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**Metabolites of Arachidonic Acid and
their Role in Inflammatory Disease**

A thesis submitted for the Degree of Doctor of Philosophy

By

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In the Faculty of Medicine
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August 1990

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DEDICATION

This thesis is dedicated to my grandparents William and Magdalene Whyte and James and Annele McDonald.

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ABBREVIATIONS

AA	arachidonic acid
ADP	adenosine diphosphate
AI	articular index
AMP	adenosine monophosphate
ARA	American Rheumatism Association
BSA	bovine serum albumin
CaI	calcium ionophore
cAMP	cyclic AMP
CO	cyclo oxygenase
cpm	counts per minute
CRP	C-reactive protein
DCC	dextran-coated charcoal
DCHA	docosaheptaenoic acid
DIP	distal interphalangeal
DMSO	dimethylsulphoxide
EFA	essential fatty acid
EPA	eicosapentaenoic acid
EPO	evening primrose oil
ESR	erythrocyte sedimentation rate
ETYA	eicosatetraenoic acid
FMLP	formyl-met-leu-phe
GCMS	gas-liquid chromatography/mass spectrometry
³ H	Tritium
Hb	haemoglobin
HEPES/HBSS	HEPES-buffered Hank's balanced salt solution
HETE	hydroxy-eicosatetraenoic acid
HLA	human leukocyte antigens
HPETE	hydroperoxy-eicosatetraenoic acid
HPLC	high performance liquid chromatography

HSP	Henoch-Schonlein purpura
¹²⁵ I	Iodine-125
IBD	inflammatory bowel disease
IC	immune complex
Ig	immunoglobulin
IL-1	interleukin-1
IV	intravenous
IQR	inter-quartile range
5-LO	5-lipoxygenase
LT	leukotriene
MSU	monosodium urate
NSAID	non-steroidal anti inflammatory drug
NSB	non-specific binding
PA	psoriatic arthritis
PBS	phosphate-buffered saline
PG	prostaglandin
PGDH	prostaglandin dehydrogenase
PGI ₂	prostacyclin
PGI ₂ -M	prostacyclin metabolite
PLA ₂	phospholipase A ₂
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PSA	ability of plasma to support PGI ₂ -like activity
PSAI	inhibition of PGI ₂ -like activity
PSS	progressive systemic sclerosis
RA	rheumatoid arthritis
RBC	red blood cell
RBL	rat basophilic leukaemia

RCS	rabbit aorta contracting substance
RD	Raynaud's disease
RF	rheumatoid factor
RIA	radioimmunoassay
RP	Raynaud's phenomenon
RS	Raynaud's syndrome
SA	specific activity
SD	standard deviation
SEM	standard error of the mean
SLE	systemic lupus erythamatosus
SRS-A	slow-reacting substance of anaphylaxis
SS	suspected secondary
TC	total counts
TX	thromboxane
VWF	vibration-induced white finger.

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SUMMARY

Arachidonic acid (AA), after it is released from cell membrane phospholipids, may be metabolized by the cyclo-oxygenase (CO) enzyme leading to formation of the prostaglandins (PG's) and thromboxanes (TX's) or by the 5-lipoxygenase (5-LO) leading to formation of the leukotrienes (LT's). These metabolites are collectively known as eicosanoids. The PG's, especially PGE₂ and PGI₂ (prostacyclin) have important roles in the development of the cardinal signs of inflammation including erythema, oedema and pain. However, PGE₂ and PGI₂ can also have anti-inflammatory actions.

Thromboxane A₂ (TXA₂) is a vasoconstrictor and a potent inducer of platelet aggregation while PGI₂ is a vasodilator and a potent inhibitor of platelet aggregation. The balance between TXA₂ synthesis by platelets and PGI₂ synthesis by vascular endothelium is thought to be an important mechanism in haemostasis while an imbalance is thought to play a role in the development of some disease states.

Leukotriene B₄ (LTB₄) is thought to mediate leukocyte behaviour during the inflammatory response since it is a potent chemokinetic and chemotactic agent for polymorphonuclear leukocytes (PMN's) and also induces adhesion, aggregation and degranulation of PMN's.

Although the individual properties of PGI₂, TXA₂ and LTB₄ are well recognized, their precise role in specific inflammatory diseases is often not known, although such information may facilitate the development of effective treatments. The remit of this thesis was to use available methodology (although not uncritically), to measure levels of these mediators in several inflammatory disease states and to examine some other aspects of their participation, with a view to assessing their role in these conditions. The effect of various therapies on production of these mediators, both *in vitro* and *ex vivo* was also examined.

The studies reported in Chapter 5 looked at the role of eicosanoids in some inflammatory vascular diseases. Theories regarding the pathogenesis of Raynaud's phenomenon (RP) and approaches to it's treatment have been numerous. This work has

shown that in RP secondary to progressive systemic sclerosis (PSS), plasma levels of PGI₂-metabolites were unexpectedly elevated. Furthermore, the platelet sensitivity to iloprost (a stable analogue of PGI₂) in these patients was shown to be decreased, but was normalized after PG therapy. The beneficial effect of PGI₂/iloprost infusion in PSS may therefore be considered as overcoming platelet resistance to PGI₂ rather than supplementing an inadequate production of PGI₂.

The production of LTB₄ from stimulated PMN's of patients with RP secondary to PSS and vibration-induced white finger (VWF) was also shown to be increased. The relevance of this observation to the development of vasospasm and vascular occlusion is discussed. The observation of increased levels in VWF supports the growing opinion that this condition is a true secondary Raynaud's syndrome. The finding of increased LTB₄ production in PSS suggests that cellular resistance to the inhibitory effects of PGI₂ may be a generalized phenomenon in this condition.

A recent advent in the treatment of RP has been the use of iloprost. The effect of transdermal iloprost on LTB₄ production from PMN's of normal volunteers and Raynaud's patients was investigated. It was concluded that iloprost had some inhibitory effect on LTB₄ production, but that its anti-platelet and vasodilatory effects may be more important in RP. The possibility that some of the beneficial effects of ketanserin (a serotonin antagonist) and stanozalol (an enhancer of fibrinolysis) in RP could be due to an effect on PMN LTB₄ production was also examined.

Henoch-Schonlein purpura (HSP) is a type of childhood vasculitis. Results are presented which show that plasma from these patients has a diminished or absent ability to support vascular PGI₂ generation. Further experiments were undertaken to determine whether this was due to reduced stimulation of PGI₂ synthesis or active inhibition.

Chapter 6 examined LTB₄ production in rheumatoid arthritis (RA) and psoriatic arthritis (PA). It was found that isolated PMN's from RA patients had an increased capacity to produce LTB₄ and that this was not secondary to non-steroidal anti-inflammatory drug (NSAID) therapy. Weak but significant correlations were found

between LTB₄ and markers for joint disease activity. Likewise, an increased capacity for LTB₄ production was observed in PMN's from PA patients. The relevance of this to both the skin and joint manifestations of the disease is discussed. No significant correlation of LTB₄ with either skin or joint indices of disease activity was observed. It was concluded from these observations that LTB₄ is unlikely to be an initiating, causal factor in chronic inflammatory conditions, but that its inhibition could, nevertheless produce an improvement in inflammatory symptoms.

A relatively new approach to anti-inflammatory therapy is the dietary manipulation of essential fatty acid (EFA's) leading to the endogenous production of PG and LT analogues with diminished inflammatory activity. Results of two double-blind, placebo-controlled studies with Evening primrose oil (EPO) (a source of di-homo- γ -linolenic acid) and fish oil (a source of eicosapentaenoic acid) in the treatment of RA and PA are presented in Chapter 7. These dietary supplements enabled RA patients to decrease or stop their NSAID therapy with no deterioration in objective markers of disease activity. In addition, the effect of EPO and EPO/fish oil on LTB₄ production from PMN's in these patients was investigated. In contrast, PA patients receiving EPO/fish oil were not very successful in discontinuing NSAID's although inhibitory effects on LTB₄ production and a stabilizing effect on PGI₂ and TXA₂ production during attempts at NSAID withdrawal were observed. The potential for the treatment of chronic inflammatory disease by EFA manipulation, is discussed.

The statistical analyses for this thesis were carried out using "STATGRAPHICS" computer package on an Amstrad PC-MD computer. The tests used were the Mann-Whitney U test (unpaired data) and the two-tailed paired Wilcoxon test (paired data). Correlations were carried out using Spearman rank correlation or linear regression analysis. Only 'p' values less than 0.05 were regarded as statistically significant.

CHAPTER 1

GENERAL INTRODUCTION

1.1 The Inflammatory Response

Inflammation is a complex biological process which occurs in tissues as a result of irritation and injury. The stimulus for this response may be chemical (for example, urate crystals), biological (microbial invasion) or direct physical trauma. It is primarily a defense mechanism, enabling fluid and white blood cells (leukocytes) to accumulate at the site of injury in order that the stimulus be eliminated and the tissue repaired. In certain circumstances however, inflammation can persist even though the initiating stimulus has disappeared and this can lead to tissue destruction.

The clinical features or "cardinal signs" of inflammation which were first noted by Celsus as early as 1 AD (Ryan & Majno, 1977) are redness, swelling, heat, pain and ultimately, loss of function (Table 1.1). These are the result of several underlying processes occurring in the soft tissue which can be divided into three major events:

(1) changes in vascular calibre and blood flow (hyperaemia), (2) increased vascular permeability and (3) infiltration of the tissues by polymorphonuclear leukocytes (PMN's), mainly neutrophils (Wedmore and Williams, 1981).

The two vascular changes which are the hallmarks of inflammation are thus hyperaemia and increased vascular permeability (Walter & Israel, 1979). Arteriolar relaxation, mediated mainly by locally acting biochemical factors, causes blood flow to increase and the tissues become engorged with blood, producing redness and heat. In the post capillary venule, the main site of permeability change in the inflammatory response,, dilation is accompanied by the formation of inter-endothelial cell gaps resulting in greatly increased permeability to plasma and plasma proteins (Ryan & Majno, 1977). This is responsible for the oedema seen at inflammatory loci, the resulting increase in tissue tension contributing to pain at the site (Table 1.1).

1.	REDNESS (Rubor)	-	hyperaemia
2.	HEAT (Calor)	-	hyperaemia
3.	OEDEMA (Tumor)	-	increased vascular permeability
4.	PAIN (Dolor)	-	irritation of pain sensitive nerve endings
5.	LOSS OF FUNCTION (Functio laesia)		

Table 1.1 The cardinal signs of inflammation and their underlying causes

The primary cellular response is the emigration of leukocytes, mainly PMN's, from the small blood vessels to accumulate at the site of inflammation. Thus, after an initial period of increased blood flow, the flow slows and PMN's pass to the periphery of the stream making contact with and adhering to the vascular endothelium and each other (aggregation). These marginated cells extend pseudopodia which are able to disrupt the junction between adjacent endothelial cells enabling the leukocytes to squeeze through (Walter & Israel, 1979). This migration occurs by an active process known as chemotaxis whereby the cells are attracted across a chemical gradient by factors released during the course of the reaction.

On arrival at the inflammatory locus, these PMN's actively phagocytose and digest bacteria, damaged cells and fibrin, as well as releasing further chemotactic factors to recruit more leukocytes (Ryan & Majno, 1977). During phagocytic activity, lysosomal enzymes and toxic oxygen metabolites are released into the surrounding tissue fluid where they are believed to contribute to the inflammatory process (Henson & Johnston, 1987). Furthermore, mediators produced at the inflammatory locus enter the circulation to reach the haemopoetic tissues where they stimulate the proliferation and release of further leukocytes which, in turn, are carried to the inflamed area (Ryan & Majno, 1977). This process can be detected clinically as a peripheral blood leukocytosis showing a higher proportion of immature cells.

1.2 Pathological Inflammation

The inflammatory reaction is initiated, amplified, perpetuated and finally resolved by a complex series of events in the micro-environment of tissues. This process is regulated by a variety of chemical mediators, some derived from the circulation and some produced by endogenous tissue or inflammatory cells. It is evident that such a process must be carefully regulated; if the initiating stimulus cannot be eliminated or if the response persists despite elimination of the stimulus, then, mechanisms originally designed for host defense may themselves cause disease. This is thought to be the basis

for the development of a chronic or pathological inflammation such as that seen in the rheumatoid joint (Currey, 1978).

Chronic inflammation is associated with a different cellular infiltrate than that seen in acute inflammation; mononuclear cells (lymphocytes and macrophages) are present along with the PMN's. The chronic picture is also one of more extensive tissue damage with resultant fibrosis and it may span a period of months or even years. The outcome is frequently permanent loss of function.

1.3 Inflammatory Mediators

1.3(a) General concept

Regardless of the inflammatory stimulus, the sequence of events which follow are always similar to the cardinal signs. This led to the realization that following injury, there was probably a release of endogenous substances which produced these effects.

One of the earliest mediators described was the vasoactive amine, histamine. Discovered by Barger and Dale in 1918, this was shown to be associated with anaphylactic shock (Owen, 1987). Subsequently, in 1929, Sir Thomas Lewis showed that injections of histamine could produce many of the features of acute inflammation and speculated that it could be one of the substances involved in the inflammatory process (Walter & Israel, 1979).

Since then, numerous factors, of diverse chemical nature, have been proposed as inflammatory mediators and, at present, there are around one hundred molecules which are claimed to influence some element(s) of inflammation. The following sections are a brief review of the major classes of inflammatory mediators and some of the more prominent mediators within them. Although the individual properties and characteristics of many mediators have been well elucidated, their contribution to, and precise role in specific inflammatory diseases is often not known. It is also important to realize that

mediators do not work in isolation; it is their interaction which leads to the fine-tuning of the inflammatory process and also makes its study so complex.

1.3 (b) Vasoactive amines

The most studied inflammatory amine is histamine. Formed by the decarboxylation of the amino acid histidine, it is present throughout body tissues, but is stored primarily in mast cells and circulating basophils (Owen, 1987). It is released in response to a number of stimuli including immunoglobulin E (IgE) (Austen, 1974), complement-derived factors C3a and C5a (Hook, Siraganian & Wahl, 1975) and direct physical trauma typified by the flare and weal of Lewis's triple response (Ryan & Majno, 1977).

Local administration of histamine elicits the classical vascular responses of acute inflammation namely, increased blood flow and content, increased vascular permeability and oedema formation (Owen, 1987). Although it does not have general leukocyte chemotactic activity it appears to be selectively chemotactic for eosinophils in low doses (Clark, Gallin & Kaplan, 1975).

Elucidation of the role of histamine has led to the clinical use of histamine antagonists to suppress inflammation associated with mast cell degranulation e.g. allergic rhinitis, the urticarias and asthma (Owen, 1987). However, the transient nature of the histamine response (Di Rosa, Giroud & Willoughby, 1971), makes it unlikely that it is involved in chronic inflammation.

1.3 (c) Kinins

The kinins are a family of small peptides formed in blood and body fluids by the action of the kallikreins on large, inactive protein precursors, the kininogens; the kallikreins themselves being formed from pre-kallikreins following the appropriate stimulus (Regoli, 1987). The concentration of kinins in blood therefore, is normally low.

The kinins are among the most potent naturally occurring peptides and exert a variety of biological actions *in vivo* and *in vitro*. The best known of the kinins is the nonapeptide bradykinin; this causes extravascular smooth muscle contraction, vasodilatation, increased vascular permeability and pain (Regoli, 1987). More recently, kinins have been shown to stimulate collagen synthesis and proliferation of human fibroblasts (Goldstein & Wall, 1984). Thus, evidence is accumulating that they could be involved in both the acute and chronic phases of inflammation.

1.3 (d) Complement-derived peptides

The complement system consists of about twenty different proteins which, once the system is activated, interact in a sequential and carefully regulated series of reactions. Activation of this complement "cascade" can occur via two different pathways, the classical pathway being initiated by the binding of immunoglobulin G (IgG) and immunoglobulin M (IgM) to antigen (Muller-Eberhard, 1975). However, it was discovered at a later date that other substances, typically polysaccharides such as those found in yeast cell walls, could activate the so-called "alternative" pathway (Muller-Eberhard, 1975). It was thus realized that complement could be involved in non-immune inflammation and the cascade is now known to give rise to a range of vascular mediators and chemotactic factors (Jose, 1987).

A central event in complement activation is cleavage of fragment C3 into two peptide fragments C3a and C3b and, similarly, activation of the fifth component results in the formation of C5a and C5b (Cochrane & Muller-Eberhard, 1968). Fragments C3a and C5a are both anaphylatoxins since they cause mast cell degranulation with release of histamine and stimulate smooth muscle contraction (Hook, Siraganian & Wahl, 1975). In addition, the C5 fragments have potent effects on leukocytes. *In vitro* these include stimulation of aggregation, degranulation and enzyme release, chemokinesis (random movement) and chemotaxis (Jose, 1987). *In vivo*, C5 factors promote neutrophil-endothelial adherence and increase microvascular permeability (Williams & Jose, 1981), two events which are known to be closely linked (Wedmore & Williams, 1981).

Fragment C3b is important for immune adherence and facilitates phagocytosis by opsonization of the pathogen (Jose, 1987). Furthermore, it increases oxidative metabolism and lysosomal enzyme secretion by phagocytes, thereby further enhancing their phagocytic and killing ability (Erhardt, 1983).

Complement is considered to be a potentially important mediator in chronic inflammation and complement-derived chemotactic factors have been reported in rheumatoid synovial fluids (Ward & Zvaiffler, 1971).

1.3 (e) Reactive metabolites of oxygen

PMN's and macrophages show rapid oxygen uptake when stimulated by phagocytosis and when they interact with immune complexes C5a and cytokines (Blake *et al*, 1987). This "respiratory burst" is a major source of highly reactive oxygen metabolites including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical (OH^{\cdot}) (Babior, 1978). These unstable metabolites, which are capable of causing breakdown of proteins, nucleic acids and other cell components (Blake, *et al*, 1987) are presumably important for bacterial killing as part of the normal defense mechanism. However, tissues at sites of PMN accumulation can become targets for inappropriate free radical attack and radical damage has been implicated in tissue injury during inflammation (Henson & Johnston, 1987).

The damaging potential of these species is related to their rate of production and their inactivation by enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (Dormandy, 1978). In addition, free radical "scavengers" such as vitamin E and thiol-containing compounds such as cysteine-rich proteins (e.g. penicillamine), have the potential to react with these metabolites to form a less reactive species (Blake, Allen & Lunec, 1987).

1.3 (f) Cytokines

The cytokines are a group of polypeptides secreted mainly by monocytes and macrophages (Billingham, 1987) and have recently attracted much attention as mediators of inflammation and for their role in defense mechanisms in general. Many cytokine activities which have been independently studied for years, for example, mononuclear cell factor (MCF), lymphocyte activating factor (LAF) and endogenous pyrogen (EP), have now been shown to have overlapping chemical, physical and biological properties and are believed to be due to the same molecule or family of closely related molecules (Dinarello, 1984). At a specially convened workshop in 1979 the name interleukin-1 (IL-1) was adopted to cover these activities and to distinguish it from another soluble product secreted by T-lymphocytes and named interleukin-2 (IL-2) (Aarden *et al.*, 1979). A vast literature on IL-1 has arisen in the past few years and the following review is a brief summary.

IL-1 is a 15,000 dalton polypeptide produced predominantly by mononuclear leukocytes, but also by many other cell types (Dinarello, 1984). Its synthesis requires appropriate cell activation and there are numerous stimuli including bacterial endotoxin, immune complexes and phagocytosis. The IL-1 thus secreted is made by de novo protein synthesis (Nordlund, Root & Wolff, 1970; Windle, Shin & Morrow, 1984) and has effects on a wide range of target tissues.

The list of biological properties of IL-1 is extensive (Table 1.2). It appears to be the initiating mediator in many host responses to infection and injury and many of its activities account for several aspects of the so-called "acute phase response". Thus IL-1 induces hypoferraemia, proteinuria, increased synthesis of hepatic acute phase proteins and fever (Dinarello, 1984). There is evidence to suggest that IL-1 is, in fact, more active at febrile temperatures (Duff, 1985). In addition, IL-1 has neutrophilia-inducing properties and seems likely to stimulate direct release of neutrophils from the bone marrow (Dale *et al.*, 1975).

BIOLOGICAL ACTIONS OF INTERLEUKIN-1

<p>Fever</p> <p>Hypozaemia/hypoferraemia</p>	<p>Chemotaxis of neutrophils, monocytes and lymphocytes</p>
<p>Increased:</p> <ul style="list-style-type: none"> Blood neutrophils Hepatic acute phase proteins Bone resorption Endothelial procoagulant Muscle proteolysis Slow wave sleep 	<p>Stimulation of PGE₂ in:</p> <ul style="list-style-type: none"> Hypothalamus Cerebral cortex Skeletal muscle Dermal fibroblast Synovial fibroblast Monocytes
<p>Proliferation of:</p> <ul style="list-style-type: none"> Fibroblasts Glial cells Mesangial cells 	<p>Augmentation of:</p> <ul style="list-style-type: none"> T-cell responses B-cell responses NK cells
<p>Increased:</p> <ul style="list-style-type: none"> IL-2 production IL-2 receptors Lymphokine production 	<p>Increases in neutrophil enzyme release and O₂</p> <p>Collagenase production</p>

Table 1.2 Biological activities attributed to interleukin - 1

To date, there is however, limited evidence that IL-1 can induce the vascular permeability changes of acute inflammation. In any case, the time taken for synthesis and release of IL-1 (about 6 hours) precludes its involvement in early oedema formation. It does, however, have a chemotactic effect on both PMN and mononuclear cells (Luger *et al*, 1983) and appears to enhance adherence of PMN's to vascular endothelium (Pohlman *et al.*, 1986).

IL-1 also seems to have a role in the repair processes which accompany many inflammatory and infectious diseases; these include the cleaning up of dead cells and debris and the replacement of lost tissue. Thus, the production of degradative enzymes such as collagenase is stimulated (Postlethwaite *et al*, 1983), as is bone resorption by osteoclasts (Gowen *et al*, 1983). Fibroblast proliferation and collagen production are also enhanced by IL-1 (Wahl, Wahl & McCarthy, 1978). However, these properties also give IL-1 the potential to mediate inappropriate tissue destruction in chronic inflammatory disease.

Finally, IL-1 appears also to have immuno-regulatory properties and can augment both T and B - lymphocyte responses (Maizel *et al*, 1981; Falkoff *et al*, 1983). The ability of IL-1 to stimulate the proliferation response of murine thymocytes to sub-optimal concentrations of plant lectins is still the basis for the quantitative assay of IL-1 and seems to occur indirectly via the production of IL-2 (Smith *et al*, 1980).

1.4 Arachidonic Acid as a Source of Inflammatory Mediators

The essential fatty acid (EFA), arachidonic acid (AA), is quite unique in that it serves as precursor for a plethora of often potent inflammatory mediators with wide-ranging effects, namely, the prostaglandins (PG's) and thromboxanes (TX's), (collectively known as prostanoids) and the leukotrienes (LT's). In 1980 the term "eicosanoid" was introduced (Corey *et al*, 1980) to cover all products of AA metabolism, the systemic name for AA being 5,8,11,14 eicosatetraenoic acid.

Eicosanoids are released by mammalian cells in response to physiological, pharmacological or pathological stimuli and, since they are not stored within cells, release is immediately preceded by biosynthesis. In most tissues, fatty acids are incorporated into complex lipids which perform a structural role in cell membranes. In mammals, the bulk of AA (and other EFA's) is esterified in the fatty-acyl chains of the membrane phospholipid fraction (Flower & Blackwell, 1976), almost exclusively in the 2-acyl position (Blackwell & Flower, 1983). Synthesis of eicosanoids must therefore be preceded by liberation of AA by the calcium-dependent enzyme phospholipase A₂ (PLA₂) (Flower & Blackwell, 1976), which cleaves at this position (Fig. 1.1). PLA₂ thus becomes a potentially important regulatory enzyme for the entire eicosanoid cascade. Evidence for the existence of an arachidonyl-specific phospholipase however, remains inconclusive (Irvine, 1982).

The enzyme systems for metabolism of EFA's are, in general, shared. This means that the different EFA's will compete with each other (the subject of chapter 7), (Crawford, 1983). Three naturally occurring eicosapolyenoic acids, with increasing degrees of desaturation, are substrates for conversion to eicosanoids; the trienoic (dihomo- γ -linolenic) acid, the tetraenoic (arachidonic) acid and the pentaenoic (eicosapentaenoic) acid. However, in most species, including man, AA is the commonest dietary EFA (red meat has a high content) and so its products, the dienoic PG's and the tetraenoic LT's usually dominate (Willis, 1981). An alternative source of AA is from anabolic desaturation and chain elongation of another EFA, linoleic acid, but it is now known that this conversion is severely rate-limited *in vivo* (Crawford, 1983).

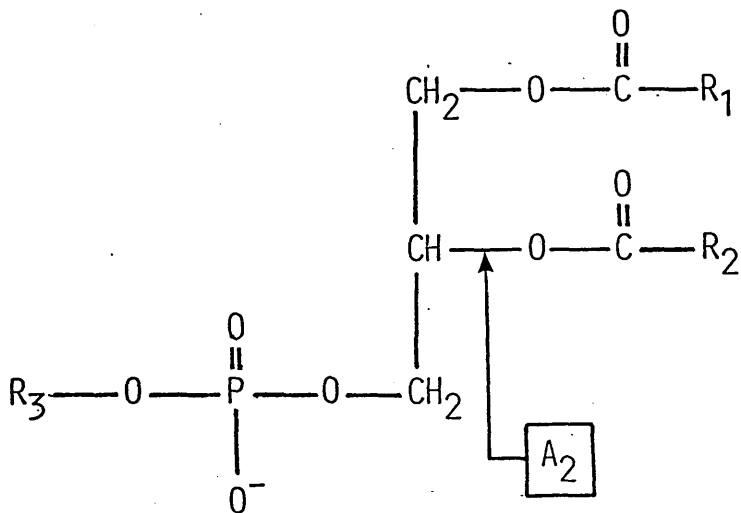


Fig 1.1 Structure of phospholipid molecule showing cleavage site of phospholipase A₂.

R₁ and R₂ - fatty acid residues
 R₃ - alcohol group

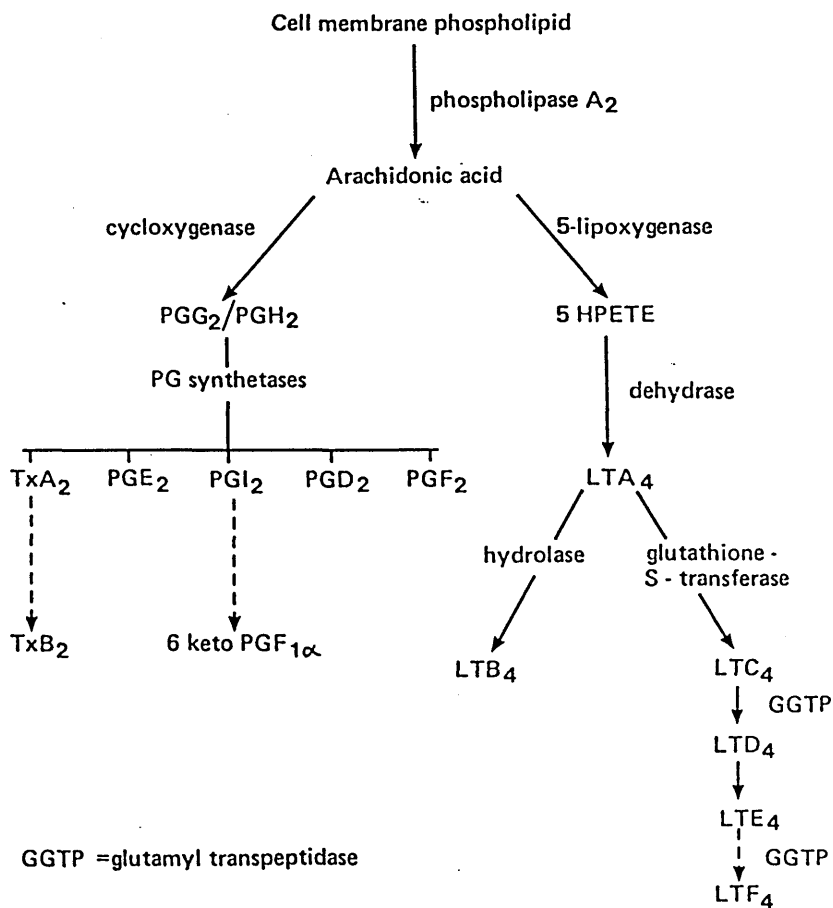


Fig. 1.2 Metabolism of arachidonic acid via the cyclo-oxygenase and 5-lipoxygenase pathways : overall scheme.

As a consequence of its predominance in mammalian biochemistry, AA and its metabolites are the most biologically significant and have therefore been the most studied. In animals, AA is metabolized by two distinct oxygenase pathways (Fig. 1.2) (Higgs *et al.*, 1981). The cyclo-oxygenase (CO), which is widely distributed in mammalian cells (Higgs & Vane, 1983) converts AA into two cyclic endoperoxides, prostaglandin G₂ (PGG₂) and prostaglandin H₂ (PGH₂), which serve as precursors for the formation of the PG's and TX's. Alternatively, AA can be metabolized by certain lipoxygenases to generate non-cyclised hydroperoxy derivatives. In the case of the 5-lipoxygenase (5-LO), the 5-hydroperoxy derivative may be further metabolized to form the LT's. Unlike the CO, the 5-LO is restricted mainly, but not exclusively, to inflammatory cells (Lewis & Austen, 1984).

The metabolites formed vary from cell to cell, depending on the distribution of the various enzymes involved. Thus, in platelets, AA is converted mainly to thromboxane A₂ (TXA₂), while in vascular endothelium, prostacyclin (PGI₂), is the main metabolite. In neutrophils, leukotriene B₄ (LTB₄) is formed via the action of the 5-LO, although CO products such as PGI₂ and TXA₂ are also formed. A more detailed description of these pathways is given in chapters 2 and 3.

It is now known that many of the products of AA metabolism are intimately involved in the inflammatory process; all the PG's, TX's and LT's play a part. However, as this thesis specifically relates to TXA₂, PGI₂ and LTB₄, emphasis has been given to these three substances in the following review.

1.5 Discovery of the Prostanoids

1.5(a) The classical prostaglandins

In 1930 Burr and Burr published their classic paper introducing the concept of the EFA's - those which had to be provided in the diet for the normal physiological functioning of animals and man. At around the same time, Kurzrok, a gynaecologist interested in artificial insemination, discovered that lipid fractions isolated from human

semen increased uterine contraction (Kurzkrok & Lieb, 1930). Unknown to the researchers at the time, these two areas of work were eventually to converge.

In 1935, Goldblatt attributed the biological activity in seminal plasma to a lipid or lipid-like substance. Von Euler (1936), who had also joined the search for the active principle, named it 'prostaglandin' supposing, wrongly, that it was produced by the prostate gland. He also reported similar activity in extracts from sheep seminal vesicles and proposed that the active component had the properties of a fatty acid.

It was some twenty years later (Bergstrom & Sjovali, 1957) that the first PG's, PGE₁ and PGF₁α were isolated from sheep vesicular gland. However, it was not until the advent of gas-liquid chromatography/mass spectrometry (GCMS) that their structure was elucidated (Bergstrom, Danielsson & Samuelsson, 1964; Van Dorp *et al*, 1964). The presence of cis double bonds in the PG's led these two groups to simultaneously suspect and subsequently confirm, with the use of radiolabelled AA, the origin of the PG's in the EFA's (Bergstrom *et al*, 1964; Van Dorp *et al*, 1964).

1.5(b) Thromboxane

In 1969 Piper and Vane demonstrated the release of a previously unknown substance from perfused guinea-pig lungs during anaphylaxis and after infusion with AA. This unstable substance was potent in causing contractions of rabbit aorta and other smooth muscle preparations and was originally known as rabbit aorta-contracting substance (RCS). Since the PG endoperoxides are also unstable and contract rabbit aorta, it was first suggested that RCS was an endoperoxide. However, this could not account for all the contractile activity observed and, moreover, the endoperoxides had a significantly longer half-life (>5 minutes) than that of RCS (<2 minutes).

These discrepancies were resolved in 1975 with the identification of an extremely labile substance released from aggregating platelets (Hamberg, Svensson & Samuelsson, 1975). This substance had a half-life of about 30 seconds at 37°C and was more active than the endoperoxides in contracting rabbit aorta. It was concluded that this

was the major constituent of RCS and, due to its ability to aggregate platelets and since its structure contained an oxane ring, it was named thromboxane. The unstable thromboxane and its stable metabolite are now known as TXA₂ and TXB₂ respectively.

1.5(c) Prostacyclin

Prostacyclin (PGI₂) was first described by Moncada and Vane (1976). The discovery arose from experiments designed to investigate the theory that damaged blood vessels might produce TXA₂ which could act synergistically with that produced by platelets. They found, however, that when microsomes from rabbit or pig aorta were incubated with PG endoperoxides an unstable substance was formed which inhibited platelet aggregation and relaxed smooth muscle, properties opposite to those of TXA₂. This substance was named PGX until its structure was elucidated (Johnson *et al*, 1976) when it was renamed prostacyclin. The discovery of PGI₂ led Moncada & Vane to speculate that this substance could be responsible for the unique ability of vascular endothelium to resist platelet adhesion and that the TXA₂/PGI₂ balance could be important for haemostasis in general (see chapter 2).

1.6 Structure and Nomenclature of the Prostanoids

The structures of some of the prostanoids are shown in Fig. 1.3. All prostanoids contain twenty carbon atoms arranged in a (bi) cyclic structure with two side chains, giving rise to the well-known "hairpin" configuration. One of these chains is terminated by a carboxyl group and the other by a methyl group which effectively means that they are cyclic fatty acids. The TX's have an oxacylohexane ring in place of the cyclopentane ring of the PG's and PGI₂ has a double ring structure.

Depending on the configuration of this cyclic part of the molecule, prostanoids are divided into various PG "families", indicated by the letters A to I, and into thromboxanes A and B. Each of these prostanoid families consists of a 1-, a 2- and a 3-series, depending on the number of double bonds in their side chains. AA gives rise to the 2-series (or dienoic) prostanoids.

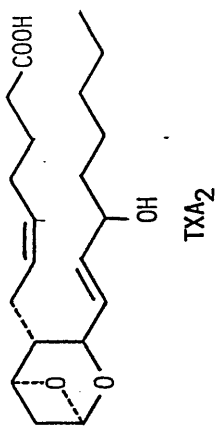
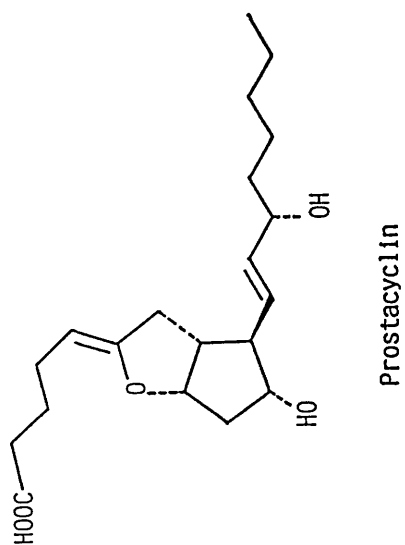
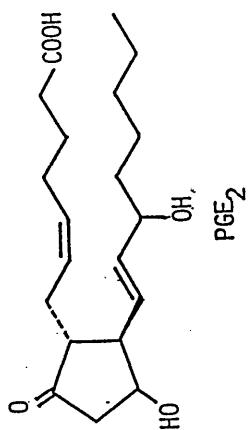
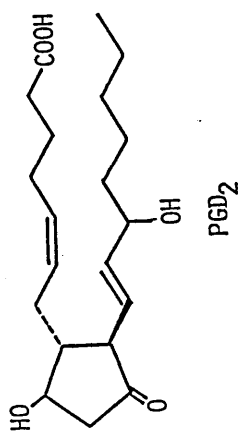
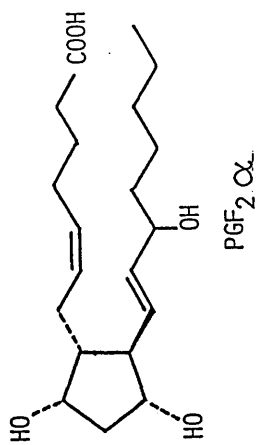


Fig. 1.3 Structures of the primary prostaglandins, prostacyclin and thromboxane A₂

1.7 Discovery of the Leukotrienes

1.7(a) Leukotriene B₄

The discovery of the LT's sprung from studies of the metabolism of AA in leukocytes. In 1976, Samuelsson and colleagues incubated ¹⁴C-labelled AA with rabbit peritoneal PMN's and found that the major metabolite formed was a new lipoxygenase product namely, 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) (Borgeat, Hamburg & Samuelsson, 1976). Further studies with rabbit PMN's showed that a number of other metabolites were formed, one of which was 5(S)-12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid (5,12 di-HETE) (Borgeat & Samuelsson, 1979a). At the same time, work with human peripheral blood PMN's showed a similar profile of metabolites and, furthermore, the biosynthesis of the 5,12 di-HETE was shown to increase dramatically when the cells were treated with the divalent cation ionophore A23187 (Borgeat & Samuelsson, 1979b).

Mechanistic studies and analysis of the stereochemistry involved, led to the hypothesis that the 5,12 di-HETE was formed via an unstable epoxide intermediate; the structure 5,6 oxido-7,9,11,14-eicosatetraenoic acid was proposed and confirmed by chemical synthesis (Borgeat & Samuelsson, 1979c). The 5,12 di-HETE and its epoxide precursor were later to be known as LTB₄ and leukotriene A₄ (LTA₄), respectively (Fig. 1.2) (Samuelsson & Hammarstrom, 1980).

In 1980, a newly discovered chemotactic and cell-aggregating substance generated by PMN's on stimulation with A23187 was shown to be identical to LTB₄ (Ford-Hutchison *et al*, 1980) and has since been shown to possess a wide range of biological activities (see chapter 3).

1.7(b) Leukotrienes C₄, D₄, E₄ and F₄

These LT's constitute a separate class from that of LTB₄. They are, in fact, peptido-LT's, having one or more amino acids linked to their FA chains via a thioether

bond at C-6. Various mixtures of these LT's are now known to make up the so-called slow-reacting substance of anaphylaxis (SRS-A).

SRS-A has been recognized as a biological activity since the work of Feldberg & Kellaway in 1938, who identified it as a smooth muscle contracting factor secreted by perfused guinea-pig lung treated with cobra venom. It is now thought that SRS-A has an important role in asthma and other types of immediate hypersensitivity reactions (Morris *et al*, 1980).

Structural elucidation of SRS-A had been limited due to lack of pure preparations. However, it had been characterized using ultraviolet (UV) absorption as a sulphur-containing polar lipid and radio-labelling experiments had suggested that it was derived from AA. In addition, the ionophore A23187 stimulated release of SRS-A from leukocytes. These observations led Samuelsson and colleagues to hypothesize that there was a relationship between SRS-A and the lipoxygenase products formed from AA in leukocytes (Samuelsson, 1983). Using mouse mastocytoma cells, which they had found to be a good source of SRS-A and a combination of chemical derivatization, high performance liquid chromatography (HPLC), UV spectroscopy and amino acid sequence analysis, they were able to make the first complete structural determination of SRS-A (Murphy, Hammarstrom & Samuelsson, 1979; Hammarstrom *et al*, 1979). The structure of SRS-A from mouse mastocytoma cells was 5-hydroxy-6-S-glutathionyl-7,9,11,14 eicosatetraenoic acid, glutathione being a tripeptide consisting of cysteine, glycine and glutamic acid. This substance is now known as leukotriene C₄ (LTC₄) (Samuelsson & Hammarstrom, 1980).

In the same year, Morris *et al* (1980) identified the corresponding cysteinylglycine derivative, later termed leukotriene D₄ (LTD₄), as the SRS product from immunologically challenged guinea-pig lung. They also showed that the structure determined was identical to that obtained non-immunologically from rat basophil leukaemia (RBL-1) cells. The cysteinyl derivative, termed leukotriene E₄ (LTE₄) was identified by Lewis *et al* (1980a) (Fig 1.2).

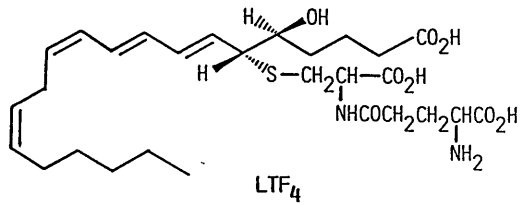
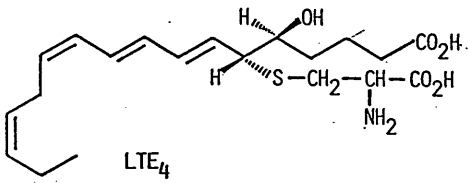
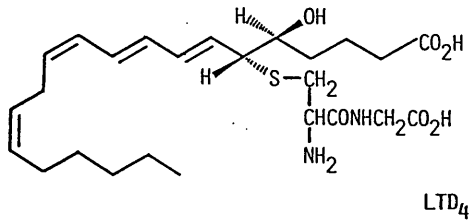
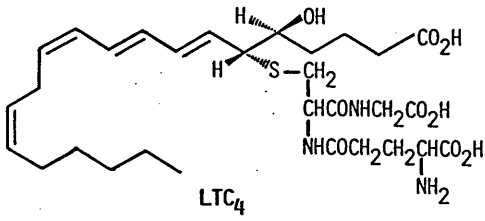
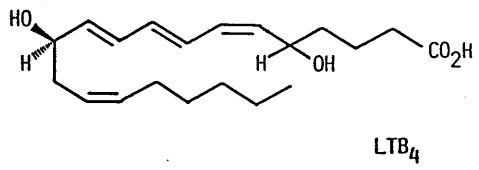
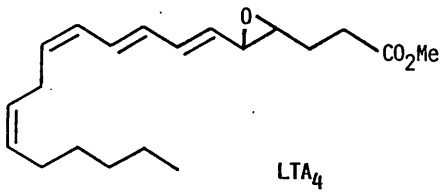


Fig. 1.4 Structures of the leukotrienes

The cysteine-containing LT's are now known to occur in a variety of different biological samples in various mixtures (Samuelsson, 1983) and the original premise that the unstable epoxide intermediate LTA₄ is also the precursor of LTC₄, has been confirmed (Hammarstrom & Samuelsson, 1980). More recently, it has been found that LTE₄ can function as an acceptor of γ -glutamic acid forming a γ -glutamyl cysteinyl derivative, named leukotriene F₄ (LTF₄) (Anderson, Allison & Meister, 1982).

1.8 Structure and Nomenclature of the Leukotrienes

The term "leukotriene" was first introduced in 1979 for compounds which are non-cyclized, C-20 carboxylic acids with one or two oxygen substituents and three conjugated double bonds (a conjugated triene) (Samuelsson *et al*, 1979). It was suggested at the same time that structurally different members of this group be distinguished by the letters A, B, C *etc.* LT's C to F have one or more amino acids linked via a thioether bond as described in the previous section.

Since other eicosapolyenoic acids (besides AA) are transformed into analogous structures, subscript numbers were introduced to denote the number of double bonds in the molecule (Samuelsson & Hammarstrom, 1980). AA gives rise to the 4-series or tetraenoic LT's, containing three conjugated double bonds and one isolated double bond (Fig 1.4). These compounds are now known, therefore, as LTB₄, C₄ and so on.

CHAPTER 2

THE PROSTANOIDS

2.1 Biosynthesis

The first step in the biosynthesis of the prostanoids from AA is the formation, by the CO enzyme system, of two short-lived intermediaries, the endoperoxides, PGG₂ and PGH₂. The initial CO transformation is the insertion of two molecules of oxygen into the fatty acid chain forming the bicyclic endoperoxide PGG₂ (Bakhle, 1983). However, many CO preparations contain a peroxidase activity which rapidly converts PGG₂ to PGH₂ by cleavage of the C-15 hydroperoxy group to form a hydroxyl group (Bakhle, 1983). This two step process was proved by Hamberg *et al.*, (1974) with the isolation, from platelets, of the two endoperoxides. The activity of the CO is dependent on the provision of non-esterified AA by the enzyme PLA₂. It has also been proposed that CO activity is sustained by the continual presence of low levels of lipid peroxide which act as an enzyme "promoter" (Warso & Lands, 1983).

The two endoperoxides thus formed by the CO serve as substrate for subsequent transformations, via various synthetase enzymes to the primary PG's (PGD₂, PGE₂, PGF_{2α}), PGI₂ and TXA₂ (Fig. 2.1). PGF_{2α} however, is suspected to be a non-enzymatic product (Bakhle, 1983). PGI₂ and TXA₂ are unstable and are rapidly converted to the stable 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}) and TXB₂ respectively (Johnson *et al.*, 1976; Hamberg *et al.*, 1975); these are the metabolites most commonly used as indices of PGI₂ and TXA₂ production. The endoperoxides can also be metabolized to a 17-carbon hydroxy acid (HHT) with the concomitant release of malondialdehyde (MDA).

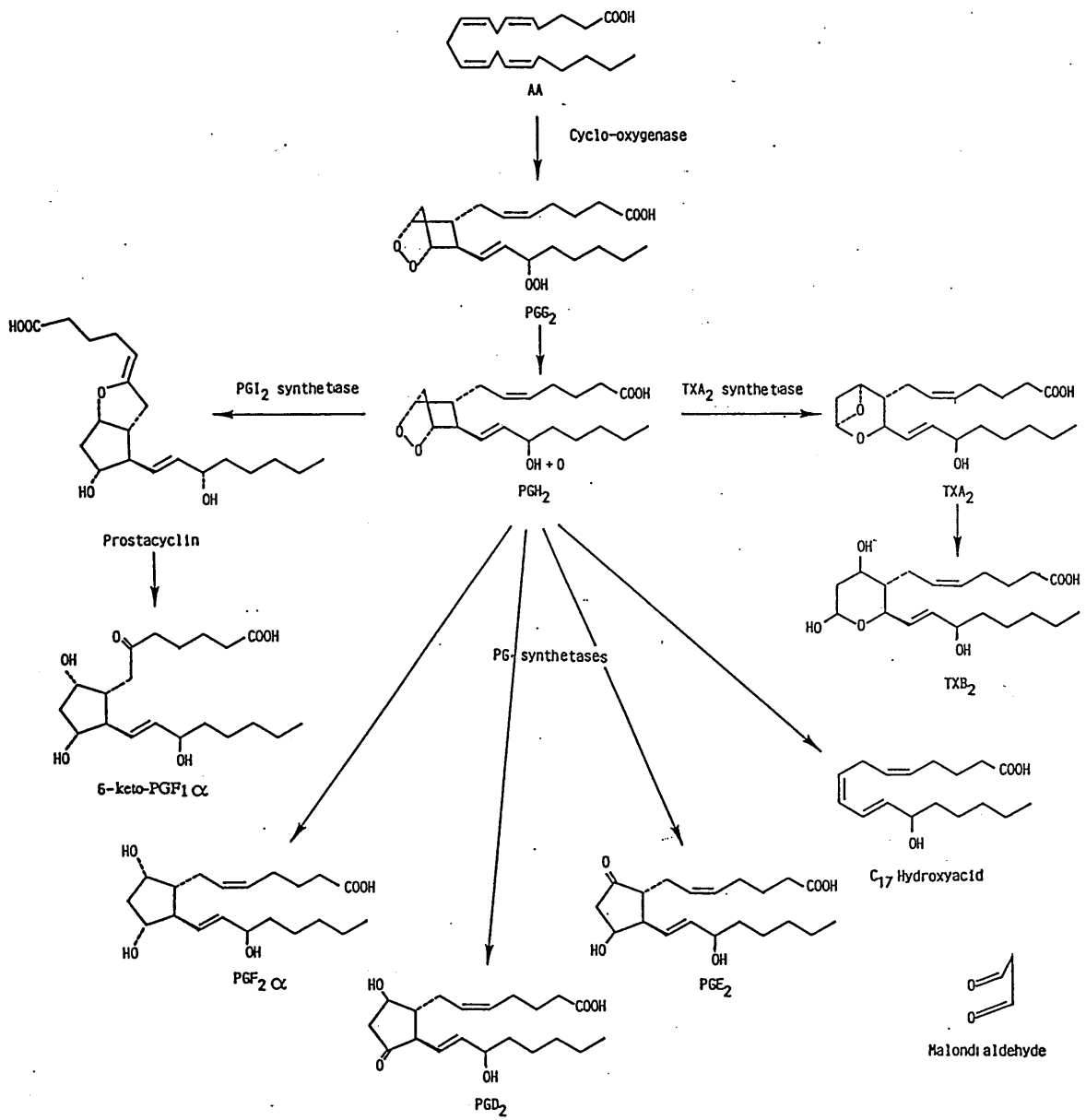


Fig. 2.1 Metabolism of arachidonic acid via the cyclo-oxygenase pathway : detailed structures and enzymes.

2.2 Sites of Synthesis

The CO enzyme seems to be ubiquitous, PG's being synthesized in every mammalian tissue with the exception of red blood cells (Samuelsson *et al.*, 1978; Sun, Chapman & McGuire, 1977). Subcellularly, CO is located in the microsomes (Samuelsson *et al.*, 1978). In most biological systems, more than one PG together with TXA₂ is the normal mixture of CO products, the exact composition of the mixture depending on cell or tissue type and species. The best example is the synthesis of PGI₂ by vascular endothelium and TXA₂ by platelets. PGI₂ is the main CO product in all vascular preparations so far tested (Samuelsson *et al.*, 1978). In contrast, platelets do not contain PGI₂ synthetase and are therefore incapable of producing PGI₂ (Bakhle, 1983). It should be noted however, that some vascular preparations such as human umbilical artery (Tuvemo, 1980) and cultured bovine endothelial cells (Ingerman-Wojenski *et al.*, 1981) can produce TXA₂.

Species differences also exist. In rat, rabbit and human lung the major CO product is PGI₂ (Alabaster, 1980; Al-Ubaidi & Bakhle, 1980) whereas in guinea-pig lung it is TXA₂ (Alabaster, 1980). Clearly, therefore, work done on animal models (guinea-pig lung has been used most frequently to study pulmonary metabolism of AA) cannot always be extrapolated to the human situation. Moreover, the profile of CO products generated in tissues is not rigid and in some cases can be changed by simply altering the substrate concentration. A low substrate concentration seems to favour production of PGI₂, whereas a high substrate concentration favours synthesis of TXA₂ (Sun *et al.*, 1977; She *et al.*, 1981).

All the major immuno-inflammatory cells (PMN's, monocytes, macrophages and lymphocytes) appear to be able to synthesize prostanoids although macrophages appear to be the major source (Humes *et al.*, 1977; Morley *et al.*, 1979). The most predominant products generated are TXA₂ and PGE₂ (Morley *et al.*, 1979). PGI₂ has been detected in macrophages and mast cells, but does not seem to be a significant product in circulating leukocytes. Stimuli for the release of PG's from leukocytes are

many and include phagocytosis, cytokines and complement (Humes *et al.*, 1977; Conti *et al.*, 1986; Clancy & Hugli, 1985).

2.3 Metabolism

The metabolism of CO products is usually through further oxidation and almost always results in marked loss of biological activity (Crutchley & Piper, 1975). The main deactivation enzymes are 15-hydroxyprostaglandin dehydrogenase (PGDH) and Δ^{13} prostaglandin reductase which catalyse the formation of dihydro-PG's (Bakhle, 1983). PGDH is an intracellular enzyme and extracellular substrate must pass across the cell membrane before inactivation can occur (Bakhle, 1983). PG metabolism is rapid, more than 90% of PGE₂ and PGF₂ α being inactivated in a single passage through the pulmonary circulation (Ferreira & Vane, 1967).

PGI₂ is a short-lived metabolite with a half-life of 2-3 minutes at physiological temperature and pH (Dusting *et al.*, 1978a), being rapidly hydrolysed to 6-keto-PGF₁ α . However, local biological inactivation by PGDH or Δ^{13} PG reductase may be a mechanism *in vivo*, the metabolites formed again being considerably less active than PGI₂ (Whittle & Moncada, 1983). The exception is that formed in hepatic tissue, 6-keto-PGE₁, which possesses anti-aggregatory activity almost equipotent with that of PGI₂ (Quilley *et al.*, 1980).

In contrast to the primary PG's PGI₂ is not inactivated in the lungs, since it is not a substrate for the membrane transfer process (Dusting *et al.*, 1978b). Furthermore, the lung has been found by some workers to continuously release PGI₂ into the arterial circulation (Gryglewski, Korbut & Ocetkiewicz, 1978; Hensby *et al.*, 1979). This, together with the lack of pulmonary inactivation, led to the suggestion that PGI₂ is a circulating hormone (Moncada *et al.*, 1978). However, other workers failed to obtain evidence of pulmonary production (Smith *et al.*, 1978; Pace-Asciak *et al.*, 1980) and in addition, Blair *et al.* (1982) have suggested that human plasma levels of PGI₂ are too low for it to function as a circulating hormone. It is now generally accepted that PGI₂ falls

instead into the category of "local hormone", being synthesized and inactivated in the same tissue or group of cells.

TXA₂ is also short-lived, having a half-life at body pH and temperature of around 30 seconds (Hamberg, Svensson & Samuelsson, 1975). Hydrolysis to the stable and inactive TXB₂ occurs spontaneously, but may be enzyme assisted *in vivo* (Bakhle, 1983). In man, β -oxidation is the main mechanism for TXB₂ degradation, 2,3-dinor-TXB₂ being the major urinary metabolite (Roberts *et al.*, 1981).

2.4 Inhibition by Aspirin

A key discovery in PG research was the finding in 1971 that drugs such as aspirin and indomethacin, which had been used empirically as anti-inflammatory drugs for many years, could selectively inhibit PG synthesis (Vane; Smith & Willis; Ferreira, Moncada & Vane). It was therefore proposed that this was the mode of action of these compounds. The mechanism of inhibition by aspirin was later shown to be the covalent acetylation of a lysine residue at the active site of the CO enzyme (Roth *et al.*, 1978). This means that aspirin has a longer lasting effect on platelets (which cannot synthesize new enzyme) than on endothelial cells, which have been shown to quickly recover their CO activity after treatment with low dose aspirin (Jaffe & Weksler, 1979). The link between aspirin and the PG's has also provided a useful research tool to help elucidate the role of PG's *in vivo*.

2.5 Biological Actions

2.5 (a) Development of the cardinal signs of inflammation.

Over the years, a substantial amount of evidence has arisen to support a role for the PG's in the development and maintenance of the signs and symptoms of inflammation. They have been demonstrated to have potent vasodilatory and hyperalgesic actions and, moreover, have been detected at inflammatory loci in sufficient concentration to cause these effects.

The vascular elements of the inflammatory response are hyperaemia and increased permeability producing redness and oedema. The E-type PG's are powerful vasodilators and give a long-lasting erythema following intra-dermal injection into human skin (Solomon, Juhlin & Kirschenbaum, 1968). PGI₂ is also a potent vasodilator with comparable potency to PGE₂ (Williams, 1979). Although PGE₂ and PGI₂ increase blood flow, PG's by themselves are poor inducers of oedema (Williams, 1979). However, they have been shown to potentiate the oedema-producing activity of bradykinin and histamine (Williams & Peck, 1977; Williams & Morley, 1973). Since these increase vascular permeability by contracting venular endothelial cells (Williams, 1983), their activity is greatly enhanced by vasodilators such as PGI₂. In addition, PG's can synergise with other mediators of increased permeability such as complement fragment C5a, to cause oedema (Williams & Jose, 1981). However, C5a has a different mechanism of action for inducing increased vascular permeability and although the exact mechanism is not known, it requires the presence of PMN's. It has been shown that increased permeability cannot be induced by C5a in animals depleted of circulating PMN's, but that responses to histamine and bradykinin remain unchanged (Wedmore & Williams, 1981).

The role of PG's in the provocation of pain also involves mediator synergism; PG's by themselves do not cause overt pain. The application of PGE₁ to a blister base (Horton, 1963) or the intradermal injection of PGE's or PGF's, both in human skin (Crunkhorn & Willis, 1971) merely induced an area which was hyperalgesic i.e. sensitive to touch. However, Ferreira (1972) found that if PGE₁ was given before sub-dermal infusion of histamine or bradykinin, overt pain resulted. Histamine and bradykinin together could not reproduce this effect, nor could they potentiate the subsequent infusion of PGE₁. Ferreira suggests that the sensitization of afferent pain receptors by PG's is responsible for this pain enhancement.

More recently, PGI₂ has also been shown to invoke hyperalgesia, being approximately five times more potent than PGE₂ in producing increased sensitivity

following injection into rat paws (Higgs, Moncada & Vane, 1978a). However, in contrast to the effects of the stable PG's which produce a cumulative, long-lasting hyperalgesia, the effect of PGI₂ is short-lived (Ferreira, Nakamura & de Abreu Castro, 1978).

Inhibition of synthesis of PG's such as PGE₂ and PGI₂ thus accounts for the reduction in erythema, oedema and hyperalgesia seen with the aspirin-like drugs (Moncada, Ferreira & Vane, 1973). In addition, because the PG's potentiate the actions of other mediators such as histamine and bradykinin, their actions are attenuated also.

Another characteristic of acute inflammation in which PG's are involved is elevated temperature, or fever. In this respect also, the PGE's are the most active. PGE₂ is a potent pyrogenic agent and elevated levels of PGE₂ have been detected in the cerebrospinal fluid (CSF) taken from pyrexia patients (Saxena *et al.*, 1979). Production of PGE₂ is thought to account for the fever induced by IL-1 (endogenous pyrogen) and some anti-pyretics appear to work by preventing PG synthesis (Dinarello & Wolff, 1982).

2.5 (b) Effect on Polymorphonuclear Leukocytes

Since one of the earliest responses to tissue injury is the influx of PMN's, the effect of AA metabolites on PMN function has received considerable attention. Reports of the effects of CO products on PMN chemotaxis have often been conflicting. However, on the whole, any effects which have been observed are weak and species specific. TXB₂ is chemotactic for mouse PMN's, but not for human cells (Kitchen, Boot & Dawson, 1978). PGE₁ is mildly chemotactic for rabbit PMN's (Kaley & Weiner, 1971) and PGF₂α, although not itself chemotactic, enhances the chemotactic responsiveness of PMN's to other stimuli (Diaz-Perez, Goldyne & Winkelmann, 1976). PGE₂ actually inhibits both chemotaxis and chemokinesis of human PMN's (Goetzl & Gorman, 1978).

It can be seen therefore, that the effects of PG's on cell movement *in vitro* are varied. Moreover, inhibition of PG production does not correlate with a reduction of leukocyte migration *in vivo* (Higgs *et al.*, 1981). Indeed, in contrast to the pro-inflammatory effects of PG's on development of the vascular responses of inflammation, their effects on leukocytes seem to be mainly inhibitory. It has therefore been suggested that the PG's could have a negative feedback role in chronic inflammation, initially aiding the development of the cardinal signs followed by a later suppressant effect (Bonta & Parnham, 1978a).

Many reports in the early 1970's indicated that the intra-cellular concentration of adenosine 3', 5'-monophosphate (cyclic AMP; cAMP) influences leukocyte function, an elevation in cAMP having a generally inhibitory effect (Bourne *et al.*, 1974). It has been suggested therefore, that this is the mechanism whereby E-type PG's suppress PMN activity and indeed, some of the effects of the PGE's can be mimicked by treating the cells with exogenous cAMP (Rivkin, Rosenblatt & Becker, 1975). PGI₂ is a particularly potent stimulator of adenylate cyclase (the enzyme catalyzing the formation of cAMP) at low concentration (Gorman, Bunting & Miller, 1977; Tateson, Moncada & Vane, 1977) and has been shown to prevent PMN chemotaxis *in vitro*, also at low concentration (Weksler, Knapp & Jaffe, 1977). In addition, PGI₂ prevents margination and adherence of PMN's in hamster cheek pouch venules (Higgs, Moncada & Vane, 1978b) and *in vitro* reduces PMN adherence to nylon fibres and cultured endothelial cells (Boxer *et al.*, 1980). Total leukocyte numbers in inflammatory exudates are at their highest when PGI₂ production is at it's lowest (Higgs & Salmon, 1979), suggesting that PGI₂ may be the most likely CO product to modulate leukocyte function.

Lysosomal enzyme release is yet another PMN function which appears to be inhibited by the PGE's and PGI₂ via an increase in cAMP (Weissmann, Dukor & Zurier, 1971). PGE₁ appears to inhibit the release of these potentially damaging enzymes both during phagocytosis and during PMN adherence to non-phagocytosable surfaces such as cartilage (Lewis, 1983). Since the PGE's and PGI₂ are found in inflammatory sites in

high enough concentration to elicit these effects, they may well modulate lysosomal enzyme release *in vivo*.

2.5 (c) Effects on Lymphocytes

A characteristic of chronic inflammation is the involvement of several different types of white blood cells including PMN's, macrophages and lymphocytes. In RA, for example, both B lymphocytes (producing antibodies) and T lymphocytes (involved in cell-mediated immune mechanisms) have been implicated in the pathogenesis.

As with PMN's, the effects of the PGE's and PGI₂ on lymphocytes is mainly inhibitory. *In vitro*, the PGE's and PGI₂ inhibit PHA-stimulated mitogenesis in lymphocytes (Smith, Steiner & Parker, 1971; Gordon, Henderson & Westwick, 1979). The E-type PG's are also recognized as inhibitors of cytokine secretion and indomethacin has been shown to enhance antigen-induced cytokine production from T-cells (Gordon, Bray & Morley, 1976).

There has been some controversy in the literature as to whether the relatively high doses of PG's used to achieve inhibition of lymphocyte function *in vitro* are physiologically unrealistic. As reflected by their concentration in inflammatory exudates however, PG's are produced locally in high concentration and may well therefore, have inhibitory effects on lymphocytes *in vivo*. In contrast, PGI₂ at high concentration may elevate cyclic guanosine monophosphate (cGMP) rather than cAMP which would result in stimulatory effects (Gordon *et al.*, 1979).

Knowledge of the PG-mediated regulation of B-lymphocyte function is limited. Some experiments indicate that certain concentrations of E-type PG's will inhibit generation of IgG and IgM antibodies (Zurier & Quagliata, 1971) while at "lower" doses they may stimulate production (Staite & Panayi, 1984).

2.6 PGI₂/TXA₂ Interaction

2.6 (a) General Concept

Since the discovery of PGI₂ and TXA₂, evidence has accumulated that these CO products, rather than the classical PG's, may be of physiological and pathological importance. TXA₂, produced by platelets, is a strong contractor of large blood vessels and induces platelet aggregation (Hamberg *et al.*, 1975). PGI₂, produced by vascular endothelium, is a strong vasodilator and the most potent inhibitor of platelet aggregation known (Bunting *et al.*, 1976). Indeed, these two compounds appear to have directly opposing actions in many systems (Table 2.1) and the PGI₂/TXA₂ ratio is thought to be a potential control mechanism for both normal haemostasis and the development of some disease states. In particular, the PGI₂/TXA₂ balance has been implicated as an important factor in the development of various thrombotic conditions in which increased platelet aggregability plays a role (see section 2.6 (f)).

PROSTACYCLIN	THROMBOXANE A₂
Anti-aggregatory	Pro-aggregatory
Vasodilator	Vasoconstrictor
Bronchodilator	Bronchoconstrictor
Cytoprotective	Cytodestructive

Table 2.1 Opposing actions of prostacyclin and thromboxane A₂

THEORY OF MONCADA & VANE

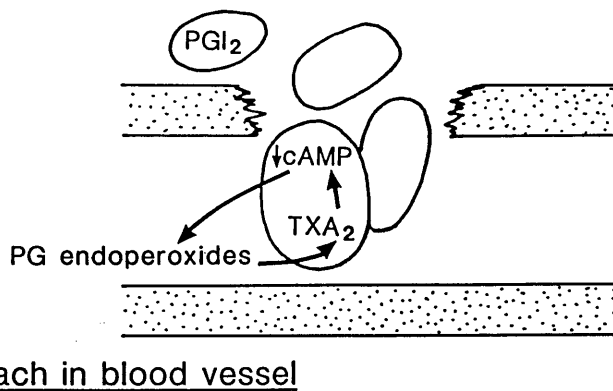
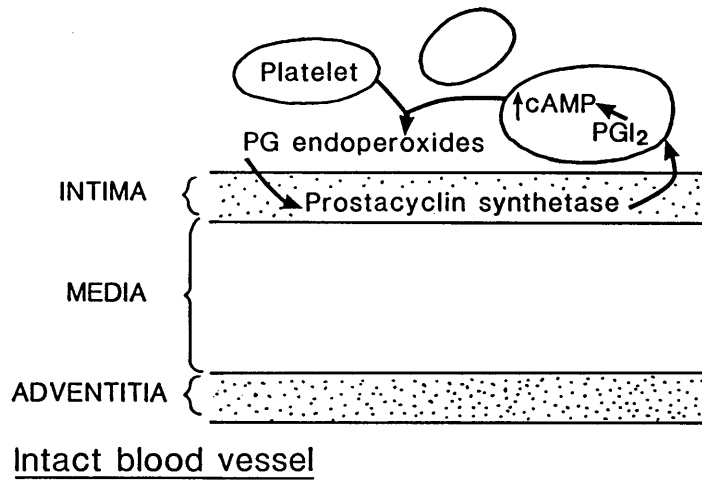


Fig. 2.2 Theory of Moncada and Vane.

2.6 (b) Effect on Platelets

In 1975, Hamberg *et al.* proposed that the newly discovered TXA₂, rather than the endoperoxides, was the platelet aggregating material contained in 'RCS'. TXA₂ is now known to be the main AA metabolite which mediates platelet aggregation and release. It should be noted however, that TXA₂-induced aggregation represents only one mechanism of platelet aggregation. Other stimulants include ADP, thrombin and collagen (de Gaetano, Bertele & Cerletti, 1987) each of which may be relevant in different *in vivo* situations.

In contrast, PGI₂ is the most potent platelet anti-aggregating agent described, being more active than either PGE₁ or PGD₂ (Gryglewski *et al.*, 1976). In addition, PGI₂ is capable of disaggregating platelet clumps in plasma, *in vitro* (Bunting *et al.*, 1976) and on collagen strips (Gryglewski *et al.*, 1978).

Moncada *et al.* (1976) have proposed that the balance between TXA₂ synthesis in platelets and PGI₂ synthesis by vascular endothelium is an important mechanism in haemostasis and that PGI₂ enables arteries to resist platelet adhesion and hence, thrombus formation (Fig. 2.2). It has been demonstrated that endothelial cells can, in fact, utilize PG endoperoxides from platelets for PGI₂ synthesis (Bunting *et al.*, 1976). However, this mechanism is probably not relevant under normal circumstances although it may play a role when the vessel wall is damaged (Needleman *et al.*, 1978).

2.6 (c) Effect on vascular tone

As with platelet function, the directly opposing vasoactive properties of TXA₂ and PGI₂ are relevant to the understanding of vascular tone and blood flow both in normal and pathological situations.

TXA₂ is highly unstable and its vascular actions can be difficult to study. It is therefore usually generated when required by rapid incubation of PGH₂ with platelet microsomes (Moncada *et al.*, 1976). TXA₂ generated in this way has been shown to

contract numerous isolated arteries including rabbit aorta, rabbit coronary arteries, human umbilical artery and bovine coronary artery (Whittle & Moncada, 1983a). Studies on perfused vascular beds *in vivo* (e.g. perfused canine mesenteric and femoral vascular beds) have demonstrated that TXA₂ causes an immediate and short-lasting vasoconstriction (Dusting *et al.*, 1978).

In contrast, PGI₂ relaxes most isolated vascular strips including rabbit coeliac and mesenteric arteries, bovine coronary artery, human and baboon cerebral arteries and lamb ductus arteriosus (Moncada & Vane, 1978). It is also a potent vasodilator in isolated perfused hearts of guinea-pig, rabbit and rat (Moncada & Vane, 1978). Because of its strong vasodilatory activity in many microvascular beds, PGI₂ is thought to be involved in the control of local blood flow and in the hyperaemic responses of tissues such as the gastric mucosa [see section 2.6 (e)]. The vasodilatory activity of PGI₂ is also evident in the pulmonary and renal circulations (Moncada & Vane, 1978).

2.6 (d) Effect on the bronchopulmonary system

TXA₂ readily contracts guinea-pig airway smooth muscle *in vitro* and following intravenous administration to the anaesthetised guinea-pig (Svensson *et al.*, 1977). Intravenous administration of a thromboxane synthetase inhibitor or thromboxane receptor antagonist in guinea-pig inhibits bronchoconstriction induced by AA or bradykinin, but not histamine or antigen-induced constriction (Greenberg, Antonaccio & Steinbacher, 1982). TX's also appear to mediate a substantial component of the bronchoconstrictor action of SRS-A in guinea-pig lung (Piper & Samhoun, 1981). In human lungs however, TXA₂ does not appear to be a significant CO product and although most work has been done on guinea-pig lung, this may not be a good model for pulmonary AA metabolism in humans (Al-Ubaidi & Bakhle, 1980).

PGI₂ is potentially able to oppose the actions of TXA₂ in the bronchopulmonary system both by its actions on the pulmonary vasculature and bronchial smooth

muscle since it has been shown to relax isolated strips of tracheal, bronchial and bronchiolar smooth muscle (Whittle & Moncada, 1983a).

2.6 (e) Cytoprotection

PGI₂ and its analogues have the ability to protect the gastro-intestinal tract from damage induced by noxious agents, typically the non-steroidal anti-inflammatory drugs (NSAID's). Although PGI₂ and its analogues are potent inhibitors of gastric acid secretion (Whittle, 1980), the anti-ulcer properties are separate since certain PG's at doses which have minimal anti-secretory activity can still protect in the rat stomach (Robert *et al.*, 1979). The term "cytoprotection" has thus been introduced to describe this protective role.

The mechanism of action of this effect is not precisely known. However, it seems to be related to the vasodilatory action of certain PG's in the gastric mucosa. Loss of this action following CO inhibition may lead to areas of local ischaemia which would then have lowered resistance to gastric acid and digestive enzymes leading potentially to ulcer formation (Whittle, 1980). The inhibition of gastric CO by NSAID's correlates closely with their ulcerogenicity (Whittle *et al.*, 1980).

These effects are, again, in contrast to those of TXA₂ which is pro-ulcerogenic. TXA₂ generated in gastric arterial blood *in situ* in the dog is a potent vasoconstrictor and, in the presence of a weak topical irritant such as taurocholate (a bile salt), causes extensive mucosal damage and ulceration within 30 minutes exposure (Whittle, Kauffman & Moncada, 1981). Likewise, the TX mimetic, epoxy-methano endoperoxide analogue, is a potent gastric vasoconstrictor and pro-ulcerogenic agent in the dog (Whittle & Moncada, 1983b).

Although the cytoprotective effect of PGI₂ in the gastric mucosa is the best documented, other areas are being explored. For example, in experimental canine liver transplantation, storage with PGI₂ has been shown to help prevent ischaemic damage (Monden & Fortner, 1982). Other studies, with platelets, indicate that PGI₂ stabilizes

cell membrane phospholipids and prolongs viability during storage (Blackwell *et al.*, 1982).

2.6 (f) PGI₂/TXA₂ Balance in pathological conditions

Several diseases have now been related to an imbalance in the PGI₂/TXA₂ ratio. In general, in diseases where there is a tendency for thrombosis to develop, TXA₂ is raised or PGI₂ is decreased or both. The opposite is found in diseases associated with an increased bleeding tendency. However, this classical picture is not always observed and in some types of vascular disease where inadequate production of PGI₂ might have been predicted, increased production has been found (see section 5.1).

In 1977 Lagarde and Dechavanne showed that platelets from patients with arterial thrombosis, deep vein thrombosis or recurrent venous thrombosis produce more PG endoperoxides and have a shortened survival time compared to normal platelets. Shimamoto *et al.* (1978) have found that platelets from rabbits made atherosclerotic produce more TXA₂ and are more sensitive to aggregating agents than controls. Similar observations have been made in platelets from patients who have survived myocardial infarction (Szczeklik *et al.*, 1978). Hirsh *et al.* (1981) have reported an increase in coronary sinus levels of TXA₂ in patients studied within 24 hours of ischaemic chest pain (angina), although it is not clear whether this is cause or effect.

Diabetes, a disease associated with formation of microvascular thromboemboli, has also been studied. Harrison, Reece & Johnson (1978) have reported reduced PGI₂ release from the aortas of diabetic rats. PGI₂ production from arteries of diabetic patients (obtained at operation) has also been measured and is said to be lowered compared to non-diabetic controls (Johnson *et al.*, 1979). In addition, Dollery *et al.* (1979) have reported that circulating levels of 6-keto-PGF_{1a} are reduced in diabetic patients with proliferative retinopathy. However, there is some controversy in this area and other workers have shown increased circulating levels of 6-keto-PGF_{1a} (McLaren, 1986) and decreased plasma TXB₂ in diabetics (McLaren, 1986; Carter & Jones, 1986).

In uraemic patients, PGI₂ production from blood vessels is increased (Remuzzi *et al.*, 1977). This is attributed to an increase in the uncharacterized "plasma factor" which stimulates vascular PGI₂ synthesis and is postulated to contribute to the bleeding tendency in these patients (MacIntyre, Pearson & Gordon, 1978). Conversely, a deficiency in this PGI₂-stimulating factor has been reported in haemolytic uraemic syndrome which is characterized by widespread thrombotic occlusions (Remuzzi *et al.*, 1978a).

Defreyn *et al.* (1981) have shown a link between a familial bleeding tendency and a partial deficiency of platelet thromboxane synthetase. The prolonged bleeding time in this case seems to have occurred due to a combination of decreased TXA₂ formation and increased PGI₂ formation.

The PGI₂/TXA₂ balance also appears to have significance in various pathological conditions of pregnancy. For instance, reduced umbilical and placental vascular PGI₂ has been observed in patients with severe pre-eclampsia (Remuzzi *et al.*, 1980) and Greer *et al.* (1985) found that maternal plasma PGI₂-M levels dropped to unrecordable levels as pregnancy-induced hypertension developed.

2.7 Interaction with other Inflammatory Mediators

2.7 (a) Introduction

Many interactions between PG's and other substances have been observed and several are believed to be important in the context of the inflammatory response. The synergism between the PG's and histamine, bradykinin and C5a and its role in the potentiation of oedema and pain has already been described in section 2.5(a). Interaction of the PG's with LT's is discussed in chapter 3.

2.7 (b) Prostaglandins and cyclic AMP

Specific binding of PG's to various cells and tissues has been correlated with the activation of adenylate cyclase and an increase in cAMP (Samuelsson *et al.*, 1978).

Many PG effects appear to be mediated by cAMP, particularly in the case of the PGE's and PGI₂ (Lewis, 1983). In turn, cAMP is able to stimulate biosynthesis of PGE which seems to suggest a positive feedback system. The "stop" signal in this system may be the desensitization of adenylate cyclase to specific agonists (Samuelsson *et al*, 1978). Many PG effects appear to be mediated by cAMP, particularly in the case of the PGE's and PGI₂ (Lewis, 1983). However, PGE actions on cell function (e.g. haemopoietic stem cell proliferation) which do not appear to be accompanied by alteration in cAMP levels have also been described (Feher & Gidali, 1974).

In platelets, inhibition of aggregation by PGI₂ has been correlated with an activation of adenylate cyclase leading to a rise in intracellular cAMP. As the most potent anti-aggregator of platelets, PGI₂ is also the most potent endogenous stimulator of adenylate cyclase (Gorman, Bunting & Miller, 1977; Tateson, Moncada & Vane, 1977) It is thought that elevated cAMP levels reduce platelet cytoplasmic calcium concentration (by stimulating removal of calcium by the dense tubular membrane system) upon which platelet shape change, aggregation and release reaction depend (Kaser-Glanzmann *et al.*, 1977).

Conversely, TXA₂ and PGH₂ reduce platelet intracellular cAMP levels, but only when these have already been raised by an adenylate cyclase stimulant such as PGE₁ (Miller, Johnson & Gorman, 1977). Basal levels of cAMP are not lowered by TXA₂.

It has also been suggested that cAMP is able to inhibit the availability of endogenous AA to CO in cultured cells (Hassid, 1982), providing a possible mechanism for negative feedback control of PGI₂ synthesis.

2.7 (c) Prostaglandins and interleukin - 1

The exact nature of the mechanism of action of IL-1 is unclear. However, it does seem to be involved with membrane perturbation and the release of AA. Chang, Gilman and Lewis (1986) have shown that IL-1 activates PLA₂ in rabbit chondrocytes and that this correlates with an increase in PGE₂ production. Similarly, Bernheim and

Dinarello (1985) have shown that IL-1 can stimulate the release of PGE₂ from human fibroblasts and Rossi *et al.*, (1985) have demonstrated an IL-1 induced production of PGI₂ in endothelial and vascular smooth muscle cells. Release of TXB₂ by human PMN's and rabbit peritoneal macrophages in response to IL-1 has also been demonstrated (Conti *et al.*, 1986).

The role of CO- derived metabolites of AA in the regulation of IL-1 production has also been examined. Kunkel and Chensue (1986) have shown that indomethacin causes a dose-dependent increase in IL-1 production in lipopolysaccharide (LPS) - stimulated murine peritoneal macrophages. In addition, a direct suppression of IL-1 was observed when exogenous PGE₂ was added.

These findings suggest a model in which the PG's may act as regulators of IL-1 production by a negative feedback mechanism. Furthermore, since macrophages produce both PG's and IL-1, they may, conceivably, control lymphocyte function by regulating their IL-1 production via the PG's.

2.7 (d) Prostaglandins and reactive metabolites of oxygen

Oxygen radicals are produced by the AA pathway, especially during the PGG₂ peroxidase step. The free radical product of this reaction, probably OH[·], appears to inactivate the CO and may therefore provide an important negative feedback role for PG synthesis (Blake *et al.*, 1987). In turn, the oxidative products of free radical reaction with lipids, lipid peroxides, are necessary at low level to activate the CO and are therefore promoters of PG production (Warso & Lands, 1983). In addition, peroxides are important in influencing whether PGI₂ or TXA₂ will predominate; high peroxide levels selectively enhance TXA₂ production by their ability to inactivate PGI₂ synthetase (Warso & Lands, 1983).

It has recently been demonstrated however, that the PGE's and PGI₂ are able to inhibit radical production in stimulated neutrophils (Szezeklik *et al.*, 1987), again

supporting the hypothesis that the PG's are initially pro-inflammatory, allowing increased radical production with later anti-inflammatory, free radical scavenging effects.

2.8 Prostanoid Receptors

Since the various prostanoids have distinct biological actions which are often observed at very low (nanomolar) concentrations, it has been hypothesized that they act via specific cell-surface receptors. As well as being specific, binding of prostanoid to receptor should be saturable, reversible and comparable in time-course and concentration to the effect produced. Using an experimental strategy of ligand-binding measurements, ranking of agonist potencies and the use of specific antagonists, many such receptors for the PG's in various tissues have now been at least partially characterized (Samuelsson *et al.*, 1975). The work carried out on PGI₂ receptors is probably that most relevant to this thesis.

The stimulation of adenylate cyclase by PGI₂ suggests that it interacts with a specific, membrane-bound receptor and attempts have been made to characterize those via which PGI₂ elicits its anti-aggregatory and vasodilatory effects. Siegl *et al.*, (1979) have identified selective binding sites of ³H-PGI₂ on human platelets. This binding was observed to be rapid (80% of PGI₂ bound in 1 minute) and is comparable to the time taken for cAMP elevation and aggregation. In accordance with other workers (Schillinger & Prior, 1980), Siegl *et al.*, have identified two distinct sub-sets of PGI₂ receptor; a small number of high affinity sites and a larger number of lower affinity sites. It has been suggested that binding to the lower affinity shows more correlation with cAMP elevation and is more likely to be the pharmacological receptor (Siegl, 1982). The relative abilities of PGI₂, PGE₁, PGE₂ and 6-keto-PGF_{1α} to displace ³H-PGI₂ reflects their potency as inhibitors of platelet aggregation *in vitro* (Siegl *et al.*, 1979). PGI₂, PGE₁ and PGE₂ appear, therefore, to share a common receptor. The exception is PGD₂ which seems to elicit its anti-aggregatory effect via a distinct receptor (Siegl, 1982).

Town *et al.*, (1982) have attempted to identify the receptors which mediate the vasodilatory effects of PGI₂ using bovine coronary arteries and the stable PGI₂ analogue, iloprost. Results are similar to those found with platelets - two receptor subsets, one high affinity and one low affinity have been identified. The dissociation constant for the high affinity site is within the concentration range in which PGI₂ and iloprost relax coronary arteries *in vitro*.

2.9 The role of the Prostanoids in Inflammatory Disease

The finding that the PG's were involved in the development of the signs and symptoms of inflammation and the discovery that anti-inflammatory drugs worked by inhibiting PG formation, established a role for them in acute inflammation. However, there have also been reports of anti-inflammatory actions for PG's, for instance, infusion of PGE₁ appears to prevent and suppress adjuvant arthritis in rats (Zurier & Quagliata, 1971) and PGE₂ inhibits cotton pellet granuloma formation in rats (Di Pasquale *et al.*, 1973). In addition, in EFA-deficient rats, where PG production is markedly reduced, the acute phase of inflammation is attenuated, but the chronic component (tissue proliferation) is enhanced (Bonta, Parnham & Adolfs, 1977). This suggests that although elevated PG levels would initially aid the development of the cardinal signs, they could, by their later suppressant effects on cellular function, help to terminate the inflammatory response. A breakdown in this mechanism could conceivably lead to the development of chronic inflammation.

The presence of PG's has been demonstrated in a wide variety of inflammatory exudates. Initially, the most likely source of PG's is the injured tissue itself, but as inflammation proceeds, infiltrating cells, especially macrophages, become an important source. However, the PG concentration in inflammatory exudates does not always increase in parallel with the accumulation of leukocytes and, in addition, there is no simple correlation between the severity of the inflammation and PG levels (Bonta & Parnham, 1978).

In 1969 Willis reported the presence of PGE₂ in carrageenin-induced exudate from the rat. Much of the early work was concerned with detection of the stable PG's, but it is now clear that PGI₂ and TXA₂ are also generated at inflammatory sites. Comparable concentrations of TXB₂ and 6-keto-PGF₁α have since been detected in carrageenin-induced exudates in the rat (Higgs & Salmon, 1979).

Inflammatory joint disease, in particular RA, has been used extensively as a model of chronic inflammatory disease; joint inflammation seems to involve most of the mechanisms which have been proposed on the basis of experimental studies and its clinical course is fairly easy to follow. Many animal models of arthritis have been studied with respect to PG production. Dumonde and Glynn (1962) developed a model resembling RA by injecting antigen into a joint in sensitized rabbits. Using this model, Blackham *et al.*, (1974) showed that levels of PGE in the synovium reached a peak after 19 hours, declining again over a chronic phase of 46 days. Floman and Zor (1976) showed that synovial tissue from rats with a laboratory-induced mono-arthritis released 5-8 times as much PGE as synovium from non-arthritic rats.

Several studies have demonstrated the presence of PG's in synovial fluid withdrawn from the joints of arthritic patients; PGE₂ appears to be the most prominent although PGE₁, PGF₂α and TXB₂ have also been detected (Sturge *et al.*, 1978; Trang, Granstrom & Lovgren, 1977). Again, however, the PG content of synovial fluid does not appear to correlate with leukocyte numbers in the synovium, nor does it seem to parallel the clinical course of RA (Bonta & Parnham, 1978). Moreover, measurement in synovial fluid is subject to error due to the necessarily invasive technique used for collection.

PG production by cultured synovial tissue has also been measured (Sturge *et al.*, 1978; Dayer *et al.*, 1976). PGE₂ and PGF₂a have both been detected and Dayer *et al.*, suggest that the main cellular source is most likely to be infiltrating macrophages or phagocytic type-A synovial lining cells which have macrophage-like properties. Bitensky *et al.*, (1981) have looked at the production of PGE₂, 6-keto-PGF₁α and TXB₂ from

both rheumatoid and non-rheumatoid synovia. However, only the TXB₂ production was significantly greater in rheumatoid tissue.

Two important aspects of chronic inflammatory joint disease are destruction of cartilage and resorption of bone. Cartilage consists mainly of glycosaminoglycan, proteoglycan and some collagen, while bone consists of collagen fibres in a mineral matrix (Lewis, 1983). Both tissues therefore, are affected by levels of the enzyme collagenase which plays a major role in the degradation of articular cartilage and other joint structures (Dayer *et al.*, 1976). Wahl *et al.*, (1977) have demonstrated that indomethacin inhibits endotoxin-stimulated collagen production from macrophages in culture and that this can be reversed by the addition of exogenous PGE₁ or PGE₂.

There are many other human inflammatory diseases besides RA, in which PG's are thought to have a role. In psoriasis for example, levels of free AA, PGE₂ and PGF_{1α} have been shown to be significantly increased in lesional epidermis compared to un-involved epidermis (Hammarstrom *et al.*, 1975). In the case of ulcerative colitis, PGE₂ levels in the rectal mucosa of patients, is double that found in normal controls (Sharon *et al.*, 1978). In most cases however, it is far from clear whether these increased levels are cause or consequence of the disease.

2.10 Inhibition of the Prostanoids and Potential for Anti-Inflammatory Therapy

2.10 (a) Introduction

There has been a search for many years for drugs which inhibit PG's or antagonize their actions. All the enzymatic conversions in the AA cascade are, in theory, potential points for drug intervention (Fig. 2.3). Presently, however, the non-steroid anti-inflammatory drugs (NSAID's) and the corticosteroids are the two main classes of drugs which are used in treatment of chronic inflammatory diseases, in particular RA .

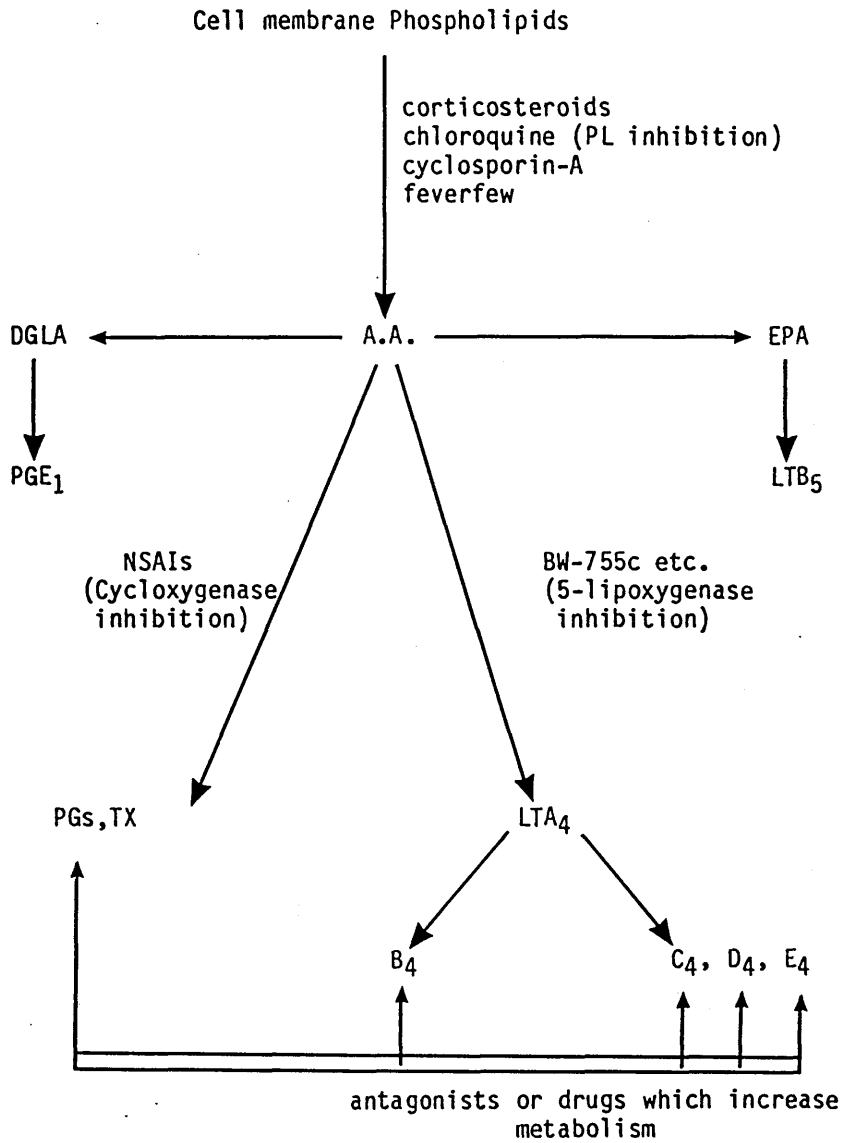


Fig. 2.3 Therapeutic manipulation of the arachidonic acid cascade.

2.10 (b) The non-steroidal anti-inflammatory drugs

The discovery that several NSAID's inhibit the formation of endogenous PG's provided a plausible explanation for their anti-inflammatory action. It is now known that these drugs selectively inhibit the CO enzyme (see section 2.4). Drugs such as indomethacin and aspirin have both analgesic and anti-inflammatory properties and in general there is good correlation between the order of potency of PG inhibitors and their anti-inflammatory effect (Higgs & Flower, 1981). However, NSAID's only provide symptomatic relief and do not reverse the underlying disease process in chronic conditions.

Since leukocytes are a major source of PG's in the early stages of inflammation, drugs which inhibit leukocyte accumulation should help to reduce PG levels in inflammatory exudates. There have been conflicting reports on the effects of NSAID's on leukocyte accumulation, but in general, drugs such as indomethacin and the salicylates enhance leukocyte migration at low doses but inhibit at high doses (Higgs *et al.*, 1981). These observations can be explained by diversion of AA substrate towards the chemotactic LO products (see chapter 3) at low doses and a non-specific inhibition of AA peroxidation at high doses. Substrate diversion to the LT's may also explain why NSAID's do not seem to modify the disease process in inflammation; analgesia and decreased oedema would result from diminished PG levels but increased LT production would allow the more chronic PMN-mediated components of inflammation to proceed unchecked.

2.10 (c) The corticosteroids

In contrast to the NSAID's, the anti-inflammatory properties of the corticosteroids (e.g. hydrocortisone, dexamethasone) cannot be explained by the inhibition of CO or any of the other PG-generating enzymes. Instead, these appear to work by preventing the release of AA from the cell membrane via the inhibition of PLA₂ and so do not work in cell-free systems (Blackwell *et al.*, 1978; Nijkamp *et al.*, 1976).

The inhibition itself appears to occur indirectly via the action of intra-cellular proteins synthesized and released after binding of the corticosteroid to specific cell membrane receptors (Blackwell *et al.*, 1980). Such inhibitory proteins have been isolated from perfused guinea-pig lung and rat peritoneal macrophages (macrocortin) and similarly from rabbit peritoneal neutrophils (lipomodulin) (Blackwell & Flower, 1983).

Effectively then, corticosteroids act as dual inhibitors, blocking both the CO and LO pathways, both of which depend on the provision of AA substrate. This explains why they are generally better anti-inflammatory agents than the NSAID's, although the advantage is off-set by the fact that they also produce more serious side effects. Experimental dual CO and LO inhibitors such as BW755C are presently being tested as anti-inflammatory agents (Salmon, Simmons & Moncada, 1983). It is hoped that these will have the steroid-like therapeutic activity (PG inhibition without potentiation of leukocyte migration) without the toxic side-effects normally associated with steroid treatment.

CHAPTER 3

THE LEUKOTRIENES

3.1 Biosynthesis

The leukotrienes (LT's) are a family of biologically active compounds derived from AA via the action of a 5-lipoxygenase (5-LO) enzyme (Fig. 3.1). In the first step, AA undergoes hydroperoxidation by the 5-LO to form a 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) (Taylor & Morris, 1983). This intermediate may be reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) or alternatively may be converted by a dehydrase to the highly unstable 5,6 epoxide intermediate, LTA₄ (Borgeat & Samuelsson, 1979c). Subsequently, LTA₄ may be hydrolysed enzymatically by epoxide hydrolase to yield the 5,12-dihydroxy-eicosatetraenoic acid (5,12-di-HETE), LTB₄ (Borgeat & Samuelsson, 1979c); this is the rate-limiting step for the pathway (Jakschik & Kuo, 1983). Alternatively, non-enzymatic hydrolysis of LTA₄ generates the less biologically active isomers of LTB₄, namely the 5,6-di-HETE's and the 6-trans LTB₄ diastereoisomers (Lewis & Austen, 1984), (Fig. 3.1).

LTA₄ is also the precursor for the formation of the cysteinyl-LT's. Addition of glutathione via glutathione-S-transferase yields LTC₄ (Murphy, Hammarstrom & Samuelsson, 1979), then, upon hydrolysis of its peptide side chain by γ -glutamyl transferase (γ -GT), LTC₄ is converted to LTD₄. Further metabolism by a dipeptidase cleaves glycine from the side chain to form LTE₄ which then has a single amino acid residue (cysteine) at C-6 (Lewis and Austen, 1984). In the presence of glutathione, LTE₄ can act as an acceptor of γ -glutamic acid to form the γ -glutamyl cysteinyl derivative, LTF₄ (Anderson *et al*, 1982). However, LTF₄ has not yet been detected in biological fluids.

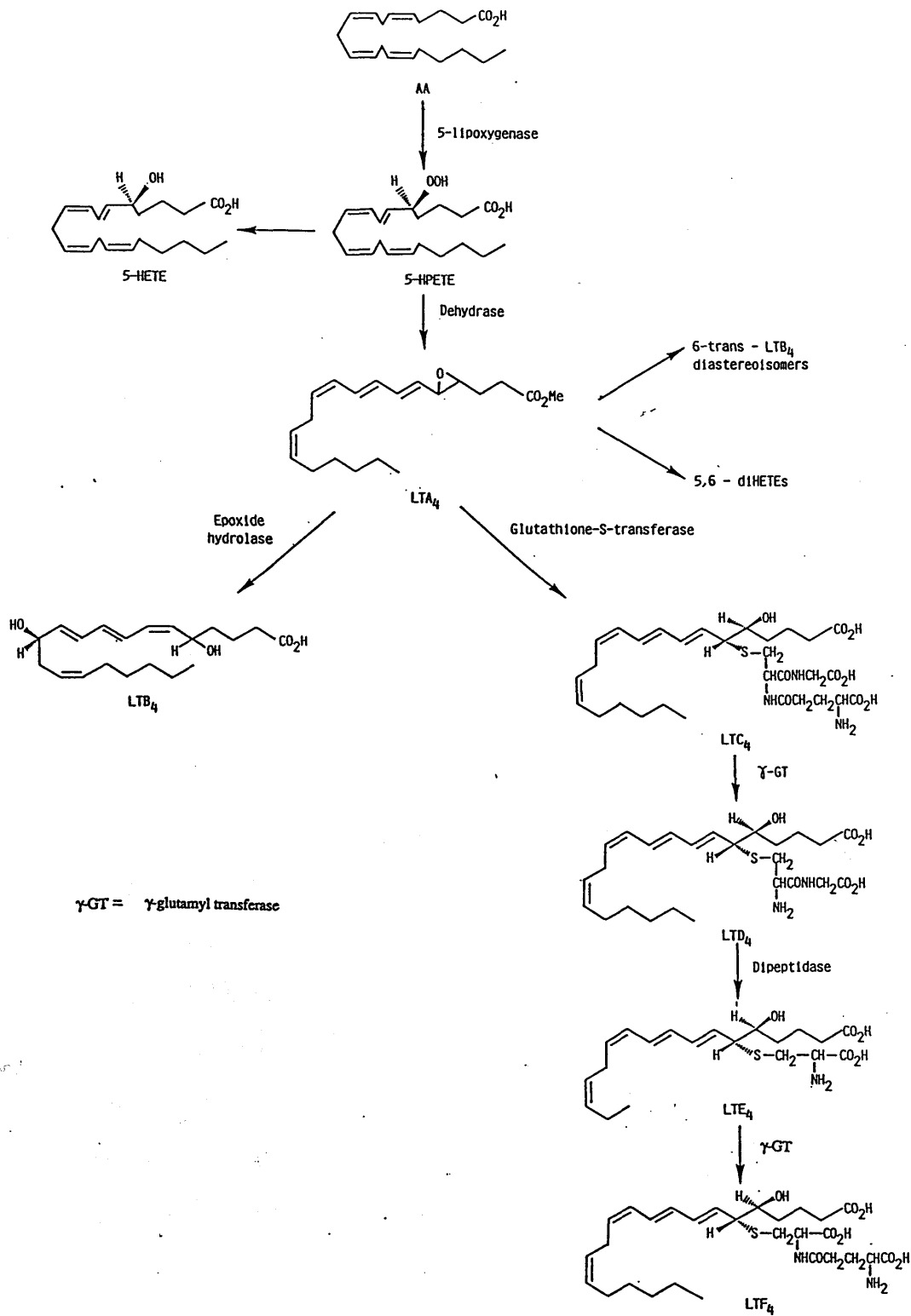


Fig. 3.1 Metabolism of arachidonic acid via the 5-lipoxygenase pathway: detailed structures and enzymes.

3.2 Sites of Synthesis

Whereas the metabolism of AA to CO products is characteristic of most cell types, the 5-lipoxygenation of AA appears to be less widely distributed but is associated with all types of leukocytes. Cells such as PMN's, monocytes and mast cells from several species release relatively large amounts of LT's when appropriately stimulated (Lewis & Austen, 1984). However, there are distinct differences in the profile of LT's formed in individual classes of leukocyte. For example, in peripheral blood PMN's (mainly neutrophils) the amount of LTB₄ produced by a given stimulus is some ten times greater than that of LTC₄. In eosinophils however, the ratio is reversed and LTC₄ is the major LT product (Lewis & Austen, 1984). In addition, there are many leukocytes in which a 12-lipoxygenase or 15-lipoxygenase is quantitatively more important (Brash, Murray & Oates, 1985). These enzymes lead to the formation of compounds such as 12-HETE and 15-HETE which may also be important in inflammation, but are largely outside the scope of this thesis.

Many other tissue types have the potential to generate LT's, including human and guinea-pig lung and guinea-pig pulmonary and coronary arteries (Piper, 1985). Piper & Galton (1984) have found that the main source of LT's from stimulated porcine pulmonary artery is the adventitia. Johnson *et al* (1985) have reported that endothelial cells do not appear to synthesise LT's, although they are able to metabolize them. Similarly, erythrocytes, which were thought to be metabolically inert in terms of eicosanoid synthesis, have been shown to transform LTA₄ into LTB₄ (Fitzpatrick *et al*, 1984).

The subcellular localization of enzymes in the LT pathway is quite complex and involves movement of intermediates between cell compartments. In RBL-1 cells for example, all the enzymes leading to the formation of LTB₄ from AA are found in the cytosol whereas the enzymes converting LTA₄ to LTC₄, D₄ and E₄ are located in the particulate fraction (Jakschik & Kuo, 1983). The exact subcellular locations have not been conclusively determined, but in a variety of cell types the γ -glutamyl transpeptidase

which converts LTC₄ to LTD₄ seems to be located in the plasma membrane (Anderson, Allison & Meister, 1982) and the dipeptidase converting LTD₄ to LTE₄ appears to be associated with the specific granules in human PMN's (Lee *et al*, 1983a). The organization of enzymes therefore, means that AA, after it is released from phospholipids, can either be converted to the prostanoids via the CO, a microsomal enzyme or to 5-HPETE by the cytosolic 5-LO. Similarly, the LTA₄ intermediate can either be a substrate for the soluble hydrolase to form LTB₄ or the particulate glutathione-S-transferase to form LTC₄. The factors controlling the fate of substrate are not entirely understood but this may be partly dependent on the nature of the stimulus causing cell activation.

3.3 Stimulation of Leukotriene Biosynthesis

Leukotrienes are not normally stored within cells but are synthesised and released after activation of the pathway by a variety of stimuli. The 5-LO is a calcium-dependent enzyme and for LT synthesis to occur therefore, there is not only a requirement for AA to be released, but also for the 5-LO to be activated (Parker & Aykent, 1982; Ochi *et al*, 1983). Hence, addition of AA to leukocytes is not usually sufficient to stimulate LT synthesis and a stimulus such as the calcium ionophore A23187 is required to activate the pathway (Borgeat & Samuelsson, 1979b). This requirement for activation of 5-LO means that the LT pathway can be inhibited either by direct inhibition of the enzyme or by agents which block activation.

Much of the *in vitro* data on LTB₄ generation have come from leukocytes stimulated with A23187. This agent is a microbial-derived carboxylic acid which is able to transport divalent cations across both cellular and intra-cellular membranes (Reed & Hardy, 1972). In leukocytes, therefore, it increases the cytosolic calcium concentration by causing an influx of extracellular calcium and also by mobilising intracellular stores of calcium.

Although A23187 is a potent stimulus for release of LT's and is often used as a research tool, it is, however, non-physiological. Other, more physiological stimuli can be used, for example, the synthetic analogue of a bacterial chemotactic peptide, N-formyl-Met Leu Phe (FMLP), the complement anaphylatoxins C3a and C5a, the phagocytic stimulus zymosan and various antigens (Bray, 1983). However, the amounts of LT produced are much less than with A23187 and indeed, the generation of LT's by many tissues would have remained elusive had A23187 not been used. It should be noted that in contrast to A23187 which activates both the PLA₂ and the 5-LO, FMLP and C5a do not induce the mobilisation of AA and only stimulate LT biosynthesis when exogenous AA is provided (Clancy, Dahinden & Hugli, 1983).

3.4 Metabolism

3.4 (a) Leukotriene B₄

The neutrophil-dependent inactivation of LTB₄ is an intra-cellular process and occurs via ω -oxidation to yield 20-OH-LTB₄ and subsequently, 20-COOH-LTB₄ (Ford-Hutchinson *et al*, 1983). This process is not observed with eosinophils or peripheral blood monocytes in adherent monolayers (Lewis & Austen, 1984). Although the 20-COOH metabolite is virtually inactive, 20-OH-LTB₄ retains significant biological activity, especially with respect to smooth muscle contraction (Ford-Hutchinson *et al*, 1983). Salmon, Simmons & Palmer (1982b) have shown that LTB₄, in the presence of human PMN's, has a half-life of 25 minutes at 37°C. However, initial rapid binding of LTB₄ to the cells, leading to loss of biological activity in the supernatant, occurs within 5-10 minutes.

3.4 (b) Leukotrienes, C₄, D₄ and E₄

The processing of LTC₄ to LTD₄ and LTE₄ by peptide cleavage represents the conversion from one active mediator to another and is not an inactivation process. However, human PMN's stimulated with phorbol myristate acetate (PMA) metabolize LTC₄, D₄ and E₄ to a mixture of biologically less active and inactive compounds by an

oxidative pathway involving hydrogen peroxide (H₂O₂) and myelo-peroxidase; hypochlorous acid (HOCl) appears to be the ultimate effector species (Lee *et al*, 1983b). Each of the three LT's gives rise to a pair of 6-trans LTB₄ diastereoisomers and it's corresponding sulphoxide derivative, also present as a pair of diastereoisomers. A third pair of minor metabolites remains unidentified (Lee *et al*, 1983b).

3.5 Biological Actions of Leukotriene B₄

3.5 (a) Effects on leukocytes

As described in chapter 1, the ability of PMN's to detect, move towards and accumulate at a site of tissue injury is an important phenomenon in the inflammatory response. This is the process in which LTB₄ seems to exert its most potent biological actions (Table 3.1) and upto 95% of the chemokinetic, chemotactic and aggregatory activity from ionophore-stimulated PMN's can be ascribed to LTB₄ (Ford-Hutchinson *et al*, 1980). Furthermore, the stereochemistry of the LTB₄ molecule is of biological importance since the two 6-trans-LTB₄ diastereoisomers, produced non-enzymatically, have considerably reduced activity compared to LTB₄ (Ford-Hutchinson *et al*, 1981).

LTB ₄	LTC ₄ , D ₄ , E ₄
PMN : Chemokinesis Chemotaxis Adherence Aggregation Degranulation Exudation of plasma Smooth muscle contraction	 Exudation of plasma Smooth muscle contraction (eg bronchoconstriction, vasoconstriction)

Table 3.1 Biological actions of the leukotrienes

As a chemokinetic and chemotactic agent LTB₄ has comparable activity (on a molar basis) to that observed for other known chemotactic factors such as C5a and FMLP (Bray, 1983). It is around 100-1000 times more active than any of the mono-HETE's (Smith, Ford-Hutchinson & Bray, 1980) and *in vitro* appears to be chemotactic for both PMN's and mononuclear cells from several species (Palmer *et al*, 1980). Like other chemotactic substances, LTB₄ appears to have the ability to enhance the expression of complement receptors on human neutrophils (Nagy *et al*, 1982).

Adhesion of PMN's to vascular endothelium is a pre-requisite for their translocation from the microcirculation into the extravascular space and their subsequent accumulation at the inflammatory site. Gimbrone, Brock & Schafer (1984) examined the effects of various LO products of AA on PMN adhesion to endothelial cells in culture and found LTB₄ to be the most effective. The endothelial cells used included those cultured from both arteries and veins of several species. LTB₄ is also a PMN-aggregating agent and induces aggregation to a greater degree and at lower concentration (10⁻¹⁰M), than any other LO product (Ringertz *et al*, 1982).

In addition to its chemotactic and aggregatory properties, LTB₄ stimulates release of lysosomal enzymes from PMN's (Feinmark *et al*, 1981). However it is relatively weak as a degranulating agent, being considerably less potent than FMLP in this respect.

There is some speculation that LTB₄ could also act as an endogenous calcium ionophore in leukocytes, giving it a potentially important role in the control of calcium-dependent cell functions (Serhan *et al*, 1982). However, this hypothesis has not been conclusively proved.

The *in vivo* actions of LTB₄ have been shown to reflect those *in vitro*. Injection of LTB₄ into the peritoneal cavity of guinea-pigs stimulates an influx of PMN's (and to a lesser extent eosinophils and macrophages) which continues upto 5 hours after treatment (Smith, Ford-Hutchinson & Bray, 1980). Extravascular accumulation of

leukocytes on application of LTB₄ has also been studied using hamster cheek pouch preparations (Bray, Ford-Hutchinson & Smith, 1981). Within 2 minutes, a reversible reduction in the number of rolling PMN's and a corresponding increase in the number of cells sticking to the vascular endothelium was observed which was followed by an increase in extravascular cell accumulation.

Leukocyte accumulation on stimulation with LTB₄ has also been observed in the anterior chamber of rabbit eye (Bhattacharjee *et al*, 1981) and human skin (Lewis *et al*, 1981). The doses of LTB₄ required for cell accumulation are relatively high (0.1-1.0 µg) but are still within physiological ranges and the effects appear to last for several hours.

3.5 (b) Effects on the microcirculation

In parallel with its ability to stimulate cell accumulation, LTB₄ also causes an increase in vascular permeability in rabbit, rat, guinea-pig and hamster (Bray, 1983). However, although LTB₄ alone has only a weak effect on plasma exudation, the response is markedly enhanced by the presence of a vasodilator such as PGE₂ (Wedmore & Williams, 1981). Furthermore, LTB₄-induced plasma leakage appears to require adhesion of PMN's to the vessel wall. This requirement has been demonstrated using animals depleted of PMN's (Wedmore & Williams, 1981) and Bjork *et al* (1983) have shown that leakage of plasma after LTB₄ application is always preceded by a marked adhesion of cells to the vessel walls. The mechanism by which adhering PMN's increase vascular permeability is not known but CO products appear not to be required since indomethacin, an inhibitor of CO, has no effect (Bjork *et al*, 1983).

3.5 (c) Effects on smooth muscle

LTB₄ contracts several smooth muscle tissues *in vitro* including guinea-pig duodenum, ileum, aorta and trachea and rat stomach and ileum (Sirois, Borgeat & Jeanson, 1981). However, in general it's effects on smooth muscle are not nearly so potent as those of the other LT's. In the case of guinea-pig lung parenchymal strips (an

in vitro preparation of peripheral airways) it does seem to be equipotent with LTC₄ and LTD₄ in causing contraction (Piper & Samhoun, 1982). However, Piper & Samhoun have also shown that this activity is blocked by pretreatment of the tissue with indomethacin and that LTB₄-induced contraction is in fact, mediated by TXA₂.

3.6 Biological Actions of Leukotrienes C₄, D₄ and E₄

3.6 (a) Effects on smooth muscle

The main biological actions of LTC₄, D₄ and E₄ are summarised in table 3.1. One of the most potent effects of these cysteinyl-containing LT's is their action on a variety of smooth muscle preparations which is typically slow in onset but long-lasting. The classical assay for SRS-A has been contraction of guinea-pig ileum where LTC₄, D₄ and E₄ are thousands of times more active than histamine, the order of potency being LTD₄ > C₄ > E₄ (Piper, 1983). Rat stomach strip and guinea-pig uterus are also contracted by these LT's (Piper & Samhoun, 1982; Weichman & Tucker, 1982) but are not as sensitive as guinea-pig ileum.

It has been recognized for many years that SRS-A has bronchoconstrictor properties (Brocklehurst, 1960) and its LT constituents (particularly LTC₄ and D₄) are now known to be potent constrictors of isolated airway smooth muscle. However, considerable species variation exists and although these LT's are, again, thousands of times more active than histamine on respiratory smooth muscle from human and guinea-pig lung (Dahlen *et al*, 1980; Lewis *et al*, 1980b), they are virtually inactive on tissues from other species, as in the case of rat trachea (Krell *et al*, 1981). LTC₄ and D₄ are often equipotent as constrictors of isolated airway smooth muscle while LTE₄ is usually less potent (Piper, 1983).

In guinea-pig *in vivo* LTC₄ and LTD₄ have a direct bronchoconstrictor action when given by aerosol but when given intravenously cause a bronchoconstriction which is inhibited by indomethacin (Schiantarelli, Bongrani & Folc, 1981; Hamel *et al*,

1982). Piper & Samhoun (1982) have shown that in guinea-pig isolated perfused lung LTC₄ D₄ and E₄ cause release of TXA₂.

Human isolated bronchus and lung parenchyma are also contracted by the cysteinyl-containing LT's (Piper, 1985), although neither TXA₂ nor PG's appear to play a major role in these tissues since indomethacin does not alter the response to LT's (Piper, 1985). In human volunteer studies, the nature of the LT-induced response in the respiratory system resembles that seen in asthma and suggests a role for these substances in allergic broncho-spastic disease (Piper, 1983).

3.6 (b) Effects on the microcirculation

In keeping with their actions on smooth muscle, LTC₄ and D₄ are also potent vasoconstrictors with actions in many different vascular beds from several species (Letts & Cirino, 1985). In particular, they appear to have potent effects in the coronary and cerebral circulations (Letts & Cirino, 1985).

Like LTB₄ leukotrienes C₄, D₄ and to a lesser extent E₄, promote plasma exudation from post-capillary venules, again at much lower concentrations than required for histamine (Piper, 1983). In guinea-pig skin, LT-induced plasma exudation is potentiated by vasodilator PG's such as PGE₂ or PGI₂ which are thought to reverse the LT-induced vasoconstriction (Peck, Piper & Williams, 1981). In contrast to LTB₄ however, increased permeability is not dependent on the presence of PMN's, but seems to occur via a direct action on the vascular endothelium (Bjork *et al*, 1983).

3.7 Leukotriene Receptors

There is a large body of both indirect and direct evidence for the presence of specific LT receptors in a variety of tissues (Bray, 1985). Radioligand binding studies have shown that, in contrast to those for the PG's, LT receptors are a heterogeneous population with widely differing species and tissue specificities.

The use of high specific activity $^3\text{H-LTB}_4$ has permitted the demonstration of two classes of LTB_4 receptors on human PMN's that differ in their affinity for LTB_4 (Goldman & Goetzl, 1984). There is some evidence to suggest that these sites may transduce different biological responses, the high affinity site mediating aggregation and chemotaxis and the low affinity site mediating degranulation (Goldman & Goetzl, 1984). In addition, FMLP and C5a do not inhibit binding of $^3\text{H-LTB}_4$ to PMN's indicating that the chemotactic receptors for LTB_4 are distinct from those of the peptide chemotactic factors (Goldman & Goetzl, 1984).

The cysteinyl-LT's also appear to exhibit a marked heterogeneity in receptor specificity and distribution. LTC_4 , D_4 and E_4 contract guinea-pig ileum with widely differing dose response relationships (Piper, 1983) while LTB_4 is effectively inactive in this tissue. Highly specific, saturable binding sites for LTC_4 have been identified on guinea-pig ileum and, since disrupted cells appear to have more binding sites than whole tissue, there is speculation that LTC_4 receptors may be largely internalized, being recruited to the plasma membrane during cell activation (Bray, 1985). This would help to explain the slow contraction in response to LTC_4 .

3.8 Interaction of Leukotrienes with other Inflammatory Mediators

3.8 (a) Other eicosanoids

The interaction between LT's and CO products has effectively been discussed in section 3.6. The effects of the LT's on the guinea-pig respiratory system have been seen to be largely due to the formation of TXA_2 and possibly other PG's. However, in human lung, studies strongly suggest that PG's and TXA_2 do not mediate the response to LT's in this case. In addition, the synergism between the LT's and the vasodilatory PG's in causing increased vascular permeability may be of importance in the formation of oedema.

The addition of AA to a suspension of human leukocytes normally induces only very small amounts of 5-LO products (Clancy *et al*, 1983). However, Maclouf *et al*

(1982) have demonstrated that co-incubation of platelets with leukocytes results in an activation of 5-LO with increased production of LTB₄. The compound responsible for the activation has been identified as 12-hydroperoxy-eicosatetraenoic acid (12-HPETE), a product of the platelet 12-lipoxygenase. Although inflammation can proceed in the absence of circulating platelets (Ubatuba, Harvey & Ferreiria, 1975), this observation suggests a possible role for platelets in the initiation or amplification of LT-modulated inflammation.

3.8 (b) Cyclic AMP

Since LTB₄ is able to induce degranulation of PMN's it was thought likely that it was able to increase cAMP production. Claesson (1982) investigated the effects of various LT's on cAMP formation in human PMN's and showed that LTA₄ and LTB₄ cause a small rise in cAMP levels. No effect was observed for LTC₄. Moreover, experimentally elevated cAMP levels were shown to lead to an inhibition of LTB₄ synthesis and enzyme release, a possible negative feedback mechanism.

In addition, inflammatory stimuli such as FMLP, C5a and ionophore A23187 have been shown to cause a small rise in cAMP levels (Claesson, 1982) also indicating a relationship between cAMP and PMN activation.

3.8 (c) Reactive metabolites of oxygen

Reactive metabolites of oxygen ("free radicals") are among the many substances released from stimulated inflammatory cells along with the LT's. Since LT's are unsaturated compounds, *in vivo* reactions between them and free radicals produced simultaneously and in close proximity, are likely.

Henderson & Klebanoff (1983) have shown that the respiratory burst in stimulated PMN's is not required for LT formation, but suggest that products of the burst may contribute by one or more mechanisms to the degradation of the LT formed. LTB₄ and LTC₄ are both rapidly inactivated by the peroxidase-H₂O₂-halide system and by

hydroxyl radicals, both products of the respiratory burst in PMN's. Neutrophils from patients with chronic granulomatous disease which lack a respiratory burst, show increased production of LTB₄ and LTC₄ compared to normal cells when stimulated with A23187.

The relative free radical "scavenging" activity of a selection of LT's *in vitro* has been examined (Chopra, Belch & Smith, 1988), the order of effectiveness being LTD₄ > LTC₄ > LTB₄. These LT's were found to be reactive towards free radicals in general rather than to the superoxide ion specifically.

3.8 (d) Interleukin-1

Another means whereby LT's can modulate inflammatory (and immunological) reactions is through their interaction with IL-1. Inhibition of IL-1 activity (standard thymocyte assay) by LO inhibitors such as eicosatetraenoic acid (ETYA) and BW755C has been demonstrated (Dinarello, Marnoy & Rosenwasser, 1983). In addition, Rola-Pleszczynski & Lemaire (1985) have shown a direct stimulation of IL-1 production from human monocytes on addition of LTB₄. Stimulation of AA lipoxygenation by IL-1 has also been demonstrated (Farrar & Humes, 1985) suggesting that LT's may play a role in the amplification of IL-1 production from human monocytes and macrophages.

3.9 Role of LTB₄ in Inflammatory Disease

In view of its biological properties it is thought that the main role of LTB₄ is to mediate at least in part, the recruitment of leukocytes during the inflammatory process. Simmons, Salmon & Moncada (1983) have established that LTB₄ is present during acute experimental inflammation in the rat. Concentrations of LTB₄ detected in the inflammatory exudate were sufficient to induce chemotaxis and degranulation of PMN's and the peak LTB₄ concentration correlated with the maximum rate of PMN influx into the exudate. However, other substances present in the exudate, such as C5a,

will also induce cell influx and it is thought likely that LTB₄ amplifies the chemotactic response in the early stages of inflammation (Simmons *et al*, 1983).

The contribution of LTB₄ to chronic inflammatory disease is less clear. However, elevated LTB₄ levels have been detected in a variety of human diseases. Klickstein, Shapleigh & Goetzl (1980) using HPLC, have detected increased levels of LTB₄ in the synovial fluid of patients with RA and ankylosing spondylitis compared to non-inflammatory osteoarthritic controls. Davidson, Rae & Smith (1983) were also able to detect LTB₄ in RA synovial fluid using a bioassay, albeit at much lower concentrations than those reported by Klickstein *et al*.

The role of LTB₄ in gout has also been investigated (Rae, Davidson & Smith, 1982). Results showed that LTB₄ is present in gouty effusions at concentrations which are greater than those found in RA synovial fluids. In addition, it was demonstrated that monosodium urate (MSU) crystals (the classical stimulus for the inflammation of gout) are able to stimulate production of LTB₄ from peripheral PMN's. Work done on MSU-induced synovitis in the dog however, has shown that there is probably no role for LTB₄ in this animal model (Carlson *et al*, 1986).

It is thought that LTB₄ may also have a role in the human inflammatory dermatoses, where PMN infiltration is often a characteristic feature. Brain *et al* (1982) have shown increased levels of LTB₄ in affected psoriatic skin compared to un-involved areas and to normal control skin. In addition, Camp *et al* (1983a) have demonstrated that intra-dermal injection of LTB₄ in human skin causes inflammatory changes accompanied by PMN infiltration.

Enhanced synthesis of LTB₄ by colonic mucosa in patients with inflammatory bowel disease (IBD) has also been reported (Sharon & Stenson, 1984). Again, this disease is characterised by infiltration of the gut mucosa with PMN's. At the same time, Sharon & Stenson showed that sulphasalazine (SASP), a drug often used in the treatment of IBD, substantially inhibited LTB₄ production in colonic mucosa.

3.10 Inhibition of the Leukotrienes and Potential for Anti-Inflammatory Therapy

The potent biological activity of LTB₄ and the other LT's has stimulated a search for inhibitors of LT synthesis (Fig. 2.3). In contrast to the CO which has fairly specific inhibitors, there are few compounds which specifically inhibit the 5-LO. However, given that inhibition of CO alone may cause substrate diversion and enhanced LT formation (see section 2.10), selective LO inhibition may result in increased PG production. If this is the case, then dual inhibitors are likely to have significant clinical advantage over single enzyme inhibitors.

In view of this new approach to anti-inflammatory therapy, much interest has been shown in the experimental dual inhibitor BW755C, a pyrazole derivative which inhibits peroxidation of AA in every tissue so far tested (Higgs & Vane, 1983). This compound has been found to be equipotent in reducing oedema, PG synthesis and leukocyte accumulation and is very similar in its anti-inflammatory effect to the corticosteroid dexamethasone (Higgs, Flower & Vane, 1979).

Another class of compounds which inhibits both CO and 5-LO pathways are the acetylenic analogues of AA (e.g. ETYA). These compounds compete with the natural substrate for enzyme but are unable to undergo peroxidation (Higgs & Vane, 1983).

Also of interest, is the fact that some natural plant products such as ginger, curcumin and onion, which have been known to possess anti-inflammatory properties for many years, have now been shown to be dual inhibitors of AA metabolism in neutrophils and platelets (Flynn, Rafferty & Boctor, 1986; Srivastava, 1986).

The investigation of the drug benoxaprofen as an anti-inflammatory agent illustrates the difficulties encountered in the interpretation of inhibitor studies *in vitro* and *in vivo*. Benoxaprofen is an effective anti-inflammatory agent in man and causes a marked improvement in psoriasis, which is normally exacerbated by NSAID's such as indomethacin (Kragballe & Herlin, 1983). As well as being a weak CO inhibitor,

benoxaprofen had been reported to be an inhibitor of 5-LO in A23187-stimulated PMN's (Myers & Siegel, 1983). The clinical effects of the drug were therefore attributed to 5-LO inhibition. However, benoxaprofen has since been reported as failing to inhibit a cell-free preparation of 5-LO from human PMN's (Masters & McMillan, 1984) and Salmon, Tilling and Moncada (1984) have shown that it failed to reduce *in vivo* synthesis of LTB₄ in an animal model of acute inflammation. These observations obviously cast doubt on the original hypothesis that the clinical effects of benoxaprofen are mediated by 5-LO inhibition. The drug has now, in any case, been withdrawn from clinical use due to side effects.

Another approach to LT inhibition has been the development of antagonists which work at the receptor level and therefore have some specificity of action. One of the best characterised is the SRS antagonist, FPL55712 (Piper & Samhoun, 1987). A number of structural analogues which block the action of LTB₄ have also been identified; these include the mono-HETE's and the LTB₄ methyl esters (Bray, 1983).

Ultimately, the therapeutic potential of inhibiting 5-LO as well as CO depends on the contribution of the LT's to disease processes. In most cases, the extent of this contribution is still unknown. However, it seems likely that the development of inhibitors for 5-LO could lead to improved drugs for the treatment of chronic inflammatory conditions.

CHAPTER 4

MATERIALS AND METHODS

4.1 Measurements of Eicosanoids

4.1 (a) Introduction

The *in vitro* effects of the eicosanoids are well documented, but in order to establish a role for them *in vivo* it is necessary to be able to measure them quantitatively in biological/pathological samples. Since the concentration of these compounds in such samples is usually low (pg-ng range), their measurement requires highly sensitive and specific assays. Techniques which have been used in the past include bioassay, GCMS, HPLC and radioimmunoassay (RIA), each of which has its advantages and disadvantages.

4.1 (b) Bioassay

Bioassay is based on the observation of an *in vitro* effect such as smooth muscle contraction or cell aggregation. For example, tens of pg's of LTB₄ activity can be measured using aggregation of rat peritoneal neutrophils (Bray, 1983). One advantage of bioassay is that a biological activity can be measured before the principle causing the effect has actually been identified. However, specificity is often difficult to achieve without the use of certain inhibitors and antagonists. In addition, bioassay cannot be used to measure levels of metabolites such as 6-keto-PGF₁ α or TXB₂ which are relatively inactive.

4.1 (c) GCMS

This is a relatively complicated, direct method of chemical analysis which is inaccessible to most laboratories, the main problem being the expense of the sophisticated equipment which is required. In addition, this method has a low sample capacity and requires a fairly large initial sample volume in comparison to other methods.

4.1 (d) HPLC

High performance liquid chromatography (HPLC) with UV detection can also be used for the measurement of eicosanoids. Indeed, HPLC played an important role in the structural elucidation of the LT's (Samuelsson, 1983). However, the main problem with this method is lack of sensitivity and, in addition, samples usually have to undergo extraction before they can be placed on the column.

4.1 (e) RIA

4.1 (e) (i) Introduction

The method chosen for measurement of eicosanoids in this work was RIA. Using this method the large numbers of samples encountered in clinical work can be assayed within a relatively short period of time and the sensitivity and specificity are better than with most other techniques. In addition, no sophisticated laboratory equipment is required other than a scintillation counter.

4.1 (e) (ii) Principle of RIA

RIA is based upon the competition of antigen (the compound being analysed), and a fixed quantity of radioactively labelled antigen for a specific antibody site. A limiting and constant amount of antibody is incubated with a constant concentration of tracer, usually so that approximately 50% of the tracer is bound to antibody. On addition of the unlabelled antigen, competition occurs between it and the labelled antigen for the limited number of binding sites on the antibody i.e. the amount of labelled antigen bound by the antibody is inversely proportional to the amount of unlabelled antigen present. The amount of unlabelled compound present in a sample can be determined by comparison with standards containing known amounts of the compound via the construction of a standard curve. Each assay must also include tubes with no antibody present to determine non-specific binding (NSB) and tubes with no unlabelled compound to give maximum binding of tracer (zero standard or B_0).

The amount of tracer bound as a percentage of B_0 ($\%B/B_0$), is calculated according to the following equation:

$$\%B/B_0 = \frac{\text{Standard or sample cpm} - \text{NSB cpm}}{B_0 \text{ cpm} - \text{NSB cpm}}$$

4.1 (e) (iii) The antibody

Probably the most important component of any RIA is the antibody. The production of an antibody with the required specificity and affinity for the compound being measured is crucial to the success of the assay.

Eicosanoids are not themselves antigenic and must first be conjugated to an antigenic molecule such as bovine serum albumin (BSA), (Salmon, 1982a). This conjugate is then mixed with Freund's adjuvant and injected into rabbits. After several booster injections to elicit a good response to the antigen, the animal is bled and the serum containing the antibody is separated and kept frozen or lyophilized. Cross-reactivity with all possible compounds of a similar structure must be checked before use and must be as low as possible (<0.2% is desirable); cross-reactivity for the metabolite to be measured must be 100%.

4.1 (e) (iv) The tracer

The isotopes most frequently used for RIA of eicosanoids are tritium (^3H) and ^{125}I (Iodine (^{125}I)). The higher the specific activity (SA) of the tracer, the smaller the mass which can be used in the assay, thus increasing sensitivity. Higher SA can be achieved with ^{125}I tracers, which also have the advantage that they do not require scintillation fluid for counting. In this respect, therefore, they are probably cheaper and easier to use. However, ^3H -tracers have a longer half-life and are safer to work with than gamma-emitters. Many high SA ^3H -tracers are now commercially available.

4.1 (e) (v) Separation method

In order to determine the amount of tracer which has been displaced from the antibody by unlabelled compound, a method for separating antibody-bound and free tracer is required. A common method of separation is adsorption of free tracer onto dextran coated charcoal (DCC) which is then centrifuged and the antibody-bound radioactivity counted in the supernatant. It is important that the separation is performed using ice-cold DCC suspended in buffer with the assay tubes in melting ice to avoid adsorption of the antibody-bound complex. In addition, the time taken to perform the addition, mixing and centrifugation of the tubes must be carefully standardized.

4.1 (e) (vi) Assay validation

There are several potential sources of error in RIA. Other factors besides presence of unlabelled antigen in the sample can affect the inhibition of binding of radioactive antigen to the antibody, including variations in pH, ionic strength, protein concentration or a significant level of cross-reacting compounds (Salmon, 1983). The specificity of an antibody is usually assessed by comparing the mass of the compound being measured with the masses of related compounds which cause 50% inhibition of binding of tracer to antibody, giving a measure of percentage cross-reactivity.

All of the assays in this work have been carried out using commercially available reagents (antibody/standard/tracer) either bought individually or in the form of RIA kits. The antibodies used therefore, are well characterized and their specificity has already been determined. Reproducibility was assessed by including a "standard" sample of known concentration in all assays performed (inter-assay variation) and by including several identical samples in the same assay (intra-assay variation). Inter- and intra- assay coefficients of variation are generally taken to be acceptable if less than 15%.

4.2 Measurement of PGI₂-Metabolites in Plasma

4.2 (a) Method of sampling

Since PGI₂ is unstable, its stable metabolites, a significant proportion of which is 6-keto-PGF_{1α} (Myatt *et al*, 1981) must be used as indices of PGI₂ production.

Measurement of PGI₂-metabolites (PGI₂-M's) was carried out on plasma. Since leukocytes can produce PGI₂ it is important that further *in vitro* generation after blood sampling is abolished. To this end, venous blood was collected into ice-cold plastic tubes containing 1:9 volumes of 3.8% w/v trisodium citrate with 3x10⁻⁵M indomethacin and 10⁻⁴M adenosine. Tubes were kept on ice and centrifuged within 1 hour at 4°C and 2500g. The plasma was then separated and stored at -70°C until assayed.

4.2 (b) Extraction of samples

It is common practice in RIA to extract plasma samples prior to assay in order to rid the sample of interfering substances and increase specificity. However, many extraction, purification and evaporation steps can add further impurities (Greaves & Preston, 1982) and where large numbers of samples are to be assayed, it may be impracticable to extract and purify every sample. Furthermore, only 40% of PGI₂-M's in plasma are 6-keto-PGF_{1α} (Myatt *et al*, 1981) and extraction of samples for this metabolite alone could give an underestimation of circulating PGI₂ levels. McLaren *et al* (1985) have shown that it is possible to obtain sensitive, specific and reproducible results for PGI₂-M's by carrying out RIA directly on unextracted plasma. On this basis, all the plasma PGI₂-M determinations for the work presented here were carried out without prior extraction of the plasma.

4.2 (c) Assay procedure

The RIA used for the determination of PGI₂-M's in plasma was based on that developed by McLaren *et al* (1985) who found that by reducing the amount of tracer and

antibody (half the amounts recommended) and by increasing equilibration time, the sensitivity of the assay could be increased so that normal plasma was brought within the limits of detection of the assay.

Reagents were purchased in the form of a 6-keto-PGF₁α [¹²⁵I] assay kit from the Institute of Isotopes of the Hungarian Academy of Sciences, Budapest. Assays were performed in duplicate, directly on undiluted plasma. All other reagents were diluted in assay buffer which consisted of phosphate buffer 50mM, pH 6.8 containing 0.05% BSA.

Reagents were prepared as suggested by the makers of the kit, except that the tracer (6-keto-PGF₁α [¹²⁵I]-iodotyrosine methyl ester, 1800 Ci/mmol, 5.4mCi) was diluted in 20 ml of assay buffer rather than 10ml. Lyophilized anti-serum was reconstituted with 10 ml of distilled water to give a solution which in the assay, bound approximately 50% of the added tracer in the absence of sample. A series of standard 6-keto-PGF₁α solutions in the range 2.5-1250 pg/ml was made by serial dilution.

Polypropylene tubes (12mm x75 mm, Luckham) were labelled in duplicate for total counts (TC), buffer NSB, plasma NSB, zero standard (B₀), standards and samples. The reagents were added to the appropriate tubes according to the protocol shown in Table 4.1, all tubes containing a final volume of 350 μl. Each assay consisted of 100 tubes, allowing construction of a standard curve and the assay of 37 unknowns in duplicate. A stock of normal pool plasma, collected in the same way as sample plasma and stored at -70°C in small aliquots was used as a control sample to assess assay reproducibility. After the addition of all the reagents, tubes were vortex mixed, covered and incubated for approximately 40 hr at 4°C.

On the third day, the tubes were placed in an ice-water bath and allowed to equilibrate for a few minutes. A suspension of 0.5% acid-washed charcoal (BDH) and 0.1% dextran grade C (BDH) in assay buffer was continuously stirred in a beaker placed in an ice-water bath. All tubes received 300 μl of this DCC suspension except the TC

tubes which received 300 μ l of assay buffer. The tubes were vortex mixed after the addition of DCC to the last tube and at 10 ± 1 minutes after DCC addition, the tubes were centrifuged at 2000 g and 4°C for 10 minutes (IEC Centra-7R, USA). Immediately after centrifugation, the tubes were placed back in the ice-water bath and the supernatants containing antibody-bound tracer were decanted into fresh tubes and counted for 1 minute each in a gamma-counter (Canberra Packard, 500 C, UK).

Reagent \ Tube	TC	Buffer NSB	Plasma NSB	Bo	Standards	Samples
Standards	-	-	-	-	200	-
Sample	-	-	-	-	-	200
^{125}I -6-keto $\text{PGF}_{1\alpha}$	100	100	100	100	100	100
Antiserum	-	-	-	50	50	50
Buffer	250	250	50	200	-	-
Pool plasma	-	-	200	-	-	-
MIX AND INCUBATE						
DCC	-	300	300	300	300	300
Buffer	300	-	-	-	-	-

Table 4.1 Protocol for RIA of PGI_2 - metabolites in plasma

(All volumes in microlitres)

4.2 (d) Assay characteristics

Table 4.2 shows the observed cross-reactivities of the antibody used in the assay (as determined by the makers of the kit) with 6-keto-PGF₁α and some related compounds.

For each assay, a standard curve was constructed by plotting the amount of tracer bound as a percentage of B₀ (% B/B₀) versus pg/ml 6-keto-PGF₁α on semi-log graph paper. The standard curve obtained over ten assays is shown in Fig. 4.1; 50% displacement of bound ¹²⁵I-6-keto-PGF₁α occurred around 13 pg of added 6-keto-PGF₁α.

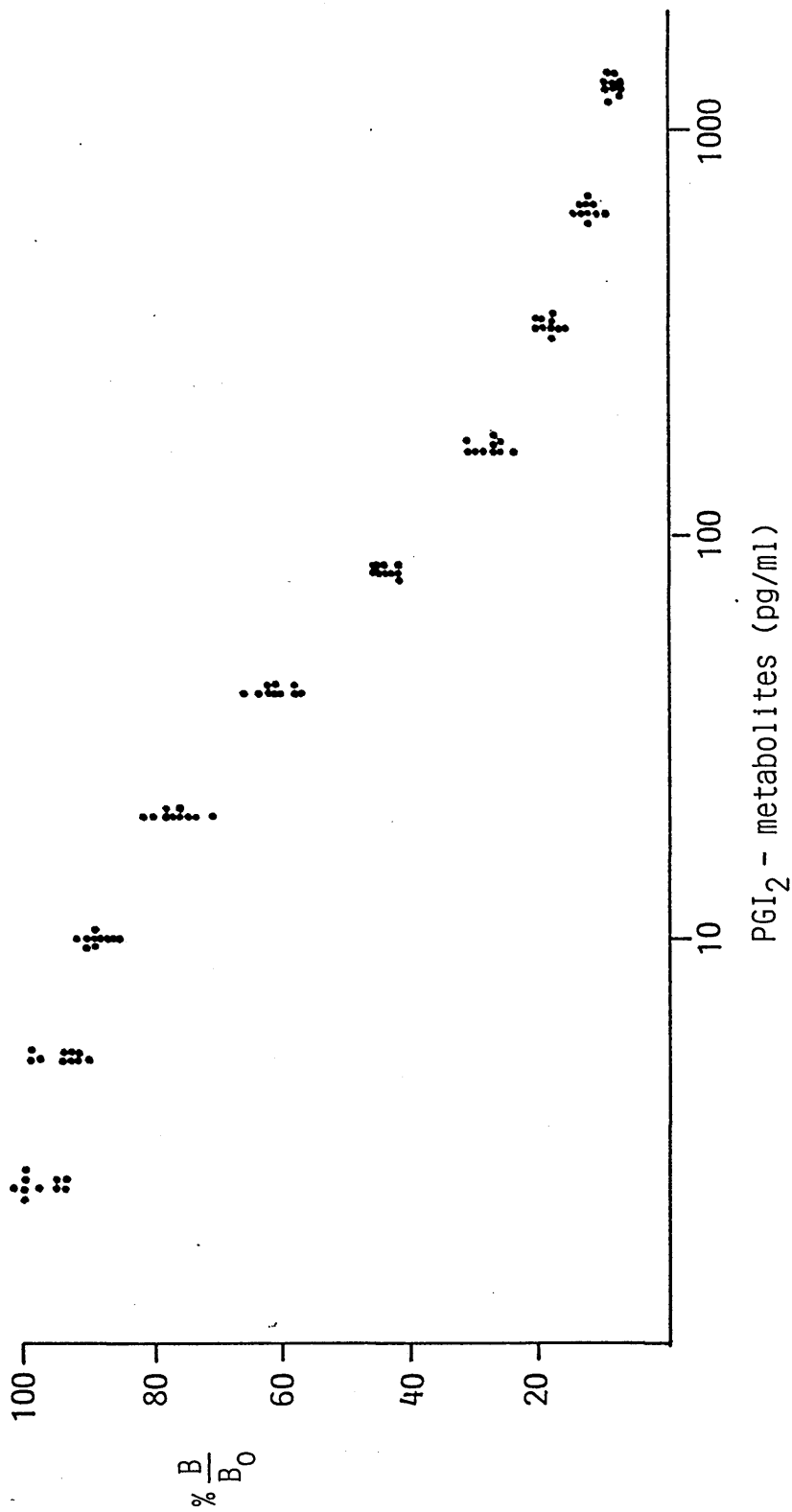


Fig. 4.1 Prostacyclin metabolite assay (Hungarian Kit). Standard curve over 10 assays.

Total counts for the assay were 10,000-12,000 cpm, NSB was typically 3% for plasma and 1% for buffer. The lower limit of sensitivity of the assay (taken as 2 standard deviations from zero-dose binding) was 3.8 pg/ml. Inter- and intra-assay variation were 4.9% and 4.0% respectively. All results were expressed as pg PGI₂-metabolites/ml.

Compound	% Cross-Reactivity
6-keto-PGF ₁ α	100
PGF ₂ α	0.8
PGE ₂	1.4
PGF ₁ α	2.0
PGE ₁	1.2

Table 4.2 Cross-reactivity of “Hungarian” antiserum with 6-keto-PGF₁α and related compounds.

4.2 (e) Normal values for 6-keto-PGF₁α in plasma

Widely differing values for plasma 6-keto-PGF₁α measured by RIA can be found in the literature (Mitchell, 1978; Kinney & Domers, 1981; Patrono *et al.*, 1982). In the early stages of RIA development for 6-keto-PGF₁α it was common to see values in the range 70-120 pg/ml for normal plasma. These values are now thought to be unrealistically high, probably due to cross-reaction of the antibodies with other PG’s. In addition, some of the extraction methods used probably contributed to falsely high levels.

More recently, levels of 5-20 pg/ml have been detected by RIA (Patrono *et al.*, 1982; Gotoh *et al.*, 1983). Even lower values (< 2 pg/ml) have been determined by GCMS (Blair *et al.*, 1982), but GCMS measures only 6-keto-PGF₁α and not the

remaining 60% of PGI₂-metabolites. These values, therefore, probably underestimate the true circulating levels of PGI₂.

Using the assay described above, the range for normal plasma in this work was 10-22 pg/ml, and the mean (SD) was 13.1 (4.0) pg/ml.

There are, however, limitations in the use of plasma assays of PGI₂-M's. Perhaps the most significant problem is that, since levels in plasma are at or even below the limit of detection of RIA, it is usually impossible to use this method when decreased PGI₂ production is suspected, for example, after NSAID's or in certain conditions such as pregnancy induced hypertension (McLaren, 1986). For this reason, since this work was done, measurement of plasma PGI₂-M is now seldom used. Instead, measurement in serum is employed, since higher levels are generated due to leukocyte production *in vitro*.

4.3 Measurement of TXB₂ in Serum

4.3 (a) Method of sampling

Due to the very short half-life of TXA₂, TXB₂, the stable breakdown product, is used as an index of TXA₂ production.

The first RIA's for TXB₂ used plasma (Granstrom & Kindahl, 1978). However, this method received much criticism due to the potential for artefactually high levels of TXB₂. Since TXA₂ is generated by platelets, any trauma associated with venepuncture will activate platelets to give increased levels. Likewise, further generation of TXA₂ may take place during the processing of the sample. Attempts were made to surmount these problems by standardizing venepuncture technique and adding a CO inhibitor (indomethacin) to the anti-coagulant. However, the method was still unsatisfactory.

An alternative method, introduced by Patrono *et al.*, (1980) uses serum. The TXB₂ measured in the serum reflects the generation of TXA₂ by platelets in response to an endogenous stimulus ie thrombin generated during blood clotting.

All TXB₂ measurements in this work were performed on serum. Venepuncture was carried out using a 19 gauge butterfly and the blood transferred into glass tubes. The amount of TXB₂ generated during blood clotting has been shown to be temperature dependent (Block *et al.*, 1987). For this reason the samples were incubated in a 37°C water bath for at least 1 hour, although one study (section 5.2) pre-dated the introduction of this methodology and serum samples in this case were left to clot at room temperature. Subsequently, the tubes were centrifuged at 2500g for 15 minutes and the separated serum stored at -70°C until use.

4.3 (b) Assay Procedure

The RIA used for the determination of TXB₂ was based on that developed by Granstrom, Kindahl & Samuelsson (1976), and used ³H-TXB₂ as a tracer. The antibody and standard were gifted by Mr. Simon Thomas, Medical Science Liaison Officer, Upjohn Ltd., Crawley and the tracer was purchased from Amersham International. All reagents were diluted in assay buffer which consisted of 0.1M phosphate buffer, pH 7.0 containing 0.1% BSA and 0.5% bovine γ -globulin.

Assays were performed in duplicate in LP3 tubes (Luckham), all serum samples being diluted 1:300 in assay buffer just prior to assay. A series of standard solutions of TXB₂ were made in the range 3.8-1000 pg/ml and the anti-serum was diluted (final 1:10,000) so that approximately 50% of added tracer bound in the absence of competing antigen. Tubes were labelled for TC, buffer NSB, serum NSB, B₀, standards and samples and reagents were added as shown in Table 4.3, all tubes containing a final volume of 400 μ l. A sample of standard pool plasma, undiluted, was included in each assay to assess inter-assay variations.

After addition of all the reagents, tubes were vortex mixed, covered and incubated overnight (16-18h) at 4°C. The following morning separation of bound and free counts was achieved by adding 300 µl of 0.1% DCC to all tubes (except TC), mixing and centrifuging in the same way as described for the PGI₂-M assay. After centrifugation, the supernatants containing the antibody-bound tracer were decanted into 10ml of scintillation fluid (Packard Insta-gel) and counted for 2 minutes in a beta-counter (Canberra Packard 460, UK).

Reagent \ Tube	TC	Buffer NSB	Serum NSB	B ₀	Standards	Samples
Standards	-	-	-	-	200	-
Sample	-	-	-	-	-	200
³ H-TXB ₂	100	100	100	100	100	100
Antiserum	-	-	-	100	100	100
Buffer	300	300	100	200	-	-
Pool Serum	-	-	200	-	-	-
MIX AND INCUBATE						
DCC	-	300	300	300	300	300
Buffer	300	-	-	-	-	-

Table 4.3 Protocol for RIA of TXB₂ in serum

(All volumes in microlitres)

4.3 (c) Assay characteristics

The standard curve obtained over six assays is shown in Fig. 4.2 . Total counts for the assay were normally 1200-1500 cpm and 50% displacement of bound ³H-TXB₂ occurred at approximately 13pg of added TXB₂ . The lower limit of sensitivity of the assay was 6 pg/ml, NSB was typically 4% for serum and 3% for buffer. Inter- and intra-assay variation were 11.2% and 5.2% respectively. All results were expressed as ng TXB₂ /ml, the mean (SD) value for normal serum (using clotting at room temperature) being 13.2 (8.6 ng/ml).

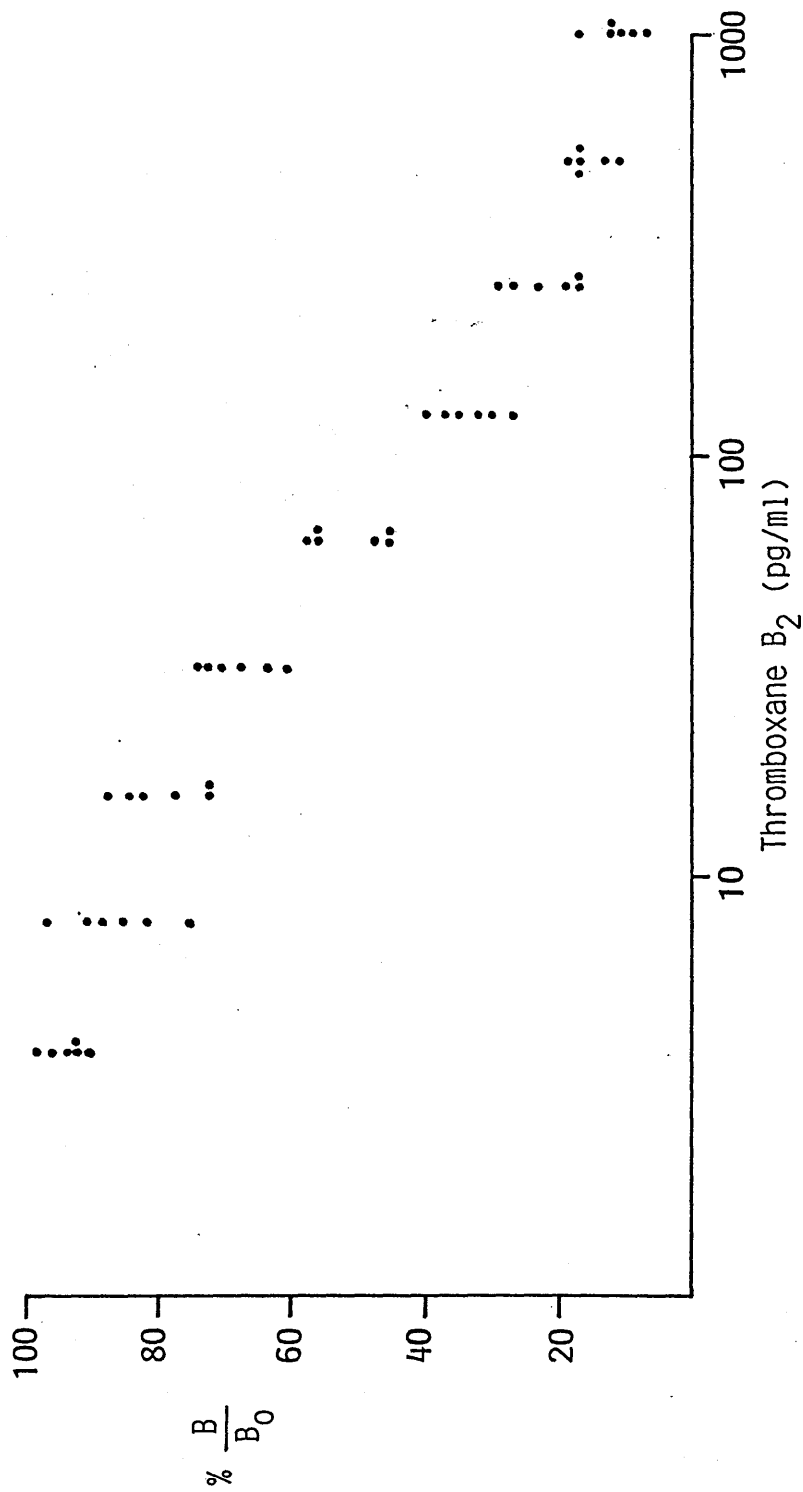


Fig. 4.2 Thromboxane B₂ Assay. Standard curve over 6 assays.

4.4 Measurement of LTB₄ Production from PMN's

4.4 (a) Preparation of PMN suspensions

The method used to separate the PMN cells from whole blood was based on that described by Boyum (1968). Venous blood (10 ml) was taken into heparin (10 units/ml blood) in plastic tubes. The blood was then mixed with 5ml of 6% w/v dextran 70 (Macrodex) and the red blood cells (RBC's) allowed to sediment under gravity for 60-90 minutes. After this time, the top layer (leukocyte-rich plasma) was removed and carefully layered onto 5 ml of Lymphoprep (9.6% w/v sodium metrizoate, 5.6% w/v ficoll; Nyegaard & Co., Oslo) which was then centrifuged at 200 g for 10 minutes to sediment the PMN's. The mononuclear cells, which remain in a band at the interface between plasma and lymphoprep, were discarded. Any remaining RBC's in the PMN pellet were removed by gentle hypotonic lysis with 0.87% ammonium chloride (10-15 minutes).

After further centrifugation (160g for 5 minutes), the PMN's were washed once in HEPES-buffered Hank's balanced salt solution (HEPES/HBSS), pH 7.4 (Flow Laboratories, Irvine, UK) and finally resuspended in 1ml of this buffer. The cells were then counted using a haemocytometer, PMN cells normally constituting >97% of the total leukocyte count. Cell viability, assessed by Trypan Blue exclusion was normally >95%.

4.4 (b) Incubation of PMN suspensions with calcium ionophore A23187

The concentration of the PMN suspensions was adjusted so that 5×10^6 cells were suspended in 0.9ml of HEPES/HBSS in a micro-centrifuge tube (Eppendorf). The suspensions were then warmed to 37°C in a water bath (2 minutes) after which 0.1ml of calcium ionophore (CaI) A23187 (Sigma Chemicals, Poole, Dorset, UK) was added as a 10 µg/ml solution in dimethylsulphoxide (DMSO)/buffer to give a final concentration of 1 µg/ml (2 µM). The final concentration of DMSO in the incubation mixtures was 0.1%. The cell suspensions were then agitated in the shaking water bath for exactly 5 minutes, the incubation being terminated by immediate centrifugation at 12000g for 30 seconds in a

microcentrifuge (Eppendorf). The cell-free supernatants were removed and stored at -70°C until use.

The dose of CaI used in stimulation and the incubation time, had been previously determined and were in agreement with those reported by others (Salmon, Simmons & Palmer, 1982a). Addition of A23187 (0.25-10.0 µg/ml) to PMN suspensions gave a dose-related increase in LTB₄ production which was maximal at 1 µg/ml (Fig. 4.3). In control incubations, where only buffer was added, no LTB₄ could be detected within the limits of the assay. Using 1 µg/ml A23187 in a time-course experiment, LTB₄ production was shown to increase from 0-5 minutes and thereafter decline, presumably due to re-uptake by the cells as a pre-requisite for ω-oxidation (Fig. 4.4).

In addition, since in many inflammatory states, pyrexia can occur (Duff, 1985), it was felt necessary to determine whether a typical febrile temperature of 39°C could affect LTB₄ production in PMN's. However, when LTB₄ formation by Ca I A23187 - stimulated cells was measured at 37°C and 39°C, there was found to be no significant difference (Fig. 4.5). It is unlikely therefore, that falsely elevated levels of LTB₄ would be encountered in patients sampled while pyrexia.

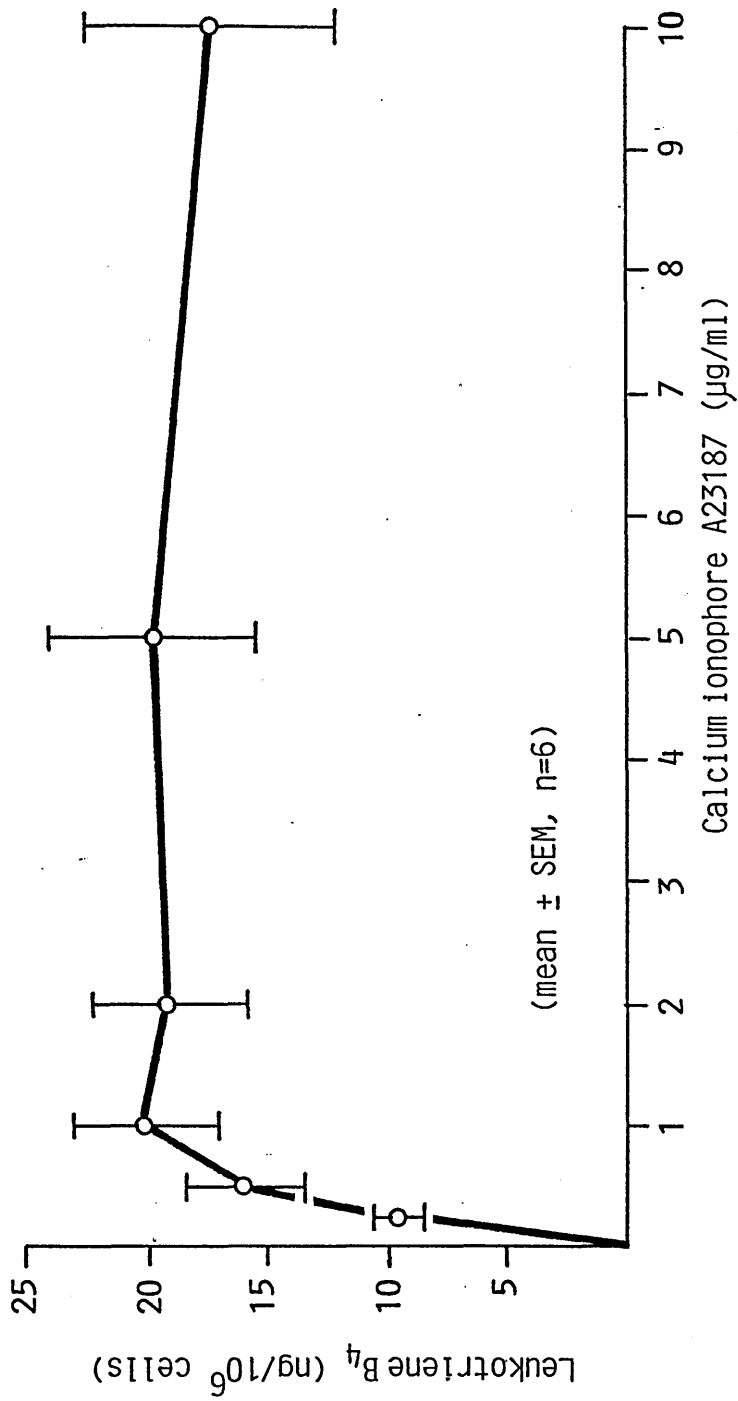


Fig. 4.3 Leukotriene B_4 production from PMN's - Dose response to calcium ionophore A23187. Each point represents the mean of six experiments.

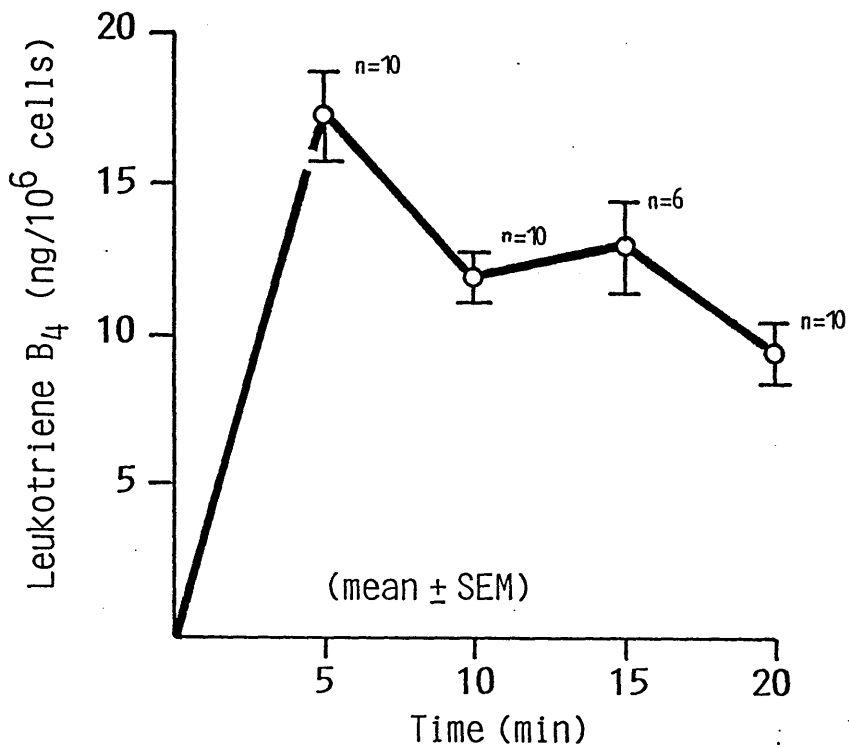


Fig. 4.4 Time course of leukotriene B₄ production from PMN's (5×10^6 cells/ml) stimulated with calcium ionophore A23187 ($1 \mu\text{g/ml}$) (n = number of experiments)

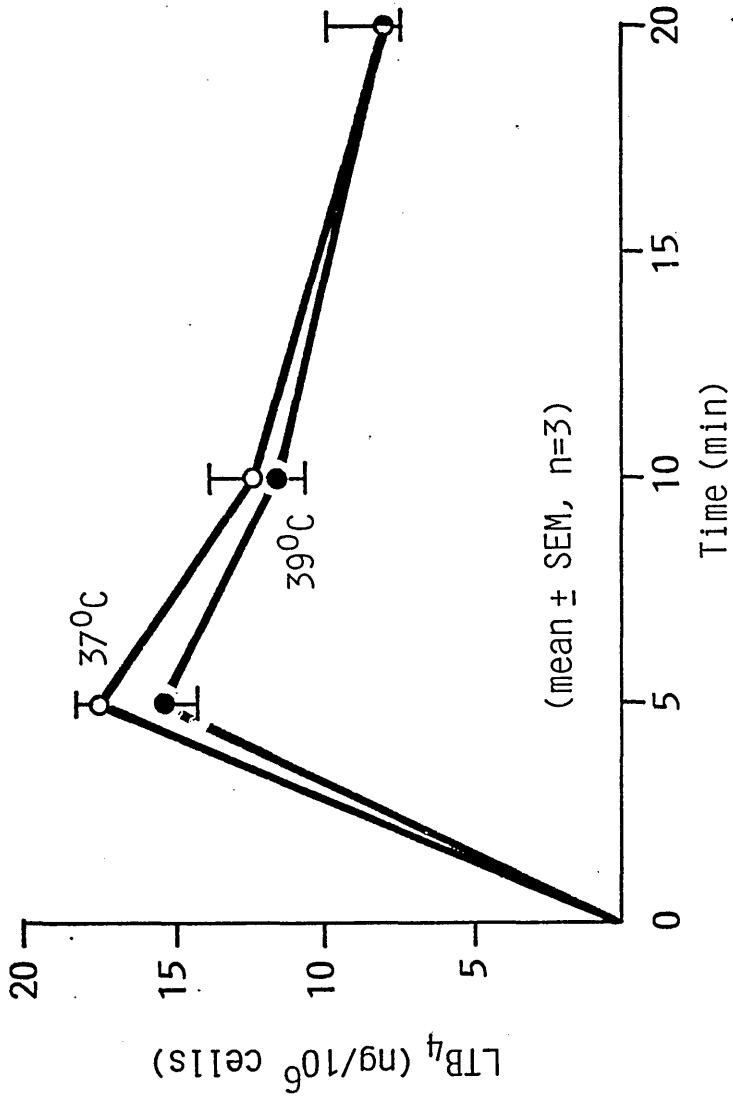


Fig. 4.5 Effect of incubation temperature on production of leukotriene B₄ by PMN's on stimulation with calcium ionophore A23187. Each point represents the mean of three experiments.

4.4 (c) Assay procedure

The RIA used for the determination of LTB₄ was based on that developed by Dr. John Salmon of Wellcome Laboratories (Salmon, Simmons & Palmer, 1982a). Antiserum to LTB₄ and LTB₄ standard were both purchased from Wellcome Diagnostics, Dartford, England. Tritiated LTB₄ ([5,6,8,9,11,12,14,15-³H] - LTB₄) was purchased from Amersham International, Amersham, UK (specific activity 210 Ci/mmol). Antiserum and standard were kept frozen at -20°C in aliquots sufficient for 1x100 tube assay.

The detailed protocol for the LTB₄ assay is shown in Table 4.4. The assay was performed directly on cell supernatants, usually diluted 1:100 in assay buffer which was 50 mM Tris-HCl, pH 8.6 containing 0.1% gelatin. All assays were carried out in 10mm x 75mm glass tubes (Samco, UK) and reagents were freshly diluted in buffer immediately prior to the performance of the assay. Antiserum was diluted 1:1000 (final 1:4000), so that approximately 50% of added tracer was bound in the absence of unlabelled LTB₄. A series of standard solutions (78-5000 pg/ml) was made by serial dilution. Stock tracer was diluted 1:100.

Test-tubes were labelled in duplicate for TC, NSB, B₀, standards and samples. Reagents were added to the appropriate tubes as shown in Table 4.4. All tubes contained a final volume of 400 µl. One assay normally consisted of 100 tubes, allowing construction of a standard curve and the assay of 40 unknowns in duplicate.

A control supernatant, used to assess assay reproducibility was included as a sample in each assay. These were generated by incubating several PMN suspensions from the same donor with CaI A23187 in the usual way. The resulting supernatants were then pooled, mixed, aliquoted into small amounts and stored at -70°C.

After the addition of all the reagents, tubes were vortex mixed and incubated overnight at 4°C. On the second day, the bound and free counts were separated as previously described in 4.2 (c), using a suspension of 2% charcoal, 0.4% dextran in

assay buffer. After centrifugation, supernatants were decanted into 10ml of 'Insta-gel' and counted for 4 minutes each in a β -counter.

Reagent \ Tube	TC	NSB	B ₀	Standards	Samples
Buffer	100	200	100	-	-
Standards	-	-	-	100	-
Samples	-	-	-	-	100
³ H-LTB ₄	100	100	100	100	100
Antiserum	100	-	100	100	100
Buffer	100	100	100	100	100
MIX AND INCUBATE					
DCC	-	200	200	200	200

Table 4.4 Protocol for RIA of LTB₄ in PMN supernatants

(All volumes in microlitres)

4.4 (d) Assay characteristics

For each assay a standard curve was obtained by plotting % B/B₀ versus pg LTB₄ /ml on semi-log graph paper. The standard curve obtained over ten assays is shown in Fig. 4.6. Total counts were approximately 4000 cpm and 50% displacement of bound ³H-LTB₄ occurred at 70 pg of added LTB₄. Non-specific binding was typically <4%; NSB in the presence of sample was similar. Assay sensitivity was 75 pg/ml; inter- and intra-assay variation were 8.6% and 6.6% respectively. Results were expressed as ng LTB₄ /10⁶ cells, the normal range being 6-37 ng/10⁶ cells. The mean (SD) was 21.9 (7.1) ng/10⁶ cells. The intra-personal variation as measured in one individual (the author) over a two year period (n=12), was 15.7%.

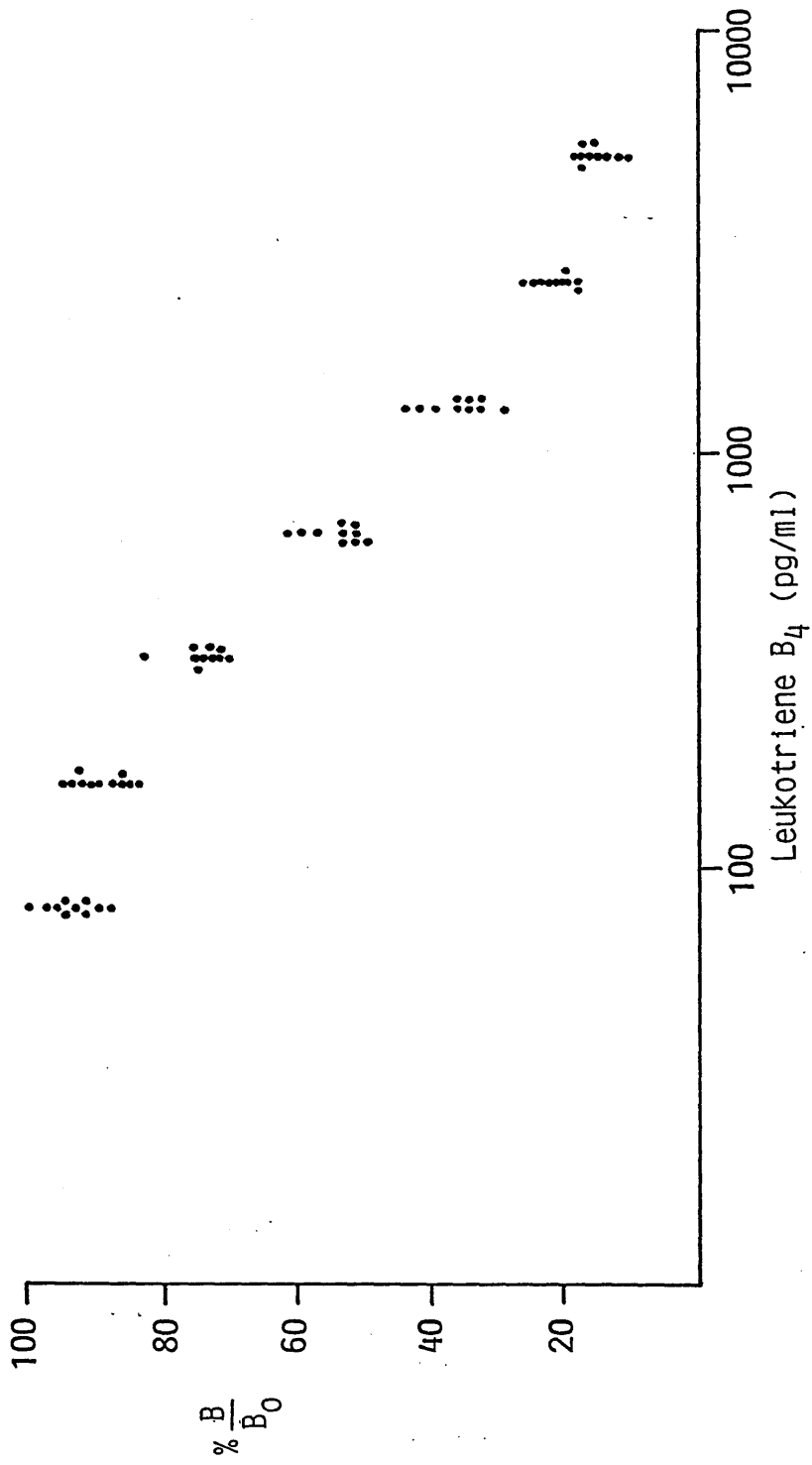


Fig. 4.6 Leukotriene B₄ assay - standard curve over 10 assays

4.4 (e) Justification for direct assay of supernatants

The measurement of LTB₄ by direct RIA of cell-free supernatants has been validated by Salmon *et al* (1982a). The assay was found to be relatively specific in that PG's, TX's and AA did not cross-react (< 0.03%); cross-reactions with LTC₄ and LTD₄ were also insignificant (0.03%). Minor cross-reactions were observed with the diastereoisomers of 6-trans LTB₄ (3.3%) and 12-HETE (2.0%). The w-oxidation products of LTB₄ did not cross-react (Salmon, Simmons & Palmer, 1982b). Table 4.5 shows a list of all the observed cross-reactivities. In addition, when purified extracts from PMN-incubations with A23187 were run on RP-HPLC, only one major peak of immunoreactive material (co-eluting with authentic LTB₄) was detected. After allowing for procedural losses, the concentration of LTB₄ determined by RIA after extraction and purification was comparable to that obtained from direct RIA (Salmon, 1985).

Compound	% Cross-reactivity
LTB ₄	100
LTC ₄	0.03
LTD ₄	0.03
Diastereoisomers of 5,12-di-OH-6,8,10-trans-14-cis-eicosatetraenoic acid	3.3
Diastereoisomers of 5,6-di-HETE	1.6
5(S), 12(S)-diHETE	0.14
5-HETE	0.03
12-HETE	2.0
11-HETE	0.04
15-HETE	<0.03
PGE ₂ and other PG's	<0.03
Arachidonic acid	<0.03

Table 4.5 Cross-reactivity of LTB₄ antiserum with LTB₄ and related compounds

4.5 Measurement of PGI₂-Metabolite Production in PMN's

4.5 (a) Assay procedure

In one study, the supernatants generated by the A23187-stimulation of PMN's were also assayed for PGI₂-M's using a [¹²⁵I] assay system purchased from Amersham. All reagents were diluted in assay buffer (phosphate buffer 0.05M, pH 6.8 containing 0.05% BSA) as instructed by the kit method. A series of working standards was prepared by serial dilution in the range 31-4000 pg/ml. The tracer, a ¹²⁵I-iodotyrosine

methyl ester (2 μ Ci) was diluted in 10ml assay buffer. Lyophilized antiserum was reconstituted by adding 10ml of distilled water.

Polypropylene tubes (12mm x 75mm, Luckham) were labelled in duplicate for TC, NSB, B₀, standards and samples. Reagents were added to the tubes as shown in Table 4.6. A sample of pooled supernatant which was stored in small aliquots at -70°C was included in each assay to assess reproducibility. All tubes contained a total volume of 500 μ l.

After addition of reagents the tubes were vortex mixed and incubated overnight at 4°C. The following morning, all tubes (except TC), received 500 μ l of 'Amerlex-M' second antibody which was provided with the kit. This, being an anti-body to the 6-keto-PGF₁ α antiserum, bound antibody-bound tracer. The tubes were then vortexed and left to incubate at room temperature for 10 minutes. After centrifugation (2000g for 10 minutes), the supernatants were decanted to leave the antibody -bound fractions which were drained and counted in a gamma-counter for 1 minute each.

Reagent	TC	NSB	B ₀	Standards	Samples
Buffer	-	400	300	200	200
Standards	-	-	-	100	-
Samples	-	-	-	-	100
¹²⁵ I-6-keto PGF ₁ α	100	100	100	100	100
Antiserum	-	-	100	100	100
MIX AND INCUBATE					
Amerlex-M second antibody	-	500	500	500	500

Table 4.6 Protocol for RIA of PGI₂-metabolites in PMN supernatants (Amersham ¹²⁵I assay)

(All volumes in microlitres)

4.5 (b) Assay characteristics

The standard curve obtained over six assays is shown in Fig. 4.7. Total counts were approximately 30,000 cpm and the added antiserum bound roughly 40% of the added tracer; 50% inhibition of binding occurred at 20 pg of added standard. The lower limit of detection of the assay was <31 pg/ml. Inter- and intra- assay coefficients of variation were 9.8% and 4.1% respectively. The specificity of the antiserum is outlined in Table 4.7.

Compound	% Cross-reactivity
6-keto-PGF ₁ α	100
PGF ₂ α	0.8
PGE ₂	0.5
PGF ₁ α	1.0
PGE ₁	0.5
TXB ₂	<0.3

Table 4.7 Cross-reactivity of antiserum in Amersham kit with 6-keto-PGF₁α and related compounds.

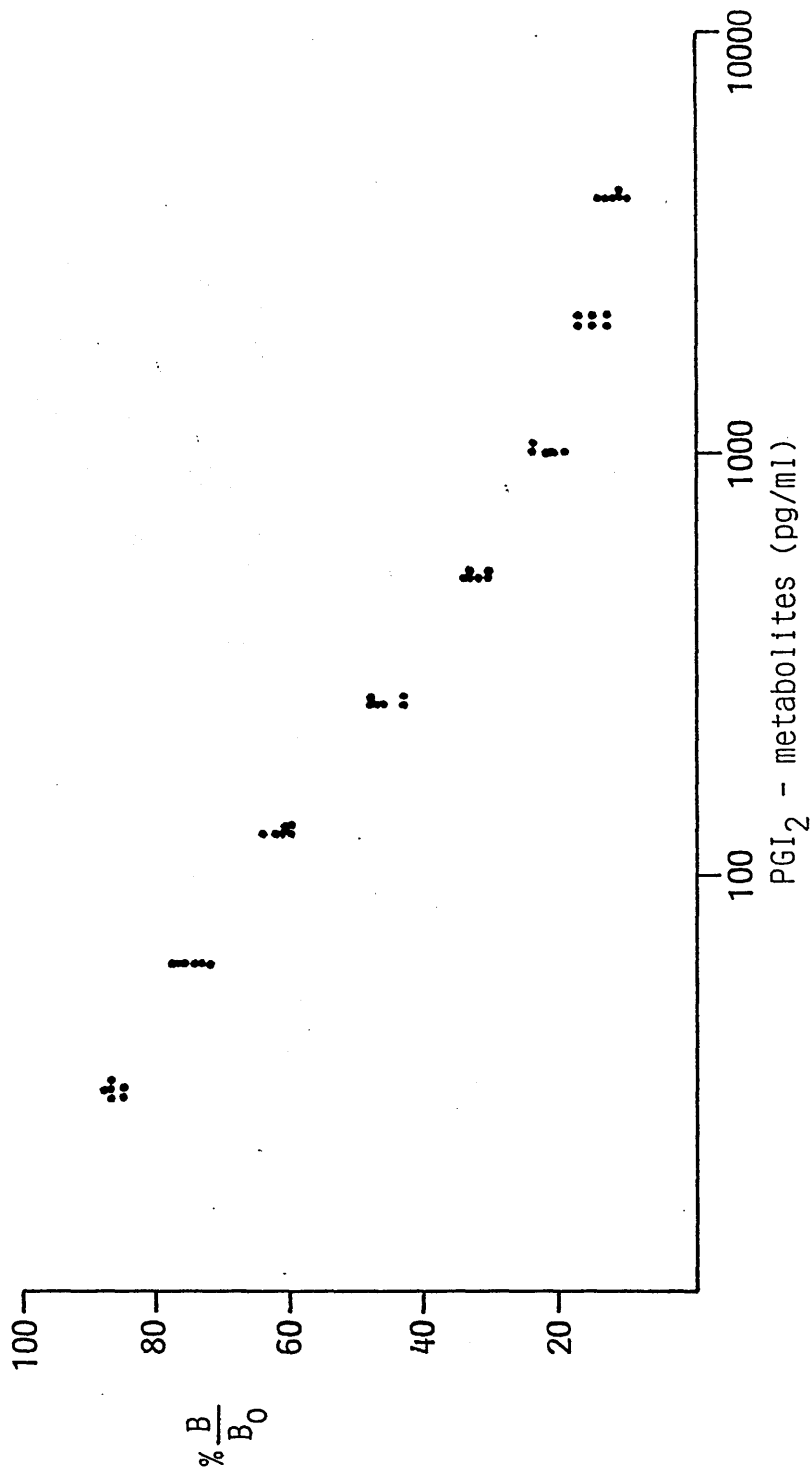


Fig. 4.7 Prostacyclin metabolite assay (Amersham Kit), Standard curve over 6 assays.

The RIA's described in the preceding sections of the chapter constitute the core of the methodology used in this thesis. Additional materials and methods which have been used are described elsewhere within the relevant sections. In particular, methods relating to the measurement of platelet sensitivity to PGI₂ and the use of umbilical artery rings to investigate the ability of plasma to support PGI₂-like activity are outlined in sections 5.1 and 5.2 respectively.

INFLAMMATORY VASCULAR DISEASE

5.1 RAYNAUD'S PHENOMENON

5.1 (a) General introduction

Raynaud's phenomenon (RP) first described over 125 years ago by Maurice Raynaud (1862), is constriction of the small blood vessels in one or several fingers or toes upon exposure to cold or sometimes to emotion. The classical manifestations are pallor of the affected digits, followed by cyanosis and rubor, although this triphasic colour change is not now thought to be essential for diagnosis. It exists in various degrees of severity in 5-10% of the population and, if severe, can cause pain, digital ulceration and even gangrene. Women are affected nine times more commonly than men (Belch & Sturrock, 1983).

There is a wide spectrum of disorders with which RP may be associated (Table 5.1). In recent years, the development of more sensitive laboratory tests has revealed that greater than 50% of patients with RP have an associated systemic disease. Over the years the nomenclature used to describe the various sub-classes of the disease has been inconsistent and confusing. In this work, the terminology used sub-divides RP into Raynaud's disease (RD), i.e. RP with no associated systemic disease and Raynaud's syndrome (RS), i.e. RP with associated disorder. These are also known as "primary" and "secondary" Raynaud's respectively. Long-term studies have shown that RD may be the precursor of systemic illness by many years.

Hand and digital blood flow is diminished during an attack of Raynaud's, although the mechanism whereby this is produced is unclear. Table 5.2 shows a list of factors which have been suggested as being important in the pathogenesis of RP. Naturally, many of the older studies were concerned with the vessel wall and the

A. Immunological and connective tissue disorder

Progressive systemic sclerosis
Systemic lupus erythematosus
Rheumatoid arthritis
Mixed connective tissue disease
Dermatomyositis and polymyositis
Hepatitis B antigenaemia

B. Occupational

Vibration white finger
Vinyl chloride workers
Cold injury (frozen food packers)
Ammunition (nitrate) workers

C. Obstructive arterial disease

Atherosclerosis
Thoracic outlet syndromes
Emboli

D. Drug induced

Beta blockers
Anti-migraine drugs
Cytotoxics
Bromocriptine
Sulphasalazine

E. Miscellaneous

Cold agglutinins
Cryoglobulinaemia
Thyroid disease
Neoplasm
Chronic renal failure
Idiopathic Raynaud's disease

Table 5.1 Disorders with which Raynaud's Phenomenon is associated

development of spasm; increased sympathetic tone, local arterial sensitivity to cold and deposition of immune complexes in the vessel wall have all been considered (Belch & Sturrock, 1983). However, more recently, attention has focussed on the contribution to decreased blood flow made by biochemical and cellular abnormalities in the blood e.g. leukocyte adherence, plasma viscosity, red cell deformability and platelet aggregation (Belch *et al*, 1987a).

Plasma	Vessel Wall	Blood Cells
Hormones	Sympathetic innervation	Leukocyte
Viscosity	Immune complexes	Erythrocyte
(fibrinogen, proteins)	Fibrinolysis	Platelet
	Factor VIII von W.factor	
	Prostacyclin	

Table 5.2 Suggested factors important in the pathogenesis of Raynaud's Phenomenon

Treatment of more severe Raynaud's whether by drugs or sympathectomy, has often been unsatisfactory in the past. However, recent work has shown that PGE₁ (Martin *et al*, 1980) and PGI₂ (Belch *et al*, 1981; Dowd *et al*, 1982) may be useful in the management of this disorder. When given intravenously, these prostaglandins have been shown to increase blood flow to the hands and to elevate hand temperature for up to six weeks after infusion.

5.1 (b) Plasma levels of PGI₂-metabolites in Raynaud's phenomenon

5.1 (b) (i) Patients and methods

Since PGI₂ is of benefit in the treatment of Raynaud's, the aim of this study was to measure endogenous stable metabolites of PGI₂ in 110 patients with RP of varying aetiology i.e. 40 patients with primary Raynaud's disease (RD), 28 patients with suspected secondary disease (SS) and 42 with secondary Raynaud's syndrome (RS); SS patients have some, but not all of the criteria required for diagnosis of a connective tissue disorder. Results were compared with 50 age, smoking and sex-matched controls.

Plasma samples were prepared and PGI₂-metabolites measured by RIA as previously described.

5.1 (b) (ii) Results

It can be seen from Table 5.3 that PGI₂-M levels were found to be elevated in patients with SS and RS compared to normal controls. In patients with RD alone and no other symptoms, levels were normal. Although the most severe group (RS) demonstrated the highest PGI₂-M's, no correlation could be found between PGI₂-M's and frequency or duration of spasm attacks as recorded in patient diaries over the two week period immediately after blood sampling.

NORMALS	RD	SS	RS
13 ± 4	10 ± 2	36 ± 7 *	55 ± 6 *

* p < 0.01 (Mann-Whitney)

Table 5.3 Plasma PGI₂-metabolite levels (pg/ml) in patients with Raynaud's phenomenon and normal controls (mean ± SD)

5.1 (b) (iii) Discussion

Given that intravenous infusion of PGI₂ is of benefit in the treatment of Raynaud's RS patients might have been expected to produce inadequate amounts of PGI₂. However, this study has shown increased levels in patients with SS and RS compared to normal controls. This finding is supported by other work. McLaren (1986) has reported increased levels of PGI₂-metabolites in diabetics with vascular disease and in patients with cardiovascular disease. In addition, PGE-metabolites have been shown to be elevated in RP (Horrobin, Jenkins & Manku, 1983).

There are a number of possible explanations for the increased levels of PGI₂ found in secondary Raynaud's. Firstly, this could be a compensatory mechanism. It is known that the endothelium produces PGI₂ in response to noxious chemical or mechanical stimuli (Ritter *et al*, 1983), this, repeated vasospastic attacks could lead to a chronic increase in PGI₂. If excess PGI₂ production is a compensatory mechanism, then infusions of PGI₂ given therapeutically would augment this.

A second possibility is that PGI₂ may be produced along with other PG's as part of the general picture of inflammation. Chronic re-exposure to increased levels may desensitize cells to the action of PGI₂ but the even higher levels achieved via infusion may be enough to allow the cells to respond normally.

The third possibility is that a cellular resistance to PGI₂ exists in RS. Prostacyclin increases intracellular cAMP which is then thought to cause a negative feedback inhibition of PG production (Horrobin, 1980). However, should this increase in cAMP not occur because of a cellular resistance, then PGI₂ production would continue unchecked. Although "resistance" could be due to an inherent receptor defect or decreased number of receptors, it could be as a result of receptor blockage by immune complexes (Moretta, Mingari & Romanzi, 1978). A pharmacological dose of PGI₂ could overcome this resistance and has been shown to normalize cAMP levels in lymphocytes from patients with systemic sclerosis (Kirby *et al*, 1980).

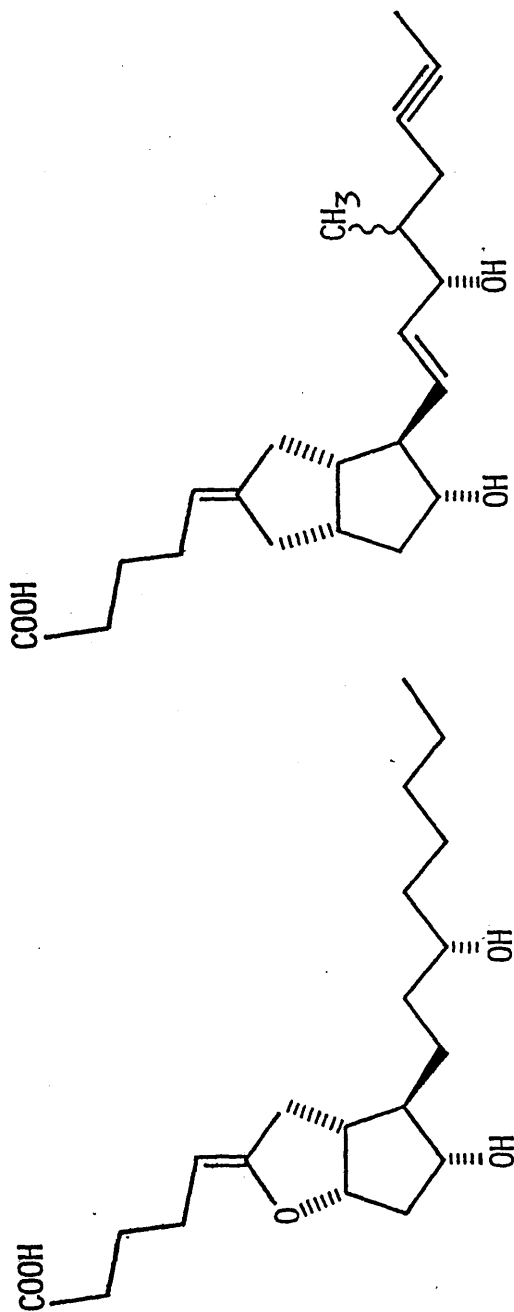
5.1 (c) Platelet sensitivity to a prostacyclin analogue in systemic sclerosis *in vitro*

5.1 (c) (i) Introduction

Progressive systemic sclerosis (PSS), is a connective tissue disease characterized by fibrotic changes involving predominantly skin and blood vessels. Over 80% of patients with PSS exhibit RP. The pathogenesis is still incompletely understood, but it has been suggested that the changes in vascular endothelium seen in the disease may be associated with an inflammatory immune response (Kahaley & Leroy, 1979). The vascular damage thus produced may contribute to the development of RP.

The previous study showed elevated levels of plasma PGI₂-metabolites in RS. One theory to explain this was that there might be abnormal sensitivity to PGI₂ in RS cells. The aim of this study was to evaluate the PGI₂ sensitivity of systemic sclerosis platelets compared to that of age- and sex-matched controls. As PGI₂ is unstable, thereby presenting methodological difficulties such as dose standardisation a stable carbacyclin analogue of PGI₂, ZK 36.374 (Schering Chemicals, Ltd) was used (Fig. 5.1). This analogue, also known as iloprost, has been shown to act via the same platelet receptors as PGI₂ both *in vitro* and *in vivo* (Sturzebecher & Losert, 1987). It is more potent than PGI₂ as an antiplatelet agent, but induces less vasodilatation (Schorr *et al*, 1981).

In addition, the PGI₂-sensitivity of platelets from patients receiving either CL115,347, a transdermally absorbed PGE analogue (Cyanamid International) or oral nifedipine, a calcium antagonist (Bayer) was determined. Both drugs are effective in the treatment of RP.



Iloprost

Prostacyclin

Fig. 5.1 Structure of prostacyclin and its stable analogue Iloprost
(ZK.36 374)

5.1 (c) (ii) Patients

Twenty-four patients (mean age 45 ± 11 years) with classical PSS, as defined by the American Rheumatism Association (A.R.A.) criteria, were enrolled in the study. Of these, 17 patients were on no drug therapy known to interfere with PG metabolism; 4 patients from this group subsequently received transdermal CL115,347 and were therefore sampled twice. Of the 7 patients remaining, one was sampled while receiving the transdermal PG (1mg/24h) and 6 were taking oral nifedipine (10 mg t.i.d.). Again, patients sat in a warm room for at least 30 minutes before blood sampling.

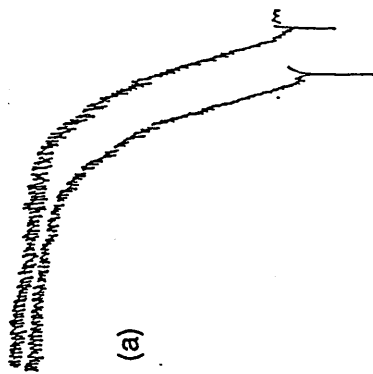
Eighteen matched controls (age 44 ± 9 years) were also sampled.

5.1 (c) (iii) Methods

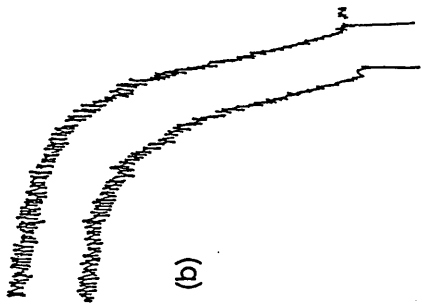
Blood (9 ml) was drawn from the antecubital fossa and anticoagulated with 1 ml of 3.2% trisodium citrate. The blood was carefully mixed and centrifuged (150 g) for 7 minutes at room temperature to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was then prepared by centrifugation of the remaining blood at 2000 g for 15 minutes. The platelet count in the PRP was standardized to approximately $250 \times 10^9/l$ by diluting with autologous plasma.

Platelet aggregation (PA), was studied in 200 μ l of PRP according to the method of Born (1962), using a two channel Malin's aggregometer connected to a chart-recorder. The aggregometer works on the principle of change in light absorbance in the PRP. Addition of an aggregating agent causes the platelets to clump thereby decreasing the turbidity of the sample and allowing more light to pass through. The transmitted light is converted to an electrical signal which results in an aggregation curve (Fig. 5.2) being generated on the chart recorder. The aggregation rate is calculated by measuring the gradient at the steepest part of the curve (arbitrary units).

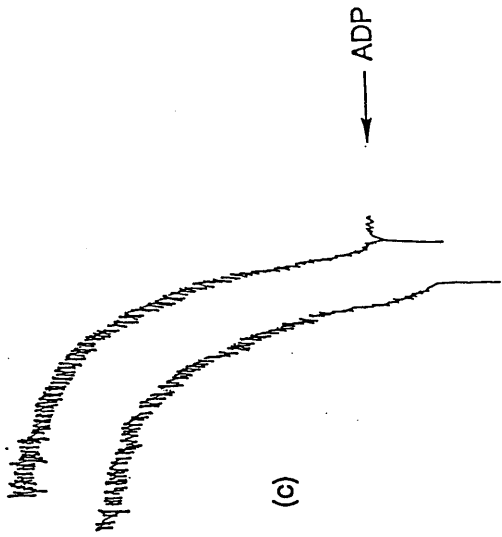
PSS PATIENT



(a)

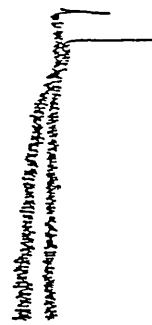


(b)

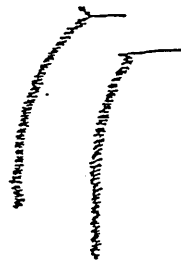


(c)

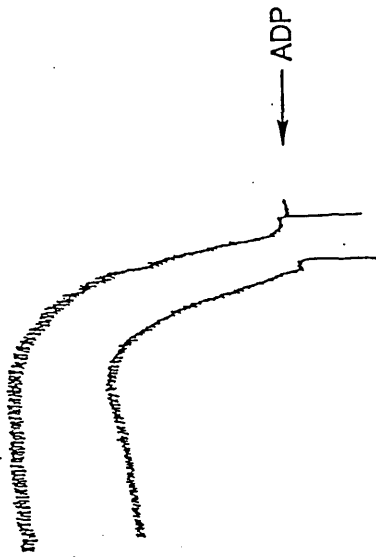
NORMAL CONTROL



(a)



(b)



(c)

Fig 5.2 Effect of Iloprost at (a) 3ng/ml, (b) 2ng/ml and saline control (c) on platelet aggregation to ADP. Typical aggregation curves for PSS patient and normal control as measured on a dual channel aggregometer. (PSS= progressive systemic sclerosis)

Each of the PRP samples being studied was incubated (with continuous stirring) in turn with 100 μ l isotonic saline, 100 μ l of iloprost (2 ng/ml) and 100 μ l of iloprost (3 ng/ml) for 3 minutes at 37°C. The dilutions of iloprost were made in saline. Following the 3 minute incubation, each sample received 3 μ l of 2 μ M adenosine-5-diphosphate (ADP) (Sigma), as an aggregating agent. Each incubation was carried out in duplicate. The doses of iloprost were compatible with those intended for therapeutic use of the drug (Belch *et al*, 1984).

The difference in rate of aggregation (gradient) obtained between iloprost-treated and saline-treated samples was expressed as a percentage inhibition of the saline control.

5.1 (c) (iv) Results

The results obtained from this study are shown in Table 5.4 and graphically, in Figures. 5.3 and 5.4.

	% Inhibition of platelet aggregation	
	2 ng/ml iloprost	3 ng/ml iloprost
NORMALS	59 \pm 17	85 \pm 11
PSS	18 \pm 13 *	49 \pm 19 *
PSS + Rx	68 \pm 18	85 \pm 15

* significantly different from normal controls (p < 0.001, Mann-Whitney)

Rx = treatment (CL 115,344 or nifedipine)

Table 5.4 Platelet sensitivity to iloprost in patients with progressive systemic sclerosis (PSS) and normal controls (mean \pm SD)

Pre-incubation of normal PRP with 100 μ l of 2 ng/ml iloprost caused 59 \pm 17% of aggregation to ADP to be inhibited. However, when the same amount of iloprost

was added to the platelets from PSS patients on no treatment, only $18 \pm 13\%$ of PA was inhibited. The same pattern was repeated at the higher dose of iloprost (3 ng/ml), $85 \pm 11\%$ inhibition of PA for normal controls and $49 \pm 19\%$ for patients (Fig. 5.3). These results were statistically significant ($p < 0.001$, Mann-Whitney).

In contrast, the platelet sensitivity to iloprost in PSS patients on either nifedipine or CL115.347 was in the normal range ($68 \pm 18\%$ and $85 \pm 15\%$) (Fig. 5.4). In an effort to show that this difference was not due to a difference between the two groups of patients, but due to the effect of treatment, four of the patients originally on no treatment were given a 2-week course of CL115,347 and then re-tested. It can be seen from Fig. 5.5 that the initial abnormal sensitivity to iloprost was normalised by this treatment.

The baseline (saline only) rate of aggregation in this study demonstrated no significant difference between PSS patients and normal controls (12.9 ± 4.2 and 12.8 ± 3.6 , respectively).

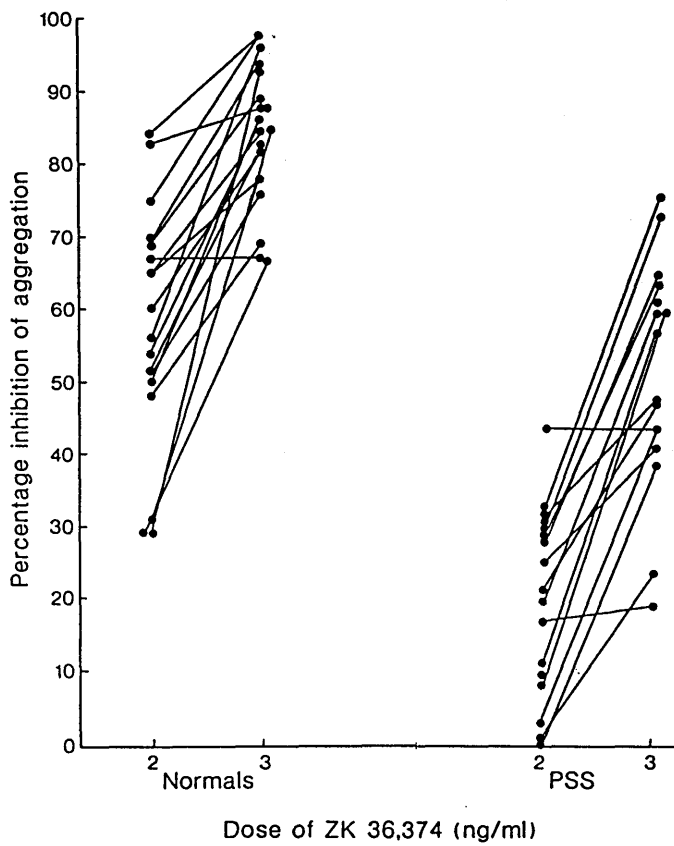


Fig. 5.3. Percentage inhibition of platelet aggregation curves by 2 and 3 ng/ml iloprost (ZK 36.374) in normal controls and PSS patients.

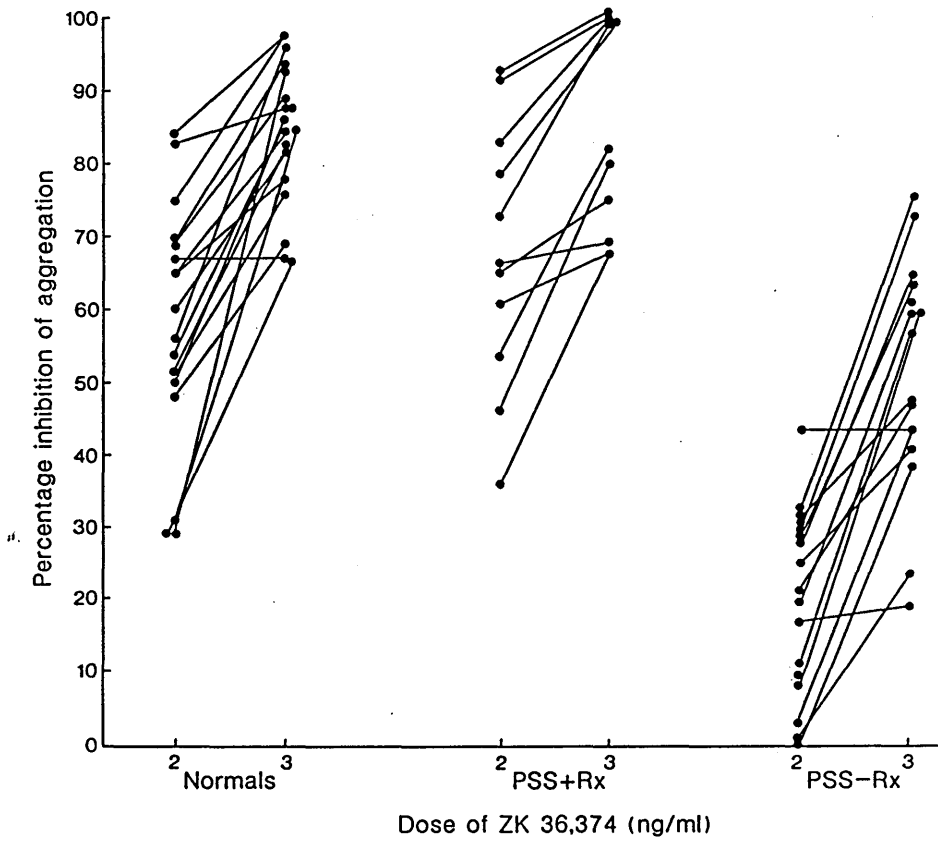


Fig. 5.4. Percentage inhibition of platelet aggregation by 2 and 3 ng/ ml iloprost (ZK 36.374) in normal controls, treated patients with PSS (PSS + Rx) and untreated patients with PSS (PSS - Rx)

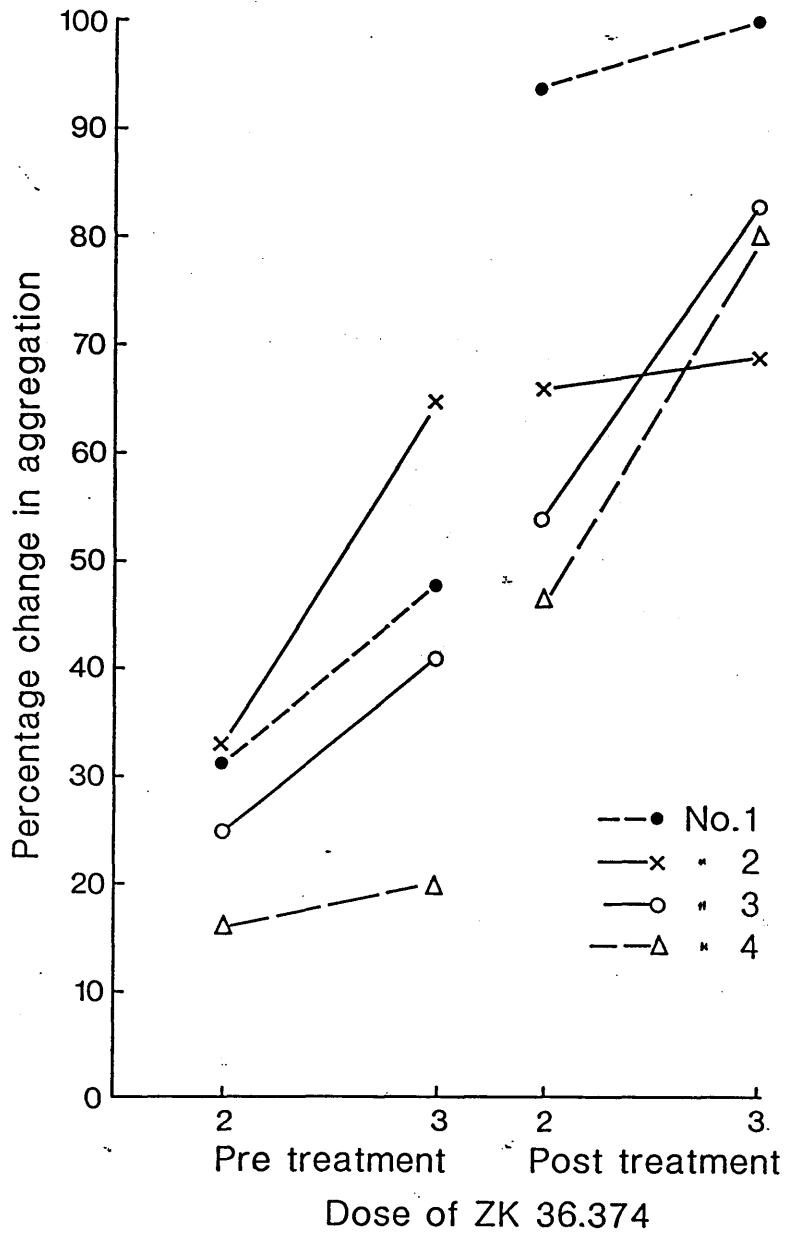


Fig. 5.5. Percentage inhibition of platelet aggregation by 2 and 3 ng/ml iloprost (ZK 36.374) in PSS patients before and after treatment with CL 115, 347.

This study demonstrated that the platelets of patients with progressive systemic sclerosis are somewhat resistant to the anti-aggregatory effects of the synthetic PGI₂ analogue iloprost. The platelets of patients with other vascular disorders have been shown to display a similar decrease in responsiveness to PGI₂ (Mehta & Mehta, 1980; Briel, Kieback & Lippert, 1984) and it is thought that this contributes to the clinical manifestations of ischaemia. Moreover, since PGI₂ sensitivity decreases even further at lower temperatures (Mikhailidis, Hutton & Dardona, 1981), aggregation of “desensitized” platelets in the microcirculation with the concomitant release of vasoconstrictor substances, could play an important role in the development of Raynaud’s phenomenon.

It appears that PG resistance may be a general phenomenon in PSS since both the lymphocyte (Kirby *et al*, 1980) and the macrophage (Whicher *et al*, 1984) in this disorder appear to display resistance to PG’s. Since PGI₂ levels have been shown to be elevated in PSS patients, it may be that a “tachyphylaxis” or “down-regulation” is occurring. A possible analogous situation has been observed in pregnancy, where PGI₂ resistance develops physiologically as the plasma levels of PGI₂ increase (Briel *et al*, 1984) and also during long-term intra-arterial infusions of PGI₂ (Sinzinger *et al*, 1981). Although in this study, the transdermally absorbed PGE appeared to normalize the response of the platelets to iloprost, it may be that pharmacological doses of PG’s only allow resistant cells to respond normally for a limited period of time, before tachyphylaxis re-occurs.

The calcium antagonist nifedipine was also shown to normalize the platelet sensitivity to iloprost. Nifedipine is known to decrease platelet activation and the subsequent release of TXA₂ either directly by its action on platelets or indirectly by its action on smooth muscle, or both (Malamet *et al*, 1985). Since TXA₂ is antagonistic to the action of PGI₂, any decrease in this mediator may enhance the action of available PGI₂.

The intracellular response to PGI₂ is mainly, if not exclusively, mediated by stimulation of the receptor-coupled adenylate cyclase system which causes an increase in cAMP and subsequently, a rise in intracellular calcium. The mechanism whereby decreased sensitivity occurs has been studied in a neuronal hybrid cell line where prolonged culture in the presence of iloprost results in a substantial loss of sensitivity to PGI₂ and PGE₁ (Leight & MacDermot, 1985). It was discovered that desensitization in this case was accompanied by a loss of high affinity receptors. It seems likely, therefore, that the phenomenon in general may be associated with “exhaustion” of PG receptors.

In conclusion, therefore, a resistance of the PSS platelet to the anti-aggregatory effects of PGI₂ has been demonstrated and could be an important factor in the development of digital ischaemia. This observation supports the hypothesis of a generalized cellular resistance in PSS, which, if it is extrapolated to other cell types, could explain some of the additional manifestations of this disease. For instance, in PSS there is augmented growth of collagen-producing fibroblasts (Botstein, Sherer & Leroy, 1982) which are normally inhibited by PGI₂. If these fibroblasts are resistant to the negative control of PG, this might explain the increased collagen production seen in this condition.

5.1 (d) Leukotriene B₄ production from PMN's in Raynaud's phenomenon

5.1 (d) (i) Introduction

Although platelets, RBC's and plasma constituents have long been recognized as being involved in the maintenance of blood flow and in the development of thrombosis, relatively little attention has been given to the possible contribution of leukocytes. Recent studies however, have noted that subjects at risk from developing ischaemic events have increased leukocyte numbers (Kostie, Turkevich & Sharp, 1984; Lowe *et al*, 1985). In addition, histopathological studies of ischaemic tissue have shown adhesion and aggregation of leukocytes, mainly PMN's, to the endothelium and migration of cells into the necrotic area (Simpson & Lucchesi, 1987). These PMN aggregates may physically obstruct blood flow and cause further tissue damage by the release of free radicals and other active substances.

Leukotriene B₄ has been shown to play an important role in the regulation of PMN behaviour. Hence, PMN's and their potential to produce LTB₄ could be important in the development of vascular occlusion and RP. The aim of this study was to investigate LTB₄ generation of PMN cells in patients with secondary RP compared to that in normal controls. However, in this instance, two distinct groups of secondary Raynaud's patients were examined; those with RP associated with systemic sclerosis and another group suffering from RP of occupational origin, also known as Vibration-Induced White Finger Syndrome (VWF). This condition affects a large proportion of the workforce exposed to vibrating tools (pneumatic drills, chain-saws, polishing tools), and can be of such severity that since 1985 it has been a prescribed disease eligible for compensation (Taylor, 1985). As yet, the pathophysiology is not fully known, but the manifestations closely resemble the spontaneous vasoconstrictive phenomena induced by exposure to cold in RP.

5.1 (d) (ii) Patients and methods

Twenty-six patients with grade 3 VWF as classified by the Taylor-Pelmeur scale (Table 5.5) were enrolled in the study. All were, or had been, employed as boilermakers exposed to vibrating machines such as chippers, grinders and buffs. Fifty five patients with secondary RP associated with systemic sclerosis were also studied. As VWF is predominantly a disease of men and systemic sclerosis a disease of women, these two groups could not be matched for sex. Results were compared to those of 59 age- and smoking-matched normal controls who had not been exposed to vibration. The comparability of the three groups is outlined in Table 5.6.

None of the subjects had taken any medication known to interfere with the test and smokers were asked to refrain from smoking from 12 midnight prior to the day of sampling.

Blood was collected and PMN's prepared and stimulated with CaI A23187 as previously described. The resultant supernatants were assayed for LTB₄ using RIA, also as previously described (section 4.4).

TAYLOR-PELMEAR SCALE

STAGE	CONDITION OF DIGITS	WORK AND SOCIAL INTERFERENCE
0	No blanching of digits	No complaints
OT	Intermittent tingling	No interference with activities
ON	Intermittent numbness	No interference with activities
1	Blanching of one or more fingertips with or without tingling and numbness	No interference with activities
2	Blanching of one or more fingers with numbness. Usually confined to Winter	Slight interference with home and social activities. No interference at work.
3	Extensive blanching. Frequent episodes Summer as well as Winter.	Definite interference at work, at home and with social activities. Restriction of hobbies.
4	Extensive blanching. Most fingers; frequent episodes Summer and Winter	Occupation changed to avoid further vibration exposure because of severity of signs and symptoms.

Table 5.5 Classification of the stages of the vibration syndrome (Taylor-Pelmeary)

	VWF	PSS	Controls
Numbers	26	55	59
Age (median (range))	49 (27-65)	43 (28-68)	35 (22-60)
Sex M:F	26:0	8:47	33:26
Smokers	17	29	25
Duration of symptoms (years, median (range))	5 (3-20)	9 (2-15)	- -
Duration of vibration exposure (years, median (range))	29 (5-49)	- -	- -

Table 5.6 Comparability of VWF, PSS and control groups

(VWF = vibration white finger
PSS = progressive systemic sclerosis)

5.1 (d) (iii) Results

Results (median (interquartile range)), are shown in Table 5.7 and graphically in Fig. 5.6.

PSS	VWF	Normal Controls
24.5 *	26.2 *	20.0
(21.0 - 29.5)	(22.0 - 28.8)	(15.0 - 25.8)

* $p < 0.001$ Mann-Whitney

Table 5.7 LTB₄ levels (ng/10⁶ cells) in stimulated PMN's from PSS, VWF and normal controls (median (IQR))

Leukotriene B₄ production from stimulated PMN's was increased in patients with VWF and PSS (both $p < 0.001$, Mann-Whitney) compared to normal controls. Comparison of the LTB₄ produced by PMN's from age-matched male and female normal subjects showed that there was no inherent difference in levels between the two sexes (Fig. 5.7).

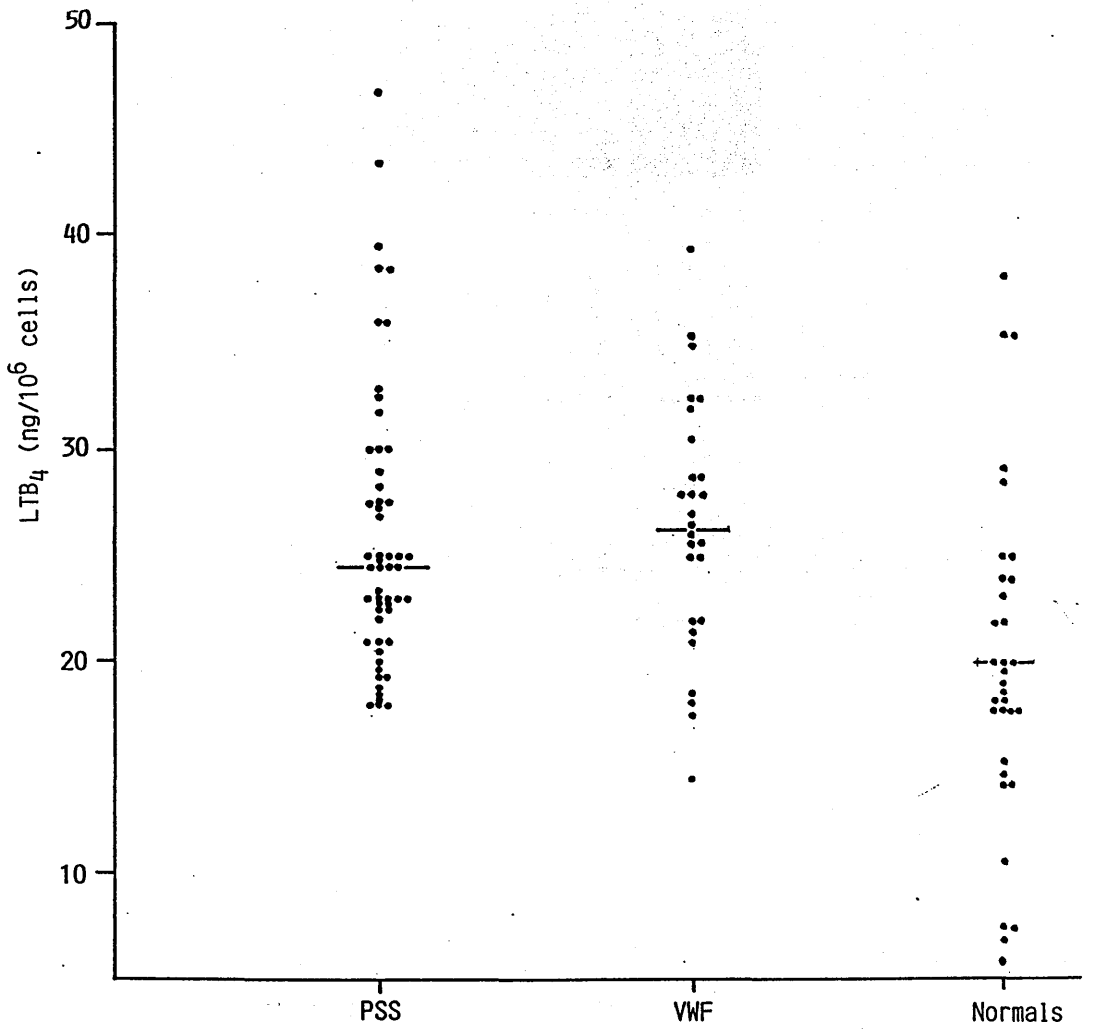


Fig. 5.6 Leukotriene B₄ production from A23187-stimulated PMN's in PSS,VWF and normals

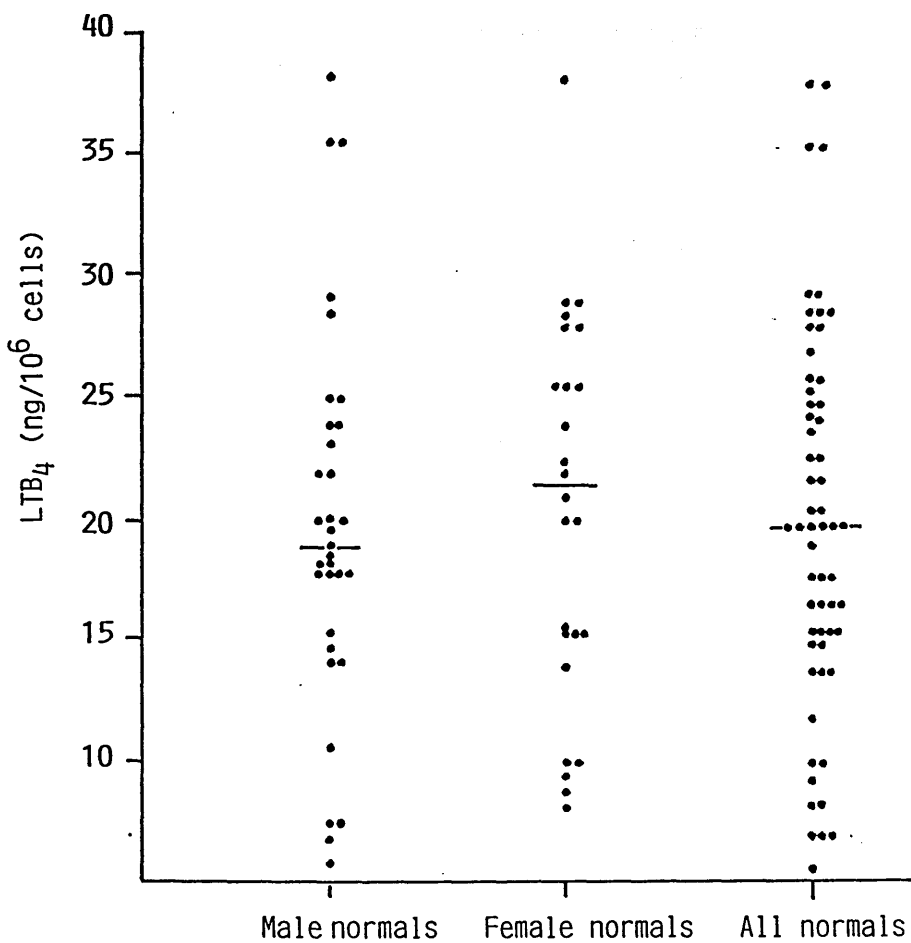


Fig. 5.7 Leukotriene B₄ production from A23187-stimulated PMN's of age-matched male and female normal controls

This study has demonstrated for the first time, an increased production of LTB₄ from PMN's of individuals with Raynaud's phenomenon secondary to systemic sclerosis and VWF. This observation appears to be consistent with the hypothesis that a generalised loss of sensitivity to PGI₂ occurs in PSS cells, since decreased sensitivity of PMN's could lead to loss of the suppressive effect of PGI₂ on LTB₄ synthesis (Claesson, Lundberg & Malmsten, 1981), resulting in increased levels.

Leukotriene B₄ has previously been shown to be a potent PMN chemotactic and aggregating agent, as well as a mediator of increased adhesion of PMN's to the endothelial lining of blood vessels. PMN's from PSS patients were, in fact, observed to be abnormally adherent during preparation. All of these effects could contribute to the development of vascular occlusion. In addition, PMN's activated in this way have been implicated in vessel wall damage via the release of other substances including lysosomal enzymes and reactive metabolites of oxygen.

It is unlikely in either systemic sclerosis or VWF, that increased potential for LTB₄ production is a primary event in the pathophysiology of these conditions. The initial effect of vibration on the digital vasculature is to stimulate proliferation of the medial coat and subsequently, the intima of the arterial wall (Belch *et al*, 1989). Although this may not be severe enough to cause arterial occlusion, the abnormal vessel wall may activate PMN's as they flow past. This would then contribute to decreased blood flow as described and may enable a vicious cycle of ischaemia, reperfusion, vessel damage, PMN activation then further ischaemia, to be set up.

The finding that LTB₄ is increased in VWF supports other evidence suggesting that this disease is a true secondary Raynaud's syndrome. Other abnormalities, such as decreased RBC deformability, increased whole blood viscosity and higher plasma factor VIII von Willebrand factor have been found in VWF as well as RS (Belch *et al*, 1989).

In summary, the increase in LTB₄ production seen in PMN's from systemic sclerosis and VWF patients could be relevant to the development of RP. The increase in LTB₄ is unlikely to be a primary event in either disease, but does suggest a common mechanism in part of their pathophysiology.

5.1 (e) Effect of transdermal iloprost on production of leukotriene B₄ from PMN's in normal volunteers

5.1 (e) (i) Introduction

The intravenous infusion of prostacyclin has proven beneficial in the treatment of patients with Raynaud's syndrome (Belch *et al*, 1981). However, intravenous administration entails some degree of inconvenience to the patient as it requires hospital admission. Although the synthetic analogue of PGI₂, iloprost, is chemically more stable than PGI₂, studies of orally administered iloprost have shown that the drug is rapidly metabolized when given in this way (Krause, Skuballa & Schulze, 1983). Any increase in dosage may merely increase side-effects.

The clinical aim of this study was to investigate the possibility of using transdermally applied iloprost, since this could be more easily and continuously applied. It was necessary in the first instance, to try the method on normal volunteers and this provided an opportunity to study the possible effect of iloprost on LTB₄ production by PMN cells *ex vivo*. This was of particular interest in view of results from the previous study which showed that LTB₄ production from PMN's was increased in RS.

5.1 (e) (ii) Study design and methods

Eight normal volunteers were enrolled. A grid (10 x 10 cm) was drawn on the skin of the back and different doses of iloprost, diluted in 70% ethanol, were applied using a fine paint brush. The area was left to dry and then occluded. Two volunteers received 0.37 µg of iloprost, two 0.57 µg, two 0.75 µg and two 1.50 µg. Subjects were sampled prior to the start of the study, and again at 1, 3, 6, 24, 48 and 72 hours later. The dose-pair receiving the highest dose of iloprost (1.50 µg) were also sampled at 12 hours. Two "control" volunteers, receiving no iloprost were to be sampled at intervals throughout the day to check for the effect of diurnal variation on blood tests. However, due to difficulties encountered in the bleeding of one of these subjects, only one "control"

was sampled. Blood was collected, PMN's isolated and after Ca ionophore stimulation, the resultant supernatants assayed for LTB₄ using RIA as previously described.

5.1 (e) (iii) Results

The LTB₄ levels determined by RIA in PMN's sampled throughout the period of transdermal application of iloprost are shown in Fig. 5.8. The results obtained are difficult to summarize for although there appears to be good correspondence in change in LTB₄ levels between dose-pairs, it is difficult to assess a clear dose effect on LTB₄ by iloprost. At all doses, a "spike" of increased LTB₄ production was seen at 0-3 hours, although this varied in magnitude. In all but one case, LTB₄ levels then appeared to fall below baseline level at some stage in the next 72 hours; this fall was earliest and most pronounced in the pair which received the highest dose of iloprost.

Levels of LTB₄ in the cells of the control subject remained essentially unchanged throughout period of measurement (Fig. 5.8).

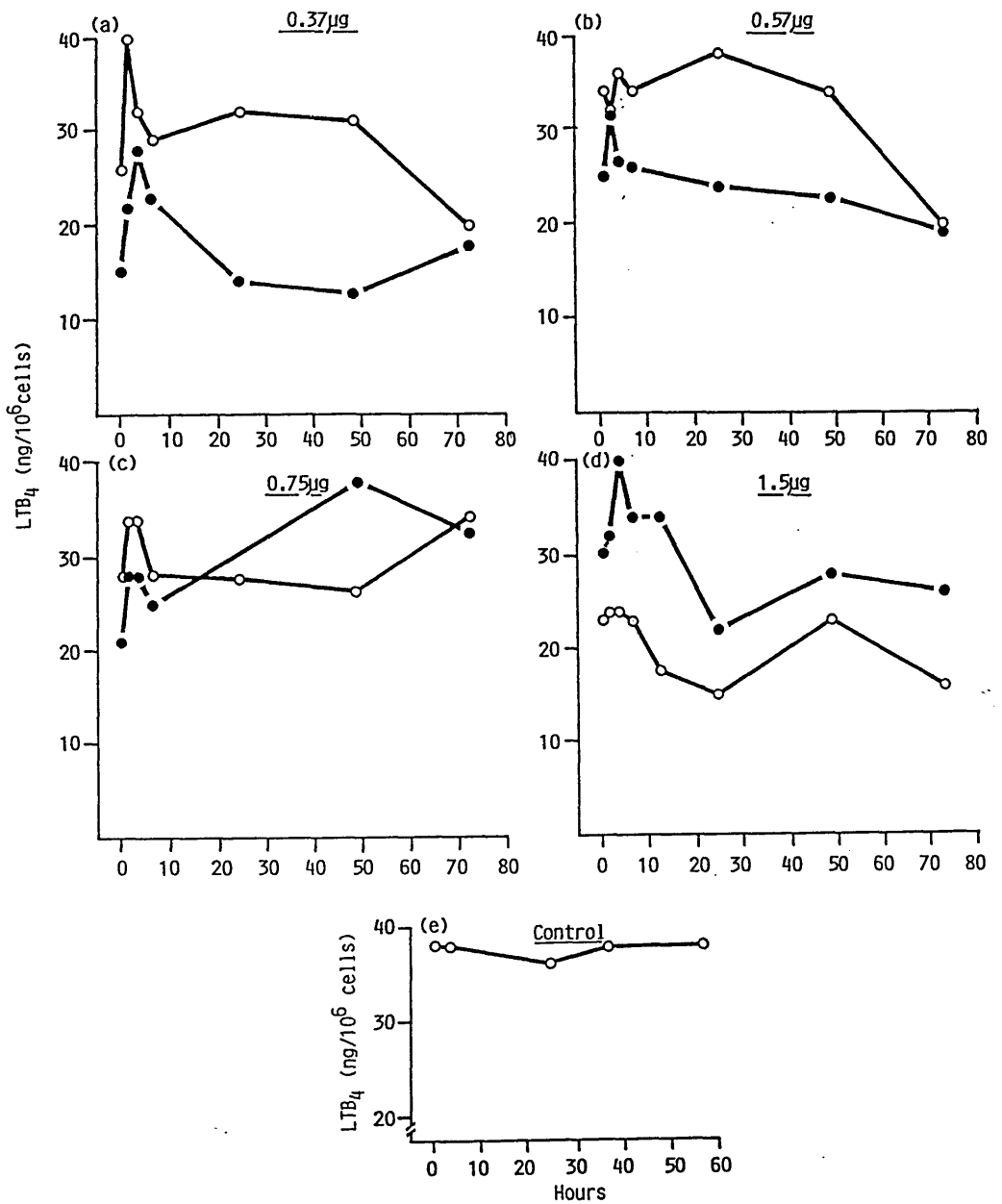


Fig. 5.8 Effect of transdermal iloprost at (a) 0.37 μg, (b) 0.57 μg, (c) 0.75 μg and (d) 1.5 μg on leukotriene B₄ production from stimulated PMN's in pairs of normal volunteers. One control volunteer (e) received no iloprost.

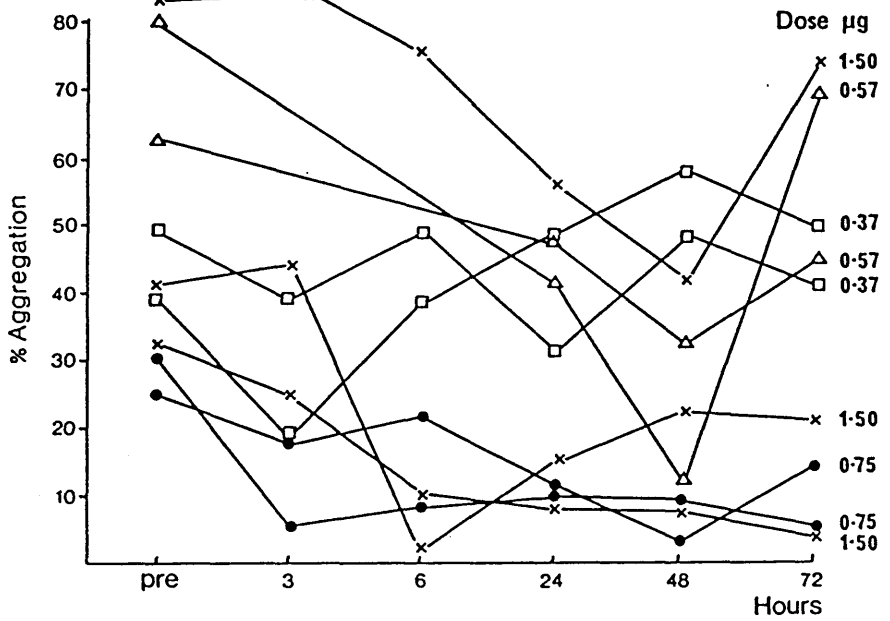


Fig. 5.9. Effect of transdermal iloprost on whole blood platelet aggregation in normal volunteers.

(Reproduced with permission from Belch *et al*, 1987b)

Drugs administered transdermally can be given long-term and patient acceptance is good. The aim of this study was to determine the efficacy of iloprost administered in this way. Parallel measurements of whole blood platelet aggregation made in the same study (Belch *et al*, 1987b), showed that the transdermally applied iloprost had a definite systemic effect since a decrease in platelet aggregation was observed at all doses and was more prolonged at the higher doses (Fig. 5.9)

Some subjects experienced a transient (but clinically insignificant) fall in blood pressure during the treatment, but other side-effects which potentially include nausea, flushing and headache, were minimal.

The reason for the initial increased production of LTB₄ observed in the first 3 hours is uncertain. However, as previously described (section 2.9), PG's have a dual effect on inflammation followed by a later suppressant effect due to elevated cAMP levels. It may be that the immediate effect of iloprost in this instance (either directly or indirectly) was PMN activation resulting in an increased potential for LTB₄ production followed by a decrease potential as cAMP levels were increased. Schillinger (1984), in early pharmacological studies of iloprost, noted that serotonin release from adherent platelets *in vitro* initially increased and then decreased after treatment with iloprost or prostacyclin. No explanation could be given for this increase at that time.

Whatever the mechanism, if this transient increased potential for LTB₄ production is a real phenomenon *in vivo*, then it may be an undesirable effect in Raynaud's patients. However, results also indicate that in the longer term (in the next few days) and at the higher doses of iloprost, PMN's may actually exhibit a decreased potential to synthesize LTB₄. This would seem to support other work, carried out *in vitro*. Claesson, Lundberg & Malmsten (1981), have demonstrated a clear inhibition of LTB₄ release from activated PMN's at pharmacological doses of PGI₂. In addition, Belch *et al* (1987c) have shown that iloprost inhibits PMN adhesion and aggregation *in vitro*, both functions mediated by LTB₄.

In summary, although the results from this study suggest that iloprost may have a suppressive effect on LTB₄ production in PMN's *ex vivo*, it's beneficial anti-platelet and vasodilatory effects are likely to be the most important in giving improved blood flow in RS patients.

5.1 (f) Effect of iloprost on LTB₄ production from PMN's *in vitro*

5.1 (f) (i) Methods

In view of the results from the previous study, it was decided to look at the *in vitro* effect of iloprost on LTB₄ production from normal PMN's. Polymorpho-nuclear leukocytes were isolated in the usual way and 5×10^6 cells were resuspended in 0.8 ml of HEPES/HBSS in polypropylene micro-centrifuge tubes (Eppendorf). To these suspensions were added either 0.1 ml of phosphate buffered saline (PBS) or 0.1ml iloprost/PBS dilutions to give final concentrations of 0.2, 2.0 and 200 μ M iloprost. Three tubes were set up for each concentration of iloprost. The cells were then pre-incubated at 37°C for 20 minutes by gentle rolling of the tubes on an automatic roller. The tubes were subsequently removed from the rollers and, after addition of 0.1 ml CaI A23187 (10 μ g/ml), were further incubated for 5, 10 or 20 minutes with vigorous shaking in a 37°C water bath. The reaction was terminated by centrifugation and the supernatants removed for measurement of LTB₄ by RIA as previously described.

It must be emphasized that even the minimum concentration of iloprost used in these *in vitro* experiments (0.2 μ M) was approximately one hundred times the plasma concentration of iloprost likely to be achieved in the transdermal study. The highest concentration used in the *in vitro* study (200 μ M) was approximately equal to the concentration which is achieved during I.V. infusion of iloprost in RS patients and which had already been shown to be clinically effective (Belch *et al*, 1981).

5.1 (f) (ii) Results

The results are shown in Fig. 5.10. Each point is the mean (SEM) of five experiments. Iloprost-treated PMN's gave the same pattern of LTB₄ production as control cells i.e. LTB₄ synthesis peaked after 5 minutes of incubation with CaI A23187 and thereafter declined. No significant difference in LTB₄ production was found between control cells and those treated with iloprost.

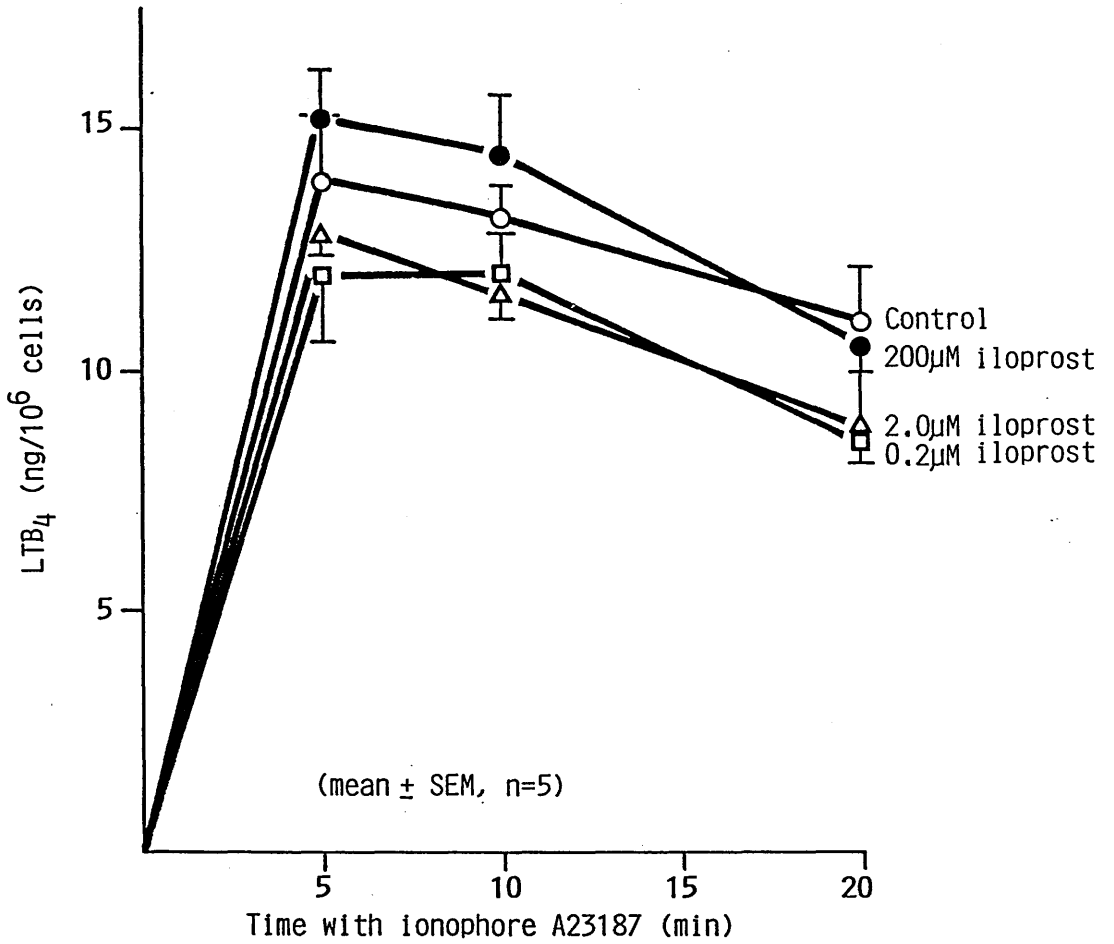


Fig. 5.10 Effect of iloprost on LTB₄ production from normal PMN's in vitro

5.1 (f) (iii) Discussion

The effect of iloprost on LTB₄ production from PMN's in this *in vitro* system, if any, was undramatic. In contrast, Claesson *et al* (1981) found that PGI₂ at pharmacological concentration (2-3 μM) gave a 55% inhibition of LTB₄ release from human PMN's activated with serum-treated zymosan. In addition, Ham *et al* (1983) reported a 50% inhibition of LTB₄ production from PMN's incubated with 100 μM PGE₁ and PGE₂ using another leukocyte activator, FMLP. It is possible therefore, that inhibition by iloprost is only observed *in vitro* when stimuli more subtle than CaI A23187 are used.

The possibility that iloprost could have a different effect from authentic PGI₂ seems unlikely, since both bind to the same receptor sites and iloprost appears to suppress LTB₄-mediated PMN functions (Belch *et al*, 1987c).

5.1 (g) Effect of transdermal iloprost on production of LTB₄ from PMN's in Raynaud's patients

5.1 (g) (i) Introduction

The clinical aim of this study was to assess the efficacy of transdermally administered iloprost in the treatment of Raynaud's phenomenon. In view of the results from the normal volunteer study (section 5.1 (e)) it was also of interest to see if LTB₄ production from isolated PMN's was altered over a treatment period of 20 weeks. In addition, it was proposed to correlate baseline LTB₄ values with the frequency and duration of spasm attacks in the 2 weeks immediately after baseline sampling.

5.1 (g) (ii) Patients and methods

Fourteen patients with RP of varying aetiology were enrolled in the study which was of the "single-blind" type i.e. only the clinician knew who was receiving which treatment. Over a period of 16 weeks patients received sequentially (4 weeks each), placebo, "low dose" iloprost (1.5 µg once daily), "middle dose" iloprost (2.0 µg once daily) and "high dose" iloprost (2.0 µg twice daily). Patients were sampled once during each of the placebo, low dose and middle dose periods and twice (weeks 2 and 4) during the high dose period. The number, duration and severity of spasm attacks was recorded by the patients in special diaries. Not all patients completed the study.

Polymorphonuclear leukocytes were isolated from blood, stimulated with CaI A23187 and the supernatants assayed for LTB₄ in the usual way.

5.1 (g) (iii) Results

The effect of the three different doses of transdermal iloprost on LTB₄ production from PMN's in Raynaud's patients is shown in Table 5.8. Values (mean(SD)) are expressed as a percentage of those obtained during the placebo phase. There was a significant decrease in LTB₄ production with low dose and middle dose iloprost (both $p < 0.03$) but not with the high dose.

		LTB ₄ (% of placebo)				
		Placebo	Low Dose	Middle Dose	High Dose	
					week 2	week 4
Mean	100	98.8*	89.8*	102.0	94.5	
(SD)		(25.0)	(14.8)	(26.8)	(28.0)	
	n = 11	n = 5	n = 5	n = 9	n = 8	

* $p < 0.03$, (Mann-Whitney)

Table 5.8 Effect of low dose (1.5 µg once daily), middle dose (2.0 µg once daily) and high dose (2.0 µg twice daily) transdermal iloprost on LTB₄ production from CaI A23187-stimulated PMN's in Raynaud's patients

However, a significant decrease in both the frequency ($p < 0.02$) and duration ($p < 0.01$) of Raynaud's attacks was observed on "high dose" iloprost (Mann-Whitney), but not at the low and middle doses.

No significant correlation could be found between LTB₄ and the duration or frequency of spasm attacks as recorded in patient diaries in the 2 weeks immediately after baseline blood sampling.

5.1 (g) (iv) Discussion

Previous studies have shown that I.V. infusion of PGI₂/iloprost is beneficial in the treatment of Raynaud's Phenomenon (Belch *et al*, 1981; Dowd *et al*, 1982). Results from this study have shown that transdermally applied iloprost is also able to produce a clinical improvement in RP patients with respect to the duration and frequency of spasm attacks. No objective parameters of disease activity (such as change in digital blood flow) were examined in this study.

It has already been demonstrated (section 5.1 (d)), that the potential for LTB₄ production in PMN's from RP patients is increased in comparison to normal controls. Since the PGI₂-sensitivity of other cells including platelets (section 5.1 (c)) and lymphocytes (Kirby *et al*, 1980) is decreased in RP, it may be that PMN's also exhibit this resistance resulting in a loss of the suppressive effect of PGI₂ on LTB₄ synthesis. In the case of the platelet, pharmacological doses of PGI₂/iloprost appear to overcome this resistance, resulting in reduced platelet aggregability. Results from this limited study suggest that the RP PMN may also respond to iloprost, resulting in decreased production of LTB₄. However, the fact that the clinical improvement in the RP patients did not coincide with the reduction of LTB₄ levels, reinforces the idea that the beneficial effects of iloprost in the treatment of RP are mediated mainly by its platelet anti-aggregatory and vasodilatory effects rather than its effects on leukocytes, but does not preclude the possibility that the inhibition of LTB₄ made some contribution..

5.1 (h) Effect of ketanserin on LTB₄ production in PMN's in Raynaud's Phenomenon

5.1 (h) (i) Introduction

Many different therapeutic approaches to the treatment of RP, based on different theories regarding its pathogenesis, have been tried. Serotonin (5-hydroxy tryptamine) is a platelet aggregant and a potent vasoconstrictor which also amplifies the constriction induced by noradrenaline and cooling (Van Nueten *et al*, 1988; Vanhoutte & Janssens, 1978). In pathological conditions where platelet activation occurs, large amounts of TXA₂ and serotonin may be released and a mutual amplification of their contractile effects on smooth muscle probably occurs (Van Nueten *et al*, 1988). It has been suggested therefore, that the local release of serotonin may be involved in the cold-induced vasospasm of RP.

Previous studies have shown that the serotonin-antagonist ketanserin (Janssen Pharmaceuticals) given intravenously, increased finger blood flow and temperature in patients with RP (Stranden, Roald & Krohg, 1982; Coffman & Cohen, 1988). Pharmacologically, Ketanserin is a selective antagonist of serotonin at S₂ receptors in blood vessels and platelets where it antagonizes serotonin-induced vasoconstriction and aggregation respectively (Van Neuten *et al*, 1981). However, orally administered ketanserin has not been consistently shown to be of benefit in RP in studies of small groups of patients (Roald & Seem, 1984; Bounameaux *et al*, 1984). For this reason, in 1986/87, a large multi-centre study (222 patients) of ketanserin in the treatment of RP was undertaken. As a result, a group of RP patients at Glasgow Royal Infirmary were enrolled in the study. This provided an opportunity to study the effect of ketanserin on LTB₄ production from PMN's in RP patients.

5.1 (h) (ii) Patients and methods

Sixteen patients were enrolled in the study after informed consent had been obtained. However, 4 patients did not fulfill the inclusion criteria of the study and were

not sampled beyond the run-in period when all patients discontinued vasoactive medications and received placebo for a period of 1 month. Thereafter, the 12 remaining patients were randomly assigned in a double-blind manner, to receive ketanserin (20 mg three times a day for 2 weeks, then 40 mg three times a day for 14 weeks) or identical placebo tablets. All patients were sampled at the end of the run-in period (time 0) after which it was intended that the patients be sampled at 2 weeks and then every 4 weeks. However, since the study took place during the winter months and included the Christmas holiday period (December-March), patient attendance was poor. In addition, on occasion PMN preparations from these patients had to be discarded due to their tendency to clump during isolation.

Both primary and secondary Raynaud's patients were included in the study. These were of the ratio 1:5 in the active treatment group and 2:4 in the placebo group. Patients kept daily diaries of frequency, duration and severity of attacks during the run-in and treatment periods.

Isolation of the PMN's and their subsequent stimulation with CaI A23187 was carried out as previously described. The resultant cell supernatants were assayed for LTB₄ using RIA also as previously described.

5.1 (h) (iii) Results

The change in LTB₄ production from A23187-stimulated PMN's over the period of ketanserin treatment is shown in Fig. 5.11. Values are expressed as a percentage of those obtained at the end of the run-in period (time 0). LTB₄ levels in the placebo group had a tendency to rise over the 16 week period while those in the ketanserin group tended to be more stable or fall. Inter-group analysis (Mann-Whitney) showed a significant difference in LTB₄ between the two groups at 8-10 weeks ($p < 0.002$) although this difference was not apparent at 14-16 weeks (Table 5.9).

	Leukotriene B ₄ (% of run-in)		
	4-6 weeks	8-10 weeks	14-16 weeks
Ketanserin Group	93.6 (21.5)	85.6* (20.6)	111.8 (37.6)
Placebo Group	113.7 (31.4)	131.6 (19.5)	123.0 (30.2)

Table 5.9 Effect of oral ketanserin on LTB₄ production from CaI A23187 - stimulated PMN's of Raynaud's patients (Mean (SD)).

* significantly different from placebo group at 8-10 weeks ($p < 0.002$, Mann-Whitney).

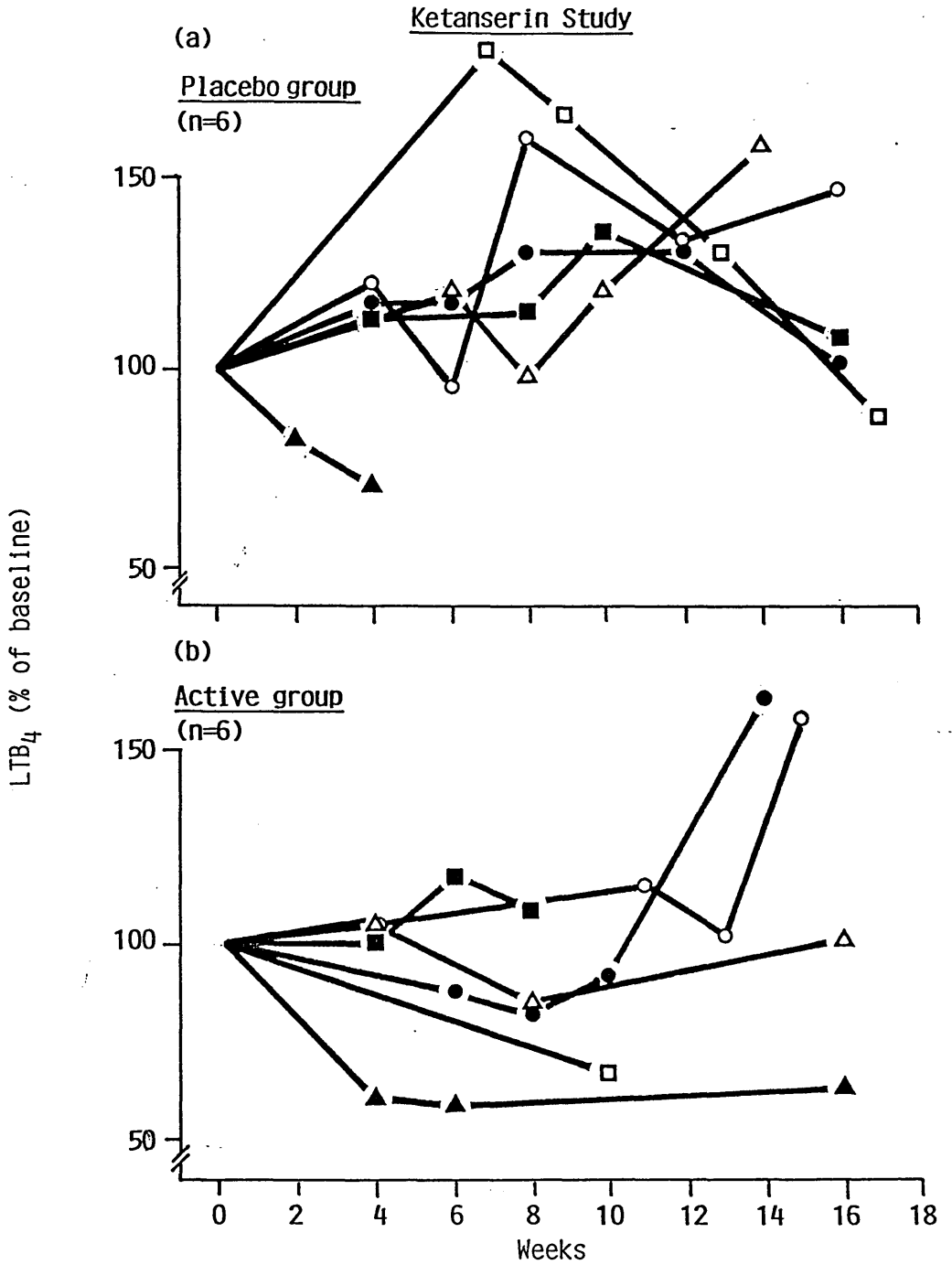


Fig. 5.11 Change in leukotriene B₄ production from stimulated PMN's of Raynaud's patients receiving (a) placebo and (b) ketanserin over a 16 week period.

5.1 (h) (iv) Clinical evaluation of ketanserin-collated results from multi-centre study

Clinical results from the multi-centre study were collated and published by Coffman *et al* (1989). Subjective assessment of treatment (patient diaries) showed that the frequency of vasospastic attacks decreased with both ketanserin and placebo, but a significantly greater reduction of 34% occurred with ketanserin compared to 18% with placebo ($p = 0.011$). There was no significant difference in duration or severity of attacks in either group.

Objective assessment was carried out by measuring digital blood flow using strain gauge plethysmography. However, no significant changes in finger blood flow were found in either group.

Global evaluation showed overall benefit with ketanserin compared to placebo whether assessed by the investigator ($p = 0.03$) or the patient ($p < 0.01$). Patients with primary or secondary RP responded similarly to treatment.

5.1 (h) (v) Discussion

In vitro pharmacological studies have demonstrated S₂-receptors in arteries and veins of the human hand which can be stimulated by serotonin and antagonized by ketanserin (Arneklo-Nobin & Owman, 1985). However, studies with ketanserin in RP have produced varying results, probably due to variation in aetiology and severity within small groups of patients. In a multi-centre study, using a large sample of RP patients, ketanserin has been demonstrated to be of benefit in RP (Coffman *et al*, 1989).

Treatment of a small group of RP patients at Glasgow Royal Infirmary as part of this larger study provided an opportunity for the effects of ketanserin on LTB₄ production by PMN's *ex vivo* to be investigated. It was notable that in the placebo group, the potential for LTB₄ production tended to increase over the study period and a significant difference was observed, compared to the ketanserin group, at 8-10 weeks. This is consistent with the fact that these patients had been withdrawn from their usual

therapy and were sampled during the winter months when vasospastic attacks are typically most frequent and severe. In contrast, in the group receiving ketanserin, LTB₄ levels appeared to be more stable, which may reflect a decreased incidence of vasospastic episodes in these patients. Thus, ketanserin, by its antagonistic action towards serotonin may affect the cycle of vasospasm, ischaemia, reperfusion, vessel damage and PMN activation which is postulated to occur in RP (Belch *et al*, 1989).

5.1 (i) Effect of stanozalol on LTB₄ production from normal PMN's *in vitro*

5.1 (i) (i) Introduction

Many patients with Raynaud's phenomenon, particularly those with secondary disease, have reduced blood fibrinolytic activity leading to raised plasma fibrinogen concentrations and possibly deposition of fibrin in the vascular endothelium (Jarrett, Morland & Browse, 1978). This is thought to contribute to impaired blood flow in RP.

In normal subjects, fibrin is removed by enzymatic degradation mediated by plasmin. Plasmin is produced by cleavage of its inactive precursor plasminogen and the rate of this reaction is determined by the relative proportion of activators and inhibitors of fibrinolysis present. Belch *et al* (1987a) have shown a decrease in plasminogen activator activity and an increase in plasma fibrinogen in patients with RS.

Stanozalol (Sterling Research) is an anabolic steroid which enhances natural fibrinolysis in both normal volunteers (Preston, 1981) and RS patients (Jarrett *et al*, 1978). Treatment of RS patients with stanozalol results in increased hand blood flow and a reduction in other symptoms. However, these changes were found to persist for at least three months after the cessation of treatment, despite the fact that plasma fibrinogen and blood fibrinolytic activity had returned to pre-treatment values.

This has led to the conclusion that stanozalol does not operate solely through a reduction in plasma fibrinogen and the concomitant decrease in blood viscosity, thus, its mode of action still remains to be conclusively determined. The aim of the study was to determine what effect, if any, stanozalol had on LTB₄ production from normal PMN's *in vitro*.

5.1 (i) (ii) Methods

Polymorphonuclear leukocytes were isolated from the blood of normal subjects as described previously. The cells (5×10^6) were resuspended in 880 μ l of HEPES/HBSS (pH 7.4) and 20 μ l of stanozalol in ethanol/buffer or 20 μ l of ethanol/buffer alone were added to the suspensions. The final concentration of ethanol in the incubation buffer was 0.7%. The final concentrations of stanozalol in the incubations were 0.16, 1.6 and 16 μ g/ml. The value of 1.6 μ g/ml corresponds approximately to the *in vivo* therapeutic dose (5-10 mg/day). The cells were then incubated for 20 minutes at 37°C by rotating the tubes gently on rollers. On two occasions, CaI A23187 (final concentration 1 μ g/ml) was added immediately after this incubation and the cells incubated for a further 5 minutes or 15 minutes in a vigorously shaking water bath at 37°C. On two other occasions, the same procedure was followed except that, after the incubation with stanozalol, the cells were pelleted and washed once with 1 ml of HEPES/HBSS before proceeding with the ionophore stimulation.

After stimulation with A23187, the cells were immediately sedimented by spinning in a micro-centrifuge for 30 seconds at 12,000 g. The resulting supernatants were assayed for LTB₄ by RIA as previously described.

5.1 (i) (iii) Results

Figure 5.12 shows the effect that pre-treatment with stanozalol had on LTB₄ production from normal PMN's stimulated with CaI A23187. In all four experiments, stanozalol at 1.6 and 16 μ g/ml caused an inhibition of LTB₄ production such that levels fell below the detection limit of the assay (<0.6 ng/10⁶ cells). In two of the experiments, stanozalol at a final concentration of 0.16 μ g/ml was included, this was found to cause an approximately 50% inhibition of LTB₄ production. Inhibition was observed at all three concentrations of stanozalol whether or not the cells were washed after stanozalol treatment.

In all cases where the PMN's were treated with stanozalol at 16 $\mu\text{g}/\text{ml}$, the cells visibly clumped. This brought into question the viability of the cells after stanozalol treatment. The viability of PMN's treated with 1.6 $\mu\text{g}/\text{ml}$ stanozalol was therefore checked using Trypan Blue exclusion and was found to be 98%.

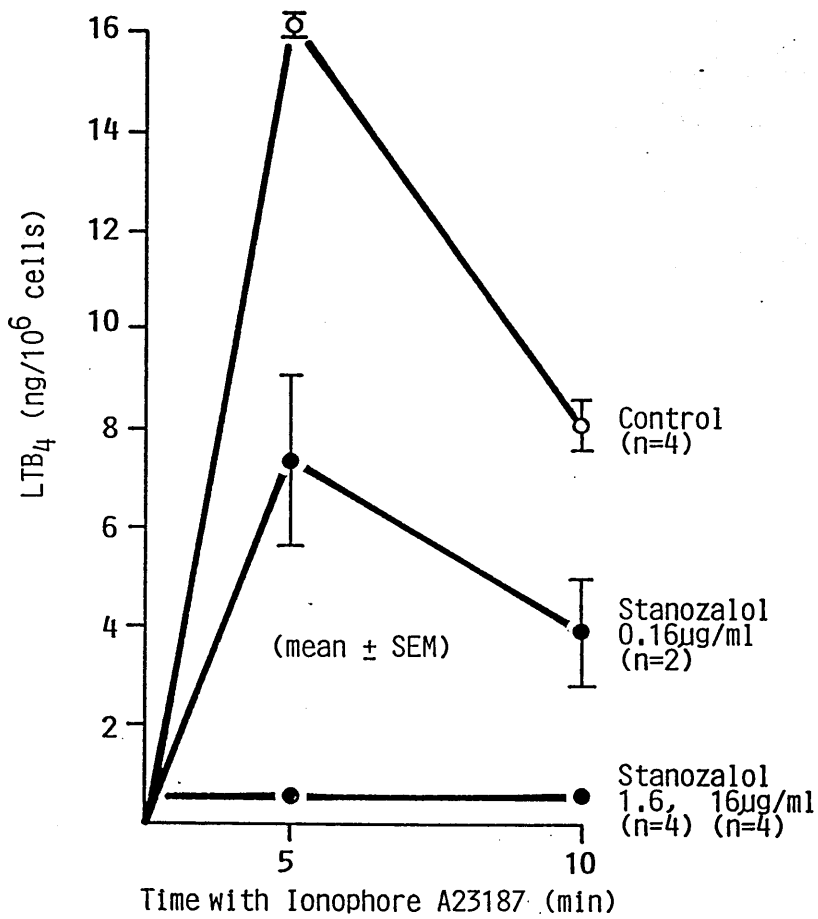


Fig. 5.12 Effect of Stanozalol on LTB₄ production from normal PMN's in vitro

This study has demonstrated that the anabolic steroid stanozalol causes a clear inhibition of LTB₄ production from ionophore-stimulated PMN's *in vitro*. It does not necessarily follow that this effect can be extrapolated to the *in vivo* situation, but the concentrations of stanozalol causing inhibition are certainly compatible with the pharmacological dose.

The fact that inhibition occurs after washing of the cells indicates that stanozalol is not merely preventing the action of the calcium ionophore, but is acting on the LTB₄ pathway itself. The possibility that stanozalol is also an inhibitor of PGI₂ synthesis in PMN's was investigated at a later date, but unfortunately, the control value (before stanozalol treatment) for the chosen donor was below the detection limit of the assay and time did not permit the experiment to be repeated. However, there is other indirect evidence which suggests that stanozalol does not inhibit PG production since it was found, by RA patients who had mistakenly stopped their NSAID's while on stanozalol, that the drug had no analgesic effect (Belch *et al*, 1986a). It seems possible therefore that stanozalol may be a specific 5-LO inhibitor although much more detailed studies would be required to demonstrate this conclusively.

Whatever the mechanism whereby stanozalol causes inhibition of LTB₄ production it may be that this is an additional mode of action contributing to its effectiveness in RP. Similarly, the effectiveness of stanozalol in treating other inflammatory diseases such as RA (Belch *et al*, 1986) may be due to its LTB₄-inhibiting action as well as its fibrinolytic effect.

Although stanozalol could be of use in other conditions where LTB₄ is implicated in the pathophysiology, its side effects, which include disordered liver function and virilization in some women, would obviously restrict its use. Presently, it is only used as a last resort treatment in RS, usually in men and post-menopausal women.

5.2 PROSTANOID ABNORMALITIES IN HENoch-SCHONLEIN PURPURA

5.2 (a) Introduction

Henoch-Schonlein purpura (HSP) is the most commonly encountered type of vasculitis in childhood. The skin lesions (purpura) are the most obvious sign, but visceral involvement makes the disease potentially more serious. In two thirds of patients arthritis and gastrointestinal involvement occur and chronic renal impairment may develop (Meadow, 1979). Although HSP may follow exposure to drugs or allergens and in some cases is preceded by an upper respiratory tract infection (Ackroyd, 1953), the exact aetiology and pathogenesis remain obscure. The primary manifestations however, are known to be due to inflammation of the small blood vessels (Meadow, 1979).

As previously mentioned, there are many reports in the literature regarding the role of PGI₂ and TXA₂ in the pathogenesis of various vasculopathies. Remuzzi *et al* first showed decreased PGI₂ production in the haemolytic uraemic syndrome (1978a) and the related disorder of thrombotic thrombocytopenic purpura (1978b). In addition, plasma from these patients had a low capacity to support PGI₂ production *in vitro* from rat aortic rings. In contrast, normal human plasma is a powerful stimulant for PGI₂ production and several other researchers have reported the existence in plasma of factor(s), as yet uncharacterized, which appear to regulate the production of PGI₂ from vascular endothelium (McIntyre *et al*, 1978; Defreyn *et al*, 1980).

The aim of this study was to examine various aspects of vascular PGI₂ metabolism and its control in the acute phase of HSP which had hitherto been uninvestigated. The work was carried out in conjunction with Dr. S. Turi and the Royal Hospital for Sick Children, Glasgow.

5.2 (b) Patients

Seventeen patients with HSP aged between 2 and 13 years (mean (SD), 5.7 (2.5) years) and 17 controls matched for age and sex were studied. The control children

were free of renal, cardiovascular, pulmonary and inflammatory disease and had been admitted for minor surgical operations. All patients had the classic purpuric rash and in 13 cases the skin lesions were associated with joint manifestations. In 8 cases gastrointestinal symptoms were observed and 8 patients had abnormal urinalysis; 3 patients had mild illness with no abnormal urinalysis or abdominal or joint manifestations.

5.2 (c) Methods

5.2 (c) (i) Sample preparation

After informed consent had been obtained from both patient and parent, 15 ml of venous blood was taken from controls and from patients during the acute phase of the disease, 1-5 days after the occurrence of the rash.

Blood for the estimation of the ability of plasma to support PGI₂-like activity (PSA) was anticoagulated with 3.2% trisodium citrate in a ratio of 9:1. Plasma was prepared within 20 minutes of collection by centrifugation for 10 minutes at 2000 g and 4°C. Plasma and serum samples for estimation of PGI₂-M's and TXB₂ respectively were prepared as previously described. All plasma and serum samples were stored at -70°C until use.

Platelet-rich plasma (PRP) for aggregation studies was prepared from normal adults by centrifugation of citrated blood at 150 g for 7 minutes at room temperature. The final platelet count was adjusted to 250-300 x 10⁹/l by dilution with autologous platelet-poor plasma.

No patient or control was taking any drug known to alter PG metabolism for at least 2 weeks before the study.

5.2 (c) (ii) Estimation of ability of plasma to support vascular PGI₂-like activity (PSA)

The ability of test plasma (patient and control) to support PGI₂-like activity was assessed by measurement of the platelet anti-aggregatory activity of the plasma after incubation with arterial rings. Remuzzi *et al* (1978 a & b) and others, have used rat or rabbit aortic rings for this type of work. The same principle was used in this case except that human umbilical artery was employed, the use of human tissue always being preferred where possible.

The umbilical arteries, which were dissected from freshly delivered umbilical cords, were freed from all surrounding tissue, cut into rings 1 mm in length and kept in Ringer's buffer, pH 7.4 at 4°C for not more than 60 minutes. In order to establish that they were functioning normally with respect to PGI₂ production, the rings (5 rings/tube) were incubated in 1 ml of glycine buffer pH 8.6 for 5 minutes at 37°C. The PGI₂-like (antiaggregatory) activity of the supernatant was tested as follows. An aliquot (100 µl) of supernatant was removed and added to 200 µl of PRP in the dual channel aggregometer. The mixture was incubated at 37°C with stirring for 1 minute, then collagen (Hormonochemie) (final concentration 2 µg/ml) was added as aggregating agent. The rate of aggregation was recorded and calculated as previously described, all aggregation measurements being carried out in duplicate. The supernatant buffer from a sample of freshly prepared rings would normally completely inhibit platelet aggregation.

In order that PSA could be measured the rings had first to be "exhausted" of PGI₂ production. This was achieved by washing the rings several times with Ringer's buffer at 37°C until little or no platelet anti-aggregatory activity could be detected. The exhausted rings were then incubated with 1 ml of test plasma at 37°C for 20 minutes and PGI₂-like activity assessed as before. The ability of each test plasma to support PGI₂-like activity was expressed by calculation of the percentage difference in inhibition of platelet aggregation obtained using the same exhausted rings, before and after addition of

the test plasma. Test plasma which had not been incubated with arterial rings was also added to PRP as a control

5.2 (c) (iii) Detection of inhibition of PGI₂-like activity (PSAI)

To detect inhibitory activity in the test plasma a modified method of Levin *et al* (1983) was used. Fresh, unexhausted umbilical artery rings (one ring per incubation) were incubated in phosphate buffered saline (PBS), pH 7.4 at 37° for 5 minutes. The PGI₂-like activity of the supernatant was compared with that produced by the same ring after 5 minutes incubation with test plasma.

5.2 (c) (iv) Preservation of PGI₂-like effect of iloprost

The preservation of the PGI₂-like effect of the stable PGI₂ analogue iloprost by test plasma was assessed as follows: iloprost at a final concentration of 2 ng/ml was incubated in 300 µl of test plasma for 5 minutes at 37°C. A 100 µl aliquot of this mixture was then added to 200 µl of PRP and after 1 minute of incubation with stirring, collagen was added and platelet aggregation was measured as before. The concentration of iloprost used gave a total inhibition of platelet aggregation when incubated with normal plasma.

5.2 (c) (v) Radioimmunoassay of plasma PGI₂-metabolites and serum TXB₂

Plasma PGI₂-metabolite and serum TXB₂ levels were measured by RIA as previously described in chapter 4. In this instance serum clotting was carried out at room temperature since this was the standard method in use at the time. It should also be noted that at the time of this study, the normal range for plasma PGI₂-M's was inexplicably higher than had previously been observed using the same assay kit (the same phenomenon was noted by other users of the kit at this time) and was probably due to a change in cross-reactivity of the antibody. However, this does not necessarily invalidate the results since patient and control samples were compared in the same assay.

5.2 (d) Results

5.2 (d) (i) Estimation of PSA

The ability of plasma to support PGI₂-like activity was reduced in 13 of the 17 patients and in 5 cases, PSA was undetectable (Fig. 5.13). The difference between the results (median (IQR)) of the patient group (16 (0-41)%) and the control group (55 (50-61.5%)) was significant ($p < 0.001$, Mann-Whitney). In addition, the results of those patients with abnormal urinalysis and gastrointestinal involvement (6.5 (0-22)%) were significantly different from the results of those patients with neither of these manifestations (36 (10-54)%), $p < 0.01$ (Mann-Whitney).

5.2 (d) (ii) Detection of PSAI

Plasma from 6 patients in which PSA was low or absent was further investigated to see whether or not a definite inhibitory activity could be demonstrated. Less PGI₂-like activity was produced by the unexhausted arterial rings when incubated with plasma from the 6 patients than with either PBS or control plasma (Table 5.10).

Case No	*PSA (%)	†PSAI	Preservation of iloprost effect
PATIENTS			
1	0	+	Normal
2	0	+	Decreased
3	0	+	Decreased
4	0	+	Decreased
5	0	+	Normal
6	23	+	Normal
CONTROLS			
1	62	-	Normal
2	57	-	Normal
3	65	-	Normal

Table 5.10 Plasma factors influencing prostacyclin (PGI₂)-like activity in Henoch-Schonlein purpura.

* Estimation of ability of plasma to support vascular PGI₂-like activity.

† Inhibition of vascular PGI₂-like activity (+ = present, - = absent)

5.2 (d) (iii) Preservation of PGI₂-like effect of iloprost

The platelet anti-aggregatory effect of iloprost was noticeably depressed after incubation with plasma from 3 of the 6 patients in which PSAI was present (Table 5.10).

5.2 (d) (iv) Plasma PGI₂-metabolites and serum TXB₂

The plasma PGI₂-metabolite concentrations (median (IQR)) of the patients with HSP were significantly lower (14.8 (9.4-25.0) pg/ml) than those of the normal controls (37.5 (28.5-39.0) pg/ml), $p < 0.001$ (Mann-Whitney) (Fig. 5.14). A positive correlation was found between PSA and PGI₂-metabolite values, $r = 0.52$, $p < 0.05$ (Spearman rank correlation).

There was no significant difference in serum TXB₂ concentration between patients (10.0 (2.0-23.8) ng/ml) and controls (9.5 (2.8-17.6) ng/ml) (Fig. 5.15). No significant correlation was found between the TXB₂ results and plasma PGI₂-metabolite results (Spearman rank correlation).

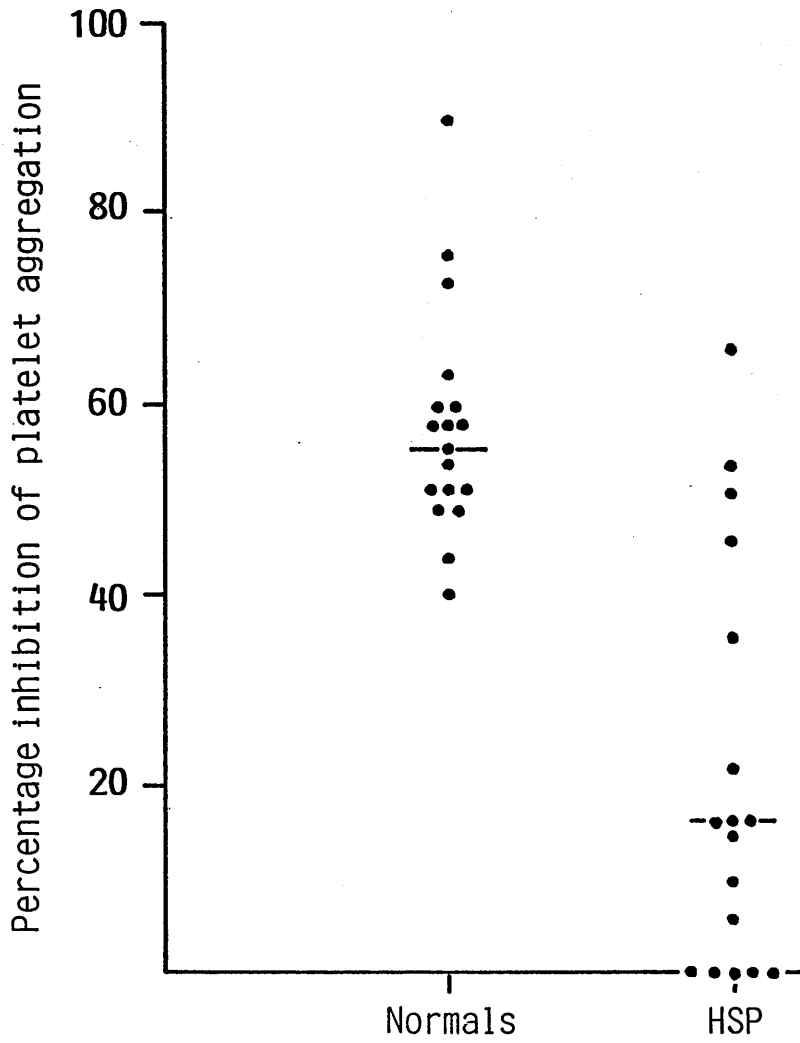


Fig. 5.13 Ability of plasma to support vascular PGI₂-like activity (PSA) in patients with Henoch-Schönlein Purpura (HSP) compared to normal controls

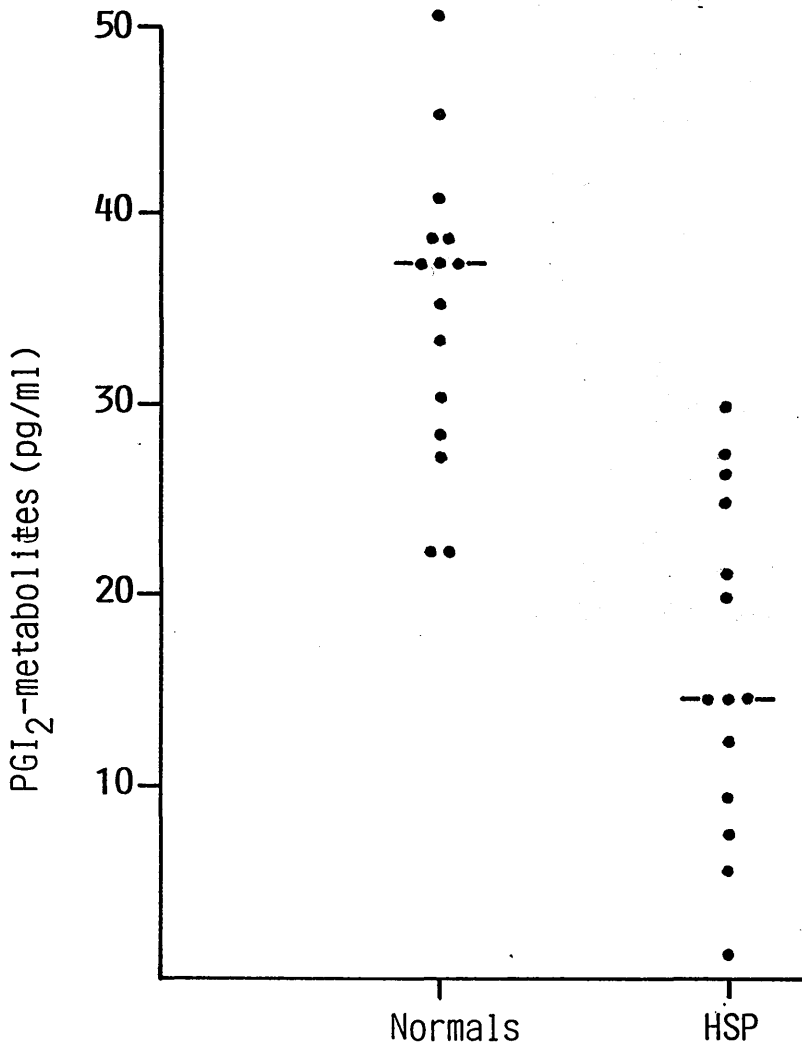


Fig. 5.14 Plasma PGI₂-metabolite concentrations in patients with Henoch-Schönlein Purpura (HSP) compared to normal controls

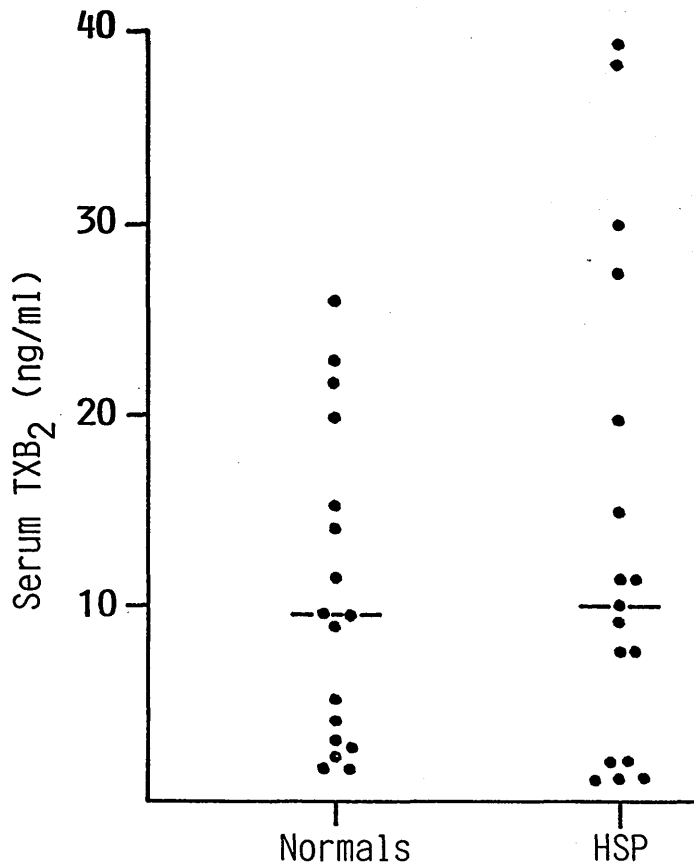


Fig. 5.15 Serum TXB₂ concentrations in patients with Henoch-Schönlein Purpura (HSP) compared to normal controls

5.2 (e) Discussion

Henoch-Schonlein purpura is a type of small vessel vasculitis. Abnormalities of vascular prostaglandin metabolism have been documented in other vasculopathies, particularly the haemolytic uraemic syndrome, but up until the time of this study HSP had not been investigated.

It has been shown that plasma from patients with HSP in the acute phase of the illness has a diminished or absent ability to support PGI₂ generation *in vitro* from human umbilical arterial rings. Absent or depressed PSA may theoretically be due to a number of factors either singly or in combination; first, a decreased ability of the plasma to stimulate PGI₂ production (Remuzzi *et al*, 1978a); secondly, the presence of inhibitory activity in the plasma either to the production (Levin *et al*, 1983) or the effect of PGI₂ (Machin *et al*, 1982); and thirdly, increased degradation of synthesized PGI₂ (Chen *et al*, 1981). All six patients in this study who had very low or absent PSA, showed the presence of PSAI. This inhibitory activity may be against PGI₂ synthesis or biological effect or, could conceivably be due to increased PGI₂ degradation. In an attempt to differentiate between these possibilities, the ability of test plasma to preserve the effect of iloprost was examined and was found to be decreased in three of the six patients studied. The concentration of iloprost used, which was significantly higher than the physiological concentration of PGI₂, was chosen because a definite anti-platelet effect was desired. It is possible, therefore, that the other patient plasma samples may not have preserved the effect of iloprost at lower (more physiological) concentrations. The effect of test plasma on the degradation of PGI₂ was not examined in this study.

On the basis of these data it would seem that in patients with low or absent PSA, inhibitory activity may exist to either PGI₂ production or PGI₂ effect or both.

It has also been shown that the HSP patients in this study had decreased plasma PGI₂-M's compared to normal controls. It is of interest, however, that two patients showed a relatively high concentration of PGI₂-M's in combination with a low

or absent PSA and plasma inhibitory activity to the effect of iloprost. This would tend to suggest the presence of a circulating inhibitor to PGI₂ effect in these cases.

No significant difference in serum TXB₂ concentrations between the patient group and the control group was found. Furthermore, there was a poor correlation between serum TXB₂ and plasma PGI₂-M concentrations. These results suggest that platelet activation does not occur in HSP. In view of the small number of patients in the study it was thought inappropriate to attempt to correlate the demonstrated abnormalities with the clinical manifestations. However, it was observed that patients whose clinical picture included abnormal urinalysis and gastrointestinal involvement, had a mean PSA that was significantly depressed compared to that of patients in whom these clinical manifestations were absent.

The above findings suggest that an abnormality of PGI₂ metabolism does occur in HSP, but that it is rather complex and heterogenous in nature. This study was a preliminary investigation and further studies will be required to define the biochemical nature of the inhibitor(s) and to establish whether low or absent PSA always reflects the presence of inhibitory activity. It seems likely, however, that these abnormalities having been shown in yet another vasculitic syndrome, are most probably a secondary manifestation of endothelial damage. However, the disturbance in PGI₂ metabolism may well be of importance in extending the primary microvascular insult.

CHAPTER 6

LEUKOTRIENE B₄ IN INFLAMMATORY JOINT AND SKIN DISEASE

6.1 Leukotriene B₄ Production by Peripheral Blood PMN's in Rheumatoid Arthritis

6.1 (a) Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease which affects mainly the peripheral synovial (articulating) joints, but is often associated with extra-articular features, and practically every tissue in the body can be involved including eyes, heart, kidneys and nervous system (Hill, 1982). It is a relatively common disease, affecting approximately 1% of the population world-wide and is 2-3 times more common in females. Peak incidence is from age 35-55 years in women and 40-60 years in man, although it may start at any age and a juvenile form is recognized (Still's Disease) (Ansell, 1982).

The clinical features of joint inflammation in RA are basically the classical signs of inflammation namely pain, redness, heat, swelling and loss of function. The established disease typically causes a symmetrical peripheral polyarthritis with early involvement of the small joints of the hands and wrists. Any synovial joint may become affected in severe disease, but in early disease, hips and the distal interphalangeal (DIP) joints of the fingers are usually spared (Thompson, 1982).

Results from biochemical and haematological screening of RA patients are frequently abnormal, but most are simply indicative of a chronic inflammatory condition and have no specific diagnostic value. However, they often give useful information about the severity of the disease and the beneficial or toxic effects of drug therapy.

A range of auto-antibodies can be found in the blood and joints of RA patients and almost all patients produce "rheumatoid factor's" (RF's) which are

immunoglobulins, mainly of the M class directed against immunoglobulin G (IgG) (Thompson, 1982). However these are not confined to RA, being found in a wide variety of diseases and even in a small proportion of the “normal” population. Conversely, RF cannot be detected in all RA sera and so a distinction is made between “sero-positive” and “sero-negative” patients. It is generally accepted that sero-positive patients (around 60%) progress to more severe erosive disease and develop more systemic features in the long term. Assay of RF thus remains useful in making a diagnosis of RA and in following the course of the disease.

Rheumatoid factor-containing immune complexes (IC's) are also found in RA, both circulating and deposited in the joints (Winchester, Kunkel & Angello, 1971). These are believed to be responsible for the chronic activation of complement in the disease and, as with RF, high titres of IC's are associated with more severe and in particular, more systemic disease. Extra-articular lesions may, in fact, represent systemic IC deposition. Erythrocyte sedimentation rate (ESR) is the most commonly measured parameter related to inflammation *per se* and is almost always elevated in RA, tending to parallel the activity of the disease. Similarly, levels of acute phase reactants, particularly C-reactive protein (CRP) have been used to assess disease activity and drug efficacy (Kendall, 1982)

In terms of pathogenesis, the focus of RA in the joint is the synovial lining. The anatomy of the typical synovial joint is shown in Fig. 6.1. Cartilage covers the bone surface to provide a cushioning effect and a smooth surface for each of movement. Enclosing the joint is a capsule of fibrous tissue which has a certain degree of elasticity to allow joint movement; this is thickened in areas of stress to form ligaments which increase joint stability. The inner surface of the joint capsule is formed by synovial tissue, made up of an inner lining of specialized cells over loose, vascular, connective tissue. The lining cells secrete synovial fluid, a viscous solution (due to its hyaluronic acid content) which lubricates the articular surfaces.

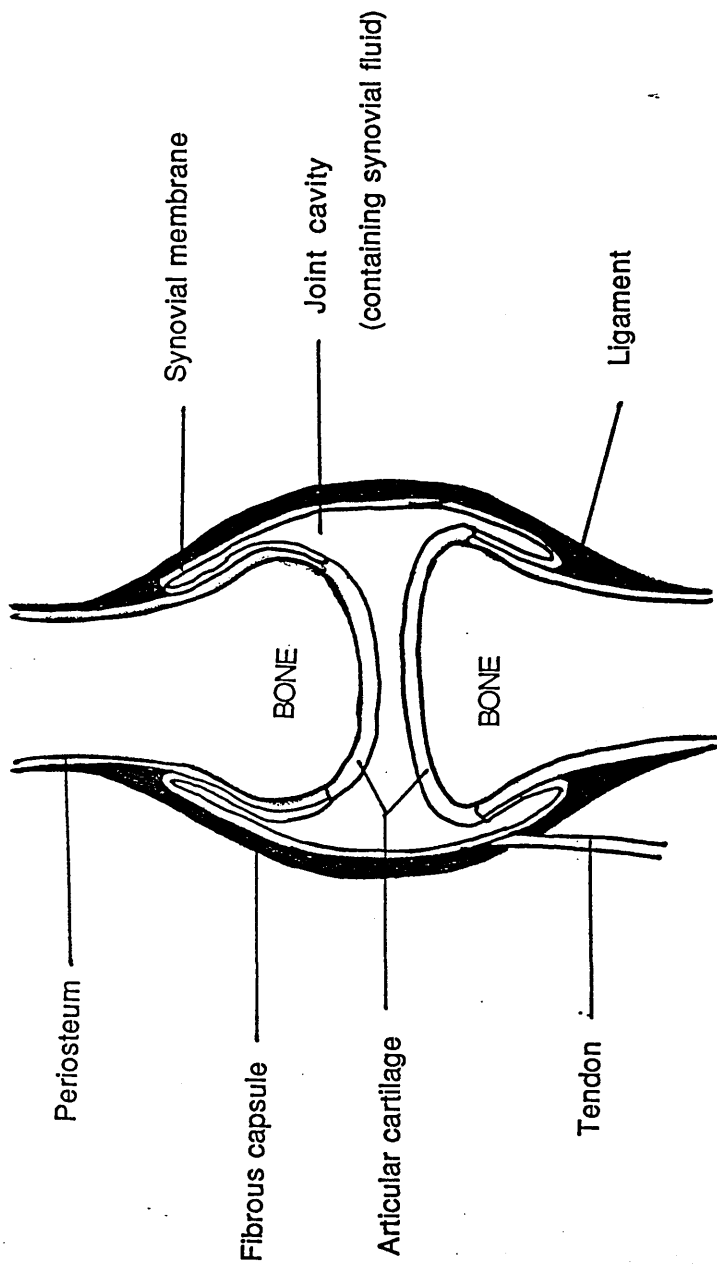


Fig. 6.1. Anatomy of a typical synovial joint.

A central event in the pathogenesis of RA appears to be the formation of IC's within the joint, which activates complement and attracts PMN's. PMN's are abundant in RA synovial fluid (Palmer, 1968) and complement levels are often decreased with a corresponding increase in chemotactic factors, particularly C5a, reflecting activation of the complement cascade (Ward & Zvaifler, 1971). Phagocytosis of IC's by PMN's results in the release of mediators which sustain the inflammation and as a result, the synovium is stimulated to proliferate as granulation tissue or 'pannus' which eventually spreads over adjacent cartilage (Mohr, Westerhellig & Wessinghage, 1981). Thus, by-products of the phagocytic process such as lysosomal proteases and reactive oxygen species, are brought into intimate contact with articular cartilage which is gradually destroyed (Weismann, 1982). Depending on the duration and severity of the disease, areas of cartilage may be completely lost and the underlying bone eroded by a similar process. Once this stage has been reached, there is significant disability and irreversible joint damage has occurred.

Despite many years of intensive research the cause of RA remains unknown. It seems likely, however, that several factors may operate together, an environmental agent provoking onset in genetically predisposed individuals. Evidence for genetic predisposition comes from family and twin studies and also from HLA-typing which shows that 60-70% of patients with sero-positive RA carry the antigen HLA-DR4 (Welsh & Black, 1983).

Infection as the cause of RA has attracted great attention and much research has been devoted to isolation of a virus or some other single, causative pathogen. It is clear that various bacterial agents *can* cause acute and chronic rheumatic disease in man; rheumatic fever, Reiter's syndrome and reactive arthritis being examples (Currey, 1978). At present, however, no single infectious agent is universally accepted as the cause of RA.

Evidence for an auto-immune disturbance in RA rests largely upon the presence of RF and other auto-antibodies. This would seem to suggest an impairment of discrimination between “self” and “non-self” and it may be that viral interaction with plasma membrane constituents is involved.

6.1 (b) Aim of study

On the basis of its actions *in vitro* (Ford-Hutchinson *et al*, 1980) and in view of the fact that it can be detected at inflammatory loci (Simmons *et al*, 1983) it is clear that LTB₄ has a role in the inflammatory process. However, there was little evidence to support the hypothesis that excess LTB₄ production contributes to the development of pathological inflammation such as that seen in RA. Klickstein *et al* (1980), using HPLC, detected increased LO products in the synovial fluid of patients with RA compared to osteoarthritic controls, findings which were supported by Davidson *et al* (1983). However the increased amounts of LTB₄ detected in these studies may merely have reflected an increased population of activated PMN's within the synovial fluid.

The aim of this study was to determine whether peripheral PMN's from RA patients when maximally stimulated, produced more LTB₄ per se than those from normal controls. The results obtained were correlated with three conventional markers for RA disease activity: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels and the Ritchie articular index (AI) (Ritchie *et al*, 1968). Furthermore, since *in vitro* studies have shown that NSAID's can potentiate the production of LTB₄ from PMN's (Higgs *et al*, 1981), the possibility that this phenomenon occurred *in vivo*, in patients taking NSAID's, was investigated.

6.1 (c) Patients and methods

One hundred and five patients, with classical or definite RA according to ARA criteria (Ropes *et al*, 1959) were enrolled in the study after informed consent had been obtained. Of these, 84 patients were taking various NSAID's, all at or near the maximum dose. Twenty one patients were not on NSAID therapy at least 2 weeks prior to sampling (median 6 weeks, range 2-24 weeks). No patient was taking any disease modifying drug.

All patients attended the out-patient department where 90 out of 105 had a Ritchie AI calculated. Twenty millilitres of blood were drawn from the antecubital fossa of these 90 patients; 5 ml, anti-coagulated with EDTA were taken for measurement of ESR (Westergren method), 5 ml of clotted blood were taken for analysis of serum CRP and 10 ml was taken into heparin for measurement of LTB₄ production from PMN's.

Fifty nine age- and sex-matched normal volunteers also had blood sampled for estimation of LTB₄ from PMN's and measurement of CRP levels.

Polymorphonuclear leukocytes were separated and stimulated with calcium ionophore A23187 as previously described. Leukotriene B₄ levels in the cell supernatants were measured by RIA, also as previously described. Measurement of ESR and CRP were carried out by the routine laboratory and AI was assessed by the clinician.

6.1 (d) Results

Table 6.1 shows the comparability between the RA patients and controls in the study. There was no significant difference in age between the two groups (Mann-Whitney). The clinical and laboratory results (medians and ranges) from RA patients are also shown in this table.

	RA	CONTROLS
Age (years)	48 (26-68)	35 (22-60)
Articular index	3 (0-20)	-
ESR (mm/h)	31 (3-99)	-
CRP (mg/l)	17 (10-88)	All <10

Table 6.1 Comparability of RA patients and normal controls (median (range)).

Figure 6.2 shows the amount of LTB₄ (ng/10⁶ cells) produced by patient and control PMN's. Cells from RA patients produced significantly higher levels of LTB₄ than those of normal controls, 27.5 (22.9-33.0) and 20.0 (14.8-25.0) ng/10⁶ cells respectively (median (IQR)), p <0.001 (Mann-Whitney).

Figure 6.3 shows the RA patient group divided according to drug therapy. There was no significant difference in LTB₄ levels between those on or off NSAID's, 28.0 (22.2-34.5) ng/10⁶ cells and 26.0 (21.0-28.5) ng/10⁶ cells, respectively (median (IQR)).

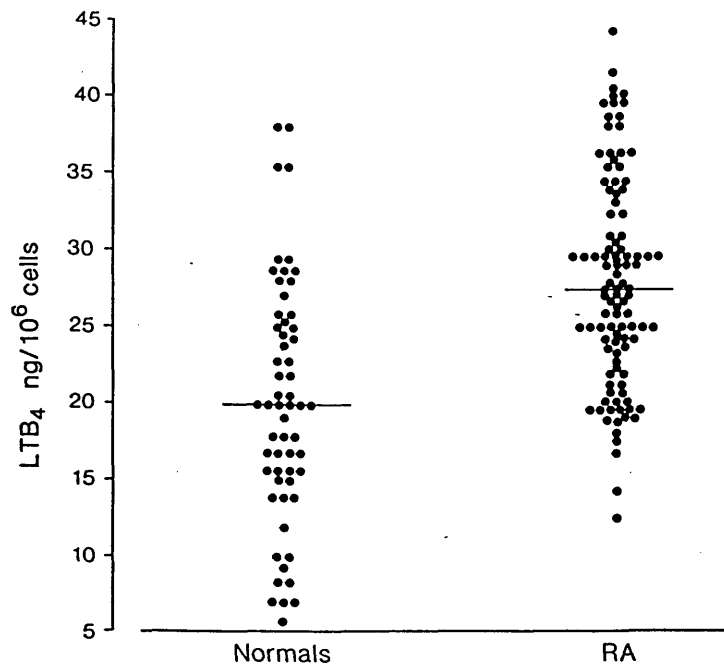


Fig. 6.2. LTB₄ production from stimulated PMN's of RA patients and normal controls.

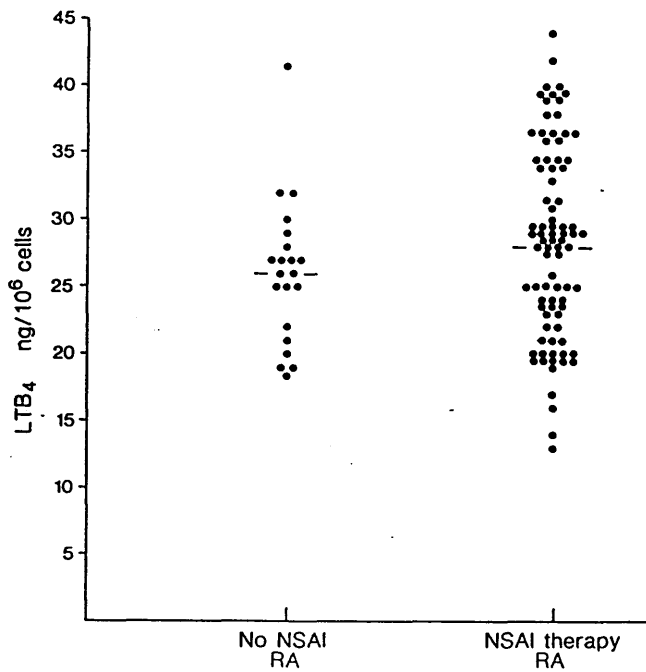


Fig. 6.3. LTB₄ production from stimulated PMNs of RA patients on and off NSAID's.

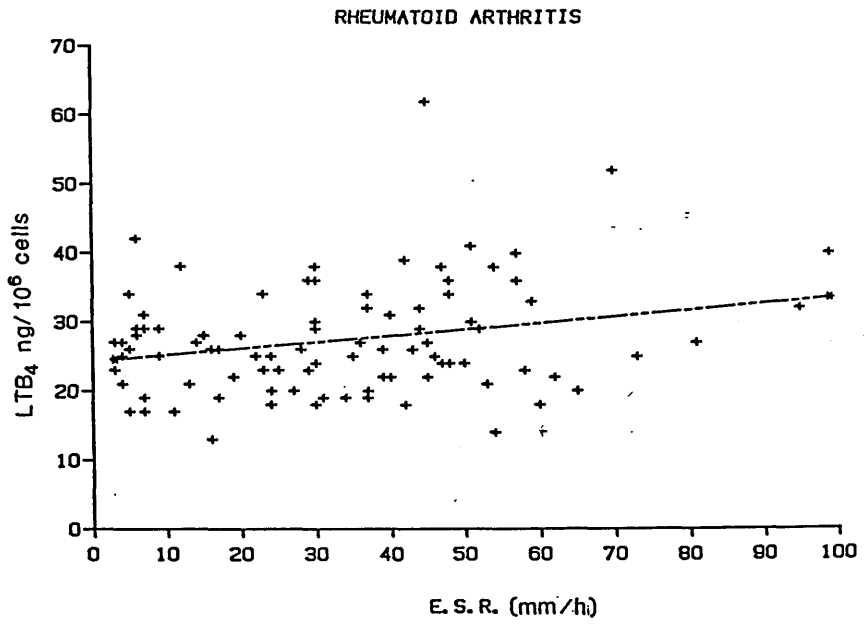


Fig. 6.4. Correlation of LTB₄ with ESR in RA patients.

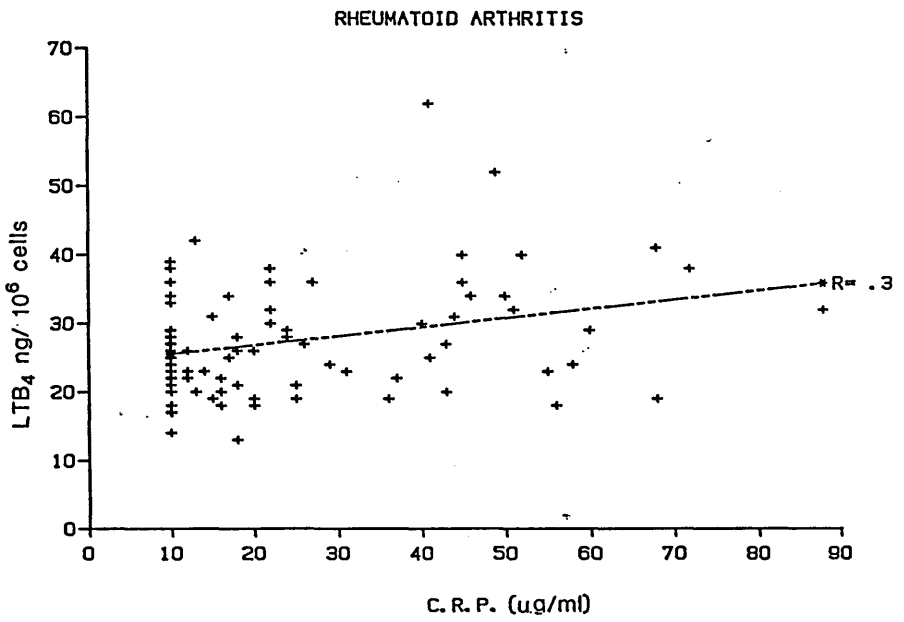


Fig. 6.5. Correlation of LTB₄ with CRP in RA patients.

Using linear regression, the Pearson's correlation coefficient (r) for LTB₄ and AI was 0.08 ($p = 0.49$). However, weak but significant correlations for LTB₄ and ESR ($r = 0.25$, $p < 0.02$) and LTB₄ and CRP ($r = 0.3$, $p < 0.03$) were observed (Fig. 6.4 and 6.5).

When these relationships were analysed using Spearman Rank Correlation, no significant correlations were observed.

6.1 (e) Discussion

Increased levels of LTB₄ have been demonstrated in synovial fluid from rheumatoid joints (Klickstein *et al*, 1980; Davidson *et al*, 1983). However, these observations do not differentiate between increased production by the RA PMN per se and increased production secondary to PMN infiltration. Results from this study showed that LTB₄ from stimulated peripheral PMN's is increased in RA suggesting a real difference between RA cells and those of normal controls. Calcium ionophore A23187 was chosen as activator in order to bypass specific receptors (Weissmann, 1982) and produce a maximal stimulation of LTB₄ synthesis in response to calcium influx, using endogenous AA. It was assumed, therefore, that this method reflected the total enzymatic capacity of PMN's from RA patients and controls to synthesize LTB₄.

The reason for the increased capacity for LTB₄ synthesis in RA PMN's is not yet clear. It has been suggested that the use of NSAID's might be a contributing factor since the potentiation of LTB₄ production by NSAID's has been demonstrated in PMN's *in vitro* (Higgs *et al*, 1981). Suggested explanations for this effect have included diversion of AA substrate towards the 5-LO pathway (Higgs *et al*, 1981) and removal of the negative regulatory effect of PG's on LTB₄ synthesis (Horrobin, 1980). However, results obtained in this study showed no difference in production of LTB₄ between those who were taking NSAID's and those who were not. This would seem to suggest that the potentiation of LTB₄ production by NSAID's whatever it's mechanism *in vitro*, does not play a part in increasing LTB₄ production from PMN's in RA. It may be possible that

substrate diversion from CO to 5-LO pathway after NSAID treatment could be significant in other cells such as macrophages where the capacities for PG and LT production are approximately equal (Chensue & Kunkel, 1983).

Analogous observations to those made in RA have been found in inflammatory bowel disease (IBD), where increased activity of LTB₄ in the gut mucosa reflects infiltration by PMN's (Sharon & Stenson, 1984). Subsequent examination of peripheral blood PMN's from IBD patients showed that increased amounts of LTB₄ were released upon stimulation (Nielsen, Ahnfelt-Rønne & Elmgreen, 1987). In addition, enhanced capacity for release of LTB₄ from PMN's in RA has also been demonstrated by other workers using a slightly different approach (Elmgreen, Nielsen & Ahnfelt-Rønne, 1987). Again, the stimulus used was CaI A23187, but LTB₄ and other metabolites of AA were estimated using radioactive labelling of endogenous AA and thin layer chromatography (TLC). These experiments showed that although RA PMN's had an increased capacity for metabolizing endogenous AA into LTB₄, the release of AA and the formation of 5-HETE and CO products were all within normal limits. Since 20-OH and 20-COOH-LTB₄ levels were also increased, suggesting that LTB₄ was not increased due to reduced metabolism via ω -oxidation, it was concluded that increased LTB₄ levels might be explained in terms of increased activity of epoxide hydrolase. The same conclusion was reached in the case of IBD (Nielsen *et al*, 1987).

Although the mechanism for increased synthesis of LTB₄ by RA PMN's is not completely understood, such an increase is likely to intensify the pro-inflammatory effects of LTB₄ within the RA synovium. By virtue of its actions on chemotaxis (Ford-Hutchinson *et al*, 1980), adherence (Gimbrone *et al*, 1984) and aggregation (Ford-Hutchinson *et al*, 1980) of PMN's, LTB₄ will theoretically induce further recruitment and accumulation of PMN's, thereby amplify and perpetuating the inflammatory response. Additionally, generation of oxygen free radicals (Weissmann, 1982) and release of lysosomal enzymes (Fienmark *et al*, 1981) both involved in local destruction of tissue would result from its cell-activating properties. It is also recognized that LTB₄ can act in

concert with the vasodilatory PG's, PGE₂ and PGI₂, to produce increased vascular permeability and exudation of plasma (Wedmore & Williams, 1981).

It is generally accepted that LTB₄ production is an undesirable consequence rather than a cause of pathological inflammation (Simmons *et al*, 1983; Clancy & Hugli, 1985). Either way, it is clear that inhibition of LTB₄ production may allow improvement in the clinical symptoms of RA. Results from this study showed only a weak correlation of LTB₄ with two conventional markers of disease activity. It is unlikely, therefore, if these markers are of real importance in the progression of RA, that lowering LTB₄ levels would have a disease-modifying effect. However, it may well decrease inflammatory *symptoms* in RA via inhibition of the cell-mediated mechanism of inflammation in which it appears to play a significant role. It has been suggested that the use of dual 5-LO/CO inhibitors in inflammatory disease might be the way forward, since these may emulate the action of the corticosteroids, but without production of steroid-like side effects (Higgs *et al*, 1979).

In conclusion, therefore this study has demonstrated that the capacity for production of LTB₄ is increased in PMN's from patients with RA; this does not appear to be dependent on NSAID therapy. There are weak but significant correlations with conventional measures of disease activity. Although there is no evidence to suggest that inhibition of LTB₄ would have a disease-modifying effect, it may however result in symptomatic improvement.

6.2 Leukotriene B₄ Production by Peripheral Blood PMN's in Psoriatic Arthritis

6.2 (a) Introduction

Psoriasis is estimated to occur in 1-2% of the population and 5-7% of people with psoriasis also have an associated polyarthritis ranging from predominantly DIP joint involvement to clinical syndromes similar to RA and ankylosing spondylitis (Wright, 1982). For many years psoriatic arthritis (PA), was considered to be essentially the coincidence of two relatively common diseases, but is now recognized as a distinct condition. In contrast to RA, PA is always sero-negative (for IgM rheumatoid factor) and there is no difference in prevalence between the sexes (Scarpa *et al*, 1984).

Although the aetiology is not completely known, heredity appears to play a major part and over 30% of patients with psoriasis have a family history of the disease. In PA, the HLA antigens associated with psoriasis (HLA-B13 and B17) and the HLA-B27 antigen, usually associated with ankylosing spondylitis, are commonly found (Wright, 1982). The pattern of disease onset is not consistent; in most cases the psoriasis appears to antedate the arthritis, while in others the arthritis precedes the psoriasis, and in some cases synchronous onset is observed (Scarpa *et al*, 1984).

The arthritis in PA has many similarities to RA in that it is a synovitis which may involve almost any joint in the body, but unlike RA it is more often assymetrical with single joints involved here and there. However, it is not usually a severe disease producing less pain and disability than the rheumatoid form and extra-articular manifestations are rare (Wright, 1982). Patients with PA require virtually the same therapy for their joint disease as those with RA, although corticosteroids are less effective.

In it's classical form, psoriasis presents as raised, erythematous, scaly lesions (plaques) and most commonly involves areas around the knees and elbows and

the scalp. Itching of the lesions is common. Histologically, these manifestations are reflected by increased basal cell proliferation, rapid epidermal turnover and infiltration of PMN's (Burton, 1985).

6.2 (b) Aim of study

Leukotriene B₄ and other LO products have been detected in increased amounts of psoriatic skin (Brain *et al*, 1984; Camp *et al*, 1983b; Grabbe *et al*, 1984) where they are believed to have a role in the pathogenesis of the psoriatic lesion. The cellular source of the LTB₄ is uncertain, but contributions are likely to come both from infiltrating PMN's and from epidermal cells which have been shown to synthesize LTB₄ *in vitro* (Fincham, Camp & Leigh, 1985).

When LTB₄ is injected into normal human skin, an infiltration of PMN's occurs (Soter *et al*, 1983) and clinically, this results in the formation of intra-epidermal microabscesses, which are a histological hallmark of psoriasis (Camp *et al*, 1984). The fact that topical application of indomethacin exacerbates the psoriatic lesion (Ellis *et al*, 1983) provides additional support for the role of LTB₄ in the pathogenesis of psoriasis since the mechanism is assumed to be diversion of AA substrate to the 5-LO pathway. Furthermore, there is an increased proliferation of keratinocytes in psoriasis and LTB₄ has been shown to stimulate DNA synthesis in cultured human keratinocytes (Kragballe, Desjarlais & Voorhees, 1985).

It can be seen, therefore, that LTB₄ has a potential role in both the skin and joint manifestations of PA. Given that PMN's from RA patients have already been shown to have an increased capacity to synthesize LTB₄, the aim of this study was to establish whether such an intrinsic abnormality in AA metabolism exists in the PMN's of patients with PA. In addition, conventional measures of joint activity (CRP, ESR, AI, duration of morning stiffness) were recorded and the degree of skin involvement was assessed using visual analogue scales (percentage body area affected, scaling, erythema, itch). The relationship between these parameters and LTB₄ levels was examined.

6.2 (c) Patients and methods

In total, 118 patients with chronic stable plaque psoriasis and inflammatory arthritis were sampled for measurement of LTB₄ from stimulated PMN's. All were sero-negative for rheumatoid factor and were on a stable dose of paracetamol or the same NSAID for at least one month before sampling. Patients undergoing treatment with systemic steroids, were excluded. All patients had skin and joint activity assessed and recorded as previously outlined. In addition, 59 age- and sex-matched normal controls had blood sampled for estimation of LTB₄ from isolated PMN's.

Polymorphonuclear leukocytes were separated, stimulated with CaI A23187 and the cell supernatants assayed for LTB₄ using RIA as previously described.

6.2 (d) Results

Comparability of PA patients and normal controls is shown in Table 6.2. Some patient clinical and laboratory measurements are also shown.

	PATIENTS	CONTROLS
AGE (years)	40 (18-76)	35 (22-60)
M : F	45 : 73	33 : 26
AI	9 (3-22)	-
ESR (mm/h)	16 (2-110)	-
CRP (mg/l)	15 (10-88)	< 10

Table 6.2 Comparability of PA patient and normal control groups (median (range)).

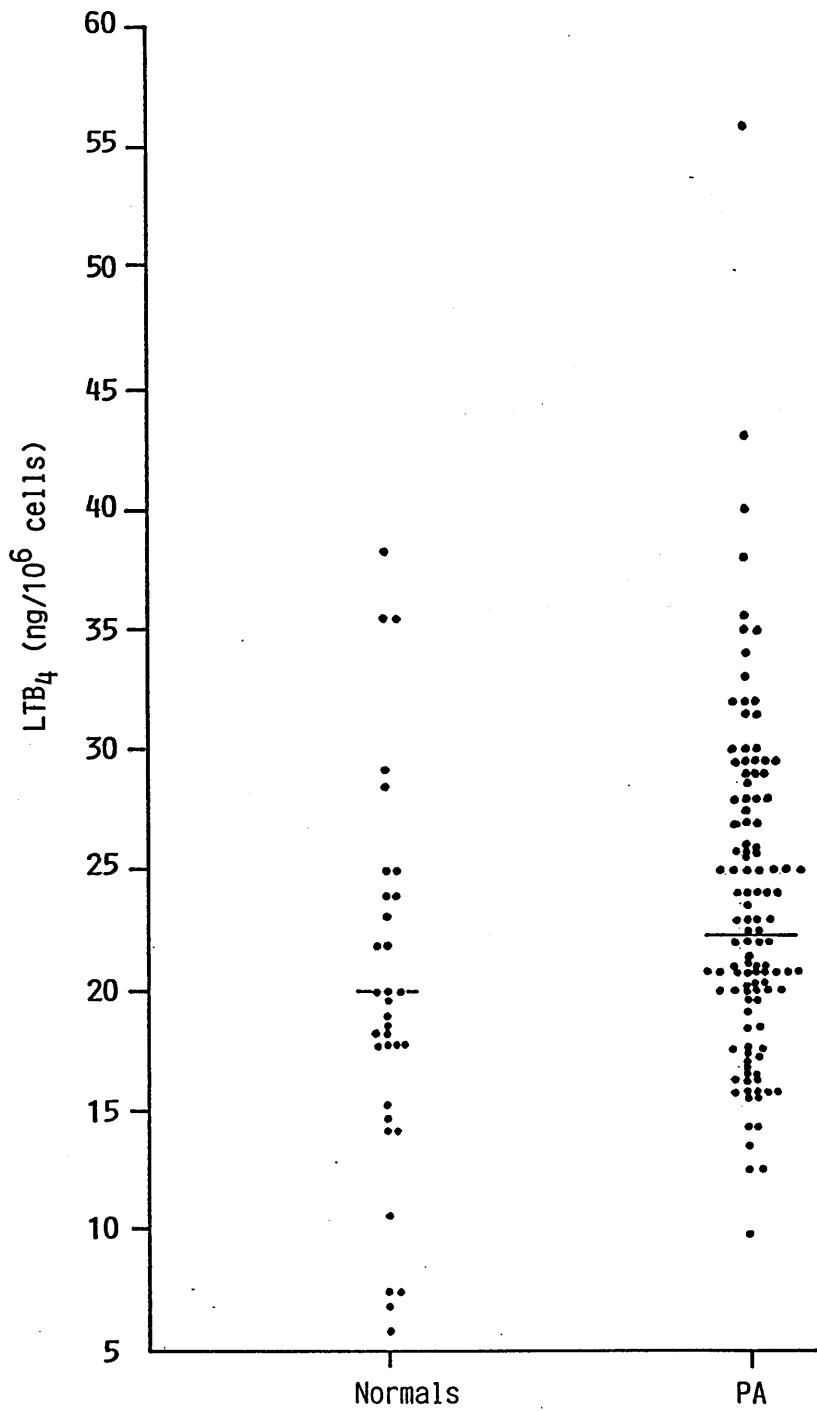


Fig. 6.6 Leukotriene B₄ production from A23187-stimulated PMN's of psoriatic arthritis patients and normal controls

The median (IQR) levels of LTB₄ from PMN's after 5 minutes stimulation with CaI A23187 in PA and control cells were 22.2 (19.6-28.0) and 20.0 (14.8-25.0) ng/10⁶ cells respectively (Fig. 6.6.). The difference was statistically significant (p <0.001, Mann-Whitney). No significant correlations were found between LTB₄ and indices of joint or skin disease activity.

6.2 (e) Discussion

There is considerable evidence that LTB₄ is involved in both the skin and joint manifestations of psoriatic arthritis. The role of LTB₄ in the pathogenesis of inflammatory joint disease has already been discussed in section 6.1. In addition, several factors support the hypothesis that LTB₄ is a participant in the pathophysiology of psoriasis. Increased levels of LTB₄ have been detected in psoriatic plaques and when LTB₄ is applied to normal human epidermis, microabscess formation is induced. It has also been demonstrated that while topical indomethacin exacerbates the psoriatic lesion, treatment with corticosteroids or putative 5-LO inhibitors such as benoxaprofen (Kragballe & Herlin, 1983) gives improvement.

Results from this study have indicated that significantly greater amounts of LTB₄ are synthesized by maximally stimulated PMN's from PA patients than by those of normal controls. Although very little work appears to have been reported in the literature on AA metabolism in psoriatic arthropathy, considerable attention has been paid to the phenomenon of disordered AA metabolism in psoriasis. Maurice, Camp & Allen (1987) have studied the metabolism of AA by peripheral blood PMN's in patients with chronic plaque psoriasis using HPLC. In contrast to the results of the present study, they found no significant difference in the amounts of LTB₄ formed by PMN's stimulated with CaI A23187 in psoriatic and control groups. Since increased LTB₄ production is observed in both RA and PA, but not in psoriasis alone, this would seem to suggest that LTB₄ is associated with inflammatory joint disease rather than inflammatory skin disease. However, Maurice *et al* (1987) did detect increased levels of the ω -oxidation products of

LTB₄ in psoriatics and, since there was no significant difference in the kinetic constants for psoriatic 20-hydroxylase, they concluded that this increase may be secondary to increased synthesis of LTB₄. It may be the case therefore that increased LTB₄ levels are only detected when they reach such a level that the 20-hydroxylase can no longer cope with the increased substrate load.

It has also been observed that levels of free AA are chronically elevated in psoriatic epidermis (Voorhees, 1983). In addition, evidence exists to suggest that CO activity in psoriatic skin is much less than that of 5-LO and 12-LO, a fact which is attributed to the presence of an endogenous CO inhibitor in psoriatic plaque which gives a chronic “indomethacin-like” effect *in vivo* (Penneys *et al*, 1975). This would result in diversion of the large amounts of free AA available to the 5-LO and 12-LO pathways. It is possible, therefore, that the intrinsic ability of PA PMN's to synthesize increased amounts of LTB₄ may be further increased after they have infiltrated the epidermis.

No significant correlations were found between LTB₄ levels and markers for joint or skin activity. This is in contrast to RA where LTB₄ did correlate with markers for joint activity, albeit weakly.

In conclusion, peripheral PMN's from patients with psoriatic arthropathy appear to have an increased capacity to synthesize LTB₄ and this may be augmented *in vivo* by the presence of an endogenous CO inhibitor. It would appear, therefore, that there is the potential for treatment of psoriasis and psoriatic arthropathy via inhibition of the 5-LO, more so since NSAID's have a clear deleterious effect on skin symptoms (Ellis *et al*, 1983).

CHAPTER 7

DIETARY MANIPULATION OF ESSENTIAL FATTY ACIDS IN INFLAMMATORY JOINT AND SKIN DISEASE

7.1 Introduction

Two groups of fatty acids which are essential for normal bodily function, cannot be synthesized *de novo* in mammalian systems and therefore must be obtained from diet. The ω -6 series is derived from linoleic acid (18 : 2 ω -6) and the ω -3 series is derived from α -linolenic acid (18 : 3 ω -3) (Willis, 1981). (In this notation the first number indicates the number of carbon atoms in the molecule, the second indicates the number of double bonds and the last refers to the position of the first double bond starting from the methyl end of the fatty acid chain.) The derivation of the eicosanoids from their precursor EFA's is illustrated in Fig. 7.1; linoleic and α -linolenic acid can be regarded as the ultimate dietary precursors of the eicosanoids. However, the standard Western diet is rich in AA (20 : 4 ω -6) and leads predominantly to the formation of the 2-series prostanoids and the 4-series LT's which, in general, have pro-inflammatory effects. It is now becoming apparent that the eicosanoid profile of cells and tissues may be changed by providing alternative substrate (via diet) for metabolism by the CO and 5-LO enzymes.

Evening primrose oil (EPO) is a naturally occurring seed oil which contains relatively large amounts of γ -linolenic acid (GLA). This can be converted to dihomo- γ -linolenic acid (DGLA; 20 : 3 ω -6), a precursor of the monoenoic PG's such as PGE₁ (Fig. 7.1) which is thought to possess anti-inflammatory properties (Zurier & Ballas, 1973; Bonta & Parnham, 1978b; Martin & Stackpool, 1981). DGLA cannot be converted to LT's as it lacks desaturation at the Δ 5 position (Willis, 1981), but it can be converted to a 15-hydroxy derivative which potently inhibits the transformation of AA to LT's (Scholkens *et al.*, 1982, Miller *et al.*, 1988) Dietary DGLA may therefore act as a competitive inhibitor for the formation of the 2-series prostanoids and the 4-series LT's to give rise to products with less inflammatory activity.

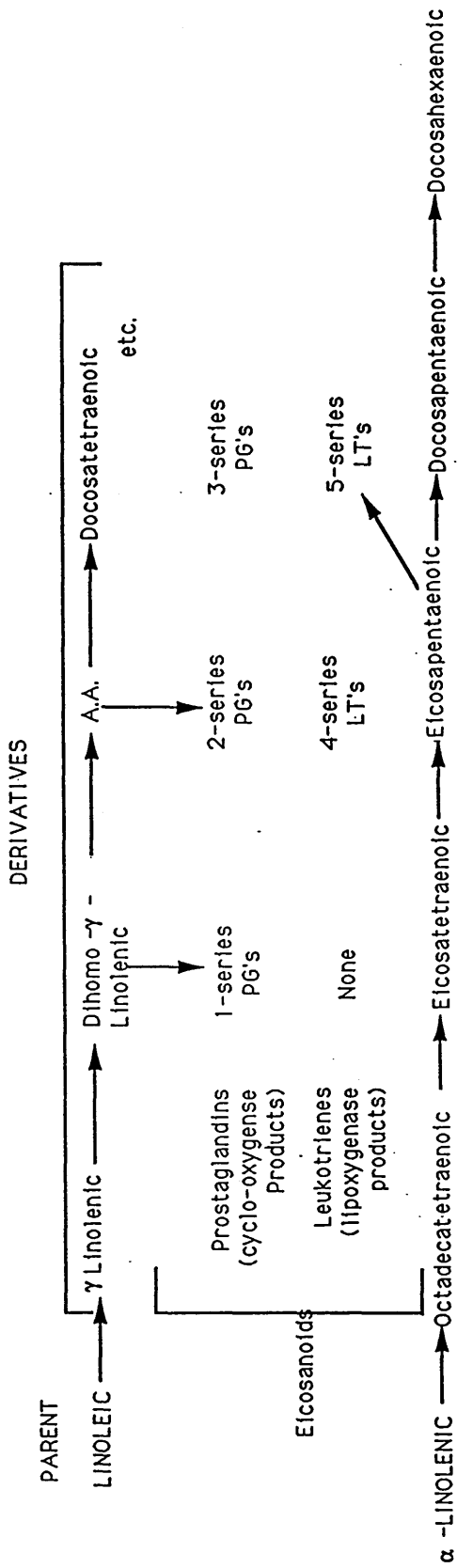


Fig. 7.1. Derivation of prostaglandins and leukotrienes from the essential fatty acids.

Several animal studies have suggested that DGLA has an overall anti-inflammatory effect. Local injection of DGLA has been shown to inhibit granuloma growth in an animal model of inflammation (Elliot *et al.*, 1986) and a diet rich in DGLA appears to decrease the chemotactic response of rat PMN's (Kunkel *et al.*, 1982). Furthermore, it has been demonstrated that EPO suppresses MSU-induced inflammation in rats (Tate *et al.*, 1988) and increases lifespan in MLR₁ mice who develop a spontaneous autoimmune disease which manifests itself as vasculitis and arthritis (Godfrey *et al.*, 1986).

Several *in vivo* and *in vitro* human studies have also suggested a beneficial effect with EPO/DGLA. Baker, Krakauer & Zurier (1986) have demonstrated that synovial cell proliferation, a hallmark of RA, is decreased *in vitro* after addition of DGLA. Wright & Burton (1982) have shown a significant clinical improvement in patients with eczema after EPO treatment. Two clinical studies with EPO in RA proved disappointing (Brown *et al.*, 1980; Hansen *et al.*, 1983), however, the doses of EPO were low (50 mg/day and 360 mg/day respectively) and the study periods short (12 weeks in both instances).

Another derived EFA, eicosapentaenoic acid (EPA; 20 : 5 ω -3) which is abundant in fish oils, can also be metabolized to eicosanoids (Fig 7.1). In humans, dietary EPA can be transformed to PGI₃ (Fischer & Weber, 1984) which has anti-aggregatory and vasodilatory properties similar to those of PGI₂ (Needleman *et al.*, 1979). In addition, small quantities of TXA₃ are formed by human platelets after dietary EPA, in conjunction with decreased TXA₂ production (Fischer & Weber, 1983). Since TXA₃ is a far less potent platelet aggregator than TXA₂ (Needleman *et al.*, 1979), the net effect of the EPA metabolites is anti-platelet and vasodilatory. Formation of the 3-series prostanoids therefore, would appear to be a means of favourably altering the PGI/TXA balance and indeed, EPA is associated with reduced incidence of cardiovascular disease (Dyerberg *et al.*, 1978). It has been suggested that the low incidence of coronary heart

disease in Greenland Eskimos may result, in part, from their high intake of marine foods rich in EPA (Dyerberg & Bang, 1979).

Metabolism of EPA via the 5-LO pathway leads to production of the 5-series LT's. In experiments with human PMN's, EPA enrichment by dietary means, followed by stimulation with CaI A23187 results in the formation of LTB₅ with a concomitant decrease in LTB₄ generation (Strasser, Fischer & Weber, 1985; Lee *et al.*, 1985). Leukotriene B₅ appears to be considerably less potent than LTB₄ (10-30 times) in causing aggregation, chemotaxis and degranulation of PMN's and in the potentiation of plasma exudation (Terano, Salmon & Moncada, 1984; Prescott, 1984).

Dietary supplementation with EPA has been shown to reduce inflammation in some, but not all, animals models. Treatment with EPA markedly reduces the severity of glomerulonephritis and it's associated mortality in NZB/NZW mice (the animal model for systemic lupus erythematosus; SLE) (Robinson *et al.*, 1985) and the manifestations of salmonella-associated arthritis induced by collagen in mice is also decreased after an EPA-rich diet (Leslie *et al.*, 1985), although adjuvant arthritis in rats is augmented by EPA (Prickett, Trentham & Robinson, 1984).

Several human studies with dietary EPA have also been carried out. Studies of normal volunteers whose diet was supplemented with EPA show a shift from LTB₄ to LTB₅ production in stimulated PMN's (Lee *et al.*, 1985; Prescott, Zimmerman & Morrison, 1985). Functional changes in leukocytes, including decreased chemotaxis and adherence, have also been observed after 6 weeks of EPA ingestion (Lee *et al.*, 1985), but not after 3 weeks (Prescott *et al.*, 1985).

Several clinical studies with EPA (with respect to inflammatory disease) have been carried out with varying results. In a placebo-controlled study, treatment of SLE patients with EPA for 12 months resulted in no significant changes in disease activity, although there did appear to be a "rebound" of symptoms after withdrawal of EPA, suggesting an effect in some individuals (Moore *et al.*, 1986). Results from studies

with RA patients have been more promising. In two studies, Kremer *et al.* (1985 and 1987) reported a clinical improvement in RA patients whose diet was supplemented with EPA. In the later study an observed decrease in joint tenderness correlated with decreased LTB₄ production from stimulated PMN's. More recently, Sperling *et al.* (1987) and Cleland *et al.* (1988) have reported improvement in disease activity and reduced LTB₄ production from PMN's in RA patients taking EPA. In two studies of psoriasis, EPA treatment resulted in a mild to moderate improvement in symptoms (Ziboh *et al.*, 1986; Bittener *et al.*, 1988).

Like EPA, docosahexaenoic acid (DCHA; 22 : 6 ω-3) is a highly unsaturated long chain fatty acid found in fish oils and marine mammals, which can be incorporated into inflammatory cell membranes and which competitively inhibits the utilization of AA by CO (von Schacky *et al.*, 1985). Unlike EPA, DCHA is a poor substrate for the 5-LO; addition of DCHA to ionophore-stimulated neutrophils *in vitro* yields only a mono-hydroxy derivative and has little, if any, effect on LT synthesis (Lee *et al.*, 1984).

7.2 Dietary Manipulation of EFA's in Rheumatoid Arthritis

7.2 (a) Aim of study

“First-line” treatment for RA is by the use of non-steroidal anti-inflammatory drugs. These act by inhibiting the CO enzyme thereby decreasing the production of pro-inflammatory prostanoids from AA. Treatment with NSAID's may therefore ameliorate the PG-mediated aspects of inflammation, but allow those dependent on 5-LO products to proceed unchecked. Furthermore, NSAID's may produce side-effects such as gastric irritation as a result of indiscriminate inhibition of PG's.

In theory, therefore, it should be possible to substitute DGLA or EPA in place of NSAID treatment since this would produce a decrease in the pro-inflammatory PG's and LT's while other, less inflammatory eicosanoids (such as PGE₁ and LTB₅)

would be increased. In addition, there should be no gastrointestinal side-effects with such therapy.

The aim of this study was to determine whether an EPO or EPO/fish oil combination (Efamol Marine) could be substituted in place of NSAID therapy without deterioration in the clinical symptoms of RA. In addition, the effects of these dietary supplements on LTB₄ production from stimulated PMN's was investigated. The study was double blind and placebo controlled.

7.2 (b) Patients

Forty-nine patients with classical or definite RA as defined by ARA criteria were enrolled in the study after informed consent had been obtained. All patients required first-line (NSAID) therapy for control of their symptoms but none were considered severe enough to warrant second-line therapy. Sixteen patients received EPO treatment taken as 12 capsules/day, providing a daily dose of 540 mg GLA; 15 patients received EPO/fish oil (12 capsules/day) providing a total daily dose of 450 mg GLA and 240 mg EPA. Eighteen patients received 12 capsules/day of placebo (indigestible liquid paraffin). In addition, all capsules contained vitamin E as an anti-oxidant (dose 120 mg/day).

All three types of capsule were supplied by Efamol Ltd. and were visually identical. They were issued to patients in a randomised, double-blind fashion.

7.2 (c) Study design

The duration of the study was 15 months. For the first 3 months, patients were instructed to take the 12 capsules of oil per day, plus their full dose of NSAID's. From 3-6 months in particular, but also up to 12 months, patients were instructed to decrease or stop their NSAID's if possible. From 12-15 months they were told to maintain, if possible, their current dose of NSAID's. Patients were only to decrease or stop NSAID's if this could be done without exacerbation of RA symptoms. At 12 months all patients received placebo capsules (12/day). As this was part of the study

design, investigators were aware of the treatment in all patients from 12-15 months; the patients, however, remained blinded. The aim of this placebo phase was to monitor relapse. The patients attended the clinic at monthly intervals for the first 6 months and thereafter at 3-monthly intervals.

7.2 (d) Clinical assessment of disease activity

Full metrological assessment was carried out by the clinician prior to the start of the study and at 3,6,12 and 15 months. The duration of morning stiffness in minutes, the grip strength of left and right hands (mmHg, mean of 3 readings) and the Ritchie articular index were noted. In addition, patients completed a 10 cm visual analogue pain scale, and the occurrence of side effects was recorded. Patients were also asked whether they obtained benefit or otherwise from the treatment.

7.2 (e) Biochemical assessment of disease activity

Blood was sampled prior to the start of the study and again at 3,6,12 and 15 months for measurement of ESR, CRP, haemoglobin (Hb) and RF. All of these estimations were carried out by routine laboratory services. In addition, samples from 0,6 and 12 months were separated and stored at -70°C for later analysis of plasma and erythrocyte membrane EFA levels to assess patient compliance. These analysis were carried out by Efamol Ltd.

7.2 (f) Measurement of LTB₄ from stimulated PMN's

Blood samples for PMN isolation and estimation of LTB₄ were taken every month from 0-6 months and thereafter every 3 months. Due to the fact that the standardised method for CaI A23187-stimulation of PMN's was established shortly after commencement of the study, only 13 patients from the EPO/fish oil group, 12 patients from the EPO group and 12 patients from the placebo group were sampled at month 0 for this estimation.

Separation of PMN's from whole blood and their subsequent stimulation with CaI A23187 was carried out as described in section 4.4. Leukotriene B₄ in the resultant supernatants was measured by RIA also as described in section 4.4.

7.2 (g) Statistical analyses

Comparability between the treatment groups was analysed using the Mann-Whitney U test. Sequential analysis were carried out using the 2-tailed paired Wilcoxon test, except for sequential LTB₄ determinations which were analysed using the Mann-Whitney U test, since paired data were not always available.

	Placebo	EPO	EPO/fish oil
Age (Years)	48 (30-74)	46 (35-68)	53 (28-73)
Duration (years)	5 (1-20)	5 (1-18)	5 (1-25)
Sex M:F	1:17	1:15	4:11
Morning stiffness (min)	31 (0-240)	47 (0-120)	31 (0-240)
Grip strength (mmHg, right hand)	106 (53-260)	116 (58-207)	105 (50-300)
Articulat index	6 (0-24)	5 (0-14)	7 (0-12)
Visual analogue scale (cm)	2.3 (0-8.0)	3.6 (0-9.5)	3.0 (0-7.3)
ESR (mm/h)	30 (3-75)	22 (4-81)	26 (4-55)
CRP (mg/l)	19 (8-76)	19 (10-43)	13 (10-38)
Hb (g/l)	11.8 (10-15.5)	12.6 (8.8-14.9)	13.8 (11.7-16.8)
RF (titre)	1:16 (0-1024)	1:128 (0-1024)	1:128 (0-1024)

Table 7.1 Comparability of the three treatment groups of RA patients at start of study (median (range)).

7.2 (h) Results

7.2 (h) (i) Clinical aspects

Table 7.1 shows the comparability of the 3 treatment groups for clinical and laboratory measurements at the start of the study. No significant differences in the patient populations were apparent. Table 7.2 shows the number of patients withdrawn from the study by 12 months. One patient out of 16 in the EPO group and 2 out of 15 in the EPO/fish oil group were withdrawn due to increasing symptoms of RA, compared to 10 out of 18 placebo patients (both $p < 0.001$, Mann-Whitney). These results are shown in Fig. 7.2 expressed as a percentage of the total numbers in each group. Subsequent results have also been expressed as a percentage of baseline so that the different group totals can be more easily compared.

	Placebo	EPO	EPO/fish oil
Increasing RA symptoms	10	1	2
Gastro-intestinal upset	0	2	0
TOTAL	10	3	2

Table 7.2 RA patients withdrawn from the Efamol study by 12 months.

Figure 7.3 shows a compilation of results illustrating the dose of NSAID's in all 3 groups prior to the start of treatment and at 3,6 and 12 months. As 1 EPO and 3 placebo patients were not taking NSAID's at the start of the study they were not included in these analyses. Eleven out of 15 EPO patients and 12 out of 15 EPO/fish oil patients reduced or stopped their NSAID's by 12 months, compared to 5 out of 15 of the placebo patients ($p < 0.003$, $p < 0.002$, $p < 0.05$ respectively, paired Wilcoxon).

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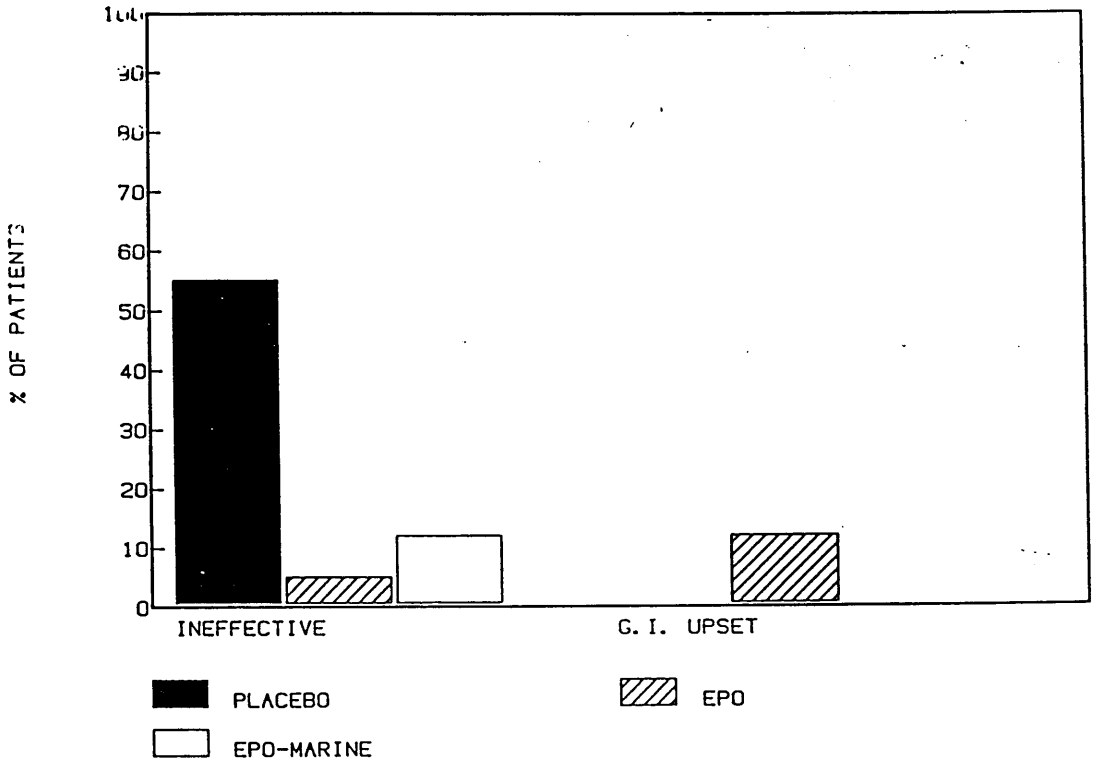
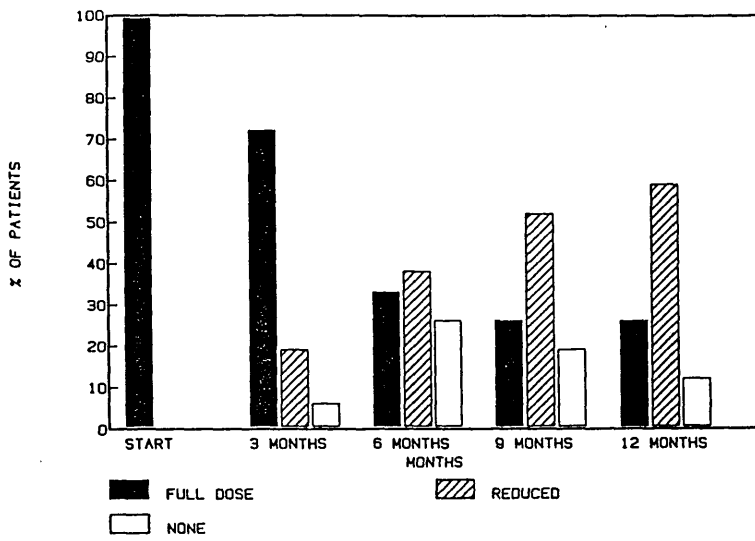
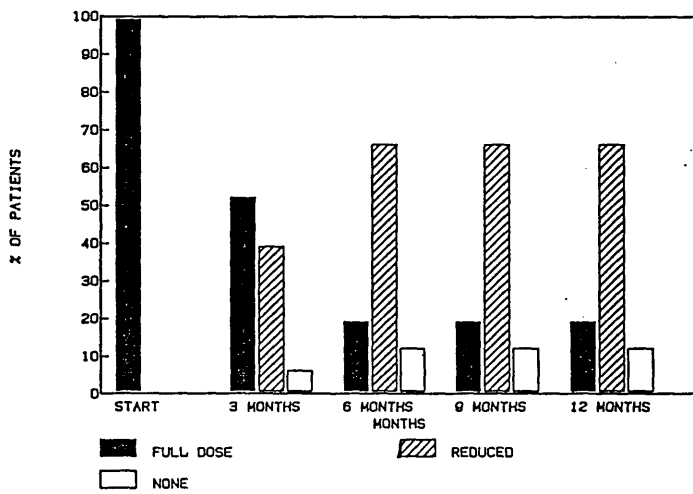


Fig. 7.2. Withdrawal from study of RA patients receiving EPO, EPO/fish oil (EPO/marine) and placebo.

EPO / NSAID DOSAGE



EPO-MARINE / NSAID DOSAGE



PLACEBO / NSAID DOSAGE

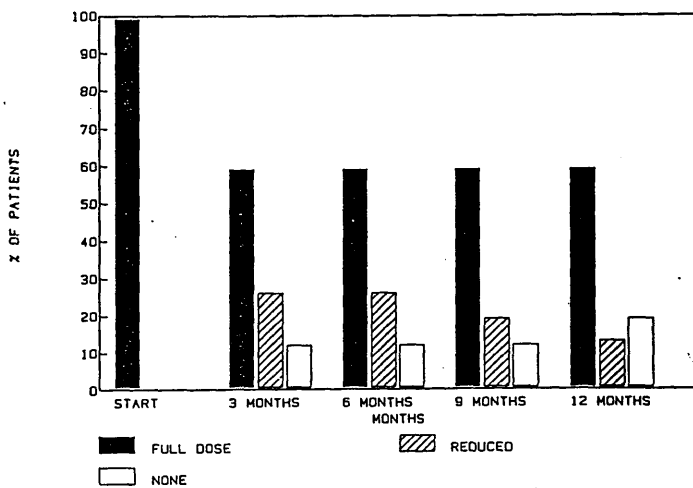
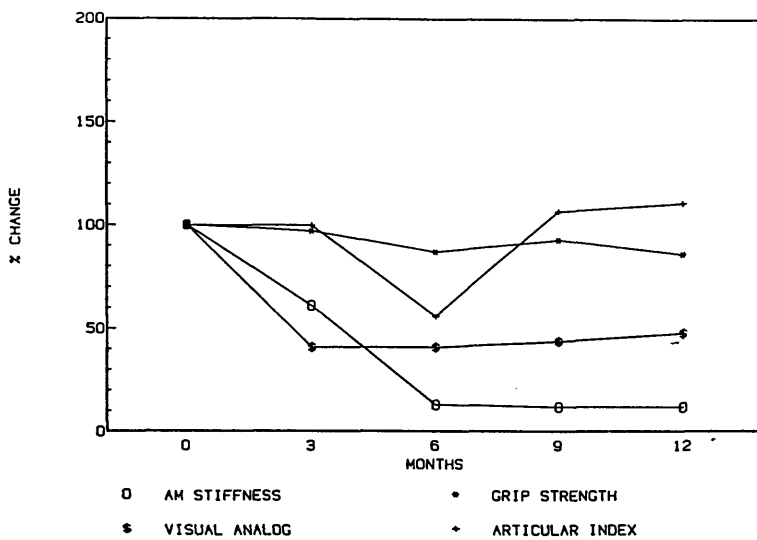
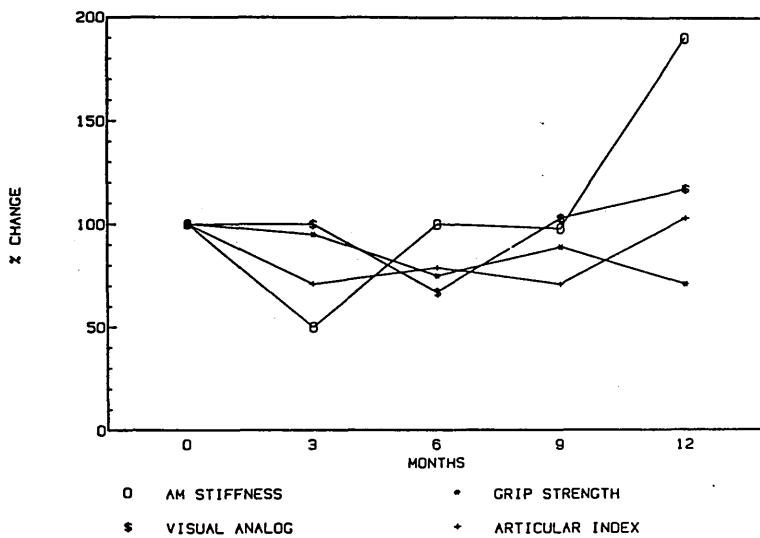


Fig. 7.3. NSAID requirement of RA patients receiving EPO, EPO/fish oil (EPO/marine) and placebo.

EPO / CLIN RESPONSE



EPO-MARINE / CLIN RESPONSE



PLACEBO / CLIN RESPONSE

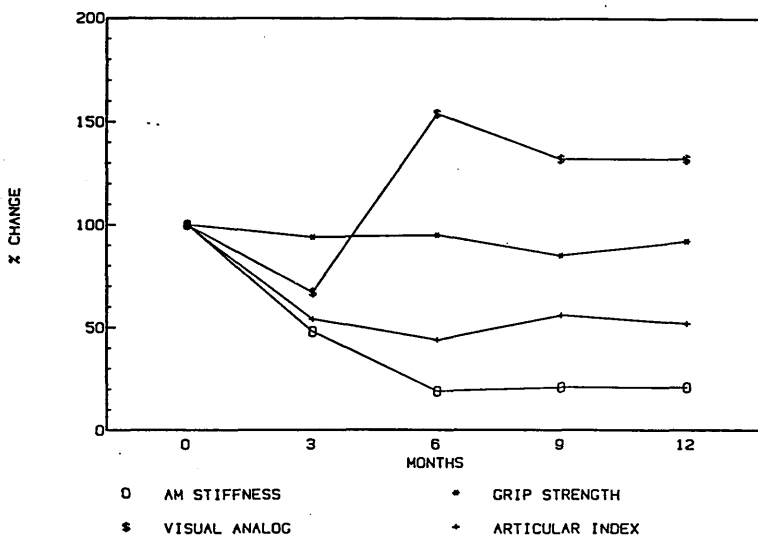
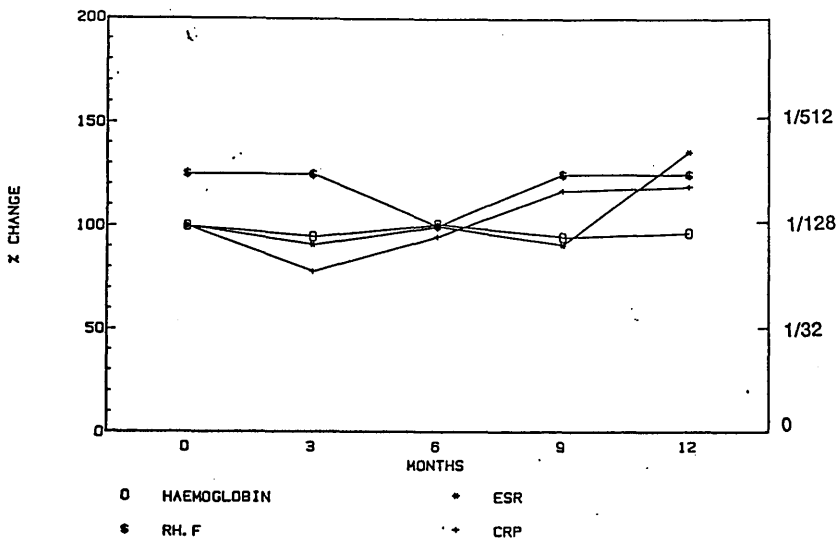
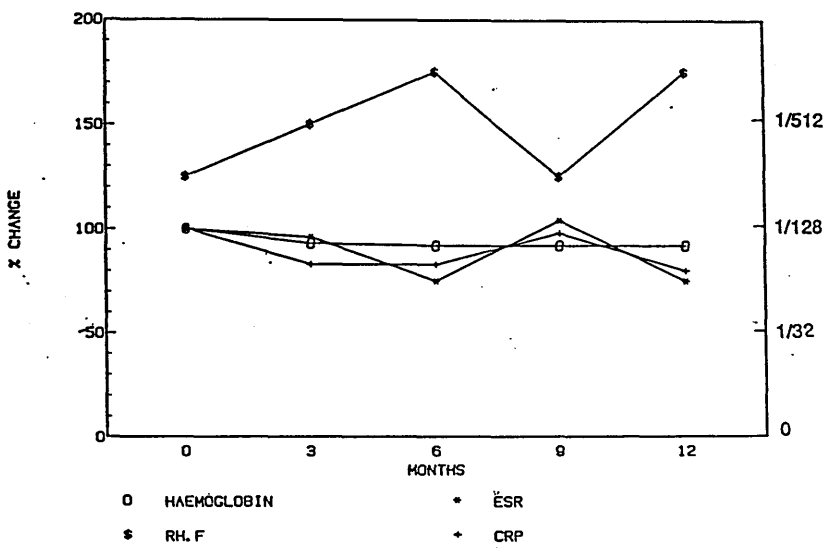


Fig. 7.4. Changes in clinical indices of disease activity in RA patients receiving EPO, EPO/fish oil (EPO/marine) and placebo.

EPO / LAB RESPONSE



EPO-MARINE / LAB RESPONSE



PLACEBO / LAB RESPONSE

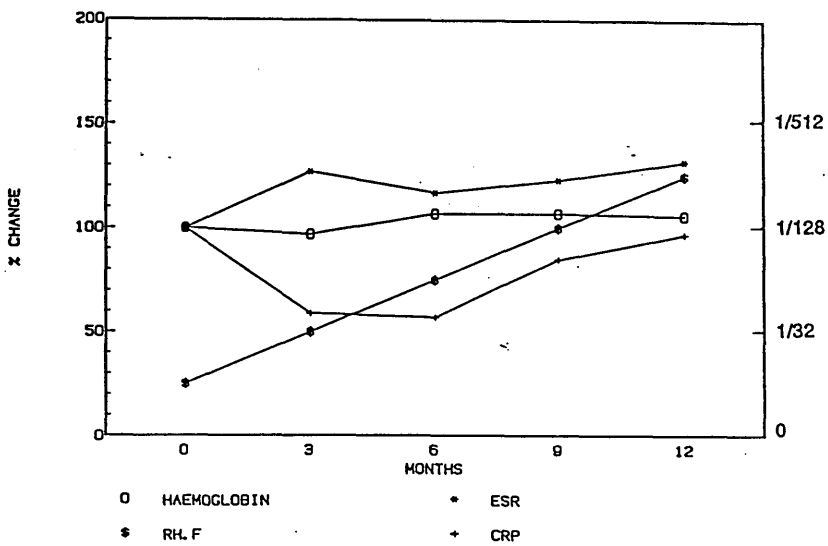


Fig. 7.5. Changes in 'laboratory' indices of disease activity in RA patients receiving EPO, EPO/fish oil (EPO/marine) and placebo.

SUBJECTIVE RESPONSE

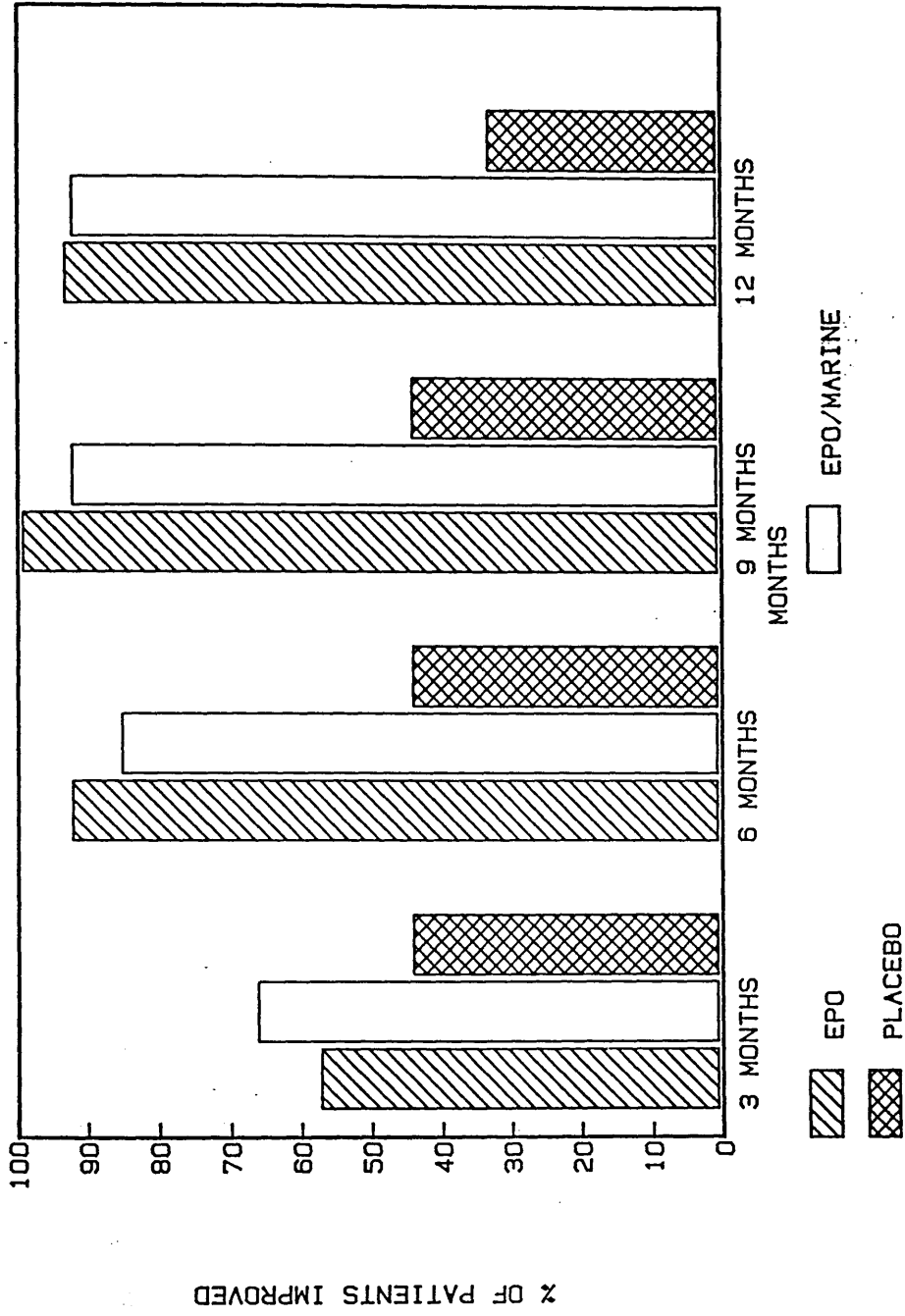


Fig. 7.6. Subjective response in RA patients receiving EPO, EPO/fish oil (EPO/marine) and placebo.

Figure 7.4 shows a compilation of results (means) for the clinical measurements in the 3 groups; no significant change in any of the four parameters measured (morning stiffness, grip strength, visual analogue and articular index) was observed over the study period.

Figure 7.5 shows the results (means) of the 3 groups for “laboratory” indices of disease activity (Hb, RF, ESR and CRP). Again, no significant change in these parameters was noted over the study period although, as with the clinical observations, the EPO group appeared to fare better.

Figure 7.6 shows the subjective response for the 3 patient groups over the study period. As one would expect (Beecher, 1955), the control group did show a marked placebo response of about 40% at 6 months. Surprisingly, this persisted throughout the study, although it fell to about 30% by 12 months. Despite there being no significant change in objective measured of disease activity, 94% of the EPO and 93% of the EPO/fish oil group felt a subjective improvement in their condition at 12 months. Most patients on active treatment recorded an increase in their general sense of well-being.

Following the 3 months placebo phase, all of the EPO patients and 80% of the EPO/fish oil patients had either returned to “baseline” or become worse. This compared to a relapse rate of only 14% in the placebo group.

Four patients in the study experienced side-effects. Two in the EPO group (one with nausea and one with gastrointestinal upset) required to be withdrawn from the study at 2 months and 1 month respectively (Table 7.2). Two patients in the EPO/fish oil group also experienced side-effects (nausea and headache) although neither was required to be withdrawn from the study.

Estimation of fatty acid levels in plasma and erythrocyte membranes by HPLC (Efamol laboratories) confirmed compliance in all subjects apart from one

EPO/fish oil patient. This patient was one of the two from this group who were withdrawn due to increasing RA symptoms.

7.2 (h) (ii) Effect on LTB₄ production

In the EPO group, LTB₄ production from A23187-stimulated PMN's appeared to fall from 1 month onwards and a significant decrease was noted at 3 months ($p < 0.02$, Mann-Whitney) and 5 months ($p < 0.05$, Mann-Whitney) (Fig. 7.7). The maximum decrease in LTB₄ observed over this period was 37%. During the placebo phase, LTB₄ levels appeared to rise again and by 21 months had returned to pre-study levels.

In the EPO/fish oil group, no significant change in LTB₄ was noted throughout the study, although levels did appear to fall during the treatment period and rise again during the placebo phase (Fig. 7.8).

In the placebo group, a significant increase in LTB₄ ($p < 0.04$, Mann-Whitney) was noted at 4 months (Fig. 7.9). No LTB₄ determinations were made between 9-12 months in this group basically due to a paucity of placebo patients continuing beyond 9 months. Figure 7.9 also shows the 5,6 and 9 month data analysed to include "last data" of the patients withdrawn after 4 months. The reason for this is discussed in section 7.2 (i) (ii).

Figure 7.10 shows the LTB₄ results for all three groups on one graph for direct comparison.

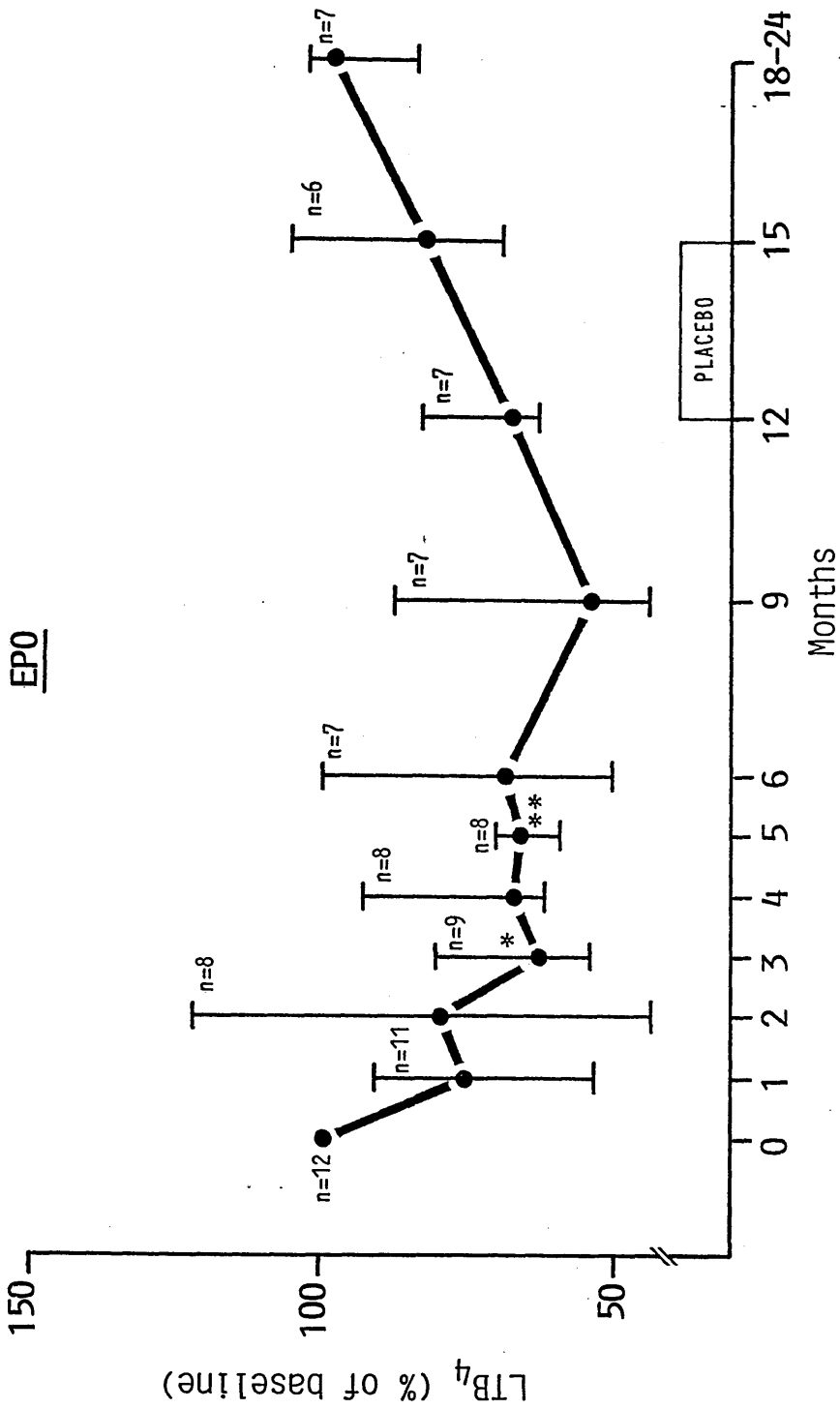


Fig. 7.7 Change in leukotriene B₄ production (median, IQR) from A23187-stimulated PMN's of RA patients receiving Evening Primrose Oil (EPO).

(n = number of patients)

*p<0.02, **p<0.05

EPO/fish oil

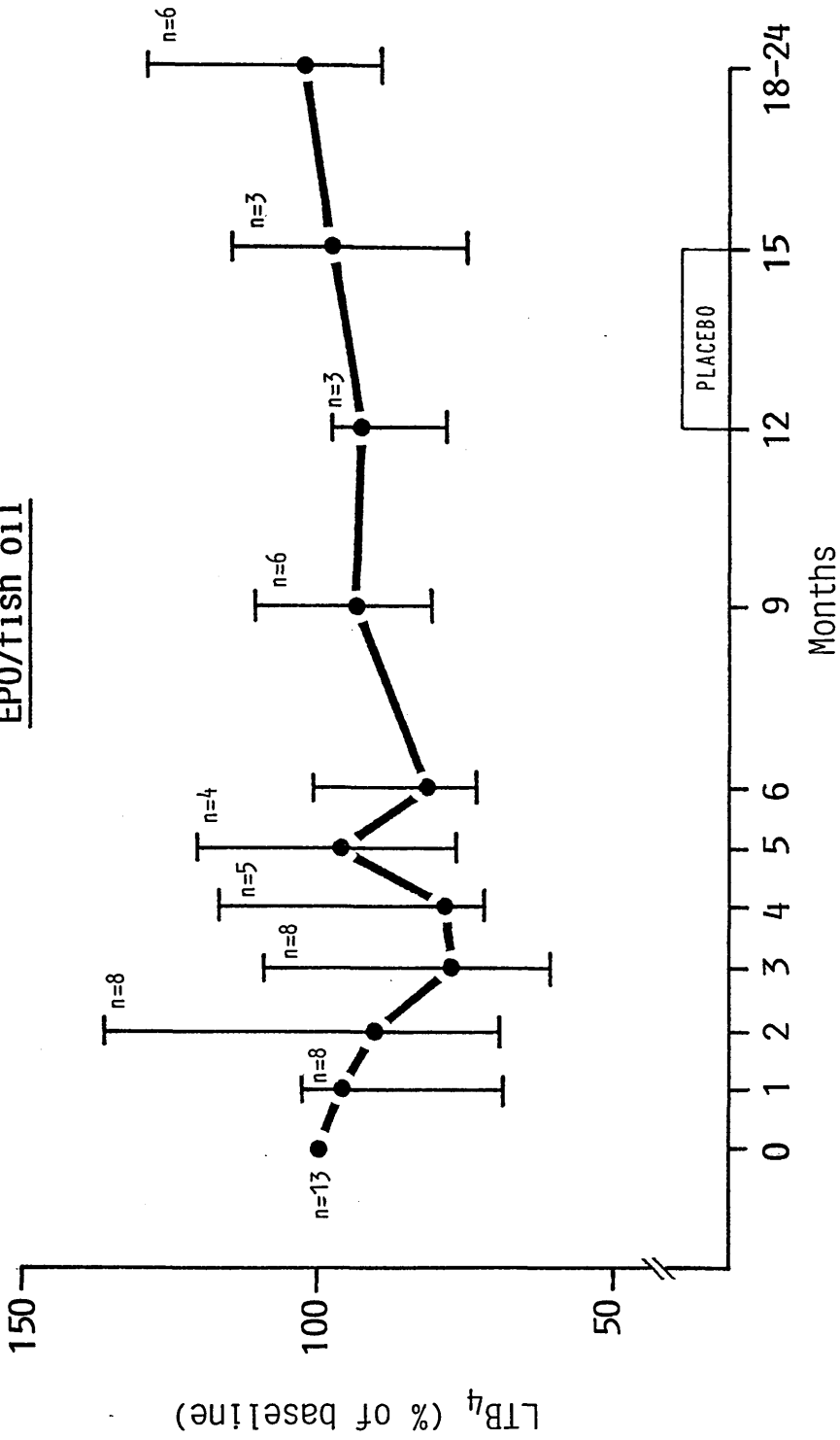


Fig. 7.8 Change in leukotriene B₄ production (median, IQR) from A23187-stimulated PMN's of RA patients receiving Evening Primrose Oil (EPO)/fish oil.
(n = number of patients)

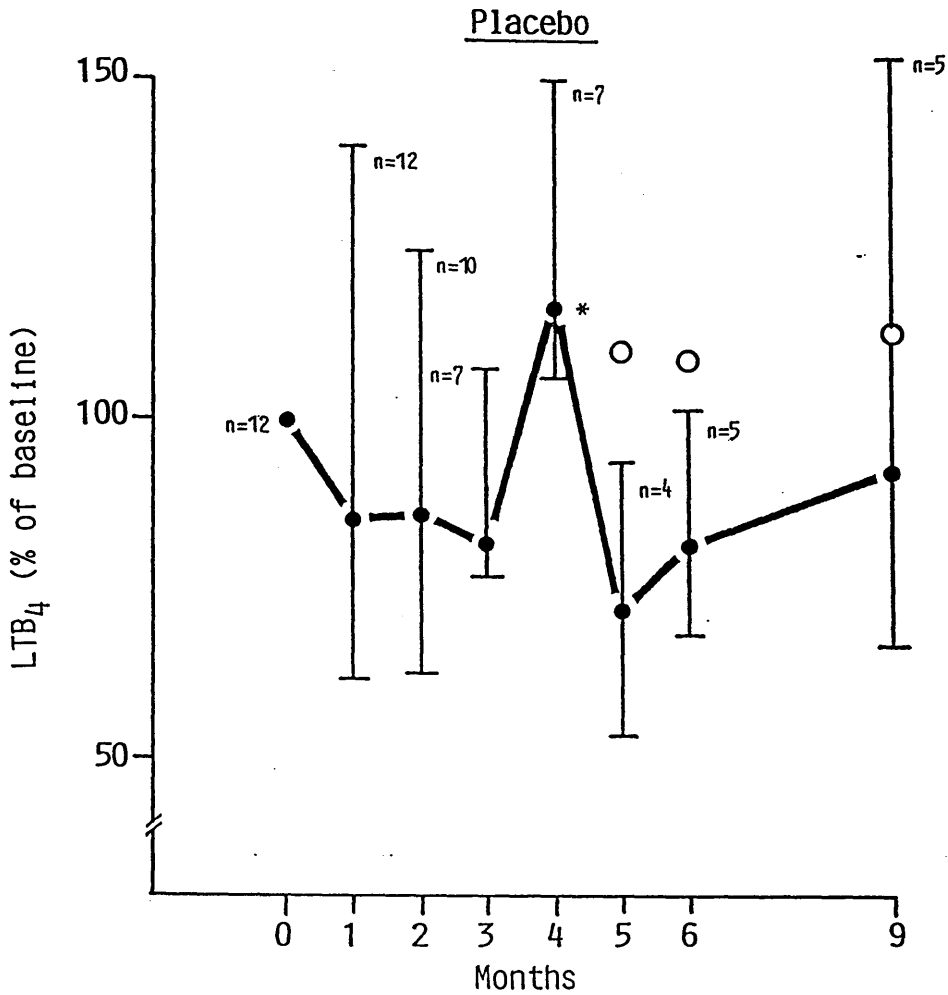


Fig. 7.9 Change in leukotriene B₄ production (median, IQR) from A23187-stimulated PMN's of RA patients receiving liquid paraffin placebo.

(n = number of patients)

* p<0.04

○ including "last data" from patients withdrawn after 4 months

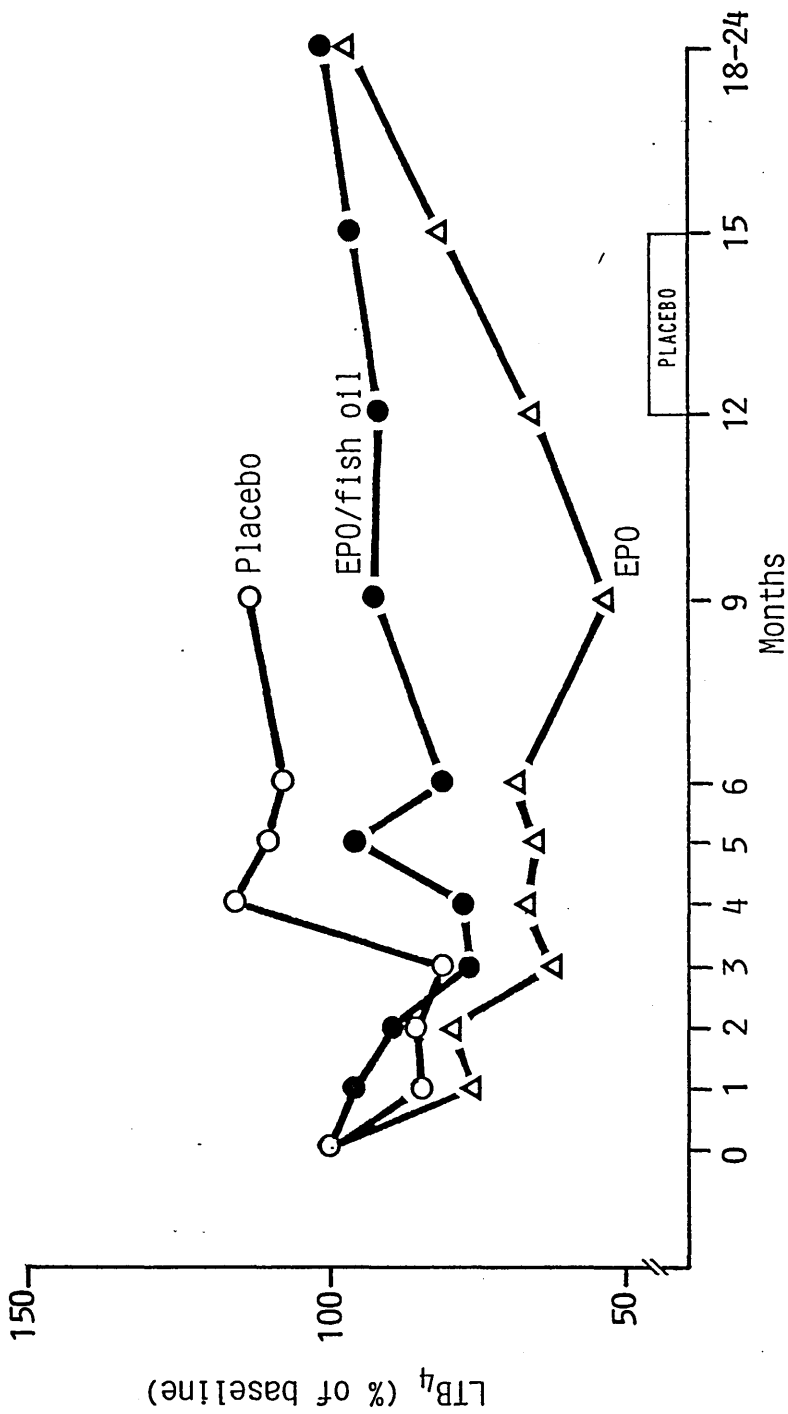


Fig. 7.10 Effect of EPO, EPO/fish oil and placebo on LTB₄ generation by stimulated PMN's of RA patients - comparison of the three treatment groups (Medians). Placebo group data for 5,6 and 9 months are shown including "last data" from 4 months. Withdrawal of NSAID's was initiated after 3 months.

7.2 (i) Discussion

7.2 (i) (i) Clinical aspects

The theory and evidence which suggests that dietary supplementation with EPO/fish oil may produce anti-inflammatory effects similar to CO inhibition by NSAID's have been outlined in section 7.1. Indeed, additional effects might be anticipated because of the inhibition of LT production not seen with the NSAID's. Furthermore fewer gastric side effects would be expected as PG's of a less inflammatory nature would still be available for gastric cytoprotection.

The results of this study have shown that it was possible for some patients with RA to decrease or stop NSAID treatment when EPO or EPO/fish oil was given. This was achieved with no deterioration in clinical or laboratory measures of RA disease activity. A decrease in NSAID dose was also observed in the placebo group, though this was much less marked. In combination with the 30% placebo subjective response, this stresses the importance of placebo controlled studies in RA.

Despite there being no objective improvement in symptoms on the active oils, there was a very definite subjective improvement, most patients reporting an improvement in their general sense of well-being. The mechanism for this is unclear, however, EPO has been used with some success in the treatment of pre-menstrual tension and is known to alter PG production in the brain (O'Brien, 1983; Brush, 1983). In addition, PGE₁ has been reported as having an anti-depressant effect (Parmigiani, 1982) and an increase in PGE₁ during EPO treatment may have been responsible for the subjective improvement witnessed in the study. It is also possible that reducing NSAID therapy may remove some of it's negative effects on the patients' well-being.

The relapse in patients during the placebo phase of the study was also examined. since only 14% of the placebo group relapsed it seems likely that spontaneous remission had occurred in the other placebo patients. In contrast, all the patients in the EPO group either felt worse or returned to pre-study level of subjective symptoms and

80% of the EPO/fish oil group relapsed on placebo. The fact that 20% of patients in the EPO/fish oil group maintained their improvement suggests that the fish oil may have had a longer lasting effect. There is some evidence to suggest that EPA is metabolized more slowly than DGLA (Tate *et al.*, 1988) and may therefore persist in cell membranes for longer. Unfortunately EFA analysis of the 15 month samples was not part of the protocol so this was never checked. However, it is also possible that DGLA incorporation into cell membranes could have been enhanced by the presence of EPA in this group since in animal studies, the tissue DGLA:AA ratio is increased substantially in animals fed a combination of GLA and EPA (Nassar *et al.*, 1986). This effect may be attributed to inhibition by EPA of the conversion of DGLA to AA (Tate *et al.*, 1988) thus increasing the amount of DGLA incorporated into cell membranes.

In two previous studies which looked at the effect of EPO on RA (Brown *et al.*, 1980; Hansen *et al.*, 1983) no clinical improvement was observed. However, both these studies used a lower dose regime of EPO and a study period of only 3 months; results from our study suggest that improvement is most marked from 6 months onwards both with respect to NSAID withdrawal and subjective improvement. In addition, the patients in these two other studies stopped NSAID's abruptly prior to the start of the studies which may have caused an initial flare in symptoms. The combination of NSAID withdrawal and short treatment period may therefore explain the negative findings. It is also possible that these two studies looked at different patient populations. All of the patients in our study had mild RA as shown by the clinical and laboratory parameters in Table 7.1. In contrast, the other two studies looked at patients with more severe disease, in particular, the group studied by Hanson *et al.* had markedly higher ranges of ESR and much lower ranges of Hb. Furthermore, these patients had been on second-line drugs such as prednisolone prior to the study and may already have had decreased LT levels, leaving little scope for further benefit via inhibition of LTB₄ by EPO.

Studies investigating the effect of EPA in RA have been more numerous. In the first of the studies by Kremer *et al.* (1985) an improvement in AI was noted at 12

weeks in the fish oil group, but not in the placebo group. In the later study (Kremer *et al.*, 1987), four other indices of disease activity were significantly improved after 14 weeks treatment. Cleland *et al.* (1988) reported similar results with a significant improvement in AI and grip strength after 12 weeks of EPA treatment. Sperling *et al.* (1987), in an open study, observed an improvement in several indices of disease activity, but only the more subjective measures (AI and patient assessment of overall disease activity) reached statistical significance. These studies however, cannot be directly compared with the present study where fish oil was used only in combination with EPO; the only valid assessment of the effect of EPA which can be made in this case is in comparison with the EPO group. Although results suggest that there was a slightly earlier response with the combined therapy (NSAID dosage and subjective response) and that the effect was preserved for longer after cessation of treatment, there was no significant difference in the clinical or laboratory response between the EPO and EPO/fish oil groups. It is possible that the addition of fish oil had no effect and the slightly lower dose of EPO used in this group was sufficient to allow NSAID withdrawal. Alternatively, it is possible that the fish oil contributed to the effect of the lower dose EPO making the two groups undistinguishable. Certainly, there is evidence in the literature to suggest a synergism between EPA and DGLA (Juan & Sametz, 1985; Nasar *et al.*, 1986; Tate *et al.*, 1988), however the design of the present study was not adequate to test for this hypothesis.

In all the other studies cited above, dietary supplementation was carried out against a background of stable NSAID (and sometimes corticosteroid) therapy, and investigators were looking for further improvement in symptoms on the dietary supplements. However, the emphasis of the study reported here was slightly different in that it's aim was to gradually replace NSAID therapy with EPO and EPO/fish oil supplements with no deterioration in RA symptoms, and this was achieved. In this respect, therefore, the EPO and EPO/fish in the present study can be considered to be stabilizing RA symptoms during NSAID withdrawal rather than giving a positive improvement.

7.2 (i) (ii) Effect on LTB₄ production

The fact that DGLA is not metabolized to LT's and that it forms a 15-OH derivative which inhibits the transformation of AA to LT's, (Willis, 1981) implicates it as a potential modulator of LTB₄ synthesis in PMN's. In this study, stimulated PMN's from the patient group treated with EPO (540 mg GLA/day), did show decreased generation of LTB₄ as measured by RIA (Fig. 7.7). This decrease was apparent by 1 month into the study and reached statistical significance by 3 months. The reason why this significant decrease (37%) was not sustained is not clear, however, it should be noted that NSAID withdrawal was initiated after 3 months and may have had some effect, albeit opposite to the predicted effect. During the placebo period and beyond (12-24 months), LTB₄ levels rose steadily to return to pre-treatment values.

Surprisingly, patients on the EPO/fish oil supplement showed less of an effect on LTB₄ production than those taking EPO alone (Fig. 7.8). Although median levels did fall during the treatment phase and rise again during the placebo phase, statistical significance was not achieved. However, it is well established in the literature that EPA is a good substrate for the 5-LO and that the efficiency of conversion of EPA to LTB₅ is similar to that of AA to LTB₄ (Lee *et al.*, 1985). Results from other studies (both clinical and normal volunteer) have demonstrated a clear inhibitory effect of EPA on LTB₄ synthesis in PMN's, the amount of LTB₄ being reduced by upto 48% at 6 weeks (Lee *et al.*, 1985; Sperling *et al.*, 1987; Cleland *et al.*, 1988). In some studies, the level of LTB₄ inhibition achieved has been shown to be disproportionate to the percentage incorporation of EPA into PMN membranes, an observation which has been attributed to inhibition of epoxide hydrolase by LTA₅ (Sperling *et al.*, 1987). In another more short-term study of 3 weeks (Prescott *et al.*, 1985), LTB₄ was shown to decrease proportionately to the synthesis of LTB₅ and it is possible that a more substantial enrichment of membranes with EPA is required before enough LTA₅ becomes available to significantly inhibit the epoxide hydrolase. It may be, therefore, that the dose of EPA employed in our study (240 mg/day) was not sufficient to have a clear inhibitory effect on

LTB₄ synthesis; in other studies where decreased LTB₄ has been demonstrated, somewhat higher doses of EPA were used (Cleland *et al.*, 3.2 g/day; Lee *et al.*, 3.2 g/day; Sperling *et al.*, 3.6 g/day).

Additionally, the daily dose of GLA in the combined therapy group (450 mg) was lower than that in the EPO-only group. Other work has suggested that a synergistic effect between DGLA and EPA may occur, which was the basis of giving a reduced daily dose of EPO in combination with fish oil. For instance, EPA is thought to inhibit the conversion of DGLA to AA, thereby increasing DGLA incorporation into cell membranes (Nassar *et al.* (1986) and in a study of MDSU-induced inflammation in rats (Tate *et al.*, 1988), a GLA enriched diet has been observed to suppress the cellular aspects of the inflammatory response whereas an EPA-enriched diet suppressed the fluid phase; a combined diet was required to reduce both phases and produced the most effective result. Within the limitations of our study however, synergy between DGLA and EPA was not witnessed, either in terms of LTB₄ inhibition or change in disease activity. The suggestion from the clinical observations that the EPO/fish oil produced an earlier and a longer-lasting response than the EPO supplement was not supported by their comparative effects on LTB₄ production.

Also to be taken into consideration is the fact that LTB₅ may be expected to exhibit a cross-reaction of around 17% with LTB₄ antiserum in the RIA (Terano *et al.*, 1984). However, the amounts of LTB₅ formed during EPA supplementation are relatively low (Cleland *et al.*, 1988) and even combined LTB₄ + LTB₅ levels have been shown to be decreased compared to LTB₄ levels prior to supplementation (Lee *et al.*, 1985; Cleland *et al.*, 1988).

The significant increase in LTB₄ at 4 months in the placebo group probably reflects the worsening of these patients following the initiation of NSAID withdrawal (Fig. 7.9). The apparent decrease at 5 months and beyond therefore, is most likely a spurious result due to removal of these patients from the analysis. For this reason, the

5,6 and 9 month results are also shown including the “last data” for patients who were withdrawn after 4 months.

7.2 (i) (iii) Conclusion

In conclusion, this study has demonstrated that it was possible to reduce or stop NSAID therapy in some RA patients, probably those with milder disease, by introducing dietary supplements of EPO or EPO/fish oil. This was achieved with no deterioration in any of the indices conventionally used to assess disease activity and in addition a subjective improvement in RA symptoms was observed.

A significant decrease in LTB_4 production from stimulated PMN's was detected after 3 months on the EPO diet, but a combination of EPO and fish oil produced a less clear inhibition of LTB_4 . This may have been due to the fact that the dose of EPA was too low to produce a decrease in LTB_4 and that the lower dose of EPO in the combined therapy was also insufficient to produce a decrease. However this could not be conclusively determined with the particular study design employed in this case.

Since LTB_4 levels decreased during the period of active treatment (when improvement was observed in both groups) and increased during the placebo phase (when most patients returned to pre-treatment level of symptoms), inhibition of LTB_4 could be one factor contributing to symptomatic improvement in patients on these dietary supplements (EFA's have other functions independent of their role as eicosanoid precursors; these are discussed in chapter 9). However, it seems unlikely that treatment with these oils would alter the course of prognosis of the disease and might be best used in clinical situations where NSAID therapy should be avoided, for example, in patients with peptic ulceration or renal impairment. It would also seem desirable that where dietary supplements are used, NSAID's are withdrawn gradually rather than stopped abruptly.

7.3 Dietary Manipulation of EFA's in Psoriatic Arthritis

7.3 (a) Aim of study

Management of the arthritis in patients with psoriatic arthropathy is often difficult, with some NSAID's exacerbating the psoriasis and an uncertain clinical response to second line drugs. An ideal therapy would be one which would benefit both the arthritis and psoriasis with a low incidence of side-effects. The observation that Eskimos have a lower incidence of psoriasis than Danes (Kromann & Green, 1980) led to speculation that dietary fish oil may be a contributory factor and two studies have subsequently suggested that fish oil is of benefit in psoriasis (Ziboh *et al.*, 1986; Bittener *et al.*, 1988). Dietary fish oil is also thought to give improvement in RA (Kremer *et al.*, 1985; Cleland *et al.*, 1988). The same rationale for the use of DGLA in the treatment of RA (section 7.1) applies in the case of PA. The aim of this study was to determine whether an EPO/fish oil combination (Efamol Marine) could be substituted in place of NSAID therapy without deterioration in the clinical symptoms of PA. In addition, levels of PGI₂-M's and LTB₄ from stimulated PMN's and serum TXB₂ were measured throughout the study.

7.3 (b) Patients

Thirty-eight patients with chronic stable plaque psoriasis and inflammatory arthritis were enrolled in the study. All patients had peripheral joint involvement plus or minus sacro-iliitis and all were seronegative for RF. Patients were on a stable dose of NSAID for at least one month prior to entering the study. Those undergoing treatment with systemic steroids were excluded.

Nineteen patients were randomly selected to receive 12 Efamol Marine capsules per day providing a total daily dose of 480 mg GLA, 240 mg EPA, 132 mg DCHA and 120 mg vitamin E. The remaining 19 patients received 12 visually identical capsules containing liquid paraffin.

7.3 (c) Study design

The duration of the study was 12 months. Patients were instructed to take their oil capsules in addition to their usual dose of NSAID's for the first 3 months. Thereafter, they were instructed to try to reduce their NSAID intake provided this produced no flare in their symptoms. From 9-12 months, all patients received placebo. The study was double-blind although investigators were aware that all patients were taking placebo in the final 3 months.

7.3 (d) Clinical assessment of disease activity

Full metrological assessment was carried out by the same investigator at the start of the study and at months 1,3,6,9 and 12 to assess change in indices of inflammatory joint disease namely grip strength, number of active joints, Ritchie AI, duration of morning stiffness and NSAID intake. Change in psoriasis was assessed using 10 cm visual analogue scales on which the clinician recorded severity of disease, percentage body area affected, scaling and erythema. The patient was asked to record, also on a visual analogue scale, whether any change in skin itch had occurred.

7.3 (e) Biochemical assessment

At each visit blood samples were taken for determination of Hb, ESR and CRP by the routine laboratories. Samples were also taken at 0,3,6,9 and 12 months for estimation of fatty acids in plasma and erythrocyte membranes to assess compliance (Efamol Laboratories).

7.3 (f) Measurement of eicosanoids

The effect of the dietary supplementation on AA metabolism was investigated by measuring LTB_4 and PGI_2 -metabolites in supernatants from CaI A23187-stimulated PMN's and TXB_2 levels in serum in both the active and placebo groups. All metabolites were measured using RIA as described in sections 4.3, 4.4 and 4.5.

7.3 (g) Statistical analyses

Comparability between the two treatment groups was analysed using the Mann-Whitney U test. Sequential analyses on individuals were performed using the two-tailed paired Wilcoxon test except in the case of eicosanoid measurements where the Mann-Whitney U test was used.

7.3 (h) Results

7.3 (h) (i) Clinical aspects

Patients in the two groups at the beginning of the study were comparable for all laboratory and clinical parameters except skin itch which was significantly worse in the active group ($p < 0.035$, Mann-Whitney) Table 7.3). Of the 39 patients enrolled in the study, 34 completed to month 9 and 32 completed to month 12. Of the 6 withdrawals, all were on active treatment - 1 patient had an exacerbation of migraine, 2 patients experienced gastro-intestinal upset, 2 patients were withdrawn because of inefficacy and 1 patient defaulted after month 6. No information regarding patient compliance (as determined by EFA analysis at Efamol Labs.) was available at the time of this report.

At month 3, the placebo group showed a significant improvement in articular index when compared to the active group, although this difference was not apparent at 6,9 or 12 months (Table 7.4). No other parameters of joint activity showed a significant change over the study period. With regard to skin symptoms, although itching was initially worse in the active group, this difference was not apparent at 3 months, suggesting a clinical improvement (Table 7.4). During the placebo phase, a worsening of skin itch in the active group was observed, but this did not reach statistical significance. All other parameters of skin severity showed no significant change over the treatment period.

In the case of 'laboratory' indices of disease activity, no significant differences were noted in ESR, CRP or Hb over the study period in either group (Table 7.5).

Table 7.6 shows the numbers of patients in the two groups who had reduced or stopped their NSAID therapy by the end of the study. Since 2 patients in the active group and 3 patients in the placebo group were not on NSAID's at the start of the study this meant that 6/17 (35%) of patients in the active group and 7/16 (44%) of patients in the placebo group had reduced or discontinued NSAID's by the end of the study.

	PLACEBO	ACTIVE
Age (yrs)	40 (25-58)	40 (18-76)
Male:Female	7:12	7:12
Duration Arthritis (yrs)	11 (2-26)	9 (1-30)
Duration Psoriasis (yrs)	16 (2-30)	16 (2-30)
Articular Index	9 (3-16)	8 (4-22)
Morning Stiffness (mins)	60 (0-240)	60 (0-720)
Skin Severity	20 (0-80)	15 (0-70)
Percentage Body Area	20 (0-77)	19 (0-81)
Itch	7.5 (0-53)	18 (0-86)*
ESR (mm/h)	16 (2-103)	15 (2-110)
CRP (N.R. <10 mg/l)	12 (10-88)	18 (10-83)
Hb (g/l)	12.7 (8.5-16.2)	12.9 (10-16.7)

* p <0.035 (Mann-Whitney U test)

Table 7.3 Comparability of active and placebo treatment groups of PA patients at start of study (Median (range)).

Month	0	3	6	9	12
Skin Itch					
Active	18 (0-86)	9 (0-55)	22 (0-66)	11 (0-70)	14 (0-70)
Placebo	*7.5 (0-53)	4.5 (0-75)	8 (0-77)	5 (0-55)	3 (0-67)
Articular Index					
Active	8 (4-22)	11.5 (0-25)	9.5 (1-28)	10 (1-27)	10 (1-32)
Placebo	9 (3-16)	*5.5 (0-18)	7 (2-33)	9 (3.20)	10 (2-35)

Table 7.4 Changes in skin itch and articular index in active and placebo groups of psoriatic arthritis patients (Median (range)).

* both $p < 0.035$ (Mann-Whitney)

MONTH	0	3	6	9	12
ESR (mm/h)					
Active	15 (2-110)	20 (2-11.4)	20 (1-57)	22 (2-77)	28 (2-72)
Placebo	16 (2-103)	16 (4-65)	17 (3-90)	20 (1-75)	20 (4-103)
CRP (mg/l)					
Active	18 (10-83)	13 (10-73)	13 (10-75)	10 (10-56)	10 (10-59)
Placebo	12 (10-88)	10.5 (10-83)	13 (10-105)	10 (10-92)	10 (10-87)
Hb (g/l)					
Active	12.9 (10-16.7)	11.9 (10.2-14.4)	12 (9.6-15.6)	12.5 (10.3-16.5)	12.5 (10.3-16.5)
Placebo	12.7 (8.5-16.2)	13 (10.5-15.3)	12 (10.3-14.4)	12.6 (9.5-13.2)	12.8 (9.6-14.0)

Table 7.5 Changes in laboratory indices of disease activity in active and placebo groups of psoriatic arthritis patients (Median (range)).

	ACTIVE	PLACEBO
Total patients	19	19
Reduced NSAID's	3	3
Stopped NSAID's	3	4
Not on NSAID's	2	3
Reduced or stopped by 12 months	6/17 (35%)	7/16 (44%)

Table 7.6 Number of psoriatic arthritis patients in active and placebo groups who reduced or stopped NSAID therapy over the 12 month study period.

7.3 (h) (ii) Eicosanoid production

The median (IQR) values for LTB₄, PGI₂-M's and TXB₂ determined in both the placebo and active groups throughout the study period are shown in Figures 7.11, 7.12 and 7.14 respectively.

Leukotriene B₄ production from A23187-stimulated PMN's in the active group tended to fall from 1 month onwards and a significant decrease (22%) was obtained at 9 months ($p < 0.035$, Mann-Whitney). Levels appeared to rise again during the 3 month placebo phase, but this did not reach statistical significance. No significant change in LTB₄ levels was detected in the placebo group throughout the 12 months (Fig. 7.11).

No significant difference in PGI₂-M production from stimulated PMN's was found in either the placebo or active groups when intra-group comparisons were made (Fig. 7.12). However, in comparison to the placebo group values in the active group appeared to fall and a significant difference between the two groups was observed at 6 months ($p < 0.035$, Mann-Whitney) (Fig. 7.13). (There had been no significant difference in PGI₂-M's between the two groups at month 0). In addition, the "rebound" effect on PGI₂-M's observed in the active group during the placebo phase suggested that the Efamol Marine was having a suppressant effect.

Serum TXB₂ levels (reflecting the generation of TXA₂ by platelets during blood clotting at 37°C *in vitro*) rose in the placebo group throughout the study period. In contrast, TXB₂ levels in the active group remained relatively constant until 9 months when patients switched to placebo and an increase was observed (non-significant). Inter-group analysis showed a significant increase in TXB₂ production in the placebo group at 6 months ($p < 0.006$) and 9 months ($p < 0.045$, both Mann-Whitney) (Fig. 7.14). There had been no significant difference in TXB₂ levels between the two groups at month 0.

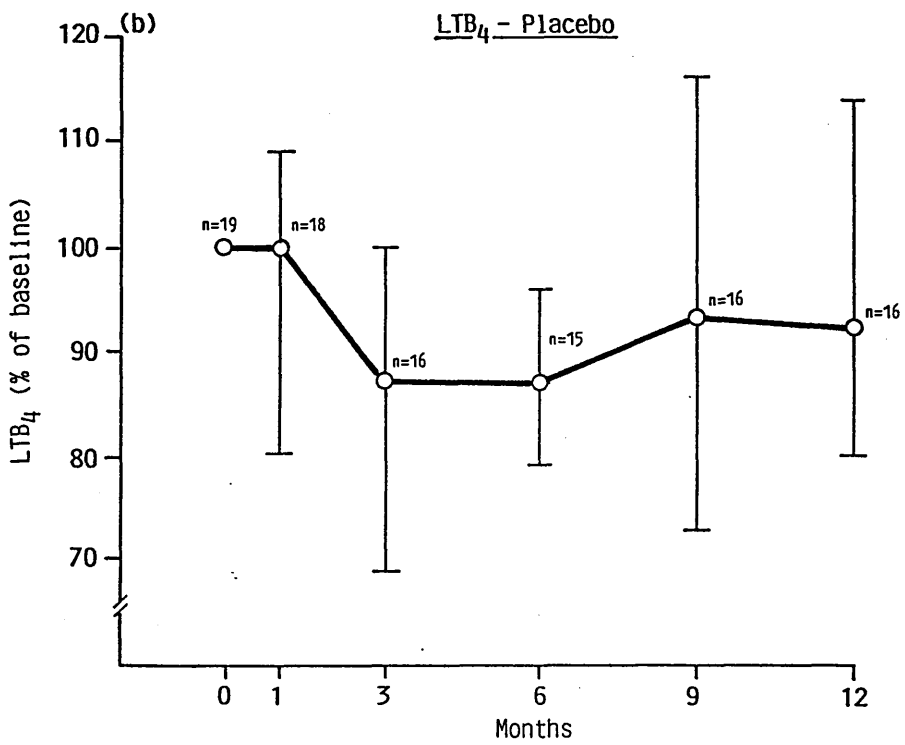
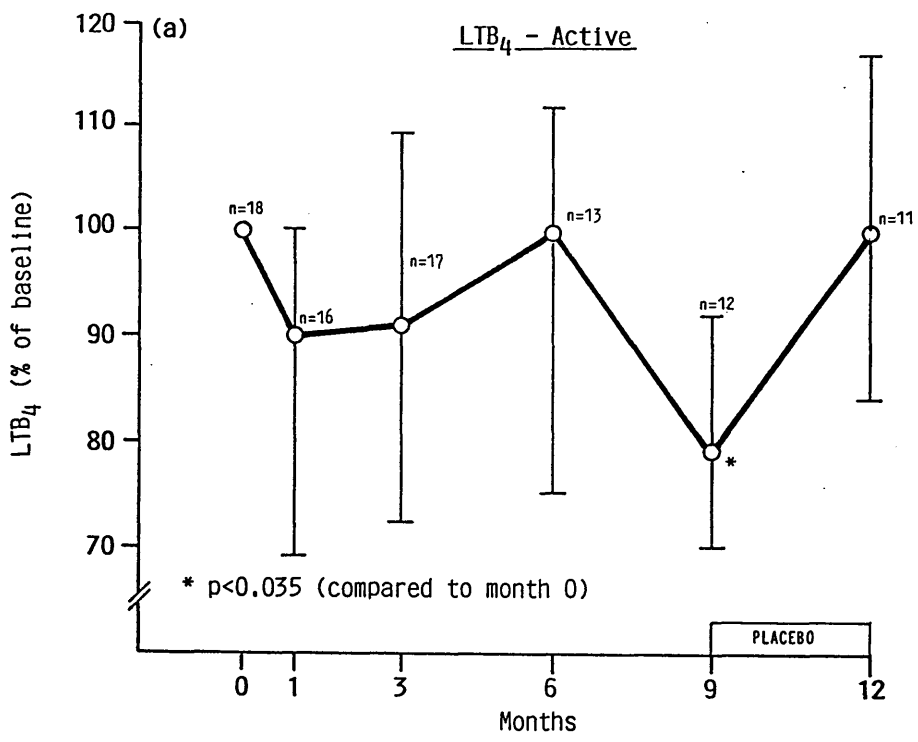


Fig. 7.11 Change in leukotriene B₄ production (median, IQR) from A23187-stimulated PMN's of PA patients on (a) active treatment (Efamol Marine) and (b) placebo. (n = number of patients)

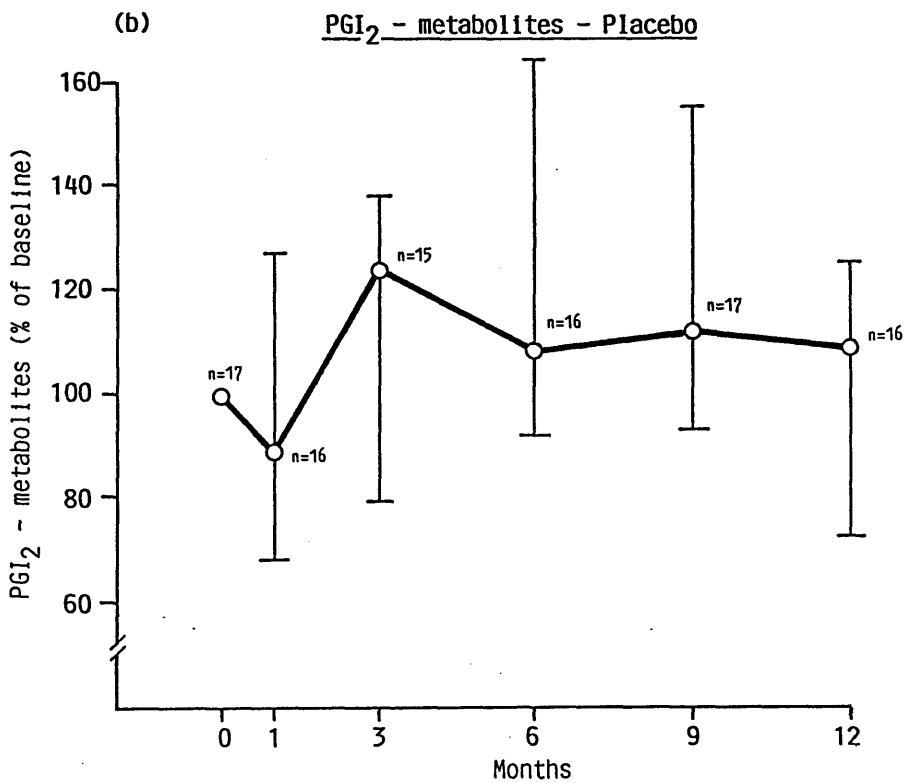
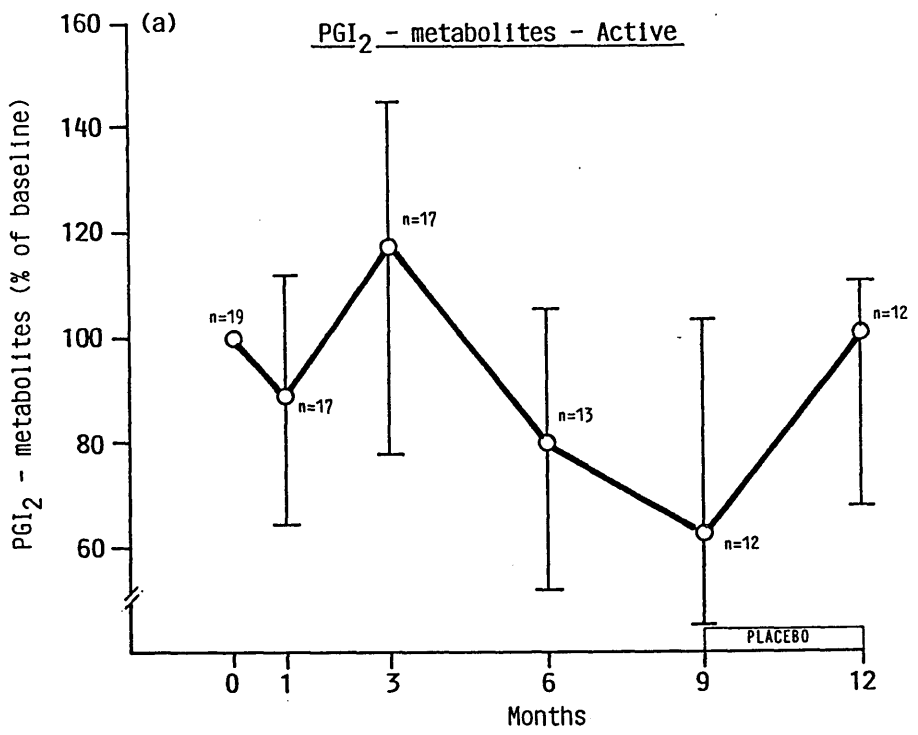


Fig. 7.12 Change in production of PGI₂-metabolites (median, IQR) from A23187-stimulated PMN's of PA patients on (a) active treatment (Efamol Marine) and (b) placebo. (n = number of patients)

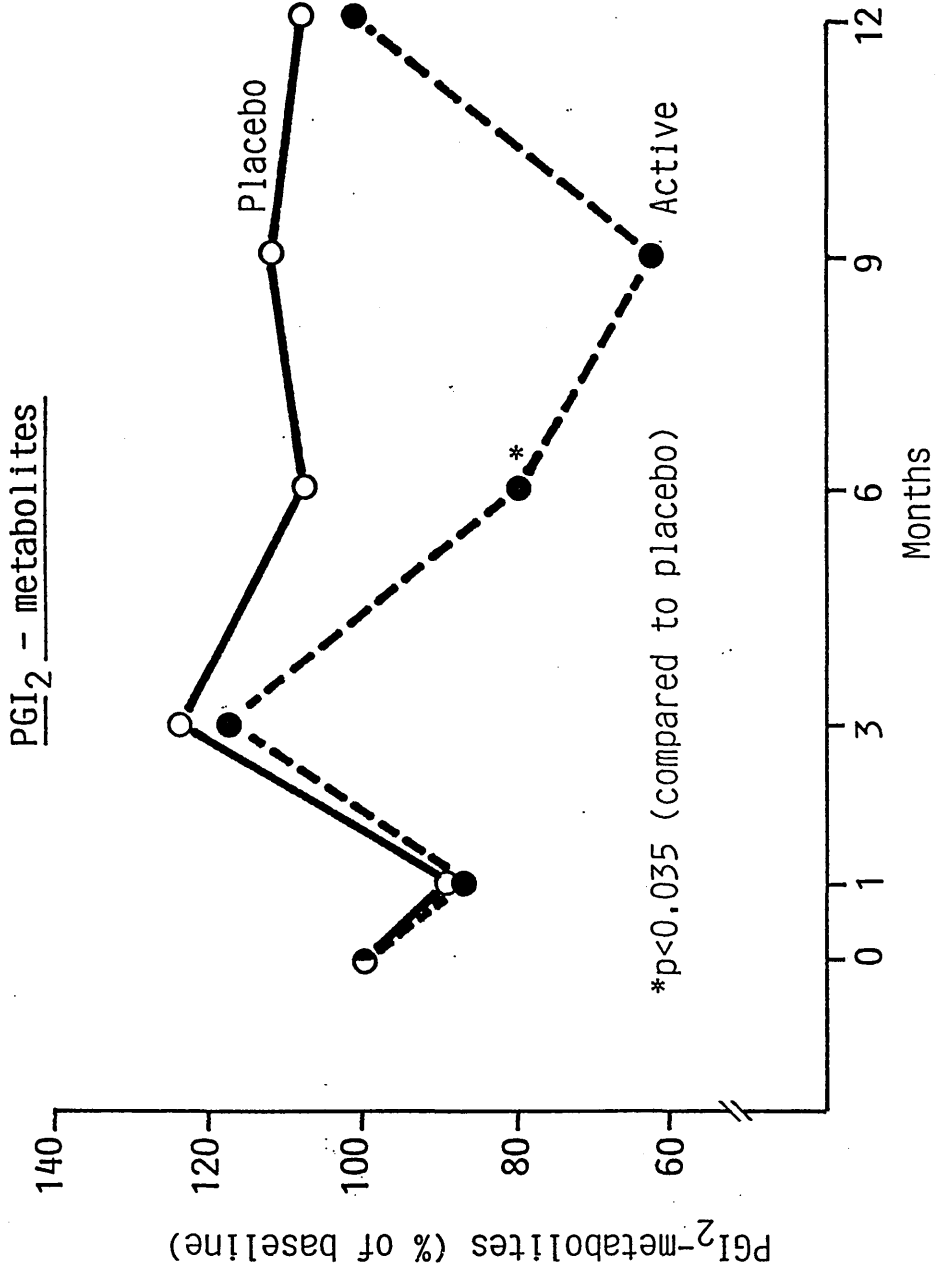


Fig. 7.13 Change in PGI₂-metabolite production from stimulated PMN's of PA patients on active treatment (Efamol Marine) and placebo. (Values shown are medians)

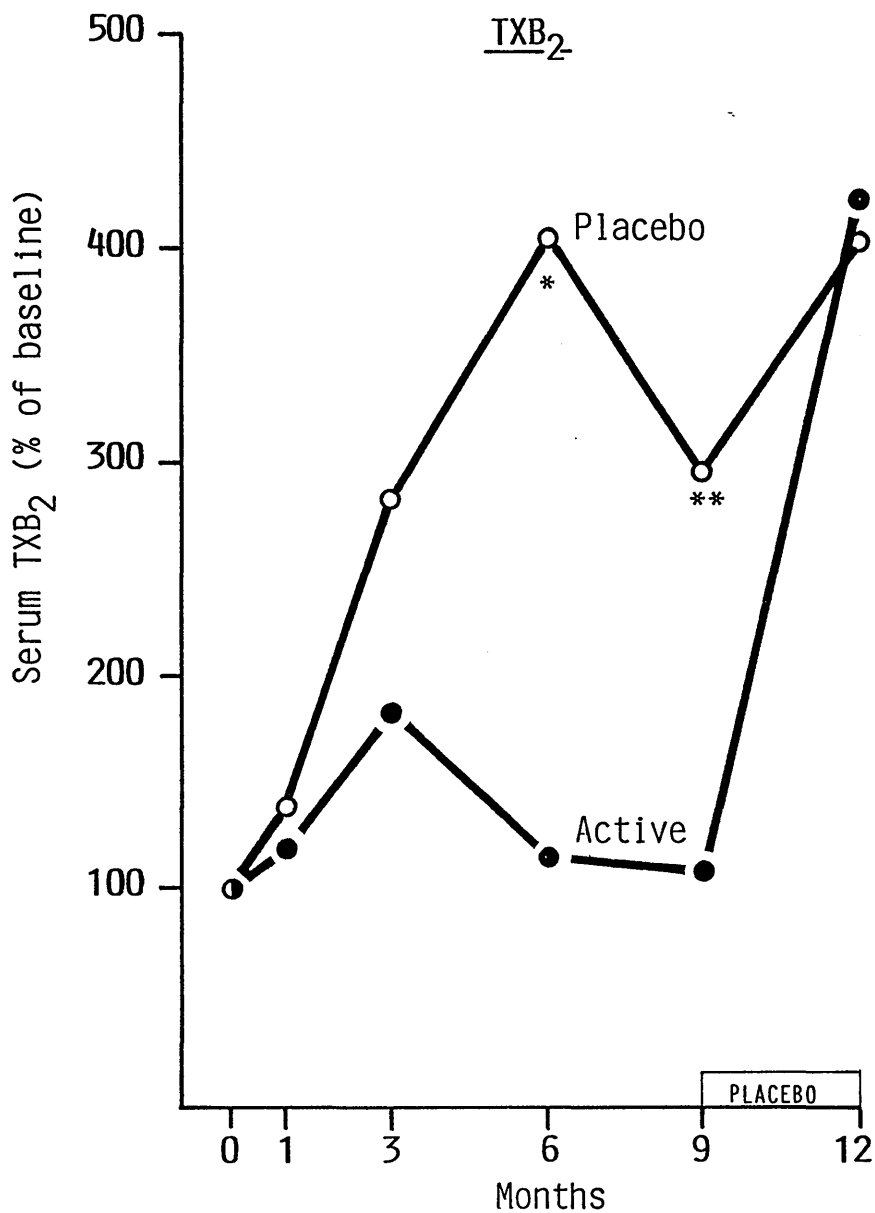


Fig. 7.14 Change in serum TXB₂ (medians) in PA patients on active treatment (Efamol Marine) and placebo.

* p<0.006

(both compared to active group)

**p<0.045

7.3 (i) Discussion

7.3 (i) (i) Clinical aspects

Since dietary supplementation with EPO and/or fish oil appears to have a beneficial effect on both inflammatory arthritis and psoriasis, when studied independently, it would seem to suggest itself as an ideal treatment for individuals with psoriatic arthropathy. However, the results from this study were somewhat disappointing in that PA patients receiving Efamol Marine were not able to significantly decrease or stop their NSAID therapy. This in contrast to the RA study in which similar doses of Efamol Marine allowed NSAID's to be withdrawn.

These studies did, however, employ somewhat lower daily doses of EPA (240 mg) in comparison to other studies where clinical improvement in arthritis or psoriasis was observed. Kremer *et al.* (1987) and Cleland *et al.* (1988) used 2.7g EPA/day and 3.2g EPA/day respectively, in the treatment of RA. In a study by Bittener *et al.* (1988), 1.8g EPA/day produced a mild to moderate improvement in psoriatic lesions. In another psoriatic study, Ziboh *et al.* (1986) demonstrated a statistically significant reduction in scaling, erythema and lesional thickness after giving large quantities of liquid fish oil equivalent to 13.5g EPA (9g DCHA) daily. An interesting observation in Ziboh's study was that although an overall statistically significant improvement was seen with the 13 patients studied, these could in fact, be divided into 8 'clinical responders' and 5 'non-responders'. Moreover, a correlation between EPA: DCHA ratio achieved (in epidermis and neutrophils) and clinical response was observed which suggested that the non-responders had incorporated relatively larger quantities of DCHA into their cell membranes.

It may be the case therefore, that higher doses of EPA are required to give clinical improvement in PA. The designers of the present study may have speculated that the effect of the relatively low dose of EPA employed would be supplemented by the presence of the EPO; this did not seem to be the case.

There was a suggestion from the results that skin itch had improved in the active group during treatment and worsened during the placebo phase. Curiously, Wright & Buron (1982), in a study of eczema, also found that itch was the first symptom to respond to low doses of EPO, whereas at higher doses, an improvement in scaling and overall severity was observed. This would also seem to suggest that larger doses of EPO/fish oil may have been more effective.

The reason for the significant decrease in the number of active joints detected in the placebo group at 3 months is not known, but may reflect a degree of spontaneous remission which sometimes occurs in patients with psoriatic arthritis (Voorhees, 1983).

7.3 (i) (ii) Effect on LTB₄ production

In agreement with the results obtained in the RA study, the effect of the EPO/fish oil supplementation on LTB₄ production from PMN's was not as great as might have been anticipated. However, the median LTB₄ level did fall during the treatment period and rise during the placebo phase in the active group. The fact that the decrease in LTB₄ only reached statistical significance by 9 months may support the idea that the dose of EPO/fish oil was not quite high enough to give a clear, early effect on LTB₄ synthesis and therefore on clinical symptoms.

7.3 (i) (iii) Effect on PGI₂-M production

The effect of an EPA-rich diet on PGI₂ production in humans is not entirely clear, but most investigators have reported unchanged or even increased amounts of PGI₂ after EPA ingestion, both in terms of urinary metabolites of PGI₂ (Fischer & Weber, 1984; von Schacky *et al.*, 1985) and production from PMN's *ex vivo* (Weber *et al.*, 1985). The *ex vivo* results appear to be in contrast to those from studies with human endothelial cells in culture, where *reduced* PGI₂ synthesis was observed after pre-incubation of the cells with EPA (Spector *et al.*, 1983). Decreased levels of PGI₂-M's after EPA supplementation have also been detected in rat aorta (Hornstra *et al.*, 1981) but animal studies are not analogous to human studies in this respect, since rats do not form PGI₃ after dietary EPA.

PGI₃-M's may be expected to cross-react with antiserum for PGI₂-M's in RIA and although the extent of the cross-reaction in the RIA used in this work is not known, results obtained can be assumed to be a measure of combined PGI₂/PGI₃-M's. PGI₁ is not formed *in vivo* (Willis, 1981), but the effect of an EPA-rich diet also seems to be increased PGI₂ generation (Belch *et al.*, 1986b).

The contribution made by DCHA to the observed effects on prostanoid metabolism after fish oil supplementation is difficult to assess. However, it has been reported as having a strong, selective inhibitory effect on the CO pathway (Corey, Shih & Cashman, 1983).

In the present study, no significant change in PGI₂-M production from A23187-stimulated PMN's within the active group itself was seen throughout the treatment period. However, in comparison with the placebo group, a significant fall in PGI₂-M's was observed at 6 months. This may have been due to a tendency for CO products, including PGI₂-M's, to rise in the placebo group during attempts to reduce or stop NSAID's from 3 months onwards. If this was the case, then the EPA/fish oil treatment appears to have stabilized this increase during NSAID withdrawal in the active

group. The “rebound” of PGI₂-M’s during the placebo phase in this group is further evidence that such a suppressant effect may have been occurring.

Results from this study, therefore, appear to be in contrast to those of others where EPA/DGLA was shown to maintain or increase levels of PGI₂ -M’s *in vivo*. However, our study is different in that another factor affecting PG production (withdrawal of NSAID’s) is in operation. The effect of this in combination with the dietary supplementation is difficult to assess. It may also be the case that the potentiation of PGI₂ -M production in PMN’s during EPO/fish oil supplementation is dependent on inhibition of LTB₄ production thereby diverting AA substrate to the CO pathway. Results have shown that a significant reduction in LTB₄ was not achieved until late in the study and was comparatively small.

In retrospect, measurement of plasma PGI₂-M’s may have been a better way to assess the effect of EPO/fish oil.

7.3 (i) (iv) Effect on TXB₂ production

There exists in the literature extensive evidence which suggests that dietary EPA has an anti-thrombotic effect both in man and animals (Siess *et al.*, 1980; Dyerberg *et al.*, 1978; Lorenz *et al.*, 1983; Culp *et al.*, 1980). Part of this anti-thrombotic effect may be attributed to the synthesis of TXA₃ which is a less potent platelet aggregant than TXA₂ (Fisher & Weber, 1983), however the main mechanism whereby it occurs, is via direct competition of EPA with the conversion of AA by CO thereby decreasing platelet TXA₂ synthesis (Needleman *et al.*, 1979). Several studies have demonstrated decreased TXA₂ and reduced platelet aggregation after dietary supplementation with EPA (Lorenz *et al.*, 1983; von Schacky *et al.*, 1985). In the latter of these studies a reduction in serum TXA₂ of 50% was observed in normal volunteers after 12 weeks on an EPA diet.

Although the effect of DGLA on TXA₂ synthesis has not been so extensively studied it would appear that this also has the potential to reduce platelet TXA₂ synthesis both in animals (Kirtland *et al.*, 1986) and in man (Belch *et al.*, 1986). Another

component of the Efamol supplement used in this study, DCHA, has also been shown to reduce TXA₂ formation in platelets (Srivastava, 1985).

The observed effects of the EPO/fish oil supplement on serum TXB₂ generation in the present study were again complicated by the fact that patients taking the supplement were simultaneously reducing their NSAID intake. Although there had been no significant difference in TXB₂ levels between the two groups at the start of the study, by 6 months, levels in the placebo group were significantly higher than those in the active group. It would appear, therefore, that while a reduction in NSAID's in the placebo group caused an increase in serum TXB₂, the same effect did not occur in the active group even though the numbers of patients reducing/stopping NSAID's was approximately equal in both groups. The increase in TXB₂ observed after the 3 month placebo phase in the active group (non-significant) is a further indication that the EPO/fish oil supplement may have been having a suppressant effect.

7.3 (i) (v) Conclusion

In conclusion, this study failed to demonstrate that NSAID's could be discontinued in PA patients treated with 12 Efamol Marine capsules daily. However, with respect to eicosanoid production, the dietary supplement did appear to stabilize the effects of NSAID withdrawal. Larger doses may be required to produce a clinical improvement in this condition.

CHAPTER 8

GENERAL DISCUSSION

8.1 Methodology

8.1 (a) Measurement of PGI₂-metabolites

It is now generally accepted that PGI₂ does not function as a circulating hormone, nevertheless, it's role as a local hormone may be crucial in maintaining haemostasis. Undoubtedly, absolute values of 6-keto-PGF_{1α}, the stable metabolite of PGI₂ normally used as an index of PGI₂ production, can be best measured by GCMS. However, it has also become apparent that PGI₂ is metabolized to a range of metabolites *in vivo* (both by 15-PGDH and by non-enzymatic means) and that estimates of PGI₂ generation by a tissue may be misleading if 6-keto-PGF_{1α} is used as the sole index of PGI₂ production.

The method used in this work for the estimation of circulating PGI₂ levels takes account of this by carrying out RIA on unextracted plasma, thereby measuring "PGI₂-metabolites" rather than 6-keto-PGF_{1α}. This method has been validated elsewhere (McLaren *et al*, 1985) and the values obtained (low pg/ml range) are now generally accepted as reflecting circulating levels of PGI₂ *in vivo*.

8.1 (b) Measurement of TXB₂

Several different approaches to the measurement of TXB₂ have been adopted over the years. It is now recognized that attempts to measure circulating levels of TXB₂ (as an index of TXA₂), by measuring plasma levels, simply reflected platelet activation during venepuncture. Since, like PGI₂, TXA₂ is not a circulating hormone, but is produced in response to local stimuli, a better approach would seem to be measurement of TXB₂ generation by platelets for a given stimulus. Consequently, the measurement of TXB₂ after *in vitro* clotting (the stimulus being endogenous thrombin) was the method used in this work. However, it has since become apparent that this method is only truly

reproducible when the incubation time and temperature are standardized usually 37°C for at least 1 hour.

In this work, one of the studies (HSP) which employed measurement of serum TXB₂ was carried out before the modified methodology was introduced and therefore, the results from this study must be treated with some reservation. However, results using the old procedure are not necessarily negated if the samples are collected and prepared as they were in this study, in a consistent manner.

8.1 (c) Measurement of LTB₄

The method of PMN stimulation used in this work, maximal stimulation with calcium ionophore A23187, is assumed to reflect the total LTB₄-synthesizing capacity of the cells. It is always possible, of course, that a different metabolic capacity could exist under conditions for stimulation occurring *in vivo*. However, the CaI method is still probably the best *in vitro* method, as it has the advantage of using endogenous AA and gives rise to levels of LTB₄ which are easily measured by RIA. The values of LTB₄ obtained in this work for normal, maximally stimulated PMN's are similar to values reported elsewhere (Lewis, 1985).

The measurement of LTB₄ by RIA directly in cell supernatants has been validated elsewhere (Salmon *et al*, 1982a) and the LTB₄ assay has been shown in this work, to be sensitive and reproducible.

8.2 Raynaud's Phenomenon

8.2 (a) PGI₂ in Raynaud's phenomenon

The discovery of PGI₂ and TXA₂, two potent mediators with opposing actions in the cardiovascular system, provided Moncada and Vane with a rationale for describing the control of normal haemostasis and the development of vascular disease. However, not surprisingly, the concept of a decreased PGI₂/TXA₂ ratio in ischaemic vascular disease is proving to be more complex than at first envisaged and it is now

apparent that in many inflammatory vascular conditions where an inadequate production of PGI₂ may have been postulated, the opposite is in fact true. The work presented here has found that this is the case for patients with secondary Raynaud's disease and has also shown that the phenomenon may be related to decreased platelet sensitivity to PGI₂ in these patients. It is still not entirely clear what the mechanism of desensitization is *in vivo*, but it may be due to a loss of specific receptors upon chronic exposure to PGI₂. If this is the case then the lack of feedback inhibition via receptor-mediated increase in cAMP may not occur allowing this "futile" PGI₂ generation to proceed unchecked. If the decreased sensitivity to PGI₂ is a generalized phenomenon in the cells of these patients then this may explain some of the other manifestations of this condition, such as increased collagen production.

As is often the case in inflammatory disease states, it is difficult to present a unified theory of pathogenesis in which cause and effect are clearly distinguishable. However, since increased plasma levels of PGI₂ were not observed in patients with primary Raynaud's disease, it seems likely that sufficient vascular damage to produce an initial compensatory elevation of PGI₂ had not yet occurred in these patients.

8.2 (b) LTB₄ in Raynaud's phenomenon

Although the role of platelets and the PGI₂/TXA₂ balance in the development of digital ischaemia has been well studied, relatively little attention has been given to role of leukocytes. Results from the study reported here have shown that isolated PMN's from patients with Raynaud's disease secondary to PSS and VWF have an increased capacity for LTB₄ production. Since LTB₄ has been shown to play an important role in many aspects of PMN behaviour including chemotaxis, aggregation and adhesion, this finding of increased LTB₄ may well be relevant to the development of digital ischaemia in these patients. PMN aggregates (like platelet aggregates) may physically obstruct blood flow and, furthermore, such activation of PMN's is likely to result in the release of lysosomal enzymes and oxygen free radicals and so perpetuate the cycle of vascular damage and PMN activation.

The finding of increased LTB₄ production from PMN's of patients with secondary Raynaud's disease is also consistent with the hypothesis that a generalised loss of cell sensitivity to the inhibitory effects of PGI₂ occurs in this condition. However, it must be stressed that this is merely a hypothesis and no work has been done here to confirm this.

Vibration-induced white finger (VWF) is increasingly being shown to be very similar in nature to the more classical types of secondary Raynaud's disease. The observation that LTB₄ production from PMN's is also increased in VWF would seem to support this idea and suggests an overlap in their pathophysiology. Although the initial source of vessel damage may differ in PSS and VWF, the self-perpetuating cycle of PMN activation and vessel damage which ensues may lead to development of systemic disease long after the initial damaging stimulus has ceased. Despite the fact that the increased production of LTB₄ and other markers for inflammation increasingly depict VWF as a true secondary Raynaud's disease, it has, since the work for this thesis was done, been removed from the prescribed list of diseases eligible for compensation.

8.2 (c) Studies with iloprost

The effectiveness of PGI₂ and its stable analogue iloprost in treating RP has stimulated investigation into less obtrusive methods of delivery, such as transdermally. Although much of the effectiveness of iloprost can be attributed to its anti-platelet effects, more evidence is emerging which suggests that some of its effects on the circulation are mediated via its inhibitory action on leukocytes. In this work, clinical studies with transdermally applied iloprost provided the opportunity to investigate the effect of iloprost on LTB₄ from PMN's *ex vivo*.

There was a suggestion from the results that LTB₄ production from PMN's of normal volunteers receiving transdermal iloprost was decreased. However, the doses used in this pilot study were cautious and in the dose-pair receiving the highest dose (even higher doses are used therapeutically in RP patients) a clear decrease in LTB₄ was observed. The initial "spike" of increased LTB₄ production in the first 3 hours of

transdermal iloprost remains unexplained. However, similar effects in other studies with iloprost are not unknown and PGI₂ is recognized as having a dual character with respect to development of the cardinal signs and its (later) inhibitory effects on cell function. Further investigation of this phenomenon is obviously required.

A longer term study of transdermal iloprost in Raynaud's patients showed that, in terms of disease activity, iloprost was successful in reducing the frequency and duration of spasm attacks. Although the treatment did appear to decrease the LTB₄ synthesizing capacity of PMN's, this decrease did not co-incide with the clinical improvement. It may still be the case that LTB₄ inhibition made some contribution to the clinical improvement, however it seems likely that anti-platelet and vasodilatory properties of iloprost were most important. An earlier study in this work showed doses of PG were able to normalize platelet sensitivity to PGI₂, however, the PMN is a less responsive cell to PGI₂ and higher levels may be required to overcome resistance in RP PMN's if it exists.

Although LTB₄ levels were increased in RP, these did not significantly correlate with subjective measures of disease activity such as frequency and duration of spasm attacks. LTB₄ could not therefore be used as any kind of diagnostic predictor in RP, and in this sense, would appear to be simply a general marker for inflammation. However, the patient numbers in this study were small, and more extensive work with larger patient groups is required to clarify the extent to which inhibition of LTB₄ can improve blood flow in RP.

Despite the results of the two *ex vivo* studies described above, inhibition of LTB₄ production by iloprost *in vitro* (although it has been reported by other authors) could not be demonstrated using the maximal stimulation method employed in this work.

8.2 (d) Other therapies

The fact that many factors are involved in the control of vascular tone is reflected in the number of different theories regarding the pathogenesis of RP and the

many different approaches to therapy. The serotonin antagonist, ketanserin is another drug used in the treatment of RP whose effectiveness can most easily be attributed to its inhibitory effects on platelet aggregation and vasoconstriction. However, results from the study of oral ketanserin in Raynaud's patients suggested that it was also having an effect on the LTB₄-synthesizing capacity of PMN's. It is most likely that this effect of ketanserin was secondary to its effects in reducing the frequency and duration of spasm attacks thus diminishing the effect of spasm on PMN activation and the potentiation of LTB₄ production.

The effectiveness of the anabolic steroid stanozalol in RP has been attributed to its fibrinolytic effect. However, there is some evidence to suggest that it does not operate solely through this mechanism. The *in vitro* study described in this work, showed that, at concentrations which are compatible with the therapeutic dose, stanozalol causes a clear inhibition of LTB₄ production from stimulated PMN's. This may therefore be an additional mode of action contributing to its effectiveness in the treatment of RP. It would obviously be desirable to confirm that this effect occurs *ex vivo* in patients receiving stanozalol treatment. It would also be of interest to establish whether stanozalol inhibits the 5-LO enzyme in a cell-free system or whether another enzyme is involved. The effect of stanozalol on PG production also requires clarification since only subjective evidence that it has no effect exists at present.

8.3 Henoch-Schonlein Purpura

Henoch-Schonlein purpura, like the haemolytic uraemic syndrome, would appear to be an inflammatory condition in which there is a deficiency of vascular PGI₂ production. Results from the preliminary study presented here indicate that probably more than one mechanism contributes to inadequate PGI₂ production and this is certainly an area in which a more thorough investigation could be carried out. Measurement of serum TXB₂ in patients with HSP suggested that platelet activation was not enhanced in this condition. However, the methodology which was used has since been modified and

repetition of the TXB₂ measurements using the standardized method for serum collection would confirm whether this observation is correct.

It is not suggested either in the case of RP or HSP that the disturbance of PGI₂ metabolism is the primary factor in pathogenesis. Rather, the disturbance is secondary to endothelial damage initiated by other factors, as yet unknown.

8.4 LTB₄ in Inflammatory Joint and Skin Disease

It has been known for several years that LTB₄ is one of the many inflammatory mediators which can be detected in the synovial fluid from rheumatoid joints. This is mainly derived from unfiltrating PMN's which accumulate at inflammatory loci. The results from the study reported here have illustrated that the LTB₄-synthesizing capacity of PMN's from RA patients is increased compared to that of normal controls and that this does not appear to occur merely as a result of NSAID therapy. If these results reflect the true *in vivo* situation, then the concern that NSAID's may inhibit the cardinal signs of inflammation, but potentiate the cell-mediated aspects of inflammation via an increase in 5-LO products may be unfounded. Nevertheless, the increased LTB₄-synthesizing capacity of RA PMN's entering the synovium is likely to intensify the inflammatory response and the use of dual CO and 5-LO inhibitors is likely to have a significant advantage over NSAID therapy.

The finding that PMN's from PA patients also have an increased capacity for LTB₄ production but that (in this case) LTB₄ levels do not significantly correlate with indices of skin or joint disease activity reinforces the hypothesis that LTB₄ is as much a product of inflammation as a cause - a so-called "inflammatory marker". Interestingly, although LTB₄ production from PMN's was found to increase in both RA and PA, another report in the literature suggests that there is no such increase in patients with psoriasis alone. This may mean that LTB₄ is more associated with inflammatory joint disease than inflammatory skin disease, however, it is more likely that raised LTB₄ simply reflects the presence of a more severe inflammatory condition in psoriatic arthropathy. The fact that the other authors found 20-hydroxylase products of LTB₄ to

be increased in psoriasis may indicate that LTB₄ production is increased, but not beyond the capacity of the 20-hydroxylase enzyme. It should be noted that 20-OH-LTB₄ does in any case, retain significant chemotactic activity (Ford-Hutchinson *et al*, 1983).

There seems to be general agreement in the literature (Simmons *et al*, 1983; Clancy & Hugli, 1985) that LTB₄, even where it is increased probably does not have an initiating patho-physiological role in inflammatory disease, but is more likely to amplify and modulate an inflammatory response which is already present. It is also usual in the literature to find that in diseases where increased production of LTB₄ has been detected, generally, no significant correlation or only a weak correlation, has been found between LTB₄ and disease activity (Maurice *et al*, 1986; Nielsen *et al*, 1987). This supports the idea that LTB₄ is a general rather than a specific marker for inflammatory disease and that its inhibition is likely to produce a reduction in inflammatory symptoms, but would not have a disease-modifying effect. In a model proposed by Clancy and Hugli (1985), the suggested initiating chemotactic stimulus at the inflammatory locus is complement fragment C5a. The fact that C5a can only stimulate LTB₄ synthesis in PMN's when exogenous AA is provided means that firstly, it is able to migrate to a site of tissue injury without the elaboration of chemotactic factors such as LTB₄ en route and secondly, the amount of LTB₄ released at the locus would initially be proportional to the amount of free AA available and hence, to the degree of tissue damage. In this model, LTB₄ would then serve to amplify the inflammatory response once it had been initiated.

It is evident that a deeper knowledge of the mechanisms controlling LTB₄ production in normal cells, and those leading to increased LTB₄ production in disease, is required. The recent identification and isolation of a 5-LO activating protein (FLAP), (Miller *et al*, 1990 ; Dixon *et al*, 1990) in the membranes of various leukocyte lines that synthesise LT's, is likely to lead to a greater understanding of the mechanisms of LT production and may also provide a new target for therapeutic manipulation.

8.5 Dietary Studies

There is, at present, a growing consumer enthusiasm for “alternative” medicine, and the use of dietary supplements of diverse nature, many of which have dubious scientific validity, is currently in vogue. However, the use of Evening Primrose oil, (a source of DGLA) and fish oil, (a source of EPA and DCHA) in the treatment of inflammatory disease has a sound scientific basis.

Horrobin (1980) has hypothesized that chronic inflammatory disease as a whole may be due to an imbalance in the AA/DGLA ratio in the modern Western diet leading to an inadequate production of PGE₁ which may be essential for the normal self-regulation of PG synthesis to occur. In this model, NSAID and corticosteroid therapy would merely aggravate the problem by having very little effect on production of the more prominent 2-series PG's, but significantly inhibiting the PGE₁ deficiency. If this is the case, then only treatment with agents which enhance PGE₁ synthesis could correct the imbalance.

The beneficial effects of EPA on the eicosanoid balance are also well recognized and, in addition, epidemiological evidence exists to suggest that a high dietary intake of fish leads to low incidence of inflammatory disease.

The influence of diet on RA (exclusion diets, fasting etc) has always been controversial and there is now an increasing volume of literature involving dietary studies with EPO and fish oil in RA. An overall assessment of these studies and comparisons with the work presented here are difficult for several reasons, 1) a wide variation in dose (especially EPA) has been used, 2) the length of these studies has varied considerably, 3) different RA patient populations have been studied, 4) in most cases the study has been carried out against a stable background of NSAID and/or corticosteroid therapy, whereas in our study, NSAID's were being withdrawn, 5) in some cases general diet was also altered and 6) the studies were not always placebo controlled. These factors have made an overall assessment of the effectiveness of the dietary supplements difficult. On the whole, however, both DGLA and EPA do seem to be of some benefit in the treatment of RA.

One possible factor contributing to their effectiveness is decreased production of LTB₄ from PMN's and indeed, decreased LTB₄ after dietary EPA at least, has now been demonstrated on several occasions.

In this work, the clinical aim was to see if the EPO or EPO/fish oil supplements could be substituted for NSAID's without deterioration in objective markers for disease activity, and, in this respect, the study was successful. However, the fact that NSAID's were being withdrawn made interpretation of the LTB₄ results more difficult. Although conventional wisdom would have predicted, if anything, a decrease in LTB₄ synthesis as NSAID's were withdrawn, results from the placebo and EPO/fish oil groups suggested the opposite.

In terms of LTB₄ inhibition, the EPO treatment appeared to be more effective than the combined therapy. The effect of the EPO and fish oil together did not appear to be additive, far less synergistic. More studies are required to clarify the situation with respect to combined therapy.

The substantial placebo effect on subjective measures of disease observed in the RA study stresses the need for placebo-controlled studies and casts some doubt on un-controlled studies which have reported improvement in the more subjective aspects of RA (eg grip strength, morning stiffness) with EPO and fish oil. A positive placebo effect may be even more likely where patients view the treatment as being a "natural" therapy rather than a "drug". In addition, the use of olive oil as placebo in some studies is dubious, since, although this mono-unsaturate is inert in terms of eicosanoid synthesis, it does have effects on membrane fluidity and cholesterol metabolism (Jantti *et al*, 1989) and certainly has the potential to affect disease activity via these other mechanisms, thus possibly obscuring any positive effect of the active EPO or fish oil treatment (Darlington & Ramsay, 1987).

Although EFA's have obvious effects on eicosanoid metabolism in PMN's, it should be appreciated that manipulation of EFA's will alter eicosanoid biochemistry in other cell types and systems. As previously mentioned, an increase in production of

PGE₁ in the brain could be responsible for the “anti-depressant” effect of EPO. In addition, EFA’s have properties which are independent of their role as eicosanoid precursors and which could contribute to their anti-inflammatory effects. One of their major roles is in the maintenance of cell membrane structure (Crawford, 1983). The unsaturated fatty acid content of a membrane determines its degree of fluidity, which in turn affects its permeability and the behaviour of membrane-bound enzymes and receptors, thus, conceivably altering the cell’s response to inflammatory stimuli. However, there is evidence to suggest that unsaturated fatty acids *per se* are not anti-inflammatory since in rats, Safflower oil (a rich source of linoleic acid) does not have an anti-inflammatory effect whereas EPO does (Tate *et al*, 1988).

In the psoriatic arthritis study, the EPO/fish oil supplement did appear to have some inhibitory effect on LTB₄ production and was able to stabilize prostanoid levels during NSAID withdrawal. Despite this, the PA patient group as a whole were not very successful in discontinuing NSAID’s, and the active group were no more successful than the placebo group. The reason for the number of placebo responders being lower in the PA patient group than the RA patient group is not known.

In conclusion, the evidence from these and other studies, taken together, suggests that EPO and fish oil may be useful treatments in the suppression of the symptoms of inflammatory joint disease in some cases. However, where there is benefit, the exact mechanism is still not completely understood due to the complex role of EFA’s in human biochemistry. It is likely, therefore, that investigation into dietary manipulation as one of the many approaches to inflammatory disease therapy, will continue to be an active area of research and may well reveal useful information regarding the role of dietary EFA’s in normal health as well as in disease.

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PLATELET SENSITIVITY TO A PROSTACYCLIN ANALOGUE IN SYSTEMIC SCLEROSIS

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SUMMARY

Vascular prostacyclin (PGI₂) regulates platelet function and blood flow. In systemic sclerosis (SS) there is increased platelet aggregation (PA) but no information is available on the platelet/PGI₂ relationship. We evaluated platelet sensitivity to a PGI₂ analogue ZK36374 in 17 SS patients and 18 controls. The percentage (%) inhibition of PA was measured at two doses of ZK36374 with saline giving the 100% baseline. In the SS group 2 ng ZK36374 produced a percentage inhibition of 19 + 14 compared to a control value of 60 + 21, and 3 ng a percentage inhibition of 47 + 21 in the SS group and 82 + 20 in the controls. In 11 SS patients treated with either prostaglandin E or nifedipine the sensitivity approached normal. These data suggest that SS platelets are less sensitive to the inhibitory effect of PGI₂ on PA. This may contribute to the vascular lesions of SS. Other cells are resistant to the effects of PGI₂ and our findings support this picture of cellular resistance.

KEY WORDS: Systemic sclerosis, Raynaud's syndrome, Prostacyclin, Platelets.

SYSTEMIC sclerosis (SS) is a connective tissue disease characterized by fibrotic changes involving predominantly skin and blood vessels (1). Raynaud's syndrome (RS) is often the presenting feature in this group of patients and can be a persistent management problem for the physician. Although it is well recognized that hand and digital blood flow is diminished (2) the pathogenesis of the Raynaud's is still incompletely understood, thus treatment has been unsatisfactory in the past.

Recent work, however, has shown that prostaglandin E₁ (PGE₁) (3) and prostacyclin (PGI₂) (4) may be useful in the management of this disorder. Intravenous infusions of these prostaglandins (PGs) have been shown to increase blood flow to the hands and to elevate hand temperatures for up to six weeks. These clinical and laboratory improvements seen following PG treatment might suggest that SS patients either produce inadequate amounts of vasodilator PGs or that they are resistant to the actions of normal or even increased amounts. A resistance to the effects of the PGs seems most likely as both our group (5) and Horrobin's group (6) have found increased levels of PGI₂ and PGE metabolites, respectively. Furthermore, Whicher *et al.* (7) demonstrated a diminished acute-phase response after PGE₁ infusion in SS, suggesting either a hepatocyte or macrophage resistance, and Kirby *et al.* (8) postulated a PG resistance of the cyclic nucleotide responsiveness of the SS lymphocyte.

The possibility of PG resistance in the SS platelet has not yet been explored. Should this resistance be found then it may explain the formation of circulating platelet aggregates (9) and occlusion of the microcirculation that occurs during a Raynaud's spasm.

The aim of this study was to evaluate the prostacyclin sensitivity of SS platelets compared to age- and sex-matched controls. As PGI₂ is unstable, therefore presenting difficulties in standardizing dose, a stable carbacyclin analogue of PGI₂, ZK36374 (Schering Chemicals Ltd.), was used (10). In addition the effect on the PGI₂ sensitivity of platelets of

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patients treated with CL115,347, a transdermally absorbed PGE analogue (Cyanamid International), and oral nifedipine was observed.

METHODS

Patients

Twenty-four patients (mean age 45 ± 11 years) with classical SS, as defined by the A.R.A. criteria, were enrolled in the study. Seventeen of these patients were on no drug therapy known to interfere with PG metabolism. Four patients subsequently received CL115,347 1000 $\mu\text{g}/24$ h transdermally and were therefore sampled twice. One further patient was also sampled whilst receiving the transdermal PG and six were taking oral nifedipine 10 mg t.i.d. After informed consent had been obtained the patient sat in a warm waiting room for at least $\frac{1}{2}$ h to ensure that no blood sample was taken during a Raynaud's attack.

Eighteen matched controls (mean age 44 ± 9 years) were also studied.

Blood tests

Blood (9 ml) was drawn from the antecubital fossa and anticoagulated with 3.2% sodium citrate (9:1, v:v). The blood was carefully mixed, and centrifuged for 7 min at 150 g at room temperature to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was prepared by centrifuging the remaining blood at 2000 g for 15 min. PRP platelet count was in general about $250 \times 10^9/\text{l}$.

Platelet aggregation (PA) was studied in 200 μl of PRP according to the method of Børn (11) using a two-channel Malin's aggregometer. Following 3 min incubation at 37°C with 100 μl of saline and two different dilutions of ZK36374 (2 ng/ml, 3 ng/ml), 3 μl of $2 \mu\text{M}$ adenosine 5-diphosphate (ADP) was added. The change in light transmission was recorded graphically and expressed as a percentage inhibition of saline control. Care was taken to standardize the procedure as suggested by Siess *et al.* (12). The doses of ZK36374 were compatible with those intended for therapeutic use of the drug (10).

RESULTS

As can be seen from Fig. 1, when 100 μl of 2 ng/ml ZK36374 is added to normal PRP, $59 \pm 17\%$ (mean \pm SD) of platelet aggregation to ADP is inhibited. However, when the same amount of ZK36374 is added to the platelets of SS patients on no treatment only $18 \pm 13\%$ of PA is inhibited. The same pattern is repeated at the higher dose of ZK36374 (3 ng/ml)— $85 \pm 11\%$ inhibition of PA for normals and $49 \pm 19\%$ for SS. These results are statistically significant ($p < 0.001$, Mann Whitney).

In contrast, the platelet sensitivity of ZK36374 in SS patients on either nifedipine or CL115,347 is in the normal range ($68 \pm 18\%$, $85 \pm 15\%$) (Fig. 2). In an effort to show that this difference was not due to a difference in SS population, four of the patients originally on no treatment were given a 2-week course of CL115,347 and then retested. As can be seen from Fig. 3 the initial abnormal sensitivity is normalized by treatment.

The baseline (saline only) rate of aggregation measured in the conventional way (% change optical density/s) demonstrated no significant difference between SS patients and normal controls (mean 12.9 ± 4.2 , 12.8 ± 3.6 , respectively).

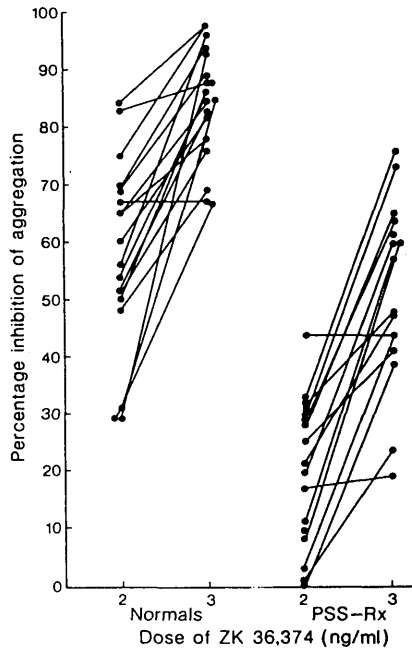


FIG. 1.—Percentage inhibition of platelet aggregation by 2 and 3 ng/ml of ZK36374 in normal controls and untreated patients with systemic sclerosis.

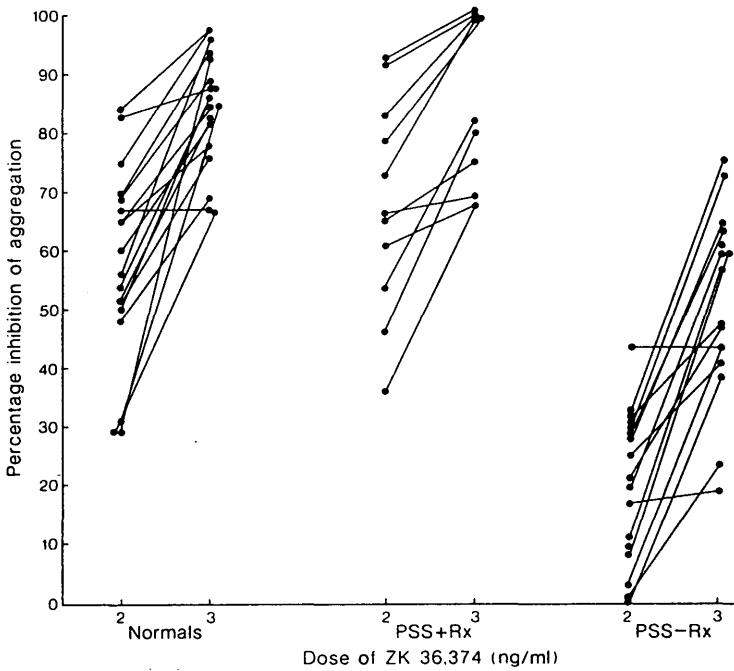


FIG. 2.—Percentage inhibition of platelet aggregation by 2 and 3 ng/ml of ZK36374 in normal controls, treated patients with systemic sclerosis and untreated patients with systemic sclerosis.

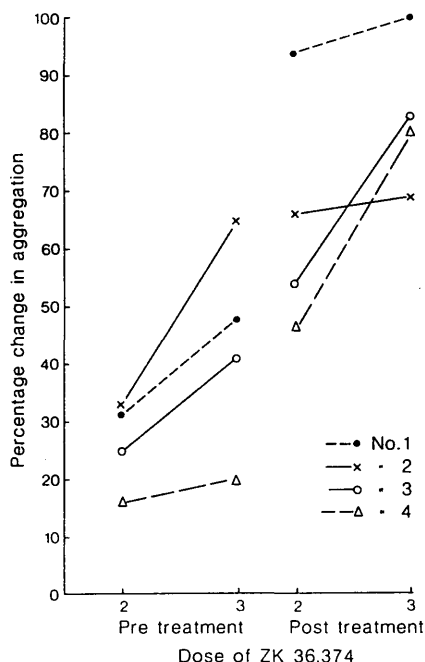


FIG. 3.—Percentage inhibition of platelet aggregation by 2 and 3 ng/ml of ZK36374 in patients with systemic sclerosis before and after treatment with CL115,347.

DISCUSSION

This study demonstrates that the platelets of patients with SS are resistant to the anti-aggregatory effects of the synthetic PGI₂ analogue, ZK36374. As PGI₂ sensitivity decreases even further at cold temperatures (13), platelet clumping in the microcirculation with release of vasoconstrictor substances could play a part in the development of Raynaud's syndrome. The platelets of patients with other thrombotic disorders have been shown to have a similar resistance to PGI₂ (14, 15) and it is thought that this contributes to the clinical manifestations of ischaemia.

Furthermore it seems likely that the SS macrophage (7), lymphocyte (8) and now the platelet are resistant to effects of PGs. Why is this? PGI₂ resistance develops physiologically in pregnancy as the plasma levels of PGI₂ increase (15). We have found increased levels of PGI₂ metabolite in these SS patients (5) and it may be that a 'tachyphylaxis' or 'down regulation' is occurring. Receptor binding studies would be required to evaluate this hypothesis. Recently conducted clinical studies (unpublished observations) have shown that chronically administered PG therapy, given in pharmacological doses, produces a similar fall-off in effect. Platelet aggregation, initially decreased by the PG, returns to baseline over a period of 6–8 weeks, as does the improved blood flow and clinical response. Chronic exposure to increased levels may desensitize the cells to the action of PGs and even higher levels obtained by pharmacological doses may be required to allow the cells to respond normally, at least for a time. Support for this can be seen with leucocytes (8) where normal cyclic nucleotide responsiveness to isoprenaline was restored after infusion of PGI₂. Similarly in our study the addition of the transdermally absorbed PGE seems to normalize

the response of the platelet to PGI₂. Oral nifedipine, via its calcium channel blocking activity, will decrease the platelet release reaction therefore inhibiting release of the vasoconstrictor thromboxane A₂ (TXA₂). TXA₂ is antagonistic to the action of PGI₂ and therefore any decrease in this substance will enhance the action of the available PGs.

In conclusion therefore, we have demonstrated a resistance of the SS platelet to the anti-aggregatory effects of PGI₂ and suggest that this may be important in the development of digital ischaemia.

These observations also support the hypothesis of a generalized cellular resistance in SS. If this is extrapolated to other cells some additional manifestations of SS could be explained, for example, in SS there is augmented growth of collagen-producing fibroblasts (16) which are normally inhibited by PG. If the fibroblasts are resistant to the negative effect of PG, this might explain the excess collagen production seen in this disease.

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height of the appliance by excessive wear. Four patients, who were asked to discontinue the use of the splint for one month suffered a relapse of their symptoms.

Discussion

dentate patients with symptoms of classical migraine on waking, or within hours of waking, occlusal splint therapy is effective. The success of treatment is also achieved without medication. The aetiological relationship of classical migraine to TMJ dysfunction syndrome remains speculative. Dental aspects of migraine seem to be important in selected patients. Other advantages of this means of therapy in selected patients are that there are no contraindications and drug therapy with its associated side effects in these patients is avoided.

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ABNORMAL BIOCHEMICAL AND CELLULAR PARAMETERS IN THE BLOOD OF PATIENTS WITH RAYNAUDS PHENOMENON

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Abstract. Finger blood flow is decreased in Raynauds Phenomenon (RP). This may be due not only to vasospasm, but also to blood abnormalities. 40 patients with Raynauds Disease (RD), 28 with suspected RP (SS) and 42 with secondary Raynauds syndrome (RS) were enrolled and compared to 50 controls. Results from base-line samples show that those with RP have abnormal haemostasis and rheology whereas those with mild Raynauds, RD do not. Blood sampled after cold challenge in 15 RS patients and 15 controls show that both groups exhibit platelet activation after emersion. The degree of activation however was much more marked in the RS patients. We have shown that abnormalities of haemostasis and rheology are found in patients likely to have endothelial damage (RS). These changes are probably a consequence rather than a cause of the disease. After cold challenge the results become more abnormal and correlate with severity of disease.

It is established that hand and digital blood flow is diminished in patients suffering from Raynaud's phenomenon (RP). However, the mechanism by which this decreased flow is produced is unclear. Many suggestions regarding the pathophysiology of RP have been studied, for example increased sympathetic tone(1), local arterial sensitivity to cold, and deposition of immune complexes in the vessel wall. An association has been found between Raynauds Syndrome (RS) and immune complexes in vinyl chloride disease. In addition, increased levels of phagocytosed immune complexes in circulating PMN leucocytes have been demonstrated and the levels of complexes correlate with systemic involvement and severity of vasospasm. All the above are concerned with vessel wall and the

development of spasm. More recently however, attention has focused on the contribution to decreased flow made by biochemical and cellular abnormalities in the blood. This study deals with the measurement of these abnormalities in patients with RP, and assessment of whether these parameters can be used as a judge of severity of disease, and to monitor improvement during treatment.

Patients

In 1932, Allen and Brown (2) emphasised the wide spectrum of disorders that may be associated with RP, and they classified patients with associated disease as having Raynauds Syndrome (RS) and those without as having Raynauds Disease (RD). In recent years the development of more sophisticated and sensitive laboratory procedures has revealed that greater than one half of patients with RP have an associated systematic disease. Furthermore, long term studies have shown that RD may be a precursor of systemic illness by many years. This had led to the delineation of a third subdivision of patients with RP, that is "suspected secondary (SS)" whereby patients have some, but not all, of the criteria required for diagnosis of a connective tissue disorder.

The data in this study obtained from 40 patients with RD, 28 patients with SS and 42 patients with RS. Results are compared to 50 age, sex and smoking matched controls.

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Table 1

Plasma levels obtained from patients with RP and normal controls (mean \pm SD)	Normals	RD	SS	RS
Fibrinogen (g/l)	2.5 \pm 0.9	2.6 \pm 1.0	3.7 \pm 1.2*	4.7 \pm 0.9**
Fibrinogen activator activity (mm ²)	86 \pm 15	83 \pm 12	65 \pm 20*	56 \pm 14**
Factor VIII:c (%)	93 \pm 20	92 \pm 18	126 \pm 20	180 \pm 42*
PGI ₂ M (pg/ml)	13 \pm 4	10 \pm 2	36 \pm 7**	55 \pm 6**

* p 0.05, ** p 0.01 (Mann Whitney)

Baseline Abnormalities**Fibrinogen and fibrinolysis**

Ability of the RP patient to digest fibrin may be impaired, and it has been suggested that this may aggravate the disease through deposition and decreased clearance of vascular endothelial fibrin (3).

In normal subjects, fibrin is removed by enzymatic degradation catalysed by plasmin. Plasmin is produced by cleavage of its inactive precursor Plasminogen, and the rate of this reaction is determined by the relative proportion of activators and inhibitors of fibrinolysis present.

Thus a decrease in plasminogen activator production from the endothelial wall would lead to increased fibrinogen (method of Clauss) (4) and plasminogen activator activity (PAA) (fibrin plate) (5) in the above group patients and control subjects. Results are displayed in Table 1, and show a decrease in PAA and increase in fibrinogen level in patients with RS. Those with RD show no abnormality and this suggests that the decreased fibrinolysis is a response to endothelial damage, that is a consequence rather than a cause of the disorder. The increase in plasma viscosity and decrease in red cell deformability produced by the elevated plasma fibrinogen, in combination with fibrin deposition round the vessel lumen, may contribute to the severity of the symptoms of RS.

Other endothelial products

In addition to plasminogen activator, factor VIII and prostacyclin (PGI₂) is also released by the endothelium. Factor VIII:c (measured by RIA) is elevated in patients with RS and normal in those with RD (Table 1). This increase probably again reflects endothelial damage, but may contribute to the hypercoagulable state seen in patients with RS.

Plasma prostacyclin metabolite levels (PGI₂M) (6) (RIA) show the same pattern, being elevated in patients with RS and normal in those with RD (Table 1). It is known that noxious physical and chemical stimuli, such as vessel spasm or infusion of hypertonic dextrose, will promote endothelial release of PGI₂. The PGI₂ thus formed has a potent vasodilator, anti-platelet effect preventing thrombosis in the affected vessel. One might expect therefore that elevated levels of PGI₂ in RS would ameliorate the symptoms of digital ischaemia. Unfortunately, the development of cellular resistance (7) to these elevated PGI₂M levels allows the activation of platelets.

Red and white blood cells

Part played by the red cell has also been studied. Its diameter of 7 μ is smaller than that of the capillaries, so flow in the microcirculation may be partially dependent of red cell hardness. Red cell deformability measured by the filtration technique (8), is decreased in patients with RS (Table III). Cold temperatures reduce red cell deformability further, as does the rigidity present in cold ulcerated fingers, so hard red cells may contribute to the decreased blood flow. In addition, Kovacs et al showed that the red cells from patients with RS were more adherent to the endothelium than in normal persons (9), and found a similar pattern for PMN leucocytes. These adherent cells could further impair blood flow.

Platelets

Platelets are activated in the circulation they aggregate and circulate in clumps, releasing vasoconstrictor substances such as thromboxane A₂ and serotonin. These two features allow good haemostasis in the presence of tissue lacerations. If, however, platelet activation occurs in the absence of haemorrhage, then inappropriate thrombosis will occur and it has been suggested that platelet activation may play a part in producing the symptoms of RS. We have measured the rate of platelet aggregation to ADP (Malins photometric aggregometer) (10), and found it to be increased in patients with RS.

Table 2

Baseline red cell and platelet results obtained from patients with RP and normal controls (mean \pm SD)

	Normals	RD	SS	RS
Red cell deformability (ratio)	1.00 \pm 0.1	1.10 \pm 0.11	1.35 \pm 0.20*	1.45 \pm 0.55***
Platelet aggregation to ADP (% change optical density/sec)	11 \pm 9	12 \pm 10	18 \pm 6	25 \pm 6
β -thromboglobulin (pg/ml)	20 \pm 8	18 \pm 11	36 \pm 10*	56 \pm 9*

* p < 0.05 (Student's test)

In addition, we have found elevated levels of the platelet release product B-thromboglobulin (RIA) in these patients (Table II). No abnormality was detected in patients with RD, suggesting that the activation seen in severe disease occurs as a result of platelet contact with damaged endothelium. It is important to note that this activation occurs despite elevated plasma PGI₂M levels. This is explained on the basis of the platelet resistance to the effects of PGI₂ seen in (7) RS.

Summary (I)

Patients with the severest form of RP, i.e. those with RS, have changes in biochemical and cellular parameters which might contribute to the decreased blood flow seen in this group of patients.

It is important to note however, because of the large standard deviations seen in the results, that it is not possible to use these measurements to diagnose the type of RP in individual patients. In addition, although the most severe group (RS) demonstrate the abnormalities, there is no correlation with any of these measurements with duration or frequency of spasm attack (diary cards) or with hand-temperature (thermocouple probe), or plethysmography (cold challenge, Medimatic SP2) results. However, we can use these abnormalities to monitor response to treatment; Our results from previous studies (11) show that those subjects given PGI₂ infusion, who demonstrate greater than 20% improvement in duration and frequency of spasm attacks, have significantly decreased platelet activation and decreased white cell adhesion compared to non-responders.

B. Abnormalities after cold-challenge

After stabilizing for one hour in a temperature controlled room (24 \pm 1°C), 15 patients with RS immersed gloved hands into water at 37°C for 10 minutes. A baseline blood sample is drawn from the antecubital fossa of one arm. Thereafter the hands are immersed in cold water (10°C) for a further five minutes and again blood is drawn, this time from the other arm. Results were compared with 15 age and sex matched controls.

Platelet Behaviour

The rate of platelet aggregation to 2 μ M ADP was measured as before, as were B-thromboglobulin levels and serum TxB₂, the stable metabolite of TxA₂ (RIA) (11). Results show that platelet activation takes place even in normal subjects; however, the changes are most marked in those with RS (Table III). An increase in red cell hardness has also been shown to occur after cold challenge.

Summary (II)

As with the baseline measurement, cold challenge results show an increase in platelet activation in patients with RS, which may contribute to the decrease in blood flow. It is interesting that, in contrast to the baseline findings, a correlation is obtained between duration and frequency of attacks and the platelet release products BTG (r=0.65) and TxB₂ (r=0.72). Furthermore, the decrease in platelet release of TxA₂ seen after treatment with nifedipine correlates well with the degree of improvement on treatment. (unpublished observations)

Table 3

Platelet behaviour before and after cold challenge in patients with RS and controls (mean \pm SD)

	Raynauds		Controls	
	Pre	Post	Pre	Post
Platelet aggregation to ADP (% change optical density/sec)	24 \pm 6	33 \pm 5	15 \pm 6	23 \pm 6
β -thromboglobulin (pg/ml)	49 \pm 10	72 \pm 9	120 \pm 17	163 \pm 22
TxB ₂ (pg/ml)	59 \pm 12	103 \pm 20	120 \pm 17	163 \pm 22

* p < 0.01 (Mann Whitney)

Conclusions

Alteration in blood biochemistry and cell behaviour might contribute to decreased blood flow. Most marked abnormalities are in those with endothelial damage (RS vs RD). These changes are probably a consequence rather than a cause of the disease. Baseline sampling not useful for individual patient diagnosis or for assessment of severity. However, they can be used as an assessment of response to certain drug treatments. Pre and post cold challenge results are also abnormal in RS and the results correlate with severity of disease and improvement on certain drug treatments.

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INPATIENT CARDIOLOGY IN A RURAL DISTRICT GENERAL HOSPITAL

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Summary: *The pattern of cardiac admissions to a rural district general hospital has been analysed. Coronary artery disease is the major disorder, both as an acute and chronic problem. Other forms of heart disease are relatively uncommon. The absence of hypertension and hypertensive heart disease is striking. The deaths were mainly related to coronary artery disease.*

Key words: Cardiac admission pattern, coronary artery disease.

CHANGING patterns of disease over the years create one of the long term challenges to medicine. During the last century and in this country, cardiac disease has undergone a great transformation. Diphtheritic heart disease has disappeared and acute rheumatic fever almost so, infectious pericarditis and its sequel of constrictive pericarditis are very rare, rheumatic heart disease is no longer the scourge it was and hypertension is now so frequently discovered and relatively successfully treated by general practitioners that hypertensive heart disease is seldom seen. On the other hand, coronary artery disease has emerged as an ever increasing threat, to become the major cardiac problem in this country today. Congenital heart lesions are better understood, more readily diagnosed and more radically and more successfully treated at earlier ages. Cardiomyopathies of viral and other causes are recognised. Aetiologies are better understood and treatments are more rational and more fundamental so that the future the pattern may slowly change. Taking stock at intervals is thus of great importance and this paper presents such an exercise in a small rural district general hospital.

Material
 During a 6 month period (April to September 1985) 104 males and 161 females were admitted to the medical wards

of Stracathro Hospital under one consultant. Forty three males and 31 females were admitted for a primarily cardiac reason — the 'acute' cardiac group, while 61 males and 130 females had a non-cardiac illness. However 30 males and 36 females in the non-cardiac group had a history of a past cardiac problem or had a co-existing cardiac disorder.

Table I

Age Distribution of the non-cardiac and cardiac admissions. The first group is subdivided into the 'non-acute cardiac group' and those totally non-cardiac. Deaths are given in brackets.

Age In Years	Male					Female				
	Totally Non Cardiac	Non Acute Cardiac	Total Non Cardiac	Acute Cardiac	Overall Total	Totally Non Cardiac	Non Acute Cardiac	Total Non Cardiac	Acute Cardiac	Overall Total
Under 20	0	0	0	0	0	8	0	8	0	8
20-29	2	0	2	0	2	7	0	7	0	7
30-39	11	0	11	0	11	9	1	10	0	10
40-49	3	2(1)	5(1)	3	8(1)	12	0	12	1	13
50-59	3	5	8	8	16	13(1)	4	17(1)	3	20(1)
60-69	3	6	9	15(2)	24(2)	9	6	15	6	21
70-79	7(1)	12(3)	19(4)	12(2)	31(6)	24(3)	12	36(3)	13(4)	49(7)
80-89	2	3(1)	5(1)	5(3)	10(4)	11(1)	12(2)	23(3)	8(1)	31(4)
90+	0	2(1)	2(1)	0	2(1)	1	1(1)	2(1)	0	2(1)
Total	31(1)	30(6)	61(7)	43(7)	104(14)	94(5)	36(3)	130(8)	31(5)	161(13)

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Effects of altering dietary essential fatty acids on requirements for non-steroidal anti-inflammatory drugs in patients with rheumatoid arthritis: a double blind placebo controlled study

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SUMMARY In rheumatoid arthritis (RA) benefit from non-steroidal anti-inflammatory drugs (NSAIDs) is mediated through inhibition of the cyclo-oxygenase enzyme, thereby decreasing production of the 2 series prostaglandins (PGs). The lipoxygenase enzyme is intact, however, allowing leucotriene (LT) production, e.g., LTB₄ (an inflammatory mediator). Treatment with evening primrose oil (EPO) which contains γ -linolenic acid (GLA) leads to production of the 1 series PGs, e.g., PGE₁, which has less inflammatory effects. Also LT production is inhibited. Eicosapentaenoic acid (EPA, fish oil) treatment provides a substrate for PGs and LTs, which are also less inflammatory. In this study 16 patients with RA were given 540 mg GLA/day (EPO), 15 patients 240 mg EPA and 450 mg GLA/day (EPO/fish oil), and 18 patients an inert oil (placebo). The aim of this study was to determine if EPO or EPO/fish oil could replace NSAID treatment in RA. The initial 12 month treatment period was followed by three months of placebo for all groups. Results at 12 months showed a significant subjective improvement for EPO and EPO/fish oil compared with placebo. In addition, by 12 months the patients receiving EPO and EPO/fish oil had significantly reduced their NSAIDs. After 3 months of placebo those receiving active treatment had relapsed. Despite the decrease in NSAIDs, measures of disease activity did not worsen. It is suggested that EPO and EPO/fish oil produce a subjective improvement and allow some patients to reduce or stop treatment with NSAIDs. There is, however, no evidence that they act as disease modifying agents.

Key words: evening primrose oil, fish oil, prostaglandins, leucotrienes.

Inflammation is a process which occurs in tissues after sublethal injury. This is followed by production of inflammatory mediators. One such group of mediators is the eicosanoids. These are formed from precursor essential fatty acids (EFAs). The most abundant EFA in the Western diet is arachidonic acid (AA), which forms the 2 series prostaglandins (PGs), e.g., PGE₂, and the 4 series leucotrienes (LTs), e.g., leucotriene B₄.^{1,2} In general these PGs and LTs have pro-inflammatory effects.³ The ingestion of a diet rich in evening primrose oil (EPO) will increase levels of γ -linolenic acid (GLA).⁴ GLA is

metabolised to dihomo- γ -linolenic acid (DGLA), which produces the monoenoic PGs, e.g., PGE₁.⁵ PGE₁ has been shown to have important anti-inflammatory effects.⁵ DGLA cannot itself be converted to LTs but can form a 15-hydroxyl derivative that blocks transformation of AA to LTs.⁶ Dietary DGLA may therefore act as a competitive inhibitor of 2 series PGs and 4 series LTs. Animal studies have shown that such a diet can both decrease inflammatory cell function and suppress inflammation.⁷⁻⁹

High dietary levels of another EFA, eicosapentaenoic acid (EPA), would provide a substrate for production of the 3 series PGs and LTs of the 5 series.^{3,10} The potency of LTB₅ in inducing leucocyte aggregation is 10% of the potency of LTB₄.¹¹

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Again, animal studies have suggested that an anti-inflammatory effect can be obtained by increasing dietary EPA.^{12 13} The effect of altering EFAs in the diet of humans with inflammatory disorders has, however, been less well studied. Wright and Burton have shown a significant clinical improvement in the manifestation of eczema in patients treated with EPO,¹⁴ and Baker, Krakauer, and Zurier have demonstrated a reduction in human synovial cell proliferation *in vitro* by addition of DGLA.¹⁵ Two clinical studies of EPO in rheumatoid arthritis (RA) proved disappointing.^{16 17} The respective doses of EPO were low and the study periods short, however.

Payan *et al* have shown a decrease in leucocyte activity in humans after treatment with EPA,¹⁰ and Kremer *et al* suggested that patients with RA had obtained benefit after such treatment.¹⁸

Another benefit might be expected from manipulating essential fatty acids in the diet of patients with RA. 'First line' treatment for RA is with non-steroidal anti-inflammatory drugs (NSAIDs). These drugs act by inhibiting the cyclo-oxygenase enzyme and therefore decreasing the production of pro-inflammatory prostaglandins from AA. AA, however, is also metabolised by another enzyme, the lipoxygenase enzyme, into the LTs.³ Treatment with NSAIDs may therefore ameliorate the PG mediated aspects of inflammation, but will allow those dependent on lipoxygenase action to proceed unchecked. Furthermore, NSAIDs may produce side effects, e.g., gastric irritation, as a result of decreased PG production. Theoretically it should be possible to substitute EPO or EPA for NSAID treatment; this would allow a decrease in the pro-inflammatory PGs and LTs metabolised from AA and, as other less inflammatory PGs (e.g., PGE₁) would be increased, there should be no gastric side effects from this therapy.

The aim of our study, therefore, was to determine whether EPO or an EPO/fish oil combination containing EPA could be substituted for NSAID therapy without any deterioration in clinical symptoms. The study was double blind and placebo controlled.

Patients and methods

PATIENTS

Forty nine patients with classical or definite RA as defined by American Rheumatism Association criteria were enrolled in the study after informed consent had been obtained. Permission for the study had been received from the regional ethical committee. All patients required first line (NSAID) therapy for control of their symptoms, but none was

considered severe enough to warrant second line therapy. Sixteen patients received EPO treatment taken as 12 capsules/day, providing a total daily dose of 540 mg GLA; 15 received EPO/fish oil capsules (12/day), providing a total daily dose of 450 mg GLA and 240 mg EPA; 18 patients received 12 capsules/day of placebo (liquid paraffin). In addition, all capsules contained vitamin E as an antioxidant (dose 120 mg/day). As it was considered that 12 capsules at one time might be inconvenient the patients were instructed to take them intermittently throughout the day. Most selected four capsules three times/day, though some patients took six twice/day or three capsules four times/day.

CAPSULES

All three types of capsules were supplied by Efamol Ltd and were visually identical. They were issued to the patients in a randomised double blind fashion.

STUDY DESIGN

The duration of study was 15 months. For the first three months of the study the patients were instructed to take the 12 capsules of oil a day, plus their full dose of NSAID. From three to six months in particular, but also up to 12 months, the patients were instructed to decrease or stop their NSAID. From 12 to 15 months they were told to maintain, if possible, the current dose of NSAID. Patients were only to decrease or stop NSAIDs if this could be done without exacerbation of RA symptoms. At 12 months all patients received placebo capsules (12/day) without vitamin E. As this was part of the study design investigators were aware of the treatment in all patients from 12 to 15 months; the patients, however, remained blinded. The aim of this placebo phase was to assess whether any improvement was due to the antioxidant and radical scavenging effect of the vitamin E,¹⁹ and also to monitor relapse. The patients attended the clinic at monthly intervals for the first six months and thereafter at three-monthly intervals.

CLINICAL ASSESSMENT

Full metrological assessment was carried out before the start of the study and at 3, 6, 12, and 15 months. The duration of morning stiffness in minutes, the grip strength of left and right hands (mmHg, mean of three readings), and the Ritchie articular index²⁰ were noted. The patients also completed a 10 cm visual analogue pain scale, and the occurrence of side effects was recorded. In addition, the patients were asked to record whether they obtained benefit or otherwise from the treatment.

BLOOD TESTS

Blood was sampled before the start of the study and

again at 3, 6, 12, and 15 months. The erythrocyte sedimentation rate (ESR) was measured, as were C reactive protein (CRP) levels. Haemoglobin (Hb) and rheumatoid factor estimation were also carried out. Samples from month 0, 6 months, and 12 months were stored at -70°C for later analysis of plasma and red cell membrane EFA levels; this enabled a check of patient compliance.

STATISTICAL ANALYSES

Comparability between the treatment groups was analysed with the Mann-Whitney U test. Sequential analyses were carried out using the two tailed paired Wilcoxon test.

Results

Table 1 shows the comparability of the different treatment groups, both for clinical and laboratory measurements, at the start of the study. No significant differences in the patient populations were witnessed. Table 2 shows the number of patients withdrawn from the study by 12 months. One patient in the EPO group and two in the EPO/fish oil group were withdrawn owing to increasing symptoms of RA, compared with 10/18 of the placebo patients (both $p < 0.001$, Mann-Whitney).

The results from all patients who were withdrawn were analysed throughout the study on an intention to treat basis. The subsequent results are expressed as a percentage of baseline so that the different group totals can be more easily compared.

Fig. 1 is a compilation of results showing the dose of NSAID in all three groups before the start of treatment and at 3, 6, 9, and 12 months. As one EPO and three placebo patients were not taking NSAIDs at the start of the study they have not been

Table 1 Comparability of groups*

	Placebo	EPO	EPO/ fish oil
Age (years)	48 (30-74)	46 (35-68)	53 (28-73)
Duration (years)	5 (1-20)	5 (1-18)	5 (1-25)
Sex M:F	1:17	1:15	4:11
Morning stiffness (min)	31 (0-240)	47 (0-120)	31 (0-240)
Grip strength (mmHg, right hand)	106 (53-260)	116 (58-207)	105 (50-300)
Articular index	6 (0-24)	5 (0-14)	7 (0-12)
Visual analogue scale (cm)	2.3 (0-8.0)	3.6 (0-9.5)	3.0 (0-7.3)
ESR (mm/h)	30 (3-75)	22 (4-81)	26 (4-55)
CRP (mg/l)	19 (8-76)	19 (10-43)	13 (10-38)
Hb (g/l)	118 (100-155)	126 (88-149)	138 (117-168)
RF (titre)	1/16 (0-1024)	1/128 (0-1024)	1/128 (0-1024)

*Median (range).
ESR=erythrocyte sedimentation rate; CRP=C reactive protein;
Hb=haemoglobin; RF=rheumatoid factor.

EPO / NSAID DOSAGE

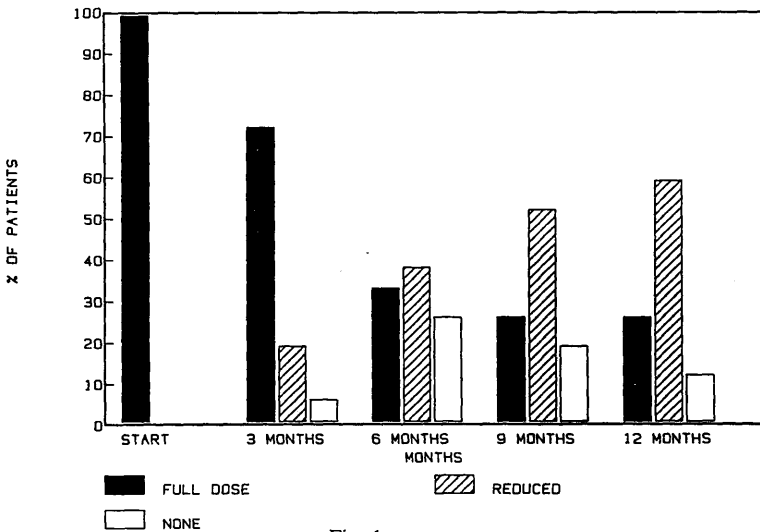


Fig. 1a

included in these analyses. Eleven out of 15 EPO patients and 12 out of 15 EPO/fish oil patients reduced or stopped their NSAIDs by 12 months, compared with five out of 15 of the placebo patients ($p < 0.003$, $p < 0.002$, $p < 0.05$ respectively, paired Wilcoxon).

Fig. 2 shows a compilation of results for the clinical measurements in all three groups. No significant change was seen over the study period.

Table 2 Patients withdrawn from the study by 12 months

	Placebo	EPO	EPO/ fish oil
Increasing RA symptoms	10	1	2
Gastrointestinal upset	0	2	0
Total	10	3	2

EPO-MARINE / NSAID DOSAGE

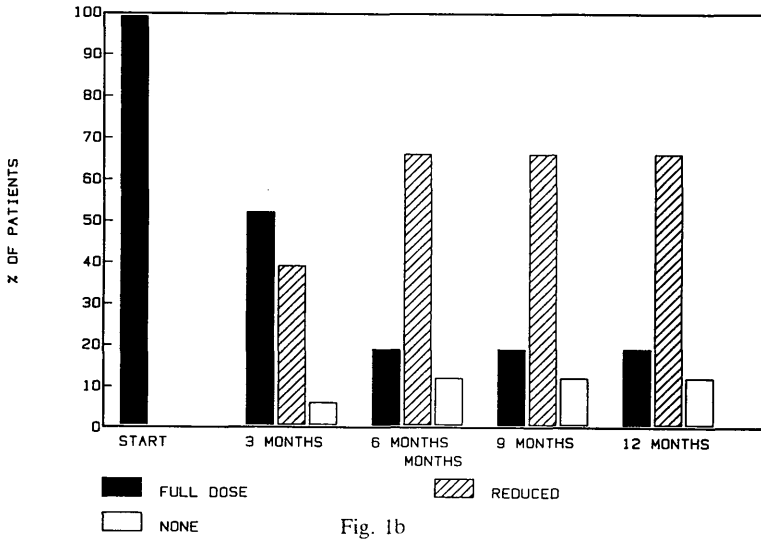


Fig. 1b

PLACEBO / NSAID DOSAGE

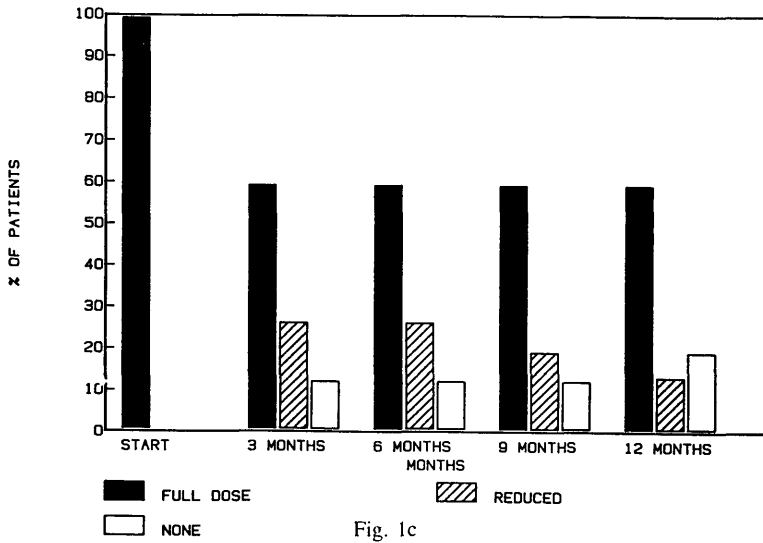


Fig. 1c

Fig. 1 NSAID treatment in the three groups of patients.

Fig. 3 shows the three sets of results from the laboratory measurements; again no significant change in results was noted over the study period, though, as with the clinical results, the EPO group appeared to fare better.

patient groups over the study period. As one would expect, the control group did show a marked placebo response of about 40% at six months. Surprisingly this persisted throughout the study, though it fell to about 30% at 12 months. Despite the absence of real change in objective measures of

Fig. 4 shows the subjective response for the three

EPO / CLIN RESPONSE

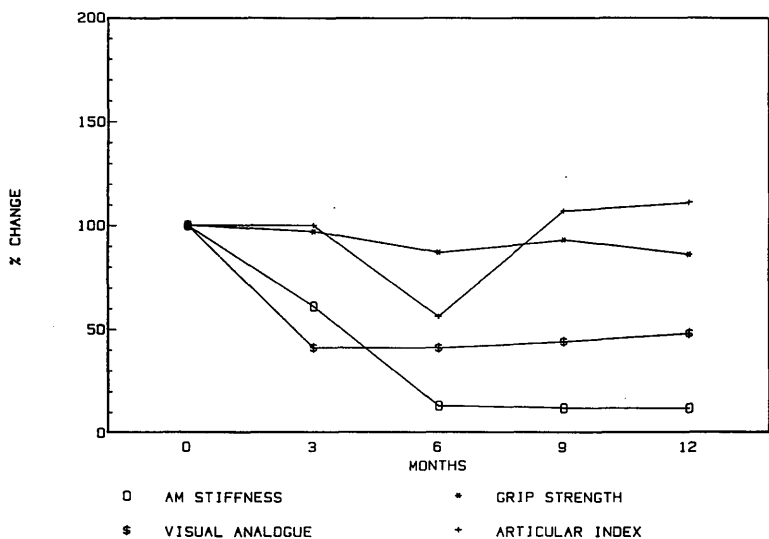


Fig. 2a

EPO-MARINE / CLIN RESPONSE

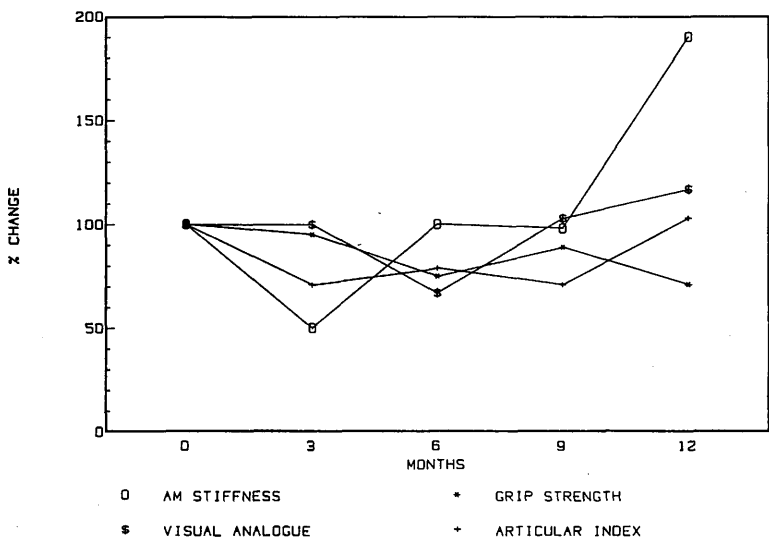


Fig. 2b

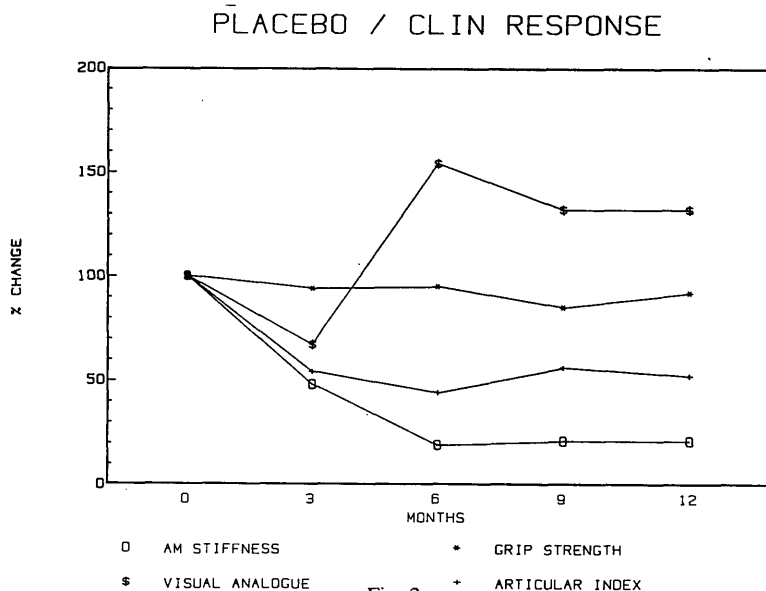


Fig. 2 Clinical measurements in the three groups of patients.

disease activity, 94% of the EPO and 93% of the EPO/fish oil group felt a subjective improvement in their condition at 12 months. Most patients on active treatment recorded an increase in their general sense of wellbeing.

After the three month placebo phase all the EPO patients and 80% of the EPO/fish oil patients had either returned to baseline or become worse. This compares with a relapse rate of only 14% in the placebo group.

Four patients experienced side effects. Two in the EPO group, one with nausea and one with diarrhoea, required to be withdrawn from the study at two and one months respectively (Table 2). Two patients in the EPO/fish oil group also experienced side effects, nausea and headache, but neither patient required to be withdrawn from the study.

Estimation of fatty acid levels by high performance liquid chromatography in plasma and red cell membranes confirmed compliance in all subjects apart from one EPO/fish oil patient. This patient was one of the two from the group who were withdrawn because of increasing RA symptoms.

Discussion

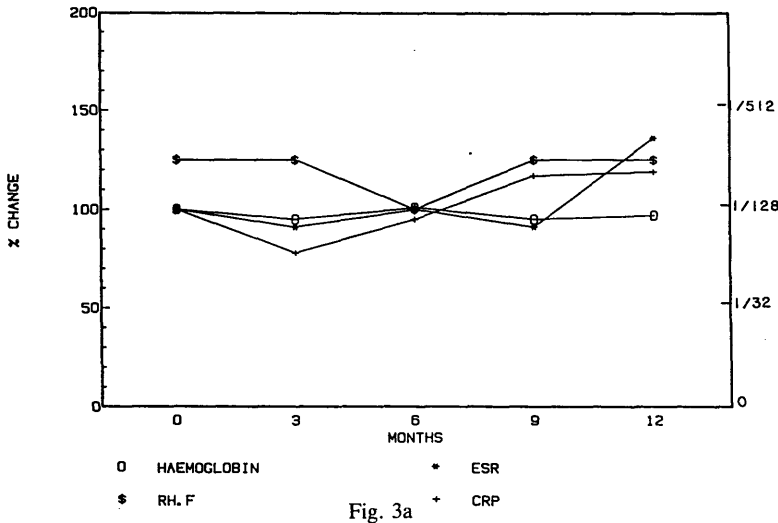
There are good theoretical reasons for suggesting that altering dietary EFA may produce anti-inflammatory effects similar to cyclo-oxygenase inhibition by NSAIDs.^{5 7 8 9 13} Indeed, additional effects might be expected because of the alteration in leuco-

triene production not seen with NSAIDs.⁶ Furthermore, fewer gastric side effects would be expected as PGs of other series, though less inflammatory, would be available for gastric cytoprotection.⁵

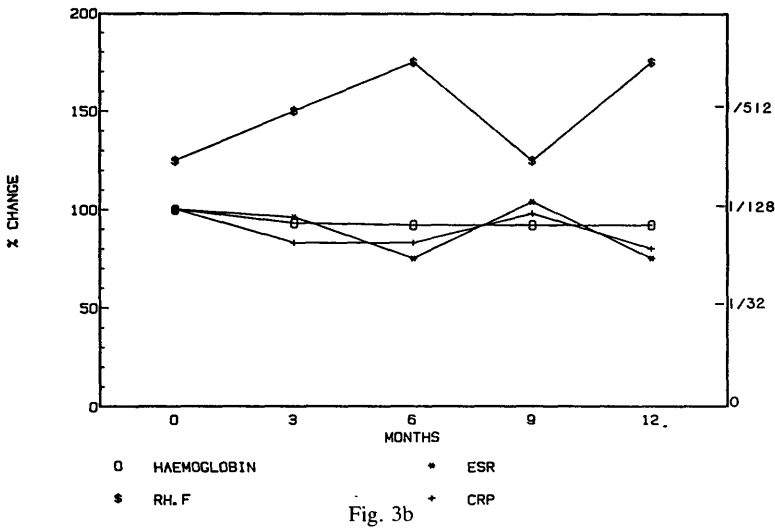
In this study we have shown that it was possible for some patients with RA to decrease or stop NSAID treatment when EPO or EPO/fish oil was given. This was achieved with no deterioration in clinical or laboratory measures of RA activity. A decrease in NSAID dose was also seen in the placebo group, though this was much less marked. In combination with the 30% placebo subjective response, this stresses the importance of placebo controlled studies in RA. Four patients (three placebo and one EPO) were not receiving any NSAID at the start of the study. These patients had stopped NSAID treatment without medical advice, having developed dyspepsia since their last clinic visit. Because of the severity of the gastrointestinal symptoms it was decided to enrol them into the study.

It was interesting that, despite the lack of objective improvement in symptoms on the active oils, there was a very definite subjective improvement. The mechanism of this is unclear, but two alternative explanations are possible. EPO has had some success in treating premenstrual tension and certainly it is known to alter brain PG production.^{21 22} PGE₁ may have an antidepressant effect,²³ and the increase in PGE₁ after EPO treatment may be responsible for the subjective response witnessed in

EPO / LAB RESPONSE



EPO-MARINE / LAB RESPONSE



the study. An alternative explanation could be that decreasing NSAID therapy may remove some negative effect of these drugs on the patient's wellbeing.

The relapse in response witnessed after substitution with placebo capsules was interesting. The fact that only 14% of the placebo group relapsed makes it very likely that spontaneous remission had occurred in the other placebo patients; all the patients in the EPO group either felt worse or returned to

pre-study level symptoms. Interestingly, although almost 80% of the EPO/fish oil group relapsed on placebo, 20% maintained their improvement. It may be that loss of EPA from the cell membrane takes longer to occur than with DGLA. Unfortunately, fatty acid analyses of the 15 month samples were not carried out so this hypothesis remains unchecked. The results from our work appear to be in contrast with those obtained by Brown *et al*¹⁶ and

PLACEBO / LAB RESPONSE

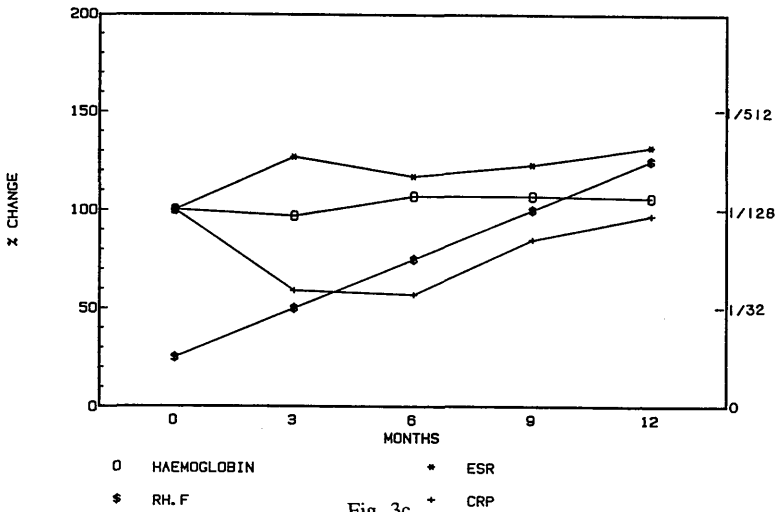


Fig. 3 Laboratory measurements in the three groups of patients.

SUBJECTIVE RESPONSE

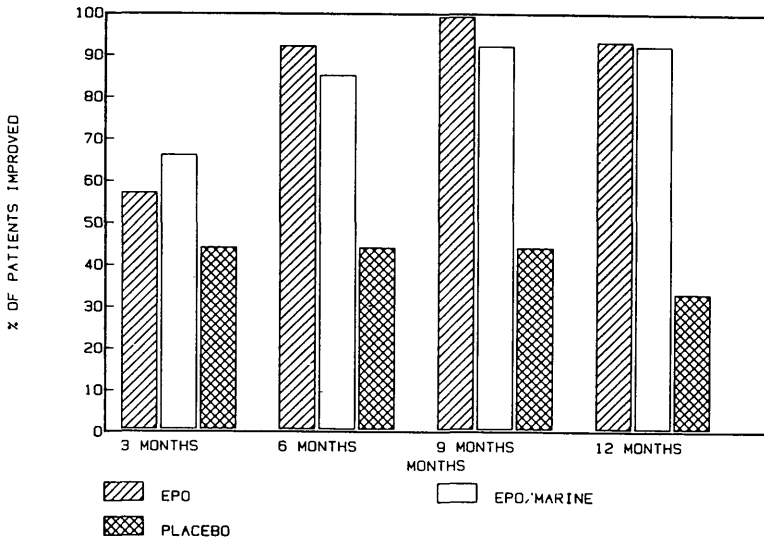


Fig. 4 Subjective response in the three groups of patients.

Hansen *et al.*¹⁷ Both these studies used a lower dosage regimen of EPO and in both the study period was only three months. As can be seen from our data, improvement is most marked from six months onwards. In addition, their patients stopped NSAIDs abruptly before the start of the studies. This may have caused a flare in the patients' symptoms in the first weeks as it is unclear how long

EFA treatment should be undertaken before alteration in PG and LT production occurs. This combination of NSAID withdrawal and short treatment period may explain the authors' negative findings. It is also possible that these two studies looked at different patient populations. All of our patients had mild RA as shown by the clinical and laboratory values in Table 1. In contrast, these other

two groups appeared to study patients with more severe disease; in particular the group studied by Hansen *et al* had markedly higher ranges of ESR and much lower ranges of Hb.¹⁷

Kremer *et al* showed benefit in RA with EPA treatment¹⁸; our work cannot be compared with this study, however, as we used EPA only in combination with EPO. Nevertheless, he did show an improvement at three months and certainly this agrees with our data, where the combined therapy did seem to produce an earlier response. There did not, however, appear to be any other differences between the EPO and the EPO/fish oil groups. It is possible that the addition of EPA had no effect and the lower dose of EPO was sufficient to allow NSAID withdrawal; it is also possible that the fish oil contributed to the effect of the EPOs, thus making the two groups indistinguishable. In retrospect a more suitable study design would appear to have been EPO versus fish oil versus placebo; previous work, however, had suggested a synergism between the two EFAs²⁴ which we wished to investigate.

In conclusion, therefore, we have shown that it is possible to decrease or stop NSAIDs in some patients with RA by introducing EPO or EPO/fish oil treatment. It should be noted, however, that, although the patients claimed a subjective improvement, there was no change in any of the measurements conventionally used to measure disease activity. It is unlikely, therefore, that long term therapy with these EFAs would alter the course or prognosis of the disease. It would seem that these oils may be best used in clinical situations where NSAID therapy should be avoided, for example in patients with peptic ulceration or renal impairment.

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Leukotriene B₄ Production by Peripheral Blood Neutrophils in Rheumatoid Arthritis

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Belch JJF, O'Dowd A, Ansell D, Sturrock RD. Leukotriene B₄ production by peripheral blood neutrophils in rheumatoid arthritis. *Scand J Rheumatology* 1989; 18: 213–219.

Leukotriene B₄ (LTB₄) is an activator of white blood cells (WBC) and it has been suggested that its inhibition may be useful in rheumatoid arthritis (RA). Its production by peripheral WBC has not yet been investigated. We measured LTB₄ production in 105 patients with RA and compared it with 59 matched controls. C-reactive protein (CRP) and ESR were measured in 90 patients and correlated with LTB₄ values. Ten millilitres of blood were drawn. Separation was undertaken to obtain polymorphonuclear leukocytes (PMN) which were stimulated with calcium ionophore, and the supernatant was frozen for radioimmunoassay of LTB₄. Results show that RA patients produce significantly higher levels of LTB₄. It has been suggested that blockage of the cyclo-oxygenase enzyme by non-steroid anti-inflammatory drugs (NSAID) leads to increased production of LT via the lipoxygenase enzyme. Twenty-one patients not taking NSAID were compared with 84 on therapy. There was no significant difference. A linear regression was used to obtain Pearson's correlation coefficients. With LTB₄ and CRP, $r=0.3$ ($p<0.003$). With LTB₄ and ESR, $r=0.25$ ($p<0.02$). Low but significant correlations with CRP and ESR were obtained.

Key words: leukotriene B₄, rheumatoid arthritis, polymorphonuclear leukocytes, erythrocyte sedimentation rate, C-reactive protein.

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The polyunsaturated essential fatty acid (EFA) arachidonic acid (AA) plays a unique role as a precursor molecule for potent mediators with far-ranging effects. The best recognised of these are the prostaglandins (PG) (1). Recently, however, the discovery of another group of biologically active derivatives of AA, the leukotrienes (LT), has provoked much interest (2). These compounds, formed via the action of the 5-lipoxygenase enzyme, have pronounced biological effects which are related to immediate hypersensitivity responses and inflammation. LTB₄ in particular would appear to play a significant role in the inflammatory process (3). Polymorphonuclear leukocytes (PMN) present the first line of defence against foreign and pathogenic stimuli. As such they have to perform several functions. LTB₄ has been shown to promote chemokinesis, chemotaxis, aggregation and degranulation of the PMN, so we know that LTB₄ plays a part in the general picture of inflammation. However, there is little evidence to support a hypothesis that excess LT production contributes to states of excess pathological inflammation such as rheumatoid arthritis (RA). Klickstein et al. (4), using high-performance liquid chromatography (HPLC), detected excess lipoxygenase products in the synovial fluid of patients with RA compared with osteoarthritic controls. These findings were supported by Davidson et al. in 1983 (5). However, these studies do not clarify whether excess LTB₄ was being produced by the PMN or whether the increased amounts of LTB₄ merely reflected an increased PMN population within the synovial fluid.

The aim of our study was therefore to determine the amount of LTB₄ produced by peripheral PMN after maximal stimulation in patients with RA compared with normal

controls. The results obtained were correlated with three conventional markers for RA disease activity: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels and the Ritchie articular index (AI).

METHODS

Patients

One hundred and five patients with classical or definite RA (ARA criteria) (7), were enrolled in the study after informed consent had been obtained. Of these, 84 patients were taking standard doses of non-steroidal anti-inflammatory drugs (NSAID). The NSAID used by these patients were various, all being taken at or near the maximum dose for the drug. Twenty-one patients were not on NSAID. They had stopped their NSAID therapy at least 2 weeks prior to sampling (median 6 weeks, range 2–24 weeks). No patient was taking any disease-modifying drug. Ethical permission for the study was obtained from the regional Ethical Committee.

Assessment

All patients attended the out-patient department, where 90 out of 105 had a Ritchie articular index calculated (6).

Blood sampling

Twenty millilitres of blood were drawn from the antecubital fossa of these 90 RA patients. Five millilitres, anticoagulated with EDTA, were taken for measurement of ESR (Westergren method). Five millilitres of clotted blood were also taken for later analysis of serum CRP ($\mu\text{g/ml}$). The final 10 ml was anticoagulated with heparin (10 μml) for measurement of LTB_4 production from PMN. A further 15 patients, making a total of 105 RA subjects, had only the LTB_4 levels measured.

LTB_4 assay

PMN cell separation was carried out by Ficoll–Hypaque centrifugation (8). The PMN were counted and then incubated at 37°C for 5 min with the calcium ionophore A23187 (1 $\mu\text{g/ml}$). Aliquots of the supernatant were then taken and stored at –70°C for later radioimmunoassay (RIA) of LTB_4 using a modification of the method devised by Salmon et al. (9). This RIA was carried out using [^3H] LTB_4 from Amersham International, Amersham, UK, and the results were expressed as $\text{ng}/10^6$ cells. In our laboratory this assay has an intra-assay variation of 6.6% and an inter-assay variation of 8.5%. The minimum detection limit is 7.8 pg. The non-specific binding of the antibody is shown in Table I.

Controls

Fifty-nine age- and sex-matched normal volunteers also had blood sampled for estimation of PMN production of LTB_4 and CRP levels.

Table I. Cross-reactivity of assay antibody

Compound	% Cross-reactivity (50% B/B ₀ replacement)
LTB_4	100
LTC_4	0.03
LTD_4	0.03
Diastereoisomers of 5,12-dihydroxy-6,8,10- <i>trans</i> -14- <i>cis</i> -eicosatetraenoic acid	3.3
Diastereoisomers of 5,6-diHETE	1.6
5(S),12(S)-diHETE	0.14
5-HETE	0.03
12-HETE	2.0
11-HETE	0.04
15-HETE	<0.03
PGE_2 and other prostaglandins	<0.03
Arachidonic acid	<0.03
LTB_5	17.0

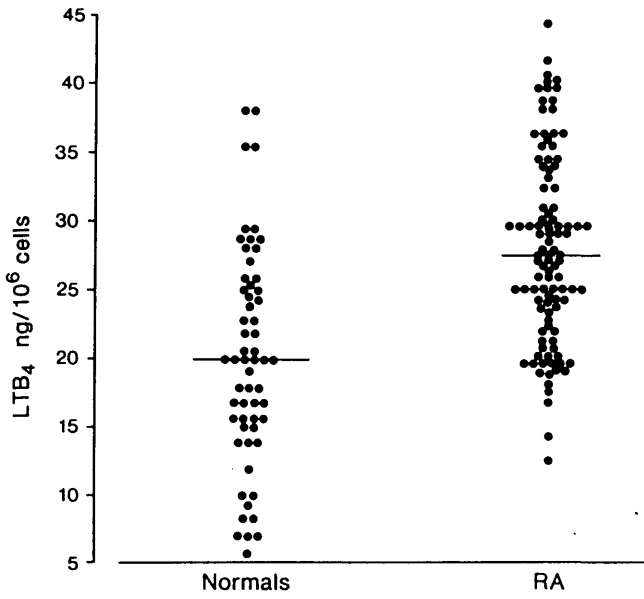


Fig. 1. LTB₄ production from PMN of RA patients and controls.

RESULTS

Table II shows the comparability between the RA patients and the controls. There were no significant differences in age between the two groups. The medians and ranges for the clinical and laboratory results from RA patients are also shown in this table.

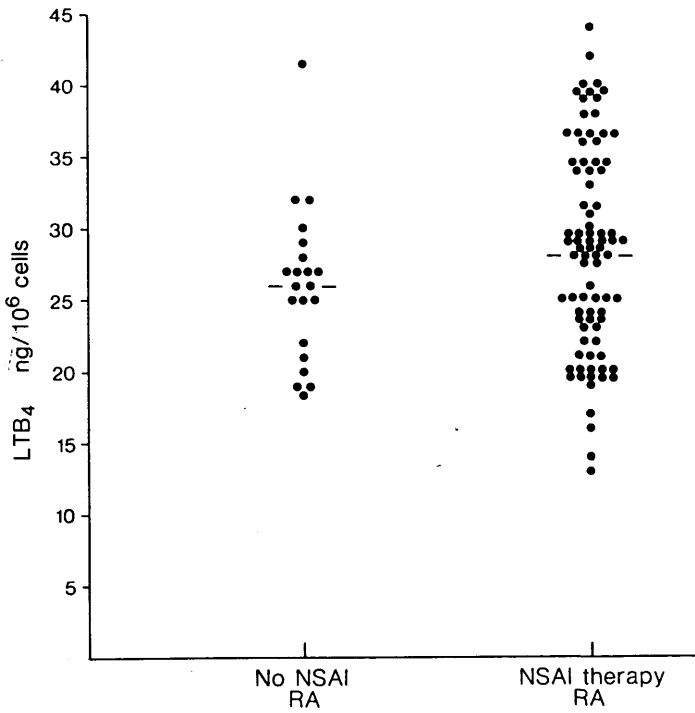


Fig. 2. LTB₄ production from PMN of RA patients on and off NSAID.

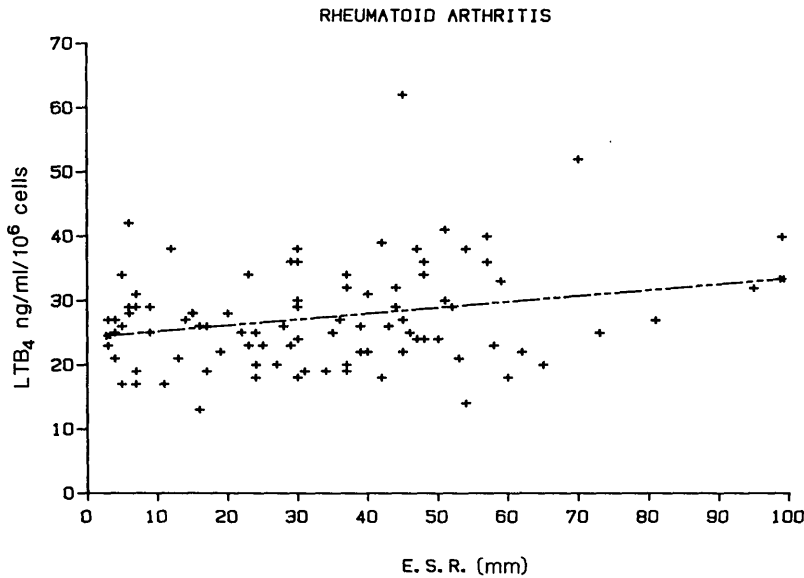


Fig. 3. Correlation of LTB₄ levels with ESR.

Fig. 1 shows the LTB₄ produced by PMN in both patients and controls. The levels from RA cells are elevated ($p < 0.002$, Mann Whitney U).

Fig. 2 shows the RA patient population divided for drug therapy. As can be seen, there is no significant difference between those on or off NSAID.

With a linear regression, the Pearson's correlation coefficient between LTB₄ and articular index has an r value of 0.08 ($p = 0.49$). The correlation between LTB₄ and ESR has an r value of 0.25 ($p < 0.02$) and the correlation between LTB₄ and CRP is $r = 0.3$ ($p < 0.003$) (Figs. 3 and 4).

DISCUSSION

Klickstein et al. (4) and Davidson et al. (5) have previously demonstrated increased levels of LTB₄ in synovial fluid from RA patients. However, these findings did not differentiate between increased production by the RA cell and increased amounts secondary to increased cell numbers. We have shown that LTB₄ production from a standard number of peripheral PMN is increased, suggesting a real difference between RA cells and those from normal controls.

Table II. Clinical and laboratory measures on RA patients and normal volunteers (median range)

	RA	Controls
Age (years)	48 (28-68)	35 (22-60)
Articular index	3 (0-20)	-
ESR (mm/h)	31 (3-99)	-
CRP ($\mu\text{g/ml}$)	17 (10-88)	All <10
LTB ₄ (ng/10 ⁶ cells)	28 (12-44)	20 (3-37)
LTB ₄ on NSAID ($n=84$)	28 (13-44)	-
LTB ₄ off NSAID ($n=21$)	26 (18-42)	-

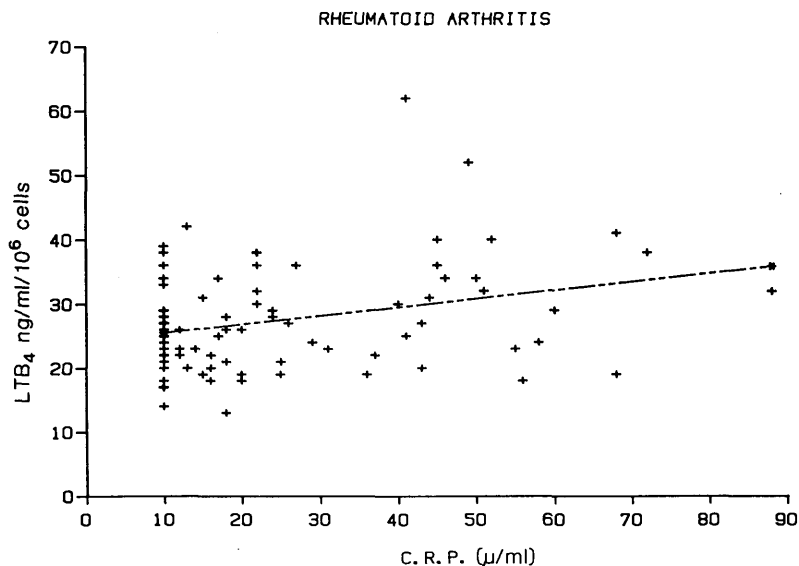


Fig. 4. Correlation of LTB₄ levels with C-reactive protein levels.

The reason for this increased LTB₄ production is not yet clear. It had been suggested that the use of NSAID might be a contributing factor (1). NSAID inhibit the cyclo-oxygenase enzyme and PG production. Thus, larger amounts of AA could be available for LT production via the 5-lipoxygenase enzyme. However, our results show no difference between those who took NSAID and those who did not. It is unlikely therefore that diversion of the substrate plays a major role in the finding of increased LTB₄ production in RA.

More recently assay systems have been devised to measure the enzymes involved in AA metabolism. An excess of either phospholipase A₂ or the 5-lipoxygenase enzyme could increase the metabolism of AA to LTB₄. Work is currently under way to assess this hypothesis and could be extended to family studies.

Even though we do not yet fully understand the mechanism of this increased LTB₄ production, such an increase may produce pro-inflammatory effects (3). In addition to the effect of LTB₄ on the PMN, there are effects on other cell types. Recently LTB₄ has been implicated in the chemotaxis of eosinophils and the release of peroxidase from these cells. LTB₄ may also be a mediator of lymphocyte responses. A number of workers (10, 11) have suggested that products of the 5-lipoxygenase pathway inhibit lymphocyte activities *in vitro*, in particular that LTB₄ reduce the lymphocyte response to mitogen. This field of study, however, has not been fully explored and reports are conflicting (12). Nevertheless it is probable that LTB₄ plays a role in modulating lymphocyte behaviour. In addition LTB₄ can interact and augment the action of other inflammatory mediators (13, 14, 15). These interactions may be important in the development and persistence of inflammation in RA.

Whether excess LTB₄ is a cause or a consequence of inflammation is unknown. It is interesting to suggest, however, that whether the excess is primary or secondary, a decrease in LTB₄ production may allow improvement in the clinical symptoms of inflammatory diseases. Our results show only a weak correlation with three conventional measures of disease activity, so it is unlikely that decreasing LTB₄ production will have any disease-modifying effect. However, such therapies may decrease the inflammatory symptoms in the same way as NSAID do via cyclo-oxygenase inhibition. The results from

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our recent study of evening primrose oil (EPO) as a treatment for RA (16) may be secondary to a decrease in LTB_4 production. EPO contains the EFA gamma-linolenic acid (GLA). In contrast to AA, GLA's metabolites are far less inflammatory; indeed GLA cannot itself be converted to LT but forms an inert 15-hydroxyl derivative that blocks transformation of AA to LT (17). LTB_5 produced from another EFA, eicosapentaenoic acid, is only 10% as potent as LTB_4 (18). This is found in fish oil, and Kremer et al. (19) have suggested that such a treatment might be useful in RA, perhaps mediating its beneficial effects through decreased LTB_4 production.

A more straightforward approach to therapy would be the development of a specific 5-lipoxygenase inhibitor. However, there are only a few compounds available and they are only now reaching the stage of clinical trials. Most of the known compounds are not selective and also inhibit the cyclo-oxygenase enzyme. It has been suggested that such a dual effect might produce the clinical benefit seen with corticosteroids (which inhibit the action of phospholipase A_2) without producing steroid-like side effects (20).

In conclusion, therefore, we have demonstrated that LTB_4 production is increased in patients with RA. This finding does not appear to depend on NSAID therapy. There are weak but significant correlations with two conventional measures of disease activity. Although there is no evidence to suggest that inhibition of LTB_4 would have a disease-modifying effect, in view of its pro-inflammatory effects we suggest that inhibition of its production might improve the clinical symptoms of RA.

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BLOOD COAGULATION AND RHEOLOGY IN PATIENTS WITH VIBRATION WHITE FINGER DISEASE

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Abstract

In vibration white finger (VWF) the abnormal response of blood vessels to cold has been clearly documented. Less fully explored has been the potential abnormalities in blood coagulation and rheology. We have investigated these in 35 patients with VWF grade III (Taylor Pelmear scale) compared to 25 matched controls. Forty-two patients with secondary Raynaud's syndrome and systemic sclerosis were used as positive controls. VWF patients had less deformable red cells than controls [1.25 (0.86-2.0) vs 1.00 (0.56-1.5), $p < 0.05$ MW], white blood cells released more leukotriene B₄ [26.0 (14.4-39.4) vs 17.2 (6.0-38.0), $p < 0.01$ MW] VWF patients had lower plasma thiols [450 (417-510) vs 480 (418-555), $p < 0.01$ MW] a trend towards brisker platelet aggregation to collagen [17 (6-32) vs 11 (3-32), $p = 0.05$ MW] higher β thromboglobulins levels [37 (20-55) vs 23 (12-41), $p < 0.01$ MW] elevated whole blood viscosity at low shear rate [20.0 (15.3-26.2) vs 17.9 (11.8-20.3) $p < 0.01$ MW] higher fibrinogens [3.1 (2.1-4.8) vs 2.6 (2.0-4.0), $p < 0.01$ MW] lower plasminogen activator activity [68 (45-95) vs 106 (64-151), $p < 0.01$ MW], and higher plasma factor VIII von Willebrand factor [175 (72-242) vs 100 (38-195) $p < 0.01$ MW]. Results in systemic sclerosis were similar to those of the patients with VWF.

We have demonstrated that VWF is a true secondary Raynaud's syndrome with abnormalities in blood clotting and rheology. Although the tests are not specific enough to be used as a diagnostic test they may contribute to decreased blood flow, and provide a rationale for the use of antithrombotic drugs in VWF.

Key words:	Vibration white finger.	Red blood cells.
	Leukotriene B ₄ .	Free radicals.
	Platelet aggregation.	B thromboglobulin.
	Blood viscosity.	Fibrinolysis.
	Factor VIII von Willebrand factor.	

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Introduction

Operators of hand-held vibrating power tools have long complained of tingling and numbness in their hands and of their fingers blanching. These episodes of vibration white finger (VWF) were first reported in the literature between 1911 and 1920 (1,2) and the link with VWF and the episodic digital ischaemia in response to cold reported by Raynaud in 1862 was quickly recognised (3). VWF is now known to be one of the secondary causes of Raynaud's Syndrome (RS).

The pathophysiology of VWF is still not fully known. Most hypotheses involve vibration induced damage to the blood vessel wall (4,5). In patients with secondary RS caused by other clinical conditions, such as connective tissue disorders (C/T), blood coagulation and rheology may be abnormal (6). It is generally agreed that these changes are not a primary phenomenon but develop later, probably as a result of blood passing over damaged blood vessels. No matter whether cause or consequence of the initial vessel damage, the resultant effects of disordered coagulation and rheology is to worsen blood flow and augment the clinical symptoms of secondary RS.

The aim of this study was to investigate aspects of blood cell and plasma behaviour in patients with VWF compared to matched controls and to patients with secondary RS and an associated C/T disorder.

Patients and Methods

Thirty-five patients with VWF were enrolled in the study. All had grade III VWF as described by Taylor et al (7), thus all had marked blanching of the digits and the condition significantly interfered with their activities at work and play. All were or had been employed as boilermakers exposed to vibrating machines such as chippers, grinders and buffs. Results were compared to 25 age, sex and smoking matched workers not exposed to vibration. As a positive control 42 patients with secondary RS associated with systemic sclerosis (SS) were also studied. Clinical details of these patients are shown in Table 1. As VWF is predominantly a disease of men and SS of women, these 2 groups could not be matched for sex. However, in this age group there is not thought to be a difference between the sexes in the tests measured.

None of the patients had taken aspirin or other medications known to interfere with the tests in the previous 2 weeks. Smokers were asked to refrain from smoking from 12 midnight prior to the day of testing. All samples were taken at the same time of day after a standard light

breakfast. Blood was taken through a 19 gauge butterfly needle with minimal venous occlusion. The following tests were performed:

Red cell deformability. 5 ml of blood were anticoagulated with edetic acid (EDTA 1.5 mg/ml) and red cell deformability was measured by a positive pressure filtration method (8). A syringe containing Tris-saline buffer solution was connected to a disposable polycarbonate filter (nucleopore, pore diameter 5 μ). A constant flow rate of 1.5 ml/min was applied and filtration pressure measured via a pressure transducer and recorded for 6 min. Washed red blood cells were suspended in buffer (haematocrit 0.05) and were then filtered the same way through the same filter. Red cell deformability was assessed by the ratio of the pressure generated by the flow of the cell suspension to the pressure generated by the flow of buffer.

Leukotriene B₄ (LTB₄). 10 ml of blood were anticoagulated with sodium heparin (10 u/ml). The polymorphonuclear leukocytes (PMNs) were separated over Ficoll Hypaque and stimulated with calcium ionophore A23187 (1 μ /ml). The resultant supernatant was aspirated and stored for later radio-immunoassay (RIA) for LTB₄ (9). As this assay was developed later than the others 25 VWF patients, 34 SS patients and 30 controls were sampled for this test.

Plasma thiols (PSH). PSH was estimated as a measure of free radical (FR) activity. This was measured using a photometric analysis using a technique first described by Ellman (10). As this assay was also a later development, 12 VWF patients, 15 SS patients and 16 normals were studied.

Platelet aggregation. 10 ml of blood were anticoagulated with 3.8% Trisodium citrate (9:1 v:v) The rate of platelet aggregation (PA) to 2 μ mol adenosine 5-diphosphate (ADP) in platelet rich plasma (PRP) was measured using the turbidimetric technique described by Born (11) (Malin photometric aggregometer). Aggregation to 1 μ g of collagen was also measured.

β thromboglobulin (BTG). This platelet release product was measured by RIA using an Amersham kit.

Blood viscosity. Blood viscosity was measured in a Contraves LS30 rotational viscometer at 37°C at high shear rate (9.45 s⁻¹) and low shear rate (0.95 s⁻¹) both corrected to a standard microhaematocrit of 0.45 (Hawksley, 13,000 g for 5 min) using regression equations derived from 200 samples (12).

Fibrinogen and fibrinolysis. 10 ml of blood was anticoagulated with 3.8% Trisodium citrate (9:1, v:v) Fibrinogen was measured using an automated coagulometer (method of Class) (13). Plasminogen activator activity was measured by estimating the area of lysis on a fibrin plate (14).

Factor VIII von Willebrand factor (fVIII vWF). Citrated blood was again used to measure fVIII vWF using the Laurell rocket technique (15).

Results

Red cell deformability. Using the pressure ratio P_i/P_b the red cells in both patient groups were harder than the cells from the controls ($p < 0.05$, Mann-Whitney U) (Table 2).

Leukotriene B_4 . LTB_4 production from stimulated WBCs was increased in both VWF and SS ($p < 0.01$, $p < 0.05$ respectively, Mann-Whitney U). This suggests a degree of activation of the WBCs and supports the hypothesis of an inflammatory element in VWF (7) (Table 2).

Plasma thiol. PSH was lower in both patient groups ($p < 0.01$, Mann-Whitney U) suggesting an excess of free radical generation (Table 2).

Platelet aggregation. There was no significant difference between the groups for PA to $2 \mu\text{m}$ ADP. However, there was a trend towards brisker PA to $1 \mu\text{g}$ collagen in the VWF group and a statistical difference between the SS patients and controls ($p = 0.05$, $p < 0.02$ respectively, Mann-Whitney U) (Table 3).

β thromboglobulin. Despite there being no statistical difference in PA in VWF there is still evidence of platelet activation, as demonstrated by the increased levels of βTG in VWF ($p < 0.01$, Mann-Whitney U). βTG is also increased in SS ($p < 0.01$, Mann-Whitney U) (Table 3).

Blood viscosity. Whole blood viscosity at low shear rate (0.95s^{-1}) is significantly increased in both patient groups ($p < 0.01$, Mann-Whitney U). There is no difference between the groups at high shear rate (9.45s^{-1}) (Table 3).

Fibrinogen and fibrinolysis. As expected from the whole blood viscosity results plasma fibrinogen levels are significantly increased in both VWF and SS ($p < 0.01$, Mann-Whitney U). Plasminogen activator activity was significantly decreased in both patient groups ($p < 0.01$, Mann-Whitney U) (Table 3).

fVIII vWF. Both VWF patients and SS patients showed a statistically significant increase in plasma fVIII vWF reflecting a degree of vascular damage in both patient groups ($P < 0.01$, Mann-Whitney U) (Table 4).

DISCUSSION

In this study we have shown abnormalities of blood coagulation and rheology in VWF similar to those found in secondary RS. These findings may be relevant. The diameter of the red blood cell is greater than some of the capillaries, so flow in the microcirculation may be critically dependent on red cell hardness. The white cell is being increasingly recognised as being important, not only in inflammation and immunity, but also in thrombosis (16). Hard white cells or clumps of aggregated WBCs may impede the circulation. Activated WBCs are less deformable and aggregate. LTB_4 is a WBC release product which promotes WBC activation (17) and we have shown this to be increased in VWF. WBCs also release noxious products, such as free radicals (FRs). FRs are prethrombotic (18) via their ability to damage the endothelium and by selectively increasing production of the platelet aggregant thromboxane A_2 . As short lived species they are difficult to measure directly. However, plasma thiol levels (PSH, mainly albumin thiol) shows the degree of oxidation of the plasma. Albumin conformation is altered by FR generation and therefore PSH is a good measure of FR activity. The decreased levels of PSH seen in VWF reflect increased FR production. Similarly, if the blood platelet is activated it aggregates and releases. We have shown increased plasma β TG in VWF. Bovenzi et al (19) also failed to show an increase in platelet aggregation in VWF patients between grade ON and grade I (Taylor Pelmear scale) (7). A more sensitive measure of platelet activation may be the β TG release into the plasma. Our results suggest, therefore, that there is an early degree of platelet activation which is not manifest by a frank increase in aggregation rate.

In a normal subject fibrin is removed by the enzymatic degradation mediated by plasmin. Plasmin is produced by cleavage of its inactive precursor plasminogen under the control of plasminogen activators. A decrease in activator activity, as shown here, may aggravate VWF through poor clearance of vascular endothelial fibrin. Fibrinogen is increased which will contribute to the elevated whole blood viscosity at low shear rate, witnessed here and also by Okada et al (20). FVIII vWF is released from the endothelium and levels have been shown to be increased in diseases in which there is vascular damage (21). It may also predispose to thrombosis by its effects on platelet aggregation and in the clotting cascade.

It is easy to diagnose VWF during an attack but it is difficult to be sure of a diagnosis when the patients are seen between attacks. As VWF is now recognised as an occupational disease, eligible for compensation, it is desirable to devise tests that will establish the diagnosis clearly. Although statistical differences are seen between the controls and patients with VWF in our tests, the degree of overlap of the results is such that disappointingly they cannot be used as a

means of diagnosis. Nevertheless, these findings may have considerable importance, as not only may they contribute to the worsening of the VWF symptoms via their deleterious effects on blood flow, but they may contribute to diseases elsewhere in the body. Elevated blood pressure and electrocardiogram abnormalities are more frequently reported in VWF patients than in controls (22) and the abnormalities described here by us may contribute to decreased coronary flow. The majority of VWF patients also suffer from a degree of industrial deafness thought to be due to noise exposure. There is, however, a synergistic effect of vibration and noise on this deafness (23) and the explanation may be that vibration produces constriction in the cochlear vessels with a contribution to the decreased flow from the rheological and blood clotting abnormalities (24). Furthermore, most of the recent advances in the treatment of Raynaud's Phenomenon have come from drugs which affect blood clotting and rheology (25). It may be, therefore, that the delineation of these abnormalities in this group of patients may give a rationale for these drugs to be studied in VWF.

In conclusion, therefore, we have demonstrated that VWF is a true secondary RS with abnormalities in blood clotting and rheology. Although the tests are not specific enough to be used as a measure of diagnosis for this disorder, they may contribute to the decreased blood flow and provide a rationale for the use of antithrombotic drugs as a treatment for severe VWF.

TABLE 1 - Clinical data and comparability of Groups

	VWF*	SS**	Controls
Numbers	35	42	25
Median age in years (Range)	49 (27-65)	43 (28-68)	44 (25-63)
Sex M/F	35:0	5:37	25:0
Smokers	23	25	19
Median duration of symptoms in years	5	9	-
(range)	(3-20)	(2-15)	-
Median duration of vibration exposure (years)	29	-	-
(range)	(5-49)		

VWF* = Vibration White Finger
 SS** = Systemic Sclerosis

Table 2 - Red cell deformability, WBC release of LTB₄ and plasma thiols

		VWF	SS	Controls
Red cell deformability	Median	1.25	1.25	1.00
(pressure ratio)	Range	(0.86-2.0)	(1.1-2.8)	(0.56-1.5)
LTB ₄ (ng/10 ⁶ cells)	Median	26.0**	23.5*	17.2
	Range	(14.4-39,4)	(14.4-44.0)	(6.0-38.0)
PSH (μML ⁻¹)	Median	450**	445**	480
	Range	(417-510)	(375-475)	(418-555)

*p<0.05; **p<0.01 (Mann Whitney U)

Table 3 - Platelet aggregation to ADP and collagen, and plasma βTG

		VWF	SS	Controls
Platelet aggregation to 2 μmol ADP	Median	17	16	15
(% change optical density/sec)	Range	(7-30)	(8-36)	(9-30)
Platelet aggregation to 1 μg collagen	Median	17	19*	11
(% change optical density/sec)	Range	(6-32)	(10-69)	(3-32)
βTG (pg/ml)	Median	37**	41**	23
	Range	(20-55)	(15-69)	(12-41)

*p<0.02; **p<0.01 (Mann Whitney U)

Table 4 - Whole blood viscosity, fibrinogen, plasminogen activator activity and fVIII vWF

		VWF	SS	Controls
Viscosity $0.95s^{-1}$ (in Pa.s)	Median	20.0*	20.1*	17.9
	Range	(15.3-26.2)	(15.8-31.9)	(11.8-20.3)
Viscosity $9.45s^{-1}$ (in Pa.s)	Median	5.71	5.92	5.79
	Range	(5.21-6.94)	3.2*	(3.12-6.66)
Fibrinogen (g/L)	Median	3.1*	3.2*	2.6
	Range	(2.1-4.8)	(1.3-6.8)	(2.0-4.0)
Plasminogen activator activity (%)	Median	68*	61*	106
	Range	(45-95)	(31-88)	(64-151)
fVIII vWF (%)	Median	175*	198*	100
	Range	(72-242)	(76-390)	(38-195)

* $p < 0.01$ (Mann Whitney U)

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