) [96].

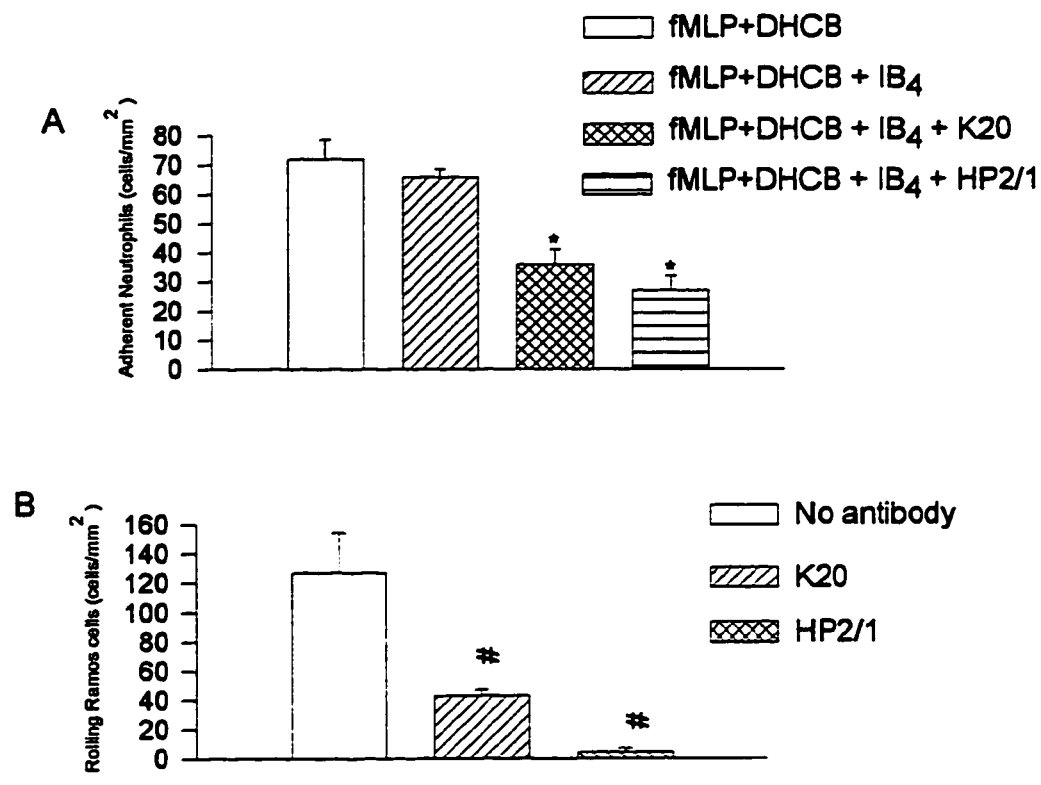


Figure 3.9: $\alpha_4\beta_1$-expressing neutrophils were allowed to settle onto VCAM-1-transfected L-cells and flow was initiated to 2 dynes/cm^2. This force did not displace the neutrophils in the presence or absence of the anti-CD18 antibody, IB_4 (panel A). Pre-treatment of neutrophils with either the anti-β_1 antibody (K20) or the anti-α_4 integrin antibody (HP2/1) significantly reduced the number of adhering cells (panel A). These concentrations of HP2/1 or K20 were sufficient to displace Ramos cells from the VCAM-1 transfected L-cells (panel B).

*$p<0.05$ relative to FMLP+DHCB value. #$p<0.05$ relative to the no antibody condition.

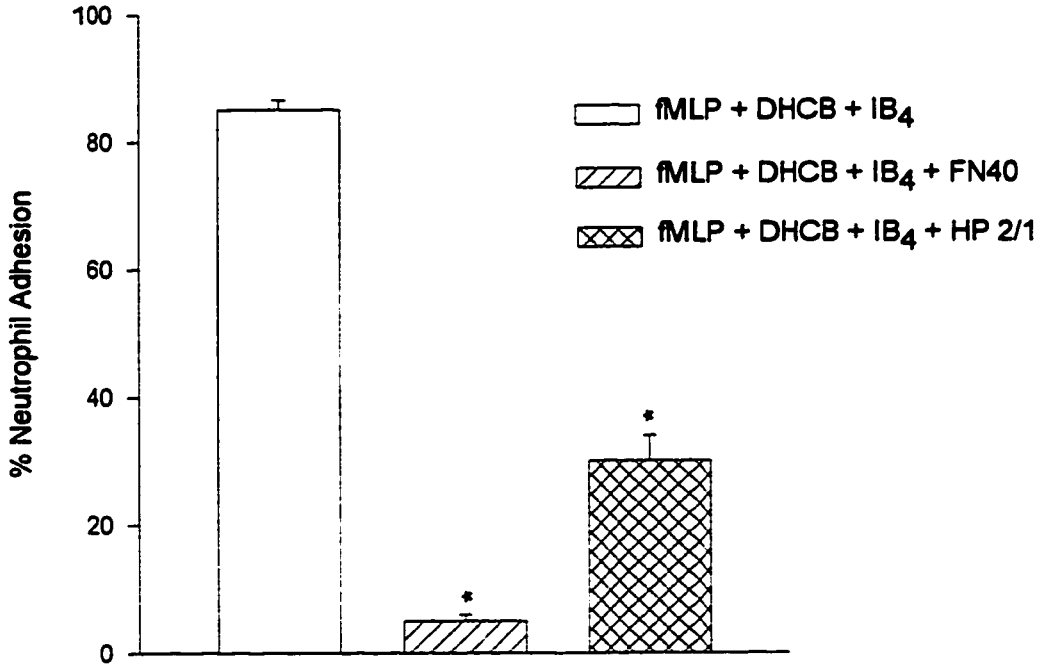


Figure 3.10: Under static conditions fibronectin served as ligand for α_4 -integrin. Neutrophils adhered avidly to fibronectin and this interaction was not reduced with pre-treatment of neutrophils with the anti-CD18 antibody, IB_4. Addition of a fibronectin fragment (FN-40) which contains the CS-1 binding motif for VLA-4 or HP2/1 (anti-α_4-integrin antibody), significantly reduced the adhesive interaction. Under flow conditions, neutrophils were unable to tether to fibronectin (not shown).

*$P<0.05$ relative to FMLP+DHCB+IB_4 value.

3.2 Discussion

Until recently, it was generally accepted that $\alpha_4\beta_1$-integrin was restricted to leukocytes other than neutrophils (the latter use β_2-integrin) and so the potential role of $\alpha_4\beta_1$-integrin as a recruiter of neutrophils has not been addressed. However, there is a growing body of evidence to suggest that neutrophils can infiltrate numerous tissues independent of β_2-integrin [72-74,98]. Moreover, certain stimuli *in vitro* can induce the expression of $\alpha_4\beta_1$-integrin on the surface of human neutrophils [12] and, at least in adjuvant-induced arthritic rats, circulating neutrophils express some $\alpha_4\beta_1$-integrin [13]. Based on these findings and on the view that $\alpha_4\beta_1$-integrin has a multi-faceted capacity to support tethering, rolling and adhesion, in this study I investigated the possibility that neutrophils could be recruited to the surface of endothelium independent of the conventional selectin and β_2-integrin pathway.

The results demonstrate that activated endothelium (TNFα for 4 hours) could recruit DHCB-treated neutrophils via the $\alpha_4\beta_1$-integrin pathway even if an anti-β_2-integrin antibody was present. However, neutrophils treated with antibodies against β_1 or α_4 could not block neutrophil adhesion unless β_2-integrin was simultaneously immunoneutralized. Clearly, the β_1 and β_2-integrin can work in unison or independently to recruit neutrophils to endothelial surfaces. Interestingly, the antibodies directed against either β_1 or α_4 that prevented neutrophil adhesion did not reduce neutrophil rolling. That particular interaction was dependent upon selectins inasmuch as addition of an E/L-selectin antibody or a selectin binding carbohydrate prevented the rolling and the associated adhesion.

These data clearly differ from the results with T-cells [8] and eosinophils [85] which have been reported to roll via $\alpha_4\beta_1$ *in vitro*. Additionally, Johnston et al. has observed a population of leukocytes that roll via α_4-integrin in chronically inflamed microvessels *in vivo* [89]. The role of $\alpha_4\beta_1$ on neutrophils is reminiscent of the $\alpha_4\beta_1$-integrin on monocytes wherein the cells adhered firmly but did not roll via this integrin [9]. The differential roles for α_4 on different cell types may reflect differences in number and/or avidity of the α_4-integrin.

The role of $\alpha_4\beta_1$-integrin on neutrophils was further elucidated by perfusing these cells over a single adhesion molecule (VCAM-1). Under the shear conditions (2 dynes/cm^2) used for endothelium, the neutrophils were not able to interact with VCAM-1, an observation previously reported for monocytes [9], T-cells and a K562 erythroleukemia cell line transfected with $\alpha_4\beta_1$ [8]. However, even a subtle reduction in shear to 1.5 dynes/cm^2 was sufficient to permit neutrophil interactions with the VCAM-1 transfected L-cells. This interaction was different from the endothelial cell interactions in that the $\alpha_4\beta_1$-expressing neutrophils tethered and immediately adhered to the VCAM-1 transfectants with essentially no discernible rolling. The difference may be related to the fact that high concentrations of FMLP in the presence of DHCB may maximally activate $\alpha_4\beta_1$ which translates into immediate adhesion following the tethering interaction. There is a precedent for this behaviour; when the avidity of $\alpha_4\beta_1$ on T-cells [8] or in this study on Ramos cells was increased with PMA, these cells also tethered and stably adhered without notable rolling.

This is the first study to demonstrate a role for a β_2-integrin-independent, $\alpha_4\beta_1$-integrin-dependent mechanism of neutrophil recruitment under shear conditions in the human system. There is a growing body of evidence to invoke a β_2-integrin-independent pathway by which neutrophils can infiltrate inflamed tissues in animal models. Doerschuk et al., [72] demonstrated that an antibody directed against CD18 entirely inhibited PMA-induced neutrophil movement out of the pulmonary microcirculation, however, the same antibody had no effect on neutrophil emigration associated with *S. pneumoniae* or hydrochloric acid. Moreover, a CD18-independent neutrophil infiltration to delayed-type hypersensitivity reactions in joints was also reported [74]. Although the role of $\alpha_4\beta_1$-integrin was not examined in either of these studies, other investigations have revealed that antibodies directed against α_4-integrin partly decreased accumulation of neutrophils into skin in a contact hypersensitivity model [99]. Since neutrophils were thought not to express α_4-integrin, the authors suggested that the neutrophil infiltration was dependent in part upon α_4-integrin expressing cells that were responsible for recruiting the neutrophils. However, an alternative explanation may be that following certain inflammatory conditions, the expression and/or activation of α_4-integrin on the surface of neutrophils may contribute to neutrophil recruitment. The data in the present study supports this view; human neutrophils can be induced to express $\alpha_4\beta_1$-integrin and use this adhesion pathway to interact effectively with endothelium even in flow conditions. Interestingly, circulating neutrophils from rats with adjuvant arthritis were positive for the α_4-integrin [13], suggesting that under at least certain specific inflammatory conditions, this adhesion

molecule may be upregulated and may underlie the CD18-independent mechanism of neutrophil infiltration.

In this study, DHCB was used in concert with either endogenous or exogenous chemotactic stimuli to maximally activate neutrophils. It is conceivable that in systemic inflammatory conditions associated with bacteraemia, sepsis and/or multiple organ failure accumulation of numerous inflammatory mediators could function like DHCB as a maximal stimulus to induce $\alpha_4\beta_1$-integrin expression on neutrophils thereby activating a secondary pathway for neutrophil infiltration. Although increased circulating levels of inflammatory mediators may shed L-selectin and selectin ligands from the surface of neutrophils, since $\alpha_4\beta_1$-integrin on neutrophils can tether to VCAM-1 at a higher shear than CD18 does to ICAM-1, this may become an important alternative pathway for the recruitment of $\alpha_4\beta_1$-expressing circulating neutrophils under shear conditions. This was further supported by the observation that $\alpha_4\beta_1$-expressing neutrophils treated with anti-selectin therapy could tether to activated endothelium although shear forces had to be reduced. Although $\alpha_4\beta_1$-integrin was less efficient at tethering to endothelium and VCAM-1 transfectants than are the selectins, in inflammatory disorders such as ischemia/reperfusion 50% reductions in shear forces are often observed [100]. Moreover, in liver and lung where neutrophils adhere to the endothelium of sinusoids and capillaries respectively, the shear forces are greatly reduced and $\alpha_4\beta_1$-integrin may also be sufficient to support tethering and adhesion.

Although in this study we used a non-physiologic or artificial stimulus (DHCB) to induce the α_4-integrin-dependent interaction, there is some data to argue that this event

may occur in the human system independent of DHCB. First, transmigration of neutrophils across endothelial monolayers in response to chemotactic agents (no DHCB) is sufficient to induce $\alpha_4\beta_1$-integrin expression [12]. This experiment suggests that as yet unidentified physiologic stimuli can indeed induce α_4-integrin expression. Secondarily, Bohnsack, et al [101] demonstrated that neutrophils lacking β_2-integrin could still adhere to laminin via β_1-integrin. Presently it is unclear how DHCB functions to induce the expression of $\alpha_4\beta_1$-integrin, however some possibilities may include a priming function, a maximal stimulus as might occur with a cocktail of pro-inflammatory molecules, or simply to maintain the α_4-integrin on the surface of neutrophils, which might rapidly be reinternalized in the absence of appropriate ligands [7].

It remains to be seen whether the CD18-independent component of neutrophil adhesion could mediate significant neutrophil recruitment *in vivo*. In this study, over the first 5 minutes, the CD18-independent component accounted for approximately 30-40% of maximal neutrophil adhesion. It is possible that over time the number of cells adhering would reach a plateau that could be similar in magnitude but delayed for CD18-independent adhesion. Nevertheless, the results of this study clearly show a role for VLA-4 in neutrophil recruitment under shear conditions and raise the possibility that this mechanism may underlie the CD18-independent recruitment of neutrophils previously described *in vivo*.

As DHCB is a pharmacological agent, the ability for neutrophils to use $\alpha_4\beta_1$ for recruitment *in vivo* remains to be determined. In the next chapter, I examined $\alpha_4\beta_1$-dependent neutrophil recruitment in the absence of DHCB.

CHAPTER 4:

UNSTIMULATED NEUTROPHILS CAN BE RECRUITED FROM WHOLE BLOOD VIA $\alpha_4\beta_1$-INTEGRIN UNDER FLOW CONDITIONS

Hypothesis : Erythrocytes will enhance neutrophil interactions with VCAM-1 under shear conditions.

Objectives:

1) To determine if unstimulated neutrophils in the presence of whole blood can be recruited onto VCAM-1.

2) To determine which shear forces are optimal for the recruitment of neutrophils to VCAM-1.

3) To determine if neutrophil recruitment is affected by changing the amount of VCAM-1 used as the experimental substrate (i.e. VCAM-1 density).

4.1 Results

In the presence of whole blood, unstimulated neutrophils are able to tether to and roll on VCAM-1 via $\alpha_4\beta_1$: To determine if red blood cells would increase the ability of neutrophils to interact with biologic substrata by an $\alpha_4\beta_1$-dependent mechanism, heparinized whole blood was perfused over soluble human VCAM-1-coated coverslips. The viscosity of whole blood is greater than that of isolated neutrophils in buffer, resulting in a higher shear force at equivalent flow rates for whole blood. The viscosity of whole blood can be approximated once the hematocrit is determined (see appendix A). Once viscosity is determined, shear forces within the flow chamber can be calculated (see chapter 2). Leukocytes where observed to roll on and adhere to VCAM-1 after 5 minutes of perfusion of whole blood over coverslips that had been coated with soluble human VCAM-1 (5 μg/ml). After whole blood perfusion, the chamber was perfused with HBSS

buffer, at which point, interacting leukocytes could be observed. Figure 4.1.A demonstrates the number of rolling leukocytes observed in separate experiments at different shear forces (20, 15, 10, 5, and 2 dynes/cm^2). Notable leukocyte rolling occurred at 15 dynes/cm^2 and was greatest at 10 dynes/cm^2, with decreases in rolling at lower shear forces. Adherent leukocytes per mm^2 of the coverslip surface are shown in 4.1.B. Peak adhesion was observed at 5 dynes/cm^2. An important observation is that when the whole blood was pre-treated with the anti-α_4 antibody, HP1/2 (2μg/ml), leukocyte rolling (Figure 4.2, Panel A) and adhesion (Panel B) were eliminated at shears of 10 and 5 dynes/cm^2.

To distinguish the types of leukocytes that where interacting with VCAM-1 after perfusion of whole blood, the coverslips where gently removed from the chambers and differentially stained (Wright-Giemsa stain). This procedure resulted in a minimal loss of interacting leukocytes as determined by comparing the number of leukocytes per mm^2 on the coverslip prior to removal from the chamber to the number after the staining procedure. Figure 4.3 shows that leukocyte ratios vary depending upon the shear force used in the experiment. Interestingly, neutrophils are apparent at all shear forces (Fig. 4.3.A), with the highest number at 15 dynes/cm^2 (14.0 ±2.6 % of total leukocytes). This occurred despite the fact that these neutrophils had not been exogenously stimulated with DHCB or other stimuli, as with the experiments using isolated neutrophils (see chapter 3). Eosinophils, which are known to have VLA-4 did not adhere at 15 dynes/cm^2 but were observed to adhere more efficiently to VCAM-1 as shear force was decreased (Fig. 4.3.B). Lymphocytes were always the most prevalent leukocyte, regardless of the shear

force (Fig 4.3.C). Monocytes were also able to adhere to VCAM-1 at all shear forces (Fig 4.3.D).

The ratio of leukocytes present on coverslips may reflect the original ratio of leukocytes in the whole blood. For example, if the majority of leukocytes in the whole blood being perfused are neutrophils, then the majority of the leukocytes recruited onto the coverslip might be expected to be neutrophils. If a higher proportion of neutrophils are recruited than is present in the blood, this would indicate that there was a preferential recruitment of neutrophils. To compare the relative recruitment efficiencies of the various leukocytes onto VCAM-1, the ratio of leukocytes recruited onto coverslips was

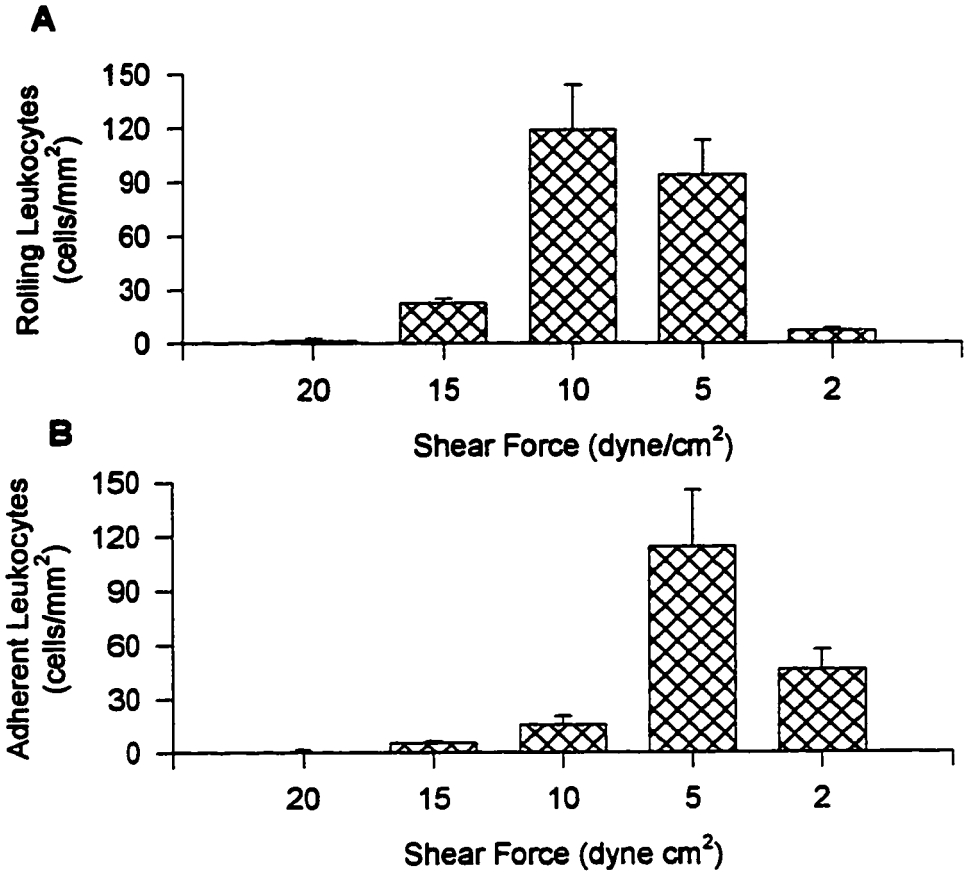


Figure 4.1: Leukocyte rolling (panel A) and adhesion (panel B) observed on soluble human VCAM-1-coated coverslips (5μg/ml) following perfusion of whole blood at different shear forces.

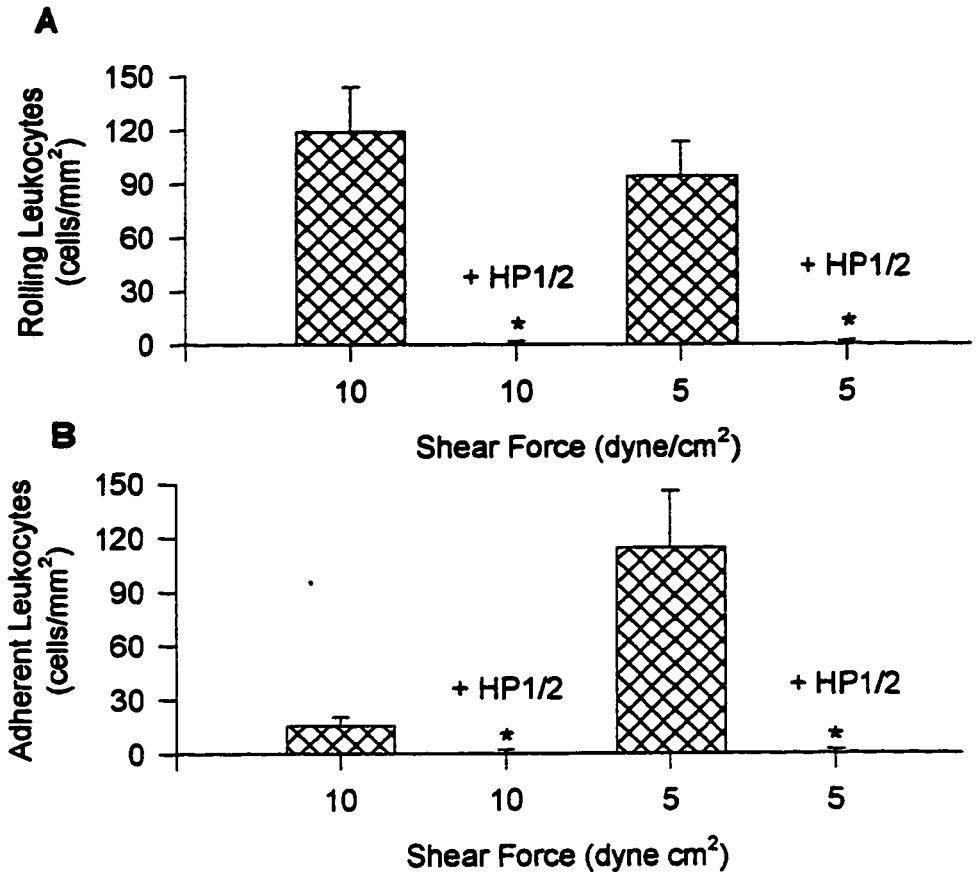


Figure 4.2: Rolling (Panel A) and adhesion (Panel B) of leukocytes recruited from whole blood was blocked by addition of anti α_4-antibody HP1/2 (2 μg/ml).

* $P<0.05$ relative to no antibody condition.

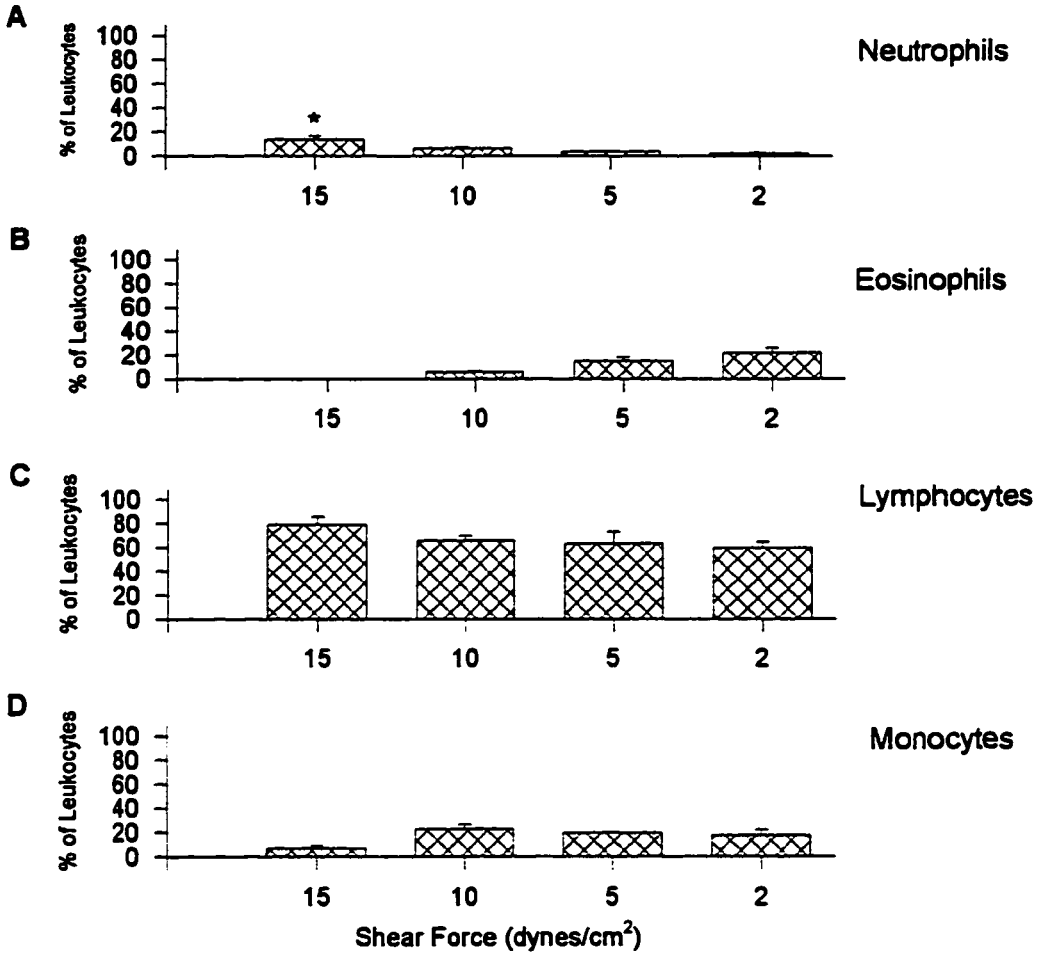


Figure 4.3: The types of leukocytes found on VCAM-1-coated coverslips after perfusion of whole blood at different shear forces. Coverslips were differentially stained and analyzed for the presence of neutrophils, eosinophils, lymphocytes, and monocytes. Data is shown as the percentage of total leukocytes found on the coverslips.

*$P<0.05$ relative to 15 dynes/cm^2 values for eosinophils and monocytes.

compared to that found in the whole blood being perfused. The recruitment factor (R-factor) was defined as percentage of leukocytes present on the coverslip over the percentage found in the whole blood. An R-factor of 1 indicates no preferential recruitment of a particular leukocyte when compared to its presence in whole blood. The higher the R-factor, the greater the preference for recruitment onto VCAM-1. Figure 4.4 shows the R-factor for different leukocytes at various shear forces. Proportionally fewer neutrophils are always recruited from whole blood (i.e. R-factor always less than 1). As shear force was decreased, the R-factor for eosinophils increased. Both lymphocytes and monocytes had R-factors greater than 1 at shear forces from 2 to 10 dynes/cm^2.

VCAM-1 density affects neutrophil recruitment from whole blood: The level of VCAM-1 expressed by activated endothelium may vary with the degree and type of inflammation present, and this may affect the amounts and types of leukocytes recruited. To test this, soluble VCAM-1 was coated onto coverslips at different densities (10.0, 5.0, 2.5, and 1.0 μg/ml), and leukocyte recruitment from whole blood was determined as before. For these experiments, the shear force was maintained at 10 dynes/cm^2. Figure 4.5.A shows the number of rolling leukocytes visible per mm^2 of the coverslips. While the number of rolling leukocytes was comparable when VCAM-1 was coated at 10.0 and 5.0 μg/ml, the observable rolling decreased sharply at 2.5 μg/ml, and was minimal at 1.0 μg/ml. The adhesion to VCAM-1 coated coverslips followed a similar trend (Figure 4.5.B).

After staining of the coverslips, the types of leukocytes recruited was determined. At VCAM-1 coating densities of 10.0, 5.0, and 2.5 μg/ml, the proportions of leukocytes

recruited from whole blood were remarkably similar, with the exception of neutrophils, which did not appreciably accumulate on coverslips coated at 2.5 μg/ml (Table 4.1). Coverslips coated at 1.0 μg/ml had very few leukocytes recruited from whole blood, and it was not possible to reliably determine leukocyte ratios.

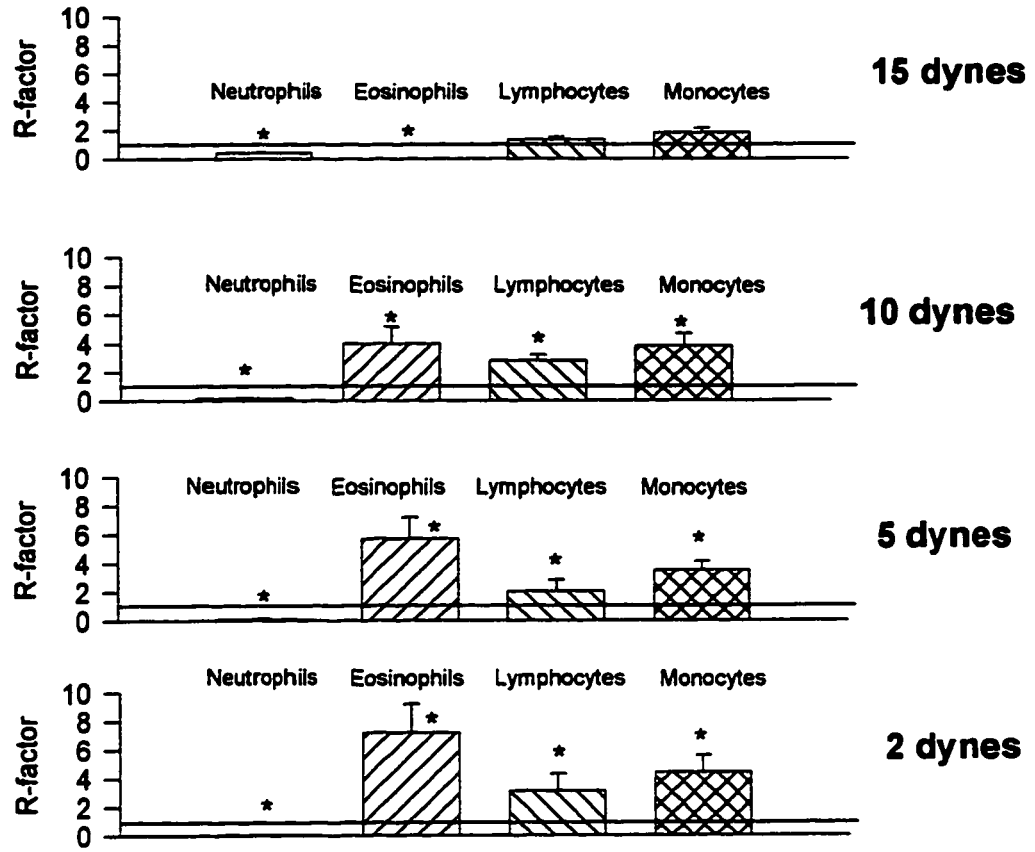


Figure 4.4: Recruitment factors (R-factors) for the various leukocytes found on VCAM-1-coated coverslips after perfusion of whole blood. The R-factor was defined as percentage of leukocytes present on the coverslip over the percentage found in the whole blood. An R-factor of 1 means that the percentage of a particular leukocyte found on the coverslip was equal to that found in the whole blood.

* $P<0.05$ relative to an R-factor value of 1.

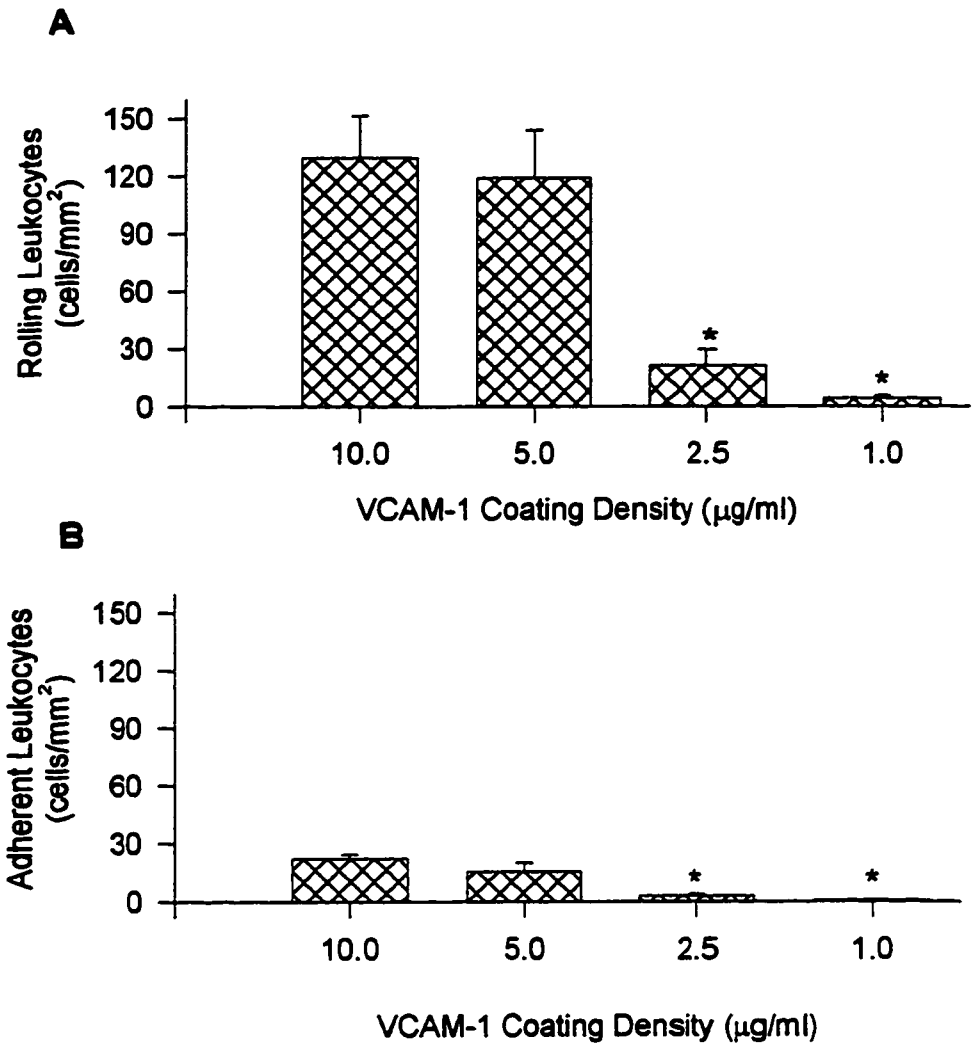


Figure 4.5: Leukocyte rolling (panel A) and adhesion (panel B) observed on coverslips coated with different concentrations of VCAM-1 following perfusion of whole blood at 10 dynes/cm^2.

*P <0.05 relative to 5.0 μg/ml

Table 4.1: Leukocyte Types on VCAM-1 Coated Coverslips.

VCAM-1	Neutrophils	Eosinophils	Lymphocytes	Monocytes
10 μg/ml	6.4 ±1.5%	3.0 ±0.6%	66.0 ±6.2%	24.5 ±4.4%
5 μg/ml	6.2 ±1.3%	5.4 ±1.4%	65.8 ±3.9%	22.6 ±3.7%
2.5 μg/ml	0.9 ±0.8%*	4.7 ±0.5%	67.7 ±6.5%	26.7 ±5.9%

Table 4.1 illustrates that leukocyte recruitment from whole blood onto coverslips coated with differing concentrations of VCAM-1 is similar, with the exception of neutrophils, which do not accumulate appreciably on coverslips coated at 2.5 μg/ml.

* $P < 0.05$ relative to 5 μg/ml .

4.2 Discussion

The previous chapter described the VLA-4-dependent recruitment of isolated neutrophils after they had been maximally stimulated with DCHB in combination with FMLP. As evidenced by the experiments in this chapter, where whole human blood was perfused over soluble VCAM-1 coated coverslips, neutrophil recruitment can occur without the need for exogenous stimulation with DHCB. This suggests that at least some circulating neutrophils possess an adhesive ligand for VCAM-1. The fact that this interaction was blocked by the addition of anti-α_4 antibody strongly suggests that the ligand is α_4-integrin.

Although neutrophil recruitment occurred on VCAM-1, the level of recruitment was always under-represented relative to their proportion in whole blood. The opposite was true for eosinophils, lymphocytes, and monocytes, which were over-represented (i.e. R-factors greater than 1) for almost all shears tested. As well, neutrophil recruitment from whole blood onto VCAM-1 is relatively small when compared to recruitment onto P-selectin and E-selectin (observations from work that is currently ongoing in our laboratory). VLA-4-dependent neutrophil recruitment may be most significant at high shear forces inasmuch as the absolute number of recruited neutrophils was greater than that for eosinophils and monocytes (but not lymphocytes) at 15 dynes/cm^2. The typical range of shear forces found in post-capillary venules is from 2 to 16 dynes/cm^2 [14], thus a VLA-4/VCAM-1-dependent mechanism of neutrophil recruitment may be very significant *in vivo*.

Alon et al. found that the tethering of isolated T lymphocytes to VCAM-1 coated coverslips was reduced when the amount of VCAM-1 was lowered from 15 to 5 μg/ml [8]. In the current study, the total amount of recruited leukocytes were decreased, but the relative amounts of eosinophils, lymphocytes, and monocytes were not affected by reducing VCAM-1 coating densities from 10.0 to 2.5 μg/ml. As opposed to other leukocyte types, the number of neutrophils recruited from whole blood was reduced when the amount of VCAM-1 coated onto coverslips was lessened to 2.5 μg/ml. This may be related to a relatively low amount of VLA-4 expressed on the surface of unactivated neutrophils[12].

In this study, it is conceivable that a subset of neutrophils are being recruited onto VCAM-1. Neutrophil precursors in the bone marrow express VLA-4, with that expression diminishing during terminal differentiation [102]. If a small percentage of neutrophils are released from the bone marrow prior to complete maturation, they might retain surface expression of VLA-4, which in turn may allow for significant neutrophil accumulation via VLA-4. Indeed, in a number of diseases, immature neutrophils are released into the circulation. During bacterial infections (for example staphylococcal, meningococcal, and pneumococcal), rheumatoid arthritis, burns, and poisonings, immature neutrophils (myelocytes, promyelocytes, and blasts) appear in the circulation [103]. This so called myeloid leukemoid reaction may be a source for VLA-4-expressing neutrophils. The blood donors for the current study were presumably healthy, thus neutrophil recruitment via VLA-4 may occur in the absence of the above listed pathologies.

Whereas DHCB-treated neutrophils were observed to tether, and immediately adhere to VCAM-1 transfectants (see chapter 3), the majority of leukocytes recruited from whole blood onto VCAM-1 were observed to roll. A limitation of the flow chamber system is that we are currently unable to determine the phenotype of the leukocytes while they are interacting with the substrate. It is possible that the leukocytes that are adherent to soluble VCAM-1-coated coverslips are neutrophils, while the rolling cells are other leukocyte types. The VLA-4 on unstimulated neutrophils in whole blood may be in an unactivated (low-avidity) state, thus permitting a rolling interaction. Alon et al. observed that rolling lymphocytes firmly adhered to VCAM-1-coated coverslips when stimulated with integrin activating agents [8].

This is the first study to use whole blood in examining leukocyte recruitment under shear conditions *in vitro*. A notable difference in the use of whole blood when compared to isolated neutrophils is the shear forces that are generated within the flow chamber, with forces being approximately 5-fold greater for whole blood at equivalent flow rates. Previous studies have demonstrated recruitment of leukocytes onto VCAM-1 coated coverslips did not occur at shears above 1.8 dyne/cm^2 [8]. Whereas FMLP+DHCB-treated neutrophils were not observed to adhere to VCAM-1 at shears above 1.5 dynes/cm^2 (chapter 3), neutrophil recruitment from whole blood was greatest at 15.0 dynes/cm^2 (14 $\pm$2.6 % of total leukocytes). The difference may be related to the presence of red blood cells, which are known to enhance leukocyte interactions with substrate. For example, Melder et al. found that lymphocyte capture by TNFα-activated endothelium *in vitro* was enhanced and occurred at higher shear forces when red blood cells were introduced into

the lymphocyte suspension [16]. As previously described in chapter 1, red blood cells may alter fluid dynamics and enhance leukocyte recruitment by forcing the leukocytes out of the main stream of flow and against the vessel wall.

We observed neutrophil recruitment after 5 minutes of whole blood perfusion. It is conceivable that even with low levels of recruitment, total neutrophil accumulation may be significant over longer time periods. Issekutz et al. has shown that VLA-4 can partially mediate neutrophil recruitment into inflamed joints and dermal inflammatory sites in the rat [13]. The results shown here suggest a potential role for VLA-4 mediated recruitment of human neutrophils *in vivo*, the significance of which remains to be determined.

CHAPTER 5

EMIGRATED RAT NEUTROPHILS ADHERE TO CARDIAC MYOCYTES VIA α_4-INTEGRINS

Hypothesis : Neutrophils can employ VLA-4 to adhere to inflamed parenchymal tissues.

Objectives:

1) To determine whether rat neutrophils use VLA-4 to adhere to freshly isolated rat ventricular myocytes, and if so, what is the ligand on the myocyte.

2) To determine if the process of emigration can alter the mechanisms by which neutrophils adhere to myocytes.

3) To determine whether the adhesion of neutrophils to myocytes can be affected by the inflammatory state of the myocyte.

5.1 Results

Circulating neutrophils adhere to cardiac myocytes via CD18. Freshly isolated neutrophils from rat blood showed minimal adherence to unstimulated rat cardiac myocytes (Fig. 5.1). When neutrophils were stimulated with fMLP, adhesion to myocytes increased significantly, an event that was totally inhibitable by addition of an anti-CD18 antibody (mAb WT-3). Stimulation of cardiac myocytes with TNFα increased the level of fMLP-stimulated neutrophil adhesion (Fig. 5.1), and this interaction was also inhibited by anti-CD18 antibody. Figure 5.2 is a photomicrograph of the system used in this study, illustrating fMLP-treated neutrophils adhering to TNFα-stimulated myocytes.

Emigrated neutrophils can adhere to cardiac myocytes via a CD18-independent mechanism. Emigrated neutrophils adhered to both unstimulated and TNFα-stimulated cardiac myocytes (Fig. 5.3, left). Once again, addition of the anti-

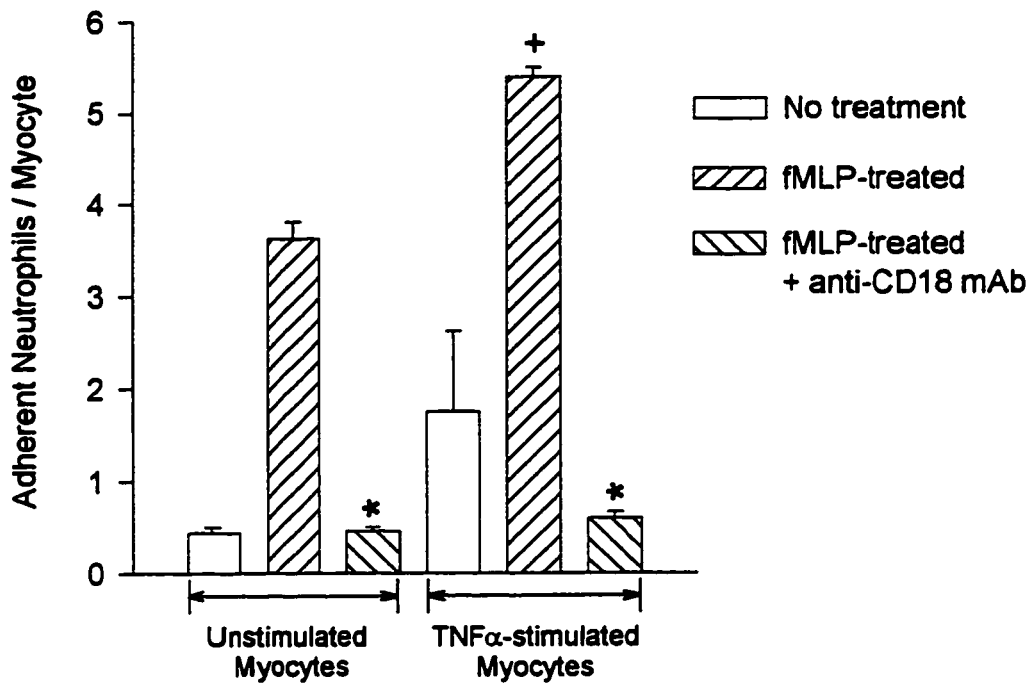


Figure 5.1: Circulating neutrophil adhesion to unstimulated or TNFα-stimulated cardiac myocytes. Neutrophils were either unstimulated or stimulated with fMLP (20μM). In some experiments, anti-CD18 antibody (WT-3, 2.0 μg/ml) was added with fMLP.

*$p<0.05$ relative to the respective fMLP-treated condition.

+$p<0.05$ relative to the fMLP-treated neutrophils on unstimulated myocytes.

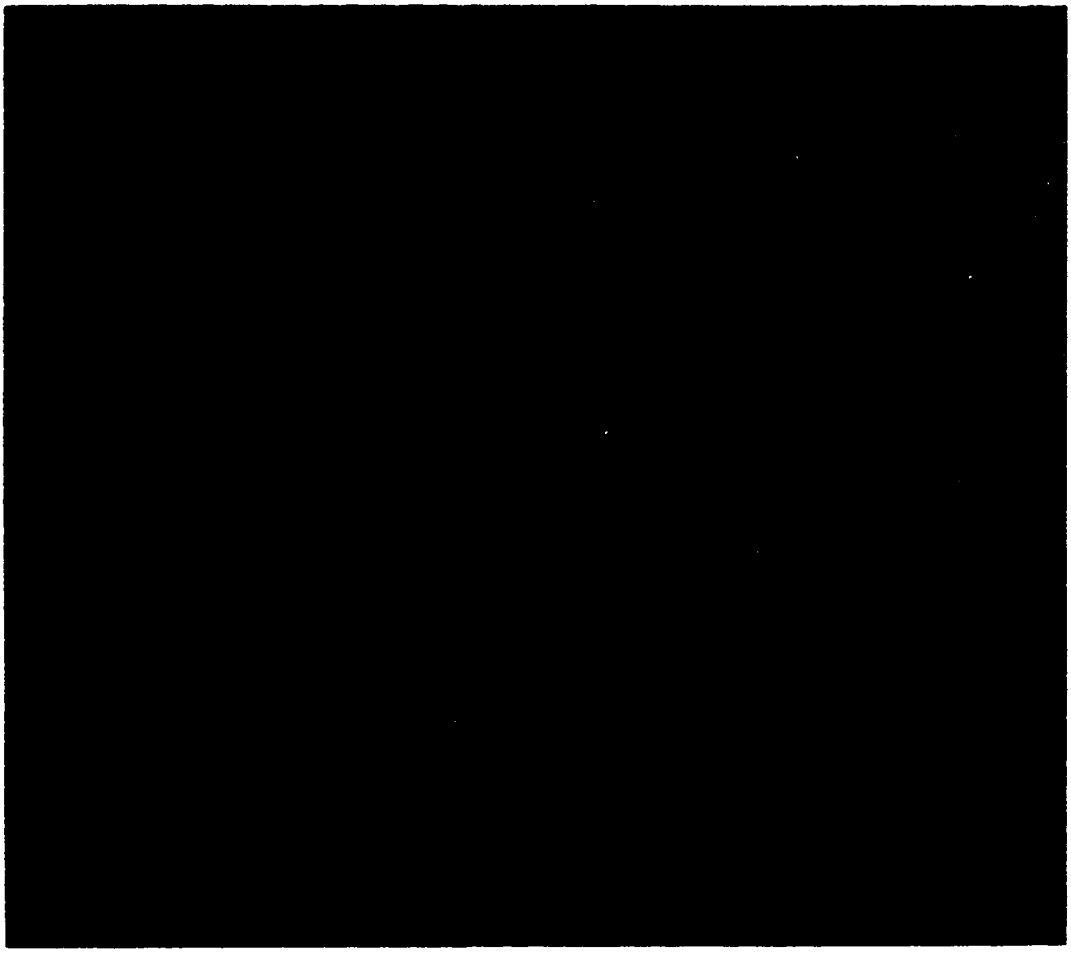


Figure 5.2: A photomicrograph demonstrating the adhesion of fMLP-treated neutrophils to TNFα-stimulated myocytes.

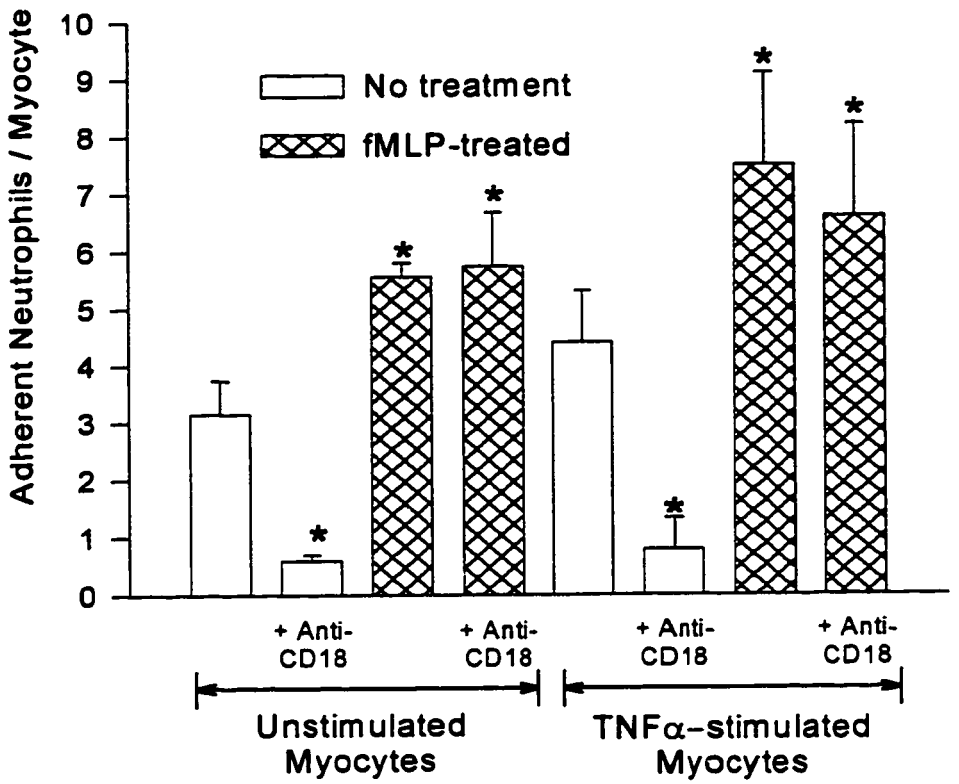


Figure 5.3: Emigrated neutrophil adhesion to unstimulated (left) and TNFα-stimulated (right) cardiac myocytes. Neutrophils were either unstimulated or stimulated with fMLP (20μM). Adhesion was determined in the presence or absence of anti-CD18 antibody (WT-3, 2.0 μg/ml). As opposed to untreated emigrated neutrophils, the adhesion of emigrated neutrophils stimulated with fMLP was not inhibited by the anti-CD18 antibody. *$p<0.05$ relative to the respective no treatment condition.

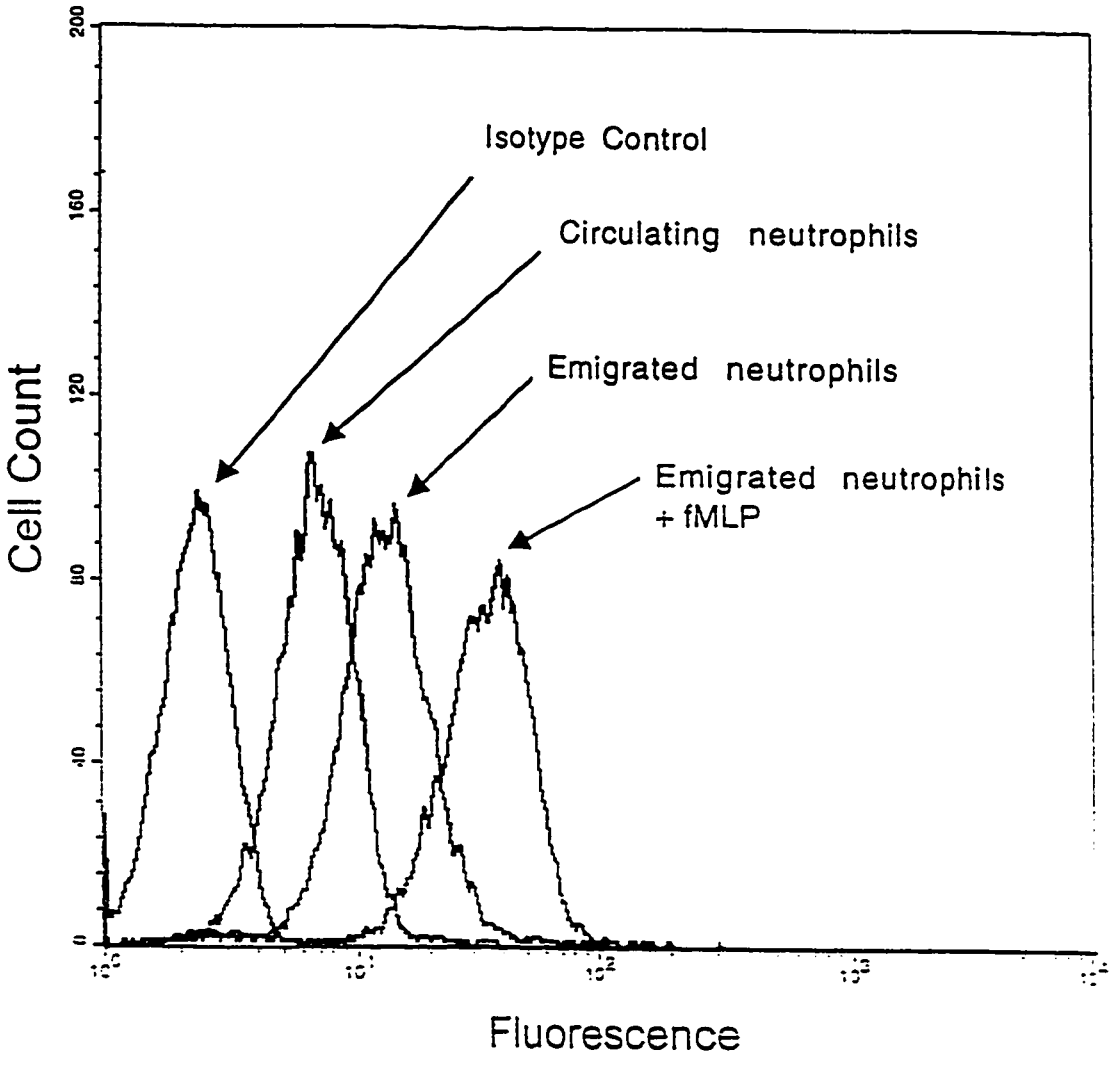


Figure 5.4: A flow cytometry histogram showing the staining for α_4-integrin on circulating and emigrated rat neutrophils. Emigration increased the level of α_4-integrin expression (indicated by a shift to the right). This level was further increased by treatment with fMLP.

CD18 antibody inhibited these interactions. When emigrated neutrophils were exposed to a dose of chemoattractant (fMLP), the adhesion to cardiac myocytes was further increased. However, the anti-CD18 antibody was unable to inhibit the adhesion of emigrated neutrophils treated with fMLP to either unstimulated or TNFα-stimulated cardiac myocytes (Fig. 5.3, right).

Emigrated neutrophils express and functionally adhere via α_4-integrin. Figure 5.4 is a flow cytometric histogram demonstrating α_4-integrin expression (staining with mAb TA-2) on circulating (from whole blood) and emigrated (elicited into the peritoneal cavity) rat neutrophils. These data confirm the observations of Issekutz et al.[13] who reported a low level of expression on circulating rat neutrophils. However, our data extends previous work inasmuch as emigration of neutrophils resulted in a two-fold increase in α_4-integrin expression (mean fluorescences; circulating neutrophils:7.0, emigrated neutrophils: 14.0). Moreover, when emigrated cells were stimulated with fMLP a further increase in α_4-integrin expression was noted (mean fluorescence: 37.9). In fact, the difference for α_4-integrin expression between circulating and re-stimulated emigrated neutrophils was more than five-fold. To determine if the CD18-independent adhesion was attributable to α_4-integrin, anti-α_4 antibody (mAb TA-2) was added to emigrated neutrophils that had been stimulated with fMLP. Whereas neither antibody had an effect, tandem addition of TA-2 and WT-3 significantly decreased adhesion (Fig. 5.5).

Neutrophil α4-integrin binds to fibronectin on cardiac myocytes to support adhesion. The CD18-independent adhesion of emigrated neutrophils to TNFα-stimulated myocytes could be inhibited if, in addition to an anti-CD18 antibody, a fibronectin

fragment (FN-40) was also added (Fig. 5.6). This effect was dose-dependent, as a higher concentration of FN-40 (10μg/ml) was more effective at inhibiting adhesion. Much like the anti-α_4-integrin data in figure 5.5, addition of FN-40 alone did not inhibit neutrophil adhesion. Whereas inhibiting α_4-integrin binding to fibronectin abrogated adhesion, addition of a polyclonal anti-rat VCAM-1 antibody (5F10) had no effect (Fig. 5.6).

CINC-gro is able to stimulate α_4-integrin-dependent adhesion of emigrated neutrophils to cardiac myocytes. To determine if other physiologic agonists are able to induce CD18-independent adhesion of emigrated neutrophils, we tested the endogenous rat chemokine, CINC-gro (10nM), a potent chemoattractant of rat neutrophils. As with fMLP-stimulation, the adhesion of CINC-gro-stimulated emigrated neutrophils resulted in significant adhesion to TNFα-stimulated cardiac myocytes, an event that was not inhibitable by anti-CD18 nor anti-α_4-integrin antibodies alone (Fig. 5.7). When anti-CD18 antibody was used in combination with the anti-α_4-integrin antibody or the fibronectin fragment (10μg/ml), significant attenuation of adhesion was observed.

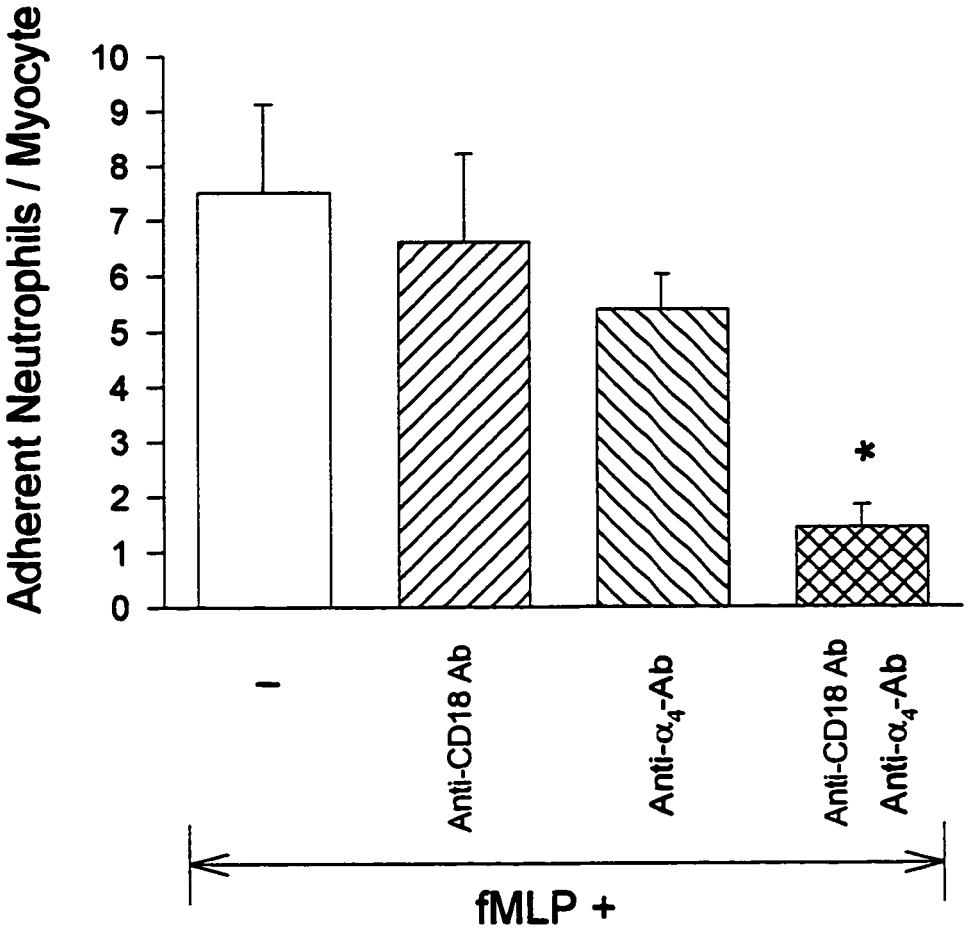


Figure 5.5: Adhesion of emigrated neutrophils stimulated with fMLP to TNFα-stimulated cardiac myocytes. Adhesion was determined in the presence or absence of anti-CD18 antibody (WT-3, 2.0 μg/ml), anti-α_4 antibody (TA-2, 2 μg/ml), or the combination of both antibodies. While addition of either antibody by itself had no effect, the combination was effective in eliminating adhesion.

*$p<0.05$ relative to the no antibody condition.

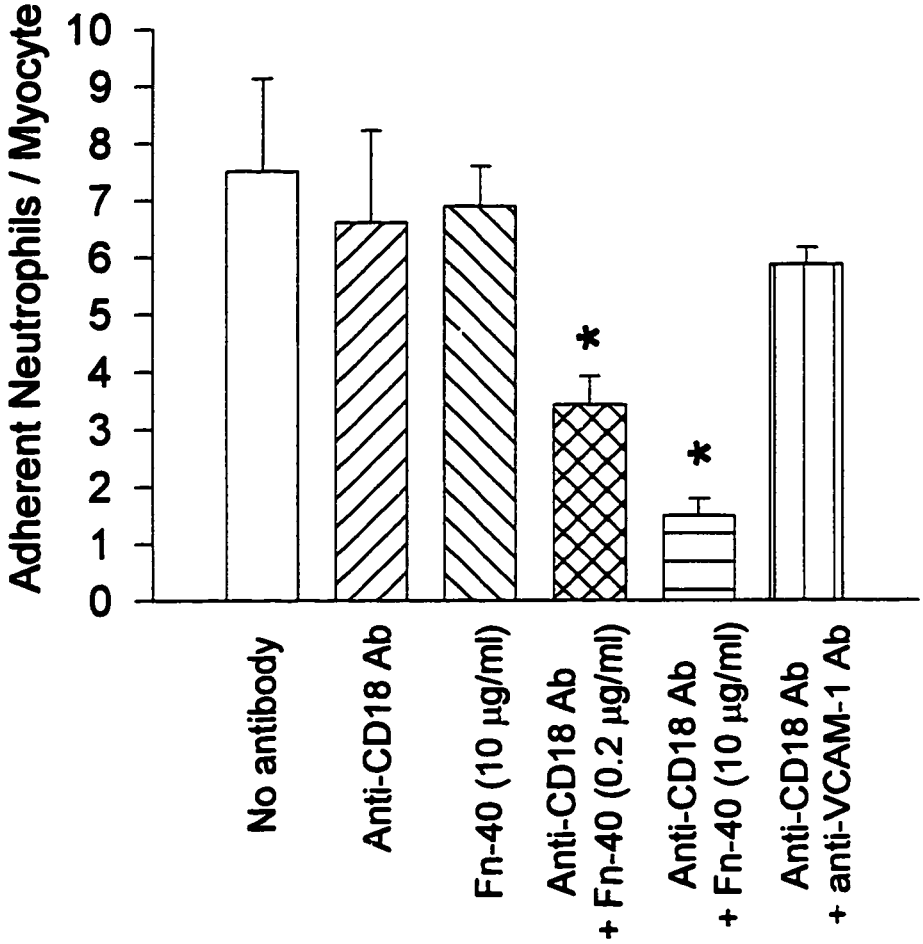


Figure 5.6: Demonstrates the adhesion of emigrated neutrophils stimulated with fMLP to TNFα-stimulated cardiac myocytes in the presence of anti-CD18 antibody (WT-3, 2 µg/ml) in combination with a fibronectin fragment (FN-40, 0.2 and 10 µg/ml) that contains the CS-1 binding region for VLA-4 or an antibody that immunoneutralizes VCAM-1 (5F10, 2 µg/ml). *$p<0.05$ relative to the no antibody condition.

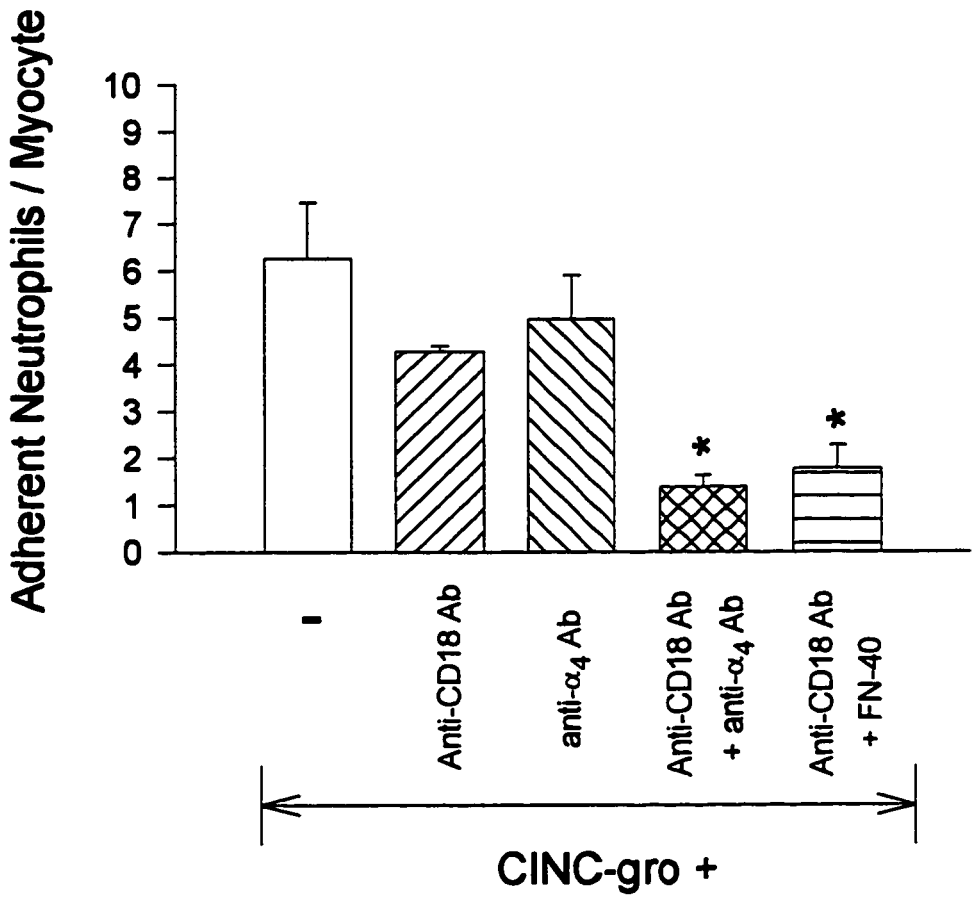


Figure 5.7: Adhesion of emigrated neutrophils stimulated with CINC-gro to TNFα-stimulated cardiac myocytes. Adhesion was determined in the presence or absence of anti-CD18 antibody (WT-3, 2.0 μg/ml), anti-α_4 antibody (TA-2, 2 μg/ml), or the combination of both antibodies. The combination of anti-CD18 antibody with FN-40 (10 μg/ml) was also performed.

*$p<0.05$ relative to the no antibody condition.

4.2 Discussion

In vitro, we have previously shown that emigration across endothelium induces the expression of the β_1-integrin, $\alpha_4\beta_1$, on human neutrophils [12]. The purpose of the present study was to determine whether neutrophils use α_4-integrin to adhere to freshly isolated ventricular myocytes, and whether this event could be influenced by the inflammatory state of the myocyte. This study is the first to demonstrate that the physiologic event of emigration alters the mechanisms by which neutrophils adhere to parenchymal cells such as cardiac myocytes. Both circulating (i.e. isolated from whole blood) and emigrated rat neutrophils adhered to rat cardiac myocytes via CD18. This occurred regardless of the inflammatory state of the myocyte. Application of the chemoattractants, fMLP or CINC-gro, to emigrated neutrophils resulted in increased adhesion. However, anti-CD18 antibody was unable to inhibit the adhesion of emigrated neutrophils treated with chemoattractant to either unstimulated or TNFα-stimulated myocytes, whereas co-administration of antibodies against CD18 and α_4-integrin did inhibit more than 80% of the adhesion. Thus, emigration induced a second adhesive mechanism on neutrophils, mediated by α_4-integrin.

These data suggest that the ligand for CD18 is constitutively expressed on rat cardiac myocytes, and is increased with inflammatory cytokine stimulation. Previous studies have established the importance of CD18 in neutrophil adhesion to cardiac myocytes under conditions where both the neutrophil and the myocyte were stimulated [19]. We have found significant adhesion when only the neutrophils were stimulated. This may be due to differences in the models used (rat vs. canine). While canine cardiac myocytes

do not express ICAM-1 unless they have been stimulated with an inflammatory cytokine [20], cultured neonatal rat myocytes are known to constitutively express ICAM-1, the level of which can be increased with TNFα-stimulation [104]. Our data suggest that this may also be the case for adult rat myocytes, although the expression of other potential CD18 ligands (e.g., ICAM-2) cannot be excluded.

The induction of α_4-integrin may strengthen the adhesive interaction between neutrophils and extravascular tissue, thus anchoring the emigrated neutrophil firmly to an inflammatory site. Alternatively, α_4-integrin may be important for extravascular adhesion when ligands for CD18 are reduced or not present. Interestingly, circulating neutrophils or emigrated neutrophils that were not re-stimulated with chemoattractant did not demonstrate α_4-integrin-dependent adhesion, despite the presence of α_4-integrin in both situations. The reasons for this may be two-fold. First, the level of α_4-integrin expression may have been insufficient to support adhesion. This observation is consistent with our flow cytometric experiments inasmuch as re-stimulation of emigrated neutrophils with fMLP increased α_4-integrin expression 5-fold. Dalton et al. has demonstrated that β_1-integrin surface expression was lost if the integrin does not engage its ligand [7]. It is conceivable that elicited neutrophils isolated from the peritoneal cavity are non-adherent, and might be expected to have reduced expression of surface integrins. Chemoattractant-stimulation may function to re-mobilize the α_4-integrin that was expressed during emigration. An alternative explanation for the need for restimulation may be that the binding affinity of the α_4-integrin may be reduced. Indeed it is well known that α_4-integrin can be in a low or high affinity state and perhaps glycogen-elicited neutrophils require

further stimulation to activate α_4-integrin. This closely mimicks the pathophysiologic condition; such increasing stimulation with chemotactic agents would occur as neutrophils emigrated towards an inflamed site. Although this has not been previously described for α_4-integrin, Hughes et al. have demonstrated that newly mobilized Mac-1 (CD11b/CD18) is capable of functioning in adhesion only if the neutrophils are subsequently exposed to an increased level of stimulus [105].

In the current study, emigrated neutrophils had to be re-stimulated with an exogenous signal. Even TNFα-stimulated myocytes were unable to deliver an activating signal to emigrated neutrophils to induce the α_4-integrin-dependent adhesion. However, this may be due to the low density of myocytes in our model versus the case in vivo, where emigrated neutrophils are closely associated with a large number of inflamed cardiac myocytes and in the presence of many interstitial cells (including mast cells and fibroblasts). These cells are known to produce many pro-inflammatory molecules in myocardial inflammation; the complement fragment C5a has been found in cardiac lymph during reperfusion of the ischemic heart [106] and bacterial products such as fMLP in bacterial associated myocarditis. Moreover, mast cells release many different stimuli including platelet activating factor, leukotrienes and TNFα, all capable of activating neutrophils. Thus *in vivo*, it is likely that emigrated neutrophils would be exposed to higher concentrations of endogenously-derived agonists than are present in our experimental model, a situation which may invoke α_4-integrin mediated adhesion.

α_4-integrin can support leukocyte adhesion by binding to VCAM-1 or to fibronectin. Anti-VCAM-1 antibody (5F10) had no effect on CD18-independent

adhesion, suggesting that VCAM-1 is not important for adherence in this model. Fibronectin is a constituent of the cardiac extracellular matrix and is found within the transverse tubules of cardiac myocytes [107]. During inflammatory episodes, immunoreactivity for fibronectin increases in affected cardiac tissue, and is associated with penetration of fibronectin into the myocytes [81]. The α_4-integrin-dependent adhesion of neutrophils to myocytes was inhibited by the addition of FN-40 (a fibronectin fragment that contains the CS-1 binding motif for α_4-integrin) (Fig.5.6). As with the anti-α_4-integrin antibody, the inhibitory effect was only revealed in the presence of anti-CD18 antibody. As fibronectin is constitutively present on heart tissue, the lack of requirement for protein synthesis may be significant during post-ischemic neutrophil influx into affected heart tissue, where emigrated neutrophils would find a readily available ligand for newly expressed α_4-integrin.

Whether this newly identified adhesive pathway is physiologically relevant remains to be resolved. To date, anti-CD18 antibodies have confirmed that β_2-integrin adhesion pathway is important in for example myocardial ischemia/reperfusion. However this is almost certainly due to the fact that neutrophils were prevented from adhering to the endothelium via CD-18 and therefore were unable to interact with the postischemic myocyte. In fact, it may not be technically feasible to resolve the importance of CD18 and α_4-integrin as adhesive mechanisms involved in parenchymal cell adhesion *in vivo*. It is interesting that the protective effect of α_4-integrin antibodies could be dissociated from leukocyte recruitment [108,109] and begs the question of whether inhibition of adhesion to parechymal cells can account for some of this protection. Related to this issue, Entman et

al. [21] have demonstrated that preventing neutrophil adhesion to myocytes with anti-adhesive therapy does reduce canine myocyte death. In our study, we saw few myocytes die, even when numerous activated neutrophils were observed to adhere to myocytes. This may be related to differences between dog and rat myocytes or incubation conditions (for example we use tuarine, an antioxidant, in our isolation procedure). The lack of myocyte death following neutrophil adhesion does not preclude the possibility that more subtle dysfunction has occured to these cells and certainly warrants further investigation.

It is apparent that the myocardial damage is due to neutrophil interaction with myocytes under various inflammatory conditions. The current study demonstrates that after emigration, CD18 continues to be important in mediating neutrophil adhesion to cardiac myocytes. However, emigration of neutrophils invokes a second adhesive mechanism, namely α_4-integrin. This emigration-dependent adhesive mechanism may require some consideration in designing drugs to inhibit neutrophil/myocyte interactions.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Recently it has become apparent that neutophils can use mechanisms other than the β_2-integrins for recruitment and extravascular adhesion. Our laboratory and other's have described the presence of β_1-integrins on neutrophils, the relevance of which has yet to be fully characterized. The work presented in this thesis was conducted to determine if $\alpha_4\beta_1$-integrin (VLA-4) has a function in neutrophil recruitment. Using an in vitro flow chamber to mimic the shear conditions found in blood vessels, $\alpha_4\beta_1$-integrin was found to support neutrophil recruitment to its endothelial ligand, VCAM-1. Isolated neutrophils (suspended in buffer), required exogenous stimulation before $\alpha_4\beta_1$-dependent recruitment was observed, whereas unstimulated neutrophils in the presence of whole blood did not. As previously observed for human neutrophils [12], rat neutrophils showed increased expression of α_4-integrin after emigration in response to inflammatory stimuli. This event was associated with the ability of rat neutrophils to adhere to cardiac myocytes via α_4-integrin. Thus $\alpha_4\beta_1$-integrin on neutrophils is a dynamic adhesion molecule, having roles in both intra- and extravascular neutrophil adhesion.

Neutrophils maximally stimulated with fMLP and DHCB were observed to adhere under shear by a mechanism involving both β_2 and β_1-integrins, resulting in enhanced recruitment. In the absence of endothelial selectins, $\alpha_4\beta_1$-dependent neutrophil recruitment was observed at low (isolated neutrophils) or high (whole blood) shear forces. Taken together, these observations suggest that $\alpha_4\beta_1$ can play a significant role in neutrophil recruitment. Under which conditions $\alpha_4\beta_1$-dependent neutrophil recruitment becomes important remains to be determined. The endothelial ligand for $\alpha_4\beta_1$-integrin is

VCAM-1 which is thought to be expressed in the vasculature only after exposure to inflammatory cytokines. However, Henninger et al. have described constitutive expression of VCAM-1 in the vasculatures of the heart, mesentery, small intestine, and brain of mice [110]. Whether this is also the case in humans or if these expression levels are significant enough to promote leukocyte recruitment is not known. With isolated neutrophils, $\alpha_4\beta_1$-integrin expression and recruitment happened only after maximal neutrophil stimulation, as might occur at inflamed tissue sites. Interestingly, $\alpha_4\beta_1$-dependent neutrophil recruitment from whole blood occurred without the need for exogenous stimulation, suggesting that maximal neutrophil stimulation may not be required for $\alpha_4\beta_1$-dependent neutrophil recruitment. Thus the types of tissues and the degree of inflammation may be important factors in determining the extent of $\alpha_4\beta_1$-dependent neutrophil recruitment.

Beyond the vasculature, neutrophils used α_4 -integrins in concert with β_2 -integrins to adhere to cardiac myocytes. Death of cardiac myocytes is associated with neutrophil adhesion, thus blocking of β_2-integrin adhesion may not be sufficient to protect against cardiomyopathy. Whether enhanced adhesion via α_4-integrin will result in increase myocyte death or dysfunction remains to be determined.

While β_2-integrin mediated neutrophil adhesion has been extensively studied, β_1-integrin function on neutrophils has only recently been described. The level of β_1–integrin expression on unstimulated neutrophils is so low as to have excluded them as possible candidates for mediating β_2–integrin-independent adhesion. Part of the reason that β_1-integrin has been difficult to detect may be related its reinternalization in the absence

ligand engagement, which has been shown to retain β_1- integrins on the cell surface [7]. Despite this, neutrophils have been shown to adhere to laminin via $\alpha_6\beta_1$ [111] and to fibronectin via $\alpha_5\beta_1$ [112]. Our results show that functional $\alpha_4\beta_1$ is present in neutrophils, with expression on most cells requiring cell activation. Further work is required to elucidate the presence and location of $\alpha_4\beta_1$ in neutrophils.

Studies designed to examine the function of $\alpha_4\beta_1$ have often employed antibodies against α_4 integrin, and concluded that inhibition of leukocyte function or recruitment in the presence of anti-α_4 antibody implicates a role for $\alpha_4\beta_1$ integrin. Some leukocytes also express $\alpha_4\beta_7$, a molecule that can interact with endothelial ligands to promote leukocyte recruitment. While $\alpha_4\beta_7$ has never been reported on neutrophils, the effects associated with the inhibition of the common α_4 subunit could be attributable to either $\alpha_4\beta_1$ or $\alpha_4\beta_7$. Similarly, the inhibitory effects of anti-β_1 antibodies could be attributable to either $\alpha_5\beta_1$ or $\alpha_6\beta_1$. What is required are reagents that specifically identify and inhibit $\alpha_4\beta_1$ per se. Some may already exist, for example, Bednarczyk et al. have made an antibody that binds an epitope on human VLA-4 comprised of a combination site of the α_4 and β_1 subunits [113]. This antibody could inhibit leukocyte adhesion to fibronectin, but was not able to bind $\alpha_4\beta_7$.

This is the first study to use whole blood for the study of leukocyte recruitment. The advantages of using whole blood are many. The leukocytes do not require isolation, a process which may lead to cell activation. This is not desirable in that some adhesion molecules, such as L-selectin, are known to be shed from leukocytes upon activation [114].

The use of whole blood allows for red cell/leukocyte interactions, which can enhance and reveal subtle interactions between leukocytes and their substrates. As well, preferential recruitment of leukocyte subtypes for different substrates can be observed. A difficulty in using whole blood is that while blood is being perfused, leukocyte interactions with substrata cannot be observed, thus the exact timing and patterns of recruitment cannot be ascertained. For example, whether leukocyte/leukocyte interactions are participating in recruitment cannot be directly observed. As well, the phenotype of interacting leukocytes cannot be determined until after the experiment, thus the types of leukocytes that are rolling or adherent are not known. A potential solution to this problem may be the use of fluorescent microscopy, where blood is treated with leukocyte-specific fluorescently labeled antibodies before perfusion through the chamber. This may allow for visualization of one or more leukocyte types while they are interacting under shear conditions.

Anti-adhesive therapies have been suggested for various inflammatory diseases. Before such therapies become effective, a complete understanding of the mechanisms used by leukocytes for recruitment is necessary. The studies herein suggest that neutrophils can bypass anti-selectin and anti-β_2-integrin therapies for recruitment and for adhesion to extravascular tissues. These studies will prompt further work investigating the role of $\alpha_4\beta_1$ in neutrophil function, such as in various models of inflammatory disease.

Bibliography

1. Fantone JC, Ward PA: Polymorphonuclear leukocyte-mediated cell and tissue injury: oxygen metabolites and their relations to human disease. *Hum Pathol* 1985;16:973-978

2. Lucchesi BR, Werns SW, Fantone JC: The role of the neutrophil and free radicals in ischemic myocardial injury. *J Mol Cell Cardiol* 1989;21:1241-1251

3. Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork A, Lucchesi BR: Reduction in the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* 1983;67:1016

4. Anderson DC: The role of β_2 integrins and intercellular adhesion molecule type 1 in inflammation, in Granger DN, Schmid-Schonbein GW (eds): *Physiology and pathophysiology of leukocyte adhesion*. New York, Oxford University Press, 1995, pp 3-42

5. Kishimoto TK, Anderson DC: The role of integrins in inflammation, in Gallin JI, Goldstein IM, Snyderman R (eds): *Inflammation: Basic Principles and Clinical Correlates*. ed2nd. New York, Raven Press,Ltd. 1992, pp 353-406

6. Albelda SM, Buck CA: Integrins and other cell adhesion molecules. *FASEB J* 1990;4:2868-2880

7. Dalton SL, Scharf E, Briesewitz R, Marcantonio EE, Assoian RK: Cell adhesion to extracellular matrix regulates the life cycle of integrins. *Mol Biol Cell* 1995;6:1781-1791

8. Alon R, Kassner PD, Carr MW, Finger EB, Hemler ME, Springer TA: The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J Cell Biol* 1995;128:1243-1253

9. Luscinskas FW, Ding H, Tan P, Cumming D, Tedder TF, Gerritsen ME: L- and P-selectins, but not CD49d (VLA-4) integrins, mediate monocyte initial attachment to TNFα-activated vascular endothelium under flow in vitro. *J Immunol* 1996;156:326-335

10. Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR: VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* 1990;60:577-584

11. Hemler ME: VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu Rev Immunol* 1990;8:365-400

12. Kubes P, Niu X-F, Smith CW, Kehrli ME, Jr., Reinhardt PH, Woodman RC: A novel β_1-dependent adhesion pathway on neutrophils: A mechanism invoked by dihydrocytochalasin B or endothelial transmigration. *FASEB J* 1995;9:1103-1111

13. Issekutz TB, Miyasaka M, Issekutz AC: Rat blood neutrophils express very late antigen 4 and it mediates migration to arthritic joint and dermal inflammation. *J Exp Med* 1996;183:2175-2184

14. Schmid-Schonbein GW, Usami S, Skalak R, Chien S: The interaction of leukocytes and erythrocytes in capillary and postcapillary vessels. *Microvasc Res* 1980;19:45-70

15. Melder RJ, Munn LL, Yamada S, Ohkubo C, Jain RK: Selectin and integrin-mediated T lymphocyte rolling and arrest on TNFα-activated endothelium: Augmentation by erythrocytes. *Biophysical Journal* 1995;69:2131-2138

16. Melder RJ, Munn LL, Yamada S, Ohkubo C, Jain RK: Selectin- and integrin-mediated T-lymphocyte rolling and arrest on TNFα-activated endothelium: augmentation by erythrocytes. *Biophysical Journal* 1995;69:2131-2138

17. Ma X, Tsao PS, Lefer AM: Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. *J Clin Invest* 1991;88:1237-1243

18. Lefer DJ, Nakanishi K, Johnston WE, Vinten-Johansen J: Antineutrophil and myocardial protecting actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion in dogs. *Circulation* 1993;88, no. 5 part 1:2337-2350

19. Entman ML, Youker K, Shappell SB, Siegel C, Rothlein R, Dreyer WJ, Schmalstieg FC, Smith CW: Neutrophil adherence to isolated adult canine myocytes-Evidence for a CD18-dependent mechanism. *J Clin Invest* 1990;85:1497-1506

20. Smith CW, Entman ML, Lane CL, Beaudet AL, Ty TI, Youker K, Hawkins HK, Anderson DC: Adherence of neutrophils to canine cardiac myocytes in vitro is dependent on intercellular adhesion molecule-1. *J Clin Invest* 1991;88:1216-1223

21. Entman ML, Youker K, Shoji T, Kukielka G, Shappell SB, Taylor AA, Smith CW: Neutrophil induced oxidative injury of cardiac myocytes. *J Clin Invest* 1992;90:1335-1345

22. Bainton DF: Developmental biology of neutrophils and eosinophils, in Gallin JI, Goldstein IM, Snyderman R: *Inflammation: Basic principles and clinical correlates.* ed2nd. New York, Raven Press, 1992, pp 303-324

23. Bokoch GM: Chemoattractant signaling and leukocyte activation. *Blood* 1995;86:1649-1660

24. Lipowsky HH: Leukocyte margination and deformation in postcapillary venules, in Granger DN, Schmid-Schonbein GW (eds): *Physiology and pathophysiology of leukocyte adhesion*. New York, Oxford University Press, 1995, pp 130-147

25. Von Ritter C, Grisham MB, Hollwarth M, Inauen W, Granger DN: Neutrophil-derived oxidants mediate formyl-methionyl-leucyl-phenylalanine-induced increases in mucosal permeability in rats. *Gastroenterology* 1989;97:778-780

26. Fiebig E, Ley K, Arfors KE: Rapid leukocyte accumulation by "spontaneous" rolling and adhesion in the exteriorized rabbit mesentery. *Int J Microcirc Clin Exp* 1991;10:127-144

27. Firrell JC, Lipowsky HH: Leukocyte margination and deformation in mesenteric venules of rat. *Am J Physiol* 1989;256:H1667-H1674

28. Kansas GS: Selectins and their ligands: Current concepts and controversies. *Blood* 1996;88:3259-3287

29. Dore M, Korthuis RJ, Granger DN, Entman ML, Smith CW: P-selectin mediates spontaneous leukocyte rolling in vivo. *Blood* 1993;82:1308-1316

30. Tedder TF, Steeber DA, Chen A, Engel P: The selectins: vascular adhesion molecules. *FASEB J* 1995;9:866-873

31. Bevilacqua MP, Nelson RM: Selectins. *J Clin Invest* 1993;91:379-387

32. Spinger TA, Lasky LA: Sticky sugars for selectins. *Nature* 1991;349:196-197

33. Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA: Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J Cell Biol* 1991;115:223-234

34. Collins PW, Macey MG, Cahill MR, Newland AC: Von Willebrand factor release and P-selectin expression is stimulated by thrombin and trypsin but not IL-1 in cultured human endothelial cells. *Thromb Haemost* 1993;70:346-350

35. Gaboury J, Granger DN, Anderson DC, Kubes P: Superoxide induces leukocyte-endothelial cell interactions by PAF, CD11/CD18 and P-selectin. *FASEB J* 1993;Abstract:1564(abstract)

36. Hattori R, Hamilton KK, McEver RP, Sims PJ: Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. *J Biol Chem* 1989;264:9053-9060

37. Jones DA, Abbassi O, McIntire LV, McEver RP, Smith CW: P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. *Biophysical Journal* 1993;65:1560-1569

38. Kubes P, Kanwar S: Histamine induces leukocyte rolling in post-capillary venules: A P-selectin-mediated event. *J Immunol* 1994;152:3570-3577

39. Bischoff J, Brasel C: Regulation of P-selectin by tumor necrosis factor-α. *Biochemical and Biophysical Research Communications.* 1995;210:174-180

40. Eppihimer MJ, Wolitzky B, Anderson DC, Labow MA, Granger DN: Heterogeneity of expression of E- and P-selectins in vivo. *Circ Res* 1996;79:560-569

41. Moore KL, Patel KD, Brehl RE, Johnson DA, Lichenstein HS, Cummings RD, Bainton DF, McEver RP: P-selectin glycoprotein ligand mediates rolling of human neutrophils on P-selectin. *J Cell Biol* 1995;128(4):661-671

42. Yang J, Galipeau J, Kozak CA, Furie BC, Furie B: Mouse P-selectin glycoprotein ligand-1: molecular cloning, chromosomal localization, and expression of a functional P-selectin receptor. *Blood* 1996;87:4176-4186

43. Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, Varki A, McEver RP: Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J Cell Biol* 1992;118:445-456

44. Olofsson AM, Arfors KE, Ranezani L, Wolitzky B, Butcher EC, Von Andrian UH: E-selectin mediates leukocyte rolling in interleukin-1-treated rabbit mesentery venules. *Blood* 1994;84:2749-2758

45. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA: Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc Natl Acad Sci USA* 1987;84:9238-9242

46. Patel KD, Moore KL, Nollert MU, McEver RP: Neutrophils use both shared and distinct mechanisms to adhere to selectins under static and flow conditions. *J Clin Invest* 1995;96:1887-1896

47. Picker LJ, Warnock RA, Burns AR, Doerschuk CM, Berg EL, Butcher EC: The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* 1991;66:921-933

48. Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC: ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature* 1991;349:796-802

49. Springer TA, Anderson DC, Rosenthal AS, Rothlein R: *Leukocyte Adhesion Molecules: Structure, Function and Regulation*, Springer-Verlag, 1990,

50. Springer TA: Traffic signals of lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell* 1994;76:301-314

51. Bosse R, Vestweber D: Only simultaneous blocking of the L- and P-selectin completely inhibits neutrophil migration into mouse peritoneum. *Eur J Immunol* 1994;24:3019-3024

52. Ma X, Weyrich AS, Lefer DJ, Buerke M, Albertine KH, Kishimoto TK, Lefer AM: Monoclonal antibody to L-selectin attenuates neutrophil accumulation and protects ischemic reperfused cat myocardium. *Circulation* 1993;88:649-658

53. Bargatze RF, Kurk S, Butcher EC, Jutila MA: Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. *J Exp Med* 1994;180:1785-1792

54. Walchek B, Moore KL, McEver RP, Kishimoto TK: Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1. A mechanism that amplifies initial leukocyte accumulation on P-selectin in vitro. *J Clin Invest* 1996;98:1081-1087

55. Hamburger SA, McEver RP: GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 1990;75:550-554

56. Diamond MS, Springer TA: The dynamic regulation of integrin adhesiveness. *Curr Biol* 1994;4:506-517

57. Anderson DC, Springer TA: Leukocyte adhesion deficiency: An inherited defect in the MAC-1, LFA-1, and p150/95 glycoproteins. *Ann Rev Med* 1987;38:175-194

58. Albelda SM, Smith CW, Ward PA: Adhesion molecules and inflammatory injury. *FASEB J* 1994;8:504-512

59. Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA: Induction by IL 1 and interferon-gamma: Tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol* 1986;137:245-254

60. Zimmerman GA, McIntyre TM, Prescott SM: Perspectives series: Cell adhesion in vascular biology. Adhesion and signaling in vascular cell-cell interactions. *J Clin Invest* 1996;98:1699-1702

61. Nathan CF, Srimal S, Farber C, Sanchez E, Kabbash L, Asch A, Gailit J, Wright SD: Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J Cell Biol* 1989;109:1341-1349

62. Huber AR, Kunkel SL, Todd III RF, Weiss SJ: Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* 1991;254:99-102

63. Muller WA, Weigl SA, Deng X, Phillips DM: PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med* 1993;178:449-460

64. Bogen S, Pak J, Garifallou M, Deng X, Muller WA: Monoclonal antibody to murine PECAM-1 (CD31) blocks acute inflammation in vivo. *J Exp Med* 1994;179:1059-1064

65. Henson PM, Johnston Jr. RB: Tissue injury in inflammation. *J Clin Invest* 1987;79:669-674

66. Engler RL, Schmid-Schonbein GW, Pavelec RS: Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. *Am J Pathol* 1983;111:98-111

67. Simpson PJ, Todd RF, Fantone JC, Mickelson JK, Griffin JD, Lucchesi BR: Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. *J Clin Invest* 1988;81:624-629

68. Weyrich AS, Ma X, Lefer DJ, Albertine KH, Lefer AM: In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. *J Clin Invest* 1993;91:2620-2629

69. Engler R, Covell JW: Granulocytes cause reperfusion ventricular dysfunction after 15-minute ischemia in the dog. *Circ Res* 1987;61:20-28(abstract)

70. Reinhardt P, Naccache PH, Poubelle PE, de Medicis R, Kehrli ME, Jr., Kubes P: Monosodium urate crystals promote neutrophil adhesion via a CD18-independent and selectin-independent mechanism. *Am J Physiol* 1996;39:C31-C39

71. Issekutz AC, Chuluyan HE, Lopes N: CD11/CD18-independent transendothelial migration of human polymorphonuclear leukocytes and monocytes: involvement of distinct and unique mechanisms. *J Leukoc Biol* 1995;57:553-561

72. Doerschuk CM, Winn RK, Coxson HO, Harlan JM: CD18-dependent and -independent mechanisms of neutrophil emigration in the pulmonary and systemic microcirculation of rabbits. *J Immunol* 1990;144:2327-2333

73. Winn RK, Harlan JM: CD18-independent neutrophil and mononuclear leukocyte emigration into the peritoneum of rabbits. *J Clin Invest* 1993;92:1168-1173

74. Issekutz AC, Issekutz TB: A major portion of polymorphonuclear leukocyte and T lymphocyte migration to arthritic joints in the rat is via LFA-1/MAC-1-independent mechanisms. *Clin Immunol Immunopathol* 1993;67:257-263

75. Takada Y, Elices MJ, Crouse C: The primary stucture of the α_4-subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. *EMBO J* 1989;8:1361-1368

76. Reszka AA, Hayaski Y, Horwitz AF: Identification of amino acid sequences in the integrin β_1 cytoplasmic domain implicated in cytoskeletal association. *J Cell Biol* 1992;117:1321-1330

77. Schleimer RP, Sterbinsky SA, Kaiser J, Bickel CA, Klunk DA, Tomioka K, Newman W, Luscinskas FW, Gimbrone Jr. MA, McIntyre BW, Bochner BS: IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. *J Immunol* 1992;148:1086-1092

78. Gao J-X, Issekutz AC: The β_1 integrin, very late activation antigen-4 on human neutrophils can contribute to neutrophil migration through connective tissue fibroblast barriers. *Immunology* 1997;90:448-454

79. Carlos TM, Schwartz BR, Kovach NL, Lee E, Rosso M, Osborn L, Chi-Rosso G, Newman B, Lobb R, Harlan JM: Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cutured human endothelial cells. *Blood* 1990;76:965-970

80. Vonderheide RH, Tedder TF, Springer TA, Staunton DE: Residues within a conserved amino acid motif of domains 1 and 4 of VCAM-1 are required for binding to VLA-4. *J Cell Biol* 1994;125:215-222

81. Froen JF, Larsen TH: Fibronectin penetration into heart myocytes subjected to experimental ischemia by coronary artery ligation. *Acta Anat* 1995;152:119-126

82. Issekutz TB, Issekutz AC: T lymphocyte migration to arthritic joints and dermal inflammation in the rat: differing migration patterns and the involvement of VLA-4. *Clin Immunol Immunopathol* 1991;61:436-447

83. Chishom PL, Williams CA, Lobb RR: Monoclonal antibodies to the integrin alpha-4 subunit inhibit the murine contact hypersensitivity response. *Eur J Immunol* 1996;23:682-688

84. Abe Y, Ballantyne CM, Smith CW: Functions of domain 1 and 4 of vascular cell adhesion molecule-1 in α_4 integrin dependent adhesion under static and flow conditions are differentially regulated. *J Immunol* 1996;157:5061-5069

85. Sriramarao P, Von Andrian UH, Butcher EC, Bourdon MA, Broide DH: L-selectin and very late antigen-4 integrin promote eosinophil rolling at physiological shear rates in vivo. *J Immunol* 1994;153:4238-4246

86. Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N: Prevention of experimental autoimmune encephalomyelitis by antibodies against α4β1 integrin. *Nature* 1992;356:63-66

87. Issekutz TB: Dual inhibition of VLA-4 and LFA-1 maximally inhibits cutaneous delayed-type hypersensitivity-induced inflammation. *Am J Pathol* 1993;143:1286-1293

88. Baron JL, Reich EP, Visintin I, Janeway, Jr. The pathogenesis of adoptive murine autoimmune diabetes requires an interaction between α_4-integrins and vascular cell adhesion molecule-1. *J Clin Invest* 1994;93:1700-1708

89. Johnston B, Issekutz TB, Kubes P: The α4-integrin supports leukocyte rolling and adhesion in chronically inflamed postcapillary venules in vivo. *J Exp Med* 1996;181:1995-2006

90. Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC: Role of α4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol* 1994;152:3282-3293

91. Ruegg C, Postiga AA, Sikorski EE: Role of $\alpha_4\beta_7$ integrin in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J Cell Biol* 1992;117:179-189

92. Lawrence MB, Springer TA: Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 1991;65:859-873

93. Abbassi O, Kishimoto TK, McIntire LV, Anderson DC, Smith CW: E-selectin supports neutrophil rolling in vitro under conditions of flow. *J Clin Invest* 1993;92:2719-2730

94. Smith CW, Rothlein R, Hughes BJ, Mariscalco MM, Rudloff HE, Schmalstieg FC, Anderson DC: Recognition of an endothelial determinant for CD18-dependent human neutrophil adherence and transendothelial migration. *J Clin Invest* 1988;82:1746-1756

95. Bouchard RA, Clark RB, Giles WR: Effects of action potential duration on excitation-contraction coupling in rat ventricular myocytes. *Circ Res* 1995;76:790-801

96. Wayner EA, Garcia-Pardo A, Humphries MJ, McDonald JA, Carter WG: Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J Cell Biol* 1989;109:1321-1330

97. Poul MA, Ticchioni M, Bernard A, Lefranc MP: Inhibition of T-cell activation with a humanized anti-beta1 integrin chain mAb. *Molecular Immunology* 1995;32:101-116

98. Arndt H, Kubes P, Grisham MB, Gonzalez E, Granger DN: Granulocyte turnover in the feline intestine. *Inflammation* 1992;16:549-559

99. Chishom PL, Williams CA, Lobb RR: Monoclonal antibodies to the integrin α_4 subunit inhibit the murine contact hypersensitivity response. *Eur J Immunol* 1993;23:682-688

100. Kubes P, Jutila M, Payne D: Therapeutic potential of inhibiting leukocyte rolling in ischemia/reperfusion. *J Clin Invest* 1995;95:2510-2519

101. Bohnsack JF, Akiyama SK, Damsky CH, Knape WA, Zimmerman GA: Human neutrophil adherence to laminin in vitro. *J Exp Med* 1990;171:1221-1237

102. Kerst JM, Sanders JB, Slaper-Cortenbach ICM, Doorakkers MC, Hooibrink B, van Oers RHJ, von dem Borne AEGK, van der Schoot CE: $\alpha_4\beta_1$ and $a_5\beta_1$ are differentially expressed during myelopoiesis and mediate the adherence of human CD34+ cells to fibronectin in an activation-dependent way. *Blood* 1993;81:344-351

103. Williams WJ, Beutler E, Erslev AJ, Lichtman MA: Hematologic abnormalities complicating medical disorders. in Anonymous*Hematology*. ed4th. New York, McGraw-Hill Pub. Co. 1990, pp 1585-1594

104. Ban K, Ikeda U, Takahashi M, Kanbe T, Kasahara T, Shimada K: Expression of intercellular adhesion molecule-1 on rat cardiac myocytes by monocyte chemoattractant protein-1. *Cardiovas Res* 1994;28:1258-1262

105. Hughes BJ, Hollers JC, Crockett-Torabi E, Smith CW: Recruitment of CD11b/CD18 to the neutrophil surface and adherence-dependent cell locomotion. *J Clin Invest* 1992;90:1687-1696

106. Dreyer WJ, Smith CW, Michael LH, Rossen RD, Hughes BJ, Entman ML, Anderson DC: Canine neutrophil activation by cardiac lymph obtained during reperfusion of ischemic myocardium. *Circ Res* 1989;65:1751-1762

107. Speiser B, Weihrauch D, Reiss CF, Schaper J: The extracellular matrix in human cardiac tissue. Part II: Vimentin, laminin, and fibronectin. *Cardioscience* 1992;3:41-49

108. Abraham WM, Sielczak MW, Ahmed A, Cortes A, Lauredo IT, Kim J, Pepinsky B, Benjamin CD, Leone DR, Lobb RR, Weller PF: α_4-integrins mediate antigen-induced late

bronchial responses and prolonges airway hyperresponsiveness in sheep. *J Clin Invest* 1994;93:776-787

109. Rabb HA, Olivenstein R, Issekutz TB, Renzi PM, Martin JG: The role of the leukocyte adhesion molecules VLA-4, LFA-1, and Mac-1 in allergic airway responses in the rat. *Am J Respir Crit Care Med* 1994;149:1186-1191

110. Henninger DD, Panes J, Eppihimer M, Russell J, Gerritsen M, Anderson DC, Granger DN: Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse. *J Immunol* 1997;158:1825-1832

111. Bohnasck JF: CD11/CD18-independent neutrophil adherence to laminin is mediated by the integrin VLA-6. *Blood* 1992;79:1545-1552

112. Bohnsack JF, Zhou X: Divalent cation substitution reveals CD18- and very late antigen-dependent pathways that mediate human neutrophil adherence to fibronectin. *J Immunol* 1992;149:1340-1347

113. Bednarczyk JL, Szabo MC, Wygant JN, Lazarovits AI, McIntyre BW: Indentification of a combiniatorial epitope expressed by $\alpha_4\beta_1$ heterodimer involved in the regulation of cell adhesion. *J Biol Chem* 1994;269:8348-8354

114. Kishimoto TK, Jutila MA, Berg EL, Butcher EC: Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* 1989;245:1238-1241

115. Fan FC, Chen RYZ, Schluessler GB, Chein S: Effects of hematocrit variations on regional hemodynamics and oxygen transport in dogs. *Am J Physiol* 1980;238:H545-H552(abstract)

Appendices

Appendix A: Viscosity Values for Various Hematocrit.

Hematocrit (%)	Viscosity (cp)
30	3.38
35	3.65
40	4.05
41	4.14
42	4.23
43	4.32
44	4.41
45	4.50
46	4.64
47	4.77
48	4.91
49	5.04
50	5.18
55	5.94
60	6.75

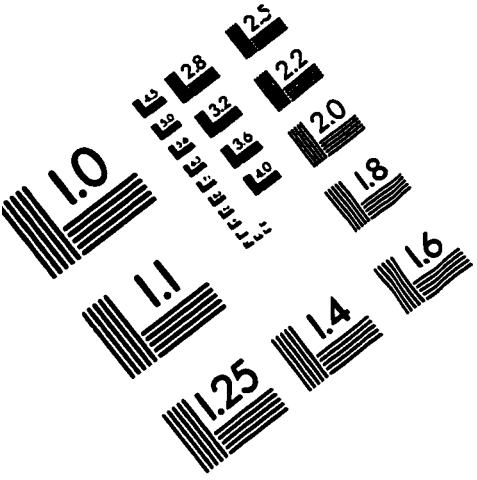
All data are taken from *in vitro* viscosity measurements at a shear rate of 200 sec [115].

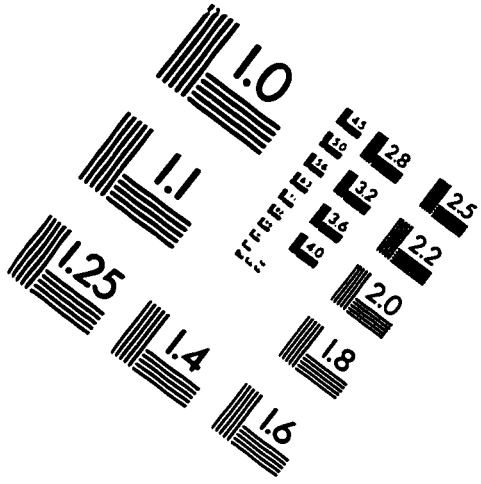
Appendix B: This thesis is partially compiled from the following manuscripts:

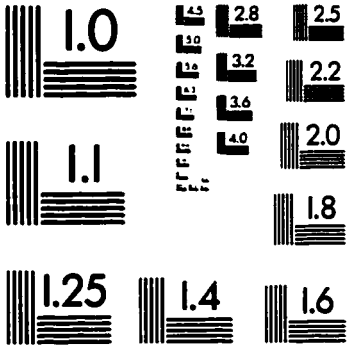
Paul H. Reinhardt, John F. Elliott, and Paul Kubes. Neutrophils can adhere via $\alpha_4\beta_1$ integrin under flow conditions. Blood, 89 (10): 3837-3846, 1997.

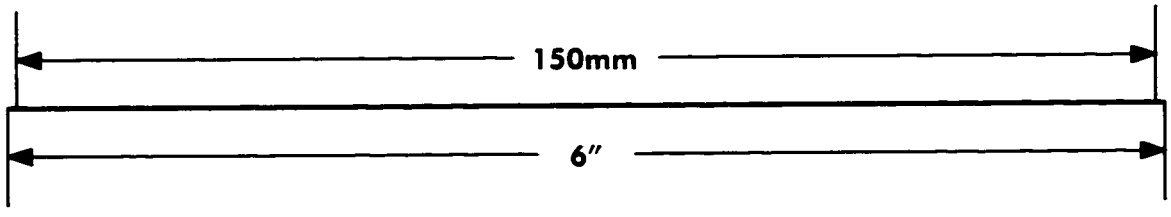
Paul H. Reinhardt, Christopher A. Ward, Wayne R. Giles, and Paul Kubes. Emigrated rat neutrophils adhere to cardiac myocytes via α_4-integrin. Circ. Res., 81: 196-201, 1997.

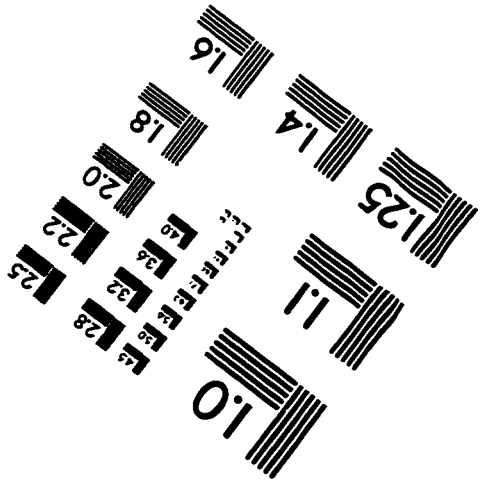
IMAGE EVALUATION
TEST TARGET (QA-3)

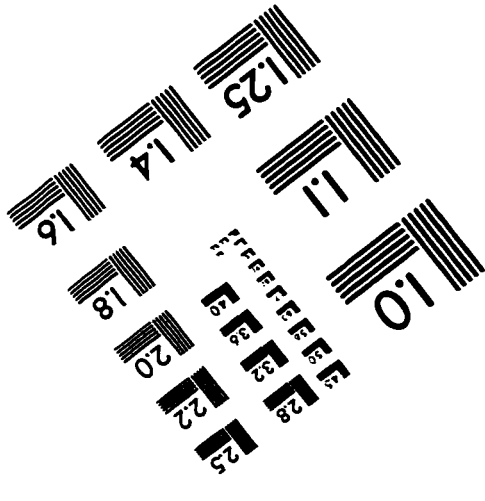












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved