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EFFECTS OF SHEAR STRESS ON PLATELET - POLYMORPHONUCLEAR LEUKOCYTE INTERACTIONS

by

Byung-Geon Rhee

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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DECEMBER, 1985
EFFECTS OF SHEAR STRESS ON
PLATELET - POLYMORPHONUCLEAR
LEUKOCYTE INTERACTIONS

by
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ABSTRACT

The effects of shear stress on platelet - polymorphonuclear leukocyte (PMNL) interactions were studied to ascertain whether mechanical trauma induces PMNL aggregation or degranulation. PMNL suspensions were exposed to shear stresses up to 150 dynes/cm² at room temperature for 1 minute. PMNL responses were monitored by the following measurements. The large particle percentage was calculated from Coulter counter data to indicate shear-induced aggregation. β-glucuronidase in the extracellular fluid was assayed to indicate azurophilic granule release. Lactate dehydrogenase (LDH) release was measured to indicate cell lysis.

PMNL suspensions in buffer did not show any aggregate formation or enzyme release at shear stresses below 150 dynes/cm² for an exposure time of 1 minute. However, supernatant plasma from sheared platelet rich plasma (PRP) initiated PMNL aggregation and β-glucuronidase release at this shear stress. In contrast,
platelet release factors, such as ADP and serotonin did not cause PMNL aggregation. These stress-induced alterations were compared with chemotactic factor and calcium ionophore A23187 induced functional changes.

The use of a cyclooxygenase inhibitor (aspirin) did not suppress the aggregation of PMNLs after shear. However, preincubation with lipoxygenase inhibitors (U-60257 and nordihydroguaiaretic acid) suppressed aggregation and enzyme release.

The shear-induced enzyme release was enhanced by cytochalasin B and heparinized samples showed greater effects than citrated samples.

In addition, the effects of shear stress on whole blood aggregation were studied. The total aggregate volume and mean channel number were calculated. The total aggregate volume increased with shear rate at 3000 - 10000 sec\(^{-1}\) for exposure times of 15 - 60 seconds. The extent of aggregation increased with both shear rate and exposure time.

Blood samples sheared at 37\(^{\circ}\)C showed much less aggregation than those sheared at room temperature. The total aggregate volume and mean aggregate size were decreased at the increased temperature. Heparinized blood was more sensitive to shear stress than citrated samples. A cyclooxygenase inhibitor (aspirin) was ineffective in
reducing shear-induced aggregation. However, a lipoxygenase inhibitor (nordihydroguaiaretic acid) was effective in reducing aggregate size.

The formation of arachidonic acid metabolites activated by shear stress was documented by reversed phase - high performance liquid chromatography (RP-HPLC). Thus, an interaction between C-12 and C-5 lipoxygenase enzymes can promote the formation of leukotriene B₄ (LTB₄) and these data strongly support the possibility of a cooperation between platelet and PMN leukocyte arachidonic acid metabolites leading to a mechanically induced cell activation.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Larry V. McIntire for his professional guidance and personal support. I would also like to thank Dr. Elizabeth R. Hall and Ms. Audrey C. Papp for their advice and assistance in the HPLC system. Last, but not least, I would like to thank my wife, Yun-Kyung, and my parents for their patience and understanding.
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# ABBREVIATIONS AND SYMBOLS

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<tr>
<td>A</td>
<td>light absorbance (β-glucuronidase assay)</td>
</tr>
<tr>
<td>ΔA</td>
<td>rate of change of light absorbance (LDH assay)</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosin diphosphate</td>
</tr>
<tr>
<td>ASA</td>
<td>acetylsalicylic acid (aspirin)</td>
</tr>
<tr>
<td>CPM</td>
<td>count per minute</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>FMLP</td>
<td>formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>HETE</td>
<td>hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HHT</td>
<td>hydroxyheptadecatrienoic acid</td>
</tr>
<tr>
<td>HPETE</td>
<td>hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>IU</td>
<td>international unit</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissolution constant</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LPP</td>
<td>large particle percentage</td>
</tr>
<tr>
<td>LT</td>
<td>leukotriene</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NDGA</td>
<td>nordihydroguaiaretic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
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<td>PG</td>
<td>prostaglandin</td>
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PGI₂ prostacyclin
PMNL polymorphonuclear leukocyte
PPP platelet free plasma
PPP platelet poor plasma
PRP platelet rich plasma
RBC red blood cell
RP-HPLC reversed phase high performance liquid chromatography
SEM standard error of the mean
Tham tris hydroxymethyl aminomethane
Tx thromboxane
U-60257 6,9-deepoxy-6,9-phenylimino-6,8-prostaglandin I₁

α : cone angle
γ : shear rate (sec⁻¹)
μ : viscosity (poise = dyne sec/cm²)
τ : shear stress (dyne/cm²)
ω : angular velocity (radians/sec)

Subscripts
N unsonicated sheared sample
NC unsonicated control sample
S sonicated sheared sample
CHAPTER I. ORIENTATION

1.1. Introduction

The rheological behavior of blood cells in the microcirculation plays a significant role in governing blood flow dynamics and material transport. Although erythrocytes constitute the major volume fraction of formed elements in blood, the leukocytes, by virtue of their larger volume and lower deformability, appear to exert significant rheological influences on blood flow in the microcirculation (4,5,25,89,148,149,150). The microrheological properties of leukocytes have been studied for developing an understanding of the dynamic behavior of these cells during flow and deformation, as well as modulating their activities in physiological and pathophysiological states (150,168,169).

The particular kind of white cells called polymorphonuclear leukocytes (PMNL) are a central component of cellular response in inflammatory reactions and of paramount importance to the immune surveillance system. These phagocytic cells contain diverse tissue destroying and microbicidal enzymes and proteins. Chemotactic factors appear pivotal in this migratory process. Many chemotactic factors have been described,
but, those derived from the fifth component of complement (e.g., C5a and des-arg-C5a) and synthetic analogues of the putative bacterial chemotactic factor (e.g., N-formyl peptides) have been most thoroughly characterized (23,27,30,32,63, 114,116,117,118). These agents, in the nanomolar concentration range, bind and activate the PMNL by interacting with distinct receptor sites to induce directional migration of the PMNL. They also stimulate other cellular functions such as aggregation, adherence, enzyme release, and the production of toxic oxygen metabolites. Thus, in addition to attracting PMNLs, chemotactic factors appear capable of modulating a number of other aspects of the inflammatory reaction. Several chemotactic factor-induced PMNL aggregation studies have been performed, initially stimulated by the observation of neutropenia in extracorporeal hemodialysis and endotoxemia (8,27,28,116,117). Neutropenia, pulmonary sequestration, and arterial hypoxemia are all transient phenomena that exhibit a time course which appears to parallel that of PMNL aggregation in vitro (30,117,118).

Fluid shearing is known to produce cell interactions and can lead to platelet and/or leukocyte aggregation (1,6,7,21,29,34,35,36,37,64,76,101,102,103,106,107,108, 134,135,136,149,163,164,165,166,170,186). Improved designs for artificial organs and prostheses require more
detailed knowledge of thromboembolic phenomena produced by mechanical trauma and biomaterial surface exposure. Shear stresses of greater than 150 dynes/cm² can be generated during blood flow through hemodialysis devices, artificial heart valves, and blood oxygenators. Unfortunately, the complicated and variable shear stress histories which cells experience in cardiopulmonary bypass and other circulatory support systems are hard to quantitate. Therefore, to generate an understanding of the role of leukocytes in embolic phenomena initially requires performing experiments in which the fluid forces are well known. In vitro studies of shear stress and surface interaction effects have normally used simpler flows, including stagnation point flow, Poiseuille flows through cylindrical capillaries, cylindrical Couette flows in rotational viscometers, and plane Couette flow (1,6,7,8,21,25,34,35,36,37,52,64,76,99,101,102,103,106, 107,108,134,135,136,140,163,164,165,166,170). In this study, a rotational cone and plate viscometer was used for the purpose of applying a known uniform shear stress to the entire sample volume.

PMNLs may participate along with platelets in hemostasis and thrombosis (1,35,76,135,136). The appearance of shear-induced PMNL aggregates has been observed in our laboratory (34,36,37,99,106,107,
108,135,136,140,165,166). Arachidonic acid metabolites appear to be involved in stimulated PMNL functional responses. Mammalian cells metabolize arachidonic acid through both the cyclooxygenase and lipoxygenase pathways. In platelets, the cyclooxygenase pathway of arachidonic acid metabolism has been implicated in these processes, through the formation of thromboxane A₂. Platelets also have a pathway involving lipoxygenase action on arachidonic acid, which forms 12S-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) and subsequently the corresponding 12S-12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) (72,92,93,96,97,98). The main pathway of arachidonic acid metabolism in PMNLs involves 5-lipoxygenation, leading to the formation of eicosanoids such as leukotrienes and other hydroxy acids (14,15,16,17,27,47,48,54,84). The discovery of leukotrienes (LTs), a new family of bioactive metabolites formed in a lipoxygenase-type reaction, and the detection of a C-15 lipoxygenase in leukocytes have indicated the existence of several lipoxygenase enzymes in blood cells. Arachidonic acid does not normally exist in free form but is usually esterified in triglycerides and membrane phospholipids. Stimulated phospholipases can release arachidonic acid, which is then enzymatically metabolized
by the 5-lipoxygenase pathway to form LTs as well as a host of other bioactive metabolites. In addition to differences in positional specificities, the heterogeneity of these enzymes is further emphasized by different reactivities. For instance, it has been shown that, in contrast to platelet suspensions which can efficiently transform exogenous arachidonic acid into 12-HETE and cyclooxygenase products, the addition of the fatty acid to preparations of human PMNLs was followed by only minor transformation of the substrate (27,84). However, the metabolism of arachidonic acid was strongly enhanced when PMNLs were incubated in the presence of arachidonic acid and divalent cation ionophore A23187, indicating clearly that the human PMNL C-5 lipoxygenase required activation for 5-HETE and LT synthesis. Maclouf et al. have shown that platelet derived 12-HPETE can stimulate this C-5 lipoxygenase (92,93). Recent studies suggest the involvement of LTs in hypersensitivity reactions and inflammation and point out the importance of a better understanding of the biochemical process involved in the control of the biosynthesis of these bioactive substances (9,17,19,20,39,40,41,42,44,45, 48,58,59,61,62,67,68,71,74,75,78,79,87,88,92,93,95,125, 135,136,143,144,151,152,153).

In this study, inhibitors of cyclooxygenase
(aspirin) and lipoxygenase (nordihydroguaiaretic acid, U-60257), synthetic chemotactic tripeptide formyl-methionyl-leucyl-phenylalanine (FMLP), and calcium ionophore A23187 were used to help in understanding the biochemical control mechanisms of shear-induced PMNL functional changes. The effect of these factors in PMNL aggregation and enzyme release in the presence or in the absence of shear-activated platelet released products suggests the occurrence of significant biochemical interactions between platelets and PMNLs in the mechanically-induced activation process. This study may be helpful in the design and evaluation of methods for reducing emboli formation in blood-contacting devices, as well as in assessing the possible significance of fluid mechanical effects in arterial diseases.

1.2. PMNL morphology and function

The leukocyte systems differ from red blood cell and platelet systems in many respects. The latter two function in the blood stream, while the primary function of leukocytes is carried out extravascularly. Therefore, the blood flow merely serves as a conduit which a white blood cell uses to get from one place to another. The circulation of a normal human being contains
approximately 5,000 to 10,000 white blood cells per \( \mu l \) of blood with individual cell volumes of approximately 300 \( \mu \) m\(^3\). For comparison, there are approximately 5,000,000 red blood cells and 300,000 platelets per \( \mu l \) of blood with individual cell volumes of 90 \( \mu \) m\(^3\) and 7.5 \( \mu \) m\(^3\), respectively. The following types of leukocytes may be distinguished in order of frequency: polymorphonuclear neutrophils, lymphocytes, monocytes, polymorphonuclear eosinophils and polymorphonuclear basophils. Lymphocyte production is carried out at several sites, but production of other white cells is limited to the bone marrow in normal human beings (33).

The morphology of PMN leukocytes is relatively complex and heterogeneous. The PMNL at rest is spherical in shape and has a segmented nucleus, in which the lobes are connected by thin nuclear filaments. The number of segments appears to increase with the age of each cell, so that the mature cells usually contain 2 to 5 segments (10). The other major intracellular particles are the granules (occupying approximately 15% of cell volume) and mitochondria (0.6% of cell volume). The granules can be seen to undergo Brownian movement in the cytoplasmic matrix. The cell membrane has numerous crestlike foldings, and the cell contains many other types of membranes in its cytoplasm (148). PMNLs possess at least
two distinct sets of enzyme rich granules known as primary (azurophilic) and secondary (specific) granules. Azurophilic granules are well equipped for both killing and digestion of microorganisms: they not only contain lysosomal hydrolases such as β-glucuronidase and α-mannosidase but also neutral proteinases such as elastase and microbicidal enzymes such as myeloperoxidase and lysozyme. On the other hand, specific granules are not well characterized. They apparently lack lysosomal hydrolases but contain lysozyme, lactoferrin, and collagenase. During phagocytosis, these granules rapidly fuse with phagocytic vacuoles where they release their contents, thereby inactivating or degrading ingested microorganisms. These actions occur intracellularly within the phagocytic vacuoles. The speed with which phagocytosis and fusion of lysosomal granules and phagosome occurs can appreciated by the observation that alkaline phosphatase (a "specific" granule enzyme) is detectable within the phagosome within 30 seconds after particles are added to PMNLs and peroxidase (a "azurophilic" granule enzyme) within 1 to 3 minutes (10).

The entire system of leukocytes is designed to defend against "foreignness". Defense against "foreignness" involves two general mechanisms: phagocytosis of a substance recognized as foreign and the
development of an immune response against a foreign substance (an antigen). Lymphocytes are not phagocytes but are specialized cells concerned with immunity, and this cellular system will be termed the "immunocyte" system. Neutrophils, monocytes, eosinophils, and basophils are cells capable of phagocytosis (the phagocytic system). However, the phagocytic and immunocytic systems are interrelated in their functions. For instance, monocytes may play a role in processing antigen as an initiating step in antibody production. Conversely, PMNLs are more efficient phagocytes for bacteria which have been coated with antibody than for bacteria without antibody.

PMNLs also have the capacity for directed motion both through selective adhesion to the vascular walls as they travel through the blood stream and through the process of chemotaxis, whereby chemical gradients induce PMNL locomotion along the capillary wall or through the connective tissue after diapedesis. Since PMNLs possess agents which lead to chemotaxis in other white blood cells, PMNL lysis itself is a sufficient initiation for PMNL sequestering and inflammation.

1.3. Arachidonic acid metabolism
Enzymatic oxidation of arachidonic acid leads to a multitude of biochemically important products. Among them are prostaglandins(PG), thromboxanes(Tx), prostacyclin(PGI₂), and leukotrienes(LT). These substances, referred to collectively as eicosanoids, constitute what has become known as the arachidonic acid cascade. Eicosanoids are extremely potent compounds with a bewildering variety of actions. Many have interesting and diverse pharmacological properties and significant medicinal potential. Some contract and others relax smooth muscle. Some promote and others prevent blood platelet aggregation. Some promote leukocyte migration and some regulate gastric secretions. These and other properties already have led to such medicinal uses as induction of labor at term, first-trimester abortion, and prevention of platelet loss in cardiopulmonary operations and kidney dialysis. Potential uses include prevention of heart attacks, and control or prevention of the symptoms of asthma.

There are two well recognized general enzymatic pathways of arachidonic acid metabolism. One is the cyclooxygenase pathway (Fig.1) and the other is the lipoxygenase pathway (Fig.2). Through the cyclooxygenase pathway, PGG₂ and PGH₂ are produced. Prostaglandins of G and H families differ only by the substituent at C-15.
The PGG series has a hydroperoxy group at C-15; the PGH series has a hydroxyl group at this position. Under the conditions of the biosynthetic incubation, the half life of the endoperoxides is only about five minutes. By inhibiting further enzymic conversions of the endoperoxides and by using rapid isolation techniques, sufficient quantities of endoperoxides were produced to permit isolation. The endoperoxide PGH₂ is a fascinating biochemical intermediate. It is susceptible to numerous enzymic and chemical transformations. Reduction converts it to PGF₂α; under nonreductive conditions it rearranges to PGE₂ and PGD₂. Also catalyzed either enzymically or nonenzymically is cleavage of the bicyclic endoperoxide into malondialdehyde (MDH) and a 17-carbon acid, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT). Despite the apparent instability in aqueous conditions and the reactivity of the molecule, the endoperoxides are stable for months once isolated, purified, and stored in a pure solvent at low temperature.

Enzymatic transformation of the endoperoxides into thromboxane A₂ is generated by the addition of arachidonic acid to platelets. TxA₂ has a profound contractile effect on a variety of smooth muscles and is a potent aggregator of platelets. It has a half life of only 30 to 40 seconds in aqueous media at physiologic
Figure 1. Cyclooxygenase Pathways of Arachidonic Acid Metabolism
Figure 2. Lipoxygenase Pathways of Arachidonic Acid Metabolism
temperature and pH and transforms to a stable product TxB₂.

As a similar way, prostacyclin is generated by the addition of arachidonic acid to endothelial cells. Prostacyclin is the most potent natural inhibitor of platelet aggregation yet discovered and also is a powerful vasodilator. It has a half life of about 3 minutes in aqueous media at physiologic temperature and pH and forms a new, more stable, but less active compound 6-keto-PGF₁α. The effects of prostacyclin on the cardiovascular system offer exciting therapeutic potential for this substance. Hydrolytic instability of prostacyclin may prevent clinical applications. However, this property is being used to advantage in one of the first medical applications envisioned for the substance. When surgery requires circulation of blood outside the body, the inhibitory effect of prostacyclin on platelet aggregation likely would be beneficial. At the same time, its short physiological life could permit the prompt resumption of aggregatory properties once administration of the compound is stopped. These observations lead to the proposal that a controlling mechanism for platelet aggregation is the balance between TxA₂ and PGI₂ formation in the cardiovascular system. Too much TxA₂ or too little PGI₂ may cause thromboembolic complications.
The lipoxygenase pathway produces a series of monohydroxylated eicosatetraenoic acids (HETEs) and leukotrienes (LTs). These include 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), a platelet product (72,92,93,97,98); 5-S-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), a PMNL product (14,15,16,54,56,84); and 15-HETE, a lymphocyte and endothelial cell product (173,174,175). The 5-lipoxygenase converts arachidonic acid to 5S-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE). 5-HPETE is hydrolyzed to 5-HETE or is converted by a dehydrase to leukotriene A₄ (LTA₄). The generated epoxide LTA₄ is unstable and converted to LTB₄ by enzymatic hydrolysis. Further, LTC₄ is formed from LTA₄ by adding glutathione, and can be metabolized to LTD₄ by elimination of its γ-glutamyl residue. The biosynthetic events leading to the generation of LTC₄ and LTB₄ are generally entirely intracellular (20,75). An LTA₄ epoxide hydrolase has been demonstrated as an extracellular enzyme in blood plasma (44), but the physiological significance is undetermined and would depend upon the extracellular generation or release of LTA₄. The evidence for cellular receptors for LTB₄ and for the sulfidopeptide leukotrienes is derived from studies of structure-function correlations and radioligand binding.
For LTB$_4$, the specificity of its interaction with human PMNL was first suggested by its 30- to 300-fold greater chemotactic potency as compared with naturally occurring isomers (48,87) and 6-trans-LTB$_4$. Saturation binding of [$^3$H]LTB$_4$ to human PMNL has been presented as additional evidence for the existence of specific receptors (58,59,83). In one study, the dissociation constant ($K_d$) for the ligand was 11-14 nM and the number of specific binding sites per cell was 26,000 - 40,000(58). In the other study, the $K_d$ was 270 nM with 386,000 specific binding sites per cell and the binding at 4°C was irreversible (83). The criteria for a stereospecific receptor for LTB$_4$ on human PMNL are not yet adequately delineated.

The biological studies indicate that the cysteinyi-containing leukotrienes (LTC$_4$, LTD$_4$, and LTE$_4$) are of importance in immediate hypersensitivity reactions, such as human asthma, through their potent effects as bronchoconstrictors and stimulators of vascular leakage (67,68,69,78, 88,113,143,144). The dihydroxy derivative, LTB$_4$, has potent effects on PMNLs related to their adhesion to postcapillary venules and extravasation as well as chemotaxis - stimulated migration to areas of inflammation (19,50,71,78,87,95, 104,125), aggregation (42,46,48,57,79,88,123,135,136,177), and degranulation
(12,40,56,61,111,112,124,136,159,180). In combination with the effects of the cysteinyl-containing leukotrienes on the increase in vascular permeability these effects are reminiscent of acute inflammatory reactions. A synergistic effect between the leukotrienes and the vasodilator prostaglandins PGE$_2$ and PGI$_2$ might also be important in the generation of inflammatory edema. These observations indicate that the leukotrienes might be of importance also in acute inflammation.

Steroids with anti-inflammatory activity prevent the release of the precursor acid, arachidonic acid, whereas cyclooxygenase inhibitors such as aspirin inhibit the transformation of this acid into PGs and Txs. The steroid-induced inhibition of arachidonic acid release, proposed to be due to formation of peptide inhibitors of phospholipase A$_2$, prevent formation of not only PGs and Txs but also LTs and other oxygenated derivatives. It is therefore conceivable that some of the therapeutic effects of steroids which are not shared by aspirin-type drugs are due to inhibition of LT formation. Figure 3 shows the formation and some biological roles of PGs, Txs, and LTs based on arachidonic acid as precursor molecule. The LT is normally stored in biological membrane structures and can be released through activation of a hydrolytic system by a variety of
Figure 3. Formation and some biological roles of prostaglandins, thromboxanes, and leukotrienes.
stimuli. Depending on the availability of active enzymes in the stimulated cell, arachidonic acid is converted into one or several biologically active molecules. A variety of stimuli can thus be converted into a multitude of compounds which can be used to regulate or mediate various cell functions. It is obvious that increased knowledge about this system will provide new possibilities to develop novel and more specific therapeutic agents.

1.4. Previous studies of mechanical trauma to blood cells.

The technology required for implantable vascular prostheses and extracorporeal circulation is now available in modern medicine. The essential function of this technology is the passage and/or processing of blood in some manner, and this inevitably requires intimate and prolonged contact between the blood and various manmade devices, e.g., pumps, valves, tubing, membranes, etc. In surgical practice blood trauma is often a limiting factor in the duration of an acute procedure. It can also cause significantly abnormal physiological states during chronic exposures such as hemodialysis. To cope with these problems will require a thorough understanding
of how blood is traumatized during circulation through artificial organs.

The majority of the research done on the subject of blood trauma has concentrated on red blood cells and platelets. Considerable data is now available on alterations produced in erythrocytes (25, 134, 170) and platelets (6, 7, 21, 29, 52, 64, 101, 102, 134, 170, 186) by shear stress. However, leukocytes have received the least attention to date in this field of research. Since these cells are the largest normally found in blood and relatively complex, leukocytes might be expected to be sensitive to mechanical trauma. The effect of shear stress on platelets and leukocytes is briefly reviewed below.

There is substantial evidence that human platelets in citrated or heparinized platelet rich plasma (PRP) when exposed to fluid shear stress in vitro undergo certain physiologic reactions, i.e., platelet aggregation, release of contents from intracellular storage organelles, and in extreme cases platelet lysis (6, 7, 21, 29, 64, 101). The alterations in platelet morphology, aggregation, and release reactions may be apparent at shear stresses as low as 100 dynes/cm² after exposure times on the order of 100 - 300 seconds
(21,64,101). Shear-induced platelet aggregation showed a maximum value at 300 - 500 dynes/cm² with an exposure time of 2 - 5 minutes (21,52). Platelet release reactions which were stimulated by mechanical trauma included the liberation of acid phosphatase, serotonin, norepinephrine, platelet factor III, and ADP (21,64). Platelet attrition due to shear stress has been measured via the liberation of lactate dehydrogenase. Colantuoni et al. showed approximately 15,000 dynes/cm² was required to lyse platelets in -0.001 seconds (29), while lower values were required for larger exposure times. Shear-induced platelet aggregation and adenine nucleotide release were significantly greater in citrated PRP stored and sheared at 24°C as compared to citrated PRP stored at 24°C or 37°C and then sheared at 37°C whereas platelet lysis was not affected by temperature (101). Recently, Reimers et al. have reported that red blood cells might potentiate platelet adherence and aggregation in different flow systems in vitro (134). Their findings indicated that both RBC-derived ADP and RBC-mediated platelet surface transport were involved in the potentiation by RBCs of platelet aggregation induced by laminar shear stress.

Several functional alterations were noted in sheared leukocytes, inducing increased adhesiveness and
aggregation, and reduced motility, phagocytosis, and hexose monophosphate shunt activity. An attempt to subject pure leukocyte suspensions to simple, well defined in vitro shearing was made by McIntire and his associates(106). They showed that significant decrease in the ability of sheared leukocytes to migrate through a Millipore filter (3 μm mean pore size) either randomly or in response to an attractive antigen (casein). Leukocyte suspensions were exposed to mechanical trauma in a Weissenberg Rheogoniometer and the range of shear rates possible for the platens was approximately 0 - 4,000 sec⁻¹. Dewitz et al. observed that the number of leukocytes in purified suspensions was significantly reduced by shear stresses exceeding 150 dynes/cm² for 10 minutes and adhesiveness was increased by exposure to shear stresses greater than 300 dynes/cm² for 2 minutes (34,36,37,99). The effect of antiplatelet agents such as PGE₁, PGF₂, theophylline, RA-8 (dipyridamole), and RA-233 on PMNLs exposed to shear stress has also been studied (107,140,165,166). Pretreatment of PMNLs with some of these agents and exposed to a shear stress of 300 dynes/cm² showed a significant reduction in cell loss, granule release, and lysis.
CHAPTER II. EXPERIMENTS

2.1. Separation Protocol

A. Preparation of polymorphonuclear leukocytes

Forty ml of whole blood was obtained from healthy donors, who were nonsmokers and were taking no medications for at least the 10 preceding days, in accordance with the principles of the declaration of Helsinki. The blood was collected in two 30 ml plastic disposable syringes, already containing 10 ml of dextran sedimentation fluid (see Appendix for formula). The blood-dextran mixture was inverted several times and underwent sedimentation for 90 minutes. After sedimentation, the leukocyte/platelet-rich supernatant was transferred to plastic tubes (Falcon, Oxnard, CA) and centrifuged at 150 x g for 10 minutes and the platelet rich supernatant was discarded. The pellet was washed twice by resuspending it in sterile, cold saline (0.85 % wt) and centrifuging at 150 x g for 10 minutes. The supernatant was pured off in preparation for hypotonic RBC lysis. 5 ml of distilled water were added rapidly to the pellet and the pellet was washed vigorously for 30 seconds. Then 5 ml of saline (1.8 % wt) were added to restore an isotonic medium for the cells. 10 ml of this
leukocyte and lysed RBC suspensions were carefully layered onto 5 ml of Ficoll-Hypaque solution (see Appendix) in a 15 ml conical-tipped Falcon plastic tube. This tube was centrifuged at 400 x g for 25 minutes. Leukocytes removed from centrifuge had been neatly fractionated, with most of the lymphocytes and monocytes at the saline-Ficoll interface and greater than 95 % PMN leukocytes at the bottom of the tube. The supernatant layers were carefully pipetted off, and the pellet was washed with 0.85 % saline. The PMN leukocyte suspensions were transferred to a clean plastic tube and centrifuged at 150 x g for 10 minutes. The final pellet was resuspended in a modified Dulbecco's phosphate buffered saline (PBS). This suspension was counted to calculate necessary dilutions and was diluted to give a final concentration of 1.0 to 2.0 x 10^7 cells per ml.

B. Preparation of platelet rich plasma (PRP)

Twenty ml of whole blood was obtained by venepuncture of healthy volunteers. 10 ml of blood was quickly and gently mixed with 1.1 ml of sodium heparin in siliconized glass tubes. The final concentration of sodium heparin (porcine mucosa type, Organon Diagnostics, W.Orange, NJ) was 10 units per ml blood. The anticoagulated whole blood was centrifuged for 10 minutes at 150 x g. The supernatant PRP was drawn off with a
pasteur pipette and transferred to a clean siliconized glass tube. The remaining fractions were centrifuged for 5 minutes at 150 x g to retrieve more PRP. Finally the RBC fraction was centrifuged for 10 minutes at 1000 x g. The resultant supernatant was platelet poor plasma (PPP) and was transferred to a separate test tube. In some experiments, part of the recovered PRP received an additional high speed spin (15000 x g for 10 minutes) to produce platelet free plasma (PFP). At the beginning of experiments, Coulter counter measurements of PRP samples confirmed normal size distributions and platelet counts in all PRP samples were adjusted to 3.0 to 4.5 x 10^8 platelets per ml by dilution with PPP.

C. Preparation of whole blood

Whole blood was drawn by venepuncture into siliconized glass tubes containing either 3.8 % sodium citrate or 100 units per ml sodium heparin (1 part anticoagulant solution to 9 parts whole blood). All blood samples were used within 4 hours after collection to insure against loss of cell viability.

2.2. Shear stress application

A rotational cone and plate viscometer (Ferranti Electronics, Model 781, Commack, NY) with 1/3 degree cone
angle was used for blood shearing experiments. This instrument (Fig.4) was capable of generating specified uniform shear rates, ranging from 100 sec\(^{-1}\) to 18,000 sec\(^{-1}\), within 1 second. To maintain the cone-plate geometry during operation, a built-in controller holds the gap between the tip of the cone and the plate to less than 10 \(\mu\) m. Before every experiment, the gap was adjusted to within this tolerance and the viscometer platens received an organic silicon (Silmacote, Sigma Chemical Co., St.Louis, MO) coating to inhibit blood cell-surface interactions. The temperature of the platen was controlled by a thermostatically controlled water bath and it was measured at three different points along the plate to confirm its value.

For analyses where the motor is used to drive the cone the shear rate, \(\dot{\gamma}\), is calculated from the equation

\[
\dot{\gamma} = \omega \cdot r/c
\]  

(2.2.1)

where \(\dot{\gamma}\) is the shear rate (sec\(^{-1}\)), \(\omega\) is the angular velocity (radians/sec), and \(c\) is the cone-plate gap at radius \(r\) and the term \(\omega \cdot r\) represents the linear velocity at that radius. Since \(c\) is linearly dependent on \(r\), the ratio \(\omega \cdot r/c\) is not a function of the radius. Furthermore, since

\[
c/r = \tan a \sim a
\]  

(2.2.2)
Figure 4. Cross section of cone-plate viscometer showing (A) rotating cone, (B) stationary plate.
C: cone-plate gap at radius r
R: radius of cone, S: cell suspension
α: cone angle, ω: angular velocity
where $\alpha$ is the cone angle, for a small angle $\alpha$ expressed in radians, equation (2.2.1) can be simplified further to yield

$$\dot{\gamma} = \omega/\alpha$$

(2.2.3)

which enables one to calculate the shear rate from the angular velocities and the cone angle. The shear stress-shear rate relationship for a Newtonian fluid (which blood approximates at shear rates higher than 500 sec$^{-1}$) obeys the following equation

$$\tau = \mu \dot{\gamma}$$

(2.2.4)

where $\tau$ is the shear stress (dynes/cm$^2$) and $\mu$ is the viscosity (poise = dynes sec/cm$^2$).

2.3. Full Protocol

A. Chemotactic factor- and ionophore-induced PMNL aggregation

Following the separation, 1 ml aliquots of PMNL suspensions were transferred to 12 x 75 mm plastic tubes to serve as the untreated controls. The other samples were incubated with either 1 $\mu$ M of FMLP or 10 $\mu$ M of A23187 for given specific time periods. In some experiments, PMNL suspensions were preincubated with
cytochalasin B (5 μg/ml) for 5 minutes and then incubated with 1 μM of FMLP. 20 μl aliquots were taken from each of the untreated and treated samples at the specific time for electronic cell counts.

B. Shear-induced PMNL aggregation

Two ml samples of PMNL suspensions were transferred to 12 x 75 mm plastic tubes to serve as the unsheared controls. 0.7 ml of sample was transferred to the viscometer using a disposable plastic pipette and sheared at 150 dynes/cm² for 1 minute. After the shear, whole sample was collected from the viscometer and transferred to plastic tubes. This was repeated for 4 times to get enough sheared sample volume. When PMNLs were mixed with PRP, there were two possible involvements of platelets. One is a physical effect of platelet particles and the other is a chemical effect of platelet release factors. To remove the physical effect, PRP was sheared at 150 dynes/cm² for 1 minute. The sheared sample was collected and centrifuged at 15,000 x g for 10 minutes to get PFP. This supernatant plasma from sheared PRP was added to PMNL suspensions (1:1 by volume). The effect of plasma proteins on the shear-induced PMNL aggregation was examined by using the PMNL suspensions mixed with PFP (1:1 by volume). The control and sheared samples were collected in the same way. The effect of agents listed in
Table 1 will be described under the Results section.

C. Aggregation assay

Control and sheared PMNL counts were monitored with an electronic particle counter (Model ZBI, Coulter Electronics, Hialeha, Florida) and a microcomputer (Apple II Plus, Apple Computer Inc., Cupertino, CA). The Coulter counter consists of two units - a counting unit and a channelyzer. Measurements of single and aggregated PMNLs required a 100 μm aperture. Table 2 provides set points and other information concerning Coulter measurements. 20 μl aliquots were taken from each of the samples and were immediately diluted into 10 ml of Isoton solution. For each sample, the particle concentration was enumerated with a lower channel of 150 μm³ (called the total or "T" particle concentration) and with a lower channel of 540 μm³ (called the aggregated or large "A" particle concentration). These names are given because with the lower channel of 150 μm³, all PMNLs in the sample were counted, whereas with a lower channel of 540 μm³, only particles larger than about 2 times the volume of an average single PMNL were enumerated. Results for a given sample of the PMNL suspension were reported as the large particle percentage (LPP = A/T x100) or the maximum large particle percentage seen over time. Figure 5 shows typical volumetric distribution curves obtained with the
Table 1. Chemical Agents Used in this Study

<table>
<thead>
<tr>
<th>Agent</th>
<th>M. W.</th>
<th>Dissolving Soln.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A23187</td>
<td>523.6</td>
<td>DMSO</td>
<td>10 μM</td>
</tr>
<tr>
<td>ADP</td>
<td>427.22</td>
<td>Water</td>
<td>2 μM</td>
</tr>
<tr>
<td>ASA</td>
<td>180.15</td>
<td>Ethanol</td>
<td>50 μM</td>
</tr>
<tr>
<td>Colchicine</td>
<td>399.4</td>
<td>DMSO</td>
<td>10 μM</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>479.0</td>
<td>Ethanol</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>FMLP</td>
<td>437.6</td>
<td>DMSO</td>
<td>1 μM</td>
</tr>
<tr>
<td>Imipramine</td>
<td>280.4</td>
<td>Water</td>
<td>1 μM</td>
</tr>
<tr>
<td>NDGA</td>
<td>302.4</td>
<td>DMSO</td>
<td>10 μM</td>
</tr>
<tr>
<td>Serotonin</td>
<td>176.2</td>
<td>Water</td>
<td>2 μM</td>
</tr>
<tr>
<td>U-60257</td>
<td>381.0</td>
<td>Trishydroxymethyl Amino Methane</td>
<td>10 μM</td>
</tr>
<tr>
<td></td>
<td>PMNL Suspension</td>
<td>Whole Blood</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Aperture</td>
<td>100 μM</td>
<td>280 μM</td>
<td></td>
</tr>
<tr>
<td>Aperture Current</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Measured Volume of Isoton</td>
<td>500 μl</td>
<td>3.4 ml</td>
<td></td>
</tr>
<tr>
<td>Sample Volume</td>
<td>20 μl</td>
<td>100 μl</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Channelizer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base Channel Threshold</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Window Width</td>
<td>70</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Count Control</td>
<td>&quot;external&quot;</td>
<td>&quot;timer&quot; (10 seconds)</td>
<td></td>
</tr>
<tr>
<td>Log Expander</td>
<td>&quot;on&quot;</td>
<td>&quot;on&quot;</td>
<td></td>
</tr>
</tbody>
</table>
Coulter counter. Nearly all the normal size of PMNLs were found in control sample(s). However a dramatic increase of large aggregated cells was shown in the activated sample(Δ). Sheared samples (and others incubated with various chemicals) were analyzed at 10 minute intervals to follow the aggregation process. The maximum large particle percentage was the peak value of LPP obtained over the 60 minute aggregation intervals.

D. Determination of enzyme release

By measuring the loss of a cytochemical enzyme activity or the release of enzymes associated with granules into the surrounding medium, two PMNL activities can be assayed; PMNL lysis (lactate dehydrogenase) and degranulation (β-glucuronidase). These assays were performed using a spectrophotometer (Gilford 2600, Gilford Instrument Lab. Inc., Oberlin, Ohio).

One ml from each of the two samples was centrifuged at 150 x g for 10 minutes, and the supernatants were transferred to clean plastic tubes. The remaining 1 ml from each sample were sonicated for 10 seconds at setting 60 with a sonic dismembrator (Artek, Farmingdale, NY). These four samples, unsheared and sheared, sonicated and unsonicated, were assayed to determine lactate dehydrogenase and β-glucuronidase release.
Figure 5. Volumetric distribution curves in Coulter counter.
Lactate Dehydrogenase assay (LDH)

Lactate dehydrogenase is a polypeptide tetramer made up from two different monomers and is a cytoplasmic enzyme in blood cells. It has been observed that human PMNLs selectively extrude lysosomal (and not cytoplasmic) enzymes from viable cells during phagocytosis (187,188). Therefore, the percentage of LDH release was used as an indicator of membrane damage and possible cell lysis. Lactate dehydrogenase activity was measured using a Gilford Diagnostics LD-P reagent with a procedure based on the spectrophotometric method of Wroblewski and LaDue (185). LDH catalyzes the reduction of pyruvate to L-lactate with concomitant oxidation of NADH to NAD.

\[
\text{LDH} \quad \text{pyruvate + NADH} \quad \longrightarrow \quad \text{L-lactate + NAD}^+ 
\]

Since the oxidation of NADH is directly proportional to the reduction of pyruvate in equimolar amounts, the LDH activity can be calculated from the rate of decrease in absorbance at 340 nm.

The Gilford Diagnostics LD-P 14 was reconstituted with 14 ml of distilled water and gently swirled to dissolve. The spectrophotometer was set to values listed in Table 3. Sample time and vacuum were adjusted for a total aspiration volume of 1.0 ml. After 1.0 ml of reagent was pipetted into a clean, dry 12 x 75 mm test
tube, 0.05 ml of each sample was added into the test tube. The contents of each tube were mixed and quickly aspirated. Distilled water was used as the reference. Absorbance was recorded after 12 seconds (A_{12}) and was read every 12 seconds for 3 minutes. ΔA/minute was calculated by subtracting A_{120} from A_{60}. Other readings were used as a check on the linear rate of reaction.

The LDH activity was expressed in International Units. One International Unit (IU) is defined as the amount of enzyme that catalyzes the transformation of 1 μmole of substrate per minute under defined conditions.

\[
\text{LDH activity (IU/l)} = \frac{\Delta A/\text{min} \times 10^3 \times 1.05}{6.3 \times 1 \times 0.05}
\]

where \(\Delta A\) = change in absorbance
6.3 = millimolar absorptivity of NADH
10^3 = conversion of milliliter to liter
1 = light path in cm
1.05 = total reaction volume in ml
0.05 = sample volume in ml

The percentage of LDH release was calculated by the expression

\[
\% \text{LDH release} = \frac{\text{LDH}_N - \text{LDH}_{NC}}{\text{LDH}_S - \text{LDH}_{NC}}
\]
<table>
<thead>
<tr>
<th></th>
<th>LDH</th>
<th>β - glucuronidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>340 nm</td>
<td>550 nm</td>
</tr>
<tr>
<td>Mode</td>
<td>Abs</td>
<td>Abs</td>
</tr>
<tr>
<td>Sample Volume</td>
<td>0.05 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Reagent Volume</td>
<td>1.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
</tr>
</tbody>
</table>
where \( \text{LDH}_\text{NC} \) equaled LDH activity of the unsonicated control sample, \( \text{LDH}_\text{N} \) was the LDH activity of the unsonicated sheared sample, and \( \text{LDH}_\text{S} \) was the LDH activity of the sonicated sheared sample.

\( \beta \)-glucuronidase assay

\( \beta \)-glucuronidase is abundant in liver, pancreas and other tissues. Increased concentrations of the enzyme may be of clinical significance in liver cell injury, diabetes mellitus, acute alcoholism, as well as in certain types of cancer and renal disease. Azurophilic granules of PMNLs also can release \( \beta \)-glucuronidase. This enzyme has been assayed by methods involving hydrolysis of various types of glucuronides. In 1967, Fishman et al. introduced a simplified procedure employing phenolphthalein glucuronic acid as substrate(43). According to this technique the complex is cleaved by \( \beta \)-glucuronidase, releasing phenolphthalein which is then determined colorimetrically. The activity of \( \beta \)-glucuronidase released by the azurophilic granules was measured with Sigma assay kit No. 325, which is similar to that proposed by Fishman et al. The essential difference was that the incubation was performed at 56°C instead of at 37°C with a significant reduction in the incubation time. \( \beta \)-glucuronidase acts on the substrate phenolphthalein
mono-β-glucuronic acid liberating free phenolphthalein. The intensity of the resulting red color in alkali is measured at 550 nm and is proportional to the enzyme activity.

The activity was measured with the following procedure. Two clear plastic tubes were used for each sample, one labelled 'sample blank' and the other labelled 'sample test'. In addition, one 'reagent blank' tube was used with each series of tests. The tubes were filled with reagents as follows:

1) 0.3 ml Acetate Buffer Solution (Sodium Acetate, 0.2 mol/liter, pH 4.5 at 25°C, Sigma stock No. 105-12) to each tube.

2) 0.1 ml Phenolphthalein Glucuronic Acid Solution (Phenolphthalein mono-β-glucuronic acid, 0.03 mol/liter, pH 4.5 at 25°C, Sigma stock No. 325-2) to the 'reagent blank' and each 'sample test' tubes.

3) 0.1 ml PBS to the 'reagent blank' and each 'sample blank' tubes.

4) 0.1 ml sample to each 'sample blank' and 'sample test' tubes.

The contents of each tube were mixed by swirling and were incubated for 1 hour in a 56°C water bath. Immediately following incubation, 2.5 ml of 2-amino-
2-methyl-1-propanol buffer (AMP, 0.1 mol/liter, pH 11 and Sodium Lauryl Sulfate, 0.2%, Sigma stock No. 325-3) was added to each tube and mixed by inversion. Absorbance(A) of each tube was measured by Gilford 2600 spectrophotometer at a wavelength of 550 nm versus distilled water as reference. The corrected absorbance(A) of 'sample test' was determined by the following:

$$\text{Corrected } A = A_{\text{sample-test}} - (A_{\text{sample-blank}} + A_{\text{reagent-blank}})$$

Using this corrected A, phenolphthalein concentration (μ g/ml) was determined from the calibration curve (Fig.6) and used to calculate activity as follows:

$$\beta - \text{Glucuronidase activity}$$

$$= \text{Phenolphthalein concentration ( } \mu \text{ g/ml) } \times \frac{3.0}{0.1}$$

$$= \text{phenolphthalein concentration ( } \mu \text{ g/ml) } \times 30$$

where 3.0 = final volume (ml) of solution

0.1 = sample volume (ml) assayed

Since this enzyme activity is linearly dependent on the corrected A, the percentage of β - glucuronidase release could then be calculated by the expression

$$\% \beta - \text{glucuronidase release} = \frac{A_N - A_{NC}}{A_S - A_{NC}}$$
Figure 6. Calibration curve for β-glucuronidase assay.
where $A_{NC}$ equaled the corrected absorbance of the unsonicated control sample, $A_N$ was the corrected absorbance of the unsonicated sheared sample, and $A_S$ was the corrected absorbance of the sonicated sheared sample.

E. High Performance Liquid Chromatography (HPLC) Analysis

Following the cell separations, 5 ml of PMNL suspensions were preincubated with 30 µl of $1^{14}C$ arachidonic acid for 10 minutes and then centrifuged at 150 x g for 10 minutes. Supernatant was discarded and the pellet was resuspended with 5 ml of PBS or 2.5 ml of PBS and 2.5 ml of supernatant plasma from sheared PRP. After the shear at 150 dynes/cm$^2$ for 1 minute, both control and sheared samples were centrifuged at 15000 x g for 10 minutes and each supernatant was transferred to clean plastic tubes. This supernatant was diluted with 9 volumes of distilled water (pH 3.0).

In preparation for reversed phase HPLC (RP-HPLC), samples were extracted using Sep-PAK C$_{18}$ cartridges (Waters Associates, Milford, MA). The extraction procedure was as follows:

1) Sep-PAK C$_{18}$ cartridges were prewet with 5 ml of methanol before use.
2) 5 ml of acetic acid (0.5 % v/v) were passed through
the column.

3) 20 ml of diluted sample were passed through the column slowly.

4) 5 ml of methanol (30 % v/v) were used to elute arachidonic acid cyclooxygenase metabolites.

5) 5 ml of methanol (90 % v/v) were used to elute arachidonic acid lipoxygenase metabolites.

These 90 % methanol eluates were evaporated under nitrogen gas stream. Residues were dissolved in 100 µl of methanol and 10 µl was injected onto the HPLC column. RP-HPLC was carried out on an 4mm I.D. µ-PAK C₁₈ column (Varian 5500, Varian Assoc. Instrument, Palo Alto, CA) with water/methanol/acetonitrile as the eluting solvent. Table 4 shows the mixing ratio and flow rate changes as functions of elution time. Fractions were collected and the radiolabel content of each tube was determined in Hydrofluor by liquid scintillation counting. Identification of the peaks was done by comparison with standards. Under these conditions, stable cyclooxygenase products 6-keto PGF₁α, TXB₂, PGF₂α, PGE₁, PGE₂, and PGD₂ were eluted at 4 - 10 minutes, LTB₄ at 20 - 22 minutes, mono HETEs at 40 - 52 minutes, and unreacted arachidonic acid at 55 - 60 minutes.
### Table 4. The Mixing Ratio and Flow Rate Changes in the HPLC

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A*</th>
<th>% B**</th>
<th>% C***</th>
<th>Flow Rate (ml/min)</th>
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<td>0.0</td>
<td>72</td>
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<td>72</td>
<td>0</td>
<td>28</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* A: distilled H₂O (0.1% HOAC), pH = 3.2
** B: CH₃OH
*** C: CH₃CN
F. Shear-induced Whole Blood Aggregation

Particle counting and size analyzing of control and sheared whole blood were performed with a Coulter counter/channeleyzer interfaced with an Apple II microcomputer. Measurement of aggregated whole blood required a 280 μm aperture. Table 2 provides set points and other information concerning the Coulter measurements. After 100 μl of suspension had been added to 20 ml of Isoton, 6 drops of a stromatolysing agent, Zap-Oglobin II (Coulter Diagnostics, Hialeha, Florida), was added to lyse the RBCs and counted within 30 seconds. Under these conditions PMNL lysis and aggregate disintegration were minimal. In the experiments described, particle size distribution was obtained as a number versus equivalent diameter curve and mean channel number was calculated using the microcomputer. Further data manipulation included background subtraction, data averaging, and the conversion from number-diameter curves to volume-diameter curves.
CHAPTER III. RESULTS

3.1. Functional changes in sheared PMNLs

A. Chemotactic factor- and ionophore A23187-induced PMNL aggregation

FMLP, as previously described by others (32,114,116,117,118,120), aggregated human PMNLs. This effect was demonstrated by an increase in the percentage of large particles formed after adding 1 μM FMLP to the suspension of PMNLs (Fig.7). Cytochalasin B, by itself, did not aggregate the cells but did significantly enhance FMLP-induced aggregation (Fig.7). In this experiment PMNLs were preincubated with cytochalasin B (5 μg/ml) for 5 minutes and then incubated with 1 μM FMLP for the specific time periods. The ionophore A23187 (10 μM) aggregated PMNLs (Fig.7) and the time course of aggregation was similar to that of shear-induced PMNL aggregation in the presence of supernatant from sheared PRP (see below). The kinetics of FMLP-induced aggregation was quite rapid and reached nearly a maximum LPP within 2 or 3 minutes. However, the time course of the ionophore A23187- and shear-induced aggregation was much slower, and a maximum value was not attained for over 30 minutes. FMLP and A23187 were dissolved in 99.8 % dimethylsulfoxide (DMSO) and cytochalasin B was prepared
Figure 7. The kinetics of the chemotactic factor and the ionophore induced PMNL aggregation. PMNLs (1x10^7 cells/ml) were incubated with 1 μM FMLP(●) and 10 μM A23187(●) for the specific time periods. PMNLs were preincubated with cytochalasin B(5 μg/ml) for 5 minutes and then incubated with FMLP(●).
in pure ethanol. The small amount of ethanol and DMSO (final concentration of 0.1 %) employed as vehicles did not alter cell viability.

B. Platelet modulation of shear-induced PMNL aggregation

Figure 8 shows the kinetics of the shear-induced PMNL aggregation. PMNLs in PBS (•), PMNLs mixed with PPP (○), PMNLs mixed with supernatant plasma from sheared PRP (♦), and the last sample preincubated with 10 μ M NDGA (■) were sheared at 150 dynes/cm² for one minute. After shearing, samples were analyzed at 10 minute intervals for one hour to follow the aggregation process. PMNL suspensions in PBS (1.0 x 10⁷ cells/ml, final concentration) did not show any aggregate formation at shear stresses below 150 dynes/cm² for one minute. However, when PMNLs were incubated in the presence of PRP, a strong stimulation of PMNL aggregation occurred in this shear stress range. PMNLs incubated with supernatant plasma from sheared PRP (1:1 by volume) would initiate PMNL aggregation after exposure to moderate shear stress (150 dynes/cm² for one minute). The effect of plasma proteins on the shear-induced PMNL aggregation was examined by using the PMNL suspensions mixed with PPP (1:1 by volume) and the maximum LPP was approximately
Figure 8. The kinetics of the shear-induced PMNL aggregation. PMNLs (△), PMNLs mixed with PFP (●), PMNLs mixed with supernatant plasma from sheared PRP (♦), and the last sample preincubated with 10 M NDGA (■) were sheared at 150 dynes/cm² for one minute. After shearing, samples were analyzed at 10 minute intervals for one hour to follow the aggregation process.
half of that observed in the presence of supernatant plasma from sheared PRP. This indicates that a substance(s) derived from shear-activated platelets plays an active role in the shear-induced PMNL aggregation. Another set of experiments has been done to show the effect of platelet release products on this PMNL aggregation (Fig.9). PMNLs in PBS(A), PMNLs mixed with supernatant plasma of sheared PRP(B), PMNLs mixed with PFP(C), PMNLs incubated with 2 μM ADP(D), and PMNLs incubated with 2 μM serotonin(E) were sheared at 150 dynes/cm² for one minute. Sheared samples (closed bar) were compared to control samples (unsheared, open bar). Data represent the mean ± SEM for 3 to 9 separate experiments, and are the maximum LPP over a one hour interval after shear exposure. Sheared platelets release ADP and serotonin which are potent platelet aggregating agents. However these platelet release factors, ADP or serotonin, did not induce PMNL aggregation at this shear stress.

To define the nature of the factor(s) responsible for the effect of sheared platelet supernatants on PMNL aggregation activity, experiments with inhibitors were performed. The inhibition of the cyclooxygenase pathway by 50 μM ASA did not suppress the aggregation of PMNLs after shear in the presence of supernatant plasma of
Figure 9. Effect of platelet release products on shear-induced PMNL aggregation. A) PMNLs in buffer alone, B) PMNLs mixed with supernatant plasma from sheared PRP, C) PMNLs mixed with PFP, D) PMNLs incubated with 2 μM ADP, and E) PMNLs incubated with 2 μM serotonin. Sheared samples (closed bar) were compared to control samples (open bar). Data represent the mean ± SEM for 3 to 9 separate experiments, and are the maximum LPP over a one hour interval after shear exposure.
sheared PRP (Fig.10B). However, preincubation with NDGA (10 μ M), an inhibitor of C-5 and C-12 lipoxygenase (51,141) and U-60257 (10 μ M), an inhibitor of C-5 lipoxygenase in human leukocytes (3,160,167), suppressed the aggregation of PMNLs(Fig.10C and 10D, respectively). PRP incubated with imipramine (1 μ M), an inhibitor of serotonin uptake, for 15 minutes was sheared and centrifuged. In this case, serotonin which was released from sheared PRP was in the supernatant. PMNLs incubated with this supernatant did not show any change in the shear-induced PMNL aggregation (Fig.10E). These data (Fig.9E and Fig.10E) suggested that serotonin had no effect on the shear-induced PMNL aggregation.

The effect of shear stress magnitude on PMNL aggregation for three different samples is illustrated in Figure 11. PMNLs in PBS(*) or PMNLs incubated with ADP(•) did not show any significant aggregate formation under shear stresses at 50, 100, and 150 dynes/cm² for one minute. However PMNLs mixed with supernatant plasma of sheared PRP (◦) showed a dramatic change in the maximum LPP which was a function of the shear stress magnitude.

The effect of platelet concentration on shear-induced PMNL aggregation is illustrated in Figure 12. When PMNL suspensions, which were mixed with supernatant
Figure 10. Effect of cyclooxygenase and lipoxygenase inhibitors on PMNL shear-induced aggregation. PMNLs were mixed with supernatant plasma from sheared PRP and incubated with A) no drugs, B) ASA (50 μM), C) NDGA (10 μM), D) U-60257 (10 μM). In E) PRP was incubated with imipramine (1 μM) and sheared and subsequently the PMNLs were mixed with this supernatant.
Figure 11. Effect of shear stress magnitude on the maximum LPP. Three different shear stresses (50, 100, 150 dynes/cm$^2$) were used to shear PMNLs in buffer (▲), PMNLs incubated with 2 μM ADP (●), and PMNLs mixed with supernatant plasma from sheared PRP (●).
plasma from sheared PRP, were sheared at 150 dynes/cm² for one minute, the maximum LPP depended on the platelet count in the PRP. When the platelet/PMNL ratio was approximately 30, the aggregation response of the PMNL was maximum. At this ratio, the total volume of platelets was nearly equivalent to that of PMNLs. Also this ratio is nearly the same as that found physiologically.

C. Shear-induced PMNL enzyme release

Figure 13 shows the effect of platelet release factors on shear-induced PMNL β-glucuronidase release. PMNLs in PBS did not release significant β-glucuronidase at shear stresses up to 150 dynes/cm² for an exposure time of one minute(A). However PMNL suspensions mixed with supernatant plasma of sheared PRP showed large amounts of β-glucuronidase release at this shear stress(B, P<0.005). Preincubation with NDGA almost completely suppressed this enzyme release even in the presence of supernatant plasma of sheared PRP(C). PFP enhanced this enzyme release by a factor of 2(D, P<0.05). Cytochalasin B treated PMNLs, mixed with supernatant plasma from sheared PRP, released nearly twice as much β-glucuronidase as untreated samples(E). When citrate was used as an anticoagulant, this shear-induced enzyme
Figure 12. Effect of platelet concentration on shear-induced PMNL aggregation. Different platelet concentrations in PRP were sheared and centrifuged. This supernatant plasma was added to the PMNL suspensions and subsequently sheared.
Figure 13. Effect of platelet release products on shear-induced PMNL β-glucuronidase release. A) PMNLs in buffer alone, B) PMNLs mixed with supernatant plasma from sheared PRP, C) PMNLs were preincubated with NDGA and mixed with supernatant plasma, D) PMNLs mixed with PFP, and E) PMNLs mixed with supernatant plasma and incubated with cytochalasin B. The pair of dashed horizontal lines represent the range of % LDH release. *: P<0.005, **: P<0.05 (Student's paired t-test, compared to value A).
release was totally suppressed indicating that Ca\textsuperscript{++} is also an essential factor.

The calcium effect on PMNL β-glucuronidase release is illustrated in Figure 14. Sheared PMNL suspensions released very small amounts of β-glucuronidase (A) and this was nearly the same amount as LDH release. Calcium ionophore A23187 caused a dose-dependent increase of β-glucuronidase release from PMNLs (B and C, P<0.005). This enzyme release from PMNLs incubated with 10 μM A23187 was approximately the same as that from sheared PMNLs mixed with sheared PRP supernatant and cytochalasin B (D). Incubation with 1 mM EDTA, a Ca\textsuperscript{++} and Mg\textsuperscript{++} chelator, almost completely inhibited shear-induced PMNL β-glucuronidase release even in the presence of supernatant plasma from sheared PRP (E). In all the cases, LDH release was less than 3% indicating that PMNLs were not lethally damaged or lysed at this shear stress. The pair of dashed horizontal lines in Figures 13 and 14 represented the range of % LDH release.

3.2. High performance liquid chromatography analysis of eicosanoids

Studies of platelet and/or PMNL arachidonate metabolites, identified by HPLC, were used to examine the
Figure 14. Calcium effect on PMNL β-glucuronidase release.
A) PMNLs in buffer alone, B) PMNLs incubated with 10 μM A23187, no shearing, C) PMNLs incubated with 20 μM A23187, no shearing, D) PMNLs mixed with supernatant plasma and incubated with cytochalasin B, E) PMNLs mixed with supernatant plasma and incubated with 1 mM EDTA.
details of arachidonic acid metabolism under shear stress. Results of a group of experiments analyzed by RP-HPLC are shown in Figures 15 through 21.

PMNL suspensions incubated with 5.5 μM 1-14C arachidonic acid were sheared and arachidonate metabolites were analyzed by RP-HPLC. No detectable amount of mono-HETEs and/or LTB₄ was produced (Fig.15). However, the PMNL generated a significantly increased quantity of LTB₄ after shearing, which appeared as a distinct peak (retention time; 21 minutes) in the presence of supernatant plasma from sheared PRP (Fig.16). When PMNL suspensions were preincubated with NDGA, there was no significant increase of LTB₄ formation after shearing (Fig.17) indicating that NDGA did produce inhibition of C-5 lipoxygenase pathway. Only 12-HETE formation was increased when PRP incubated with 1-14C arachidonic acid was exposed to shear stress (Fig.18). That this metabolite was 12-HETE, not 5-HETE was confirmed by using the thin layer chromatography (Table 5). Only two major peaks were found in the thin layer chromatogram and these were identified by comparison with standards. The Rf values of 5-HETE, 12-HETE, and arachidonic acid were 0.54, 0.66, and 0.77 respectively. Very little cyclooxygenase products were detected after shear stress stimulation of platelets. The amount of TxB₂
formation after shear stress stimulation of platelets measured by radioimmunoassay was less than or equal to 10 ng/10⁸ platelets. The counts per minute (CPM) range corresponding to this concentration was nearly the same as the background CPM. PMNLs and 1-¹⁴C arachidonic acid were incubated with the ionophore A23187 in the presence of sheared PRP supernatant. In this case, production of LTB₄ by PMNLs after shearing increased even more dramatically. It is noteworthy that 5-HETE production was increased and 15-HETE formation was decreased (Fig.19). The use of PRP instead of sheared PRP supernatant showed nearly the same extent of arachidonic acid metabolites formation (Figures 20 and 21).

3.3. Shear-induced whole blood aggregation

The volumetric distribution curves of sheared whole blood are illustrated in Figure 22. Citrated blood samples were sheared at 3000 sec⁻¹(○), 5000 sec⁻¹(△), 7500 sec⁻¹(○), and 10000 sec⁻¹(●) for an exposure time of 30 seconds. The total aggregate volume was calculated in a size window of 14 - 80 μm equivalent spherical diameter. These particles were bigger than single leukocytes and represented aggregates of platelets and/or leukocytes. The total aggregate volume would be
Figure 15. RP-HPLC chromatogram of the metabolites of arachidonate formed in PMNLs incubated with \( 1 - ^{14}\text{C} \) arachidonate. Control(○) and after shearing at 150 dynes/cm\(^2\) for one minute(△).
Figure 16. RP-HPLC chromatogram of the metabolites of arachidonate formed in PMNLs incubated with 1-14C arachidonate in the presence of supernatant plasma from sheared PRP.
Figure 17. RP-HPLC chromatogram of the metabolites of arachidonate formed in PMNLs preincubated with $1\cdot^{14}$C arachidonate and with NDGA in the presence of supernatant plasma from sheared PRP.
Figure 18. RP-HPLC chromatogram of the metabolites of arachidonate formed in PRP incubated with $1^{-14}C$ arachidonate.
Table 5. The R_f values of Thin Layer Chromatography

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<th>Standards</th>
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<td>5-HETE</td>
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</tr>
<tr>
<td>12-HETE</td>
<td>0.66</td>
</tr>
<tr>
<td>Arachidonic acid</td>
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<td>PRP + AA**</td>
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<td>peak 2</td>
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* Solvent System: ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (100:50:20:100, v/v, upper phase)

** Sheared at 150 dynes/cm² for an exposure time of 1 minute.
Figure 19. RP-HPLC chromatogram of the metabolites of arachidonate formed in PMNLs incubated with the ionophore A23187 and $l - ^{14}C$ arachidonate in the presence of supernatant plasma from sheared PRP.
PMNL + AA + PRP

150 dynes/sq.cm for 1 min.

Figure 20. RP-HPLC chromatogram of the metabolites of arachidonate formed in PMNLs incubated with 1-14C arachidonate in the presence of PRP.
PMNL + AA + NDGA + PRP

150 dynes/sq.cm for 1 min.

Figure 21. RP-HPLC chromatogram of the metabolites of arachidonate formed in PMNLs preincubated with 1-14C arachidonate and with NDGA in the presence of PRP.
underestimated when a significant number of particles were bigger than 80 μm in diameter (upper sizing window). This was observed when blood samples were sheared at a high shear rate and for long exposure times. In these cases, the actual total aggregate volume would remain constant at its maximum value, even though the measured volume in our size window decreased. The mean aggregate size varied from 17.1 μm (3000 sec\(^{-1}\) for 30 seconds) to 33.2 μm (10000 sec\(^{-1}\) for 30 seconds) with increasing the shear rate.

Citrated blood samples were sheared for 15 seconds, 30 seconds, and 60 seconds at shear rates ranging from 3000 to 10000 sec\(^{-1}\) and the results are shown in Figures 23, 24, and 25 respectively. Before shearing, samples were incubated with none (open bars), 50 μM ASA (slashed bars), or 10 μM NDGA (dotted bars). Samples sheared at 3000 sec\(^{-1}\) for 15 or 30 seconds showed a small amount of aggregation. Eventually, a significant part of the total aggregate volume extended beyond our upper sizing window with increasing shear rate and/or exposure time. An arrow-head is marked whenever the size distribution curves extend beyond upper sizing window, indicating that a number of very large aggregates were not included in the volume calculation. This effect resulted in a decrease of measured total aggregate volume at shear
Figure 22. Volumetric distribution curves of sheared whole blood. Citrated blood samples were sheared at 3000 sec$^{-1}$($\circ$), 5000 sec$^{-1}$($\Delta$), 7500 sec$^{-1}$($\triangle$), and 10000 sec$^{-1}$($\bullet$) for an exposure time of 30 seconds. The particle diameter was calibrated by measuring latex particles of known diameters at the same Coulter counter settings.
rates above 10000 sec\(^{-1}\) for 15 and 30 seconds (Figures 23 and 24, respectively) or at shear rates above 5000 sec\(^{-1}\) for 60 seconds (Figure 25). As can be seen in Figure 22, the size distribution curves gradually shifted toward bigger-size channels under these conditions. To characterize the kinetics of aggregate formation and leukocyte-platelet interactions, we treated blood samples with ASA (inhibitor of cyclooxygenase) or NDGA (inhibitor of lipoxygenase). Samples were incubated with these drugs for 30 minutes before shearing. Although quantitative aspects of the actual aggregation histogram at a specific shear rate and an exposure time were different from donor to donor, the overall conclusions obtained by comparing results from sheared and control samples from each donor were the same. When blood samples were incubated with NDGA, the inhibitory effect of shear-induced aggregation was usually significantly greater than that of ASA. Typical results from such experiments with a shear rate of 7500 sec\(^{-1}\) for 15 seconds and 30 seconds are shown in Figures 23 and 24, respectively. However, at a low shear rate, 5000 sec\(^{-1}\), ASA and NDGA treated blood samples showed nearly the same total aggregate volume indicating that arachidonic acid metabolism was probably not stimulated at a low shear rate and therefore the inhibition effects of the various
Figure 23. Aggregation kinetics of sheared whole blood. Citrated blood samples were sheared for 15 seconds at shear rates ranging from 3000 to 10000 sec⁻¹. Before shearing, samples were incubated with no drugs (open bars), 50 μM ASA (slashed bars), or 10 μM NDGA (dotted bars). Arrow symbols (+) indicate that significant numbers of aggregates formed were larger than 80 μm in diameter. Data represent the mean ± SEM for 4 to 7 separate experiments.
Figure 24. Aggregation kinetics of sheared whole blood. Citrated blood samples were sheared for 30 seconds. Experimental procedures and presentation symbols are the same as described in the legend to Figure 23.
Figure 25. Aggregation kinetics of sheared whole blood. Citrated blood samples were sheared for 60 seconds. Experimental procedures and presentation symbols are the same as described in the legend to Figure 23.
enzyme pathways were not so significant. This inhibitory effect was also not clear for the case of high shear rate (10000 sec\(^{-1}\)) or long exposure time (60 seconds) in this study. In these cases, volumetric distribution curve extended beyond upper sizing window which resulted in a decrease of measured total aggregate volume. Mean aggregate volume was calculated by measuring mean channel number of the distribution curve to elucidate actual aggregate profile. Table 6 shows the effect of shear stress and both drugs on the mean channel number change. The mean channel number was often slightly decreased in the presence of ASA, but NDGA treated samples normally showed much greater effects. In Figure 23, the total aggregate volume of ASA treated samples sheared at 10000 sec\(^{-1}\) was nearly the same as that of NDGA treated one. However the mean channel number changed from 45.1 ± 1.4 (ASA) to 38.7 ± 3.6 (NDGA). That is, the mean aggregate size varied from 25 \(\mu\) m (ASA) to 21 \(\mu\) m (NDGA). This represents a 41 % decrease in the mean aggregate volume. The same effect was observed at 5000 sec\(^{-1}\) for 60 seconds shearing(Figure 25). The volumetric distribution curves shifted toward smaller aggregates. This indicates that lipoxygenase products (12-HETE, 5-HETE, and LTs) may play a positive role in the shear-induced whole blood aggregation.
Table 6. Effect of Shear Stresses on the Mean Channel Number

<table>
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<th>30 sec</th>
<th>60 sec</th>
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<td>3000 sec(^{-1})</td>
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<td>26.8 ± 2.4</td>
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<tr>
<td></td>
<td>ASA</td>
<td>—</td>
<td>—</td>
<td>36.6 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>NDGA</td>
<td>—</td>
<td>—</td>
<td>30.8 ± 4.9</td>
</tr>
</tbody>
</table>
| 5000 sec\(^{-1}\) | Control    | 28.4 ± 4.3 | 32.9 ± 2.5 | 58.1 ± 2.4  
|             | ASA        | 25.3 ± 0.2 | 30.8 ± 3.6 | 60.0 ± 0.4  
|             | NDGA       | 24.0 ± 1.6 | 32.3 ± 2.2 | 54.7 ± 1.8  |
| 7500 sec\(^{-1}\) | Control    | 40.2 ± 2.1 | 49.3 ± 4.6 | 56.9 ± 0.7  
|             | ASA        | 37.1 ± 2.5 | 49.1 ± 5.2 | 55.9 ± 0.8  
|             | NDGA       | 32.9 ± 2.1 | 36.6 ± 4.1 | 54.7 ± 2.5  |
| 10,000 sec\(^{-1}\) | Control    | 47.1 ± 1.1  
|             | ASA        | 45.1 ± 1.4  
|             | NDGA       | 38.7 ± 3.6  

\(a\) mean channel number, mean ± SEM

\(b\) arrow symbols (†) indicate that significant numbers of aggregates formed were larger than 80 µm in diameter.
Figure 26 shows the effect of two anticoagulants (citrate vs. heparin) on aggregation kinetics of sheared whole blood. Blood samples in citrate or heparin were sheared for 30 seconds at shear rates ranging from 3000 sec\(^{-1}\) to 10000 sec\(^{-1}\). Heparin treated blood samples were more sensitive to shear stress than citrate treated blood samples. They formed a significant number of large aggregates, which were beyond our upper sizing window even at a shear rate of 5000 sec\(^{-1}\). For heparinized blood samples, the measured total aggregate volume in our size window decreased as shear rate increased, indicating that bigger aggregates were formed at higher shear rates. Blood samples incubated with NDGA exhibited a larger total aggregate volume than those incubated without drugs or with ASA. This suggests that NDGA treated samples may have weaker binding forces between platelet aggregates and leukocytes. This resulted in the volumetric distribution curves shifted back into our size window, and the appearance of a bigger measured aggregate volume.

The effect of temperature on aggregation kinetics of sheared whole blood is illustrated in Figure 27 and Table 7. Citrated blood samples were sheared for 30 seconds at room temperature or 37 °C. The total aggregate volume and mean aggregate size decreased as temperature increased. Blood samples sheared at 37 °C showed much less
Figure 26. Effect of anticoagulants on aggregation kinetics of sheared whole blood. Blood samples in citrate or heparin were sheared for 30 seconds at shear rates ranging from 3000 to 10000 sec\(^{-1}\). Experimental procedures and presentation symbols are the same as described in the legend to Figure 23.
aggregation than those sheared at room temperature. In addition, the shape of size distribution curves for sheared blood samples at 37 °C was relatively flat, indicating that aggregate sizes scattered in a wide range. The effect of both inhibitors (ASA and NDGA) was nearly the same as that seen at room temperature.
Figure 27. Effect of temperature on aggregation kinetics of sheared whole blood. Citrated blood samples were sheared for 30 seconds at room temperature or 37°C. Experimental procedures and presentation symbols are the same as described in the legend to Figure 23.
Table 7. Effect of Temperatures on Shear-Induced Whole Blood Aggregation

<table>
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<tr>
<th>Temperature (°C)</th>
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<th>25</th>
<th>30</th>
<th>35</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Aggregate Volume ( (x \ 10^6 \ \mu m^3/\mu l) )</td>
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<td>2.94</td>
<td>2.61</td>
<td>1.57</td>
<td>1.55</td>
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<tr>
<td>Mean Channel Number</td>
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<td>52.5</td>
<td>47.8</td>
<td>32.6</td>
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<tr>
<td>Mean Aggregate Diameter (μm)</td>
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<td>28.7</td>
<td>25.9</td>
<td>18.7</td>
<td>17.2</td>
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</table>

All runs were sheared at 7500 sec\(^{-1}\) for an exposure time of 30 sec.
CHAPTER IV. DISCUSSION

This thesis consists of two parts. The first part examined the shear stress effects on isolated PMNL and PRP interactions to determine the clear relationships between these two cells. Shear-induced PMNL aggregation, degranulation, and arachidonic acid metabolism has been studied. The second part was a study of shear stress effects on platelet-leukocyte interactions in whole blood. By using the results from the first part, more directed and extensive work in whole blood system can be done. The effect of shear stress, exposure time, temperature, and anticoagulant were investigated with respect to the total aggregate volume change.

4.1. Effect of shear stress on platelet-PMNL interactions

In this study, we have shown that a substance(s) derived from platelet activation in a shear field may activate the C-5 lipoxygenase of human blood PMNLs in vitro, and therefore, induce the aggregation and degranulation of PMNLs. However there is an activation(shearing) requirement for PMNL aggregation and degranulation even though the cells have been incubated with supernatant plasma of sheared PRP (Figures 9B and
13B, respectively). PMNLs incubated with PFP show some shear-induced aggregation (Fig. 9C) and degranulation (Fig. 13D). This may be due to plasma protein binding and to a lesser extent viscosity changes. The differences between Figures 9B and 9C indicate that a substance(s) derived from shear activated platelets plays an active role in this PMNL functional changes.

Sheared platelets release ADP (102) and serotonin (64) which are potent platelet aggregating agents. However, 2 μM ADP and 2 μM serotonin do not cause PMNL aggregation (Figures 9D and 9E, respectively). Redl et al. (133) recently demonstrated that platelets augment granulocyte aggregation by a serotonin and TxA2 independent mechanism and it was not necessary for platelets to be intact for them to augment granulocyte aggregation. Indeed, platelet lysates and the supernatant fluids therefrom proved equally efficacious. 10 μM NDGA and 10 μM U-60257 totally suppressed the shear-induced PMNL aggregation to that level seen in a mixture of PMNL and PFP (Figures 10C and 10D, respectively). This indicates that lipoxygenase- rather than cyclooxygenase-pathway products are important in platelet-PMNL interactions. It is hypothesized that the platelet derived PMNL activating substance may be 12-HPETE. Shear-induced aggregation of platelets produces the release of
arachidonic acid from cellular phospholipids and the subsequent metabolism of the arachidonic acid by cyclooxygenase and lipoxygenase systems. It is interesting that in shear activation, a substantial portion of the free arachidonic acid in platelets is transformed by a lipoxygenase to 12-HPETE and subsequently 12-HETE (Fig. 18). This 12-HPETE may activate the C-5 lipoxygenase in PMNLs, and therefore, lead to increased release of C-5 lipoxygenase products, such as 5-HETE and leukotrienes. Figure 28 shows a hypothetical scheme of the platelet-PMNL interactions that may lead to increased release of PMNL C-5 lipoxygenase products.

The C-5 lipoxygenase is present in human PMNLs in an inactive form and must be activated to catalyze the dioxygenation of arachidonic acid. Evidence that the lipoxygenase pathway might be involved in PMNL activation comes from the studies showing that rabbit exudate PMNLs are stimulated directly by exogenous arachidonic acid to release HETEs and LTB₄ and to aggregate (14,15,27,119,120,121). Previous observations have linked arachidonic acid and its oxygenated metabolites to degranulation of neutrophils (61,111,112). Smolen and Weissman demonstrated that only high concentrations of indomethacin (shown to inhibit mainly cyclooxygenase and to a lesser extent lipoxygenase) also hampered part of
Figure 28. Hypothetical scheme of platelet and PMN leukocyte lipoxygenase interactions induced by mechanical stimulation.
the secretory effects of FMLP, whereas lower concentrations (inhibiting only the cyclooxygenase) did not affect enzyme release (161). These findings are compatible with a role of lipoxygenase products for neutrophil degranulation. However, the addition of arachidonic acid alone to human blood PMNL suspensions does not induce significant synthesis of C-5 lipoxygenase products (27). The divalent cation ionophore A23187 is known to be a potent activator of the synthesis of LTs. Its action probably involves the stimulation of Ca\(^{++}\) flux and the subsequent activation of the Ca\(^{++}\) dependent phospholipases and C-5 lipoxygenase. It thus appears that the release of arachidonic acid (following phospholipase stimulation) and the activation of the C-5 lipoxygenase are prerequisites for the synthesis of 5-lipoxygenase products in human blood PMNLs. One possible mode of action of stress-induced mechanical activation is a membrane stretching induced increased calcium flux. Volpi et al. have shown that the addition of arachidonic acid to neutrophils will cause a rapid and significant increase in the permeability of the plasma membrane to calcium (179). This hypothesis is supported by the similarity of the ionophore A23187-induced (Fig.7) and shear-induced (Fig.8) PMNL aggregation time course curves. Inhibition of shear-induced \(\beta\) - glucuronidase
release by citrate (Fig.13) and EDTA (Fig.14) strongly suggests that calcium is essential for PMNL degranulation.

Leukotriene biosynthesis requires enzymes that are distributed among different subcellular compartments and it appears that their biosynthesis involves movement of intermediates between these compartments (20,75). Among these leukotrienes, LTB₄ is known to be a very potent chemotactic factor and to induce PMNL aggregation and degranulation. In the case of LTB₄ formation, the action of a soluble cytosolic hydrolase has been proposed to be the rate limiting step (75). Consequently, intermediate substrates such as 5-HPETE or LTA₄ must accumulate, decay via alternate enzymatic and nonenzymatic processes, or be released from cells, such as PMNLs. In the latter instance, a plasma enzyme capable of metabolizing extracellular LTA₄ to LTB₄ seems reasonable. In fact, recent studies suggest that an extracellular enzymatic transformation of LTA₄ into the potent chemotaxin, LTB₄, may be involved (44,45). Plasma enzymatic activity responsible for LTB₄ production may play a general role in modulating the inflammatory response. Hafstrom et al. showed that LTB₄ induced a release of lysosomal enzymes in cells treated with cytochalasin B, but also in untreated cells. This is a response which differs from
FMLP, where release is completely dependent on the presence of cytochalasin B (61). Therefore our observations that shear stress could induce PMNL enzyme release in the absence of cytochalasin B supports our hypothesis that LTB₄ formed in a shear field stimulates PMNL degranulation.

Quantitative confirmation of lipoxygenase involvement in the shear-induced PMNL functional changes was obtained using RP-HPLC. Very small amounts of di-HETEs were detectable when PMNL suspensions were sheared in buffers alone (Fig.15). The formation of LTB₄ was greatly increased after shearing PMNL in the presence of supernatant plasma from sheared PRP (Fig.16). When the PMNLs were stimulated both by the ionophore A23187 and shearing, LTB₄ and 5-HETE production increased dramatically (Fig.19). However, preincubation of NDGA with PMNL suspensions totally suppressed the increment of LTB₄ formation after shearing (Figures 17 and 21). This again supports the hypothesis that these C-5 lipoxygenase products induce PMNL aggregation and degranulation. Sheared PRP exhibited greatly increased synthesis of 12-HETE with surprisingly little TxB₂ production (Fig.18). This indicates that shear stress stimulation of platelets may produce quite different arachidonic acid metabolism than that seen with direct chemical stimuli,
such as thrombin or collagen. This platelet derived 12-HETE (12-HPETE) may be the mediator which stimulates the C-5 lipoxygenase activity in human PMNLs during shear-induced aggregation and degranulation. In this HPLC system, only PMNL and platelet arachidonic acid metabolism was studied. Erythrocytes and other leukocyte components (monocytes and lymphocytes) involvement in the shear-induced arachidonic acid metabolism of these cells should be quantitated. Reimers et al. reported that red blood cells may augment platelet-platelet interaction or platelet aggregation in laminar flow, and that this was associated with chemical (RBC-derived ADP) and physical (platelet surface transport) mechanisms(134). RBCs may affect the C-12 lipoxygenase activity in platelets during this process. Fitzpatrick et al. showed that human erythrocytes are not metabolically inert in terms of eicosanoid biosynthesis. One of their constitutive enzymatic capabilities is conversion of LTA₄ into LTB₄ (45). LTB₄ production may be increased in the presence of erythrocytes. 15-HETE production from lymphocytes has been reported to serve as an inhibitor of platelet and leukocyte lipoxygenase enzymes(141,173,174). It has been shown that the C-15 lipoxygenase also interacts with the C-5 lipoxygenase to produce a 5S,15S diHETE (93). The formation of 5S,12S diHETE and of the 5S,15S diHETE
reflects the interactions between lipooxygenase enzymes in
different blood cells. Marcus et al. have established
that cell components of a thrombus share precursors and
intermediates of the lipooxygenase and cyclooxygenase
pathway with concomitant, stimulus-specific production of
new metabolites (96,97). These biochemical aspects of
arterial and venous thrombosis indicate cell-cell
communication via eicosanoids. Recently they have shown
a new metabolite of arachidonic acid, formed during
interaction between thrombin or collagen stimulated
platelets and unstimulated neutrophils. That was
confirmed to be 12S,20 diHETE. Their experiments showed
that platelet stimuli known to occur in vivo might
initiate metabolic interactions between different cell
types via the arachidonic acid pathway (98).

The platelet/PMNL ratio also appears to be important
(Fig.12). When the ratio is approximately the same as
found under physiological conditions, the aggregation
response of the PMNL is maximum. It is possible that, in
addition to the platelet/PMNL ratio, the cell
concentration in the incubation medium also is an
important parameter. This has been observed in studies
examining the possibility of platelet-derived
endoperoxides serving as substrates for endothelial cell
prostacyclin synthase (97).
More work should be done to elucidate the control mechanisms responsible for production of these metabolites during cell-cell interactions, and functional consequences thereof for the thrombotic and hemostatic, as well as the inflammatory process.

4.2. Effect of shear stress on platelet-leukocyte interactions in whole blood

In this study, the kinetics of shear-induced whole blood aggregation have been presented as a function of shear rate and exposure time. Particles in a viscometric flow tend to travel along the fluid lines and rotate with a frequency proportional to the fluid shear rate. As a result of rotation, the particles experience a periodic surface stress and will deform periodically if nonrigid. Above a threshold shear rate, blood cells would undergo membrane fatigue failure and the ensuing leakage of chemicals (ADP, serotonin, and 12-HETE from platelets; 5-HETE and leukotrienes from leukocytes) which could activate the blood cells, causing aggregation. As shown in Figure 22, much bigger aggregates were generated at higher shear rates and the size distribution curves gradually shifted toward bigger size channels. The aggregates formed during and after shear stress exposure
may be composed primarily of platelets. However involvement of leukocytes appears to have contributed significantly to the volume of aggregates (35,76). Several groups have shown that platelet derived eicosanoids (12-HPETE and 12-HETE) activated the C-5 lipoxygenase in PMNLs, and therefore, lead to increased release of LTB₄ (55,92,93,96). This suggests the potential role of release products from one cell type in modulating the biological and mechanical response of other blood cells. In this study, the possible involvement of leukocytes on the process of platelet aggregation to shear stress is postulated.

To characterize the kinetics and biochemical mechanism of aggregate formation and leukocyte-platelet interaction, blood samples were treated with inhibitor of cyclooxygenase(ASA) or lipoxygenase(NDGA). For shear rates up to 7500 sec⁻¹ for 15 or 30 seconds shearing, the volume distribution curves do not extend beyond our upper sizing window. At a low shear rate, 5000 sec⁻¹, ASA and NDGA treated blood samples showed nearly the same total aggregate volume. At 7500 sec⁻¹, the total aggregate volume of NDGA treated blood sample was dramatically decreased. ASA treated sample showed much less effect than NDGA treated ones. This indicates that activation of arachidonic acid metabolism was not great at low shear
rate and the inhibition of the various enzyme pathways did not show much effect. However arachidonic acid metabolism was stimulated at higher shear rates and lipoxygenase products seemed to be more important in shear-induced whole blood aggregation than cyclooxygenase products. Interestingly, the total aggregate volume of ASA treated and NDGA treated samples was nearly the same at a shear rate of 10000 sec\(^{-1}\) for 15 seconds. However the mean channel number changed from 45.1 (ASA treated sample) to 38.7 (NDGA treated sample) (Table 6). That is, the mean aggregate size varied from 25 μ m (ASA) to 21 μ m (NDGA). This represents a 41 % decrease in mean aggregate volume. The same effect was observed at 5000 sec\(^{-1}\) for 60 seconds shearing. The mean aggregate size decrease for NDGA treated samples may represent detachment of leukocytes from platelet aggregates. Lipoxygenase products may strengthen the binding effect of leukocytes and platelets or may modulate platelet aggregation in the shear field. ADP, released from platelets, has been proposed to be the major mediator of shear-induced aggregation in PRP (102). ADP applied externally has been shown to increase platelet stickiness and these ADP-stimulated platelets form aggregates at relatively low shear stress (186). Recently, Jen and McIntire have shown that ADP released from sheared blood
cells is involved not only in the aggregate formation process but also in maintenance of aggregate stability (76). The effect of lipoxygenase products on the shear-induced whole blood aggregation should be further studied.

Heparinized blood samples were more sensitive to shear stress than citrated blood samples. Heparinized blood samples form large aggregates even at a low shear rate of 3000 sec\(^{-1}\) for an exposure time of 30 seconds. The difference between these two anticoagulants is that citrate chelates Ca\(^{++}\) in plasma but heparin inhibits other coagulation factors (mainly by acceleration of anti-thrombin III activity). This indicates Ca\(^{++}\) may be an important factor in shear-induced whole blood aggregation. This was also supported by the similarity of the ionophore A23187-induced and shear-induced PMNL aggregation time course curves (Figures 7 and 8). Furthermore, most aggregates formed in heparinized blood were much less reversible than those formed in citrated sample, when incubated with ASA or NDGA. Ca\(^{++}\) may strengthen the binding between platelet aggregates and leukocytes.

When citrated whole blood samples were exposed to a shear rate of 7500 sec\(^{-1}\) for 30 seconds, the total
aggregate volume was a maximum in our sizing window at room temperature and this was decreased as temperature increased. At 37 °C, this value was nearly half of the maximum value. Also mean aggregate size was dramatically decreased with increasing temperature. The reasons for these effects are not completely elucidated, but may be important with regard to the physiological interpretation of in vitro aggregation results. There are at least two possibilities. First, shear-induced aggregates formed at 37 °C are unstable and immediately start to disaggregate due to increased cellular kinetics. Second, cellular viscoelastic properties or receptor exposure may change due to temperature and this results in blood cells which are less sensitive to shear stress at 37 °C than at room temperature.

Our conclusions are: (a) that exposure of whole blood to higher shear stress and longer exposure time produces bigger aggregates of platelets and leukocytes, (b) that lipoxygenase products (12-HETE, 5-HETE, and LTs) are involved in whole blood aggregate formation and platelet-leukocyte interactions in the shear field, (c) that heparinized blood was more sensitive to shear stress than citrated blood, (d) that total aggregate volume and mean aggregate size were decreased with increasing temperature, for citrated whole blood.
4.3. Future studies

Much work remains to be done in order to understand the biochemical mechanisms involved in shear-induced cell-cell interactions. The results of this study indicate that lipoxygenase products of platelets and PMNLs are important in shear-induced PMNL aggregation and degranulation. However, the effect of these lipoxygenase products on shear-induced whole blood aggregation remains unclear. Erythrocytes and other leukocyte components (lymphocytes and monocytes) possible involvement in shear-induced whole blood aggregation should be elucidated. Erythrocytes may augment platelet aggregation or PMNL aggregation by releasing ADP(134) or increasing LTB₄ formation from LTA₄(45), respectively. 15-HETE produced from lymphocytes may decrease the shear-induced aggregation by inhibiting the platelet and PMNL lipoxygenase. The effect of erythrocytes and lymphocytes on platelet-PMNL interactions via eicosanoids remains to be elucidated.

An additional possible study is the shear-induced cell-cell interactions involving endothelial cells and smooth muscle cells. During vascular occlusion, platelets, PMNLs, endothelial cells, and arterial smooth muscle cells are brought into close apposition, affording
the biochemical interchange of metabolic products such as precursors and intermediates of the eicosanoid pathways. Direct interactions between platelets and endothelial cells via the cyclooxygenase pathway have been demonstrated. In those studies platelet-derived endoperoxides were metabolized by endothelial cells to form PGI$_2$(97). Migration of smooth muscle cells from the medial to intimal wall of blood vessels is an early response to endothelial injury in the development of atherosclerosis. Platelet-derived eicosanoid metabolites may stimulate this migration process. A study of eicosanoid metabolism in the interactions between cultured smooth muscle cells, platelets, PMNLs, and endothelial cells may contribute to understanding the genesis of thrombosis and atherosclerosis.
SUMMARY

The results of experiments described in this text elucidated basic mechanisms of arachidonic acid and eicosanoid metabolism involved in cell-cell interactions under shear. Our studies suggest important roles for the platelets(C-12) and leukocytes(C-5) lipoxygenases in shear-induced PMNL functional changes and shear-induced whole blood aggregation. These data support the concept of platelet-leukocyte interactions due to mechanical trauma and add new data concerning the potential role of release products from one cell type in modulating the biological and mechanical response of another blood cells. These may contribute to the development of models for thrombosis, acute inflammation, and atherosclerosis. An understanding of the mechanisms involved should serve as a basis for formulating new therapeutic approaches to vascular diseases and to control of embolism seen in artificial organ implantations.
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APPENDIX

Dextran Sedimentation Fluid with Heparin

1.25\% (w/v) Glucose (M.W. 180) = 0.0694 \text{ M} (12.5\text{g/l})

3.00\% (w/v) Dextran (Macrodex 70, Pharmacia Laboratories, N.J.) 188,000d

0.638\% (w/v) NaCl = 0.109 \text{ M} (6.38\text{g/l})

30 units Heparin per ml.

Ficoll-Hypaque Leukocyte Separation Fluid

9.1 g Ficoll (Pharmacia Laboratories, N.J.) (M.W. 400,000)

120 ml distilled H$_2$O

Filtration

Add to above:

30 ml Hypaque-sodium 50\% (w/v) (Winthrop Laboratories, New York, N.Y.)

= 6.07\% (w/v) Ficoll and 10\% (w/v) Hypaque in final solution.

0.85\% NaCl with 0.85 mM Citrate (0.179 g/l)

1.8\% NaCl (9.0 g NaCl/500ml H$_2$O)