Effects of Temperature and Anticoagulant on the in vitro quantitation of Leukocyte Expressed Mac-1' and Post-traumatic Assay to Predict the Development of ARDS

by

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in the

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Youngstown State University

November, 1997

Effects of Temperature and Anticoagulant on the in vitro quantitation of Leukocyte Expressed Mac-1

and

Post-traumatic Assay to Predict the Development of ARDS

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Tracy S. Pitt

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Approvals:

Thesis Advisor — 11/25/97 Date

Committee Member — 11/25/97 Date

Committee Member — 11/25/97 Date

Dean of Graduate Studies — 12/1/97 Date

Abstract

Adult Respiratory Distress Syndrome (ARDS) is post-traumatic disease state in which the lungs are compromised, resulting in aberrant function. The objective of this study is to determine if there are differences in expression of the cell adhesion molecule Mac-1 on white blood cells for ARDS patients compared to patients who do not develop ARDS. Control studies first examined the effect of anticoagulant and incubation temperature on the ***in vitro*** expression of Mac-1. Blood from healthy volunteers was collected into tubes containing either EDTA, heparin or oxalate and incubated at room temperature for 0, 5, 10, 15, 30 and 60 minutes before being placed on ice (prevents in vitro stimulation). Subsequently, the leukocytes were indirectly labeled with fluorescence conjugated antibodies, isolated and then analyzed by flow cytometry. Based on the control study results it was determined that incubating blood samples at room temperature in EDTA, followed by placing the sample on ice within 15 minutes would produce minimal effects on Mac-1 expression in vitro. Therefore EDTA was chosen as the best anticoagulant for patient studies. Patient studies involved collecting blood from trauma patients in EDTA containing tubes at 0, 6, 12, 18, and 24 hours after initial trauma. The patient analysis utilized a protocol similar to the control studies. ARDS patients were found to show increased Mac-1 expression in EDTA anticoagulated blood when compared to non-ARDS patients. Therefore, these studies suggest that Mac-1 is differentially expressed in post-traumatic patients who will develop ARDS when compared to patients who do not develop ARDS.

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May 5,1997

Dr. Diana Fagan
Department of Biological Sciences
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Dear Dr. Fagan:

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

We wish you well in your study.

Sincerely,

Dr. Peter J. Kasvinsky
Dean of Graduate Studies

cc

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

HM Health Services
Youngstown, Ohio

Institutional Review Board

Application for Expedited Review

Project Title: Trauma Induced Activation of the Mononuclear Phagocyte System (MPS)
Approval #93-005

1. Brief Description of Study:

See attached

2. Under which expedited review category does this study fall?
- **See** category descriptions on reverse side of page.

3. Attach a ropy of the complete research study protocol, informed consent document, pertinent bibliography or references, all data collection forms, current vitae of **each investigator and any other appropriate documents.**

5/2/96 — Date

Vincent W. Vanek — Principal Investigator, Vincent W. Vanek, M.D

DECISION REGARDNG PROPOSED STUDY

On the basis of the information presented, this study:

✓ does qualify for an expedited **review,** is granted approval # 12 and will be. reported to the IRB at their next **meeting** on June 26, 1996. If study **extends** beyond one year, renewal application due to IRB on __________

_____ docs not **qualify** for **an expedited** review; a complete **application** should be presented to the **IRB** for a full board review.

5-1-96 — Date

[signature] — Chairperson, IRB
Chatrchai Watanakunakorn, M.D.

ST. ELIZABETH HOSPITAL MEDICAL CENTER

INVESTIGATIONAL REVIEW BOARD

DECISION OF PROPOSED INVESTIGATIONAL STUDY

TO: Dr. Vanek
Principal Investigator

APPROVAL #: 93-005

The St. Elizabeth Hospital Medical Center Investigational Review Board has reviewed the proposal submitted by Dr. Vanek entitled Trauma Induced Activation of the Mononuclear Phagocyte System (Change to Entry Criteria of Study - 7/8/94)

Please be advised that with respect to 1) the rights and welfare of the individual(s), 2) the appropriateness of the methods to be used to secure informed consent, and 3) the risks and potential medical benefits of the investigation, the committee considers the project:

(X) FULLY ACCEPTABLE, WITHOUT RESERVATION

( ) ACCEPTABLE, CONTINGENT ON MINOR REVISIONS

( ) NOT ACCEPTABLE FOR REASONS NOTED

REMARKS:

7/13/94
Date(s) of Committee Review

7/13/96
Date of Approval

Committee Chairman
ROBERT G. SLATING, M.D.
Senior VP, Medical Affairs

Source of Support: ( ) Departmental or other ( ) Outside funding

Are any of the following involved? ( ) Yes, those underlined ( ) No

Minors
Fetuses
Abortuses
Pregnant Women
'Prisoners
Mentally Retarded
Mentally Disabled

cc: Chairman, Clinical Department

List of Figures

	Page number
Figure 1: Mac-1 antigen	5
Figure 2: Extravasation	11
Figure 3: Inderect fluorescence antibody labeling	24
Figure 4: Flow cytometer	28
Figure 5: Gating and leukocyte isolation	31
Figure 6: Sample isolation and fluorescence detection	34
Figure 7: Oxalate/Mac-1/Neutrophils (experiment 1)	38
Figure 8: Oxalate/Mac-1/Monocytes (experiment 1)	40
Figure 9: Oxalate/Mac-1/Neutrophils (experiment 2)	42
Figure 10: Oxalate/Mac-1/Monocytes (experiment 2)	44
Figure 11: Heparin/Mac-1/Neutrophils (experiment 1)	47
Figure 12: Heparin/Mac-1Monocytes (experiment 1)	49
Figure 13: Heparin/Mac-1/Neutrophils (experiment 2)	51
Figure 14: Heparin/Mac-1/Monocytes (experiment 2)	53
Figure 15: Heparin/Mac-1/Neutrophils (experiment 3)	55
Figure 16: Heparin/Mac-1/Monocytes (experiment 3)	57
Figure 17: EDTA/Mac-1/Neutrophils (experiment 1)	60
Figure 18: EDTA/Mac-1/Monocytes (experiment 1)	62
Figure 19: Patient Mac-1/Neutrophils	65
Figure 20: Patient A/Mac-1/Monocytes	67
Figure 21: Patient B/Mac-1/Neutrophils	70
Figure 22: Patient B/Mac-1/Monocytes	72
Figure 23: Patient C/Mac-1/Neutrophils	74
Figure 24: Patient C/Mac-1/Monocytes	76
Figure 25: Patient D/Mac-1/Neutrophils	79
Figure 26: Patient D/Mac-1/Monocytes	81
Figure 27: Patient E/Mac-1/Neutrophils	83
Figure 28: Patient E/Mac-1/Monocytes	85
Figure 29: Patient F/Mac-1/Neutrophils	88
Figure 30: Patient F/Mac-1/Monocytes	90
Figure 31: Percent highest PMA-response/Neutrophils	92
Figure 32: Percent highest PMA-response/Monocytes	95

List of Abbreviations

ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine triphosphate
BAL	Bronchoalveolar Lavage fluid
CAM	Cell Adhesion Molecule
CD	Cluster of Differentiation
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetate
FACS	Fluorescence Activated Cell Sorter
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
HCl	Hydrochloric Acid
ICAM-1	Intracellular Adhesion Molecule-1
IgG	Immunoglobin G
IL	Interleukin
Mac-1	Glycoprotein expressed on leukocyte surface.
MF	Mean Fluorescence
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaH_2PO_4	Monobasic Sodium Phosphate
Na_2HPO_4	Dibasic Sodium Phosphate
NaOH	Sodium Hydroxide
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PMA	Phorbol Myristate Acetate
SSC	Side Scatter
TNF	Tumor Necrosis Factor
Mac-1	Cell Adhesion Molecule involved in the process of extravasation

Table of Contents

	Page number
I. Introduction	1-18
II. Materials and Methods:	
a. antibodies and chemicals	19
b. solutions	19-20
c. controls	21
d. general protocol	21-25
e. control studies	25-26
f. patient studies	26
g. flow cytometric analysis	26-35
h. data analysis	35
III. Results:	
a. control studies	36-63
b. patient studies	63-95
IV. Discussion:	
a. control studies	96-103
b. patients studies	103-110
V. Works Cited	111-118

I. INTRODUCTION:

This research focuses on one consequence of an unrestrained human inflammatory response; trauma-induced Adult Respiratory Distress Syndrome (ARDS). Prior to any discussion of the pathophysiological process of ARDS, it is important to consider the role of inflammation. Because the development of an immune response takes time, the nonspecific inflammatory response has evolved to provide immediate and short term protection against tissue damage or Infection by pathogens (66). There are two characteristics which are hallmarks of inflammation; redness of the tissue due to increased blood supply, and swelling caused by increased vascular permeability leading to increased exudation of fluid, including plasma proteins and white blood cells (59). These two effects of the inflammatory process help promote the healing process, primarily by bringing white blood cells (scavengers) to the site. There are many different types of inflammation, all of which depend on the types of mediators and the inflammatory cells that participate. The various types of inflammation can be classified as: non-specific, allergic, immune complex mediated, and cell mediated (1). The non-specific inflammatory response will be the focus of this discussion because it is the type seen in trauma cases (1). In response to a sudden injury, the acute inflammatory response functions to repair the damaged tissues (59). Since the intensity of the acute response is positively correlated to the severity of injury (59), traumatic injury could activate an intense inflammatory response. In such an event, the acute inflammatory response could continue and develop into a chronic inflammatory situation, whch is a pathophysiological (disease) condition (50). The pathways by whch

the inflammatory response function are very complex, especially considering the diversity, redundancy, and number of mediators involved. One such mediator, the complement system, consists of numerous soluble peptides (C1-C9) that are activated via an enzyme cascade (34). Complement activation serves a two-fold purpose; destruction of invading pathogens, and chemotactic signaling for leukocyte (neutrophils and monocytes) extravasation (24). During tissue damage (trauma), the alternate pathway of the complement cascade is activated and many peptide fragments are produced (1). C5a and C3a are two anaphylatoxins which are produced as a result of the peptide cleavages that occur during complement activation. These two anaphylatoxins, in addition to other mediators, cause increased vascular permeability, allowing phagocyte influx into the inflamed tissue (1). Once the white blood cells have been stimulated by mediators such as histamine, they themselves begin producing cytokines, leukotrienes, and prostaglandins which control the magnitude and duration of the inflammatory response (1, 24, 59). These substances contribute to the recruitment and accumulation of leukocytes at the site(s) of inflammation. At this point, phagocytic white blood cells have begun infiltrating the site of inflammation and have also begun exerting their effects. If this acute phase of inflammation persists and becomes chronic, self tissue damage may result due to the activity of neutrophils and monocytes/macrophages.

Cell adhesion molecules (CAM) represent a diverse group of molecules found on the surface of all cells within the body. The functions of cell adhesion molecules in the body include cell-extracellular matrix interactions and both homotypic and heterotypic

cell-cell interactions. A vast array of processes within the body are under control of CAM's; for instance, embryonic development, learning and memory, viral infections, and cancer metastasis (18). There are four major classes of CAM's found on the various cells w i t h the body: integrins, selectins, cadherins, and immunoglobin-like adhesion receptors (18). These studies focus on one specific subclass of the integrin family.

The integrins constitute a diverse group of membrane associated molecules (Fig. 1). All members of the integrin family are α/β heterodimeric transmembrane glycoproteins, where the β subunits have the same molecular weight and the α subunits are variable in size. It has been found that there are at least 8 different β subunits and at least 14 different α subunits (31). The association between the α and β subunits is restricted in that only certain α subunits can bind to only certain β subunits (31). This research effort focuses on the β_2 integrins, since they are specific for leukocytes. There are three α subunits that can associate with the β_2 chain: namely α_M, α_L and α_X (31). The most relevant integrin to this paper is Mac-1, an $\alpha_M\beta_2$ integrin (31) (Table I). Mac-1 is the predominate integrin expressed on polyrnorphonuclear leukocytes (neutrophils) and monocytes (52), and these two cells are the predominate cells involved in the inflammatory response. Mac-1 levels have been found to increase on the surface of these two white blood cells when they are stimulated (33, 61), indicating a role for monocyte and neutrophil Mac-1 in the inflammatory response. Other functions of neutrophil Mac-1 include: homotypic adhesion to other neutrophils, phagocytosis of opsonized (coated with antibody to enhance phagocytosis) particles, chemotaxis

Figure 1: Diagrammatic representation of the Mac-1 antigen expressed on the surface of leukocytes. This heterodimeric polypeptide is composed of two subunits, designated α_m (140 kd) and β_2 (100 kd), which are noncovalently associated. Both subunits of Mac-1 are transmembrane proteins with a large extracellular domain and a short cytosolic domain. The cytosolic domain is involved in signal transduction pathways.

Figure 1.

Mac-1: membrane bound receptor complex

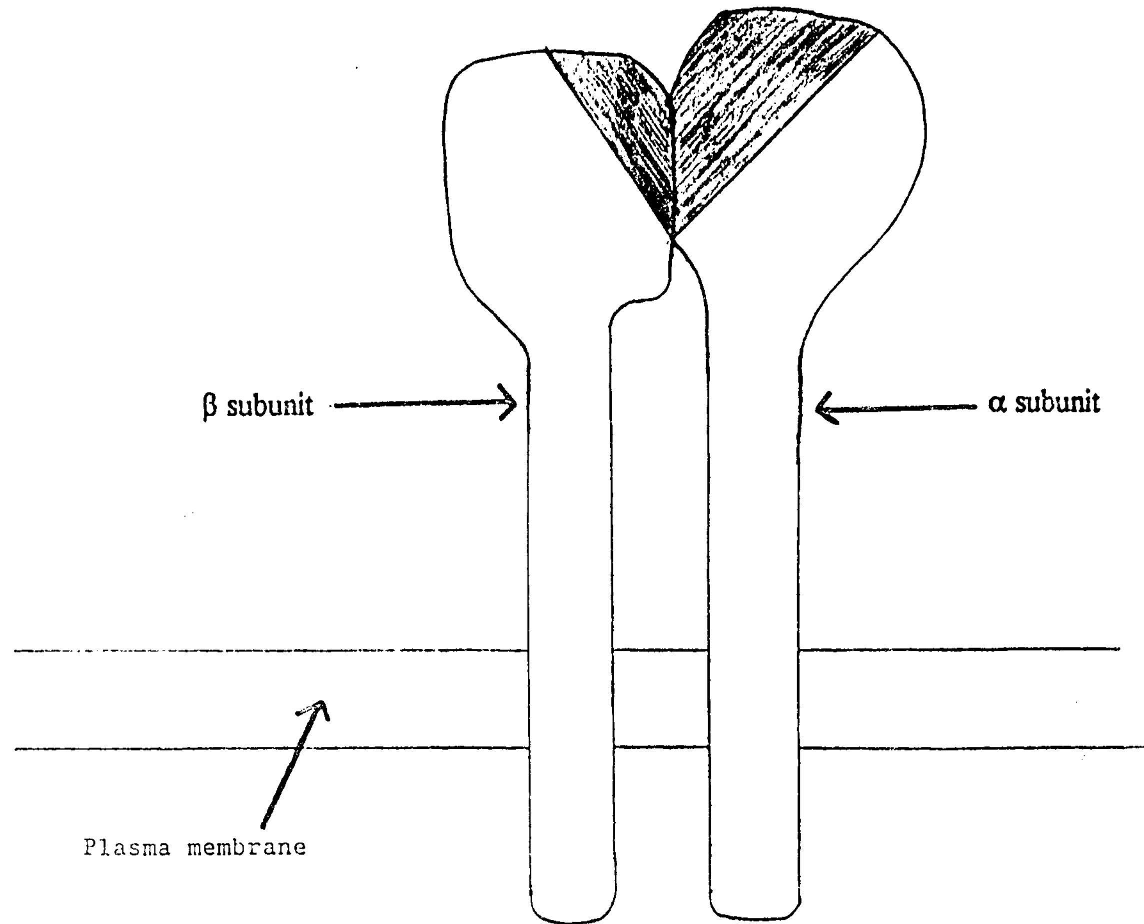


Table I: List of ligands, cellular distribution, and other names for the Mac-1 surface antigen.

Table I:

	Ligands for:	distribution:	other names:
Mac-1	ICAM-1 (CD54) fibrinogen factor X C3bi fibronectin bacterial peptides	mononuclear cells granulocytes macrophages natural killer cells	CD11b/CD18 $\alpha_M\beta_2$ CR3 C3b Mo-1

by binding transiently to extracellular matrix proteins, Antibody dependent cell cytotoxicity (recognize Fc portion of antibodies bound to target cells and cause cell lysis) and oxidative burst (release of reactive oxygen oxidants) (2, 27, 33, 36, 56). The list of ligands for Mac-1 is extensive and includes: Intracellular Adhesion Molecule-1 (ICAM-1, CD54), C3bi, Lipopolysaccharide (LPS), Fibrinogen, and Factor X (33, 52, 61).

In the presence of certain pro-inflammatory stimulatory signals, the white blood cells (ie. monocytes and neutrophils) become activated. This results in CAM expression changes and the mediation of their effects. C5a, bacterial peptides, platelet-activating factor, leukotriene B4, tumor necrosis factor-α, interleukin-1, and interleukin-8 are the main pro-inflammatory molecules involved in increasing the surface expression of Mac-1on neutrophils and monocytes (8, 32, 36, 58, 65, 69). Two cytolunes that are associated primarily with traumatic injury are interleukin-1 (Il-1) and tumor necrosis factor alpha *(TNF-a),* which are responsible for inflammatory mediated recovery of the individual (50). These signals stimulate not only monocytes and neutrophils to increase surface expression (upregulation) of Mac-1, but also stimulate the endothelium to upregulate P- and E-selectin and ICAM-1. The most important molecules in this process are the cytokines. Cytokines are chemicals produced by and secreted from cells of the immune/inflammatory response which act locally to modulate the activity and functioning of the immune/inflammatory response. Additionally important is C5a, which is a peptide fragment produced secondary to complement activation. One role of C5a in inflammation is that it acts as a chemotactic signal for neutrophils. A

chemotactic signal is an insoluble chemical that indicates a direction of movement that the white blood cells are to follow. Also, C5a stimulates the release of toxic substances from the neutrophils' arsenal of antimicrobial substances (69). In response to these chemical mediators of activation, Mac-1 is rapidly mobilized from intracellular storage pools. For example, TNF-α is secreted by immune cells in response to bacterial invasion. Subsequently, TNF-α binds to TNF receptors on white blood cells and causes increases in intracellular calcium levels (calcium is mobilized from intracellular storage granules) (52). The calcium signal causes Mac-1 storage granules to move towards the membrane, fuse with the membrane, resulting in Mac-1 insertion into the membrane (43).

The main influence Mac-1 has on neutrophils relates to their emigration from the vasculature and into the tissue spaces (52). Investigating Mac-1 is important because of its role in facilitating the development of this inflammatory response (33). During an inflammatory response the white blood cells begin "rolling" along the endothelium in a process termed rolling adhesion, next they attach firmly and finally they move into the tissue spaces (extravasation) (Fig. **2A).** The process of rolling adhesion is due to L-selectins, a member of the selectin family of CAM's, transient binding interactions with endothelial cell expressed P- and E-selectin (9). E-selectin is the primary molecule upregulated by cytokine activity and is the primary mediator of monocyte and neutrophil extravasation during inflammation *(57)*. L-selectin is constitutively expressed on resting monocytes and neutrophils in high numbers, but is shed from the cell upon activation. Mac-1, on the other hand, is present in low numbers

Figure 2: A).The process of extravasation. White blood cells begin rolling along the endothelium due to interactions between L-selectin and E- or P-selectin. The cells next adhere firmly to the endothelial cell due to binding interaction between Mac-1 and ICAM-1. Following a change in CAM expression, the cell moves between the endothelial cells and enters the tissue spaces. B). Diagrammatic representation of the binding events between endothelial expressed CAM's and leukocyte expressed CAM's. (see text for details)

Figure 2A.

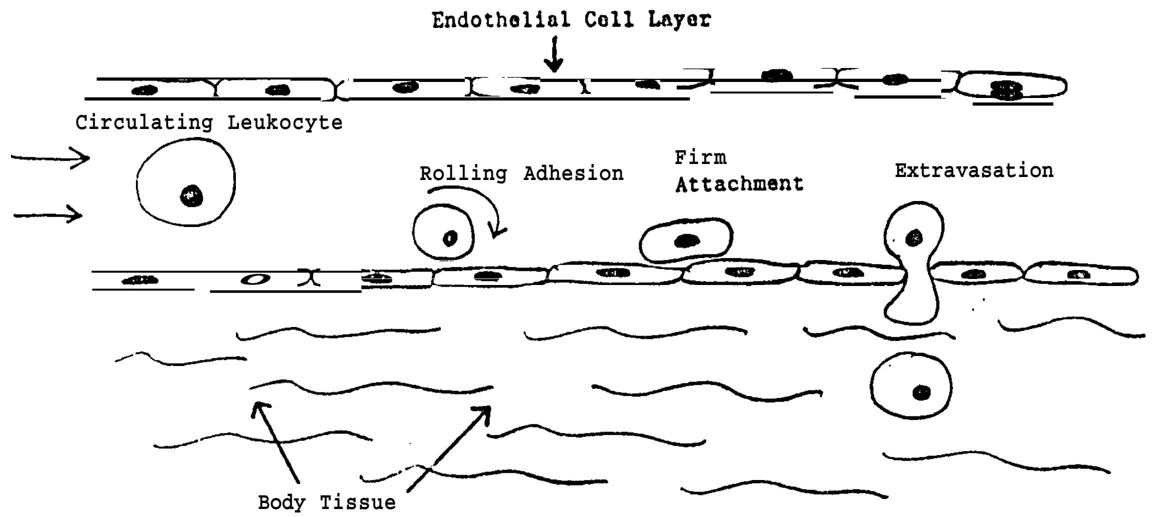
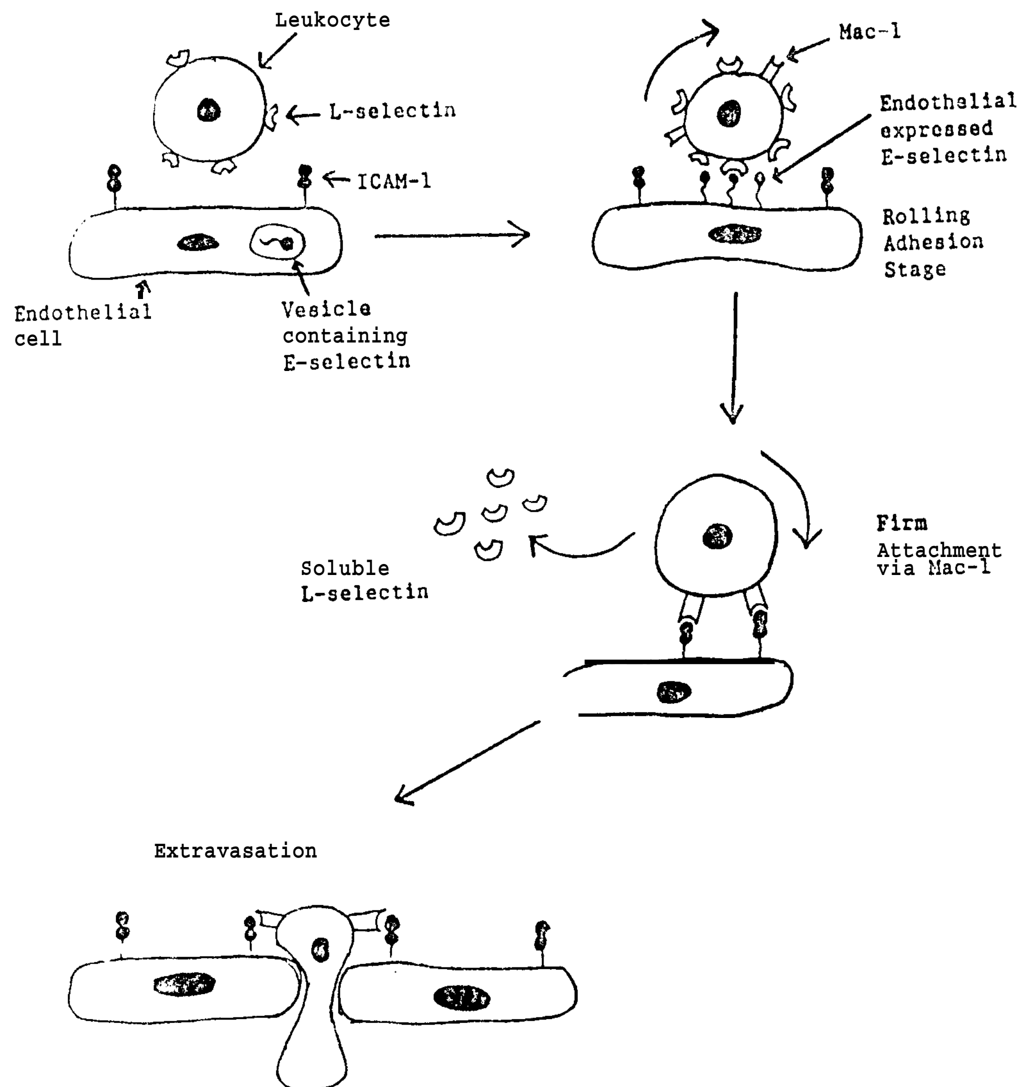


Figure 2B.



on the surface of leukocytes and is upregulated in response to changes in the individual's inflammatory state (presence of stimulatory signals). When white blood cells are stimulated to increase Mac-1 concentrations on the cell, it enables the white blood cells to adhere firmly to the endothelium by high avidity binding between Mac-1 and ICAM-1 (9). Endothelial cells are simultaneously stimulated to increase the numbers of ICAM-1 on their lumenal surface by the same signals that stimulate leukocytes (12, 52). After the firm attachment via the Mac-1/ICAM-1 interactions, L-sel is cleaved from the cell surface. This change in CAM expression enables the cells to squeeze between the endothelial cells and enter the tissue spaces in a process termed extravasation (Fig. 2B) (32, 36). In fact, cross-linking L-selectin can signal CD18-dependent cell arrest at the site of inflammation. This means that binding of L-selectin to its ligand causes Mac-1 to increase its avidity for ICAM-1, thereby allowing the firm attachment to the endothelium followed by extravasation (26). The importance of extravasation is that it enables white blood cells to enter areas where they are needed, thus effectively countering any invading pathogen that must be destroyed.

In addition to Mac-1's role in extravasation, it's role in homotypic aggregation facilitates the massive accumulation of neutrophils at the intravascular site, further compounding the magnitude of cellular influx into the inflammatory site. The early stages of traumatic injury are associated with the establishment of a chemotactic gradient which, in combination with the upregulation of adhesion molecules, could be responsible for the accumulation and sequestration of leukocytes in the microvascular spaces, and

extravasation of leukocytes into the alveolar spaces (14). Both the homotypic and heterotypic cell-cell binding characteristics promote the excessive accumulation of white blood cells characteristic of many inflammatory disease states, including ARDS (56).

The polymorphonuclear leukocyte (neutrophil) and the monocyte are the cells which need to be investigated because of their role in inflammation, and presumably in the development of inflammatory disease states. Since Mac-1 is involved in the process of monocyte and neutrophil extravasation, it is possible that there are time dependent changes in Mac-1 expression prior to ARDS development. Several factors have implicated the neutrophil in the pathogenesis of ARDS. For instance, bronchoalveolar lavage (BAL) fluid from ARDS patients exhibits increases in the total number of lung neutrophils and it contains increased quantities of neutrophil secretory products such as elastase and collogenase (14, 62). Additionally, 50-60 % of all intravascular neutrophls are sequestered within the lung vasculature, thus providing a large intravascular pool of inflammatory mediators in close apposition to the lungs. Also, ARDS patients have increased numbers of neutrophils in the lung tissue spaces (65) indicating that neutrophils may be the primary mediator of lung parenchymal cell injury. Furthermore, it has been found neutrophils harvested from inflamed peritoneal spaces in experimental models contain increased expression of Mac-1 (32), and that blocking monoclonal antibodies which abolish Mac-1 activity attenuate the self tissue damage seen in these animal models (65). Therefore we speculate that expression levels of Mac-1 on these two cell types in patients who ultimately develop ARDS will vary significantly from those levels seen in healthy

controls. That is, increased expression of Mac-1 may be responsible for the accumulation of neutrophils and macrophages in the lungs of ARDS patients. The possible mechanism for monocyte involvement in ARDS is that, following activation, they migrate into the tissues and differentiate into macrophages. It has been found that patients who develop ARDS produce significantly greater amounts of macrophage secreted IL-8 (chemotactic for neutrophils) found in the bronchoalveolar lavages (BAL) (20). This finding indicates that tissue macrophages may contribute to ARDS pathogenesis since positive correlation's between IL-8 and BAL neutrophil content in ARDS patients have been noted (25). This information suggests that stimulation of macrophages results in secretion of cytokines that direct white blood cells to the site of inflammation. ARDS patient monocytes significantly increase production of TNF-α and IL-6 (6, 8). This is important since TNF-a and IL-6 have profound effects on neutrophil activity and endothelial cell integrity (49). That is, they stimulate the neutrophils and cause the blood vessels to become "leaky", thereby allowing activated neutrophils to enter the lungs. Lastly, ARDS patient BAL fluids were found to cytotoxic for normal, cultured lung parenchymal cells (62), indicating the potential for lung destruction by various factors, such as neutrophils, found within the lungs.

If neutrophils are recruited to the lungs through increased Mac-1 expression, tissue damage may be caused by any of the numerous weapons that neutrophils' possess to protect against invading pathogens. There are two arms to the neutrophil arsenal: membrane-associated mechanisms and cytosolic mechanisms (65). The membrane associated mechanism consists of a membrane bound enzyme called

nicotinamide adenine dinucleotide phosphate (NADPH). When proinflammatory signals stimulate the neutrophil, NADPH catalyzes a reaction that produces superoxide radicals (63, 65). Following its production, the superoxide radical is consumed in a reaction that produces hydrogen peroxide (63, 65). Both of these short lived products are nonetheless able to react with biological substrates, resulting in host cell destruction. The most notable of these chemicals is produced by a third reaction which uses hydrogen peroxide to produce hypochlorous acid, a long-lived radical with cytolytic effects (63, 65). The second means by which a neutrophil can cause damage utilizes the cytosolic mechanism. Stimulation of the neutrophil causes intracellular storage granules to fuse with the membrane. In this case, the granules contain any of a number of proteolytic enzymes, bactericidal proteins, or lysozyme (63, 65). The most important concern here are the proteolytic enzymes elastase, collagenase, and gelatinase (65). These enzymes are capable of breaking down normal components of the bodies tissues and thus causing tissue damage.

As a result of these numerous and complex interactions, the inflammatory response is initiated to exert it's effects, which are normally protective. Inflammation is usually a self limiting process that is confined to a specific site of action, then turned off. However, there are instances where this response can cascade "out of control". An unrestrained inflammatory response can thus cause the tissue damage that has been characterized in many disease states; including rheumatoid arthritis, lupus erythematous, diabetes mellitus, ulcerative colitis, and others (33, 63).

Adult respiratory distress syndrome is a condition defined by diffuse damage to lung tissue presumably as an early manifestation of the systemic (whole body) inflammatory response (3, 64). There are two common pathways for the development of ARDS: sepsis and trauma (10, 44). We are more concerned with the latter. ARDS is a condition which manifests as diffuse pulmonary infiltrates (as seen on chest X-rays), hypoxemia (low oxygen concentrations in the blood), high permeability pulmonary edema, dyspnea (difficulty breathing), tachypnea (rapid breathing), anxiety, inspiratory crackles upon auscultation (listening to sounds within the body, usually with aid of a stethoscope), and ultimately decreased lung compliance (ability to fill lungs with air) (3, 10, 28, 44, 62). In the past this disease was associated with an extremely high mortality rate. Even with recent advancements in critical care medicine, which have brought a decline in morbidity caused by this condition, the mortality rate remains 50 percent (10, 22, 58). Others have reported the mortality rate to be between 22% - 84%. This difference is attributable to discrepancies in the definition of ARDS (3). Thus it is apparent that a clear cut definition of ARDS has not been definitively established. ARDS appears to develop as a result of fluid accumulation in the lungs secondary to neutrophil accumulation in the lungs, blood vessel permeabilization/destruction, and compounding leakage of fluid into the lungs (55). The microvascular injury has been proposed to manifest subsequent to the inflammatory response. For instance, the initiating event (ie. trauma) is believed to increase mononuclear activity with a subsequent release of pro-inflammatory cytolunes. The result of cytokine release and accumulation is neutrophil and endothelial cell

activation. This activation promotes increased white blood cell adherence to the endotheliurn, followed by the concomitant release of oxidants and proteases resulting in microvascular collapse, decreased functioning of the lungs and ultimately organ failure (10, 58).

To investigate to possibility that ARDS can be detected prior to its onset by testing for Mac-1 levels on neutrophils and monocytes, blood samples were obtained fiom trauma patients at 0, *6,* 12, 18, and 24 hours post-trauma. These samples were then analyzed to determine if the level of Mac-1 on trauma patient (ARDS) white blood cells Qffered significantly fiom levels seen in trauma patients that Qd not develop ARDS. This analysis is possible because of antibodies, conjugated to a fluorescence marker, that will bind to Mac-1 proteins on the white blood cell surface. The leukocytes fiom the two patient populations were saturated with antibody directed against Mac-1. Since these antibodies produce fluorescence when exposed to light, the relative numbers of Mac-1 glycoprotein can be measured quantitatively. The projected outcome is a significant increase in Mac-1 levels on monocytes and neutrophils of ARDS patients versus non-ARDS patients.

Anticoagulants are added to blood collected fiom patients to prevent blood fiom clotting. Therefore, examination of the effects of various anticoagulants upon the expression levels of Mac-1 in vitro was examined. Three anticoagulants were considered plausible candidates for the collection of patient blood, namely EDTA, heparin, and oxalate. Previous studies in this laboratory have shown that when leukocytes are collected at room temperature in EDTA they express elevated levels of Mac-1. However, in vitro stimulation of white blood

cells can be prevented by placing the blood sample on ice (55). The result of the cells becoming stimulated during blood collection is the accumulation of data that does not accurately reflect the in vivo environment, since they are substantially altered with regard to Mac-1 surface expression. Since conclusions will be made regarding the in vivo environment, the patient samples must not be altered by in vitro collection techniques.

The goal of this study is to find a correlation between cell adhesion molecule expression and the development of ARDS. If one considers the possible benefits of early detection and aggressive treatment of this mortal disease state, the importance of finding an early detection test can be considered urgent. Being able to prevent this often fatal disease could have profound implications in the treatment of trauma patients.

II. MATERIALS AND METHODS:

A. ANTIBODIES AND CHEMICALS

Mouse monoclonal antibodies directed against human Mac-1(αMac-1) and L-sel (αL-sel) were donated by Dr. Robert Rothlein, Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, Connecticut). αMac-1 and αL-sel concentrations were 5.96 mg/ml and 11.40 mg/ml, respectively. Fluorescein isothiocyanate (FITC) labeled goat anti-mouse antibody, FACS lysing solution, and the control antibodies [CD45-FITC/CD14-phycoerythrin (PE), CD3-FITC/CD19-PE, and Gamma-1-FITC/Gamma-2a-PE] were purchased from Becton Dickinson (San Jose, California). Phorbol myristate acetate (PMA), dimethylsulfoxide (DMSO), sodium chloride (NaCl), and sodium azide were purchased from Sigma Chemical Company (St. Louis, Missouri). Monobasic sodium phosphate (NaH_2PO_4) was purchased from Fisher Chemical Company (Fairlawn, New Jersey) and anhydrous, dibasic sodium phosphate (Na_2HPO_4) from Mallinckrodt Chemical Company (Paris, Kentucky).

B. SOLUTIONS

10X Phosphate Buffered Saline (PBS)

Monobasic sodium phosphate (1.28g), dibasic sodium phosphate (5.97g) and 200ml of water were mixed. Following dissolution of the chemicals, the pH was adjusted to 7.4 using 0.1N HCI or 0.1N NaOH.

Next, 43.83g of NaCl was added and allowed to dissolve. Finally, the mixture was brought up to 500 ml using Milli Q water and the solution was stored at room temperature.

PBS-Azide

Sodium azide (1.0g) was dissolved in 100 ml of 10X PBS plus 900 ml Milli Q distilled water. The solution was immediately filtered and stored at 4°C.

Paraformaldehyde

Paraformaldehyde (20 mg) and 10X phosphate buffered saline (20 ml) were mixed and brought up to a volume of 200 ml using Milli-Q distilled water. The paraformaldehyde crystals were dissolved by placing the mixture on a heating plate (in a water bath) and heating to 56°C with gentle stirring. The solution was allowed to cool to room temperature in the water bath. The pH was adjusted to 7.4 using 0.1N HCL or 0.1N NaOH. The solution was immediately filtered and stored at 4°C.

Lysis Buffer

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

Phorbol Myristate Acetate (PMA)

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

C. CONTROLS

Incubation of unstimulated or PMA-stimulated cells with 100 ul whole blood with CD45(FITC)/CD14(PE) antibody allowed us to identify monocyte and neutrophil populations, based on FITC and/or PE levels on the different cell types. The level of nonspecific antibody binding to the cells Fc receptor or binding by any other mechanism was demonstrated by a negative control (IgG1 and IgG2a antibody) to 100ul of unstimulated blood (5). A third control (quenching control) was also used for the purpose of calibrating the flow cytometer. CD3/CD19 antibody was added to 100ul of unstimulated blood. This control is important for separating FITC signals from PE signals. All control antibodies were added as 20ul aliquots, as indicated by Becton Dickinson protocol for 5 X 10^5 cells.

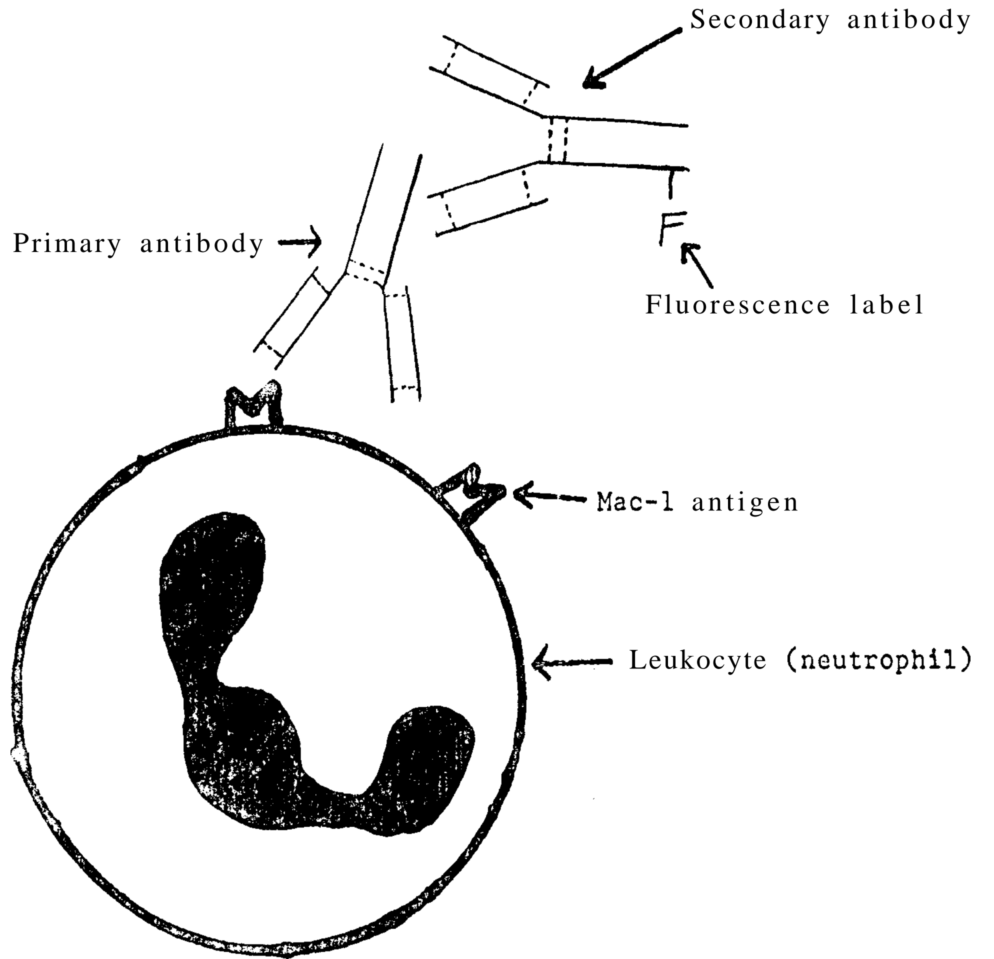
D. GENERAL PROTOCOL

A peripheral blood sample was collected in vacuum tubes containing anticoagulant and placed on ice (4^oC) to prevent ex *vivo* (outside the body) stimulation of the white blood cells (51, 55). Following collection, 1X PBS (121 ul) was mixed with stock PMA (2 ul of 1 mM in DMSO). Subsequently, an aliquot of blood (990ul) was incubated with 10ul diluted phorbol myristate acetate for 10 minutes at 37^oC yielding a final PMA concentration of 0.16 uM (the remainder of the blood was kept on ice to prevent stimulation) (68). PMA stimulates monocytes and neutrophils by activating the protein kinase C second messenger pathway, resulting in maximal expression of Mac-1 on the cell surface (68). PMA-stimulated blood is used for comparison with

the patient/trial data since neutrophils and monocytes experience large increases in Mac-1 expression in response to stimulation by PMA (41). During the incubation period, 20 ug of αMac-1 or 20 ug of αL-sel was pipetted into duplicate Falcon tubes. αMac-1 and αL-sel are monoclonal antibodies directed against purified human Mac-1 or L-sel glycoproteins, respectively. The αMac-1 used here (R15.7) is an antibody directed against the CD18 subunit of Mac-1 (11). Unstimulated or stimulated blood (100 ul) was added to the duplicate tubes containing antibody. The tubes were vortexed immediately for 3 seconds allowing for complete mixing of the white blood cells and the antibodies. A 15 minute incubation allowed for complete binding of antibody to all available binding sites on the white blood cells. The cells were then washed twice using 2 ml of ice cold phosphate buffered saline containing sodium azide (PBS-azide). The importance of the PBS-azide wash is two-fold; first, washing will remove any unbound antibody and secondly, the sodium azide disrupts the cytochrome oxidase system within the cells thereby eliminating the cells ability to produce ATP via electron transport. This prevents endocytosis and loss of antigen from the cell surface. Furthermore, using ice cold PBS and the temperature at which the cells are centrifuged also helps to prevent endocytosis. PBS-azide was added to the cells, which were then centrifuged at 1100 x g for 8 minutes (4°C). The supernatant fluid was aspirated off the cells using a disposable pipette. After completion of two washes, 1 ug of the secondary antibody (FITC-labeled goat-anti mouse antibody) is added followed by immediate vortexing (5). The secondary antibody binds to the Fc domain of the primary antibody, thereby effectively tagging each CAM with fluorescence (Fig. 3). The

Figure **3:** Indirect antibody labeling of surface antigens (Mac-1). The primary antibody is a mouse monoclonal antibody directed against human Mac-1 (M). The Fab segment (binding specificity) binds to the surface antigen. The secondary antibody is a goat monoclonal antibody directed against mouse antibodies. The Fab segment of the goat **anti-**mouse antibody recognizes and binds to the Fc portion of the primary antibody. A fluorescence marker (FITC) is conjugated to the Fc portion of the secondary antibody.

Figure 3.



fluorescence marker absorbs light of one wavelength and emits light of another wavelength. Its presence on the cells can be detected as well as quantified using flow cytometry. An incubation period of 15 minutes in the dark allowed for complete antibody binding to occur. Performing this incubation in the dark is important since any light will activate the fluorescence marker and, after enough time, abolish it's ability to emit fluorescent light. Subsequent to the incubation period, the red blood cells were lysed using 2 ml of FACS lysing solution (68) for 10 minutes. This solution disrupts the integrity of red blood cell membranes and essentially breaks the cells apart, leaving the white blood cells for study. The white blood cells were centrifuged at 1100 x g for 8 minutes ($4^{o}C$) and the supernatant was discarded. The cells were washed one time in PBS-azide as previously described. The cells were resuspended in 400 ul of paraformaldahyde and stored at $4^{o}C$ in the dark. Paraformaldehyde is a chemical that cross-lmks the protein molecules on the surface of all cells. In doing this, the cells become rigid and unable to change morphologically in any way (32). Therefore, the integrity of the cells is maintained until the cells could be analyzed using flow cytometry.

E. CONTROL STUDIES

Peripheral blood samples were collected from healthy volunteers into vacuum tubes containing either ethylenediamine tetraacetate (EDTA), oxalate, or heparin. An aliquot of blood (100 ul) was immediately removed and processed as the "0" minute time point (see general protocol). Meanwhile, 300 ul aliquots were removed and placed on ice at timed intervals following blood collection (5, 10, 15,

30, and 60 minutes). At the completion of the 0 minute time point the rest of the timed samples were processed following the protocol previously described. The purpose was to determine effects of the various anticoagulants and length of time at room temperature on cell adhesion molecule expression.

F. PATIENT STUDIES

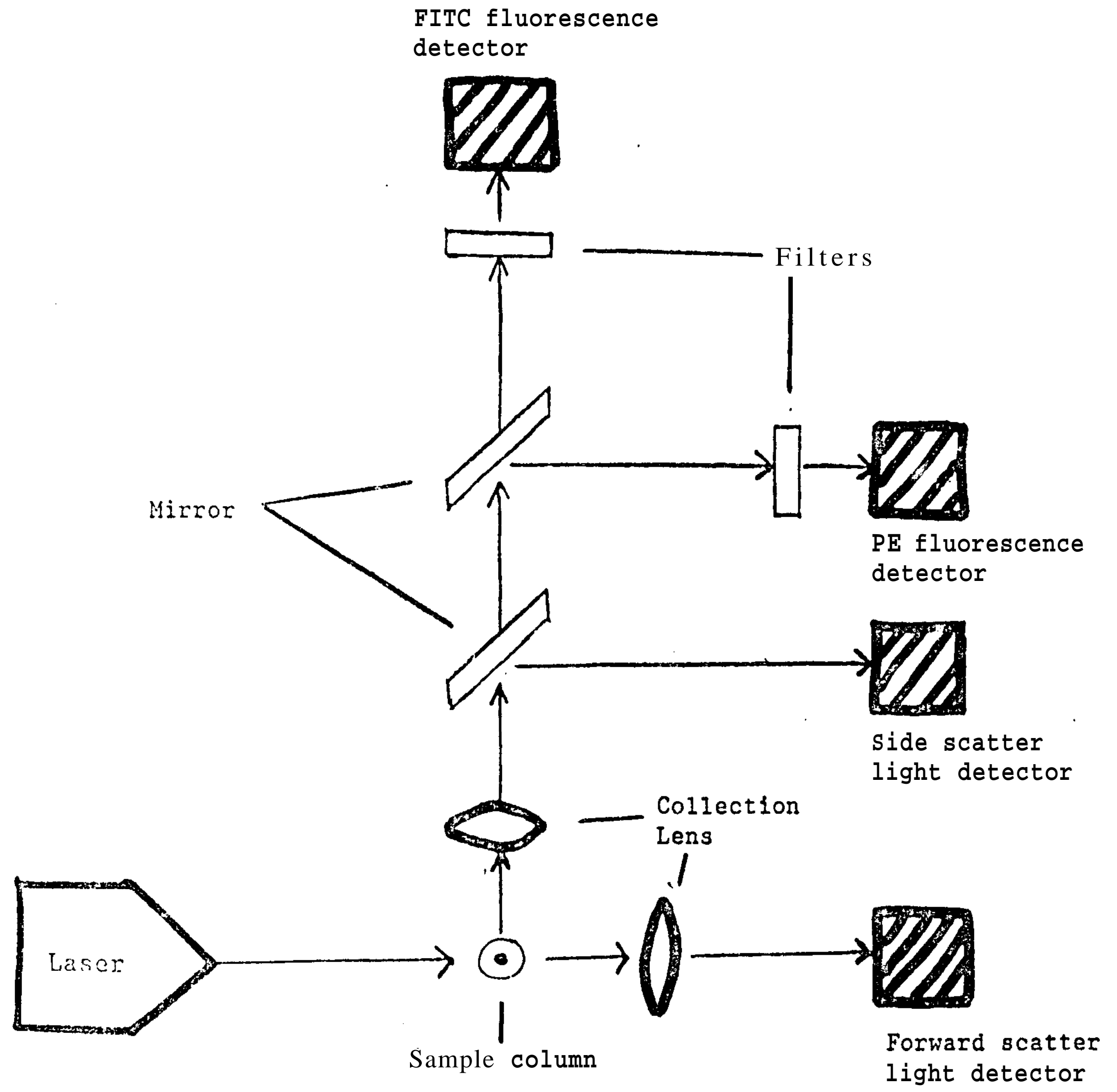
Patients were enrolled at the discretion of the on-call trauma surgeon at St. Elizabeth's Medical Center. These patients were between the ages of 18 and 65 years of age. Their injuries were severe enough that the probability of their condition deteriorating into Adult Respiratory Distress Syndrome was high. Once a patient was enrolled in the study and informed consent was obtained fiom the patient or a close family member, one EDTA vacuum tube of blood was collected. These samples were collected at 0, 6, 12, 18, and 24 hours post-trauma. The zero hour sample was collected fiom blood samples obtained for routine lab tests, then placed on ice immediately. The remaining samples were collected by the attending nurse in SICU as part of routine lab work, were put on ice, and were processed immediately following the procedure described in the general protocol.

G. FLOW CYTOMETRIC ANALYSIS

The cells were aspirated into the flow cytometer and processed by Dr. Diana Fagan using a Becton Dickinson flow cytometer (Fig. 4) with LYSIS II software at St. Elizabeth's Hospital. A flow cytometer is an instrument that can detect fluorescence levels on individual white blood cells (35). It has been noted that the antibody used in this study

Figure 4: Diagrammatic representation of the mechanism by which a flow cytometer functions. The sample (single white blood cell) is passed through the sample column. A beam of light (laser) is directed at the sample. The light is scattered either forward (FSC) or to the side (90° SSC) depending on the size and granularity of the cell, respectively. The amount of light deflected by these means is detected by two light detectors. Secondly, the beam of light activates the fluorescence markers (FITC or PE) attached to the cells. This causes the marker to emit fluorescent light which can be measured by two different detectors for FITC or PE.

Figure 4.

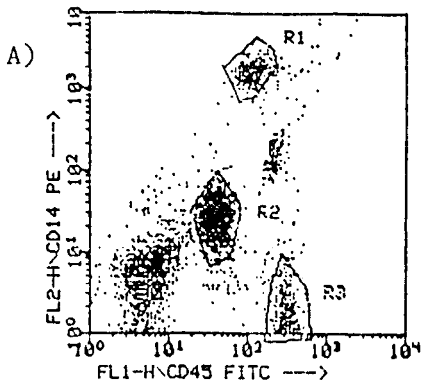


(R15.7) is useful for flow cytometric analysis of increasing Mac-1 expression on the surface of white blood cells (11). A flow cytometer is also used to identify cell types based on the morphological features of the various cell types. Each cell passes through a column, at which time a beam of light is directed at the cell. There is a fluorescence detector that can quantitate the amount of fluorescence being emitted as well as 2 other light detectors. One of these light detectors interprets forward scatter (FSC) of the incident beam and the other detects side scatter (SSC). In fact, Identification of specific white blood cells can be effectively accomplished through the use of light scattering measurements (15). Forward scatter patterns are a result of the cells size, whereas the side scatter pattern results from differences in cellular granularity. In order to analyze the data collected by the light detectors, the desired cell populations must be identified by electronic gating (Fig. 5). Following acquisition, the cells were gated using two criteria. First, the control cells were identified as being monocytes or neutrophils by the surface expression of CD45 and CD14 (protein molecules that are differentially expressed on the cell's surfaces) (35). Monocytes and neutrophils have similar levels of CD45 on their surface; however monocytes have much higher quantities of CD14 than other white blood cells (Fig. 5A) (5). Secondly, identification was also made possible based on morphological characteristics of the two cell types. Monocytes and neutrophils are similar in size, however neutrophils are much more granular than are monocytes. Therefore, monocytes and neutrophils have similar levels of FSC (X-axis) but neutrophils have higher levels of SSC (Y-axis) (Fig. 5B). Electronic gates were then drawn around the white blood cell populations based on; one, their

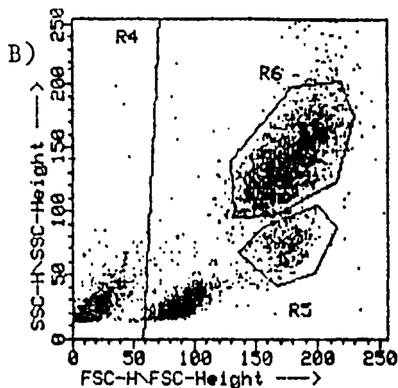
Figure 5: A) Dot plot of CD45 (x-axis) and CD14 (y-axis) labeled white blood cells. B) Dot plot of FSC (x-axis) versus SSC (y-axis). After both of these plots have been established, gating is performed. First, an electronic gate is drawn around monocytes (R1 in part A). Second, an electronic gate is drawn around neutrophils (R2 in part A). Third, an electronic gate is drawn around lymphocytes (R3 in part A). Fourth, an electronic gate is drawn around all white blood cells (R4 in part B). Fifth, an electronic gate is drawn around monocytes (R5 in part B). Lastly, an electronic gate is drawn around neutrophils (R6 in part B). Next, two dot plots are established which show the monocytes found in gates R1 and R4 (C) and neutrophils found in R2 and R4 (D). From these dot plots, the gates can be modified to exclude and unwanted cells. E and F) After modification of the gates, two additional dot plots are established showing the isolated monocyte and neutrophil populations (E and F, respectively). The dot plots (E and F) are then used to select the parameters of the gates. G) Shows the parameters for the monocyte populations. In this particular study, 248 monocytes(R1) versus 8 neutrophils (R2) and 9 lymphocytes (R3) were examined. H) 2141 neutrophils, 17 monocytes, and 2 lymphocytes were examined. The importance of this procedure is that the gates established here will be the same gates used to examine sample cells labeled with aMac-1.

Figure 5:

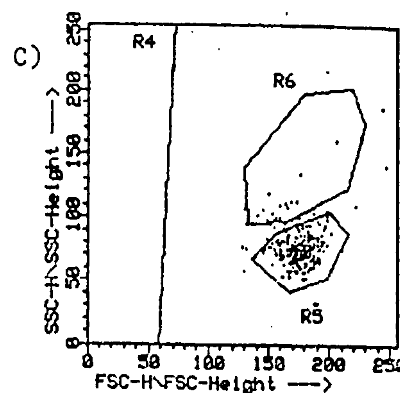
A)



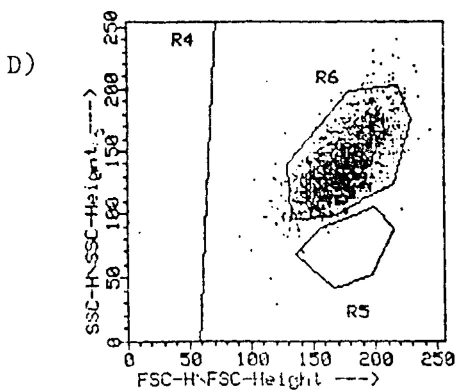
B)



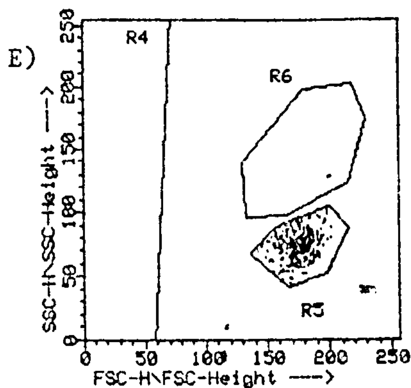
C)



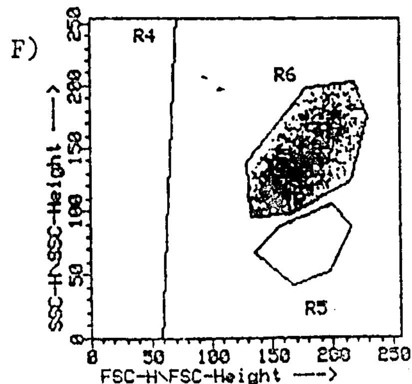
D)



E)



F)



G) Gate Stats

File: /25/JAT001 Sample: A T 001
Date: 3/26/97 Gate G5= R5
Selected Preference: Arithmetic/Linear
Total= 5000 Gated= 308

Gate		Events	% Gated	% Total
1	G1	248	80.52	4.96
2	G2	8	2.60	0.16
3	G3	9	2.92	0.18
4	G4	308	100.00	6.16
5	G5	308	100.00	6.16
6	G6	0	0.00	0.00
7	G7	248	80.52	4.96
8	G8	8	2.60	0.16

H) Gat-Stat~

File: /25/JAT001 Sample: JAT
Gate: 3/26/97 Gato G6= R6
Selected Preference: Arithmet
Total= 5000 Gated= 22

Gate		Events	% Gated	5 Tot
1	G1	17	0.77	0.
2	G2	2141	96.35	42.
3	G3	2	0.09	0.
4	G4	2222	100.00	44.
5	G5	0	0.00	0.
6	G6	2222	100.00	44.
7	G7	17	0.77	0.
8	G8	2141	96.35	42.

surface expression of CD45/CD14 and two, their light scattering properties (FSC versus SSC) (Fig. 5: A & B). Subsequently, each cell type (ie. monocytes or neutrophils) were identified based upon their presence within the established gates in both scenarios and isolated in a dot plot (Fig. 5: C & D) (35). Using these dot plots, the gates can be modified to exclude cellular contamination within the gates. The result of this process is the establishment of gates which will be subsequently used to examine samples. Processing the sample tubes begins at this point. The sample neutrophils and monocytes are plotted on a FSC vs. SSC dot plot (Fig. 6A) The gates that the cells fall within are the gates established using CD45/CD14 and FSC vs. SSC. Once the sample cells have been selected in this manner, the computer can then be told to look at each cell found within a gate, and subsequently show how much fluorescence (αMac-1) is present on each cell. The result is fluorescence levels depicted in the form of a single parameter histogram (Fig. 6B). Movement of the histogram to the right along the x-axis indicates the level of fluorescence is increased due to increased levels of the labeled molecule. The y-axis indicates the number of cells found at each level of fluorescence. Using this procedure, monocytes and neutrophils can be reliably isolated. and their levels of αMac-1 fluorescence can be determined and, more importantly, quantitated (5).

During the analysis, samples which were stimulated may contain a high proportion of cells that move out of their established gate. Moving out of a gate refers to the populations of monocytes and neutrophils which, due to degranulation, shft in light scattering properties relative to the unstimulated control. To accommodate for this, PMA-stimulated blood (100 ul) was mixed with CD45/CD14

Figure 6: A) FSC versus SSC dot plot of a patient sample. The neutrophils and monocytes fall within the previously established gates (see figure 5 for details). The computer is then able to indicate the levels of fluorescence on each cell in each population of cells. B) Histogram showing relative fluorescence intensities of monocytes (top) and neutrophils (bottom). Below each histogram are the statistics of each population. Our results were based on the mean fluorescence intensities (Mean).

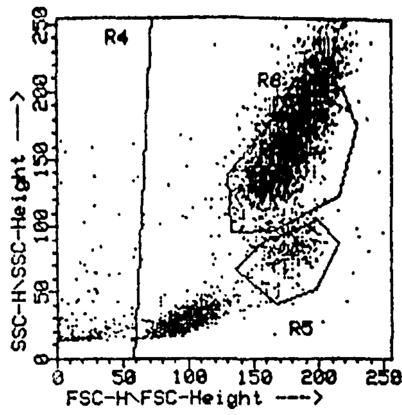
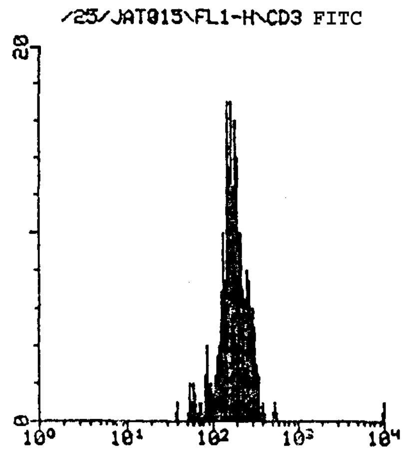


Figure 6B.



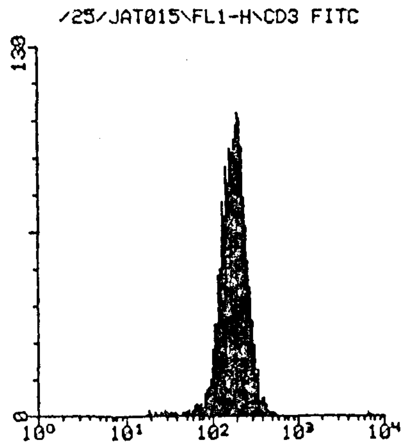
/25/JAT015\FL1-H\CD3 FITC

--- Arithmetic Histogram Statistics for /25/JAT015 ---

Selected Preferences: Arithmetic/Linear

Parameter FL1-H CD3 FITC Gate G5= R5

M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV %
0	1.00, 9646	271	100.00	17	148.55	218.70	171.54	578.65	>100.0



/25/JAT015\FL1-H\CD3 FITC

--- Arithmetic Histogram Statistics for /25/JAT015 ---

Selected Preferences: Arithmetic/Linear

Parameter FL1-H CD3 FITC Gate G6= R6

M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV %
0	1.00, 9646	2249	100.00	107	205.35	169.75	177.83	146.72	77.32

antibody during control tube preparations. Hence, this sample was used to set gates for those samples whose cells had moved "out" of the gates established using the unstirnulated blood - CD45/CD14 sample.

H. DATA ANALYSIS

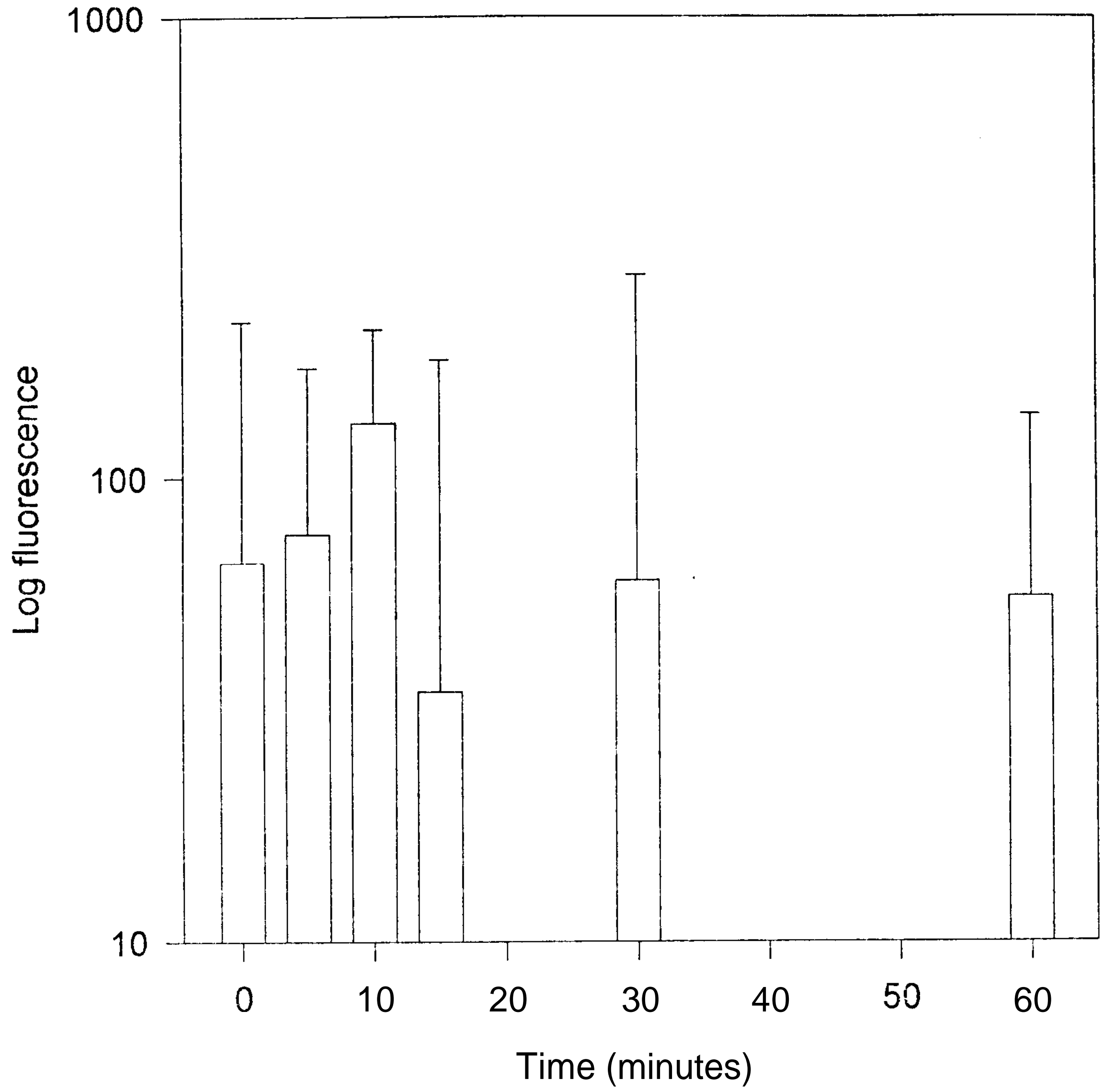
The results were expressed as mean fluorescence values as determined by flow cytometric analysis. The mean fluorescence (MF) values were averaged from duplicate trials then graphed using sigma plot graphing program. Any calculations were made using MF values. Any values that are given for comparison are likewise MF values.

III. RESULTS :

A. CONTROL STUDIES

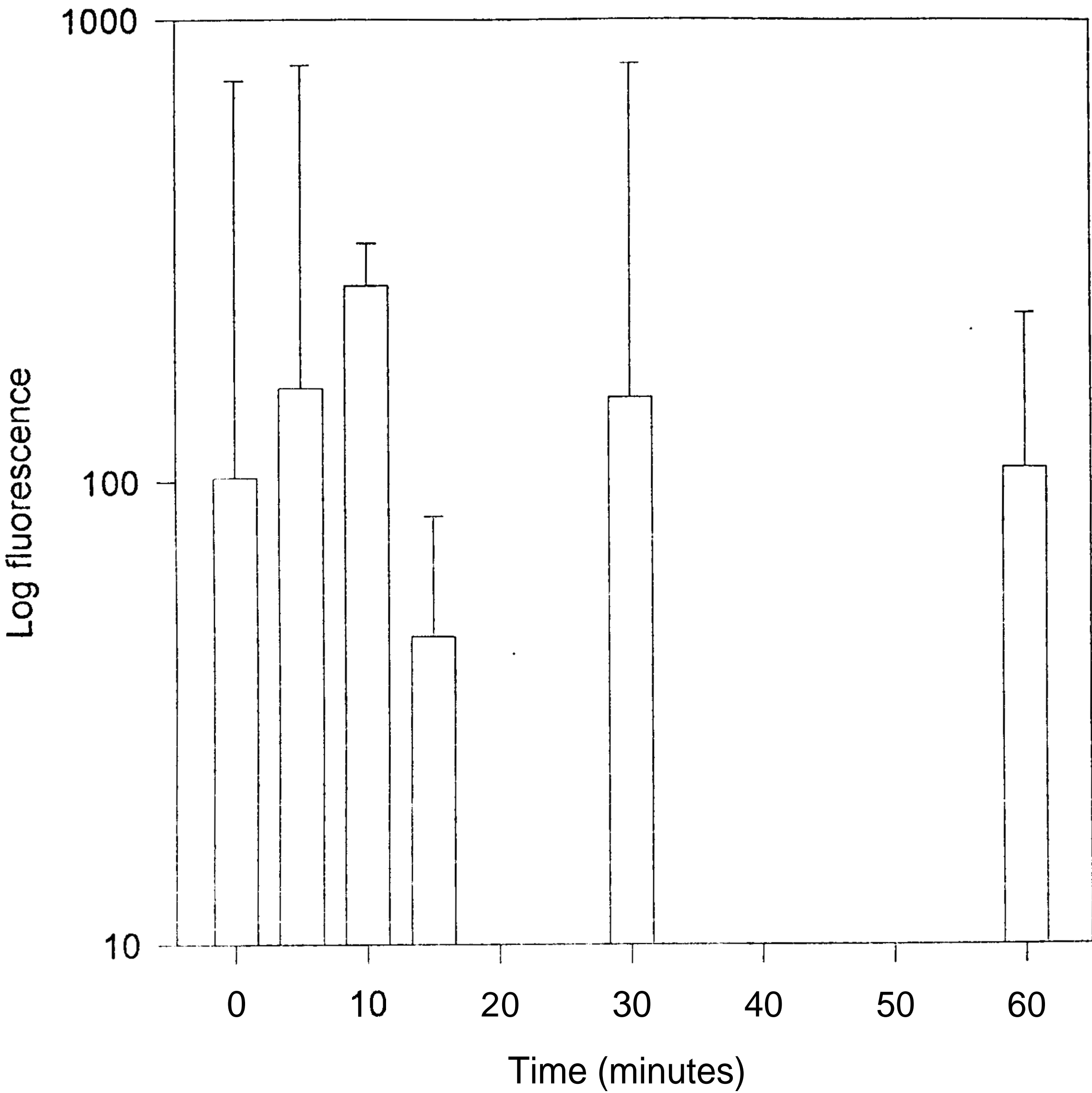
To determine if oxalate would be the best choice of anticoagulant for measuring Mac-1 levels on white blood cells, blood was collected in oxalate-containing vacuum tubes and processed as described in the materials and methods section. The purpose of these experiments was to examine Mac-1 expression as indicated by increased or decreased binding of monoclonal antibody to Mac-1. The time points for this study were as follows, 0, 5, 10, 15, 30, and 60 minutes post-draw. The zero (0) minute sample was drawn and processed immediately. During this time, 300 ul aliquots were removed from the room temperature sample at the corresponding time points and placed on ice ($4^{o}C$). At completion of the zero (0) minute sample, the remainder of samples were processed. Two separate experiments were performed in this manner. Our data from experiment 1 indicates that in the presence of oxalate, Mac-1 levels did not substantially change over a 60 minute period of incubation at room temperature (Fig's. 7-10). Similar results are seen when comparing Mac-1 expression on monocytes and neutrophils (Fig's. 7 and 8). Duplicate trials using oxalate were difficult to decipher since they yielded inconsistent levels of Mac-1 expression over time (Fig's. 7 and 9). For instance, neutrophil expression of Mac-1 was 64% higher at zero minutes for experiment 2, and was relatively higher at each corresponding time point. Experiment 2 also exhibited a much more profound increase in expression at 5 minutes. Additionally, Experiment 1 showed decreasing expression from 10 to 15 minutes, whereas experiment 2 showed a slight increase in expression. The most notable finding however, was an increase in

Figure 7: Bar graph representing αMac-1 mean fluorescence values for neutrophils isolated from oxalate anticoagulated whole blood. The x-axis represents time after blood collection in minutes, ranging fiom 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.



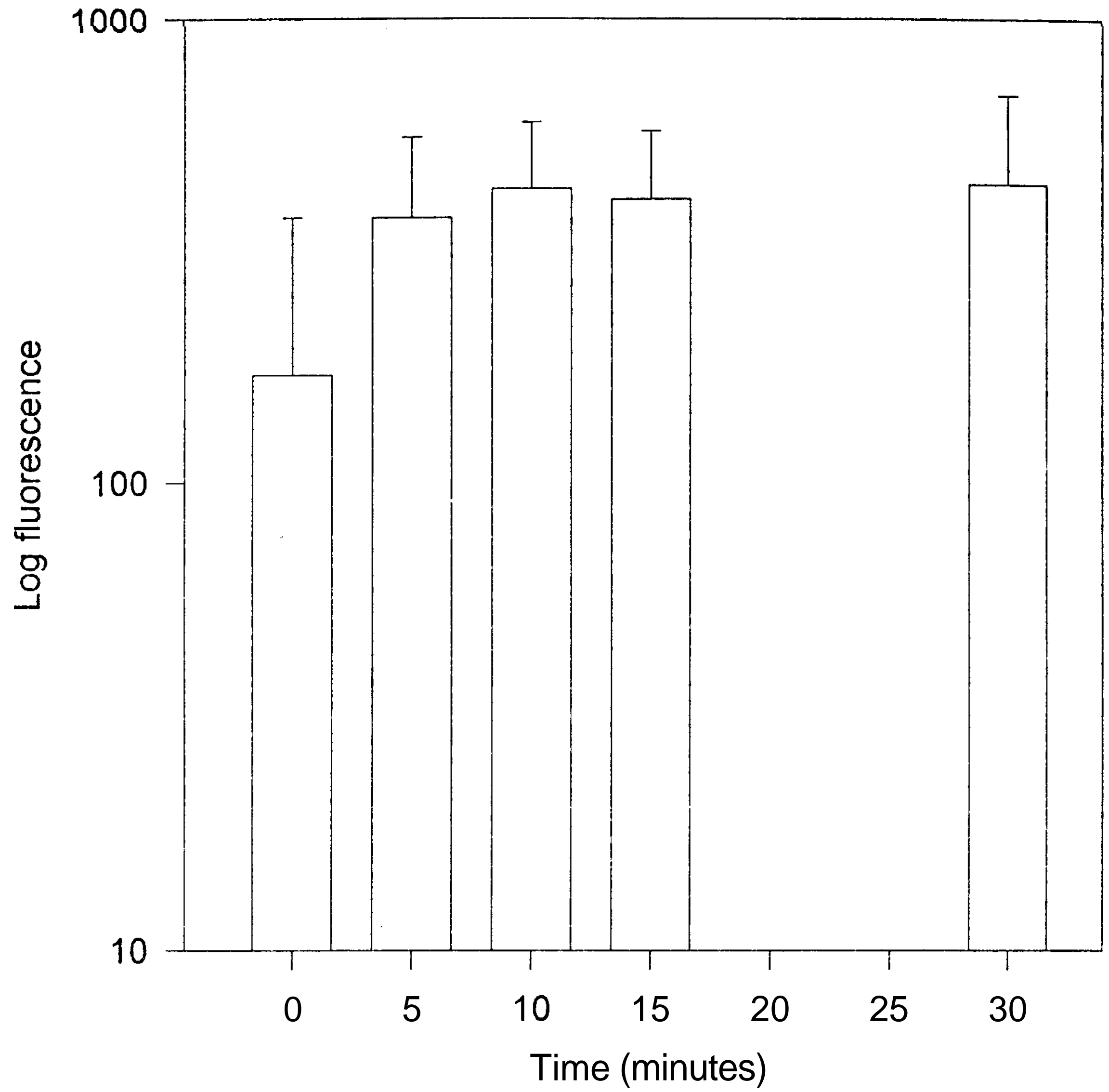
PMA-stimulated fluorescence = 81.8
Experiment 1 (TPO)

Figure 8: Bar graph representing αMac-1 mean fluorescence values for monocytes isolated from oxalate anticoagulated whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.



PMA-stimulated fluorescence = 232.9
Experiment 1 (TPO)

Figure 16: Bar graph representing αMac-1 mean fluorescence values for monocytes isolated from heparinized whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 30. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.



PMA-Stimulated = 1355.3

Experiment 3 (DDA2)

Figure 15: Bar graph representing αMac-1 mean fluorescence values for neutrophils isolated from heparinized whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 30. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.

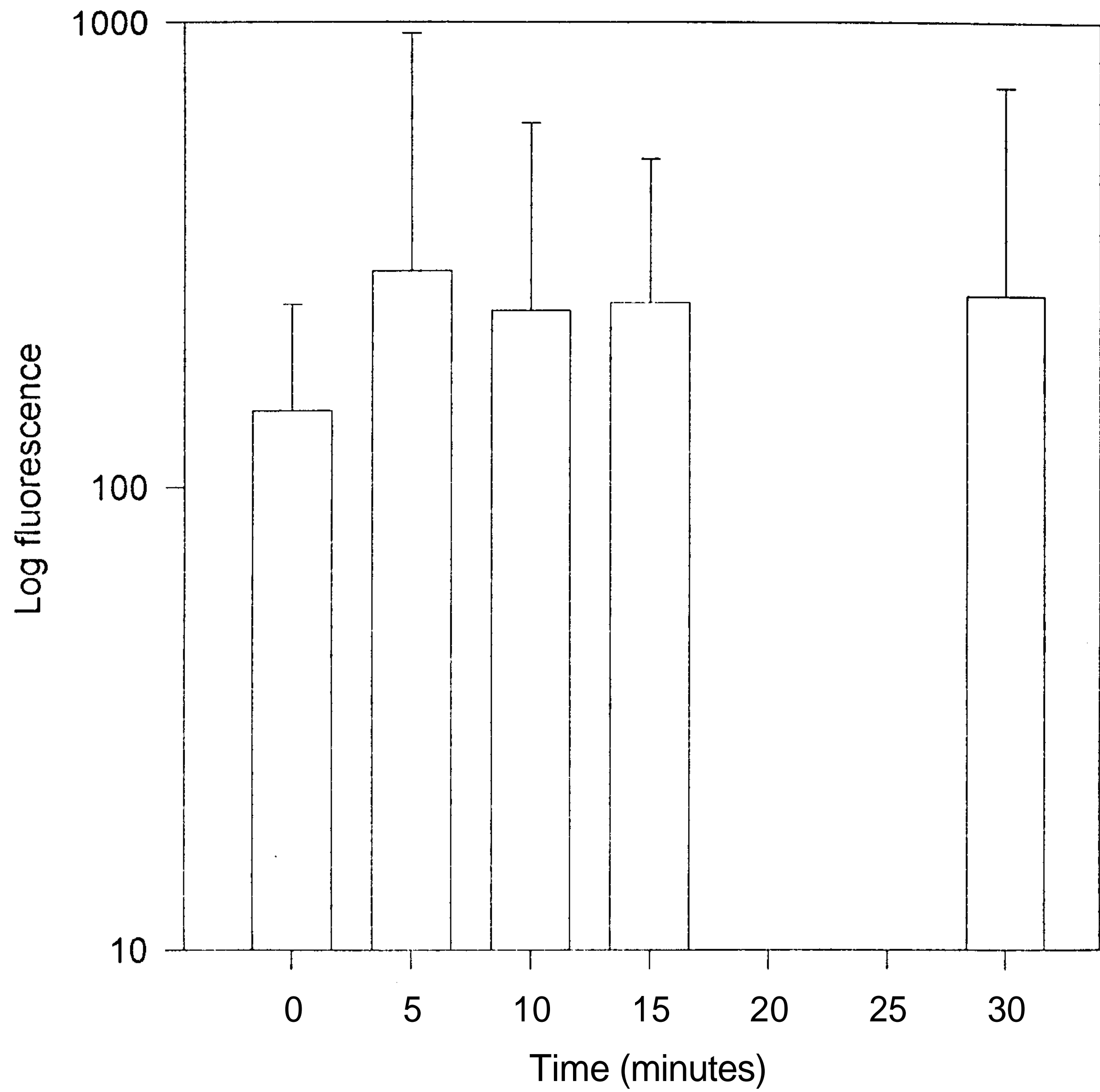
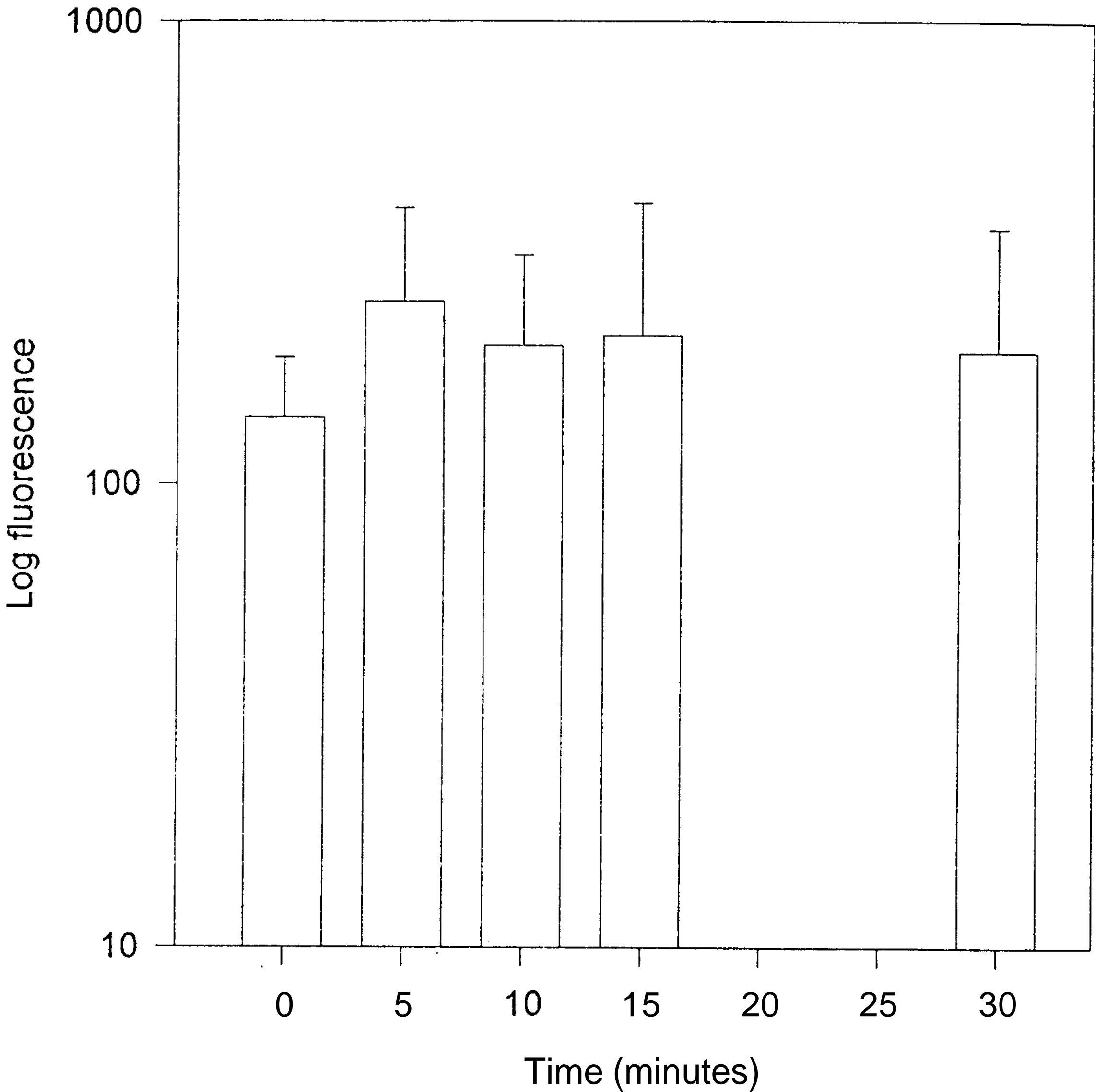

Heparin/Mac-1/Monocytes
1000
100
10
Log fluorescence
0
5
10
15
20
25
30
Time (minutes)
PMA-Stimulated = 840.4
Experiment 2 (DDA1)

Figure 14: Bar graph representing αMac-1 mean fluorescence values for monocytes isolated from heparinized whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 30. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.

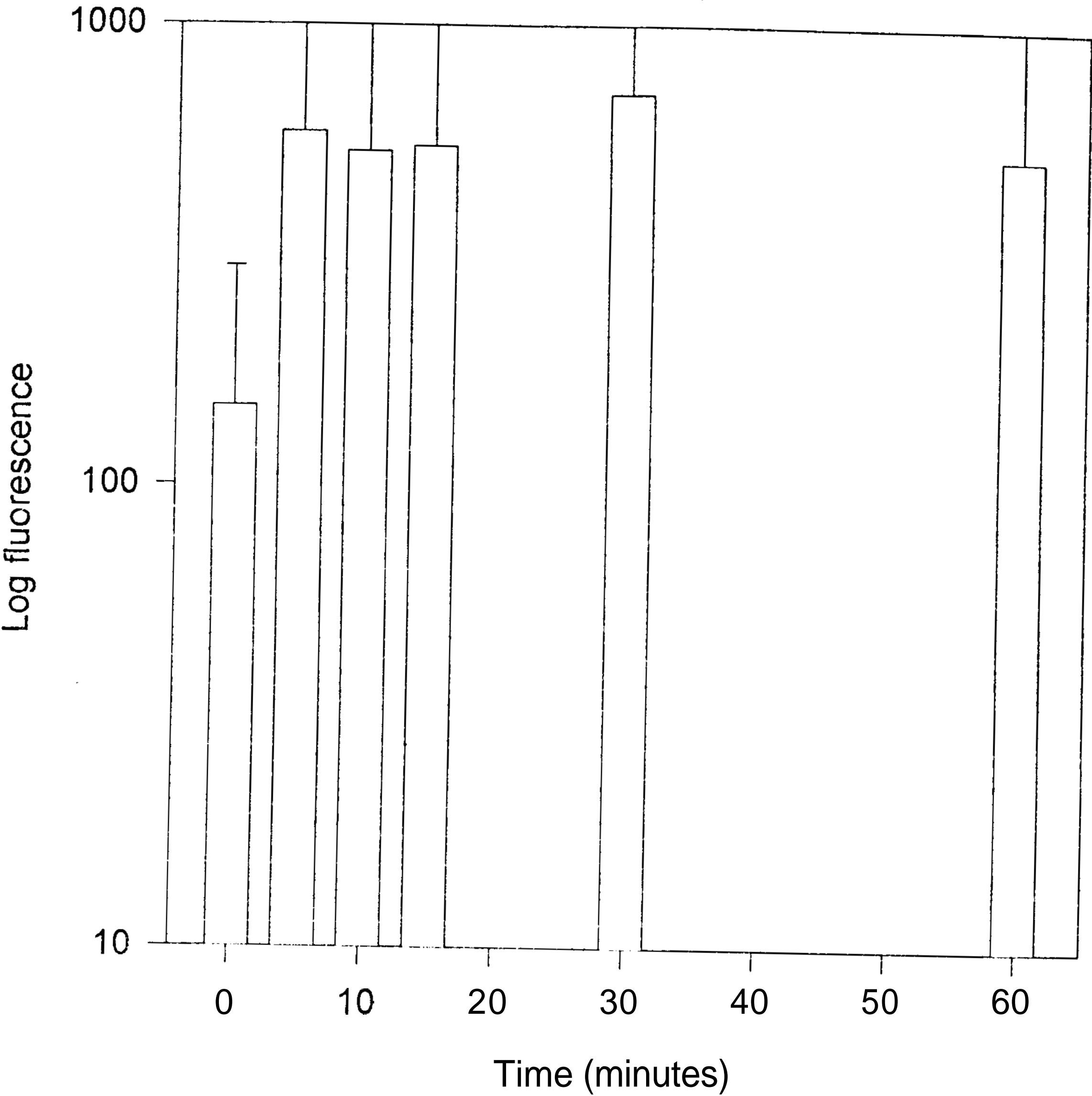
Heparin/Mac-1/Neutrophils



PMA-Stimulated = 1198.5

Experiment *2* (DDA1)

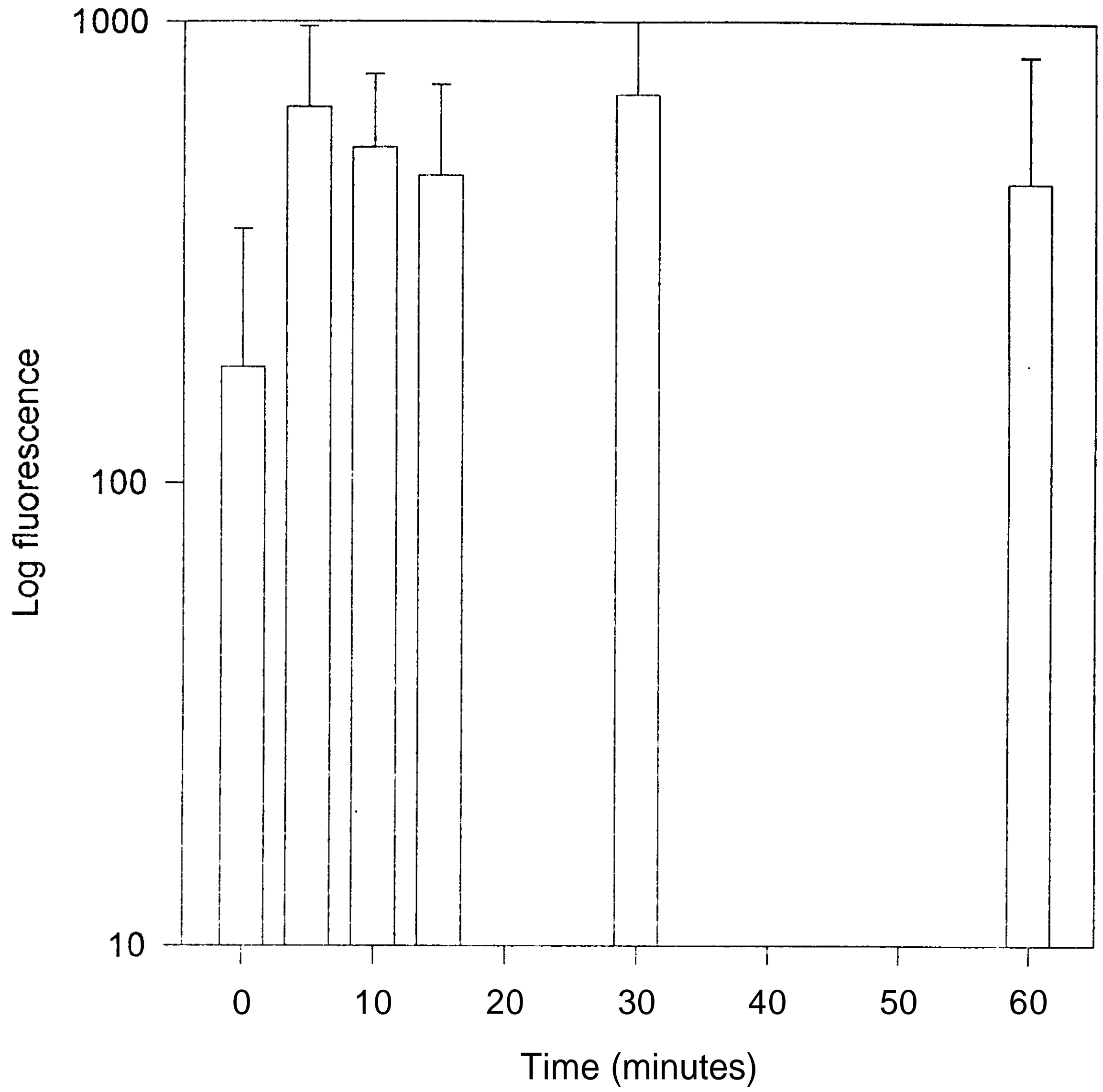
Figure 13: Bar graph representing αMac-1 mean fluorescence values for neutrophils isolated from heparinized whole blood. The x-axis represents time after blood collection in minutes, ranging fiom 0 to 30. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.



PMA-stimulated fluorescence = 276

Experiment 1 (KXM)

Figure 12: Bar graph representing αMac-1 mean fluorescence values for monocytes isolated from heparinized whole blood. The x-axis represents time after blood collection in minutes, ranging fiom 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.



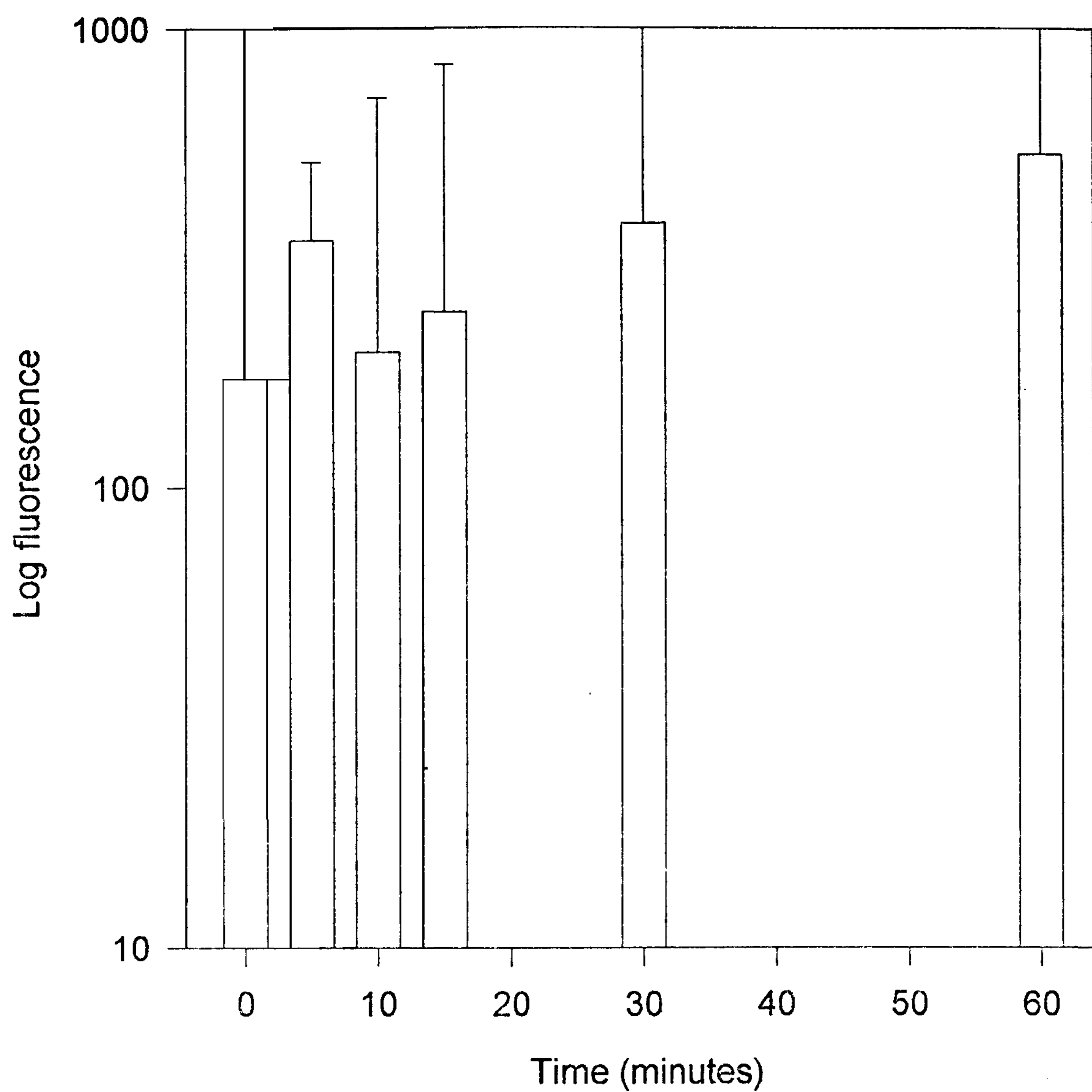
PMA-stimulated fluorescence = 564.5

Experiment I (KXM)

Figure 11: Bar graph representmg aMac-1 mean fluorescence values for neutrophils isolated from heparinized whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.

expression at 5 minutes, which was seen in both experimental trials. Moreover, both neutrophil and monocyte unstimulated levels of Mac-1 expression at 10 minutes post-draw were higher than the Mac-1 levels found on cells stimulated with phorbol esters. Based on the information presented here, blood cells incubated in oxalate expressed Mac-1 inconsistently and in greater numbers within 5 minutes, therefore the investigation was redirected towards heparin.

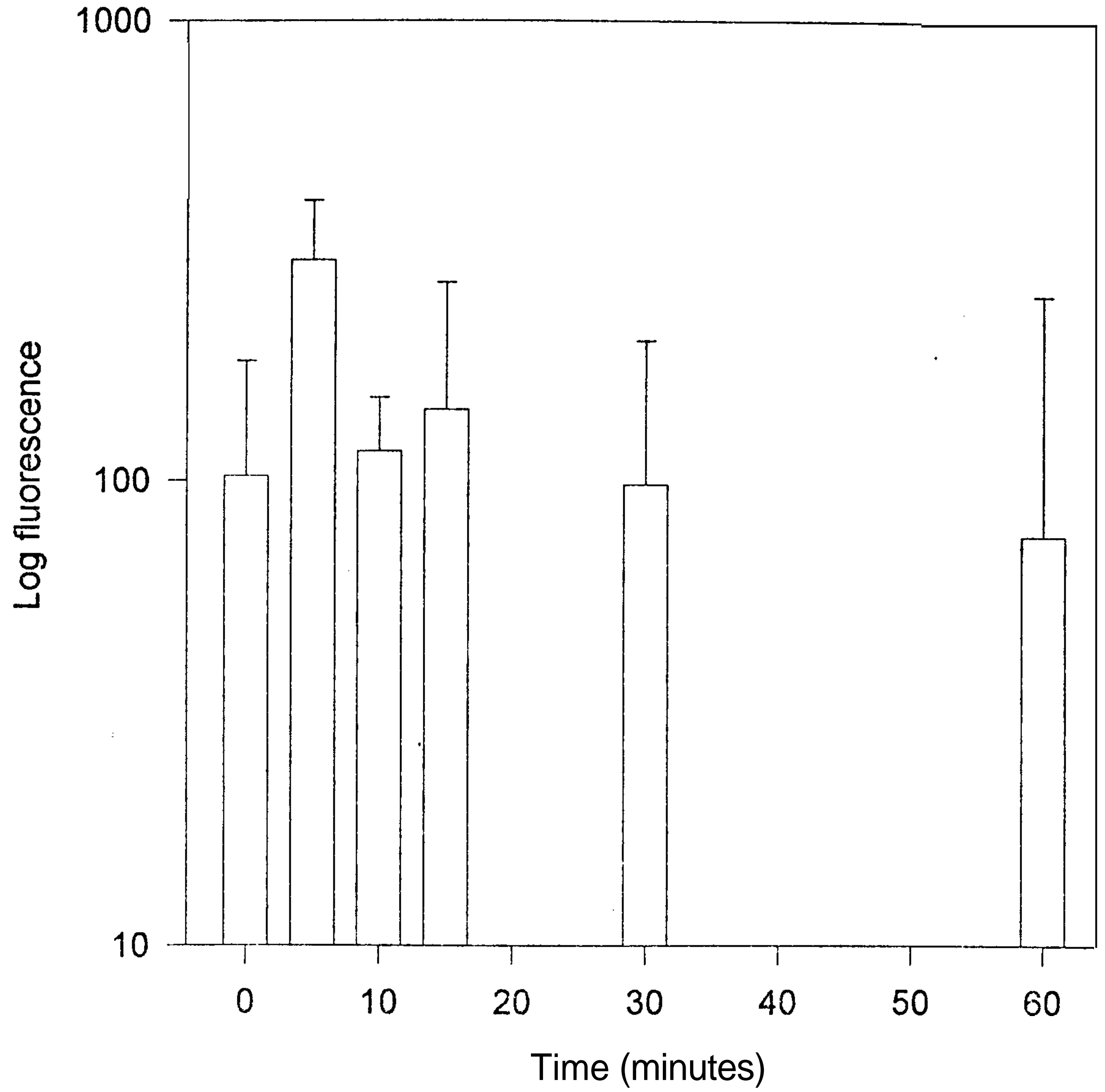
To investigate the usefulness of heparin as a patient study anticoagulant, blood was collected in heparin and processed as described in the materials and methods section. The data obtained from three studies utilizing heparin (Fig's.11-16) showed trends similar to oxalate data. Experiment 1 was processed with samples from 0 to 60 minutes. Even though there was not a substantial difference between the time points, this trial showed that the levels of Mac-1 on the cells increased within 5 minutes for both monocytes and neutrophils (25% and 27%, respectively) (Fig's. 11 and 12). In fact, unstimulated monocyte and neutrophil Mac-1 expression was higher than the stimulated samples by 5 minutes, and remained higher throughout the duration of the experiment (unstimulated neutrophil expression was lower at one time point, 10 minutes, but only very minimally). Moreover, the increased expression generally remained elevated throughout the duration of each study. Experiment 2 (Fig's. 13 and 14) and experiment 3 (Fig's. 15 and 16) showed slightly different results. The increased expression seen at 5 minutes was also noted in experiments 2 and 3, however the highest levels of unstimulated Mac-1 expression was not as high as the level on PMA stimulated cells. Because of the increases in Mac-1 levels seen within 5 minutes during



PMA-stimulated fluorescence = XXX
Experiment 2 (DBT)

Figure 10: Bar graph representing αMac-1 mean fluorescence values for monocytes isolated from oxalate anticoagulated whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.

Oxalate/Mac-1/Neutrophils

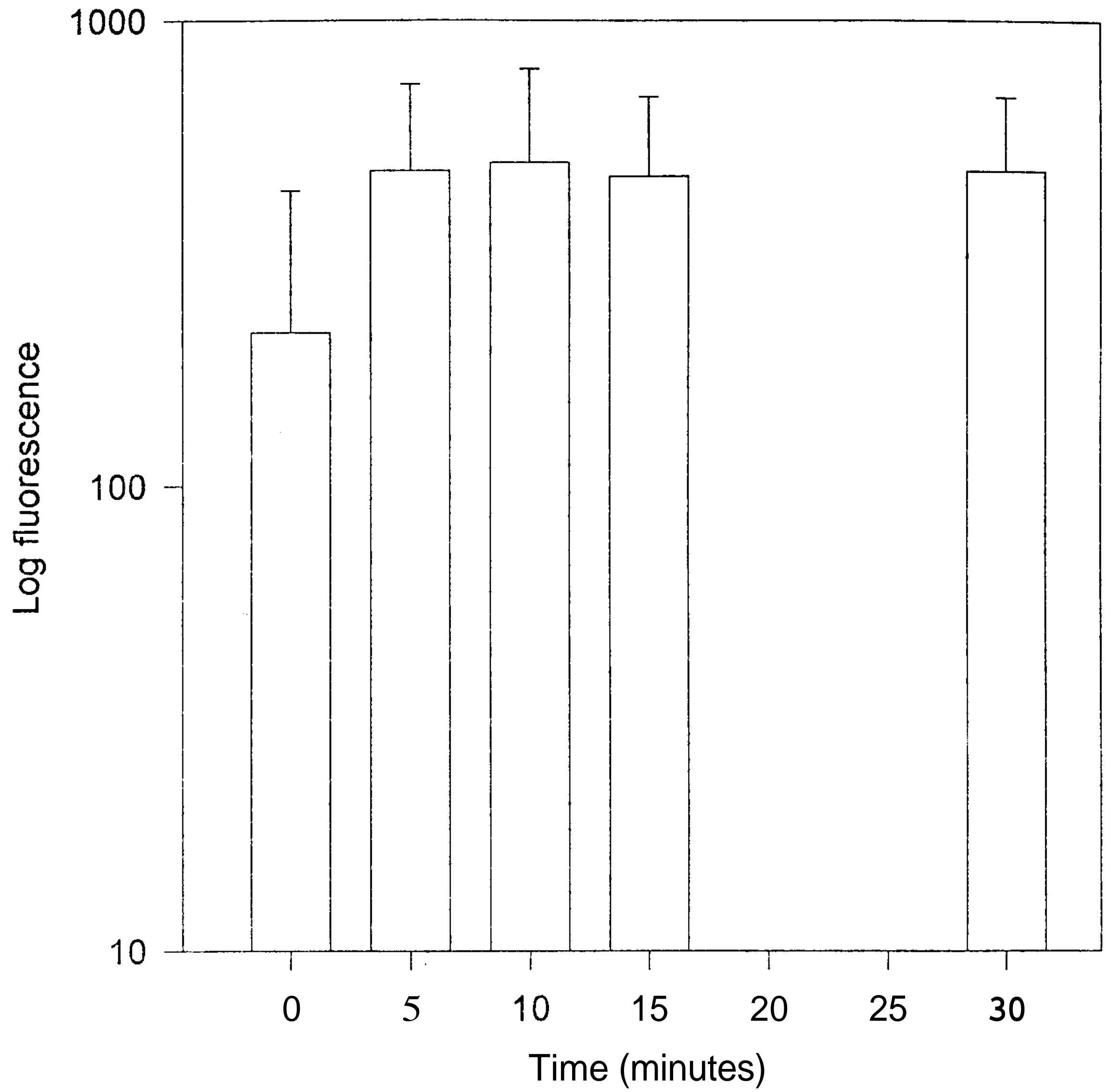


PMA-stimulated fluorescence = XXX

Experiment 2 (DBT)

Figure 9: Bar graph representing αMac-1 mean fluorescence values for neutrophils isolated from oxalate anticoagulated whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.

Heparin/Mac-1/Monocytes



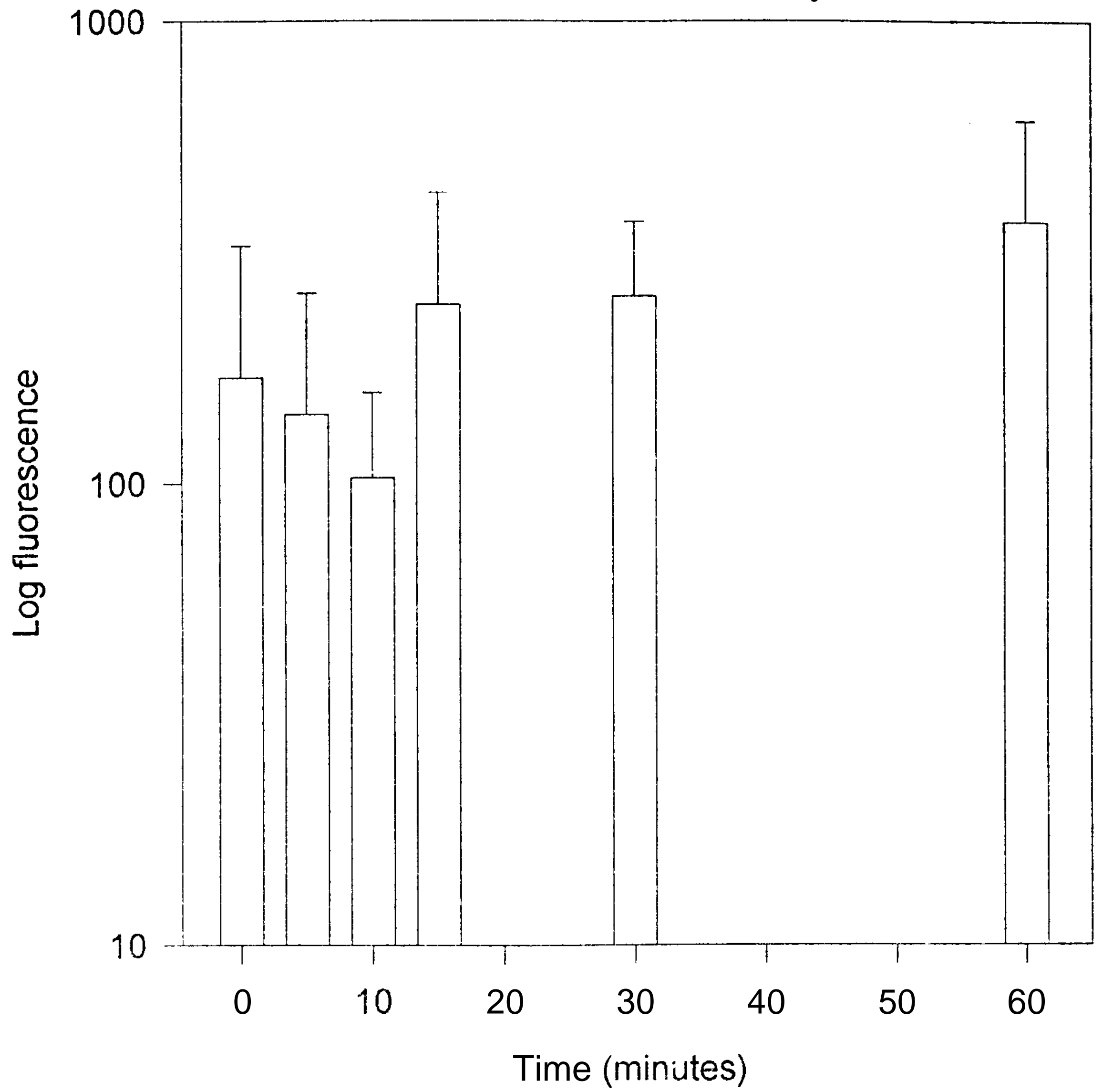
PMA-Stimulated = 521.1

Experiment 3 (DDA2)

experiment 1, experiment 2 and experiment 3 were processed fiom 0 to 30 minutes. Both experiments 1 and 2 (monocytes and neutrophils for each) had PMA-stimulated values that were substantially higher than any of the unstimulated values (ie. at 5 minutes 246.7 is the highest value for neutrophils, while the stimulated value was 1198.5; Fig. 13) (also see Fig's. 14-16). The responses seen on neutrophils and monocytes were similar in appearance for all three experiments, that is, they increased in Mac-1 expression fiom 0 (zero) to 5 minutes and remained relatively constant.

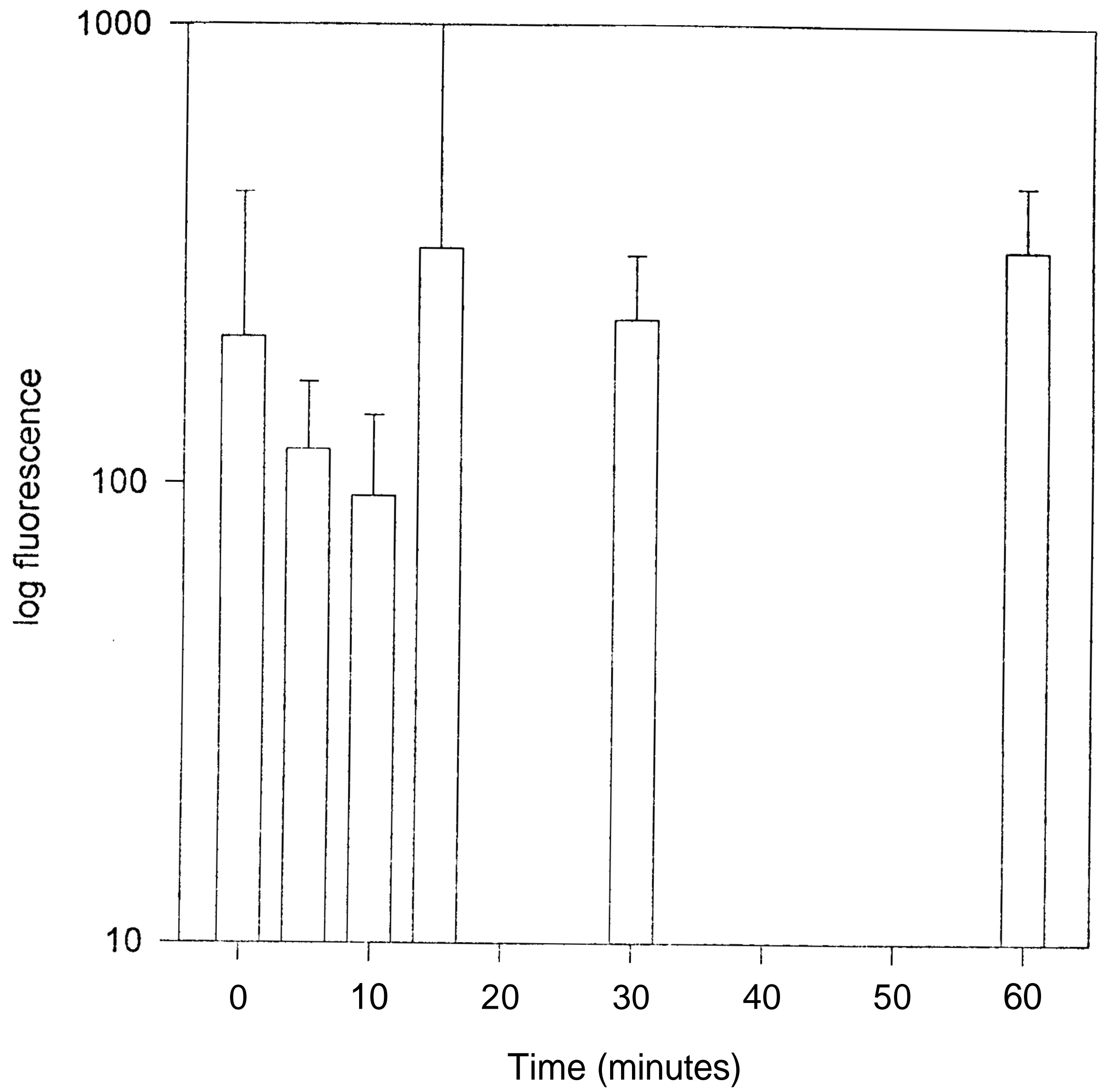
To examine the effects of EDTA on Mac-1 expression when used as an anticoagulant blood was collected into tubes containing EDTA, and one experiment was performed. This test procedure was performed using the same protocol as that used for the other anticoagulants. Data obtained from this study (Fig's. 17 and 18) showed that Mac-1 expression decreased slightly at 5 and 10 minutes post-draw. At approximately 15 minutes the white blood cells showed slight increases in the levels of Mac-1 expression, however, there was not a substantial difference between the values of any time point, for neither neutrophils nor monocytes. Also, the mean fluorescence value of PMA-stimulated samples for neutrophils was substantially higher than any of the unstimulated values at all time points. Monocytes exhibited PMA-stimulated expression levels that were also higher than the unstimulated samples, but less than that seen with neutrophils, and only prior to the 15 minute sample. The trends in Mac-1 expression seen were the same for both monocytes and neutrophils. Furthermore, the standard deviations were smaller than those seen when oxalate or heparin was used as the anticoagulant. Mac-1 expression levels on

Figure 17: Bar graph representing αMac-1 mean fluorescence values for neutrophils isolated from EDTA anticoagulated whole blood. The x-axis represents time after blood collection in minutes, ranging from 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging from 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.



PMA-stimulated fluorescence = 646
Experiment 1 (TSP)

Figure 18: Bar graph representing αMac-1 mean fluorescence values for monocytes isolated from EDTA anticoagulated whole blood. The x-axis represents time after blood collection in minutes, ranging fiom 0 to 60. The y-axis represents the mean fluorescence units on a logarithmic scale, ranging fiom 10 to 1000 log fluorescence units. Standard error of the mean is represented by error bars.



PMA stimulated fluorescence = 222
Experiment 1 (TSP)

leukocytes collected in EDTA or heparin were initially (zero time point) similar, and higher than the initial levels seen when using oxalate (compare Figures 7, 11 and 17).

III. RESULTS:

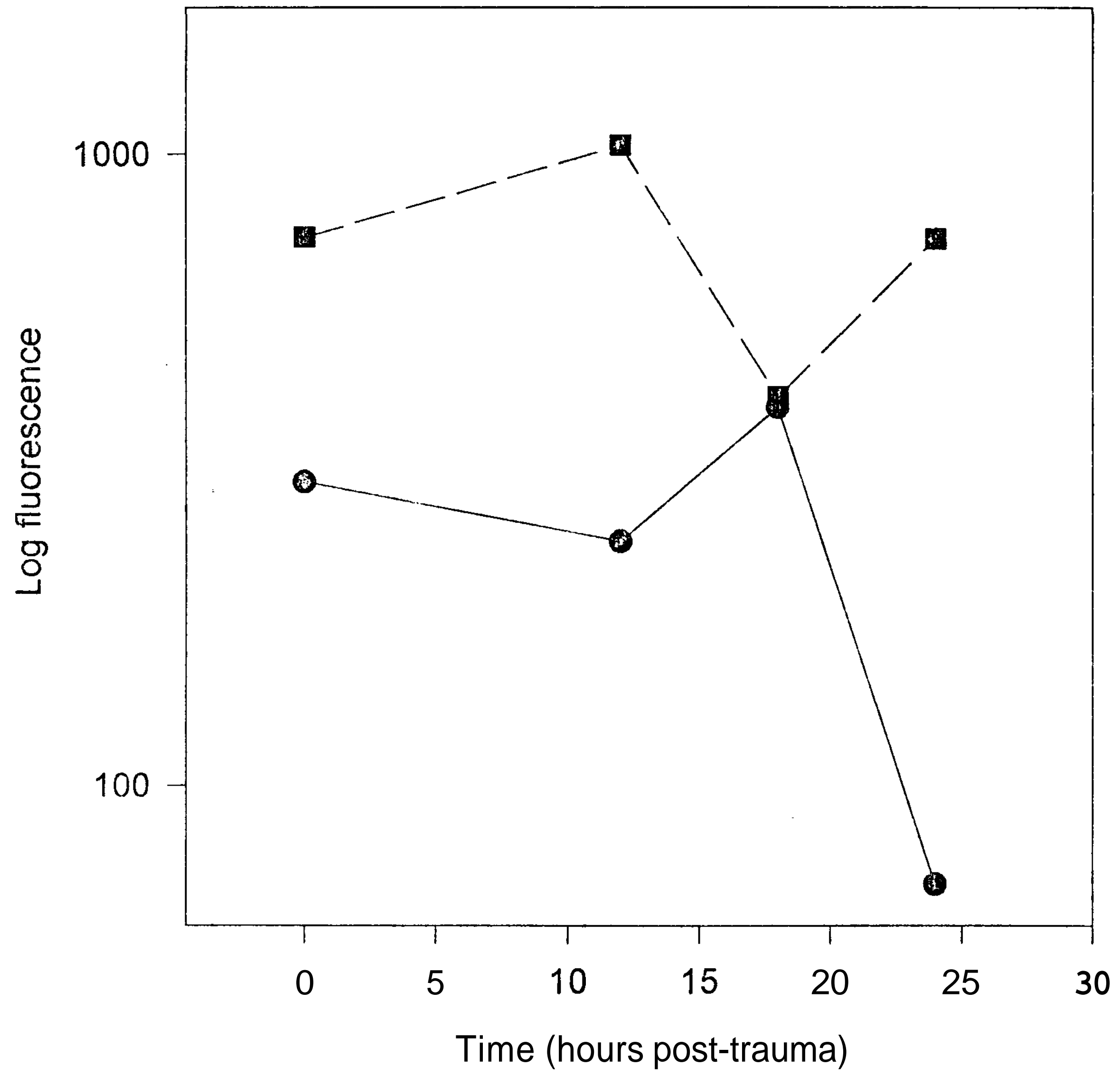
B. PATENT STUDIES

Analyzing trauma patient white blood cells for expression of Mac-1 may possibly identify some correlation between patients who do or do not develop ARDS. To investigate this hypothesis, blood was collected from trauma patients, at various time points (0, 6, 12, 18 and 24 hours), after the initial traumatic insult. Following blood collection, the cells were labeled with an antibody directed against Mac-1 and a fluorescence tag that indicates the amount of Mac-1 on the cell surface (see materials and methods for complete protocol). Patients were placed into one of three categories depending on the extent of lung injury subsequent to hospital admission: ARDS, acute lung injury, and non-ARDS. Two trauma patients, A and B, were categorized as ARDS patients (n = 2) and their leukocytes were examined for Mac-1 expression. For comparison, a patient with acute lung injury (n = 1) and patients that did not develop ARDS (n = 3) were studied.

Patient A's unstimulated samples exhibited increased expression of Mac-1 on both monocytes and neutrophils at 18 hours post-trauma (Fig's. 19 and 20), however, this increase was much less extreme for neutrophils than monocytes. At the earlier time points, neutrophil expression of Mac-1 was higher than seen on monocytes, hence the seemingly smaller increase at 18 hours. At 18 hours post-trauma, the levels of Mac-1 on unstimulated leukocytes reached the same level as

Figure 19: Line graph showing αMac-1 mean fluorescence values for patient A (ARDS) neutrophils isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient A (ARDS) / Neutrophils (Mac-I)

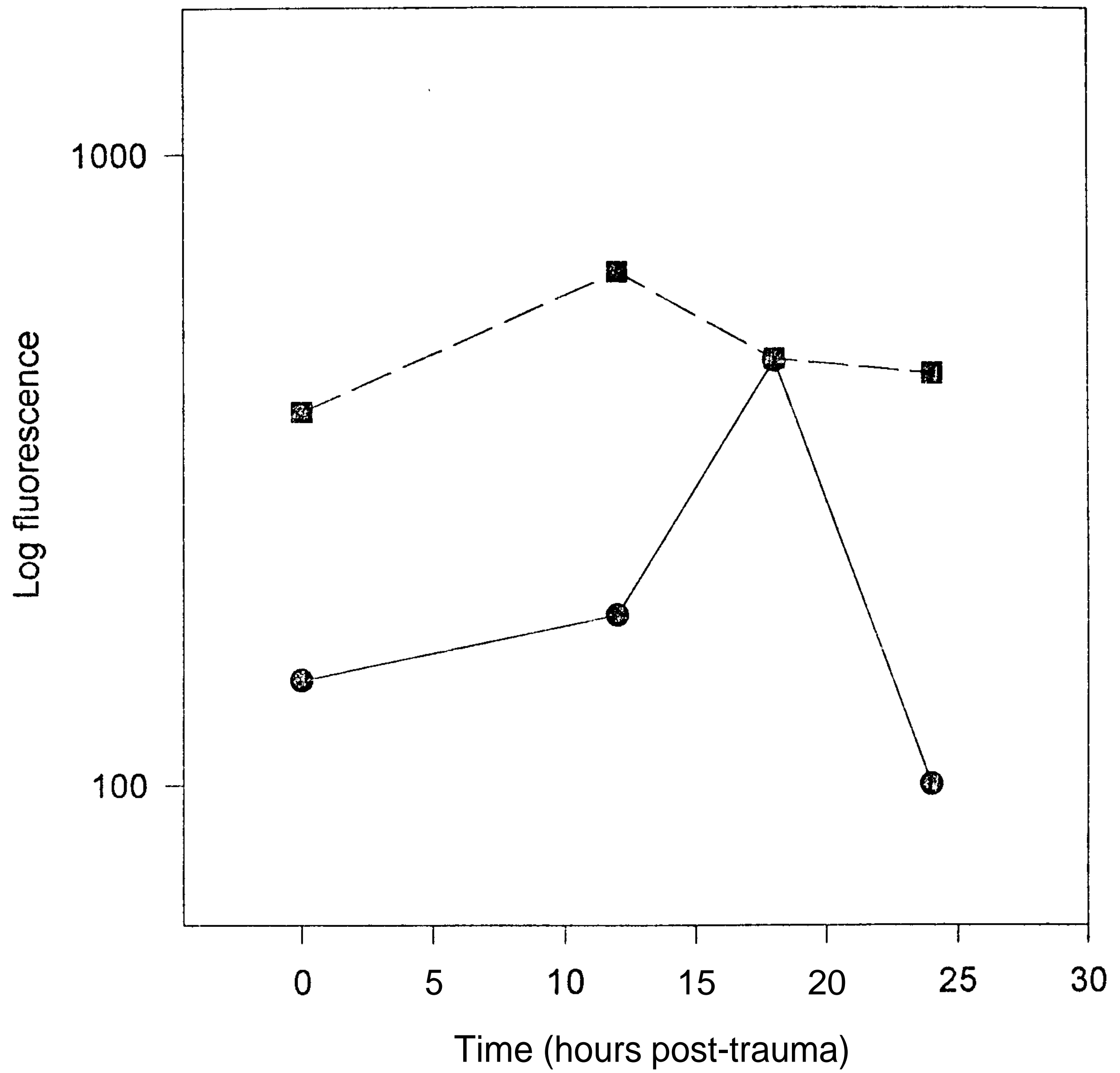


PMA-Stimulated - dashed line (squares

Unstimulated - solid line (circle)

Figure 20: Line graph showing αMac-1 mean fluorescence values for patient A (ARDS) monocytes isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstirnulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient A (ARDS) / Monocytes (Mac-1)



PMA-stimulated = dashed line (squares
unstimulated = soloid line (circles)

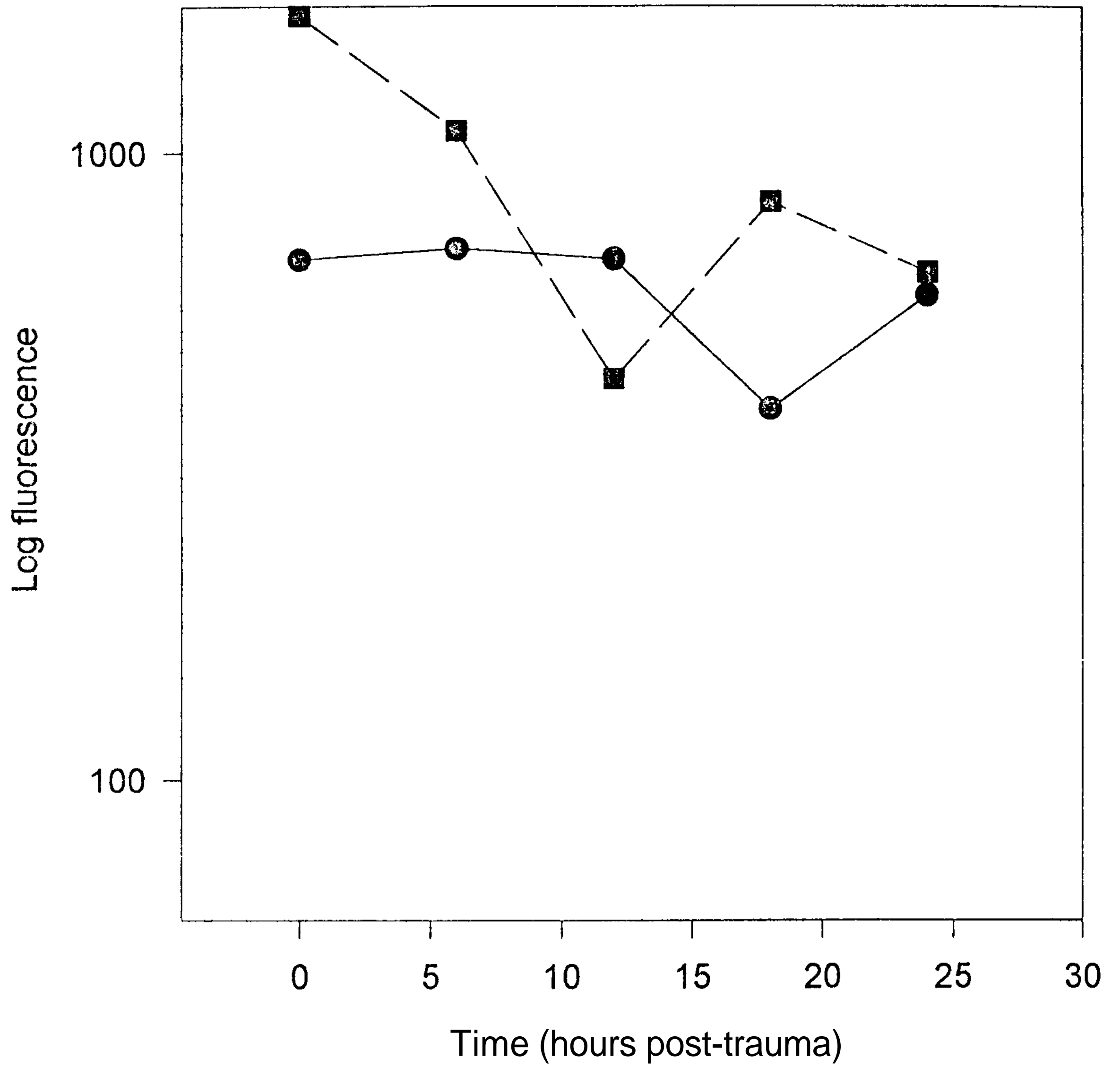
those of PMA-stimulated cells. Every time point other than 18 hours showed what is expected, unstimulated levels below the PMA-stimulated levels.

Patient B's unstimulated samples showed high levels of Mac-1 expression at all time points throughout the duration of sample analysis (Fig's. 21 and 22). Patient B had much higher Mac-1 expression levels on both unstimulated monocytes and unstimulated neutrophils at all time points when compared to patient A. (Patient A neutrophils had an average value of 252.4 whereas patient B neutrophils had an average value of 599.4). Levels of Mac-1 on patient B's cells were even higher than levels attainable using a phorbol ester to stimulate the cell. The predominant observation to note is that both ARDS patients (A and B) exhibited maximal or near maximal levels of Mac-1 expression on the surface of both monocytes and neutrophils. Moreover, The range of expression levels in ARDS patients seem to fall into a range above 400 fluorescence units (ie. 18 hours for patient A and all time points for patient B). These responses seem to be more robust for neutrophils than monocytes.

Patient C did not progress to ARDS, however this patient did develop a condition known as Acute Lung Injury (ALI) (n = 1), which is often considered mild ARDS. The germane finding was that Mac-1 expression on patient C's unstimulated white blood cells were similar to stimulated levels at most time points, and were also at levels similar to ARDS patients (around 500-600 fluorescence units) (Fig's. 23 and 24). These "trends" were exhibited by both monocytes and neutrophils. Patient C unstimulated neutrophil expression of Mac-1 was lower than those levels attainable by stimulating with phorbol esters, whereas

Figure 21: Line graph showing αMac-1 mean fluorescence values for patient B (ARDS) neutrophils isolated fiom EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging fiom 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging fiom 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophl mean fluorescence values are represented by circles and a solid line.

Patient B (ARDS) / Neutropkils (Mac-I)

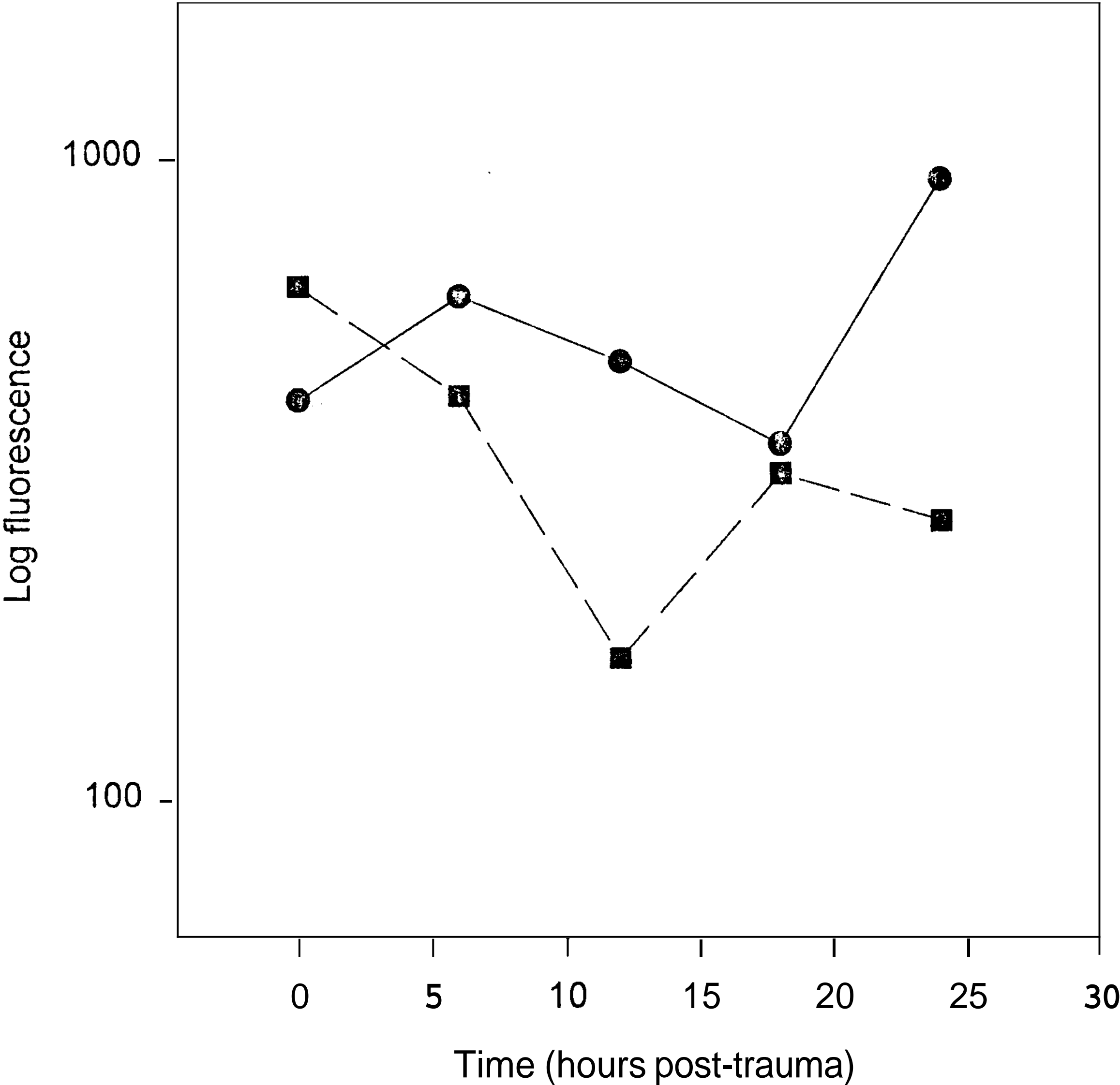


PMA-Stimulated = dashed line (squares:

Unstimulated = solid line (circles)

Figure 22: Line graph showing αMac-1 mean fluorescence values for patient B (ARDS) monocytes isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient B (ARDS) / Monocytes (Mac-1)

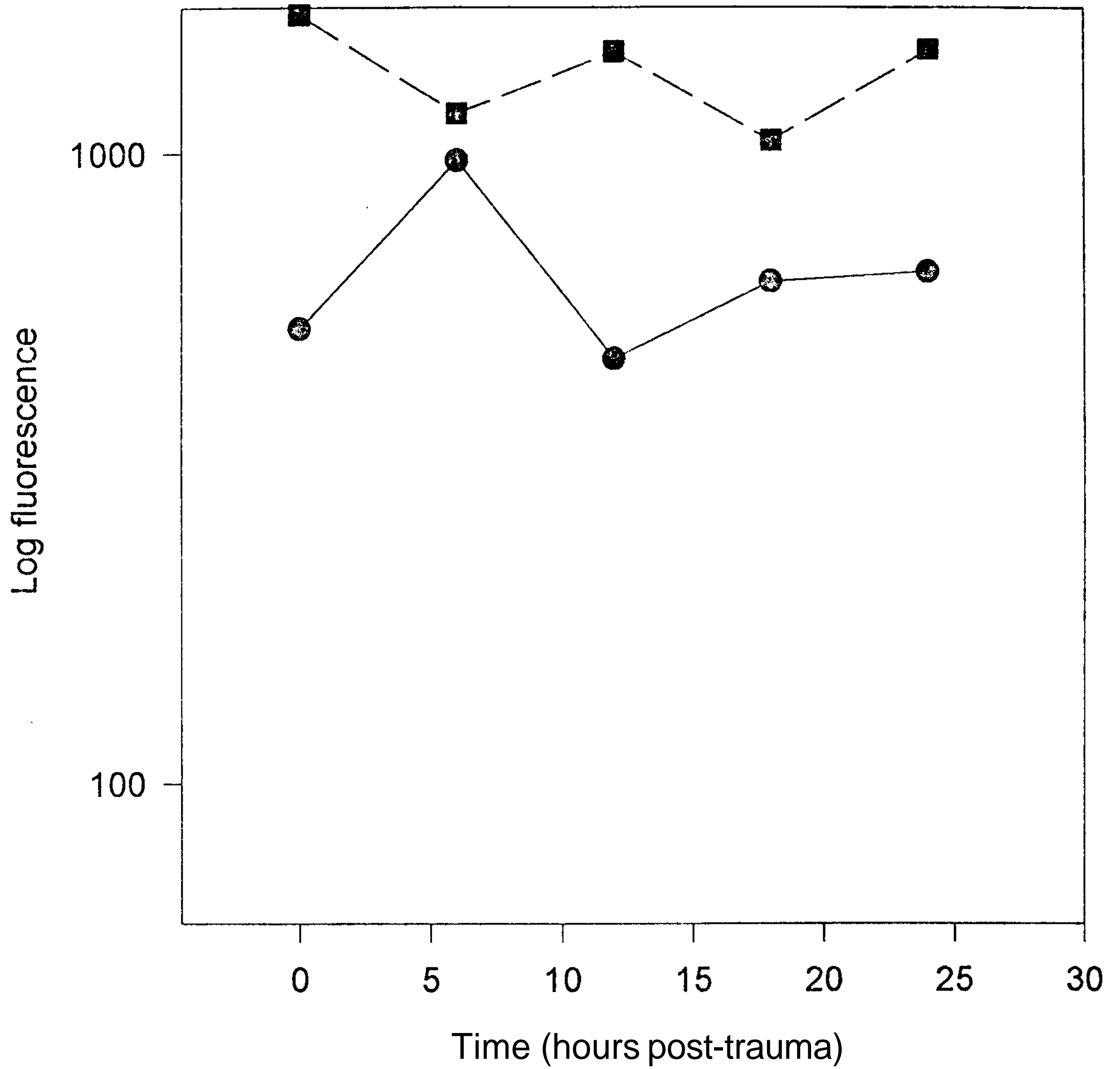


PMA-Stimulated = dashed line (squares)

Unstimulated = solid line (circles)

Figure 23: Line graph showing αMac-1 mean fluorescence values for patient C (Acute Lung Injury) neutrophils isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient C (ALI) / Neutrophils (Mac-I)

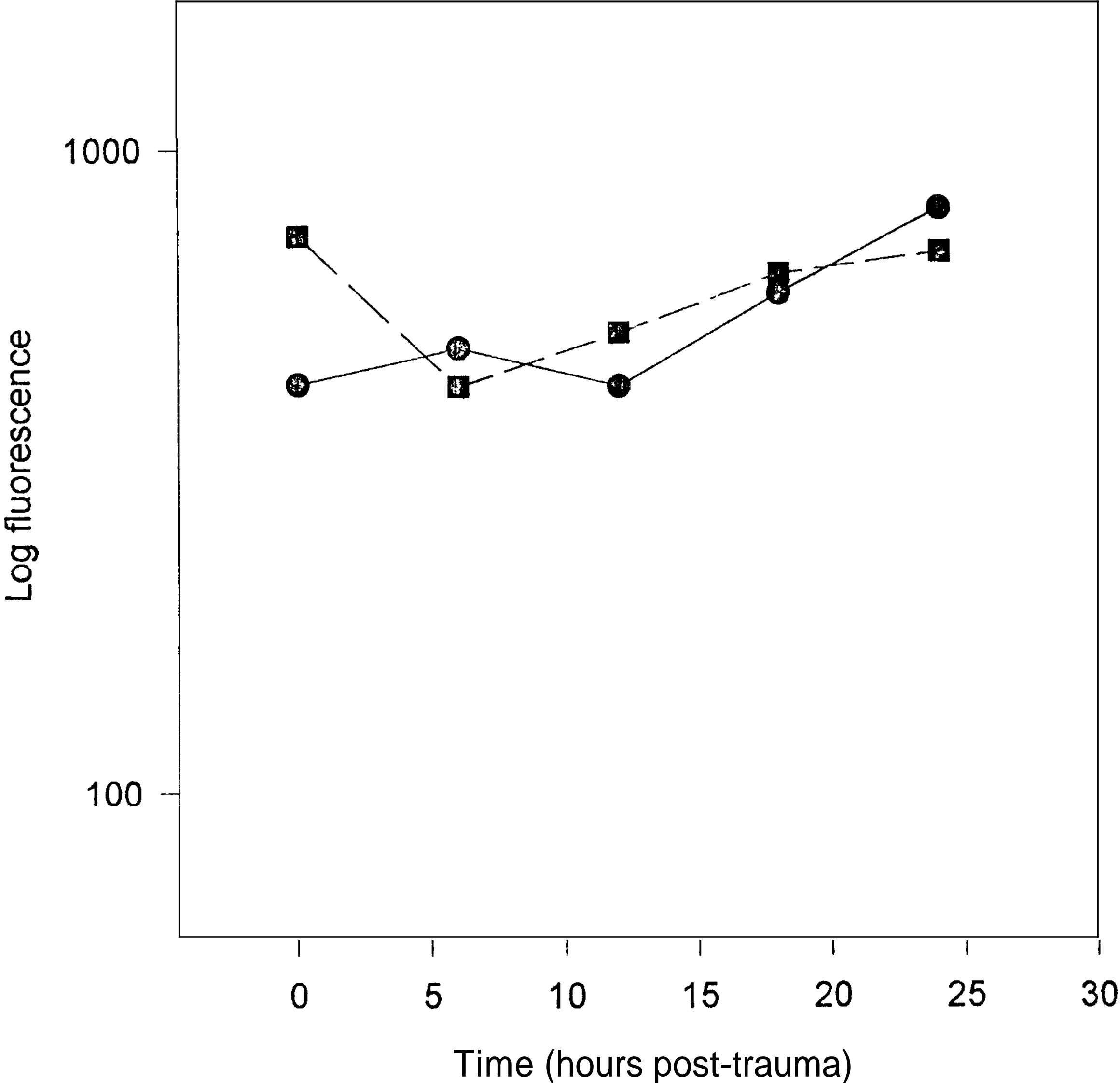


PMA-Stimulated = dashed line (syuares)

Unstimulated = solid line (circles)

Figure 24: Line graph showing αMac-1 mean fluorescence values for patient C (Acute Lung Injury) monocytes isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient *C* (ALI) / Monocytes (Mac-1)



PMA Stimulated - dashed line (square:

Unstimulated - solid line (circles)

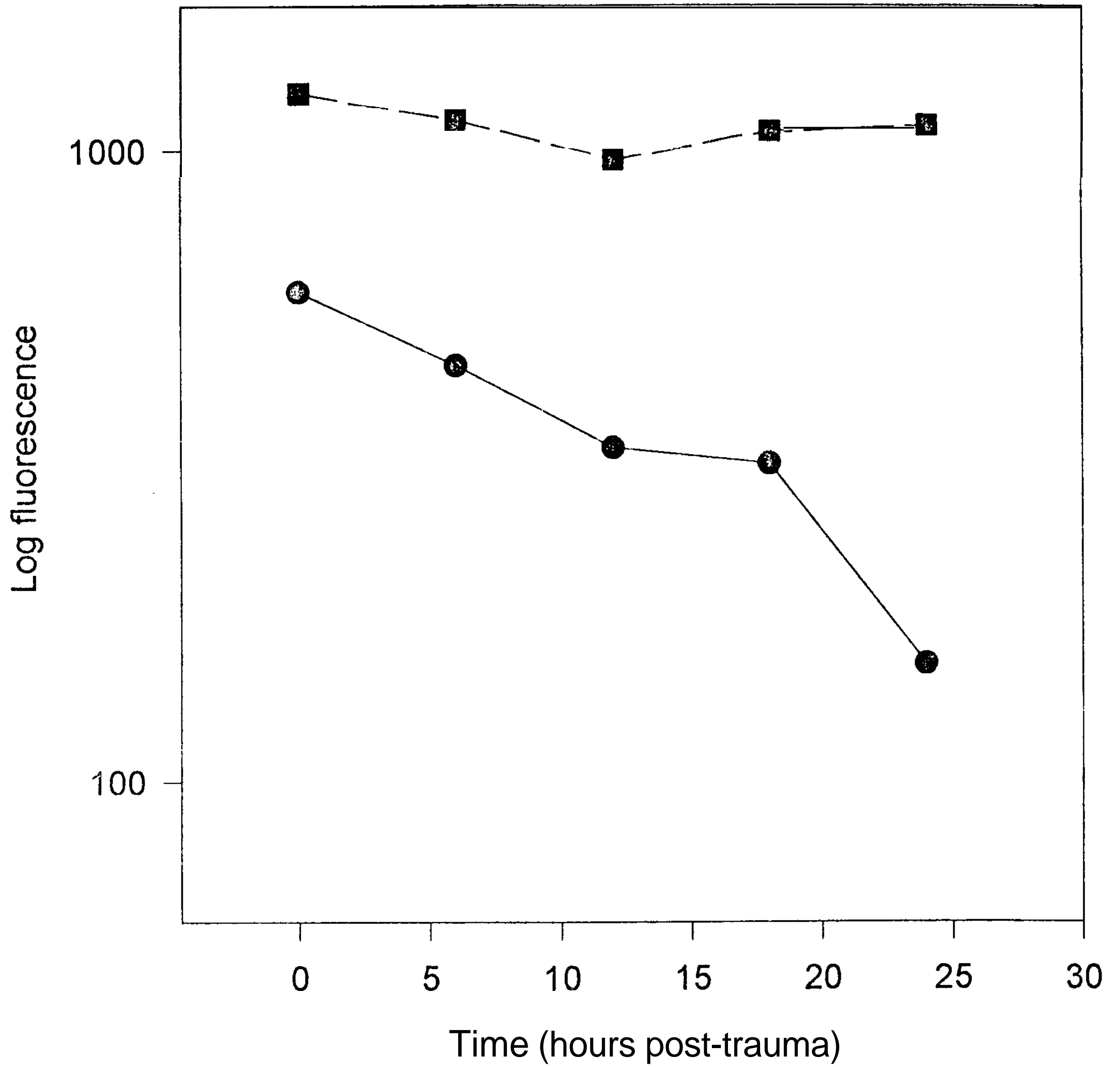
monocyte unstimulated values were almost the same as PMA-stimulated levels at all time points.

In order to compare the findings pertaining to ARDS patients, three trauma patients (D, E and F) who did not develop ARDS were also examined, and were classified as non-ARDS patients (n = 3). Unstimulated samples from patient D (Fig's. 25 and 26) exhibited Mac-1 expression at the later time points which were more characteristic of healthy controls (see reference 47). Mac-1 up-regulation in unstimulated cells did not occur to the extent that was seen in the 3 lung injury patients (2 ARDS and 1 ALI). In fact, it appeared that the fluorescence levels were decreasing towards baseline levels. Early increases were seen in neutrophrl Mac-1 expression, but most importantly, these levels declined relative to stimulated samples with time. Monocytes, on the other hand, showed expression levels at 0 hours which were slightly lower than for neutrophils. However, the monocyte levels remained constant throughout the duration of the study, and were sub-maximal as seen by comparison to the PMA-stimulated samples. The fluorescence levels on monocytes fell below 400 at 18 and 24 hours post-trauma and neutrophils at 12, 18, and 24 hours post-trauma.

Mac-1 levels from the white blood cells of a second non-ARDS patient (patient E) are shown in Fig's. 27 and 28. The trends shown on the data plot were the most similar to the values seen in control studies. The Mac-1 levels seen on PMA-stimulated white blood cells are hrgher than unstimulated and the Mac-1 levels on both remain relatively constant. Neutrophrls showed Mac-1 expression levels similar in number to those seen in patient D. At 6, 12, and 18 hours the

Figure 25: Line graph showing αMac-1 mean fluorescence values for patient D (non-ARDS) neutrophils isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient D (non-ARDS) / Neutrophils (Mac-1)

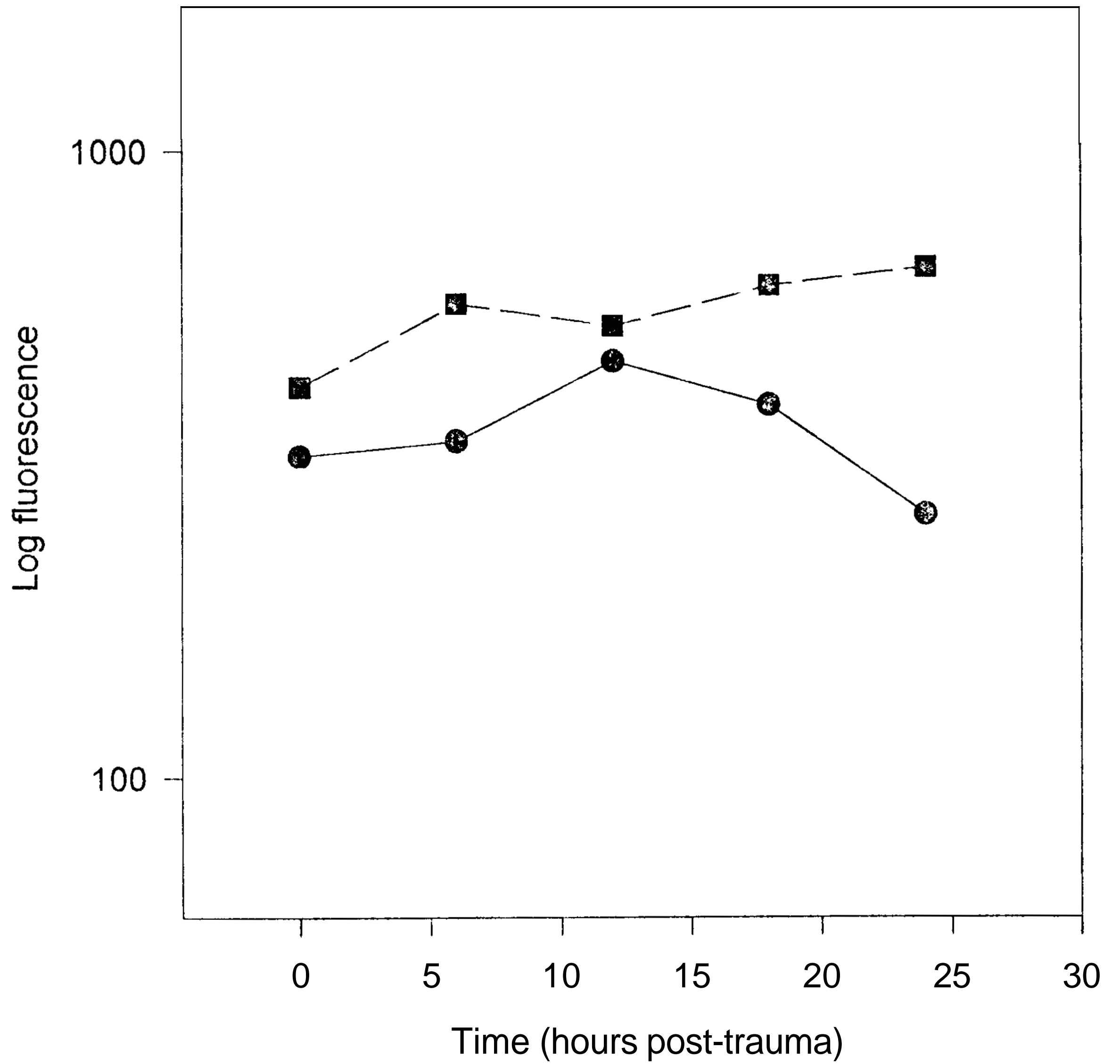


PMA-stimulated = dashed line (squares

unstimulated = solid line (circles)

Figure 26: Line graph showing αMac-1 mean fluorescence values for patient D (non-ARDS) monocytes isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

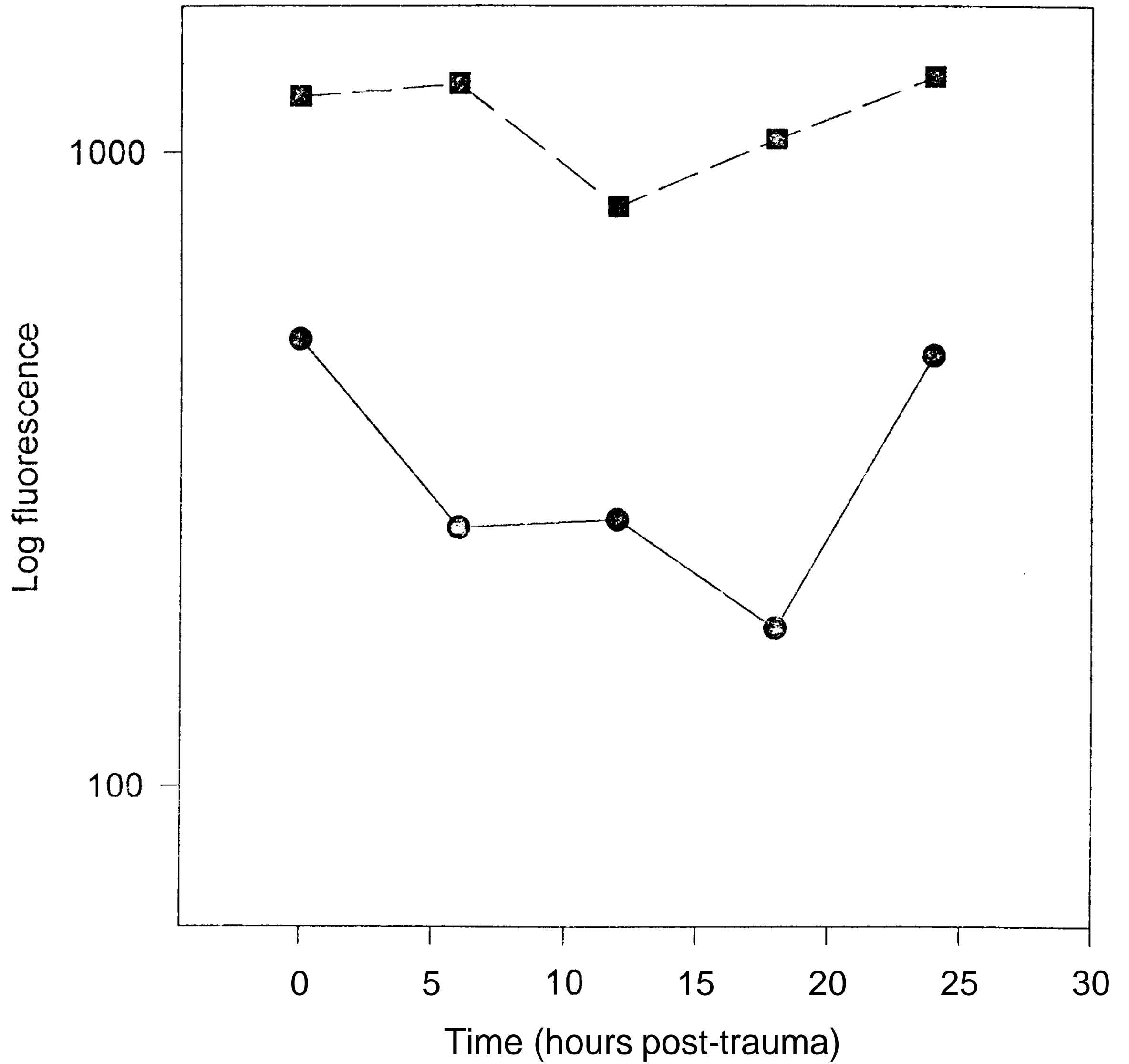
Patient D (non-ARDS) / Monocytes (Mac-1)



PMA Stimulated = dashed line (squares
unstimuiated - solid line (circle)

Figure 27: Line graph showing αMac-1 mean fluorescence values for patient E (non-ARDS) neutrophils isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging fiom 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient E (non-ARDS) / Neutrophils (Mac-1)

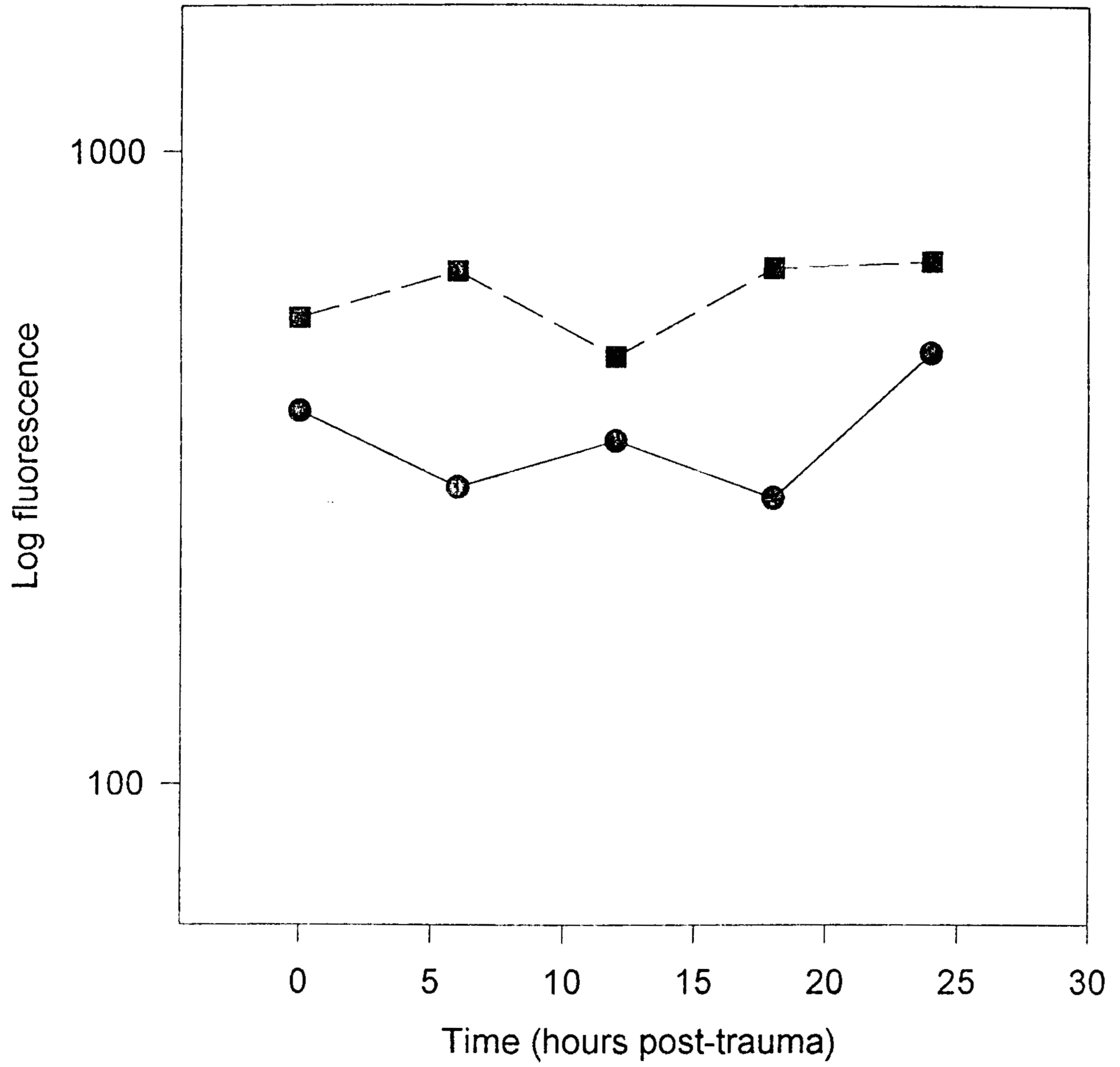


PMA-Stimulated = dashed line (squares

Unstimulated = solid line (circle)

Figure 28: Line graph showing αMac-1 mean fluorescence values for patient E (non-ARDS) monocytes isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

Patient E (non-ARDS) / Monocytes (Mac-4)



PMA-Stimulated = dashed line (square:
Unstimulated = solid iine (circle)

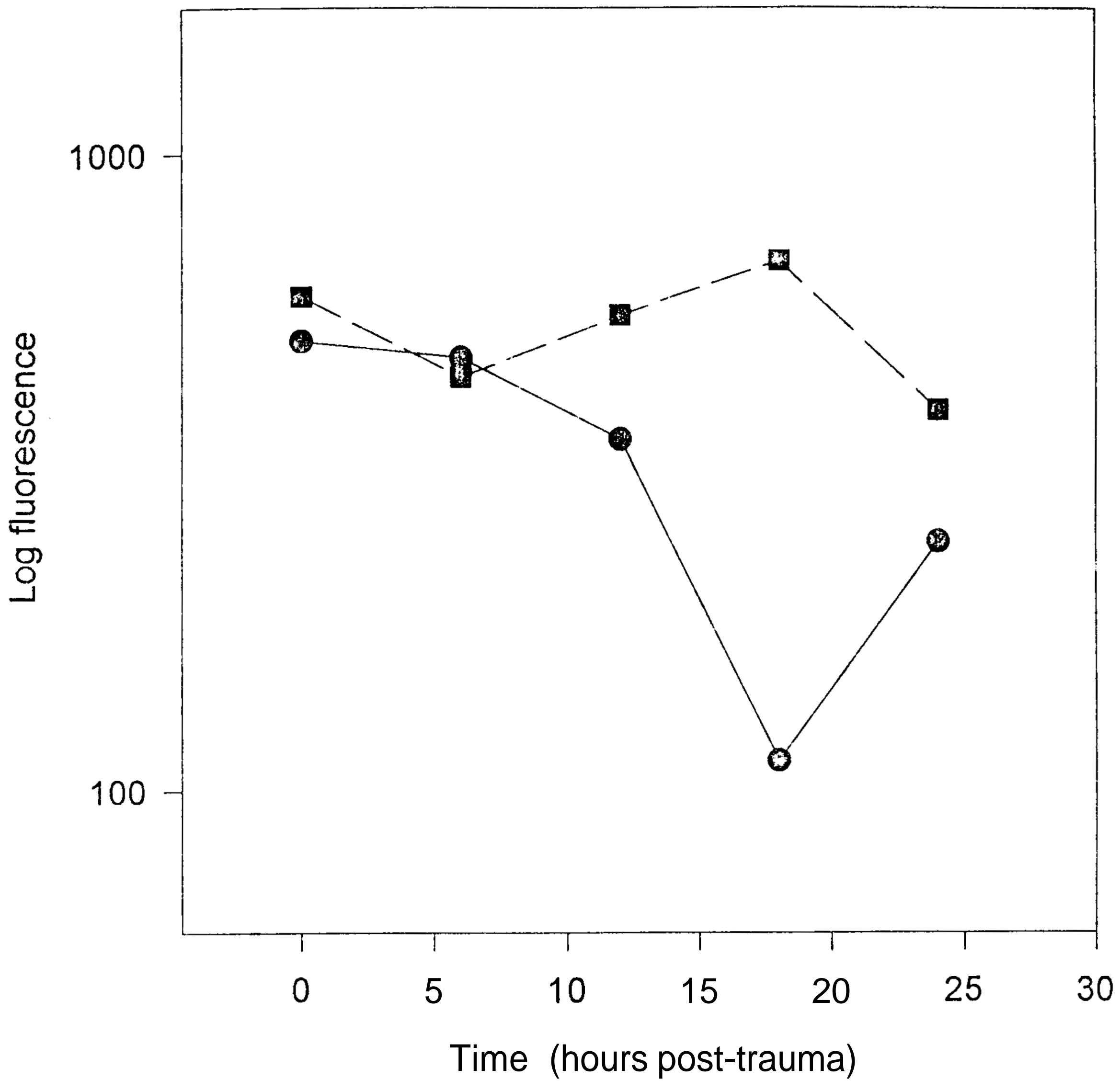
fluorescence levels were much lower than 400, and the average value of all time points was 336.0. Monocytes showed higher levels of Mac-1 on the unstimulated cells, however, all time points except 24 hours were below 400 fluorescence units, average was 358.8. Additionally, the difference between the unstimulated values and PMA-stimulated values were larger for neutrophils than monocytes, nonetheless unstimulated values were lower than PMA-stimulated values for both cell types at all time points.

Cells fiom patient F had Mac-1 levels that are similar to the other non-ARDS patients (Fig's. 29 and 30). Initially the levels of Mac-1 expression were somewhat high (around 450 fluorescence units at zero hours), but decreased at later time points. The drop was most substantial at 18 hours post-trauma. Also, the values of florescence fell into a range below 400 log fluorescence units at 12, 18, and 24 hours post-trauma for both neutrophils and monocytes. Also, the values for the PMA-stimulated cells were lower than those seen with the lung injury patients.

In figure 31 the unstimulated cell data fiom all trauma patients (n = 6) was graphed as percent highest PMA-response. Neutrophils (Fig. 31) showed a response that provides an insightful look at the potential for traumatic, pre-ARDS screening. At 18 hours, both ARDS patients and the ALI patient had values that were above those of the non-ARDS patients (n = 3). There was some variability however in the percent highest response at 0, 6, and 12 hours post-trauma. Also, fiom 18 to 24 hours one ARDS patient and two non-ARDS patients were showing increases in the percent highest response while one ARDS and one non-ARDS patient were showing decreases.

Figure 29: Line graph showing αMac-1 mean fluorescence values for patient F (non-ARDS) neutrophils isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophrl mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophrl mean fluorescence values are represented by circles and a solid line.

Patient F (non-ARDS) / Neutrophils (Mac-1)

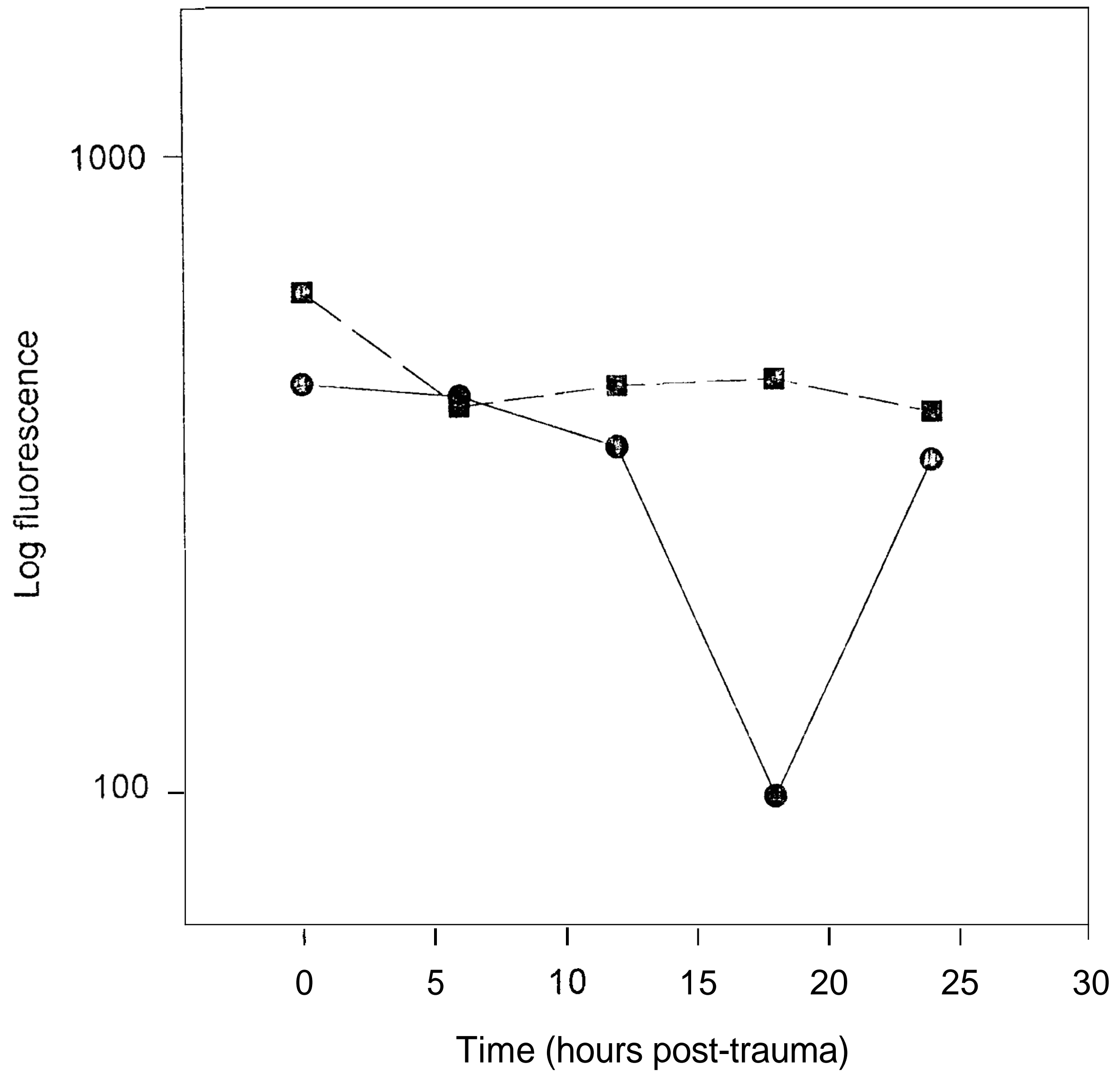


PMA-Stimulated = dashed line (squares

Unstimulated = solid line (circles)

Figure 30: Line graph showing αMac-1 mean fluorescence values for patient F (non-ARDS) monocytes isolated from EDTA anticoagulated whole blood. The x-axis represents time in hours post-trauma, ranging from 0 to 24. The y-axis represents mean fluorescence values plotted on a common log scale, ranging from 60 to 1600. PMA-stimulated neutrophil mean fluorescence values are represented by squares and a dashed line. Unstimulated neutrophil mean fluorescence values are represented by circles and a solid line.

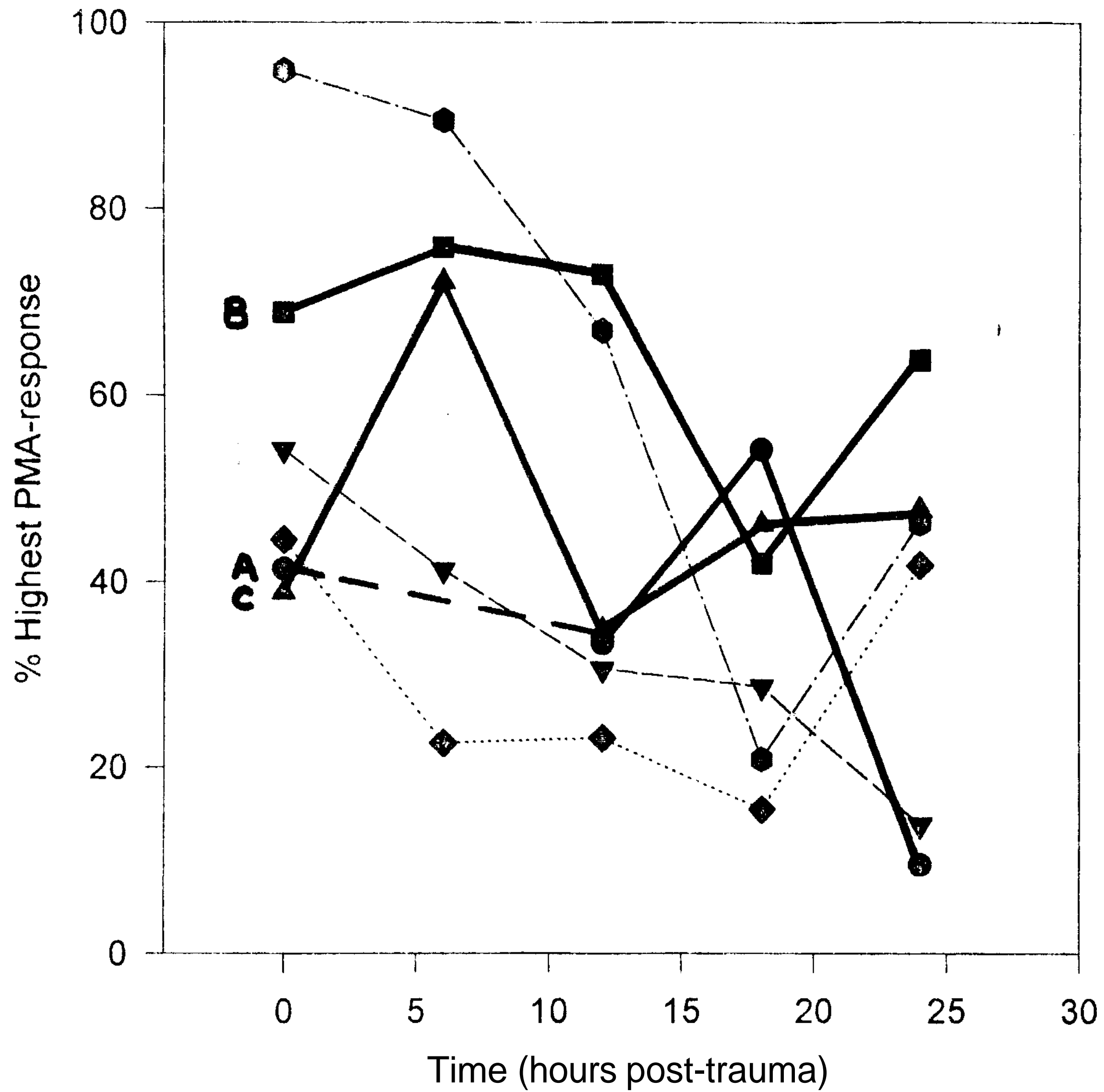
Patient F (non-ARDS) / Monocytes (Mac-I)



PMA Stimulated = dashed line (square:

Unstimulated = solid line (circles)

Figure 31: Line graph comparing percent highest PMA-response for ARDS, Acute Lung Injury and non-ARDS patient neutrophils. For each patient, the highest values of PMA-stimulated Mac-1 expression were used as a constant denominator. The numerator was the Mac-1 mean fluorescence value for unstimulated neutrophils at each time point. Following division, the value was multiplied by 100 to yield a percentage. The x-axis represents time post-trauma, ranging fi-om 0 to 24 hours. The y-axis represents the percent highest PMA-response, ranging fi-om 0% to 100%. ARDS patients are highlighted in red, the Acute Lung Injury patient in blue, and the non-ARDS patients in green.

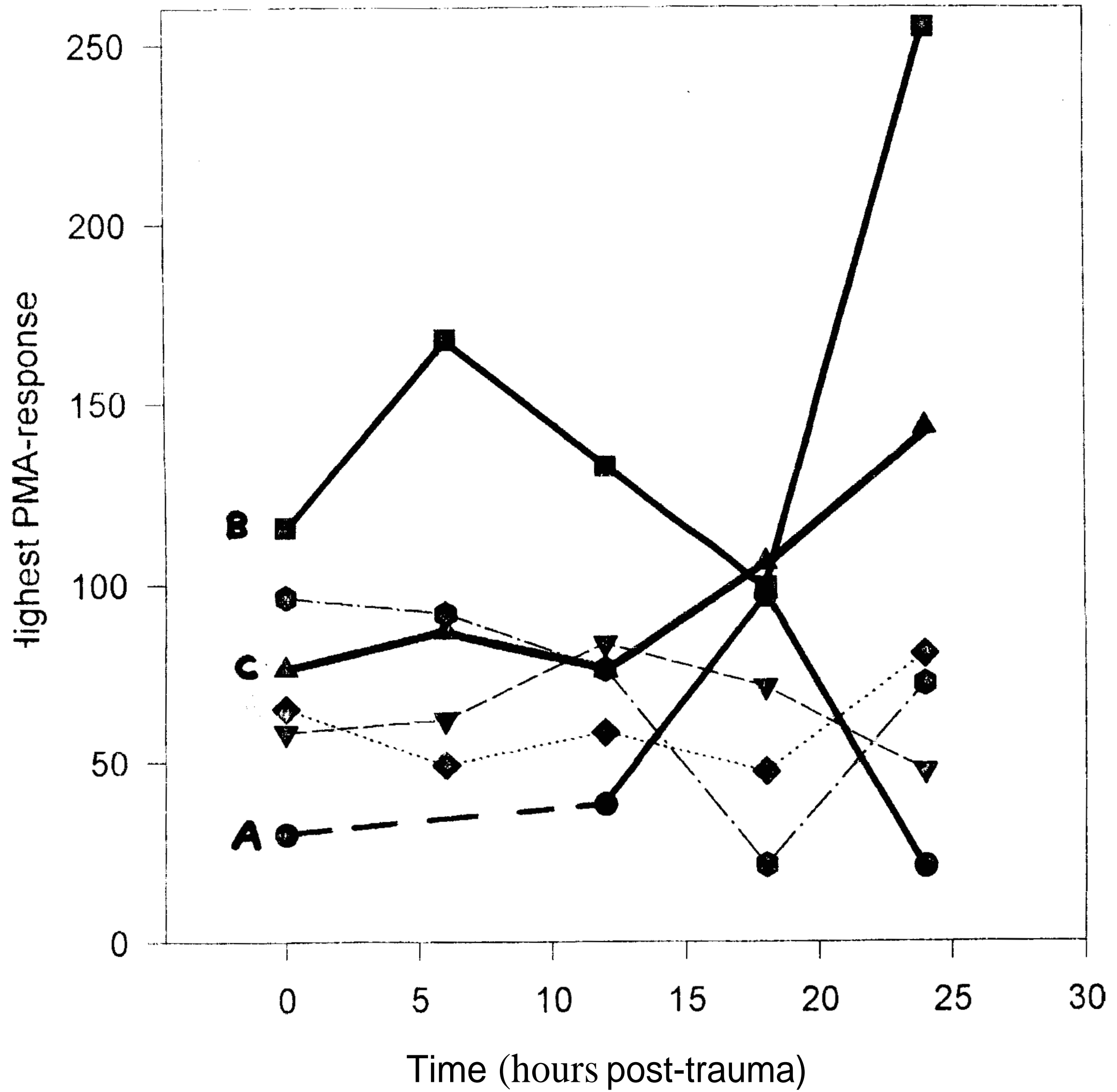


ARDS patients (A and B) - Highlighted in red
Acute Lung Injury patient (C) - Highlighted in blue
Non-ARDS patients (D, E, and F) - Highlighted in green

Monocytes (Fig. 32) showed similar reposes at 18 hours post-trauma. Variability at earlier time points (0, 6, and 12) as well as at 24 hours were also similar to those trends seen when examining neutrophils. The only discernible difference between the two was that monocyte values were much higher than the corresponding neutrophil values, often reachmg values well over 100%. Monocyte and neutrophil responses for each patient as an individual were similar in appearance.

Figure 32: Line graph comparing percent highest PMA-response for ARDS, Acute Lung Injury and non-ARDS patient monocytes. For each patient, the highest values of PMA-stimulated Mac-1 expression were used as a constant denominator. The numerator was the Mac-1 mean fluorescence value for unstimulated monocytes at each time point. Following division, the value was multiplied by 100 to yield a percentage. The x-axis represents time post-trauma, ranging from 0 to 24 hours. The y-axis represents the percent highest PMA-response, ranging fi-om 0% to 260%. ARDS patients are highlighted in red, the Acute Lung Injury patient in blue, and the non-ARDS patients in green.

Monocytes: ARDS versus Non-ARDS Patients



ARDS patients (A and B) - Highlighted in red
Acute Lung Injury patient (C) - Highlighted in blue
Non-ARDS patients (D, E, and F) - Highlighted in green

IV. DISCUSSION:

A. CONTROL STUDIES

There are many factors that influence leukocyte expression of Mac-1, such as: the time and temperature at which blood is maintained post-collection, cell washes, fixation, lysis, and anticoagulant used (40). Stimulation of white blood cells *in vitro* (outside the body) is an undesirable consequence of blood collection. Because we are interested in a true representation of Mac-1 levels *in vivo* (within the patient), all factors associated with blood collection and processing needed to be examined before analyzing patient samples. With the exception of the anticoagulant and amount of time at room temperature, all other collection and processing techniques for our assay had been resolved by preceding graduate students (47). Furthermore, Bray and Landay stated that whole blood lysis procedures, such as our protocol, are the best for flow cytometric analyses of antibody labeled leukocytes. This method involves anticoagulating blood, mixing it with monoclonal antibodies, followed by red blood cell lysis (5). The control portion of this study thus represents an important prelude to the patient studies. These studies thus examine the effect of EDTA, heparin, or oxalate, and the amount of time anticoagulated blood remains at room temperature on the levels of leukocyte expressed Mac-1.

One concern when selecting an anticoagulant is hospital collection procedures. Ethical considerations do not allow for blood collection from patients prior to obtaining consent from the patient. Therefore, a means of obtaining blood from a preexisting collection would increase our ability to conduct scientific studies on trauma

patients. The three anticoagulants which were tested each have advantages as well as disadvantages, and are each used for specific hospital tests based on their advantages. The mechanism by which EDTA inhibits clotting is by chelating or binding calcium ions. Since calcium ions are needed for clot formation, clotting can be inhibited. EDTA-anticoagulated blood does not show distorted or aberrant WBC morphologies (23). Because of this, EDTA is used for whole blood hematology studies including: complete blood counts, white blood cell differentials, and platelet counts (21, 39). Blood samples on ice and in either EDTA or heparin were shown by Repo *et. al.* to contain low proportions of PI (propidium iodide) positive white blood cells (high PI proportions indicate that membrane injury has occurred) (55).

Heparin inhibits clotting by inactivating thrombin and thromboplastin (proteins that mediate clotting) (21). Various tests for which heparin is used include: potassium measurements, glucose, blood urea nitrogen, creatine, and electrolytes (23, 39). Heparin's disadvantages include platelet and blood cell aggregation, therefore the cellular components of the blood could aggregate (40). The concern is that if cellular components aggregate, the numbers of cells examined would be lessened while the fluorescence intensity would increase for each event.

Oxalate functions to inhibit clotting by binding calcium ions (21), but in contrast to EDTA, it can distort cellular morphology (23, 64). It is possible that the adverse effects on leukocyte morphology could have caused the variability found in our study. Oxalate is used for tests such as glycolytic inhibition tests (23 pg. 66). Oxalate was not believed to be a good anticoagulant for blood collection since it has

been tested little, if at all, by other researchers. In fact, there is an overwhelming interest in the effects of EDTA and heparin on CAM expression in vitro. Because of this and our results, the focus was directed towards EDTA and heparin.

The first concern when selecting the best anticoagulant is that anticoagulants may have secondary effects that could effect the isolation and examination of leukocytes by flow cytometry. For example, if a particular anticoagulant were to cause neutrophil degranulation, it's light scattering properties would be altered and analyses by flow cytometry would be effected. Doornbos et. al. has provided evidence that monocytes and neutrophils can be reliably separated based on their light scattering properties when collected in EDTA (15). This may be due to the fact that EDTA minimally influences cellular morphology. This is important since ow gating procedures (see flow cytometry in material and methods) involve the isolation of monocyte and neutrophl populations based on light scattering properties. Nicholson et. al. noted that various commercial lysing preparations (including FACS lysing solution from Becton Dickinson) are optimized when EDTA is used as the anticoagulant (RBC's are effectively lysed in EDTA-anticoagulated blood) (48). This information is enlightening since we are using a commercial lysing procedure (FACS lysing solution) and because we want to avoid leukocyte contamination with RBC's. Paxton and Bendele also performed experiments to determine whether EDTA or heparin were best suited for flow cytometry. They collected blood samples in tubes (EDTA or heparin) stored at $4^{o}C$, and looked at various cell surface markers, such as CD45/14, CD3, CD4, etc., and determined that

neither EDTA nor heparin are significant factors that adversely effect flow cytometric analyses (51). Based on this information, it is feasible to use either anticoagulant for flow cytometric determinations.

Another concern with an assay involving antibody labeling is whether or not all of the white blood cells are effectively labeled. McCarthy and Macey found that the percentages of neutrophils which stained positively with αCD11b were greater than 90% when either EDTA or heparin was used. However, the percentages of monocytes effectively labeled was less than 90% when heparin was used and greater than 90% when EDTA was used (40). Both monocytes and neutrophils show the greatest percentages of labeled cells if EDTA was used versus heparin. One disadvantage of using EDTA for leukocyte labeling experiments is that a calcium ion (Ca^{2+}) chelating agent (removes free Ca^{2+}) may influence the antigenicity of Ca2+ dependent epitopes (specific binding sites) such as on Mac-1 (CD11b subunit binding sites) (40). In a study by McCarthy *et. al.*, anticoagulated (EDTA or heparin) blood was rapidly cooled to $4^{o}C$, labeled with antibody, then analyzed by flow cytometry. It was found that neither anticoagulant (EDTA, or heparin) nor cation presence had effects on the mean fluorescence intensity of Mac-1 (41). Furthermore, Macey *et. al.* showed that CD11b expression was higher when divalent cations are absent, which further supports the notion that divalent cations are not crucial for antibody labeling during quantitative assays. EDTA has been implicated to be a good anticoagulant for antibodies that do not required divalent cations by Repo *et. al.* (55).

The next point to consider for this study is whether anticoagulants have any inherent ability to affect Mac-1 expression on

leukocyte surfaces. Macey et. al. studied the effect of anticoagulant choice on Mac-1 (CD18/CD11b) expression. Briefly, they collected blood from healthy volunteers into tubes containing either EDTA or heparin. The leukocytes were fixed immediately, the RBC's were lysed, the cells labeled with fluorochrome conjugated monoclonal antibody and then analyzed using FACS scan (Becton Dickinson) LYSIS II program. Their findings indicated that anticoagulant choice had no effect on Mac-1 expression when labeling either the CD18 or CD11b subunits of Mac-1 with antibody (37). This information is important to us since our monoclonal antibody (αMac-1) binds to the CD11b subunit of Mac-1. Based on their report, binding interactions are not effected by either EDTA nor heparin. Similarly, we noted that Mac-1 levels are approximately the same in magnitude at 0 minutes after collection in either EDTA or heparin. Contradictory results were reported by Repo and coworkers who found Mac-1 intensity to be much higher when heparin was used as an anticoagulant versus the others which were tested (ie. EDTA) (55). They used Ficoll-isoated monocytes and neutrophils which were directly labeled with αCD11b-PE, all of which was done at $4^{o}C$. A study by El Habbal et. al. may explain this discrepancy. They obtained samples immediately (0 minutes), incubated the cells at room temperature ($37^{o}C$), and processed them at 0, 10, 30, 60, and 120 minutes. The processing included FITC imrnunofluorescent staining followed by flow cytometric analysis. They noted that there was a gradual increase in Mac-1 expression over time in both EDTA and heparin, however the increase was more profound in heparin. In fact, their data reached statistical significance at 15 minutes for heparin, and 30 minutes for

EDTA (16). This information indicates that stimulation occurs in *vitro* in either anticoagulant, however the stimulation that does occur is more substantial when heparin is used. The different results seen in the previous studies may reflect a difference in processing time. Samples left longer at room temperature may have become stimulated, resulting in different Mac-1 levels when comparing EDTA and heparin anticoagulated samples. While ow data indicated no difference in Mac-1 expression when the blood was labeled immediately, increased Mac-1 expression could be seen within 5 minutes at room temperature in heparinized blood and within 15 minutes for EDTA anticoagulated blood at room temperature.

As indicated, it has been found that leukocytes become stimulated (increase Mac-1 expression) when the cells are kept at room temperature in vitro, in any anticoagulant (51, 68). McCarthy *et. al.* directly labeled (Fluorescein labeled antibody only) white blood cells collected in EDTA. The cells were isolated using a (histopaque) centrifugation gradient at 20-22°C, washed twice at room temperature (37°C), labeled at 4°C, and promptly analyzed by flow cytometry (40). They reported that the mean fluorescence intensity of Mac-1 molecules on monocytes and neutrophils significantly increased if blood was maintained at room temperature for 1 hour. McCarthy *et. al.* additionally reported that EDTA and heparin are both satisfactory anticoagulants for the in vitro quantification of Mac-1 on whole blood leukocytes at 4°C (40). Their results indicate that Mac-1 expression did not increase significantly within the first 60 minutes if the cells were collected and maintained at 4°C. It is probable that the process of white blood cell separation using a centrifugation gradient, in itself,

causes some stimulation (upregulation of Mac-1). To ascertain whether or not the same increase in Mac-1 levels are seen at room temperature when a gradient is not used and also if ice could prevent stimulation, blood was collected into anticoagulated tubes (EDTA or heparin) by McCarthey *et. al.* and cooled to 4°C. The leukocytes were labeled with monoclonal antibody in whole blood samples, followed by RBC lysis and fixation of the cells. They found that at 4°C the mean fluorescence intensity on all leukocyte subclasses was not affected by anticoagulant choice (41).

The results presented in this thesis are similar to those of Bateman *et. al.* who analyzed blood from healthy volunteers collected in EDTA or heparin. The main difference between our study and theirs was the fact that their manipulations were carried out at room temperature. They found that Mac-1 expression was similar when EDTA or heparin were used as anticoagulants (4). The results of El Habbal *et. al.* directly support the observation that heparin effects the expression of Mac-1 to a greater degree than does EDTA. They found that leukocytes experience more substantial increases in Mac-1 expression when anticoagulated with heparin versus EDTA (16). This information supports our conclusion that EDTA would serve as the best anticoagulant for patient sample collection since it would not adversely effect the expression of Mac-1 in and of itself.

Data from our laboratory using oxalate anticoagulated blood showed variable levels of Mac-1 expression at each time point when comparing two separate experiments, and was therefore concluded to adversely affect the immunophenotype (antigen expression) of white blood cells. Data collected using heparinized blood, on the other hand,

showed that Mac-1 levels were similar when comparing three separate trials. however, Mac-1 levels appear to increase with incubation at room temperature when heparin is used as the anticoagulant. Mac-1 expression in EDTA anticoagulated blood samples did not increase within 15 minutes of collection at room temperature. We found that temperature of the sample after collection was an important variable in Mac-1 quantitation. Blood samples that were anticoagulated with EDTA, and incubated at room temperature (37°C) for up to 15 minutes showed minimal changes in Mac-1 expression, whereas those samples collected in heparin were stimulated to a greater degree within 5 minutes of room temperature exposure. Our results indicate that heparin contributes to leukocyte stimulation (Mac-1 upregulation) to a greater extent than does EDTA. The data obtained in our study indicates that in vitro activation (increased Mac-1 expression) of white blood cells occurs to a lesser extent when samples are collected in EDTA compared to those collected in heparin. Data we gathered while collecting blood in oxalate was interpreted to be unreliable since the leukocytes demonstrated increased Mac-1 and because the results varied between two trials. In reviewing the information presented by our laboratory and others, EDTA was chosen as the anticoagulant for use in the patient study.

IV. DISCUSSION:

B. PATIENT STUDIES

Activation of neutrophils and monocytes results in increased surface expression of Mac-1 and an increase in avidity for it's adhesion partners, namely ICAM-1 (17). Laurent _et. al._, in addition to others,

has shown that neutrophils fkom patients with ARDS have increased Mac-1 expression on their surface (7, 33, 64). There is then a possibility that a time dependent increase in Mac-1 expression may occur in trauma patients who develop ARDS. αCD18 is a monoclonal antibody directed against the CD18 subunit of Mac-1 and LFA-1, and is effective in preventing activated neutrophils fkom binding to activated endothelial cells (express ICAM-1) (29). This information indicates that blocking the CD18 complex inhibits the action of Mac-1 and LFA-1, thereby preventing the fim adherence necessary for extravasation to occur. In fact, Issekutz and Issekutz examined the effect of antibody directed against LFA-1 and against Mac-1 during inflammatory reactions in a rat model. Inflammation was stimulated by intradermal injections of endotoxin and the effects of various antibodies against CD18 antigens (including Mac-1 and LFA-1) were examined. Their findings indicated that both antibodies individually were effective inhibitors of neutrophil binding to stimulated endothelial cells in these rat Inflammatory models. Furthermore, the combination of αMac-1 and αLFA-1 significantly inhibited adherence (30). These studies suggest that LFA-1 and Mac-1 are cooperatively involved in the extravasation process.

Petruzelli *et. al.* confirmed the development of an antibody that binds to the β2 subunit of Mac-1 and LFA-1, thereby stimulating the cell. They used this antibody to examine the functions of Mac-1. Purified ICAM-1 (counter-receptor for Mac-1) was adhered to petri dishes while neutrophils were incubated with activating and blocking monoclonal antibodies, then the number of cells bound to ICAM-1 were counted on a high power field. Their findings suggest that

activating the cell with an activating antibody increased neutrophil binding to ICAM-1, and in the presence of an antibody to block Mac-1 activity, there was partial blockage of neutrophil binding (only partial because LFA-1 is also needed to bind to ICAM-1) (53). These findings mimic the in vivo activation of white blood cells and implicate Mac-1 as a mediator of extravasation.

Increased levels of Mac-1 could lead to increased neutrophil and monocyte emigration. Increases in Mac-1 expression found on leukocytes in our study seem to be more robust for neutrophils than monocytes. This observation, along with the fact that others have implicated neutrophils as a mediator of lung injury (32, 62, 69), provide a possible mechanism by which lung injury occurs in ARDS. Chollet et. al. showed that nitric oxide inhalation improves arterial oxygenation in ARDS patients (ARDS patients exhibit decreased arterial oxygenation due to the decreased functioning of the lungs). Moreover, the neutrophils from nitric oxide treated patients showed decreased levels of Mac-1 and also decreased the spontaneous production of hydrogen peroxide (6). Nitric oxide may ameliorate ARDS due to its influence on the levels of leukocyte expressed Mac-1. The possible mechanism is two-fold: one, decreased Mac-1 levels could potentially prevent neutrophil accumulation in the lung and two, the decrease in Mac-1 may be related to a decreases in hydrogen peroxide release. Based on this information, there are two possible contributions by Mac-1 in mediating lung injury, namely extravasation and hydrogen peroxide release via Mac-1.

Nagase et. al. showed that animal models treated with hydrochloric acid (induces lung injury) showed significant reductions in

lung injury when antibody (αICAM-1) was used to block Mac-1 and ICAM-1 binding interactions (46). Since ICAM-1 is the counter receptor for Mac-1 it is possible that Mac-1 may, in part, be responsible for ARDS pathogenesis. Vedder *et. al.* examined CD18 (part of the Mac-1 heterodimeric complex) function in rabbits with ischemia-reperfusion injury (model of hemorrhagic shock and resuscitation). After this induced injury, animals were treated with αCD18 thereby blocked the activity of CD18. They found that CD18 plays a role in the development of multiple organ failure (ARDS-like condition affecting organs of the body), however lung injury was less dependent on CD18 (60). One possible explanation for this finding is that the CD11b subunit of Mac-1 may be responsible for leukocyte extravasation. However, Horgan *et. al.* found that experimentally induced lung injury in rabbits was reduced in those animals treated with αCD18 but not αCD11b (29). The conflicting information presented by these authors needs to be further studied in order to conclusively determine the exact roles of the CD18 and CD1b subunits.

In another experimental model of lung injury, Fabian *et. al.* examined pigs that were anesthetized and ventilated, followed by a traumatic insult (blunt trauma). The pigs were hemorrhaged (volume of blood was removed) and then resuscitated (volume of removed blood was reinfused) 60 minutes later. After 3 days the animals were challenged with endotoxin (lipopolysaccharide) (19). This model is relevant to our study of ARDS since trauma patients generally develop infections, such as pneumonia, secondary to trauma. This process therefore results in a situation analogous to trauma cases seen in

emergency rooms. Data collection involved immunofluorescence (FITC-αCD18) and detection by flow cytometry. They found that animals treated with αCD18 (blocks CD18 function) prior to trauma showed significant neutrophilia (increased circulatory neutrophil content) versus controls up to 72 hours. The blockade of CD18 action prevented neutrophils from emigrating into the tissues hence the increase in vascular neutrophil content. The elimination of neutrophil influx into the lungs prevents the tissue destruction caused by the neutrophils, thereby ameliorating the lung injury. Treatment with αCD18 was also associated with a decreased mortality rate in the trauma animals. Finally, bronchoalveolar lavage (lung fluid) showed increased levels of neutrophils which was decreased by pre-traumatic treatment with αCD18 (19). The experiments of Endemann *et. al.* added an interesting twist. They found that Mac-1 upregulation on leukocytes and the appearance of activation dependent epitopes were not affected by the presence of leumedins (anti-inflammatory drugs that prevent neutrophil adherence to endothelial cells), however adherence was inhibited (17). Endemann suggested that adherence is mediated by constitutively expressed Mac-1 whereas upregulated Mac-1 is involved in chemotaxis. If this is true, our study is still relevant in that chemotaxis could play a role in the exacerbation of ARDS. Not only do white blood cells need to enter the tissue spaces, they also need to move through the tissues to their site of action.

Since ARDS patient's lungs have conclusively been proven to contain higher total numbers and higher percentages of neutrophils (7, 42, 54, 62), it is possible that Mac-1 is responsible for this accumulation. In studies performed by White-Owen *et. al.*, trauma

patients were enrolled into their study and blood was collected in EDTA vacuum tubes within 48 hours of patient injury. Following collection, the neutrophils were isolated and labeled (αCD11b-PE) by irnmunofluorescence. They found that the percentages of neutrophils positive for Mac-1 (CD11b/CD18) increased significantly ($p < 0.005$) when blood was collected within 48 hours. Also, peripheral blood smears showed that greater than 98% of the cells in the injured patient were mature neutrophils (64). This study suggests that ARDS patients have higher levels of Mac-1 expression when compared to non-ARDS patients.

To test whether leukocytes express different levels of Mac-1 in post-traumatic patients who develop ARDS versus those patients who do not, blood samples were drawn for this study at 0, 6, 12, 18, and 24 hours following the initial traumatic insult and then analyzed for Mac-1 expression. The protocol used for this study was unique in that post-traumatic/pre-ARDS white blood cells were examined, while other studies have addressed various situations during established ARDS. Our results indicate that ARDS patient white blood cells have higher post-traumatic Mac-1 expression than do non-ARDS patients, especially at later time points. Of interest to us were the levels seen at 18 hours post-trauma. Cunningham stated that acute lung injury develops within 24 hours in 80% of patients at risk, ARDS develops 24 to 48 hours later (10). Therefore it is possible that Mac-1 upregulation at 18 hours post-trauma could be a situation of "priming the pump" for ARDS development.

The data obtained from patients who ultimately developed ARDS ($n = 2$) subsequent to trauma shows increased Mac-1 levels, as

expected for activated cells, when compared to non-ARDS patients (n = 4). ARDS patient neutrophils and monocytes exhibited Mac-1 levels which were higher than those seen in control patients. Healthy controls (data not shown, see 47) showed "uniform" levels of Mac-1 expression throughout the course of study (consistent mean fluorescence values at all time points). Non-ARDS patients show levels that are initially elevated but then decline towards levels characteristically seen in healthy controls around 12 hours post-trauma. These early increases in Mac-1 levels were comparable in magnitude to the levels seen in ARDS patients. However, the increased expression is likely due to the initial traumatic insult and the concomitant activation of inflammation. At 12 to 24 hours post-trauma, the levels of Mac-1 expression on non-ARDS leukocytes decreased toward baseline values. In these patients the inflammatory response is restrained and does not appear to proliferate out of control, hence a more homeostatic state is achieved. Mac-1 levels for ARDS patients were consistently elevated at 18 hours or 24 hours post-trauma. PMA stimulation of these white blood cells was not as effective at increasing Mac-1 levels in the ARDS patient group.

This study demonstrates that trauma patients show post-traumatic Mac-1 expression that differs from that of non-ARDS patients. It is possible that inhibition of leukocyte adherence by blocking Mac-1 function could ameliorate the pathophysiological conditions persistent in ARDS. In future studies it would be interesting to examine LFA-1 expression and to characterize its post-traumatic expression. It has been demonstrated that in animal models of lung injury, pretreatment with αLFA-1 attenuates the gas exchange

anomalies characteristic of lung injury (groups without pretreatment had significantly increased BAL neutrophil content) (45). In a separate experiment by Mulligan *et. al.* it was shown that intravenous treatment with αMac-1 had no protective effects in a lung injury model, however αLFA-1 did. Moreover, αMac-1 administered intratracheally did have protective effects in the same animal model (45). Based on these observations, it is important that we consider the influence of LFA-1 and include its findings in our future patient trials. Cytokine levels in the plasma also need to be characterized. Much of the research on cytokine levels examined BAL fluid levels (25, 42, 54, 69) and plasma levels (7, 8) after ARDS had been established, not before. Taken together this data shows something abnormal occurring with regards to Mac-1 expression in trauma patients. Therefore, post-traumatic expression of Mac-1 may be a significant factor in predicting which patients will develop ARDS.

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