

**Maintaining Neutrophils at Low Temperature
During *in Vitro* Manipulation Improves
Accuracy of Cell Adhesion Molecule
Analysis**

By

Daniel T. Malleske, BS

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Maintaining Neutrophils at Low Temperature During *in Vitro*
Manipulation Improves Accuracy of Cell Adhesion Molecule
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Daniel T. Malleske, BS

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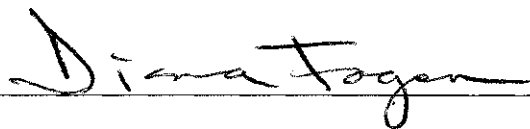


4/14/00

Daniel T. Malleske, Student

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Approvals:



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Diana Fagan, Thesis Advisor

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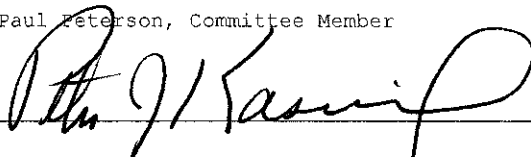
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Paul Peterson, Committee Member

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Peter J. Kasvinsky, Dean of Graduate Studies

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ABSTRACT

Cell adhesion molecules play crucial roles in many disease entities including the adult respiratory distress syndrome. Understanding their function and regulation depends on accurate methods of *in vitro* analysis. The studies presented in this thesis were specifically designed to test the effects of various conditions during leukocyte collection and processing that may alter cell adhesion molecule expression. Using specific monoclonal antibodies and flow cytometry the patterns of expression of five cell adhesion molecules were analyzed; these included, ICAM-1 (CD54), LFA-1 α (CD11a), LFA-1 β (CD18), MAC-1 (CD11b), and L-selectin. From this investigation, several critical factors were determined to affect cell adhesion molecule expression *in vitro*; these included, the choice of anticoagulant, the method of leukocyte isolation, and most importantly, the temperature at which cells were stored and processed. The leukocyte processing technique developed here will be applied to future investigations of patterns of cell adhesion molecule expression in trauma patients that may identify patients at risk for developing the adult respiratory distress syndrome.

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LIST OF ABBREVIATIONS

Ab	Antibody
ARDS	Adult Respiratory Distress Syndrome
C3a, 4a, 5a	Complement Components
CAM	Cell Adhesion Molecule
CD	Cluster of Differentiation
DMSO	Dimethylsulfoxide
E- (selectin)	Endothelial Selectin
EDTA	Ethylenediaminetetraacetic acid
f.w.	Formula Weight (atomic weight)
FACS	Fluorescence Activated Cell Sorter
FITC	Fluoresceinisothiocyanate
FMLP	f-Methionyl-Leucyl-Phenylalanine
g	Gram(s) or Gravity
GlyCAM-1	Glycosylated CAM 1
HCAM	Homing associated CAM
HCl	Hydrochloric Acid
HOCl	Hypochlorous Acid
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferon
IL	Interleukin
L- (selectin)	Leukocyte Selectin
LFA-1	Leukocyte Function Associated antigen 1

LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
M	Molar
Mab	Monoclonal Antibody
Mac-1	Macrophage antigen 1
MadCAM	Mucosal Adhesion CAM
mg	microgram
mM	Millimoles
N	Normal
Na ₂ HPO ₄	Sodium Monophosphate
NaH ₂ PO ₄ ·H ₂ O	Sodium Bisphosphate Monohydrate
NaN ₃	Sodium Azide
NaOH	Sodium Hydroxide
ng	Nanogram
N- (terminal)	Nitrogen terminus of a Peptide Chain
P- (selectin)	Platelet Selectin
PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
pH	Negative Logarithmic Expression of Hydrogen ion Concentration
PMA	Phorbol Myristate Acetate
PMN	Polymorphonuclear Leukocyte
sLe ^a	Sialyl Lewis a

sLe ^x	Sialyl Lewis x
TCR	T Cell Receptor
TNF	Tumor Necrosis Factor
VCAM	Vascular Cell Adhesion Molecule
VLA-4	Very Late Antigen 4
μg	Micrograms
μl	Microliters
μm	Micrometer

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Chapter I

INTRODUCTION

The inflammatory response is one of the most complex systems of cellular and chemical interactions in the vertebrate body, comparable to the labyrinthine tangle of cellular connections and neurohormones found in the nervous system. Without this process the body is virtually unable to defend itself against the smallest microbial invader. Even the slightest defect in this array of receptors and hormones leaves the host susceptible to a multitude of infectious and injurious agents. As the search to untangle this maze of pathways continues, each new finding brings us gradually closer to understanding the correlation between immunological events and human disease.

Inflammation is a complex series of chemical and cytological interactions central to the immune system's response to tissue injury and bacterial invasion. The inflammatory response consists of chemically mediated activation of vascular endothelium and immune cells, with subsequent vasodilatation, increased vascular permeability, and migration of leukocytes into the inflammed tissue. These result in the classic signs of inflammation first

described in the second century A.D. by the Roman physician, Aulus Celsus: edema and swelling (*tumor*), secondary to increased vascular permeability; redness (*rubor*), due to vasodilatation; heat (*calor*), from increased blood flow; and pain (*dolor*), as the swelling compresses nerve endings. During the acute phase of this process, neutrophils enter tissue beds and begin to release cytotoxic antimicrobial substances, which, under pathologic conditions, also damage host tissues (Weiss, 1989). The life threatening complications of these events have warranted much investigation into the steps involved in this process, particularly, those leading to leukocyte migration and cytotoxin release (Windsor, 1993; Weiss, 1989).

Leukocyte migration may be conceptualized with a four-step model proposed by Eugene Butcher (1991). Following tissue injury, the events of this model include, (1) rolling adhesion or tethering, (2) leukocyte activation, (3) arrest or stable adhesion, and (4) transendothelial migration or diapedesis (Luscinskas, et al., 1994; Butcher, 1991). Each of these events involves intricate regulatory mechanisms that will be summarized individually.

When tissues are injured by trauma or bacterial invasion a variety of inflammatory mediators are released by local histiocytes and mast cells and by recruited lymphocytes and monocytes (Welbourn and Young, 1992). Inflammatory mediators include plasma enzyme mediators such as bradykinin released from the clotting cascade, fibrinopeptides like thrombin produced during thrombus formation, fibrin degradation products resulting from fibrinolysis, and components of the complement system such as C3a, C4a, and C5a (collectively referred to as the anaphylatoxins because of their role in anaphylactic or allergic reactions) (Vercellotti, *et al.*, 1989; Skogen, *et al.*, 1988; Senior, *et al.*, 1986; and Hugli, 1981). There are lipid-derived chemoattractants such as the phospholipid platelet activating factor (PAF) and those derived from arachadonic acid metabolism, the prostaglandins and leukotrienes (Tonnesen, *et al.*, 1989). Prostaglandins and leukotrienes are produced by catabolism of arachadonic acid through the cylooxygenase and lipoxygenase pathways, respectively (Rochels and Busse, 1984; Movat, *et al.*, 1984; Klausner, *et al.*, 1989). Cytokines are small polypeptides consisting of 70 - 80 amino acid residues that serve as cell regulators, as chemotactic signals, or both. They are released by all leukocytes, vascular endothelium, and some

from fibroblasts. Each may exert a variety of effects on its target cell with many having overlapping functions (Turner, 1992).

Cytokines act locally to stimulate the endothelial cells of vascular beds in the injured tissue (Butcher, 1991). Two cytokines, interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF- α), as well as lipopolysaccharide (LPS) from the cell walls of gram negative bacteria are key to this activation process (Welbourn and Young, 1992; Turner, 1992; Luscinskas, *et al.*, 1991, Tonnesen, *et al.*, 1989; Thornhill, *et al.*, 1990). Stimulation from chemical mediators results in the expression of receptor molecules on the luminal surfaces of endothelial cells, these are necessary for the recruitment of phagocytic cells and lymphocytes (Luscinskas, *et al.*, 1994; Tonnesen, *et al.*, 1989; Butcher, 1991; Thornhill, *et al.*, 1990). These specific receptors are known as cell adhesion molecules (CAMs). Expression of CAMs results in increased avidity of endothelial cells for circulating leukocytes (Springer, 1990). Some CAMs may also undergo biochemical modification thereby increasing affinity for their ligand (Turner, 1992).

Cell adhesion molecules are transmembrane glycoproteins that are integral to the inflammatory

process. There are four families of CAMs associated with leukocyte-endothelial cell interactions: the integrin family, the immunoglobulin superfamily, the selectin family, and the addressins (Laffon and Gonzalez-Amaro, 1995; Springer, 1990). Alternatively, other authors have referred to the addressin family as homing associated cell adhesion molecules or HCAMs (Turner, 1992), and the "mucin-like" family (Kuby, 1994). The following discussion will present a brief description of each family according to their role in the leukocyte migration model.

The first stage of the model proposed by Butcher describes the process of leukocyte rolling, or tethering. This initial attachment to the endothelial surface is mediated by the selectin family of cell adhesion molecules and serves to slow leukocyte transit through the venules (Bevilacqua and Nelson, 1993). Selectins are glycoprotein molecules composed of an N-terminal lectin domain followed by an epidermal growth factor-like repeat, a variable number of complement regulatory-like domains, a transmembrane segment, and finally, a short cytoplasmic sequence at the carboxyl terminus (Bevilaqua and Nelson, 1993; Lasky, 1992). The lectin-like domain binds to carbohydrate ligands in a calcium dependent interaction (Geng, *et al.*, 1990). These ligands belong to the

addressin family and include several molecules. Those found on leukocytes are sialyl Lewis X (sLe^x) and sialyl Lewis A (sLe^a), CLA/HECA-452, CD15, CD66, and PGSL-1. The high endothelial venules of lymph nodes and mucosal surfaces express PGSL-1 and GlyCAM-1, which serve as lymphocyte trafficking receptors. Lastly, endothelial cells present sLe^x and sLe^a on their luminal surfaces (Lasky, 1992; Springer, 1990). Selectin-addressin interactions are weak and transient, and under flow conditions (as in blood vessels) bonds are readily formed, broken, and reformed allowing the cell to tumble or 'roll' along vessel surfaces where these molecules are expressed (Luscinskas, *et al.*, 1994; Mayadas, *et al.*, 1993; Lawrence and Springer, 1991). However, unless a second, more stable adhesion is formed the cells will soon detach and return to circulation (Luscinskas, *et al.*, 1994; Hakkert, *et al.*, 1991).

To date, there are three described members of the selectin family: P-selectin, E-selectin, and L-selectin (Elangbam, *et al.*, 1997; Laffon and Gonzalez-Amaro, 1995). P-selectin (CD62P, PADGEM, or GMP-140) co-localizes with von Willibrand factor, an essential platelet receptor, in the alpha-granules of megakaryocytes and platelets, and in the Weibel-Palade bodies of vascular endothelial cells

(Mayadas, *et al.*, 1993; McEver, *et al.*, 1989). Expression is induced by chemoattractants like thrombin, histamine, complement, platelet activating factor (PAF), endotoxin, and oxygen free radicals, and facilitates adhesion of phagocytes to endothelium at sites of tissue injury and inflammation (Dore, *et al.*, 1993; McEver, *et al.*, 1989). E-selectin (CD62E or ELAM-1) is neither stored nor constitutively expressed; rather, it is synthesized *de novo* by endothelial cells in response to stimulation by IL-1, TNF, or endotoxin (Laffon and Gonzalez-Amaro, 1995; Schleimer, *et al.*, 1992; Bevilacqua, *et al.*, 1987 and 1989). Lastly, L-selectin (CD62L, LECAM-1, or MEL-14/LAM-1), constitutively present on all granulocytes, monocytes, and certain populations of lymphocytes, and known to play an integral role in lymphocyte trafficking, is the primary selectin responsible for leukocyte rolling (Laffon and Gonzalez-Amaro, 1995; Luscinskas, *et al.*, 1994; Beveilacqua and Nelson, 1993). L-selectins localize on the apical surfaces of leukocyte microvilli thereby facilitating their contact with the endothelium (Erlandsen, *et al.*, 1993; von Andrian, *et al.*, 1993). Stimulated endothelium will express E and P selectins. These have terminal sLe^x residues to which L-selectin will bind allowing the cell to begin to roll under flow conditions (Picker, *et al.*, 1991;

Lawrence and Springer, 1991). Rolling significantly slows leukocyte transit and allows for more stable, integrin-mediated bonds to form (Bienvenue and Granger, 1993). However, L-selectin binding alone is not sufficient for leukocyte diapedesis (Lawrence and Springer, 1991; Kishimoto, *et al.*, 1991).

The second and third events in the model, leukocyte activation and stable adhesion, respectively, will be discussed together as they are integrally related. Leukocyte activation is mediated by chemoattractants or by direct cell contact causing expression of activation-dependent receptor molecules. These molecules will subsequently provide for more stable leukocyte-endothelium adhesion and diapedesis (Butcher, 1991). The activation dependent molecules belong to the integrin family of CAMs (Butcher, 1991; Springer, 1990). Integrins are heterodimeric molecules consisting of a unique alpha chain, which determines ligand specificity, and a common beta (CD18) chain that defines the subfamily. Integrins allow for binding to other cell surface molecules as well as to extracellular matrix elements such as collagen, laminin, and fibronectin (Laffon and Gonzalez-Amaro, 1995; Turner, 1992). To date, there are eight identified beta subfamilies, b1-b8, with b1, b2, b3, and b7 being the best

defined; however, b1, b2, and b7 are the most pertinent to the study of leukocyte migration (Elangbam, *et al.*, 1997). The beta 1 integrins include the very late activation (VLA) molecules such as VLA-4 ($\alpha_4\beta_1$). The beta 2 integrins of interest are LFA-1 ($\alpha_L\beta_2$ or CD11a/CD18), Mac-1 ($\alpha_M\beta_2$ or CD11b/CD18), and p150,95 ($\alpha_X\beta_2$ or CD11c/CD18). Lastly, the most extensively studied member of the beta 7 family is LPAM-1 ($\alpha_4\beta_7$) (Elangbam, *et al.*, 1997; Turner, 1992). Each of these molecules have been demonstrated to interact with specific immunoglobulin superfamily (Ig) molecules in the process of endothelial adhesion and diapedesis (Laffon and Gonzalez-Amaro, 1995).

Immunoglobulin superfamily CAMs are composed of one or more extracellular domains bearing homology to receptor immunoglobulins, the T-cell Receptor (TCR) for instance, found on lymphocytes (Laffon and Gonzalez-Amaro, 1995). Examples of Ig CAMs include: ICAM-1 and ICAM-2, which recognize the beta 2 integrins; VCAM-1, a receptor for VLA-4; and MadCAM-1, expressed on high endothelial venules, binds lymphocyte LPAM-1 facilitating lymphocyte trafficking (Elangbam, *et al.*, 1997).

The importance of selectins in leukocyte capture and rolling along activated endothelium has already been discussed. As mentioned, without an activating signal,

leukocytes bound through L-selectin simply detach and re-enter circulation. However, with appropriate stimulation of the leukocyte, integrins will be expressed, firm adhesions formed, and the cell will exit the blood vessel into the tissue bed. Some of the most frequently discussed neutrophil activators include: chemoattractant molecules like platelet activating factor (PAF), leukotriene B₄ (LTB₄), complement component C5a, LPS (endotoxin), formyl-methionyl-leucyl-phenylalanine [FMLP (bacterial proteins contain formylated-methionone N-terminal moieties which are not synthesized by eukaryotic cells, thus, are recognized as foreign)]; and the cytokines, TNF- α and interleukin-8 (IL-8) (Patrick, *et al.*, 1997; Niwa, *et al.*, 1996; Richard, *et al.*, 1994; Wyman, *et al.*, 1987). Upon stimulation by these molecules, three changes in neutrophil physiology occur that are consistent with activation: 1) shedding of L-selectin, 2) upregulation of Mac-1, and 3) initiation of the neutrophil respiratory burst with production of oxygen free radicals (discussed later) (Condliffe, *et al.*, 1996).

As mentioned, PMN activation occurs through either direct contact, as with PAF expressed on the membranes of activated endothelial cells, or through chemical mediators like TNF or FMLP. Much evidence suggests that this is actually a two-step process. Carefully controlled studies

have revealed that a priming signal, resulting in physiological changes at the level of signal transduction (Daniels, *et al.*, 1994), enables the cell to respond faster and more effectively to a second, activating stimulus (Condliffe, *et al.*, 1996; Wyman, *et al.*, 1987). Platelet activating factor expressed on endothelial cells is central to this process *in vivo* (Hill, *et al.*, 1994; Condliffe, *et al.*, 1996). In addition, PAF has been shown to play a dual role as either the primer or the activator depending on the presence of a prior stimulus: for example, PAF may activate a TNF- α primed cell, or it may prime neutrophils for subsequent activation by FMLP (Hill, *et al.*, 1994; von Asmuth, *et al.*, 1995; Condliffe, *et al.*, 1996).

Priming events, such as the binding of rolling neutrophils to PAF or exposure to FMLP, result in shedding of L-selectin and increased expression of the CD18 integrin Mac-1 (Lorant, *et al.*, 1991; Furie, *et al.*, 1991). Shedding of L-selectin occurs intravascularly, prior to diapedesis (Kuhns, *et al.*, 1995), via enzymatic cleavage of the extracellular component proximal to the membrane surface (Kahn, *et al.*, 1994). Expression of Mac-1 is constitutive at low levels, but shows significant upregulation (*i.e.*, increased expression) upon stimulation of the cell (Erlandsen, *et al.*, 1993). These receptors

are not confined to the apices of microvilli like L-selectin; rather, they are more evenly distributed over the cell surface (Borregard, *et al.*, 1994; Erlandsen, *et al.*, 1993). The inducible stores of Mac-1 are located in cytoplasmic granules within PMNs and translocate to the cell surface immediately upon cell stimulation (English and Graves, 1992). Furthermore, binding of adherent cells to PAF not only induces this upregulation, but, more importantly, also leads to a conformational change in Mac-1 from an inert state to a functional protein (Condliffe, *et al.*, 1996). Interaction between Mac-1 and endothelial ICAM-1 is essential for diapedesis and activation of the respiratory burst (Shappell, *et al.*, 1990). In fact, in studies using monoclonal antibodies to block Mac-1, activating chemokines fail to induce transmigration and production of oxygen free radicals in primed neutrophils (Entman, *et al.*, 1992; Jaeschke, *et al.*, 1991). In summary, with stable adhesion to Mac-1, the activated neutrophil may exit the vasculature and enter the interstitial environment. Once in the tissues, it may interact with other chemoattractant molecules leading to accentuation of its cytotoxic potential (Hill, *et al.*, 1994).

Neutrophils destroy invading organisms by employing two separate cytotoxic systems. The first, referred to as the neutrophil respiratory burst, is a membrane bound enzyme system capable of generating reactive oxygen metabolites which destroy cell membranes. The second is a stock of cytoplasmic granules containing proteinases, microbicidal peptides, and other enzymes (a complete review of these systems is provided in Windsor, 1993 and Weiss, 1989). The respiratory burst is generated concomitant with stimulation of phagocytosis. As the phagocytic vacuole forms, membrane bound NADPH oxidase catalyzes electron transfer from reduced NADPH in the cytoplasm to molecular oxygen dissolved in the engulfed extracellular fluid. This results in the production of two molecules of superoxide, $O_2\cdot^-$ (the dot symbolizes the extra, reactive electron) (Windsor, 1993; Weiss, 1989). The resultant rapid consumption of oxygen is referred to as the 'respiratory burst' (Thomas, *et al.*, 1988). Next, cytosolic superoxide dismutase converts two molecules of superoxide into hydrogen peroxide, H_2O_2 (Windsor, 1993; Thomas, *et al.*, 1988). Upon fusion of a lysosome with the phagocytic vacuole, the lysosomal enzyme myeloperoxidase catalyzes the reaction converting hydrogen peroxide into highly bactericidal hypochlorous acid, $HOCl$, which lyses cells via

oxidative damage of cell membrane components (Kettle and Winterbourn, 1990; Schraufstatter, et al., 1990; McKenna and Davies, 1998). In contrast, the cytoplasmic granules are storage vacuoles which fuse with the forming phagocytic vesicle emptying collagenase, elastase, gelatinase, and other proteolytic enzymes into the extracellular fluid (Weiss, 1989). During the phagocytic process, leakage of oxygen radicals and enzymes into the extracellular environment may result in untoward destruction of healthy tissue (Windsor, 1993; Weiss, 1989).

Neutrophil induced tissue injury affects several organ systems (Soriano, et al., 1999; Entman, et al., 1992; Jaeschke, et al., 1991), but the most critical injury occurs to the lungs. Neutrophil induced lung injury results in a complex pathologic process known as the Adult Respiratory Distress Syndrome, or ARDS (Windsor, 1993). Clinically, ARDS is defined by acute respiratory failure with progressive hypoxemia, decreased pulmonary compliance, and radiographic evidence of diffuse pulmonary infiltrates (Chollet-Martin, et al., 1996). The underlying pathology responsible for this presentation is an accumulation of protein and neutrophil rich fluid in the alveolar spaces (Ashbaugh, et al., 1967). Neutrophils are found to sequester in three separate pools in the body: in the bone

marrow, in the blood, and in the tissues. At any time, approximately 54% of those neutrophils in the intravascular pool are adherent to the vascular endothelium, a process called margination. Thirty-three percent of these marginate in the pulmonary vasculature (MacNee and Selby, 1993). Based on these statistics, it is easy to foresee the impact neutrophils may have on lung function in a pathological state.

The exact progress of events leading to ARDS is, at present, poorly understood. The inciting events range from any cause of tissue ischemia to leukemia; however, sepsis and massive trauma are most frequently indicated (Lillington and Redding, 1986). Activated neutrophils are implicated as important mediators of lung damage by their high content in blood and bronchoalveolar lavage fluid (BAL) collected from ARDS patients (Zimmerman, *et al.*, 1983). Likewise, high concentrations of the cytokines TNF- α , IL-8 (a neutrophil chemotactic peptide), and IL-6 (a neutrophil activator and endogenous pyrogen) are also present in BAL fluid from ARDS versus non-ARDS patients (Chollet-Martin, *et al.*, 1992 and 1996). Tumor necrosis factor-alpha is believed to be a significant mediator in the development of this condition (Mulligan, *et al.*, 1995; Chollet-Martin, *et al.*, 1992). Peter Ward describes

several pathways by which sepsis leads to TNF- α production by tissue macrophages (Ward, 1997). In his algarhythms, deposition of antibody-antigen complexes in the lung leads to activation of the complement system with production of C5a, a powerful chemoattractant. Macrophages are subsequently activated by C5a and begin producing TNF- α and IL-1. It should be noted that C5a itself is also a potent chemotactic and activating factor for neutrophils (Wyman, *et al.*, 1987; Richard, *et al.*, 1994). Endotoxin too may directly activate both neutrophils and macrophages (Chollet-Martin, *et al.*, 1992; Condliffe, *et al.*, 1996). These diffuse to local vascular endothelial cells, which are in turn stimulated to express selectins and ICAM-1. This is followed by recruitment, activation, and diapedesis of neutrophils leading to tissue destruction by oxidative and enzymatic agents.

During major trauma, blood loss and shock lead to tissue hypoxia. With successful resuscitation, these ischemic tissues are suddenly reperfused with oxygenated blood. Through a poorly understood mechanism, this process of ischemia and reperfusion leads to the activation of the cytoplasmic enzyme, xanthine oxidase, within affected endothelial cells (Zulueta, *et al.*, 1997). Xanthine oxidase generates reactive oxygen metabolites which

stimulate local production of PAF and LTB₄ capable of activating passing neutrophils (Kurose, *et al.*, 1997). In the lungs, oxygen metabolites increase IL-1 and TNF- α production by local macrophages (Schwartz, *et al.*, 1995). Ultimately, activated neutrophils will accumulate, and ischemia-reperfusion induced microvascular injury will result (Carden, *et al.*, 1998).

In addition to oxidative damage, a growing body of evidence implicates the granule enzyme, elastase, as a prime effector of pulmonary microvascular injury. Some trials even suggest that oxygen radicals may play only a minor role in endothelial injury as compared to elastase or other proteolytic enzymes (MacGregor, *et al.*, 1997). Studies comparing serum levels of neutrophil elastase in trauma victims at the time of hospital admission have revealed significant increases in those patients who developed ARDS as compared to those who did not progress to ARDS (Donnelly, *et al.*, 1995). Additional investigations showed that elastase levels remained elevated throughout the course of hospitalization in those who developed ARDS (Gando, *et al.*, 1997). Other workers observed both *in vitro* and *in vivo* that neutrophil elastase mediates proteolytic degradation of endothelial cell cadherins (cell adhesion molecules involved in maintaining static cell-cell

adhesion), thereby compromising microvascular integrity (Carden, *et al.*, 1998). Studies involving animal models of ARDS further support these observations. In these trials, animals receiving the neutrophil elastase inhibitor ONO-5046 demonstrated a significant decrease in microvascular permeability and alveolar accumulation of neutrophils (Miyazaki, *et al.*, 1998; Sakamaki, *et al.*, 1996). These compelling data underscore the need for continued study of the role of enzyme mediators in lung injury, as well as the contribution of oxygen radicals.

Whether oxygen radicals, proteolytic enzymes, or some combination of the two prove to be ultimately responsible, further study of chemoattractants, cell adhesion molecules, and neutrophils and their role in lung injury is essential. The experiments presented in this thesis were developed to investigate the effects of neutrophil isolation and processing on cell adhesion molecule expression. These techniques will be applied to future investigations of patterns of cell adhesion molecule expression in trauma patients at risk for developing the adult respiratory distress syndrome.

Chapter II

MATERIALS AND METHODS

MATERIALS

Monoclonal Antibodies: Monoclonal antibodies against ICAM-1 (CD54), LFA-1 α (CD11a), LFA-1 β (CD18), Mac-1 (CD11b), and L-selectin (CD62L) were generously donated by Dr. Robert Rothlein, Boehringer Ingleheim Pharmaceuticals, Inc. (Ridgefield, CT). Monoclonal antibodies against VLA-4 were purchased at a stock concentration of 0.2 mg/mL from AMAC (Westbrook, ME). Simultest Leuco Gate (CD45-FITC and CD14-PE), Simultest controls (gamma 1- FITC and gamma 2a-PE), Simultest CD3-FITC/CD19-PE, FACS Lysing Buffer, and Cali Brite beads (unlabeled and FITC and PE-conjugated) were all purchased from Becton-Dickinson (San Jose, CA). Additional reagents, which include NaH₂PO₄·H₂O, anhydrous Na₂HPO₄, NaCl, paraformaldehyde, and sodium azide, NaN₃, were purchased from Sigma Chemical Company (St. Louis MO). Dimethylsulfoxide (DMSO) and phorbol myristate acetate (PMA) were purchased from Fisher (Fair Lawn, NJ).

Preparation of reagents:

10X Phosphate Buffered Saline (PBS): Stock solutions of 10X PBS were prepared by dissolving 1.28g NaH₂PO₄·H₂O (f.w.

120.0) and 5.97g anhydrous Na_2HPO_4 (f.w. 142.0) in 200 ml of MilliQ water. The pH was adjusted to 7.4 using either 0.1 N NaOH or 0.1 N HCl as necessary. Following pH correction, 43.83g NaCl (f.w. 58.44) were added and the mixture was brought to a final volume of 500 ml using MilliQ water and was stored at room temperature.

1X PBS: Stock 10X PBS was diluted to a 1x preparation by mixing 100 ml of 10X PBS into 900 ml of MilliQ water. The solution was stored at 4°C and filtered using a 0.2 μm membrane filter prior to each use.

PBS/Azide: A 100 ml aliquot of 10X PBS and 1.0 g of NaN_3 (f.w. 65.01) were mixed with 900 ml of MilliQ water. The solution was then stored in the dark at 4°C. PBS/Azide was filtered using a 0.2 μm membrane filter prior to each use. The solution remains stable for one week at 4°C.

1X FACS Lysing Buffer: A 50 ml aliquot of FACS Lysing Buffer was diluted in 450 ml of MilliQ water and stored at room temperature. Lysing buffer was filtered prior to each use using a 0.2 μm membrane filter.

Dilute Phorbol Myristate Acetate (PMA): One millimole of PMA (f.w. 616.8) was dissolved in 2 μl of dimethylsulfoxide. From this stock PMA, a 2 μl aliquot was added to 121 μl of filtered 1X PBS. Next, 10 μl of this solution were diluted 1:100 in the blood sample to be analyzed.

Paraformaldehyde: A solution of paraformaldehyde was prepared by adding 20 mg of paraformaldehyde (f.w. 90.0 in trimer form) to 20 ml of 10X PBS. This mixture was then brought to 200 ml with MilliQ water. The solution was heated to 56°C in a water bath then slowly allowed to cool to room temperature. The mixture was adjusted to a pH of 7.4 using either 0.1 N NaOH or 0.1 N HCl as necessary and stored at 4°C. Paraformaldehyde is stable for two weeks at 4°C.

METHODS

Sample collection and processing: Blood was obtained from healthy volunteers via peripheral venipuncture and collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. Aliquots of this blood were either processed immediately or incubated for varying lengths of time at room temperature or on ice (4°C) prior to processing. Blood incubated on ice underwent all subsequent processing on ice; this included the use of all solutions, incubations, washings, and centrifugations at 4°C. A stimulated control was prepared by adding 10 µl of Dilute PMA to 990 µl of whole blood (100 ng/ml or 0.01% final concentration), and then incubated at 37°C for 15 minutes. Samples containing 100 µl of either unstimulated or stimulated blood were incubated in polycarbonate test tubes containing monoclonal antibodies against either ICAM-1 (20 µg), LFA-1α (20 µg), LFA-1β, (20 µg), Mac-1 (20 µg), L-selectin (20 µg), or VLA-4 (2 µg) for 15 minutes. For ice-incubated samples, the antibodies were added to the test tubes and brought to 4°C prior to adding the blood. PBS/azide (2 ml) was then added to each tube and immediately vortexed for 3 seconds (vortexing was kept brief to minimize mechanical stimulation of the leukocytes) followed by centrifugation at 400 x g for 8 minutes. This wash was performed twice and the supernatants were discarded each time. Next, a 4 µl (1.0 µg) aliquot of FITC-

conjugated goat anti-mouse immunoglobulin was added to each tube. The tubes were immediately vortexed for 3 seconds and allowed to incubate in the dark for 15 minutes. Following this incubation, a 2 ml aliquot of FACS lysing buffer, room temperature or chilled to 4°C as indicated, was added to each tube. The tubes were immediately vortexed for 3 seconds followed by incubation in the dark for 10 minutes. Each tube was then centrifuged at 400 X g for 8 minutes, the supernatants were discarded, and 2 mL of PBS/azide were added to each tube. The samples were washed as previously described. Following the last wash, a 400 µl aliquot of chilled paraformaldehyde was added to each tube and immediately vortexed for 3 seconds. The labeled and fixed samples were stored in the dark at 4°C and analyzed by flow cytometry within 24 hours of processing.

Controls: Immunoglobulins directed against CD45 and CD14 (FITC and PE-labeled, respectively) were utilized to specifically identify monocyte populations. CD45 or leukocyte common antigen is present on all leukocytes, and CD14 is expressed in high density on monocytes (Loken, et al, 1990). Immunoglobulins directed against CD3 (the T cell receptor) and CD19 (a common B cell specific antigen) (likewise FITC and PE-labeled) were used to assess overlapping signals from FITC and PE emissions, a process known as compensation (Bray and Landay, 1989). Murine Gamma 1/Gamma 2a immunoglobulins (FITC and PE labeled,

respectively) served as the negative control. The antibodies against CD45/CD14, murine Gamma 1/Gamma 2a, and CD3/CD19 were standardized by Becton Dickinson for use at 20 μ l per 10^6 cells. FITC conjugated goat anti-mouse immunoglobulin (secondary antibody) was likewise standardized for use at 4 μ l per 10^6 cells.

Data acquisition and analysis: Cell samples were analyzed using a Becton Dickinson FACScan flow cytometer. Prior to sample analysis, autocompensation correction must be performed. Compensation eliminates overlapping photosignals from FITC and PE. Fluorescence compensation is set using a series of FITC-labeled, PE-labeled, and unlabeled glass beads (Cali Brite beads). These are placed in tubes such that one tube contains unlabeled beads and a second tube contains a mix of unlabeled, FITC-labeled, and PE-labeled beads (Bray and Landay, 1989). The unlabeled beads are necessary to determine the amount of autofluorescence (non-specific fluorescence) present, and the labeled beads are used to establish FL1 (FITC) versus FL2 (PE) (this information was provided by Becton Dickinson in the Simultest reference guide). Assessment of compensation correction is also accomplished through the use of the CD3/CD19 gating control where CD3 bears FITC and CD19 bears the PE label.

Once autocompensation has been completed the cell samples may be acquired. Within the flow cytometer the

cells pass single file through a laser beam. Photodetectors sense the light deflected from each cell (scatter). The scatter pattern of the light is then recorded as the degree of forward (low) angle and orthogonal (side) light scatter. Forward scatter is roughly proportional to the size of the cell and orthogonal scatter is equivalent to the granularity of the cell (McCoy, et al., 1990). Utilizing the whole blood lysis procedure described previously, three leukocyte populations can be determined by CD45/14 density and by forward and orthogonal light scatter. Figure 1 demonstrates the typical appearance of each cell population as identified by flow cytometry. Monocytes display a high density of CD14 (R1) and intermediate forward and orthogonal scatter (R5). Lymphocytes present a lower density of CD14 (R2) and minimal forward and orthogonal scatter (R4). Granulocyte populations present with even lower CD14 (R3) and high but heterogeneous forward and orthogonal scatter (R6) (McCoy, et al., 1990). A histogram was created and statistics calculated automatically for each gated population of cells selected for analysis (figure 2). On each histogram, the peak-channel fluorescence represents the maximum level of fluorescence obtained per 1000 cells analyzed.

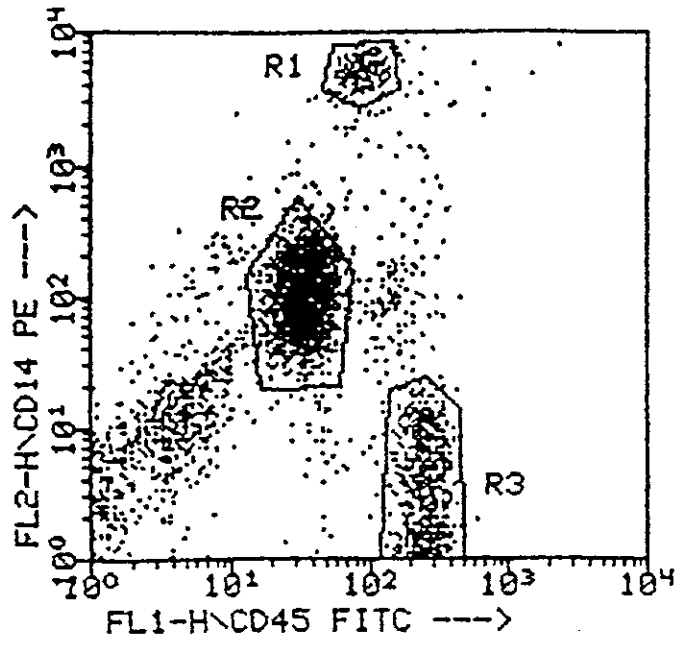
Statistics: Statistical analysis was applied to data presented in figure 10 using the paired students t-test. Calculations were performed on SigmaStat software for

Windows. Histograms were generated using SigmaPlot for
Windows.

Figure 1: Identification of Leukocyte Populations by
CD45/14 Expression and Light Scattering
Characteristics

Figure 1 demonstrates the typical appearance of leukocyte populations as identified by flow cytometry. Monocytes display a high density of CD14 (R1) and intermediate forward and orthogonal scatter (R5). Lymphocytes present a lower density of CD14 (R2) and minimal forward and orthogonal scatter (R4). Granulocyte populations present with even lower CD14 (R3) and high but heterogeneous forward and orthogonal scatter (R6). Flow cytometric analysis was performed on a Becton Dickinson FACScan Flow Cytometer. Cell populations were identified based on CD45/14 expression and light scattering properties. Gating of these populations was done manually.

/16/BJ0001



/16/BJ0001

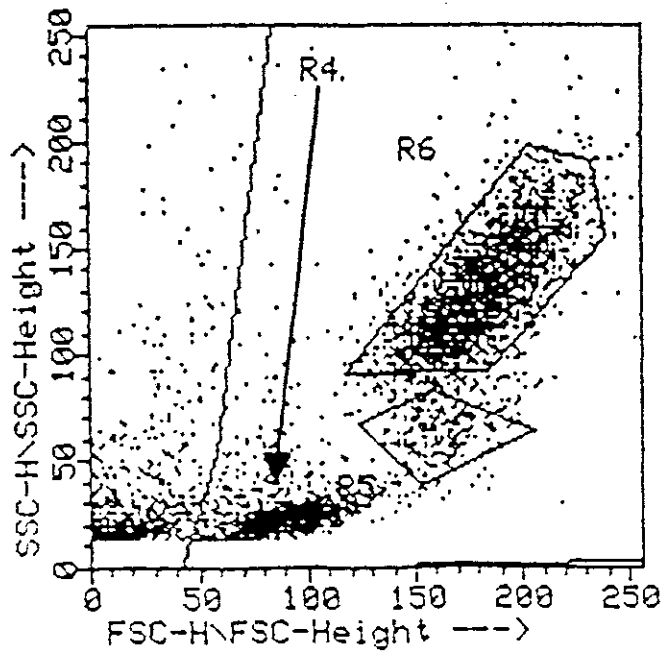
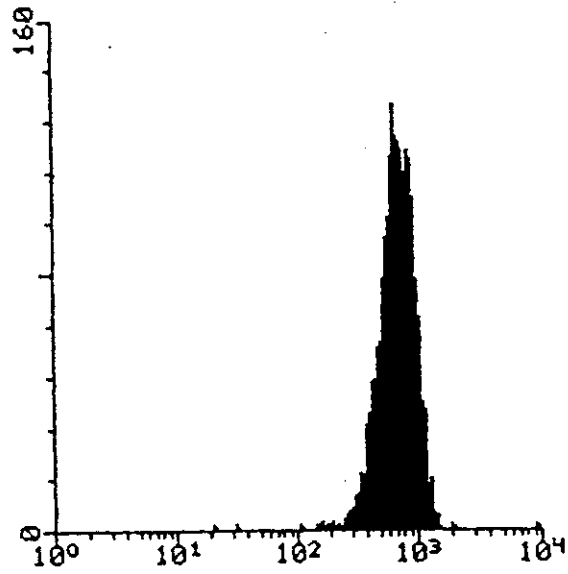


Figure 2: Histogram Analysis of Immunofluorescence from
Gated PMNs

A histogram was created and statistics calculated automatically for each gated population of cells selected for analysis. On each histogram, the peak-channel fluorescence (PkCh1) represents the maximum level of fluorescence obtained per 1000 cells analyzed by flow cytometry.

/16/BJ0023\FL1-H\CD3 FITC



/16/BJ0023\FL1-H\CD3 FITC

--- Arithmetic Histogram Statistics for /16/BJ0023 ---

Selected Preferences: Arithmetic/Linear

Parameter FL1-H CD3 FITC Gate G6= R6

M	Left,Right	Events	%	Peak	PkCh1	Mean	Median	SD	CV %	
0	1.00,	9646	2682	100.00	134	649.38	721.37	697.83	268.50	37.22

RESULTS

The purpose of these experiments was to test for the occurrence of specific trends in cellular adhesion molecule (CAM) expression as influenced by various collection methods. The first series of experiments used specific monoclonal antibodies (described in Methods) and flow cytometry to evaluate patterns of expression of five different cell adhesion molecules: ICAM-1 (CD54), LFA-1 α (CD11a), LFA-1 β (CD18), MAC-1 (CD11b), and L-selectin. Very late antigen-4 (VLA-4) is not expressed on neutrophils, but is present on mononuclear cells (Schleimer, *et al.*, 1992; Thornhill, *et al.*, 1990); therefore, anti-VLA-4 monoclonal antibodies were used to assess the presence of mononuclear cell contamination of neutrophil isolates. Each trial plotted represents the maximum level of fluorescence (equivalent to CAM expression) obtained per 1000 cells analyzed by flow cytometer. Figure 3 illustrates the findings of a single trial involving a healthy volunteer. A blood sample was drawn into Vacutainer tubes containing EDTA and 100 μ l aliquots were taken for separate analysis.

Figure 3: CAM Expression on Unstimulated and Stimulated PMNs

Peripheral blood was drawn via venipuncture from a healthy volunteer into Vacutainer tubes containing EDTA. The anticoagulated blood was separated into two fractions, an unstimulated fraction and a stimulated fraction. Stimulation of the cells was obtained by incubation with 10 μ l of PMA solution (described in Methods) at 37°C for 15 minutes. Aliquots of 100 μ l of whole blood, either stimulated or unstimulated, were either labeled immediately or after 2 hours of incubation at room temperature. Cells were labeled by incubation with monoclonal antibodies specific for a selected cell adhesion molecule as indicated on the abscissa of the graph. Each preparation was then subjected to a second incubation with FITC-conjugated goat anti-mouse IgG. After incubation, red cells were lysed by the method described (Methods), and fluorescence of the remaining leukocytes was measured using a Becton Dickinson FACScan flow cytometer. Each bar plotted represents the level of CAM expression on PMNs as peak-fluorescent intensity in arbitrary units. These results were obtained from a single trial with blood from one donor. Statistical analysis was not performed due to small sample size.

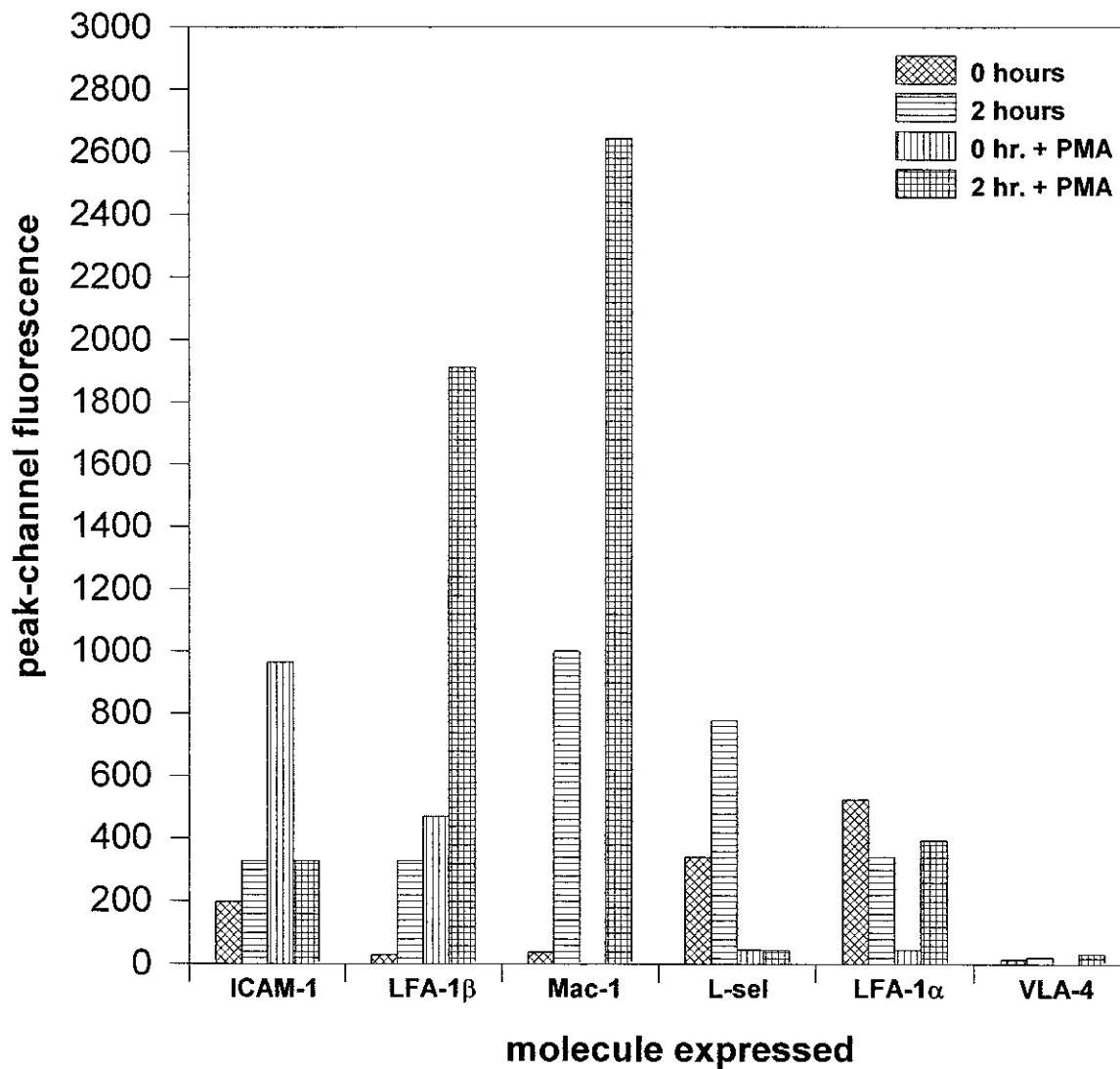
Cross-hatched fills = cells analyzed at 0 hours

Horizontal fills = cells analyzed at 2 hours at room
temperature

Vertical fills = PMA stimulated cells analyzed
Immediately (0 hours)

Checkered fills = PMA stimulated cells analyzed after
2 hours at room temperature

Figure 3: CAM Expression on Unstimulated and Stimulated PMNs



One aliquot was processed immediately, time = 0 hours, and is represented by the bars with cross-hatched fills in the figure. A second aliquot was allowed to sit at room temperature for 2 hours prior to processing and is represented by the bars with horizontal fills in the figure. A third aliquot was stimulated with dilute phorbol myristate acetate (PMA) for 15 minutes at 37°C and processed immediately, time = 0 hours, and is represented by the bars containing vertical fills in the figure. The 0 hour PMA stimulated samples for both MAC-1 and VLA-4 were lost during processing, thus, no data appears for these points on the graph. The fourth aliquot was stimulated with PMA then allowed to sit at room temperature for 2 hours prior to processing; the bars containing checkered fills represent it.

The first molecule studied, ICAM-1, shows an increased expression between the 0 hour and 2 hour unstimulated trials of about 1.5 fold (fluorescence = 198 at 0 hours and 328 at 2 hours); however, the 0 hour PMA stimulated trial shows an increase in expression three times greater than the unstimulated levels (fluorescence = 328 at 2 hours and 965 at 0 hours + PMA). The 2 hour PMA stimulated trial shows results identical to those of the 2 hour unstimulated trial which may be indicative of either a time dependant or

transient mechanism of ICAM-1 up regulation, or may simply reflect experimental error. The next molecule, LFA-1 β , represents the CD18 component of LFA-1, and is common to all integrin molecules (LFA-1, Mac-1, and p150, 95). It showed a marked increase in expression between the 0 hour trial with a fluorescence of 29 and the 2 hour trial with a fluorescence of 328. PMA stimulation at the 0 hour time point showed only minimal increase in expression with a peak fluorescence of 470 whereas the 2 hour PMA stimulated sample reached a peak fluorescence of 1911. Mac-1 expression showed a pattern similar to that of LFA-1 β as would be expected since the LFA-1 β antibody binds CD18, the beta chain of both of these integrin molecules. The sample which was allowed to sit at room temperature for 2 hours (fluorescence = 1000) shows a dramatic increase over the 0 hour trial (fluorescence = 38), and the 2 hour PMA stimulated sample (fluorescence = 2642) shows a 2.6 fold increase over it. With L-selectin, the most obvious change is the shedding of this molecule following PMA stimulation. An unexpected doubling in peak fluorescence was noted between the 0 hour (fluorescence = 340) and 2 hour (fluorescence = 777) unstimulated trials. Analysis of the CD11a (alpha) subunit of LFA-1 revealed a marked decrease

in expression with PMA stimulation at 0 hours (fluorescence = 44) as compared to the 0 hour unstimulated sample (fluorescence = 523). A small decrease in expression, about 1.5 fold, was noted between the 0 hour unstimulated and 2 hour unstimulated (fluorescence = 340) trials. When comparing the 2 hour PMA stimulated (fluorescence = 392) and the 0 hour stimulated trials, an increased expression is observed possibly suggesting a time dependency. Lastly, VLA-4 levels are shown to be low indicating relatively little contamination of the sample with mononuclear cells.

A second experiment, similar to the first, was conducted to investigate the effects of incubating blood on ice prior to processing. Figure 4 shows the results of a single trial involving blood collected from a healthy volunteer. A peripherally drawn blood sample was collected as previously described and 100 μ l aliquots were analyzed at 0 hours (horizontal fills), after sitting for two hours at room temperature (vertical fills), and after two hours on ice (checkered fills). Additionally, PMA stimulated samples were analyzed at 0 hours (empty bars) and after an incubation period of 2 hours on ice (diagonal fills). Differing slightly from the data presented in figure 1, here ICAM-1 shows little change between the 0 hour (fluorescence = 294) and the 2 hour (fluorescence = 269)

Figure 4: CAM expression on Unstimulated and Stimulated PMNs

Blood was collected from a healthy volunteer into Vacutainer tubes containing EDTA. The anticoagulated blood was separated into two fractions, unstimulated and stimulated (prepared as previously described). Aliquots of 100 μ l were taken from the unstimulated fraction were either labeled immediately, after 2 hours of incubation at room temperature, or after 2 hours on ice. Samples from the stimulated fraction were either labeled immediately or after 2 hours on ice. Labeling and cytometric analysis were performed as described in figure 3, and the results were displayed as follows:

Horizontal fills = cells analyzed at 0 hours

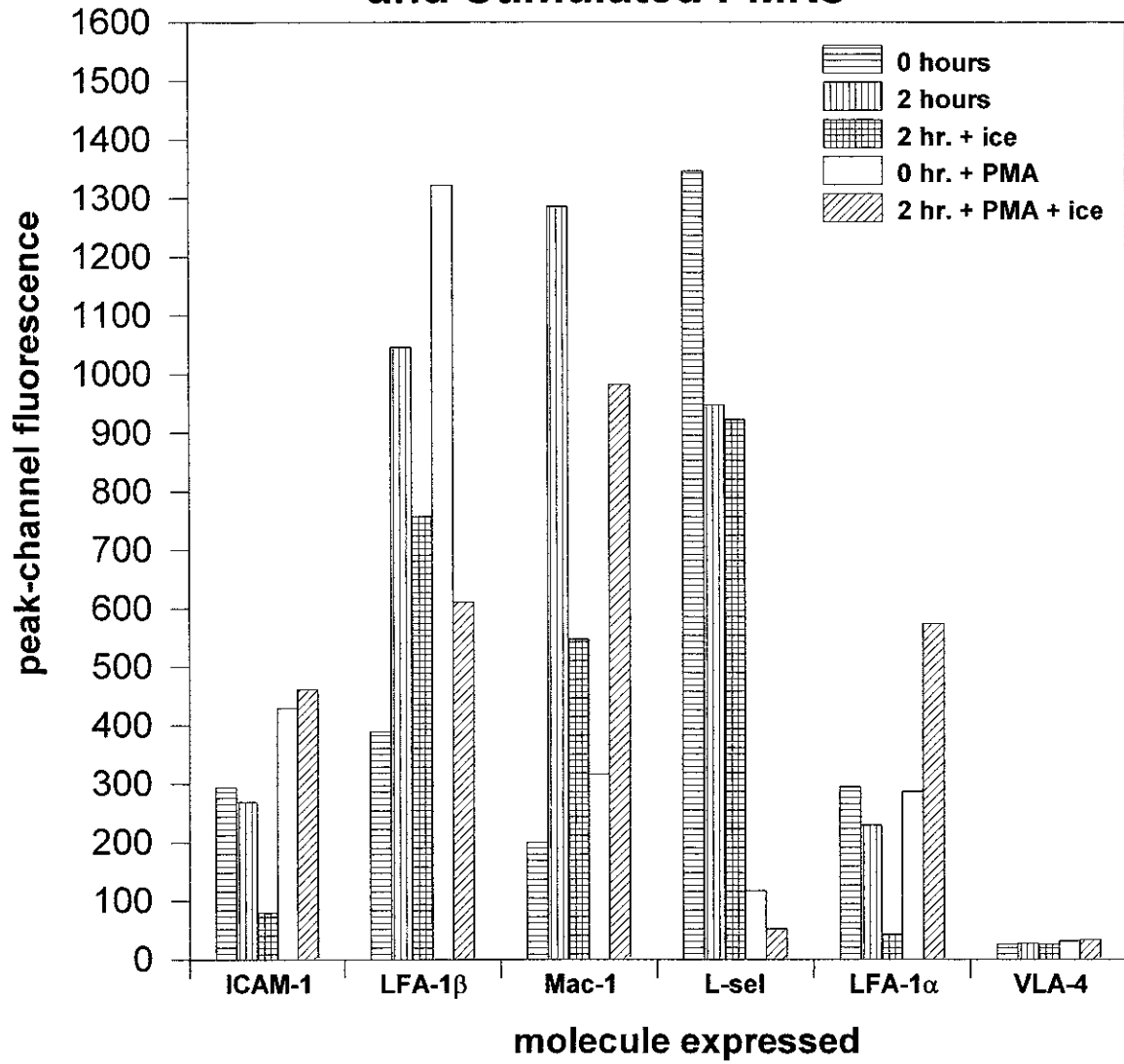
Vertical fills = cells analyzed at 2 hours at room temperature

Checkered fills = cells analyzed after 2 hours on ice

Empty Bars = PMA stimulated cells analyzed at 0 hours

Horizontal Fills = PMA stimulated cells analyzed after 2 hours on ice

Figure 4: CAM Expression on Unstimulated and Stimulated PMNs



room temperature trials. Placing the sample on ice resulted in a 3.5 fold decrease in fluorescence to a value of 80. PMA stimulation resulted in a 1.5 fold increase in fluorescence (fluorescence = 429) over the unstimulated samples. Placing the PMA stimulated sample on ice for two hours had little effect (fluorescence = 461). LFA-1 β demonstrated an increase in peak fluorescence among the 0 hour and 2 hour unstimulated trials from 389 to 1046, respectively. Placing the sample on ice appears to have dampened this rise with peak fluorescence reaching only 757. PMA stimulation of the sample induced a peak in fluorescence to 1322. Placing the stimulated sample on ice again resulted in an attenuation of expression with fluorescence reaching only 610, a result comparable to the unstimulated sample on ice. Mac-1 expression increased nearly 6.5 times in samples remaining at room temperature for 2 hours as compared to those processed immediately (fluorescence = 1286 and 200 respectively). Placing the sample on ice attenuated this rise with fluorescence reaching only 547. PMA stimulation induced an increase in Mac-1 expression (fluorescence = 316) although to a lesser degree than was seen at two hours unstimulated. The PMA sample placed on ice for two hours showed a more dramatic increase in expression (fluorescence = 982), but not to the

extent of the unstimulated 2 hour sample. L-selectin levels were diminished over time and to a far greater extent with PMA stimulation. Peak fluorescence decreased between the 0 hour (fluorescence = 1346) and 2 hour (fluorescence = 947) trials by a factor of 1.4. Placing the sample on ice had little effect (fluorescence = 922). PMA stimulation induced an 11.5 fold decrease in fluorescence to 117. Placing the PMA stimulated aliquot on ice for 2 hours had no effect on the loss of L-selectin (fluorescence = 55). LFA-1 α showed a slight decrease from a fluorescence of 294 at 0 hour to a fluorescence of 229 at 2 hours. Incubation on ice over two hours resulted in a more pronounced drop in fluorescence to 43, nearly a 7-fold drop. PMA stimulation of the sample had little effect on expression (fluorescence = 286) and placing stimulated samples on ice resulted in an increase of expression (fluorescence = 573). VLA-4 levels remained low indicating little contamination of the samples with mononuclear cells.

Figure 5 displays the mean results of a duplicate trial performed on a healthy volunteer. Blood was collected and aliquoted as described previously. Samples were processed at 0 hours (checkered fills), 2 hours at room temperature (horizontal fills), and at 2 hours on ice (vertical fills). ICAM-1 demonstrates a fall in peak

Figure 5: CAM Expression on Unstimulated PMNs

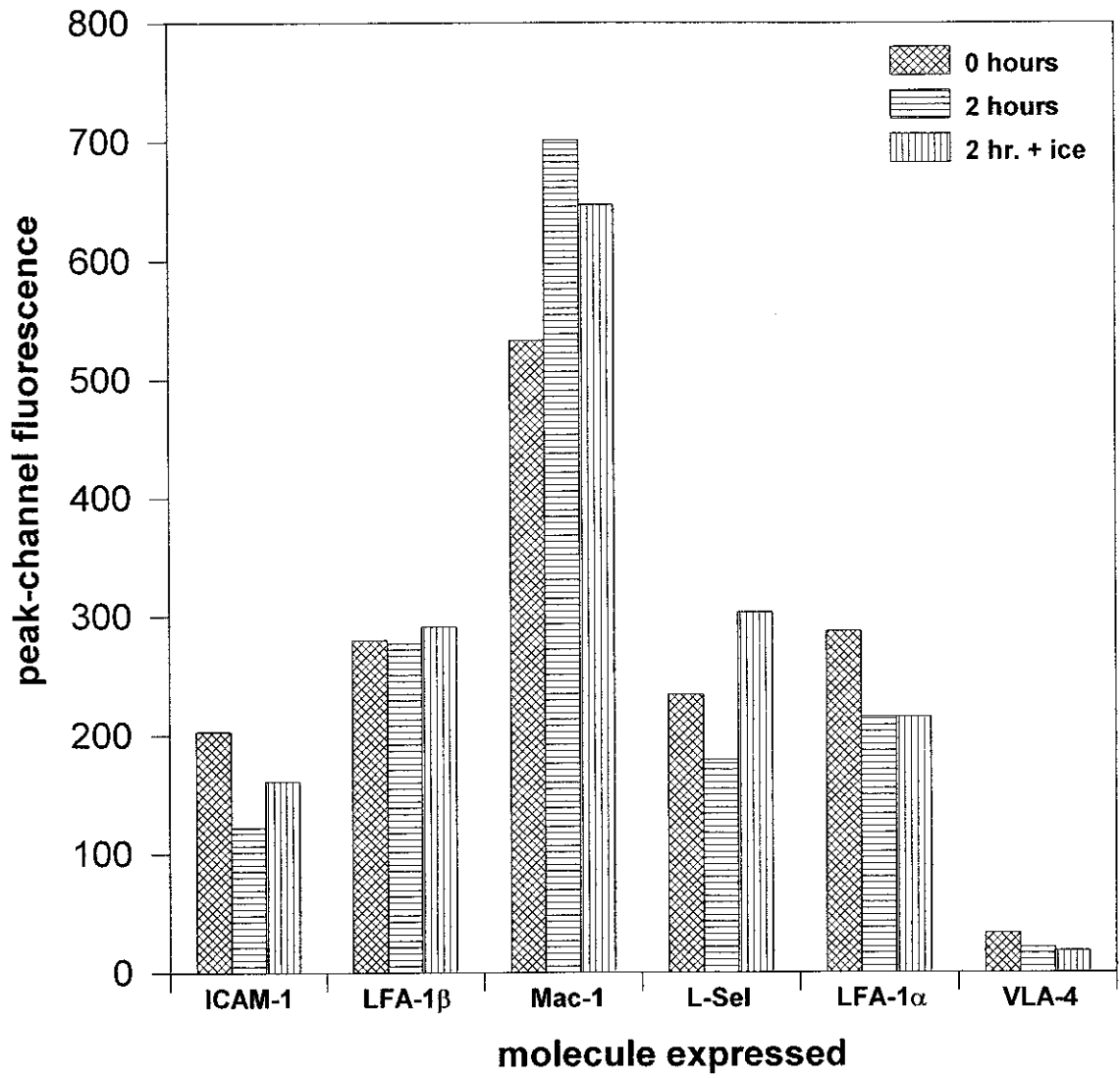
Blood was collected from healthy volunteers and processed as previously described (figure 3). In this experiment, there were no PMA stimulated trials. Three samples were analyzed for each CAM listed at 0 hours, after 2 hours of incubation at room temperature, and after 2 hours of incubation on ice. These results were obtained from single trials performed on blood from two donors. Statistical analysis was not performed. The results were presented as follows:

Cross-hatched fills = cells analyzed at 0 hours

Horizontal fills = cells analyzed after 2 hours at
room temperature

Vertical fills = cells analyzed after 2 hours on ice

Figure 5: CAM Expression on Unstimulated PMNs



fluorescence between 0 and 2 hours (fluorescence = 203 and 111, respectively) with minimal change at 2 hours on ice (fluorescence = 161). Overall, changes in ICAM-1 in this trial were minimal, consistent with the previous experiments. LFA-1 β showed no change over time (fluorescence = 280 at 0 hours, 278 at 2 hours, and 292 at 2 hours on ice). MAC-1 demonstrated a moderate elevation in fluorescence between 0 and 2 hours (fluorescence = 534 and 702, respectively). Incubation for 2 hours on ice yielded results similar to 2 hours at room temperature (fluorescence = 647). With L-selectin, peak fluorescence fell from 234 at 0 hours to 179 at 2 hours. Maintaining the sample on ice produced results similar to the 0 hour aliquot (fluorescence = 303). LFA-1a showed a moderate drop over 2 hours with and without ice incubation (fluorescence = 287 at 0 hours and 215 at both 2 hours and 2 hours on ice). VLA-4 levels remained low indicating little mononuclear cell contamination.

Figure 6 represents the averaged results of the previous three experiments for all time points excluding the PMA stimulated trials. Samples analyzed at 0 hours are represented by checkered fills, those analyzed at 2 hours by horizontal fills, and those at 2 hours on ice by vertical fills. Both ICAM-1 and L-selectin revealed

Figure 6: CAM Expression on Unstimulated PMNs

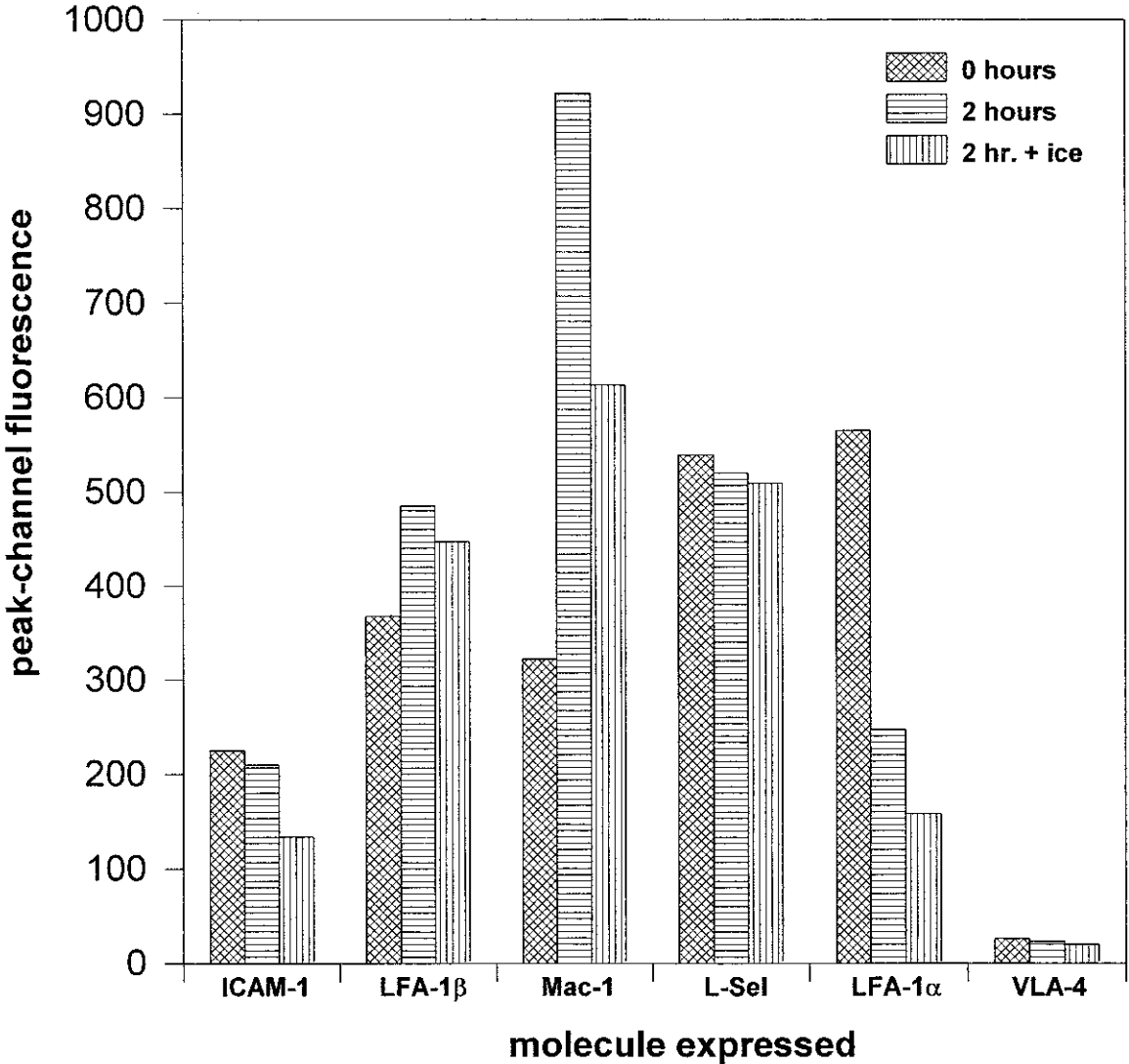
Figure 6 represents the averaged results of the previous three experiments for all time points excluding the PMA stimulated trials. Statistical analysis was not performed. The results were presented as follows:

Cross-hatched fills = cells analyzed at 0 hours

Horizontal fills = cells analyzed after 2 hours at
room temperature

Vertical fills = cells analyzed after 2 hours on ice

Figure 6: CAM expression on Unstimulated PMNs



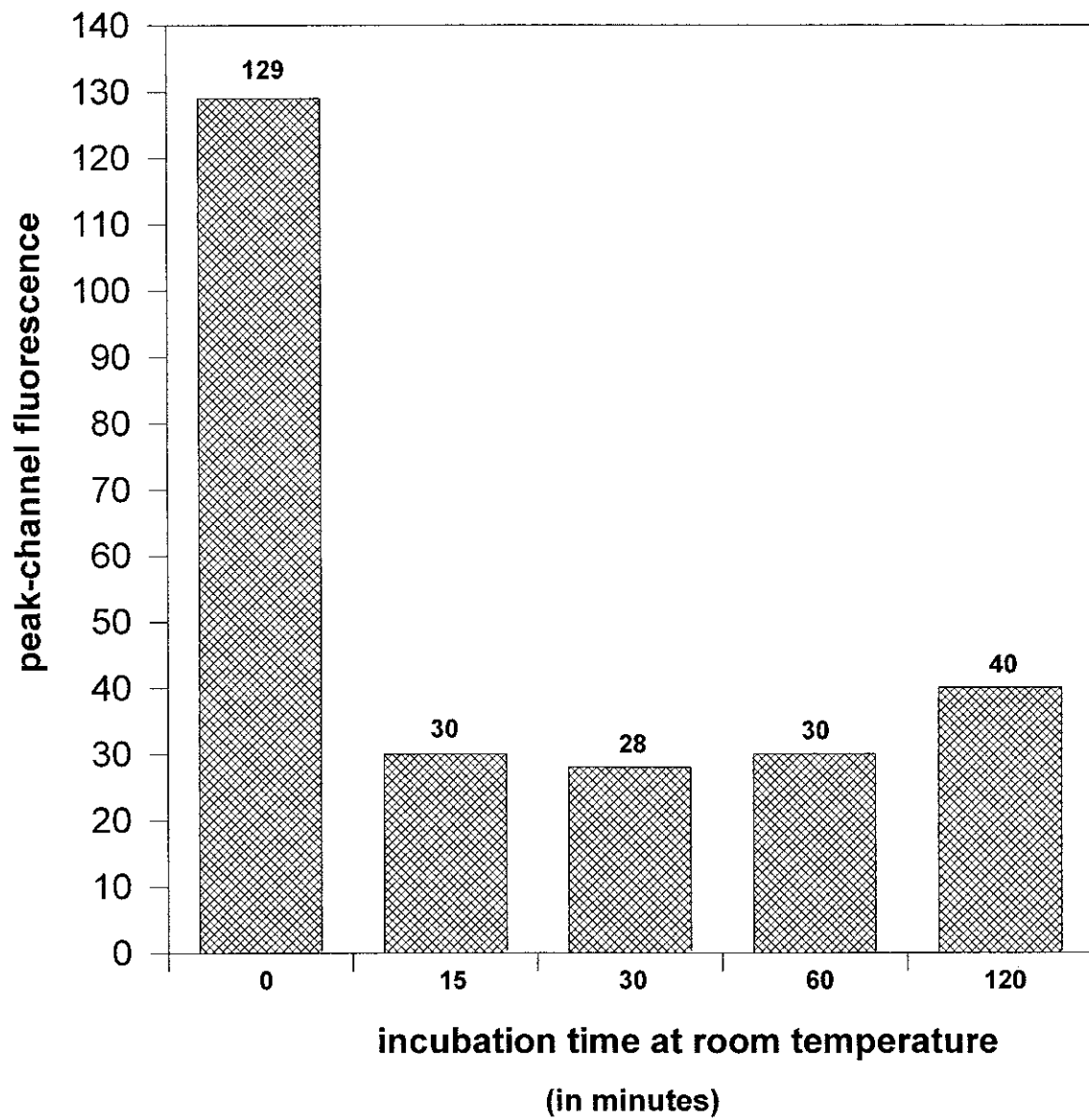
essentially no change over time irregardless of incubation on ice. LFA-1 β and Mac-1 again move in the same direction, as expected, with expression of both increasing over two hours. Mac-1 shows the most dramatic change with a nearly three fold increase in fluorescence between 0 and 2 hours (fluorescence = 326 and 922 respectively). Incubation on ice had relatively little effect on LFA-1 β , but did seem to attenuate the rise in expression of Mac-1 by about one third (fluorescence = 613). LFA-1 α showed a 50% decrease in expression between 0 and 2 hours, and a greater than 70% fall at two hours on ice (fluorescence = 565, 264, and 158, respectively). Finally, VLA-4 levels have remained consistently low.

The next series of experiments was designed to more closely examine short term fluctuations in L-selectin expression over time. A single healthy volunteer was selected for each trial, blood was collected as previously described, and cells were labeled for L-selectin using specific monoclonal antibodies. For the first experiment in this series 100 μ l aliquots of labeled blood were processed at 0 minutes, 15 minutes, 30 minutes, 60 minutes, and 120 minutes. The results of this study were presented in figure 7. At 0 minutes a peak fluorescence of 129 was

Figure 7: L-selectin Expression on Unstimulated PMNs

Blood was collected and processed as previously described; however, in this experiment, the cells were only labeled with monoclonal antibodies specific for L-selectin. Individual samples were then incubated at room temperature for 0, 5, 10, 15, and 30 minutes prior to being placed on ice. Two hours after collection, the samples were removed from ice and all were processed simultaneously. A histogram was generated presenting results obtained from a duplicate trial with blood from one donor. Peak-channel fluorescence for each sample analyzed was indicated over the corresponding bar on the graph. Statistical analysis was not performed.

Figure 7: L-selectin Expression on Unstimulated PMNs



obtained. This level dropped sharply at 15 minutes to 30 and remained low through 120 minutes. Peak levels of fluorescence were indicated above each bar on the graph.

Figure 8 presents the results of duplicate trials from a single healthy volunteer analyzed at 0, 5, 10, 15, and 30 minutes at room temperature. Differing from the previous experiment, L-selectin remained relatively stable over the first 15 minutes with the first suggestion of shedding becoming evident at 30 minutes. A stimulated control was included for comparison. Peak levels of fluorescence were indicated on the graph above each bar.

The last experiment performed proceeded as described previously. These data are presented in figure 9. Results were found to be similar to the previous experiment in that L-selectin expression fluctuated only slightly over 30 minutes. Again, the stimulated control was included for comparison. Peak levels of fluorescence were noted above each bar.

Figure 10 shows an average of the results obtained in the previous two experiments. A paired student's *t*-test was performed on these data and the change in fluorescence over 30 minutes was not found to be significant (mean $p > 0.05$). The change that occurred with PMA stimulation was found to be significant (mean $p = 0.0001$).

Figure 8: L-selectin Expression on Unstimulated PMNs vs.
Stimulated Control

Blood was collected and processed as in figure 7. A histogram was generated presenting results obtained from a duplicate trial with blood from one donor. Peak-channel fluorescence for each sample analyzed is indicated over the corresponding bar on the graph. Statistical analysis was not performed.

Figure 8: L-selectin Expression on Unstimulated PMNs vs. Stimulated Control

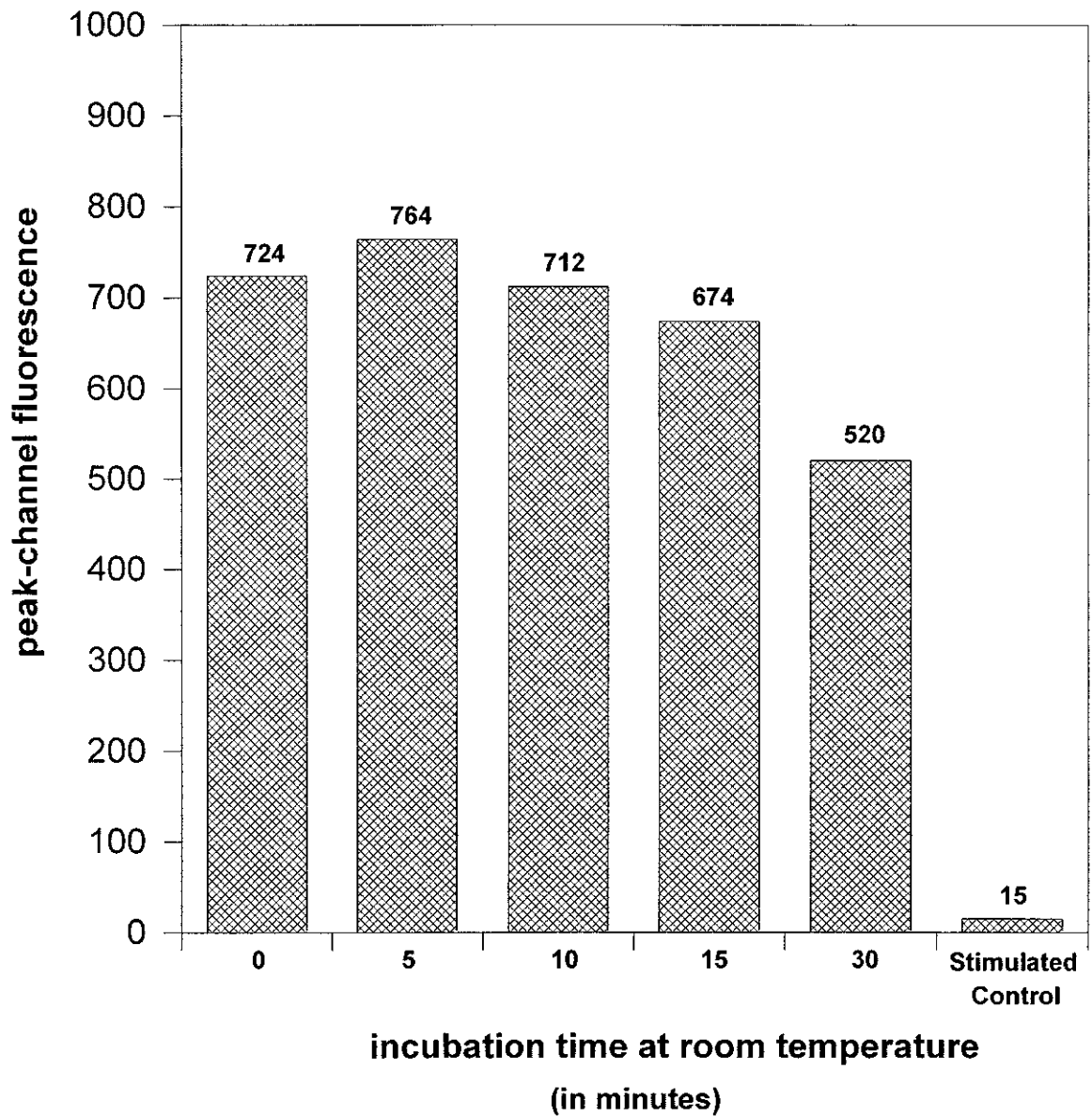


Figure 9: L-selectin Expression on Unstimulated PMNs vs.
Stimulated Controls

Blood was collected and processed as in figure 7. A histogram was generated presenting results obtained from a duplicate trial with blood from one donor. Peak-channel fluorescence for each sample analyzed is indicated over the corresponding bar on the graph. Statistical analysis was not performed.

Figure 9: L-selectin Expression on Unstimulated PMNs vs. Stimulated Control

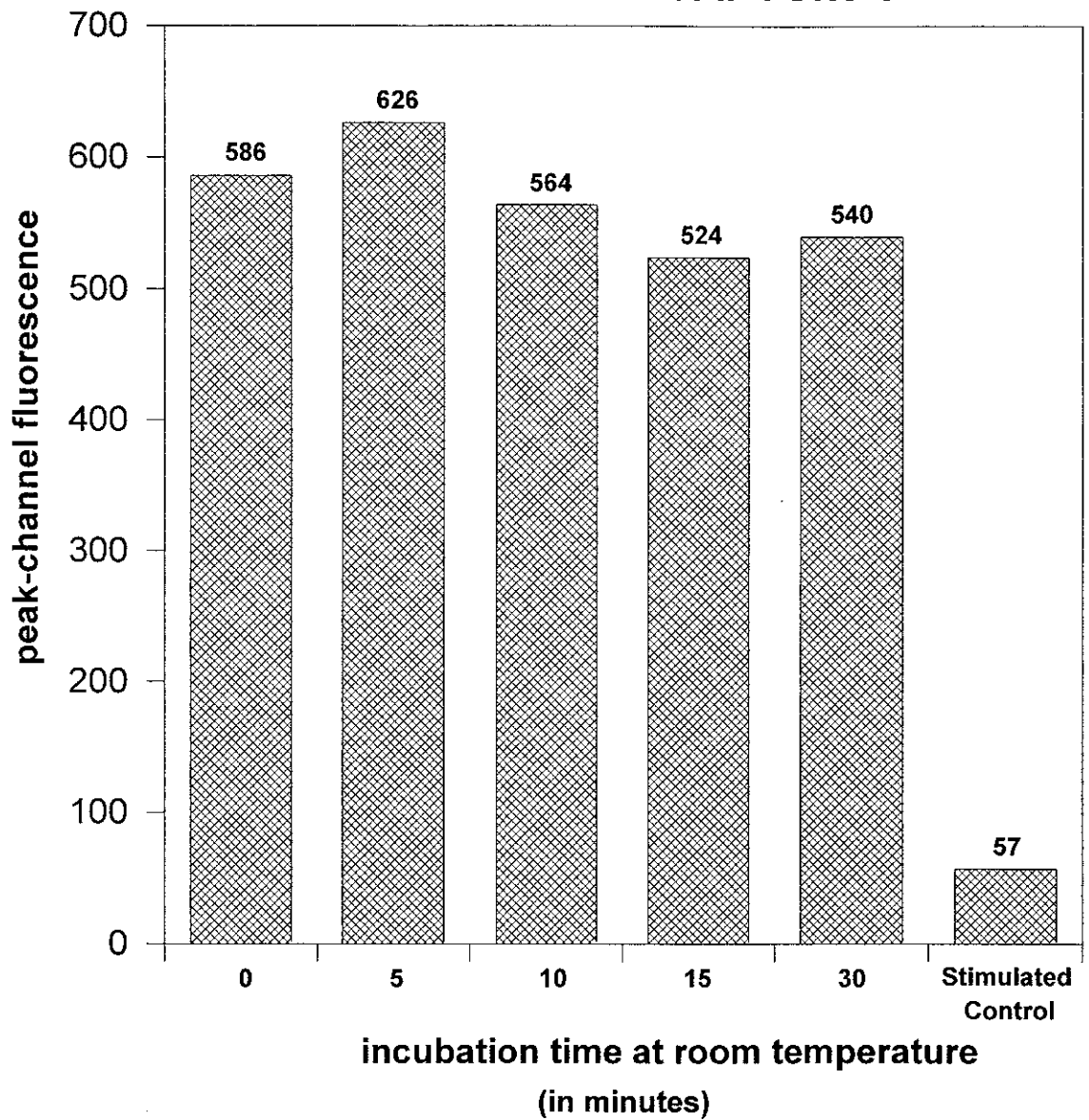
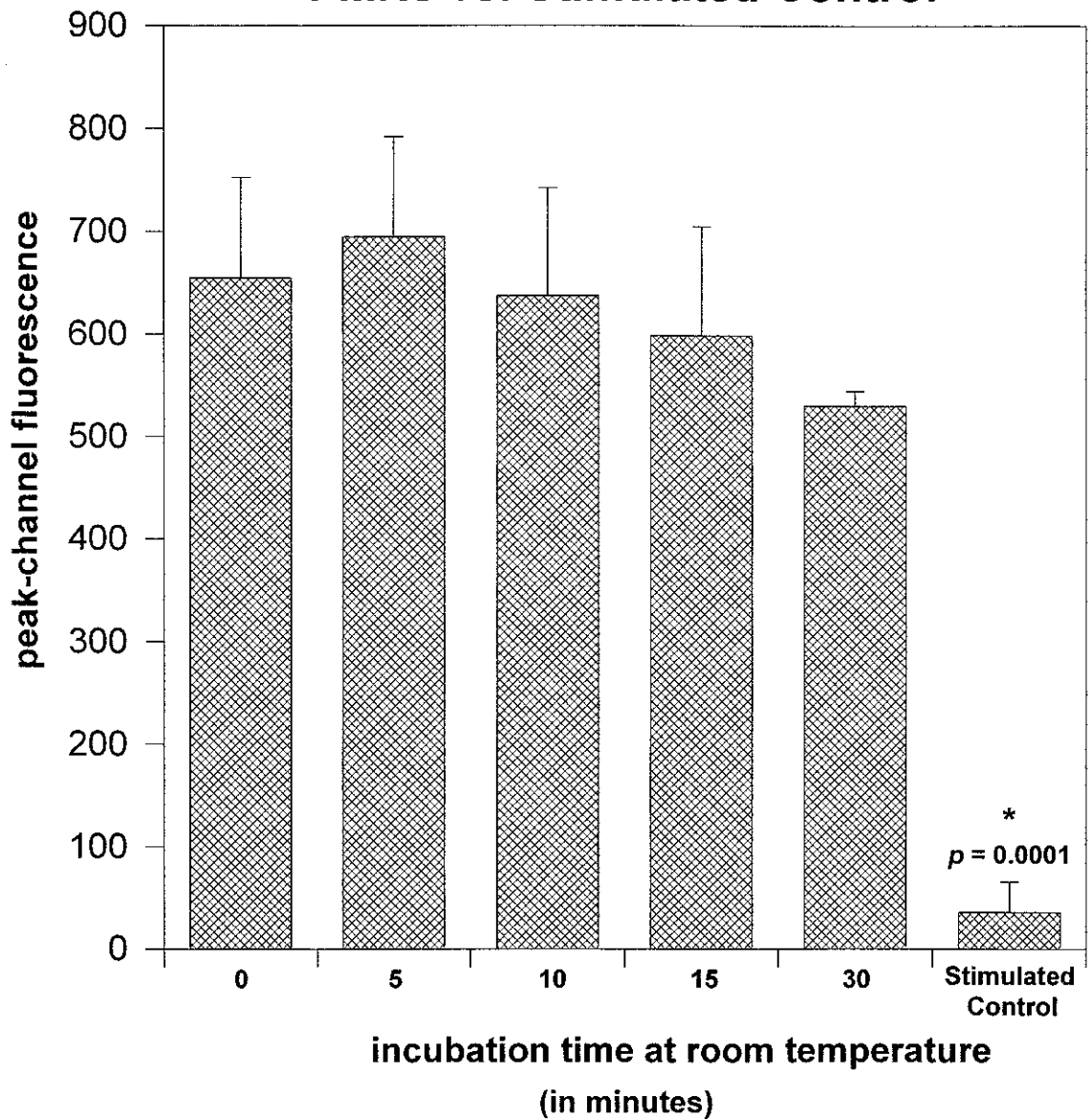


Figure 10: L-selectin Expression on Unstimulated PMNs
vs. Stimulated Control

This figure represents the averages of the results obtained in experiments 8 and 9. A paired student's t-test was performed on these data and the change in fluorescence over 30 minutes was not found to be significant (mean $p > 0.05$). The change that occurred with PMA stimulation was found to be significant (mean $p = 0.0001$). Peak-channel fluorescence for each sample analyzed is indicated over the corresponding bar on the graph.

Figure 10: L-selectin Expression on Unstimulated PMNs vs. Stimulated Control



DISCUSSION

Cell adhesion molecules play crucial roles in many disease entities including rheumatoid arthritis, graft rejection, reperfusion injury, and adult respiratory distress syndrome. Understanding their function and regulation has become a leading area of interest in biomedical science; yet this field is still in its infancy. The development of accurate methods of collection and preparation of leukocytes is crucial to furthering our understanding of these molecules.

The first step in the analysis of cell adhesion molecules is the collection of whole blood from a test subject. The choice of anticoagulant used to prevent clotting of the sample is critical. Most of the authors cited used either EDTA (Hed, *et al.*, 1988; Macey, *et al.*, 1992; Heikko, *et al.*, 1993; Lundahl, *et al.*, 1995) or sodium citrate (Youssef, *et al.*, 1995) anticoagulants. However, at least one article demonstrated a significant upregulation of Mac-1 on monocytes collected in heparin as compared to EDTA regardless of leukocyte isolation technique (red cell lysis of whole blood sample vs. density

gradient centrifugation on Ficoll-Paque) or temperature (4°C vs. room temperature defined as 20°C for 15 minutes) (Lundahl, et al., 1995). Prior studies performed in our lab (unpublished) looked at blood collected in four different anticoagulants, EDTA, sodium citrate, potassium oxalate, and heparin. Neutrophils were isolated and labeled as described in Methods at 0 hours room temperature, after 2 hours at room temperature, and after 2 hours on ice. It was observed that neutrophils from EDTA, sodium citrate, and potassium oxalate blood showed similar, expected results (i.e., basal level of expression at 0 hours, increased at 2 hours, near basal levels on ice). However, cells from heparinized blood consistently revealed elevated levels of expression on ice as compared to 0 or 2 hour room temperature trials. A generalized upregulation of Mac-1 on cells from heparinized versus EDTA blood for each incubation was not noted as previously described by Lundahl, et al. (1995). A typical plot of the data from one of these early studies is shown in figure 11. Based on these observations and literature support, all blood was collected in EDTA. It was recommended, however, that blood samples anticoagulated with EDTA should be processed within twenty-four hours of collection. If not, granulocytes, when examined by flow cytometry, may show changes in

Figure 11: Effect of Anticoagulants on Mac-1 Expression

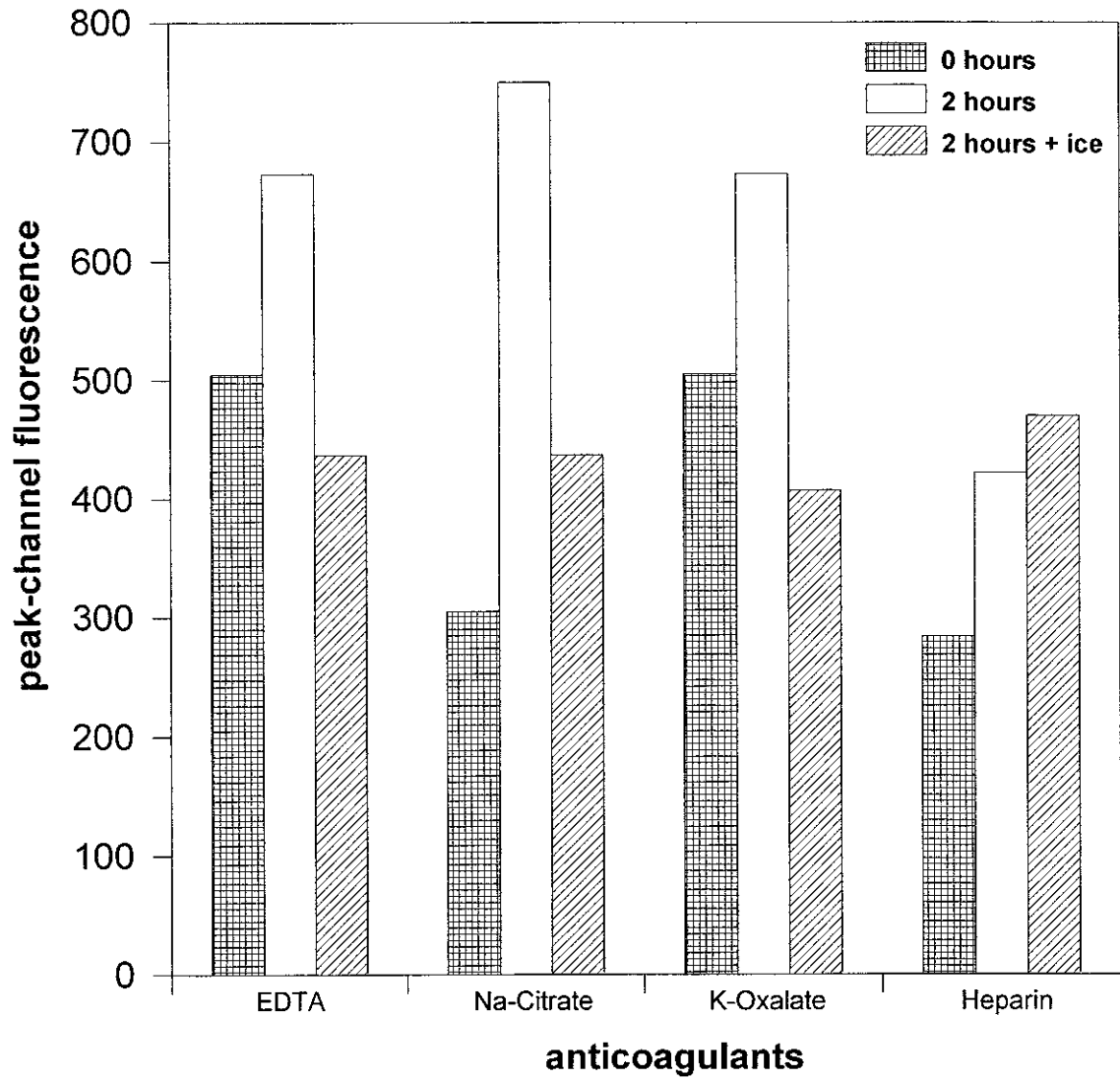
Data presented in this figure represents typical findings of previous studies performed to investigate the effects that different anticoagulants might have on Mac-1 expression. Blood was collected from a healthy volunteer into Vacutainer tubes containing EDTA, sodium citrate, potassium oxalate, or heparin. One sample from each tube was processed immediately (0 hours). Additional samples were incubated for either 2 hours at room temperature or two hours on ice. The blood was processed and labeled for Mac-1 as previously described.

Checkered Fills = samples processed immediately following collection

Empty Bars = samples processed after 2 hours at room temperature

Horizontal Fills = samples processed after 2 hours on ice

Figure 11: Effect of Anticoagulants on Mac-1 Expression on PMNs



orthogonal light scattering possibly leading to inaccurate representation of neutrophil populations (McCoy, *et al.*, 1990).

The next consideration was to select the best method for processing the cells. Two very commonly utilized cell separation procedures were density gradient centrifugation and dextran sedimentation. However, preparation by either of these methods was well documented to modulate significant upregulation of Mac-1 and other CAMs on neutrophil and monocyte populations (Heikki, *et al.*, 1993; Macey, *et al.*, 1992). Another manner of isolating leukocytes described by Hed, *et al.* (1988) lysed erythrocytes in an ammonium chloride solution without prior separation. For this preparation the authors collected whole blood in EDTA and hemolyzed 100 μ l aliquots by incubation in 0.15 M NH_4Cl at 4°C for 5 minutes. The leukocyte preparations were then washed with phosphate buffered saline (PBS) supplemented with 0.15 M EDTA (binds ambient calcium) and 0.002% sodium azide, NaN_3 , (interrupts electron transport), thereby arresting calcium and energy dependent cellular processes such as the mobilization of Mac-1 stores to the cell surface. The leukocyte preparations were then labeled for immunoassay with appropriate primary and secondary monoclonal antibodies by

30-minute incubations with each at 4°C. The utility of sodium azide in arresting energy dependant cellular processes is described in early literature on leukocyte preservation by Pinthanond and Petchlai (1975). These investigators applied NaH₃ in concentrations as low as 0.001% to cells isolated from whole blood by dextran sedimentation and red cell lysis with NH₄Cl, and fixed with 2.5% glutaraldehyde. This technique proved sufficient to maintain highly stable leukocyte preparations at both 4°C and room temperature for up to 3 months. We adopted a protocol similar to Hed, *et al.* for our studies; however, instead of NH₄Cl, we selected FACS lysing buffer (supplied by Becton Dickinson and recommended for use with Becton Dickinson FACScan flow cytometers), a detergent based lysing solution, to perform erythrocyte lysis on whole blood. In addition, Hed, *et al* performed red cell lysis prior to labeling leukocyte CAMs. We, however, labeled the leukocytes in whole blood prior to lysing the red cells, similar to the method described by Pinthanond and Petchlai. This did not appear to have a significant effect on the results of our study, as findings were consistent with those of Hed, *et al.* as will be discussed later. Other technical differences involved the formulation of the PBS/azide solution. The solution used in our laboratory

was not supplemented with EDTA, and sodium azide was used at a final concentration of 0.01% versus 0.001%. In subsequent studies by Youssef, *et al.* (1995), isolated leukocytes using either FACS lysing buffer in a protocol similar to ours, or by methylcellulose sedimentation followed by density gradient centrifugation and hypotonic lysis of red cells. In their report, 100 μ l aliquots of whole blood collected in sodium citrate were incubated with primary antibody for 30 minutes at 4°C, washed with PBS containing 0.01% sodium azide, then incubated for 30 minutes with secondary antibody at 4°C. Erythrocytes were lysed with a 1/10 dilution of FACS lysing solution and the isolated leukocytes were stored in Becton Dickinson FACS fixative buffer (PBS with 2% D-glucosan and 2.6% formaldehyde, this is not the same fixative used in our studies) at 0 to 4°C until analyzed. The other population of cells isolated by sedimentation, density gradient centrifugation, and red cell lysis were not labeled for flow cytometric analysis until after isolation. Their findings showed that erythrocyte sedimentation followed by density gradient centrifugation and hypotonic lysis resulted in significant upregulation of Mac-1 and shedding of L-selectin, especially when performed at 37°C. Leukocytes isolated using FACS lysing buffer showed no

significant change in these molecules unless blood initially stored on ice was subjected to rewarming prior to preparation. This phenomenon was not assessed in our studies. The authors did note that a small residual of unlysed red cells remained when lysis was performed with cold FACS lysing buffer. A minor inconvenience encountered in our lab as well that did not seem to impair analysis in either study.

Should CAM analysis prove clinically useful, it would not be likely that a patient's blood would be processed immediately following collection. It was therefore necessary to determine what effect allowing cells to stand at room temperature or on ice prior to processing would have on CAM expression. These results were compared to PMA stimulated samples in order to determine whether changes, if any, in CAM expression resembled changes seen with stimulated samples. The success of PMA as an activator of protein kinase C, which mediates leukocyte activation via phosphorylation of calcium regulatory proteins, was described in early literature by Kishimoto, *et al.* (1989). In these studies, neutrophils from murine bone marrow were analyzed following incubation for 1 hour at 37°C with a number of physiologic mediators of neutrophil activation; these included, C5a (10^{-8} M), TNF (10 U/ml), LTB₄ (10^{-8} M),

LPS (10 µg/ml), IL-1 (1 µg/ml), and PMA (100 ng/ml). It was found that PMA elicited a more complete upregulation of Mac-1 and downregulation of gp100^{MEL-14} (L-selectin) at lower concentrations than any of the other agents.

The majority of the literature concerning the effects of cell preparation procedures on CAM expression focused on Mac-1 and, to a lesser extent, on L-selectin. Changes in expression of these molecules were among the earliest detected in response to inflammatory stimuli. This made them likely targets for early diagnosis or prevention of inflammatory illnesses. Our experiments confirm the findings of other studies suggesting that cells remaining at room temperature prior to or during processing showed dramatic upregulation of Mac-1, similar to stimulated levels. Review of the literature shows this finding to be consistent irregardless of anticoagulant or leukocyte isolation technique used. Lundahl, *et al.* (1995) collected whole blood samples in either EDTA or heparin which were then placed immediately on ice (4°C) or kept at room temperature (20°C). Samples were taken from these at 1, 5, and 60 minutes for analysis. Monocyte isolation was carried out by either ammonium chloride-mediated red cell lysis at 4°C or by density gradient centrifugation on Ficoll-Paque at both 4°C and 20°C. Samples prepared by

NH₄Cl-mediated lysis were washed (PBS/azide) and centrifugation performed at 4°C. Density gradient centrifugation-prepared cells were washed with RPMI medium and centrifuged at either 4 or 20°C. Immunostaining for flow cytometry was performed for all samples at 4°C. At each time and temperature combination, samples prepared by NH₄Cl-mediated lysis showed significantly lower levels of Mac-1 expression than did monocytes prepared by density gradient centrifugation. As mentioned previously, cells prepared by either method from blood collected in heparin showed significantly increased Mac-1 expression versus cells from EDTA blood. The work by Youssef, *et al.* (1995) (previously discussed) demonstrated this change in Mac-1 expression within 15 minutes of incubation at 37°C regardless of the leukocyte isolation technique employed (*i.e.*, whole blood preparation utilizing FACS lysing buffer vs. sedimentation, density gradient centrifugation, and hypotonic red cell lysis). The study by Hed, *et al.* (previously described) suggested that upregulation of Mac-1 had occurred after only 2.5 minutes of incubation at 20°C, 25°C, and 37°C. In contrast, incubation at 4°C or 15°C for up to 30 minutes showed no significant change in Mac-1 expression.

Our studies, like those presented, revealed a dramatically attenuated rise in Mac-1 expression over time on cells maintained on ice; however, we also observed that expression consistently remained slightly above baseline levels at two hours. A similar observation was made in a study by Macey, *et al.* (1992). These authors reported a progressive but not statistically significant rise in Mac-1 levels on cells collected in EDTA, isolated by whole blood lysis of red cells, and analyzed after incubation on ice for 10, 20, 30, and 60 minutes. Forsyth and Levinsky (1990) provided another important observation. They demonstrated that cells subjected to cooling (collected and incubated at 4°C) and subsequent re-warming (cell preparation at 37°C) showed increased surface expression of integrin molecules (LFA-1, Mac-1, and p150, 95) as compared to a reference (control) sample; however, the reverse situation was not observed. It is important to note that blood for this study was collected in heparin and separated by density gradient centrifugation followed by dextran sedimentation and hypotonic lysis of residual red cells, a processes known to upregulate integrins. However, these data were interpreted as per cent change in antigen expression as compared to a reference sample collected and maintained throughout processing at 37°C. This sample

showed no significant difference in integrin expression as compared to a parallel sample collected and maintained at 4°C. This would suggest that exposing cells to changes in temperature from cold to warm during processing might play a significant role in cell stimulation. This study does not account for any upregulation of integrin molecules due to the effect of heparin or the process of leukocyte isolation, which have both been shown to induce this change. At any rate, this study makes the important recommendation that blood stored on ice prior to preparation must be kept at 4°C throughout processing, and until the cells are formalin fixed. As a final consideration, one author observed that simply storing samples on crushed ice was not sufficient to prevent augmentation of Mac-1 levels. Instead, drawing blood into pre-chilled tubes and storage on crushed ice in a layer of ice water was recommended for better results (Heikko, *et al.*, 1993).

The next molecule we studied was LFA-1 β , which, as previously indicated, represents the CD18 component of the integrin molecule LFA-1 and Mac-1. In our studies, changes in its expression paralleled those of Mac-1, though not to the same magnitude. This finding was explained by considering changes in total CD18 (Mac-1 + LFA-1 β) in

relation to individual changes in Mac-1 and LFA-1 α . Antibodies recognizing LFA-1 β likely bound the CD18 component of Mac-1 as well. Thus, as Mac-1 expression increased over time (raising total CD18), LFA-1 β appeared to rise also. However, since LFA-1 α was seen to decrease over time, the portion of total CD18 contributed by LFA-1 also decreased. A rise in Mac-1 of a greater magnitude than the fall in LFA-1 would result in a net increase in total CD18, but to a lesser extent than the elevation in Mac-1 alone, thereby explaining the changes observed in LFA-1 β .

Data presented for L-selectin revealed relatively stable expression with only a slight drop observed over two hours, regardless of incubation temperature. The literature supported these findings indicating that L-selectin expression by granulocytes is stable at room temperature and on ice when collected in either EDTA or sodium citrate and prepared by red cell lysis of whole blood (NH₄Cl or FACS lysing solution) (Stibenz and Buhner, 1994; Lundahl, *et al.*, 1995; Youssef, *et al.*, 1995). It was further demonstrated that L-selectin expression remained stable even with density gradient centrifugation, but only if performed at 4°C (Stibenz and Buhner, 1994; Lundahl, *et al.*, 1995; Youssef, *et al.*, 1995).

Due to the relative paucity of data on short-term changes in L-selectin expression, additional trials were conducted to look at data collected at shorter, room temperature incubations. The first experiment in this series suggested that cells became activated and shed L-selectin after 15 minutes at room temperature. However, initial peak-fluorescence was low at 129, as compared to our typical 0 hour findings of approximately 300-700, suggesting that these cells may have become stimulated during processing. Two subsequent studies were performed to further investigate these findings by looking at expression at 0, 5, 10, 15, and 30-minute incubations at room temperature. The results of these trials suggested that L-selectin was stable through 15 minutes with the first sign of shedding occurring at 30 minutes. Taking the average of the data from these two trials for statistical analysis, the change at 30 minutes was not found to be significant ($p > 0.05$) as compared to earlier time points. Stimulation with PMA did result in significant ($p = 0.0001$) loss of L-selectin as compared to all unstimulated time points.

Results obtained for measures of ICAM-1 at all incubation times (0 hours and 2 hours), both at room temperature and on ice, revealed a fair amount of

variability between trials making individual studies difficult to interpret; however, when these data were averaged together, expression appeared to have remained relatively stable over time regardless of temperature. Compared to trials with PMA stimulated samples, little change occurred. LFA-1 α showed a decreased expression over time, possibly enhanced on ice. These data too were somewhat variable between experiments and interpretation was difficult; however, other workers had described considerable donor-to-donor and preparation-to-preparation variability among beta 2 integrins on neutrophils analyzed by immunofluorescence flow cytometry (Tonnesen et al., 1989). In addition, PMA stimulation did not appear to produce the expected upregulation of this molecule. This too was addressed by Tonnesen suggesting that all β 2 integrins may show considerable donor-to-donor variability in response to stimulation (Tonnesen et al., 1989). Unfortunately, there was little discussion in the literature of the effects of ex vivo manipulation of neutrophils on ICAM-1 and LFA-1 α expression. It was, however, reasonable to expect that all integrin molecules would respond in a similar manner under similar test conditions. It was also conceivable that our findings were

consistent with actual events relating to these molecules or they may simply have reflected errors from inexperienced handling of the cells.

In conclusion, the study of cell adhesion molecules is an important frontier in modern biomedical science. The continued search for better methods of analysis is vital to its advancement. The method described here suggested several critical factors necessary for obtaining an accurate representation of CAM expression *in vitro*; these included, the choice of anticoagulant, the method of leukocyte isolation, and most importantly, the temperature at which cells were stored and processed. EDTA was one of the most commonly cited and reliable anticoagulant agents; therefore, it was the anticoagulant of choice. Both NH_4Cl and the FACS lysing buffer have proven effective at lysing red cells without modulation of leukocyte surface antigens. Our method described the use of the FACS lysing buffer from Becton Dickinson. It was recommended that blood be placed on ice with a layer of ice water immediately after collection, and for samples to remain on ice throughout processing. In addition, all washings were performed with chilled PBS or PBS/azide solutions, and the centrifuge was refrigerated to 4°C. Finally, cells were fixed with chilled

formalin and stored at 4°C for preservation until analyzed by flow cytometry.

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APPENDIX

Appendix 1:

Approval for the use of human subjects



Youngstown State University / One University Plaza / Youngstown, Ohio 44555-309

Dean of Graduate Studies

(330) 742-309

FAX (330) 742-158

E-Mail: amgrad03@ysub.yzu.ed

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,

A handwritten signature in black ink, appearing to read 'Peter J. Kasvinsky'.

Dr. Peter J. Kasvinsky
Dean of Graduate Studies

cc

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