

The Effect of Anticoagulants on White Blood Cell L-selectin Levels

by
Tracy L. Smith

**Submitted in Partial Fulfillment of the Requirements
for the Degree of
Master of Science
in the
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Program**

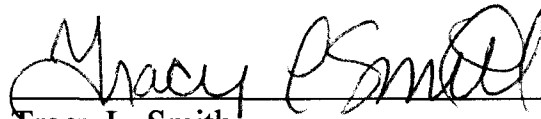
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Tracy L. Smith


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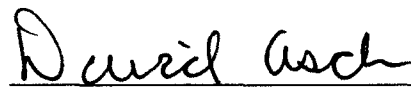

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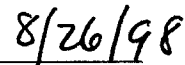

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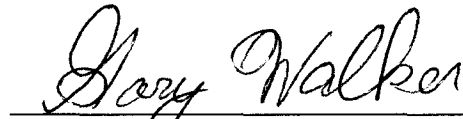
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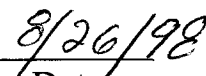

Dr. Diana Fagan

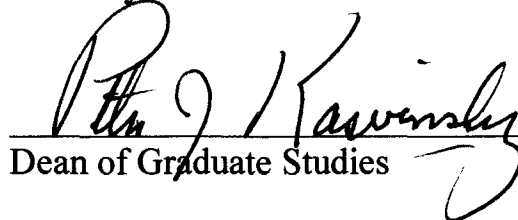

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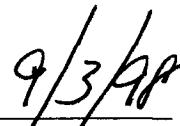

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ABSTRACT

Cell Adhesion Molecules (CAM's) are proteins embedded in the membranes of cells that bind to carbohydrates found on other cells. During an immune response, CAM's mediate the initial attachment of cells to the vessel wall. Tethering and "slow rolling" of the cell through the blood vessel occurs next, followed by movement of the cell through the vessel wall to the site of infection. L-selectin is a specific CAM that is initially involved in attachment of the white blood cell to the vessel wall. In order for the cell to squeeze through the blood vessel and migrate towards the site of infection, L-selectin must be shed. Stimulation of the white blood cells can be monitored by quantitating L-selectin levels on the cells. To study the white blood cells, it is important to use an anticoagulant that will prevent clotting of the blood, but not stimulate the cells. These studies compare the effects of four well known anticoagulants; EDTA, Potassium Oxalate, Sodium Citrate and Heparin on white blood cell expression of L-selectin molecules. Blood was drawn from 7 volunteers into a vacuum tube containing one of the anticoagulants. Blood samples were removed at various timepoints up to one hour after collection and placed on ice. At one hour the cells were incubated with fluorescently labeled antibodies that bind specifically to L-selectin. Lysis buffer was added to lyse the red blood cells, leaving only the white blood cells. Paraformaldehyde was used to preserve the cells until they could be analyzed using a flow cytometer. The flow cytometer counts the cells one at a time using light patterns that deflect off of the cells. The fluorescence associated with the cells was measured to determine the L-selectin levels. The study showed that incubation in EDTA caused the least stimulation of the cells over time and therefore was the best anticoagulant to use when studying L-selectin.

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I would like to take this opportunity to thank my advisor, Dr. Diana Fagan for all of the time and energy she put into helping me with my research and into preparing this written thesis. Her knowledge and experience has been beneficial both inside and outside of the laboratory. I am grateful for having had an opportunity to work with her and wish her the best in her future endeavors.

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A thank-you also goes to my family and friends for their encouragement and support. I would especially like to thank my mother for always pushing me to strive to reach my goals. She showed me that a woman could move mountains in today's world and for that I am truly grateful. I hope that I didn't drive her too close to the edge during these last two months when I was writing this thesis. In addition, I need to thank my grandmother for always being there for me. She has kept me sane over the years (especially during the writing of this thesis).

Finally I want to thank God for giving me both the intelligence and the drive to pursue my studies. Without him, none of this would have been possible.

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LIST OF ABBREVIATIONS

ANOVA	one way repeated measures analysis of variance
ARDS	Acute Respiratory Distress Syndrome
CAM	cell adhesion molecule
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
E-selectin	endothelial selectin (CAM)
FITC	fluorescein isothiocyanate (fluorescent tag)
GAM	goat anti-mouse (2 ^o antibody)
GLyCAM	(CAM) mucin-like
ICAM	intracellular adhesion molecule (CAM)
Ig	immunoglobulin
IL-1	interleukin-1
LAD	Leukocyte Adhesion Deficiency
LFA	lymphocyte function associated antigen (CAM)
L-selectin	leukocyte selectin (CAM)
mAb	monoclonal antibody
MAdCAM	(CAM) mucin-like
Mac-1	(CAM) integrin
MF	mean fluorescence
PBS	phosphate buffered saline
PE	phycoerythrin (fluorescent tag)
PECAM	platelet endothelial cell adhesion molecule (CAM)
PFM	paraformaldehyde
PMA	phorbol myristate acetate (mitogen)

P-selectin	platelet selectin (CAM)
RBC	red blood cell
SEM	standard error of the mean
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule (CAM)
VLA	very late antigen (CAM)
WBC	white blood cell

INTRODUCTION

Inflammation:

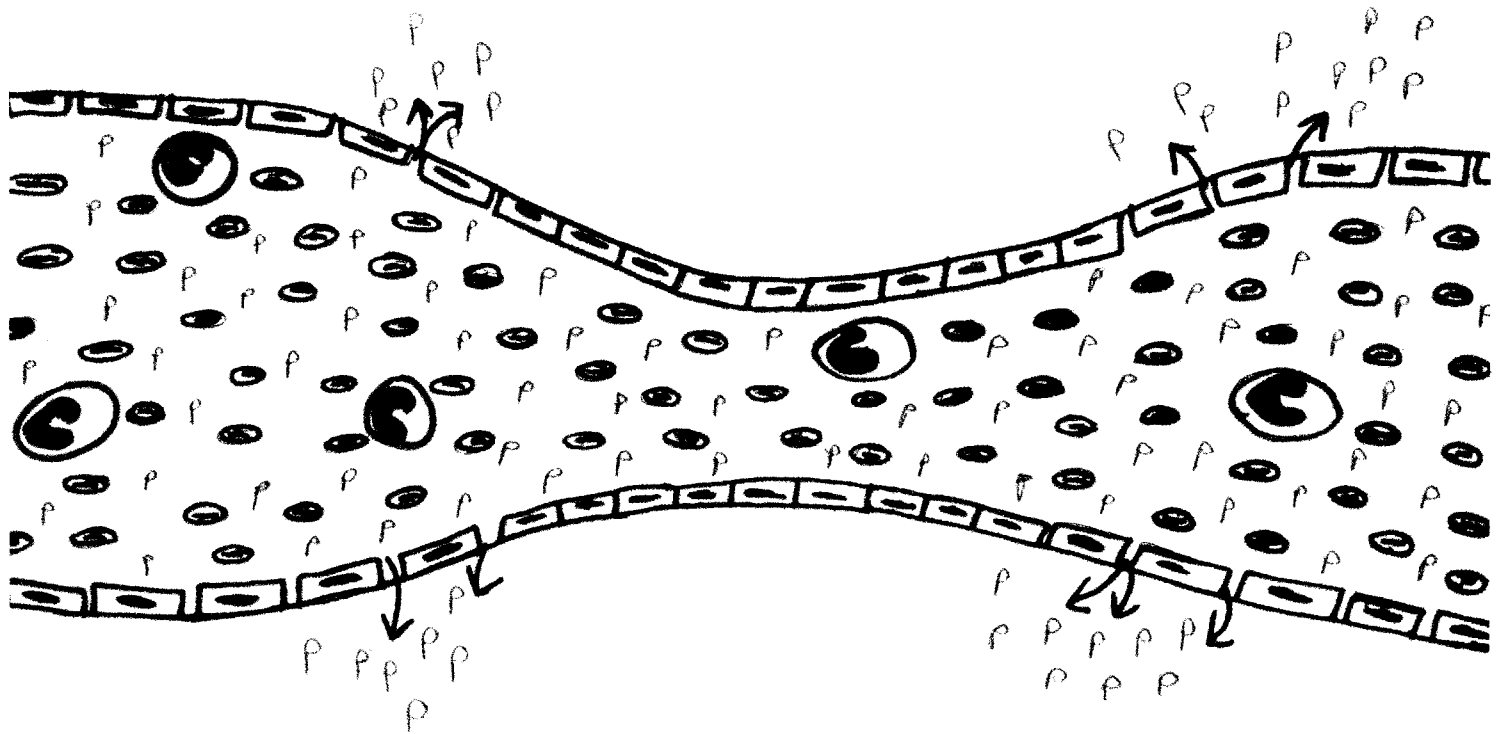
Cornelius Celsus, a first century A.D. writer, described the four cardinal signs of inflammation as rubor, tumor, calor and dolor (redness, swelling, heat and pain).⁽²⁷⁾ These cardinal signs of inflammation signal 3 major events that take place during an inflammatory response. The first of these is vasodilation. This is an increase in the diameter of the blood vessel that occurs at the arterial level as a result of the constriction of the veins at the site of injury. Capillary engorgement results and is responsible for tissue redness and heat. The next event to occur is increased vascular permeability. This leads to the loss of fluid, the congestion of blood cells and the adhesiveness of phagocytic cells (Figure 1). Exudate (high protein fluid lost from the blood vessel) accumulates in the tissue which leads to edema (swelling of the tissue). The third event is the influx of phagocytic cells. As the white blood cells (monocytes and neutrophils) roll along the blood vessel wall more slowly, they are exposed to chemotactic factors which activate them. This activation of the white blood cells leads to their emigration between the cells of the blood vessel wall and into the tissue.

There are two types of immune responses launched by the body in its defense against infectious disease. Acquired, or specific immunity includes cell mediated, humoral and allergic responses.⁽²⁷⁾ Innate, or nonspecific immunity is the second type of immune response. Nonspecific immunity is the focus of this research because it involves the basic resistance of an organism to disease.⁽²⁷⁾ Neutrophils and monocytes are phagocytic cells (cells that

Figure 1:

This is a pictorial representation of a blood vessel responding to a local infection. Vasodilatation (blood vessel wall increases in diameter) first occurs at the arterial level as a result of the constriction (blood vessel wall decreases in diameter) of the veins at the site of injury. Capillary engorgement results as well as increased vascular permeability. This leads to the loss of fluid, the congestion of blood cells and increased adhesiveness of phagocytic cells. Exudate (high protein fluid lost from the blood vessel) accumulates in the tissue which leads to edema (swelling of the tissue). As the white blood cells (monocytes and neutrophils) roll along the blood vessel wall more slowly, they are exposed to chemotactic factors which activate them. This activation of the white blood cells leads to their emigration between the blood vessel wall and into the tissue.

Figure 1



- represents red blood cells
- P represents plasma (exudate in the tissues)
- represents white blood cells

ingest and destroy exogenous material) which act to destroy and remove inflammatory stimulus and in the case of monocytes, clean up the cellular and tissue debris prior to tissue repair. Neutrophils are the first cells to respond to an inflammatory stimulus and provide a rapid, relatively non-specific defense mechanism. Following emigration (the movement of the cell from the blood vessel into the tissue), they have the shortest life span in tissues. They respond 30-40 minutes after the initial inflammatory stimulus as opposed to monocytes which usually respond 6-18 hours later. Neutrophils are terminally differentiated, compared to monocytes which can divide and differentiate for a specific “acquired” defense.⁽²⁵⁾ Neutrophils are the most motile of the leukocytes and are present in the largest concentration in the blood. In the non-specific inflammatory response, the intensity of the response is positively correlated to the injury. Occasionally the immune system will overreact and this can lead to an attack on the host itself. This is observed in ARDS (Acute Respiratory Distress Syndrome) which will be discussed in more detail later.

Cell Adhesion Molecules:

CAM's are proteins embedded in the membranes of cells that bind to carbohydrates found on toxins, viruses, bacteria and other cells. In addition to being the receptor that causes binding during an infection or an immune response, CAM's help cells bind to one another and initiate cell-cell interactions. There are four types of CAM's involved in white blood cell adherence events: selectins, integrins, Immunoglobulin superfamily and

sialomucins. The studies described herein examine a member of the selectin family of CAM.

There are three members in the selectin family: endothelial selectin (E-selectin), platelet selectin (P-selectin) and leukocyte selectin (L-selectin).⁽³⁷⁾ They contain 2 alpha helices, 2 antiparallel beta sheets (one of which is formed by 2 strands and the other by 3 strands) and 2 intact disulfide bonds formed by cysteine residues.⁽⁴⁾ There are several domains within the selectin structure. (Figure 2) The first domain consists of a calcium dependent lectin responsible for cell-cell contact with surface carbohydrates. The carbohydrate binding lectins are believed to be responsible for the adhesive functions of these proteins. Another domain has an epidermal growth factor (EGF) region responsible for ligand recognition. The EGF region contains a series of consensus repeats, not unlike those in complement and complement regulatory proteins and may bind directly to the protein portion of a glycosylated ligand. Lastly, there is a transmembrane domain and a cytoplasm tail.

Selectins are expressed on resting WBC's and are released from the cell membrane upon activation of a protease.⁽²⁶⁾ Both E- and P- selectins are found on endothelial cells, while L-selectin is expressed on resting white blood cells. The selectin ligand is not present on resting endothelial cells, but is expressed on the endothelial cells following an inflammatory signal (Figure 3).⁽⁴⁶⁾ The selectins bind to sialylated, fucosylated and in some cases sulfated carbohydrates. All selectins seem to recognize a sialylated carbohydrate determinant on their counterreceptors.⁽¹⁷⁾ E- and P- selectin recognize separate but closely related structures to the tetrasaccharide sialyl-Lewis^X and its isomer sialyl-Lewis^a. The carbohydrate ligand for

Figure 2:

This is a pictorial representation of an L-selectin molecule. The soluble portion of the selectin molecules contain an amino end group, a calcium dependent lectin domain, a single domain with homology to epidermal growth factor and a series of repeats related to compliment binding proteins. The non-soluble portion of the selectin molecule that is shed during the process of extravasation contains an anchor and a cytoplasmic tail.

Figure 2

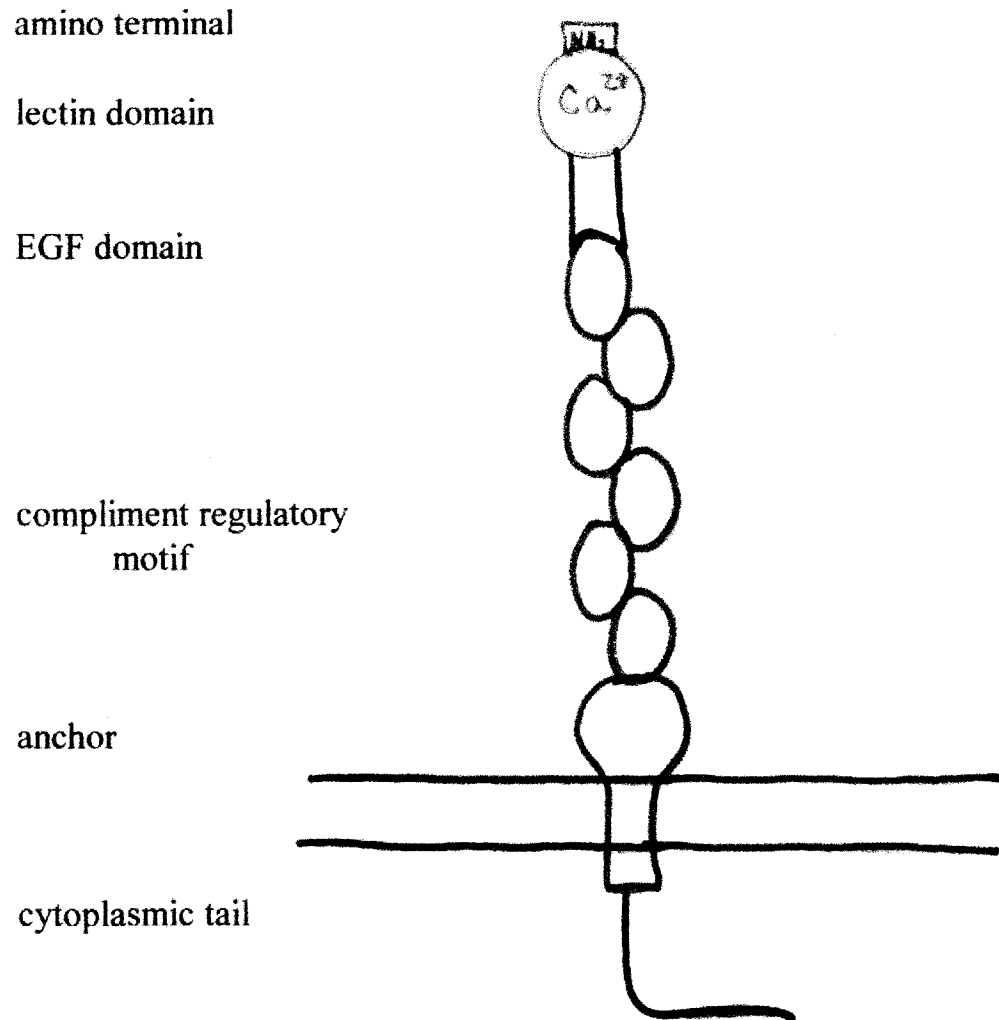
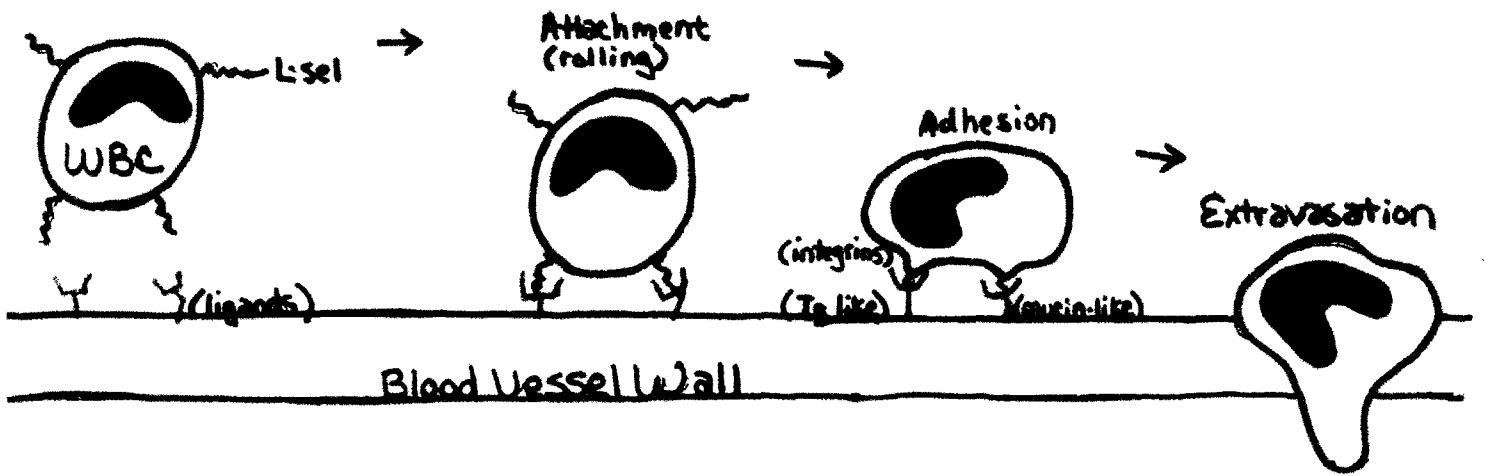


Figure 3:

During an infection, chemoattractants (chemical signals) call the white blood cell out of circulation within the blood vessel. The endothelial cells are then activated and express a mucin-like molecule which acts as a ligand for L-selectin. The ligand stimulates weak, selectin-mediated interactions with the white blood cell which tether the neutrophil along the blood vessel wall. The neutrophil proceeds to "roll" along the endothelium at a slower rate than the flow of blood. The chemoattractants in the endothelium can then activate the white blood cell which then expresses integrins on its surface. Immunoglobulin-like cell adhesion molecules, which are expressed on the endothelium tightly bind with the integrins on the neutrophil, causing the white blood cell to arrest. Once the cell is arrested, margination (a process where white blood cells bind to the endothelial wall of the capillary) occurs. Integrin binding then stimulates the once rounded neutrophil to flatten out and begin to squeeze through the endothelial tissue. This process, extravasation, results in neutrophil migration into the inflamed tissue where they can then phagocytose (engulf and eliminate) particulate antigens.

Figure 3



L-selectin is related to sialyl-Lewis^X and sialyl-Lewis^a and also contains sialic acid and sulfate. (Table 1) Research done by L.A. Lasky shows that carbohydrates that lack the appropriate sialic acid linkage, fucose linkage or both, result in weak binding instead of high affinity binding. The importance of these fucose linkages have also been demonstrated in the genetic defect LADII. The patients who have LADII cannot synthesize fucose, therefore, they lack functional E and P selectin ligands. These patients show decreased neutrophil migration through the endothelium. This ultimately leads to increased bacterial infections.(49) L-selectin deficiency also results in defects in leukocyte rolling and migration, and delayed leukocyte recruitment into inflamed tissue.(11)

The main role of the selectins is to mediate the initial attachment of cells to the endothelium during an immune response. Initially, a chemotactic signal calls white blood cells to the site of an inflammatory response, Weak, selectin mediated interactions tether the neutrophils to the blood vessel wall (Figure 3). The neutrophil interacts with the endothelium through L-selectin's carbohydrate, calcium dependent ligand. Since this adhesion is weak, the neutrophil proceeds to "roll" along the endothelium at a rate slower than the flow of blood.(30) While rolling along the endothelium, the neutrophils become activated by the chemoattractants released from or associated with the endothelial cells.

Chemoattractants are chemicals that attract white blood cells. Chemoattractants released by the endothelial cells may activate the white blood cells further and cause them to adhere more tightly to the endothelium. The importance of chemotactic agents was shown in T.A. Springer's in vitro experiments using a flow chamber (a plastic slide layered with purified cell

Table 1:

L-selectin is found on white blood cells (monocytes and neutrophils) but has many different names. This table gives the different names of L-selectin as well as the different ligands that activate it.

Table 1

L-selectin

<u>Names</u>	<u>Ligand</u>	<u>Cellular distribution</u>
MeI-14	Sialyl Lewis ^X	lymphocytes
LECAM	GlyCAM-1	neutrophils
LAM-1	CD34	monocytes
TQ1	MAdCAM-1	
CD62L	P-sel	
Leu8	Sgp200	
gp90mel		

adhesion molecules that was mounted and inverted in a phase contrast microscope).⁽¹⁾ Isolated leukocytes were allowed to migrate through the flow chamber, coated with intracellular adhesion molecule (ICAM-1) and a member of the selectin family (P-selectin). Under these conditions, the cell rolls along the surface normally in the absence of chemoattractant. Cells allowed to move through in the presence of chemoattractant would stop rolling, spread and firmly attach to ICAM-1.^(1,2,16,18,49) (ICAM-1 is an extracellular protein with immunoglobulin-like receptors. It binds to stimulated integrin proteins on the white blood cell and facilitates cell migration into the tissue during an immune response.) This procedure was repeated using P-selectin alone, ICAM-1 alone or both P-selectin and ICAM-1 together. The results showed that the selectin-mediated step is required for subsequent adhesion steps. Neutrophils would attach to and form sticky, rolling adhesions only when the selectin was present in addition to the ICAM-1. P-selectin interacted with L-selectin on the neutrophil initially, causing the neutrophil to tether. Adherence of the neutrophil on the other hand, occurred downstream in the presence of ICAM-1.^(1,18)

The second group of cell adhesion molecules, crucial in initiating an immune response, are integrins. Integrins are noncovalently linked heterodimers of an alpha and a beta chain which are transmembrane proteins with cytoplasmic tails. Two of the integrin subgroups are important in leukocyte adhesion to the endothelium. The first subgroup, B1 integrins, adhere cells to the extracellular matrix. Within this group, very late antigen-1 (VLA-1) through very late antigen-6 (VLA-6) are important in causing "slower rolling of the cell" and adhesion.⁽³²⁾ VLA-4 mediates "slower rolling than L-selectin." The slow rolling of monocytes and neutrophils allows them

to be exposed to chemotactic factors which activates them.⁽³¹⁾ Following cell activation, the second subgroup (B2 integrins), expressed on the surface of monocytes and neutrophils, undergo a conformational change.⁽³¹⁾ The B2 integrins allow the adherence of white blood cells to other cells. Lymphocyte function associated antigen (LFA-1), Mac-1 and p150/95 are B2 integrins. Integrins firmly attach to CAMs (members of the Ig superfamily) located on endothelial cells and arrest the rolling of the white blood cells.⁽⁴⁷⁾ This attachment facilitates the migration of the white blood cell through the blood vessel wall.

The leukocyte B2 integrins as well as L-selectin are essential in the neutrophil extravasation process.⁽⁴⁷⁾ Increased expression of Mac-1 (a B₂ integrin) is seen on monocytes and neutrophils following cell activation. This up regulation of Mac-1 allows the leukocyte to bind to the endothelium and migrate to the site of infection. Inversely proportional to this up regulation of Mac-1 is the down regulation or shedding of L-selectin.⁽²⁶⁾ This shedding of L-selectin from the leukocyte is important because the separation allows the leukocyte to crawl toward the site of infection once it has squeezed through the endothelium.⁽⁴¹⁾

The third group of cell adhesion molecules, the Ig superfamily proteins, are essential in the binding and subsequent migration of the white blood cell during an immune response. Ig superfamily proteins are "integral plasma membrane proteins with immunoglobulin domains in the extracellular regions, single transmembrane regions and short cytoplasmic tails".⁽¹¹⁾ There are four cell adhesion molecules in this family: intercellular adhesion molecule-1 (ICAM-1), intercellular adhesion molecule-2 (ICAM-2), vascular cell adhesion molecule-1 (VCAM-1), and platelet endothelial cell adhesion

molecule-1 (PECAM-1). ICAM-1 on endothelial cells bind to both Mac-1 and LFA-1 (another B_2 integrin) on neutrophils and plays a role in adhesion, activation, arrest and transmigration of the neutrophil through the endothelium. The cell adhesion molecules in the Ig superfamily are regulated by the presence of secreted proteins (cytokines). The inflammatory cytokines, tumor necrosis factor (TNF- α) and interleukin-1 (IL-1), released from activated macrophages, stimulate the synthesis of Ig-like CAM by endothelial cells. This allows binding of neutrophils and monocyte integrins and stimulate the movement of WBCs into the tissue.

The fourth family of cell adhesion molecules is the sialomucins. They are found on the endothelial tissue and act as a ligand for L-selectin. Members of this family include GlyCAM-1, MAdCAM-1 and CD34. These molecules act as a signal in activating L-selectin and calling the white blood cell out of circulation and into the tissue.^(8,20) These molecules have regions that are rich in O-linked sugars that provide an extended structure for the exposure of multiple terminal sugars. For example, MAdCAM-1 is a particular sialomucin which also contains Ig-like regions. This provides an additional binding site for integrin receptors. As a result this family is believed to have multiple adhesive functions.

Summary of Cell Adhesion Molecule Effects:

The cell adhesion molecules work together to initiate the emigration of white blood cells into the tissue.⁽⁵⁰⁾ Selectins are expressed on resting WBC's and are cleaved upon activation by a protease. Both E- and P-

selectins are found on the endothelial cells, while L-selectin is expressed on resting white blood cells. The selectin ligand is not present on resting endothelial cells, but is expressed on the endothelial cells following an inflammatory signal (Figure 3).(24) The main role of the selectins is to mediate the initial attachment of cells to the endothelium during an immune response. Initially, a chemotactic signal activates the mucin-like molecules found on the endothelial tissue. The sialomucins then act as a ligand (a molecule that binds to a receptor) for L-selectin.(8) L-selectin is then activated and weak, selectin-mediated interactions tether the neutrophil to the blood vessel wall (Figure 3), causing the cell to roll along the endothelium at a rate slower than the flow of blood.(28) While rolling along the endothelium, the neutrophil becomes activated by the chemoattractants released from or associated with the endothelial cells. Integrins are then expressed on monocytes and neutrophils and act as adhesion receptors for the Ig superfamily located on the endothelium. Tight binding transpires, the white blood cell arrests and margination, a process where white blood cells (monocytes and neutrophils) bind to the endothelial cell wall of the capillary ensues (Figure 3).(6,7) Integrin binding then stimulates the once rounded neutrophil to flatten out and begin to squeeze through the endothelial tissue. This process, extravasation, results in neutrophil migration into the inflamed tissue where they can then phagocytose (engulf and eliminate) particulate antigens.(1,3,4,15,21,23,42)

The importance of cell adhesion molecules is best illustrated in the inflammatory response of patients with leukocyte adhesion deficiency (LAD). This disorder results when integrin B2 (CD18) molecules of white blood cells are missing. The most significant symptom of this disease is recurrent

bacterial infections that result from the leukocytes inability to sufficiently attach to the endothelial cells. In this case, the white blood cells cannot migrate into the tissue to engulf the bacteria.(14)

Acute Respiratory Distress Syndrome:

In many metabolic pathways, it is the first step or the rate determining step that is regulated. L-selectin has been implicated as initiating the first identifiable step in an immune response because of its involvement in the initial contact of peripheral blood leukocytes with vascular epithelium.(38) Rolling of the cells along the endothelium is the first in a series of steps leading ultimately to the host defense in responding to antimicrobial agents. It is therefore anticipated that by understanding the functioning of L-selection, particularly its expression on neutrophils, and the chain of events leading to an immune response, that the resulting pathway leading to an immune response might be controlled.

The need for ways to regulate inflammatory responses (or white blood cell responses) is obvious in many of the immune response diseases, particularly in Acute Respiratory Distress Syndrome (ARDS). ARDS is acute lung injury and respiratory distress which results from increased permeability of the lung tissue, pulmonary edema (fluid accumulation), severe hypoxemia (low oxygen delivery to blood), multilobar infiltrates (fluid in the lungs) and reduced pulmonary compliance (inability to expel carbon dioxide) due an unregulated inflammatory response.(10,12,29,38) ARDS most commonly occurs in patients who have sepsis, have aspirated gastric contents, or

suffered a severe physical trauma. In ARDS, the inappropriate migration of cells into the tissue can lead to increased release of cytotoxic compounds and unnecessary tissue and blood vessel damage.

A clinical study involving trauma patients admitted through St. Elizabeth Hospital Medical Center and in conjunction with Northeastern Ohio University College of Medicine (NEOUCOM) is underway in Dr. Diana Fagan's lab. The purpose of this study is to examine the role of CAM's in the development of ARDS as it relates to trauma patients. In order to study the role of CAM's and the development of ARDS it is necessary to collect patient blood in an anticoagulant that will effectively keep the cells separated over time while not interfering with or causing cell stimulation.

MATERIALS

Materials:

Specific antibodies against L-selectin and Mac-1 were generously donated by Dr. Robert Rothlein of Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, Connecticut). Vacutainers containing EDTA, heparin, potassium oxalate and sodium citrate were purchased from Beckton Dickinson Vacutainer Systems (Franklin Lakes, New Jersey). Reagents from Sigma Chemical Company (St. Louis, Missouri) include NaCl, sodium azide, phorbol myristate acetate (PMA) and paraformaldehyde (PFM). FACS lysing solution, Simultest (anti-CD45-FITC/anti-CD14-PE), Simultest control (IgG₁-FITC/IgG_{2a}-PE), Simultest (anti-CD3-FITC/anti-CD19-PE) and Goat anti-mouse-FITC (GAM) were purchased from Beckton Dickinson (San Jose, California). Anhydrous (dibasic) Na₂HPO₄ was purchased from Mallinkrodt (Paris, Kentucky) and anhydrous (monobasic) NaH₂PO₄ was purchased from Fisher Scientific (Fair Lawn, New Jersey).

Reagents:

(All reagents were filtered using a 0.2 um filter prior to use.)

10X Phosphate Buffered Saline (PBS)

Monobasic sodium phosphate (0.021M) and dibasic sodium phosphate (0.084M) were dissolved in 200 ml of Milli Q water. The pH was adjusted to

7.4 using either 0.1 N HCL or 0.1 N NaOH. NaCl (1.50M) was added, allowed to dissolve and the solution brought up to 500 ml using Milli Q water. The solution was then stored at room temperature.

Paraformaldehyde

Paraformaldehyde (1%) and 10X PBS (20 ml) were mixed and brought up to a volume of 200 ml using Milli Q water. The solution was heated to 57°C in a water bath to dissolve the paraformaldehyde then cooled to room temperature. The pH was adjusted to 7.4 using 0.1 N HCL or 0.1 N NaOH. The solution was then immediately filtered and stored in the dark at 4°C for up to 2 weeks.

Lysis Buffer

FACS lysis buffer (50 ml of 10X) was brought up to 500 ml using Milli Q water.

Phorbol Myristate Acetate (PMA)

PMA stock (1 mM in DMSO) was diluted 1:60 in 1X PBS.

METHODS

Procedure:

Blood from a healthy volunteer was drawn into a vacuum tube (vacutainer) containing one of the anticoagulants; Heparin, Citrate, Oxalate or EDTA. (Figure 4). Separate tubes were labeled and 20 ug of anti-L-Selectin monoclonal antibody (mAb) was placed in each. Each time point was run in duplicate. The time points were 0 (processed immediately after drawing the blood) 0i (placed on ice immediately after drawing the blood), 5, 10, 15, 30 and 60 minutes. All samples were placed on ice at the time indicated (following collection) and processed together at one hour. A stimulated sample was incubated with 6.6 micromolar PMA at 37°C for 15 minutes to maximally stimulate the cells and to serve as a frame of reference for interpreting the stimulation incurred at the various time points. PMA is a mitogen that non-specifically activates the cells.

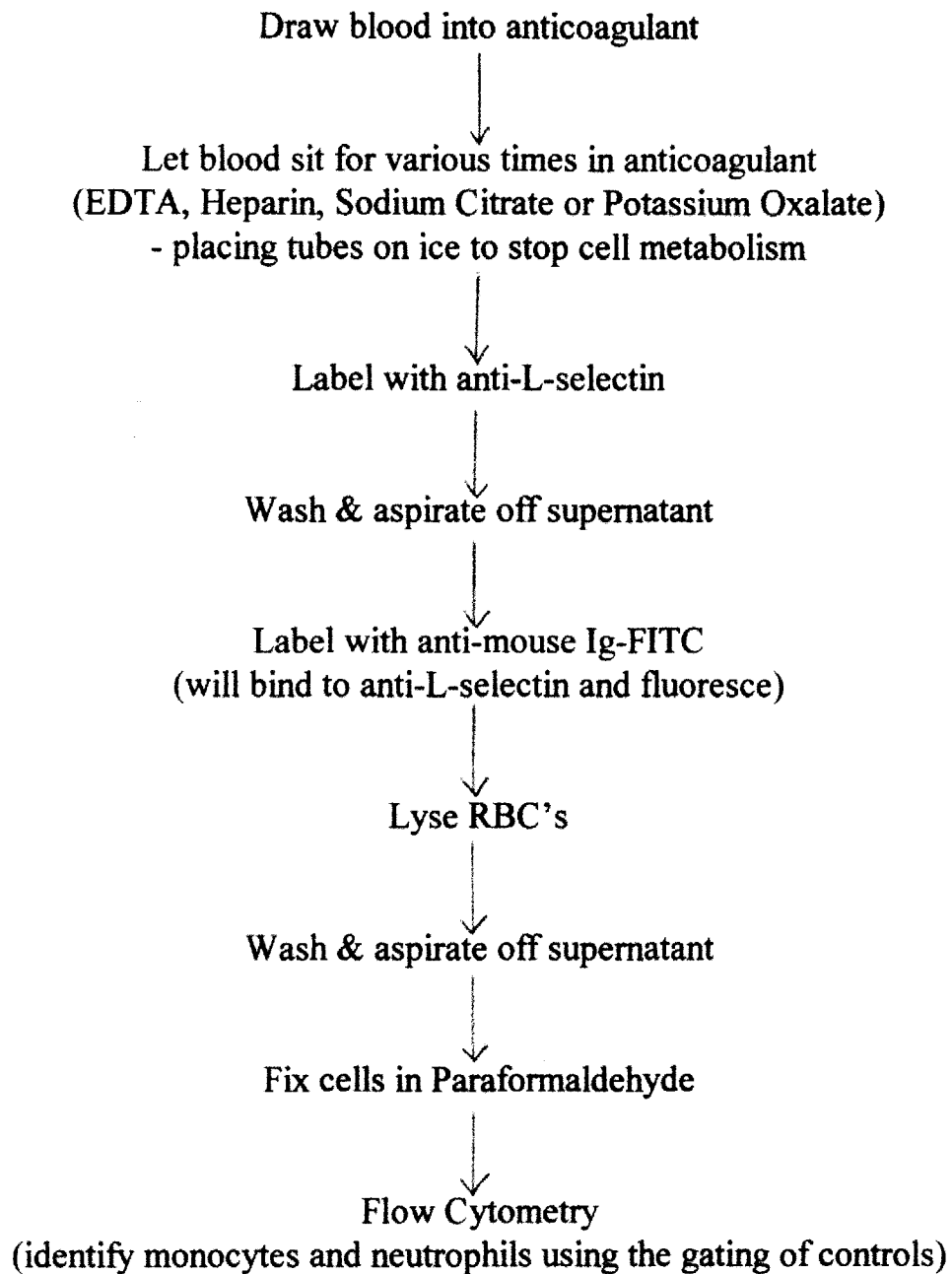
The blood samples were placed on a rocker at room temperature. At each time point, 200 microliters was placed on ice. The exception was the 0 time point which was processed immediately. After the 60 minute time point had been aliquoted, 100 micro liters from each time point was then pipetted and vortexed with the 20 micrograms of anti- L-Selectin (antibody directed against L-Selectin) in duplicate. Unstimulated and stimulated blood samples were kept on ice to be used as controls to be processed in a later step.

The cells were then incubated with antibody for 15 minutes at room temperature. They were then washed twice by suspension in 2 ml of filtered 1x PBS followed by centrifugation at 300 x g at 4°C for 8 minutes to pellet

Figure 4:

Flowsheet indicating the step by step protocol involved in preparing the blood for analysis during the control study.

Figure 4



the cells. The supernatant containing unbound antibody was then pipetted off and discarded. After the wash, 4 microliters of FITC-conjugated goat anti-mouse immunoglobulin was added and vortexed. The cells were then incubated in the dark for 10 minutes at room temperature. At this time, the four controls labeled A, B, C and D were also incubated in the dark for 10 minutes. Control A contained 20 microliters anti-CD14-PE/anti-CD45-FITC with 100 micro liters of unstimulated blood, Control B contained 20 microliters IgG₁/IgG_{2a}-FITC with 100 microliters of unstimulated blood, Control C contained 20 microliters of anti-CD3-PE/anti-CD19-FITC with 100 microliters of unstimulated blood and Control D contained 20 microliters of anti-CD14-PE/anti-CD45-FITC with 100 microliters of stimulated blood. These controls allowed calibration of the flow cytometer and gating of the cell populations. Following the incubation, 2 ml. of filtered lysis buffer was added to lyse the red blood cells (RBC's), leaving just the white blood cells. The tube was then vortexed and the cells incubated in the dark at room temperature for 10 minutes. They were then centrifuged at 300 x g at 4°C for 8 minutes to remove the lysed cells and washed twice with PBS, as described previously. Paraformaldehyde in PBS (400 micro liters of 1%) was then added to the cells and vortexed to resuspend the cells and to evenly fix them in solution. They were then stored at 4°C in the dark until analysis could be performed on the flow cytometer.

Data Analysis:

The Beckton Dickinson FACScan flow cytometer at St. Elizabeth Hospital was utilized for data analysis. The samples labeled with fluorescent (FITC-conjugated) goat anti-mouse Ab were passed through the detector and a total of 5,000 cells were counted (Figure 5). The physical characteristics of the cell were measured as the cell passed through the machine and deflected light according to the size and granularity of the cell.⁽⁵⁾ Cells were measured by forward scatter (FSC) of light which is proportional to the size of the cell and side scatter (SSC) of light which is proportional to the cell's granularity.⁽³⁴⁾

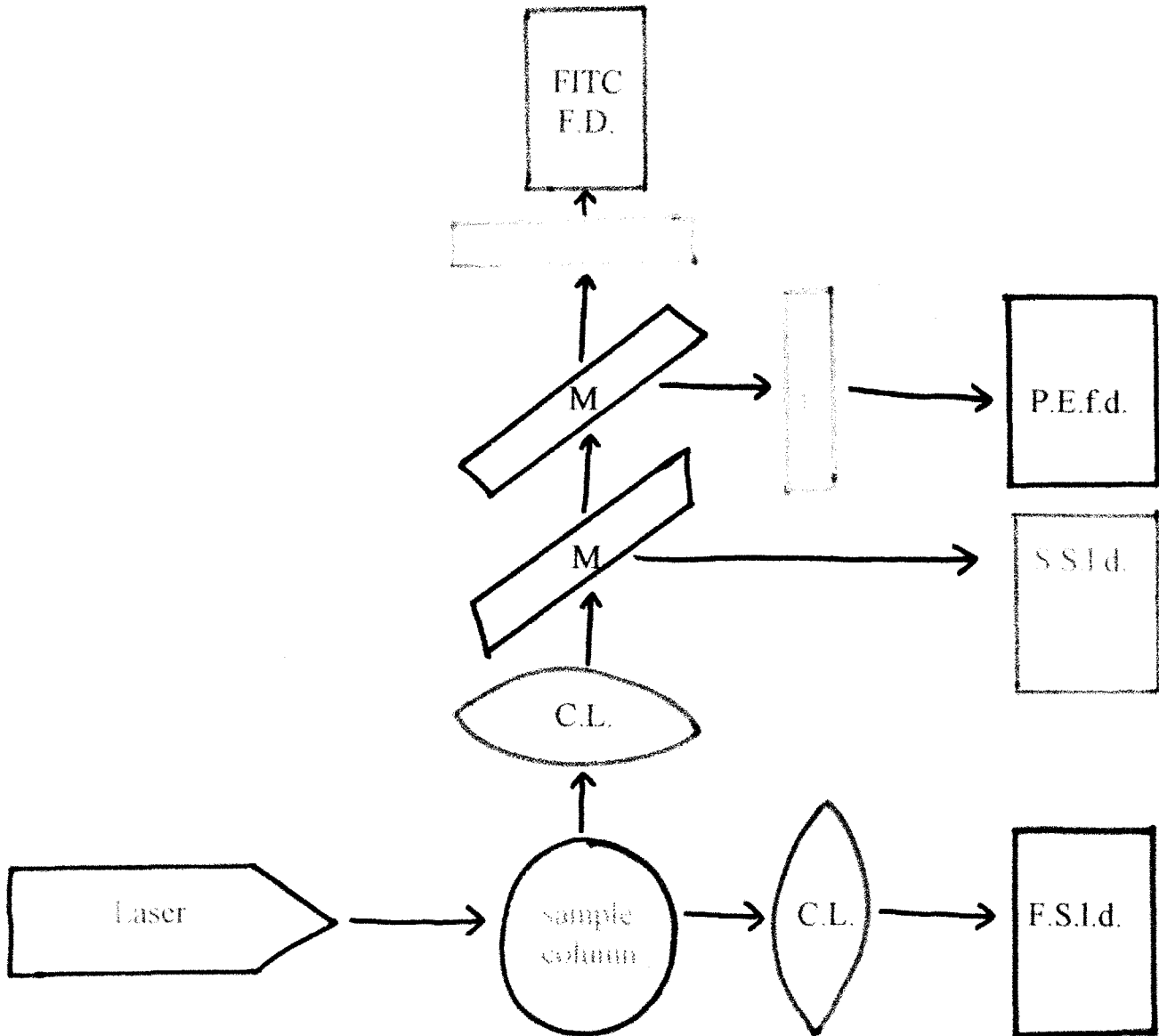
Controls A and D (anti-CD14-PE/CD45-FITC) were used for identification of the different cell types by fluorescent-labeled cell surface molecules. Monocytes have high levels of CD14, resulting in bright staining with PE and moderate levels of CD45 that results in staining with FITC. In Figure 6, cells are grouped according to their surface levels of CD14 and CD45. In Figure 6, monocytes are represented by (R1). Monocytes also have extensive forward and side scatter (see (R5) in Figure 7). The second group of cells (R2) are granulocytes or neutrophils, with dim staining of both CD14 and CD45. Lymphocytes (T cells, B cells and NK cells) have low forward and side scatter. These are represented by (R3) in Figure 6 and (R4) in Figure 7.

Control B (IgG₁/IgG_{2a}-FITC) was the negative control and determines the amount of nonspecific binding of antibody to the cells. Control C (anti-CD3-PE/CD19-FITC) was used to set compensation of the fluorescence detectors. Compensation is required when using two or more spectral color

Figure 5:

This diagram shows the mechanism by which a flow cytometer works. White blood cells are passed through the sample column one at a time (a total of 5,000 cells are counted) and a laser beam is directed at the sample. Depending on the physical characteristics of the cell, the light is either deflected forward (large cell) or to the side (granular cell). The amount of light which is deflected by the cell is detected by the 2 light detectors. In addition the forward and side scatter, the laser beam activates the fluorescence markers (PE or FITC) which are attached to the cell. This activation causes the markers to emit fluorescent light which can be measured by one of the 2 different fluorescence detectors (either FITC or PE).

Figure 5



FITC F.D. = FITC fluorescence detector

filter = filter

M = mirror

P.E.f.d = PE fluorescence detector

S.S.I.d = Side Scatter light detector

C.L = Collection lens

F.S.I.d = Forward Scatter light detector

Figure 6:

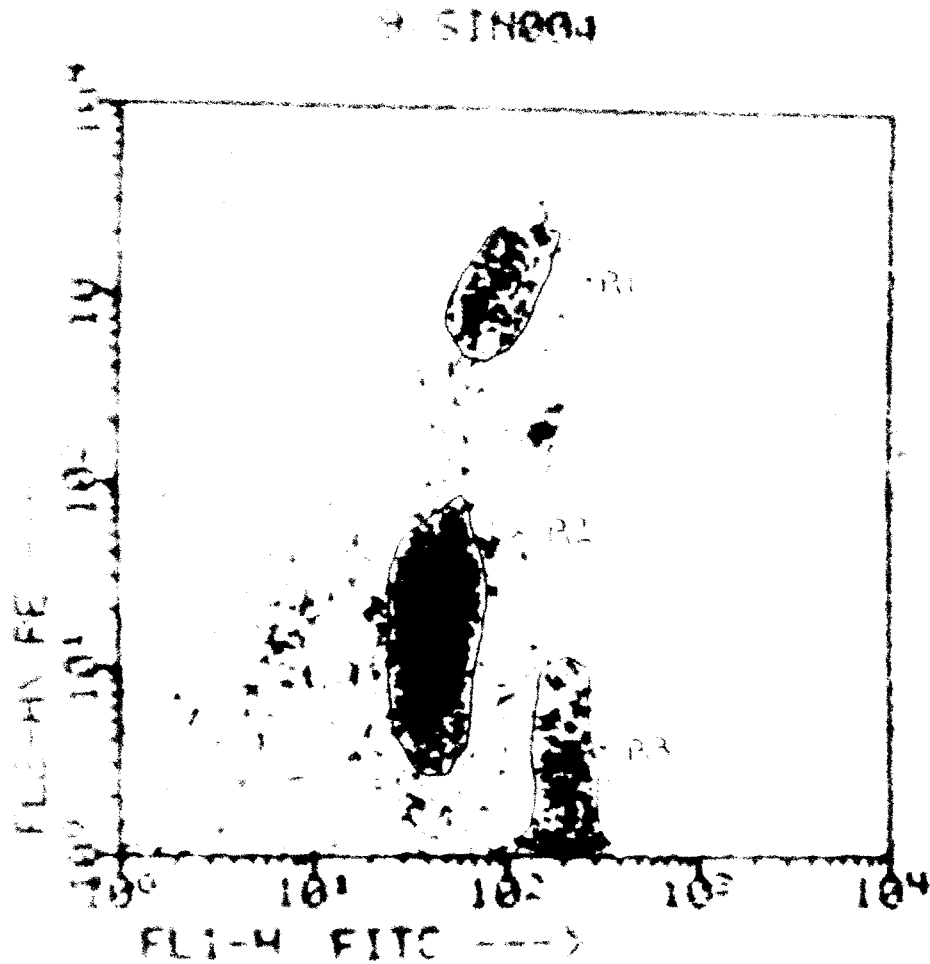
This figure is a cytogram which shows separation of the cell population by fluorescence. The Y axis indicates staining with PE (cells that have CD14 on the surface), Cells that stain brightly with PE are near the top of the graph. The X axis indicates staining with FITC (cells that have CD45 on the surface). Cells that stain brightly with FITC are to the right of the graph.

Monocytes (R1) stain brightly with both stains.

Neutrophils (R2) stain intermittently with both stains.

Lymphocytes (R3) stain brightly with FITC and poorly with PE.

Figure 6



This figure shows separation of the cells based on fluorescence
The Y axis indicates staining with PE (cells that have CD14 on the surface)
cells that stain brightly with PE are near the top of the graph
The X axis indicates staining with FITC (cells that have CD45 on the surface)
cells that stain brightly with FITC are to the right of the graph

Monocytes (R1) stain brightly with both stains
Neutrophils (R2) stain intermittently with both stains
Lymphocytes (R3) stain brightly with FITC and poorly with PE.

Figure 7:

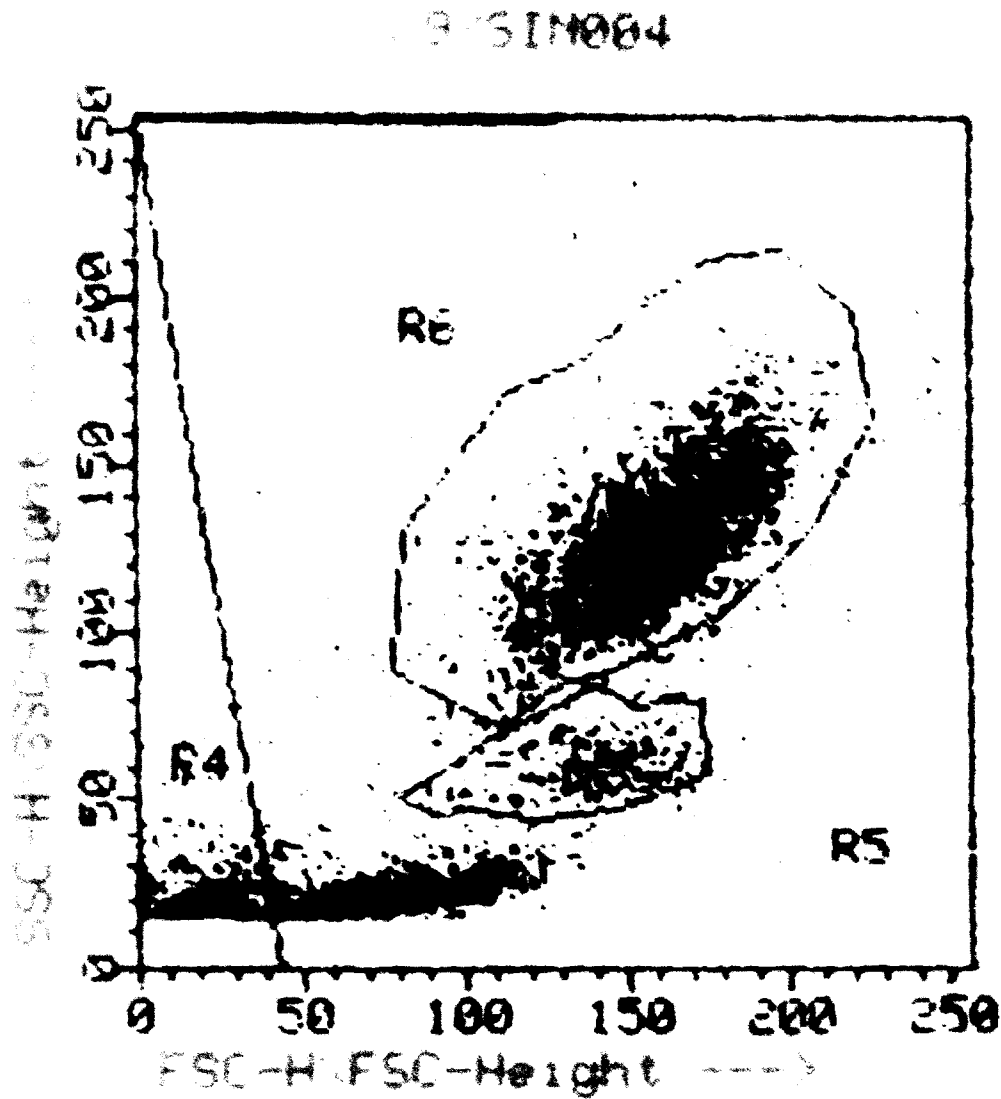
This figure is a cytogram which shows separation of the cells based on forward and side scatter. The Y axis represents side scatter or the cell's granularity. Cells with increased granularity are found near the top of the graph. The X axis indicates forward scatter or the size of the cell. Larger cells are found on the right side of the graph.

Monocytes (R5) have high forward and side scatter.

Neutrophils (R6) have high forward and low side scatter.

Lymphocytes (R4) have low forward and side scatter.

Figure 7



This figure shows separation of the cells based of forward and side scatter
The Y axis represents side scatter or the cells granularity.
cells with increased granularity are found near the top of the graph
The X axis indicates forward scatter or the size of the cell.
larger cells are found on the right side of the graph

Monocytes (R5) have high forward and side scatter
Neutrophils (R6) have high forward and low scatter
Lymphocytes (R4) have low forward and side scatter

analysis (fluorescence signals). In this case, we were using both Phycoerythrin (PE) and Fluorescein isothiocyanate (FITC) to examine the cells. The FITC signals were subtracted from the PE signal to eliminate any overlap.(5)

In order to examine the data, gates were drawn around the different cell types. The purpose of drawing these gates is to identify the population of cells to be analyzed. In gating, a polygon is drawn around the monocytes (R5) and neutrophils (R6) as seen in Figure 7. This is a depiction of a cytogram of forward scatter versus side scatter for control sample A. Once these gates are created, they are used for analysis of subsequent samples in the same experiment.

Cells are separated and classified using gates, based on their physical characteristics (size and granularity). Controls A and D (anti-CD14-PE/anti-CD45-FITC) assist in the identification of cells within these gates based on fluorescent labeling. PE is a green dye bound to an antibody that labels CD14 expressed on the surface of cells and FITC is a yellow-orange dye bound to an antibody that labels CD45 on the cell surface. Monocytes have large quantities of both CD14 and CD45 on their surface, monocytes therefore stain brightly with both PE and FITC. Neutrophils have an intermediate quantity of both CD14 and CD45 on their surface, therefore the neutrophils do not stain as brightly as the monocytes. Lymphocytes have significant quantities of CD45, but no CD14 on their surface, they therefore stain brightly with FITC but not PE. In Figure 6, cells that express high levels of CD14 (stain brightly with PE) are found near the top of the graph along the Y axis (R5). Likewise, cells that stain dimly with PE are found near the bottom of the graph along the Y axis (R3). Cells that express high levels of

CD45 (stain brightly with FITC) appear furthest to the right along the X axis (R1 and R3). Cells that stain poorly with FITC appear further to the left along the X axis (R2). The identification of these cells with fluorescence (PE and FITC) is useful in establishing purity or contamination of cells within the gates established using forward and side scatter (Figure 7). In figure 7, forward scatter (cell size) and side scatter (cell granularity) are used to isolate the cell populations, namely monocytes (R5) and neutrophils (R6). Monocytes (R5) have high forward and side scatter and neutrophils have low forward and low side scatter. In figure 7, cells with high forward scatter are found near the top of the graph along the Y axis. Cells with high side scatter are found to the right of the graph along the X axis. The fluorescent labels can then be used as in Figures 8 and 9 to estimate the purity of the cells identified by gates R5 and R6 (light scatter). Figure 8a shows the disbursement of the fluorescently labeled monocytes on the graph, particularly how many fall in the (R5) gate. Figure 9a shows the disbursement of the fluorescently labeled neutrophils, particularly how many fall in the (R6) gate. Figure 8b shows the monocytes which fall in the (R5) gate only. Statistics can then be derived (Figure 8c) based on the cells present within the (R5) gate. In Figure 8c, the first column identifies the different gates/cell populations that were established. Column 2 indicates the number of cells counted for each gate. Column 3 shows the percentage of the cells within a gate that belong to a particular cell population. Column 4 illustrates the percentage of cells (monocytes, neutrophils and lymphocytes), out of the total number of cells counted (5,000 cells), that fall in the (R5) gate. Figure 9b shows the neutrophils which fall in the (R6) gate only and subsequently Figure 9c shows the statistics derived from the cells present in the (R6) gate. These gates were

Figure 8a:

This figure shows the disbursement of the fluorescently labeled monocytes. Assessment of purity/contamination can then be visualized by observing where the monocytes are located relative to the gates.

Figure 8b:

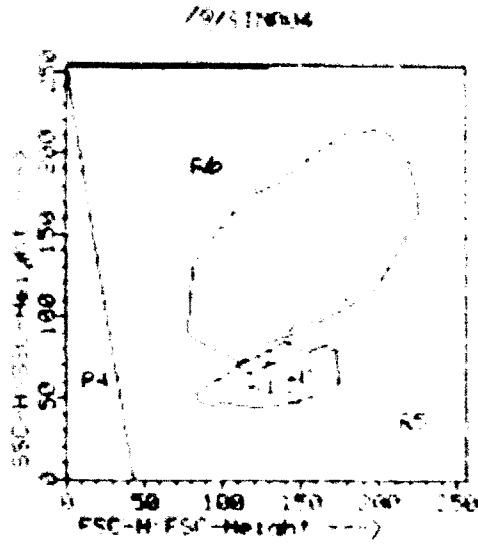
This figure shows all the fluorescently labeled cells which are located within the R5 gate.

Figure 8c:

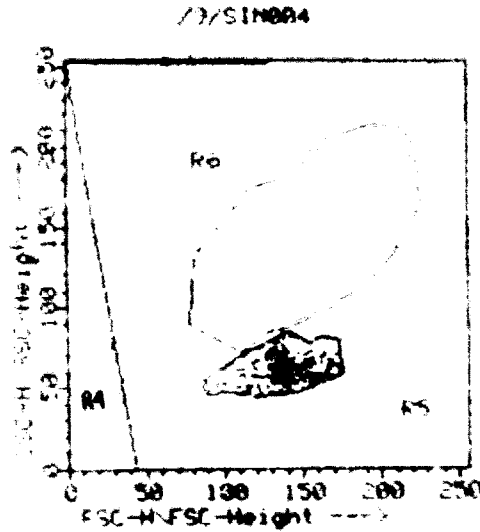
The statistics which are generated based on the cell populations which are located in R5 in Figure 8b. This identifies the purity/contamination of the gate R5 in comparison to other cell populations also found in the gate. Vertical column 1 identifies the different cell populations (gate 1= monocytes, gate 2= neutrophils, gate 3= lymphocytes, etc..). Column 2 identifies the number of cells, within a cell population, found within the R5 gate. Column 3 represents the percentage of that cell population relative to the other cell populations found within the R5 gate. Column 4 illustrates the percentage of cells for each cell population found within the R5 gate relative to the number of cells counted for the sample (5000 cells).

Figure 8

8a)



8b)



8c)

79/SIN004

----- Gate Stats -----

File: 9/SIN004 Sample: SIN 004

Date: 9-11-97 Gate G5= R5

Selected Preference: arithmetic/Linear

Total= 5000 Gated= 407

Gate	Events	% Gated	% Total
1 01	250	61.43	5.00
2 02	21	5.16	0.42
3 03	8	1.97	0.16
4 04	407	100.00	8.14
5 05	407	100.00	8.14
6 06	0	0.00	0.00
7 07	250	61.43	5.00
8 08	21	5.16	0.42

Figure 9a:

This figure shows the distribution of the fluorescently labeled neutrophils. Assessment of purity/contamination can then be visualized by observing where the neutrophils are located relative to the gates.

Figure 9b:

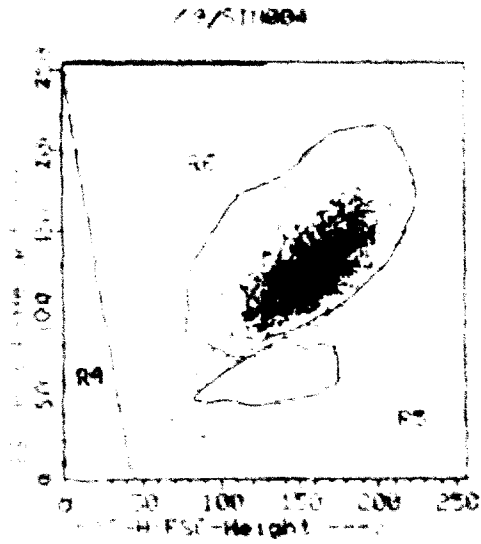
This figure shows all the fluorescently labeled cells which are located within the R6 gate.

Figure 9c:

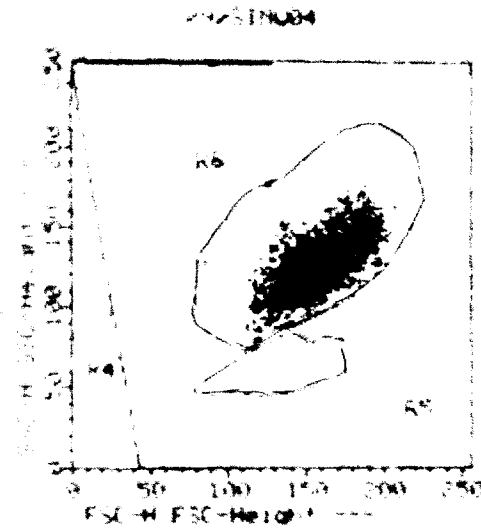
The statistics which are generated based on the cell populations which are located in R6 in Figure 9b. This identifies the purity/contamination of the gate R6 in comparison to other cell populations also found in the gate. Vertical column 1 identifies the different cell populations (gate 1= monocytes, gate 2= neutrophils, gate 3= lymphocytes, etc.). Column 2 identifies the number of cells, within a cell population, found within the R6 gate. Column 3 represents the percentage of that cell population relative to the other cell populations found within the R6 gate. Column 4 illustrates the percentage of cells for each cell population found within the R6 gate relative to the number of cells counted for the sample (5000 cells).

Figure 9

9a)



9b)



9c)

79/SIN004

----- Gate Stats -----

File: 79 SIN004 Sample: SIN 004

Date: 9-11-97 Gate: G6=R6

Selected Preference: Arithmetic: Linear

Total= 5000 Gate= 2497

Gate	Events	% Gated	% Total
0-01	24	0.96	0.48
0-02	2267	90.75	45.32
0-03	18	0.40	0.20
4-04	2497	100.00	49.94
5-05	0	0.00	0.00
6-06	2497	100.00	49.94
7-07	24	0.96	0.48
7-08	2267	90.75	45.32

then applied to each time point and subsequently identified monocytes and neutrophils from the blood sample based on fluorescence.

A histogram (Figure 10) was then generated showing the amount of fluorescence per cell (monocyte or neutrophil) that was labeled with anti-L-selectin and an FITC goat anti-mouse immunoglobulin. The level of fluorescence is shown on the horizontal (X) axis. The number of cells is on the vertical (Y) axis. The amount of (FITC) that appears on the cell surface was measured and from this a histogram is generated that shows the mean or average fluorescence for the sample. The mean fluorescence for each sample was recorded and averaged between the duplicate samples of the same time point. High fluorescence on the histogram represents high levels of CAM's on the cell surface.

Data Interpretation:

To minimize normal variation between subjects, the data was expressed as a percentage of the zero timepoint: $(\text{Average Fluorescence of Timepoint} / \text{Average Fluorescence of 0 Timepoint}) \times 100 = \% \text{ of 0 Timepoint}$. The zero timepoint (0), since it was processed immediately, should reveal the least alteration in CAM expression of all the timepoints. As a result, the zero timepoint (0) represents 100% fluorescent expression for that experiment and all other timepoints are referenced as a percent of that fluorescence.

Once the fluorescence (% of 0 timepoint) is determined, the mean fluorescence (MF) and standard error of the mean (SEM) was calculated for each timepoint (Tables 2 & 3). Neutrophil and monocyte L-selectin

Figure 10a:

This figure shows separation of monocyte and neutrophil populations based on forward and side scatter.

Figure 10b:

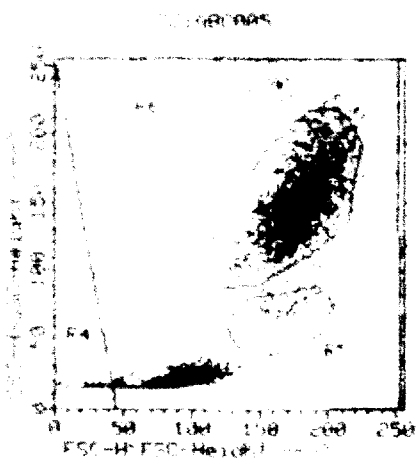
Histogram and statistics for the monocyte population in the blood sample shown in Figure 8a. The histogram is indicative of the fluorescent cells within the gate. The statistics reveal how many cells contain fluorescence out of the number of cells counted. Mean fluorescence is used to determine the L-selectin levels for each blood sample.

Figure 10c:

Histogram and statistics for the neutrophil population in the same blood sample. It shows fluorescent shift with the shift moving to the left (decreased fluorescence) as the cells become stimulated over time. The statistics indicate how many cells were counted and the mean fluorescence of the sample. The mean fluorescence is used to determine the L-selectin levels for each blood sample.

Figure 10

10a



10b

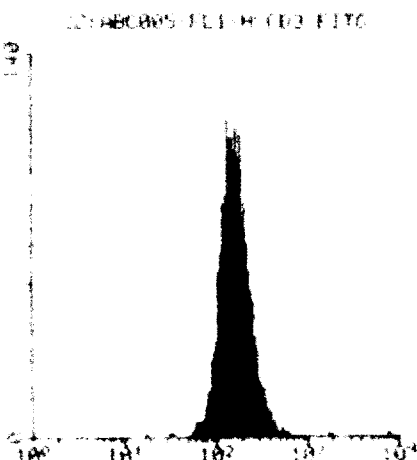


22:ABCB005 F11-H CD3 FITC

--- Arithmetic Histogram Statistics for 22:ABCB005 ---
 Selected Preferences: Arithmetic/Linear
 Parameter: F11-H CD3 FITC Gate: G4-R5

N	Left	Right	Events	%	Peak	PeakH	Mean	Median	CP
0	1.00	9646	100	100.00	11	171.59	173.09	170.75	176.1

10c



22:ABCB005 F11-H CD3 FITC

--- Arithmetic Histogram Statistics for 22:ABCB005 ---
 Selected Preferences: Arithmetic/Linear
 Parameter: F11-H CD3 FITC Gate: G6-R6

N	Left	Right	Events	%	Peak	PeakH	Mean	Median	CP
0	1.00	9646	2433	100.00	115	177.05	174.73	159.62	169.1

Table 2:

This table shows the mean fluorescence (% of 0 timepoint) of neutrophils and the standard error margin for each timepoint for each of the 4 anticoagulants. The vertical columns represent the mean and SEM for each anticoagulant (heparin, EDTA, sodium citrate and potassium oxalate). The horizontal rows represent the timepoints (0,0i,5,10,15,30,60 and Stim.)

Table 2

Mean Fluorescence (expressed as % zero timepoint) and Standard Error of the Mean for Neutrophils in 4 Anticoagulants

	Heparin		EDTA		Citrate		Oxalate	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	100	0	100	0	100	0	100	0
0i	83	0	93	14	82	14	60	11
5	57	23	91	9	90	9	47	17
10	57	23	97	9	78	9	44	18
15	57	21	95	11	81	9	40	14
30	62	28	101	10	65	11	40	16
60	63	27	96	10	88	10	43	15
Stim	5	3	10	2	11	1	60	26

Table 3:

This table shows the mean fluorescence (% of 0 timepoint) of monocytes and the standard error of the mean for each timepoint for each of the 4 anticoagulants. The vertical columns represent the mean and SEM for each anticoagulant (heparin, EDTA, sodium citrate and potassium oxalate). The horizontal rows represent the timepoints (0,0i,5,10,15,30,60 and Stim.)

Table 3

Mean Fluorescence (expressed as % zero timepoint) and Standard Error of the Mean for Monocytes in 4 Anticoagulants

	Heparin		EDTA		Citrate		Oxalate	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
	0	100	0	100	0	100	0	100
0i		89	0	67	10	85	3	93
	5	62	27	76	9	87	8	84
	10	62	25	80	7	77	7	87
	15	68	33	63	9	67	5	61
	30	69	33	86	14	56	11	87
	60	65	32	71	12	71	9	89
Stim		17	8	27	11	29	6	109
								26

expression was evaluated separately for each anticoagulant used. A one way repeated measures analysis of variance (a statistical program) was performed to compare mean fluorescence for each timepoint (separately for each anticoagulant) and assign a numeric value (p-value) based upon statistical significance. Statistical significance indicates a difference between the fluorescent values not expected by chance. A p-value (Table 4) less than 0.05 indicates that L-selectin expression was significantly altered over time.

Table 4:

This table shows the p-values from a one way analysis of variance for both monocytes and neutrophils in each of the 4 anticoagulants. The fluorescent values (% of 0 timepoint) for each experiment were entered into the computer and compared. The experiments assigned a numeric value based upon statistically significant relationships not expected by chance. A p-value (in this case) of less than 0.05 indicates cell expression that was significantly altered over time in the presence of anticoagulants.

Table 4

ANOVA analysis for Monocytes and Neutrophils
in each of the 4 Anticoagulants
(p values)

	monocyte	neutrophil
Heparin	0.574	0.174
EDTA	0.151	0.799
Citrate	0.0179	0.12
Oxalate	0.507	0.000964

* A (p value) less than 0.05 indicates that there is a statistically significant difference in cell expression (fluorescence) over time.

RESULTS

To determine which anticoagulant would be the best for measuring L-selectin levels on white blood cells, blood was collected into tubes containing one of each anticoagulant and processed as described in the procedure section (Figure 4). The purpose of this study is to examine the change in L-selectin expression over time by measuring the binding of monoclonal antibodies to L-selectin. The timepoints for this study are: 0, 0i, 5, 10, 15, 30, and 60 minutes post collection. The 0 minute sample was drawn and processed immediately, whereas the 0i minute sample was immediately placed on ice. At each timepoint, 300 aliquots were removed from the room temperature sample and placed on ice. Following the removal of the 60 minute sample, all samples were processed together except the 0 minute sample. The results for the experiments involving the 4 anticoagulants; heparin, EDTA, potassium oxalate and sodium citrate, represent the mean of 4(EDTA), 3(heparin), 3(oxalate) or 3(citrate) experiments. The results are then expressed as a percent of the 0 timepoint.

The effect of incubation in the presence of heparin (Figures 11 & 12) upon the L-selectin levels on white blood cells was examined. In neutrophils (Figure 11), there was a 40% decrease in fluorescence (indicating decreased levels of L-selectin) within the first 5 minutes of incubation (Table 2). A 40% decrease in fluorescence was also observed in the monocytes (Figure 12 & Table 3). However, due to variability among samples, the decrease in L-selectin levels was not statistically significant by ANOVA analysis (neutrophil $p = 0.574$, monocyte $p = 0.174$). As indicated, a large SEM (Tables 2 & 3) of greater than 20% was present for both. The SEM is

Figure 11:

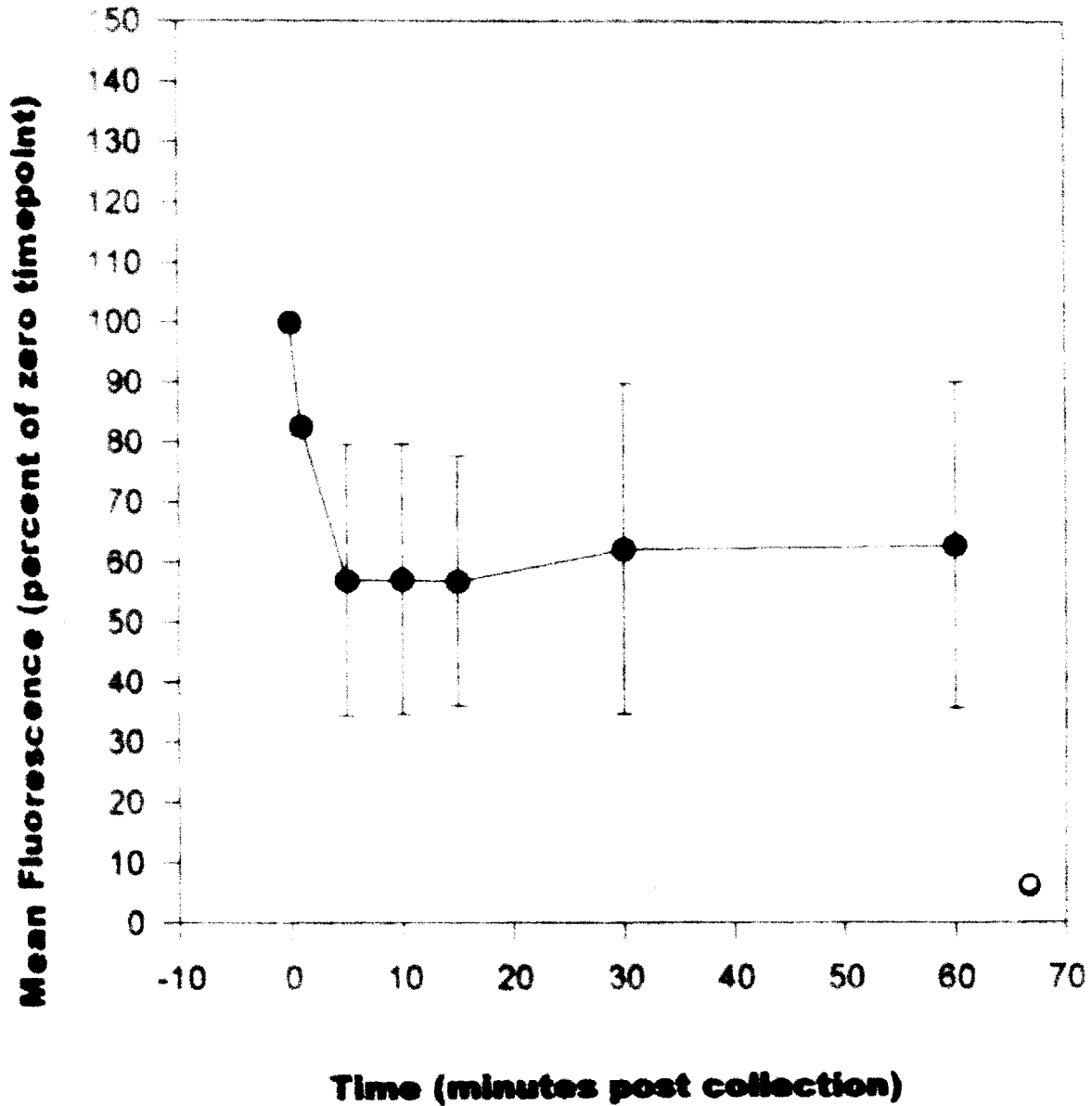
Mean fluorescence (L-selectin) levels on neutrophils collected in heparin, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection. (labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 11

Neutrophils Collected in Heparin



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

Figure 12:

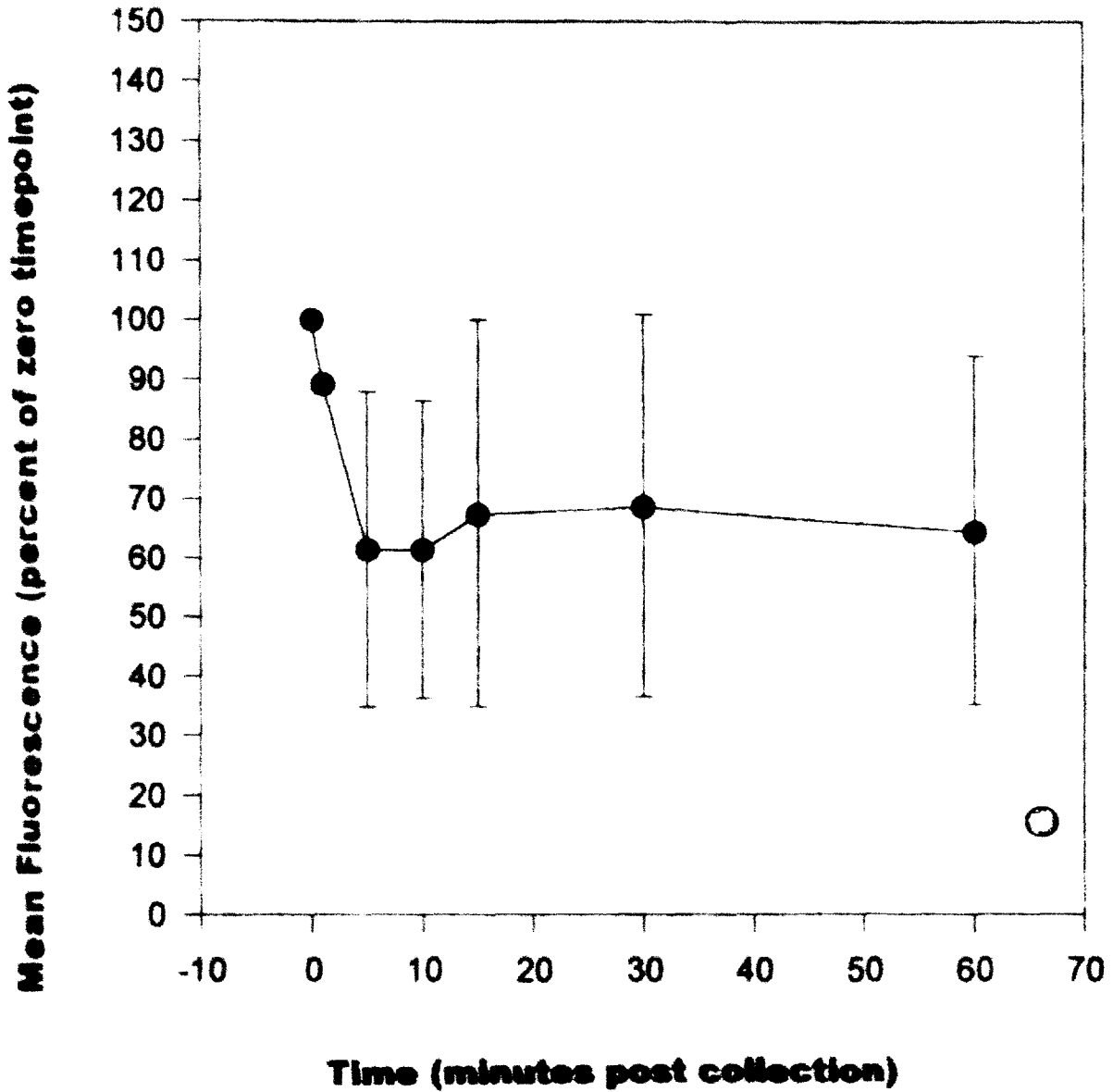
Mean fluorescence (L-selectin) levels on monocytes collected in heparin, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection. (labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 12

Monocytes Collected in Heparin



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

also represented by error bars on the graphs (Figures 11 & 12). This reveals inconsistent expression of L-selectin on the cells between repeated experiments.

EDTA (Figures 13 & 14) was next evaluated to determine its influence on L-selectin levels. Figure 13 shows L-selectin expression on neutrophils over time. No significant variation was seen in L-selectin levels at the various time points ($p = 0.151$). At five minutes post collection, a less than 10% decrease in fluorescence is observed (Table 2). What is even more impressive is that at 60 minutes, L-selectin continues to show less than a 10% decrease in cell expression. The SEM is also less than 10% which indicates that the experiments were consistently reproducible. The monocytes however, didn't respond as well as the neutrophils (Figure 14), although no significant variation was seen in the monocyte L-selectin levels ($p = 0.799$). In the first 5 minutes, 25% of the cells showed a decrease in fluorescence (Table 3). At 60 minutes, almost 30% of the cells showed a decrease in L-selectin levels. As seen with the neutrophils, the SEM was low, indicating that the results are consistently reproducible.

The effect of potassium oxalate on L-selectin expression was next examined for the neutrophils (Figures 15 & 16). Neutrophil L-selectin expression was significantly decreased in the presence of potassium oxalate ($p = 0.00096$). Table 2 shows that there was almost a 55% decrease in fluorescence within the first 5 minutes and almost a 60% decrease at 60 minutes. The SEM was low, indicating that the L-selectin expression on the neutrophils was relatively consistent in the repeated experiments. The monocytes (Figures 16 and Table 3) revealed greater than a 15% decrease in L-selectin within the first 5 minutes and less than 15% at 60 minutes. This

Figure 13:

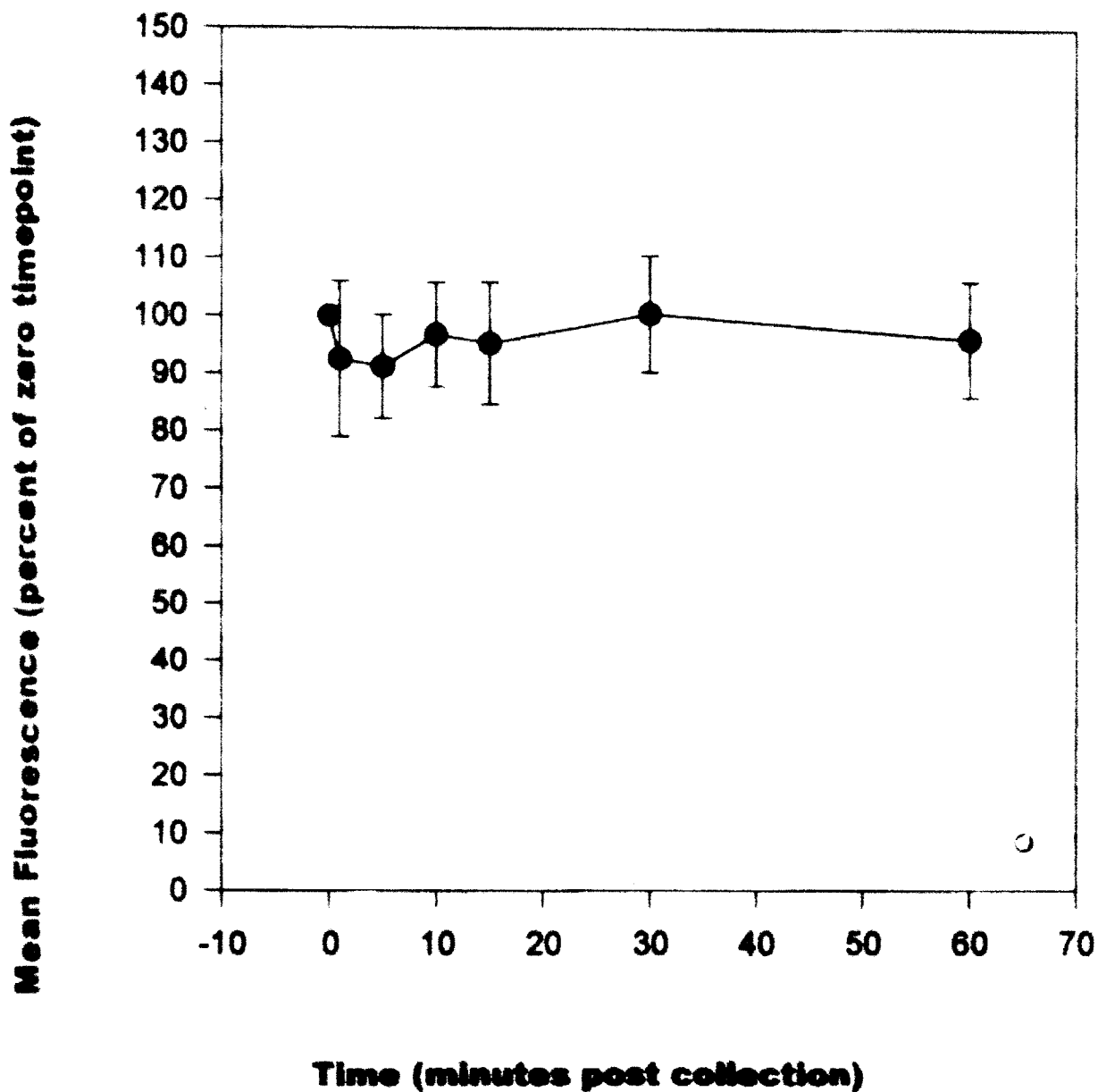
Mean fluorescence (L-selectin) levels on neutrophils collected in EDTA, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection. (labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 13

Neutrophils Collected in EDTA



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

Figure 14:

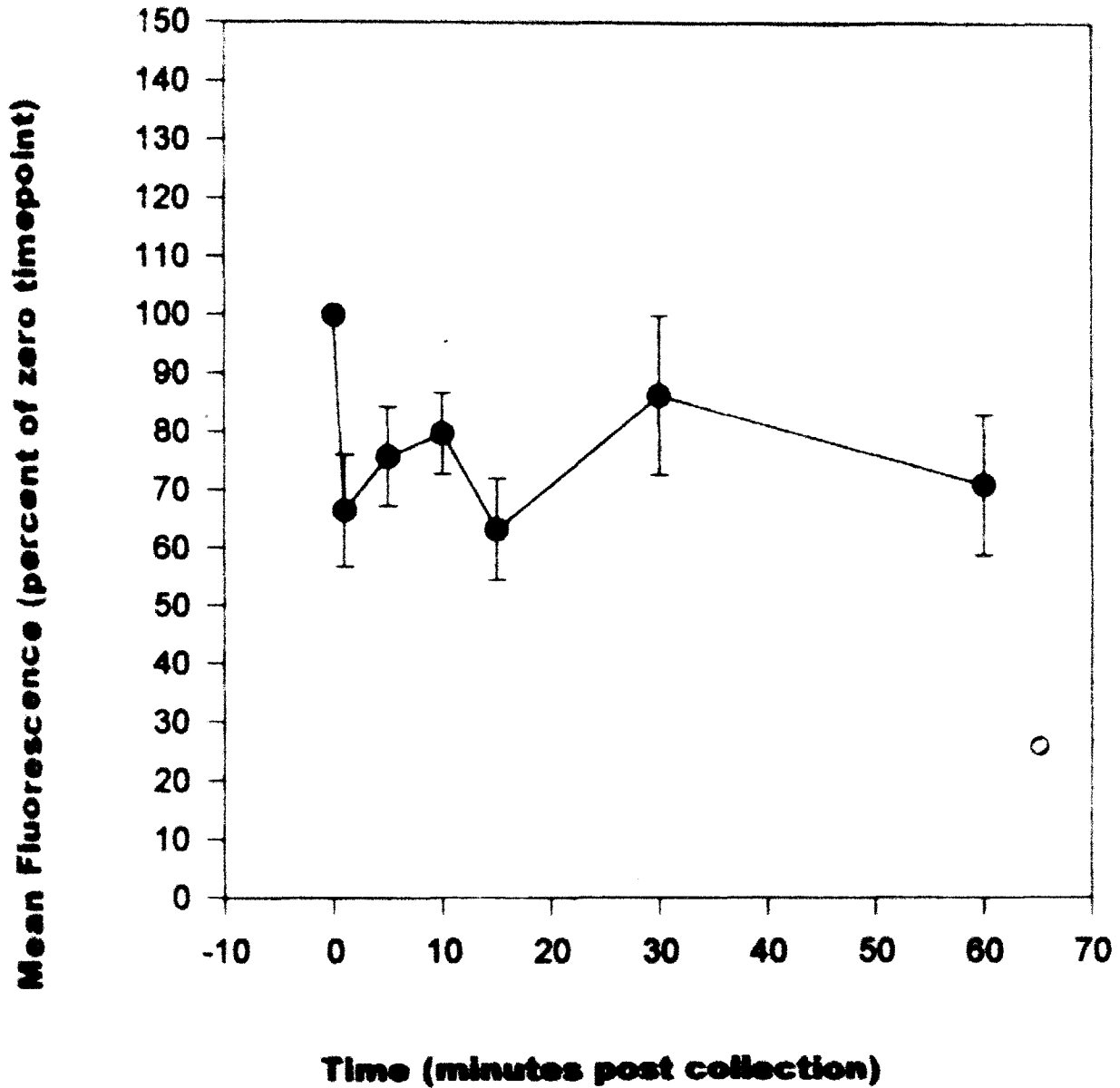
Mean fluorescence (L-selectin) levels on monocytes collected in EDTA, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection. (labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 14

Monocytes Collected in EDTA



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

Figure 15:

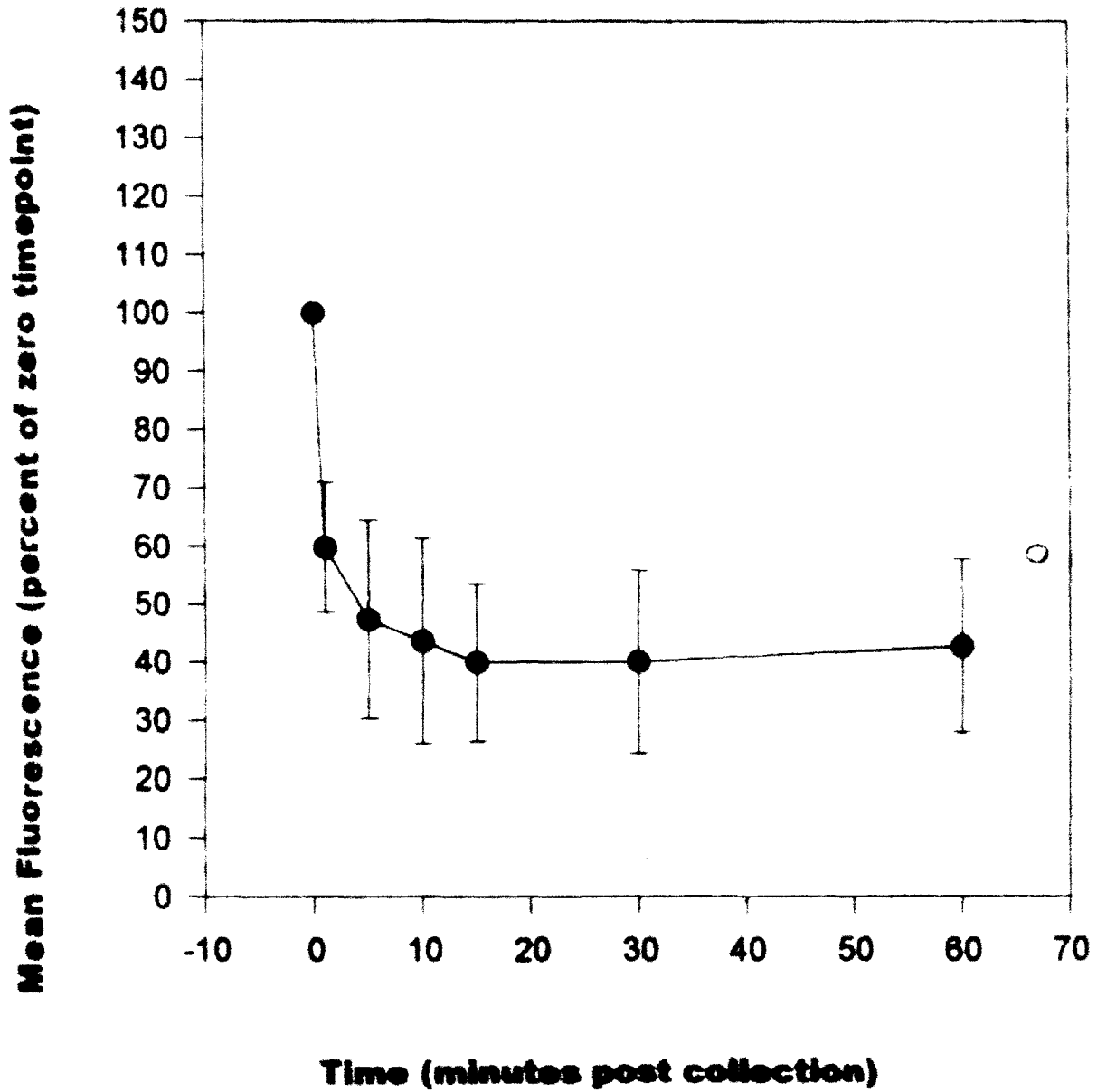
Mean fluorescence (L-selectin) levels on neutrophils collected in potassium oxalate, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection.
(labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 15

Neutrophils Collected in Potassium Oxalate



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

Figure 16:

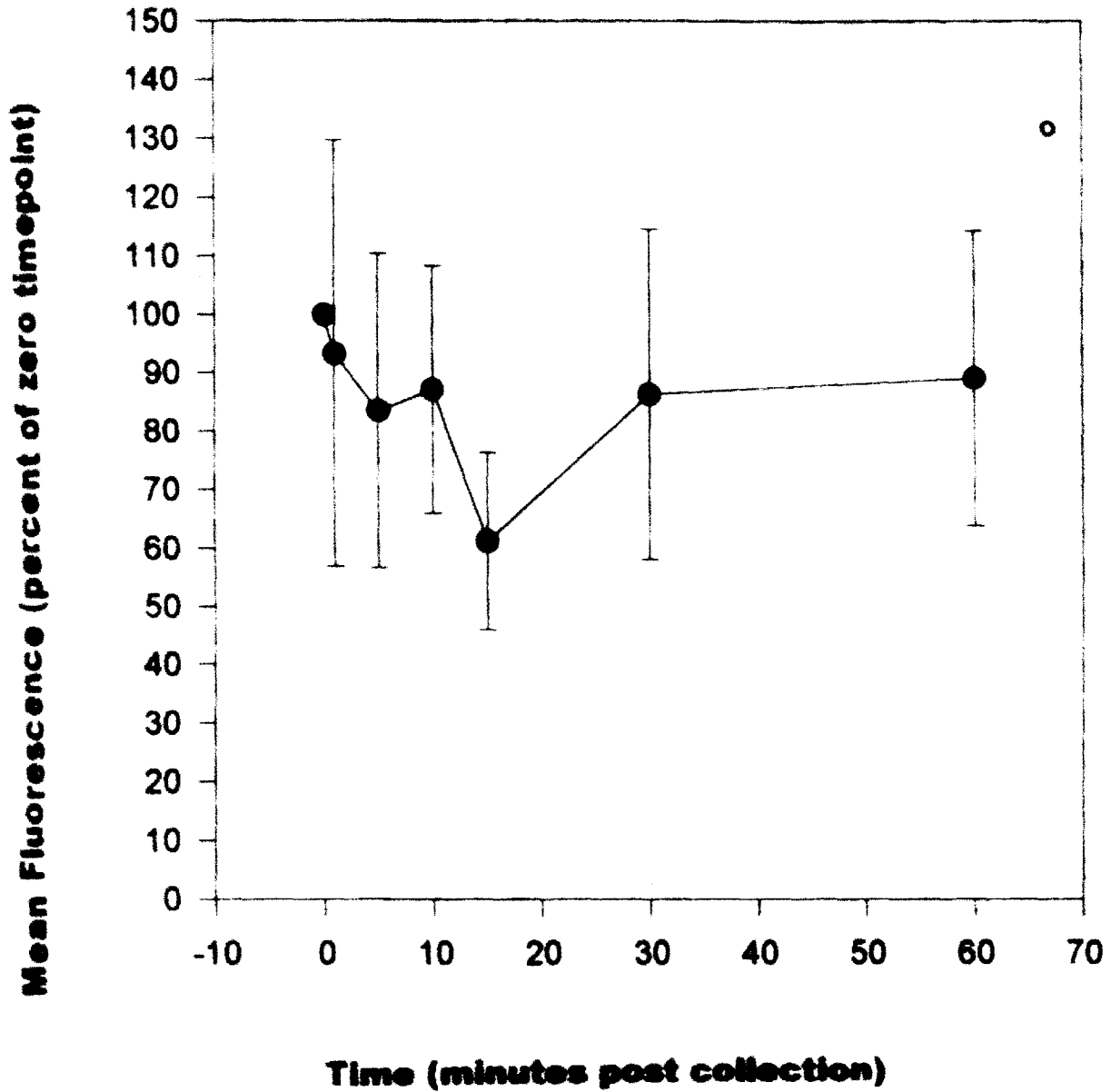
Mean fluorescence (L-selectin) levels on monocytes collected in potassium oxalate, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection. (labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 16

Monocytes Collected in Potassium Oxalate



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

decrease in L-selectin levels however, was not statistically significant ($p = 0.507$). On monocytes, the L-selectin levels were inconsistently reproducible in repeated experiments using potassium oxalate (SEM greater than 10%).

Sodium citrate's effect on L-selectin levels on neutrophils were examined last (Figure 17 & Table 2). No significant variation was seen ($p = 0.120$), however a 10% decrease in fluorescent expression was observed within the first 5 minutes. After 60 minutes, there was less than a 15% decrease in L-selectin expression. L-selectin levels fluctuated between the timepoints, however, with a 45% decrease seen in L-selectin expression at 30 minutes. The SEM (Table 2) for L-selectin expression on neutrophils was reproducible in repeated experiments. Monocytes collected in sodium citrate, had significantly decreased L-selectin expression over time ($p = 0.0179$). Within the first 5 minutes, fluorescent levels on cells decreased by almost 15%. At 60 minutes, the fluorescent levels decreased by 30%. The SEM (Table 3), was low (8%), indicating that L-selectin expression on monocytes was expressed consistently in repeated experiments.

The four anticoagulants (heparin, EDTA, potassium oxalate and sodium citrate) were reviewed to examine their effect on L-selectin levels on both unstimulated (0 timepoint) and maximally stimulated (PMA) neutrophils and monocytes (Tables 5 and 6). In Figures 19 and 20, no significant variation in L-selectin levels on maximally stimulated (PMA) neutrophils or monocytes in the 4 different anticoagulants was observed. Likewise, the anticoagulant had no effect on L-selectin levels on unstimulated (0 timepoint) neutrophils or monocytes in heparin, EDTA or sodium citrate. L-selectin expression on unstimulated (0 timepoint) neutrophils and monocytes were decreased by 60-65% in potassium oxalate when compared to all other

Figure 17:

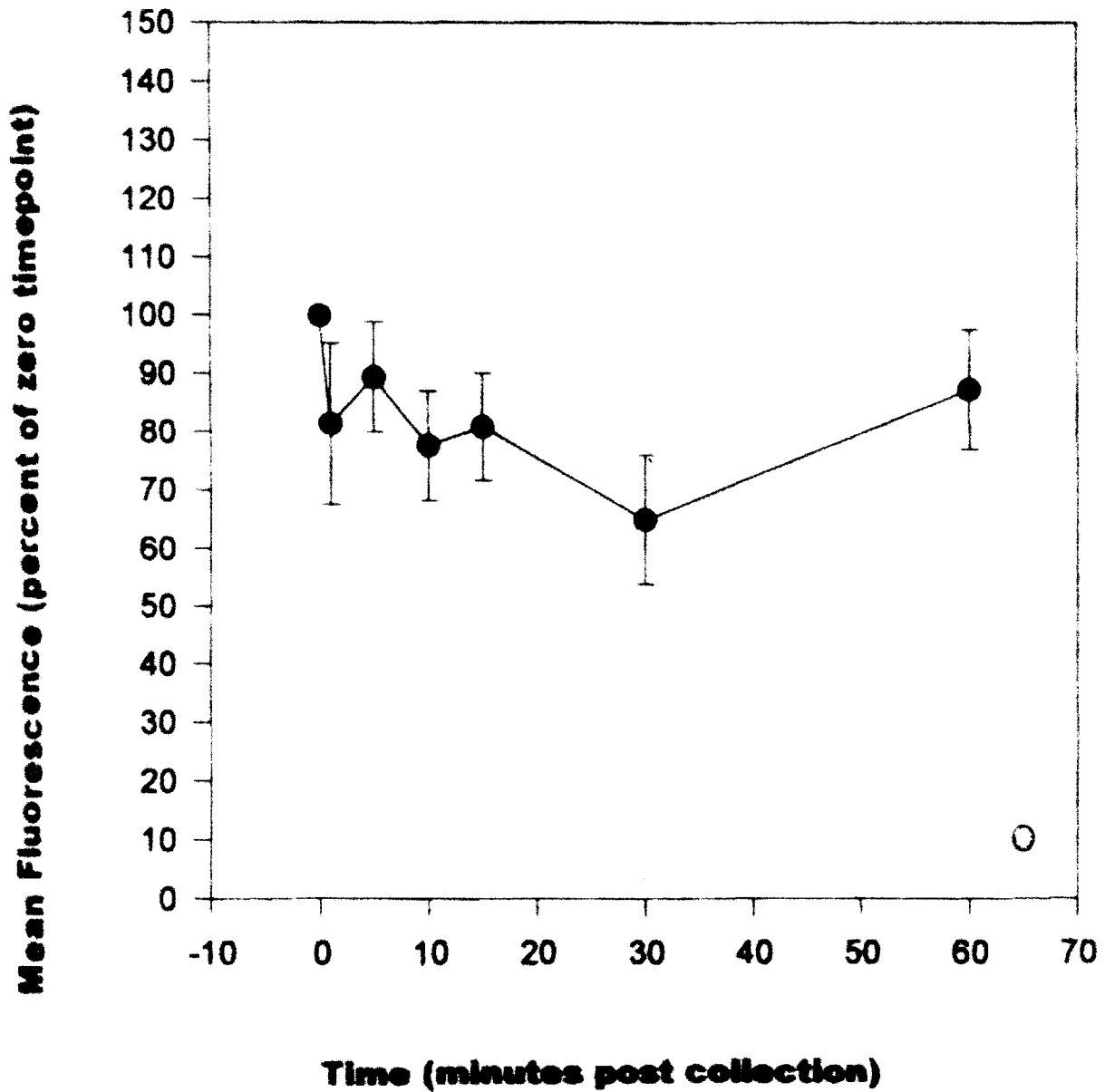
Mean fluorescence (L-selectin) levels on neutrophils collected in sodium citrate, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection.
(labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 17

Neutrophils Collected in Sodium Citrate



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

Figure 18:

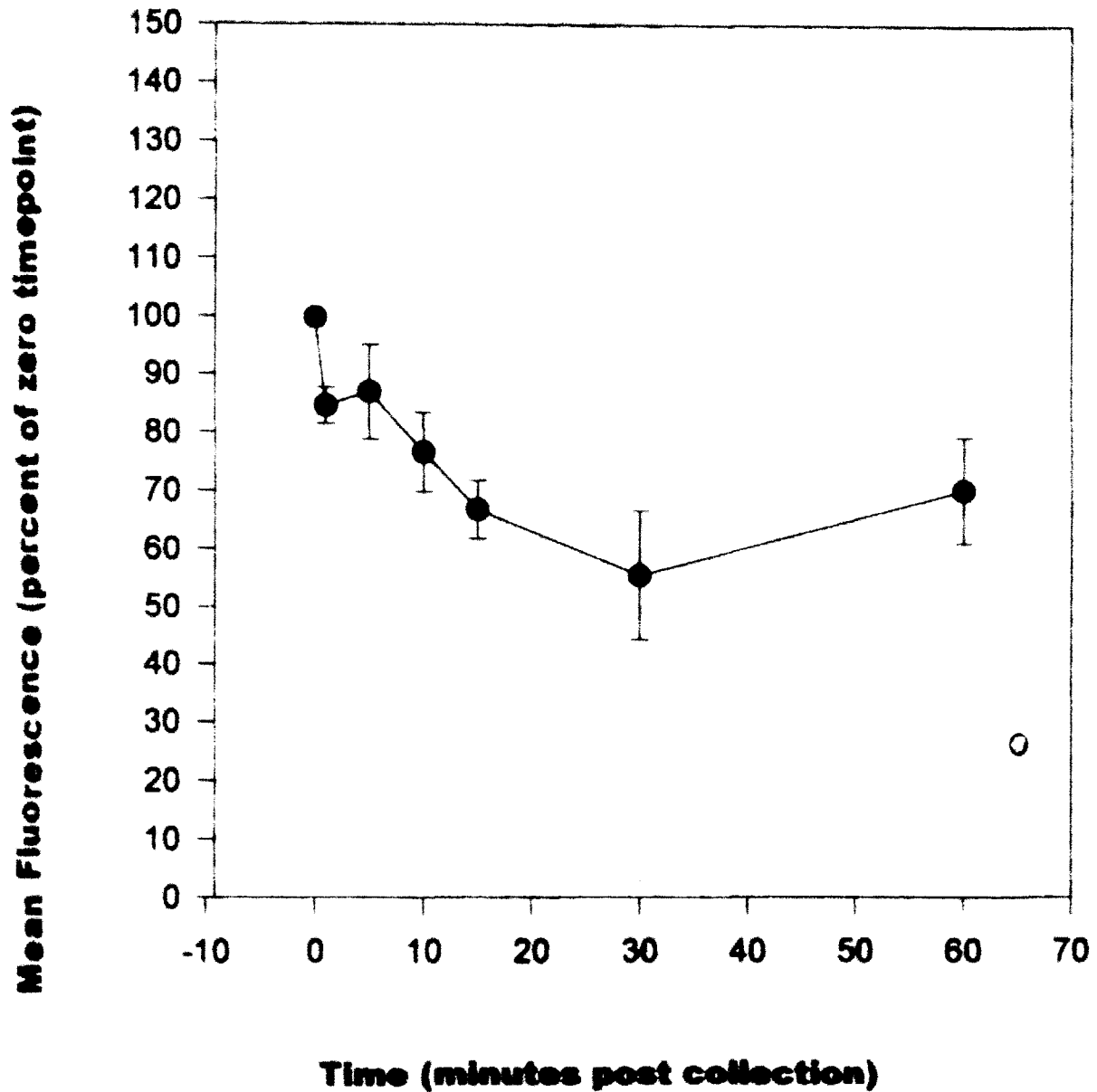
Mean fluorescence (L-selectin) levels on monocytes collected in sodium citrate, expressed as percent of the zero timepoint. The results represent the mean \pm SEM of four experiments. Error bars show the variation in L-selectin levels for the 4 experiments. Black circles represent unstimulated timepoints and the white circle represents the simulated control.

0 minute time was the blood processed immediately after collection.
(labeled 0 timepoint)

1 minute time was the blood placed on ice immediately after collection. (labeled 0i timepoint)

Figure 18

Monocytes Collected in Sodium Citrate



Black Circles- Unstimulated Timepoints
White Circle- Stimulated Control

Table 5a:

The mean of L-selectin levels on unstimulated neutrophils (zero timepoint) for all experiments in all four anticoagulants (heparin, EDTA, sodium citrate and potassium oxalate).

Table 5b:

The mean of L-selectin levels on maximally stimulated neutrophils (PMA stimulated) for all experiments in all four anticoagulants (heparin, EDTA, sodium citrate and potassium oxalate).

Table 5a

Mean L-selectin Levels on Unstimulated Neutrophils

	Heparin	EDTA	Citrate	Oxalate
1	518	388	497	104
2	650	627	635	293
3	579	558	647	187
4		624		

experiments

Table 5b

Mean L-selectin Levels on Maximally Stimulated Neutrophils

	Heparin	EDTA	Citrate	Oxalate
1	60	36	50	57
2	8	60	60	51
3	10	82	82	200
4		38		

experiments

Table 6a:

The mean of L-selectin levels on unstimulated monocytes (zero timepoint) for all experiments in all four anticoagulants (heparin, EDTA, sodium citrate and potassium oxalate).

Table 6b:

The mean of L-selectin levels on maximally stimulated monocytes (PMA stimulated) for all experiments in all four anticoagulants (heparin, EDTA, sodium citrate and potassium oxalate).

Table 6a:

The mean of L-selectin levels on unstimulated monocytes (zero timepoint) for all experiments in all four anticoagulants (heparin, EDTA, sodium citrate and potassium oxalate).

Table 6b:

The mean of L-selectin levels on maximally stimulated monocytes (PMA stimulated) for all experiments in all four anticoagulants (heparin, EDTA, sodium citrate and potassium oxalate).

Table 6a

Mean L-selectin Levels on Unstimulated Monocytes

experiments		Heparin	EDTA	Citrate	Oxalate
	1	430	393	456	227
	2	510	488	461	211
	3	533	426	657	105
	4		386		

Table 6b

Mean L-selectin Levels on Maximally Stimulated Monocytes

experiments		Heparin	EDTA	Citrate	Oxalate
	1	187	70	101	98
	2	11	107	84	181
	3	141	259	282	* 1481
	4		36		

Figure 19:

This bar graph shows the effect of anticoagulants (EDTA, heparin, sodium citrate and potassium oxalate) on the mean fluorescence of the unstimulated neutrophils (0 timepoint) versus the mean fluorescence of the maximally stimulated neutrophils (PMA stimulated).

An asterisk suggests that there is a significant difference ($p = 0.00136$) when using multiple measures one way analysis of variance in L-selectin expression (fluorescence).

Figure 19

NEUTROPHIL L-SELECTIN

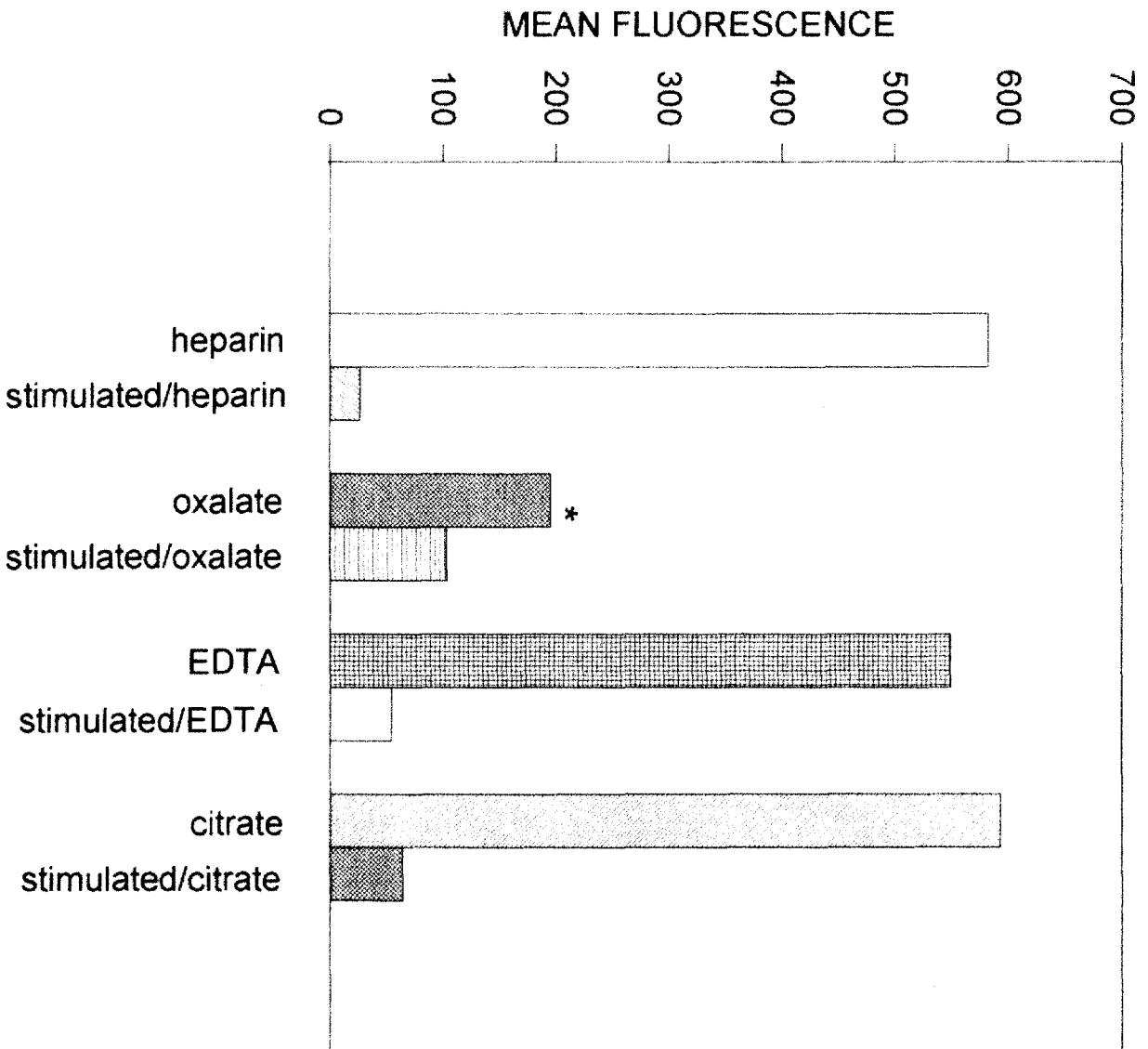


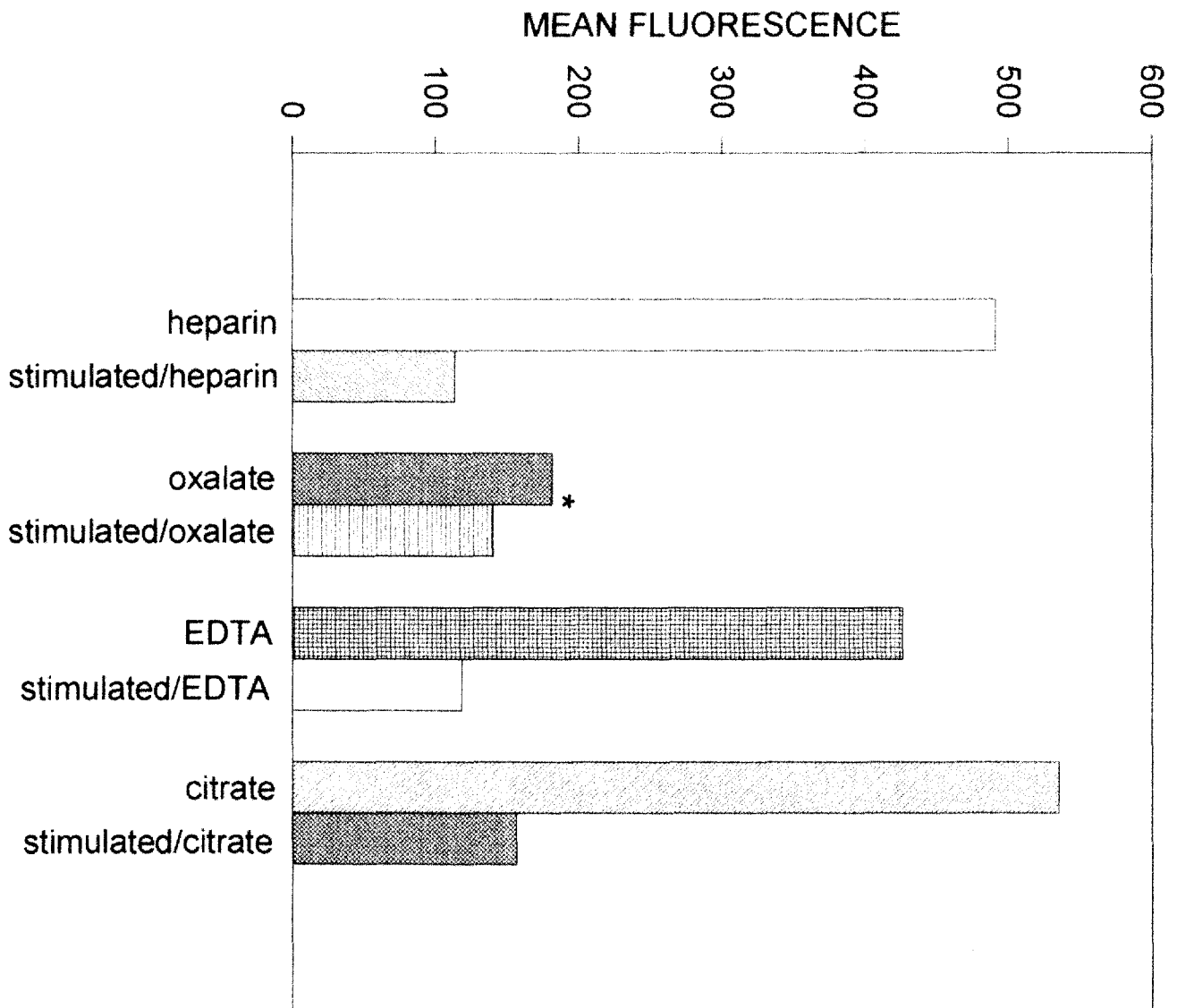
Figure 20:

This bar graph shows the effect of anticoagulants (EDTA, heparin, sodium citrate and potassium oxalate) on the mean fluorescence of the unstimulated monocytes (0 timepoint) versus the mean fluorescence of the maximally stimulated monocytes (PMA stimulated).

An asterisk suggests that there is a significant difference ($p = 0.00169$) when using multiple measures one way analysis of variance in L-selectin expression (fluorescence).

Figure 20

MONOCYTE L-SELECTIN



anticoagulants. Using multiple comparisons analysis of variance, a significant decrease in L-selectin levels on both unstimulated neutrophils and monocytes (neutrophils $p = 0.0014$, monocytes $p = 0.0017$) was observed. There was no obvious effect on antibody binding when heparin, EDTA or sodium citrate was used, but decreased antibody binding may be causing the decrease in L-selectin levels in potassium oxalate.

DISCUSSION

The purpose of the ARDS study is to examine the role of cell adhesion molecules (Mac-1 and L-selectin) in the development of ARDS in trauma patients. The stimulation of these cells outside the body during testing would be undesirable. In preparation for the clinical ARDS study, many issues concerning the preservation of the cell adhesion molecules (Mac-1 and L-selectin) in vitro needed to be resolved. How long the blood could sit at room temperature, what type of anticoagulant to use and even the normal cellular expression of CAM's were examined. To determine whether the time of day (day or night) had any influence on CAM expression within the body, an early study in our lab examined the diurnal variation on various CAM's.⁽⁴⁰⁾ Since blood is to be collected from patients in the ARDS study over a consecutive 24 hour period, diurnal variation in CAM's was a valid concern. To examine CAM levels over a 24 hour period, a heparin lock was inserted into the antecubital vein of volunteers. Blood was collected and labeled antibodies (anti-L-selectin, anti-Mac-1, anti-LFA-1alpha, anti-LFA-1beta, anti-VLA-1 or anti-VCAM-1) every 6 hours over a consecutive 24 hour period. Blood for each timepoint was processed immediately. The results showed that CAM expression (L-selectin and Mac-1) was not significantly affected by diurnal (time of day) fluctuation.

Another concern that needed to be addressed in the ARDS study was the subject of patient consent. A majority of trauma patients admitted into the ARDS study may be unable to give their immediate consent for being involved in the study. Since it is neither legal, nor appropriate to draw blood from a patient for research purposes without his or her consent, another

approach had to be adopted. Upon admission into the emergency room, routine blood work is collected by the laboratory for complete blood counts and various other screenings. It was determined that blood left over from this collection could be used for the initial timepoint without violating patient rights and Institutional Review Board policies. Once the hospital laboratory removes the blood they need for their blood screening, our lab then recovers the remaining anticoagulated blood. For the patient to continue to be in the study, written consent must be obtained by either the patient or family member prior to the second timepoint (6 hours post trauma). If this is not achieved, the patient is discontinued from the study and any data obtained is destroyed.

The recovery of patient blood by our lab from the hospital's laboratory usually does not occur immediately following blood collection from the patient. The time and temperature at which blood could sit prior to the processing of the blood by our lab therefore, needed to be established. Previous studies in our lab suggest that blood stored at 4°C show less Mac-1 expression and more L-selectin expression over time than cells stored at room temperature. In these studies, blood was collected from volunteers and divided into two portions.⁽⁴⁸⁾ Blood processed immediately was compared to blood processed after two hours at room temperature or two hours at 4°C. Differences in CAM expression following incubation were examined. White blood cells were labeled with antibodies directed against one of the cell adhesion molecules; L-selectin, Mac-1, LFA-1alpha, LFA-1beta, VLA-1 or VCAM-1. The results showed that blood stored at 4°C had the least variation in CAM expression. However, even on ice the cells eventually (within 2

hours) developed a stimulated phenotype with increased Mac-1 and decreased L-selectin expression.

There are contradictory opinions about the most optimal temperature and anticoagulant to use when performing flow cytometric analysis on blood samples. Bray and Landay claim that blood stored at room temperature is preferred to 4°C because of the significant loss of helper T-cells which occurs in blood stored at 4°C.⁽⁵⁾ On the other hand, research by Macey *et al.* indicates that blood kept at 4°C is preferred to blood kept at room temperature because blood at room temperature shows an increase in the amount of forward scatter by granulocytes. This suggests that cells are being stimulated.⁽³⁶⁾ In the experiments by Macey and coworkers, blood was drawn into an anticoagulant (citrate, EDTA or heparin), fixed with formaldehyde, labeled with anti-L-selectin, lysed and observed using flow cytometry. The anticoagulated blood was kept at room temperature or stored on ice for timepoints ranging from 1 minute to 60 minutes prior to the processing of the blood. The blood processed at 1 minute (room temperature and 4°C) showed minimal shedding of L-selectin on white blood cells. The blood processed at subsequent timepoints however, showed decreasing levels of L-selectin expression on the cells with a more significant decrease in L-selectin expression on cells stored at room temperature.⁽³⁶⁾

Similar results were observed in research by Yousseff and coworkers.⁽⁵¹⁾ In these studies, blood was drawn into sodium citrate and cell adhesion molecules on neutrophils (both in whole blood and on isolated neutrophils) were examined. Anticoagulated blood was kept at either room temperature or 4°C prior to the processing of the blood. The results indicated that L-selectin on neutrophils in whole blood kept at either room temperature

or 4°C were not significantly shed. L-selectin from isolated neutrophils, kept at room temperature, was shed up to 30% compared to L-selectin on neutrophils stored at 4°C.(51) Additional research by Paxton and Bendele also agrees that an increase in temperature causes the greatest variation in samples.(43) In this study, CD3, CD4, CD8, and CD19 were examined in blood that was anticoagulated (using EDTA, citrate or heparin) and stored at 4°C, room temperature or 37°C up to 36 hours prior to processing the blood. Cells processed immediately at room temperature provided a baseline to compare cell surface marker expression at the different times and temperatures. CD3, CD4 and CD8 all showed increased expression with increasing temperatures, while CD19 showed decreased expression with increasing temperatures. Their conclusion was that cells become stimulated over time at increasing temperatures.

Since the choice of anticoagulant affects CAM expression in flow cytometry, the advantages and disadvantages of various anticoagulants were considered. The four most commonly used anticoagulants in the hospital setting are heparin, sodium citrate, potassium oxalate and EDTA.(17,19) Heparin is a mucopolysaccharide that inactivates the blood clotting chemicals thrombin and thromboplastin (which prevents platelet aggregation). Blood tests for which heparin is preferred are electrolyte levels, liver functions tests, metabolic enzyme assays, creatinine, blood glucose levels and isoenzyme levels. A disadvantage in using heparin is that it binds to platelets and other cells and acts to block inositol triphosphate receptors and calcium transport ATP-ase (it may enhance platelet aggregation).(13,33)

Potassium oxalate prevents coagulation by binding calcium ions, but at the same time it can also distort cellular morphology and shrink the red blood

cells.(17) Another disadvantage in using oxalate is that it may cause platelets to clump together. Oxalate is usually used in blood tests such as glycolytic inhibition tests where physical characteristics and morphology of the blood cells are not examined.

Sodium citrate functions to inhibit clotting by removing calcium ions and forming insoluble calcium salts. A disadvantage in using citrate is that it is a liquid instead of a powder and as a result, the dilution of the blood specimen may cause hematology cell counting to be low.(36) Citrate is used in tests such as prothrombin time tests, partial thromboplastin time tests and pro-times (bleeding time).

EDTA is a chelating agent which effectively aggregates platelets. In addition, it also effects the calcium dependent channels. Blood effectively anticoagulated with EDTA does not show distorted or irregular white blood cell morphologies. (33) EDTA is used for studies involving whole blood cell counts, white blood cell differentials, platelet counts, hemoglobin and hematocrit counts.

Mac-1 and L-selectin are the two cell adhesion molecules being investigated in the ARDS Clinical study. It was necessary therefore, to determine which anticoagulant interfered the least in cell expression for both CAM's (Mac-1 and L-selectin). All anticoagulants have both advantages and disadvantages. We were therefore concerned how these effects might influence antibody labeling and leukocyte examination by flow cytometry. The goal for in vitro sample preparation is to maintain all cellular components as they were in vivo. According to Bray *et.al.*, the recommended procedure for sample preparation is labeling of the blood with specific monoclonal antibodies, followed by whole blood lysis, preferably with a commercial

lysing preparation.⁽⁵⁾ Sodium azide is used to inhibit ATP generation and to prevent capping and internalization of cell surface markers. However, fixation with aldehyde is thought to be a more reliable method of stabilization and preservation. This produces optimal results and minimal contamination between cell populations. This contradicts Macey's research which supports the fixation of cells prior to labeling and which favors examining cells in whole blood. The majority of labs however, including the company that produces the antibodies (Boehringer Ingelheim Pharmaceuticals Inc.), suggest that RBC lysis and fixation of cells following antibody labeling is the preferred method. This is also the opinion held by our lab and as a result, it is the procedure followed in earlier work as well as in current research.

To determine the best anticoagulant to use in the clinical ARDS study, our lab investigated the effect of the various anticoagulants on Mac-1 expression.⁽⁴⁴⁾ Blood was collected from volunteers into one of four anticoagulants (EDTA, heparin, potassium oxalate and sodium citrate). Blood was incubated for various lengths of time up to 1 hour and CAM expression was observed and compared. It was determined from this study that EDTA caused the least alteration in Mac-1 expression.

In our experiments, the expression of L-selectin levels in blood processed immediately (0 timepoint) versus blood placed on ice immediately (0i) was evaluated. It is obvious that, even at 0°C, L-selectin levels decrease over time. Fluorescent labeling of cells decreases 10% (in EDTA) and 40% (in potassium oxalate) when comparing cells processed immediately (0) to cells placed on ice and processed at 60 minutes (0i). The 0 timepoint shows the highest L-selectin levels followed by 0i, which behaves similarly to the other timepoints placed on ice. All timepoints show similar decreases in

L-selectin when compared to the zero timepoint. In all anticoagulants, this study shows that blood processed immediately (0), acts as a better reference point in determining maximum L-selectin expression than blood placed on ice immediately (0i). Blood placed on ice immediately (0i) exhibits decreased L-selectin expression.

In these studies it was found that blood collected in EDTA caused the least variation in L-selectin expression on white blood cells over time ($p > 0.05$). In neutrophils, there was a decrease in L-selectin expression, of less than 10%, after a 60 minute incubation at room temperature. In monocytes, this decrease in expression was less than 30%. For both monocytes and neutrophils the SEM was less than 10% indicating that repeated experiments in EDTA are consistently reproducible. Blood collected in Heparin showed a large decrease in L-selectin levels on monocytes and neutrophils (40%) after an incubation at room temperature for 60 minutes when compared to blood processed immediately (0 timepoint). This difference was not statistically significant ($p > 0.05$) however, due to the large SEM (greater than 20%). The inconsistent expression of L-selectin on monocytes and neutrophils in repeated experiments using heparin made heparin a poor choice of anticoagulant for the ARDS study. L-selectin expression in sodium citrate was consistent in repeated experiments, but it was not considered to be the best choice of anticoagulant to measure L-selectin levels on white blood cells. L-selectin levels on neutrophils decreased by 15% after 60 minutes at room temperature. The SEM was low and this was not considered to be statistically significant ($p = 0.12$), but this was not true of the monocytes. On the monocytes, L-selectin levels decreased by 30% after 60 minutes. The SEM remained less than 10%, but the decrease in L-selectin was statistically

significant ($p = 0.0179$). Potassium oxalate was the least appropriate anticoagulant to use for measuring L-selectin levels because of its possible interference on antibody binding. L-selectin expression on monocytes decreased by only 15% after 60 minutes ($p = 0.507$), and the SEM was less than 10%. L-selectin levels on neutrophils after 60 minutes incubation at room temperature however, were decreased by almost 60%. This was a statistically significant decrease ($p = 0.000964$).

Anticoagulant choice preferred in other labs was divided between EDTA and heparin for investigating monocytes and neutrophils using flow cytometry. In flow cytometry itself, Doornbos *et. al.* suggests that monocytes and neutrophils can be efficiently separated based on their light scattering properties when collected into EDTA.⁽¹³⁾ This may occur because EDTA does not distort cellular morphology which can alter light scattering properties. In work done by Nicholson and coworkers, blood was drawn from volunteers into two tubes containing one of three anticoagulants (EDTA, heparin and citrate). Blood was processed immediately (looking at cell surface markers; CD3, CD4, CD8 and CD19 on white blood cells) and compared to blood processed 1 and 2 days post collection.⁽³⁹⁾ Heparinized blood was considered to be more stable (had the least variability in cell surface marker expression) at room temperature than blood anticoagulated with either EDTA or sodium citrate. Heparinized blood could sit for up to 3 days and still retain cell surface marker expression similar to that which is found in blood processed immediately.^(35,39) Over time, blood anticoagulated with EDTA at room temperature yielded mononuclear fractions contaminated with granulocytes. Blood anticoagulated with sodium citrate at room temperature preserved the cellular components well, but

because citrate is a liquid additive, it tended to dilute the specimen. This resulted in lower cell counts.⁽⁴⁰⁾ Nicholson and coworkers however, believe that EDTA is a better choice of anticoagulant for flow cytometry if the blood is processed immediately.⁽³⁹⁾ In addition, they found commercial lysing agents to be optimized when blood is anticoagulated in EDTA as opposed to heparin or citrate.⁽³⁹⁾ The combination of commercial lysing product and EDTA eliminated contamination by erythrocytes and mononuclear cells. This information is relevant to our investigation since our protocol also involves the use of commercial lysing preparation.

Research done by Macey *et. al.* (previously discussed for establishing the temperature at which blood samples could sit), suggests that incubation in heparin decreases L-selectin expression on cells.⁽³³⁾ Blood was drawn into an anticoagulant (EDTA, heparin or citrate), fixed with formaldehyde, labeled with anti-L-selectin, lysed and observed using flow cytometry. The anticoagulated blood was kept at room temperature or stored on ice for timepoints ranging from 1 minute to 60 minutes prior to the processing of the blood. The blood processed at 1 minute proved to be minimally affected by the anticoagulant, but the blood processed at subsequent timepoints showed decreasing levels of fluorescence on the cells. Fluorescent intensity tended to be even lower in heparin than in either EDTA or citrate. The observations in Macey's research are similar to the findings in our investigation. The use of an anticoagulant (EDTA, heparin and citrate) appeared to have no effect on L-selectin levels on monocytes and neutrophils in blood processed immediately. In our studies, potassium oxalate did decrease the L-selectin levels on both unstimulated monocytes and neutrophils, but this was not tested by Macey and coworkers. In Macey's studies, L-selectin expression on

both monocytes and neutrophils (over time) was found to be lower in heparin (40%) than in either EDTA (10%) or citrate (15%).

One concern in selecting an anticoagulant for a clinical study involves effective antibody labeling. In a study performed by McCarthy *et. al.*, blood was drawn into EDTA, citrate or heparin.⁽³⁵⁾ Histopaque centrifugation was used to separate the monocytes and neutrophils. Cells were labeled with antibody against Mac-1 and L-selectin, but not fixed. They found that greater than 90% of neutrophils stained positively with antibodies directed against L-selectin when EDTA, heparin or citrate were used. The percentage of monocytes that were effectively labeled was less than 90% when heparin or citrate were used compared to greater than 90% when EDTA was used. According to this study, EDTA was preferable to heparin and citrate in expressing the greatest percentage of labeled monocytes and neutrophils.

Work done by El Habbal *et. al.* agrees with this finding and goes on to suggest that heparin may actually stimulate the expression of CAM's on neutrophils.⁽²²⁾ Blood was drawn from volunteers into an anticoagulant (EDTA, heparin and citrate) and kept at 37°C for various timepoints (0, 10, 30, 60 and 120 minutes) prior to processing the blood. They found that cells anticoagulated in heparin versus blood anticoagulated in EDTA had a higher binding intensity of the monoclonal antibodies (anti-Mac-1 and anti-L-selectin). The results however, also show shedding of L-selectin over time, with the shedding being more marked in heparin. At 120 minutes in heparin, neutrophils showed a 95% decrease in L-selectin expression. Neutrophils in EDTA showed a 15% decrease in L-selectin expression and in citrate, L- selectin expression was decreased by 20%. They concluded that the use of EDTA and citrate as an anticoagulant is “more appropriate” and

that “use of heparin may cause false positive results” if used to monitor the stimulation of cell adhesion molecule expression on neutrophils.⁽²²⁾ Repo *et. al.* also concluded that L-selectin was significantly shed from neutrophils in heparin when compared to L-selectin shed from neutrophils in citrate.⁽⁴⁵⁾ The research by El Habbal and associates and Repo and coworkers agrees with the results obtained in our lab. Over time, L-selectin levels on neutrophils in heparin were in fact lower than L-selectin levels in either EDTA or citrate. Citrate would be a suitable anticoagulant to use for investigating L-selectin levels on neutrophils, but EDTA would be even better suited.

The results in all the studies mentioned seem to suggest that cells left at room temperature over time will become stimulated. Our study does not dispute this, in fact, our results are similar to those presented in El Habbal’s research. The main difference is that their research was carried out at 37°C while our incubations were performed at 25°C. Their results support the observation that heparin effects the expression of L-selectin more significantly than EDTA or citrate. El Habbal’s data and data from this study suggest that L-selectin is shed more rapidly from white blood cells when heparin is the anticoagulant.

Based on these studies, it has been established that EDTA is the best choice of anticoagulant for studies examining L-selectin. When blood is collected in EDTA, L-selectin levels remain fairly consistent over time when measured by flow cytometry. These studies showed a less than 10% decrease in L-selectin expression on neutrophils and less than a 30% decrease in L-selectin on monocytes (at room temperature), 60 minutes post collection in

EDTA. In addition reproducible measurements were seen in repeated trials when EDTA was used as the anticoagulant.

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May 5, 1997

Dr. Diana Fagan
Department of Biological Sciences
UNIVERSITY

Dear Dr. Fagan:

The Human Subjects Research Committee of Youngstown State University has reviewed the revised Informed Consent Form you provided for your Protocol HSRC #97-7, "Effect of Time and Anticoagulant on the Expression of Human Leukocyte Cell Adhesion Molecules," and determined that it now fully meets the guidelines. Therefore, I am pleased to inform you that your project has been approved.

We wish you well in your study.

Sincerely,

Dr. Peter J. Kasvinsky
Dean of Graduate Studies

cc

c: Dr. Paul Peterson, Chair,
Department of Biological Sciences
HSRC Committee Members