The Effect of Blood Collection Methods on the Expression of Monocyte Cellular Adhesion Molecules

by

Erin L. Skornicka

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Erin L. Skornicka — Date

Approvals:

Diana Fagan 11/26/96

Dr. Diana Fagan — Date

David Asch 11/22/96

Dr. David Asch — Date

Paul C. Peterson 11/25/96

Dr. Paul Peterson — Date

Peter J. Kasvinsky 12/5/96

Dean of Graduate Studies — Date

ABSTRACT

Cellular adhesion molecules (CAMs) play an important role in the inflammatory response by allowing white blood cells to adhere to the endothelium and then migrate into infected tissues. The goal of this study is determine the effect of blood collection techniques on the ability to detect changes in these molecules. In this study, the effects of time (0 hour versus 2 hours), temperature (room temperature versus $4^{0}C$), and anticoagulants (EDTA, potassium oxalate, sodium citrate, and heparin) were examined to see which blood collection techniques retained accurate results when compared to samples that were processed immediately. Cells, both unstimulated and stimulated with PMA, from healthy volunteers were labeled with various antibodies against CAMs (anti-ICAM-1, LFA-1beta, L-selectin, Mac-1, LFA-1alpha, and VLA-4) followed by FITC-labeled goat anti-mouse antibody then analyzed using a flow cytometer. From these experiments, the time of incubation and temperature of storage seem to influence the expression of CAMs. The anticoagulant does not seem to play an important role in altering CAM expression. In these studies, the best storage medium for monocytes when examing Mac-1 expression was sodium citrate on ice.

ACKNOWLEDGEMENTS

I would like to take this opportunity to personally thank all of my committee members for their time and effort in helping me with my education. I want my advisor, Dr. Diana Fagan, to know that I appreciate all of her time and advice while helping me through this project. A special thank you goes out to my lab partner, Daniel Malleske, for always adding excitement to the lab and to my life. I would also like to acknowledge my best friend, LeeAnn Henry, for providing moral support through my entire graduate career. I also am grateful for having such wonderful and caring parents to help guide me through life. The love and support of my entire family has made all the difference.

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ABBREVIATIONS

ARDS --------> Adult Respiratory Distress Syndrome

CAM ---------> Cellular Adhesion Molecule

CD ----------> Cluster of Differentiation

EDTA --------> Edetate

E-selectin --> Endothelial-Selectin

FITC --------> Fluorescein Isothiocyanate

ICAM --------> Intracellular Adhesion Molecule

IL ----------> Interlukin

LAD ---------> Leukocyte Adhesion Deficiency

LFA ---------> Leukocyte Functional Antigen

LPS ---------> Lipopolysaccharide

L-selectin --> Leukocyte-Selectin

MCP ---------> Monocyte Chemotatic Peptide

PE ----------> Phycoerythrin

PMA ---------> Phorbol Myristate Acetate

P-selectin --> Platelet-Selectin

TNF ---------> Tumor Necrosis Factor

VCAM --------> Vascular Cell Adhesion Molecule

VLA ---------> Very Late Antigen

INTRODUCTION

The Inflammatory Response

The human body has a great capability to heal itself in response to certain conditions that injure tissues, such as a wound or exposure to harmful microorganisms which may invade the tissues. Tissue damage triggers a series of events which leads to the healing process. One of the first events to occur is the diameter of blood vessels near the infection enlarge while some capillaries further away from the affected area constrict. These events lead to the symptoms of inflammation in which the tissue begins to swell and turn red along with an increase in tissue temperature. Along with the vasodilation of the capillaries, an influx of white blood cells also occurs at this site. The vessels increase their permeability to allow the movement of various leukocytes, mainly phagocytic cells, from the capillaries to the surrounding tissues. This emigration involves cellular adherence to the endothelial wall to allow movement of the phagocytic cells between capillary endothelial cells into the tissue. The phagocytic cells at the site of inflammation are then able to engulf microorganisms and also release lytic enzymes to rid the microorganisms from the body.

The inflammatory response brings many cell types to the infected site. The first and most predominant cells to appear are the granular neutrophils. These are active

phagocytic cells containing lytic enzymes. After approximately six to eight hours, agranular monocytes begin to accumulate near the same site. Monocytes contribute to the inflammatory response by removing pathogenic microorganisms and repairing damaged tissue. These cells are able to mature in the tissues and differentiate into macrophages which also are involved with the immune response (Lundahl and Hed, 1994).

Cell Adhesion Molecules

Cellular adherence seems to be an important step in the inflammatory process. Without increased binding to endothelial cells, the phagocytic cells are not able to get to their final destination to destroy invading microorganisms. The molecules responsible for the adherence of white blood cells to the vascular endothelium are cellular adhesion molecules (CAMs), carbohydrate-binding proteins found on the surface of cells. The CAMs belong to three different families of proteins: the immunoglobulin superfamily, the selectins, and the integrins. The immunoglobulin superfamily contains proteins on the surface of the cell that are involved in either antigen recognition (C1-type) or cellular adhesion (C2-type). The immunoglobulin-like cellular adhesion proteins contain immunoglobulin-like domains with conserved cysteine sequences. Two important members of this family are intracellular adhesion molecule-1 (ICAM-1) and vascular

cell adhesion molecule-1 (VCAM-1). These molecules, when present on the surface of endothelial cells, contribute to the adhesion of leukocytes to the endothelium (Carlos and Harlan, 1994). ICAM-1 appears to affect recruitment and adhesion of monocytes during an immune response (Navab *et al.*, 1994).

Along with the immunoglobulin superfamily, the selectins also play a role in cellular adhesion during inflammation. The family of selectins contain several domains in their structure. One domain consists of a calcium dependent lectin domain, another domain has homology to epidermal growth factor, and also has a series of repeats related to the complement binding proteins (Carlos and Harlan, 1994). The three important members of this family are endothelial-selectin (E-selectin), platelet-selectin (P-selectin), and leukocyte-selectin (L-selectin). Both E-selectin and P-selectin are expressed on endothelial cells, while L-selectin is expressed on white blood cells. The selectins main role is to "mediate the initial attachment to the endothelium, the so-called rolling, which precedes a more firm adhesion..." (Lundahl and Hed, 1994). Scientists have demonstrated that white blood cells at the beginning of inflammation do not adhere tightly to the endothelium, instead the adhesion events cause the white blood cells to roll along the capillaries at a slower rate than the flow of the blood (Lasky, 1992).

This process of rolling is mediated by L-selectin and its carbohydrate ligand found on the epithelial cells.

The final category of the CAMs are the integrins which are heterodimeric glycoproteins. These molecules have an alpha-chain containing three metal-binding sites and a beta-chain containing cysteine rich repeats (Kuby, 1994). The integrin superfamily has a total of eight subfamilies differing in the structure of their beta chain. Only two of the subfamilies are involved in leukocyte adhesion to the endothelium. The $beta_1$ subfamily adheres cells to extracellular matrix components. Its members consist of very late antigen-1 (VLA-1) through very late antigen-6 (VLA-6) (Yurochlo *et al.*, 1992). The $beta_2$ subfamily adheres cells to other cells, and its members consist of leukocyte functional antigen-1 (LFA-1), Mac-1, and p150/95 (Yurochko *et al.*, 1992). These molecules are expressed in activated cells and help to facilitate white cell emigration from the capillaries during an inflammatory response. Once the leukocytes begin their rolling along the endothelium, then the $alpha_4beta_1$ integrins allow the cells to begin to roll along the endothelium, therefore permitting the $beta_2$ integrins to firmly attach the white blood cells onto the surface of the endothelial cells (Luscinskas *et al.*, 1994). Once the white blood cells are in the proper position, they are then able to migrate through the endothelium into the tissue and carry out their

functions.

The importance of the CAMs in the inflammatory response has been demonstrated in patients with leukocyte adhesion deficiency (LAD). This disorder results when cellular adhesion molecules of white blood cells are absent. The most prominent symptom of this disorder is recurrent bacterial infections resulting from the inability of the leukocytes to sufficiently attach to the endothelial cells. When this happens, the white blood cells are not capable of migrating into the tissue where the bacteria are located (Lasky, 1992).

In order for monocytes or neutrophils to contribute to the inflammatory response, the cellular adhesion molecules on both the monocytes or neutrophils and the endothelial cells need to be expressed on the cell surface. In general, selectins are present constitutively and shed after cell activation, while integrins and the immunoglobulin-like cellular adhesion molecules are synthesized after exposure to an activating stimulus. The CAMs belonging to the immunoglobulin superfamily are regulated by the presence of cytokine secreted proteins involved in cell-to-cell communication. Tumor necrosis factor-alpha (TNF-alpha) and interlukin-1 (IL-1) are two cytokines that are released from activated macrophages. Lipopolysaccharide (LPS) is found on the cell wall of gram negative bacteria and may stimulate the synthesis of

cellular adhesion molecules. All of these substances, when released during the inflammatory response, are capable of increasing the expression of ICAM-1 and VCAM-1 on endothelial cells (Luscinskas *et al.*, 1994). The cytokines, IL-1 and TNF-alpha, are also responsible for the secretion of interlukin-8 (IL-8) from macrophages. IL-8 aids in the adherence of white blood cells to the endothelium (Kuby, 1994).

Monocyte Cell Adhesion Molecule Expression

L-selectin, a member of the selectin family, plays an important role in the monocyte response to inflammation. Monocytes constitutively express L-selectin, however, the synthesis of the receptor on the endothelial cells must be induced before monocytes are able to adhere to the vascular endothelium. IL-4, a cytokine synthesized by T helper cells, seems to increase both VCAM-1 and the L-selectin ligand, which is composed of the carbohydrates $Lewis^x$ (CD15) and sialyl $Lewis^x$ (CD15s) (Luscinskas *et al.*, 1994).

Once the selectins have slowed the process of the leukocytes through the capillaries down, the integrins are required to continue the inflammation process by firmly attaching the monocytes to the endothelium. Two subfamilies of the integrins are involved in the movement of monocytes from the blood to the tissue. The initial integrins are the $alpha_4beta_1$ integrins, namely the VLA

subfamilies, which promote arrest. Afterwards, the $beta_2$ integrins, namely LFA-1, Mac-1 and p150, allow attachment of the monocytes to the endothelium to become more secure so that the monocytes are induced to move through the endothelium into the surrounding tissue (Luscinskas *et al.*, 1994). One of the important integrins on monocytes is Mac-1. The levels of monocyte Mac-1 have been measured, and its expression demonstrated to increase upon activation of the cells (Navab *et al.*, 1994 and Kishimoto *et al.*, 1989). This upregulation of Mac-1 plays a significant role in the inflammatory response, allowing monocytes to migrate to site of infection. More importantly is the fact that after cellular activation, L-selectin is downregulated from the surface of the monocytes. This process of shedding L-selectin allows for the separation of the monocytes from the endothelium once adhesion has occurred (Navab *et al.*, 1994). This process becomes important because the monocytes need to continue to move to reach their final destination.

The cellular adhesion molecules present on the surface of mononuclear phagocytes not only participate in the attachment to the endothelial cells for emigration into the tissues but also are necessary for cell cytotoxicity (Bernasconi *et al.*, 1991). The CAMs that are involoved with cytoxicity include a subfamily of the integrins. Upon the activation of monocytes by TNF and IL-1, these cells

are capable of adhering to tumor cells and then eliminating them from the body. The $beta_2$ subfamily is necessary for the monocytes to adhere to tumor cells prior to eliminating the tumor. This process has been tested by blocking the beta2 chain of the integrin with antibodies. By doing so, the monocytes are then greatly inhibited in their ability to interact with tumor cells (Bernasconi *et al.*, 1991).

Acute Respiratory Distress Syndrome

Cellular adhesion molecules play an important role in the inflammatory process. Without CAMs, leukocytes are unable to migrate through tissues. However, when macrophages are exposed to endotoxins, endotoxin causes stimulation of the macrophages which leads to increased levels of phagocytosis and increased levels of CAMs. Lytic enzymes are released during phagocytosis by phagocytic cells which may also destroy healthy cells as well as infected cells. The activated macrophages also produce IL-1 and TNF-alpha. This increase in the levels of IL-1 and TNF-alpha leads to septic shock which causes decreased blood pressure, fever, and blood clots to form in organs (Kuby, 1994).

Inappropriate activation of endothelial cell expression of CAMs may play an important role in adult respiratory distress syndrome (ARDS). ARDS refers to "diffuse, acute parenchymal lung injury that results in

increased permeability, pulmonary edema, severe hypoxemia and mechanical abnormalities" (Chollet-Martin *et al.*, 1992). Following a trauma or sepsis, the inflammatory response may be initiated, enabling a large number of white blood cells to mistakenly transmigrate into the lungs. Upon examination of the lungs after ARDS has developed, the interstitium found within the lungs contains a large number of phagocytic cells (Donnelly *et al.*, 1994). During the progression of ARDS, the endothelial cells in the tissue become damaged then edema occurs in the alveoli of the lungs.

Endotoxin, which is produced by the release of dead gram negative bacterial cell walls into the circulation, causes the expression of CAMs and a "progressive and uncontrolled whole body inflammation" (Welbourn and Young, 1992). Macrophages are one cell type that are activated in response to endotoxin. As previously discussed, activated macrophages release TNF and IL-1. The cytokines then increase adhesion molecule expresssion on the endothelium. With this increase in CAMs, white blood cells more readily attach to the endothelium, enabling more white blood cells to move into the tissues containing endotoxin (Welbouin and Young, 1992). Niehaus has postulated "that the pathophysiologic progression of the sepsis-induced ARDS represents a biphasic activation of the mononuclear phagocyte system" (1993). The first phase involves the

production of monokines secreted into the blood, which releases a large number of monocytes from the bone marrow. Some of these monocytes go to the lung microvasculature and then move into the lung to mature into tissue macrophages. The next phase results from increasing numbers of blood-borne particulates, possibly bacteria. If this occurs while the hyperactivated macrophages are in the microvasculature, the macrophages phagocytize the blood-borne particulates and release excess cytotoxins. These cytotoxins cause injury to the microvasculature endothelium and also induce pulmonary edema (Niehaus, 1993).

To determine the role that CAM expression has in the development of subsequent lung injury, clinical studies are planned to examine the levels of CAMs on monocytes of patients entering the surgical intensive care unit at St. Elizabeth's Hospital as a result of a traumatic injury. In future studies, the blood of these patients will be collected and the expression of CAMs will be examined to determine if there is any correlation between CAM expression and the development of ARDS. One of the problems associated with doing a clinical study is that patient consent is needed before any blood can be obtained. In most trauma patients that progress to ARDS, the subject is unconscious and cannot give his/her consent. However, when patients are brought into the emergency room, blood is drawn for cell counts and this previously collected blood

could ethically be used for a clinical study without the requirement of patient consent. However, this presents a problem of determining which blood collection techniques keep the necessary data accurate. Many different factors can affect the quality of a blood sample: anticoagulant, storage conditions and specimen age are just a few (Bray and Landay, 1989).

In this study, we have used flow cytometry to analyze the effect of collection mehods on CAM expression on monocytes. First, we have looked at the effect of specimen age by examining blood samples processed immediately after collection and samples processed two hours after collection. We then examined the effect of storing the blood at $4^{0}C$ for two hours versus storage at room temperature. In the final experiment, we have examined the effects of four different anticoagulants, namely EDTA, potassium oxalate, sodium citrate, and heparin, on CAM expression.

MATERIALS

Antibodies against ICAM-1, LFA-1 alpha, LFA-1 beta, L-selectin and Mac-1 were contributed by Dr. Robert Rothlein from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, Connecticut). Antibodies against VLA-4 (0.2 mg/mL) were purchased from AMAC (Westbrook, Maine). Cali Brite beads (unlabeled, FITC, and PE), FACS lysing solution, Simultest Leuco Gate (CD45 FITC, CD14 PE), Simultest control (Gamma 1 FITC, Gamma 2a PE), Simultest CD3 FITC/CD4 PE, and Simultest CD3 FITC/CD19 PE were all purchased from Becton Dickinson (San Jose, California). Specific reagents from Sigma Chemical Company (St. Louis, MO) were Dimethyl sulfoxide (DSMO), NaCl, sodium azide, and Phorbol Myristate Acetate (PMA). Anhydrous Na_2HPO_4 was purchased from Mallinckrodt (Paris, Kentucky). Na $H_2PO_4H_2O$ was purchased from Fisher Scientific (Fair Lawn, New Jersey). Isoton II solution was purchased from Baxter Scientific, Inc. (Deerfield, IL).

REAGENTS

Sodium phosphate (0.01M) and sodium chloride (0.15M) were used to produce 1XPBS which was stored at 4^0C. Sodium azide (0.1 mg/mL) in PBS was stored in the dark at 4^0C for up to one week and was filtered with a 0.2 um filter before use. Paraformaldehyde (0.1mg/mL) was placed in a final volume of 200 mL of PBS. This solution was heated to 56^0C in a water bath in order to dissolve the paraformaldehyde.

This paraformaldehyde was stored in the dark at 4^0C for up to two weeks and was filtered before use. 10X buffered lysing solution containing less than 50% diethylene glycol and less than 15% formaldehyde (Becton Dickinson Immunocytometry Systems in San Jose, CA) was diluted to 1X lysis buffer which was stored at room temperature.

METHODS

Blood samples from healthy subjects were drawn directly into vacuum tubes containing an anticoagulant. The samples were divided equally into two or three parts by placing each part into a separate tube. In the first experiment, blood was divided into two equal amounts. One portion was processed immediately while the other portion sat for two hours at room temperature and then was processed. In some experiments a third portion sat on ice before processing. Stimulated samples were incubated with 6.6 micromolar PMA at 37^0C for 15 minutes. At the end of the incubation period, 100 uL of stimulated or unstimulated blood was added to test tubes with 20 ug/test of the appropriate antibody (anti-ICAM-1, LFA-1beta, Mac-1, L-selectin, LFA-1alpha, or VLA-4). All tubes were incubated for 15 minutes at room temperature. The cells were then washed twice with 2 mL of PBS Azide and centrifuged for 8 minutes at 300 X g. After each wash, the supernatant was removed and discarded. At this time, 4 uL of a fluorochrome-labeled secondary antibody (FITC-labeled goat

anti-mouse immunoglobulin Becton Dickinson) was added to all tubes. The FITC-labeled goat anti-mouse immunologlobulin is directed against the Fc portion of the specific cell surface marker antibodies already placed on the monocytes. The fluorochrome (fluorescent dye) used in the experiments was fluorescein. At this time, 100 uL of unstimulated blood was added to control antibodies (CD45/CD14, gamma1/gamma2alpha, and CD3/CD19). Only CD45/CD14 antibody was also used to label 100 uL of the stimulated blood. Both samples and control tubes were incubated for 15 minutes in the dark at room temperature. In order to remove the red blood cells, 2 mL of lysis buffer was added to all tubes, and the tubes were immediately vortexed for 3 seconds. The tubes again were incubated in the dark for 10 minutes at room temperature. The tubes were then centrifuged for 8 minutes at 300 X g and the supernatant was discarded. Another wash was performed on the cells using 2mL of PBS Azide, and the cells were centrifuged for 8 minutes at 300 X g. Once the supernatant was discarded from the final centrifugation, the white blood cells were fixed by the addition of 400 uL of paraformaldehyde, vortexed immediately and stored in the dark at 4^{0}C.

STATISTICS

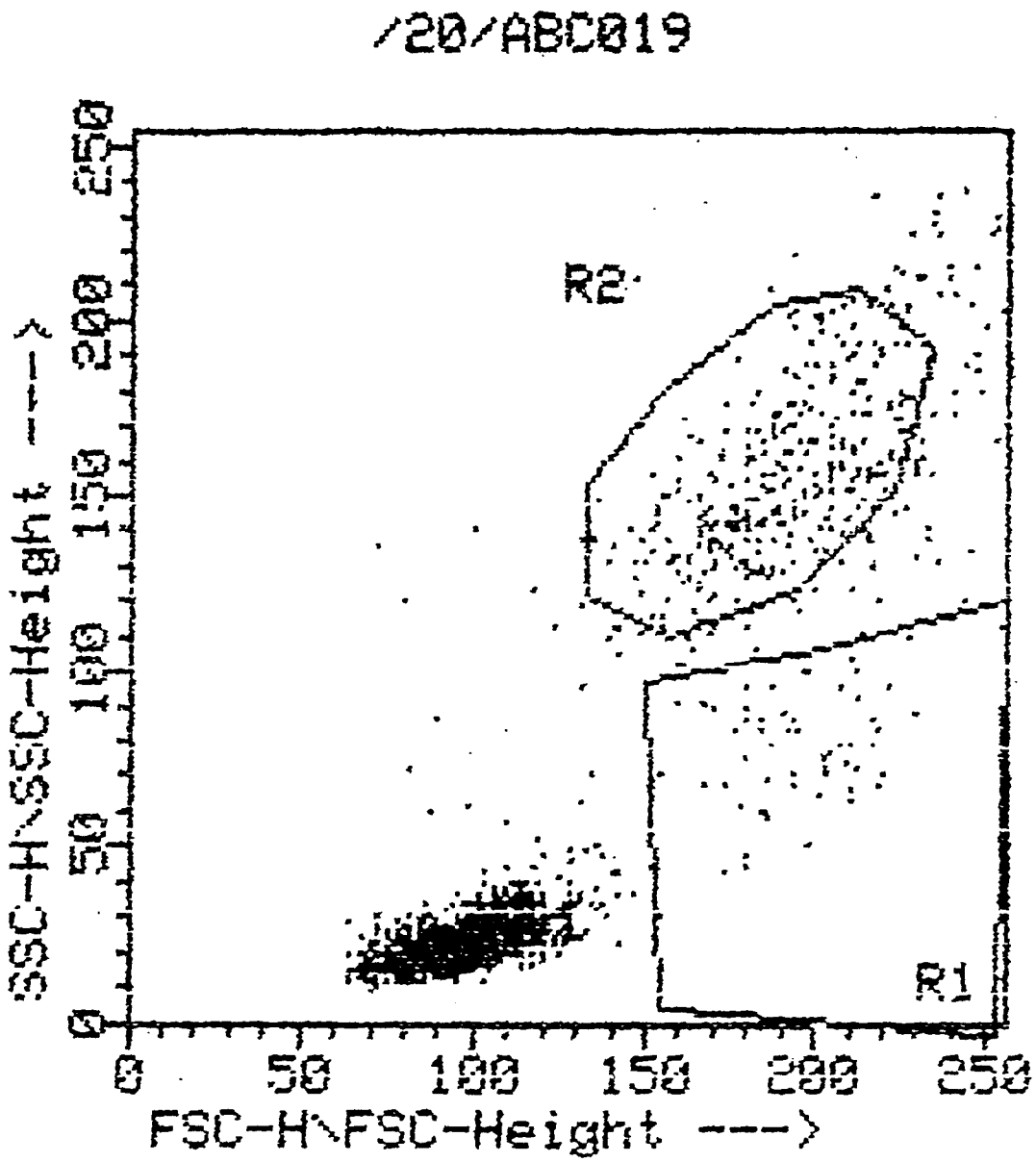
A paired t-test (Sigma Stat 3.10, 1988) was performed using data from experiments #2 and #3 to examine Mac-1

expression on monocytes collected and stored in EDTA at the various time points and temperatures.

DATA ANALYSIS

A Becton Dickinson flow cytometer was used to examine the blood samples. Flow cytometry is the process in which the physical characteristics of cells are measured while cells, in a fluid, pass in a single file stream through a narrow glass tube (Shapiro, 1995). As the cells flow through the machine, they pass through a laser beam which causes light scattering patterns to be produced. These patterns are measured, allowing the cells to be separated into different populations. The forward scatter of light is proportional to the cell's size while the side scatter of light is proportional to the cell's granularity. Using these light patterns, four different grouping of cells are created (Figure 1). The first group of cells consists of the granulocytes or neutrophils (R2) which exhibit high forward (size) and side (granularity) light scatter patterns. The next group of cells are the monocytes (R1) with high forward (size) and intermediate side (granularity) scatter. The lymphocytes (T cells, B cells and NK cells) have low forward and side light scatter. The remaining cells include RBCs, platelets and various debris that all present very low forward and side scatter patterns of light (McCoy et al., 1990).

"The scattered light generated by cells passing through the laser is collected by photodetectors which convert the photon pulses into electronic signals. Further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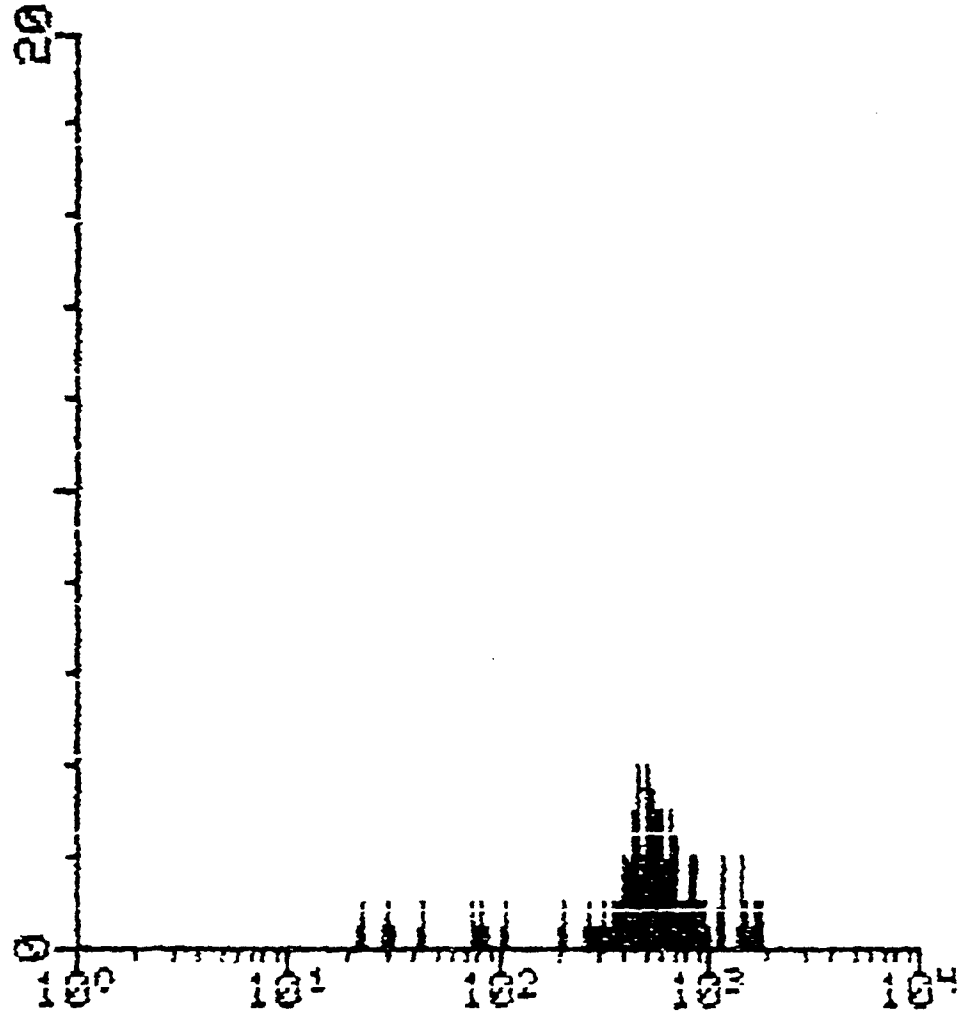
SSC-H\SSC-Height --->
0
50
100
150
200
250
R2
R1
FSC-H\FSC-Height --->

electronic and computational processing results in a graphic display and statistical analysis of the measurements being made" (Ormerod, 1990). In order to begin analyzing data, gates are drawn around the appropriate cell population, monocytes, to group the cells together for futher examination. The aim in setting gates is to exclude all extraneous cells from the analysis (Bray and Landay, 1989). The procedure begins by drawing a polygon around the monocyte population on the graph. The first sample to be analyzed was an aliquot of control blood from which the computer depicted a cytogram of forward scatter (FSC) versus side scatter (SSC). A polygon was then drawn around the population of monocytes as seen in figure 1. Once this gate was created, a similar gate was used to analyze each subseqent sample. For each sample that was analyzed, a histogram, which displays the data graphically, (Figure 2) along with statistics (Figure 3) were produced from the polygon (R1) of monocytes. The histogram shows the amount of fluorescence per monocyte labeled with an anti-CAM and an FITC goat anti-mouse immunoglobulin on the horizontal axis versus the number of cells on the vertical axis. In these histograms, we are measuring the amount of fluorescent dye, namely fluorescein isothiocyanate (FITC), that appears on the surface of the cells. From the histogram, statistics can be generated which present the mean or average fluorescence for each sample. High fluorescence from the histograms indicates

/20/ABC019\FL1-H\Gamma-1 Control FITC



/20/ABC019\FL1-H\Gamma-1 Control FITC

--- Arithmetic Histogram Statistics for /20/ABC019 ---

Selected Preferences: Arithmetic/Linear

Parameter FL1-H Gamma-1 Control FITC Gate G1= R1

M	Left,Right	Events	%	Peak	PkChl	Mean
0	1.00, 9646	63	100.00	4	453.16	583.20

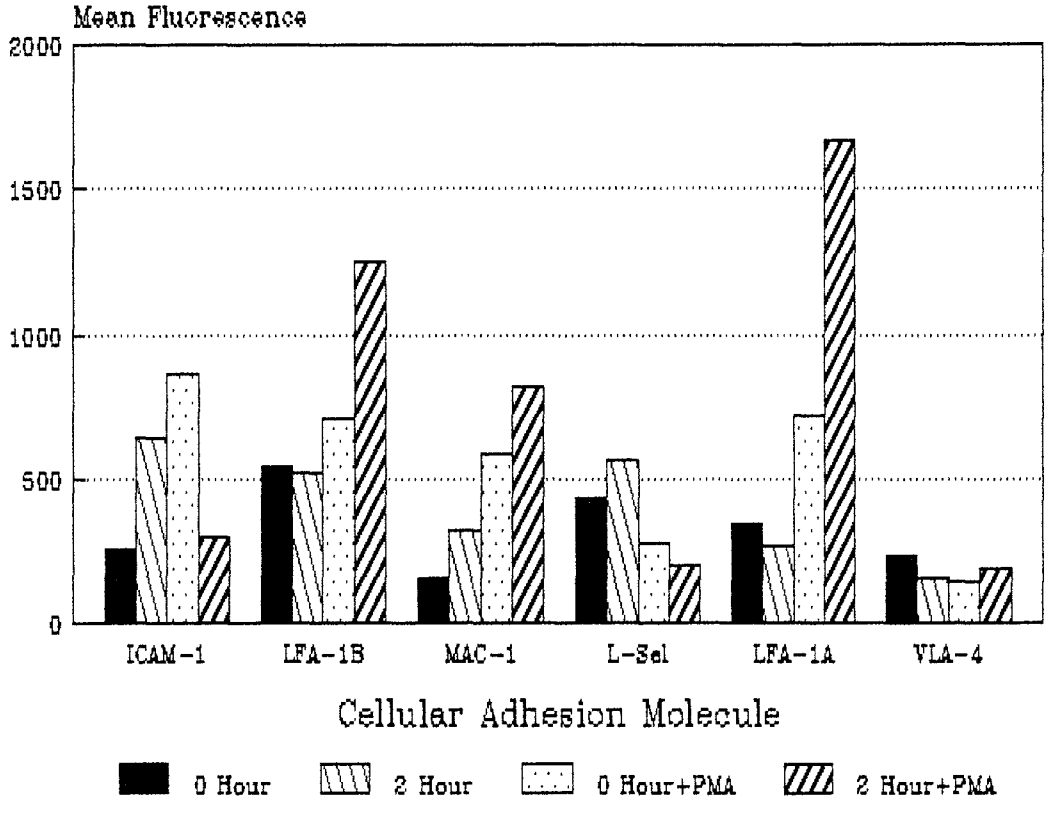
Median	SD	CV %
504.81	383.48	65.75

that the cellular adhesion molecule is present in large quantities on the surface of the monocytes.

RESULTS

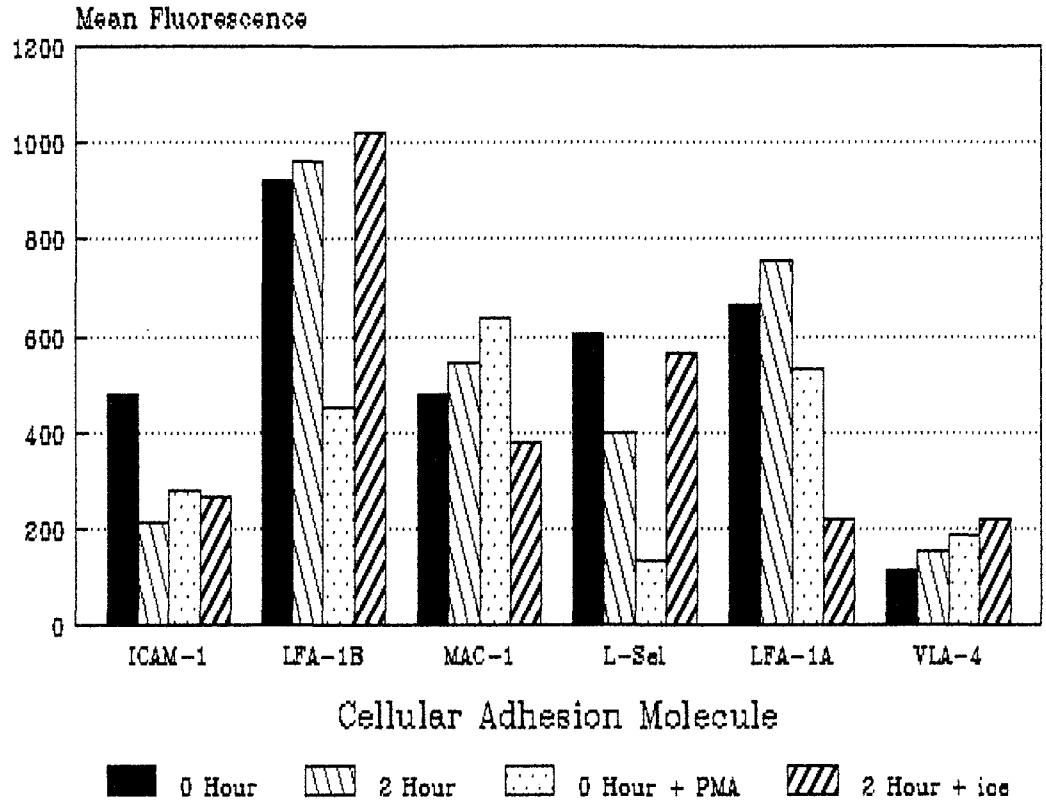
The expression of CAMs on monocytes was examined at the time of collection and at two hours after collection using both unstimulated cells and cells stimulated with PMA (Figure 4). The results show the presence of six different CAMs on the monocyte cell surface: ICAM-1, LFA-1beta, Mac-1, L-Sel, LFA-1alpha, VLA-4. No apparent effect of incubation time occurred for LFA-1beta, L-selectin, and LFA-1alpha. However, a large increase in ICAM-1 expression was seen with time and a moderate increase was seen in the levels of Mac-1. VLA-4 showed a slight decrease in mean fluorescence with time. Comparing stimulated cells with unstimulated cells, a large increase was seen in mean fluorescence for all CAMs except L-selectin and VLA-4, whose values decreased. When examining the effect of PMA after 2 hours, LFA-1beta, Mac-1, and LFA-1alpha increased in mean fluorescence over that seen with PMA at the 0 time point. To summarize the results seen in experiment 1, all samples responded to PMA with altered expression of the CAM. The cells incubated for 2 hours at room temperature in the prescence of EDTA displayed a moderately activated phenotype of approximately half that seen in PMA activated cells. Cells stimulated with PMA following a 2 hour incubation at room temperature responded more vigorously than these activated with PMA at the time of collection.

In the second experiment, the same parameters were

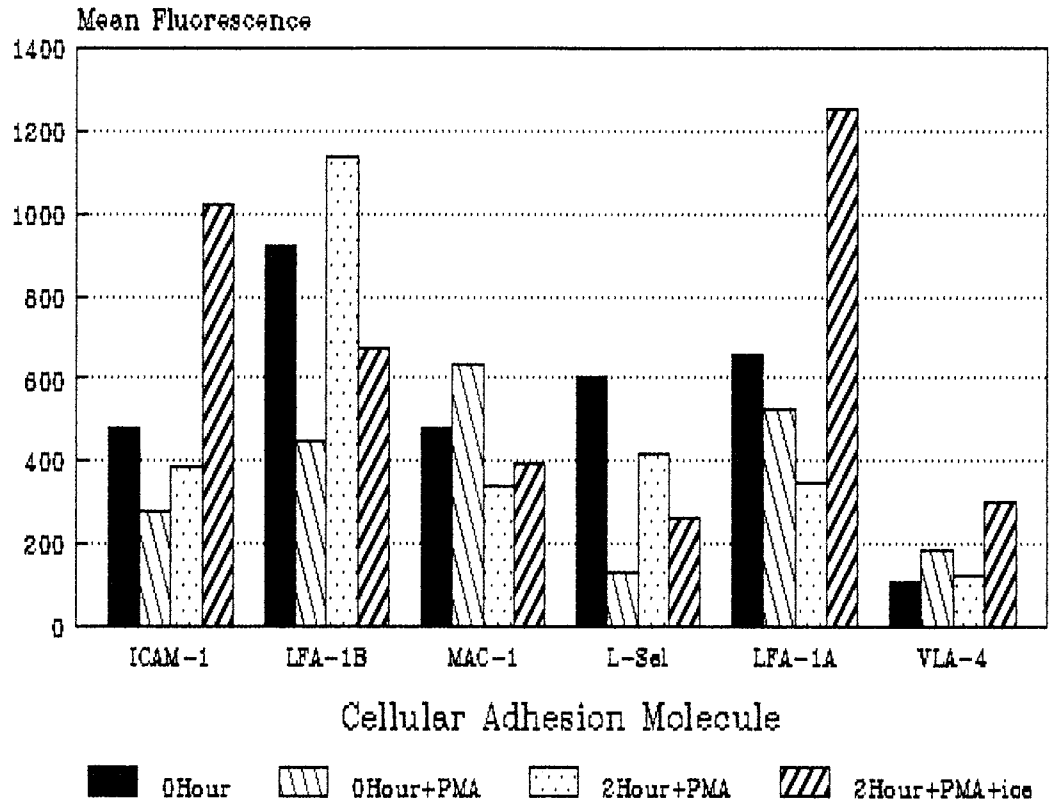

Mean Fluorescence
2000
1500
1000
500
0
ICAM-1
LFA-1B
MAC-1
L-Sel
LFA-1A
VLA-4
Cellular Adhesion Molecule
0 Hour
2 Hour
0 Hour+PMA
2 Hour+PMA

examined, however, a change in the temperature of incubation was also included in this experiment to see if placing the blood samples on ice versus staying at room temperature influenced the effect of blood storage on CAMs (Figures 5a and 5b). A slight increase was seen in the levels of CAMs over time for Mac-1 and LFA-1alpha, as seen in experiment 1 (Figure 4). Increases were also noted in CAM expression with the 2 hour incubation for LFA-1beta and VLA-4. A decrease in the levels of CAM expression with time was seen for ICAM-1 and L-selectin. The effect of 2 hours on ice on ICAM-1, LFA-1beta, L-selectin, and VLA-4 seems to increase expression of the CAMs as compared to 2 hours at room temperature, while decreasing expression of Mac-1 and LFA-1alpha. The cells that were stimulated with PMA increased cellular expression of Mac-1 and VLA-4 and decreased expression of ICAM-1, LFA-1beta, L-selectin, and LFA-1alpha as compared to 0 hour nonstimulated cells. The PMA stimulated cells that were incubated for 2 hours at room temperature seemed to have a higher expression of ICAM-1, LFA-1beta, and L-selectin as compared to the 0 hour PMA stimulated cells. Mac-1, LFA-1alpha, and VLA-4 all decreased expression upon the 2 hour incubation with PMA stimulated cells. Ice had an effect on PMA stimulated samples; however, the results varied. All CAMs, except Mac-1, had an increased mean fluorescence in stimulated cells stored with ice compared to control stimulated cells at 0 hour. To summarize the results seen in experiment #2,

5 A.

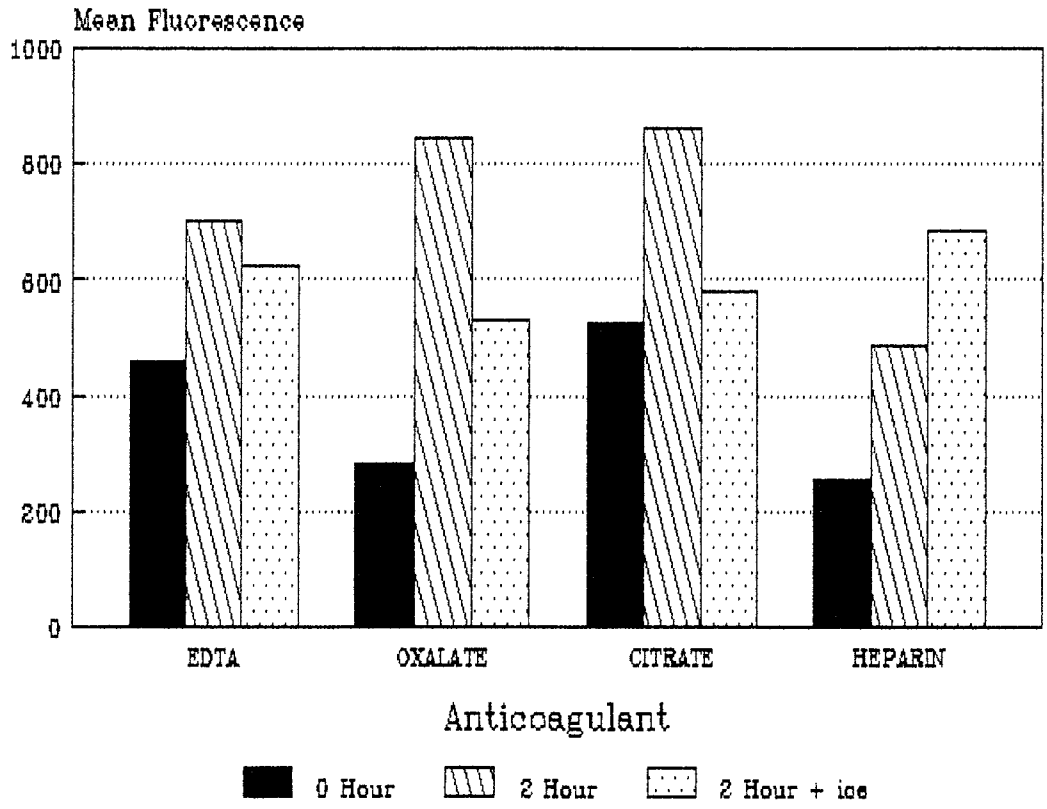

Mean Fluorescence
1200
1000
800
600
400
200
0
ICAM-1
LFA-1B
MAC-1
L-Sel
LFA-1A
VLA-4
Cellular Adhesion Molecule
0 Hour
2 Hour
0 Hour + PMA
2 Hour + ice

5 B.


Mean Fluorescence
1400
1200
1000
800
600
400
200
0
ICAM-1
LFA-1B
MAC-1
L-Sel
LFA-1A
VLA-4
Cellular Adhesion Molecule
0Hour
0Hour+PMA
2Hour+PMA
2Hour+PMA+ice

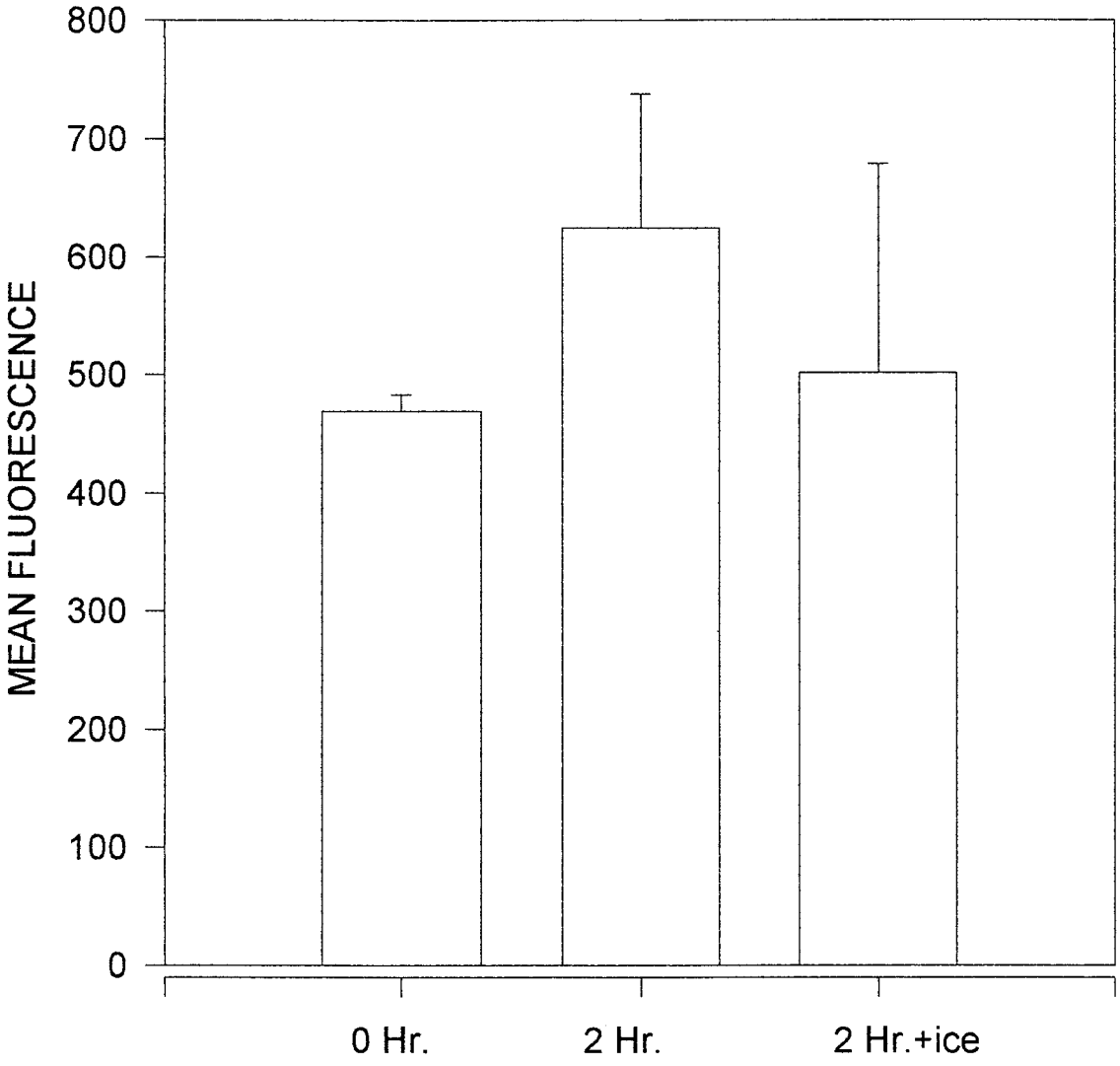
once again all samples responded to PMA with altered expression of the CAM. The cells incubated for 2 hours at room temperature in the presence of EDTA displayed CAM expression simular to that seen with incubation for 2 hours at 4^0C. Cells stimulated with PMA following a 2 hour incubation at room temperature responded much differently than those cells stimulated with PMA and incubated on ice for 2 hours.

After examining the effects of time and temperature, yet another variable comes into play -- the anticoagulant. To determine if anticoagulants other than EDTA have an effect on CAM expression, the cells collected in EDTA, oxalate, citrate, or heparin were tested for Mac-1 expression at the time of collection, at 2 hours after collection when stored at room temperature and at 2 hours after collection when stored in ice. In Figure 6, the general trend for all the anticoagulants was to see increased levels of Mac-1 upon sitting for 2 hours at room temperature. Ice seemed to decrease Mac-1 expression when compared to incubation at room temperature, therefore prevented activation of the cells to some degree. Sodium citrate on ice appears to be the best storage medium to eliminate increased CAM expression indicating the prevention of cell activation. Potassium oxalate on ice appears to have a moderate potential for preventing activation, and ice has only a slight effect on samples


Mean Fluorescence
1000
800
600
400
200
0
EDTA
OXALATE
CITRATE
HEPARIN
Anticoagulant
0 Hour
2 Hour
2 Hour + ice

collected in EDTA. In the samples collected in heparin, ice didn't appear to prevent activation of the monocytes.

The average of experiments #2 and #3 is seen in Figure 7. When the blood samples were collected in EDTA, an increase in Mac-1 expression was seen with an incubation of 2 hours at room temperature. When the samples were stored in EDTA at $4^{0}C$ for two hours, the increased expression of the Mac-1 was limited and kept more like freshly collected blood. The t-test performed on this data did not demonstrate any significance to this observation.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DISCUSSION

In this study, methods for collecting blood were examined in order to determine the optimal conditions for preserving CAM levels on monocytes. The effects of time, temperature and various anticoagulants on the expression of CAMs were studied. The ultimate goal will be to study these CAMs in trauma patients who might progress to acute respiratory distress syndrome (ARDS). ARDS refers to a disease "with pulmonary oedema, decreased lung compliance and arterial hypoxia" (Welbourn and Young, 1992). ARDS may result from an inappropriate inflammatory response following a trauma, resulting in a large number of phagocytic cells transmigrating into the lungs. Cellular adhesion molecules (CAMs) may play a role in this response by allowing the cells to enter the tissues.

Since trauma patients are often unconscious and unable to give consent for using their blood, studies may have to be done on blood that has already been collected for other purposes, such as cell counts. The most commonly used anticoagulant is EDTA, and the blood is usually stored at room temperature. One set of researchers noted that the most commonly used anticoagulant in blood samples for cell typing in flow cytometry is heparin because these samples are more resistant to change at room temperature than samples stored in EDTA (Bray and Landay, 1989). Another group of scientists also examined anticoagulants for

immunophenotyping and determined that heparin, not EDTA, after 48 hours, yeilded similar results to fresh specimens when stored at room temperature (Nicholson *et al.*, 1993). As blood collected in heparin is not often available, the data found in this study looked at the effects of other anticoagulants and time of storage at room temperature on the detection of CAM expression on monocytes.

The levels of six different CAMs (ICAM-1, LFA-1beta, Mac-1, L-sel, LFA-1alpha, and VLA-4) on the monocyte cell surface were measured using flow cytometry. The blood sample was allowed to sit for two hours at room temperature before processing and compared to controls that were processed immediately. In the first two experiments, the blood was collected in EDTA, one sample was altered by stimulation with PMA (a nonspecific activator). Once the cells are activated, they begin to either lose or gain certain CAMs so that the cells can migrate through the tissues.

In the first two experiments (Figures 4 and 5), similar findings for Mac-1 and L-selectin expression were found. Upon stimulation of monocytes with PMA, the levels of Mac-1 were increased while the levels of L-selectin were decreased. These results concur with Lundahl and Hed's findings that upon stimulation of monocytes an up-regulation of Mac-1 and a down-regulation of L-selectin

occurs (1994). Different findings for VLA-4 expression were found in the first two experiments. In the first experiment, VLA-4 levels decreased upon stimulation with PMA. However, in the second experiment, the levels increased upon stimulation. According to Bauvios *et al.*, VLA-4 decreases upon PMA stimulation in a promonocytic cell line, therefore the first experiment concurred with this study (1992). LFA-1alpha and LFA-1beta also have different results upon stimulation with PMA in the first two experiments. According to the literature, both LFA-1s should increase expression with PMA (Matikainen and Hurme, 1994). Therefore the first experiment followed the expected pattern while the second experiment did not. These results could have been due to technical error. ICAM-1 should decrease in response to stimulation of cells (Pigott *et al.*, 1992). The first experiment did not follow what the literature states. However, the second experiment seemed to hold the correct response since ICAM-1 decreased its expression in the stimulated cells.

In the first two experiments, the samples were processed immediately and following a 2 hour incubation at room temperature. Unstimulated cells that were incubated at room temperature for 2 hours had increased levels of ICAM-1, Mac-1 and L-selectin when compared to cells that were processed immediately. The levels of LFA-1beta, LFA-1alpha, and VLA-4 were decreased in the first experiment

after incubation at room temperature for 2 hours. In the second experiment, the levels of LFA-1beta, Mac-1, LFA-1alpha and VLA-4 were also increased while the levels of ICAM-1 and L-selectin decreased on unstimulated cells. The first two experiments have inconsistent results for ICAM-1 expression on unstimulated cells. LFA-1beta expression had no significant change upon the two hour incubation at room temperature in both experiments. Mac-1 consistently increased in both cases upon the two hour incubation for 2 hours. The three final CAMs (L-selectin, LFA-1alpha, and VLA-4) had inconsistant results between the first two experiments when examining both stimulated and unstimulated cells. From these experiments, it appears that the cells are becoming activated by sitting at room temperature since many of the CAM levels are half of what is seen in stimulated cells. Scientists have previously demonstrated that samples analyzed up to 36 hours had accurate cell blood count (number of lymphocytes) parameters compared to controls that were analyzed immediately. The parameters that were examined were CD3 (cluster of differentiation 3), CD4, CD8, and CD19 which are cell surface markers found on T lymphocytes and B lymphocytes (Paxton and Bendele, 1993). However, the CD molecules that have been examined previously may not be regulated by cell activation, giving different results.

In experiments #2 and #3, a second variable,

temperature, was examined to determine its effects on CAM expression. In experiment #2, L-selectin had a decrease in CAM expression upon 2 hour incubation at room temperature, when compared to the control that was processed at 0 hour. When the blood sample was placed on ice for the 2 hour incubation, the level of L-selectin expression was back to baseline. In experiment #3 which examined Mac-1 expression, the levels of its expression increased when compared to the control that was stored in EDTA. Moreover, Mac-1 expression was enhanced in both experiment #2 and #3 upon the 2 hour incubation at room temperature. The blood samples that were placed on ice for the 2 hour incubation allowed the Mac-1 expression in the last two experiments to decrease towards baseline. L-selectin and Mac-1 expression are the most sensitive indicators of cell activation (Kishmoto, 1989). Bray and Landay have concluded that room temperature storage of blood is preferred to $4^{0}C$ in phenotypic studies, which look at the physical properties of the cells, ie. cell markers (Bray and Landay, 1989). Their studies were done on lymphocytes, and they were not examining CAMs. This study is interested in examining CAMs found on neutrophils and monocytes, which may respond differently to the blood storage conditions. Another study by Paxton and Bendele showed that temperature, if at or below room temperature, did not change the expresson of cell surface markers (CD3, CD4, CD8, and CD19) on blood samples of T lymphocytes and B lymphocytes for analysis by

flow cytometry (Paxton and Bendele, 1993). Their studies were performed on lymphocytes and examined CD markers not CAMs. In this study, temperature did seem to affect the expression of CAMs by either increasing or decreasing expression of the CAMs.

In this study, placing the samples on ice appeared to allow the levels of L-selectin and Mac-1 to be preserved in both stimulated and unstimulated cells. The blood samples that were kept on ice also conserved the level of ICAM-1 on unstimulated cells. When the blood samples in this study were placed on ice for the 2 hour incubation, cell activation appeared to be prevented as indicated by changes in CAM expression that was seen with storage at room temperature.

Several anticoagulants were examined to determine their effects upon CAM expression. Each anticaogulant that was used prevents coagulation, however, the chemicals affect the cells differently. EDTA removes calcium to prevent blood clotting. EDTA prevents platelet aggregation and is usually used for general hematological studies (Flynn, 1994). Sodium citrate binds calcium to prevent clotting, and this anticoagulant is used for coagulation studies (Flynn, 1994). Potassium oxalate also removes calcium to stop clotting, and it is used for glucose analysis of blood (Flynn, 1994). Heparin inactivates

thrombin and thromboplastin which prevents blood clotting. Heparin is used when plasma or whole blood is needed since it does not affect erythrocyte size (Flynn, 1994).

This study demonstrates that when unstimulated blood is colleced in EDTA, Mac-1 expression is increased upon stimulation with PMA and also by a 2 hour incubation at room temperature in all three experiments. When the sample is place at 4^0C for two hours, a lowered expression of Mac-1 is found as compared to the 2 hour incubation at room temperature as seen in the last two experiments. Different anticoagulants seem to influence Mac-1 expression on monocytes because variable levels were found among the four tested anticoagulants (EDTA, oxalalte, citrate, and heparin).

In our experiments, EDTA, oxalate and citrate used as an anticoagulant gave similar results with Mac-1 expression increasing upon a two hour incubation at room temperature. Two hour incubation at 4^0C resulted in decreased response. The expression of Mac-1 on cells incubated at 4^0C was similar to that seen on cells processed immediately at 0 hour. Incubation on ice prevented the apparent activation of cells seen after 2 hours at room temperature. An increase in the expression of Mac-1 also occurred in the prescence of heparin when the cells were incubated at room temperature for 2 hours and placing the sample at 4^0C

enhanced the expression of Mac-1 further. According to Bray and Landay, 1989, "heparin is the most commonly used anticoagulant for obtaining samples for immunotyping." However, another study has concluded that "the choice of anticoagulant is not as significant a factor as variation on temperature" (Paxton and Bendele, 1993). This study doesn't agree with heparin being the choice of anticoagulant for examining CAM levels since EDTA, oxalate and citrate, not heparin, appeared to preserve CAM expression when the blood was stored on ice for the 2 hour incubation. Temperature of storing the blood samples for examing CAM levels does appear to be an important factor because different results occurred in this study when the sample sat for 2 hours at room temperature versus 2 hours at $4^{0}C$.

This study has shown that CAM expression on monocytes is altered by allowing blood samples to sit in an anticoagulant 2 hours. In these experiments, the results indicate that the temperature at which the blood is incubated affects the expression of CAMs more than the type of anticoagulant. The best temperature to store blood samples used to examine CAM levels appears to be $4^{0}C$ instead of room temperature. In order to fully understand the best way to collect and store blood samples for observing CAM expression, more studies need to be done with shorter time points to determine more precisely the length

of time cells may sit before processing to obtain reliable results. In these experiments, the effects of certain anticoagulants (EDTA, oxalate and citrate) on CAM expression did not seem to be important variables for storing blood samples for analysis of monocytes by flow cytometry. However, heparin does not seem to be a good anticoagulant for storage because it is unable to prevent cellular activation at either temperature.

The time and temperature of incubation does effect CAM expression on monocytes when examined by flow cytometry. According to the results with Mac-1, monocytes that sit at room temperature for 2 hours have increased expression of Mac-1, however, placing the samples on ice for the 2 hours appeared to decrease this activation. The 2 hour incubation at either temperature still altered CAM expression when compared to those samples that were processed immediately, but the samples that were stored at $4^{0}C$ seemed to maintain CAM expression better than those samples stored at room temperature.

Clinical studies have been proposed to examine the expression of CAMs in trauma patients to determine if the levels of CAMs on monocytes and neutrophils are altered in patients that progress to ARDS. If the levels of CAMs on cells can be correlated to the progression to ARDS, possible therapies may be developed that are directed

toward the ligands of the CAMs. Inhibition of white blood cell emigration from the vasculature might be an effective method for inhibiting the inflammatory process. It is also possible that a correlation may be seen between the expression of CAMs after trauma and the development of ARDS. If this is true, measurement of CAM levels might be used to determine patients that are at risk for the syndrome.

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THE EFFECT OF STORAGE IN ANTICOAGULANT ON LEUKOCYTE CELL ADHESION MOLECULES

CONSENT FORM

I have been asked to participate in a research study of the effect of storage time and anticoagulants on the expression of cell adhesion molecules on white blood cells. Adhesion molecules direct white blood cells to the site of an inflammatory response. Future studies will measure changes in the expression of cell adhesion molecules on white blood cells as an indicator of an inflammatory response in a patient population. As the blood examined may have to be collected prior to analysis, it is important to know the effect of storage conditions on the expression of these molecules on the cells.

If I agree to participate in this study, I will be asked to donate 5 ml (approximately 2 tsp) of blood. The blood will be collected by venipuncture (drawn from the vein using a needle and blood collection equipment). There are minimal risks associated with venipuncture. These include the possibility of bruising and infection at the site of venipuncture. The study will be conducted at no cost to me. I will receive no personal benefit from participating in the study.

I understand that I am free to decline participation in this study. My participation will be kept confidential. The informed consent form will be the only record of my participation and it will remain in a restricted site. My name will not be used in any written report.

I understand that I may contact Dr. D. Fagan at (330) 742-1554 if I have any questions about this research. I may also call Dr. S. Ellyson, chair of the IRB office, at (330) 742-3403 if I have any questions about my rights as a subject. I have carefully read this document and all my questions have been answered. I am 18 years of age or older and voluntarily consent to participate in this study.

Name:_____

Signature:_____

Date:_____

Consent obtained by:

Name:_____

Signature:_____

Date:_____

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

7/13/94
Date of Approval

Robert G Slating
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

HM Health Services
Youngstown, Ohio

Institutional Review Board

Application for Expedited Review

Project Title: Trauma Induced Activation of the Mononuclear Phagocyte System (MPS)
Approval 493-005

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 copy 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 REGARDING 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 _____.

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

5-1-96 — Date

Chatrchai Watanakunakorn, M.D. — Chairperson, IRB
Chatrchai Watanakunakorn, M.D.

Categories of Expedited Review

Research projects involving the following procedures may be reviewed using an expedited process, that is review and approval by the IRB chairperson or an IRB sub-committee:

1. collection of hair and nail clippings in a non-disfiguring manner, deciduous teeth and permanent teeth if patient care indicates a need for extraction;
2. collection of excreta and external secretions including sweat, uncannulated saliva, placenta removed at delivery and amniotic fluid at the time of rupture of the membranes prior to or during labor;
3. recording of data from subjects 18 years of age or older using non-invasive procedures routinely employed in clinical practice. This includes the use of physical sensors that are applied either to the surface of the body or at a distance, and do not involve input of matter or significant amounts of energy into the subject or an invasion of the subject's privacy. It also includes such procedures as weighing, testing sensory acuity, electrocardiography, electroencephalography, thermography, detection of naturally occurring radioactivity, diagnostic echography and electroretinography. It does not include exposure to electromagnetic radiation outside the visible range, such as x-rays, microwaves, etc.;
4. collection of blood samples by venipuncture, in amounts not exceeding 450 milliliters in an eight-week period and no more often than two times per week, from subjects 18 years of age or older who are in good health and not pregnant;
5. collection of both supra and subgingival dental plaque and calculus, provided the procedure is not more invasive than routine prophylactic scaling of the teeth and the process is accomplished in accordance with accepted prophylactic techniques.
6. voice recordings made for research purposes, such as investigations of speech defects;
7. moderate exercise by health volunteers;
8. study of existing data, documents, records, pathological specimens or diagnostic specimens;
9. research on individual or group behavior or characteristics of individuals, such as studies of perception, cognition, game theory or test development where the investigator does not manipulate subjects' behavior and the research will not involve stress to subjects;
10. research on drugs or devices for which an investigational new drug exemption (IND) or an investigational new device exemption (IDE) is not required; if randomization is involved, a full board review is required;
11. survey research or interview procedures involving no more than two of these conditions:
    a. responses are recorded in such a manner that the human subjects can be identified, directly or through identifiers linked to the subjects;
    b. subjects' responses, if they became known outside the research could reasonably place the subject at risk of criminal or civil liability or be damaging to the subjects' financial standing or employability;
    c. the research deals with sensitive aspects of the subjects' own behavior, such as illegal conduct, drug use, sexual behavior or use of alcohol;
12. minor modifications or additions to existing approved studies;
13. continuing review of activities which the IRB determined in earlier full board review could have expedited continuing review.