THE ROLE OF THE NEUTROPHIL IN THE DEVELOPMENT OF ARDS

by

ELYCE F. NICASTRO

Submitted in Partial Fulfillment of the Requirement

for the Degree of

Master of Science

in the

Biological Sciences Program

Diana Fagan, Ph.D. 8/15/94

Advisor Date

8/17/94

Dean of the Graduate School Date

YOUNGSTOWN STATE UNIVERSITY

August, 1994

THE ROLE OF THE NEUTROPHIL IN THE DEVELOPMENT OF ARDS

by

ELYCE F. NICASTRO

I hereby release this thesis to the public. I understand this thesis will be housed at the Circulation Desk of the University library and will be available for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

Elyce F Nicastro — 8/15/94

Elyce F. Nicastro — Date

Approvals:

Diana Fagan, Ph.D. — 8/15/94

Dr. Diana Fagan — Date

James F. Toepfer — 8/15/94

Dr. James Toepfer — Date

David Asch — 8/15/94

Dr. David Asch — Date

[signature] — 8/17/94

Dean of Graduate Studies — Date

THESIS APPROVAL FORM

THESIS TITLE: The Role of the Neutrophil in the Development of ARDS.

AUTHOR: Elyce F. Nicastro

DEGREE: Master of Science

ADVISOR: Dr. Diana L. Fagan

COMMITTEE MEMBERS:	ACCEPT	REJECT
Diana Fagan, Ph.D.	✓	
Dr. Diana Fagan		
James R. Toepfer	✓	
Dr. James Toepfer		
David Asch	✓	
Dr. David Asch		

DATE: 8/15/94

1 copy to accompany library copy
1 copy to advisor
1 copy to departmental files
1 copy to graduate school

ORAL EXAMINATION APPROVAL FORM

Elyce F. Nicastro has successfully defended a thesis entitled The Role of the Neutrophil in the Development of ARDS by oral examination on August 15, 1994.

COMMITTEE MEMBERS:	ACCEPT	REJECT
Diana Fagan, Ph.D.	✓	
Dr. Diana Fagan		
James R. Toepfer	✓	
Dr. James Toepfer		
David Asch	✓	
Dr. David Asch		

ABSTRACT

The Role of the Neutrophil in the Development of ARDS

Elyce F. Nicastro

Master of Science

Youngstown State University, 1994

We investigated the levels of various cell adhesion molecules (CAMs), ICAM-1, LFA-1 beta, Mac-1, L-selectin, LFA-1 alpha, and VLA-4, found on white blood cells. Recruitment of neutrophils to the site of an immune response is dependent upon the expression of CAMs on the cell. In particular, the neutrophil was examined because of its proposed role in the development of acute respiratory distress syndrome (ARDS). The neutrophil is the first white blood cell summoned to a site of injury. To better understand results seen in patients at risk of developing ARDS, we first established normal control levels of the various CAMs. For each experiment, normal patients were used. Blood was collected at 1, 3, 6, 9, 12, 18, and 24 hour time points. Unstimulated and PMA-stimulated blood was examined at each time point. Addition of the primary antibody against the various CAMs was followed by the addition of the secondary antibody, FITC anti-mouse Ig. The cells were analyzed by flow cytometry to determine the fluorescence seen in the neutrophil cell population. Histograms showing level of fluorescence detected on the cells were then derived for the neutrophil population. A paired t-

test was performed to compare the unstimulated and PMA-stimulated cells at specific time points. A significant difference was found in Experiment 1 for ICAM-1, Mac-1, and L-selectin but was not supported by data from the other two experiments. In Experiment 2, LFA-1 alpha exhibited a significant difference which was not supported by the other two experiments.

Normal controls were also done to establish if diurnal variation occurred over time. The repeated measures one way ANOVA analysis was done to determine if a significant statistical difference had occurred. Statistical differences were only found in Experiment 1 which could be indicative of diurnal variation. However, this finding was not supported by the subsequent experiments.

Future studies will examine the effect of CAMs in the development of ARDS. A better understanding of the role of CAMs in the onset of inflammatory responses may aid in the development of compounds able to modify the immune response and reduce the incidence of tissue injury.

ACKNOWLEDGEMENTS

I would like to thank the following individuals: Dr. James Toepfer and Dr. David Asch for their time and suggestions while evaluating my thesis, and especially Dr. Diana Fagan for being my graduate advisor. Her guidance, patience, and time were much appreciated.

With deepest appreciation, I thank Scott for always listening and making sacrifices while I completed this work.

Special thanks go to my parents, John and Sharon, for their constant support and encouragement during the writing process and throughout all of my life. Their belief in me has always made things easier. Thanks also to my brothers, Jason and Lee, who give their own unique support. Lastly, thanks to my Gram and Grandpa whose love has been a constant force in my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab	antibody
ARDS	acute respiratory distress syndrome
CAM	cell adhesion molecule
FITC	fluorescein isothiocyanate
FSC	forward scatter
Ig	immunoglobulin
IL-1	interlukin-1
LAD	leukocyte adhesion deficiency
LPS	lipopolysaccharide
mAb	monoclonal antibody
mL	milliliter
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
MSOF	multiple system organ failure
MSF	multisystem failure
NK	natural killer
PE	phycoerythrin
PBS	phosphate buffered solution
PF	paraformaldehyde
PMA	phorbol myristate acetate
PMN	polymorphonuclear
sL-selectin	shed L-selectin
SSC	side scatter
TNF	tumor necrosis factor
uL	microliter

CHAPTER I

INTRODUCTION

Multiple organ failure (MOF), also referred to as multiple organ dysfunction syndrome (MODS), multiple system organ failure (MSOF), and multisystem failure (MSF), is a syndrome identified by a sequential decay of vital organs with severity that quickly worsens. MOF affects people who have undergone a severe trauma; consequently their inflammatory immune response does not function properly. In contrast to a normal person, a trauma patient's inflammatory immune response goes on "unchecked" causing damage to various organs and tissues. The main organ systems affected are the "pulmonary, cardiovascular, gastrointestinal/hepatic, renal, neurologic, and hematolic/immunologic systems" (Brass, 1994). Acute respiratory distress syndrome (ARDS) has similar symptoms resulting in injury to the lungs. ARDS often precedes the development of MOF. ARDS occurs rapidly and injures the lungs so severely that a 50-90% mortality rate exists. Its development may not be seen until hours or even days following an initial trauma (Weiland et al., 1986; Donnelly et al., 1993). There is no set definition for ARDS because it is affiliated with many etiologies (Atkins et al., 1994). The main characteristic of ARDS is pulmonary edema (Fowler et al., 1987). Pulmonary edema refers to an excessive buildup of fluid within the lungs' air spaces. ARDS' first

"pathophysiologic" attribute is "diffuse alveolar capillary membrane injury" (Zimmerman et al., 1983). With ARDS, the lung is unable to function properly. The exact cause of ARDS is unknown. Common risk factors that may make a patient susceptible to ARDS include "aspiration of gastric contents, multiple transfusions, near drowning, sepsis, and trauma" (Lillington and Redding, 1992).

ARDS and MOF probably result from recruitment of neutrophils to various organs, such as the lungs, followed by activation of the leukocytes (St. John and Dorinsky, 1993). Neutrophils are the most numerous of the leukocytes (about 70%); they are also referred to as polymorphonuclear cells (PMNs). Overall, neutrophils are "nonspecific, easily activated phagocytic cells, and the first line of defense" in the body against foreign material (Osborn, 1990).

Various studies have indicated the neutrophil's importance in the development of ARDS. Patients with ARDS have numerous neutrophils sequestered in their lungs in contrast to a normal person (Weiland et al., 1986; Repine, 1992). In patients or animals who become septic, neutrophils have been shown to sequester rapidly in the lungs (Windsor et al., 1993). In several animal models, injury related to ARDS was prevented by the removal of neutrophils as shown by bronchoalveolar lavage, a technique to sample human and animal distal brochoalveolar spaces (Repine and Beehler, 1991). Following the introduction in the lung of an inflammatory

stimulus (phorbol myristate acetate), functional leukocytes may be retrieved by bronchoalveolar lavage for up to twenty four hours. The principal cell type found in the lavage of animals given an inflammatory stimulus in the lung are neutrophils. As the severity of ARDS in humans increases, the number of neutrophils recovered by lavage also increases (Martin et al., 1991). Fowler and coworkers found 72+/-10% PMN in lavage fluid from patients who developed ARDS versus 40+/-10% PMN from patients who did not develop ARDS (1987). Weiland and coworkers found neutrophils to comprise 67.6+/-9.8% of the bronchoalveolar lavage from ARDS patients (1986). In patients suffering from ARDS, serial bronchoalveolar lavage was done to determine the number of neutrophils sequestered in the lungs. In six of these patients, a decline in the number of neutrophils was seen concomitant with recovery in the patients (Weiland et al., 1986).

Neutrophils may be attracted to a tissue and attach to endothelial cells at the site of an immune or inflammatory response. If the white blood cells are activated and phagocytizing particles, toxic compounds, such as proteases and other oxygen species, may be released and cause organ injury (St. John and Dorinsky, 1993). If patients become septic (destruction of tissues by bacteria or bacterial toxins) due to an infection or release of bacteria from the gastrointestinal tract, neutrophils are activated by endotoxin, lipopolysaccharides found in the cell walls of all

gram negative bacteria. Lipopolysaccharides are complex molecules composed of both lipid and polysaccharide. Endotoxins have a direct effect on how pathogenic the bacteria is. Endotoxin is only released when the bacterial cell is broken down or the cell dies and subsequently deteriorates. When animals' lungs were injected with endotoxin, neutrophils entered the lungs. Endotoxin has deleterious effects on the lungs which may result from stimulation of neutrophils to produce superoxide and elastase which in turn disrupt the microvascular barrier (Welbourn and Young, 1992; Atkins et al., 1994). When endotoxin is found in human blood, there is a direct correlation between endotoxin levels and the development of ARDS. However, endotoxin alone may not be sufficient as removal of neutrophils prior to endotoxin injection, eliminates the subsequent development of lung injury in various animal models (Welbourn and Young, 1992).

Tumor Necrosis Factor (TNF) is a cytokine that is made by monocytes and macrophages in response to bacterial endotoxin, interlukin 1 (IL-1), and mitogens (Stephens et al., 1988). TNF may be an important mediator in the development of ARDS, as infusion of TNF into the lungs results in the accumulation of neutrophils in the lungs. In these studies, the neutrophils can also be shown to attach to the capillaries in the lung (St. John and Dorinsky, 1993). TNF also causes neutrophils to make elastase, superoxide ion, and hydrogen peroxide (Welbourn and Young, 1992). These compounds can

cause injury to the microvasculature and to the tissues (Skolnick, 1990). Oxygen radicals that are produced are not stable and are very reactive (Windsor et al., 1993). These oxygen radicals cause injury to the lung. Elastase is made only by neutrophils; whereas oxidants are produced by other cells in addition to neutrophils, such as macrophages and endothelial cells. Elastase is not toxic if the lung has not been exposed to oxidants; however in combination with oxidants, elastase can have deleterious effects on the lungs (Repine, 1992). Lavage fluid taken from ARDS patients shows elevated amounts of elastase and superoxide anion when compared to normal controls (Zimmerman et al., 1983).

A critical step in neutrophil recruitment to a site of inflammation is the interaction of adhesion molecules on circulating cells with their ligands on vascular endothelium adjacent to the inflammatory site (von Andrian et al., 1992). Leukocyte recruitment and attachment to endothelial cells during inflammation involves three main steps. In the first step, cell adhesion molecules belonging to the selectin family mediate a low affinity interaction resulting in slower movement, often described as rolling, along the vascular wall. Rolling is thought to act as a homing mechanism that determines the tissue that the lymphocyte will migrate into (Lasky, 1992). Monoclonal antibodies against L-selectin reduced human neutrophil rolling. In addition, because antibodies to L-selectin block endothelial rolling and

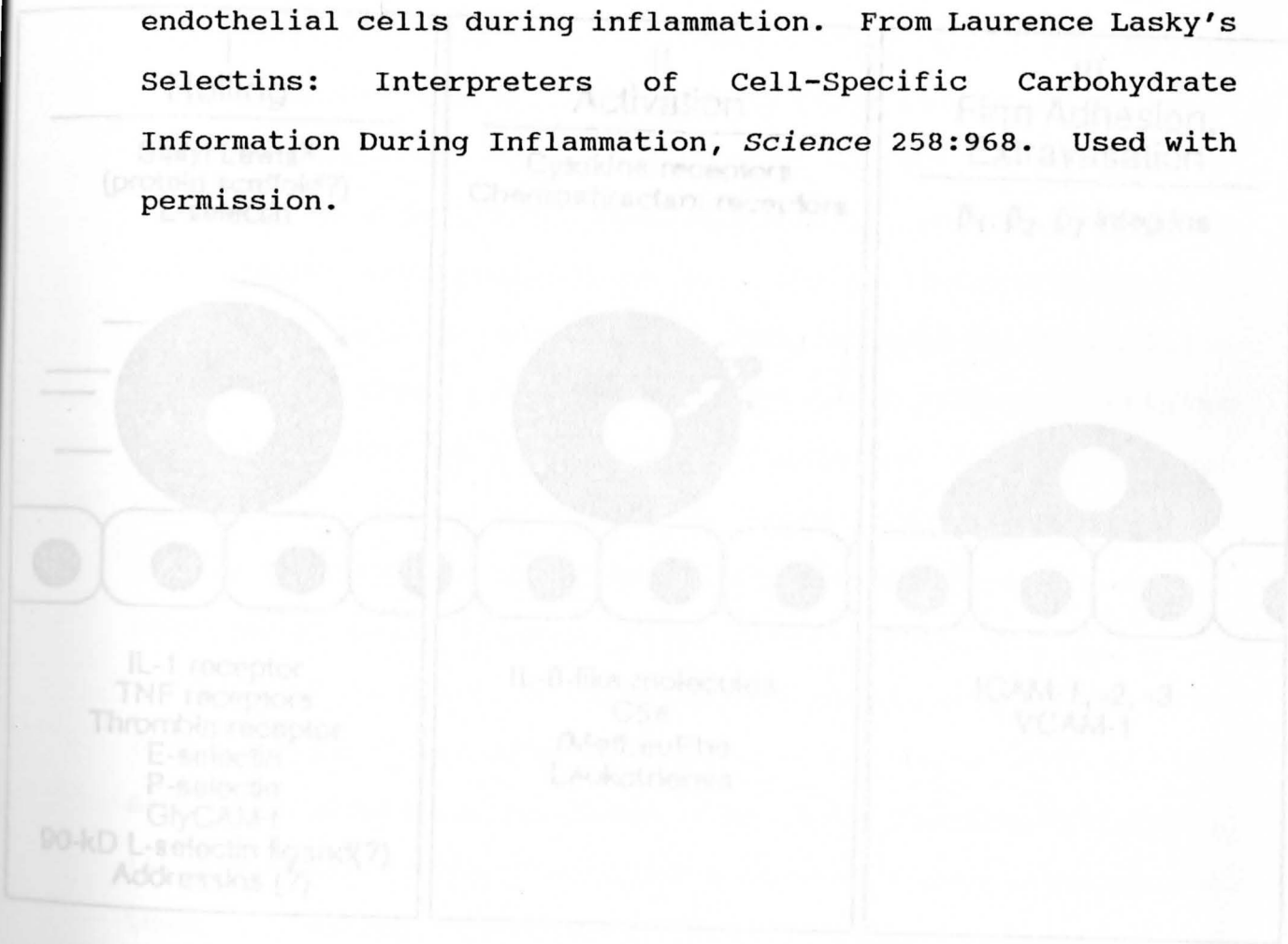
inflammation, it is thought that rolling is needed before inflammation can occur *in vivo* (von Andrian et al., 1992).

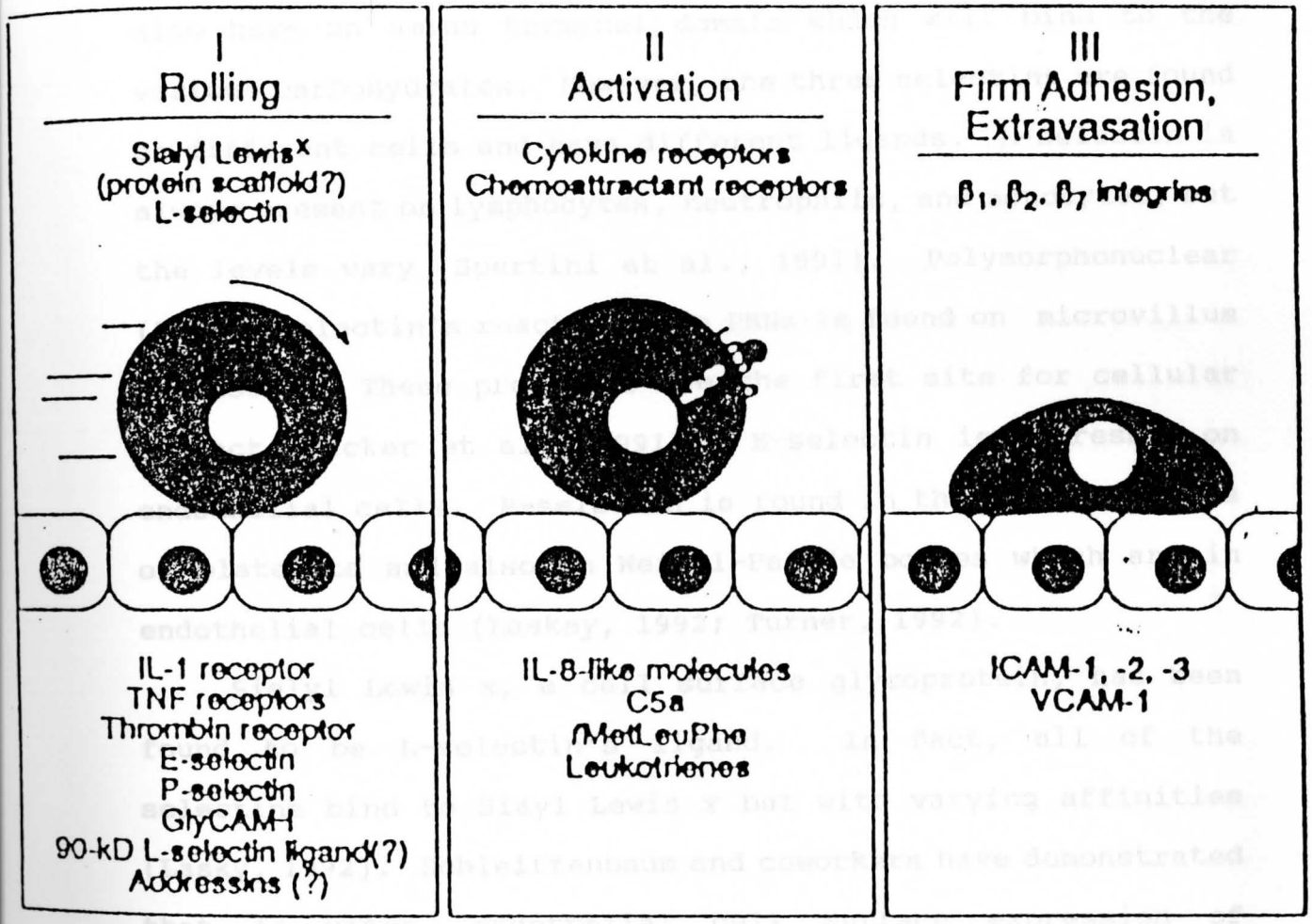
Stimulation of the neutrophil occurs in the second step. The activation is controlled by concentration gradients of the chemotactic factors C5A, f-Met-Leu-Phe, and Leukotrienes. Activation of the cells results in the expression on the cell of cell adhesion molecules belonging to the integrin family. The final step is accomplished when leukocyte integrins bind to their ligands on the endothelium. In contrast to the low affinity event in step one, this step is a high affinity adhesion event. Change in the leukocytes' shape and extravasation also take place in this step (Lasky, 1992). Extravasation of white blood cells from the blood into the surrounding tissues occurs following an injury, burns, inflammation, or an allergy (see Figure 1). In summary, the receptor on neutrophils responsible for rolling seems to be L-selectin but probably involves other selectins. The integrins are responsible for high affinity binding which results in movement of the cells into the tissues (von Andrian et al., 1992).

The first group of CAMs discussed above were the selectins. These molecules include three glycoproteins found on cell surfaces, all having lectin domains. Lectins are carbohydrate binding proteins; they are widely distributed and perform a great assortment of functions. The three members of the selectin family are: L-selectin (LECAM-1, human Leu8,

Figure 1: Model of leukocytes during inflammation.

The figure depicts leukocyte recruitment and attachment to endothelial cells during inflammation. From Laurence Lasky's Selectins: Interpreters of Cell-Specific Carbohydrate Information During Inflammation, *Science* 258:968. Used with permission.




I
Rolling
Sialyl Lewis^x
(protein scaffold?)
L-selectin
IL-1 receptor
TNF receptors
Thrombin receptor
E-selectin
P-selectin
GlyCAM-1
90-kD L-selectin ligand(?)
Addressins (?)
II
Activation
Cytokine receptors
Chemoattractant receptors
IL-8-like molecules
C5a
fMetLeuPhe
Leukotrienes
III
Firm Adhesion,
Extravasation
β_1, β_2, β_7 integrins
ICAM-1, -2, -3
VCAM-1

murine Mel-14, LAM), E-selectin (ELAM-1), and P-selectin (GMP140/PADGEM). Similarities among the selectins include mediation of regional inflammatory responses which occurs when the selectins recognize cell specific carbohydrates. They all also have an amino terminal domain which will bind to the various carbohydrates. However, the three selectins are found on different cells and have different ligands. L-selectin is always present on lymphocytes, neutrophils, and monocytes, but the levels vary (Spertini et al., 1991). Polymorphonuclear (PMN) L-selectin's reactivity on PMNs is found on microvillus processes. These processes are the first site for cellular contact (Picker et al., 1991). E-selectin is expressed on endothelial cells. P-selectin is found in the alpha granules of platelets and also in Weibel-Palade bodies which are in endothelial cells (Laskey, 1992; Turner, 1992).

Sialyl Lewis x, a cell surface glycoprotein, has been found to be L-selectin's ligand. In fact, all of the selectins bind to Siayl Lewis x but with varying affinities (Lasky, 1992). Schleiffenbaum and coworkers have demonstrated that *in vitro*, endothelial cell surface expression of ligand(s) for L-selectin is seen only when the endothelial's cell surface is exposed to inflammatory cytokines, such as IL-1, TNF, and LPS (1992).

Whereas E-selectin within hours can increase with inflammatory activation of the cell with IL-1, TNF, or LPS, P-selectin can be increased in minutes on the surface of

platelets following thrombin activation. In contrast, L-selectin levels usually decrease when the cell is activated (Lasky, 1992). L-selectin on mouse and human PMNs and lymphocytes can be downregulated with LPS, PMA, TNF, and IL-1. The decline in L-selectin expression following activation may be the result of the cell surface shedding of L-selectin. Cytokines and mitogens (such as LPS, PMA, TNF, and IL-1) cause L-selectin to be lost from the surface of leukocytes after the cells have adhered to the endothelium. Contact with the same cytokines and mitogens in the bloodstream can also cause L-selectin to be downregulated prior to contact with endothelial cells. As a result, the leukocytes will remain in the bloodstream (Salmi and Jalkanen, 1992).

L-selectin shedding from the cell's surface following cellular activation may be an important mechanism for regulation of the inflammatory response (Simon et al., 1992). L-selectin can be shed from a neutrophil's or a monocyte's surface in five to fifteen minutes. L-selectin shedding from a lymphocyte's surface takes slightly longer, fifteen to thirty minutes is required (Palecanda et al., 1992). High L-selectin plasma levels are found due to the shedding. Simon and coworkers have found shed L-selectin (sL-selectin) to have an inhibitory effect on adhesion mediated by L-selectin, supporting the hypothesis that inflammation may be influenced by sL-selectin. Leukocytes in the tissues lacked L-selectin on their surface, suggesting that L-selectin may be lost from

the leukocytes' surface during migration into the tissues (Simon et al., 1992; Schleiffenbaum et al., 1992). In this case, shedding may be a required step prior to leukocyte extravasation into tissues.

The second group of CAMs are the integrins which are a large family of transmembrane glycoproteins. The Beta-1 integrins are referred to as the VLA subfamily and have six members. They are found on a variety of lymphoid and nonlymphoid cells. Of the Beta-1 family, only VLA-4 (CD49d/CD29) appears to play a role in inflammation. VLA-4 is found on fibroblasts, monocytes, and lymphocytes. Its ligands are fibronectin (matrix protein) and VCAM-1 (Immunoglobulin gene superfamily member). The Beta-2 integrins include: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1, Mo1, CR3 [Complement receptor 3]), and CD11c/CD18 (p150,95; Leu15, CR4 [Complement receptor 4]). CD11 refers to the alpha chain which has a direct effect on the affinity of the binding to various ligands. CD18 is the common beta chain shared by all three which is 94 kD (Arnout, 1990). The alpha chain varies for each member, CD11a/CD18's is 180 kD, CD11b/CD18's is 170 kD, and CD11c/CD18's is 150 kD (Larson and Springer, 1990).

Beta-2 integrins are found only on leukocytes although their abundance is determined by the type of cell and the cell's state of differentiation. Overall, for resting monocytes, CD11a/CD18> CD11b/CD18> CD11c/CD18; on tissue macrophages, CD11c/CD18> CD11a/CD18> CD11b/CD18; and on

granulocytes, CD11b/CD18>> CD11a/CD18 or CD11c/CD18. CD11a/CD18 is found on all types of leukocytes. All three integrins can be found on natural killer cells, monocytes, and macrophages (Arnout, 1990). Overall, they are referred to as the leukocyte integrins and are involved in numerous leukocyte adhesion events. They are also important in inflammation and various immune responses (Larson and Springer, 1990).

Whereas CD11b/CD18 and CD11c/CD18 are stored in granulocytes and monocytes and can have their surface expression increased within minutes, CD11a/CD18 is not stored extensively intracellularly (Arnout, 1990). With cell activation, the intracellular pools of CD11b/CD18 (Mac-1) and CD11c/CD18 (p150,95) are brought to the surface of the cell (Kishimoto et al., 1989). F-met-leu-phe, which is a neutrophil activator, had the ability to increase the surface level of CD11b/CD18 and also increase binding to its ligand, ICAM-1. In contrast, the levels of CD11c/CD18 increase when extravasation occurs, probably in response to cell activation. However, following extravasation of the neutrophil into the tissue, the levels of CD11b/CD18 decrease (Larson and Springer, 1990).

The two ligands for CD11a/CD18 (LFA-1) are ICAM-1 (CD54) and ICAM-2, both members of the Immunoglobulin supergene family (proteins having structural homology with the immunoglobulins). This CAM/ligand combination is unique, as it is rare for members of these two families to interact

(Larson and Springer, 1990). ICAM-1 is an antigen found on leukocytes, endothelial, and epithelial cells. IL-1, TNF, and endotoxin induce ICAM-1 expression but not ICAM-2 expression. CD11b/CD18's (Mac-1) various ligands include iC3b, Factor X, fibrinogen, ICAM-1, polysaccharides, and Leishmania glycoprotein 63. Competition for binding to CD11b/CD18 occurs between fibrinogen, factor X, and iC3b. CD11b/CD18's principal ligand is iC3b and binding to this ligand is seen with unstimulated leukocytes. Currently, iC3b is CD11c/CD18's (p150,95) only known ligand and binding occurs with resting cells (Arnout, 1990).

The Beta-2 integrins (CD11/CD18 complex) have a key role in the attachment between leukocytes and endothelial cells (St. John and Dorinsky, 1993). The adhesiveness of neutrophils for the endothelium is blocked when monoclonal antibodies (mAbs) against CD11a/CD18 (LFA-1) or CD11b/CD18 (Mac-1) are used. Adhesion is almost totally stopped when mAbs against both CD11a/CD18 and CD11b/CD18 are used (Osborn, 1990). The importance of the CD18 complex is demonstrated by a disease found in humans, Leukocyte Adhesion Deficiency (LAD). LAD is a rare, inherited disease in which the CD18 complex's expression is lessened or completely absent. These patients suffer from bacterial infections that are recurrent and severe (Turner, 1992). This appears to occur because in patients with LAD, the neutrophils cannot migrate into the sites of inflammation (Osborn, 1990).

An *in vitro* experiment revealed that stimulation of leukocytes caused CD11b/CD18 to increase and also caused L-selectin to be shed from the cell's surface. In addition, CD11b/CD18 is upregulated but L-selectin is lost when movement into the tissues occurs. In humans, PMNs located in the tissue went into the airways following an allergen challenge. An allergen is any antigen that produces an allergy in a hypersensitive person. In this experiment, short ragweed antigen, dust mite, and timothy grass were used as the allergens. In humans, incubation of cells with monoclonal antibodies (mAbs) against CD11b/CD18 and L-selectin resulted in inhibition of cell adhesion when anti-L-selectin mAb was used, while anti-CD11b/CD18 mAbs stopped endothelial transmigration (Georas et al., 1992). This supports the hypothesis that selectins are involved in rolling and integrins are needed for transmigration.

Simon and coworkers propose that the accumulation of neutrophils is due to the binding of L-selectin and Beta-2 integrins to their ligands (1992). When L-selectin was shed in rabbits, the neutrophils could not identify endothelial cells that were inflamed even though Beta-2 integrins were being expressed (von Andrian et al., 1992). Work done by Kishimoto and coworkers found neutrophils located "within the lumen or walls of small vessels associated with inflamed tissues" were CD11b/CD18 (Mac-1) positive and murine L-selectin (Mel-14) positive. But when the neutrophils

extravasated to the area around the tissue, the neutrophils were CD11b/CD18 positive and murine L-selectin negative. This demonstrates that the Mel-14's (murine L-selectin) downregulation occurs rapidly *in vitro* and *in vivo* (1989). Spertini and coworkers conducted experiments demonstrating that 85-90% of adhesion of lymphocytes to the endothelium was mediated by L-selectin, VLA-4, and/or the CD11/CD18 complex. When mAbs to L-selectin, CD11/CD18 complex, and E-selectin were used, approximately 90% of the neutrophils were inhibited from binding to the endothelium (1991).

Because of the high mortality rate associated with ARDS, it would be extremely beneficial to susceptible patients if the development of ARDS could be prevented. It has been proposed that the normal bacteria that is found in the intestines can be released into the blood when hypovolemia (a decrease in the amount of circulating blood) proceeds a trauma (Deitch et al., 1988). An increase in the cell adhesion molecules and endotoxin have a direct affect in ARDS that results. In this project, we wanted to determine the specific cells that are recruited into the lungs and why they become sequestered in the lungs during the progression of ARDS. In particular, these studies examine the neutrophil population because we and others propose that the neutrophil, which is the first leukocyte summoned to defend the body, can actually harm the body. As a result of stimulation from cytokines, neutrophils, responding as the initial cell for defense,

sequester at the inflammatory site (Windsor et al., 1993). After the neutrophils are recruited to the lungs, they begin to adhere to them and consequently cause damage (Repine, 1992). A type of positive feedback loop occurs; the neutrophils that are summoned to aid the body secrete substances that in turn will summon more neutrophils and so on. The neutrophils are capable of releasing substances, such as "oxygen radicals, degradative enzymes, arachidonic acid metabolites, and platelet activating factor", that have the ability to disturb the "microvascular barrier" (Atkins et al., 1994). Elastase, produced by neutrophils, can destroy the host environment. Windsor and coworkers assert that the neutrophil has the ability to negotiate the kind of injury which leads to ARDS (1993). Bronchopulmonary lavage fluid has been shown to have neutrophil activators (Repine, 1992; Donnelly et al., 1993). A number of investigators showed as the severity of ARDS increased, so did the number of neutrophils (Martin et al., 1991; Fowler et al., 1987; Weiland et al., 1986). Martin and coworkers report that 81.3+/-9.9% of the 43.4+/-37.8 x 10^6 leukocytes obtained from lavage of ARDS patients were neutrophils (1991).

Our studies examine the cell adhesion molecules (CAMs) found on neutrophils. When more is known about the induction of CAMs required for recruitment of neutrophils to the alveolar spaces, we may be able to identify which of these are induced during the development of ARDS and if the time course

of expression of these molecules correlates with the induction of disease. [3] Glycoproteins that inhibit CAM-ligand interactions may be potential targets for the development of drugs that may interfere with the development of inappropriate immune responses.

TABLE 1

CAM	CELL	LIGAND
CD11a/CD18 (LFA-1)	PMN/mono/macrophage	ICAM-1 (CD54) ICAM-2
CD11b/CD18 (Mac-1)	PMN/mono/macrophage (activated)	iC3b Factor X Fibrinogen ICAM-1
CD11c/CD18 (p150,95)	PMN/mono/macrophage (activated)	iC3b
L-selectin (LECAM, (murine Mel-14)	Leukocytes	Siayl Lewis x
VLA-4 (CD49d/CD29)	mono/lymphocytes/ fibroblasts	VCAM-1

CHAPTER II

MATERIALS AND METHODS

Materials

Antibodies against VLA-4 (.2 mg/mL) were bought from AMAC (Westbrook, Maine). Antibodies against ICAM-1, LFA-1 alpha, LFA-1 beta, Mac-1, and L-selectin were donated to us by Dr. Robert Rothlein, Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, Connecticut). FACS lysing solution, Cali Brite beads (unlabeled, FITC, and PE), Simultest Leuco Gate (CD45 FITC, CD14 PE), Simultest control (Gamma 1 FITC, Gamma 2a PE), Simultest CD3 FITC, CD16 PE, Simultest CD3 FITC, CD4 PE, and Simultest CD3 FITC, CD19 PE were all purchased from Becton Dickinson (San Jose, California). Other reagents from Sigma (St. Louis, MO) included DMSO, NaCl, sodium azide, and PMA. $NaH_2PO_4H_2O$ was purchased from Fisher (Fair Lawn, New Jersey). Anhydrous Na_2HPO_4 was purchased from Mallinckrodt (Paris, Kentucky). Isoton II solution was purchased from Baxter Scientific Inc. (Deerfield, IL).

Preparation of Reagents

Diluted PMA: PMA (FW 616.8) stock was equal to 1mM in DMSO. 4 uL of 1mM PMA was added to 56 uL of 1X PBS and then diluted in blood to a final concentration of 6.6 uM (3.7 ug/uL).

10X Phosphate Buffered Saline (PBS): 1.28 g of $NaH_2PO_4H_2O$ and

5.97 g of anhydrous Na_2HPO_4 were dissolved in 200 mL of milliQ water. The pH was adjusted to 7.4, using .1M NaOH or HCl as needed. 43.83 g of NaCl was added and then dissolved. The final solution was brought up to 500 mL using milliQ water and stored at room temperature.

1X PBS: 100 mL of the 10X PBS solution was mixed with 900 ml of milliQ water and stored at 4°C.

PBS/Azide: 100 mL of 10X PBS was mixed with 900 mL milliQ water and 1 g of sodium azide. The solution was stored in the dark at 4°C and filtered with a 0.2 mm filter before use. PBS/Azide solution was stable for one week.

1X Lysing Buffer: 50 mL of 10X lysis buffer was added to 450 mL of milliQ water and stored at room temperature.

Paraformaldehyde: 20 mL of 10X PBS and 20 mg of paraformaldehyde were mixed with 200 mL of milliQ water. To dissolve the paraformaldehyde, the solution was heated at 56° C in a water bath then allowed to cool slowly to room temperature. The pH was adjusted to 7.4, using .1 M NaOH or HCl as needed. It was filtered immediately after preparation and before each use, then stored at 4°C for up to two weeks.

Methods

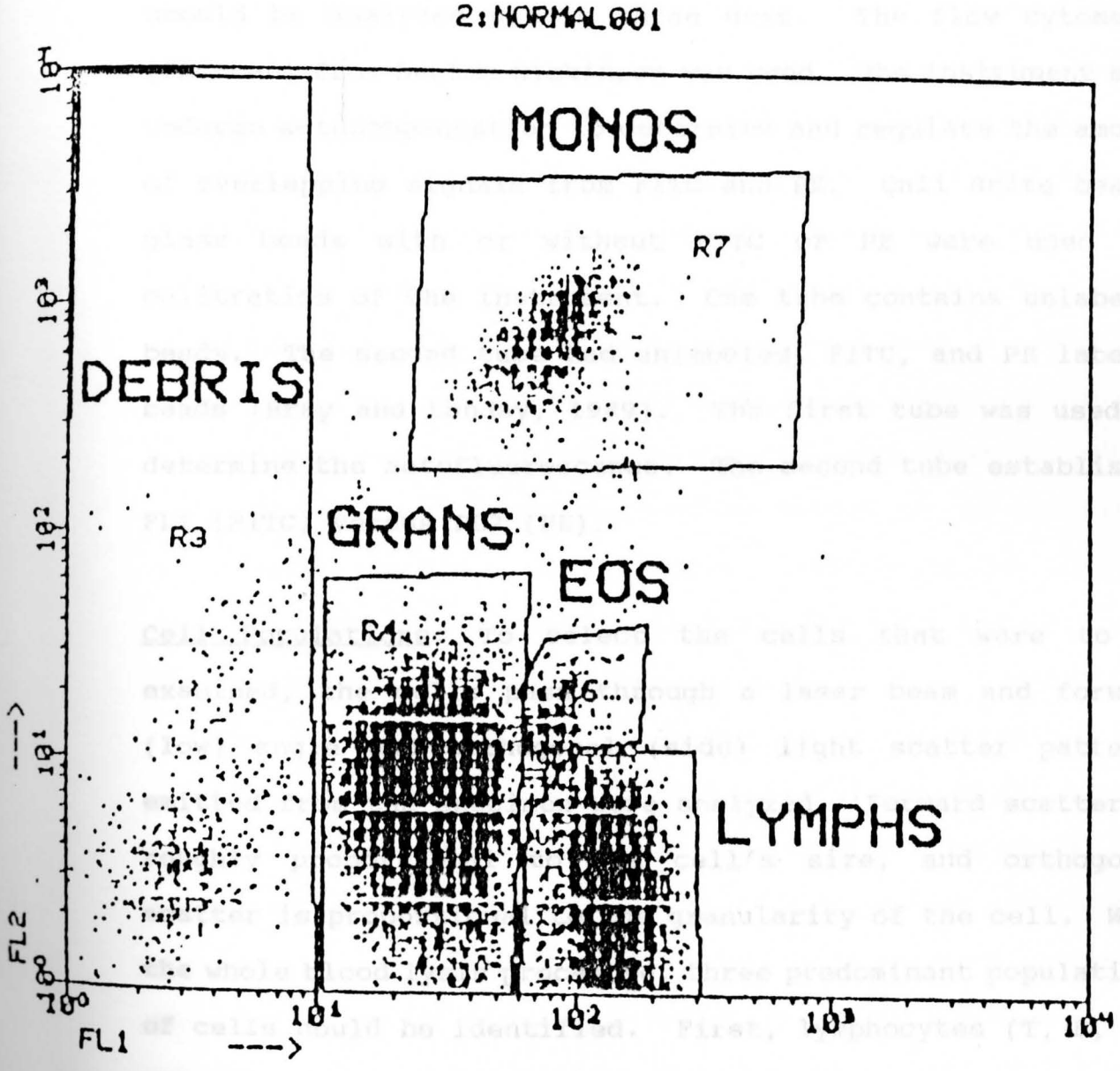
Sample Collection and Processing: Blood was obtained from the patient through a Heplock and collected into Vacutainer tubes containing the anticoagulant ethylenediaminetetraacetic acid. The blood was processed immediately. PMA (14.8 ug) was added to 600 uL blood and placed at 37°C for 15 minutes (PMA stimulated cells). Stimulated and unstimulated cells (100 uL of whole blood) were incubated with 20 ug of one of the following antibodies: ICAM-1, LFA-1 beta, Mac-1, L-selectin, LFA-1 alpha, and VLA-4. All tubes were then incubated for 15 minutes at room temperature. The cells were washed with PBS azide then centrifuged at 300 xg for 5 minutes, and the supernatant was discarded. 4 uL of FITC-labeled goat anti-mouse Ig was then incubated with the cells for 15 minutes at room temperature in the dark. Lysis buffer (2mL) was added to all of the tubes to lyse the red blood cells. After addition of the lysis buffer, the tubes were immediately vortexed on low for three seconds then incubated for 10 minutes at room temperature in the dark. The tubes were then centrifuged at 300 xg for 5 minutes. After centrifugation, the supernatant was discarded. The cells were then washed once with PBS azide and centrifuged at 300 xg for 5 minutes. After centrifugation, the supernatant was discarded and the cells were fixed by the addition of 400 uL of paraformaldehyde. The tubes were stored in the dark at 4°C.

Positive and Negative Controls: Antibodies against CD45/CD14, Gamma 1/Gamma 2a, and FITC anti-mouse Ig were standardized by Becton Dickinson for use in the experiment at 20 uL per 5 x 10^5 white blood cells. Gamma 1/Gamma 2a (FITC and PE-labeled antibodies directed against mouse immunoglobulin classes gamma 1 and gamma 2a) were used as a negative controls to determine background fluorescence and nonspecific staining of the cell. Positive controls are needed to determine if the reagents and labeling procedures are satisfactory for each experiment. Cells from a normal donor were drawn and prepared identically to the patient sample (Bray and Landay, 1989). The positive controls used were anti-CD45/CD14 immunoglobulins (FITC and PE-labeled) and anti-CD4/CD8 immunoglobulins (FITC and PE-labeled). CD45 can be found on all normal leukocytes. CD45 is displayed on lymphocytes at the highest concentration, followed by monocytes and then neutrophils. CD14 is seen on both neutrophils and monocytes, but monocytes exhibit much more CD14 than do neutrophils. By comparing FITC-CD45 labeled Ab to PE-CD14 labeled Ab, five different cell population can be distinguished (Loken et al., 1990) (see Figure 2). In the figure, R3 represents cellular debris, R4 represents the neutrophils, R5 represents lymphocytes (T, B, and natural killer cells), R6 represents the eosinophils, and R7 represents the monocytes.

Figure 2: 5 cell populations with FL1 versus FL2.

The figure shows the 5 cell populations that are observed when fluorescent labeling with FITC-labeled anti-CD45 Ab is plotted on the X axis (FL1) and PE-CD14 labeled anti-CD14 Ab is plotted on the Y axis (FL2).

Abbreviations: MONOS = monocytes, GRANS = neutrophils, EOS = eosinophils, and LYMPHS = lymphocytes.


2:NORMAL001
MONOS
R7
DEBRIS
R3
GRANS
EOS
R4
R6
LYMPHS
FL2 --->
FL1 --->
10^0
10^1
10^2
10^3
10^4

Data Analysis: When preparing the sample, the aim is to conserve the cellular components as they were *in vivo*. Cells should be analyzed within three days. The flow cytometer (FACScan) from Becton Dickinson was used. The instrument must undergo autocompensation to determine and regulate the amount of overlapping signals from FITC and PE. Cali Brite beads, glass beads with or without FITC or PE were used for calibration of the instrument. One tube contains unlabeled beads. The second tube had unlabeled, FITC, and PE labeled beads (Bray and Landay, 1989). The first tube was used to determine the autofluorescence. The second tube established FL1 (FITC) versus FL2 (PE).

Cell Populations: To select the cells that were to be examined, the cells pass through a laser beam and forward (low) angle and orthogonal (side) light scatter patterns emitted from the specimen were analyzed. Forward scatter is roughly proportional to the cell's size, and orthogonal scatter is proportional to the granularity of the cell. With the whole blood lysis procedure, three predominant populations of cells could be identified. First, lymphocytes (T, B, and natural killer cells) display low forward and orthogonal scatter. Second, the monocytes show intermediate forward and orthogonal scatter. Lastly, neutrophils display high forward and orthogonal scatter (McCoy et al., 1990).

Gating: The first step in the analysis of the cells was to gate for the appropriate population of cells. Gating was done to select the population of cells that will be analyzed by the computer (McCoy et al. , 1990). In gating, a box or polygon was drawn around the cell population to be analyzed. The positive control of CD45 FITC (FL1) versus CD14 PE (FL2) was first. Gate R1 was used to designate the monocyte population; gate R2 was used to designate the PMN (neutrophil) population as identified by CD45/CD14 staining (see Figure 3). The same sample was then used to plot forward scatter (FSC) versus side scatter scatter (SSC) (see Figure 4). R4 was drawn around the cell population that corresponded to monocytes, as identified by computer analysis of CD45/CD14 labeling in the previous figure. Gate R5 was used to indicate the cell population on the scatter graph identified as cells gated in R2 (neutrophils by CD45/CD14 staining). The monocytes and neutrophils were examined for overlapping cell populations, and R5 is adjusted to include the maximum amount of neutrophils with the minimum amount of monocyte contamination. This was then compared to the fluorescent-labeled cell population in order to determine the percentage of the total neutrophils (96%) and the total percentage of contamination from monocytes (3%) (see Figure 5). The computer then used this to formulate a histogram (Carter, 1990). Once the gate for the neutrophils was established, the same gate was used to analyze each control or patient sample. For each sample, a histogram and accompanying

Figure 3: FL1 (FITC-CD45) versus FL2 (PE-CD14).

This figure depicts gating of the monocyte population (R1) and the neutrophil population (R2) that is performed when fluorescent labeling with FITC-CD45 (FL1) is plotted on the X axis versus PE-CD14 (FL2) on the Y axis.

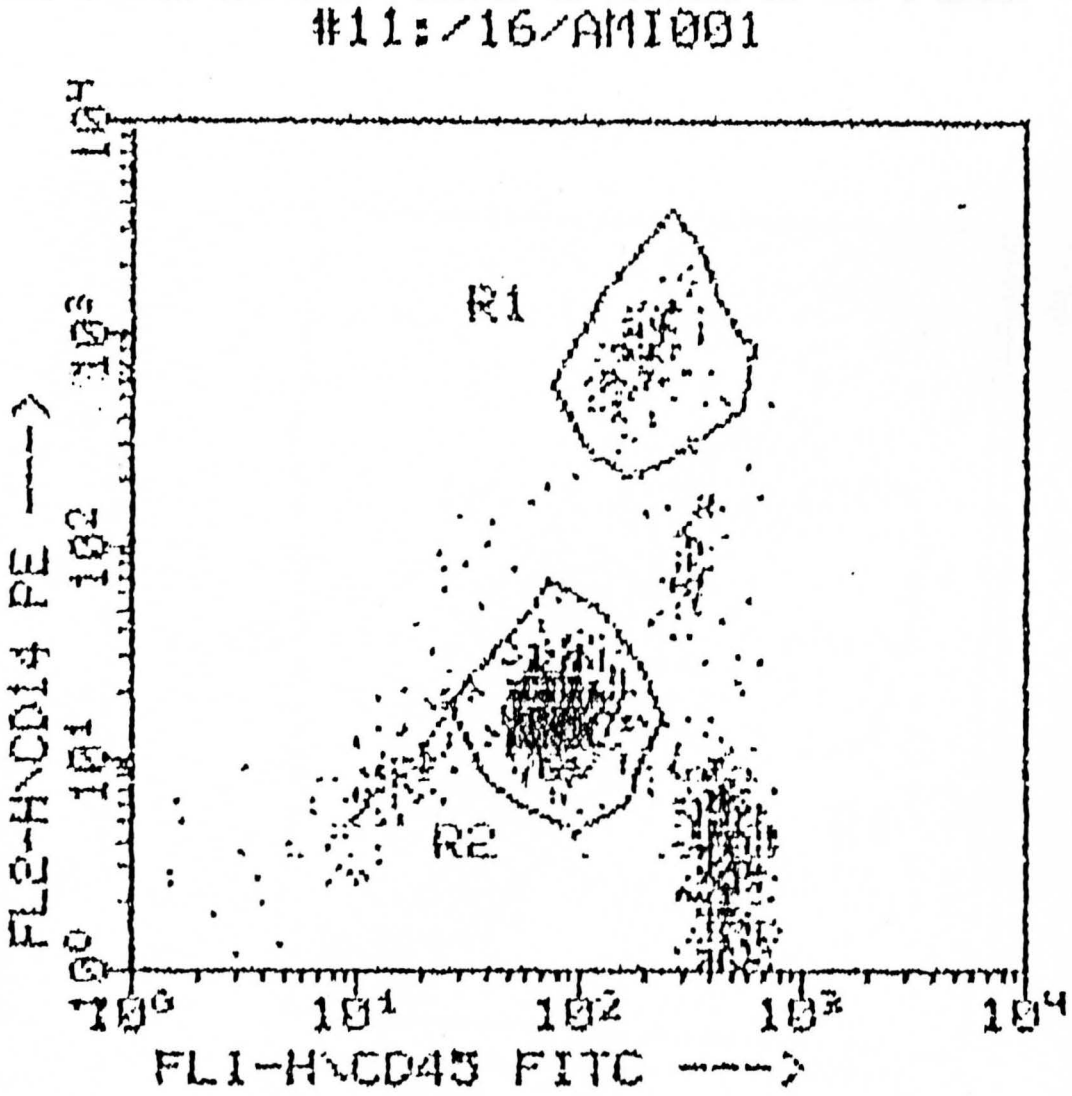

#11:/16/AM1001
R1
R2
FL2-H\CD14 PE ---->
FL1-H\CD45 FITC ---->
10^0
10^1
10^2
10^3
10^4

Figure 4: FSC versus SSC.

This figure depicts gating of the monocyte population (R4) and the neutrophil population (R5) when forward scatter is plotted on the X axis and side scatter is plotted on the Y axis.

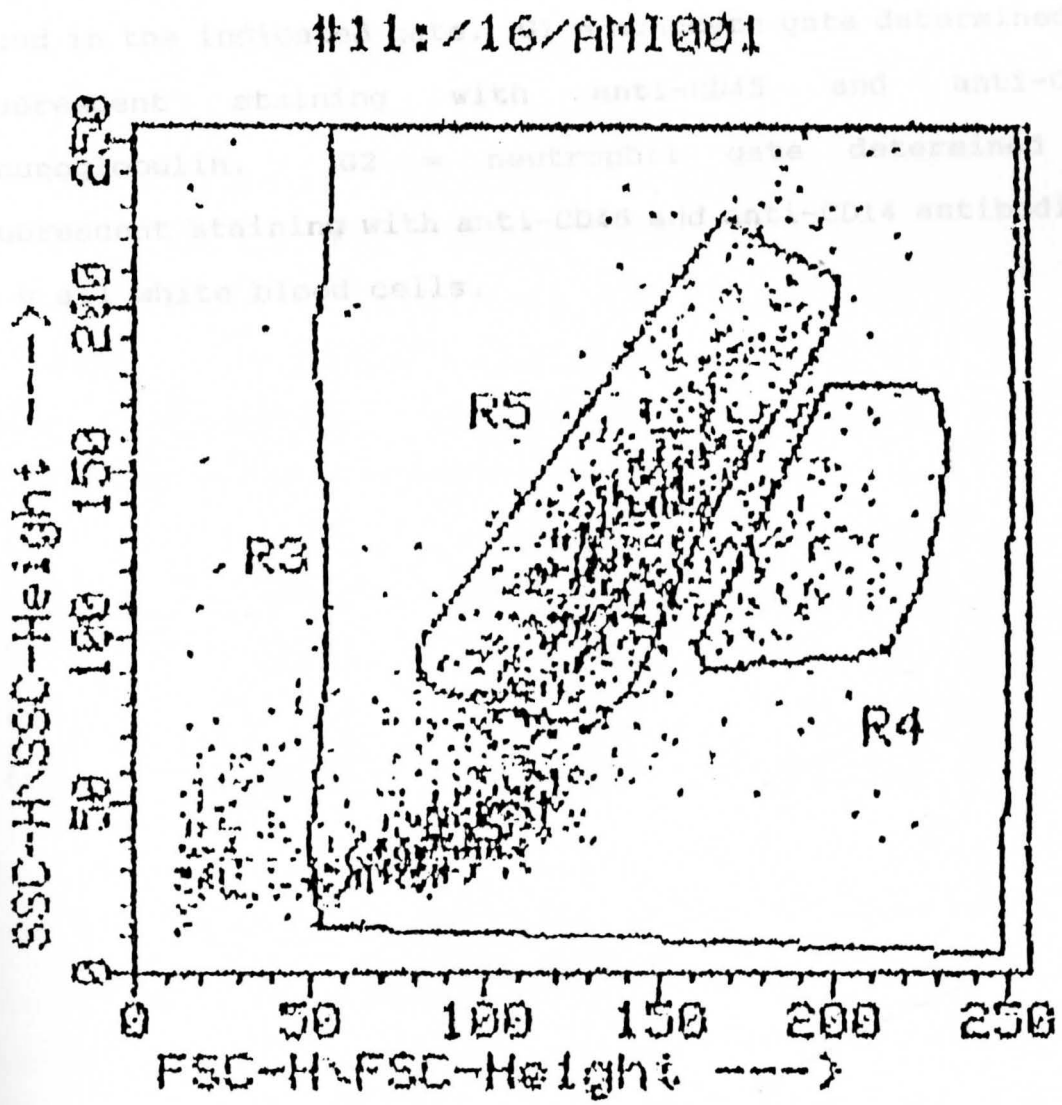

#11:/16/AM1001
SSC-H\SSC-Height --->
FSC-H\FSC-Height --->
R3
R4
R5
0
50
100
150
200
250

Figure 5: Statistics from FSC versus SSC.

The figure shows the statistics generated from neutrophils found inside of gate R5, as shown in Figure 4. Events = number of cells found within the indicated gate (G1-G5). % Gated = percent of total cells found in gate R5 that are also found in the indicated gate. G1 = monocyte gate determined by fluorescent staining with anti-CD45 and anti-CD14 immunoglobulin. G2 = neutrophil gate determined by fluorescent staining with anti-CD45 and anti-CD14 antibodies. G3 = all white blood cells.

#11:/16/AM1001

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

File: #11:/16/AM1001 Sample: AM

Date: 7/16/94 Gate 05= R5

Selected Preferences: Arithmetic/

Total= 2000 Gated= 890

Gate	Events	% Gated	% Total
1 G1	26	2.92	1.30
2 G2	852	95.73	42.60
3 G3	890	100.00	44.50
4 G4	0	0.00	0.00
5 G5	890	100.00	44.50

statistics were generated. The histogram displays the amount of fluorescence per cell on the X axis versus the number of cells on the Y axis. Statistics can then be generated from the histogram which give the peak channel reflecting the highest value of fluorescence obtained (see Figure 6).

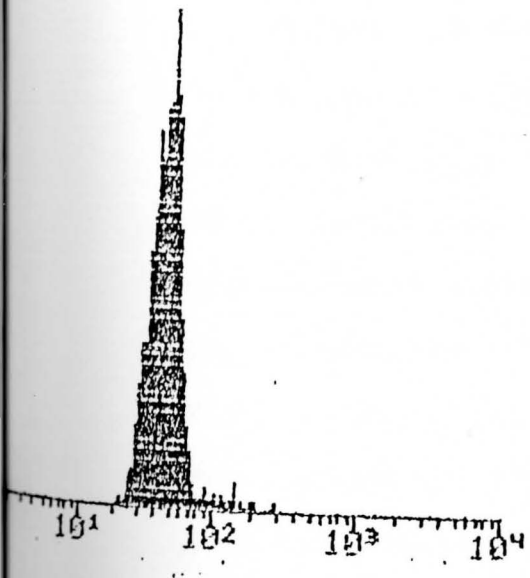
Experiments: Three experiments were conducted. The procedure for all three was identical. Results throughout the three were fairly consistent. We encountered some difficulties with the samples which forced us to drop certain time points. LFA-1 beta was not included in the first experiment due to insufficient antibody. In Experiment 1, three time points, 9, 18 and 24 hours, were dropped from the graphs due to the addition of the wrong secondary antibody to these samples. In Experiment 2, three time points, 9, 12, and 18 hours, were also left off of the experiment due to the crystallization of the paraformaldehyde. The paraformaldehyde crystals were identified as cells by the flow cytometer producing unsatisfactory results.

Statistics: The Sigma Stat program from Jandel Scientific was used to examine the data. For the first two experiments, the three patient samples were compared for statistical differences. The curves of the means for the unstimulated and stimulated for each antibody were analyzed using the one way repeated analysis of variance. When a statistical difference

Figure 6: Histogram and statistics.

This figure depicts the histogram generated from fluorescent cells found within gate R5. The statistics were generated from the histogram and indicate the fluorescence intensity found on the largest population of cells.

1:/16/AMI003\FL1-H\CD3 FITC



#11:/16/AMI003\FL1-H\CD3 FITC
--- Arithmetic Histogram Statistics for #11
Selected Preferences: Arithmetic/Linear
Parameter FL1-H CD3 FITC Gate G5= R5

M	Left,Right	Events	%	Peak	PkChl
0	1.00, 9646	900	100.00	70	46.90

was found, an asterisk was placed above the time point that was greater. An explanation is provided in the figure legend. A paired t-test was performed to check the unstimulated time points versus the stimulated time points. A statistical difference is denoted by a lower case "a" when $p<.05$. An explanation is provided in the figure legend.

CHAPTER III

RESULTS

In these studies, we investigated the levels of the cell adhesion molecules ICAM-1, LFA-1 beta, Mac-1, L-selectin, LFA-1 alpha, and VLA-4 found on unstimulated and *in vitro* stimulated white blood cells over a twenty four hour period. These studies also examined if diurnal variation had any affect on the CAMs.

ICAM-1: Our studies demonstrate consistently high levels of ICAM-1 on both unstimulated and PMA-stimulated neutrophil populations. In Experiment 1, we saw some variation in the curve over time, with the 12 hour time point in the unstimulated cells appearing to be significantly higher than the 1 and 3 hour time points ($p<.05$) (see Figure 7). However, this finding is not supported by the two subsequent experiments in which no significant variation was observed (see Figures 8 and 9). Experiment 1 also showed some increase in ICAM-1 molecules on PMA-stimulated cells. However, this was seen only at the 3 hour time point and is not supported by the data from experiments 1 and 2.

LFA-1 beta: Only two of our experiments examined LFA-1 beta levels on neutrophils. No variation in the curves was noted in either experiment (see Figures 10 and 11). In both, the

Figure 7: ICAM-1 levels, Experiment 1.

For each control patient, data was generated from blood collected at 1, 3, 6, and 12 hours. Addition of the primary Ab directed against ICAM-1 was followed by the addition of the secondary antibody, FITC anti-mouse Ig, as described in Methods. The cells were analyzed by flow cytometry to determine the fluorescence seen in the neutrophil cell population. Histograms were derived from neutrophil gating, as described in Methods. The mean (+/- SEM) of the fluorescent peak channel from three patients is shown. Log fluorescence is plotted against time (hours). The open squares represent stimulated (+PMA) cells, and the open circles represent unstimulated cells. Statistical variance in the curves was determined by Repeated Measures One Way ANOVA analysis. Significant differences ($p<.05$) are denoted by an asterisk. Significance of altered CAM levels resulting from *in vitro* stimulation with PMA was determined by a Students T-test with an "a" representing a p value of $<.05$.

EXPERIMENT 1, ICAM-1

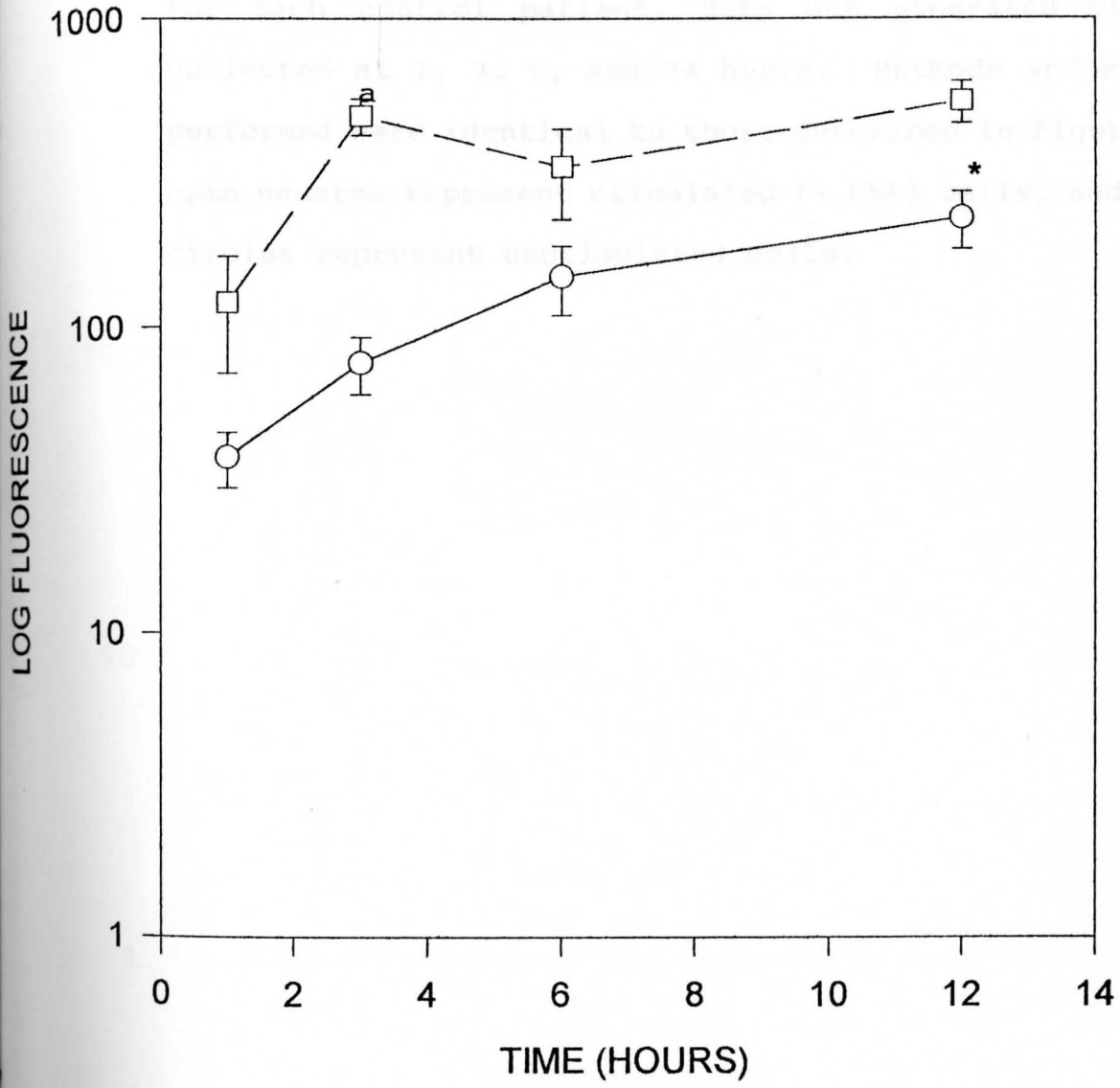


Figure 8: ICAM-1 levels, Experiment 2.

For each control patient, data was generated from blood collected at 1, 3, 6, and 24 hours. Methods and statistics performed were identical to those described in Figure 7. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

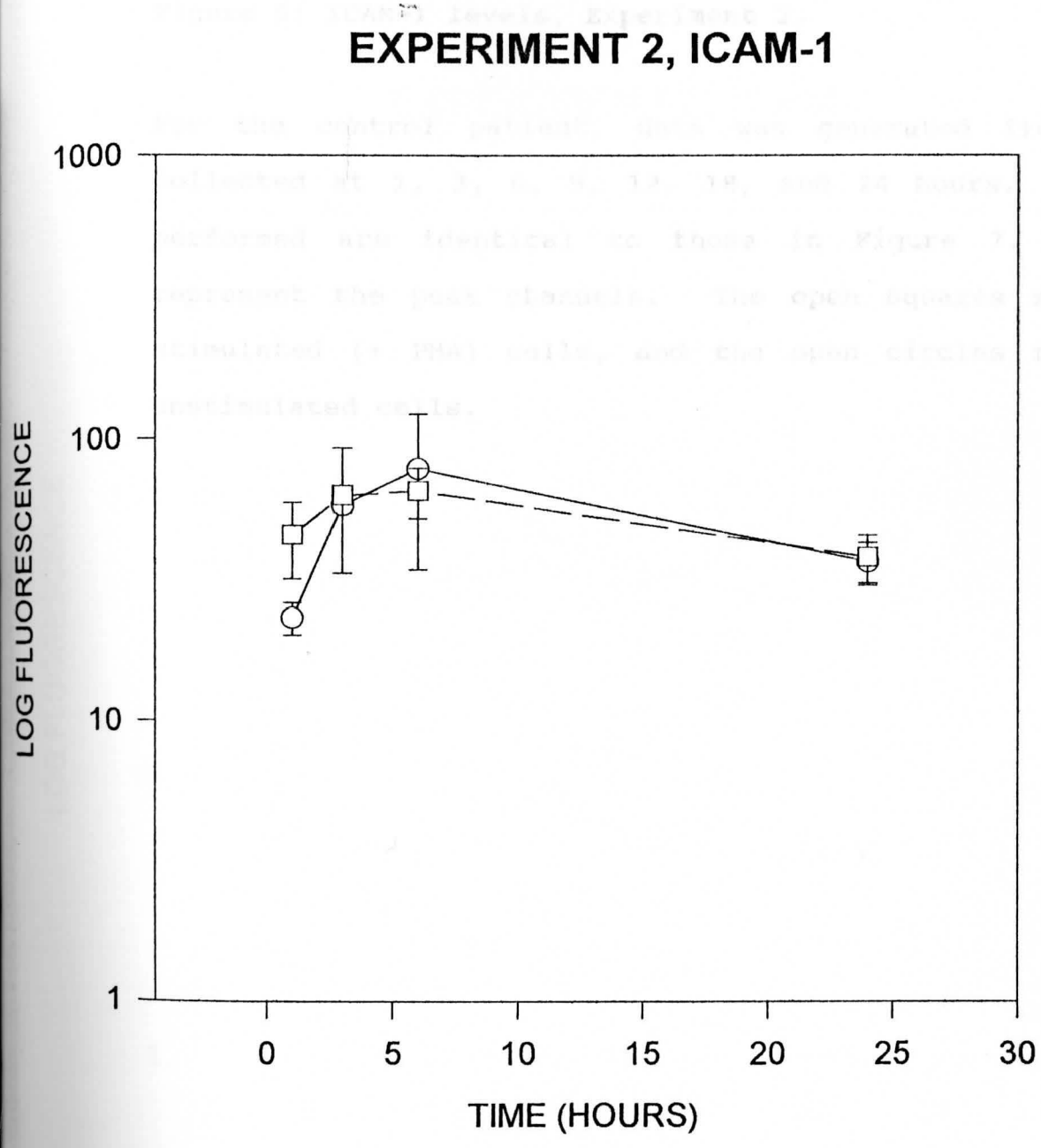

EXPERIMENT 2, ICAM-1
LOG FLUORESCENCE
1000
100
10
1
0
5
10
15
20
25
30
TIME (HOURS)

Figure 9: ICAM-1 levels, Experiment 3.

For the control patient, data was generated from blood collected at 1, 3, 6, 9, 12, 18, and 24 hours. Methods performed are identical to those in Figure 7. Points represent the peak channels. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

EXPERIMENT 3, ICAM-1

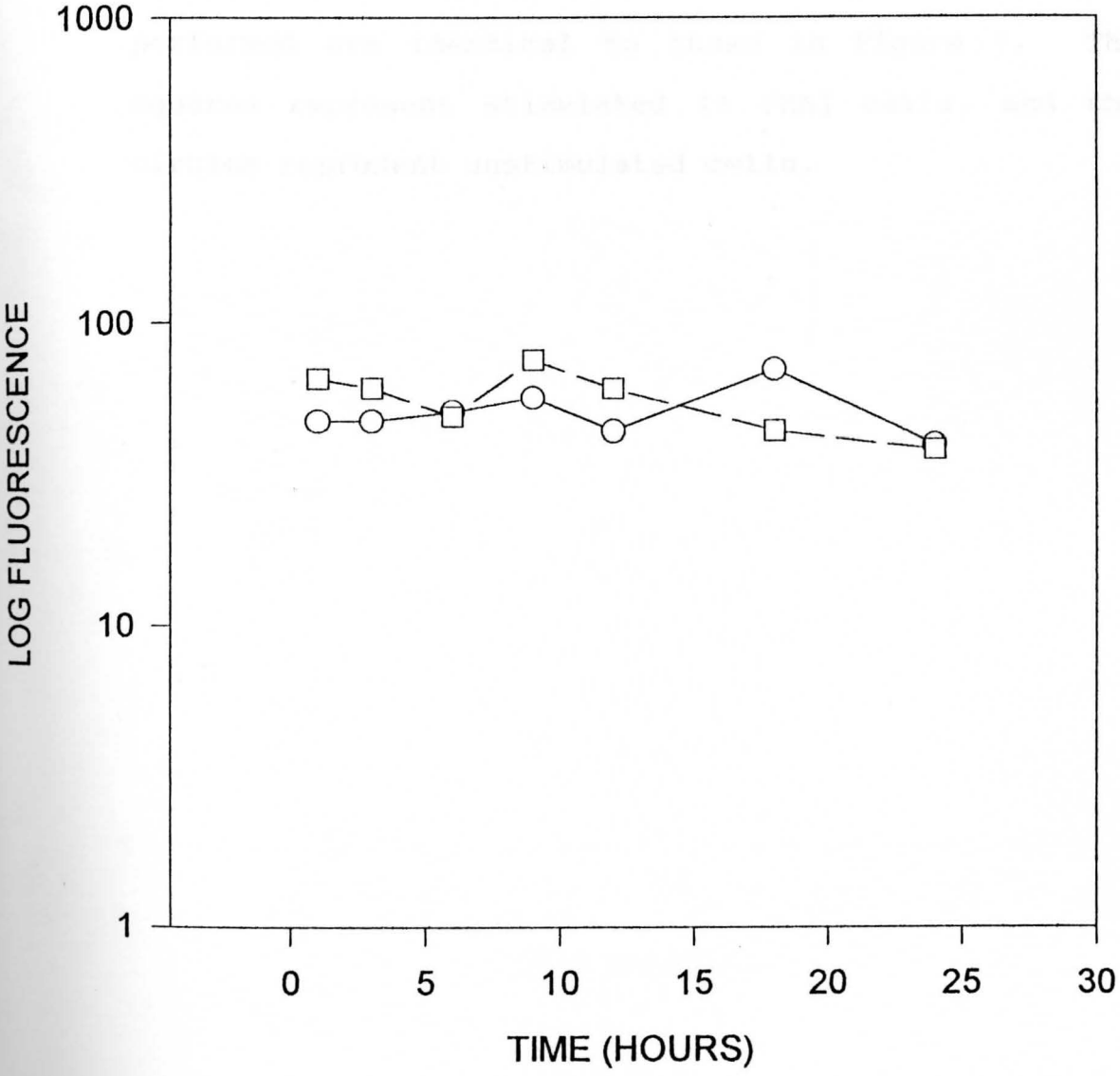


Figure 10: LFA-1 beta levels, Experiment 2.

For each control patient, data was generated from blood collected at 1, 3, 6, and 24 hours. Methods and statistics performed are identical to those in Figure 7. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

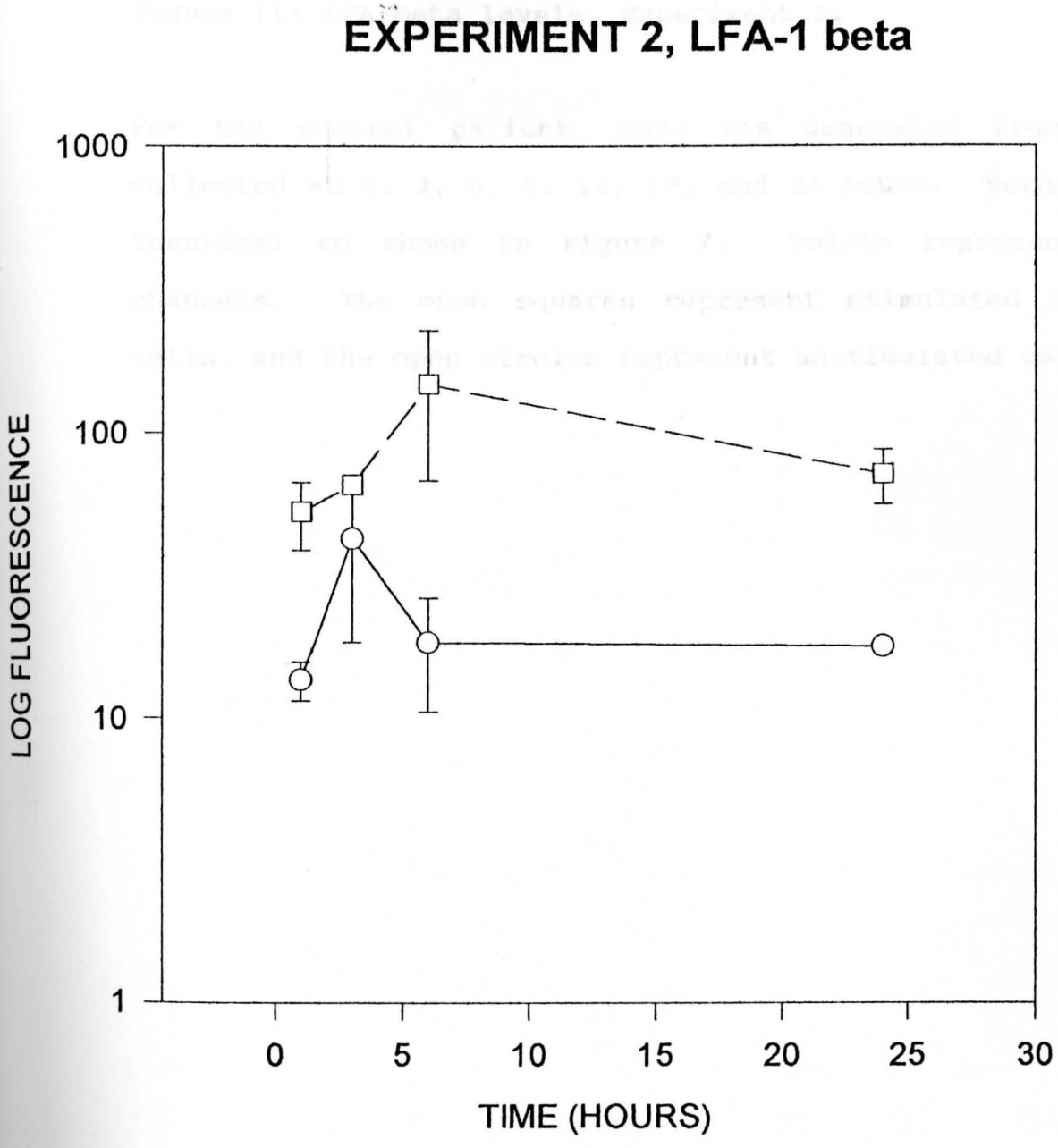
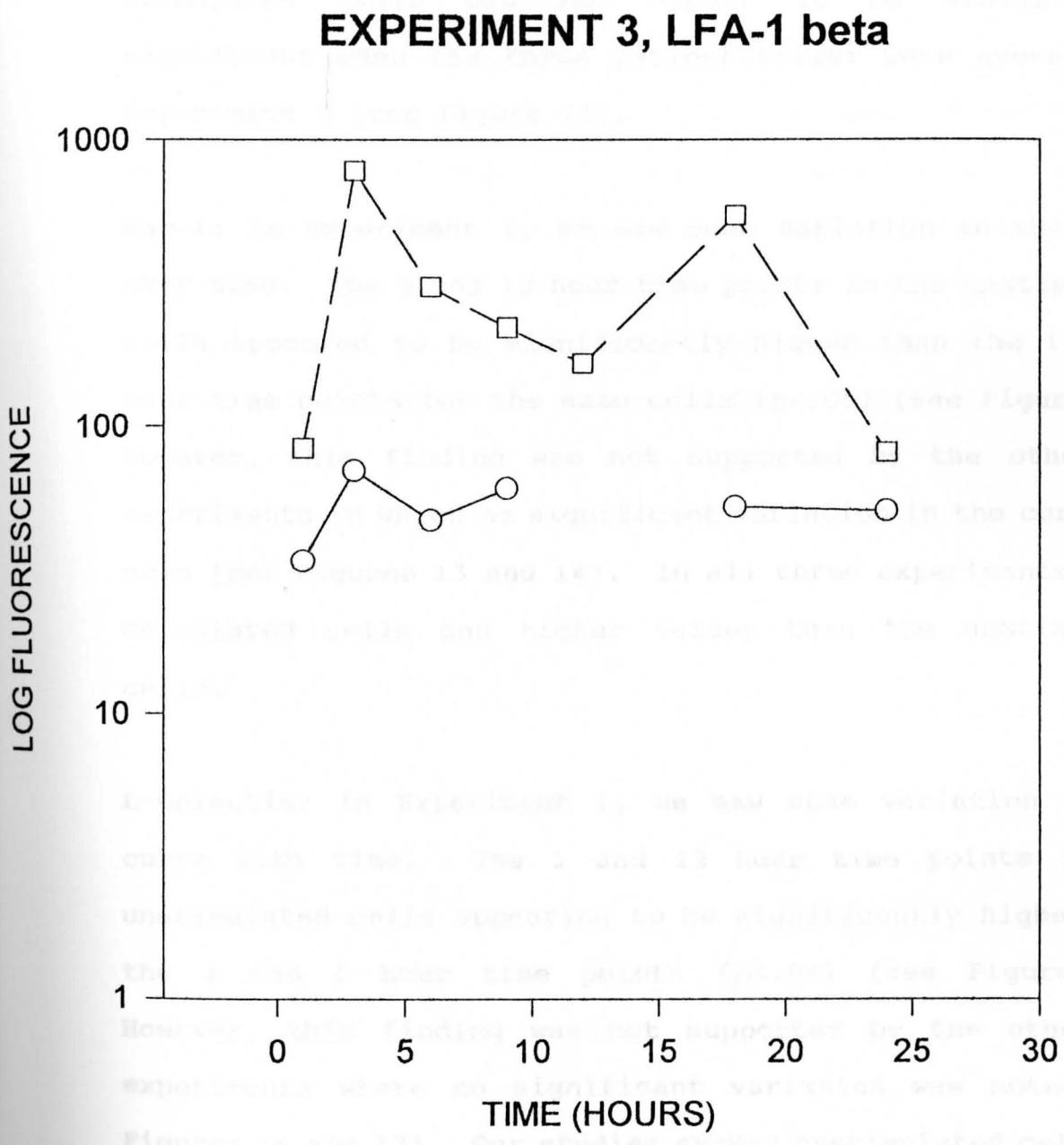

EXPERIMENT 2, LFA-1 beta
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

Figure 11: LFA-beta levels, Experiment 3.

For the control patient, data was generated from blood collected at 1, 3, 6, 9, 12, 18, and 24 hours. Methods are identical to those in Figure 7. Points represent peak channels. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.


EXPERIMENT 3, LFA-1 beta
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

PMA-stimulated cells had higher values than the unstimulated cells. However, the increase in LFA-1 beta molecules on stimulated cells did not appear to be statistically significant when the three patient values were averaged in Experiment 2 (see Figure 10).

Mac-1: In Experiment 1, we saw some variation in the curve over time. The 3 and 12 hour time points in the unstimulated cells appeared to be significantly higher than the 1 and 3 hour time points for the same cells ($p<.05$) (see Figure 12). However, this finding was not supported by the other two experiments in which no significant variation in the curve was seen (see Figures 13 and 14). In all three experiments, PMA-stimulated cells had higher values than the unstimulated cells.

L-selectin: In Experiment 1, we saw some variation in the curve with time. The 3 and 12 hour time points in the unstimulated cells appearing to be significantly higher than the 1 and 6 hour time points ($p<.05$) (see Figure 15). However, this finding was not supported by the other two experiments where no significant variation was noted (see Figures 16 and 17). Our studies showed unstimulated cells had higher values than the PMA-stimulated cells in all three experiments.

Figure 12: Mac-1 levels, Experiment 1.

For each control patient, data was generated from blood collected at 1, 3, 6, and 12 hours. Methods and statistics are identical to those in Figure 7. Significance of altered CAM levels resulting from in vitro stimulation with PMA was determined by Students T-test with an "a" representing a p value of <.05. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

EXPERIMENT 1, Mac-1

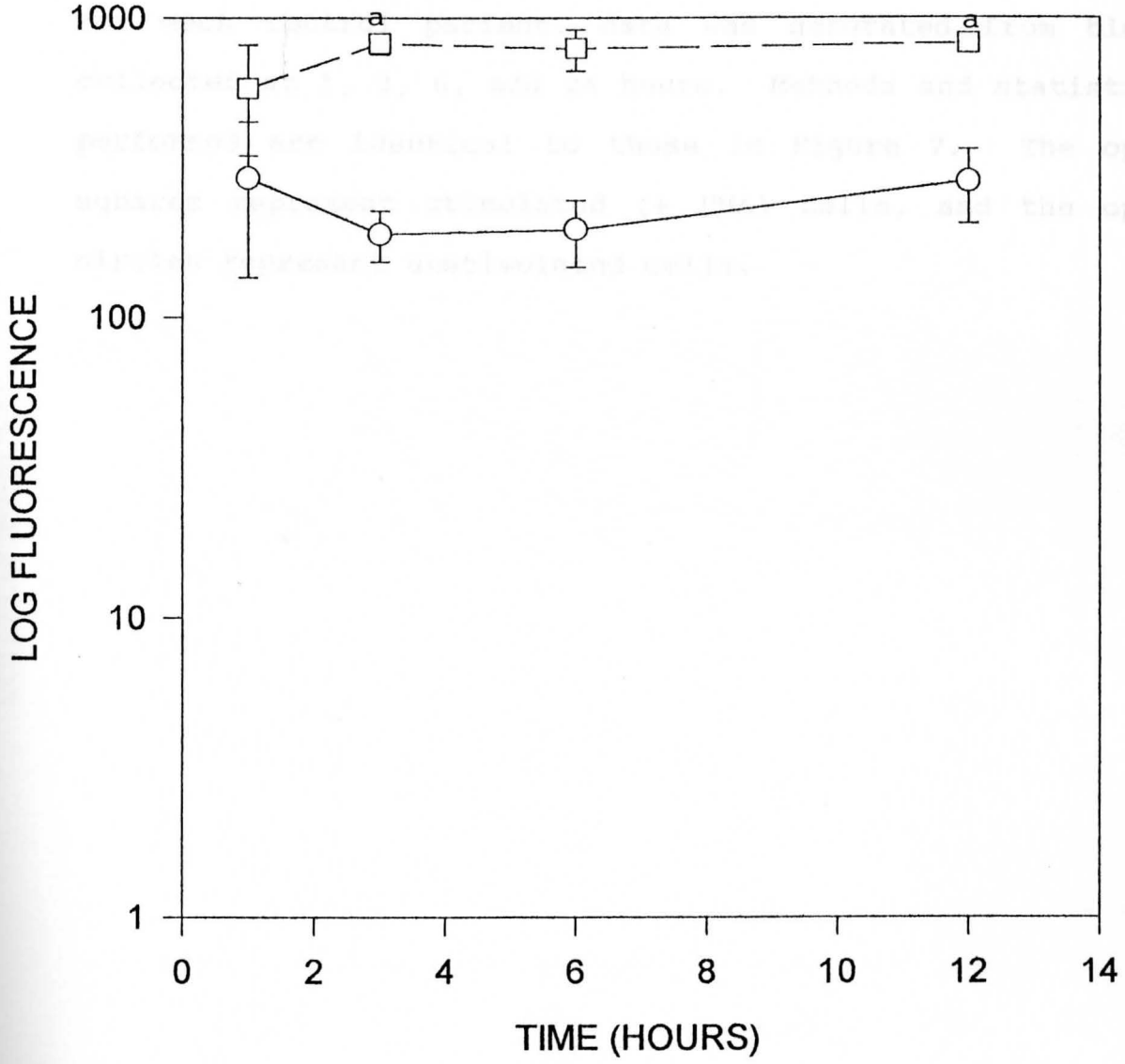
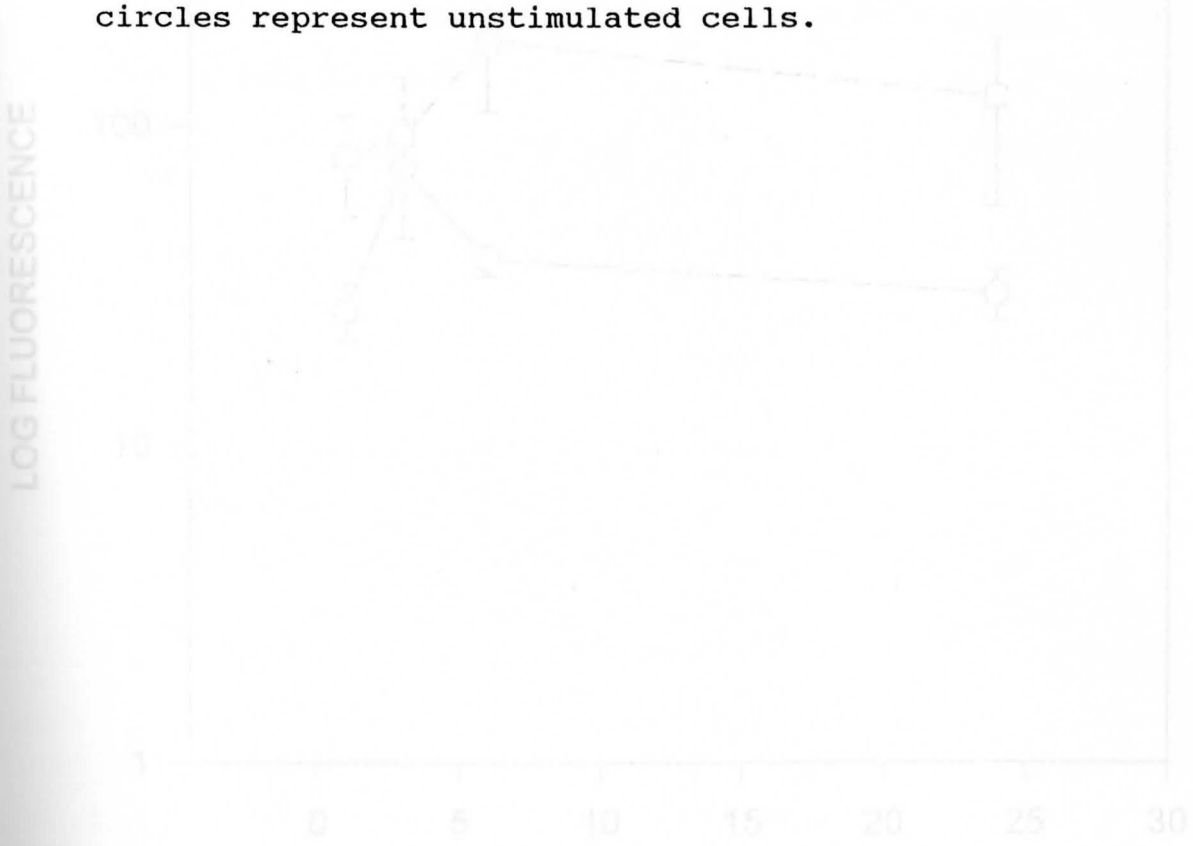


Figure 13: Mac-1 levels, Experiment 2.

For each control patient, data was generated from blood collected at 1, 3, 6, and 24 hours. Methods and statistics performed are identical to those in Figure 7. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.



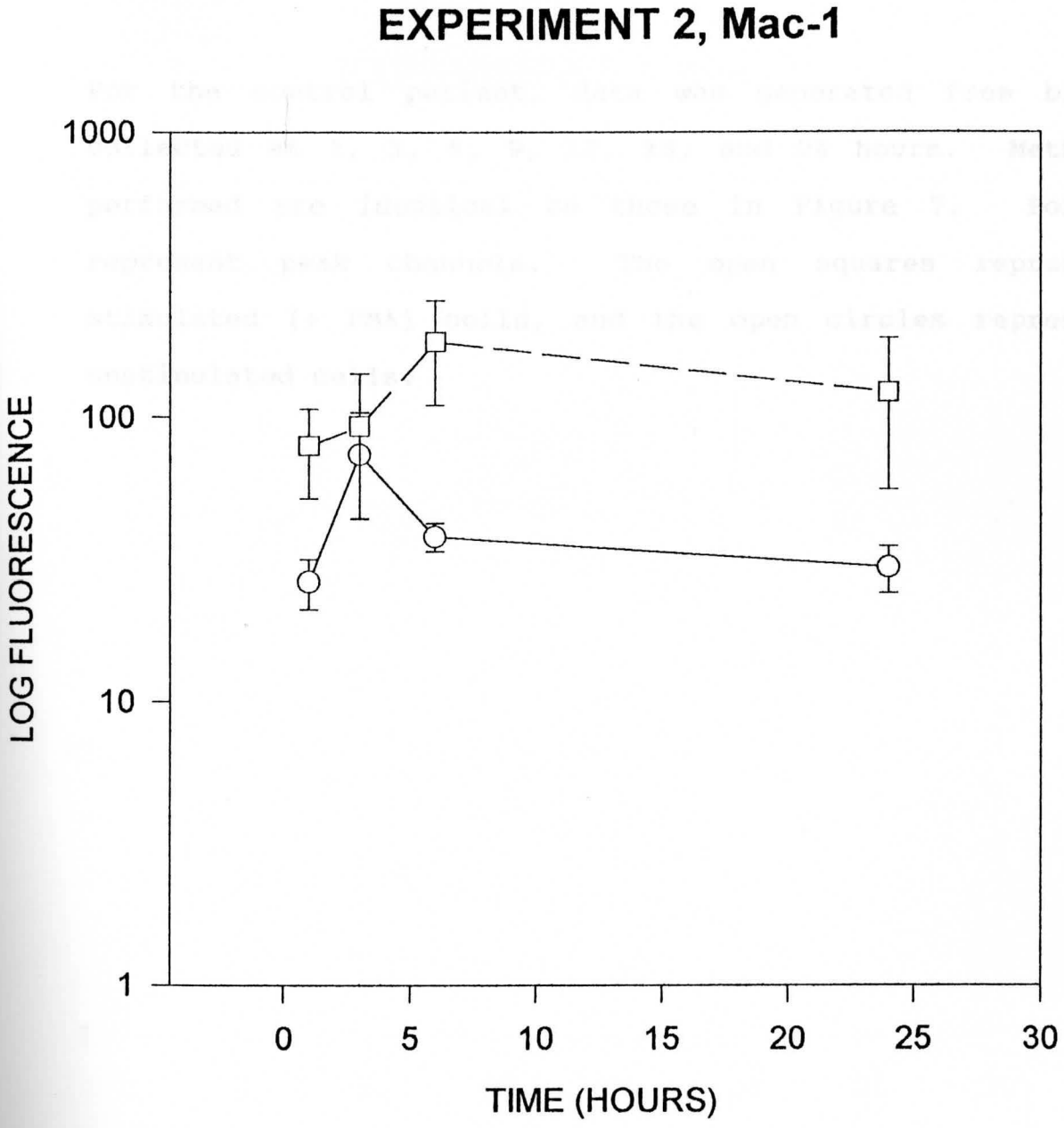

EXPERIMENT 2, Mac-1
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

Figure 14: Mac-1 levels, Experiment 3.

For the control patient, data was generated from blood collected at 1, 3, 6, 9, 12, 18, and 24 hours. Methods performed are identical to those in Figure 7. Points represent peak channels. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

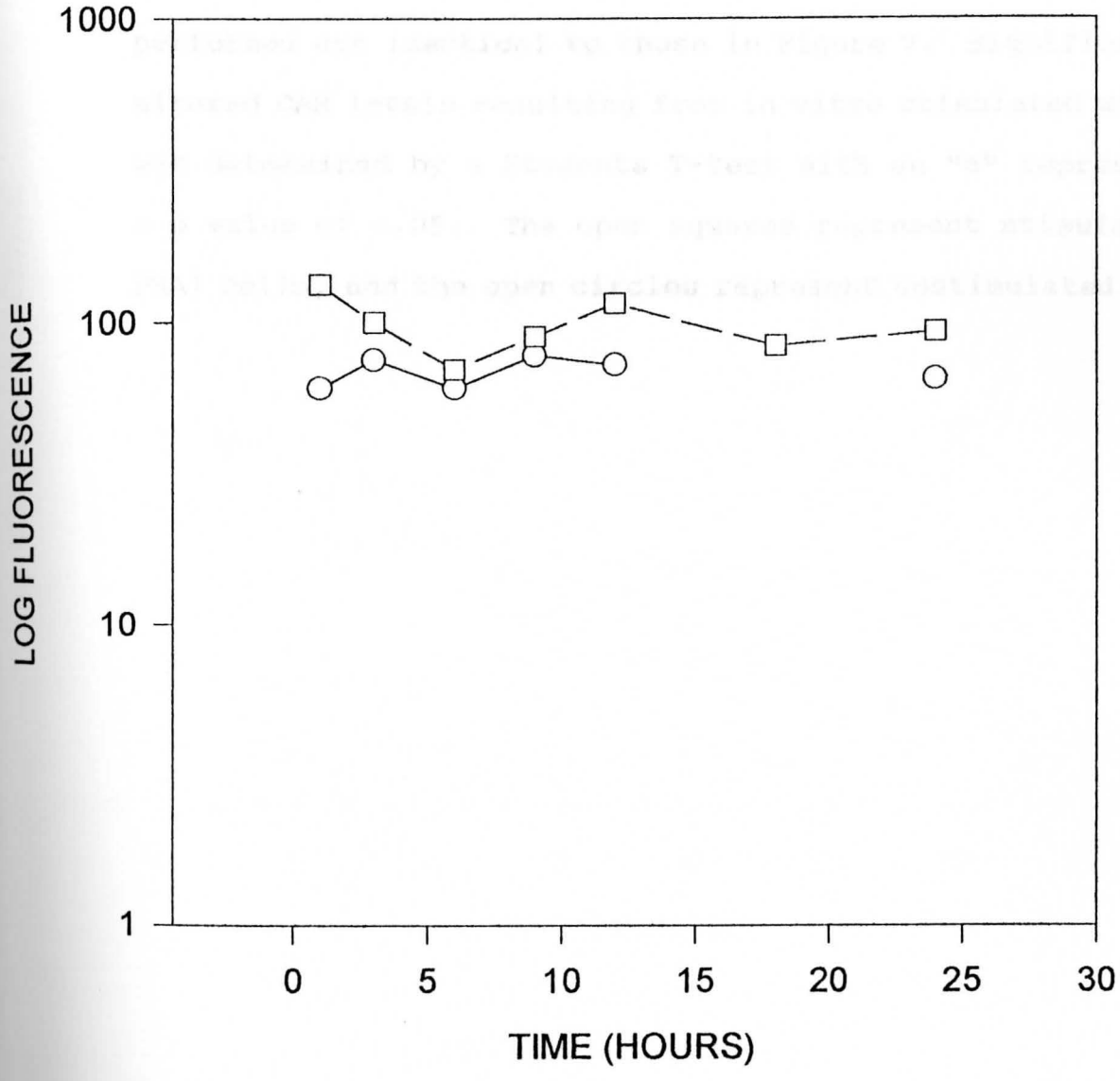

EXPERIMENT 3, Mac-1
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

Figure 15: L-selectin levels, Experiment 1.

For each control patient, data was generated from blood collected at 1, 3, 6, and 12 hours. Methods and statistics performed are identical to those in Figure 7. Significance of altered CAM levels resulting from in vitro stimulated with PMA was determined by a Students T-test with an "a" representing a p value of <.05. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

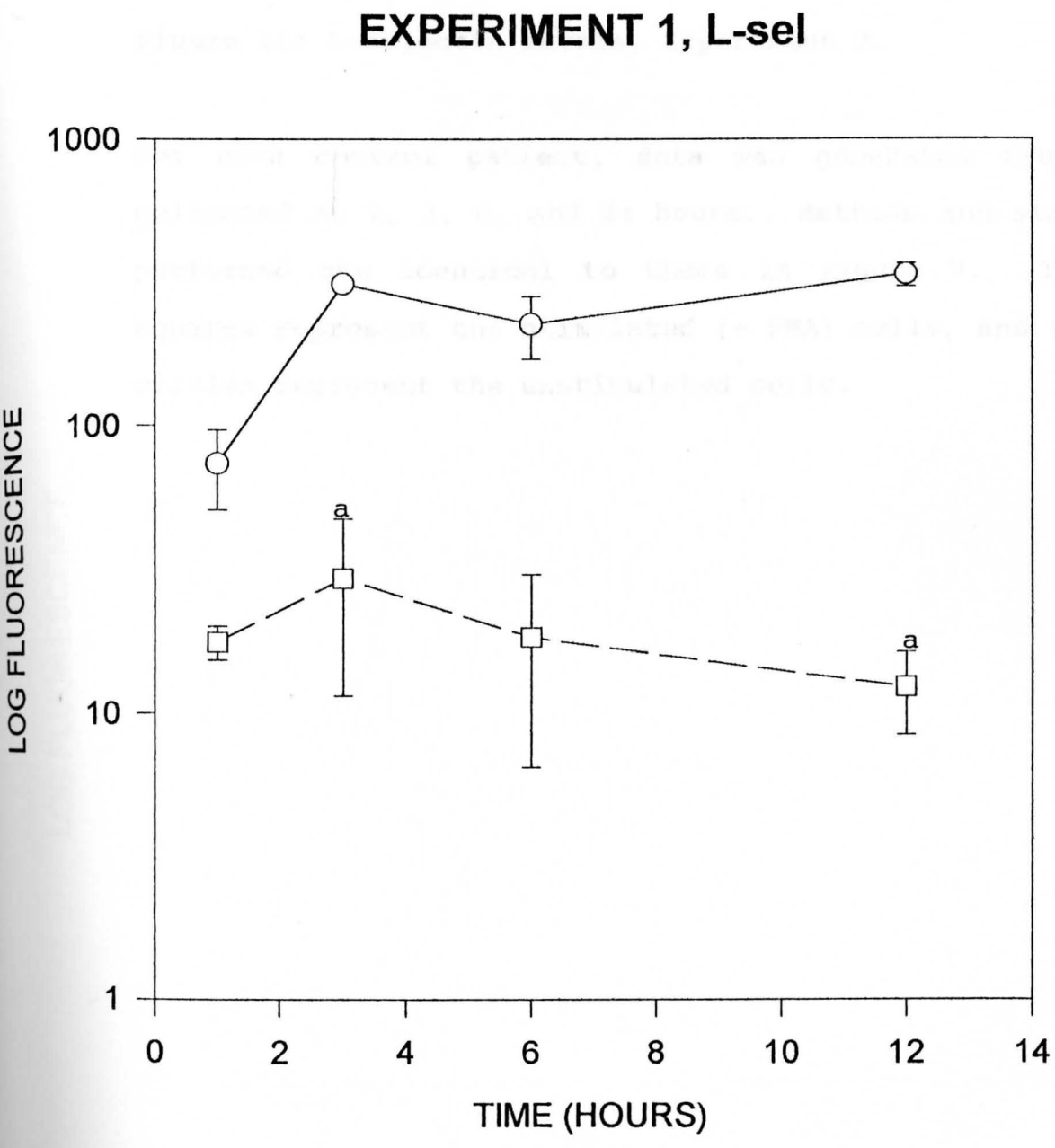

EXPERIMENT 1, L-sel
1000
100
10
1
LOG FLUORESCENCE
a
a
0
2
4
6
8
10
12
14
TIME (HOURS)

Figure 16: L-selectin levels, Experiment 2.

For each control patient, data was generated from blood collected at 1, 3, 6, and 24 hours. Methods and statistics performed are identical to those in Figure 7. The open squares represent the stimulated (+ PMA) cells, and the open circles represent the unstimulated cells.

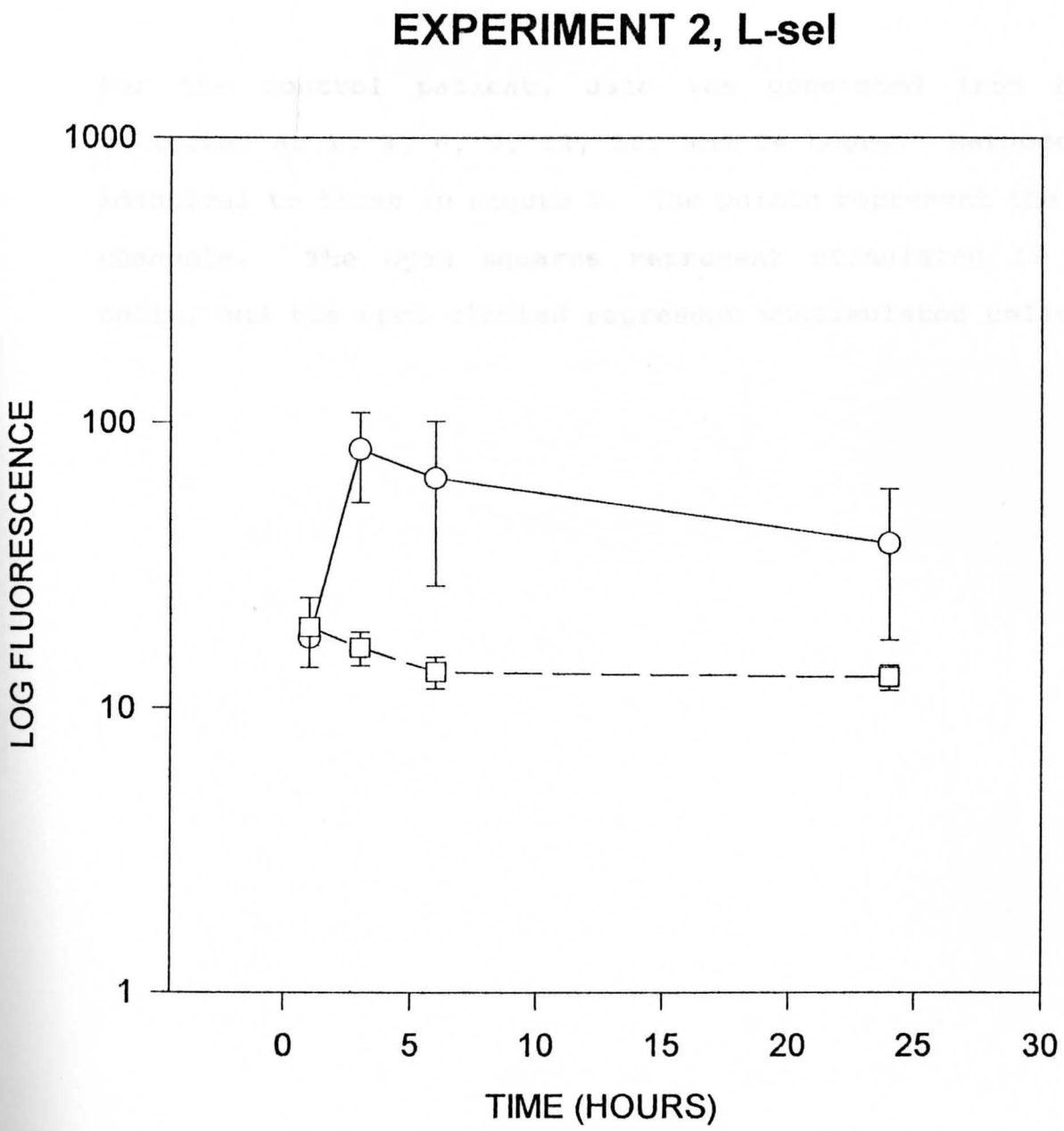
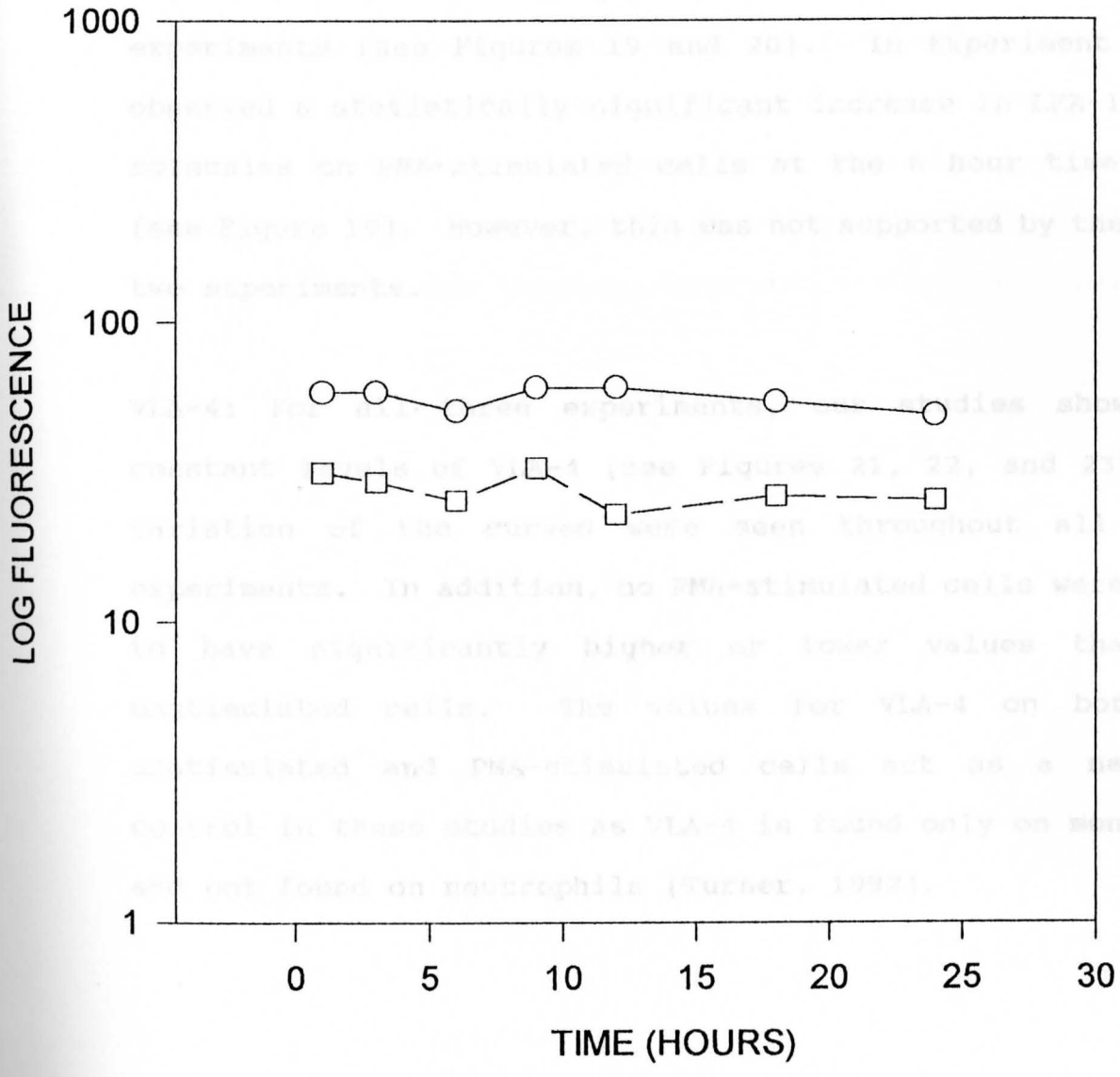

EXPERIMENT 2, L-sel
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

Figure 17: L-selectin levels, Experiment 3.

For the control patient, data was generated from blood collected at 1, 3, 6, 9, 12, 18, and 24 hours. Methods are identical to those in Figure 7. The points represent the peak channels. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.


EXPERIMENT 3, L-sel
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

LFA-1 alpha: In Experiment 1, a statistically significant variation was seen in the curve; an increase in LFA-1 alpha molecules was noted at the 3, 6, and 12 hour time points for both the unstimulated and the PMA-stimulated cells (see Figure 18). However, this finding was not supported by the other two experiments (see Figures 19 and 20). In Experiment 2, we observed a statistically significant increase in LFA-1 alpha molecules on PMA-stimulated cells at the 6 hour time point (see Figure 19). However, this was not supported by the other two experiments.

VLA-4: For all three experiments, our studies show low, constant levels of VLA-4 (see Figures 21, 22, and 23). No variation of the curves were seen throughout all three experiments. In addition, no PMA-stimulated cells were found to have significantly higher or lower values than the unstimulated cells. The values for VLA-4 on both the unstimulated and PMA-stimulated cells act as a negative control in these studies as VLA-4 is found only on monocytes and not found on neutrophils (Turner, 1992).

Figure 18: LFA-1 alpha levels, Experiment 1.

For each control patient, data was generated from blood collected at 1, 3, 6, and 12 hours. Methods and statistics performed are identical to those in Figure 7. Statistical variance in the curves was determined by Repeated Measures One Way ANOVA analysis. Significant differences are denoted by an asterisk. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

EXPERIMENT 1, LFA-1 alpha

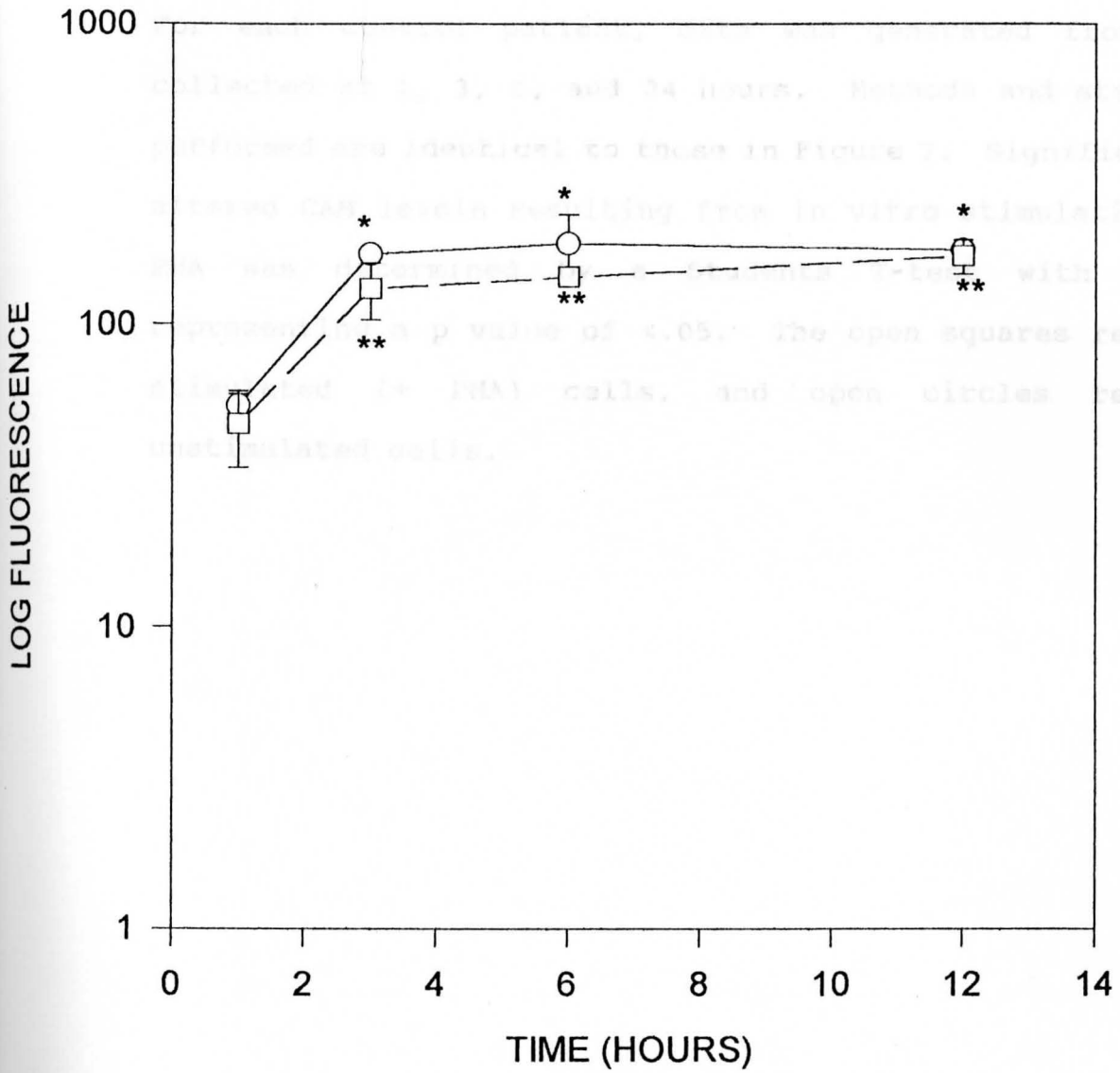


Figure 19: LFA-1 alpha levels, Experiment 2.

For each control patient, data was generated from blood collected at 1, 3, 6, and 24 hours. Methods and statistics performed are identical to those in Figure 7. Significance of altered CAM levels resulting from in vitro stimulation with PMA was determined by a Students T-test with an "a" representing a p value of <.05. The open squares represent stimulated (+ PMA) cells, and open circles represent unstimulated cells.

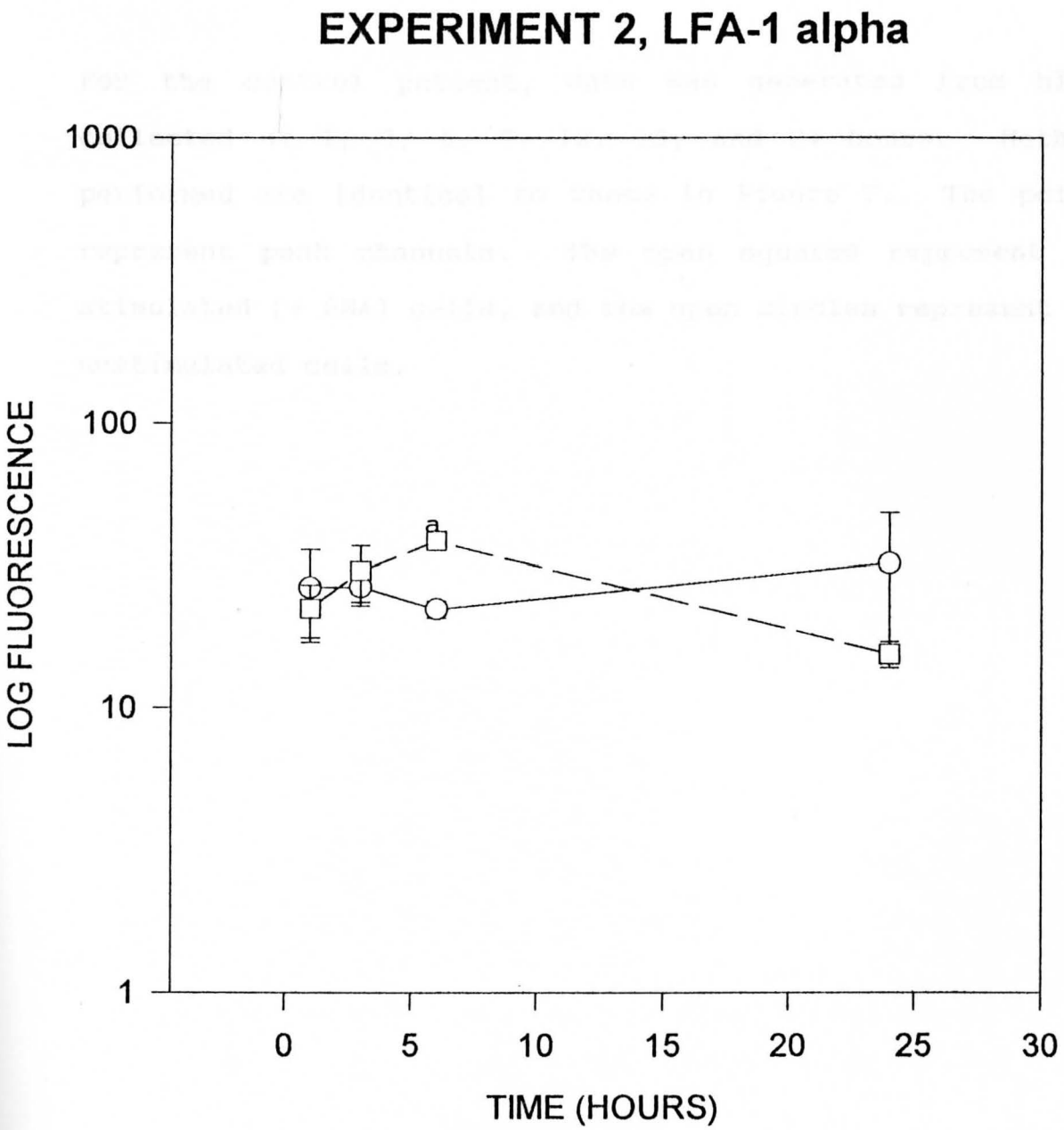

EXPERIMENT 2, LFA-1 alpha
1000
100
10
1
LOG FLUORESCENCE
a
0
5
10
15
20
25
30
TIME (HOURS)

Figure 20: LFA-1 alpha levels, Experiment 3.

For the control patient, data was generated from blood collected at 1, 3, 6, 9, 12, 18, and 24 hours. Methods performed are identical to those in Figure 7. The points represent peak channels. The open squares represent the stimulated (+ PMA) cells, and the open circles represent the unstimulated cells.

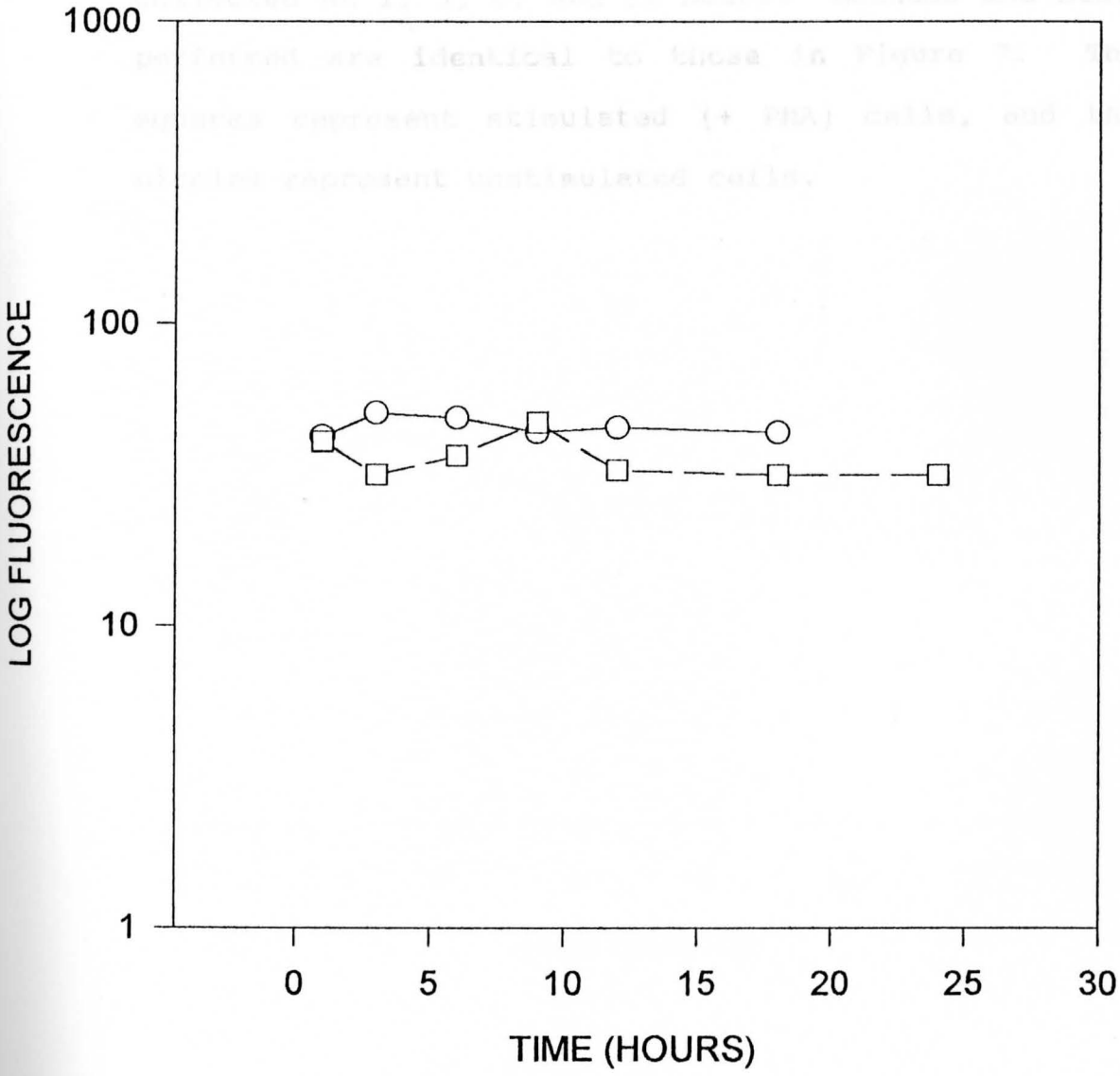

EXPERIMENT 3, LFA-1 alpha
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

Figure 21: VLA-4 levels, Experiment 1.

For each control patient, data was generated from blood collected at 1, 3, 6, and 12 hours. Methods and statistics performed are identical to those in Figure 7. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

EXPERIMENT 1, VLA-4

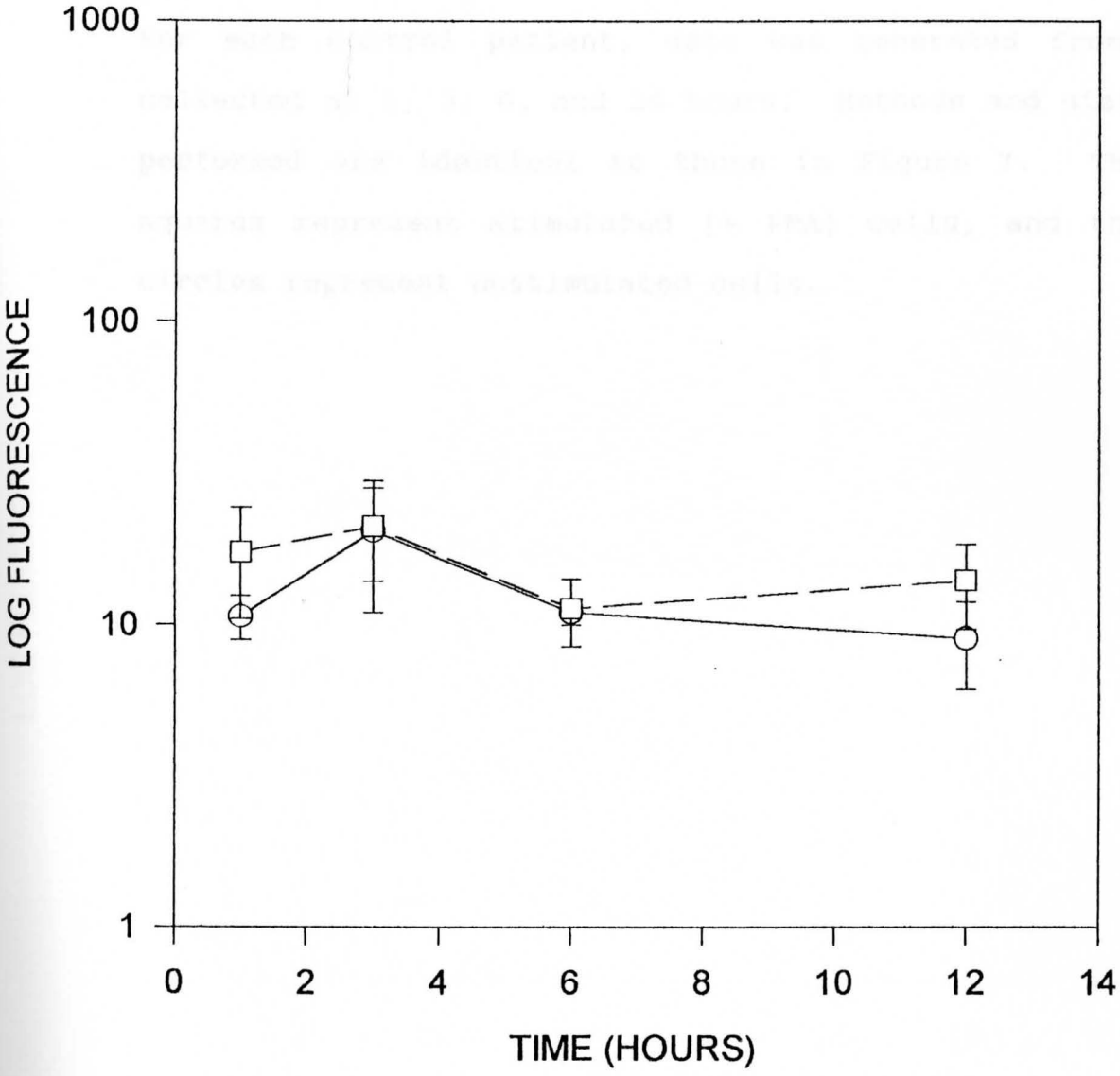


Figure 22: VLA-4 levels, Experiment 2.

For each control patient, data was generated from blood collected at 1, 3, 6, and 24 hours. Methods and statistics performed are identical to those in Figure 7. The open squares represent stimulated (+ PMA) cells, and the open circles represent unstimulated cells.

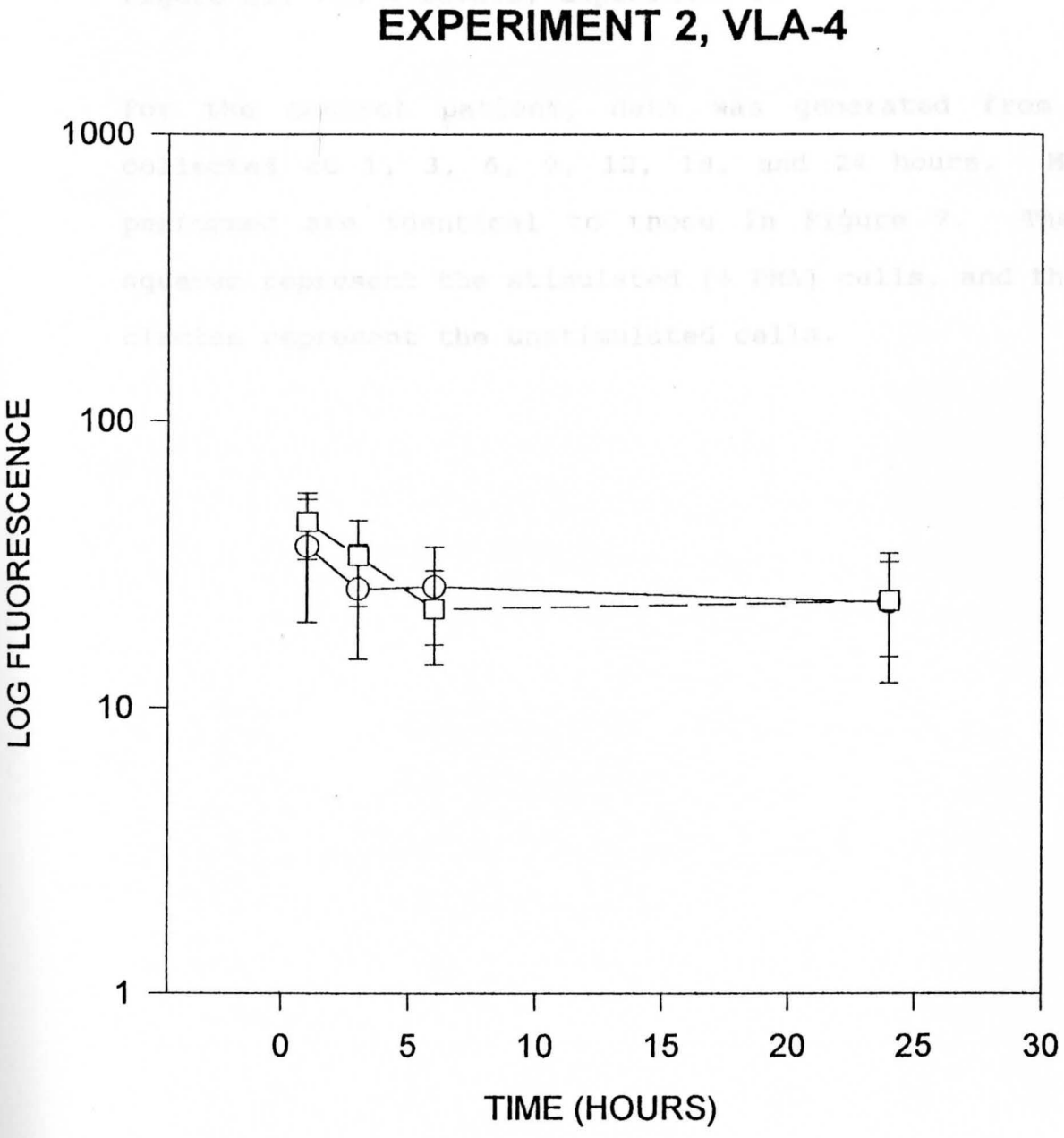
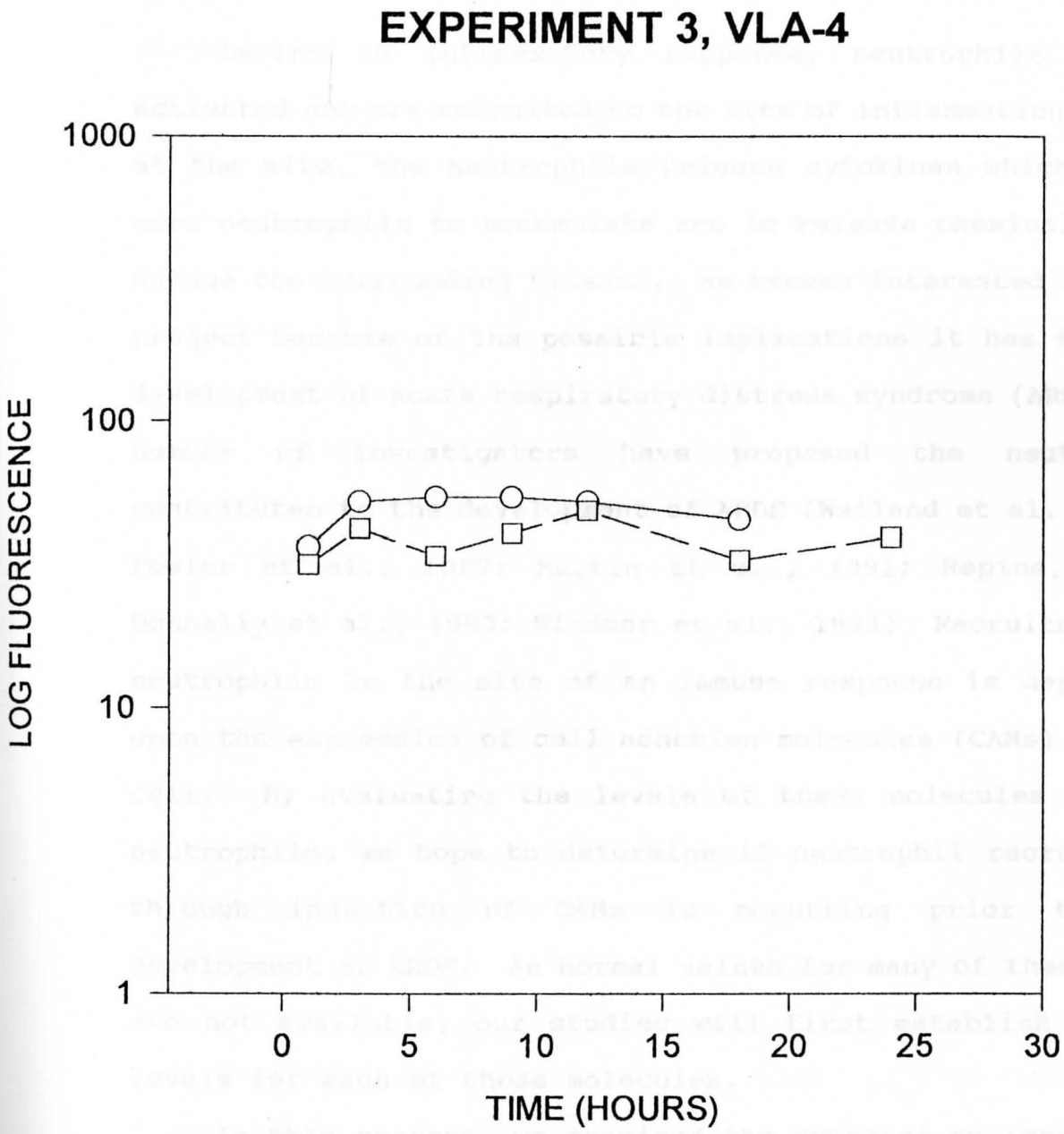

EXPERIMENT 2, VLA-4
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

Figure 23: VLA-4 levels, Experiment 3.

For the control patient, data was generated from blood collected at 1, 3, 6, 9, 12, 18, and 24 hours. Methods performed are identical to those in Figure 7. The open squares represent the stimulated (+ PMA) cells, and the open circles represent the unstimulated cells.


EXPERIMENT 3, VLA-4
1000
100
10
1
LOG FLUORESCENCE
0
5
10
15
20
25
30
TIME (HOURS)

CHAPTER IV

DISCUSSION

During an inflammatory response, neutrophils become activated and are recruited to the site of inflammation. Once at the site, the neutrophils release cytokines which cause more neutrophils to accumulate and to release chemicals that damage the surrounding tissues. We became interested in this project because of the possible implications it has for the development of acute respiratory distress syndrome (ARDS). A number of investigators have proposed the neutrophil contributes to the development of ARDS (Weiland et al., 1986; Fowler et al., 1987; Martin et al., 1991; Repine, 1992; Donnelly et al., 1993; Windsor et al., 1993) Recruitment of neutrophils to the site of an immune response is dependent upon the expression of cell adhesion molecules (CAMs) on the cell. By evaluating the levels of these molecules on the neutrophils, we hope to determine if neutrophil recruitment through induction of CAMs is occurring prior to the development of ARDS. As normal values for many of these CAMs are not available, our studies will first establish normal levels for each of these molecules.

In this project, we examined the presence or absence of the cell adhesion molecules, ICAM-1, LFA-1 beta, Mac-1, L-selectin, LFA-1 alpha, and VLA-4, on neutrophils. We measured the levels of CAMs found on unstimulated and PMA-stimulated

neutrophils using a flow cytometer. To control for possible endotoxin contamination in our reagents, we examined both unstimulated and PMA-stimulated cells. L-selectin levels in normal controls should act as an extremely sensitive indicator of cell activation either through exposure to endotoxin, PMA, or other stimuluses. L-selectin is shed very rapidly in stimulated cells (Kishimoto et al., 1989). In unstimulated, normal controls, incubation of cells with PMA should result in decreased levels of L-selectin. It is also likely that cells are activated *in vivo* following sepsis. In the presence of normal controls, a lack of response to PMA-stimulation *in vitro* could indicate activation of patient samples *in vivo*. The ability to detect cell activation *in vivo* could be useful in the prediction of patient susceptibility to developing ARDS.

A factor influencing the results could be the diurnal or circadian rhythm that is related to the continual fluctuations in the levels of hormones over a twenty four hour period. We looked at normal patient samples in order to observe any effects caused by diurnal variation. These hormone oscillations r0 '3 to light exposure and activity levels of the individual. Some hormones that undergo circadian variation in their levels are retinoic acid (Nau, 1993), thyrotropin (Stewart et al., 1994), somatostatin, somatomedins, melatonin, growth hormone, and cortisol (Sherwood, 1989). Growth hormone has a characteristic diurnal

pattern. During the day, low, constant levels are seen. But at night, after deep sleep, the levels increase then drop again as the night progresses. Cortisol and melatonin levels also follow the pattern exhibited by growth hormone (Sherwood, 1989). Diurnal variation has also been reported to affect retinoic acid compounds (Nau, 1993) and secretions of thyrotropin (Stewart et al., 1994).

There is reason to believe that these hormone fluctuations may influence white blood cell levels or the state of activation of these cells in normal individuals. Several hormones whose levels follow a circadian rhythm have been shown to affect white blood cells. Cortisol has been found to have a variety of effects on white blood cells including alterations in monocyte function and reduction of monocyte numbers (Vagnucci and Winkelstein, 1993). Retinoic acid has been reported to increase the levels of cell adhesion molecules CD11a (LFA-1) and CD11b (Mac-1) (Matikainen and Hurme, 1994). Normal controls have also been shown to have diurnal variations in their lymphocyte subsets (McCoy et al., 1990; Vagnucci and Winkelstein, 1993). CD4 positive T cells and lymphocyte counts have also shown diurnal variation (Vagnucci and Winkelstein, 1993; Holodniy et al., 1994), so there is a possibility that other white blood cells, such as neutrophils, will exhibit this pattern.

To determine if diurnal variation of CAMs exists, normal samples were drawn over a period of 24 hours and levels of

CAMs plotted for each time point. One way repeated measures analysis of variance (ANOVA) was completed on the data. This statistical program examined the unstimulated and the PMA-stimulated curve separately to determine any significant variation in either curve. If no statistical difference is present, no diurnal variation is indicated. A statistical difference reflects a variance which is statistically significant and could result from diurnal variation. ICAM-1 (control) and LFA-1 alpha (control and stimulated) showed significant variation in Experiment 1, as reflected in the statistical difference indicated. However, in Experiment 2, no statistical difference was noted, so diurnal variation was not likely to be a contributing factor for any of the data.

Kishimoto and coworkers examined Mac-1 and L-selectin levels in PMA-stimulated neutrophils using identical Abs and methods as those used for these experiments (1989). In their studies, Mac-1 levels increased with PMA stimulation, and L-selectin levels were shown to decrease. Kahn and coworkers reported a decrease in L-selectin levels when neutrophils were activated (1994). In addition, Devery and coworkers reported activated murine PMNs exhibited an increase in Mac-1 and a decrease in L-selectin (1994).

In our studies, a paired t-test was performed to compare the unstimulated and PMA-stimulated cells at specific time points. A significant difference was found in Experiment 1 for ICAM-1, Mac-1, and L-selectin but was not supported by

data from the other two experiments. In Experiment 2, LFA-1 alpha exhibited a significant difference which was not supported by the other two experiments. Our results, while not statistically significant were, however, consistent with Kishimoto's data throughout the three experiments. When Mac-1 and L-selectin levels for individual patients were examined, we saw a consistent increase in Mac-1 levels with PMA stimulation and a consistent decrease in L-selectin levels. Our work was also consistent with these results throughout all three experiments.

Silber and coworkers examined blood lymphocytes during a delayed type hypersensitivity reaction and saw no change in CD11a (alpha chain, LFA-1 alpha) or CD18 (beta chain, LFA-1 beta) (1994). If this finding is extended to neutrophils, our results for CD11a (LFA-1 alpha) were more consistent with those of Silber and coworkers than were our results for CD18 (LFA-1 beta). However, their study examined a T cell mediated response rather than an inflammatory response. For other cell adhesion molecules, the data available in the literature is scarce. ICAM-1 and L-selectin levels on lymphocytes were examined during an inflammatory response and reported to increase (Seymour et al., 1993). This data may not be relevant for neutrophils; however, as the L-selectin levels have been reported to decrease on neutrophils (see above). VLA-4 is found only on monocytes, so the levels should have been very low as seen in our data (Turner, 1992).

It is our hope that this information will allow detailed analysis of patient populations and comparison to normal controls. Future studies will examine the effect of CAMs in the development of ARDS. A better understanding of the role of CAMs in the onset of inflammatory responses may aid in the development of compounds able to modify the immune response and reduce the incidence of tissue injury.

BIBLIOGRAPHY

Arnaout, Amin A. 1990. Structure and Function of the Leukocyte Adhesion Molecules CD11/CD18. *Blood* 75(5):1037-1050.

Atkins, Patricia J., Mary E. Egloff, and David C. Williams. 1994. Respiratory consequences of multisystem crisis: The adult respiratory distress syndrome. *Crit Care Nurs Q* 16(4):27-38.

Brass, Nancy J. 1994. Predisposition to multiple organ dysfunction. *Crit Care Nurs Q* 16(4):1-7.

Bray, Robert A. and Alan L. Landay. 1989. Identification and Functional Characterization of Mononuclear Cells by Flow Cytometry. *Arch Pathol Lab Med* 113:579-590.

Carter, N.P. 1990. Measurement of cellular subsets using antibodies, pp 45-67, in Ormerod, M.G. (ed), Flow Cytometry: a practical approach, Oxford: New York: Oxford University Press.

Deitch, E.A., W. Bridges, J. Baker, J.W. Ma, L. Ma, M.B. Grisham, D.N. Granger, R.D. Specian, and R. Berg. 1988. Hemorrhagic Shock-Induced Bacterial Translocation is Reduced by Xanthine Oxidase Inhibition or Inactivation. *Surgery* 104(2):191-198.

Devery, J.M., N.J. King, and C.L. Geczy. 1994. Acute inflammatory activity of the s100 protein CP-10. Activation of neutrophils *in vivo* and *in vitro*. *J. Immunol*. 152(4):1888-97.

Donnelly, Semas C., Robert M. Strieter, Steven L. Kunkel, Alfred Walz, Cloin R. Robertson, David C. Carter, Ian S. Grant, Anthony J. Pollok, and Christopher Haslett. 1993. Interlukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *The Lancet* 341:643-47.

Fowler, Alpha A., Thomas H. Hyers, Bernard J. Fisher, Daniel E. Bechard, Robert M. Centor, and Robert O. Webster. 1987. Cell Populations and Soluble Mediators in the Air Spaces of Patients at Risk. *Am Rev Respir Dis* 136:1225-1231.

Georas, Steve N., Mark C. Lui, Walter Newman, L. Dawson Beall, Becky A. Stealy, and Bruce S. Bochner. 1992. Altered Adhesion Molecule Expression and Endothelial Cell Activation Accompany the Recruitment of Human Granulocytes to the Lung after Segmental Antigen

Challenge. *Am. J. Respir. Cell Mol. Biol.* 7:261-269.

Holodniy, Mark, Larry Mole, Mark Winters, and Thomas C. Merigan. 1994. Diurnal and Short-Term Stability of HIV Virus Load as Measured by Gene Amplification. *Journal of Acquired Immune Deficiency Syndromes* 7(4):363-368.

Kahn, J., R.H. Ingraham, F. Shirley, G.I. Migaki, and T.K. Kishimoto. 1994. Membrane proximal cleavage of L-selectin: identification of the cleavage site and a 6-kD transmembrane peptide fragment of L-selectin. *J. Cell Biol.* 125(2):461-70.

Kishimoto, Takashi Kei, Mark A. Jutila, Ellen Lakey Berg, and Eugene C. Butcher. 1989. Neutrophil Mac-1 and MEL-14 Adhesion Proteins Inversely Regulated by Chemotactic Factors. *Science* 245:1238-41.

Larson, Richard S. and Timothy A. Springer. 1990. Structure and Function of Leukocyte Integrins. *Immunological Review* 114:181-217.

Lasky, Laurence A. 1992. Selectins: Interpreters of Cell-Specific Carbohydrate Information During Inflammation. *Science* 258:964-969.

Lillington, Glen A. and Gregory J. Redding. 1992. What you need to know about ARDS. *Patient Care* 26(9):221-228, 235-6.

Loken, Michael R., Jeanne M. Brosnan, Bruce A. Bach, and Kenneth A. Ault. 1990. Establishing Optimal Lymphocyte Gates for Immunophenotyping by Flow Cytometry. *Cytometry* 11:453-459.

Martin, Thomas R., Brent P. Pistorese, Leonard D. Hudson, and Richard J. Maunder. 1991. The Function of the Lung and Blood Neutrophils in Patients with the Adult Respiratory Distress Syndrome. *Am Rev Respir Dis* 144:254-262.

Matikainen, S. and M. Hurme. 1994. Comparison of retinoic acid and phorbol myristate acetate as inducers of monocytic differentiation. *Int. J. Cancer.* 57(1):98-103.

McCoy, J. Phillip, John L. Carey, and John R. Krause. 1990. Quality Control in Flow Cytometry for Diagnostic Pathology. *Am J Clin Pathol* 93(Suppl 1):S27-37.

Nau, H. 1993. Embryotoxicity and teratogenicity of topical

retinoic acid. *Skin Pharmacol.* 6(Suppl 1):35-44.

Osborn, Lauralee. 1990. Leukocyte Adhesion to Endothelium in Inflammation. *Cell* 62:3-6.

Palecanda, Aiyappa, Bruce Walchek, D. Keith Bishop, and Mark A. Jutila. 1992. Rapid activation-independent shedding of leukocyte L-selectin induced by cross-linking of the surface antigen. *Eur. J. Immunol.* 22:1279-1286.

Picker, Louis J., R. Aaron Warnock, Alan R. Burns, Claire M. Doerschuk, Ellen L. Berg, and Eugene C. Butcher. 1991. The Neutrophil Selectin LECAM-1 Presents Carbohydrate Ligands to the Vascular Selectins ELAM-1 and GMP-140. *Cell* 66:921-933.

Repine, John E. and Connie J. Beehler. 1991. Neutrophils and Adult Respiratory Distress Syndrome: Two Interlocking Perspectives in 1991. *Am Rev Respir Dis* 144:251-252.

Repine, John E. 1992. Scientific perspectives on adult respiratory distress syndrome. *The Lancet* 339:466-469.

Salmi, Marko and Sirpa Jalkanen. 1992. Regulation of L-selectin expression on cultured bone marrow leukocytes and their precursors. *Eur. J. Immunol.* 22:835-843.

Schleiffenbaum, Boris, Oliver Spertini, and Thomas F. Tedder. 1992. Soluble L-selectin Is Present in Human Plasma at High Levels and Retains Functional Activity. *The Journal of Cell Biology* 119(1):229-238.

Seymour, G.J., E. Gemmell, R.A. Reinhardt, J. Eastcott, and M.A. Taubman. 1993. Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. *J. Periodontal Res.* 28(6 Pt 2):478-86.

Sherwood, Lauralee. 1989. Human Physiology. St. Paul: West Publishing Company.

Silber, A., W. Newman, K.A. Reimann, E. Hendricks, D. Walsh, and D.J. Ringler. 1994. Kinetic expression of endothelial adhesion molecules and relationship to leukocyte recruitment in two cutaneous models of inflammation. *Lab Invest* 70(2):163-75.

Simon, Scott I., J. David Chambers, Eugene Butcher, and Larry A. Sklar. 1992. Neutrophil Aggregation is $Beta_2$-Integrin and L-Selectin-Dependent In Blood and

Isolated Cells. *The Journal of Immunology* 149(8):2765-2771.

Skolnick, Andrew. 1990. Inflammation-Mediator Blockers May Be Weapons Against Sepsis Syndrome. *JAMA* 263(7):930-931.

Spertini, Oliver, Francis W. Luscinskas, Geoffrey S. Kansas, J. Michael Munro, James D. Griffin, Michael A. Gimbrone Jr., and Thomas F. Tedder. 1991. Leukocyte Adhesion Molecule-1 (LAM-1, L-selectin) Interacts with an Inducible Endothelial Cell Ligand to Support Leukocyte Adhesion. *The Journal of Immunology* 147:2565-2573.

St. John, Roy C. and Paul M. Dorinsky. 1993. Immunologic Therapy for ARDS, Septic Shock, and Multiple Organ Failure. *Chest* 103:932-43.

Stephens, Kenton E., Akitoshi Ishizaka, James W. Larrick, and Thomas A. Raffin. 1988. Tumor Necrosis Factor Causes Increased Pulmonary Permeability and Edema. *Am Rev Respir Dis* 137:1364-1370.

Stewart, R.E., J.S. Stevenson, and J.E. Minton. 1994. Serum hormones in response to estradiol and (or) progesterone in ovariectomized cows after thyroidectomy. *Domest. Anim. Endocrinol.* 11(1): 13-24.

Turner, Marc L. 1992. Cell Adhesion Molecules: A Unifying Approach to Topographic Biology. *Biol. Rev.* 67:359-377.

Vagnucci, Anthony H. and Alan Winkelstein. 1993. Circadian Rhythm of Lymphocytes and Their Glucocorticoid Receptors in HIV-Infected Homosexual Men. *Journal of Acquired Immune Deficiency Syndromes* 6(11):1238-1247.

Weiland, Jeffrey E., W. Bruce Davis, John F. Holter, Jeannette R. Mohammed, Paul. M. Dorinsky, and James E. Geadek. 1986. Lung Neutrophils in the Adult Respiratory Distress Syndrome. *Am Rev Respir Dis* 133:218-225.

Welbourn, C.R.B. and Y. Young. 1992. Endotoxin, septic shock, and acute lung injury: neutrophils, macrophages, and inflammatory mediators. *Br. J. Surg.* 79:998-1003.

Windsor, A.C.J., P.G. Mullen, A.A. Fowler, and H.J. Sugerman. 1993. Role of the neutrophil in adult respiratory distress syndrome. *Br. J. Surg.* 80:10-17.

von Andrian, Ulrich H., Peter Hansell, J. David Chambers, Elaine M. Berger, Ivo Torres Filho, Eugene C. Butcher, and Karl-E. Arfors. 1992. L-selectin function is required for $Beta_2$-integrin-mediated neutrophil adhesion at physiological shear rates in vivo. *Am. J. Physiol.* 263:H1034-H1044.

Zimmerman, Guy A., Attilio D. Renzetti, and Harry R. Hill. 1983. Functional and Metabolic Activity of Granulocytes from Patients with Adult Respiratory Distress Syndrome. *Am Rev Respir Dis* 127:290-300.