PLATELET-NEUTROPHIL INTERACTION:

NEUTROPHILS MODULATE PLATELET FUNCTION

Richard William FAINT

1992

A thesis submitted to the

University of London

for the degree of Ph.D

The Department of Haematology
University College and Middlesex School of Medicine
University College London
98 Chenies Mews
London WC1E 6HX

ABSTRACT

Platelets are vital for the arrest of bleeding following tissue injury. Neutrophils play an important role in host defence against bacterial infection. In the milieu of the acute inflammatory response both cellular and non-cellular elements may interact to modify behaviour. Evidence suggests that leukocytes may play an active role in the modulation of platelet function. This interaction may be abnormal in certain pathological states.

This study examined the effect of purified neutrophils upon both washed platelets and platelet-rich plasma, as well as in whole blood *in vitro*. Neutrophils were found to alter platelet behaviour by several mechanisms. These included transcellular metabolism of eicosanoids. Neutrophils utilized platelet-derived arachidonate to release increased amounts of leukotrienes. Other arachidonate metabolites resulted from platelet-neutrophil interaction and these differed quantitatively and qualitatively from those arising from either cell-type alone.

Another mechanism was the release of a nitric oxide-like factor by neutrophils. Nitric oxide inhibits platelet adhesion and aggregation via guanylate cyclase stimulation. Platelet ATP secretion and TXB_2 release were also impaired. The effect of authentic nitric oxide in whole blood was investigated. Platelet aggregation in whole blood, in the presence of L-arginine (the substrate for nitric oxide synthesis), was found to be inhibited by a nitric oxide-like factor.

Neutrophils, under different conditions, were potent inducers of platelet calcium flux, aggregation and secretion. This activity was mediated by a neutrophil-derived protease, most likely to be cathepsin G.

These different mechanisms of platelet-neutrophil interaction were investigated in patients with Multiple System Organ Failure Syndrome, where clinical observations have implicated both platelet and neutrophil involvement. Some patients exhibited platelet-neutrophil interaction abnormalities.

The interaction of platelets with neutrophils may help to explain some of the pathophysiological events associated with different clinical states.

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ACKNOWLEDGMENTS

I wish to express my gratitude to the following people who have helped me to accomplish this study.

Professor S.J. Machin for supervision and helpful criticism.

Dr. I.J. Mackie for helpful discussions and advice.

Dr. P.J. Roberts for valuable criticism and discussion, as well as for culturing HL-60 cells.

Dr. M. Mython for selecting patients for study, recording clinical information and obtaining blood samples.

Helen, my wife, for constant encouragement and for typing part of the manuscript.

ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ARDS	Adult Respiratory Distress Syndrome
ATP	Adenosine 5'-triphosphate
Disodium EDTA	Disodium ethylenediaminetetra-acetic acid
Disodium EGTA	Disodium ethyleneglycoltetra-acetic acid
DMSO	Dimethyl sulphoxide
EDRF	Endothelium-derived relaxing factor
FMLP	N-formyl-Met-Leu-Phe
HBSS	Hank's Balanced Salt Solution
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroxyperoxyeicosatetraenoic acid
HPLC	High Performance Liquid Chromatography
LT	Leukotriene
MSOF	Multiple System Organ Failure
NAD	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NDGA	Nordihydroguaiaretic acid
ODS	Octa-decyl silica
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PF4	Platelet factor 4
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PMNs	Polymorphonuclear leukocytes (neutrophils)
PG	Prostaglandin
RIA	Radioimmunoassay
ßTG	ß-Thromboglobulin
TX	Thromboxane

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Haemostasis ensures that blood remains in a fluid state, yet is able to respond rapidly to any interruption in the system to prevent excessive blood loss. It involves a complex interaction between different blood cells, plasma factors and the vessel wall. Platelets are important for maintaining the integrity of apparently uninjured vessels as they passively circulate the vascular tree which is lined by a monolayer of endothelial cells.

When injury to the vessel occurs platelets become exposed to procoagulant surfaces causing them to respond in several distinct ways: by adhesion, shape change, aggregation, the secretion of three different types of granules (dense granules, alpha granules and lysosomes) and arachidonate liberation. However, for complete haemostasis, the coagulation system must be activated on the surface of aggregated platelets to form a meshwork of fibrin. This consolidates the platelet plug and renders it impermeable.

The mechanism by which blood loss is staunched and any invading microorganisms are repelled is exquisitely coordinated and culminates in the formation of a precisely localized haemostatic plug. The degree of platelet activation is dynamically adjusted by the balance of actions of a diverse array of excitatory and inhibitory signals to which the platelet surface is exposed. Platelets have specific plasma membrane receptors that recognise and organise these various stimuli. Initiated by receptor occupancy, the positive and negative extracellular signals are quickly translated into

complex biological responses via a limited number of intracellular secondary messengers through processes of signal response coupling.

Haemostasis represents a specialised inflammatory response to tissue injury. The development and resolution of the haemostatic process involves participation of other inflammatory cells, namely, leukocytes. Considerable evidence indicates that platelets or platelet products can contribute to the accumulation of leukocytes in the clot and to their activation. Conversely, activated leukocytes can alter platelet function.

The immune response to infection and tissue damage consists of two basic components: the non-specific and the specific immune response. While the specific immune response is capable of recognition and an amplified secondary response, non-specific immunity refers to the protective roles of such diverse systems as the unbroken skin and phagocytosis, which are incapable of a secondary response. The inflammatory process is a part of the non-specific immune response.

Inflammation is initiated by vasoactive amines such as histamine, serotonin, and the kinin polypeptides. Venular sphincters constrict and capillaries dilate while the kallikrein-kinin system increases both vascular permeability and the adherent properties of the venular endothelium. As a result, fluid and fibrinogen leave the permeable vessels creating a fibrin network via the action of thrombin, thus containing invading bacteria. The adherent endothelial vessel wall traps phagocytes and allows their emigration into tissues. Kinins also aid leukotaxis.

Inflammation brings serum into contact with invading microorganisms, aiding in their destruction by such non-specific compounds as betalysin (lethal to Gram-positive organisms), and the complement cascade (lethal to Gram-negative organisms and a promoter of neutrophil phagocytosis). Complement activation leads to bacteriolysis and

also the release of cleavage products with even greater activity. C3a and C5a, for example, have specific receptors on cell membranes effecting, among other things, vascular permeability. C3a, C5a and C567 also have powerful chemotactic properties for polymorphonuclear leukocytes (PMNs). Finally, inflammation results in the release and circulation of a variety of mediators with strong immunological activity, such as the prostaglandins and leukotrienes, some of which also regulate lymphocyte function.

Within minutes of tissue damage or microbial invasion PMNs adhere to blood vessel walls and then emigrate into the tissue. Diapedesis is unidirectional with no return of cells to the circulation, and is facilitated to a large degree by complement. PMNs are very active in phagocytosis, a process augmented by opsonins, which include complement and antibody. The intracellular destruction of phagocytised bacteria by PMNs is facilitated by a "respiratory burst" of cellular activity, which includes: increased glycolysis and lactate production; a fall in the pH of phagocytic vacuoles; increased oxygen consumption; increased hexose monophosphate shunt activity; increased NADPH oxidation; increased hydrogen peroxide and superoxide production; and increased membrane lipid synthesis.

If inflammation cannot contain a local infection, invading microorganisms are carried to regional lymph nodes via the lymphatics. Here, fixed macrophages phagocytise and have the potential to kill microoganisms where PMNs have failed, as they contain a very different enzyme reportoire. The fixed macrophages residing in the spleen, lymph nodes, liver (Kupffer cells), lung (alveolar macrophages), and skin (Langerhans cells) are known collectively as the reticuloendothelial system. These cells exist in vast numbers making the macrophage a key cell in the immune response. Blood borne macrophages or monocytes are capable of phagocytosis and chemotaxis like PMNs. However, all macrophages can undergo activation after antigen exposure, making them even more

efficient in phagocytosis and intracellular killing. Lymphocytes can trigger macrophage activation by means of cellular secretions (lymphokines). Macrophages can likewise influence lymphocyte response via monokines (such as interleukin-1 (IL-1)).

The second major division of immune reactivity is the specific response of the lymphocyte. The lymphocyte population consists of basically two separate response systems: the T-cell system, which is responsible for cell-mediated immunity and much of immunoregulation, and the B-cell system, which is responsible for antibody production. B and T cells arise from a common bone marrow precursor, but then mature via different pathways. About 80% of the circulating pool of lymphocytes are T-cells and 12-15% are B-cells. The remaining lymphocytes do not fit clearly into either category. When stimulated by antigen, T-cells undergo blast transformation and proliferate to form: memory cells, which live many years; effector cells, capable of a variety of responses, including cytotoxicity; regulatory (helper and suppressor) cells, which influence almost every activity of the immune response, including PMNs.

The regulatory activities of these specialised T-cells are mediated through the elaboration of lymphokines (such as interleukins 2 and 3 (IL-2 and IL-3)). B-cells are responsible for humoral immunity, the main characteristic of which is the production of specific antibodies by the plasma cell progeny of activated cells. B-cells can secrete five types of antibody, although it appears that only three (Immunoglobulin (Ig) G, IgM and IgA) participate in immunity to invading pathogens. The response of these cells and their products is specific and avid, and often performed along with the products of the complement cascade.

Although each facet of the immune response has traditionally been studied in isolation from the whole, it is important to note that in reality all components of the

immune response undergo complex interactions. Whatever affects one component of the immune response will either directly or indirectly affect others.

1.2 Platelet responses

Resting platelets are discoidal cells of 2-3μM in diameter which circulate in the blood for 6-10 days. Platelets originate in the bone marrow as anucleate fragments of the cytoplasm of giant megakaryocytes. They only contain mitochondrial DNA. Typically, platelets have a large number of secretory granules, a small number of mitochondria, a circumferential band of microtubules and two different membrane systems called the open canalicular system (OCS, a surface-connected network of channels continuous with the plasma membrane, which extends throughout the interior of the platelet) and the dense tubular system (intramembranous channels). The process of arresting blood loss by adhering to sites of vascular injury and to each other produces a haemostatic platelet plug. This is the result of the activation of various responses such as adhesion, shape change, aggregation, secretions and arachidonate liberation. The degree of response elicited depends on the number of stimulating agents and their concentration in the media surrounding the platelets. *In vitro* studies of platelet responses use platelet-rich plasma (PRP) which is prepared by centrifugation of anticoagulated whole blood. Platelets may also be washed and resuspended in a suitable buffer for study.

1.2.1 Adhesion

Resting platelets adhere to almost all foreign surfaces to which they are exposed. Such platelet adhesion can occur without morphological change (Turitto and Baumgartner, 1982). Increased platelet adhesion occurs when the foreign surface is coated with plasma

proteins, such as fibrinogen and von Willebrand factor (Zucker and Vroman, 1969). This adhesion is an interaction between platelets and the protein coat on the sticky surface (Packham *et al*, 1969). The initial adhesion may be followed by further platelet activation if the sticky surface exposes collagen fibrils or if the platelets are stimulated in other ways. Inactive platelets may detach or remain adhered to the surface (Baumgartner *et al*, 1976).

1.2.2 Shape change

Activation by stimulating agents induces a rapid transition in platelet shape from discoidal cells to spheres with pseudopods. This response occurs within a few seconds. It is energy dependent requiring ATP, but is independent of extracellular calcium ions (Holmsen, *et al*, 1974). This change in platelet morphology is probably isovolumetric (Feinberg *et al*, 1974) although some studies showed a small increase (about 20%) in the apparent volume of platelets (Holme and Murphy, 1980). Ultrastructurally, the circumferential band of microtubules disappears, and actin filaments and single microtubules appear in the newly formed pseudopods (Castle and Crawford, 1979). During shape change, previously unavailable receptors (glycoprotein IIb-IIIa) for the binding of fibrinogen are exposed on the platelet surface. Calcium (Ca^{2+}) dependent binding of fibrinogen to their receptors is necessary for platelet aggregation (Bennett and Vilaire, 1979). Shape change can be studied *in vitro* as an increase in the optical density of a platelet suspension or as a decrease in light scattering since discoidal platelets scatter more light than spherical ones.

1.2.3 Aggregation

When spherical, activated platelets (with exposed fibrinogen receptors) are brought into close contact in a medium containing calcium ions (Ca^{2+}) and fibrinogen, they adhere to each other and form aggregates (Born and Cross, 1964; Deykin *et al*, 1965; Marguerie *et al*, 1980). This response is slower than shape change, taking two to three minutes for completion. Aggregation may be inhibited by metabolic inhibitors, such as 2-deoxyglucose and antimycin A, as can shape change (Holmesen *et al*, 1974). After initial shape change there is no further decrease in the ATP levels (a measurement of the cells energy status). Therefore aggregation may be a passive process which occurs after shape change and fibrinogen receptor exposure. Aggregation may be reversible or irreversible depending on the potency and concentration of the stimulating agent. A biphasic aggregation response may be obtained with certain weak agonists which induce a primary phase (primary aggregation) followed by a second phase (secondary aggregation) caused by a positive feedback mechanism.

During *in vitro* experiments, platelets are brought into close proximity by rapid stirring of the platelet suspension. The response, aggregation, is quantified with a special photometer (aggregometer; Born, 1962) as a relative decrease in optical density.

1.2.4 Secretion

Platelets contain three different types of secretory granules: dense granules, alpha granules and granules that contain acid hydrolase (primary lysosomes). The secretory process involves exocytosis with fusion of the granules to the surface-connected open canalicular system (Holme *et al*, 1973, 1974). As platelets lack a nucleus they are unable to resynthesise the granules and their contents. This is in contrast to other secretory cells.

The secretion of all three types of granules may be inhibited by ATP deprivation (metabolic inhibition). The granules differ from each other in the strength of stimulus required to cause secretion. For instance, selective secretion of alpha granules may be induced by low levels of some agonists (such as collagen) without simultaneous secretion from other granules (Kaplan *et al*, 1979). Also, lysozomal secretion only occurs after stimulation with the most potent agonists, whereas secretion of both alpha and dense granules may be induced by all known platelet agonists (Mills *et al*, 1968). In addition, the kinetics of secretion of the three granule types may vary. Acid hydrolase secretion is only about 60% complete after two minutes, whereas secretion of dense granules and alpha granules is virtually complete within one to two minutes (Holmsen and Day, 1970).

1.2.5 Dense granule secretion

The dense granules in human platelets are 250-300nm in diameter and contain electron-opaque material consisting of ions and low molecular weight compounds: Ca^{2+}, inorganic phosphate, inorganic pyrophosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin (5-hydroxytryptamine) and low concentrations of catecholamines (Meyers *et al*, 1982). These constituents are virtually unexchangeable with their cytosolic counterparts (Reimers *et al*, 1977), except for serotonin which is freely exchangeable with extracellular serotonin through active transport over both the plasma and granule membranes (Wilson and Salganicoff, 1981). Dense granule ATP and ADP are metabolically inactive and account for about two thirds of total cell ADP and ATP content (Ugubilk *et al*, 1979). The major platelet granules and their contents are shown in Table 1.1.

Table 1.1 Major factors secreted by platelets.

Lipid membranes	Alpha granules	Dense granules	Lysosomes
Arachidonic acid	*specific*	Calcium	Acid hydrolases
	ß-Thromboglobulin	Serotonin	
Endoperoxides:	Platelet Factor 4	ATP	
PGG_2	PDGF	ADP	
PGH_2		GTP	
	non-specific	GDP	
Thromboxane A_2	Fibrinogen	phosphate	
	Factor V		
12-Hydroxy acids:	Factor VIII		
12-HPETE	α_2-Antiplasmin		
12-HETE	Thrombospondin		
	Albumin		
PAF	Fibronectin		
	Kallikrein		

1.2.6 Alpha granule secretion

The alpha granules have a diameter of 300-500nm and contain proteins which can be subdivided into three groups: a) platelet-specific proteins (platelet factor 4 (PF4), platelet-derived growth factor (PDGF), ß-thromboglobulin (ß-TG)); b) coagulation factors (fibrinogen, factor V, factor VIII/von Willebrand factor); c) other proteins (thrombospondin, fibronectin, albumin, α_2-antiplasmin, histidine-rich glycoprotein, high molecular weight kininogen, immunoglobulin, α_1-antitrypsin and α_2-macroglobulin).

1.2.7 Lysosome secretion

The morphology of primary lysosomes is very similar to that of alpha granules. They can be distinguished by histochemical techniques. Lysosomes secrete acid hydrolases (with an optimum pH range of 3.5-5.5) which can be separated into groups: a) acid proteases (endo- and exopeptidases); b) acid glycosidases (such as ß-hexosaminidase and ß-glucuronidase); c) acid phosphatase; d) aryl sulphatases. Acid hydrolase secretion

requires potent agonists, and is slow and incomplete. The different acid hydrolases are secreted to different extents. ß-Hexosaminidase is secreted most completely (about 60%). The secreted enzymes may be involved in clearing platelet thrombi, inactivation and degradation of heparin and possibly host defence, including anti-inflammatory functions.

1.2.8 Arachidonate liberation

Stimulated platelets release arachidonic acid from phospholipids by the hydrolytic action of phospholipases. Phosphatidylinositol, phosphatidylcholine and phosphtidylethanolamine are the most likely substrates (Bills *et al*, 1977). This arachidonic acid is oxygenated via the cyclooxygenase and lipoxygenase pathways to different active products. The cyclooxygenase pathway involves conversion of arachidonate to prostaglandin G_2 (PGG_2) and peroxidation of PGG_2 to prostaglandin H_2 (PGH_2); both steps are catalyzed by cyclooxygenase (Hamberg and Samuelsson, 1974). This enzyme is inhibited by non-steroidal anti-inflammatory drugs. For example, inhibition is irreversible with aspirin and reversible with indomethacin. Inhibition by aspirin involves acetylation of the active site of the enzyme (Roth *et al*, 1975). PGG_2 and PGH_2 may be converted to the unstable thromboxane A_2 (TXA_2) by thromboxane synthetase (Hamberg *et al*, 1975). PGG_2, PGH_2 and TXA_2 are potent platelet activators. Thus, the response to platelet agonists which liberate arachidonic acid is amplified by this positive feedback mechanism. TXA_2 is rapidly and non-enzymatically hydrated to inactive TXB_2.

The platelet lipoxygenase pathway results in the formation of 12-hydroxy-eicosatetraenoic acid (12-HETE) which is weakly chemotactic for leukocytes (Turner *et al*, 1975).

1.3 Physiologically important platelet agonists

Many different agents are able to elicit platelet activation as shown in Table 1.2. These include collagen, thrombin, ADP, adrenaline, prostaglandins, thromboxane, serotonin and platelet-activating factor (PAF).

Table 1.2. Major platelet-activating agents

Adenosine diphosphate (ADP)	Ristocetin
Adrenaline	Factor VIII
Collagen	Antiplatelet antibody
Thrombin	Immune complexes
Thromboxane A_2	Polylysine
Endoperoxides	Endotoxin
Platelet-activating factor (PAF)	Viruses
Serotonin	Glass
Vasopressin	Latex particles
Snake venom proteases	Trypsin

Collagen. This insoluble, major connective tissue glycoprotein is of great importance for haemostasis. Upon vessel damage, collagen fibrils become exposed on the subendothelium and comprise the initial agonist during platelet activation *in vivo*. Collagen fibrils differ from other physiological agonists because they are in an insoluble state. Platelets therefore have to adhere to the fibrils before activation can occur. There are different sub-types of collagen in the vessel wall. Each differs from the other with regard to their tertiary and quaternary structure. These subtypes are located at specific sites within the vascular wall: types I and III in the interstitial layer and types IV and V in the basement membrane (Chung and Miller, 1974). Type I and type III are very potent platelet agonists, whereas

other types only have very low stimulatory effects (Trelstad and Carvallo, 1979).

Thrombin. At a site of vascular injury, active α-thrombin is liberated by enzymatic cleavage of inactive prothrombin (Factor II). As well as its important enzymatic action on fibrinogen which terminates the coagulation cascade, thrombin is also the most potent platelet agonist *in vivo* and induces all platelet responses even when the cyclooxygenase pathway is blocked. Thrombin rapidly binds to specific receptors on the platelet surface (Tollefson *et al*, 1974). Two types of receptor have been identified: high affinity and low affinity.

ADP. There is normally no ADP in an accessible state in plasma. It may be released from damaged cells, such as erythrocytes, during vascular injury. The main source of ADP for platelet stimulation is secretion from platelet dense granules during activation. ADP induces shape change, aggregation and secretion of both alpha and dense granules. Its activity is largely dependent upon an active cyclooxygenase pathway being present. Two types of ADP receptors seem to be present on the platelet surface: one type conveying the platelet-stimulatory signal and the other type is coupled to the inhibitory adenylate cyclase system (Macfarlane *et al*, 1983).

Adrenaline. Adrenaline, liberated from the adrenal medulla, is normally present in plasma. It is a weak to moderate strength agonist. Unlike other physiological agonists adrenaline induces aggregation without causing an initial shape change. Adrenaline binds two different subtypes of receptor: α_2- and $ß_2$-adrenoreceptors (Grant and Scrutton, 1979). The stimulatory action of adrenaline is coupled to the α_2-receptor, whereas the $ß_2$-receptor is weakly coupled to the adenylate cyclase system (Kerry and Scrutton, 1983).

Prostaglandins and thromboxanes. Stimulatory prostaglandins (PGH_2 and PGG_2) and thromboxanes (TXA_2) produced during platelet activation from liberated arachidonate form

an important positive feedback mechanism. These agonists are of moderate to strong potency, and bind to specific receptors on the platelet surface (Armstrong *et al*, 1983).

1.4 Neutrophil responses

The main function of human neutrophils is to sense, approach and destroy invading microorganisms, in particular pyrogenic bacteria. They are an essential defence against these organisms.

Neutrophils are also increasingly implicated as mediators of tissue damage in inflammatory diseases, ranging from rheumatoid arthritis and myocardial reperfusion injury to respiratory distress syndromes, blistering skin disorders and ulcerative colitis (Malech & Gallin, 1987). In each of these diseases, as well as a variety of other acute inflammatory disorders, important components of the pathological processes are being attributed to the neutrophil's ability to release a complex assortment of agents that can destroy normal cells and dissolve connective tissues. Although these toxins normally defend the host against invading microbes, the neutrophil has little intrinsic ability to differentiate between foreign and host antigens and relies on other arms of the immune system (such as antibodies, complement and cytokines) to select its targets. If normal host tissues are inappropriately identified as foreign or damaged structures, the appropriate receptors on the plasma membrane of the neutrophil will be engaged, eliciting the cells destructive potential. Neutrophils are one of the most mobile mammalian cells, constantly leaving the vascular compartment. They crawl into tissues to exert their function and are able to sense distant microorganisms and actively move in their direction to engulf them. The killing of microorganisms in phagocytic vesicles occurs by release of microbicidal compounds from various populations of granules and the generation of highly reactive

oxygen metabolites.

The frequent and severe infections that occur in persons whose neutrophils are deficient in number or function testify to the central role of PMNs in host defence against microorganisms. The ultrastructure of a neutrophil reveals a cell extremely rich in various populations of granules, poor in mitochondria and rough endoplasmic reticulum, and with a dystrophic nucleus. Neutrophils are terminally differentiated cells, unable to replicate and are able to synthesize only small amounts of protein. PMNs kill bacteria and fungi by oxidative and non-oxidative mechanisms. The former depend on oxidants whose production follows the activation and assembly of an NADPH oxidase on the PMNs plasma membrane. These include hydrogen peroxide, hypochlorite, chloramines and hydroxyl radicals (Clarke, 1990).

The non-oxidative mechanisms reflect the actions of potent antimicrobial polypeptides residing within the PMNs cytoplasmic granules, which may be selectively released from different types of granule. The products released from granules and the active oxygen species alone or in combination are potently bactericidal.

1.4.1 Adherence and migration

In normal subjects PMNs represent the majority of intravascular leukocytes. There is also a large reservoir of PMNs in the lung vasculature and more specifically in the capillary bed. Some of these PMNs appear to be in intimate contact with the endothelial cells to form a "marginating pool". Adherence to the endothelium is probably the initial and prerequisite step in PMNs migration towards the alveolar lumen (Worthen and Henson, 1983).

Different substances can enhance PMNs adherence to the endothelium both *in vitro*

and *in vivo*. Among these substances the complement components C3a and C5a, and bacterial products such as endotoxin and peptide fragments of bacterial cell walls are potent inducers of PMNs adherence (O'Flaherty *et al*, 1978). Secretory products of phagocytes such as LTB_4, IL-1, TNF_α and lactoferrin and platelet-derived products have also been reported to enhance PMNs adherence (Palmblad *et al*, 1981; Boogaerts *et al*, 1982; Deuel *et al*, 1982; Gamble *et al*, 1985; Bevilacqua *et al*, 1985). Adhesion molecules expressed on neutrophil membranes have been shown to play a critical role in adherence. These integrins belong to a family of structurally and functionally related glycoproteins comprised of α and ß subunits. There are three types of α chain: IIa (LFA-1), IIb ($C3b_i$/Mac1) and IIc (p150,95). A membrane glycoprotein of PMNs called Mol or CD18 forms the ß chain. These complexes have been shown to be critical for adherence (Harlan *et al*, 1985, Arnaout *et al*, 1983).

A surface molecule called intercellular adhesion molecule (ICAM-1) present on endothelial cells has been shown to recognize the CD18 complex (Smith *et al*, 1988). The regulation of surface expression of CD-18 on PMNs and ICAM-1 on endothelial cells may be crucial for transendothelial migration of PMNs. *In vitro* observations have shown that PMNs as well as monocytes adhere to endothelial cells by a mechanism of indentation of the cell surface with multiple pseudopods (Migliorisi *et al*, 1987). Once they have adhered to the endothelium and if appropriately stimulated by a chemotactic gradient, PMNs begin to migrate either between or through endothelial cells towards their target for phagocytosis.

1.4.2 Chemotactic receptor activation

Chemoattractants may be products of microbial metabolism (such as formyl

peptides; N-formyl-methionyl-leucyl-phenylalanine (FMLP) is widely used to study neutrophil activation) or derive from the interaction of the immune and/or inflammatory system with microorganisms (for instance C5a, LTB_4, PAF). Chemoattractants activate neutrophils by interacting with specific cell surface receptors. Very low (nanomolar) concentrations of such chemoattractants induce movement of neutrophils towards the microorganism. At higher (μM) concentrations, that is, close to the microorganism or in the phagocytic vesicle, chemoattractants activate the microbicidal mechanism of neutrophils causing the release of granular enzymes and production of toxic oxygen radicals.

About one third of the granules in the cytoplasm of mature human PMNs contain myeloperoxidase (MPO) and because they appear first during the PMNs maturation in the bone marrow they have been called "primary" granules. Also, as primary granules, when immature, have an affinity for the azure components of dyes, they are also referred to as "azurophil" granules. Mature PMNs contain about 1500 azurophil granules (Damiano, *et al* 1988). The azurophil granules of PMNs show considerable heterogeneity of structure, density and composition (Rice, *et al*, 1987). These granules contain most of the cells antimicrobial effectors, including defensins, cathepsin G, lysozyme, azurocidin, and bactericidal/permeability increasing factor (BPI). The azurophil granules also contain hydrolases such as elastase, collagenase and ß-glucuronidase.

The peroxidase negative granules, generally referred to as secondary or "specific" granules, are produced after the azurophil granules in maturing cells. Such granules contain about half of the PMNs lysozyme and all of its stores of lactoferrin, vitamin B_{12} binding protein, adhesin receptors, chemoattractant receptors and gelatinase. In addition, the specific granules contain cytochrome b_{558} (also known as Cyt b_{-245}), a major

component of the PMNs NADPH oxidase complex (Segal & Jones, 1979).

The internal contents of PMNs granules probably remain inert so long as the surrounding granule membrane remains intact. Degranulation, the fusion of granules with the peripheral or perivacuolar plasma membrane, can insert membrane or internal contents into the cell or phagosomal membrane and deliver granule contents into the extracellular or phagosomal compartments. Degranulation of azurophil and specific granules can occur independently (Wright *et al*, 1977) and opsonic factors may influence which granules fuse to phagosomes (Joiner *et al*, 1989).

1.4.3 Proteolytic enzymes

Phagocytosis of microorganisms triggers the release of granule enzymes into the phagocytic vacuole. There are a dozen or so antimicrobial polypeptides that are packaged within the cytoplasmic granules of human PMNs. The PMNs applies these antibiotic molecules conjointly and in high concentration. The ensuing combat occurs in a structure called the phagocytic vacuole. As the principal peptide antibiotics in human azurophil granules exist in concentrations of 1-5 $\mu g/10^6$/PMNs, fusion of only 1% of these granules to a phagosome 1μm in diameter should result in intraphagosomal concentrations as high as 20-100 mg/ml for each polypeptide, (Lehrer and Granz, 1990).

The ability of PMNs and other phagocytes to sequester microorganisms in such vacuoles can also deprive the ingested organisms of nutrients, subject them to an unfavourable pH, and expose them to powerful oxidants.

Among the neutral serine proteases, PMNs elastase represents quantitatively the major component of the azurophil granules (3 $\mu g/10^6$ cells) (Campbell, 1986). This enzyme has been investigated extensively because of its potential role in emphysema.

PMNs elastase can cleave elastin, collagen, fibrinogen, fibronectin and proteoglycans. Elastase as well as the other PMNs proteases (cathepsin G and collagenase) can also activate and inactivate the complement components C3 and C5a, thereby regulating the inflammatory reaction (Goetzl, 1975). The major inhibitor of PMNs elastase in serum is α_1-proteinase inhibitor (α_1-antitrypsin).

The properties of cathepsin G, the chymotrypsin like enzyme of PMNs, are poorly defined. In addition to its depolymerizing effect on collagen, cathepsin G can degrade proteoglycans, can enhance the proteolyis induced by elastase and can activate gelatinase (Senior & Campbell, 1983).

Three additional neutral proteases are present in specific granules: plasminogen activator (PA), collagenase and heparanase. PA activates other enzymes mainly through the conversion of plasminogen to plasmin. Collagenase is stored in its latent form in specific granules. Its activation depends on the oxidative reaction, suggesting a combined effect of oxidants and enzymes in PMNs functions. Characterisation of the PMNs collagenase activity demonstrated preferential degradation of Type I collagen compared to Type III collagen (Horwitz, *et al*, 1977).

PMNs also contain a metalloproteinase that is active on basement membrane and is able to cleave α_1-proteinase inhibitor. PMNs gelatinase is also a metalloenzyme which degrades Type V collagen and renders the interstitial matrix more sensitive to subsequent attacks by other proteases. A similar role has been assigned to the more recently characterised PMNs heparanase. This is spontaneously released in the absence of any stimuli and specifically hydrolyzes heparan sulphate thereby rendering the interstitium more suitable for cell migration (Matzner, *et al*, 1985).

The differential release of these various enzymes by PMNs is probably critical in

inflammation. The major contents of neutrophil granules are shown in Table 1.3.

Table 1.3 Neutrophil granules and their major contents.

Primary (azurophil)	Secondary (specific)	Tertiary
Microbicidal agents		
Lysozyme	Lysozyme	
Myeloperoxidase		
Defensins		
Cationic proteins		
BPI agent		
Proteases		
Elastase	Collagenase	Gelatinase
Cathepsin G	Heparanase	
Acid hydrolases		
N-Acetylglucuronidase		
Cathepsins B and D		
ß-Glucuronidase		
ß-Glycerophosphatase		
Other		
C5a-Inactivating factor	Lactoferrin	
	Vitamin B_{12} BP	
	Plasminogen activator	

1.4.4 The NADPH oxidase system and oxygen metabolites

The phagocyte NADPH oxidase system is a membrane-associated enzyme complex that participates directly in the generation of at least three oxygen metabolites: the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH.). While the oxidase lies dormant in unstimulated neutrophils, triggered cells rapidly activate the enzyme system and begin to shuttle electrons from cytosolic NADPH to oxygen dissolved in the extracellular fluid. Under most conditions, one molecule of oxygen acts as an acceptor for a single, donated electron, resulting in the generation of one molecule of the superoxide anion (the dot in $O_2^{\cdot -}$ represents an unpaired electron, which makes the product a free radical, whereas the additional negative charge donated by the electron gives the

oxygen molecule a net charge of -1).

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^{\cdot -} + NADP^+ + H^+$$

In turn, two molecules of $O_2^{\cdot -}$ interact spontaneously (dismutation reaction) to generate one molecule of H_2O_2.

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Both $O_2^{\cdot -}$ and H_2O_2 can react with a number of important biological substrates (Fridovich, 1986), but intact neutrophils are somewhat limited in their ability to use either metabolite alone to cause extracellular damage. Thus, under most conditions the preferred substrate for one $O_2^{\cdot -}$ molecule is a second $O_2^{\cdot -}$ molecule and few other substances can compete with the already fast spontaneous disumation reaction (Fridovich, 1986). Secondly, although H_2O_2 is a stable oxidant that can exert a number of damaging effects, neutrophils themselves consume most of this metabolite and only a small part of the generated H_2O_2 can be detected in the extracellular pool (Test & Weiss, 1984). As the bulk of $O_2^{\cdot -}$ generated by the triggered neutrophil dismutes to H_2O_2 and this molecule in turn is quickly catabolized, the end product of oxidative metabolism depends on the fate of consumed H_2O_2. Thus, a second enzyme in the neutrophil granules, myeloperoxidase, in combination with H_2O_2 can oxidize halides (Cl^-, Br^-, I^- or thiocyanate) to their corresponding hypohalous acids (HOX). As the plasma concentration of Cl^- is more than a thousand times that of the other halides, the H_2O_2-myeloperoxidase system probably uses Cl^- at most sites *in vivo* to form hypochlorous acid (HOCl). Due to the high reactivity of HOCl, it does not actually accumulate in biological systems but instead almost instantaneously disappears in multiple reactions with available substrates (Test & Weiss, 1986). Readily oxidised microbial proteins, such as those with iron-sulphur centres or

biologically important sulphydryl groups, are among the many likely targets (Rosen & Klebanoff, 1989). HOCl participates in the generation of a derivative group of oxidants known as the chloramines (Grisham *et al*, 1984).

The list of oxygen metabolites generated in the phagocytosis-dependent respiratory burst has expanded to include hypohalides, especially hypochorite (OCl^-), formed through activity of the azurophilic granule enzyme myeloperoxidase; superoxide anion ($O_2.^-$), the initial conversion product of the consumed oxygen; and hydroxyl radical, a highly potent oxidant formed by the interaction of $O_2.^-$ and H_2O_2 in the presence of iron or copper (Haber-Weiss reactions) or between H_2O_2 and iron (Fenton reaction). More recently identified are chloramines, formed by the reaction of hypochlorite with ammonia or amines.

1.4.5 Bioactive lipids

Endogenous arachidonic acid is metabolized by PMNs and converted into different biologically active lipids through the cyclooxygenase and lipoxygenase pathways. Among the lipoxygenase metabolites, leukotriene B_4 (LTB_4) is probably the predominant secretory product of the PMNs. Other lipoxygenase metabolites released by PMNs include 5-HETE and 15-HETE and small amounts of leukotriene C_4 (LTC_4; Borgeat and Samuellson, 1979). Upon binding to specific membrane receptors LTB_4 induces different PMNs functions, such as chemotaxis, aggregation and adherence to endothelial cells (O'Flaherty *et al*, 1981). Compared to LTB_4, 5-HETE appears to be a minor stimulus of PMNs activity, but it can potentiate the stimulation induced by other mediators (Walshe *et al*, 1981). The common inactivation pathway of LTB_4 or 5-HETE is the ω-oxidation with the production of 20-OH-LTB_4 and 20-COOH-LTB_4, or 5s-20s-diHETE respectively

(Hansson *et al*, 1981; O'Flaherty *et al*, 1986). Despite reports suggesting the release of prostaglandins and thromboxanes by PMNs, the contribution of cyclooxygenase metabolites to PMNs secretory products is probably minor as compared to the products of the lipoxygenase pathway (Walshe *et al*, 1981; Goldstein *et al*, 1978).

In vitro release of platelet activating factor (PAF) by PMNs can be induced by various stimuli (Henson, 1981; Braquet and Rola-Pleszczynski, 1987). PAF is known to mediate numerous functions in platelets, mononuclear phagocytes, lymphocytes, eosinophils, PMNs and endothelial cells, and therefore may represent a major intermediate mediator between PMNs and other inflammatory cells.

1.5 Intracellular Regulatory Processes in Platelets and Neutrophils

Physiological platelet (or neutrophil) activation or inhibition is initiated when an extracellular signal molecule interacts with the cell surface. This interaction involves a ligand-receptor coupling wherein structurally and functionally heterogeneous molecules bind to one or more specific membrane receptors. With platelets there is remarkable chemical heterogeneity between the different extracellular signals that regulate platelet function. They include plasma constituents such as thrombin, plasmin and catecholamines; vascular products such as prostaglandin I_2 (PGI_2), endothelium derived relaxing factor (EDRF), and collagen; platelet products such as ADP and TXA_2; and stimuli derived from multiple blood cell and vascular sources such as PAF, von Willebrand factor and prostaglandin endoperoxides. Extracellular signals may be grouped as "strong agonists" (thrombin, collagen, prostaglandin endoperoxides, TXA_2 and PAF); "weak agonists", which depend on secretion to effect a full response (for example, ADP, adrenaline and serotonin); and antagonists (such as PGI_2 and EDRF).

Neutrophil activation is commonly initiated by the binding of ligands (e.g. chemoattractants) to their specific membrane receptors. Receptor engagement stimulates the generation of intracellular signals mediated by G-proteins which lead to cell specific effector functions (Gilman, 1987). The activity of G-proteins are modulated by bacterial toxins. Cholera toxin catalyzes the ADP-ribosylation of G_s reducing the GTPase activity and thereby causing a persistent activation of adenylate cyclase. Pertussis toxin catalyzes the ADP ribosylation of G_i inducing uncoupling of the receptor and thus terminating the hormone signal. Thus neutrophils and other leukocytes contain a unique pertussis/cholera toxin sensitive G-protein that mediates the chemoattractant induced cell-activation (Becker *et al*, 1985).

The major activating pathway uses a combination of second messengers derived from enzyme-linked hydrolysis of inositol phospholipids: inositol 1,4,5-triphosphate (IP_3) and *sn*-1,2-diacylglycerol (DG; Berridge, 1987). The major inhibitory signalling pathway uses the second messenger cyclic AMP. These pathways are initiated by occupancy of cell surface receptors for specific extracellular signals. Second messengers of both pathways are generated internally by activation of membrane-associated enzymes through receptor-linked changes in a family of signal transducing GTP-binding regulatory G-proteins. In each case, the activated signal-generating enzymes convert highly phosphorylated precursor molecules into intracellular second messengers, for instance phospholipase C (PLC) cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and DG, whereas adenylate cyclase converts ATP into cyclic AMP. The second messengers formed by these reactions exert their intracellular actions by inducing conformational changes in target proteins either directly or indirectly by activation of protein kinases: IP_3 releases calcium (Ca^{2+}) which binds to calmodulin

and causes activation of Ca^{2+}/calmodulin-dependent protein kinases (Marjerus *et al*, 1986), DG causes activation of protein kinase C (Berridge *et al*, 1987), and cyclic AMP causes activation of cyclic AMP-dependent protein kinases. The signal transduction pathways involved during platelet activation are illustrated in Figure 1.1.

1.5.1 G-proteins

Platelets have three functionally distinct, although not necessarily structurally unique, guanine nucleotide-binding regulatory (G) proteins (Neer and Clapham, 1988): G_p couples activating ligand-receptor interactions to stimulation of phosphoinositide-specific PLC; G_s couples inhibitory ligand-receptor interactions to activation of adenylate cyclase; and, G_i couples some platelet agonist-receptor interactions to inhibition of adenylate cyclase. Each of these G-proteins is a heterotrimeric complex (comprised of α-, ß-, and γ-subunits), which has GDP tightly bound to the α-subunit in the basal state. After receptor-ligand coupling occurs, GDP dissociates from the complex. Cytoplasmic GTP, in the presence of magnesium (Mg^{2+}), then binds to the open guanine nucleotide binding site on the α-subunit. This binding of GTP to the α-subunit results in dissociation of the α-subunit from the α/ß/γ complex. The dissociated G_α.GTP then interacts with an enzyme "signal amplifier" within the matrix of the plasma membrane to convert phosphorylated precursors into second-messengers molecules. In platelets, $G_{p,\alpha}$.GTP stimulates PLC-mediated hydrolysis of PIP_2 to DG and IP_3, while $G_{s,\alpha}$. GTP stimulates adenylate cyclase-mediated conversion of ATP to cyclic AMP. The ß/γ component remains a single, functional unit which reassociates with the α-subunit as the intrinsic GTPase activity of the α-subunit hydrolyses GTP to GDP, thus terminating the G protein-mediated signal. The ß/γ component may also be the relevant G protein moiety regulating inhibition of

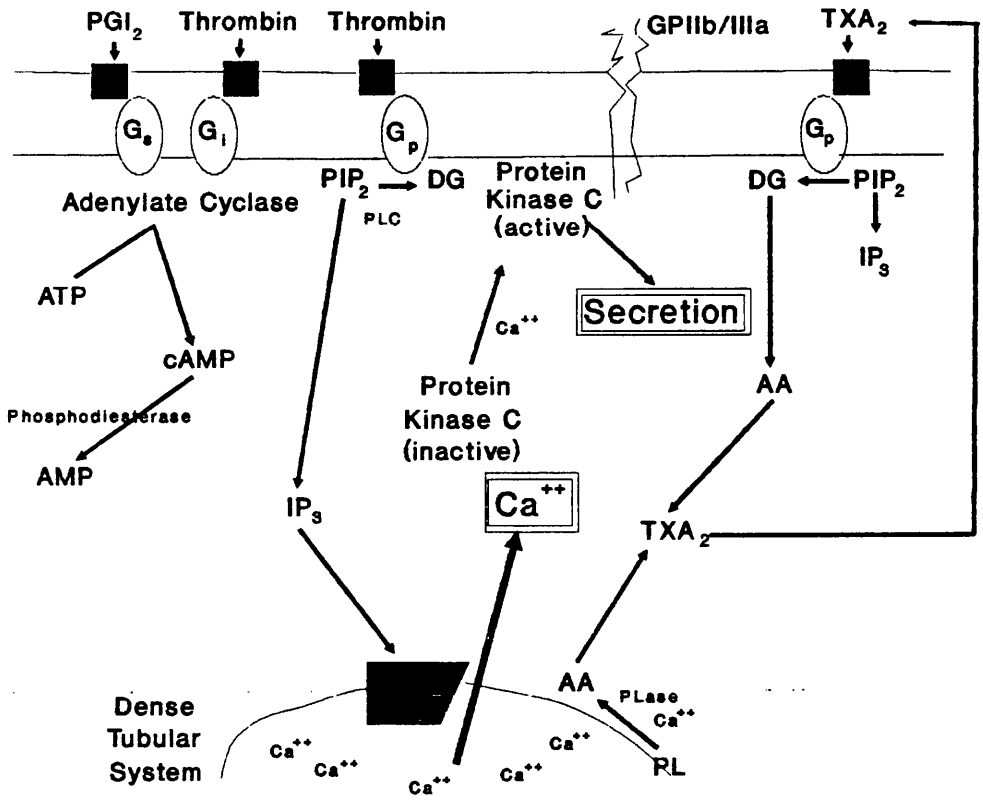

Figure 1.1. Signal transduction during platelet activation. Platelet activation is initiated by an agonist, such as thrombin, which binds to specific receptors on the cell surface. A cascade of intracellular second messengers is started which includes inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG). IP_3 releases calcium ions (Ca^{++}) from the platelet dense tubular system, thereby raising the cytosolic free calcium concentration. Diacylglycerol activates protein kinase C, switching it to the plasma membrane and triggering granule secretion and fibrinogen receptor exposure on the GPIIb/IIIa complex. Arachidonate (AA) formation by phospholipase A_2 is triggered by rising cytosolic free calcium and this generates thromboxane A_2 (TXA_2) which diffuses out of the cell, interacts with receptors on the platelet surface and causes further platelet activation.

adenylate cyclase (Casey and Gilman, 1988).

1.5.2 Inositol phosphates and cell signalling

In 1953 Hokin and Hokin reported the enhanced turnover of the phosphorylinositol group of phosphatidylinositol following the stimulation of various cells and tissues (Hokin and Hokin, 1953). They demonstrated in experiments using [^{32}P]phosphate, that acetylcholine-stimulated amylase secretion in pancreatic cells was accompanied by a dramatic increase in phospholipid turnover. It later became apparent that this catabolism of inositol phospholipids occurs in many cell types as a response to various signals. Later it was suggested that this stimulated metabolism may be related to receptor function. More specifically it was proposed that inositide metabolism was coupled to the activation of receptors that mobilise intracellular calcium (Michel, 1975). The hydrolysis of phosphatidylinositol 4,5 bisphosphate to yield IP_3 has been confirmed as the key event that causes the mobilisation of intracellular calcium following the stimulation of receptors. Cells contain a variety of soluble and membrane-bound enzymes that may be specific for phosphatidylinositol 4,5 bisphosphate in the presence of GTP or may hydrolyse phosphoinositol 4-monophosphate under certain conditions.

D-*myo*-inositol 1,4,5-triphosphate is the second messenger responsible for the release of calcium from non-mitochondrial stores (probably a compartment of the endoplasmic reticulum). It was initially thought that IP_3 was inactivated via specific 5-phosphate hydrolysis. However, further phosphorylation/dephosphorylation pathways have been discovered in various cell types. IP_3 is phosphorylated to inositol 1,3,4,5-tetrakisphosphate, which may regulate the entry of calcium into cells (Irvine and Moore, 1986). Specific binding sites for tetrakisphosphate have been demonstrated

(Bradford and Irvine, 1987) indicating that it too has second messenger status. Inositol 1,3,4,5-tetrakisphosphate is hydrolysed by the same 5-phosphatase to inositol 1,3,4-triphosphate which in turn may be phosphorylated to inositol 1,3,4,6-tetrakisphosphate. Further phosphorylation leads to the occurrence of inositol 1,3,4,5,6- pentakisphosphate (IP_5) and phytate (IP_6) in some mammalian cells. Dephosphorylation of tris- and tetrakisphosphates results in the generation of various inositol bis- and monophosphates whose hydrolysis back to *myo*-inositol may be perturbed by lithium ions. The significance of many of these compounds *in vivo* remains unclear but they may help regulate diverse cellular functions such as proliferation and membrane permeability.

1.5.3 Diacylglycerol and protein kinase C

In stimulated human platelets and neutrophils, PLC generated DG is released into the matrix of the plasma membrane and then rapidly recycled back into the phosphoinositides through its phosphorylation to phosphatidic acid by the enzyme diacylglycerol kinase. Membrane-bound DG triggers the translocation of inactive protein kinase C (PKC) from the cytosol to the membrane. PKC is then activated in the presence of calcium (Ca^{2+}) and phosphatidylserine. DG increases the affinity of inactive PKC for calcium so that a slight increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is required to effect PKC activation. PKC can be activated directly and independently of PLC by the *in vitro* use of phorbol esters or synthetic DG. Such experiments have demonstrated that PKC activation is associated with aggregation, secretion, metabolism of arachidonic acid and activation of neutrophil NADPH-oxidase. In combination with coincident platelet activation by calcium ionophore these direct PKC activators cause synergistic responses

(Kaibuchi *et al*, 1983). Physiological and pharmacological activation of PKC is associated with phosphorylation of a 47kD protein which serves as a useful marker of PKC activation (Sano *et al*, 1983).

Certain inhibitory events counterbalance the factors responsible for calcium mediated platelet activation. Foremost is the generation of cyclic AMP, a molecule that antagonizes calcium dependent activation events. In addition, the 5-phosphatase that converts IP_3 to IP_2, and consequently inactivates the IP_3 signal for calcium flux, is activated by PKC. This feedback inhibition by PKC regulates the calcium pathway of platelet activation from which the PKC pathway initially diverges (Connolly *et al*, 1986).

1.5.4 Inhibition by cyclic nucleotides

Activation of platelets and neutrophils is counterregulated by biochemical processes which attenuate or prevent agonist-induced responses. Cyclic AMP is of paramount importance and is produced in these cells as a consequence of extracellular signals such as prostaglandin I_2.

Cyclic AMP. Cyclic AMP decreases thrombin binding to human platelets and with this inhibits formation of a signal transducing complex (Lerea *et al*, 1987). Cyclic AMP inhibits the formation of DG and IP_3 by PLC (Knight and Scrutton, 1984). Cyclic AMP may reduce the DG signal for PKC activation by increasing its metabolism to phosphoinositides (Lapetina, 1986); it also directly inhibits the activity of PKC (De Chaffoy de Courcelles *et al*, 1987). Most importantly cyclic AMP antagonizes calcium mediated responses by influencing the uptake and release of calcium from the dense tubular system of platelets (Feinstein *et al*, 1983).

Cyclic GMP. Guanylate cyclase converts GTP to cyclic GMP following platelet or

neutrophil stimulation. Increasing platelet cyclic GMP with sodium nitroprusside or nitric oxide inhibits subsequent platelet responses to agonists, possibly by affecting the activation of PLC (Mellion *et al*, 1981). Physiological activation of guanylate cyclase may be directed by EDRF, which may be nitric oxide (Palmer *et al*, 1987). EDRF inhibits agonist-induced platelet responses through selective activation of guanylate cyclase (Furlong *et al*, 1987). This is independent of cyclic AMP which appears to act synergistically with EDRF to effect inhibition of agonist-induced platelet aggregation (Macdonald *et al*, 1988). Under physiological conditions, cyclic GMP may serve to inhibit, or more likely to dampen the platelet response to agonists.

1.6 Cell-cell interactions

The milieu of vascular injury causes different cell-types to be in very close proximity to each other. Under such conditions both cellular elements, as well as released or secreted non-cellular elements, have an increased probability of triggering, inhibiting or modifying responses of other cells. Considerable evidence indicates that platelets or their products can contribute to the accumulation of leukocytes in a clot and to their activation. Conversely, activated leukocytes can alter platelet function. Similarly, both platelets and leukocytes are able to interact with vascular endothelial cells.

Several areas of interaction between platelets and neutrophils have been identified. These include eicosanoid metabolism, PAF formation, nitric oxide metabolism, PF4 and platelet-derived growth factor (PDGF) induced neutrophil chemotaxis and platelet activation induced by neutrophil proteolytic enzymes.

1.6.1 Eicosanoid metabolism

"Eicosanoids" is the collective name for unsaturated lipids derived from arachidonic acid ($C_{20:4\ n-6}$), or similar polyunsaturated fatty acid precursors, via the cyclooxygenase, lipoxygenase or epoxygenase metabolic pathways. This group of compounds includes prostaglandins, thromboxanes, leukotrienes, lipoxins and various hydroxy- and hydroperoxy-fatty acids.

The term "prostanoid" relates to those products of cyclooxygenase pathway that contain the 5-membered cyclopentane ring characteristic of the theoretical parent structure prostanoic acid. As well as the prostaglandins, thromboxanes which contain an oxane ring, are usually included in the prostanoid group as they are also derived from the cyclooxygenase pathway (Samuelsson *et al*, 1978; Moncada, 1982).

The lipoxygenase products, such as leukotrienes, lipoxins and various hydroxy- and hydroperoxy-fatty acids (Needleman *et al*, 1986), differ structurally from prostanoids. Leukotrienes are conjugated trienes produced by the action of 5-lipoxygenase. Lipoxins A and B are members of the group of trihydroxy tetraenes.

The other more recently described group is the NADPH-dependent cytochrome *P*-450 epoxygenase metabolites (Capdevila *et al*, 1982).

Interest in all these compounds centres around their ubiquity, biological activity and the fact that many of them have diverse and potent pharmacological activities that may also be of physiological or pathological significance. The biosynthesis of eicosanoids requires non-esterified (free) arachidonic acid (or similar polyunsaturated fatty acid) as precursor. This is provided from membrane phospholipids by the action of phospholipase A_2, although alternative sources and routes of supply may exist. The arachidonate cascade for eicosanoid formation is shown in Figure 1.2.

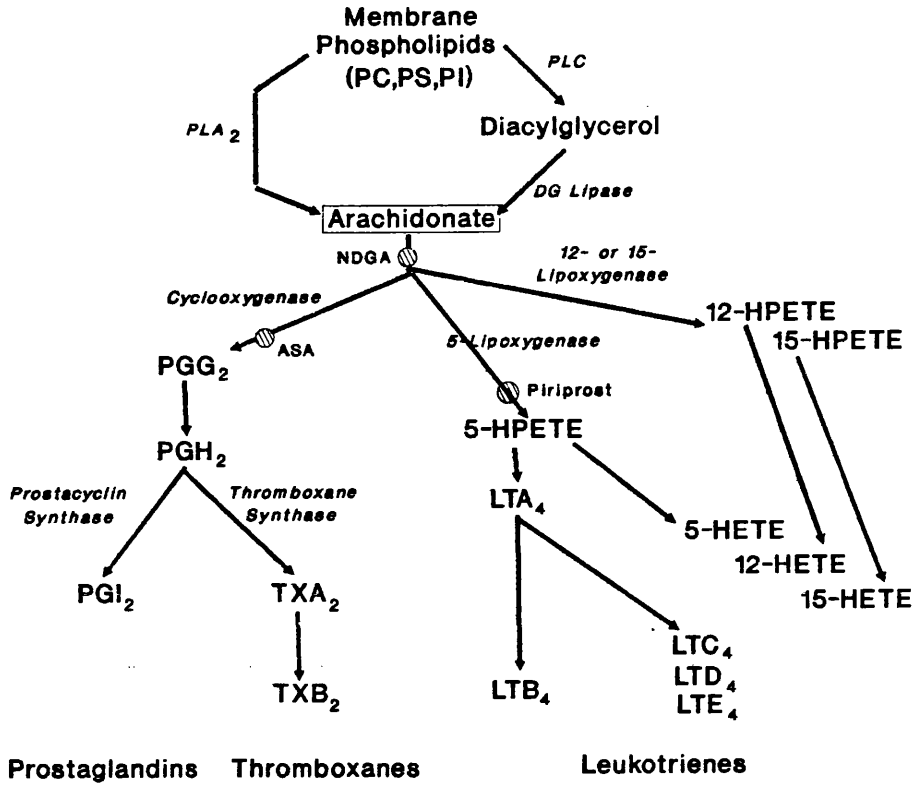

Figure 1.2. The arachidonate cascade.

The concept that eicosanoid precursors and intermediates can be metabolized by different cell types stems from several of their unique biological properties: (i) they are not stored in tissues but are transitory biological substances (autocoids) synthesized only in response to stimulation; (ii) eicosanoid production is qualitatively tissue specific, and these labile lipid autocoids exert their effects in the immediate microenvironment of the cell; (iii) eicosanoids enhance or synergize the inherent functional capacity of a tissue without being the sole modulator of a particular cell function. The functional integrity of a cell is rarely abolished by pharmacological inhibition, rather cell responsiveness is attenuated. Such properties can be seen with regulation of blood flow, smooth muscle relaxation or contraction, and platelet stimulation or inhibition. It can be seen therefore

that a physiological or pathological stimulus is potentially capable of eliciting responses from different cell types either because the stimulus is "recognised" by multiple cells or because it induces release of multiple precursors or intermediates which can be shared and metabolized simultaneously. At an inflammatory site or thrombus, which may include leukocytes, platelets, endothelial cells, or smooth muscle cells, eicosanoids are able to modulate multicellular events.

1.6.2 Classification of eicosanoid mediated cell-cell interactions (Marcus AJ, 1986)

Type I. The sharing of a common precursor by two or more cells in close proximity. Some cells are capable of synthesizing their own eicosanoid precursors, but can also acquire these precursors from a nearby cell. This results in the production of more end products than the cell could have produced individually (Marcus, 1986). An example of this is the utilization of platelet-derived endoperoxides by aspirin-treated endothelial cells for the production of prostacyclin (Marcus *et al*, 1980). Platelets radiolabelled with arachidonate were incubated with a suspension of unlabelled aspirin-treated endothelial cells. Stimulation with thrombin, collagen or calcium ionophore resulted in the production of radiolabelled prostacyclin. This could only have originated from labelled platelets. Also, platelets in these mixtures were unable to aggregate because of the prostacyclin generated.

Another example of this type of interaction is the metabolism of platelet arachidonate by stimulated neutrophils for the synthesis of LTB_4 (Marcus *et al*, 1982). A mixture of platelets prelabelled with arachidonate and unlabelled neutrophils stimulated with calcium ionophore produced radiolabelled LTB_4 and 5-HETE. These products cannot be synthesized by platelets alone as they do not possess 5-lipoxygenase. This phenomenon may be of clinical significance as aspirin-treated platelets continue to release arachidonate.

This could then interact with other cells such as activated neutrophils and serve as a source of proinflammatory mediators such as LTB_4 and 5-HETE.

Lipoxin A_4 formation by platelets in the presence of purified LTA_4 (from neutrophils) has also been demonstrated (Serhan and Sheppard, 1990). Platelet 12-lipoxygenase and its ω-6-oxygenase activity were implicated in this process.

Another form of Type I interaction is where a given cell-type is unable to form a precursor endogenously but possesses mechanisms for further processing the precursor if it can be obtained from another stimulated cell-type in the microenvironment. For example, incubation of human platelets with radiolabelled LTA_4 in the presence of reduced glutathione resulted in the production of radiolabelled LTC_4. As platelets contain glutathione-S-transferase, the exogeneously provided LTA_4 was directly converted to LTC_4 (Pace-Asciak *et al*, 1986). Red cells have also been shown to be capable of conversion of exogenous LTA_4 into LTB_4 (Fitzpatrick *et al*, 1984).

Type II The capability of one cell to transform an eicosanoid from another cell into a new product which neither cell can synthesize alone. Type II cell interactions may be subdivided according to whether one or both cell-types have been activated. If both cell-types have been activated then, for example, a suspension of tritiated arachidonate-labelled platelets stimulated by calcium ionophore (also a neutrophil agonist) in the presence of unlabelled neutrophils produce 5s-12s-dihydroxyeicosatetraenoic acid (5s-12s-diHETE). 12-HETE released from platelets is converted to 5s-12s-diHETE by neutrophil 5-lipoxygenase which has also been activated by calcium ionophore (Marcus *et al*, 1982). If purified 12-HETE is used to replace the platelets then the same result occurs. Similarly if radiolabelled 5-HETE is added to stimulated platelets then again 5s-12s-diHETE is formed. If only one of the cell-types is activated, for example tritiated

arachidonate-labelled platelets (with thrombin or collagen, which do not stimulate neutrophil eicosanoid release) in the presence of unlabelled neutrophils, a labelled compound appears: 12s-20s-dihydroxyeicosatetraenoic acid (12s-20s-diHETE), a novel compound that cannot be synthesized by by platelets or neutrophils alone. In this cell-cell interaction, unstimulated neutrophils hydroxylate released platelet 12-HETE to 12s-20s-diHETE.

Type III. The ability of an eicosanoid synthesized by one cell-type to act as an agonist or as an inhibitor for another cell. It can be demonstrated under certain experimental conditions that peptide containing leukotrienes (LTC_4, LTD_4 and LTE_4) can induce biological effects attributable to induction of cyclooxygenase products in tissues and organs (Piper, 1983). Similarly, 12-HETE, 5-HETE and 15-HETE can each independently inhibit prostacyclin release from stimulated endothelial cells (Hadjiagapiou and Spector, 1986).

Another example is the synthesis of LTB_4 by neutrophils upon stimulation with released platelet 12-HPETE (Maclouf *et al*, 1982). Interestingly, lipoxygenase-deficient platelets from patients with myeloproliferative disorders do not significantly stimulate LTB_4 synthesis during platelet-neutrophil interactions because they have defective 12-HETE production (Kanaji *et al*, 1986). This defect may result in poor responsiveness at sites of thrombosis or inflammation in patients with platelet lipoxygenase deficiency.

1.6.3 PAF-acether formation

In neutrophils, PAF-acether (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) and arachidonic acid metabolites (such as leukotrienes) share a common biochemical pathway (Sisson *et al*, 1987). Choline-containing phospholipids as well as

1-O-alkyl-2-arachidonyl-GPC may serve as a substrate for phospholipase A_2 (Chilton *et al*, 1984). The phospholipase A_2 released arachidonic acid is subsequently converted to LTB_4, while lysophosphatidyl choline or 1-O-alkyl-2-lyso-GPC are converted to PAF by acetylation. PAF-acether has a wide spectrum of biological activity; it is, in particular, a potent platelet and neutrophil agonist. PAF-acether evokes different symptoms of acute inflammation, asthma, and thrombosis. It was initially described as a mediator of immediate hypersensitivity (Benveniste *et al*, 1972). It is formed by various cell-types, mostly proinflammatory ones, under various immune and non-immune stimuli. The same biosynthetic pathway is operative in both platelets and neutrophils. Cooperation between platelets and neutrophils for PAF-acether formation has been reported (Cöeffier *et al*, 1990). When washed platelets and neutrophils were incubated together *in vitro* and stimulated with thrombin and opsonized zymosan, respectively, they formed more than twice as much PAF-acether as did either platelets or neutrophils stimulated separately. This was found to be due to platelet contribution of lyso-PAF-acether to neutrophils which subsequently converted it to PAF-acether. This cell-cell interaction is of interest since PAF-acether is formed by and acts on platelets and neutrophils and represents a molecular basis for potent amplification of inflammatory reactions.

1.6.4 PF4- and PDGF-induced neutrophil chemotaxis

Several components in the coagulation process are reported to be chemotactic for leukocytes *in vitro*, these include: kallikrein, fibrin fragments, Hageman factor fragments and tissue plasminogen activator. The physiological role of these chemotaxins is unclear. Platelets themselves may contribute a variety of chemotactic mediators of inflammation when they release preformed vasoactive substances, synthesize lipid chemotaxins or

enzymatically cleave complement to form peptide chemotaxins (Turner *et al*, 1977; Weksler and Coupal, 1973). The platelet alpha granule proteins PDGF and PF4 are also chemotaxins (Deuel *et al*, 1982; Goldberg *et al*, 1980). PDGF is best known as a competence factor for fibroblast and vascular smooth muscle cell proliferation but at very low concentrations (low picomolar) it is chemotactic. It is postulated that the major function of such chemotaxis is the recruitment of cells to a site of healing. PDGF binds strongly to the extracellular matrix, is very stable and so could have protracted biological effects. PF4 is a highly cationic protein that antagonizes heparin. On release from platelets it is rapidly taken up by the endothelium where it enters the vascular wall. PF4 is chemotactic for both neutrophils and monocytes and may help direct these cells into the vessel wall following tissue damage (Deuel *et al*, 1981). PF4 has also been reported to cause a conformational change in elastin so that additional elastase-sensitive sites are exposed for enzymatic degradation. Human leukocyte elastase levels are raised in diseases such as chronic pulmonary obstructive disease and pneumonia. Platelet activation is also enhanced making it possible for local release of these products in inflammatory foci. This may contribute to the breakdown of lung elastin in these diseases, where oxidative inhibition of α_1-proteinase inhibitor, the major inhibitor of elastase, is also enhanced (Lonky and Wohl, 1981).

1.6.5 Neutrophil-derived oxygen metabolites and proteolytic enzymes.

Platelet function is altered by neutrophil oxygen metabolites. When platelets are exposed to phagocytosing neutrophils they exhibit reduced aggregation. Hydrogen peroxide may be the soluble effector of this response as it is reversed by catalase (Levine *et al*, 1976). In the presence of myeloperoxidase and a halide, very low concentrations of

hydrogen peroxide induce platelet serotonin release (Clark and Klebanoff, 1979). Superoxide also induces serotonin release and acts synergistically with thrombin to activate platelets (Handin *et al*, 1977).

Enzymes released from neutrophils alter platelet reactivity and can affect the activity of factors released by platelets. Exposure of fibrinogen receptors on the platelet membrane is essential for fibrinogen binding and subsequent platelet aggregation. Platelets treated with proteolytic enzymes can aggregate spontaneously. This may be one cause of thrombocytopenia in sepsis or acute leukaemia. In both of these conditions high levels of proteolytic enzymes may be detected. Low concentrations of neutrophil elastase in particular have been shown to cause fibrinogen receptor exposure with spontaneous aggregation in the presence of fibrinogen (Kornecki *et al*, 1986). This aggregation could not be inhibited by prostacyclin or other agents that raise cyclic AMP unlike the reversible aggregation which normally occurs when fibrinogen receptors are exposed on intact platelets by physiological agonists. At physiologically relevant concentrations human leukocyte elastase has also been reported to inhibit stimulated platelet aggregation and serotonin release. This loss of function was related to proteolytic inactivation of high-affinity thrombin receptors and cleavage of glycoprotein Ib on the platelet membrane (Brower *et al*, 1985).

Dallegri and co-workers (Dallegri *et al*, 1989) reported that platelets were able to inhibit the chemiluminescence response of neutrophils activated with phorbol myristate acetate. This inhibition of neutrophil chemiluminescence could be prevented by pulsing the platelets with carmustine, a glutathione cycle inhibitor. The carmustine (BCNU) preventable platelet-induced inhibition of chemiluminescence may reflect the consumption of neutrophil-derived hydrogen peroxide by the platelet glutathione cycle with subsequent

impairment of hypochlorous acid production. Platelets may therefore be able to limit the oxidant production by neutrophils and so protect vascular structures from neutrophil-mediated oxidative stresses.

1.6.6 Nitric oxide metabolism

The contribution of the vascular endothelium to the regulation of blood vessel tone and permeability, blood coagulation and platelet reactivity is well documented. The release of prostacyclin has powerful vasodilator effects (Moncada *et al*, 1976) and exerts its inhibitory action upon platelets by stimulating adenylate cyclase and thereby raising cyclic adenosine monophosphate (cyclic AMP; Gorman *et al*, 1977).

The discovery of the obligatory role of the endothelium in the vascular relaxation induced by acetylcholine and the demonstratiom that a humoral factor was involved in this action led to the identification of many stimuli which induce endothelium dependent relaxation and the description of a number of inhibitors of this mechanism. This extremely labile, vasoactive substance was named "endothelium-derived relaxing factor" (EDRF). The discovery of EDRF (Furchgott and Zawadski, 1980) has sparked further interest in the search for endogenous modulators of both vascular smooth muscle and platelet function. A number of substances are now known to lead to the release of EDRF, including calcium ionophores, adenine nucleotides and bradykinin. Speculation about the structure of EDRF continued until 1986 when Furchgott and Ignarro suggested that it may be nitric oxide (.N=O). Nitric oxide has indeed been shown to account for the action of EDRF (Palmer *et al*, 1987). Using a bioassay consisting of a strip of smooth muscle superfused by effluent from a column containing porcine aortic endothelial cells immobilized on microcarriers, they showed that release of EDRF from bradykinin-

stimulated cells led to relaxation in the smooth muscle strip. A number of indirect experiments showed the structure of EDRF to be consistent with the chemical properties of nitric oxide. Then using chemiluminescence detection, they observed directly the formation of .N=O. Nitric oxide is synthesised from the terminal guanidino nitrogen atom(s) of L-arginine (Sakuma *et al*, 1988; Palmer *et al*, 1988). The identification of EDRF as nitric oxide suggests a mechanism of action for the vasodilatory drugs such as amyl nitrate and nitroglycerin. These drugs, used since the nineteenth century, will produce nitric oxide in the presence of thiols such as glutathione thereby circumventing the generation of nitric oxide from its physiological substrate. Nitric oxide relaxes vascular smooth muscle (Griffith *et al*, 1984), inhibits platelet adhesion (Radomski *et al*, 1987) and platelet aggregation (Furlong *et al*, 1987). Nitric oxide, being a lipophilic molecule readily permeates the plasma membrane forming a complex with the haem group of soluble guanylate cyclase. The nitric oxide-haem complex activates soluble guanylate cyclase thereby elevating cyclic guanosine 3'5' monophosphate (cyclic GMP) levels with a subsequent inhibition of platelet aggregation (Mellion *et al*, 1981). Cyclic GMP probably exerts its action by affecting the activation of phospholipase C and so decreasing the intracellular free calcium concentration.

The action of nitric oxide may be characterised pharmacologically. It is destroyed by oxygen and superoxide anions (Rubanyi and Vanhoutte, 1986; Gryglewski *et al*, 1986) but not by other reactive oxygen species. Hence superoxide dismutase potentiates the effects of nitric oxide. Selective cyclic GMP phosphodiesterase inhibitors, such as M&B22,948, also potentiate the effect of nitric oxide by blocking hydrolysis of cyclic GMP. On the other hand, haemoglobin, ferrous ions and methylene blue inhibit the effect of nitric oxide through a mechanism not involving superoxide.

Macrophages (Hibbs *et al*, 1987) and more recently neutrophils have been shown to be capable of releasing a factor *in vitro* that has the pharmacological profile of nitric oxide. The formation and release of nitric oxide by neutrophils has been demonstrated by direct nitric oxide-specific chemiluminescence (Schmidt *et al*, 1989; Wright *et al*, 1989); by inducing vascular smooth muscle relaxation (Rimele *et al*, 1988); and by inhibition of platelet aggregation (McCall *et al*, 1989, Salvemini *et al*, 1989).

The L-arginine pathway for nitric oxide production underlies a variety of other biological processes, including cytotoxicity of phagocytic cells (Hibbs *et al*, 1987) and neurotransmission (Garthwaite *et al*, 1989).

1.7 Pathogical implications of platelet-neutrophil interactions

Over the past twenty years the role of the platelet and red cell in thrombosis has become increasingly recognized, as has the importance of plasma factors such as fibrinogen. Similarly, the role of neutrophils in the inflammatory response to ischaemia and tissue damage is better understood. However, less attention has been paid to the way in which different cell types interact. Platelet interactions with other cell lines such as, neutrophils, macrophages, endothelial cells and fibroblasts suggest major mechanisms that include: release of platelet agents that promote neutrophil chemotaxis or cellular adhesiveness, thereby entrapping these potent effector cells at sites of injury; release of platelet factors that serve as substrates for production of non-platelet vasoactive substances by other cell lines; and production by platelets and other cell types of membrane protective agents.

One situation where platelet-neutrophil interactions have been implicated is in the pathophysiology of septic shock and Multiple System Organ Failure Syndrome (MSOF).

1.7.1 The role of platelet-neutrophil interactions in septicaemic shock.

The microcirculation is the primary target organ to undergo both morphological and functional change following trauma and sepsis. The initial pathomorphological and pathophysiological disturbances following trauma (including tissue destruction and ischaemia-reperfusion injury) are caused by non-bacterial inflammation of the specific organ. This may later be a place of least resistance for subsequent inflammation caused by bacteria with endotoxin (or other bacterial toxins) as the main stimulants. In addition, the impaired specific immune response (immunosuppression) aggravates the situation (Faist *et al*, 1987). As a result MSOF develops. It may involve all vital organs and is frequently fatal (Goris *et al*, 1985).

Adult Respiratory Distress Syndrome (ARDS) is frequently associated with MSOF. ARDS is a form of acute non-cardiogenic oedamatous lung injury with a mortality rate approaching 100% if two other organs are involved.

The presence of an endotoxaemia is a common observation in the pathogenesis of MSOF and results in multisystem activation. However, the underlying mechanisms that initiate lung and other tissue injury remain obscure. Much research has focused on the role of vasoactive substances (such as complement, fibrin, prostaglandins, thromboxane and leukotrienes) and cellular agents (such as platelets, neutrophils, macrophages and endothelial cells). There is a complex interrelationship of these factors in the pathophysiology of both MSOF and ARDS. Available evidence implicates both the platelet and neutrophil as key components in the pathogenesis of acute septicaemic shock.

Post-mortem examination has shown sequestration of platelets and neutrophils interlaced with a fibrin mesh, in the pulmonary circulation adjacent to areas of endothelial injury following ARDS (Pietra *et al*, 1981). The presence of increased quantities of

degranulated neutrophils on bronchoalveolar lavage fluid from patients with ARDS further substantiates their location to to regions of alveolar capillary damage (McGuire *et al*, 1982). Animal models also suggest the involvement of neutrophils in pulmonary endothelial damage. Animal models of lung oedema and injury are attenuated in animals made neutropenic prior to infusion of agents, such as endotoxin, which produce pulmonary oedema (Heflin and Brigham, 1981).

Administration of *E.coli* lipopolysaccharide (endotoxin) to rats causes hyporesponsiveness to several contractile agents such as noradrenaline and vasopressin (Fink *et al*, 1985; Schaller *et al*, 1985). Nitric oxide has an important role in determining vascular function and reactivity. There is some indirect evidence that nitric oxide production is increased in certain pathological conditions. For example, urinary excretion of nitrate, a stable product of nitric oxide, is increased in patients during infection and in animals administered lipopolysaccharide (Stuehr and Marletta, 1985). Nitric oxide is a potential mediator of the hypotension and vascular failure seen in septicaemic shock. Modification of vascular activity by endothelium-derived nitric oxide is due to an increase in cyclic GMP content of smooth muscle (Holzmann, 1982).

Recent results from Fleming and colleagues suggest that endotoxin-induced hyporeactivity results from activation of an L-arginine dependent pathway producing a nitric oxide-like factor in non-endothelial cells (Fleming *et al*, 1990; Julou-Schaeffer *et al*, 1990). However, the exact mechanism of endotoxin-induced release of nitric oxide in circulatory shock *in vivo* remains to be investigated, as does the source of nitric oxide (whether from endothelial cells, activated macrophages or neutrophils).

Substances released by neutrophils and platelets such as oxygen radicals, eicosanoids and proteolytic enzymes have properties which could account for many of the

observed symptoms of MSOF. When generated in the pulmonary vasculature these substances may directly damage the endothelium, causing a permeability defect, promoting vasoconstriction and recruiting additional platelets, neutrophils and other leukocytes to an inflammatory site. Early in the course of lung injury platelets are sequestered in the lung. During aggregation platelets release substances that promote both bronchorestriction and vasoconstriction thereby contributing to pulmonary hypertension and ventilation perfusion mismatch. However, whether platelets do indeed participate in lung injury or are just innocent bystanders is still uncertain. Nevertheless available data does support the following hypothetical mechanisms for a platelet role in MSOF.

During the course of MSOF and ARDS platelets are stimulated to aggregate by circulating factors or localized pulmonary inflammation. During aggregation, platelets release substances that promote vasoconstriction (such as TXA_2 and serotonin), thereby contributing to pulmonary hypertension and bronchoconstriction, which worsen ventilation-perfusion relationships. Platelet products may not directly damage alveolar-capillary membranes, but the generated vasoconstriction may augment lung oedema by hydrostatic mechanisms in the setting of an underlying permeability defect. Platelets entrapped in the lung may activate complement and further release metabolites of arachidonic acid, serotonin and platelet-specific proteins that recruit neutrophils, stimulating them to release toxic substances. Neutrophil-induced injury may be further amplified by the bioconversion of platelet arachidonic acid and 12-HETE through the neutrophils 5-lipoxygenase pathway, producing novel factors that damage the alveolar-capillary membrane and aggregate additional platelets and neutrophils. The enhanced production of LTB_4 by neutrophils in the presence of platelets could also serve to recruit and aggregate more neutrophils.

Endothelial cells injured in this sequence of events in turn release PAF acether, attracting additional platelets and neutrophils to the pulmonary vascular bed. These activated platelets not only cause further damage, but also supply various growth factors required by the endothelium to sustain injury and generate repair. Platelet release of endoperoxides can be further metabolized by the endothelial cells to PGI_2, thereby promoting vasodilation and limiting platelet aggregation. If injury progresses unabated, mitogens (such as PDGF) released by platelets in a wound healing effort may stimulate excess fibroblast deposition of collagen with subsequent pulmonary fibrosis.

Much about ARDS and MSOF remains unclear but different studies confirm that no single pathway leads to acute oedematous lung injury. Rather, a diverse array of plasma factors, cell-types and additional undefined agents interact in a complex fashion to promote vascular membrane damage and respiratory failure.

1.8 Aims of thesis

This thesis aims to further investigate the interaction between platelets and neutrophils, in particular, the affect of neutrophils upon platelet function. Several potential pathways for interaction will be examined including eicosanoid metabolism by platelets in the presence of neutrophils and the action of neutrophil-derived nitric oxide release upon platelets.

Platelet-neutrophil interactions in washed, purified suspensions and in anticoagulated blood will be investigated.

Finally, the role of platelet-neutrophil interactions in blood from patients with, or at risk from, MSOF will be examined.

CHAPTER 2

METHODS

2.1 Blood samples, collection and handling.

Samples. Blood samples were obtained from apparently healthy, human volunteers and from critically ill patients under intensive care. Ethical permission was obtained for sampling from all patients.

Reagents. All reagents were from Merck Ltd, Poole, Dorset.

Anticoagulants: *tri*-sodium citrate, this was prepared as a 0.108M solution in distilled water;

Acid-citrate-dextrose (ACD), pH 4.5, comprised of:

66mM *tri*-sodium citrate, 85mM citric acid.H_2O, 111mM D-glucose.

Method. Blood samples were obtained from an anti-cubital vein by fresh venepuncture using a 21-gauge needle. Blood was collected into plastic tubes containing *tri*-sodium citrate (1 part anticoagulant to 9 parts blood) for studies using whole blood and platelet-rich plasma or into ACD (1 part anticoagulant to 6 parts blood) for studies involving washed cells.

Blood was kept at room temperature and experiments performed within three hours of collection.

All handling of blood was kept to a minimum and performed with polypropylene tubes and pipettes.

2.2 Full blood, differential, platelet and leukocyte counts

These were performed with the Coulter S+IV (Coulter Electronics Ltd, Luton, Beds.) cell counter. Some manual differential counts were performed on Romanowsky stained peripheral blood films or purified neutrophil samples.

2.3 Preparation of platelet-rich plasma (PRP)

Blood samples anticoagulated with either ACD or *tri-* sodium citrate were centrifuged at 170*g* for 10 minutes. PRP was removed with a plastic pasteur pipette and stores at room temperature in a capped tube. The residual blood was then either centrifuged at 2700*g* for 15 minutes and the platelet-poor plasma collected, or it was used to prepare washed leukocytes.

2.4 Preparation of washed platelets.

2.4.1 Gel filtration

Reagents. All reagents were from Merck Ltd, Poole, Dorset, unless stated otherwise.

Sepharose 2B-CL (Pharmacia Ltd, Milton Keynes, Bucks.). This gel was packed into a 60ml plastic column.

Calcium-free Tyrodes buffer, pH 7.4, containing per litre: sodium chloride, 8g; potassium chloride, 0.2g; disodium hydrogen orthphosphate.$2H_2O$, 0.065g; magnesium chloride.$6H_2O$, 0.415g; sodium hydrogen carbonate, 1g; D-glucose, 1g; bovine serum albumin (essentially fatty acid free; Sigma Chemical Company, Poole, Dorset), 2g.

Method. The Sepharose 2B-CL was pre-equilibrated with the Tyrodes buffer containing albumin. PRP (5-10ml) was then laid onto the column and allowed to enter the gel before adding more Tyrodes buffer. The washed platelets were eluted in the void volume and

collected by visual observation of the change in opacity of the column effluent.

2.4.2 Centrifugation with inhibitors

Reagents. All reagents were from Merck Ltd, Poole, Dorset, unless stated otherwise.

Calcium-free Tyrodes buffer, pH 6.5.

Disodium EDTA: 100mM in Tyrodes buffer.

Prostacyclin analogue (ZK36,374; Schering Chemicals, Munich, Germany): 100ng/ml in saline.

Method. PRP was diluted 1:1 (%) with Tyrodes buffer which contained 10ng/ml ZK36,374 and 10mM disodium EDTA at room temperature. The diluted PRP was placed in plastic conical-bottomed centrifuge tube and centrifuged at 850*g* for 10 minutes. The supernatant was discarded and the pellet resuspended in buffer containing inhibitors. The platelets were again centrifuged. This process was repeated a further two times. The platelets were finally resuspended in calcium-free Tyrodes buffer, pH 7.4, without inhibitors.

2.5 Preparation of washed, purified neutrophils

Reagents. All reagents were from Merck Ltd, Poole, Dorset, unless stated otherwise.

Dextran T500 (Pharmacia Ltd, Milton Keynes, Bucks.) was dissolved in saline to give a 10% solution.

Phosphate buffered saline (PBS), pH 7.4, containing per litre: sodium chloride, 8g; potassium chloride, 0.2g; disodium hydrogen phosphate, 1.15g; potassium dihydrogen phosphate, 0.2g; D-glucose, 1g.

Histopaque-1077 (Sigma Chemical Company, Poole, Dorset).

Hanks balanced salt solution without calcium (HBSS; Sigma Chemical Company, Poole, Dorset), pH 7.4.

Method. Blood was collected into ACD anticoagulant and centrifuged at 170*g* for 10 minutes. The platelet-rich plasma was removed. Neutrophils were then washed essentially according to the method of Boyum (Boyum *et al*, 1968. The blood was diluted approximately twofold with PBS. 10% Dextran T500 in saline was then added to give a final concentration of 1%. The sample was then sedimented for 1 min/ml, for up to 30 minutes. The supernatant, leukocyte-rich plasma, was collected and the cells centrifuged at 170*g* for 5 minutes. The supernatant was removed and discarded. The cells were resuspended in 10ml of PBS and layered onto 5ml Histopaque-1077 and centrifuged at 400*g* for 20 minutes at room temperature. The supernatant was discarded and the pellet resuspended with 2ml of PBS. The red cells were hypotonically lysed with 6ml of ice-cold distilled water for 45 seconds followed rapidly by 2ml of 3.5% saline. The tube was topped up with PBS to 20ml and centrifuged immediately at 170*g* for 5 minutes. The cells were then washed twice with PBS at 170*g* for 5 minutes before finally being resuspended in Hanks balanced salt solution. The cells were store over ice and warmed to 37°C before use.

Quality control. Neutrophil purity was determined by Romanowsky staining on a film or cytospin. Only samples exceeding 95% neutrophils were used.

Cell viability. This was determined by trypan blue exclusion (0.1% in saline) and was greater than 95%.

2.6 Methods for studying platelet function

2.6.1 Turbidometric platelet aggregation

Reagents. All reagents were from Sigma Chemical Company, Poole, Dorset, and stored at -20°C unless stated otherwise.

ADP (grade III), a 10mM stock solution in saline; sodium arachidonate, a 20mM stock solution in distilled water; calcium ionophore A23187, a 1mM stock solution in dimethyl sulphoxide (DMSO); PAF-acether, a 1mg/ml stock solution in chloroform; and Formyl-met-leu-phe, a 1mM stock solution in DMSO. Collagen, a 1mg/ml stock solution, stored at 4°C (Hormon-Chemie, Munich, Germany). Bovine thrombin, a 10 units/ml stock solution in saline (Parke-Davis Ltd). Endoperoxide analogue U46619, a 250μg/ml stock solution in sodium carbonate buffer (Biomol Ltd, St.Albans, Herts.). Leukotriene B_4, a 50μg/ml stock solution in ethanol and stored under argon (Cascade Ltd, Reading, Berks.).

Method. Platelet aggregation was studied using citrated blood and either PRP or washed platelets were prepared. The platelet count was adjusted to $200x10^9/l$ with either autologous PPP when using PRP or with buffer when using washed platelets. Turbidometric platelet aggregation was studied both in the absence and presence of varying numbers of purified, washed neutrophils.

The extent of aggregation was measured with a dual channel lumiaggregometer (Chronolog Corporation, Havertown, PA, USA) which allowed simultaneous assay of ATP secretion. The aggregometer's sample wells were warmed to 37°C and the stirring speed set to 900rpm. The aggregometer signal was calibrated on the chart recorder as follows: 0.3ml PPP or buffer was placed in one cuvette, while 0.27ml PRP or washed platelets were placed in another. The cuvette containing no platelets was used to set the chart recorder to 90% maximum response and the cuvette containing platelets to 10%

maximum response. When the effect of neutrophils on platelet function was studied the change in light transmission caused by neutrophils was compensated for by including the appropriate number of neutrophils in the PPP or buffer used to set the 90%, as well as the PRP or washed platelets used to set the 10% response. After a steady baseline had been obtained, 30µl of an aggregating agent was added and the response measured. Some experiments included addition of agents that modified either platelet or neutrophil responses. Samples were incubated with these compounds prior to addition of a platelet aggregating agent.

Calculation. The length of the lag phase, rate of aggregation and extent of aggregation were measured. Normal ranges were determined for each agonist used.

2.6.2 Whole blood impedance aggregation

Reagents. Stock solutions of agonists were prepared as described (section 2.6.1) with one exception: arachidonic acid (Sigma Chemical Company, Poole, Dorset) was dissolved in ethanol, that had previously been bubbled with nitrogen, to a concentration of 200mM and stored at -70°C. Just before use this was diluted to 40mM.

Method. Platelet aggregation in citrated whole blood samples was measured by electrical impedance (Cardinal and Flower, 1980) using a Model 540 impedance aggregometer (Chronolog Corp., Havertown, PA, USA). Plastic cuvettes containing 950µl of blood were placed in the heating block of the aggregometer and warmed to 37°C. A teflon coated stir bar was included and the sample stirred at 600rpm. An electrode consisting of two fine platinum wires set a fixed distance apart was placed into the sample. A standard resistance of 5 ohms was applied to the system to enable calibration of the chart recording. Some samples were pre-incubated for various lengths of time with either inhibitors or promoters

of platelet aggregation. After a steady baseline was achieved aggregation was induced with 25µl of reagent injected directly into the sample. Impedance changes were recorded at chart speed of 2cm/min. Normal ranges were determined for each agonist used.

Calculation. Three measurements were taken: the lag period between addition of agonist and beginning of aggregation wave; the rate of aggregation, which was assessed by drawing a tangent to the initial exponential part of the response, and measuring its length over a 1 minute strip of the chart-recording; and the extent of response, which was determined by measuring the vertical height between the minimum point of the trace after addition of agonist and the maximum height in chart paper units. This was then converted into a resistance (ohms) by referring to the calibration response.

2.6.3 Measurement of adenine nucleotides in ethanol extracted samples.

Reagents. Reagents were from Merck Ltd, Poole, Dorset. Disodium EDTA solution, 100mM, in distilled water.

TCA/disodium EDTA solution: Equal volumes of 8mM disodium EDTA and trichloroacetic acid (20%$^{w}/_{v}$ in distilled water).

Ethanol.

Sample buffer, pH 7.75, comprising per litre: TRIS, 12.1g and disodium EDTA, 0.744g.

Assay buffer, pH 7.75, comprising per litre: TRIS, 12.1g.

Bovine serum albumin (BSA) buffer comprising 10mg BSA (Fraction V; Sigma Chemical Company, Poole, Dorset) in 10 ml assay buffer.

0.4M magnesium sulphate and 1.3M potassium chloride in 100ml distilled water.

Phosphoenolpyruvate, (Sigma Chemical Company, Poole, Dorset) 100mM, in 10ml distilled water.

Pyruvate kinase (Sigma Chemical Company, Poole, Dorset; Type II, ammonium sulphate suspension).

Luciferase (Pharmacia Ltd, Milton Keynes, Bucks.) dissolved in 10ml distilled water per vial.

ATP standard (Pharmacia Ltd, Milton Keynes, Bucks.) dissolved in distilled water to give a 10μM stock solution. This was further diluted in saline to give working solutions of 1.0, 0.5, 0.25, 0.125 and 0.063μM.

ADP standard (Sigma Chemical Company, Poole, Dorset) dissolved in saline to give a stock solution of 100μM. This was further diluted with saline to give a 0.5μM working solution.

Preparation of test samples. *Total platelet ATP/ADP samples.* 0.2ml of PRP or washed platelet samples were mixed with an equal volume of TCA/disodium EDTA, and left for 10 minutes on ice. The samples were then centrifuged for 10 minutes at 2000*g*. 10μl of supernatant was then added to 490μl sample buffer and then frozen until assay.

Granule released ATP/ADP samples. Platelets (270μl) were aggregated with 30μl of different agonists for 4 minutes. The reaction was stopped with 30μl of 100mM disodium EDTA. The sample was centrifuged at 2000*g* for 5 minutes. 100μl of the supernatant was mixed with absolute alcohol and vortex mixed before further centrifugation at 10000*g* for 5 minutes. 10μl of the sample supernatant was then diluted with 490μl of sample buffer and frozen until assay.

Method. *Active buffer preparation.* This was prepared by mixing 90μl BSA buffer with 10μl pyruvate kinase, 84μl magnesium sulphate/potassium chloride solution and 16μl phosphoenolpyruvate in a plastic tube on ice. 20μl of this active buffer was added to 330μl sample buffer in a cuvette.

Inactive buffer preparation. This was prepared by mixing 20.34ml assay buffer with 504µl magnesium sulphate/ potassium chloride solution, 60µl pyruvate kinase and 96µl phosphoenolpyruvate in a glass tube. This mixture was then placed in boiling water bath for 30 minutes. It was then cooled and 350µl placed in cuvettes.

Assay. Luminescence was measured with a single well luminometer (Pharmacia Ltd, Milton Keynes, Bucks.). Up to six pairs of active and inactive buffer cuvettes were prepared at a time as described above. 100µl of luciferase was added to each cuvette. The cuvette was placed in the luminometer and the background luminescence measured. 50µl of test or ATP standard was then added to each pair of cuvettes and a reading taken of the inactive cuvettes. Luminescence was then measured in the active cuvettes.

Calculation. Inactive cuvettes measured ATP. Active cuvettes measured ATP plus ADP. A graph of luminescence response (minus background) was plotted against ATP standard concentration. The ATP and ATP plus ADP concentrations were extrapolated from the graph. The concentrations in nmol/10^9 platelets were obtained after the following corrections for dilution and platelet count:

$$\text{released ATP} = \text{ATP 1} \times 1000/P \times 27500/225,$$

$$\text{total ATP} = \text{ATP 1} \times 100000/P,$$

where ATP 1 = µmol ATP from standard curve and P = platelet count (x10^9/l).

Released and total ADP were calculated the same way but the ATP concentration was subtracted from the ATP+ADP concentration to give the ADP result.

2.6.4 Direct measurement of ATP secretion by PRP and washed platelets.

Reagents. Chronolume reagent (Chronolog Corp., Havertown, PA, USA): each vial contained 0.2mg luciferin, 22000 units D-luciferase, magnesium sulphate, human serum

albumin, stabilizers and buffer. This was reconstituted with 1.25ml of distilled water.

ATP standard (Chronolog Corp., Havertown, PA, USA): each vial contained 2μmol lyophilized ATP. This was reconstituted with 5ml saline.

Method. Aggregation was performed with a lumiaggregometer (Chronolog Corp., Havertown, PA, USA) as previously described (section 2.6.3), except that 250μl of PRP or washed platelets were used and 20μl Chronolume reagent added prior to addition of agonist. After addition of agonist both aggregation and luminescence (representing ATP secretion) were monitored. The luminescence gain setting was adjusted to give maximum pen excursion. Once aggregation was complete 5μl of ATP standard was added and further luminescence (representing 2nmol ATP) was recorded.

Calculation. The following formula was used to calculate ATP release:

$$L_{test}/G_{test} \times G_{standard}/L_{standard} \times \text{concentration of test}$$

where L_{test} =luminescence of test,

G_{test} =gain of test,

$G_{standard}$ =gain of standard,

$L_{standard}$ =luminescence of standard,

2.7 Measurement of intracellular calcium flux

Reagents. Hepes-Buffered Saline (HBS), pH 7.4, comprising per litre 140mM NaCl, 2.7mM KCl, 0.1% BSA, 0.1% glucose, 3.8mM HEPES.

100mM disodium EGTA in HBS.

100mM magnesium chloride in saline.

100mM calcium chloride in saline.

Dimethyl sulphoxide.

Triton X-100 (Sigma Chemical Company, Poole, Dorset) in distilled water.

Aequorin (Dr.J.Blinks, Mayo Foundation, New York, USA). Aequorin was prepared by dissolving 1mg in 333µl calcium free water containing 7mM disodium EGTA.

Equipment. The platelet ionized calcium aggregometer (PICA; Model 660, Chronolog Corp., Havertown, PA, USA) measured shape change and aggregation at the same time as luminescence in the same sample. An amplifier-integrator (Model 610) attached to the PICA allowed selective suppression of background luminescence and integration of the luminescence signal over the range 000 to 999. A nine position switch selected the photomultiplier amplification over a linear 400-fold range. Overrange light signals were attenuated by the insertion of a 1% neutral density filter into the luminescence light path.

Method for loading cells with aequorin (Yamaguchi *et al*, 1986). Blood was collected into ACD anticoagulant and centrifuged twice for PRP. Platelets were washed by centrifugation with inhibitors (section 2.4.2) and resuspended in 90µl of HBS with 5mM disodium EGTA. 10µl of 3mg/ml aequorin was added. DMSO was added stepwisely in 1µl portions over 7.5 minutes to a final concentration of 6%. The time delay between successive steps was 1.5 minutes. The platelet suspension was incubated for 2 minutes at room temperature before being diluted with 10 volumes of HBS. After a further 2 minutes the platelets were centrifuged at 10000*g* for 10 seconds. This was repeated twice and the pellet finally resuspended in HBS containing Ca^{2+} and Mg^{2+} ions (1mM). In some experiments washed neutrophils were loaded with aequorin by the above method.

Method for measuring intracellular calcium flux. Aequorin-labelled platelets were added to 1ml plastic cuvettes in the PICA. Unlabelled neutrophils were then added to give a final volume of 950µl. HBS was used as a diluent to standardize cell concentrations. The cells were prewarmed to 37°C and stirred at 900rpm in the PICA. A steady baseline

was obtained before adding 25µl of agonist through a light-proof rubber septum. The gain setting for luminescence was adjusted to give a maximum deflection on the chart recorder. Luminescence (representing intracellular calcium flux) and optical aggregation were then monitored. Incorporated aequorin was measured in a fresh, unstimulated sample immediately afterwards by lysing the cells with 0.1% Triton X-100 and measuring the luminescence response. This luminescence value represented L_{max}. All intracellular calcium flux experiments were performed within 1 hour of aequorin-loading to reduce the effects of aequorin decay.

Calculation for quantitizing aequorin curves. A standard curve for calibration of light signals was generated and was essentially as described by Blinks and co-workers for microinjected preparations (Blinks *et al*, 1982). The logarithmic ratio of luminescence (L) obtained upon cell stimulation and the luminescence (L_{max}), obtained when all the aequorin in the preparation was suddenly exposed to a saturating calcium concentration (1mM) by 0.1% Triton X-100 induced cell lysis, was compared to a calibration curve (Log L/L_{max} versus Log $[Ca^{2+}]$) generated *in vitro* using the same lot of aequorin. The Log $[Ca_i^{2+}]$ indicated by a particular Log L/L_{max} was read from the calibration curve (Figure 2.1).

2.8 Cyclic GMP radioimmunoassay

Sample preparation.

Materials. All reagents were from Merck Ltd, Poole, Dorset. Trichloroacetic acid (10% in water); 1,1,2 trichlorotrifluoroethane (Freon); tri-*n*-octylamine.

Method. The reactions of samples containing either platelets or neutrophils alone or mixtures of platelets and neutrophils were terminated and extracted as described (Martin *et al*, 1988). Briefly, 1 volume of 10% $^{w}/_{v}$ trichloroacetic acid (TCA) was added and the

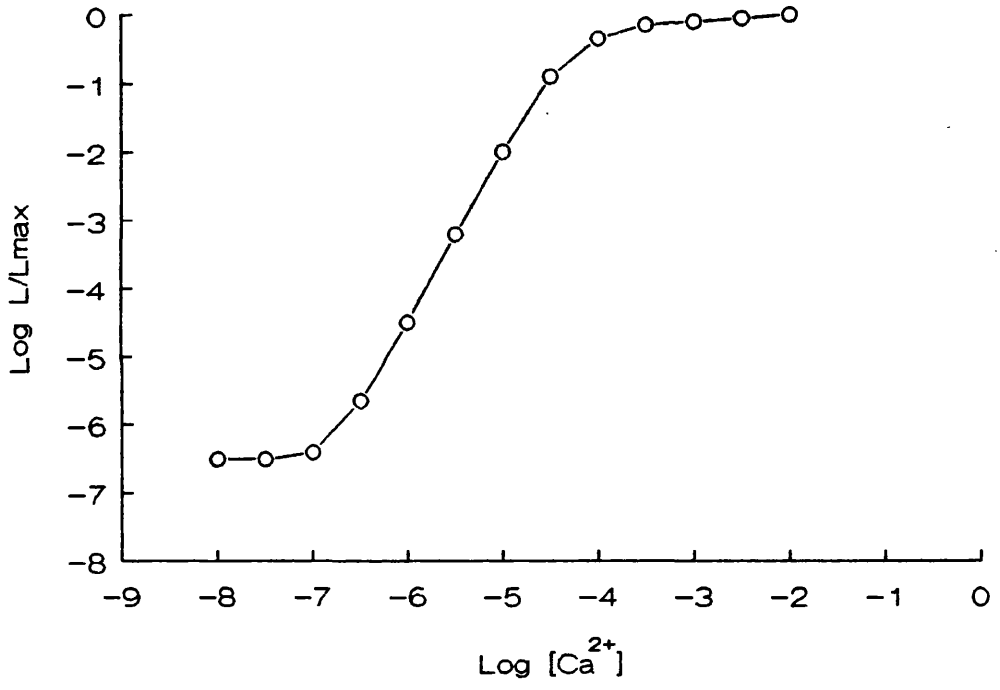

Figure 2.1 Calibration curve for determining intracellular [Ca^{2+}] in washed, purified platelets using aequorin luminescence signals.

sample centrifuged at 2000*g* for 10 minutes. TCA was neutralized (to pH 5.5-6.0) by washing the sample with an equal volume of 1,1,2 trichlorotrifluoroethane (Freon): tri-*n*-octylamine (1:1 $^{v}/_{v}$). The aqueous phase was then stored at -70°C until assay.

Radioimmunoassay procedure.

Reagents. All reagents were part of a kit from Amersham International plc, Amersham, Bucks.

0.05M Tris-EDTA buffer, pH 7.5, containing 4mM disodium EDTA.

Cyclic GMP antiserum.

[8-^{3}H Guanosine 3',5' cyclic phosphate (cyclic GMP); 80pmol containing 59 kBq.

Cyclic GMP standards. These were 8, 4, 2, 1 and 0.5 pmol/100µl in Tris-EDTA buffer.

Blank reagent.

Ammonium sulphate (60% saturated solution).

Method. Polystyrene tubes were labelled in duplicate as follows: total count (TC), zero standard tubes (B_0), standards A-D and samples. 100µl of assay buffer was pipetted into the TC tubes. 100µl of blank reagent was added to the B_0 tubes. 50µl of [^{3}H] cyclic GMP was added to all tubes. 100µl of each cyclic GMP standard was then added to the relevent tubes as was 100µl of each sample to be assayed. 50µl of cyclic GMP antiserum was pipetted into the B_0, standard and sample tubes. All tubes then contained a total of 200µl. Each tube was then thoroughly mixed and then incubated at 4°C for about 1.5 hours. Next the unbound cyclic GMP was separated from the complex. All tubes were placed on ice. 1ml of assay buffer was placed into the total count tubes. 1ml of ammonium sulphate, which was constantly stirred and on ice was then pipetted into all other tubes. All tubes were then incubated on ice for approximately 5 minutes, remixed, and then centrifuged at 4°C and 2000*g* for 10 minutes. Each supernatant was then decanted and the tubes

allowed to drain. 1.1ml of distilled water was then added to all tubes. The tubes were then mixed until the precipitate was dissolved. 1ml aliquots were then with 5ml of scintillation fluid (Optiphase "HighSafe II", Pharmacia Ltd, Milton Keynes, Bucks.). The amount of [^{3}H] cyclic GMP in each tube was then determined by beta scintillation counting (LKB-Wallac 1212 Rackbeta, Pharmacia Ltd, Milton Keynes, Bucks.).

Calculation. The average counts per minute (cpm) were determined for each set of replicated tubes. Percentage binding was then calculated from cpm as follows:

$$\% \text{ Binding}/B_o = (\text{standard or sample}) / (B_o)$$

A linear standard curve was then generated by plotting the %Binding/B_o as a function of the standard concentrations. This gave a linear standard curve with an intercept of 1.0 on the ordinate. Cyclic GMP (pmol per tube) for each sample was determined and then converted to pmol/10^9 platelets. Cyclic GMP levels obtained for neutrophils alone were subtracted from those obtained for platelets in the presence of neutrophils to give cyclic GMP as pmol/10^9 platelets.

Quality control. The intra-assay coefficient of variation for 10 samples was 6.8%.

2.9 Preparation of authentic nitric oxide solution

Materials. Distilled water (Analar grade; Merck Ltd).

Helium gas ("CP" grade; British Oxygen Company, Guildford, Surrey).

Nitric oxide gas (99% purity; Merck Ltd, Poole, Dorset).

Method. 40ml of water was placed in an air-tight Wheaton flask and degassed with helium for 45 minutes through a 19 gauge needle. A volume of 10ml nitric oxide gas was then injected into the degassed water. This gave a saturated solution with an estimated concentration of about 1.12mM nitric oxide which was stored on ice. The solution was

prepared fresh each day and used within six hours.

Methods for assay of eicosanoids

2.10 Thromboxane B_2 radioimmunoassay

Reagents. All reagents were part of a kit from Amersham International plc, Amersham, Bucks.

Dextran-coated charcoal.

Assay buffer, comprising PBS, gelatin and thimerosal.

[^{3}H]-thromboxane B_2. Approximately 1μCi, 37kBq in assay buffer.

Thromboxane B_2 antiserum diluted in assay buffer.

Thromboxane B_2 standards. 3.0, 1.1, 0.4, 0.13 and 0.05ng/ml aliquots in assay buffer.

Sample collection and preparation. Samples containing washed platelets, neutrophils or a mixture of both were activated with various agonists as described under turbidometric aggregation methods. After 4 minutes activity was terminated by the addition of 10mM disodium EDTA (final concentration). Samples were placed on ice for 10 minutes and then centrifuged at 10000*g* for 5 minutes. 100μl of each supernatant was then mixed with 100μl of ice cold ethanol to precipitate protein and placed on ice for a further 10 minutes. Samples were then centrifuged at 10000*g* for 5 minutes. The supernatant was then diluted 1/10 with assay buffer and frozen at -70°C until assay.

Radioimmunoassay procedure. Polystyrene tubes were labelled in duplicate as follows: total count (TC), non-specific binding tubes (NSB), zero standard tubes (B_0), standards A-E and samples. 200μl of assay buffer was pipetted into the TC and NSB tubes. 100μl of assay buffer was added to the B_0 tubes. 100μl of [^{3}H] thromboxane B_2 was added to all tubes. 100μl of each thromboxane B_2 standard was then added to the relevent tubes,

as was 100µl of each sample to be assayed. 100µl of thromboxane B_2 antiserum was pipetted into the B_0, standard and sample tubes. All tubes then contained a total of 300µl. Each tube was then thoroughly mixed and then incubated at 4^0C for about 16 hours. Next the unbound thromboxane B_2 was separated from the complex. All tubes were placed on ice. 1ml of assay buffer was placed into the total count tubes. 1ml of dextran-coated charcoal, which was constantly stirred and on ice was then pipetted into all other tubes. All tubes were then incubated on ice for approximately 9 minutes, remixed, and then centrifuged at 4°C and 1000*g* for 10 minutes. Each supernatant was then decanted into 5ml of scintillation fluid (Optiphase "HighSafe II", Pharmacia Ltd). The amount of [^{3}H] thromboxane B_2 in each tube was then determined by beta scintillation counting (LKB-Wallac 1212 Rackbeta, Pharmacia Ltd).

Calculation. The average counts per minute (cpm) were determined for each set of replicated tubes. Percentage binding was then calculated from cpm as follows:

% Binding/B_o = (standard or sample x NSB) / (B_o-NSB)

A standard curve was then generated by plotting the logit %Binding/B_o as a function of the $\log_{10}$ standard concentration. This gave a linear standard curve from which pg of thromboxane B_2 per tube for each sample was determined and then converted to ng/10^9 platelets.

Quality control. The intra-assay coefficient of variation for 10 samples was 5.3%.

2.11 6-keto prostaglandin $F_{1\alpha}$ radioimmunoassay

Reagents. All reagents were part of a kit from Amersham International plc, Amersham, Bucks.

Dextran-coated charcoal.

Assay buffer, comprising PBS, gelatin and thimerosal.

[^{3}H]-6-keto prostaglandin $F_{1\alpha}$. Approximately 1μCi, 37kBq in assay buffer.

6-keto prostaglandin $F_{1\alpha}$ antiserum diluted in assay buffer.

6-keto prostaglandin $F_{1\alpha}$ standards. 3.0, 1.1, 0.4, 0.13 and 0.05ng/ml aliquots in assay buffer.

Sample collection and preparation. Sample containing washed platelets, neutrophils or a mixture of both were activated with various agonists as described under turbidometric aggregation methods. After 4 minutes activity was terminated by the addition of 10mM disodium EDTA (final concentration). Samples were placed on ice for 10 minutes and then centrifuged at 10000*g* for 5 minutes. 100μl of each supernatant was then mixed with 100μl of ice cold ethanol to precipitate protein and placed on ice for a further 10 minutes. Samples were then centrifuged at 10000*g* for 5 minutes. The supernatant was then diluted 1/10 with assay buffer and frozen at -70°C until assay.

Radioimmunoassay procedure. Polystyrene tubes were labelled in duplicate as follows: total count (TC), non-specific binding tubes (NSB), zero standard tubes (B_0), standards A-E and samples. 200μl of assay buffer was pipetted into the TC and NSB tubes. 100μl of assay buffer was added to the B_0 tubes. 100μl of [^{3}H]-6-keto prostaglandin $F_{1\alpha}$ was added to all tubes. 100μl of each 6-keto prostaglandin $F_{1\alpha}$ standard was then added to the relevent tubes, as was 100μl of each sample to be assayed. 100μl of 6-keto prostaglandin $F_{1\alpha}$ antiserum was pipetted into the B_0, standard and sample tubes. All tubes then contained a total of 300μl. Each tube was then thoroughly mixed and then incubated at 4°C for about 16 hours. Next the unbound 6-keto prostaglandin $F_{1\alpha}$ was separated from the complex. All tubes were placed on ice. 1ml of assay buffer was placed into the total count tubes. 1ml of dextran-coated charcoal, which was constantly stirred and on ice was

then pipetted into all other tubes. All tubes were then incubated on ice for approximately 9 minutes, remixed, and then centrifuged at 4°C and 1000*g* for 10 minutes. Each supernatant was then decanted into 5ml of scintillation fluid (Optiphase "HighSafe II", Pharmacia Ltd). The amount of [^{3}H] 6-keto prostaglandin $F_{1\alpha}$ in each tube was then determined by beta scintillation counting (LKB-Wallac 1212 Rackbeta, Pharmacia Ltd).

Calculation. The average counts per minute (cpm) were determined for each set of replicated tubes. Percentage binding was then calculated from cpm as follows:

% Binding/B_o = (standard or sample x NSB) / (B_o-NSB)

A standard curve was then generated by plotting the logit %Binding/B_o as a function of the $\log_{10}$ standard concentration. This gave a linear standard curve from which pg of 6-keto prostaglandin $F_{1\alpha}$ per tube for each sample were determined and then converted to ng/10^9 cells.

Quality control. The intra-assay coefficient of variation for 10 samples was 5.9%.

2.12 Leukotriene B_4 radioimmunoassay

Reagents. All reagents were part of a kit from Amersham International plc, Amersham, Bucks.

Standard LTB_4. A 4ng/ml solution in assay buffer. This was diluted with assay buffer to give the following standard solutions: 200pg, 100pg, 50pg, 25pg, 12.5pg, 6.2pg, 3.1pg and 1.6pg per tube.

[5,6,8,9,11,12,14,15(n)-^{3}H] LTB_4. Approximately 0.5µCi, 18.5 kBq in 250µl of methanol: water: acetic acid (60:40:0.01 $^v/_v$), pH 5.6. This was mixed with 12.5ml of assay buffer.

LTB_4 antiserum. This was dissolved in 12.5ml assay buffer.

Assay buffer. This consisted of 0.05M TRIS:HCl buffer, pH 8.6, containing 0.1% $^w/_v$

gelatin.

Dextran-coated charcoal in 0.05M Tris:HCL buffer, pH 8.6, containing 0.1% $^{w}/_{v}$ gelatin.

Sample preparation. Samples containing washed neutrophils, platelets or a mixture of both were quenched with 10mM disodium EDTA (final concentration) 4 minutes after stimulation with different agonists. Samples were then rapidly centrifuged for 1 minute and 10000*g*. Supernatants were then frozen at -70°C until assay. LTB_4 was then assayed directly in these supernatants after a 1/100 dilution with assay buffer.

Radioimmunoassay procedure. Polystyrene tubes were labelled in duplicate as follows: total count (TC), non-specific binding tubes (NSB), zero standard tubes (B_0), standards A-H and samples. 200µl of assay buffer was pipetted into the TC and NSB tubes. 100µl of assay buffer was added to the B_0 tubes. 100µl of each standard was pipetted into the appropriate tubes. 100µl of each sample was added in duplicate to the appropriate tubes. 100µl of [^{3}H] LTB_4 was added to all tubes. 100µl of antiserum was pipetted into all tubes except for the NSB and TC tubes. All tubes were vortex mixed and incubated at 4°C for 16 hours. Next the unbound LTB_4 was separated from the complex. All tubes were placed on ice. 200µl of assay buffer was placed into the total count tubes. 200µl of dextran-coated charcoal, which was constantly stirred and on ice was then pipetted into all other tubes. All tubes were then mixed and incubated at room temperature for 5 minutes. They were then remixed, and centrifuged at 4°C and 2000*g* for 10 minutes. Each supernatant was then decanted into 5ml of scintillation fluid (Optiphase "HighSafe II", Pharmacia Ltd). The amount of [^{3}H] LTB_4 in each tube was then determined by beta scintillation counting (LKB-Wallac 1212 Rackbeta, Pharmacia Ltd).

Calculation. The average counts per minute (cpm) were determined for each set of replicated tubes. Percentage binding was then calculated from cpm as follows:

% Binding/B_0 = (standard or sample x NSB) / (B_0-NSB)

A standard curve was then generated by plotting the logit %Binding/B_0 as a function of the $\log_{10}$ standard concentration. This gave a linear standard curve from which pg of LTB_4 per tube for each sample was determined and then converted to ng/10^7 neutrophils.

Quality control. The intra-assay coefficient of variation for 10 samples was 3.8%. The inter-assay coefficient of variation was 5.3% (n=4).

2.13 High Performance Liquid Chromatography (HPLC) of eicosanoids

All reagents were from Merck Ltd, Poole, Dorset, unless stated otherwise. Some studies required prelabelling of either platelets or neutrophils with [1-^{14}C] arachidonic acid.

2.13.1 Preparation of [1-^{14}C] sodium arachidonate

[1-^{14}C] arachidonic acid (1.85MBq/ml; Amersham International plc, Amersham, Bucks.) was converted to its sodium salt: its ethanol diluent was dried under nitrogen and the sides of the vial were washed with degassed hexane. This solvent was evaporated and the arachidonate was resuspended in 50µl 0.01M Na_2C0_3 plus 150µl deionised water (both degassed). Contact was maintained by agitation for 15 mins at room temperature. Finally 3ml of buffer (Tris 15mM, sodium chloride 134mM, glucose 5mM and BSA 0.01%, pH 7.4) was added.

As a final check as to how much [^{14}C] sodium arachidonate was added to the platelets, a 3µl sample was removed for scintillation counting.

2.13.2 Labelling of platelets with [1-^{14} C]-sodium arachidonate. Blood samples were collected into ACD and PRP collected. Platelets were concentrated about tenfold by layering the PRP onto Histopaque-1077 (Sigma Chemical Company, Poole, Dorset) and

centrifuging at 500*g* for 10 minutes. Platelets were resuspended in 1ml autologous plasma and transferred to a screw-tight tube. 100µl (0.02MBq) of[1-^{14}C]-sodium arachidonate was added and the cells incubated at 37°C with gentle mixing for 45 minutes. The cells were then cooled at room temperature for 10 minutes and then filtered through a Sepharose 2B column, as previously described (section 2.4.1). Platelets were counted for both number and radioactivity. About 50-60% of radioactivity was incorporated into platelets by this method.

2.13.3 Labelling of neutrophils with [1-^{14}C] sodium arachidonate. Blood samples were collected into ACD and neutrophils purified and washed as previously described (section 2.5). The washed neutrophils were resuspended in 1ml of Hanks Balanced Salt Solution. The cells were incubated with 100µl (0.02MBq) of [1-^{14}C] sodium arachidonate for 30 minutes at 37°C, then diluted with 10ml of buffer and washed twice by centrifugation for 5 minutes at 170*g*. The neutrophils were then counted for both number and incorporated radioactivity. Viability by Trypan blue dye exclusion was also checked and this exceeded 90%. Typically, 70-80% recovery of radioactivity was obtained.

2.13.4 Incubation and activation of samples. Radiolabelled platelets were mixed with unlabelled neutrophils or vice versa. Different ratios of platelets to neutrophils were tested for eicosanoid release. Control samples containing only one cell-type were also prepared.

Samples containing neutrophils in the absence or presence of platelets were incubated at 37°C for 5 minutes with continuous stirring at 900rpm in an aggregometer. Incubation mixtures contained 1mM of both calcium and magnesium ions. Some samples contained added inhibitors of eicosanoid synthesis. Samples were stimulated with different platelet and neutrophil agonists and the activity was terminated after 4 minutes with either

formic acid or 3 volumes ice cold ethanol and put on ice for 10 minutes. The internal standard, 500ng (1500 pmol) PGB_2, was added at this stage to each sample. The samples were centrifuged at 4°C for 10 minutes and 2000*g* to remove the precipitate. The supernatants were collected and made to 15% ethanol with degassed water. The samples were then acidified to pH 3 with glacial acetic acid and frozen at -70°C until extraction and HPLC.

2.13.5 Extraction of arachidonate metabolites.

1. Method according to Verhagen *et al*, 1986.

Reagents. All reagents were "HiPerSolv" grade from Merck Ltd, Poole, Dorset, unless stated otherwise.

Octadecylsilica (ODS) minicolumns (Waters Corp., Milton Keynes, Bucks. or J.T.Baker Ltd, Hayes, Middx.);

Methanol, 0.1% disodium EDTA in water;

water; ethanol ("Analar" grade).

Method. The ODS minicolumn was prepared by washing with 20ml ethanol, followed by 10ml water and 10ml 0.1% EDTA. The column was not allowed to dry out.

The sample was added slowly to the pre-wetted column and drawn through the column by a vacuum pump at 15 mmHg pressure. The column was then washed with 10ml water. Eicosanoids were eluted with 5ml methanol into a polypropylene tube. The samples were dried under a stream of nitrogen at 37°C, resuspended in 200µl methanol:water (70:30 $^{v}/_{v}$) and stored at -70°C until assay by HPLC.

2. Method according to W.S.Powell, 1982.

Reagents. Octadecylsilica (ODS) minicolumn (Waters Corp. or J.T.Baker Ltd), 15% ethanol in water (HPLC grade), 0.1% disodium EDTA in water (HPLC grade), hexane (HPLC grade), ethyl acetate (HPLC grade), water (HPLC grade).

Method. The ODS minicolumns were pre-wetted with 20ml ethanol, followed by 10ml water and 10ml 0.1% disodium EDTA. The sample was applied slowly to the ODS minicolumn. This was followed by successive washes with 10ml 15% ethanol and 10ml hexane. The eicosanoids were then eluted with 10ml ethyl acetate into polypropylene tubes. The samples were dried under a stream of nitrogen at 37°C and resuspended in 200µl methanol:water (70:30 $^{v}/_{v}$) and stored at -70°C until assay by HPLC.

3. Method according to M.Van Rollins *et al*, 1980.

Reagents. Ethyl acetate; methanol; water.

Method. Metabolites of arachidonic acid were extracted twice with 3 volumes of ethyl acetate containing 1g of sodium chloride. Recovery of ^{14}C was about 90%. The combined extracts were pooled and shaken with a vortex mixer after addition of 1g sodium sulphate. They were then centrifuged for 15 minutes at 4°C and 2000*g*. After evaporation to dryness under nitrogen the samples were stored in 200µl methanol/water (70/30 $^{v}/_{v}$) at -70°C until assay.

2.13.6 HPLC of eicosanoids.

Equipment. HPLC equipment comprising either: two 110a pumps, single fixed wavelength detector (Model 160, preset to 280nm or 235nm), sample injector with 20µl

loop, gradient controller and mixer, chart recording integrator (all from Beckman Ltd, High Wycombe, Bucks.) and fraction collector (Pharmacia Ltd, Milton Keynes, Bucks.) or quaternary pump, variable wavelength (190nm-420nm) scanning diode array detector, sample injector with 20µl loop, computer-controlled gradient and data handling system, and fraction collecter (All Waters Corp., Milton Keynes, Bucks.).

Ultrasphere ODS (250mm x 4.6mm) stainless steel column (Beckman Ltd, High Wycombe, Bucks.).

1. Method according to J.Verhagen *et al*, 1984.

Materials. Water, tetrahydrofuran, methanol, acetic acid (all HPLC grade), disodium EDTA. The mobile phase used was:

tetrahydrofuran:methanol:water:acetic acid, (25/30/45/0.1 %), pH 5.5. with 0.1% EDTA in the aqueous phase. Ammonia was added to adjust the pH.

Method. The sample (20µl) was applied to the pre-equilibrated column which had a flow rate of 0.9ml/minute. The effluent was monitored at 280nm. The peaks obtained were co-eluted with authentic leukotriene standards for more positive identification. The peaks were also identified by wavelength scanning. The peak corresponding to leukotriene B_4 was collected and the solvent evaporated. The samples were then diluted in radioimmunoassay buffer and subjected to a highly specific competitive radioimmunoassay for leukotriene B_4 as previously described (section 2.12).

2. Method according to M.Van Rollins *et al*, 1980.

Materials. Acetonitrile; water; orthophosphoric acid ("Analar" grade).

Method. The sample (20µl) was injected onto the pre-equilibrated column at a flow rate

of 1ml/minute. Separations were performed using programmed increases in proportions of acetonitrile in aqueous orthophosphoric acid (pH 2.0). For the first 63 minutes after injection of sample the acetonitrile concentration was 30.5% (%$_v$). Linear increases of acetonitrile concentration commenced at 63, 78, 114 and 134 minutes to 44% over 15 minutes, 48% over 10 minutes, 66% over 5 minutes and to 95% over 5 minutes, respectively. The column was equilibrated at 30.5% acetonitrile for 30 minutes before re-use. Radiochromatograms were plotted from radioactivity content of 1ml fractions. The counting efficiency for ^{14}C was 90% and background levels of radioactivity did not vary significantly. The peaks obtained were co-eluted with authentic eicosanoid standards for identification. The peaks were also identified by on-line wavelength scanning with the Waters HPLC.

2.13.7 Quantitation of eicosanoids by ultra violet absorbance.

HPLC was used to quantitate arachidonic acid metabolites, using either UV or radioactivity counting. Lipoxygenase products were quantitated on the basis of UV absorbance, whereas prostaglandins and other cyclooxygenase products were measured only in experiments where radioactive arachidonate was used as substrate. In order to quantitate eicosanoids by HPLC an internal standard (prostaglandin B_2: extinction coefficient 28650, absorption maximum 278nm) was added to correct for differences in recovery between samples. The column eluate was monitored and the area under the peaks integrated electronically. The extinction coefficients of various eicosanoids are listed in Table 2.1.

The concentrations of leukotrienes and monohydroxy acids were determined by comparison of their peak areas with that of the internal standard PGB_2, after correction

for the difference in extinction coefficients, as shown below (Powell *et al*, 1982). The sensitivity of this method was about 1ng.

Amount of X (pmol)=

$$\frac{[\text{peak area (X)}] \times [28650] \times [\text{amount of } PGB_2 \text{ (pmol)}]}{[\text{peak area } PGB_2] \times [\text{molar extinction coefficient (X)}]}$$

Table 2.1 Ultraviolet absorption characteristics of some arachidonic acid metabolites

COMPOUND	ABSORPTION MAXIMUM (nm)	MOLAR EXTINCTION COEFFICIENT
LTB_4	270	39500
6-*trans*-LTB_4	270	44000
6-*trans*-12-*epi*-LTB_4	270	44000
5*s*-12*s*-diHETE	278	40000
LTC_4	280	40000
LTD_4	280	40000
LTE_4	280	40000
5-HETE	235	30500
12-HETE	237	30500
HHT	232	33400
Prostaglandin B_2	278	28650

2.14 Statistical analysis

Statistical analysis was performed as indicated in the text. Some results were analysed by Student's paired "t" test (as stated in legend). Other results with non-Gaussian distribution or where distribution was uncertain due to a small number of experiments

were analysed by non-parametric tests (Wilcoxon's signed rank test). Probability values with $p<0.05$ were taken as statistically significant. Confidence intervals (for non-parametric data) were calculated when comparing results from patients with control samples.

CHAPTER 3

PLATELET AGGREGATION IS INHIBITED BY A NITRIC OXIDE-LIKE FACTOR RELEASED FROM HUMAN NEUTROPHILS *IN VITRO*

3.1 Introduction

In 1986 Furchgott and Ignarro independently suggested that endothelium-derived relaxing factor was nitric oxide (individual talks presented at the Fourth Symposium on the Mechanisms of Vasodilation, Rochester, Minnesota, July 11, 1986; Ignarro, 1989). This inference was soon confirmed experimentally (Palmer *et al*, 1987). The biochemical pathway that leads to the formation of NO was identified from earlier work with macrophages. Macrophages produce nitrate and nitrite (Stuehr and Marletta, 1985) which are derived from the semi-essential amino acid L-arginine and their production is blocked by non-metabolizable arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA; Hibbs *et al*, 1987). Nitric oxide intermediate in this pathway has now been shown to derive from one of the terminal guanidino-nitrogen atoms of L-arginine both in endothelial cells (Palmer *et al*, 1988) and macrophages (Marletta *et al*, 1988). The source of the oxygen atom in NO remains unclear. The enzyme responsible, nitric oxide synthetase, is cytosolic, NADPH-dependent, and leads to the formation of stoichiometric amounts of L-citrulline and nitric oxide (Palmer and Moncada, 1989). Tetrahydrobiopterin is a cofactor for the oxidation of L-arginine and the first step in its catalytic conversion is via L-hydroxyarginine (Tayeh and Marletta, 1989).

The best understood biological role of nitric oxide is its participation in the control

of vascular resistance and platelet function. The stimulation of soluble guanylate cyclase by NO and the subsequent rise in cellular concentrations of cyclic GMP mediates profound vasorelaxation and inhibition of platelet adhesion and aggregation (Ignarro, 1989). Experiments with L-NMMA confirm the central role of basal NO release for the acute control of blood pressure and flow *in vivo* (Vallance *et al*, 1989). However, basal EDRF activity seems to be absent in human veins.

The demonstration of NO release by macrophages and more recently by neutrophils may have important implications regarding current concepts of the immune system. Macrophages and neutrophils also produce superoxide anions which rapidly inactivate nitric oxide, indicating a complex interaction between these two radicals. The killing of tumour cells and certain bacteria by macrophages seems to involve NO, probably through inhibition of mitochondrial electron transport, the Krebs cycle and DNA synthesis (Hibbs *et al*, 1988; Granger and Lehninger, 1982).

The chemical properties of NO are uniquely suited as a local modulator of cellular responses. As a small lipophilic molecule, NO readily diffuses throughout its cells of origin and into adjacent cells of various types where it activates guanylate cyclase and thereby elevates intracellular cyclic GMP. Endothelium-derived NO raises cyclic GMP in the underlying smooth muscle and nearby blood platelets, thereby resulting in vasorelaxation and inhibition of platelet adhesion and aggregration to the endothelial surface. In this manner NO communicates with nearby cells, calling on them to act in a complementary manner to increase local blood flow and reduce platelet aggregation and thrombosis. The very short half-life of NO, together with the ability to bind haemoglobin and other haemoproteins, ensures a highly localized action.

It is not certain whether the NO released from phagocytes has a role in causing

vascular smooth muscle relaxation and inhibition of platelet adhesion and aggregation. However, the fact that platelets and leukocytes come into such close proximity, especially when the haemostatic process is activated means that phagocyte-derived NO could well modulate local platelet reactivity. Another function of phagocyte-derived NO could be cytotoxic, although the mechanism is unclear. One possibility is that NO reacts with iron and iron-sulphur containing proteins and other metalloproteins, thereby effecting their degradation (Hibbs *et al*, 1988; Stuehr and Nathan, 1989). In addition, NO derived from vascular endothelium or other cells may stimulate cyclic GMP formation in phagocytic cells and thereby signal cytotoxic functions such as phagocytosis and lysosomal enzyme secretion (Smith and Ignarro, 1975). Thus, NO may have a widespread physiological role in local cell-to-cell communication by acting as a potent but labile transcellular signalling agent.

The formation and release of nitric oxide by neutrophils has been demonstrated by direct nitric oxide-specific chemiluminescence (Schmidt *et al*, 1989; Wright *et al*, 1989); by induction of vascular smooth muscle relaxation (Rimele *et al*, 1988); and by inhibition of platelet aggregation (McCall *et al*, 1989; Salvemini *et al*, 1989).

This chapter aims to further investigate the effect of both resting and activated neutrophils upon platelet function in normal, healthy individuals *in vitro* and to identify any inhibitory factors observed. This study was a prelude to investigating the effect of nitric oxide upon platelet function both in washed cells from patients with Multiple System Organ Failure and also in a "whole blood" environment.

3.2 METHODS

Blood samples. Venous blood was collected from up to sixteen apparently healthy donors

(who had not taken any drugs in the preceding 14 days) into acid-citrate-dextrose anticoagulant (ACD; section 2.1).

Preparation of washed platelets. See section 2.4.1.

Preparation of washed, purified neutrophils. See section 2.5.

Preparation of authentic nitric oxide solution. See section 2.9.

Fixation of neutrophils. Some neutrophil preparations ($5x10^9$/l) were fixed with glutaraldehyde. Cells were fixed with 0.25% glutaraldehyde for 5 minutes and washed three times with buffer prior to use.

Turbidometric platelet aggregation. See section 2.6.1. Some platelet samples had superoxide dismutase, haemoglobin, L-arginine, M&B22,948, apyrase or other potential modifiers of activity added 1 minute prior to neutrophil addition. Some neutrophil samples were preincubated with L-canavanine for 50 minutes prior to incubation with platelets. Neutrophils were coincubated with platelets for 1 minute prior to addition of agonist. Platelets were stimulated with different concentrations of thrombin, collagen, ADP, U46619, ristocetin or arachidonic acid as detailed in section 3.3. Some of these compounds can also activate neutrophils. Some neutrophil samples were specifically stimulated with either formyl-Met-Leu-Phe (FMLP) or leukotriene B_4 (LTB_4) upon addition to platelets. Aggregation was measured in the absence or presence of varying numbers of neutrophils ($1\text{-}32x10^9$/l). Samples containing platelets (as well as superoxide dismutase, haemoglobin or other aforementioned compounds) but not neutrophils, were aggregated simultaneously with platelet-neutrophil samples to act as control.

Platelet ATP release. This was assayed by luciferin/luciferase chemiluminescence on incubation mixtures terminated with disodium EDTA and extracted with ice-cold ethanol. See section 2.6.3.

Thromboxane B_2 and 6 keto Prostaglandin $F_{1\alpha}$ (6ketoPGF$_{1\alpha}$). See section 2.10 and 2.11 respectively.

Cyclic GMP. See section 2.8.

Leukotriene B_4. See section 2.12.

Statistics. Results were expressed as the mean and the standard error of the mean (SEM). Either Student's paired t-test or Wilcoxon's signed rank sum test were used to determine the significance of differences between samples and $p<0.05$ was taken as statistically significant.

3.3 RESULTS

The effect of unstimulated, washed, purified neutrophils upon platelets stimulated with different agonists. Figure 3.1 shows that unstimulated neutrophils partially inhibited platelet aggregation stimulated by all agonists except ristocetin. Glutaraldehyde-fixed neutrophils did not significantly inhibit platelet aggregation. The absence of inhibition by neutrophils of platelets stimulated with ristocetin is probably because this agonist, unlike the others tested, causes von Willebrand factor receptor-mediated platelet agglutination and "primary" aggregation. Both arachidonic acid and calcium ionophore A23187 also activate neutrophils and these samples showed further attenuation of platelet aggregation. These results indicate the release from neutrophils of one or more compounds able to diffuse into or be actively taken up by platelets leading to at least partial inhibition of platelet function.

Thrombin-induced platelet aggregation and ATP release in the presence of increasing numbers of neutrophils. When platelets were aggregated in the presence of increasing numbers of neutrophils ($1\text{-}32\times10^9$/l) both aggregation and ATP release declined as

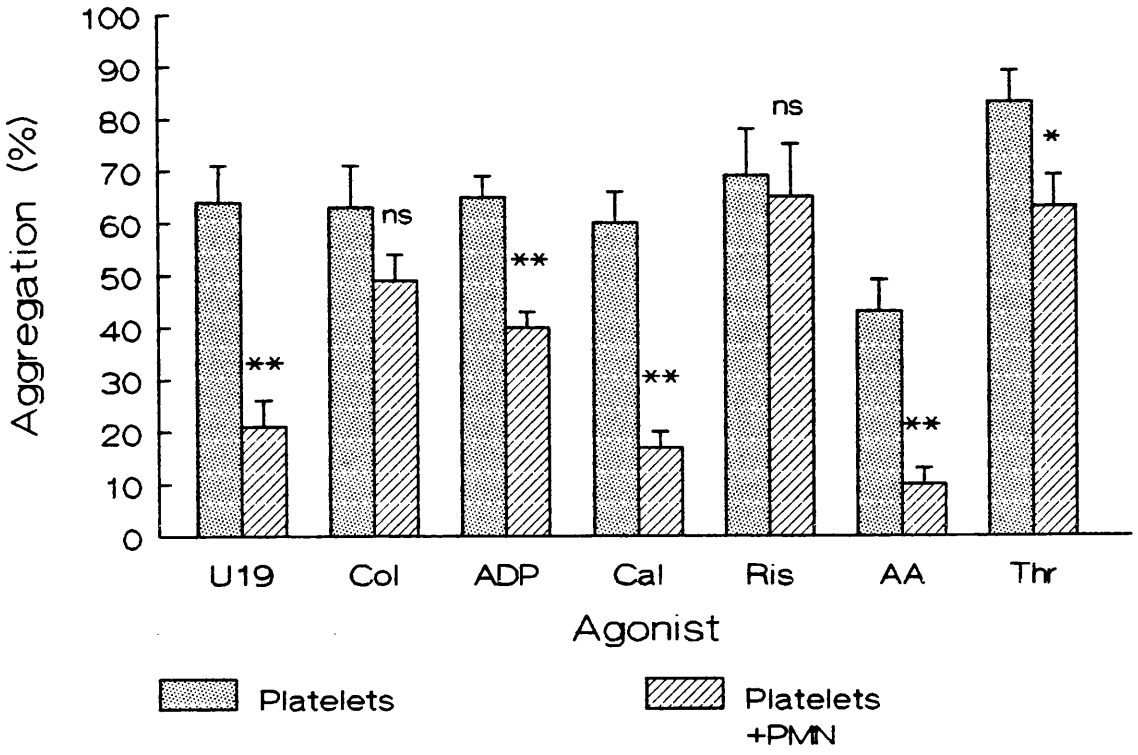

Figure 3.1. The effect of unstimulated, washed, purified neutrophils upon platelets stimulated with different agonists: U46619 (U19), 2.5μg/ml; collagen (Col), 10μg/ml; ADP (ADP), 10μM; calcium ionophore A23187 (CaI), 2.5μM; ristocetin (Ris), 1.25mg/ml; arachidonic acid (AA), 0.5mM; and thrombin (Thr), 0.1u/ml. Platelets ($200x10^9$/l) were incubated at 37°C with neutrophils ($5x10^9$/l) and stirred for 2 minutes prior to addition of agonist. Results show the mean and SEM for 5 separate experiments and were not statistically significant (ns), $p<0.05$ (*) or $p<0.01$ (**).

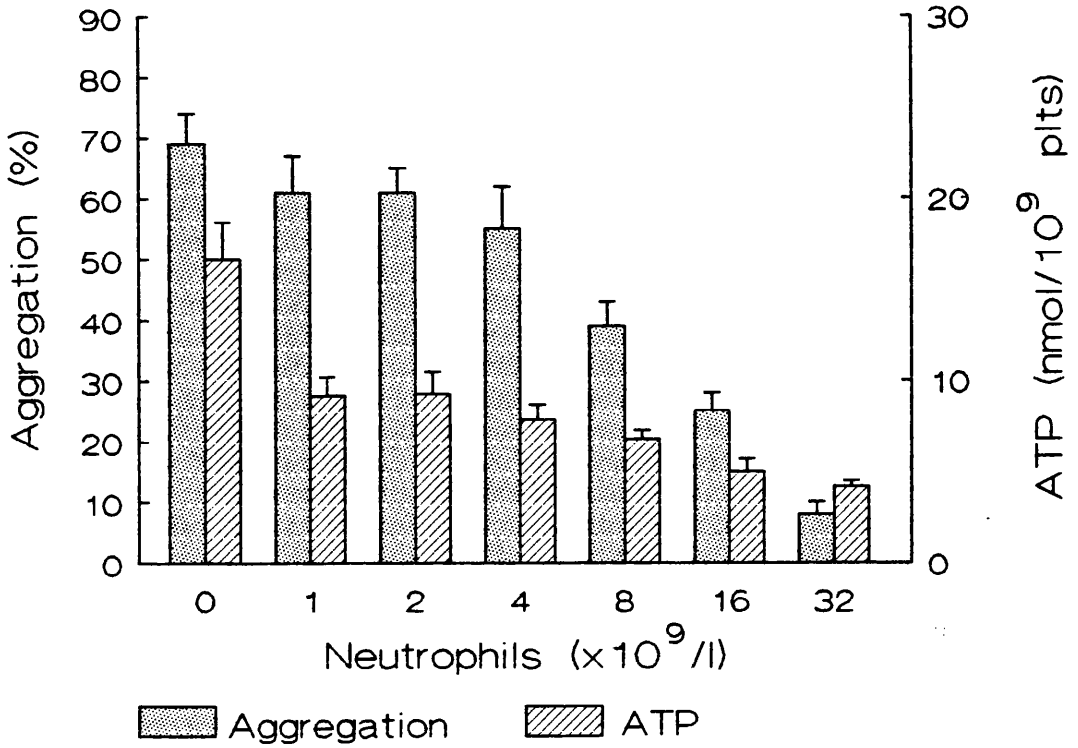

Figure 3.2. The effect of neutrophil concentration on 0.05u/ml thrombin-induced platelet aggregation and ATP secretion. Washed platelets were incubated in the presence of increasing numbers of neutrophils for 1 minute prior to platelet stimulation. Results represent the mean and SEM of four separate experiments.

neutrophil concentration increased (figure 3.2). ATP release was apparently more sensitive to inhibition by neutrophils than was aggregation.

The effect of different concentrations of thrombin on nitric oxide inhibitory activity. Platelets aggregated with thrombin concentrations above 0.1u/ml were less susceptible to inhibition by both NO and the neutrophil-derived factor at the concentrations used in these experiments (figure 3.3). The activity of authentic NO was prevented by purified haemoglobin (10μM) and enhanced by M&B22948 (1μM), a selective cyclic GMP phosphodiesterase inhibitor. This indicates both the authenticity of the NO and its mode of action via guanylate cyclase stimulation.

The effect of different concentrations of nitric oxide on platelet aggregation to thrombin. Nitric oxide (20μl or more) was able to completely abolish platelet responses to low doses of thrombin (0.05u/ml or less). Higher concentrations of thrombin (above 0.1u/ml) caused platelet aggregation which although reduced was not absent (figure 3.4). This may indicate a role for nitric oxide (and therefore cyclic GMP) in controlling low level platelet stimulation rather than overt activation, although it certainly had a dampening effect on even the highest agonist concentrations.

Characterisation of neutrophil inhibitory action on platelets. Figure 3.5 shows that glutaraldehyde fixed neutrophils at a concentration of $2x10^9/l$ had no inhibitory effect on platelet aggregation to 0.025u/ml thrombin, whereas viable neutrophils at the same concentration gave significant inhibition ($p<0.01$). When superoxide dismutase (60u/ml) was added to the platelet suspension prior to neutrophil addition and stimulation then inhibition of aggregation by neutrophils was more pronounced ($p<0.001$). This was statistically highly significant. Similarly, M&B22,948 (1μM), a specific inhibitor of cyclic GMP phosphodiesterase, enhanced inhibition of platelet aggregation. L-arginine (200μM)

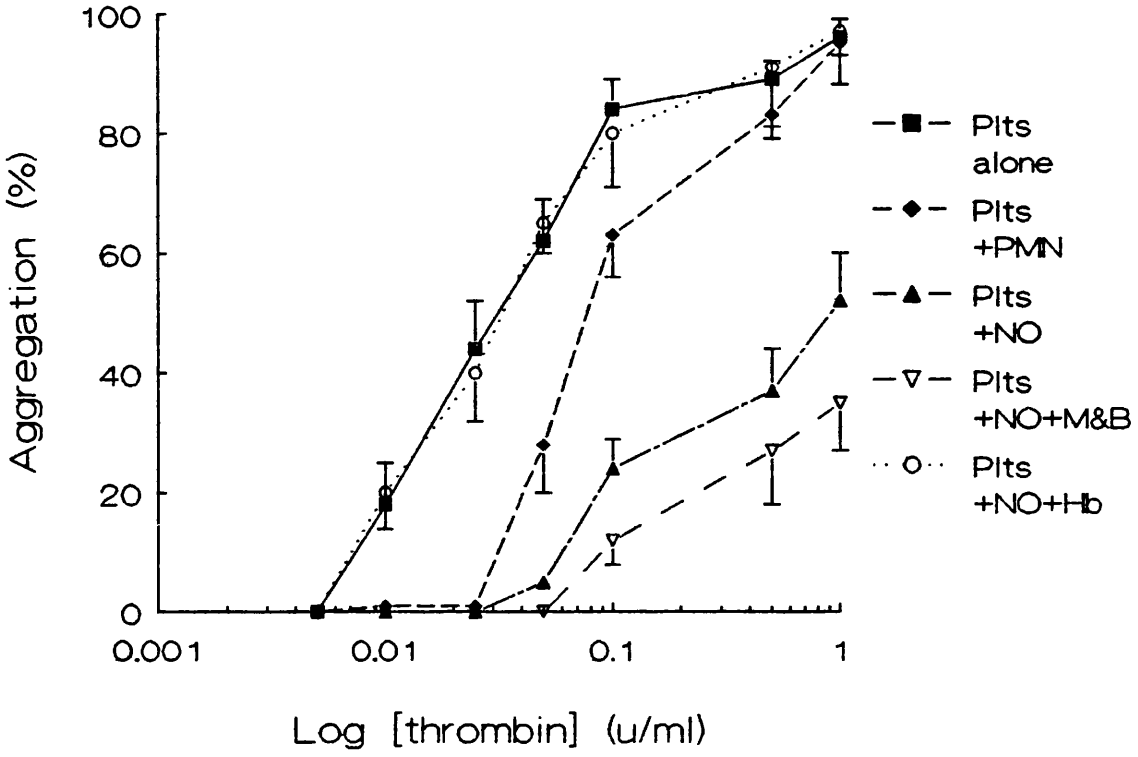

Figure 3.3. Platelet aggregation induced by different concentrations of thrombin in the absence or presence of either $2.5x10^9$/l unstimulated neutrophils (Plts+PMN) or 20µl authentic NO (Plts+NO). Neutrophils were preincubated with platelets for 1 minute, while NO addition immediately preceded addition of thrombin. The action of authentic NO (20µl) was attenuated by 10µM free haemoglobin (Plts+NO+Hb) and enhanced by 1µM M&B22948 (Plts+NO+M&B). Neither haemoglobin nor M&B22948 affected platelet aggregation by themselves at these concentrations. Results show the mean and SEM of 3 experiments.

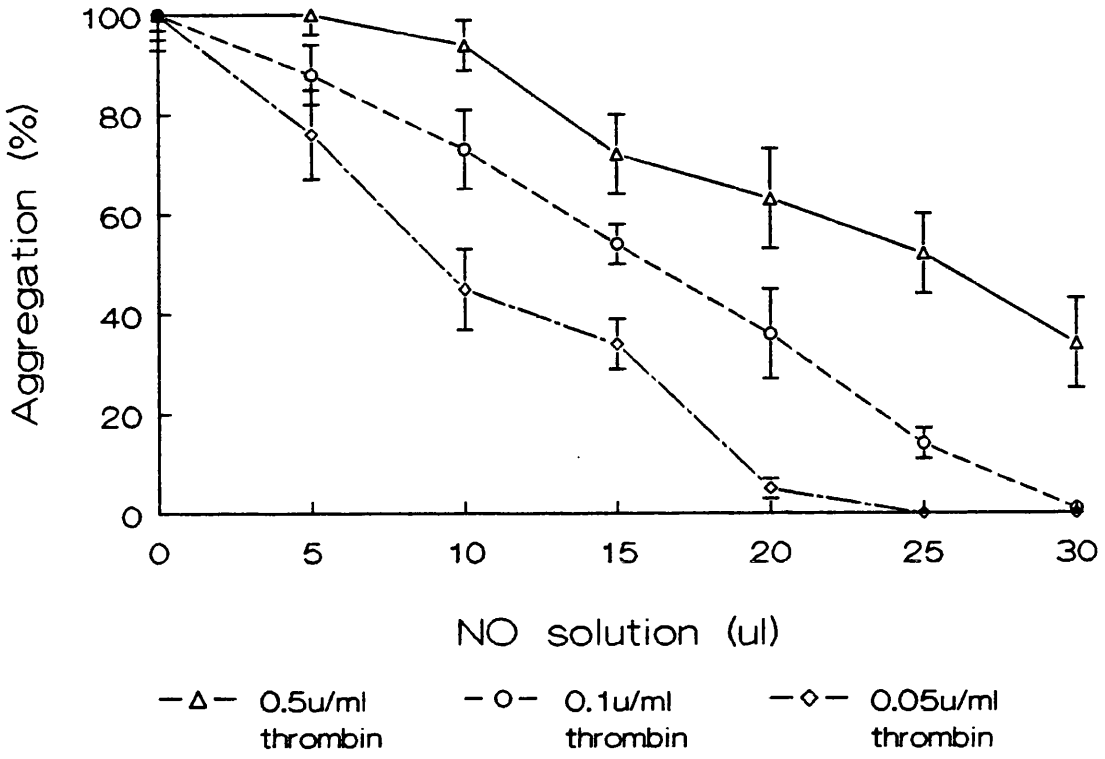

Figure 3.4. The effect of different concentrations of authentic NO on platelet aggregation to 0.05, 0.10 and 0.05u/ml thrombin. Results represent the mean and SEM of 4 separate experiments.

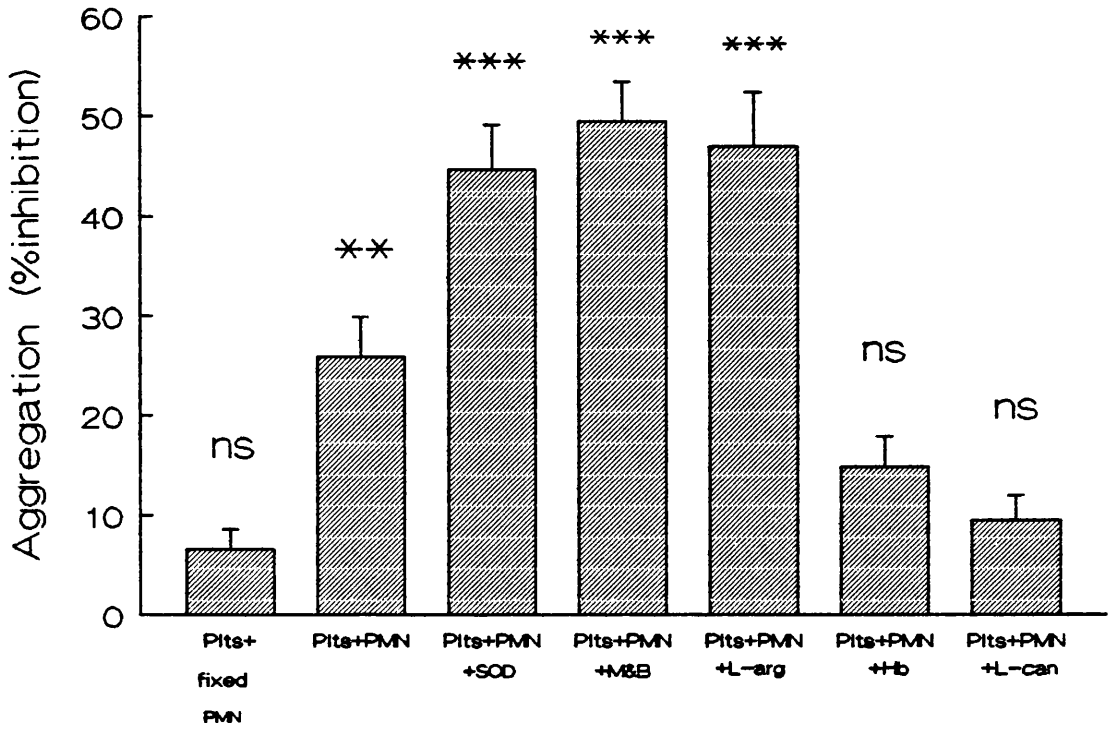

Figure 3.5. Characterisation of a neutrophil-derived inhibitory factor on thrombin (0.025u/ml) induced platelet (Plts) aggregation in the presence of $2x10^9$/l neutrophils (PMN) by either superoxide dismutase (SOD), M&B22,948 (M&B), L-arginine (L-arg), haemoglobin (Hb) or L-canavanine (L-can) added. Results show the mean and SEM for ten separate experiments. Student's paired t-test was performed and the results were either not significant (ns), $p<0.01$ (**) or $p<0.001$ (***).

the presumed substrate for nitric oxide production also enhanced inhibition. However, free haemoglobin (10μM) and L-canavanine (1mM), a structural analogue of L-arginine, both independently reduced the inhibition of platelet aggregation caused by neutrophils.

Platelet ATP release. Viable neutrophils ($2.5x10^9$/l) diminished platelet ATP release ($p<0.035$) as shown in figure 3.6. Superoxide dismutase slightly enhanced this effect ($p<0.017$), whereas haemoglobin abolished inhibition of ATP release by neutrophils. Neutrophils did not release detectable amounts of ATP (data not shown).

Thromboxane B_2 and 6-ketoPGF$_{1\alpha}$ release. Neutrophils alone did not release detectable amounts of thromboxane B_2. However when viable neutrophils were incubated with thrombin-stimulated platelets they attenuated platelet thromboxane B_2 release as seen in figure 3.7. Gluteraldehyde fixed neutrophils had no effect on platelet thromboxane B_2 release. The stable breakdown product of prostacyclin, 6-keto-PGF$_{1\alpha}$, was not detected in any samples. Neither did inhibition of prostaglandin production by incubation with indomethacin (5μM) prevent neutrophil inhibition of platelet aggregation (data not shown). These data indicate that the inhibition of platelet aggregation was not due to the release of prostacyclin by neutrophils.

Platelet cyclic GMP in the presence of neutrophils. Sodium nitroprusside (1μM) and authentic nitric oxide caused large increases in intracellular cyclic GMP levels in platelets (figure 3.8). Neutrophils caused an increase in platelet cyclic GMP and this was augmented by superoxide dismutase. Haemoglobin prevented the rise in platelet cyclic GMP caused by neutrophils.

When neutrophils were stimulated with FMLP (1μM) 1 minute before the stimulation of platelets with 0.025u/ml thrombin (figure 3.9) then they further increased platelet cyclic GMP concentration. Platelet cyclic GMP was increased further in the

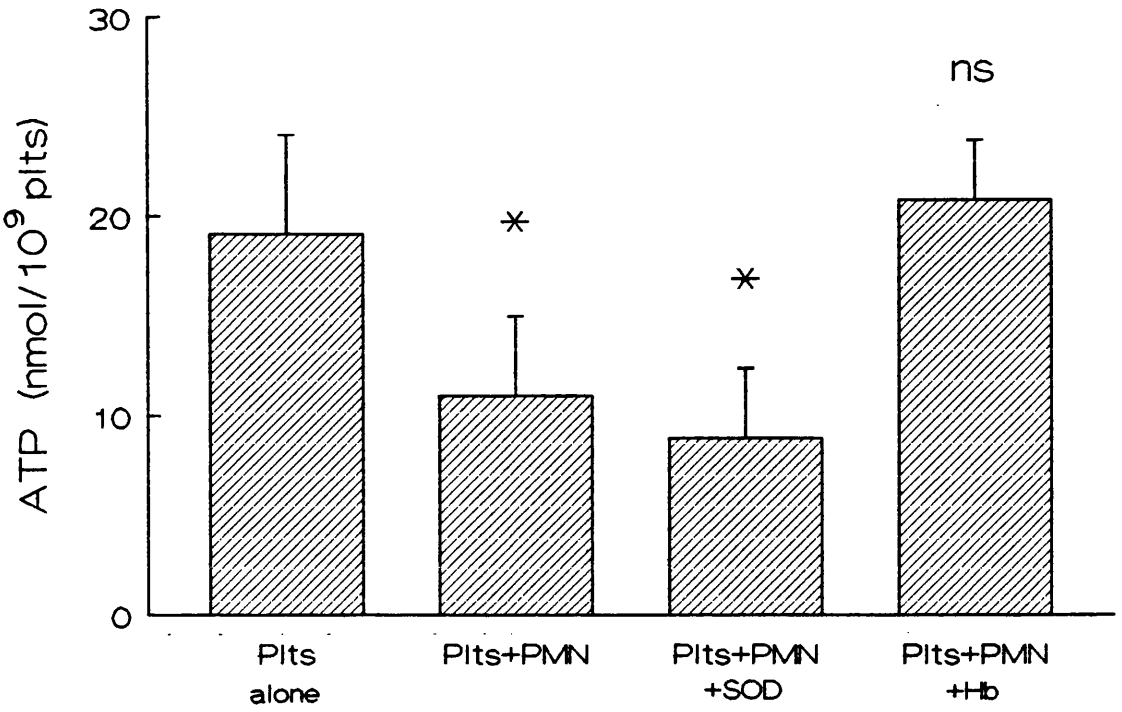

Figure 3.6. The effect of $2.5x10^9$/l neutrophils (PMN) on 0.025u/ml thrombin-induced platelet ATP secretion in the presence of either superoxide dismutase (SOD) or haemoglobin (Hb). Results indicate the mean and SEM of five separate experiments and were either not statistically significant (ns) or $p<0.05$ (*) by Student's paired t-test.

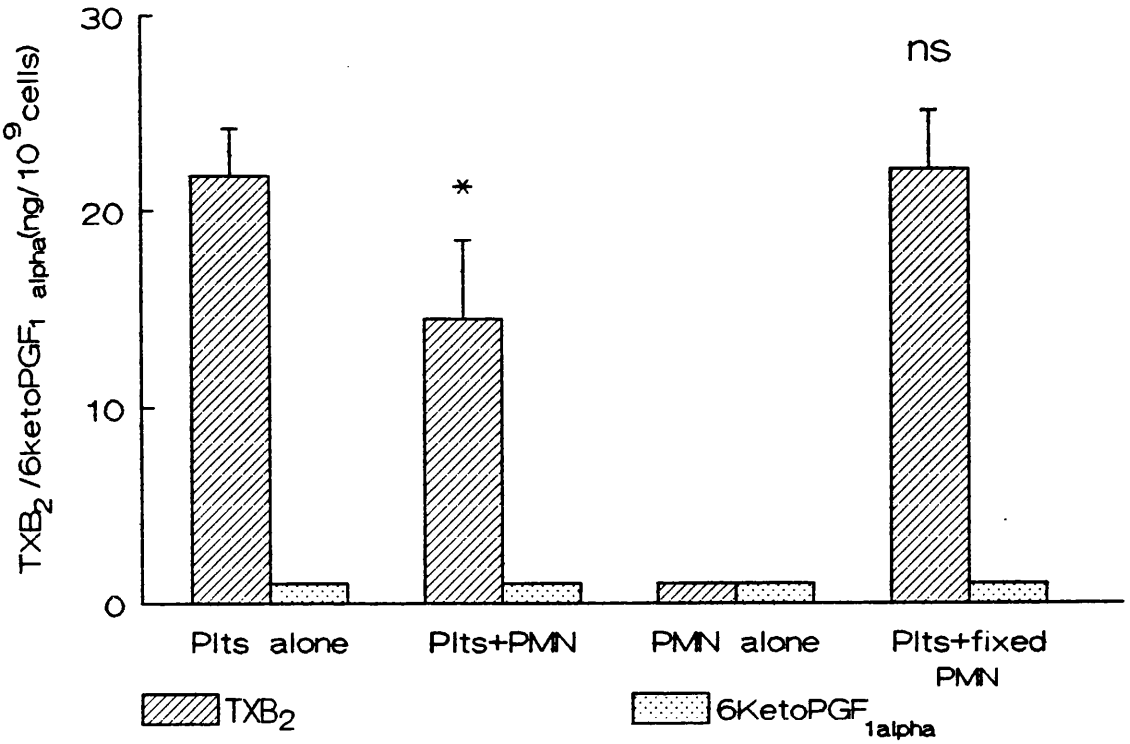

Figure 3.7. Thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6ketoPGF$_{1alpha}$) release from platelets alone (Plts alone), neutrophils alone (PMN alone), platelets and neutrophils (Plts+PMN) or platelets and fixed neutrophils (Plts+fixed PMN) stimulated with 0.025u/ml thrombin. Results indicate mean and SEM from five separate experiments and were either not statistically significant (ns) or $p<0.05$ (*) by Student's paired t-test.

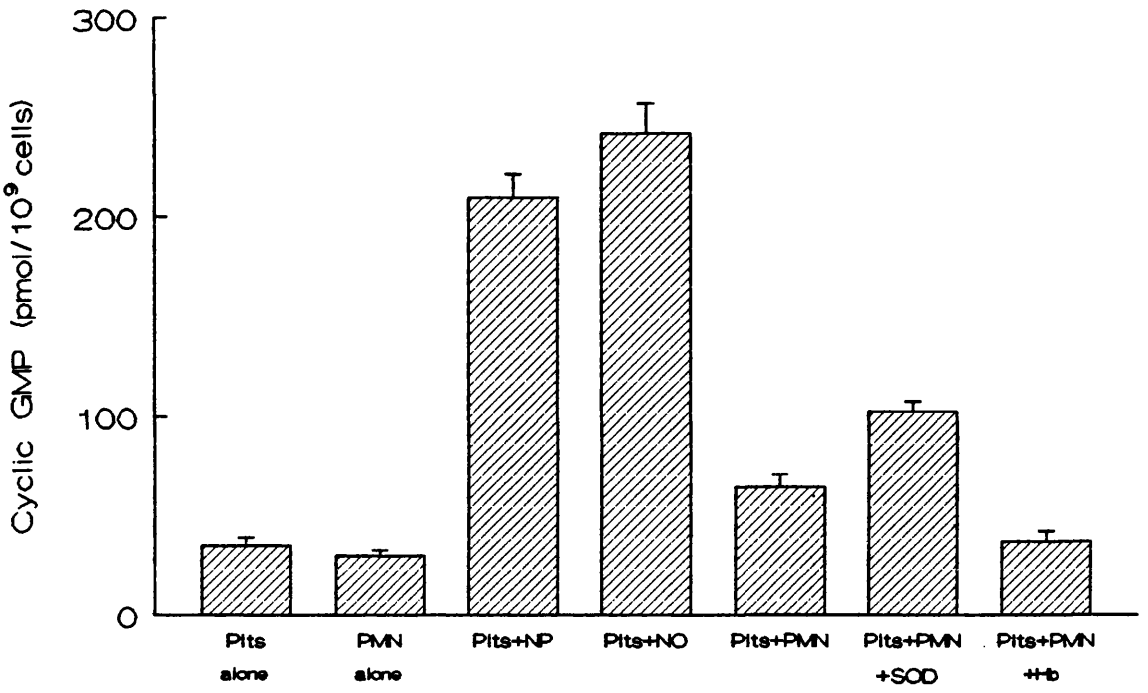

Figure 3.8. The effect of neutrophils ($2.5x10^9/l$) on platelet cyclic GMP concentration. Platelets (Plts) were stimulated with 0.025u/ml thrombin in the absence or presence of either superoxide dismutase (SOD) or haemoglobin (Hb). Platelets were also stimulated in the presence of either sodium nitroprusside (NP) or authentic nitric oxide (NO). Results indicate the mean and SEM of three separate experiments.

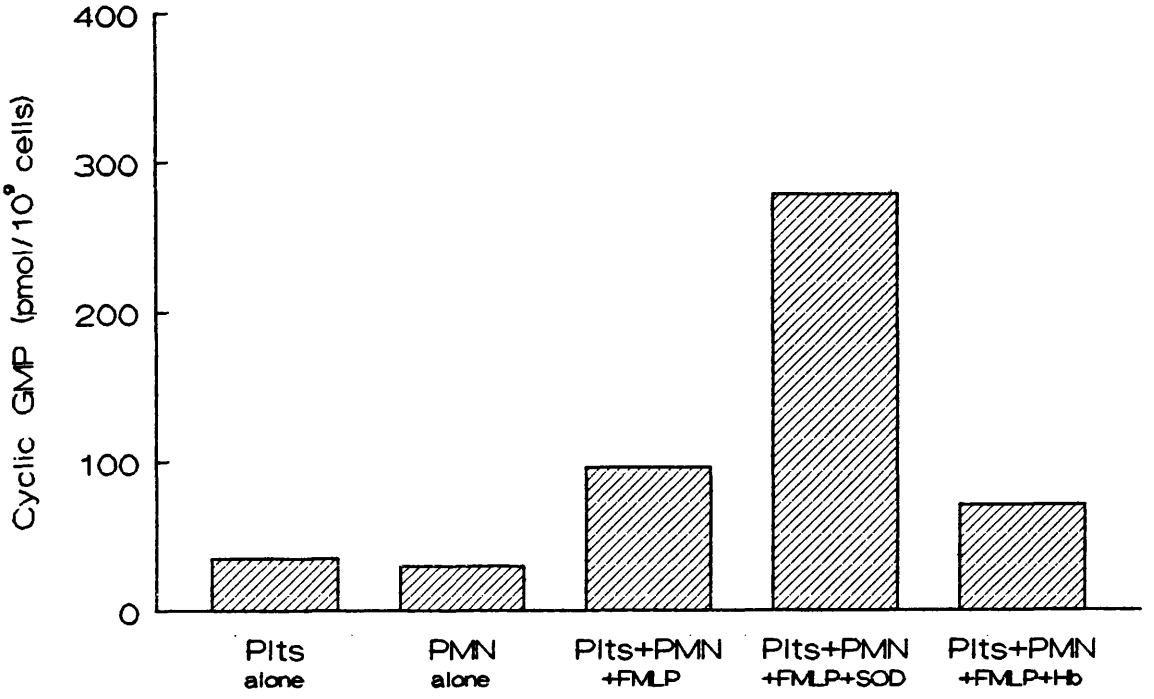

Figure 3.9. The effect of neutrophils ($2.5x10^9$/l) stimulated with FMLP (1μM) on platelet cyclic GMP concentration. Platelets were stimulated with 0.025u/ml thrombin in the absence or presence of superoxide dismutase (60u/ml; SOD) or haemoglobin (10μM; Hb). Results indicate the mean of two separate experiments.

presence of FMLP-stimulated neutrophils compared to platelets in the presence of unstimulated neutrophils. Superoxide dismutase caused a significant enhancement of the effect of activated neutrophils on platelet cyclic GMP. This is because superoxide dismutase binds to superoxide anions so preventing them from destroying nitric oxide. Haemoglobin lessened the effect of activated neutrophils on platelet cyclic GMP.

Stimulation of neutrophils with LTB_4. LTB_4 had no effect on the aggregation of platelets alone as shown by figure 3.10. Thrombin stimulated platelet aggregation was unaffected by fixed neutrophils to which LTB_4 had been added. However when viable neutrophils were stimulated with LTB_4 (50ng/ml) then inhibition of platelet aggregation was more pronounced than with unstimulated neutrophils.

The inhibition of platelet aggregation by neutrophils with different agonists was not completely prevented by inhibition of NO synthesis. Figure 3.11 demonstrates that N^G-N^G-nitro-L-arginine, an inhibitor of NO synthesis, did not completely restore platelet aggregation in the presence of neutrophils. This suggested the presence of at least one other inhibitory factor.

Characterisation of another neutrophil-derived inhibitor. The partial inhibition of aggregation to collagen could not be prevented by either catalase or superoxide dismutase indicating that the inhibitor was not another oxygen free radical (figure 3.12). Similarly, aspirin (100μM) treated neutrophils were still able to antagonize platelet aggregation. The inhibition of neutrophil 5-lipoxygenase by Piriprost (40μg/ml), a specific inhibitor, partially restored aggregation, but this was not statistically significant. Apyrase, an enzyme able to degrade ADP, did significantly restore platelet aggregation when added to platelet-neutrophil mixtures. Apyrase obviously did not completely restore platelet aggregation as it is an inhibitor in its own right causing the degradation of ADP released from platelets.

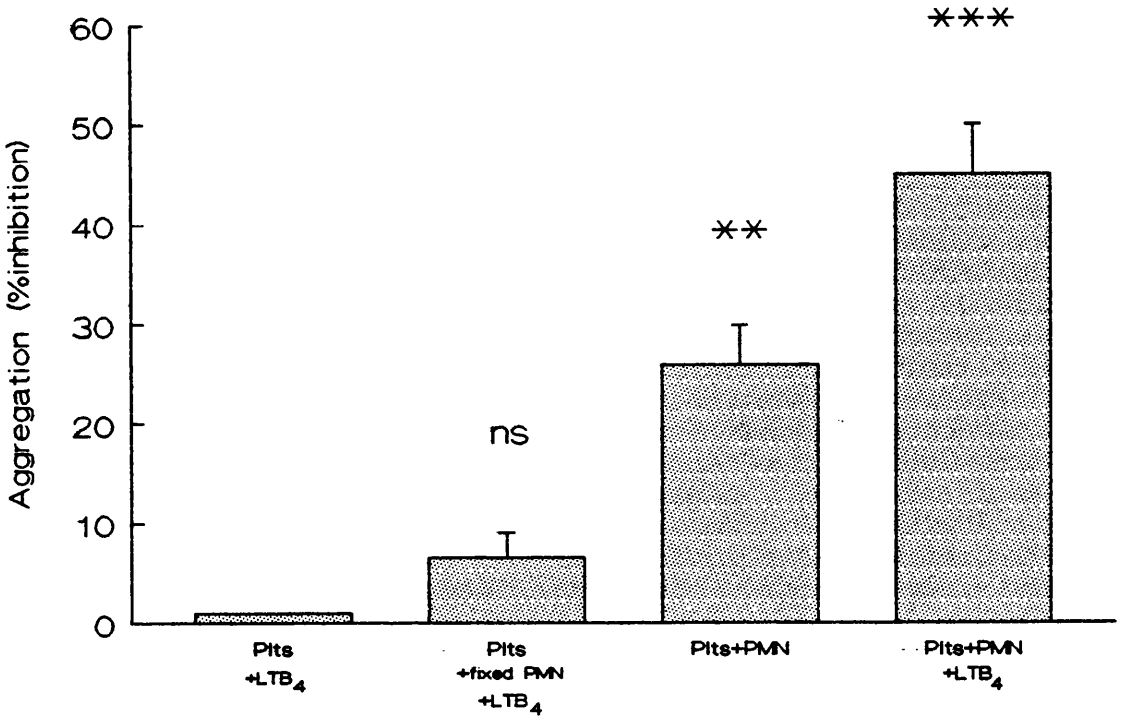

Figure 3.10. The effect of 50ng/ml LTB_4 stimulated neutrophils on 0.025u/ml thrombin-induced platelet (Plts) aggregation. Neutrophils were either viable (PMN) or fixed (fixed PMN). Results show the mean and SEM of five separate experiments and were either not statistically significant (ns), $p<0.01$ (**) or $p<0.001$ (***) when compared to platelets alone (Plts) by Student's paired t-test. LTB_4 stimulated neutrophils were significantly different from unstimulated neutrophils ($0.05 > p > 0.01$).

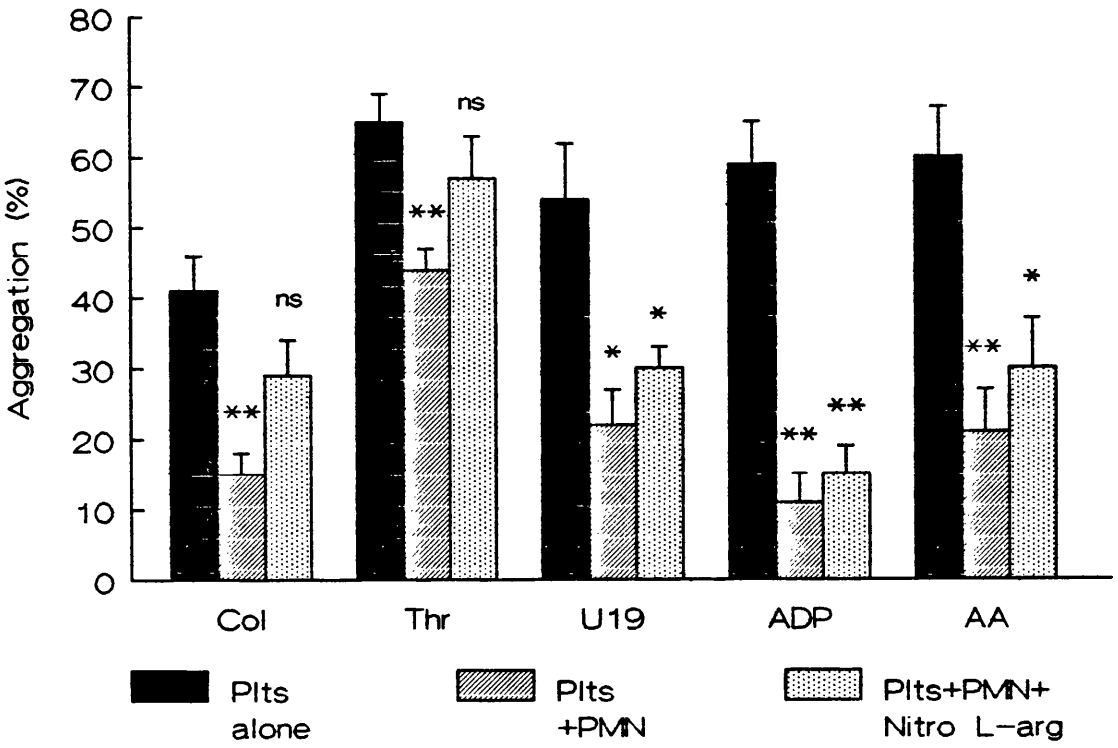

Figure 3.11. The failure of N^G-nitro L-arginine, an inhibitor of NO synthesis to completely restore platelet aggregation in the presence of neutrophils. Platelets were stimulated in the absence (Plts alone) or presence of neutrophils (Plts+PMN) with collagen (Col), 10μg/ml; thrombin (Thr), 0.1u/ml; U46619 (U19), 1.25μg/ml; ADP (ADP), 10μM; or arachidonic acid (AA), 0.5mM. N^G-Nitro L-arginine (100μM) was added in another set of experiments to prevent any inhibition due to NO. Results show the mean and SEM of 4 experiments and were not statistically significant (ns), $p<0.05$ (*) or $p<0.01$ (**).

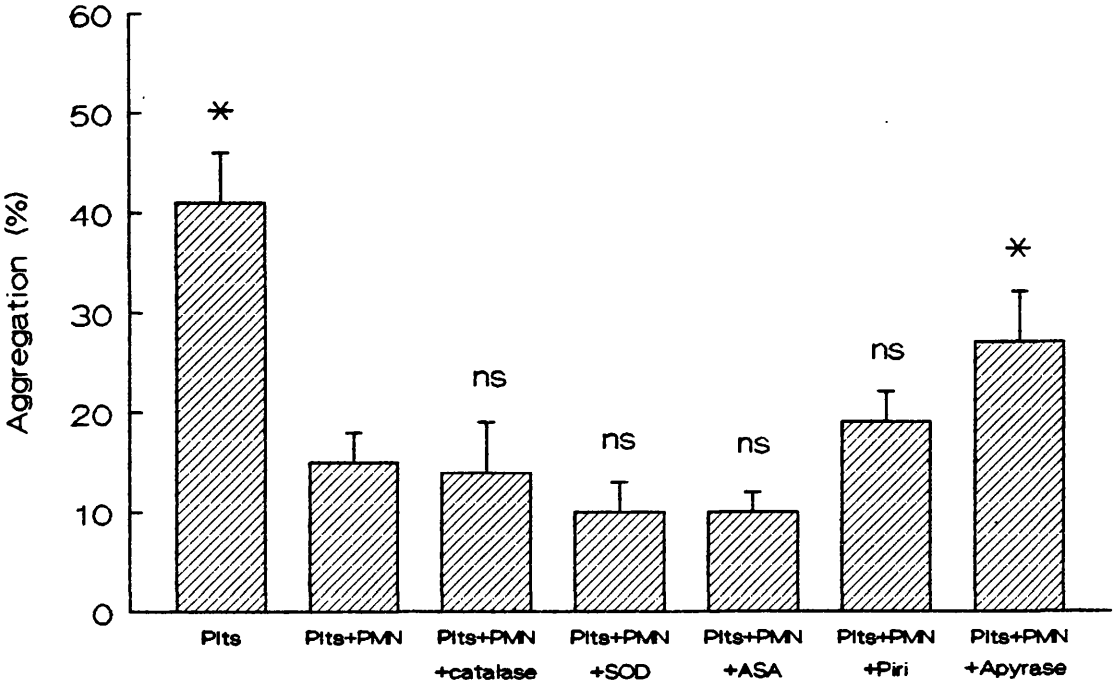

Figure 3.12. The preliminary characterisation of another neutrophil-derived inhibitor of platelet function. Platelets were stimulated with 1μg/ml collagen in the absence (Plts) or presence (Plts+PMN) of neutrophils ($5x10^9$/l). The following were added to mixed cell suspensions: catalase (100u/ml); superoxide dismutase (SOD), 60u/ml; aspirin (ASA), 100μM; Piriprost (Piri), 40μg/ml; and apyrase (10u/ml). Results indicate the mean and SEM of 3 experiments and were statistically not significant (ns) or $p<0.05$ (*).

The action of neutrophil-derived adenosine on platelet aggregation. Figure 3.13 shows that when neutrophils were stimulated with FMLP (1μM) then their inhibitory activity was enhanced compared to unstimulated neutrophils. Adenosine deaminase (6u/ml) preincubated with mixed suspensions of platelets and neutrophils reversed neutrophil-mediated inhibition of collagen activated platelets. Adenosine deaminase had no effect on platelets alone. These results suggest the involvement of neutrophil-derived adenosine in mediating the inhibition of platelet aggregation and/or the metabolism of platelet-derived ADP to adenosine. Figure 3.14 shows a dose response curve for collagen-activated platelets in the presence of adenosine. Its reversal by adenosine deaminase was demonstrated.

Inhibition of platelet aggregation by supernatants from neutrophils. Figure 3.15 shows that the supernatant of FMLP-stimulated neutrophils has some inhibitory activity as does the supernatant from a mixed platelet and neutrophil suspension stimulated with FMLP. However, the supernatant from a suspension of thrombin and FMLP-stimulated platelets and neutrophils inhibited platelet aggregation the most. FMLP-stimulated neutrophils alone, with added ADP, also gave further platelet inhibition. This indicated the metabolism of ADP to adenosine (as adenosine deaminase abolished it) by neutrophils.

DISCUSSION

The presence of human neutrophils in stimulated, washed platelets suspensions (as well as in platelet-rich plasma) inhibited platelet aggregation in a manner that was dependent on neutrophil concentration and to an extent on period of incubation. This effect was seen with most platelet agonists. Neutrophils had no effect on the platelet agglutination associated with ristocetin stimulation; although the "primary" aggregation

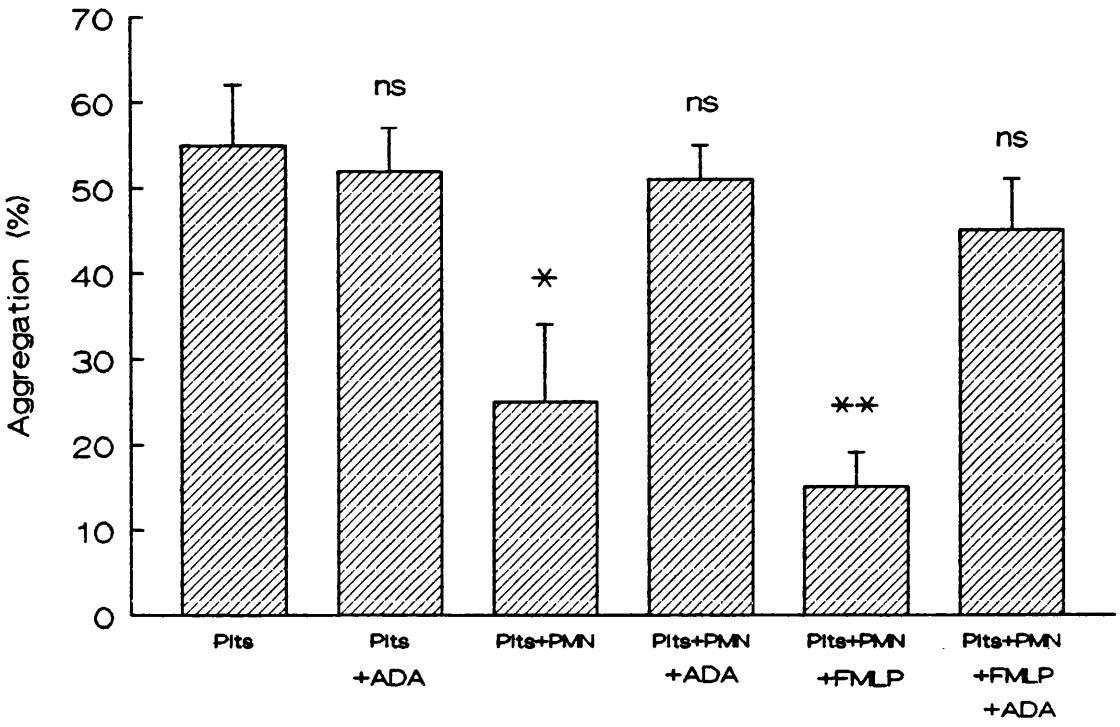

Figure 3.13. The aggregation of platelets to 1μg/ml collagen in the absence (Plts) or presence of neutrophils (Plts+PMN). Adenosine deaminase (ADA; 6u/ml) prevented neutrophil inhibition of platelet aggregation. FMLP-stimulated neutrophils further inhibited platelets, but this too was largely prevented by adenosine deaminase. Results show the mean and SEM of 4 experiments and were statistically not significant (ns), $p<0.05$ (*) or $p<0.01$ (**).

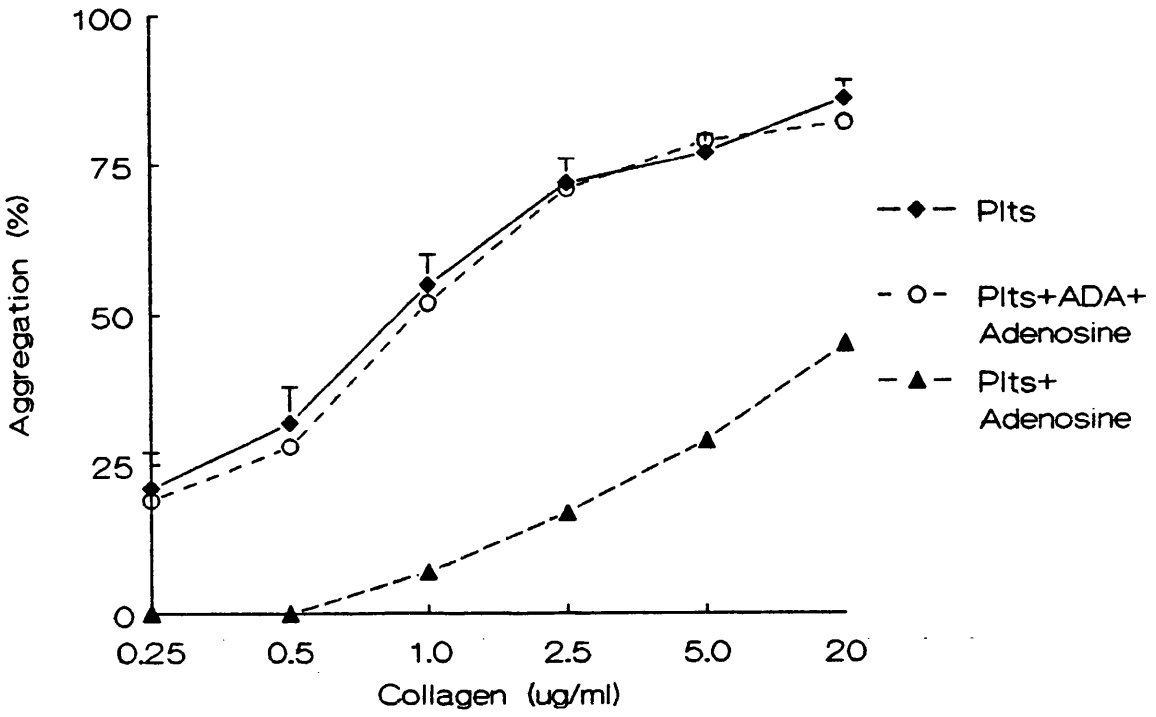

Figure 3.14. A dose response curve for platelet aggregation induced by collagen in the absence (Plts) or presence of either adenosine (100μM) or adenosine and adenosine deaminase (ADA; 6u/ml). Results show the mean and SEM of 5 experiments.

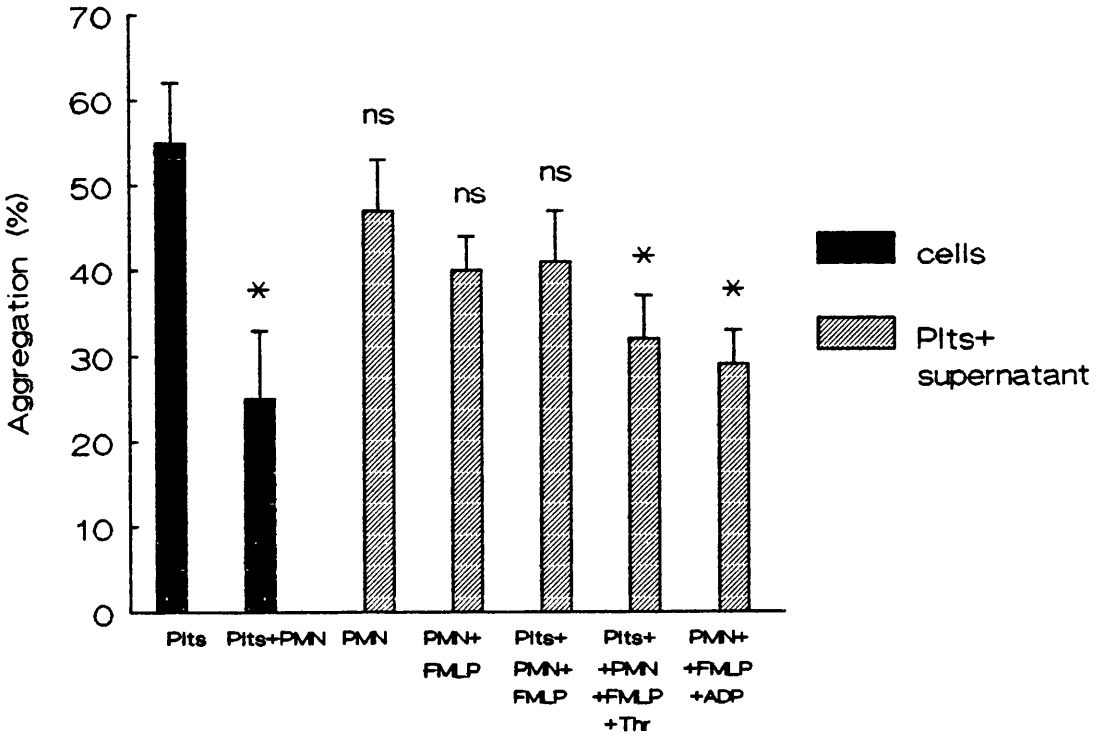

Figure 3.15. The activity of different supernatants (1:10 $^{v}/_{v}$) on platelet aggregation induced by 1μg/ml collagen. All supernatants were prepared 2 minutes after stimulation by rapid centrifugation (10000*g*) and placed on ice. 30μl supernatant was added to washed platelets. Supernatants were from unstimulated neutrophils (PMN); neutrophils stimulated with 1μM FMLP (PMN+FMLP); platelets and FMLP-stimulated neutrophils (Plts+PMN+FMLP); 0.1u/ml thrombin stimulated platelets and FMLP-stimulated neutrophils (Plts+PMN+FMLP+Thr); and FMLP-stimulated neutrophils in the presence of 2.5μM ADP. Results show the mean and SEM of 5 experiments and were statistically not significant (ns) or $p<0.05$ (*).

element was inhibited. The absence of inhibition by neutrophils of platelets stimulated with ristocetin is probably because this agonist, unlike the others tested, causes von Willebrand factor receptor-mediated platelet agglutination (Howard and Firkin, 1971). This suggests that the neutrophil-derived inhibitors were affecting platelet metabolism rather than platelet membrane receptors.

Human neutrophils have recently been shown to modulate platelet aggregation *in vitro* by releasing a factor with the pharmacological profile of nitric oxide (McCall *et al*, 1989; Salvemini *et al*, 1989).

As with platelet aggregation, inhibition of ATP secretion was dependent upon neutrophil concentration. ATP release was apparently more sensitive to inhibition by neutrophils and may be due to the more sensitive nature of the assay compared to turbidometric platelet aggregation.

Neutrophil inhibition of platelet function was most pronounced when platelets were stimulated with submaximal concentrations of agonist (such as 0.05u/ml thrombin). At concentrations of agonist that normally gave maximum aggregation (0.50u/ml thrombin), the presence of unstimulated neutrophils in physiological numbers (20-40 platelets per neutrophil) had less effect. High concentrations of authentic NO solution were able to virtually abolish platelet aggregation at all agonist concentrations, including those that normally caused maximum aggregation.

Inhibition of aggregation did not occur when platelets were stirred in the presence of neutrophils fixed with glutaraldehyde. Therefore inhibition was not due to non specific steric or particle effect.

When free haemoglobin was added to samples it attenuated the inhibitory activity of neutrophils. As haemoglobin abolishes the biological action of nitric oxide by oxidizing

it to nitrate the data would support the concept that neutrophils are producing nitric oxide. Reduced as well as oxidized haemoproteins react with and bind to nitric oxide, forming a relatively stable nitrosyl-haemoprotein complex that is unable to penetrate the plasma membrane. Therefore haemoproteins cannot antagonize the effects of nitric oxide liberated intracellularly (Ignarro, 1989).

Superoxide anions released by activated neutrophils also destroy nitric oxide. The inhibitory activity of neutrophils was enhanced by superoxide dismutase, which dismutes superoxide to hydrogen peroxide which is then further catabolized. This is also suggestive of nitric oxide production.

Nitric oxide activates guanylate cyclase directly and so causes intracellular cyclic GMP accumulation (Salvemini *et al*, 1989). This was shown in platelets either by using authentic nitric oxide or sodium nitroprusside which releases nitric oxide. Nitric oxide is either spontaneously released or metabolized from sodium nitroprusside. The specific cyclic GMP phosphodiesterase inhibitor, M&B22,948, potentiated the inhibitory action on platelets demonstrating that the mechanism of inhibition of platelet aggregation is via increased levels of cyclic GMP.

L-arginine promoted the inhibitory activity of neutrophils, presumably because it provided further substrate for nitric oxide production (Sakuma *et al*, 1988). However, nitric oxide production is generally not thought to be substrate limited and the use of washed cells may have made this artefactually different. L-canavanine, a structural analogue of L-arginine attenuated inhibition when preincubated with neutrophils.

These experiments indicate a basal release of a platelet inhibitor by unstimulated, resting neutrophils which is likely to be nitric oxide. However, it is possible that the purification and experimental procedures resulted in a mild neutrophil activation leading

to NO release.

Neutrophils at the concentration used antagonised both ATP secretion and thromboxane B_2 release without complete inhibition. This is consistent with the mechanism of guanylate cyclase activation which serves to dampen rather than completely inhibit the platelet response to agonists (Kroll and Schafer, 1989).

Activation of neutrophils with FMLP just prior to thrombin induced platelet aggregation caused further inhibition of platelet aggregation and increased cyclic GMP accumulation compared to the addition of unstimulated neutrophils. This was also significantly potentiated by superoxide dismutase. When activated by FMLP, neutrophils release reactive oxidizing agents as well as nitric oxide. Superoxide anions, in particular, are able to destroy nitric oxide. The addition of superoxide dismutase binds the superoxide anions, probably leading to a net release of nitric oxide, over and above that at rest. This would cause further platelet inhibition.

Similarly, LTB_4 stimulated neutrophils caused further significant inhibition of platelet aggregation compared to unstimulated neutrophils. LTB_4 has been reported to cause little superoxide anion release at the concentration used (McCall *et al*, 1989).

The physiological importance of nitric oxide release by neutrophils with subsequent inhibition of platelet aggregation has yet to be determined but may prove to be an important contributing factor for the control of thrombosis. When platelets and neutrophils come into contact *in vivo* the release of nitric oxide could act to restrict the onset and propagation of thrombotic stimuli. Nitric oxide could itself be controlled by superoxide anion release either simultaneous with nitric oxide release or at a later stage when neutrophils have been more fully activated. Haptoglobin-haemoglobin complexes in the plasma could also restrict nitric oxide activity (Edwards *et al*, 1986). Nitric oxide may

regulate superoxide anion levels in blood, especially during weak stimulation of neutrophils as might occur during margination and emigration. A net release of nitric oxide may facilitate neutrophils in leaving the vascular system without endothelial damage. When greater stimuli are encountered, neutrophils may be more fully activated with increased superoxide anion generation. This may lessen the effectiveness of nitric oxide in favour of microbicidal activity.

As well as producing NO, neutrophils are apparently responsible for at least one other platelet inhibitory mechanism. This was evidenced by the inability of N^G-nitro-L-arginine (an inhibitor of NO synthesis) to completely restore full platelet aggregation to mixed cell suspensions. This second inhibitor was not inhibited (or enhanced) by superoxide dismutase or catalase. It appears, therefore, not to be another oxygen free radical. The inhibitor was aspirin-insensitive as was NO. Inhibition of 5-lipoxygenase, also had little effect. However, apyrase, an ADP scavenger, significantly lessened the degree of platelet inhibition caused by neutrophils. This suggests the modification or utilization of ADP and possibly other nucleotides.

Ecto-nucleotidases have been documented to be present in endothelial cells and are seemingly potent regulators of plasma nucleotide levels, especially in the microcirculation (Pearson *et al*, 1980). Platelets contain a large pool of adenine nucleotides which are secreted upon activation. ADP is a potent platelet stimulant, whereas ATP competitively inhibits platelet aggregation. Adenosine is the end product of adenine nucleotide degradation by ectonucleotidases. Adenosine inhibits both platelet and neutrophil stimulation to agonists by raising cyclic AMP levels (Packham *et al*, 1969). 20% of neutrophil ADPase activity has been found to be localized to the cell surface (Smith and Peters, 1985), giving neutrophils significant thromboregulatory potential. The results presented here support this

idea (figures 3.12-15) and as in the case of endothelial cell ecto-ADPase, so the neutrophil enzyme may metabolize ADP released from platelets or damaged tissues. This would then inhibit platelet activation and recruitment.

The activity of adenosine deaminase in abolishing this inhibition points to adenosine synthesis by neutrophils either from platelet-derived ADP metabolism or as a separate pathway. This could also form a negative feedback mechanism for neutrophil activation.

FMLP-stimulated neutrophils had greater inhibitory activity than unstimulated neutrophils, however, this was also effectively abolished by adenosine deaminase under these experimental conditions. The inhibitory activity of different supernatants, although not as potent as platelet suspensions containing intact neutrophils, did nevertheless show the presence of a stable inhibitor. This inhibitor could not have been labile NO; as demonstration of NO synthesis requires its continual release from intact cells. The addition of ADP to FMLP-stimulated neutrophils (in the absence of platelets) provided a supernatant which, when added to stimulated platelets, had enhanced platelet inhibitory activity. This again suggests the generation of adenosine neutrophil ADPase.

The importance of neutrophil-derived adenosine as an inhibitor of platelet function has recently been reported (Zatta *et al*, 1991). Neutrophil ecto-nucleotidases may be important regulators of vasoactive and platelet-active nucleotide concentrations, especially in areas of impaired blood flow such as those within a forming thrombus.

The neutrophil has been widely implicated in the mediation of both host defence and tissue destructive events in inflammatory diseases. The demonstration of nitric oxide release and exogenous nucleotide metabolism by neutrophils may also confer upon them functions that influence haemostasis and thrombosis by regulation of platelet function.

CHAPTER 4

THE INHIBITION OF WHOLE BLOOD AGGREGATION *IN VITRO* BY AN ENDOGENOUS NITRIC OXIDE-LIKE FACTOR

4.1 INTRODUCTION

Platelet aggregation studies can be performed by use of optical, impedance, or particle counting methods. Optical aggregometry is the most common method used, but is limited by the need for centrifugation and separation of platelets from other blood cells, which may modulate platelet behaviour. Also, as platelets are heterogenous in size, density and metabolic activities (Frojmovic & Milton, 1982) it is possible that a sub-population is lost during centrifugation. Methods measuring platelet aggregation in whole blood overcome these disadvantages. The impedance aggregometry technique (Cardinal & Flower, 1980) measures the increase in impedance across two platinum electrodes placed in a blood sample. Platelet aggregation measured in this way has "platelet" aggregates on the electrode that also contain erythrocytes and leukocytes (Joseph *et al*, 1989). This suggests that other cell-types may modulate platelet activity.

This study aims to investigate the effect of authentic nitric oxide and sodium nitroprusside (a nitric oxide donor) on platelet aggregation in whole blood *in vitro*. Whole blood, although anticoagulated, provides a more physiological medium to study the effects of nitric oxide than does a washed cell suspension. Another aim was to investigate the possibility that blood cells may produce nitric oxide themselves in a "whole blood" environment. Blood monocytes and neutrophils have previously been demonstrated to

release a nitric oxide-like factor in washed cell suspensions. More recently still, an L-arginine/nitric oxide pathway has been demonstrated in washed human platelets (Radomski *et al*, 1990). L-Arginine, the presumed substrate for nitric oxide synthesis, when added to washed platelets alone caused inhibition. The factor responsible for this was characterised pharmacologically and found to be nitric oxide. Nitric oxide synthase was also demonstrated to be present and to be activated upon platelet stimulation. This pathway may contribute to regulation of platelet aggregation. Electronic impedance aggregometry has been used to demonstrate that rat aortic rings, added to blood, released endothelium-derived relaxing factor *in vitro* that inhibited platelet aggregation (Bhardwaj *et al*, 1988).

4.2 METHODS

Blood samples. These were collected into *tri*-sodium citrate from apparently healthy volunteers (see section 2.1).

Platelet aggregation. This was recorded by the electrical impedance technique (see section 2.6.2) in either "whole blood" or platelet-rich plasma ($200x10^9$/l; see section 2.3) to which washed, purified red cells were added.

The response to different concentrations of collagen was recorded in the absence or presence of one or more of the following: L- or D-arginine (10-1000μM), M&B22948 (1μM), pentoxifylline (500μM), N^G-nitro-L-arginine (100μM), and purified red blood cells. All compounds were preincubated with whole blood or platelet-rich plasma for 5 minutes before addition of agonist. Authentic nitric oxide and sodium nitroprusside (60μM) were added immediately prior to agonist.

Red cell purification. After platelet-rich plasma collection, the remaining citrated blood

was centrifuged at 2000*g* for 10 minutes. The platelet-poor plasma and buffy coat were removed and the remaining cells resuspended in Hanks Balanced Salt Solution. This process was then repeated twice, except that the cells were centrifuged at 170*g*. The red cells were then diluted 1:4 in buffer, layered over Histopaque-1096 (Sigma) and centrifuged at 400*g* for 20 minutes. This effectively removed any remaining platelets or leukocytes.

Authentic nitric oxide. This was prepared as described (section 2.9).

Statistics. Results were analysed using Wilcoxon's signed rank test for non-parametric data (section 2.14).

4.3 RESULTS

The effect of authentic nitric oxide and sodium nitroprusside on aggregation in whole blood. Sodium nitroprusside partially inhibited aggregation when added immediately prior to collagen (see figure 4.1), but this did not reach statistical significance (p=0.172). However, preincubation with M&B22948 significantly furthered inhibition of impedance aggregation (p=0.011) compared to the control (5μg/ml collagen only). This was also significantly different from the partial inhibition by nitroprusside (p=0.011). When added more than 30 seconds apart from agonist, nitroprusside was unable to cause inhibition suggesting perhaps that the donated nitric oxide had been exhausted and a steady state regained.

Authentic nitric oxide (10-100μl/ml) had no effect upon collagen-induced aggregation even at low doses of agonist (1μg/ml) and after the addition of M&B22948.

The effect of purified red cells upon nitric oxide-induced inhibition of aggregation. When platelet-rich plasma was stimulated with different concentrations of collagen it was dose-

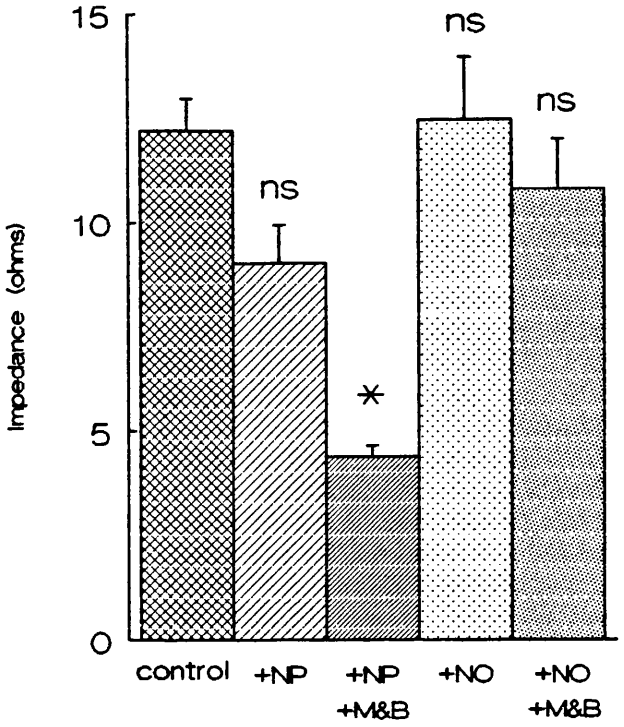

Figure 4.1. Aggregation was induced in citrated whole blood by 5μg/ml collagen. Either sodium nitroprusside (60μM) or authentic nitric oxide (50μl) were added to samples immediately prior to agonist. Samples were stimulated with collagen alone (control); collagen with nitroprusside (+NP); collagen with nitroprusside and M&B22948 (+NP+M&B); collagen with authentic nitric oxide (+NO); or collagen with authentic nitric oxide and M&B22948 (+NO+M&B). Results indicate the mean and SEM for 6 separate experiments and are shown as either not significant (ns) or $p<0.05$ (*).

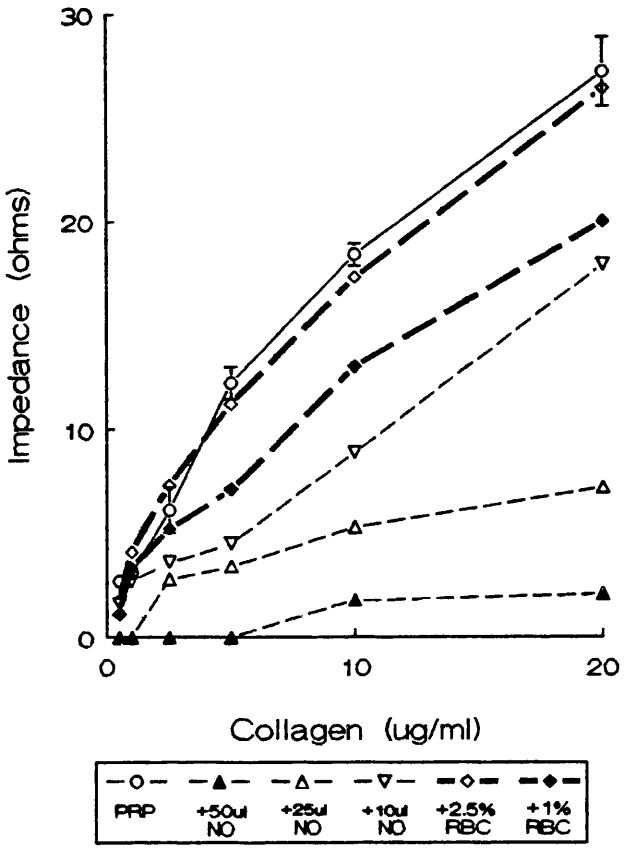

Figure 4.2. Aggregation was induced by 0.5-20μg/ml collagen in platelet-rich plasma (PRP) in the absence or presence of different nitric oxide concentrations (+50μl NO, +25μl NO and +10μl NO respectively). The effect of two different concentrations (1.0 and 2.5% $^{v}/_{v}$) of purified red cells on inhibition by 50μl nitric oxide was also observed (+2.5% RBC and +1%RBC). Results show the mean of 3 experiments.

dependently inhibited by nitric oxide (figure 4.2). Inhibition was virtually complete when 50µl nitric oxide was added, even at the highest concentration of collagen. However, the addition of only 2.5% $^{v}/_{v}$ of red cells completely abolished the effect of 50µl nitric oxide. 1% $^{v}/_{v}$ red cells partially abolished the activity of nitric oxide (50µl).

The identification of nitric oxide-like activity in whole blood. When aggregation was induced with collagen (5µg/ml), in citrated whole blood, in the presence of 250µM L-arginine there was a significant (p=0.004) inhibition of aggregation (figure 4.3). This was significantly enhanced by M&B22948 compared to both the control (p=0.002) and the L-arginine inhibited samples (p=0.033). Pentoxyfylline, a specific cyclic AMP phosphodiesterase inhibitor, had no detectable enhancing effect on aggregation. D-arginine did not have any discernable effect on collagen induced aggregation.

L-arginine, but not D-arginine, dose dependently inhibited aggregation induced by collagen (figure 4.4; 5µg/ml). The IC_{50} was 438±57µM (mean±SEM). This was significantly enhanced by M&B22948 to 296±67µM.

Inhibition induced by L-arginine (250µM) was effectively abolished by preincubation with N^{G}-nitro-L-arginine, a competitive inhibitor of nitric oxide synthesis (figure 4.5).

DISCUSSION

Inhibition of platelet aggregation by L-arginine has been previously demonstrated although the exact mechanism remained unknown (Houston *et al*, 1983). It has subsequently been shown that platelets possess a pathway for nitric oxide synthesis (Radomski *et al*, 1990). This was demonstrated using both washed platelets and whole blood anticoagulated with hirudin. Platelets were counted after stimulation with

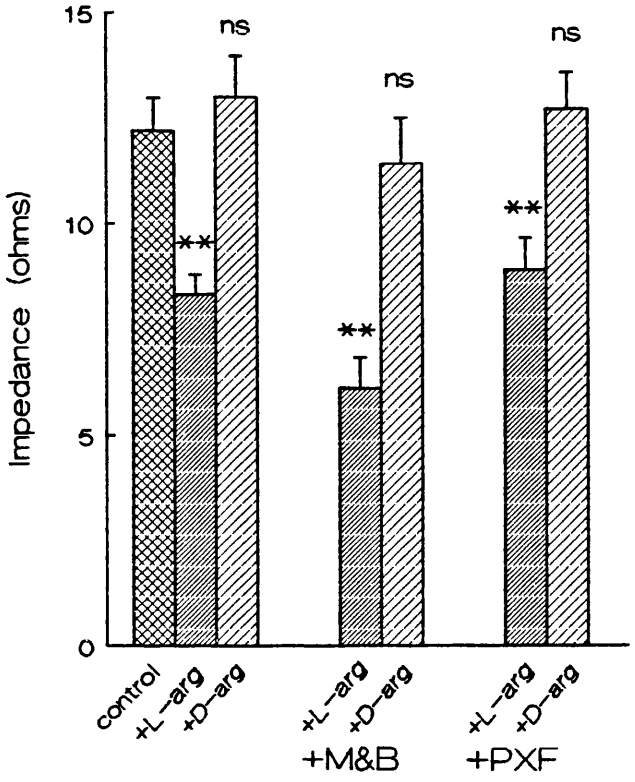

Figure 4.3. Aggregation was induced by 5μg/ml collagen (control) in citrated whole blood. The addition of 250μM L-arginine (+L-arg) 3 minutes prior to collagen addition resulted in significant inhibition of aggregation (p=0.0036). D-arginine (+D-arg) had no significant effect (p=0.5447). Inhibition of aggregation was enhanced by M&B22948 (+L-arg+M&B; p=0.0023); this was also significantly different from inhibition by L-arginine alone (p=0.033). Again D-arginine, with M&B22948, was not significantly different from the control (D-arg+M&B; p=0.2832). Pentoxifylline did not significantly enhance inhibition caused by L-arginine (+L-arg+PXF) and had no effect in the presence of D-arginine (+D-arg+PXF). Results show the mean and SEM of 6 experiments and were either not significant (ns) or $p<0.01$ (**).

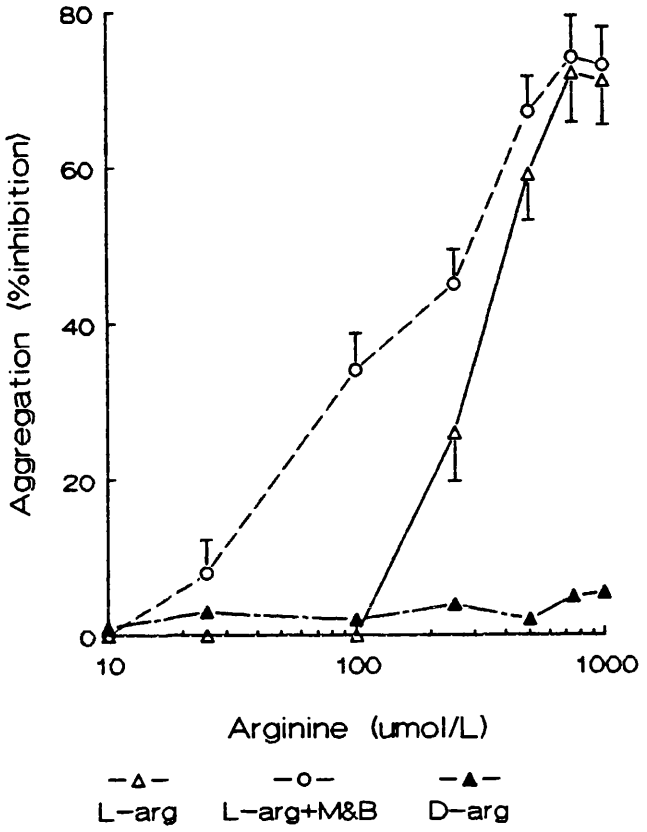

Figure 4.4. The inhibition of aggregation induced by 5μg/ml collagen in whole blood by 10-1000μM L-arginine in the absence (L-arg) or presence of M&B22948 (L-arg+M&B); or by 10-1000μM D-arginine (D-arg). Results show the mean and SEM of 3 experiments.

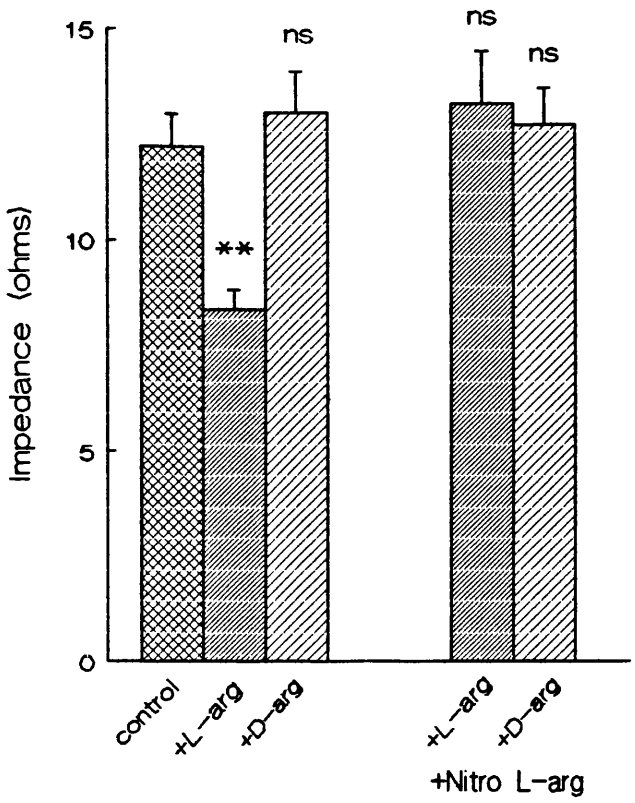

Figure 4.5. The effect of N^G-nitro-L-arginine (100μM), a competitive inhibitor of nitric oxide synthesis, on aggregation induced by 5μg/ml collagen in the presence of either L-arginine (L-arg) or D-arginine (D-arg). Aggregation induced by 5μg/ml collagen alone (control) or in the presence of either L-arginine (L-arg) or D-arginine (D-arg) are shown for comparison. Results show the mean and SEM of 6 experiments and were either not significant (ns) or $p<0.01$ (**).

0.01-3.0μg/ml collagen by a whole blood platelet counter.

This study not only confirms these findings but is the first to demonstrate the release of a nitric oxide-like factor in citrated whole blood stimulated with collagen and measured by electrical impedance aggregometry. Authentic nitric oxide was found to have no significant effect on aggregation induced by 5μg/ml collagen. Even the addition of M&B22948, a specific cyclic GMP phosphodiesterase inhibitor, failed to enhance any inhibition caused by nitric oxide. Sodium nitroprusside at high concentrations (60μM) had significant inhibitory effects on whole blood aggregation. This effect was short lived and could only be detected when nitroprusside was added immediately prior to agonist. It was enhanced by M&B22948. This suggests that the donated nitric oxide was either at a much higher concentration or was afforded some protection by the nitroprusside molecule allowing it to effect platelet inhibition. Nevertheless, the inhibition resulting from nitroprusside disappeared within 20-30 seconds as collagen-induced aggregation after this time was normal.

The inability of authentic nitric oxide to inhibit aggregation in whole blood seems to be due to the large number of red cells present. Even at concentrations of 1-2% $^{v}/_{v}$, red cells abolished the inhibition caused by authentic nitric oxide upon collagen-induced aggregation in platelet-rich plasma. Nitric oxide is a very lipophilic molecule and probably entered the red cells where it could be inhibited by haemoglobin. Free haemoglobin was absent from the supernatants of the purified red cell preparation. Red cells have previously been demonstrated to inhibit nitric oxide-like activity in a different system (Evans *et al*, 1989).

The addition of L-arginine, the substrate for nitric oxide synthesis, significantly inhibited collagen-induced aggregation. It was unable to completely abolish aggregation

even at concentrations as high as 1000μM. D-arginine had no detectable effect upon platelet aggregation suggesting that the inhibition was not due to simple osmolarity or pH changes. M&B22948 enhanced the inhibition of aggregation induced by L-arginine, but not D-arginine. This would indicate that L-arginine was causing inhibition via guanylate cyclase stimulation. Pentoxiphylline, a cyclic AMP phosphodiesterase inhibitor, did not enhance inhibition caused by L-arginine. At high concentrations of L-arginine inhibition was barely enhanced by M&B22948 and even appeared to plateau out. This may be because the nitric oxide synthesising potential had been saturated and the substrate, L-arginine, was in excess. In these experiments the IC_{50} for L-arginine was almost double that reported by Radomski and co-workers in whole blood. This could be for either of two reasons: firstly, they used lower collagen concentrations to induce aggregation (up to 3μg/ml); and secondly, their method of measuring aggregation (by cell counting) may be more sensitive than the impedance technique used in this study.

The inhibition of aggregation induced by L-arginine was abolished by preincubation of whole blood with N^G-nitro-L-arginine, a competitive inhibitor of L-arginine-mediated nitric oxide synthesis. This suggests that the L-arginine was not causing inhibition by itself, but was indeed being metabolised to a nitric oxide-like factor.

L-arginine is present in high concentrations in human blood (about 50-140μM; Glass *et al*, 1986). This would provide ample substrate for nitric oxide synthesis *in vivo*. Radomski and co-workers (Radomski *et al*, 1990) have reported that L-arginine did not increase basal levels of cyclic GMP in unstimulated platelets but did so when they were first stimulated with collagen. This suggests that nitric oxide synthesis occurs only once the cells have been stimulated. This could then act as an important negative feedback mechanism to down-regulate platelet aggregation. It is uncertain whether activation of the

cell is necessary to stimulate L-arginine uptake or nitric oxide synthase, although high concentrations of L-arginine in blood may down-regulate its uptake by the cell. The inability of authentic nitric oxide to inhibit aggregation in whole blood indicates that platelets themselves may synthesise nitric oxide intracellularly for autoregulation when stimulated in the presence of excess L-arginine. Alternatively, the shear rates induced *in vitro* by stirring could facilitate membrane-membrane contact between platelets and other nitric oxide releasing cells such as neutrophils. This may protect nitric oxide from degradation by red cells. Similarly, *in vivo*, the streaming characterisitics of flowing blood mean that platelets stream towards the perimeter of a vessel, closer to the endothelium, whereas red cells stream along the centre of a vessel. This may allow nitric oxide to exert a powerful but local platelet regulating activity. L-arginine could also be utilised by endothelial cells and phagocytes as well as platelets for nitric oxide synthesis.

CHAPTER 5

NEUTROPHILS STIMULATE PLATELET INTRACELLULAR CALCIUM FLUX AND AGGREGATION *IN VITRO.*

5.1 INTRODUCTION

Neutrophils are endowed with the capacity to produce oxidative and non-oxidative mediators (such as proteases) that exert potent microbicidal and, in some circumstances, tissue destructive effects (Weiss, 1989).

The milieu of the acute inflammatory response contains both cellular and non-cellular components that trigger and modify the release of these neutrophil-derived agents. High concentrations of bacterial chemotactic peptides, activated complement components, or antibody fixed to non-phagocytosable tissues stimulate the release of reduced oxygen products and granular proteases from invading neutrophils. Recent studies have demonstrated the importance of interaction between plasma antiproteinases (such as α_1-proteinase inhibitor) and neutrophil proteases and oxyradicals in determining the effects of these inflammatory cells on connective tissues (Weiss, 1986).

Platelet activation induced by stimulated neutrophils has been demonstrated in animals and humans (Cöeffier *et al*, 1987; Oda *et al*, 1986; Virella *et al*, 1982). Another report (Del Maschio *et al*, 1990) showed that neutrophils challenged with different agonists induced platelet activation. Activation was unaffected by PAF-receptor antagonists in mixed cell suspensions containing FMLP-stimulated neutrophils, suggesting the lack of PAF involvement. Similarly, aspirin, at concentrations completely blocking

TXA_2 synthesis, did not influence platelet activation by neutrophils. The activation of platelets by neutrophils was dependent on pretreatment of the neutrophils with cytochalasin B. This compound enhances secretion of granule-bound material to the cell exterior. This suggests that a lysozomal enzyme was mediating platelet aggregation and secretion.

This study further investigated the platelet-neutrophil interaction responsible for activation of platelets. Washed cell suspensions, platelet-rich plasma with added washed neutrophils, and whole blood were examined for platelet activation by neutrophils stimulated with different agonists.

This phenomenon may be an important modifier of platelet function if it occurred *in vivo* at a site of trauma and inflammation.

5.2 METHODS

Samples collection. Blood was collected into ACD anticoagulant (6:1$^{v}/_{v}$) from apparently healthy volunteers who had taken no drugs within the preceding 10 days.

Samples for PRP and whole blood aggregation were collected into *tri*-sodium citrate (see section 2.1).

Preparation of washed platelets. PRP was collected after centrifugation (see section 2.3. Washed platelets were prepared by Sepharose 2B gel filtration (see section 2.4.1).

Preparation of washed, purified neutrophils. Neutrophils were purified and washed (see section 2.5).

Turbidometric platelet aggregation. Platelet aggregation (section 2.6.1) and calcium flux were recorded with a platelet ionized calcium aggregometer (PICA). Platelets ($200x10^9$/l) or platelets mixed with neutrophils ($5x10^9$/l) were incubated at 37°C for 2 minutes with

1mM Ca^{2+}, 0.25g/l fibrinogen and 2.5μg/ml cytochalasin B. Aggregation and luminescence were monitored upon addition of agonist (FMLP, LTB_4, PAF, endotoxin) for up to 20 minutes. The effect of neutrophil elastase and cathepsin G (Sigma Chemical Company) on washed platelet aggregation was measured. Some samples were preincubated for 5 minutes with soya bean trypsin inhibitor or colchicine (both from Sigma Chemical Company), to see if neutrophil induced platelet aggregation could be modified.

Preparation of antiproteinase-deficient plasma. Citrated platelet-poor plasma from apparently healthy individuals was incubated with the following different specific antibodies: sheep anti-human α_1-antitrypsin (Serotec, Kiddlington, Oxon.), rabbit anti-human α_2-macroglobulin, anti-rabbit C_1-esterase inhibitor, anti-rabbit antithrombin III (all from Dako Ltd, High Wycombe, Bucks.). 10μl of antibody was incubated with 1ml of plasma for 16 hours at 4°C. Samples were then centrifuged at 10000*g* for 30 minutes and supernatants collected.

Platelet intracellular calcium flux. Platelets were loaded with the photoprotein aequorin according to the method of Yamaguchi *et al*, 1987 (see section 2.7).

Whole blood impedance aggregation (section 2.6.2) was recorded in the absence or presence of cytochalasin B after addition of FMLP or PAF.

Adenine nucleotides. These were assayed in ethanol extracted samples from washed cell preparations (see section 2.6.3).

Cultured HL-60 cells. These cells are a human promyelocyte cell line isolated from a patient with promyelocytic leukaemia and were cultured by Dr. P.J.Roberts according to the method of Collins *et al*, 1977. The cells were induced to undergo morphological and functional maturation after incubation with 1.25% DMSO for 11 days.

5.3 RESULTS

Platelet activation by stimulated neutrophils. Washed neutrophils after incubation with cytochalasin B (2.5μg/ml) for 2 minutes, in the presence of platelets (ratio 1:40), were stimulated with FMLP (1μM), LTB_4 (50ng/ml), or PAF (2μM). These agonists (except for PAF) had no detectable effect on platelets alone. However, neutrophils were able to cause platelet calcium flux, aggregation and nucleotide secretion (see figures 5.1, 5.2, 5.3 and 5.4). Neutrophils, when studied in the absence of platelets, accounted for less than 5% of observed aggregation. Cytochalasin B had no detectable effect on platelets alone at this concentration. When incubated with neutrophils for less than 1 minute, platelet activation by neutrophils was reduced. PAF directly activated platelets alone, but this was enhanced by neutrophils. Platelet activation was dependent on both neutrophil (Figure 5.5) and agonist concentration (data not shown). Endotoxin (100μg/ml), another potential neutrophil agonist, failed to cause platelet activation even at higher (1000μg/ml) concentrations.

Characterisation of neutrophil-derived platelet stimulating factor. The supernatant of FMLP-stimulated neutrophils retained some platelet stimulatory activity, suggesting a stable, non-cellular, release factor (figure 5.6). This activity was not reduced when neutrophils were preincubated with aspirin, piriprost or NDGA indicating that it was not due to either a cyclooxygenase or lipoxygenase product. Soya bean trypsin inhibitor (50u/ml) significantly reduced the neutrophils potential to activate platelets and abolished it completely when added to neutrophil supernatants. Similarly, colchicine (1μM) when added to cytochalasin B treated neutrophils greatly reduced their platelet activating ability.

Time course of the neutrophil-derived platelet stimulating factor. When FMLP-activated neutrophils were added to platelets at increasing time intervals after stimulation their platelet activating ability was maximal after 2 minutes and declined thereafter (figure 5.7).

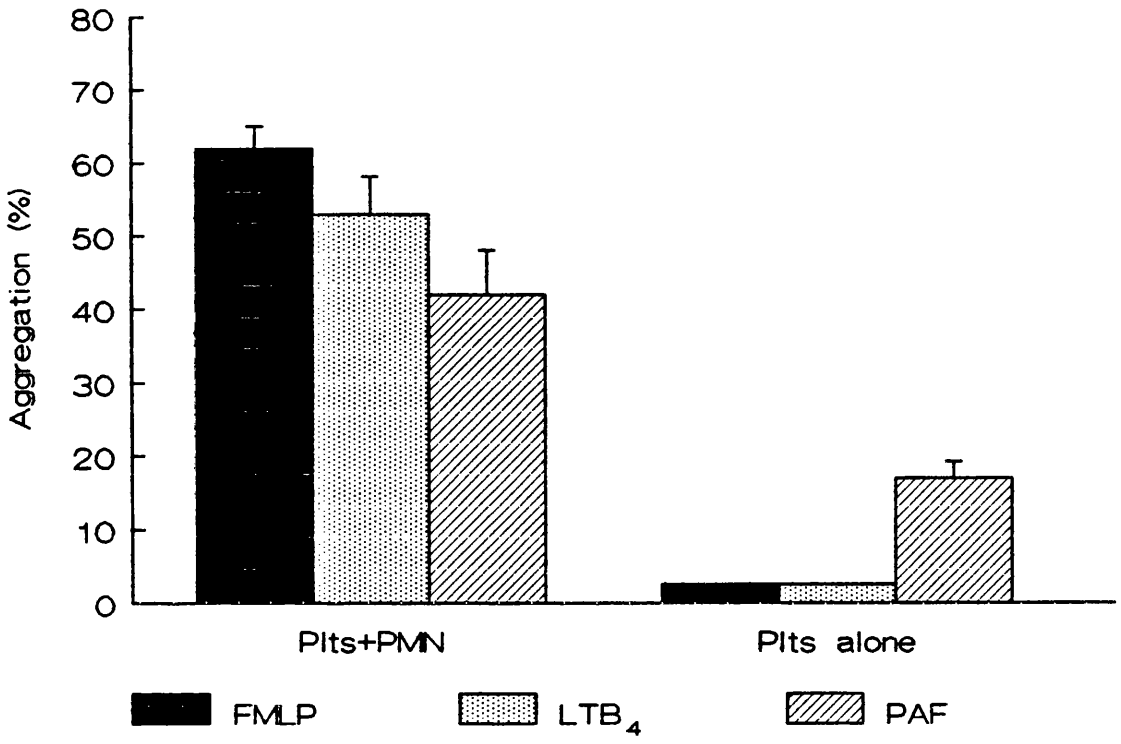

Figure 5.1. Platelet aggregation induced by 1μM FMLP, 50ng/ml LTB_4 or 2μM PAF in samples containing platelets alone (Plts alone) or platelets and neutrophils (Plts and PMN). Results indicate the mean and SEM of 10 experiments. Stimulated neutrophils alone had less than 5% aggregation.

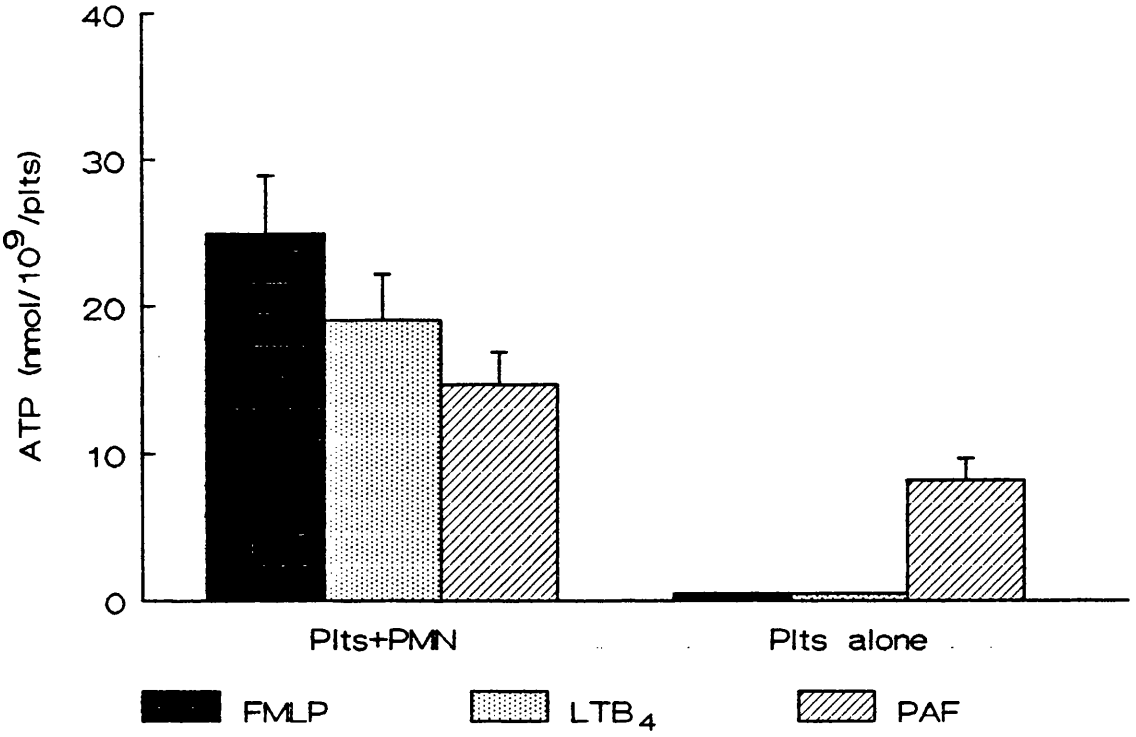

Figure 5.2. Platelet ATP secretion induced by 1μM FMLP, 50ng/ml LTB_4 or 2μM PAF in samples containing platelets alone (Plts alone) or platelets and neutrophils (Plts and PMN). Results indicate the mean and SEM of 10 experiments. Stimulated neutrophils alone showed no detectable ATP secretion.

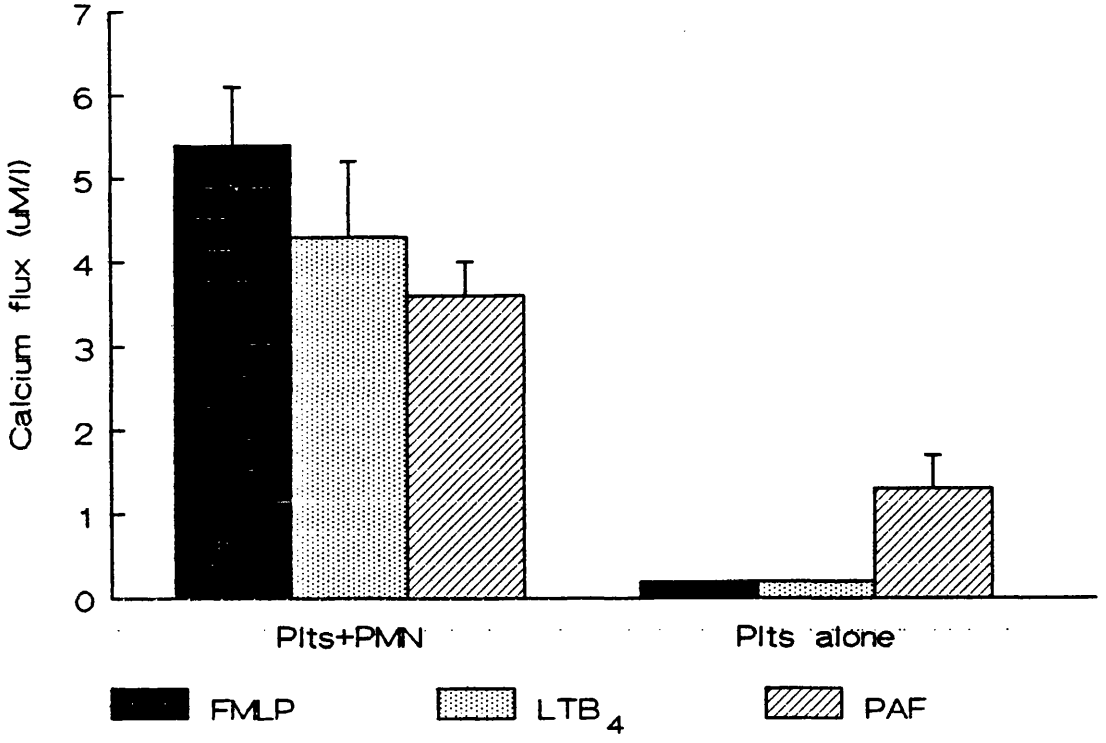

Figure 5.3. Platelet calcium flux induced by 1μM FMLP, 50ng/ml LTB_4 or 2μM PAF in samples containing platelets alone (Plts alone) or platelets and neutrophils (Plts and PMN). Results indicate the mean and SEM of 10 experiments.

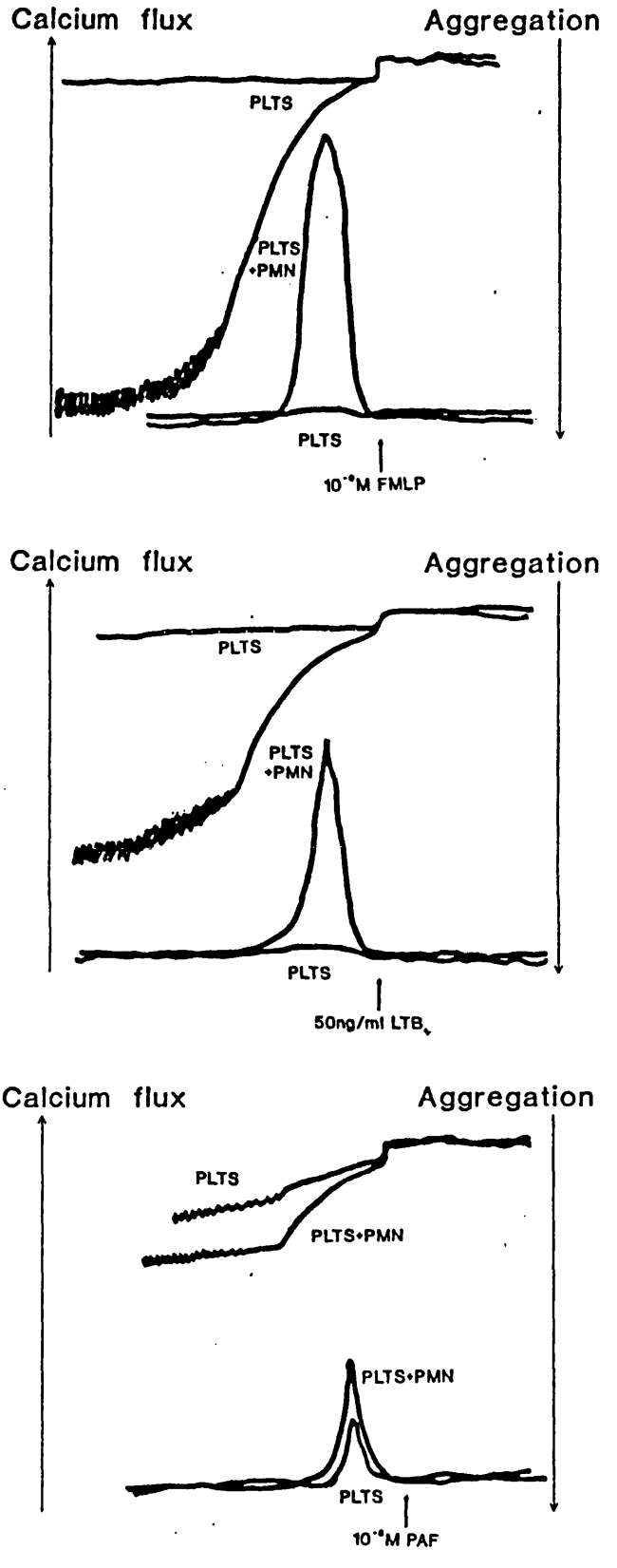

Figure 5.4. Representative traces of platelet calcium flux and aggregation.

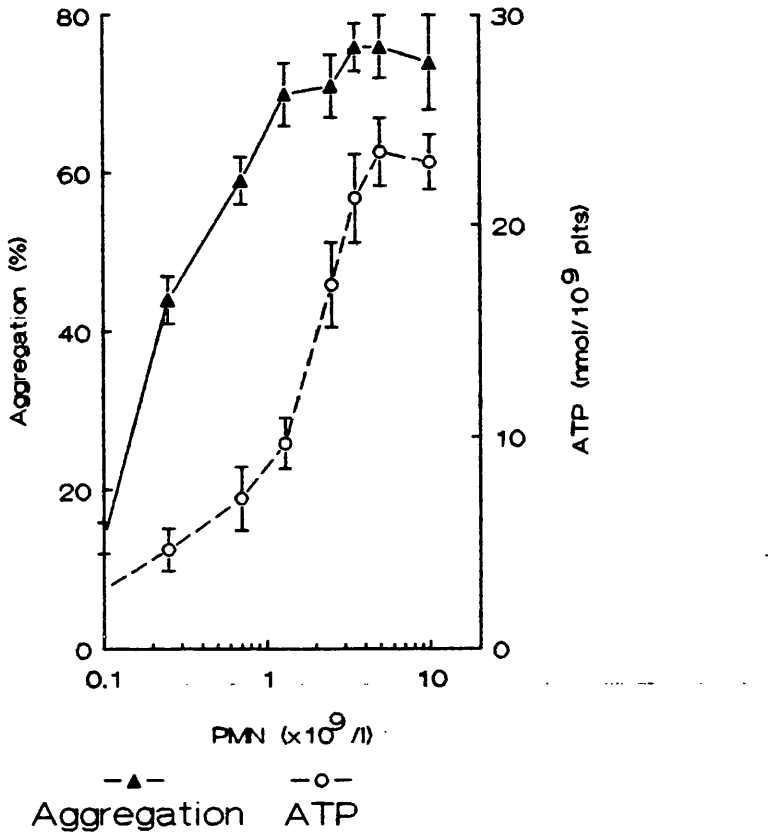

Figure 5.5. The effect of increasing neutrophil concentration on platelet aggregation. Neutrophils (0-10x10^9/l) were stimulated with 1μM FMLP. Results show the mean and SEM of 4 experiments.

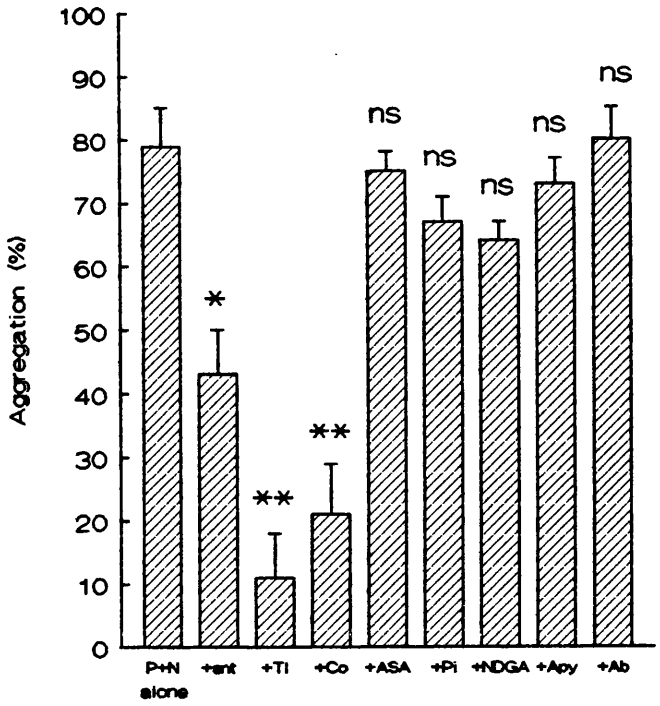

Figure 5.6. The effect of various compounds on neutrophil-induced platelet aggregation. Neutrophils were stimulated with 1μM FMLP. Results show the mean and SEM of 4 experiments. P+N is platelets with neutrophils; +SNT is platelets with 30μl activated neutrophil supernatant; +TI is P+N with 50μg/ml soya bean trypsin inhibitor; +Co is P+N with 1μM colchicine; +ASA is P+N with 1mM aspirin; +Pi is P+N with 40μg/ml Piriprost; +NDGA is P+N with 1μM NDGA; +Apy is P+N with 10u/ml apyrase; +Ab is P+N with 1/100 $^{v}/_{v}$ anti-elastase. Results show the mean and SEM of 3-8 experiments and were not statistically significant (ns), $p<0.05$ (*) or $p<0.01$ (**).

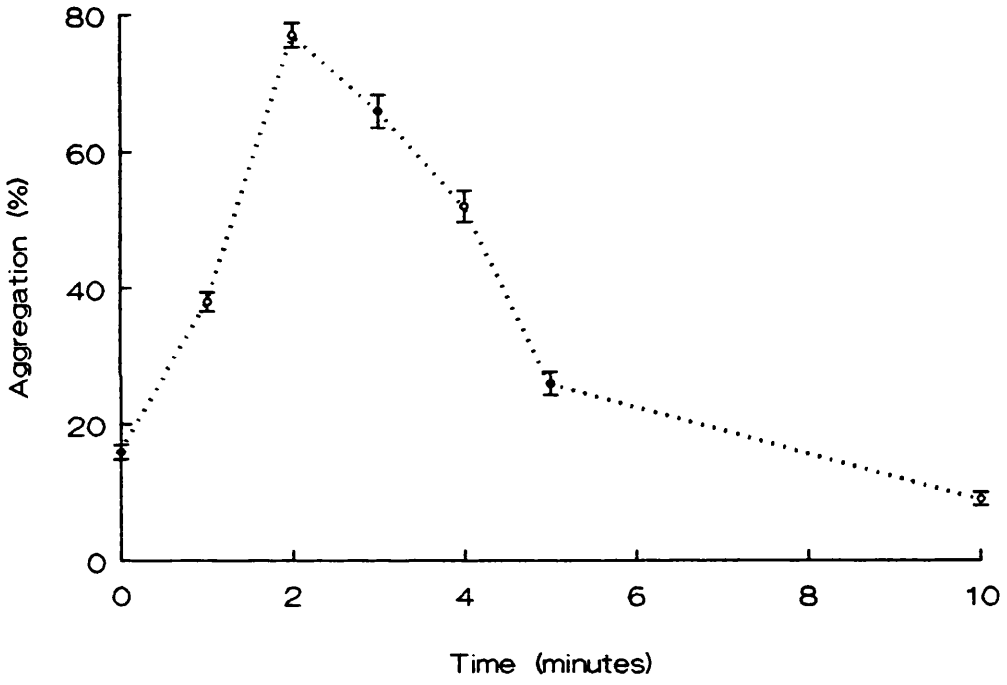

Figure 5.7. The addition of washed platelets to neutrophils at intervals of up to 10 minutes after stimulation of neutrophils with FMLP (1μM). Results show the mean and SEM of 3 experiments.

This suggests that the activating factor is only partially stable in the presence of neutrophils before it is inhibited or degraded itself.

Platelet activation by neutrophil-like HL-60 cells. Both uninduced and DMSO-induced HL-60 cells were able to activate platelet aggregation when stimulated with LTB_4 or PAF (figure 5.8). However, the extent of platelet aggregation was less compared to that of neutrophils. FMLP-stimulated HL-60 cells were unable to activate platelets.

Platelet activation by neutrophils in plasma and whole blood. Neutrophil-induced platelet activation was not detectable in platelet-rich plasma to which washed neutrophils were added or in citrated whole blood. Figure 5.9 shows that neutrophils and supernatant from activated neutrophils were able to cause platelet aggregation in the presence of diluted plasma. The neutrophil supernatants were more susceptible to inhibition by plasma than were mixed platelet and neutrophil suspensions.

Identification of the inhibitor present in plasma of the neutrophil-derived platelet activator. Platelet-poor plasma was incubated with specific antibodies directed to α_1-antitrypsin, α_2-macroglobulin, antithrombin III or C_1-esterase inhibitor. These depleted plasmas were then tested for reduced inhibitory activity on either platelet and neutrophil suspensions. Figure 5.10 shows that only plasma depleted of α_1-antitrypsin no longer inhibited neutrophil-induced platelet aggregation.

The effect of purified neutrophil elastase and cathepsin G on platelet calcium flux and aggregation. Cathepsin G was a strong platelet agonist, stimulating both calcium flux and aggregation. The maximal response was obtained at 10μg/ml (Figure 5.11.). Elastase, on the other hand, was unable to stimulate either platelet calcium flux or aggregation in the 1-1000μg/ml range. In fact it had inhibitory activity on 1μg/ml collagen induced platelet aggregation.

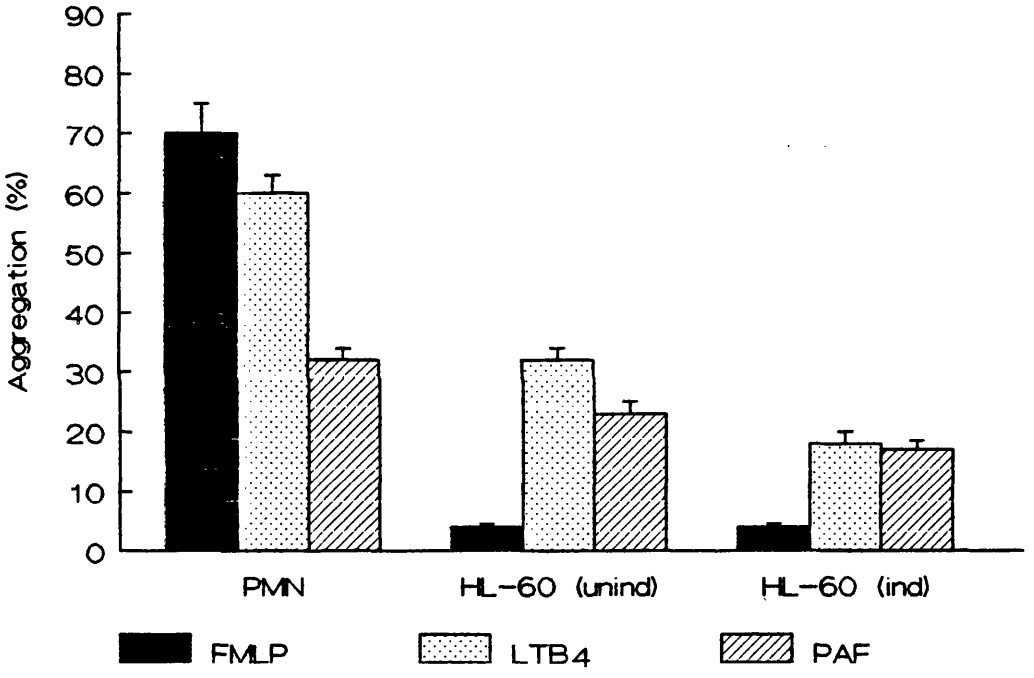

Figure 5.8. The stimulation of platelet aggregation by $5x10^9/l$ neutrophils or HL-60 cells (uninduced or induced with 1.25% DMSO for 11 days) stimulated with 1μM FMLP. Results show the mean and SEM of 3 experiments.

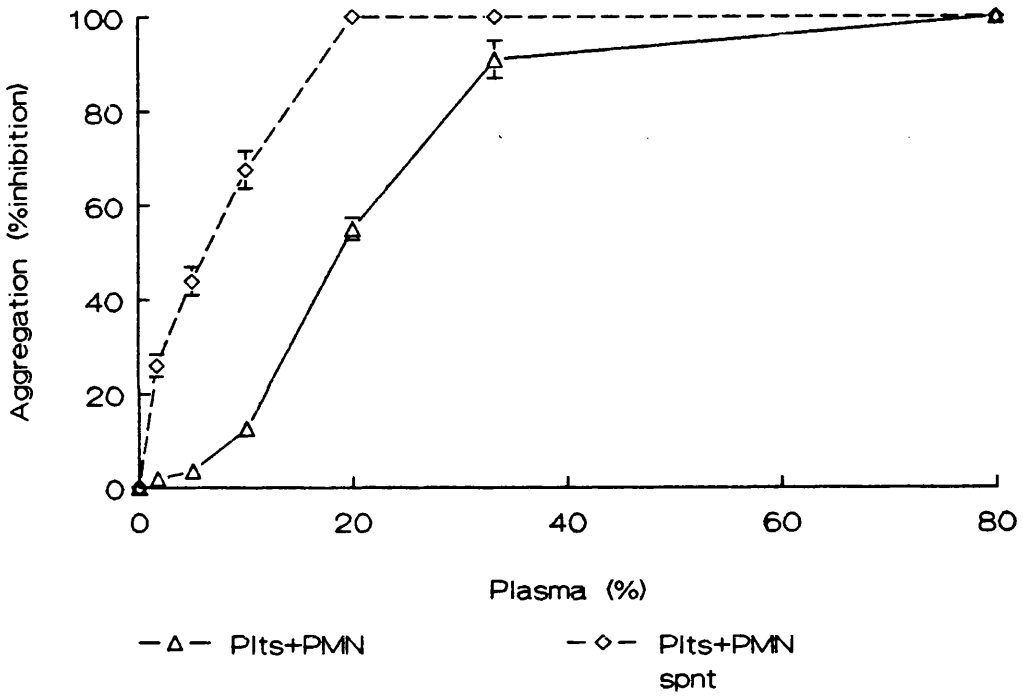

Figure 5.9. The effect of different concentrations of platelet poor plasma on neutrophil-induced platelet aggregation. Samples contained either mixed suspensions of platelets and neutrophils (Plts+PMN) or platelets with 30µl of FMLP-stimulated neutrophil supernatant (Plts+PMN spnt). Results show the mean and SEM of 3 experiments.

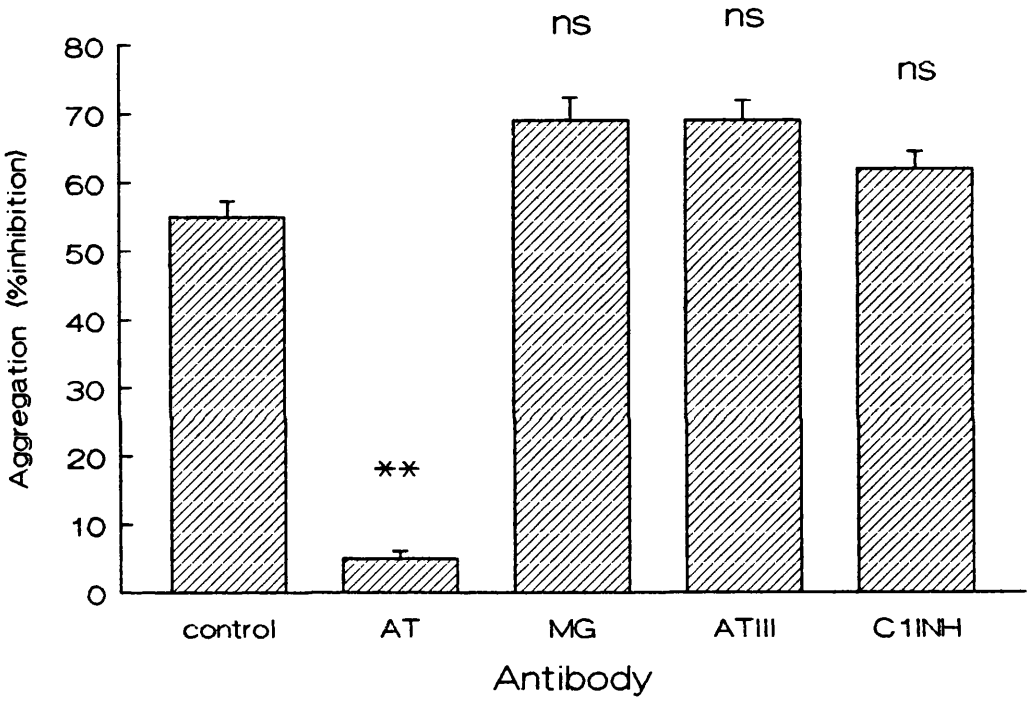

Figure 5.10. The effect of plasma pretreated with different specific antibodies on neutrophil-induced platelet aggregation. Neutrophils were stimulated with 1μM FMLP, either in 60μl untreated plasma (control) or aliquots of plasma depleted, by using specific antibodies, to the following compounds: α_1-antitrypsin (AT), α_2-macroglobulin (MG), antithrombin III (ATIII) and C_1-esterase inhibitor (C1INH). Results show the mean ans SEM of 3 experiments and were either not statistically significant (ns) or $p<0.01$ (**).

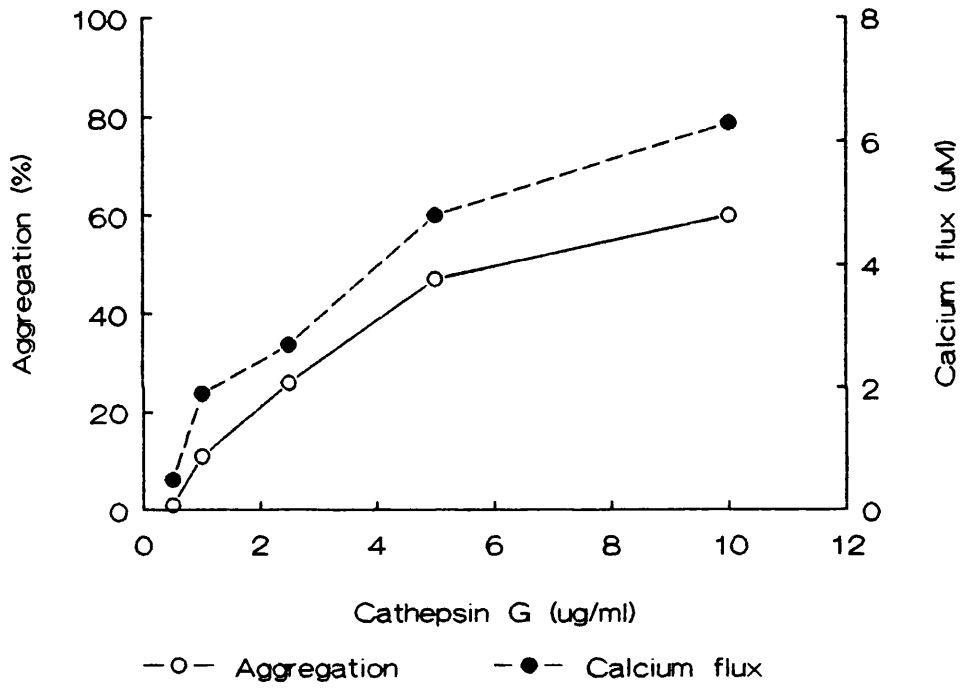

Figure 5.11. The effect of purified human neutrophil cathepsin G on platelet calcium flux and aggregation. Results show the means of 2 separate experiments.

5.4 DISCUSSION

This *in vitro* study confirms previous reports (Chignard *et al*, 1986; Del Maschio *et al*, 1990) demonstrating that activated human neutrophils are able to stimulate platelet function in washed cell suspensions. The increase in light transmission provoked by FMLP, LTB_4 or PAF was significantly greater than that obtained by stimulation of either cell-type alone. Both FMLP and LTB_4 were unable to elicit platelet activation in the absence of neutrophils. The extent of platelet activation positively correlated with the concentration of each agonist and also with the number of washed neutrophils in the mixed suspension.

The fungal metabolite, cytochalasin B, was essential for the observation of neutrophil-induced platelet activation. Cytochalasin B, by itself, at the concentration used had no observable effect on platelet function. Cytoplasmic microtubules and microfilaments are the main components of the cytoskeleton in mammalian cells. Cytochalasin B reversibly interferes with the microfilamental system of neutrophils strongly influencing chemotaxis, phagocytosis, oxidant generation and lysosomal enzyme release (Bourgoin *et al*, 1990). Cytochalasin B facilitates the exocytosis of neutrophil granules to the extracellular environment when neutrophils are activated. These granules, especially the azurophilic granules which contain myeloperoxidase and neutral serine proteases are important for digestion. The addition of colchicine to neutrophils promotes microtubule disassembly, inhibiting phagocytosis, chemotaxis and degranulation. Colchicine-treated neutrophils were less effective at platelet activation. This was probably due to the failure of degranulation.

The identification of the factor responsible for platelet activation was ascertained by Chignard and coworkers to be a soluble factor present in supernatants of FMLP-

stimulated neutrophils. It was stable for up to 10 minutes at 37°C and its concentration proportional the number of neutrophils. The factor was destroyed by short incubation at 90°C and also inhibited by a specific serine protease inhibitor. Del Maschio and coworkers also showed that by adding platelets to neutrophils at various time intervals after neutrophil stimulation, platelet calcium flux, aggregation and TXB_2 release were maximal within the first few minutes of neutrophil activation. Platelet activation progressively decreased after this time. This study confirms these observations also indicating that the biological activity present in neutrophil supernatants is a protease, and that it is quickly destroyed by the presence of neutrophils from which it was released. Neither aspirin, Piriprost or NDGA prevented this neutrophil-derived effect upon platelets, so the activating factor is unlikely to be an eicosanoid. Soya bean trypsin inhibitor did attenuate platelet activation in the presence of neutrophils and abolished it when neutrophil supernatant was added instead of neutrophils themselves.

The reduced ability of supernatants from activated neutrophils to cause platelet activation indicates the importance of direct cell-cell interaction in amplifying the response. Other neutrophil products may then enhance the response initiated by proteases. Several other products of activated neutrophils, such as oxygen radicals, have been reported to activate platelets (Clarke & Klebanoff, 1980).

Purified cathepsin G and elastase, two neutral serine proteases contained in azurophil granules of neutrophils, have previously been shown to stimulate platelet function (Selak *et al*, 1988; Bykowska *et al*, 1983). In this study, however, purified neutrophil elastase was unable to provoke platelet aggregation at the concentrations used. Also, antibodies to elastase did not inhibit activation of platelets by neutrophils. Purified cathepsin G, on the other hand, was found to be a strong platelet agonist directly causing

platelet calcium flux and aggregation. This is in agreement with Selak and coworkers (Selak *et al*, 1988) and supports the hypothesis that there is a specific cathepsin G receptor on platelets (Selak & Smith, 1990).

The inability of both aspirin and apyrase to inhibit platelet activation by neutrophils suggests that neither arachidonic acid metabolites nor ADP are directly responsible for the response, although they may amplify it. Nevertheless, initial stimulation of platelets by neutrophils appears to be independent of platelet secondary amplification pathways.

HL-60 cells are an immature granulocyte (promyelocyte) cell line. These cells too had some platelet activating ability indicating that this property may arise early in a cells development. HL-60 cells have azurophil granules, whether induced with DMSO or not, but they entirely lack secondary granules (Collins *et al*, 1977). This shows that the neutrophil-derived platelet agonist originates from azurophil granules.

Neutrophils were unable to stimulate platelet aggregation in either platelet-rich plasma or citrated whole blood under these experimental conditions. This finding indicates the presence of a naturally occurring inhibitor in plasma. When plasma was added to washed platelets and either neutrophil suspensions or neutrophil supernatants the extent of aggregation was directly related to the plasma concentration. At a plasma concentration of 30-40%, neutrophil activation of platelets was completely inhibited. Plasma therefore contains saturating concentrations of this inhibitor at physiological ratios of platelets and neutrophils. The addition of different antibodies to plasma prior to its addition to mixed cell suspensions demonstrated that activation of platelets by neutrophils was restored in plasma depleted of α_1-proteinase inhibitor (α_1-antitrypsin). This inhibitor is likely to be responsible for the absence of neutrophil-stimulated platelet aggregation in platelet-rich

plasma and whole blood. The neutrophil-derived protease was less susceptible to inhibition when neutrophils, rather than neutrophil supernatant, was present. This may be because the close apposition of platelets with neutrophils in stirred suspensions results in membrane to membrane contact. This could create a microenvironment in which the soluble platelet activating protease discharges onto adherent platelets, thus being protected from antiproteinases (Evangelista *et al*, 1991).

The onset and propagation of haemostasis and inflammation could be influenced by platelet-neutrophil interaction. These *in vitro* studies required cytochalasin B-pretreatment of neutrophils prior to stimulation to activate platelets. A protease, such as cathepsin G, is responsible for platelet activation. It is possible to speculate that *in vivo* neutrophils are primed for activation by cytokines, such as GM-CSF. Stimulation of neutrophils by chemotactic factors could result in activation of platelets aswell. Regurgitation during phagocytosis occurs when degranulation, a rapid response to stimulation, begins before closure of a phagocytic vacuole, thus releasing granule contents into the immediate environment as well as into the developing vacuole. When platelets are in close proximity to activated neutrophils at a localised site of tissue injury or inflammation platelets may be recruited by these cells, further amplifying the response to trauma. Platelets could in turn enhance neutrophil-endothelial adhesion and neutrophil-induced cytotoxicity (Boogaerts *et al*, 1982). In some pathological states, when neutrophils are greatly or inappropriately activated, widespread platelet activation may follow as a direct result.

CHAPTER 6

INTERACTION BETWEEN PLATELETS AND NEUTROPHILS FOR EICOSANOID PRODUCTION

INTRODUCTION

The initial focus of eicosanoid research was on metabolites produced by individual cell-types upon activation. However, given that eicosanoids are autocoids that exert their biological influence in the microenvironment, a multitude of cell-types in close proximity may have the potential for metabolising precursors, intermediates and end products of this pathway. Human neutrophils from peripheral blood may physically interact with platelets under different circumstances, including haemostasis, inflammation, and a variety of vascular disorders. Lipoxygenase-derived products have been implicated in each of these settings and this study investigates the formation of some of these products during coincubation of purified platelets and neutrophils.

Leukotriene B_4 (LTB_4) is a potent proinflammatory mediator derived from arachidonic acid through the 5-lipoxygenase pathway. LTB_4 possesses a number of biological activities both *in vitro* and *in vivo*. Its most important activity lies in its chemotactic and chemokinetic effects on phagocytes, and it is likely that LTB_4 plays an important role in host defence as well as in inflammatory diseases (Ford-Hutchinson, 1990).

Apart from the obvious role of platelets in haemostasis, evidence that cooperation between platelets and neutrophils may contribute to local inflammation in different

pathophysiological situations is accumulating (Weksler, 1988). Moreover, platelets release arachidonic acid metabolites, PAF, PDGF and PF4 which can affect neutrophil functions, including adhesion, chemotactic activity, secretion and superoxide anion generation (Weksler, 1988). Additionally, activated platelets have been shown to adhere to neutrophils (Larsen *et al*, 1989). Such an interaction could either result in the elimination of activated platelets and/or the recruitment of platelets to inflammatory sites where they may facilitate metabolic or functional modulation. Platelet-neutrophil interaction can lead to the transcellular metabolism of eicosanoids (Marcus *et al*, 1982) and the formation of products not generated by either cell-type alone (Marcus *et al*, 1987). This study examined eicosanoid production by platelets and neutrophils; either when incubated separately or together, as well as when either one or both cell-types were activated. The release of eicosanoids in whole blood stimulated with different agonists was also investigated.

METHODS

Blood samples. These were collected from normal, healthy volunteers into ACD, *tri*-sodium citrate or heparin (10u/ml). See section 2.1.

Preparation of platelet-rich plasma and washed platelets. See sections 2.3 and 2.4.1.

Preparation of washed, purified neutrophils. See section 2.5.

Radiolabelling of platelets and neutrophils. See sections 2.13.2 and 2.13.3.

Turbidometric platelet aggregation. See section 2.6.1.

Whole blood impedance aggregation. See section 2.6.2.

Incubation of samples. Washed platelet and neutrophil suspensions were mixed to give a cell suspension containing $1x10^7$/ml neutrophils and between $50x10^6$/ml and $1000x10^6$/ml platelets. Calcium chloride and magnesium chloride were added at final concentrations of

1mM each. The cells were then preincubated at 37°C with stirring for 5 minutes. The cells were then stimulated for different intervals with various agonists. Incubations were stopped with 3 volumes of ice-cold ethanol containing 500ng PGB_2 and placed on ice. Precipitated protein was removed by centrifugation at 2000*g* for 10 minutes at 4°C. Samples were then stored at -70°C until extraction of eicosanoids.

Samples of whole blood (10ml; anticoagulated with heparin or citrate) were preincubated at 37°C for 5 minutes with mixing before addition of agonist. Samples were then stimulated and continuously mixed for different time intervals before addition of a final concentration of 10mM disodium EDTA. Samples were then cooled on ice for 5 minutes before centrifugation at 2000*g* for 10 minutes. Platelet-poor plasma was then collected and 3 volumes of ice-cold ethanol containing 500ng PGB_2 added to precipitate protein. After centrifugation samples were stored at -70°C until extraction of eicosanoids (see also section 2.13.4).

Extraction of eicosanoids. Three different methods were used depending on sample volume and type. All three methods gave comparable results (see section 2.13.5).

HPLC of eicosanoids. See sections 2.13.6 and 2.13.7. Different mobile phases were used to give optimal separation of either cyclooxygenase or lipoxygenase products. Lipoxygenase metabolites were quantitated by measuring integrating the area under the peak. The amount of each metabolite was calculated by reference to the internal standard PGB_2. Recovery of PGB_2 was used to correct for losses during the procedure. The overall rate of recovery was 65±8% (mean±SD). Values were expressed as picomoles (pmol) of metabolite produced by $1x10^7$ neutrophils.

Reproducibility was tested by measuring LTB_4 production in the same individual on 5 separate occasions over 5 weeks. The mean value was 127 pmol/10^7 cells, with a

coefficient of variation of 16%.

Eicosanoids were identified either by coelution with standards or by their characteristic ultra violet spectra.

Leukotriene B_4 radioimmunoassay. See section 2.12. In order to confirm the identity of LTB_4 and to validate its quantitation by HPLC the fraction of solvent eluting from the column that contained LTB_4 was collected from 16 samples for analysis by RIA. 1ml aliquots of each peak were evaporated to dryness under nitrogen and then reconstituted in 5ml of RIA buffer. The amount of LTB_4 in the HPLC solvent fraction as measured by RIA was compared to the amount (pmol) given by integration of the appropriate peak at 280nm. There was good correlation between the values obtained using the two methods (r=0.87).

RESULTS

Eicosanoid production by radiolabelled washed platelets. Washed platelets, previously radiolabelled with ^{14}C-sodium arachidonate, were stimulated with 20μg/ml collagen for 4 minutes. Figure 6.1a shows the main products were TXB_2, HHT, 12-HETE and unincorporated arachidonate. Aspirin (1mM) effectively inhibited metabolites of cyclooxygenase (Figure 6.1b). Aspirin-treated platelets released more 12-HETE and arachidonate.

Eicosanoid production by radiolabelled washed neutrophils. Washed neutrophils were stimulated with 2.5μM calcium ionophore A23187 for 4 minutes. Eicosanoids were extracted with ethyl acetate as for washed platelets. The major products were 5-HETE, LTB_4 and the oxidized metabolites of LTB_4 (COOH- and OH-LTB_4). There were no detectable cyclooxygenase metabolites (Figure 6.2a). Preincubation of cells with NDGA

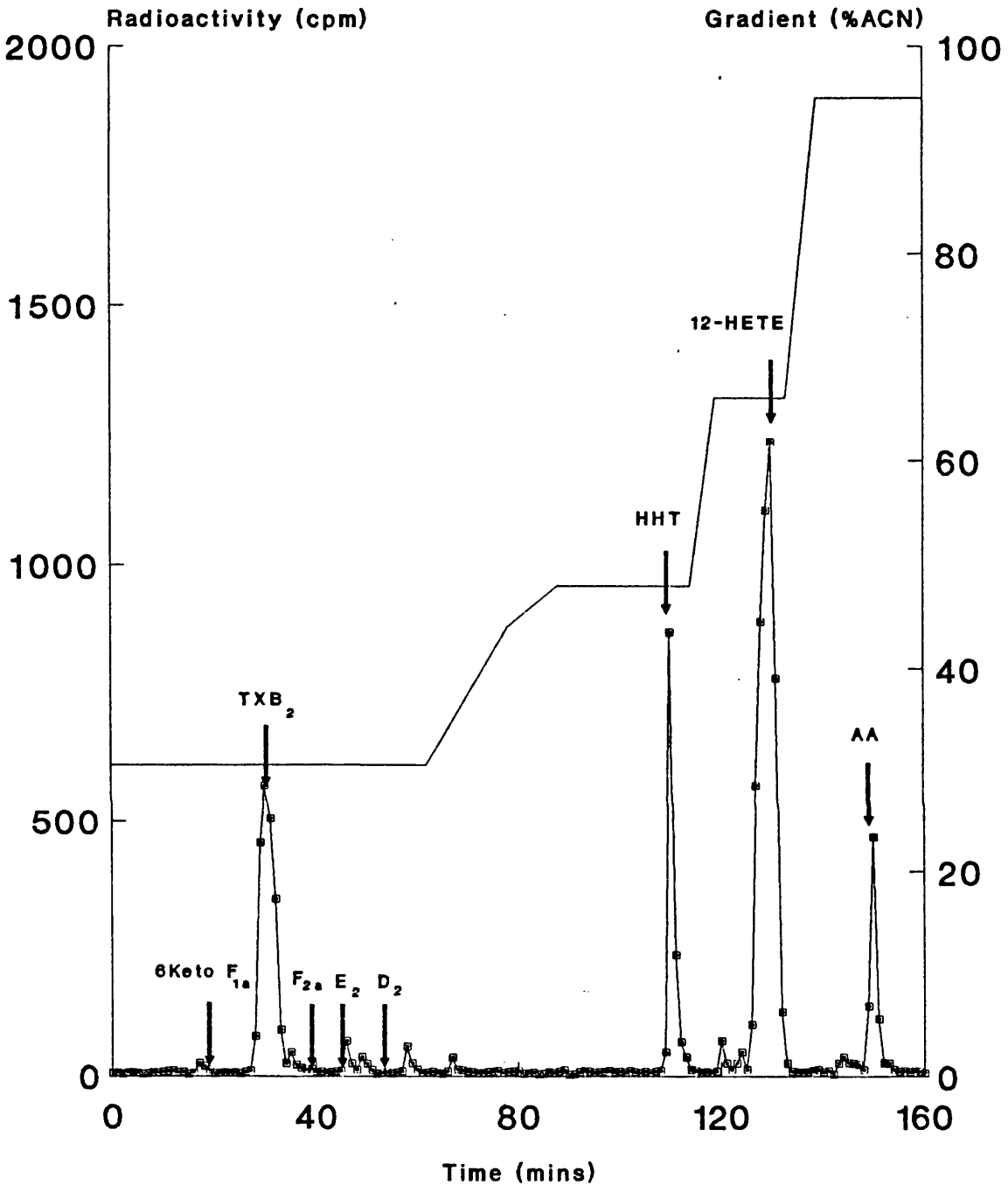

Figure 6.1a. A typical eicosanoid profile from washed platelets radiolabelled with ^{14}C-sodium arachidonate and stimulated with 20μg/ml collagen for 4 minutes (n=10). Samples were extracted with ethyl acetate and resolved by HPLC on a gradient of 30.5-95% acetonitrile in aqueous orthophosphoric acid (pH 2.3).

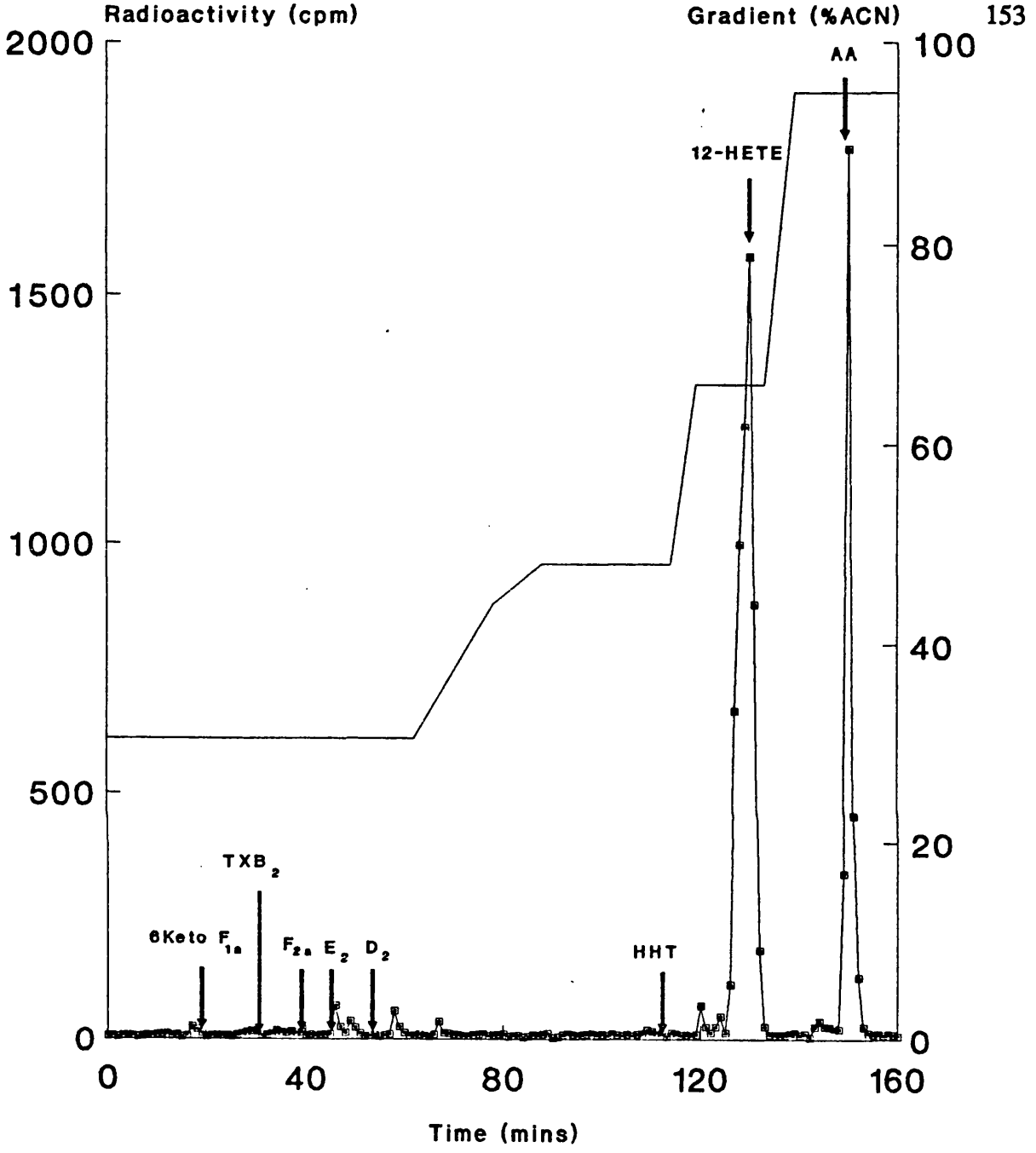

Figure 6.1b. A typical eicosanoid profile from washed platelets radiolabelled with ^{14}C-sodium arachidonate and stimulated with 20μg/ml collagen for 4 minutes in the presence of 1mM aspirin (n=10). Samples were extracted with ethyl acetate and resolved by HPLC on a gradient of 30.5-95% acetonitrile in aqueous orthophosphoric acid (pH 2.3).

a) PMN+A23187

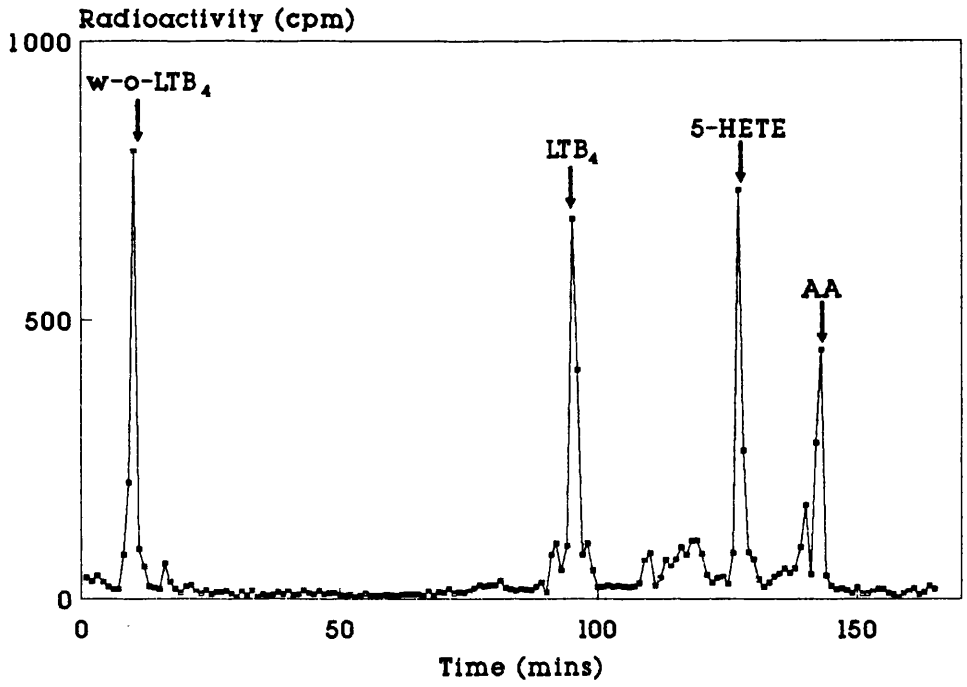

Figure 6.2a. A typical eicosanoid profile from washed neutrophils radiolabelled with ^{14}C-sodium arachidonate and stimulated with 2.5μM calcium ionophore A23187 for 4 minutes (n=8). Samples were extracted with ethyl acetate and resolved by HPLC on a gradient of 30.5-95% acetonitrile in aqueous orthophosphoric acid (pH 2.3).

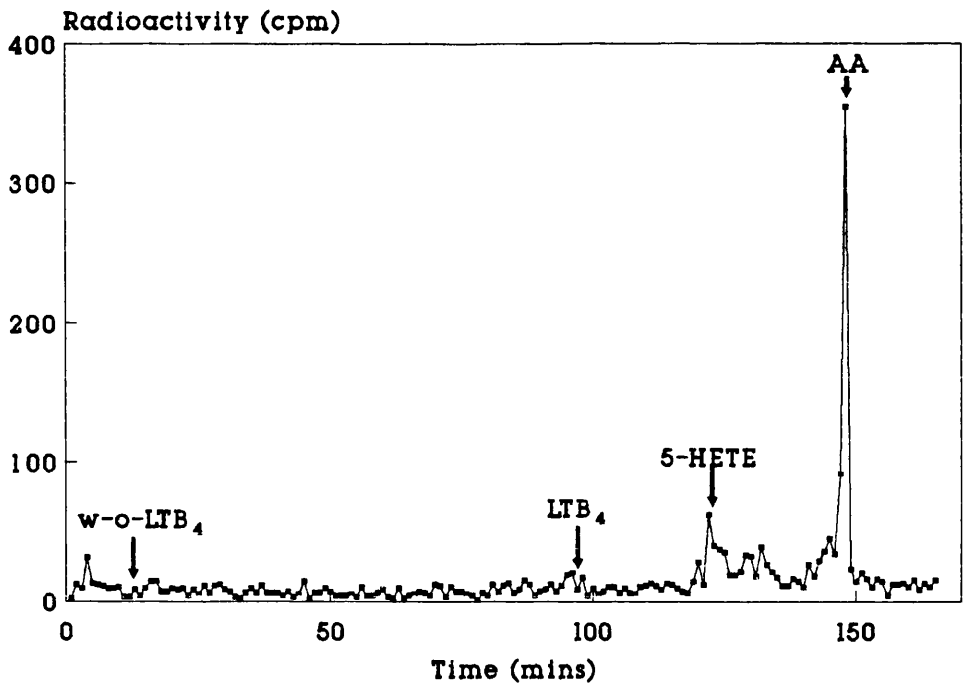

Figure 6.2b. A typical eicosanoid profile from washed neutrophils radiolabelled with ^{14}C-sodium arachidonate and stimulated with 2.5μM calcium ionophore A23187 for 4 minutes (n=2) in the presence of 10μM NDGA. Samples were extracted with ethyl acetate and resolved by HPLC on a gradient of 30.5-95% acetonitrile in aqueous orthophosphoric acid (pH 2.3).

c) PMN+A23187+Piriprost

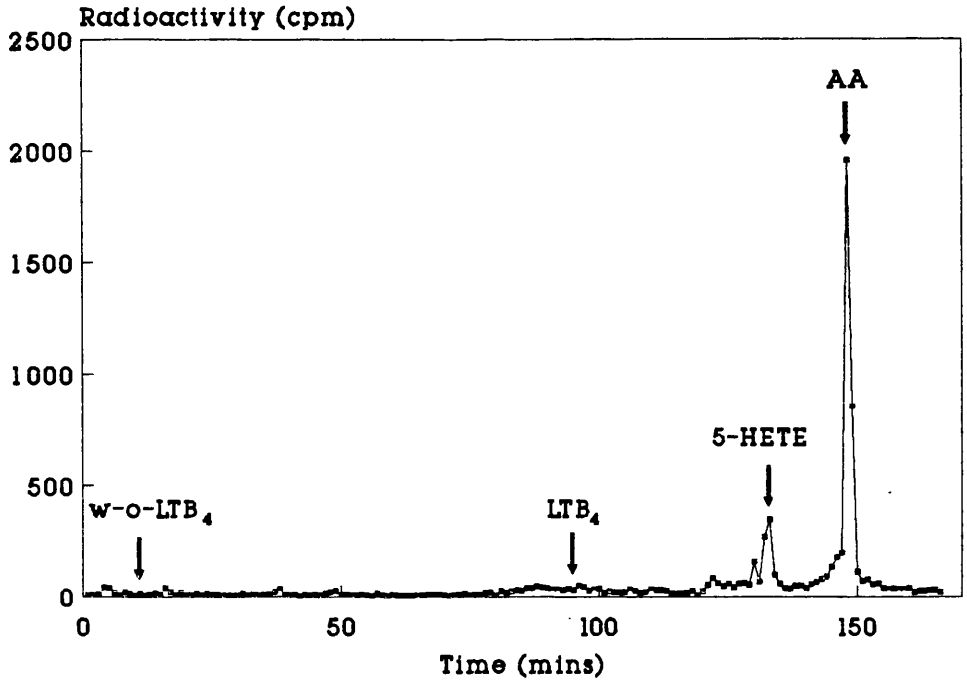

Figure 6.2c. A typical eicosanoid profile from washed neutrophils radiolabelled with ^{14}C-sodium arachidonate and stimulated with 2.5μM calcium ionophore A23187 for 4 minutes (n=2) in the presence of 40μg/ml Piriprost. Samples were extracted with ethyl acetate and resolved by HPLC on a gradient of 30.5-95% acetonitrile in aqueous orthophosphoric acid (pH 2.3).

d)PMN+A23187+ASA

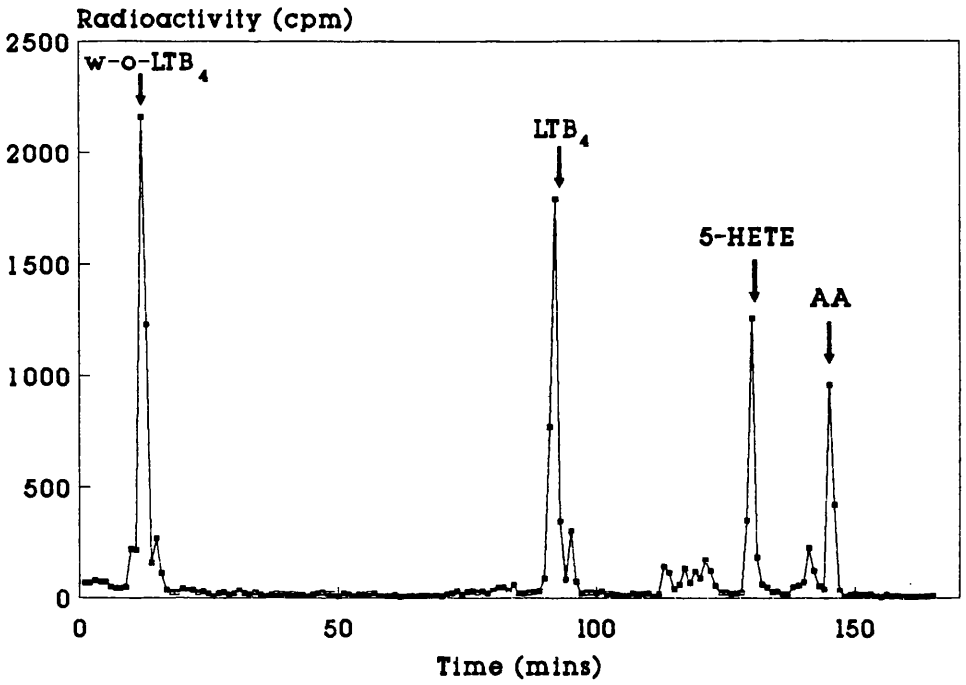

Figure 6.2d. A typical eicosanoid profile from washed neutrophils radiolabelled with ^{14}C-sodium arachidonate and stimulated with 2.5μM calcium ionophore A23187 for 4 minutes (n=2) in the presence of 1mM aspirin. Samples were extracted with ethyl acetate and resolved by HPLC on a gradient of 30.5-95% acetonitrile in aqueous orthophosphoric acid (pH 2.3).

(10μM) effectively inhibited all eicosanoid production (Figure 6.2b). Piriprost (40μg/ml), a specific 5-lipoxygenase inhibitor prevented synthesis of 5-HETE, LTB_4 and oxidized metabolites of LTB_4 (Figure 6.2c). Aspirin had no detectable effect on eicosanoid production by neutrophils (Figure 6.2d).

Identification of leukotrienes by their characteristic absorption spectra. Figure 6.3 shows the typical absorption curves for LTB_4, COOH-LTB_4, OH-LTB_4 and LTC_4. Each compound gives a characteristic peak with two shoulders, which allow positive identification. LTB_4 absorbed maximally at 270nm with shoulders at 260nm and 281nm. All sample peaks were compared with those of authentic standards.

Time course for LTB_4 production by calcium ionophore-stimulated washed neutrophils. Neutrophils reached maximum LTB_4 production approximately 4 minutes after stimulation with 2.5μM calcium ionophore A23187. LTB_4 concentration declined after 4 minutes as its oxidized metabolites continued to increase (Figure 6.4).

Coincubation of washed platelet and neutrophil suspensions. ^{14}C-arachidonate labelled platelets were preincubated with unlabelled neutrophils prior to stimulation with 2.5μM calcium ionophore A23187 for 4 minutes. The activation of neutrophils in the presence of different platelet concentrations (100-800x10^9/l) resulted in enhanced LTB_4 production. This increase in LTB_4 concentration was proportional to the number of platelets. Some of the LTB_4 produced was radiolabelled indicating that its precursor was platelet-derived (Figure 6.5a,b and c) as neutrophils were not radiolabelled. Platelets by themselves were unable to produce LTB_4. 5s-12s-diHETE (6-*trans*-8-*cis*-12-*epi*-LTB_4) was only produced in samples containing both platelets and neutrophils. This peak contained radiolabelled metabolite. The enhancement of LTB_4 synthesis and related metabolites is shown in figure 6.6.

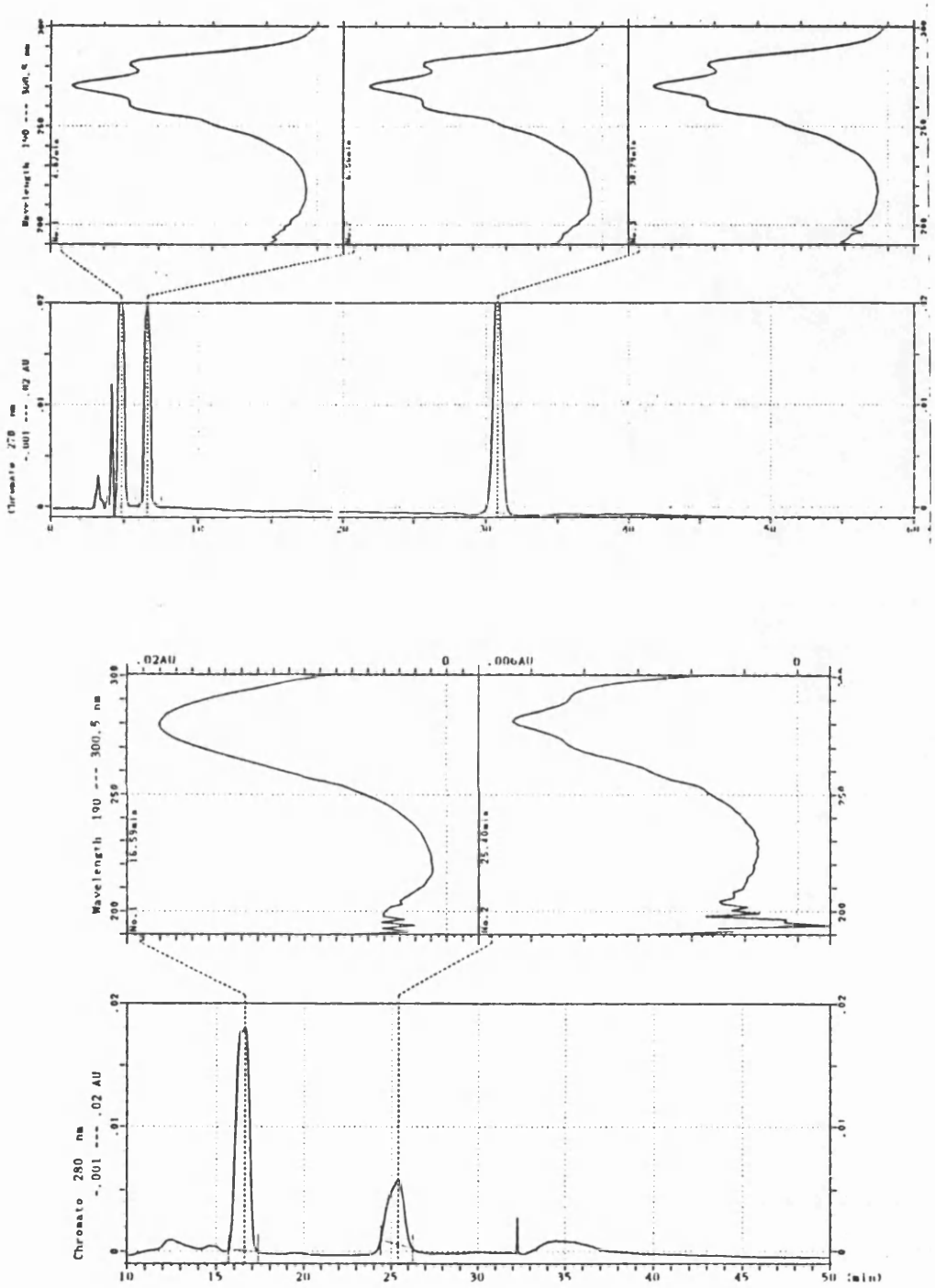

Figure 6.3. Representative absorption curves and chromatograms for standard eicosanoids. Samples (50ng of each) were chromatographed isocratically with methanol:water:acetic acid (67:33:0.1), pH 6.25, with a flow rate of 0.75ml/min. Eicosanoids identified were: from top left, OH-LTB_4, COOH-LTB_4, LTB_4, PGB_2 and LTC_4. Chromatograms were recorded at 270nm and peaks eluant scanned from 190-300nm.

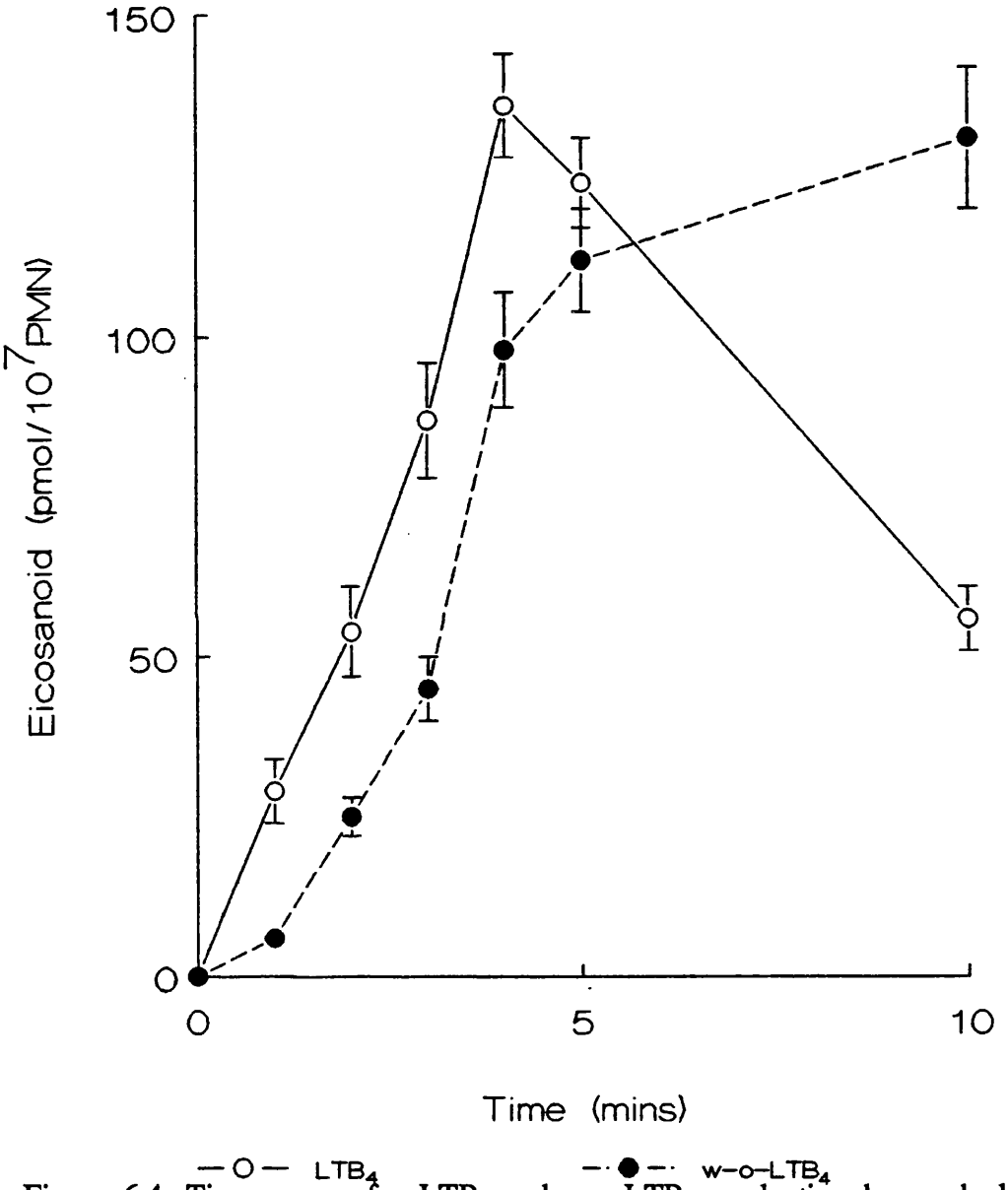

Figure 6.4. Time course for LTB_4 and w-o-LTB_4 production by washed neutrophils stimulated with 2.5μM calcium ionophore A23187. Results show the mean and SEM of 6 separate experiments.

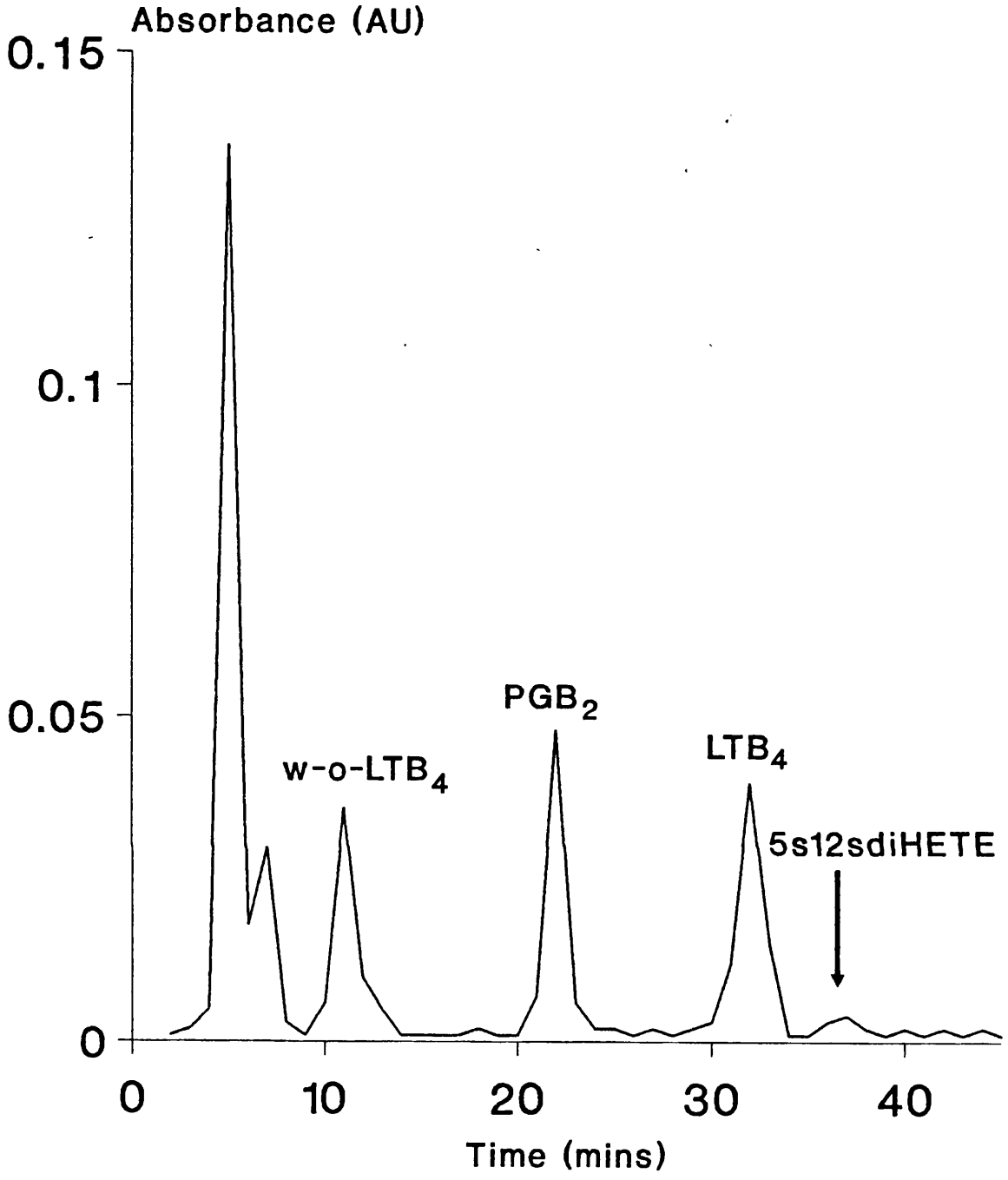

Figure 6.5a. A representative chromatogram of eicosanoids released by washed, purified neutrophils ($10x10^9$/l; n=24) stimulated with 2.5μM calcium ionophore A23187 for 4 minutes. Eicosanoids were resolved isocratically with a mobile phase of methanol:tetrahydrofuran:water:acetic acid (30:25:45:0.1), pH 5.5.

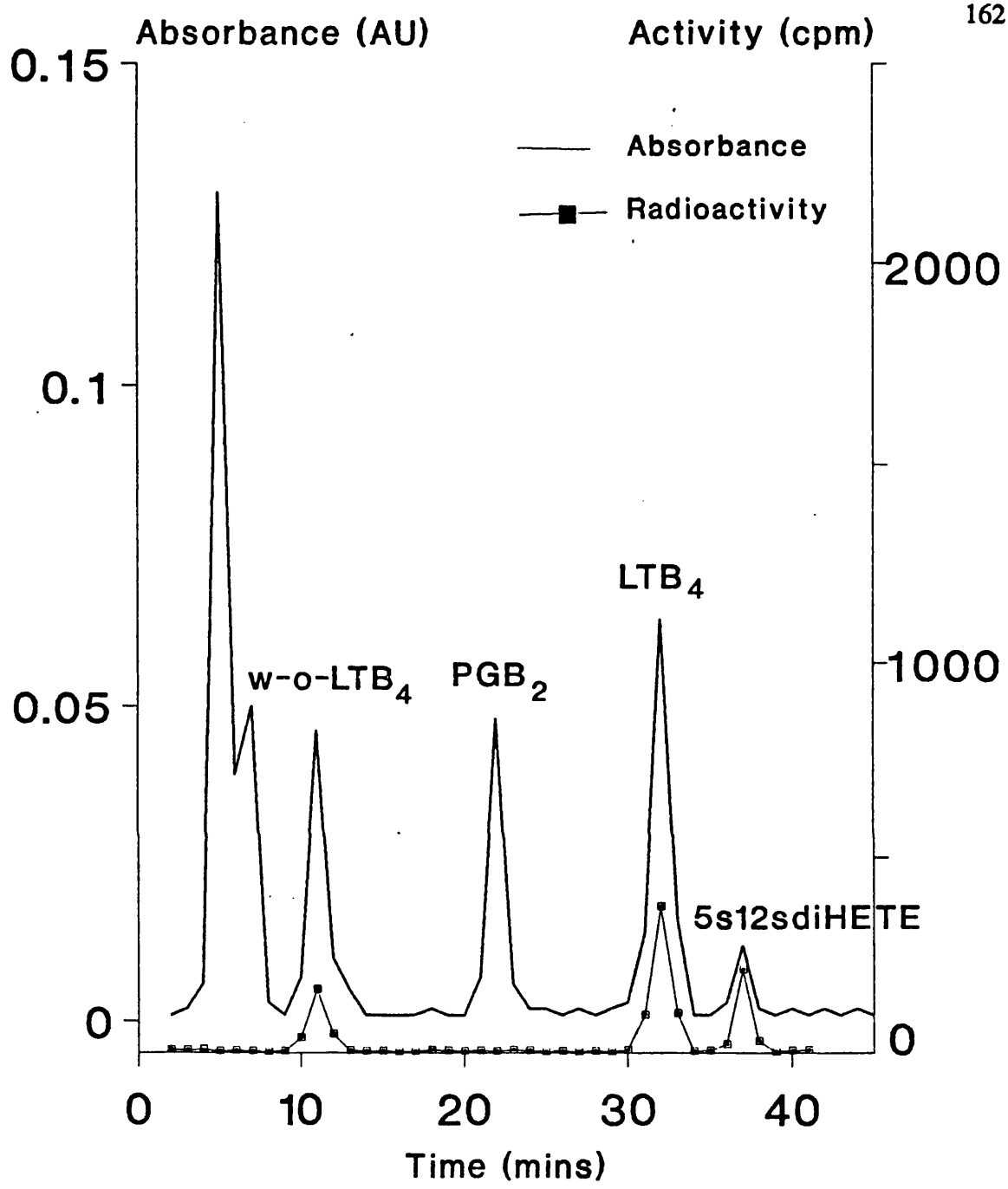

Figure 6.5b. Coincubation of ^{14}C-sodium arachidonate labelled washed platelets ($200x10^9$/l) with unlabelled washed neutrophils ($10x10^9$/l). Cells were stimulated with 2.5μM calcium ionophore A23187 for 4 minutes. Eicosanoids were resolved isocratically with a mobile phase of methanol:tetrahydrofuran:water:acetic acid (30:25:45:0.1), pH 5.5. The trace shown is representative of 8 separate experiments.

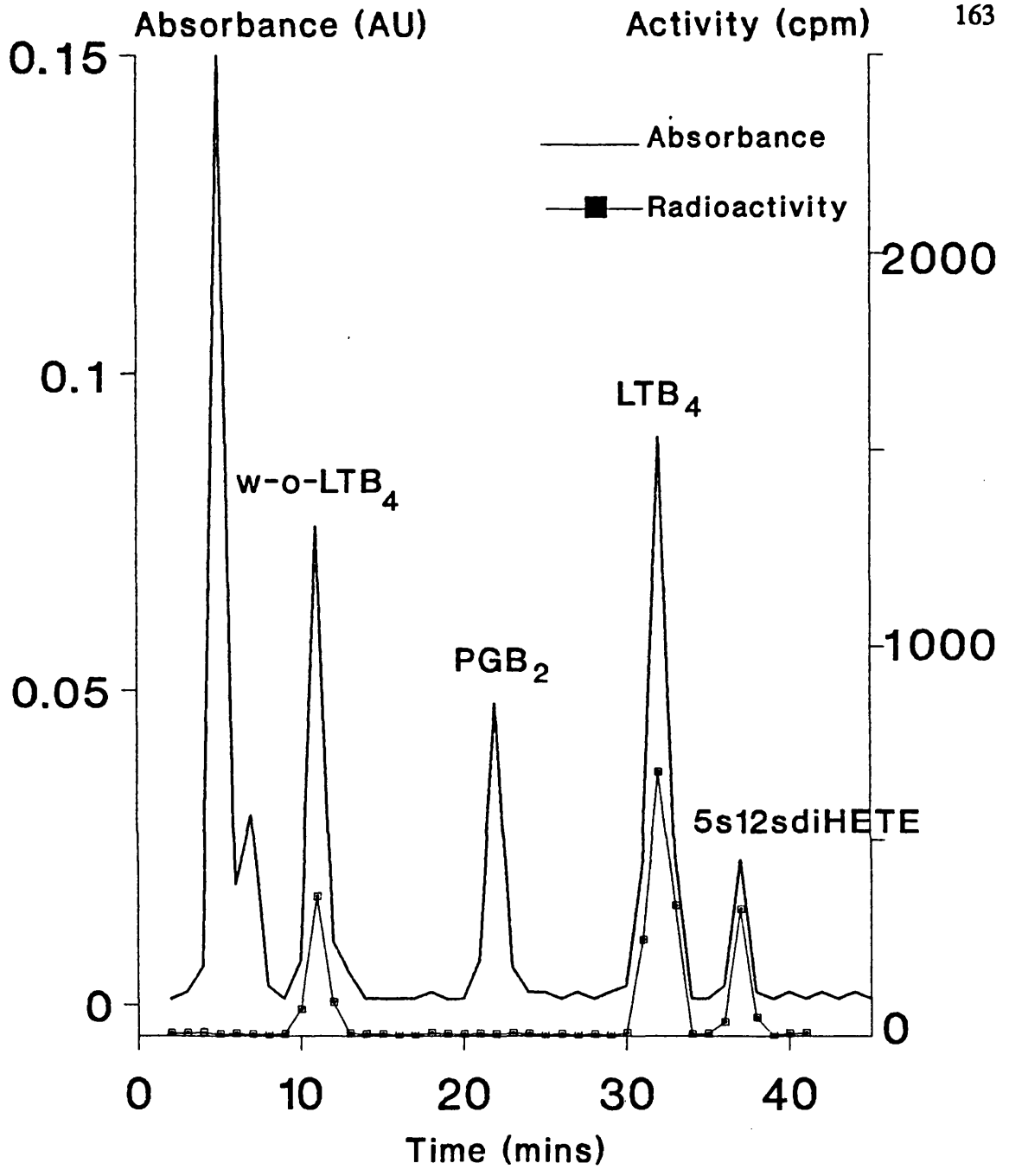

Figure 6.5c. Coincubation of ^{14}C-sodium arachidonate labelled washed platelets ($800x10^9$/l) with unlabelled washed neutrophils ($10x10^9$/l). Cells were stimulated with 2.5μM calcium ionophore A23187 for 4 minutes. Eicosanoids were resolved isocratically with a mobile phase of methanol:tetrahydrofuran:water:acetic acid (30:25:45:0.1), pH 5.5. The trace shown is representative of 8 separate experiments.

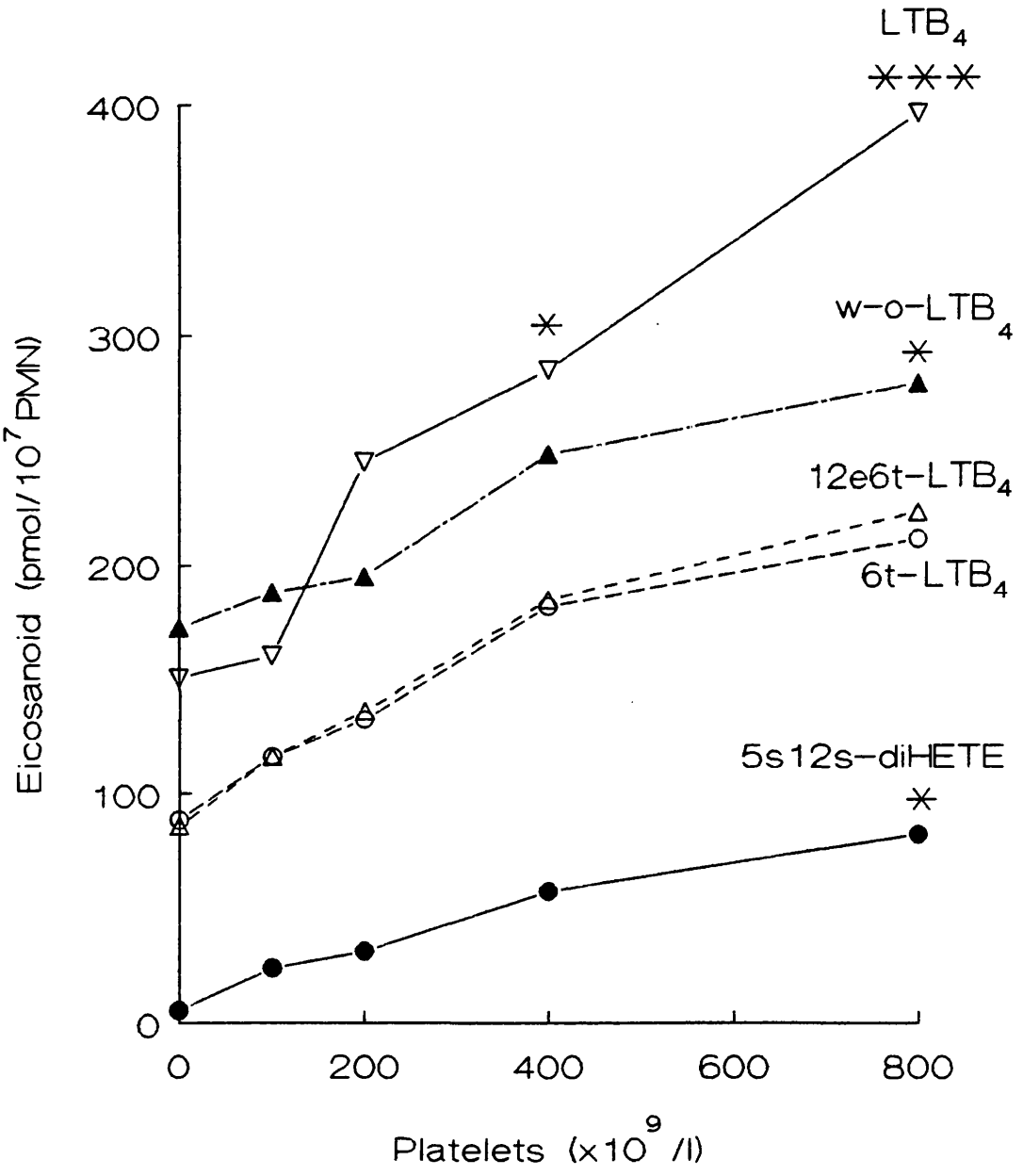

Figure 6.6. Coincubation of ^{14}C-sodium arachidonate labelled washed platelets (100-800x10^9/l) with unlabelled washed neutrophils (10x10^9/l). Cells were stimulated with 2.5μM calcium ionophore A23187 for 4 minutes. Results show the mean and SEM of 8 experiments and were not significant (unlabelled), $p<0.05$ (*) or $p<0.001$ (***).

LTB_4 production in whole blood: concentration of agonist. LTB_4 formation in heparin anticoagulated blood stimulated with 10-50μM calcium ionophore was maximal at a concentration of 40μM. Heparin anticoagulated blood supported more LTB_4 synthesis than citrate anticoagulated blood (Figure 6.7). This is probably because eicosanoid production, in particular that of LTB_4 by 5-lipoxygenase is calcium dependant. At concentrations of calcium ionophore A23187 above 30μM, some haemolysis was observed. This was probably due to the ionophore itself, rather than the DMSO vehicle which never exceeded 0.5% $^v/_v$. Substantially more agonist was required to stimulate eicosanoid release in whole blood compared to washed cells.

LTB_4 production in whole blood: time course with 20μM calcium ionophore A23187. LTB_4 release was maximal at 30 minutes in both heparin and citrate anticoagulated blood (Figure 6.8). Again it was substantially greater in heparinized blood. LTB_4 concentration declined slowly after 30 minutes. This was probably due to its degradation by oxidation. However, levels remained at about 200pmol/10^7 neutrophils even after 120 minutes. LTB_4 was not detectable in unstimulated blood as measured by HPLC.

The effect of specific inhibitors on eicosanoid release in heparin anticoagulated blood. Figure 6.9 shows the release of different eicosanoids upon stimulation with 20μM calcium ionophore A23187. Inhibition of cyclooxygenase by aspirin (1mM) inhibited TXB_2 release, but appeared to cause a significant rise in LTB_4 and its metabolites. The inhibition of leukotriene synthesis by Piriprost (40μg/ml) had no detectable effect on TXB_2 release. Low concentrations of 5s-12s-diHETE were detectable in stimulated whole blood.

The effect of collagen on eicosanoid release in heparin anticoagulated blood. Collagen (20μg/ml) failed to cause any detectable LTB_4 release although some oxidized metabolites of LTB_4 were detected (Figure 6.10). Stimulation of blood with both collagen and FMLP

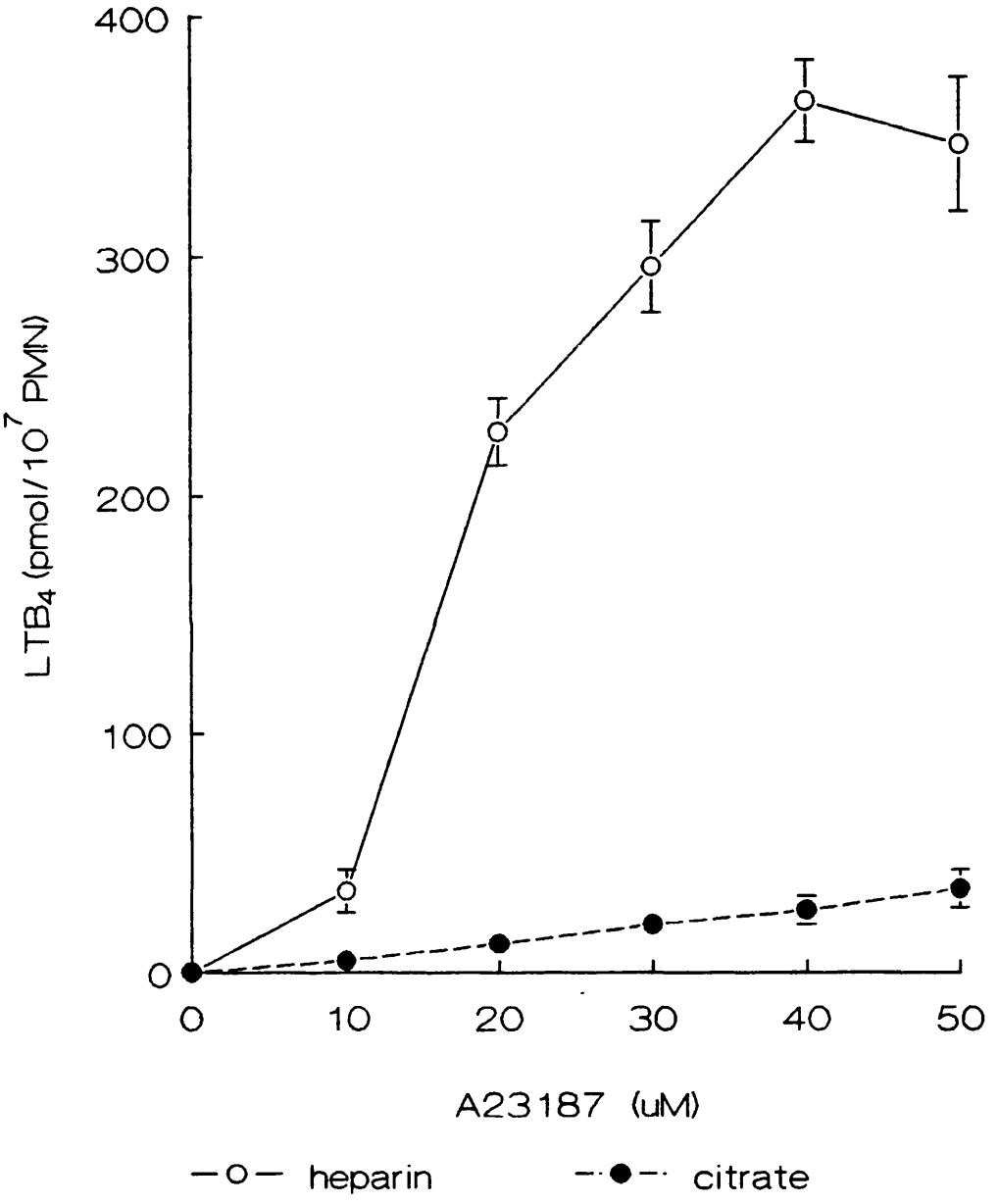

Figure 6.7. Dose response curve for LTB_4 release by either citrate or heparin anticoagulated blood stimulated by 0-50μM calcium ionophore A23187 for 30 minutes. Results show the mean and SEM of 3 separate experiments.

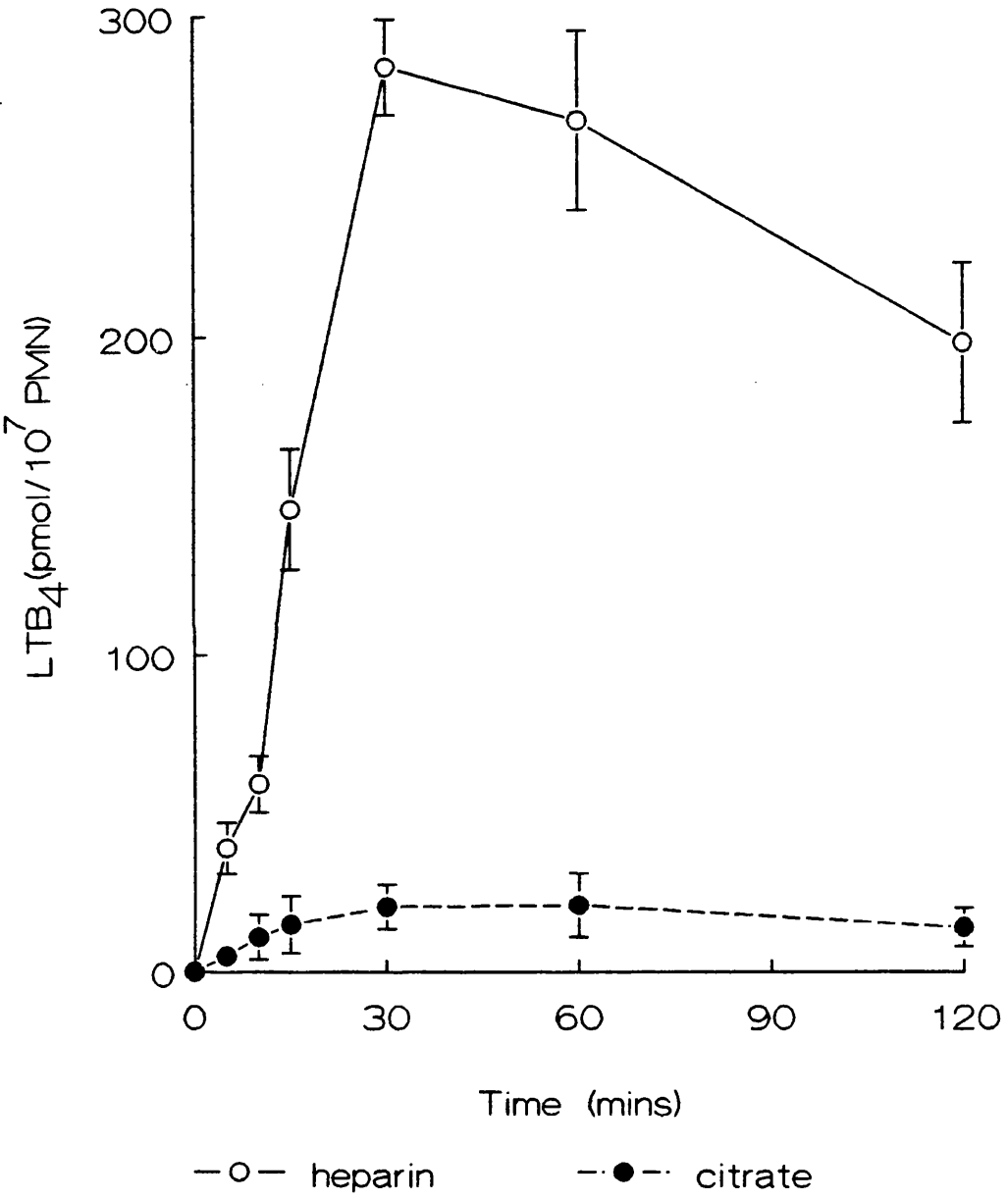

Figure 6.8. Time course for LTB_4 release by either citrate or heparin anticoagulated blood stimulated with 20μM calcium ionophore A23187 for 0-120 minutes. Results show the mean and SEM of 3 separate experiments.

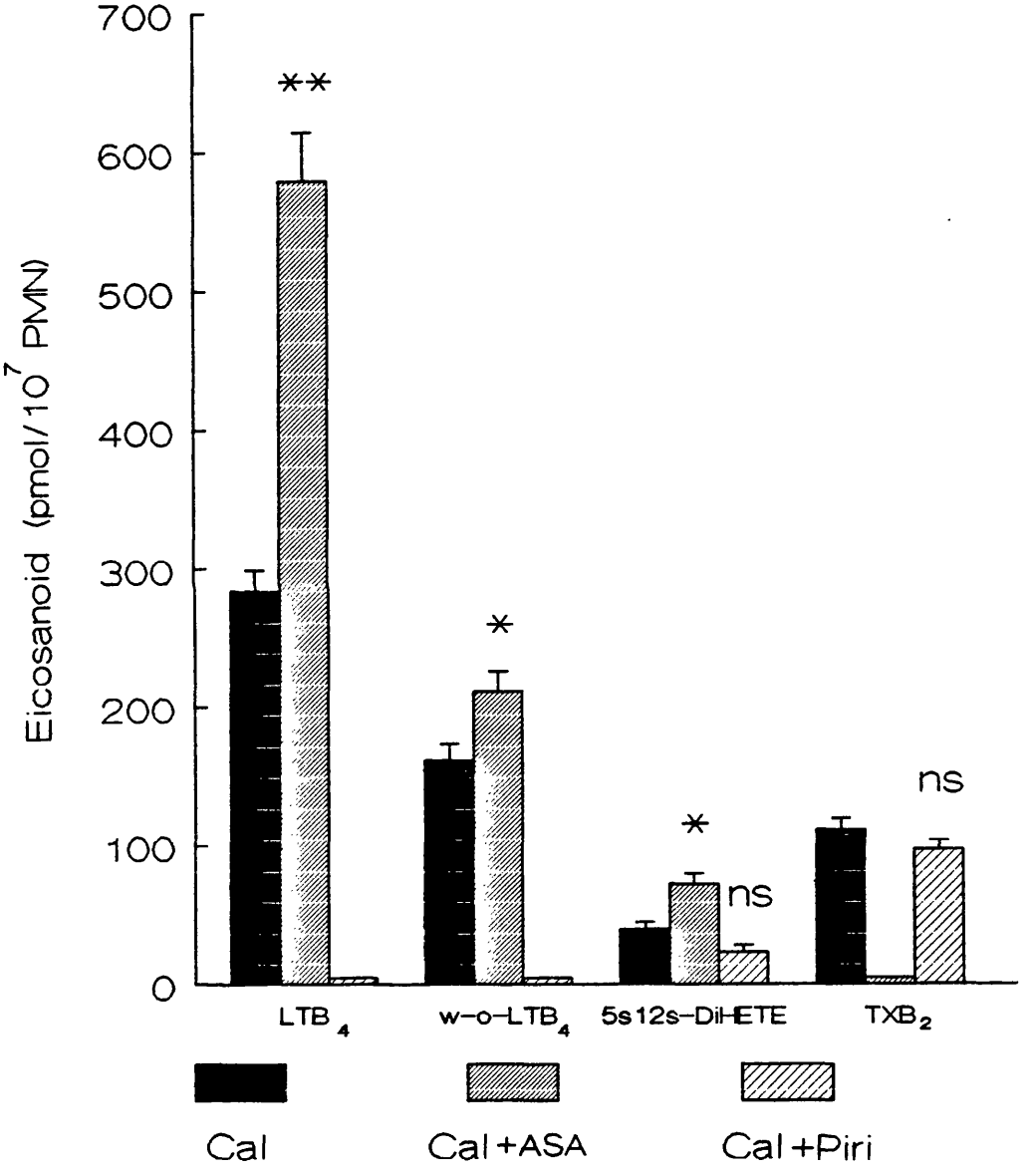

Figure 6.9. Release of different eicosanoids *in vitro* by stimulation of blood with 20μM calcium ionophore A23187 (CaI) for 30 minutes. Some samples were preincubated with aspirin (1mM; CaI+ASA) or Piriprost (40μg/ml; CaI+Piri). Results show the mean and SEM of 4 separate experiments and were not statistically significant (ns), $p<0.05$ (*) or p,0.01 (**).

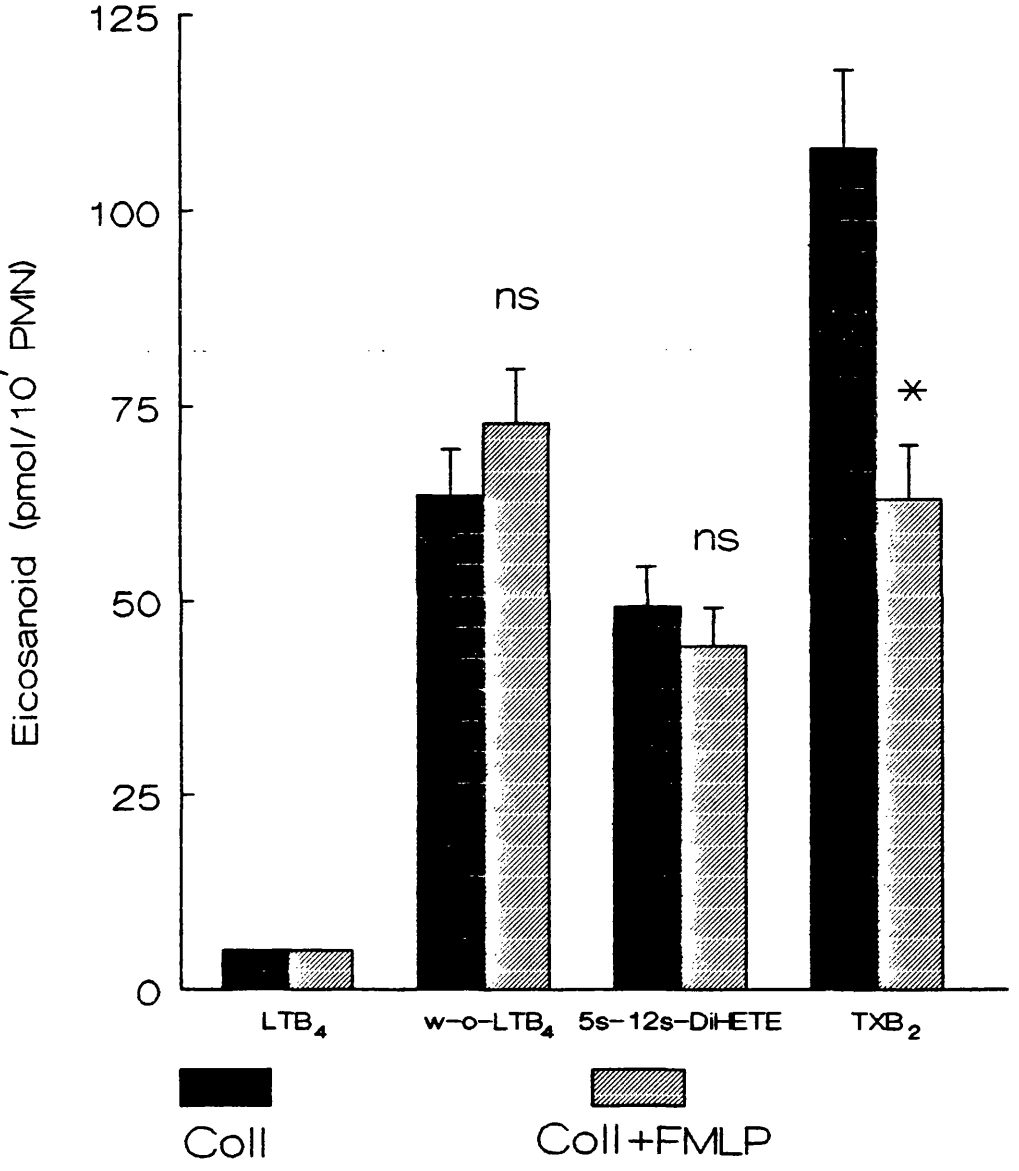

Figure 6.10. Release of different eicosanoids *in vitro* by stimulation of blood with 20μg/ml collagen for 30 minutes in the absence (Coll) or presence (Coll+FMLP) of co-stimulation with 10μM FMLP. Results show the mean and SEM of 4 separate experiments and were either not statistically significant (ns) or $p<0.01$ (*).

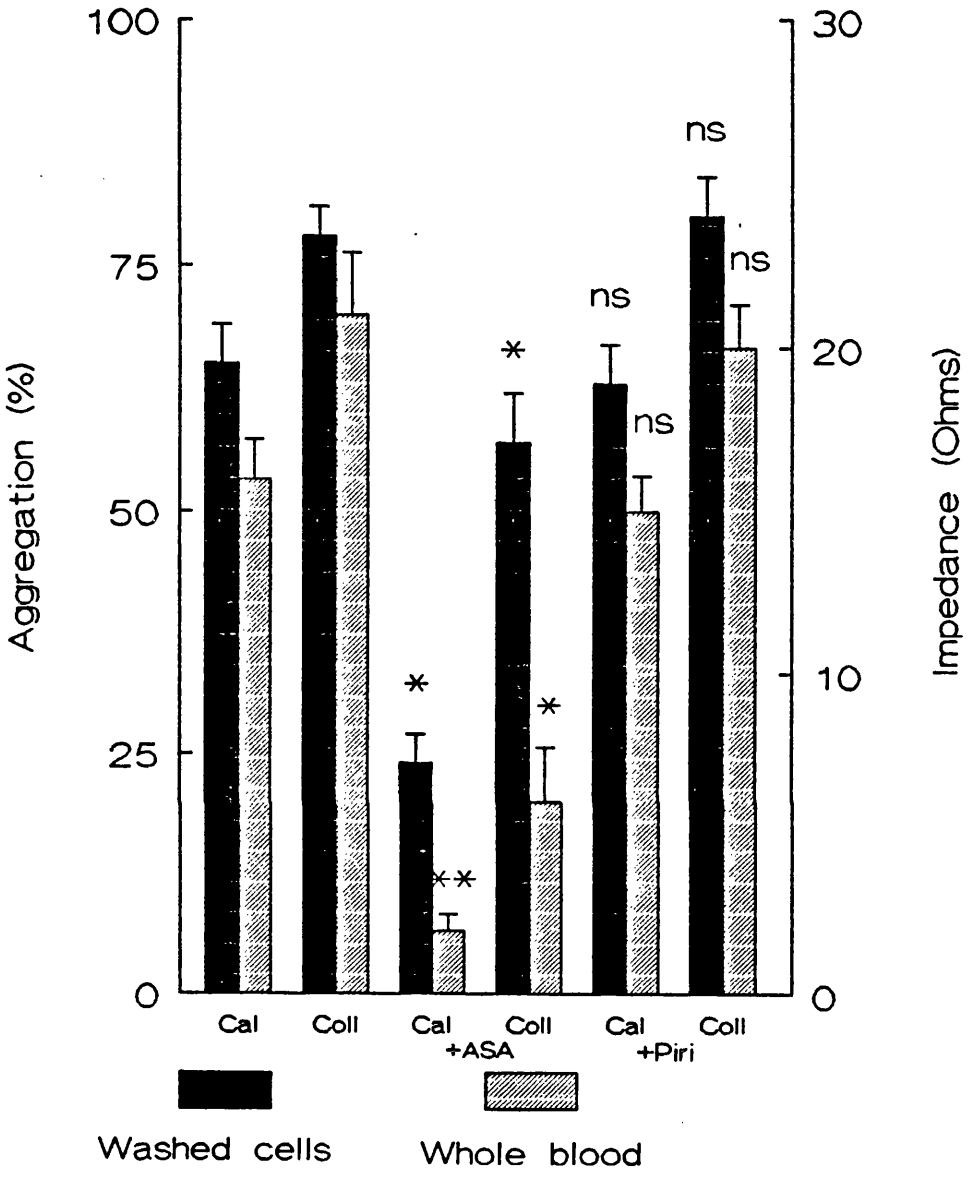

Figure 6.11. The effect of either cyclooxygenase or 5-lipoxygenase inhibition upon aggregation in washed platelet and neutrophil mixtures ($200x10^9$/l platelets and $2x10^9$/l neutrophils) and heparin anticoagulated whole blood. Aggregation was induced by either 20μg/ml collagen or 2.5μM calcium ionophore A23187 (20μM in whole blood). Maximum aggregation was measured by impedance (ohms) with blood and turbidometry (% aggregation) with washed cells. Some samples were preincubated with either 1mM aspirin (+ASA) or 40μg/ml Piriprost (+Piri). Results show the mean and SEM of 5 experiments and were not statistically significant (ns), $p<0.05$ (*), or $p<0.01$ (**).

(10μM) also failed to stimulate LTB_4 synthesis. FMLP did cause some reduction in TXB_2 concentration in blood stimulated with collagen. Blood stimulated with FMLP alone did not cause any detectable release of eicosanoids.

The effect of 5-lipoxygenase inhibition on platelet aggregation. The inhibition by aspirin of both collagen- and calcium ionophore A23187-induced aggregation in both whole blood and washed systems was observed. The inhibition of 5-lipoxygenase by Piriprost, with the subsequent inability to produce leukotrienes had no effect on platelet aggregation *in vitro* (Figure 6.11). Piriprost had no effect on aggregation induced in either heparin or citrate anticoagulated blood.

DISCUSSION

Eicosanoid metabolism has been shown to be modulated by cell-cell interactions where precursors, intermediates and end products can be processed by different cells in close proximity and under different stimulatory conditions (Marcus, 1988).

Platelet activation results in the liberation of arachidonate, the oxygenation of which is catalysed by two intracellular enzymes. The cyclooxygenase is particle bound and inhibited by aspirin. It promotes formation of thromboxane A_2, which is rapidly converted to stable and inactive thromboxane B_2 (TXB_2). Cyclooxygenase also forms 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malonaldehyde (MDA). The platelet 12-lipoxygenase is cytoplasmic and formation of its oxygenation product 12s-hydroxy-5,8-cis,10-trans,14-cis-eicosatetraenoic acid (12-HETE) continues after aspirin treatment (Marcus, 1984). This study confirmed the presence of metabolites formed by cyclooxygenase in activated platelets and their inhibition by aspirin. 12-HETE release was found to increase after aspirin treatment indicating that cyclooxygenase inhibition provided

extra substrate for 12-lipoxygenase activity. Similarly, aspirin treatment of platelets resulted in greater release of unincorporated radiolabelled arachidonate.

Washed, purified neutrophils upon stimulation produced metabolites of 5-lipoxygenase: 5-HETE, LTB_4 and ω-oxidized metabolites of LTB_4. Neutrophils produced no detectable cyclooxygenase metabolites after stimulation with calcium ionophore A23187. This is consistent with previous reports (Marcus AJ, 1990).

NDGA, a dual cyclooxygenase and lipoxygenase inhibitor effectively inhibited eicosanoid synthesis by neutrophils. Similarly, Piriprost (Sun and McGuire, 1983), a specific 5-lipoxygenase inhibitor, abolished 5-HETE, LTB_4 and LTB_4 metabolite release. On the other hand aspirin had no detectable effect on neutrophil eicosanoid release.

Neutrophil preparations mixed with different numbers of platelets resulted in an altered eicosanoid profile. Stimulated neutrophils have previously been shown to metabolise platelet-derived 12-HETE *in vitro* by 2 different mechanisms (Marcus *et al*, 1982; Marcus *et al*, 1984). Stimulated neutrophils, in which 5-lipoxygenase has been activated, convert platelet 12-HETE to 5s,12s-dihydroxy-6-trans, 8-cis, 10-trans, 14-cis, eicosatetraenoic acid (5s-12s-diHETE; Marcus *et al*, 1982). In contrast, unstimulated neutrophils metabolise platelet 12-HETE to 12s-12,20-dihydroxy-5,8,10,14-eicosatetraenoic acid (12s-20s-diHETE; Marcus *et al*, 1984). 12-HETE is ω-hydroxylated by neutrophils via a cytochrome P-450 enzyme system (Marcus *et al*, 1987).

This study confirms the appearance of a product found only in platelet and neutrophil mixtures as opposed to either cell-type alone. Both cell-types were activated and the compound eluted with authentic 5s-12s-diHETE. The physiological role of this compound is uncertain, but it may be a means of removing 12-HETE which is known to have weak chemotactic activity for neutrophils.

The incubation of platelets with neutrophils caused a significant increase in neutrophil LTB_4 production when both cell-types were incubated. This increase in LTB_4 was dependent on platelet concentration. The LTB_4 was radiolabelled, indicating that platelets served as donors of arachidonate for conversion by 5-lipoxygenase. LTB_4 is a potent inflammatory mediator and its amplification by platelets could have proinflammatory consequences if it occurred *in vivo*.

The investigation of eicosanoid production by stimulated whole blood was found to be greatest in heparin anticoagulated blood. Citrated blood was less able to support LTB_4 release in particular, presumably due to a lack of calcium ions.

In washed, purified neutrophil preparations stimulated with calcium ionophore A23187 LTB_4 release was maximal at 4 minutes and rapidly metabolised by ω-oxidation. This confirms previous findings (Salmon *et al*, 1982). In whole blood, LTB_4 was released in a dose dependant method in response to calcium ionophore A23187. However, in contrast to washed cells LTB_4 release took longer to reach maximum, achieved higher concentrations and was relatively stable. This may be due to one or more of the following reasons: firstly, LTB_4 may be stabilized by binding to plasma albumin; secondly, the presence of other active cell-types such as platelets or monocytes may provide arachidonate as substrate for neutrophils; and finally, LTB_4 may be directly released from monocytes (Williams *et al*, 1984) or from erythrocytes in response to LTA_4 released from neutrophils (Fitzpatrick *et al*, 1984).

Following activation with calcium ionophore A23187 aspirin-treated whole blood was found to contain significantly higher concentrations of LTB_4 than non-aspirin treated blood. Again, as with washed cells, inhibition of cyclooxygenase by aspirin may cause increased availability of arachidonate for metabolism by 5-lipoxygenase. Oxidized

metabolites of LTB_4, as well as 5s-12s-diHETE were also significantly higher in aspirin treated blood. This suggests that aspirin, although an anti-inflammatory drug, may under certain conditions promote synthesis of certain proinflammatory leukotrienes. Piriprost was an effective inhibitor of 5-lipoxygenase in blood *in vitro*.

5-Lipoxygenase products could not be detected in collagen stimulated blood. Collagen seems not to activate neutrophil 5-lipoxygenase. Collagen is however a potent activator of platelets and cyclooxygenase. Low levels of 5s-12s-diHETE were present in blood. This indicates some transcellular eicosanoid metabolism.

The addition of FMLP to collagen stimulated blood did not significantly alter leukotriene release. FMLP is an effective agonist for purified neutrophils due to an upregulation of specific receptors during preparation, whereas in whole blood these receptors are not sufficiently expressed. It remains a relatively weak, but more physiological stimulator of eicosanoid synthesis. In blood, FMLP has been shown to be effective at stimulating respiratory burst activity only if cells are primed with cytokines (Jaswon *et al*, 1990). This would be an area for future study. FMLP stimulation of cytokine-primed cells results in greater eicosanoid release (Fiore & Serhan, 1990). This may be amplified by collagen stimulation of platelets.

Platelet aggregation, in both washed cells or blood was unaffected by Piriprost inhibition of 5-lipoxygenase. Although the amplification of LTB_4 release from neutrophils by platelets may modulate inflammation, it appears that platelet function relating to haemostasis is unaffected.

The modulation of eicosanoid release via transcellular metabolism may be important at inflammatory sites. The analysis of these interactions in inflammatory disease may lead to a clearer understanding of the pathophysiology of these conditions.

CHAPTER 7

A PRELIMINARY INVESTIGATION OF PLATELET-NEUTROPHIL INTERACTIONS IN MULTIPLE SYSTEM ORGAN FAILURE

INTRODUCTION

The syndrome of Multiple System Organ Failure (MSOF) is a leading cause of death in the intensive therapy unit (Machiedo *et al*, 1981). Even with advances in intensive care medicine the mortality from MSOF, when fully established, and when 3 or more organ systems are involved for more than 3 days, remains greater than 98% (Knaus *et al*, 1985). MSOF is frequently associated with Adult Respiratory Distress Syndrome (ARDS) which is now regarded as the pulmonary manifestation of MSOF. This state of affairs has directed research towards the prevention of MSOF. The prevention of MSOF after surgery has been attempted by increasing oxygen transport to supranormal levels with the aim of avoiding tissue hypoxia. This has resulted in reduced mortality and incidence of MSOF (Shoemaker *et al*, 1988). The management of ITU patients with established MSOF has also included this strategy of treatment (Edwards *et al*, 1989).

Multisystem activation is a common denominator in the pathogenesis of MSOF. However, the underlying mechanisms that result in uncontrolled activation of inflammatory pathways remain obscure. Vascular leakage which accompanies sepsis and MSOF is caused by a complex mechanism in which a variety of mediators are involved. Arachidonic acid metabolites, toxic oxygen species and proteinases can be considered as final mediators which, acting as executive instruments, directly affect the endothelial and

epithelial barriers.

Much research has focused on the role of vasoactive substances (such as complement, fibrin, prostaglandins, thromboxane and leukotrienes) and cellular agents (such as platelets, neutrophils, alveolar macrophages and endothelial cells). There is a complex interrelationship of these factors in the pathophysiology of MSOF and available evidence implicates both the neutrophil and the platelet as key components. Post mortem examination has shown sequestration of platelets and neutrophils interlaced with a fibrin mesh in the pulmonary circulation adjacent to areas of endothelial injury following ARDS. The presence of increased quantities of degranulated neutrophils in bronchoalveolar lavage fluid from patients with ARDS further substantiates their location to regions of alveolar-capillary damage.

Substances released by neutrophils and platelets such as oxygen radicals, eicosanoids and proteolytic enzymes have properties which could account for many of the observed symptoms of MSOF. When generated in the pulmonary vasculature these substances may directly damage the endothelium, causing a permeability defect, promote vasoconstriction and recruit additional platelets, neutrophils and other leukocytes to an inflammatory site. Early in the course of lung injury platelets as sequestered in the lung. Activated platelet release products which promote both bronchoconstriction and vasoconstriction thereby contributing to pulmonary hypertension and ventilation perfusion mismatch.

Neutrophil-induced injury may be furthered by the conversion of platelet-derived arachidonate through the neutrophil 5-lipoxygenase pathway.

Nitric oxide release by endothelial cells and perhaps leukocytes may be an important physiological agent in the control of vascular tone and platelet adhesion and

aggregation. Little is known about the role of nitric oxide in pathophysiological states. However, nitric oxide abnormalities might account for disorders of vascular reactivity that characterise a wide variety of disease states.

This study aims to further investigate the role of platelet-neutrophil interactions in patients both at risk from, and with MSOF. Changes in platelet-neutrophil interactions may help to explain some pathophysiological events.

METHODS

Patient selection criteria. Dr. M. Mython selected appropriate patients for this study and collected blood samples. Patients either with established MSOF, or considered to be at risk of developing it were eligible for study.

The following criteria were used for the diagnosis of ARDS: radiological evidence of interstitial or alveolar infiltrate or both, usually sparing the costrophrenic angles; hypoxaemia on mechanial ventilation with PaO_2 <9.0 kPa on FiO_2 ≥0.6 or PaO_2/FiO_2 <15; PAWP <18 mmHg.

Patients were also eligible for study if they could be included in one of the following at risk categories.

(i) Patients with sepsis defined as evidence of serious bacterial infection. Two or more of the following must have been present: a) rectal temperature >39°C or white blood count >12000/ml; with at least one of the following: b) positive blood culture of an accepted pathogen; c) a strongly suspected or proved source of systemic infection; d) gross pus in a closed space; e) unexplained systemic arterial hypotension (systolic pressure <80mmHg); f) systemic vascular resistance <800 dyne sec^{-1} cm^{-1}; g) unexplained metabolic acidosis. (ii) Severe pancreatitis. (iii) Hypertransfusion: patients requiring >10 units of blood (whole

or packed cells) in <24 hours. (iv) Chest trauma resulting in flail chest or pulmonary contusion with a PaO_2 of <9 kPa with the patient receiving an FiO_2 >0.4. (v) Multiple fractures, defined as one of the following: a) unstable pelvic fracture; b) two or more major long bone fractures; c) pelvic fracture and a major long bone fracture. (vi) Cardiopulmonary bypass.

Patients were scored for organ failure according to the method of Goris (Goris, 1986).

Table 7.1. Clinical diagnosis of patients studied.

PATIENT	DIAGNOSIS
AC	GI bleed/Hypertransfusion/Hepatic failure
DT	Multiple trauma/ARDS
AG	Sepsis
AP	Post CABG
AH	Hepatobiliary surgery/Hypertransfusion
IL	Post aortic aneurysm repair/ARDS/MSOF
GC	Post CABG/ARDS/MSOF
AA	Sepsis

The following exclusion criteria were observed: patients under 18 years of age; patients considered to be in a terminal state; pregnancy; patients with coagulopathies (including a platelet count $<80x10^9/l$) and any administration of an investigational drug within the past 30 days.

Blood samples, collection and handling. See section 2.1.

Preparation of PRP. See section 2.3.

Preparation of washed platelets. See section 2.4.1.

Preparation of washed, purified neutrophils. See section 2.5.

Turbidometric platelet aggregation. See section 2.6.1.

The effect of nitric oxide release from patients neutrophils. This was studied with normal platelets from healthy volunteers who had taken no drugs in the preceding 10 days. Neutrophils from these donors were used simultaneously as a control. Some samples had superoxide dismutase, haemoglobin, L-arginine or N^G-nitro-L-arginine added 1 minute prior to neutrophil addition. Platelets were stimulated with submaximal (70-80% of maximum) concentrations of bovine thrombin (Diagnostic Reagents; 0.025u/ml). Aggregation was measured in the absence or presence of neutrophils ($5x10^9$/l). Neutrophils were coincubated with platelets for 1 minute prior to addition of agonist. This coincubation time was found to be optimal for neutrophil inhibitory activity. Samples containing platelets (as well as superoxide dismutase, haemoglobin or other aforementioned compounds) but not neutrophils, were aggregated simultaneously with platelet-neutrophil samples to act as control.

Platelet activation by stimulated patients neutrophils. Platelets ($200x10^9$/l) or platelets mixed with neutrophils ($5x10^9$/l) were incubated at 37°C with 1mM Ca^{2+}, 0.25g/l fibrinogen and 2.5μg/ml cytochalasin B. Aggregation and luminescence were monitored upon addition of agonist (FMLP, LTB_4, PAF and thrombin) for up to 20 minutes. Samples from apparently healthy volunteers were run simultaneously with patients samples.

Statistical analysis. Results from patients samples were compared with those from apparently healthy volunteers (controls) by Wilcoxon's Signed Rank Sum test for non-parametric data. Confidence intervals (95%) for non-parametric data were also used to compare results from patients with those from controls. The aggregation results were correlated with the MSOF score for each patient.

RESULTS

The effect of nitric oxide release from patients neutrophils. Washed neutrophils from patients with or at risk from MSOF were able to partially inhibit the aggregation of normal platelets stimulated with 0.05u/ml thrombin. This was statistically significant (see table 7.2). Inhibition of aggregation was enhanced by both superoxide dismutase (see table 7.3) and L-arginine (see table 7.4). This inhibition caused by the neutrophils was equivalent in both groups. Both N^G-nitro-L-arginine (see table 7.5) and haemoglobin (see table 7.6) were able to prevent this inhibitory activity. Neutrophils from the patients studied were able to release nitric oxide under these experimental conditions. These results from both individual patients and the group as a whole showed no significant difference to neutrophils from normal individuals. 95% Confidence intervals comparing neutrophils from "controls" with patients are shown in table 7.7. There was no correlation detected between extent of aggregation and MSOF score.

Platelet activation by stimulated patients neutrophils. When the neutrophils from patients with or at risk from MSOF were stimulated with either FMLP or LTB_4 they were found to be less able to aggregate normal platelets than neutrophils from normal, healthy volunteers. These differences were statistically significant (see tables 7.9 and 7.10). PAF-stimulated neutrophils (from both groups) and platelets showed no significant differences (see table 7.11). The range of responses to PAF were wide. Thrombin-stimulated platelets in the presence of either "control" neutrophils or patients neutrophils just failed to show a clear significant difference (see table 7.12). These results suggest that neutrophils from the control group were antagonizing platelet aggregation in some way. 95% Confidence intervals comparing the effect on platelet aggregation of neutrophils from "controls" and neutrophils from patients is shown in table 7.7. There was

again no significant correlation detected between the extent of aggregation and MSOF score.

Table 7.2 A comparison of the effect of washed neutrophils from either normal subjects (control) or MSOF patients on platelet aggregation induced by 0.05u/ml thrombin. Platelets from healthy volunteers were aggregated alone (Plts.alone) or in the presence of neutrophils (Plts.+PMNs).

NAME	DATE	MSOF SCORE	AGG^N (%) Plts.alone	PATIENT AGG^N (%) Plts.+PMNs	CONTROL AGG^N (%) Plts.+PMNs
AC	30/10	6	71	61	58
AC	1/11	3	65	55	58
DT	13/9	4	71	60	58
DT	14/9	6	69	59	60
DT	20/9	3	78	67	65
DT	25/9	3	81	70	71
AG	6/11	4	72	61	61
Median			71	61	60
Mean			72.4	61.9	61.6
SD			5.4	5.1	4.9
SE			2.1	1.9	1.9
Wilcoxon's Signed Rank Sum Test				0.018	0.018

Table 7.3 A comparison of the effect of washed neutrophils from either normal subjects (control) or MSOF patients on platelet aggregation induced by 0.05u/ml thrombin in the presence of superoxide dismutase (60u/ml). Platelets were aggregated alone (Plts.alone) or in the presence of neutrophils (Plts.+PMNs).

NAME	DATE	MSOF SCORE	AGG^N (%) Plts.alone	PATIENT AGG^N (%) Plts.+PMNs	CONTROL AGG^N (%) Plts.+PMNs
AC	30/10	6	72	51	52
AC	1/11	3	64	50	49
DT	13/9	4	73	51	54
DT	14/9	6	68	52	48
DT	20/9	3	76	49	53
DT	25/9	3	79	50	50
AG	6/11	4	70	48	52
Median			72	50	52
Mean			71.7	50.3	51.1
SD			5.0	1.4	2.2
SE			1.9	0.5	0.8
Wilcoxon's Signed Rank Sum Test				0.018	0.018

Table 7.4 A comparison of the effect of washed neutrophils from either normal subjects (control) or MSOF patients on platelet aggregation induced by 0.05u/ml thrombin in the presence of L-arginine (100μM). Platelets were aggregated alone (Plts.alone) or in the presence of neutrophils (Plts.+PMNs).

NAME	DATE	MSOF SCORE	AGG^N (%) Plts.alone	PATIENT AGG^N (%) Plts.+PMNs	CONTROL AGG^N (%) Plts.+PMNs
AC	30/10	6	70	58	53
AC	1/11	3	63	42	39
DT	13/9	4	75	50	56
DT	14/9	6	71	49	45
DT	20/9	3	60	42	42
DT	25/9	3	70	50	54
AG	6/11	4	67	45	47
Median			70	49	47
Mean			68.0	48.0	48.0
SD			5.1	5.6	6.5
SE			1.9	2.1	2.5
Wilcoxon's Signed Rank Sum Test				0.018	0.018

Table 7.5 A comparison of the effect of washed neutrophils from either normal subjects (control) or MSOF patients on platelet aggregation induced by 0.05u/ml thrombin in the presence of nitro-L-arginine (10μM). Platelets were aggregated alone (Plts.alone) or in the presence of neutrophils (Plts.+PMNs).

NAME	DATE	MSOF SCORE	AGG^N (%) Plts.alone	PATIENT AGG^N (%) Plts.+PMNs	CONTROL AGG^N (%) Plts.+PMNs
AC	30/10	6	72	71	65
AC	1/11	3	68	62	71
DT	13/9	4	80	75	81
DT	14/9	6	75	77	79
DT	20/9	3	69	73	67
DT	25/9	3	71	74	75
AG	6/11	4	77	78	70
Median			72	74	70
Mean			73.1	72.9	72.6
SD			4.4	5.3	6.0
SE			1.7	2.0	2.3
Wilcoxon's Signed Rank Sum Test				0.753	0.786

Table 7.6 A comparison of the effect of washed neutrophils from either normal subjects (control) or MSOF patients on platelet aggregation induced by 0.05u/ml thrombin in the presence of haemoglobin (10μM). Platelets were aggregated alone (Plts.alone) or in the presence of neutrophils (Plts.+PMNs).

NAME	DATE	MSOF SCORE	AGG^N (%) Plts.alone	PATIENT AGG^N (%) Plts.+PMNs	CONTROL AGG^N (%) Plts.+PMNs
AC	30/10	6	70	66	73
AC	1/11	3	67	68	64
DT	13/9	4	83	84	81
DT	14/9	6	79	81	72
DT	20/9	3	73	70	77
DT	25/9	3	74	69	69
AG	6/11	4	71	67	63
Median			73	69	72
Mean			73.4	72.1	71.3
SD			5.5	7.2	6.6
SE			2.1	2.7	2.5
Wilcoxon's Signed Rank Sum Test				0.866	0.764

Table 7.7 95% Confidence intervals for the effect of nitric oxide release by "control" neutrophils and patients neutrophils on platelet aggregation.

	Difference between medians	95% Confidence Interval
Thrombin alone	1	-2.0 to 2.5
Thrombin+SOD	-2	-4.0 to 2.0
Thrombin+L-arg	2	-4.0 to 4.0
Thrombin+nitro-L-arg	4	-6.0 to 7.0
Thrombin+haemoglobin	-3	-7.0 to 6.5

Table 7.8 95% Confidence intervals for the stimulation of "control" neutrophils and patients neutrophils with subsequent platelet aggregation.

	Difference between medians	95% Confidence Interval
FMLP	-7	1.5 to 28.5
LTB_4	-6.5	6.0 to 17.5
PAF	21	-38.0 to 12.5
Thrombin	11.5	0 to 23.0

Table 7.9 A comparison of different patients (patient) washed neutrophils with neutrophils from apparently healthy volunteers (control) on platelet aggregation. Neutrophils ($5x10^9/l$) were incubated with normal, washed platelets ($200x10^9/l$) prior to stimulation with 1μM FMLP.

NAME	DATE	MSOF SCORE	PATIENT Aggregation (%)	CONTROL Aggregation (%)
AP	22/1	0	61	69
AH	24/1	0	60	69
IL	29/1	8	68	66
IL	31/1	9	61	66
IL	5/2	9	59	80
IL	12/2	11	13	65
GC	14/2	8	68	60
GC	19/2	6	53	59
GC	21/2	7	63	72
AA	5/3	5	45	79
median			60.5	67.5
mean			55.1	68.5
SD			16.30	7.01
SE			5.15	2.22
Wilcoxon's Signed Rank Sum Test	0.025			

Table 7.10 A comparison of different patients (patient) washed neutrophils with neutrophils from apparently healthy volunteers (control) on platelet aggregation. Neutrophils ($5x10^9/l$) were incubated with normal, washed platelets ($200x10^9/l$) prior to stimulation with 50 ng/ml LTB_4.

NAME	DATE	MSOF SCORE	PATIENT Aggregation (%)	CONTROL Aggregation (%)
AP	22/1	0	58	72
AH	24/1	0	60	78
IL	29/1	8	68	66
IL	31/1	9	64	74
IL	5/2	9	59	76
IL	12/2	11	18	36
GC	14/2	8	24	44
GC	19/2	6	65	64
GC	21/2	7	56	62
AA	5/3	5	46	60
median			58.5	65
mean			51.8	63.2
SD			17.35	13.77
SE			5.49	4.35
Wilcoxon's Signed Rank Sum Test	0.013			

Table 7.11 A comparison of different patients (patient) washed neutrophils with neutrophils from apparently healthy volunteers (control) on platelet aggregation. Neutrophils ($5x10^9/l$) were incubated with normal, washed platelets ($200x10^9/l$) prior to stimulation with 2μM PAF.

NAME	DATE	MSOF SCORE	PATIENT Aggregation (%)	CONTROL Aggregation (%)
AP	22/1	0	71	29
AH	24/1	0	20	30
IL	29/1	8	40	71
IL	31/1	9	21	15
IL	5/2	9	76	63
IL	12/2	11	34	68
GC	14/2	8	83	15
GC	19/2	6	23	18
GC	21/2	7	64	20
AA	5/3	5	51	19
median			45.5	24.5
mean			48.3	34.8
SD			24.04	23.10
SE			7.60	7.30
Wilcoxon's Signed Rank Sum Test	0.203			

Table 7.12 A comparison of different patients (patient) washed neutrophils with neutrophils from apparently healthy volunteers (control) on platelet aggregation. Neutrophils ($5x10^9/l$) were incubated with normal, washed platelets ($200x10^9/l$) prior to stimulation with 0.05u/ml thrombin.

NAME	DATE	MSOF SCORE	PATIENT Aggregation (%)	CONTROL Aggregation (%)
AP	22/1	0	51	70
AH	24/1	0	5	51
IL	29/1	8	69	61
IL	31/1	9	32	36
IL	5/2	9	46	69
IL	12/2	11	50	58
GC	14/2	8	43	54
GC	19/2	6	60	68
GC	21/2	7	29	33
AA	5/3	5	35	27
median			44.5	56
mean			42.0	52.7
SD			17.96	15.73
SE			5.68	4.98
Wilcoxon's Signed Rank Sum Test	0.059			

DISCUSSION

The precise mechanism of vascular hyporesponsiveness that accompanies sepsis and MSOF is not yet clearly defined. This vascular failure is a major factor in determining the clinical outcome of these states (Groeneveld and Thijs, 1986). Nitric oxide has been implicated in the loss of vascular responsiveness induced by endotoxin (Julou-Schaeffer *et al*, 1990; Gray *et al*, 1991). Endotoxin was found to increase the release of nitric oxide by endothelial cells (Salvemini *et al*, 1989).

This study on patients either with MSOF or at risk of developing MSOF examined the effect of neutrophil-derived nitric oxide upon platelet aggregation. Although only a small number of patients were studied no evidence was found of enhanced nitric oxide activity. Nitric oxide was previously found to be released by "resting" or unstimulated neutrophils and so an up regulation of nitric oxide synthesis in patients with septicaemia may occur or alternatively, a lack of nitric oxide could result in platelet hyperaggregability. However, neutrophil-derived nitric oxide from the patients studied inhibited aggregation of normal platelets to the same extent as neutrophils from normal, healthy "controls". There was no correlation between clinical state and the effect of neutrophil-derived nitric oxide on platelet aggregation. Endothelium-derived nitric oxide is more likely to be the major modifier of vascular responsiveness *in vivo* and it is here that endotoxin is likely to have its effect. In this study neutrophils were washed and purified prior to being mixed with platelets. The washing procedure would have removed any plasma factors which may provide a stimulus for neutrophil activation. Further experiments utilizing the whole blood impedance aggregation technique for examining nitric oxide activity may provide more positive information. This would have the advantage of being able to study the aggregation of the patient's own platelets. In these

experiments samples from patients consistently had platelet counts of less than $100x10^9/l$ making them unsuitable for washing because of the poor yield obtained.

Platelet activation by neutrophils exposed to chemotactic agents has been reported (Del Maschio *et al*, 1990). The results reported here indicate that washed, purified neutrophils from the patient group were less able to cause platelet activation than normal "control" neutrophils when stimulated with chemotactic agents. Neutrophil activation with either FMLP or LTB_4 resulted in significantly less platelet activation. Despite the small number of patients studied confidence interval analysis suggests this difference between the groups was real. These findings suggest that the neutrophils had been at least partially activated *in vivo* and were thus less able to respond fully to *in vitro* activation. This neutrophil-derived stimulation of platelets under these experimental conditions is likely to be mediated by neutral proteases such as cathepsin G (Selak *et al*, 1988). Activation by PAF, an agonist of both platelet and neutrophils, was found to cause variable degrees of platelet aggregation in both groups studied. Consequently no statistical difference between "control" and patient groups was detected. Stimulation of normal platelets with a relatively low concentration of thrombin was slightly different between the two groups although this just failed to reach statistical significance.

In MSOF and related clinical states both neutrophils and platelets become activated by different mediators of inflammation. The underlying disease (such as multiple trauma and sepsis) strongly alters the responsiveness, function and population of peripheral granulocytes. Previous studies have demonstrated impaired neutrophil capacity to generate LTB_4 whereas the metabolism of LTB_4 into its ω-oxidized products was increased (König *et al*, 1990).

The exact mechanism of the pathogenesis of MSOF and septic shock remains to

be established. However, a role for both neutrophils and platelets in the ensuing inflammation is likely. Neutrophils and other phagocytes possess a wide array of inflammatory mediators, which if uncontrolled could explain many of the clinical features. Nitric oxide release, if not checked, may also lead to pathological vasodilatation and tissue damage.

CHAPTER 8

SUMMARY AND CONCLUSION

8.1 Summary

Neutrophils release factors that are able to inhibit aggregation and secretion from washed platelets *in vitro*. One of these inhibitory mechanisms could be attributed to a nitric oxide-like factor (Chapter 3). Nitric oxide was characterised pharmacologically with agents known to either inhibit or potentiate its activity. Inhibition of platelet activity was mediated via nitric oxide stimulation of guanylate cyclase and the resultant increase in cyclic GMP concentration. This inhibition was dependent upon neutrophil concentration and viability. Neutrophils fixed with glutaraldehyde were unable to modify platelet aggregation. Neutrophils were unable to completely abolish platelet activity in these experiments. Similarly, high concentrations of platelet agonists, such as thrombin, were able to overcome the inhibition from neutrophil-derived nitric oxide. These results suggest that nitric oxide may serve to dampen platelet activity, acting as a fine control. Authentic nitric oxide was able to completely abolish platelet responses at higher concentrations of agonist, presumably because it was at a far higher concentration to that released by neutrophils.

The addition of intact red cells to washed platelets effectively neutralized the activity of added authentic nitric oxide (Chapter 4). Anticoagulated whole blood was also found to have the potential for nitric oxide-like activity. L-arginine, but not D-arginine, was able to dose dependently inhibit aggregation induced by collagen. This activity could

be enhanced by M&B22,948, a specific cyclic GMP phosphodiesterase inhibitor, but not by pentoxifylline, a cyclic AMP phosphodiesterase inhibitor. Conversely, N^G-nitro-L-arginine, an inhibitor of nitric oxide synthase abolished the activity of L-arginine.

Neutrophils were also found to inhibit platelet aggregation by the formation of adenosine (Chapter 3). A neutrophil-derived ADPase metabolized platelet-derived ADP to adenosine and so limit the extent of platelet aggregation.

As well as being able to inhibit platelet activity, neutrophils were also able to stimulate platelet activation under different conditions. Neutrophils stimulated with chemotactic factors in the presence of platelets were able to initiate platelet intracellular calcium flux, aggregation and secretion. The factor responsible for these effects was found to be a serine protease. Purified, neutrophil cathepsin G elicited similar platelet responses to the addition of stimulated neutrophils.

Platelet-derived eicosanoid metabolism was altered in the presence of neutrophils (Chapter 6). Neutrophils utilized arachidonate from platelets to significantly increase LTB_4 release. Platelet-derived 12-HETE release was also modified resulting in the formation of 5s-12s-diHETE. This compound was formed by a mechanism involving transcellular eicosanoid metabolism. Eicosanoid formation was also studied in anticoagulated whole blood stimulated with different agonists. Heparin was a better anticoagulant than *tri*-sodium citrate for studying eicosanoids due to their calcium dependence. Treatment of blood with specific inhibitors of eicosanoid synthesis altered the profile of eicosanoids produced by other pathways. Aspirin-treated stimulated blood was found to produce significantly more LTB_4.

A preliminary study of patients with, or at risk of developing, Multiple System

Organ Failure Syndrome failed to detect any differences between "control" and patient's neutrophils ability to inhibit aggregation of washed platelets (Chapter 7). However, washed neutrophils from the patients when stimulated with either FMLP or LTB_4 were less able to cause platelet aggregation when compared with "control" neutrophils from normal, healthy volunteers.

8.2 Conclusion

Microscopic examination of haemostatic platelet plugs and arterial thrombi have been consistently shown to contain neutrophils. They have often been considered to play a passive role in both the formation of the platelet plug and the subsequent repair process. However, there is now mounting evidence implicating neutrophils in the biochemical and functional modulation of platelet reactivity. Neutrophils, by virtue of this evidence can also be described as "thromboregulators", as well as possessing a diverse array of functions in the inflammatory response. The interactions of neutrophils with platelets depend on the experimental conditions used. Thus neutrophils can down-regulate platelet activity by releasing nitric oxide which would be augmented by endothelium-derived nitric oxide. Alternatively, the action of neutrophil ADPase on released ADP to form adenosine could inhibit platelet recruitment and activation. Neutrophils are also able to up-regulate platelet activity by at least one mechanism. This is the release of a serine protease, most likely to be cathepsin G.

Platelets may also either augment or suppress neutrophil responses at inflammatory sites. The transcellular metabolism of platelet-derived arachidonate and its products, such as 12-HETE, result in compounds both quantitatively and qualitatively different from those produced by either cell-type alone. These lipid mediators may in turn effect

neutrophil chemotaxis and platelet aggregation. Increased local LTB_4 would promote neutrophil chemotaxis, aggregation and degranulation.

Experiments investigating platelet-neutrophil interactions in disease indicate some differences from responses of healthy individuals. The extreme stimulation of neutrophils that occurs in sepsis and endotoxaemia could be a major contributor to platelet activation, which in turn may promote further neutrophil activity.

In vivo indications of platelet-neutrophil interaction are difficult to identify because such mechanisms are first of all, likely to be localized to sites of trauma, and secondly many of the agents involved are labile. Further studies should aim to find ways of examining the different elements in blood and the way they interact under both physiological and pathophysiological conditions. Although the use of whole blood *in vitro* provides a more physiological environment in which to study cell function, the role of the vessel wall and its relationship with cells, such as platelets and neutrophils, also needs to be taken into account. Nevertheless, experiments presented here indicate that both platelets and neutrophils possess mechanisms that are able to strongly modulate each other and their microenvironment.

CHAPTER 9

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