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Leukotriene B_4 and platelet-activating factor: assessment of biological significance in neutrophil trafficking

par

Hanan Attia El Imam

Faculté de pharmacie

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présenté par:

Hanan Attia El Imam

A été évalué par un jury composé des personnes suivantes:

Dr. Patrice Hildgen
Président-rapporteur

Dr. Sylvie Marleau
Directeur de recherche

Dr. Pierre Borgeat
Co-directeur de recherche

Dr. Patrick P. McDonald
Membre externe du jury

Mémoire accepté

SUMMARY

In order to delineate the role of the lipid mediators, platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) in regulating polymorphonuclear neutrophil (PMN) and plasma extravasation at sites of acute inflammation, we used selective and potent PAF and LTB_4 receptor antagonists in a rat dermal inflammation model. Briefly, rats were injected subcutaneously with filgrastim (Neupogen^{MD}) for 9-11 days before the experiment in order to raise the number of PMN circulating in the blood. Rats were then pretreated orally with UK-74,505 or SR-27417 (PAF receptor antagonists) and/or CP-105,696 (LTB_4 receptor antagonist). Agonists under investigation, including LTB_4 and PAF were injected intradermally at duplicate sites in each rat. Myeloperoxidase, an enzymatic marker contained in PMN azurophilic granules, was used to assess local PMN accumulation while the Evans blue dye was used to quantify plasma extravasation. In some experiments, local microcirculatory blood flow was assessed by laser-doppler blood flowmetry.

In a series of experiments, we studied the role of PAF and/or LTB_4, as well as their potentially co-operative effect on PMN accumulation. To this end, rats were pretreated with either a selective PAF or LTB_4 receptor antagonist, or with both drugs, prior to intradermal injections of PAF and LTB_4 to induce cutaneous inflammation. Interestingly, the results show an additive inhibitory effect of LTB_4- and PAF-receptor antagonists on PAF-elicited PMN accumulation, suggesting a role for LTB_4 in regulating, at least in part, PAF-induced PMN extravasation at the blood-endothelium interface. In contrast, we did not observe an additive effect of the drugs when LTB_4 was used as a stimulus to elicit PMN accumulation in rat skin. In this particular series of experiment, the LTB_4 receptor antagonist CP-105,696 inhibited LTB_4-stimulated PMN accumulation by 79% at the dose used, which may have precluded the observation of co-operative effect of agonists. Further studies are undergoing to elucidate this point.

In a second series of experiments, we investigated the role of PAF and/or LTB_4 in the chemotactic effect of a number of chemically unrelated soluble mediators on PMN accumulation to dermal inflammatory sites. Our results support that PAF and/or LTB_4 contribute, at least in part, to dermal inflammation elicited by TNF-α and zymosan-activated plasma, the latter used as a source of $C5a_{desarg}$. In contrast, the lipid mediators PAF and LTB_4 do not appear to contribute to IL-8-elicited PMN chemotaxis *in vivo* in rats. These results further support our working hypothesis that LTB_4 and PAF biosynthesis at the blood-endothelium interface may act in an aurocrine or paracrine fashion to regulate events that are crucial to the PMN transmigration process. Inasmuch as plasma extravasation is a phenomenon accompanying PMN diapedesis to the inflammatory sites, we have assessed the contribution of PAF and/or LTB_4 to plasma exudation elicited by inflammatory mediators including PAF, LTD_4 and substance P. Our results support that PAF and/or LTB_4 contribute, at least in part, to plasma extravasation elicited by LTD_4.

The results obtained in this work support a role for PAF and/or LTB_4 in regulating PMN extravasation elicited by a number of inflammatory mediators at the blood-endothelial interface. Furthermore, PAF and LTB_4 may co-operate to this aim, inasmuch as PAF and LTB_4 receptor blockade had an additive effect in reducing PMN accumulation elicited by either agonist *in vivo*. Our observations open a new perspective to further investigate the role of these lipid mediators in pathologic inflammatory settings.

Key words: chemotaxis, dermal inflammation, laser-doppler, LTB_4, myeloperoxidase, oedema, PAF, PMNs accumulation, rats.

RÉSUMÉ

Dans le but d'étudier le rôle des médiateurs lipidiques, notamment le facteur d'activation plaquettaire (PAF) et le leucotriène B_4 (LTB_4) dans la régulation de la migration des neutrophile polymorphnucléaire (PMN) et de l'extravasation plasmatique aux sites d'inflammation aiguë, nous avons utilisé des antagonistes sélectifs des récepteurs du PAF et du LTB_4 dans un modèle d'inflammation dermique chez le rat. Brièvement, les rats ont reçu des injections sous-cutanées de filgrastim (Neupogen^{MD}) pendant une période de 9 à 11 jours avant de débuter le protocole expérimental afin d'augmenter le nombre des PMNs circulants dans le sang. Les rats ont été prétraités oralement avec des antagonistes sélectifs des récepteurs du PAF (UK-74,505 ou SR-27417) et/ou par un antagoniste sélectif des récepteurs du LTB_4 (CP-105,696). Les agonistes pro-inflammatoires à l'étude, incluant le LTB_4 et le PAF, ont été injectés par voie intradermique en duplicata sur la région dorsale de chaque rat. La myelopéroxidase, une enzyme contenue dans les granules azurophiles des PMNs a été utilisée comme marqueur afin de déterminer la quantité de PMNs présents dans les biopsies, tandis que le bleu d'Evans a été utilisé pour quantifier l'œdème tissulaire. Dans quelques expériences, nous avons déterminé le flux sanguin microcirculatoire à l'aide d'un moniteur laser-doppler «laser-doppler blood flowmetry ».

Dans une première série d'expérience, nous avons étudié le rôle du PAF et/ou du LTB_4 et leur effet coopératif potentiel chez les rats prétraités avec un antagoniste sélectif du PAF et/ou du LTB_4. Les résultats ont montré qu'il y a un effet inhibiteur additif des antagonistes du LTB_4 et du PAF sur la migration extravasculaire des PMNs induite par le PAF, ce qui suggère une rôle régulateur pour le LTB_4 dans la régulation de la migration des PMNs à l'interface neutrophiles-cellules endothéliales au niveau des vaisseaux sanguins. Par contre, nous n'avons pas observé d'effet additif des antagonistes lorsque le LTB_4 a été utilisé comme stimulus pour induire l'accumulation dermique des PMNs. Dans cette dernière série d'expériences, l'antagoniste CP-105,696, sélectif pour les récepteurs du LTB_4 administré seul , a réduit de façon importante l'accumulation des PMNs induite par le LTB_4 (79%), ce

qui a pu limiter l'observation d'une action coopérative entre les agonistes. Des études sont en cours présentement pour évaluer cette possibilité.

Dans une deuxième série d'expériences, nous avons étudié le rôle du PAF et du LTB_4 dans l'effet chimiotactique de plusieurs médiateurs solubles de nature chimique différente. Nos résultats montrent que le PAF et/ou le LTB_4 contribuent, du moins en partie, à l'effet chimiotactique induit par le TNF-α et le plasma activé par le zymosan (ZAP) utilisé comme source de $C5a_{desarg}$. À l'opposé, les médiateurs lipidiques PAF et LTB_4 ne semblent pas contribuer à la chimiotaxie des PMNs induite par l'IL-8 *in vivo* chez le rat. Ces résultats supportent notre hypothèse de travail selon laquelle la biosynthèse du LTB_4 et du PAF au niveau de l'interface leucocytes-cellules endothéliales, et leurs effets de nature autocrine ou paracrine, peuvent moduler certains événements dans le processus de transmigration des PMNs. Étant donné que l'extravasation du plasma est un phénomène qui accompagne la diapédèse des PMNs au site inflammatoire, nous avons étudié la contribution du PAF et/ou du LTB_4 dans l'exsudation de plasma induite par des médiateurs inflammatoires tels que le PAF, le LTD_4 et la substance P. Nos résultats montrent que le PAF et/ou LTB_4 contribuent, au moins en partie, à l'extravasation plasmatique induite par le LTD_4.

Les résultats obtenus dans ce travail appuient un rôle du PAF et du LTB_4 dans la régulation de l'extravasation des PMNs induite par plusieurs médiateurs inflammatoires au niveau de l'interface neutrophile-endothélium vasculaire. De plus, le PAF et le LTB_4 peuvent coopérer ensemble à cette fin. Nos observations ouvrent de nouvelles perspectives pour l'étude du rôle des ces médiateurs lipidiques dans des situations pathologiques inflammatoires.

Mots clés : chimiotaxie, inflammation dermique, laser-doppler, LTB_4, myelopéroxidase, œdème, PAF, accumulation des PMNs, rats.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HPETE:	5-hydroperoxyeicosatetranoic acid
5-LO:	5-lipoxygenase
AA:	arachidonic acid
ARDS:	adult respiratory distress syndrome
BAL:	bronchoalveolar lavage
BLT:	LTB_4 receptors
Ca^{2+}:	calcium
CINCs:	cytokine-induced neutrophil chemoatracttants
CMC	carboxymethyl cellulose
COX:	cyclo-oxygenase
$cPLA_2$:	cytosolic PLA_2
CysLTs:	cysteinyl leukotrienes
DNA:	deoxyribonucleic acid
ECM:	extracellular matrix
ECs:	endothelial cells
E-selectin:	endothelial selectin
FAs:	fatty acids
FLAP:	5-lipoxygenase activating protein
fMLP:	N-formyl-methionyl-leucyl-phenylalanine
G-CSF:	granulocyte-colony stimulating factor
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GRK:	G-protein-coupled receptor kinase-1
H_2O_2:	hydrogen peroxide
HOCl:	hypochlorous acid
I.V.:	intravenous
I/R:	ischemia and reperfusion
ICAM-1:	intercellular adhesion molecule-1
IgE:	immunoglobulin E
IL-1:	interleukin-1

IL-3:	interleukin-3
IL-5:	interleukin-5
IL-6:	interleukin-6
IL-8:	interleukin-8
IP_3:	inositol trisphosphate
LAD-1:	leukocyte adhesion deficiency syndrome-1
LFA-1:	lymphocyte-associated function antigen-1
LO:	lipoxygenase
LPS:	lipopolysaccharide
L-selectin:	leukocyte selectin
LTA_4:	leukotriene A_4
LTB_4:	leukotriene B_4
LTC_4:	leukotriene C_4
LTD_4:	leukotriene D_4
LTE_4:	leukotriene E_4
LTs:	leukotrienes
Lyso-PAF AcT	acetyl-CoA:lyso-PAF acetyltransferase
Lyso-PAF:	1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine
MAC-1:	macrophage antigen-1
MCP-1:	macrophage chemoattractant protein-1
MDP:	muramyl dipeptide
MIP-1α:	macrophage inflammatory protein-1 alpha
MPO:	myeloperoxidase
$NO^{\bullet}$:	nitric oxide
O_2^{-}:	superoxide anion
$OONO_2^{-}$:	peroxynitrite
PAF:	platelet-activating factor
PAF-AH:	PAF acetylhydrolase
PAF-PCT:	PAF-phosphocholine transferase
PAFR:	PAF receptor
PECAM-1:	platelet endothelial cell adhesion molecule-1

PGE_2:	prostaglandin E_2
PGH_2:	prostaglandin H_2
PGHS:	prostaglandin H synthase
PGI_2:	prostaglandin I_2 (prostacyclin)
PKC:	protein kinase C
PLA_2:	phospholipase A_2
PLC:	phospholipase C
PMNs:	polymorphonuclear neutrophils
PPAR-α:	peroxisome proliferator-activated receptor-α
P-selectin:	platelet selectin
RNS:	reactive nitrogen species
ROS:	reactive oxygen species
RPA:	reversed passive Arthus
S.C:	subcutaneous
SCR:	short consensus repeat domain
SOD:	superoxide dismutase
$sPLA_2$:	secretory PLA_2
SRS-A:	slow reacting substance of anaphylaxis
TNF-α:	tumor necrosis factor- α
TXA2:	thromboxane A_2
VCAM-1:	vascular cell adhesion molecule-1
VLA-4:	very late antigen-4
ZAP:	zymosan-activated plasma

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CHAPTER I: INTRODUCTION

Inflammation

1.1. The inflammatory response

Inflammation, a complex homeostatic reaction of the body, is a localized, protective response to trauma or microbial invasion that destroys, dilutes, or walls-off the injurious agents and the injured tissue. This response requires innate immunity and, in some cases, an adaptive immune response, which are the two main integral components of the host's defence system. Innate immunity not only acts as the first line of defence against noxious material, but after recognition of an appropriate stimulus, it provides the necessary signals to instruct the adaptive immune system to mount a response. In turn, the adaptive response relies on the innate immune system to provide the necessary effectors, in the form of phagocytes and granulocytes, to deal with the initiating stimulus (Lawrence et al., 2002).

These responses involve cellular and molecular mechanisms and are orchestrated in such a manner as to protect the organism from further insult and to return normal functions to the tissues (Gauldie, 1991). Early investigators considered inflammation as a primary host defence system. However, inflammation may also lead to debilitating diseases, such as arthritis and gout. Hence, the end point of an inflammatory reaction may be beneficial or harmful.

Macroscopically, inflammation is characterized in the acute form by five classic signs: (a) **Heat** *(calor);* increase in temperature is seen only in peripheral parts of the body, such as the skin. It is due to increased blood flow (hyperaemia) through the region, resulting in vascular dilatation and the delivery of warm blood to the area. (b) **Redness** *(rubor);* an acutely inflamed tissue appears red, due to dilatation of small blood vessels within the damaged area. (c) **Swelling** *(tumor);* swelling results from oedema, the accumulation of fluid in the extravascular space. (d) **Pain** *(dolor);* results partly from the stretching and distortion of tissues due to inflammatory oedema and, in particular, from pus under pressure in an abscess cavity. (f) **Loss of function**

(functio laesa); movement of an inflamed area is consciously and reflexly inhibited by pain (**figure 1.1**). These signs have been known since the ancient Greek and Roman era (Dennis et al., 1976; Gauldie, 1991; Walker and Fantone, 1994).

1.1.1. The phases of inflammation

Inflammation can be divided into several phases. The earliest, gross event of an inflammatory response is temporary vasoconstriction, e.g., narrowing of blood vessels caused by contraction of smooth muscle in the vessel walls, which can be seen as blanching (whitening) of the skin. This is followed by several phases that occur over minutes, hours and days later, as following:

- **Acute vascular response** follows within seconds of the tissue injury and last for some minutes. It results from vasodilation and increased capillary permeability due to alterations in the vascular endothelium, which leads to increased blood flow (*hyperaemia*) that causes redness (*erythema*) and the entry of fluid into the tissues (*oedema*).
- **Acute cellular response** takes place over the next few hours. This phase occurs if there is sufficient damage to the tissues, or if infection has occurred. The appearance of granulocytes, particularly neutrophils, in the tissues is the specific sign of this phase. If the vessel is damaged, fibrinogen and fibronectin are deposited at the site of injury, platelets aggregate and become activated, and the red cells stack together to help stop bleeding and aid clot formation. The dead and dying cells contribute to pus formation.
- **Chronic cellular response** may follow during the next few days. It is characterized by the appearance of a mononuclear cell infiltrate composed of macrophages and lymphocytes. The macrophages are involved in microbial killing and in clearing up cellular and tissue debris. Macrophages also seem to be very important in tissue remodeling.
- **Resolution,** during which the normal tissue is restored as the blood clots are removed by fibrinolysis where possible, or scarring as a result of in-

Figure 1.1. Cardinal signs of inflammation

This cartoon depicts five Greeks representing the cardinal signs of inflammation: heat, redness, swelling, pain and loss of function, which are as appropriate today as they were when first described by Celsus more than 2000 years ago. This figure was commissioned by D.A.W. and drawn by P. Cull for the Medical Illustration Department at St Bartholomew's Medical College.

From **Lawrence et al.,** *Nature Reviews Immunology* **2**, 787-795, 2002.

filling with fibroblasts, collagen, and new endothelial cells (ECs). Generally, by this time, any infection will have been overcomed.

In acute inflammation, the hydrostatic pressure in postcapillary venules may overcome the osmotic pressure of plasma proteins. Therefore, fluid and low molecular substances have the tendency to penetrate into the surrounding area. The increased capillary permeability for plasma proteins is the key factor for the production of inflammatory exudate. In the interstitial area, high-molecular weight proteins may be split into smaller fragments that participate in the raising of the osmotic pressure of interstitial fluid.

Cellular exudate is formed during the second and the third phase of inflammation, e.g., acute and chronic cellular responses. During the former, neutrophils are prevalent, whereas mononuclear cells (macrophages and lymphocytes) are predominant in later phases. Cell composition of exudate differs not only depending on the phase of inflammation, but also on the type of inflamed tissue and on the factors triggering the inflammatory process. Central effector and regulatory functions in acute inflammation rely on neutrophils. Eosinophils and basophils may also be involved. So, a number of different cell types are potentially recruited into the area where damage has occurred, and these are responsible for inactivation and removing of invading infectious agents and damaged tissues, as well as for inducing the formation of new tissue and reconstructing the damaged cell matrix, including basement membranes and connective tissue. A new blood supply to the area is also established during the repair process (Martinez-Hemandez, 2001).

1.1.2. The response to injury and infection

The cells of the immune system are widely distributed throughout the body, but, if an infection or tissue damage occurs, it is necessary to concentrate them and their products at the site of injury. As discussed above, three major events occur during this response:

- An increased blood supply to the tissue "in danger" (vasodilatation). The inflamed tissue looks like containing a greater number of vessels.
- Retraction of the endothelial cells (ECs), allowing larger molecules than usual to escape from the capillaries, and thus, allowing the soluble mediators of immunity to reach the site of inflammation.
- Migration of leukocytes out of the capillaries into the surrounding tissues (Walker and Fantone, 1994).

Inflammatory responses must be well-ordered and controlled. Therefore, a wide variety of interconnected cellular and humoral (soluble) mechanisms are activated when tissue damage and infection occur. Cells and inflammatory mediators participating in these events are detailed in the following paragraphs.

1.2. Cells participating in acute inflammation

1.2.1. Mast cells and basophils

Mast cells are localized within the connective tissue of the body, whereas basophils are present in low number in the circulation. They play a central role in a variety of inflammatory and allergic conditions; they are able to release potent inflammatory mediators such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid from the dense cytoplasmic granules into extracellular tissues by degranulation (Fantone and Ward, 2001). The degranulation could be induced by: (a) a physical destruction such as mechanical trauma; (b) chemical substances, such as toxins and proteases; (c) cell mediators, peptidases and peroxidases such as cationic proteins derived from eosinophils and neutrophils; (d) immune mechanisms which may be immunoglobulin (Ig) E-dependent or IgE-independent. Both, eosinophils and basophils contain high affinity receptors (FcR1) for IgE on their surface which can be triggered during the IgE-dependent mechanism.

In the IgE-independent way, the anaphylatoxins C5a, C3a and C4a, formed during activation of complement, trigger degranulation through C5a cell surface receptors.

1.2.2. Eosinophils

The eosinophil is a terminally differentiated, end-stage leukocyte that resides predominantly in the submucosal tissue and is recruited to sites of specific immune reactions, including allergic diseases. Its cytoplasm is filled with large eosinophilic granules and its nucleus is almost always bilobed although trilobed forms are sometimes seen (Bainton, 1999). The granules are the principal identifying feature of eosinophils. They contain four distinct cationic proteins which exert a range of biological effects on host cells and microbial targets: major basic protein, eosinophil cationic protein, eosinophil derived neurotoxin, and eosinophil peroxidase. In addition, histaminase and a variety of hydrolytic lysosomal enzymes are also present in the large specific granules (Peters et al., 1986). Recently, it has been recognized that eosinophils are capable of elaborating cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and IL-5, (Kita et al., 1991; Desreumaux et al., 1993) as well as chemokines such as eotaxin (Ponath et al., 1996), which play a role in inflammatory responses. Eosinophils also synthesize lipid inflammatory mediators, in particular leukotriene C_4 (LTC_4) and platelet-activating factor (PAF). Both mediators contract airway smooth muscle, promote the secretion of mucus, alter vascular permeability and elicit eosinophil and neutrophil infiltration (Lewis and Austen, 1984; Lee et al., 1984).

1.2.3. Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (PMNs) represent 50 to 60% of the total circulating leukocytes in the normal adult human and constitute the "first line of defence" against infectious agents or "non-self" substances that penetrate the body's physical barriers. Their targets include bacteria, fungi, protozoa, viruses, and tumor cells. The neutrophils are spent in three environments, including bone marrow, blood

and tissues. Bone marrow is the site of the important processes of the proliferation and terminal maturation of neutrophilic granulocytes before being released to the circulation (Bainton, 1999).

Neutrophils are terminally differentiated cells rich in cytoplasmic granules and containing a lobulated chromatin-dense nucleus with no nucleolus; the nucleus of the mature neutrophil is segmented into three to five interconnected lobes. The bone marrow of a normal healthy adult produces more than 10^{11} neutrophils per day and more than 10^{12} per day in settings of acute inflammation. Upon release from the bone marrow to the circulation, the cells are in a non-activated state and have a half-life of only 4 to10 h before marginating along the vessel walls and entering tissue pools, where they survive for 1 to 2 days. Cells of the circulating and marginated pools can exchange with each other. Senescent neutrophils are thought to undergo apoptosis (programmed cell death) prior to removal by tissue macrophages (Savill et al., 1993). The rate of neutrophil egress from the bone marrow is controlled by several factors, such as infection, stress and also by two colony-stimulating factors, granulocyte-colony stimulating factor (G-CSF) and GM-CSF. Both factors direct the production and differentiation of bone marrow progenitor cells. The rate of the neutrophil differentiation can increase as much as 10-fold during state of stress and infection (Cannistra and Griffin, 1988).

1.2.3.1. PMN granules

The initial classification into two major types of neutrophil granules was based on their content in myeloperoxidase. However, the granules can be subdivided into four distinct populations, azurophil, specific, gelatinase, and secretory, as summarized by Borregaard and Cowland (1997). These populations (**table 1.1**) have been identified by cytochemical, immunocytochemical, and cell fractionation procedures.

Azurophil granules are known to contain myeloperoxidase (MPO), an antibacterial enzyme, and other numerous antimicrobial agents, lysozyme, and lysosomalenzymes (Bretz and Baggiolini, 1974). Defensins, azurophil-derived bacterial factors, and bactericidal permeability-increasing protein are bactericidal factors have been found in some azurophil granules (Rice et al., 1987; Gabay et al., 1986; Weiss and Olsson, 1987). In the azurophil granules of human PMNs, cathepsin G, elastase, proteinase-3, and azurocidin are antimicrobial proteins which collectively could be called "serprocidins" and are closely related to serine proteases with microbicidal activity (Gabay et al., 1989). **Specific granules**, peroxidase-negative granules, which by definition do not contain peroxidase, contain lysozyme (Cramer and Breton-Gorius, 1987), lactoferrin (Bretz and Baggiolini, 1974), B_{12}-binding proteins (Kane and Peters, 1975), and others as shown in **table 1.1** (Bainton, 1999). The limiting membrane of specific granules and/or intracellular vesicles serves as a reservoir of receptors and other proteins involved in adherence, signal transduction, and functional activation of microbicidal pathways (Bainton et al., 1987). **Gelatinase granules** are a subgroup of small, peroxidase-negative specific granules. They are defined by their high content of gelatinase (Borregaard and Cowland, 1997). **Secretory granules** were originally discovered as highly mobilizable intracellular vesicles that contain alkaline phosphatase on their luminal surface (Borregaard et al., 1990). Although many studies have assumed that alkaline phosphatase was a plasma membrane marker, it seems instead to be in a cytoplasmic organelle, distinguishable from azurophil-, specific-, and gelatinase-containing granules, which is easily mobilized to the surface(Kobayashi and Robinson, 1991).

Among azurophil granule constituents, MPO is a critical enzyme converting hydrogen peroxide (H_2O_2) to hypochlorous acid. Together with hydrogen peroxide and a halide cofactor, MPO system forms the most effective microbicidal and cytotoxic mechanism of leukocytes. MPO is responsible for the characteristic green color of pus (Klebanoff, 1999).

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Membrane CD63 CD68 V-type H^+-ATPase	**Membrane** CD11b Cd15 antigens CD66 CD67 Cytochrome b_{558} FMLP-R Fibronectin-R G-protein subunit Laminin-R NB-1 antigen 19-kd protein 155-kd protein Rap1, Rap2 SCAMP Thrombospondin-R TNF-R Urokinase-type plasminogen activator-R VAMP-2 Vitronectin-R	**Membrane** CD11b Cytochrome b_{558} Diacylglycerol-deacylating enzyme FMLP-R SCAMP Urokinase-type plasminogen activator-R VAMP-2 V-type H^+-ATPase	**Membrane** Alkaline phosphatase CR1 Cytochrome b_{558} CD11b CD14 CD16[a] FMLP-R SCAMP Urokinase-type plasminogen activator-R V-type H^+-ATPase VAMP-2 CD10, CD13, CD45[a] C1q-receptor[a] DAF[a]
Matrix Acid β-glycerophosphatase Acid mucopolysaccharide α_1-Antitrypsin α-Mannosidase Azurocidin/CAP37/ heparin-binding protein Bactericidal permeability increasing protein β-Glycerophosphatase β-Glucuronidase Cathepsins Defensins Elastase Lysozyme Myeloperoxidase N-acetyl- β-glucosaminidase Protinase-3 Sialidase Ubiquitin-protein	**Matrix** β_2-Microglobulin Collagenase Gelatinase hCAP-18 Histaminase Heparanase Lactoferrin Lysozyme NAGL Urokinase-type plasminogen activator Sialidase SGP28 Vitamin B_{12}-binding protein	**Matrix** Acetyltransferase β_2-Microglobulin Gelatinase Lysozyme	**Matrix** Plasma proteins (including albumin and tetranectin)

Table 1.1. The content of human neutrophil granules and secretory vesicles

From **Bainton**, Developmental biology of neutrophils and eosinophils. *In Inflammation: Basic Principle and Clinical Correlates, 3rd ed.*, 1999.

1.2.3.2. PMNs in host defence

The major role of neutrophils is to phagocytose and destroy infectious agents or cells, but they also limit the growth of some microbes, thereby buying time for adaptive (specific) immunological responses.

Phagocytosis is a complex process that involves several biochemical steps. Phagocytosis is triggered upon binding of opsonized or unopsonized microorganism through opsonin receptors (for complement fragments and antibodies) or through nonspecific glycosylated receptors that recognize certain lectins on target microorganism (Smith, 1997).

During phagocytosis, cytosolic granules (lysosomes) fuse with the invaginating plasma membrane (around the engulfing microorganism) to form a phagolysosome into which they release their contents, thereby creating a highly toxic microenvironment **(figure 1.2)**. This normally prevents the release of the microbicidal components into the extracellular milieu. However, some targets may be too large to be phagocytosed, resulting in frustrated phagocytosis in which no phagosome is formed, and which may lead to the secretion of the cytosolic granules content into the extracellular milieu, where the targets may be killed (Klebanoff, 1999).

The other microbicidal mechanism is the oxidative mechanism (oxidative burst), so called because of the 50-100 fold increase in O_2 consumption which results in the production of the superoxide anion (O_2^-) and other cytotoxic reactive oxygen species (ROS) and, potentially, reactive nitrogen species (RNS). O_2^- is formed, initially, by the reduction of molecular oxygen by a single electron that originates from NADPH; this process is catalyzed by the combined action of the plasma membrane NADPH oxidase and cytochrome b_{558}. Although O_2^- may contribute to microbial killing, additional more potent ROS are generated from this precursor such

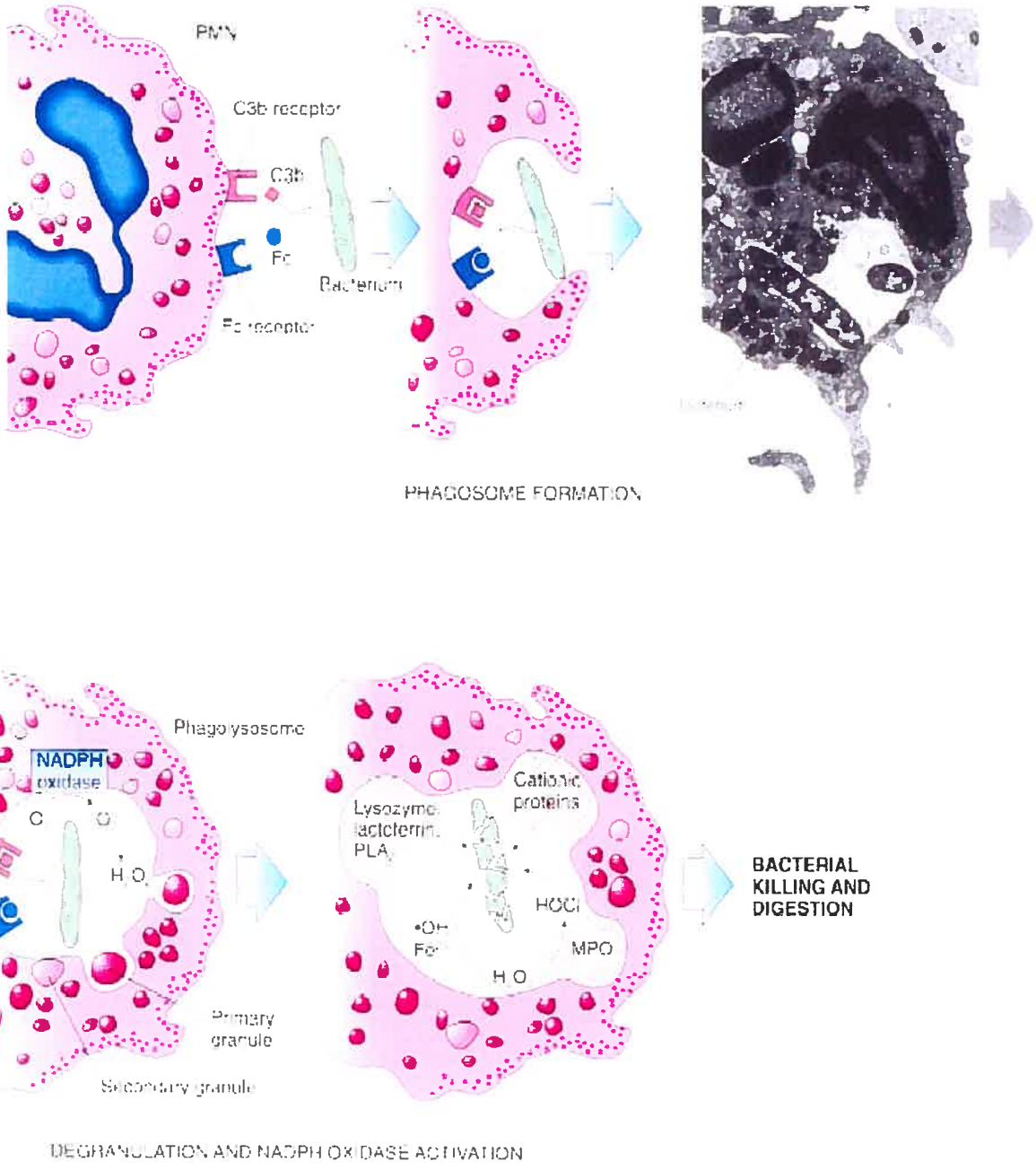

Figure 1.2. Mechanism of polymorphonuclear neutrophil bacterial phagocytosis

From **Fantone and Ward**, *Inflammation. In Essential Pathology, 3rd ed.*, 2001.

as H_2O_2 which is formed by the spontaneous dismutation of O_2^- and/or the catalytic action of superoxide dismutase (SOD) (Smith, 1994). H_2O_2 react with the abundant Cl^- ions taken up from extracellular fluid to generate hypochlorous acid (HOCl), a reaction catalyzed by MPO contained in azurophil granules (Smith, 1997).

Nitric oxide ($NO^\bullet$), which derived from the guanidino nitrogen in the conversion of L-arginine to L-citrulline, may contribute to the microbicidal activity of neutrophils by reacting with ROS to form secondary cytotoxic species such as peroxynitrite ($OONO_2^-$) (McCall et al., 1989).

1.2.3.3. PMNs and host tissue damage

While neutrophils are essential to host defence, they have also been implicated in many pathologic inflammatory conditions and in ischemia-reperfusion injury (Weiss, 1989; Ricevuti et al., 1991).

Host tissue damage may occur through several independent mechanisms. These include: premature activation during migration, extracellular release of toxic products during the killing of microbes or during removal of infected or damaged host cells, and debris as a first step in tissue remodeling, or as a failure to terminate acute inflammatory responses (Smith, 1994; Smith, 1997).

For example, neutrophils have been implicated in the pathology of the adult respiratory distress syndrome (ARDS) because of large influx of these cells into the lung and the associated tissue damage caused by oxidants and hydrolytic enzymes released from activated neutrophils (Martin et al., 1991). Activation of the neutrophils by immune complexes in synovial fluid contributes to the pathology of rheumatoid arthritis (Robinson et al., 1992). Chronic activation of neutrophils may also initiate tumor development because some ROS generated by neutrophils damage deoxyribonucleic acid (DNA) *in vitro* (Weitzman and Gordon, 1990).

1.2.3.4. PMN priming

Neutrophils may show three distinct states: dormant, primed, and activated. Priming means the neutrophil's functional responses (e.g., ROS production, chemotaxis) to a stimulus are amplified by previous exposure of the cell to a priming agent. The priming agent, at low concentration, does not normally cause a noticeable functional response (Guthrie et al., 1984; Bass et al., 1987; Swain et al., 2002). Furthermore, there is also evidence that primed neutrophils can return to a resting state (Kitchen et al., 1996).

The priming agents include bacterial products like lipopolysaccharide (LPS), muramyl dipeptide (MDP) and peptidoglycan, lipotechoic acid and N-formyl-methionyl-leucyl-phenylalanine (fMLP); products from fungi like glucan and mannan; lipid mediators like leukotriene B_4 (LTB_4) and PAF; and cytokines like interleukin-8 (IL-8) and tumor necrosis factor- α (TNF-α) (Pabst, 1994).

Neutrophil priming is thought to play a key role in host defence process, and the regulation of priming may be essential for host survival. A number of studies have suggested that priming may be a good indicator of clinical disease activity. Enhanced neutrophil ROS production has been reported in patients with infection (Solberg et al., 1982). Primed neutrophils have also been found in the blood of trauma patients (Zallen et al., 1999), as well as in patients with chronic inflammatory disease such as rheumatoid arthritis (Eggleton et al., 1995).

1.2.3.5. Mechanisms of PMN extravascular accumulation

Circulating leukocytes can migrate from vessels into tissues under both normal and pathologic circumstances. The neutrophil migration from vasculature occurs by a multistep process **(figure 1.3)**, dictated by sequential activation of adhesive proteins and their ligands on both leukocytes and ECs (von Andrian et al., 1991). In postcapillary venules, the blood flow slows following injury and leukocytes

migrate to the endothelial surface. Initiation of the migration begins with the "capture" of PMNs from flowing blood by the vessel wall, and this is followed by their "rolling" along the vessel wall.

1.2.3.5.1. Capture and rolling of leukocytes

Both the capture and rolling of leukocytes along the vessel wall is due to the reversible binding of transmembrane glycoprotein adhesive molecules called selectins, which are found on both PMNs and ECs. Selectins have three major domains, of which the extracellular lectin domain is involved in ligand binding, an epidermal growth factor-like domain and a varying numbers of short consensus repeat domain (SCR) that keep selectins away from the cell surface (Crockett-Torabi and Fantone, 1995). There are three types of selectins; the ***leukocyte selectin*** (L-selectin) appears to be critical for the rolling process. In addition, there are two endothelial selectins, the ***platelet selectin*** (P-selectin), which is rapidly and transiently expressed on ECs, and the ***endothelial selectin*** (E-selectin), which appears a few hours later. The endothelial selectins are expressed only when appropriate inflammatory stimuli are present and interact with their PMN counterpart, which is a complex glycoprotein called P-selectin glycoprotein ligand-1 (PSGL-1), belonging to the family of sialomucines (McEver and Cummings, 1997; Wagner and Roth, 2000).

1.2.3.5.2. Integrin-mediated firm adhesion

Cytoplasmic domains of bound L-selectin and PSGL-1 are linked to signal transduction pathways that lead to integrin activation in PMNs (Crockett-Torabi and Fantone, 1995). Integrin activation is associated with the next step of the migration process, which is the firm adhesion of the leukocyte on ECs surface **(figure 1.3)**. The

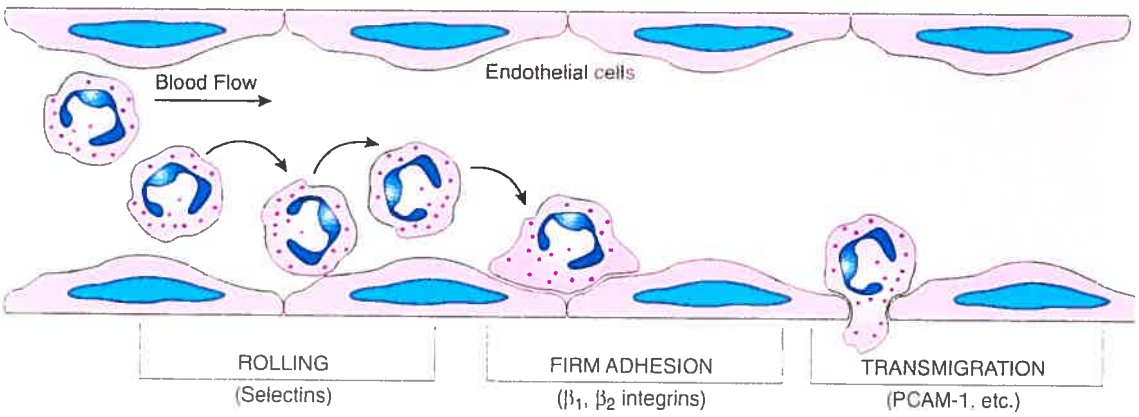

Figure 1.3. Mechanism of leukocyte transmigration
From **Fantone and Ward**, *Inflammation. In Essential Pathology. 3rd ed.*, 2001.

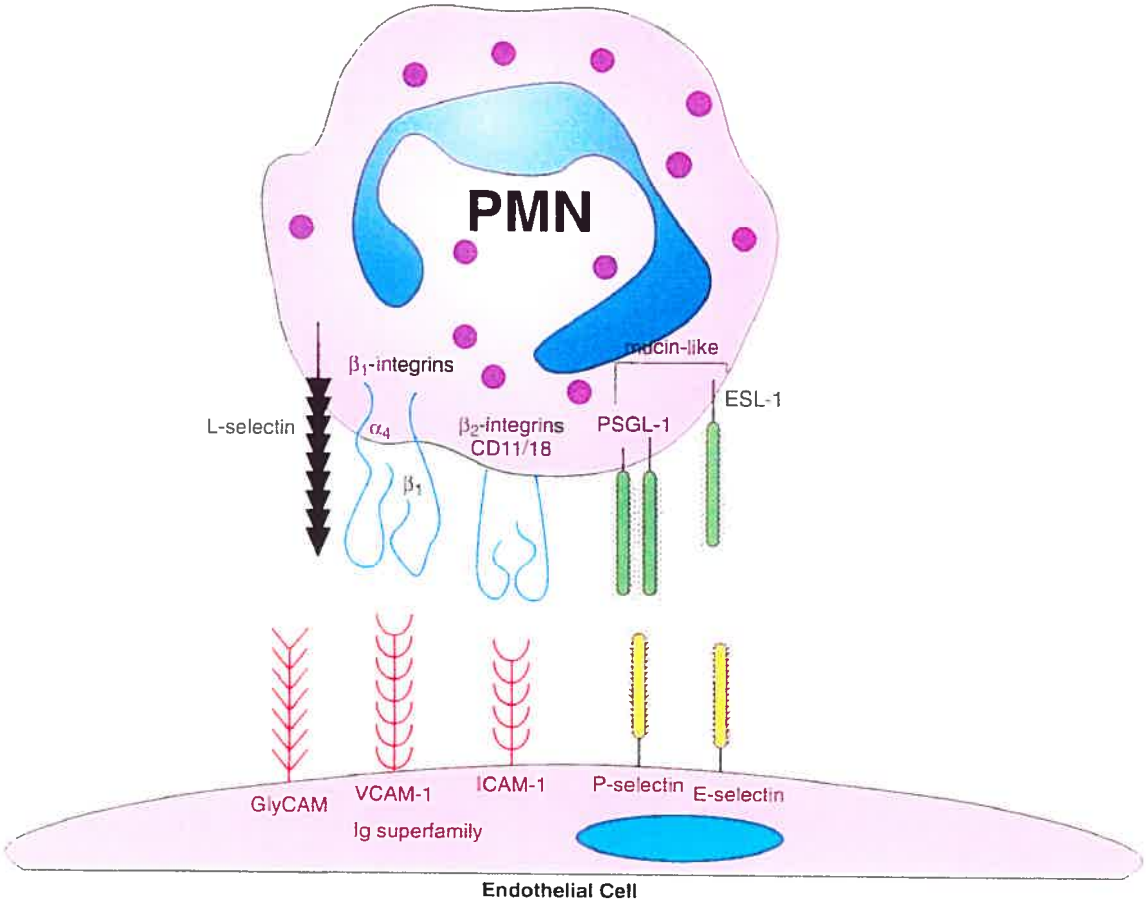

Figure 1.4. Leukocyte and endothelial adhesion molecules
From **Fantone and Ward**, *Inflammation. In Essential Pathology, 3rd ed.*, 2001.

integrins are a group of heterodimeric transmembrane glycoproteins found on PMNs and other hematopoietic cells that mediate cell-cell or cell-extracellular matrix (ECM) adhesion. There are 18 different integrins α-chains and 8 different β-chains which pair together in specific pattern depending on the cell type (Travis et al., 2003). PMN binding to activated endothelium is mediated mainly by two integrins: macrophage antigen-1 (MAC-1), also known as $\alpha_M\beta_2$ or CD11b/CD18, and lymphocyte-associated function antigen-1 (LFA-1), also known as $\alpha_L\beta_2$ or CD11a/CD18 **(figure 1.4)**. The importance of the integrin-mediated adhesion process is illustrated in patients with leukocyte adhesion deficiency syndrome (LAD-1), characterized by the absence of the β_2 (CD18) chain, which show severe recurrent, and life-threatening bacterial infections secondary to an inability to locally recruit PMN (Anderson and Springer, 1987).

The major counterligands of integrins belong to the *immunoglobulin (Ig)-liked superfamily of adhesive molecules,* characterized by the presence of one or more immunoglobulin homology regions in their structure. *Intercellular adhesion molecule-1* (ICAM-1), a member of this superfamily, is an important complementary endothelial ligand for MAC-1, exhibiting low constitutive presentation on EC membrane which is markedly induced by exposure of ECs to inflammatory cytokines (Iigo et al., 1997). LFA-1 can also bind to ICAM-1, but it has higher affinity to a related protein, ICAM-2, a ligand to which MAC-1 binds with low affinity (Wagner and Roth, 2000). Vascular cell adhesion molecule-1 (VCAM-1) is also an Ig-like molecule on ECs, but it binds selectively to β_1-integrins such as $\alpha_4\beta_1$ integrin also called very late antigen-4 (VLA-4), which is critical for the migration of monocytes and eosinophils. However, VLA-4 has also been identified in both activated human and rat PMNs and may mediate VCAM-1-dependent adhesion of PMNs to endothelium *in vitro* (Davenpeck et al., 1998). A summary of integrin, selectin and Ig-like superfamily of adhesion molecules is found in **table 2.1** (Horton, 1996).

Family	Homology region in receptor	Examples	CD no. (cluster of differentiation)	Ligands	Recognition motif in ligand/counter-receptor	Extracellular matrix components with shared homology
Integrin	PEGG (all β chains) I domain (CD11,α1α2)	gpIIbIIIa LAF-1	CD41/61 CD11/18	Blood proteins. Cell counter-receptors (e.g.ICAM)	RGD, KQAGDV	Collagen VI, von Wilebrand factor protein (Integrin I domain)
		αvβ3 α2β1 α4β1	CD51/61 CD49b/29 CD49d/29	Matrix, blood proteins Collagen Fibrioectin	RGD DGEA EILDV	
Ig superfamily	Ig fold	ICAMS, VCAM N-CAM CD2	CD54 etc.	Heterophylic interaction Homophylic LFA-3 counter-receptor	Multiple KYSFNYDGSE	Perlecan (Ig fold) Fibronectin, tenascin, thrombospondin (N-CAM type III repeat)
Selectins	C-type lectin EGF repeat Complement regulatory protein domain	L-, P- and E-selectin CD62	CD62	Glycam-1, PSGL-1, CD34 etc.	Sialyl Le^x (CD15) etc.	Aggrecan, versican (lectin), Laminin, tenascin, thrombospondin, aggrecan versican (EFG repeat)
Mucins	Mucin side chain	Leukosialin	CD43 CD34	Selectins		Muc-1
CD36 Family		Platelet gpIV	CD36	Thrombospondin, collagen	SVTCG (for thrombospondin)	Aggrecan, versican, link protein
CD44	Hyaluronidate- binding site		CD44	Hyaluronic acid, etc.		Aggrecan, versican, link protein
Cadherins	LDRE repeat (IIO-AA module)	E-, N-cadherin		Homophylic	HAV	

Table 1.2. Classes of cell adhesion receptors and their ligands

Modified from **Horton,** *Molecular biology of cell adhesion molecules,* 1996.

1.2.3.5.3. PMN diapedesis

Subsequent to the firm adhesion, diapedesis of the PMN at endothelial cells junctions occur in a platelet endothelial cell adhesion molecule-1 (PECAM-1) or CD31-dependent manner. PECAM-1 is found on PMNs, platelets and ECs, serve as its own ligand and form homodimers with molecules on opposing cells. PECAM-1 is hypothesized to be a homing receptor to localize the transendothelial route for the migrating PMN. Treatment of the PMN or endothelial monolayers with an antibody to PECAM-1 blocks transmigration *in vitro* (Muller et al., 1993).

1.2.3.5.4. PMN migration in the extravascular space

PMNs possess proteases capable of digesting collagen, laminin, and other extracellular components present in the vascular wall. Adhesion and migration are accompanied by the release of PMN-derived proteases (Hanlon et al., 1991). However, Mackeral et al. found that protease inhibitors are ineffective in stopping PMN migration through intact endothelial monolayers and basement membranes *in vitro.* Thus, the requirement for protease release is uncertain (Mackarel et al., 1999).

Interaction of PMN β_1–integrin with ECM proteins is important for transit of PMNs through the extravascular milieu. For example, PMN migration through the lung or synovial fibroblast barriers has been shown to require not only CD18 (β_2) but also VLA-4, VLA-5 ($\alpha_5\beta_1$), VLA-6 ($\alpha_6\beta_1$) and VLA-9 ($\alpha_9\beta_1$), which are the most highly expressed β_1–integrins in PMN, to bind the ECM components (Shang and Issekutz, 1997).

PECAM-1 is also involved in the extravascular transit of leukocytes. But unlike the homotypic interaction between PECAM molecules that mediate homing and diapedesis, migration through the subendothelial environment requires

heterophilic binding of leukocytic PECAM-1 to an unidentified ligand (Wakelin et al., 1996).

1.3. Inflammatory mediators

When leukocytes reach the site of infection or inflammation, they release mediators which control leukocyte accumulation and activation of neighboring cells.

Inflammatory mediators are soluble molecules that act locally at the site of tissue damage and infection, and at more distant sites. These mediators may be low molecular weight proteins like cytokines and chemokines, hormones like neuroendocrine hormones, growth hormone and prolactin, or bioactive lipids like arachidonic acid and their metabolites (Smith, 1994).

Inflammatory mediators can be divided into exogenous and endogenous mediators. **Exogenous mediators,** such as bacterial products and toxins, evoke powerful responses. For example, LPS can trigger complement activation, resulting in the formation of the anaphylatoxins C3a and C5a which cause vasodilation and increase vascular permeability. LPS also activates the Hageman factor, leading to activation of the coagulation and fibrinolytic pathways as well as the kinin system. In addition, it elicits T cell proliferation, and has been described as a superantigen for T cells. **Endogenous mediators** of inflammation are produced from the immune system (innate and adaptive) itself, as well as from plasma. For example, they can be derived from molecules that are normally present in the plasma in an inactive form, such as peptide fragments of the complement, coagulation, and kinin systems. Mediators of inflammatory responses are also synthesized by a number of cell types that contain them as preformed molecules within storage granules, e.g. histamine, or which can be rapidly synthesized when they are required, for example, metabolites of arachidonic acid such as LTB_4, LTC_4 and leukotriene D_4 (LTD_4).

Inflammatory mediators can also be classified into early and late phase mediators (White, 1999). **Early phase mediators** are important in acute inflammation and include histamine, serotonin and other vasoactive substances. They are produced by mast cells and platelets. In addition, lipid mediators, chemoatractants (e.g. C5a) and cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-α belong to the early phase mediators. **Late phase mediators** are found 6-12 hours after initiation of inflammation. They are responsible for the regulation of vascular events. The later vascular events are mediated, at least in part, by products of arachidonic acid such as prostaglandin E_2 (PGE_2), LTD_4 and LTC_4.

Mediators which accumulate at local inflammatory sites such as skin blisters are somewhat different from those released following intravenous endotoxin. Mediators detected in blister fluid within 3 to 5 h of the inflammatory response include LTB_4, C5a, IL-8 and IL-6. In contrast, IL-1β, GM-CSF, and TNF-α are not detected until after 8 hr in the blister. After i.v. endotoxemia, serum TNF reaches a peak level in 90-120 minutes and subsequently declines, to become undetectable within 4-6 h (Wang and Tracey, 1999).

1.3.1. Histamine and Serotonin

They are the most important vasoactive mediators stored in mast cell and basophil granules; both of them are also present in human platelets. **Histamine** is largely complexed to mucopolysaccharides such as heparin. Histamine has diverse biological functions including local dilatation of small vessels, widespread arteriolar dilatation, local increased vascular permeability through ECs contraction, contraction of nonvascular smooth muscle, chemotaxis for eosinophils, and blockade of T lymphocyte function (Pearce, 1991). **Serotonin** is also capable of increasing vascular permeability, dilating capillaries and producing contraction of nonvascular smooth muscle.

1.3.2. Cytokines and Chemokines

Cytokines are soluble polypeptides that mediate and regulate various aspects of inflammation. The most important pro-adhesive cytokines that are present during most inflammatory responses are TNF-α and IL-1. The primary cellular sources of these mediators are macrophages and monocytes, and LPS is perhaps their most important inducer (Di Girolamo et al., 1997). Neutrophils also synthesize and secrete small amount of some cytokines including IL-1, IL-6, TNF-α, and GM-CSF, which may act in an autocrine or paracrine manner (Lloyd and Oppenheim, 1992). PMNs respond to TNF-α by activating and expressing integrins, producing PAF and other mediators, and releasing granule contents. Likewise, ECs can respond to TNF-α exposure by mobilizing E-selectin, up-regulating ICAM-1 and activating procoagulant pathways (Burke-Gaffney and Hellewell, 1996). It was found that during inflammation and endotoxemia, PMNs release from their membrane a soluble TNF-α receptor (p55) that can bind and effectively inactivate circulating TNF-α (Wagner and Roth, 2000). IL-1 exerts similar physiological effects (Scholz et al., 1996). Thus, the early appearance of TNF-α and IL-1 in plasma during inflammation is likely critical for the capture and firm adhesion of PMNs to vascular endothelium.

Chemokines are a superfamily of low molecular weight cytokines with selective chemotactic properties. They have been subdivided into families on the basis of the relative position of their cysteine residues. The largest families are α-chemokine (cysteine-X amino acid-cysteine or CXC) and β-chemokine (cysteine-cysteine or CC). IL-8, a member of the CXC superfamily, as well as macrophage inflammatory protein-1 alpha (MIP-1α), macrophage chemoattractant protein-1,-2, and -3 (MCP-1,-2,-3) and RANTES, which are members of the C-C superfamily, function primarily as chemoattractant molecules for macrophage and circulating leukocytes (Luster, 1998). Chemokines were first identified *in vitro* and initially thought to be produced by activated macrophages and monocytes. However under the proper conditions, their production and release has been elicited from neutrophils, endothelium, and a variety of parenchymal cells *in vitro*. For example, activation of

neutrophils with particulate stimuli such as zymosan induces the generation of the CXC chemokine IL-8 (Au et al., 1998). Further studies suggest that these cells produce chemokines in animal models (Rovai et al., 1998). Chemokines are thought to provide the signals that convert the low-affinity, selectin-mediated interaction into the higher-affinity, integrin-mediated interaction that lead to extravasation of leukocytes (Luster, 1998).

Rodents do not have an IL-8 analog and, instead, possess cytokine-induced neutrophil chemoatracttants (CINCs). CINC-1, MIP-2, CINC-2α and CINC-2β are released from LPS-stimulated rat macrophages *in vitro* and possess similar ability to elicit chemotaxis and degranulation of PMNs (Nakagawa et al., 1996). Rodents respond to human IL-8 and the i.v. administration of IL-8 inhibits PMN migration to extravascular sites of inflammation in rabbits (Ley et al., 1993). Another *in vivo* study showed that eosinophil accumulation in response to IL-1β was significantly suppressed in rats treated with an anti-human IL-8 mAb DM/C7 (Sanz et al., 1995).

Although LPS, TNF-α, and IL-1 are not chemoatactic for PMNs, their exposure to cultured cells can induce the production of chemoattractants such as IL-8 and PAF (Burns et al., 1997).

1.3.3. Products of the complement system

The complement system is a potent mechanism for initiating and amplifying inflammation. This is mediated through activated fragments of complement components. C3a, C4a and C5a are proteolytic products of the serine proteases of the complement system. These anaphylatoxins are polypeptides containing approximately 75 amino acid residues. The C-terminal arginine in the molecule of C3a is of fundamental importance for its biological activity, whereas removal of the C-terminal arginine of C5a ($C5a_{desArg}$) only decreases its biological activity.

The anaphylatoxins have powerful effects on blood vessel walls, causing contraction of smooth muscle and an increase in vascular permeability. C5a is extremely potent at stimulating neutrophil chemotaxis, adherence, respiratory burst generation and degranulation (Kohl and Bitter-Suermann, 1993). Ligation of the neutrophil C5a receptor at dermal skin sites is followed by mobilization of membrane arachidonic acid which is primarily metabolized to LTB_4, another potent chemoattractant for neutrophils and monocytes, as observed in rabbits (Marleau et al., 1999).

1.3.4. Lipid mediators

Lipid mediators are a class of lipid molecules derived from the metabolism of membrane phospholipids. These include PAF and the vast array of eicosanoids, which are derived from the metabolism of arachidonic acid **(figure 1.5)**.

1.3.4.1. Leukocyte phospholipases

During inflammation, cellular phospholipases, especially phospholipase A_2 (PLA_2) and C, are activated and degrade phospholipids to induce mobilization of fatty acids (FAs) from the membrane lipid pool for the synthesis of lipid mediators at the site of cellular damage or inflammation (Heller et al., 1998). The FAs composing the normal inflammatory cell membrane are saturated FAs, monounsaturated FAs, and polyunsaturated FAs, the latter composed of omega-3 and omega-6 FAs, which includes arachidonic acid (AA).

AA, the mother substance of pro-inflammatory eicosanoids, has a short half-life and can be metabolized by two major pathways, the cyclo-oxygenase (COX) and lipoxygenase (LO) pathways. The COX pathway leads to the production of prostaglandin (PG) H_2 which can be further metabolized to PGE_2, PGI_2 (prostacyclin) and thromboxane A_2 (TXA_2). These eicosanoids have potent local effects in the

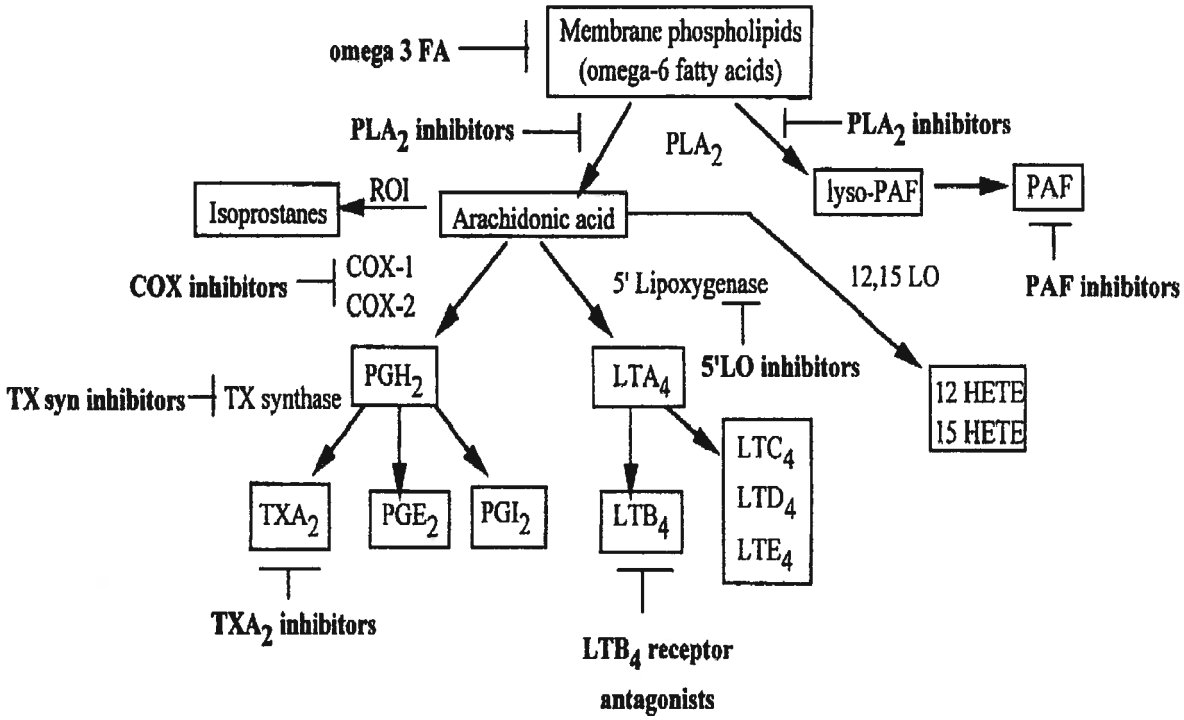

Figure 1.5. Biosynthesis of lipid inflammatory mediators

From **Bulger and Maier** *Crit Care Med.*, 28(4 Suppl):N27-36, 2000.

manifestation of inflammation. LO pathways lead to the production of leukotriene A_4 (LTA_4) which is further metabolized into a number of leukotrienes: LTB_4, LTC_4, LTD_4 and leukotriene E_4 (LTE_4), which have potent pro-inflammatory effects. Phospholipids can also be metabolized by induction of PLA_2 activity to lyso-PAF which is then converted to PAF (Braquet et al., 1991; Bulger and Maier, 2000).

PLA_2 is a key enzyme in the production of lipid mediators from membrane phospholipids as it catalyzes the hydrolysis of the sn-2 position of membrane phospholipids to release unsaturated fatty acids. PLA_2 enzymes fall into four broad categories: the group IV cytosolic PLA_2 ($cPLA_2$) calcium (Ca^{2+})-dependent enzymes; the low molecular weight, secretory enzymes ($sPLA_2$), including many groups like IB, IIA, IIC, IID, IIE, IIF, III, V, X and XII; the Ca^{2+}-independent, group VI enzymes; and the selective acetyl hydrolases of PAF, groups VII and VIII PLA_2 (Dennis, 1997; Diaz and Arm, 2003). $cPLA_2$ is present in most cells and tissues including PMN and monocytes, where it is critical to the production of PAF and AA, thereby playing an important role in both rapid and prolonged cellular response occurring during inflammatory processes. PLA_2 activity is increased in response to norepinephrine, adenosine, bradykinin, PAF, TNF, and IL-1β stimulation (Anderson et al., 1994). In summary PLA_2 groups and their properties are found in **table 1.3** (Six and Dennis, 2000).

Phosphatidylinositol-bisphosphate is a major phospholipid in eukaryotic cells that can be hydrolyzed by PLC to diacylglycerol which is further metabolized to AA by diacylglycerol lipase (Fantone and Ward, 2001).

1.3.4.2. Prostaglandins (PGs)

PGs have been detected in almost all experimental models of inflammation and clinical inflammatory conditions. For instance, the major PGs found in the synovial fluid of patients with arthritis are PGE_2 and PGI_2. In leukocytes, they are synthesized *de novo* from membrane-released AA when cells are activated by

Group		Initial/common sources	Alternate names employed	Size (kDa)	Ca^{2+} effects	Characteristics
IV	A	Human U937 cells/ platelets RAW 264.7/rat kidney	$cPLA_2\alpha$	85	< μM; membrane translocation	C2 domain, α/β-hydrolase regulatory phosphorylation
	B	Human pancreas/liver heart/brain	$cPLA_2\beta$	114	< μM; membrane translocation	C2 domain, α/β-hydrolase
	C	Human heart/skeletal muscle	$cPLA_2\gamma$	61	None	Prenylated, α/β-hydrolase
VI	A-1	$P388D_1$ macrophages, CHO	$iPLA_2$ or $iPLA_2$-A	84-85	None	Short splice, 8 ankyrin repeats
	A-2	Human B-lymphocytes, testis	$iPLA_2$-B	88-90	None	Long splice, 7 ankyrin repeats
	B	Human heart/skeletal muscle	$iPLA_2\gamma$ or $iPLA_2$-2	88	None	membrane-bound
VII	A	Human/mouse/porcine bovine plasma	PAF-AH	45	None	Secreted, α/β-hydrolase Ser/His/Asp triad in VHA and B
	B	Human/bovine liver/kidney	PAF-AH (II)	40	None	Intracellular, myristoylated
VIII	A	Human brain	PAF-AHIb α_1 (subunit of trimer)	26	None	Intracellular, G protein fold Ser/His/Asp triad, dimeric
	B	Human brain	PAF-AHIb α_2 (subunit of trimer)	26	None	Same as VIII A; active as hetreodimer or homodimer

Table 1.3. Superfamily of PLA_2 enzymes

From **Six and Dennis.** *Biochim. Biophys. Acta*, 1488, 1-19, 2000.

mechanical trauma or by specific cytokines, growth factors, or other stimuli, and act as autocrine and paracrine mediators.

1.3.4.2.1. Biosynthesis of PGs in leukocytes

At the endoplasmic reticulum and nuclear membrane, AA released by $cPLA_2$ is presented to prostaglandin H synthase (PGHS), also known as COX for cyclo-oxygenase, to form an intermediate prostaglandin, PGH_2 (Funk, 2001). PGHS exists as two isoforms referred to as PGHS-1 (COX-1) and PGHS-2 (COX-2) (Smith et al., 2000). In simplistic terms, COX-1 is the enzyme responsible for basal, constitutive prostaglandin synthesis, whereas COX-2 is an inducible form of cyclooxygenase stimulated in the setting of acute inflammation in response to various cytokines, endotoxin and mitogens. Both enzymes catalyze the insertion of molecular oxygen into arachidonic acid at C11 and C15 result in formation of PGG_2, and then, catalyze the reduction of PGG_2 to PGH_2 which is further metabolized by PG synthases into PGD_2, PGE_2, and PGF_2. The cellular expression pattern of each synthase may profoundly influence the type of PG produced by particular cell. For example, platelets produce predominantly TXA_2; ECs produce PGI_2 and mast cell produce PGD_2 (Griffiths, 1999). In addition, monocytes produce mainly PGE_2 through human peripheral blood mononuclear cells produce a factor (MCF) (Robinson et al., 1979).

The effect of PGE_2 results from its binding to PGE_2 receptor subtypes EP2 and EP4, which stimulate production of the second messenger cAMP (Yoshikai, 2001).

1.3.4.2.2. Role of PGs in inflammation

PGs generated by the PLA_2/COX pathway play a dichotomous role in acute inflammation. PGE_2 at physiological concentrations may be pro-inflammatory during the early phase of acute inflammation, while other PGs may regulate the resolution of

acute inflammation. PGE_2 for instance, produced by macrophages (and ECs), contribute to the local vasodilation. PGE_2 also downregulate macrophage response to cytokine stimulation (Fink, 1998). PGE_2 concentration at inflammatory sites may determine whether its effect is pro- or anti-inflammatory; high concentrations of PGE_2 may rather suppress the increased vascular permeability elicited by histamine, bradykinin, and C3a, thus preventing inflammatory reaction (Yoshikai, 2001).

In dermal inflammation, application of PGE_2 or PGI_2 alone increases blood flow, but does not promote oedema. However, co-injection of either agent with chemotactic mediators such as LTB_4, IL-8, PAF-α or bradykinin causes profound plasma protein leakage (Wedmore and Williams, 1981). PGs also modulate leukocyte function. For instance, PGE_2, PGD_2 and PGI_2 all inhibit PMN activation *in vitro*, as measured by chemotaxis and superoxide production (Wheeldon and Vardey, 1993). The inhibitory effect is associated with increase in cAMP formation. PGE_2 is also a potent inhibitor of monocyte activation as it inhibits TNF production *in vitro*. *In vivo*, administration of COX-inhibitors enhances the release of TNF-α in response to administration of LPS (Pettipher and Wimberly, 1994).

In addition, PGs may regulate lymphocyte function via modulation of the EP2 and EP4 receptor expression during acute inflammation (Griffiths, 1999). For example, PGE_2 inhibits T-cell proliferation, cytokine production, and T-cell migration through a cAMP-mediated mechanism, involving EP2 or EP4 receptors.

1.3.4.3. Leukotrienes

The term "leukotriene" (LTs) refers to the cellular source (leukocytes are one of the major sources) as well as the conjugated triene that characterizes their structure (Samuelsson et al., 1979). Leukotrienes are formed from the catalytic oxygenation of 20-carbon unsaturated fatty acids, mainly arachidonic acid. Leukotrienes can be divided into two different classes, based upon their chemical structure and biological activity;

- The cysteinyl leukotrienes (CysLTs), namely LTC_4, LTD_4, and LTE_4, containing different amino acid residues
- The dihydroxy-derivative leukotriene B_4 (LTB_4)

Briefly, CysLTs were originally described as the slow reacting substance of anaphylaxis (SRS-A) because of its slow and sustained smooth muscle contracting abilities. LTB_4 (5S,12R-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid) was isolated and purified in 1978 from neutrophils upon activation by calcium ionophores such as A23187 (Borgeat and Samuelsson, 1979a), and characterized as a major AA metabolite in rabbit polymorphonuclear leukocytes (Borgeat and Samuelsson, 1979b).

1.3.4.3.1. Leukotriene biosynthesis in leukocytes

The limiting step in the generation of LTs is the enzymatic release of AA from the cell membrane phospholipids by PLA_2. The first committed step in LTs formation from AA is through the enzymatic action of 5-lipoxygenase (5-LO) that requires cell activation and influx of intracellular and extracellular calcium (Wong et al., 1991). Stimulation of neutrophils results in the translocation of 5-LO, a cytosolic or nuclear soluble enzyme, to the nuclear envelope where it colocalizes with the 5-lipoxygenase activating protein (FLAP). FLAP is an integral membrane protein necessary for leukotriene synthesis, initially thought to act as a docking protein for 5-LO. Its role appears to be more complex, and may include efficient conversion of AA to leukotrienes by handing the substrate to 5-LO (Abramovitz et al., 1993).

5-LO catalyzes the insertion of reactive oxygen into arachidonic acid at C-5 leading to formation of 5-hydroperoxyeicosatetranoic acid (5-HPETE). This unstable lipid hydroperoxide may be either reduced by peroxidase to the hydroxy metabolite (5-HETE) or can be stereospecifically dehydrated by a second 5-LO catalyzed step to eicosatetraenoic acid (LTA_4) **(figure 1.6)** (Sirois and Borgeat, 1988). LTA_4 undergoes transformation by one or more metabolic pathways depending on the cellular context; hydrolysis of LTA_4 by the LTA_4 hydrolase occurs in the cytoplasm,

and potentially in the nucleus, yielding LTB_4, a potent neutrophil chemoattractant (Peters-Golden and Brock, 2001). LTA_4 conjugation with glutathione at the nuclear envelope yield LTC_4, a step catalyzed by LTC_4 synthase. LTC_4 can be metabolized by a γ-glutamyltransferase to LTD_4, a cysteinyl-glycyl conjugate that in turn can be further metabolized by ubiquitous peptidases to the cysteinyl containing LTE_4.

Neutrophils have been shown to synthesize large amounts of LTB_4, possessing 5-LO and LTA_4 hydrolase, whereas eosinophils and mast cells preferentially synthesize LTC_4, according to the presence of 5-LO and LTC_4 synthase within these cells. However, the co-incubation of different cell types was shown to greatly influence, both, the quantitative and the qualitative profile of the leukotrienes produced (Maclouf and Murphy, 1988). These changes were shown to depend on the ability of neutrophils to export the unstable intermediate LTA_4 to neighboring cells, which may not have the primary oxidative enzyme, namely the 5-LO, but possess either LTA_4 hydrolase (erythrocytes) or LTC_4 synthase (platelets, endothelial cells and vascular smooth muscle cells).

1.3.4.3.2. Leukotriene receptors and signaling pathways

Leukotrienes act at distinct cell surface G protein-coupled receptors; the receptors for LTB_4 are called BLT receptors and the CysLTs receptors are designated CysLT receptors. BLT receptors are highly expressed in human leukocytes (Goldman and Goetzl, 1984). In neutrophils, two BLT receptors subtypes have been identified. Indeed, Yokomizo et al. (2000) identified the second leukotriene B_4 receptor, BLT_2, from humans and mice and found that the BLT_2 gene closely located to the BLT_1 gene in both humans and mice. In addition, the BLT_1 receptor may show two different affinity states which have been linked to different functional responses. The high-affinity receptor state (BLT_1) transduces the functions of chemotaxis and adhesion, whereas the low-affinity receptor state mediates the secretion of granule content and superoxide generation. In addition, LTB_4 binds to a nuclear receptor, PPAR-α (peroxisome proliferator-activated receptor-α), which enhances the

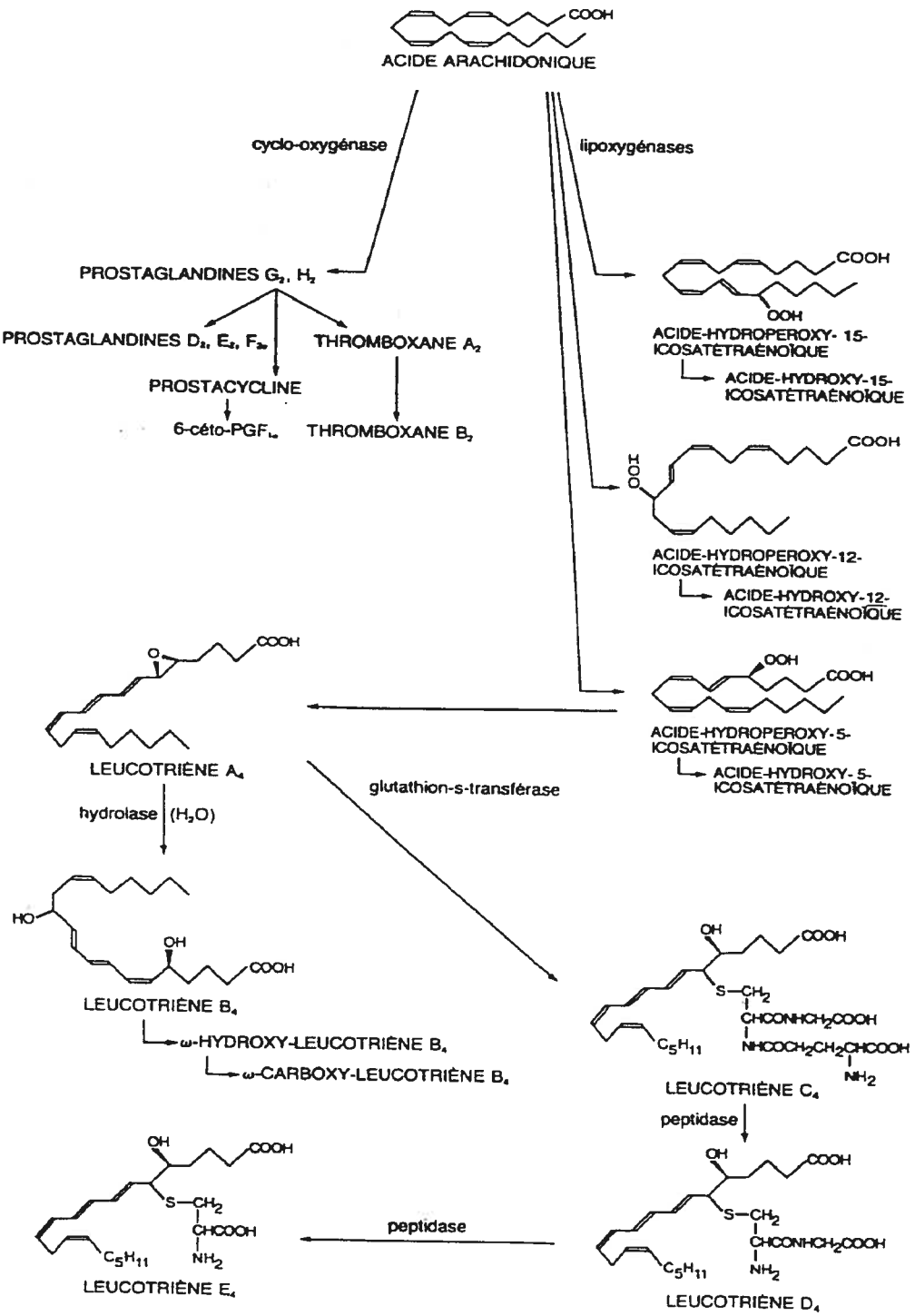

Figure 1.6 . Metabolism of arachidonic acid by 5-LO enzyme and biosynthesis of leukotrienes

From **Sirois and Borgeat**, 1988.

and potentially in the nucleus, yielding LTB_4, a potent neutrophil chemoattractant (Peters-Golden and Brock, 2001). LTA_4 conjugation with glutathione at the nuclear envelope yield LTC_4, a step catalyzed by LTC_4 synthase. LTC_4 can be metabolized by a γ-glutamyltransferase to LTD_4, a cysteinyl-glycyl conjugate that in turn can be further metabolized by ubiquitous peptidases to the cysteinyl containing LTE_4.

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inactivation and plays a role in LTB_4 clearance during inflammation (Devchand et al., 1996).

On the basis of primarily functional studies in smooth muscle, the CysLTs receptors are divided into two subtypes; $CysLT_1$ mediates the action of LTD_4 and LTE_4, whereas $CysLT_2$ interacts with LTC_4 (Penrose et al., 1999).

Binding of leukotrienes to its receptors induce intracellular signaling by activation of PLC leading to production of inositol triphosphate (IP_3) and diacyglycerol (DAG) from the plasma membrane. IP_3 causes the release of Ca^{2+} from the endoplasmic reticulum and produces IP_4 which results in Ca^{2+} influx from the extracellular space. DAG, together with intracellular Ca^{2+}, results in the activation of protein kinase C (PKC) which plays an important role in the activation of neutrophils, macrophages, and lymphocytes (Bulger and Maier, 2000).

LTB_4 receptors undergo homologous desensitization, a control mechanism that potentially limits its signaling actions. Continuous exposure of circulating PMNs to a steady-state concentration of LTB_4 resulted in a homologous desensitization to LTB_4-induced neutropenia (Sirois et al., 1983; Marleau et al., 1993).

1.3.4.3.3. Role of leukotrienes in inflammation

1.3.4.3.3.1. Pathophysiological effects

LTB_4 stimulates neutrophil chemokinesis and chemotaxis, enhances neutrophil-endothelial interaction, and stimulates neutrophil activation, leading to degranulation and release of inflammatory mediators including PAF and LTB_4 itself, lysosomal enzymes and superoxide (Palmblad et al., 1981). LTB_4 also stimulates IL-6 production by human monocytes as well as the production of other cytokines such as GM-CSF, IL-1 and TNF (Brach et al., 1992; Stankova and Rola-Pleszczynski, 1992).

The chemotactic activity of LTB_4 has been shown *in vivo* following injection in rat peritoneal cavity, resulting in substantial accumulation of macrophages and PMNs (Smith et al., 1980). Intradermal application of LTB_4 or injection into the interior chamber of rabbit eye showed accumulation of PMNs at the site of injection (O'Flaherty et al., 1981). Intratracheal instillation of LTB_4 in humans increased the total number of neutrophils recovered by bronchoalveolar lavage (BAL) (Martin et al., 1989).

In vivo, LTB_4 has the potential to increase vascular permeability, possibly as a result of PMNs activation (Bray et al., 1981; Bjork et al., 1982). *In vitro,* LTB_4 increases 5-LO activation in human PMNs in an autocrine fashion (McDonald et al., 1994).

The CysLTs are involved in the regulation of vascular tone and permeability. The intratracheal administration of LTC_4 and LTD_4 results in a significant increase in pulmonary capillary permeability leading to pulmonary oedema (Woodward et al., 1983).

1.3.4.3.3.2. Identification of leukotrienes at lesional sites

Leukotrienes are present in the biologic fluids of inflammatory lesions in several disease states. LTB_4 is found in the rectal fluid of patients with inflammatory bowel disease, the skin lesions of patients with psoriasis, and in the synovial fluid of patient with rheumatoid arthritis (Davidson et al., 1983; Tsuji et al., 1999) and gout (Rae et al., 1982; Penrose et al., 1999). In patients with rheumatoid arthritis, the elevated levels of LTB_4 in the synovial fluid correlate with the increase number of synovial fluid leukocytes, immune complexes, and rheumatoid factor (Ahmadzadeh et al., 1991). CysLTs are present in serum, bronchial lavage fluid, and urine of patients with bronchial asthma (Wardlaw et al., 1989) and others diseases including allergic rhinitis and conjunctivitis (Bisgaard et al., 1985).

Supporting a role of LTs in clinical inflammatory conditions, 5-LO inhibitors improved symptoms in many disease disorders such as ulcerative colitis, psoriasis (Black et al., 1990), and asthma (Israel et al., 1993).

1.3.4.4. Platelet-activating factor

1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) is a potent mediator produced by a number of inflammatory cells including platelets, PMNs, macrophages, ECs and epithelial cells, which present specific receptors for PAF, functioning in both a paracrine and autocrine fashion. As with eicosanoids, PAF is synthesized when inflammatory cells are activated, and its synthesis and subsequent secretion are closely regulated. For example, PAF, as LTB_4, is synthesized in large amounts upon activation of PMNs by calcium ionophores (A23187) or by phagocytic stimuli such as zymosan or inflammatory microcrystals (Borgeat and Samuelsson, 1979a; Riches et al., 1990; McDonald et al., 1994).

1.3.4.4.1. Platelet-activating factor synthesis in leukocytes

PAF may be synthesized through either the *remodeling* or *de novo* enzymatic pathways (**figure 1.7**). PAF synthesis through the remodeling pathway is initiated by activation of a $cPLA_2$ enzyme that hydrolyzes 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine to 1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine (lyso-PAF) and arachidonate. The remodeling pathway appears to be the principle mechanism of the synthesis of PAF in stimulated neutrophils, monocytes and ECs in various inflammatory and allergic disorders. The second and final step is the acetylation of lyso-PAF by acetyl-CoA:lyso-PAF acetyltransferase (lyso-PAF AcT) to form PAF. This step is also regulated since lyso-PAF AcT is activated by phosphorylation (Peplow, 1999; Prescott et al., 1999). The formation of PAF via the remodeling pathway is always accompanied by the release of arachidonic acid that can be converted into a variety of potent bioactive eicosanoid mediators.

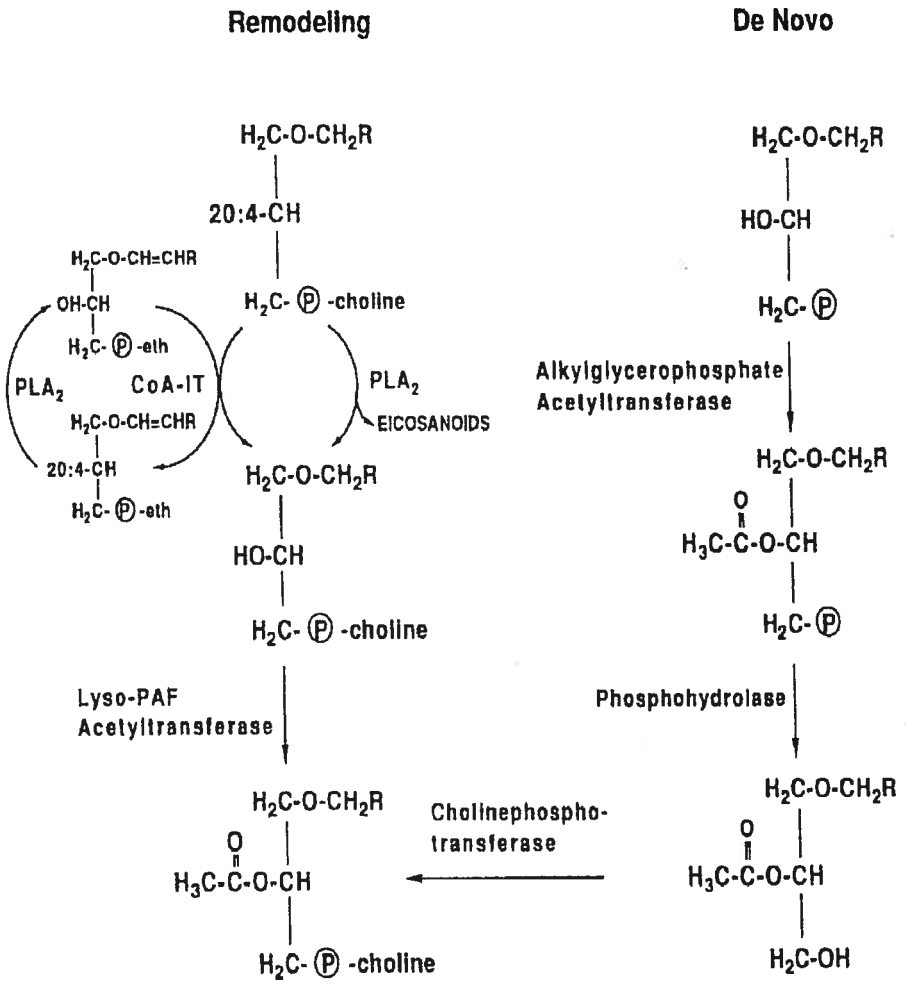


Figure 1.7 Pathways of platelet-activating factor biosynthesis

From **Prescott et al.**, Platelet-activating factor. *In Inflammation: Basic Principle and Clinical Correlates, 3rd ed.*, 1999.

The second mechanism of PAF synthesis, *de novo* pathway, starts with 1-*O*-alkyl-sn-glycero-3-phosphate which is acetylated by the alkylglycerophosphate acetyl transferase enzyme, followed by removal of the phosphate by a phosphohydrolase enzyme and its replacement with phosphocholine, the last reaction catalyzed by PAF-phosphocholine transferase (PAF-PCT) (Peplow, 1999). The enzymes in this pathway are constitutively active and regulated by the availability of substrate.

PAF synthesized via the remodeling pathway is secreted into the fluid phase by human monocytes and eosinophils. In contrast, PAF synthesized by endothelial cells via the same pathway is translocated to the plasma membrane and retained on the cell surface.

Degradation of PAF occurs by hydrolysis of the sn-acetyl residue to generate lyso-PAF and acetate. This reaction is catalyzed by PAF acetylhydrolase (PAF-AH) (**figure 1.8**). PAF-AH belongs to group VI of PLA_2.

1.3.4.4.2. Platelet-activating factor receptors

The action of PAF is mediated through a member of G protein-coupled receptors. The receptors from several species have been cloned and characterized at the molecular level. PAF acts by binding to a specific receptor (PAFR) which is a "7-membrane-spanning" receptor and induces intracellular signaling via PLC-β activation and turnover of phosphatidylinositol. As consequence, there is an increase in intracellular calcium and activation of PKC (Peplow, 1999).

PAFR undergoes homologous desensitization, a control mechanism that potentially limits its signaling actions. Desensitization of the PAFR is accompanied by phosphorylation of the PAF receptor (Takano et al., 1994; Ali et al., 1994). In summary, PAFR can be desensitized in multiple ways. Homologous desensitization of PAFR uses two mechanisms: phosphorylation of the receptor (probably by a G-

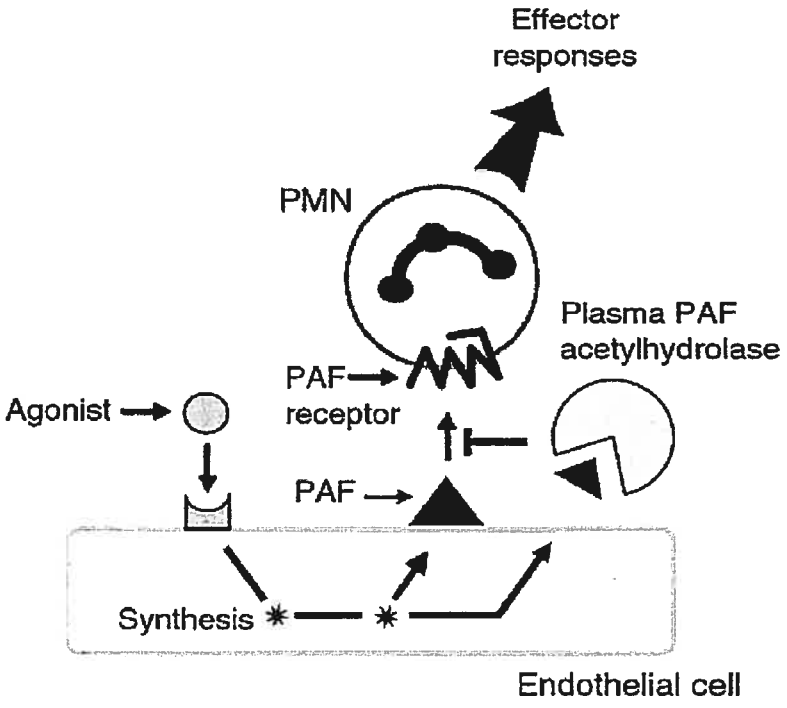


Figure 1.8. Platelet-activating factor signaling pathways

From **Zimmerman et al.**, *Critical Care Med.*, 30(5 Suppl):S294-301, 2002.

protein-coupled receptor kinase-1, GRK) and inactivation of the key downstream effector PLC-β3 by PKC. Heterologous desensitization of the PAFR uses a third mechanism: phosphorylation of the receptor by PKC (Prescott et al., 2000).

1.3.4.4.3. Role of PAF in inflammation

PAF has been implicated in the pathogenesis of both acute inflammation and hypersensitivity disorders. PAF is a potent activator of human neutrophils and stimulate neutrophil adhesion, lysosomal enzyme release, generation of reactive oxygen species and eicosanoids (Smith et al., 1984). In inflammation, rapid activation of ECs change their surface properties to become adhesive for leukocytes, with P-selectin expression facilitating leukocyte tethering (Mayadas et al., 1993). The leukocytes that have been tethered are then activated by PAF, which is produced and displayed on the ECs surface (Zimmerman et al., 1992). The *in vitro* demonstration that P-selectin and PAF cooperatively mediate the adhesion of PMNs to the activated ECs is supported by *in vivo* studies showing that antibodies to P-selectin and PAF receptor antagonists suppress the inflammation (Coughlan et al., 1994).

PAF can activate human monocyte cells by increasing the synthesis of chemokines, cytokines, tissue factor, and other mediators (Zimmerman et al., 2002). Thus PAF stimulates the release of several cytokines including IL-1 and TNF, resulting in significant amplification of the inflammatory process.

In vivo, PAF was shown to trigger aggregation and accumulation of platelets and to induce subsequent changes in local blood flow at the site of experimental thrombosis and vascular injury (Golino et al., 1993). Platelets aggregation is mediated by inside-out signaling of the integrin $\alpha_{IIb}\beta_3$ and consequent binding of fibrinogen (Shattil et al., 1998), indicating that the PAF receptor is linked to this prothrombotic intracellular pathway.

Work from a variety of sources has suggested that PAF is a mediator of sepsis. For example, PAF antagonists block endotoxin-induced recruitment of platelets to the lung (Beijer et al., 1987). PAF is also involved in inflammatory bowel disease and its inhibition by specific antagonists may have a potential therapeutic benefit in treatment of these inflammatory diseases (Nassif et al., 1996).

PAF could be implicated in the pathogenesis of chronic arthritis considering that PAF antagonists have beneficial effects in a model of joint injury (Palacios et al., 1999). Other investigators found PAF in bronchoalveolar lavage (BAL) fluid from ARDS patients (Matsumoto et al., 1992). In addition, clinical trial have suggested that PAF receptor antagonist may be beneficial in treating pancreatitis (Kingsnorth et al., 1995).

1.4. Aims of the present research project

Experimental evidence suggests that lipid mediators play an important role in regulating PMN extravasation at the blood endothelium interface by regulating, for instance, vascular permeability, vasodilation, and/or endothelial cell hyperadhesiveness for neutrophils (Marleau et al., 1999).

The goal of the present study is to delineate the role of PAF and LTB_4 in neutrophil trafficking in a model of dermal inflammation in rats. The local injection of certain lipid mediators, including LTB_4 and PAF in the dorsal skin of male Sprague-Dawley rats, stimulates PMNs and/or ECs to facilitate the transmigration process of PMNs. In addition, LTD_4, by causing ECs retraction may also contribute to PMNs extravasation. In order to delineate the role of these lipid mediators and their potentially synergistic or additive effects on leukocyte migration, we have used selective LTB_4 and PAF receptor antagonists in a dermal inflammatory model.

Briefly, rats were pretreated with either a LTB_4 and/or PAF receptor antagonist after which neutrophil accumulation was elicited by intradermal agonist injections. The mediators under study included LTB_4, PAF, TNF-α, IL-8 and ZAP. PMN accumulation to skin sites has been assessed by the myeloperoxidase (MPO) assay. In additional studies, we have assessed the role of lipid mediators on plasma extravasation and microcirculatory blood flow using the same compounds. The drug used included the selective LTB_4 receptor antagonist (CP-105,696) and selective PAF receptor antagonists (UK-74,505 and SR-27417).

Hence, this research project enabled us to further delineate the role of lipid mediators in leukocyte trafficking by elucidating;

1. The role of LTB_4 in PMN emigration, plasma extravasation and microcirculatory changes in blood flow elicited by PAF or other soluble agonists (ZAP, IL-8 and TNF-α);
2. The role of PAF in PMN emigration, plasma extravasation and microcirculatory changes in blood flow elicited by LTB_4 or other soluble agonists (ZAP, IL-8 and TNF-α);
3. The potentially synergistic or additive effects of lipid mediators, including LTB_4, PAF and LTD_4 in PMN emigration, plasma extravasation and microcirculatory changes in blood flow elicited by soluble agonists including PAF, LTB_4 and other soluble agonists.

CHAPTER II: MATERIALS AND METHODS

2.1. Chemicals

Neupogen^{MD} (Filgrastim) was obtained from Amgen Canada Inc. (Mississauga, Ontario). Bovine serum albumin (BSA) (low endotoxin), casein, dimethyl sulphoxide (DMSO), hexadecyltrimethylammonium bromide (HTAB), hydrogen peroxide (H_2O_2), N,N-dimethylformamide (DMF) 99.9%, PAF, substance P, 3,3',5,5'-tetramethylbenzidine (TMB) and zymosan were purchased from Sigma-Aldrich Company (St. Louis. MO). LTB_4, LTD_4 and PGE_2 were purchased from Cayman Chemical (Hornby, Ontario, Canada). Evans Blue was obtained from Fisher Scientific Company (Fair Lown, New Jersey). Dextran T-500 and Percoll were obtained from Pharmacia Canada Inc. (Baie d'Urfé, Qc, Canada), and modified Hank's Balanced Salt Solution (HBSS) and HEPES buffer were from Gibco Life Technologies (Grand Island, NY). Human recombinant (hr) IL-8 and hrTNF-α were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). UK-74,505 (4-(2-chlorophenyl)-1,4-dihydro-3-ethoxycarbonyl-6-methyl-2-[4-(2-methylimidazo[4,5-c]pyrid-1-yl)phenyl]-5-[N-(2-pyridyl)carbamoyl]pyridine) and (+)-1-(3*S*,4*R*)–[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]-cyclopentan carboxylic acid (CP-105,696) were kind gifts from Pfizer Central Research (Sandwich, Kent, UK) and (Groton, USA), respectively. SR-27417 [N-(2-dimethylamino-ethyl)-N-(3-pyridinylmethyl)[4-(2,4,6-triisopropyl-phenyl)thiazol-2-yl]amine] was a kind gift from Sanofi Recherche (Toulouse, France). Solutions for parenteral administration, such as the Evans blue solution, were made from pyrogen-free, sterile 0.9% NaCl (Baxter Corporation Laboratories, Toronto, Ontario, Canada).

2.2. Animals

Male Sprague-Dawley rats (90-100g) were purchased from Charles River (St-Constant, Québec, Canada). They were maintained in cages with free food and water for 9-11 days before doing any experimental work. During this period of time, rats were injected subcutaneously (s.c.) with 5 μg/kg G-CSF (Neupogen^{MD}) diluted with

0.9% NaCl. Neupogen^{MD} was injected until the day of the experiment in order to increase the number of circulating PMN in the rats, resulting in a proportional increase in the local accumulation of PMN in response to agonist challenge. The increased PMN numbers at local sites facilitated assessment of the effect of drugs on agonist-induced PMN accumulation in a quantitative manner.

2.3. Isolation and purification of rat peritoneal neutrophils

Rat PMNs were obtained by a modification of the method described by Bokoch and Gilman (1984). Animals were injected intraperitoneally (i.p.) with 10 ml of 5% sodium caseinate. The peritoneal exudates were collected 6 h later in 30 ml heparinized saline (10 units/ml). Neutrophils were purified using a Percoll gradient, the neutrophil rich band was collected and contaminating red blood cells (RBC) removed by hypotonic lysis. The purified neutrophils were greater than 98% pure, contaminating cells being mononuclear cells. The cell suspension was diluted to 0.5 x 10^6 PMN/ml with acetate buffer containing 0.5% HTAB, aliquoted and kept at -20°C until assayed for myeloperoxidase (MPO) activity in a PMN standard curve.

2.4. Preparation of zymosan-activated plasma (ZAP)

ZAP, a source of $C5a_{desarg}$, was prepared by incubating heparinized (10 units/ml) rat plasma with zymosan (5 mg/ml) for 30 min at 37°C. Zymosan was removed by centrifugation (2 x 10 min at 3000 g) and ZAP was stored in aliquots at -20°C.

2.5. Experimental protocol

The drugs (PAF and LTB_4 antagonists) were dissolved in appropriate vehicles and administered to the rats orally at specific times before the intradermal (i.d.) injections of agonists. CP-105,696 was dissolved in 0.5% carboxymethyl cellulose

(CMC) and administered 16 h prior to i.d. agonist injections. UK-74,505 was dissolved in 0.1N HCl, diluted with sterile water and administered 2 h prior to i.d. agonist injections. SR-27417 was dissolved in sterile water and administered 2 h prior to i.d. agonist injections. Control rats received an oral administration of vehicle.

Rats were sedated by a s.c. injection of Hypnorm (fentanyl-fluanisone, Janssen Pharmaceutica, Beerse, Belgium) (0.15 ml), the dorsal skin of the rats was shaved, and the sedated animals were placed on heating pads.

2.5.1. Agonist preparation

The agonists under investigation, LTB_4, PAF, IL-8, TNF-α, LTD_4 and substance P, were dissolved as followed:

- LTB_4, PAF, PGE_2 and LTD_4 were initially dissolved in ethyl alcohol and diluted with 0.1% HBSS/BSA (final % of solvent is $\leq 0.1\%$).
- IL-8 (25 µg) and TNF-α (50 µg) were dissolved in sterile water and Substance P in sterile 0.9% NaCl before diluting with 0.1% HBSS/BSA.
- ZAP, prepared as described above, was diluted in 0.1% HBSS/BSA and used at concentrations of 3 and 30%.

All agonists were co-injected with 250 pmol PGE_2, a potent vasodilator, to enhance PMN extravasation and facilitate measurements of MPO activity.

2.5.2. Measurement of PMN accumulation in rat skin

PMN accumulation in response to i.d. injected agonists was quantified by measuring MPO activity in skin biopsies. The agonists, freshly prepared from stock solutions, were injected i.d. (50 µl per site) at duplicate sites in the dorsal skin of the rat **(figure 2.1)** according to a well-balanced and randomized site injection plan. The agonists under study included LTB_4 (500 pmol/site), PAF (1.9 nmol/site), ZAP (3 and

Figure 2.1. Intradermal injections of agonists in the rat dorsal skin

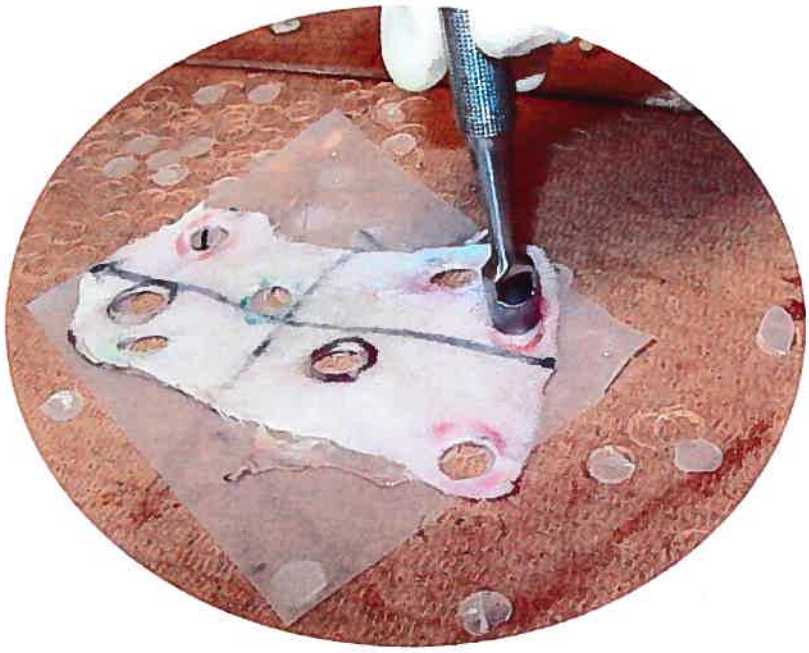


Figure 2.2. Punching of the skin biopsies

30%), IL-8 (50 pmol/site), TNF-α (50 pmol/site) and vehicle (HBSS/BSA 0.1%). After a 2 h period to allow PMN accumulation, the rats were anaesthetized with a solution of ketamine (Vetrepharm-Canada Inc. Belleville, ON) (90 mg/kg) and xylazine (Bimeda-MTC Animal Health Inc. Cambridge, ON) (10 mg/kg); a mid-line incision was performed on the thorax and 1 ml of blood was taken from the thoracic aorta to determine total and differential leukocyte counts. The rats were sacrificed with an overdose of pentobarbital (Euthanyl, Schering Canada Inc. Pointe-Claire, Québec) injected directly into the heart, and the dorsal skin was harvested and cleaned of excess blood and fat. The skin biopsies were punched out (11 diam. i.d.) **(Figure 2.2)**, weighed, and frozen at -80°C until assayed for MPO activity.

2.5.2.1. MPO enzymatic assay

The MPO assay was performed by a modification of the method described by Stark et al. (1992). Briefly, the biopsies were thawed and homogenized in 100 mM of acetate buffer pH 6, containing 0.5% HTAB and 20 mM EDTA. The homogenates were sonicated on ice for 15 s, frozen (-80°C), subjected to three freeze-thaw cycles, and then centrifuged (15°C, 2000 X g for 15 min). The supernatants are filtered and diluted with the acetate buffer solution. A 3.2 mM stock solution (10 µl) of TMB (in DMSO) was added to 75 µl of the filtrate, and the solutions were preincubated at 37°C for 5 min. The enzymatic reaction was started by the addition of 50 µl of a solution of 1.0 mM H_2O_2 in 0.2 M acetate buffer pH 6, and incubated at 37°C for 5 min. The reaction was terminated by the addition of 100 µl of 0.2 M sodium acetate (pH 3.0). The absorbance of the samples was determined at 630 nm and the number of PMN per site was calculated according to a standard curve from rat PMN obtained from the peritoneal cavity. The results are expressed as PMN/site.

2.5.3. Measurement of local oedema formation in rat skin

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability and oedema formation. The procedure of measuring

local oedema was identical to that of PMN accumulation except for the following: Evans blue (100mg/kg) solution in sterile 0.9% NaCl (10% w/v) was injected i.v. in the jugular vein. Five minutes later, the agents under study, freshly prepared in HBSS/BSA 0.1%, were injected i.d. (50 µl per site). The agonists included PAF (1.9 nmol/site), LTD_4 (500 pmol/site), substance P (100 pmol/site) and vehicle (HBSS/BSA 0.1%). The animals were sacrificed 30 min after the i.d. injections. A blood sample (1 ml) was obtained immediately prior to sacrifice and plasma was prepared by centrifugation (3000 X g, 10 min) and kept at -20°C until assayed for Evans blue. The dorsal skin was subsequently removed and the injected sites were punched out (11 diam. i.d.).

2.5.3.1. Evans blue assay

Evans blue was extracted by incubating the minced skin sites in 0.25 ml DMF at room temperature for 48 h and quantified by measuring the absorbance at 630 nm. Evans blue in the plasma was quantified, following dilution with water, by measuring the absorbance at the same wavelength. The concentration of Evans blue in skin site and plasma was determined using a standard curve of Evans blue. Plasma extravasation in the skin was expressed in terms of µl of plasma, and calculated by dividing the concentration of Evans blue in the skin site per its concentration in 1 µl of plasma.

2.5.4. Measurement of the microcirculatory blood flow in rat skin

The day before the experiment, rats were sedated by injecting Hypnorm (0.15 ml) s.c. and the dorsal region of the rats was shaved carefully to remove the hair. The experimental protocol started by inducing rat anaesthesia with an i.p. injection of a Ketamine/Xylazine (90:10) solution (0.1 ml).

Skin blood flow was measured noninvasively with a laser-doppler blood flowmetry (Periflux system 5000) **(figure 2.3)**. Using this technique, basal

microvascular blood flow of all skin sites was measured and only those with similar basal flow were used for intradermal injections. The area of measurement was 1 mm^2 and the depth of the laser beam penetration was approximately 0.5-1.5 mm. The agonists under investigation included PAF (3.8 nmol/site), LTB_4 (1000 pmol/site) and vehicle (HBSS/BSA 0.1%). The local microvascular blood flow was recorded before injection of the agonists, and again following i.d. injections of 100 µl of freshly prepared agonists, incubated at 37°C for 1 min before injection. The optic fibre probe was placed about 2 mm from the circumference of the injection site delineated with a color marker, and changes in the local microvascular blood flow responses were measured directly after agonist injections and monitored for 5 minutes.

Results are shown as representative tracing of the vasodilator responses to various agonists in control rats and in rats treated with the PAF antagonist (SR-27417). The rats were allowed to recover and were used again after at least one week of washout period before another blood flow experiment.

2.6. Statistical analysis

All results are expressed as mean ± SEM. Statistical significance was assessed by a one-way ANOVA with Student-Newman-Keuls for multiple comparisons as a post-test, using GraphPad InStat version 3.05 Software (San Diego, California, USA). P values < 0.05 were considered statistically significant.

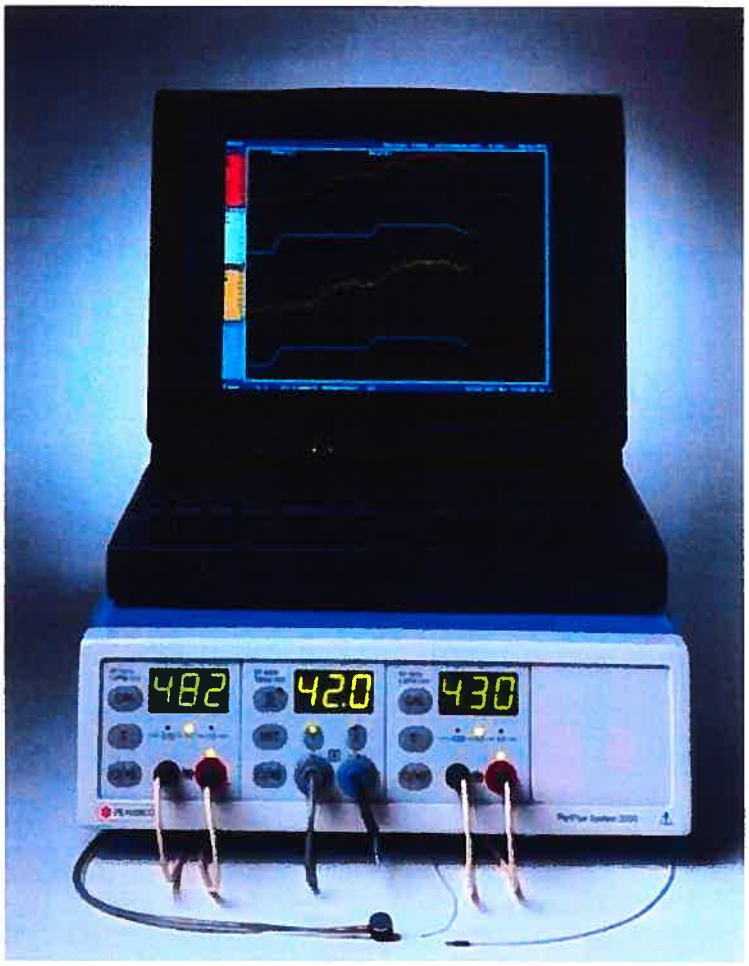


Figure 2.3. Laser-doppler apparatus
Periflux system 5000 (Perimed AB, Stockholm, Sweden)

CHAPTER III: RESULTS

3.1. Preliminary experiments

3.1.1. Dose-dependent effect of a LTB_4 antagonist on LTB_4-induced PMN accumulation in rat skin

Prior studies in our laboratory have shown a dose-dependent inhibition of LTB_4 -induced PMN accumulation by a selective LTB_4 antagonist, CP-105,696. The doses of 10 and 30 mg/kg significantly reduced PMN accumulation, induced by LTB_4 (500 pmol/site), by 43 % and 77 % respectively **(figure 3.1)**.

3.1.2. Dose-dependent effect of selective PAF antagonists, on PAF-induced PMN accumulation in rat skin

Preliminary experiments were performed to assess the dose-dependent inhibition of the selective PAF antagonist UK-74,505. UK-74,505, administered at a dose of 2.5 mg/kg, tended to reduce PAF-elicited PMN accumulation by 28%, while a dose of 5 mg/kg was found to inhibit PMN accumulation by 61% *($P < 0.05$)* **(figure 3.2).**

Similar studies were performed to determine the optimal dose of SR-27417, a potent and selective PAF receptor antagonist. As shown in **figure 3.3**, SR-27417, at a dose of 0.1 mg/kg, did not significantly reduce PMN accumulation (16%, $P > 0.05$), but optimally inhibited PMN accumulation induced by PAF at a dose of 1 mg/kg by 65% *($P < 0.05$)*.

In both of these preliminary experiments, the dose of the intradermal agonist PAF was of 950 pmol/site. In addition, no PGE_2 was used as vasodilating agent.

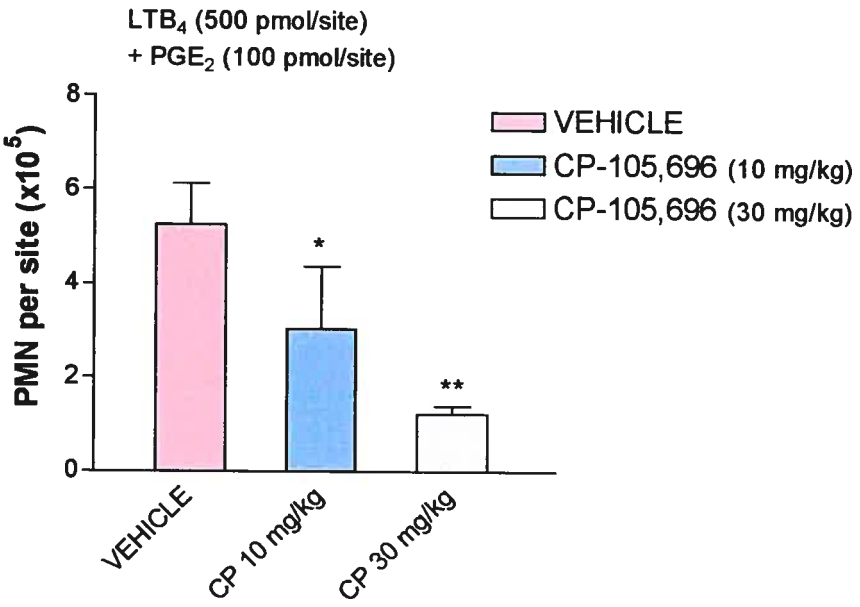


Figure 3.1. Dose-dependent inhibition of LTB_4-elicited PMN accumulation by pretreatment of rats with CP-105,696.
Data represent the mean ± SEM of 8 sites obtained from 4 rats.

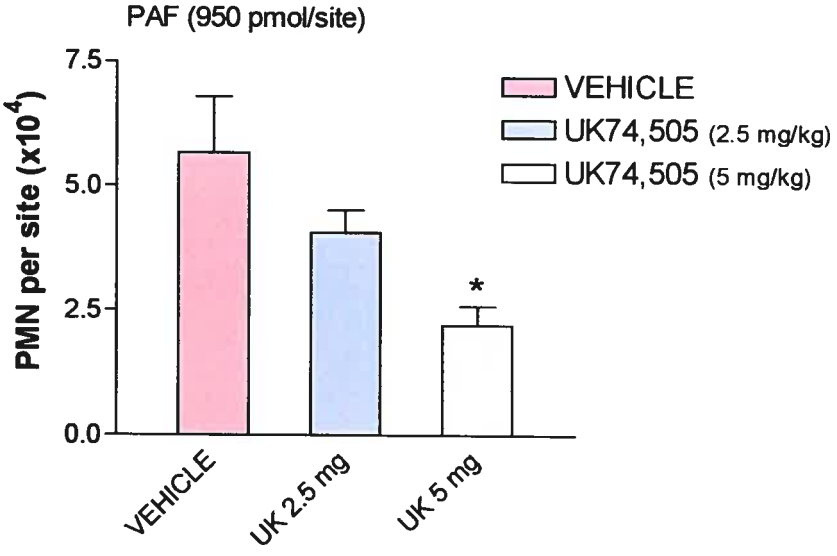


Figure 3.2. Dose-dependent inhibition of PAF-elicited PMN accumulation by pretreatment of rats with UK-74,505.
Data represent the mean ± SEM of 4-8 sites obtained from 2-4 rats.

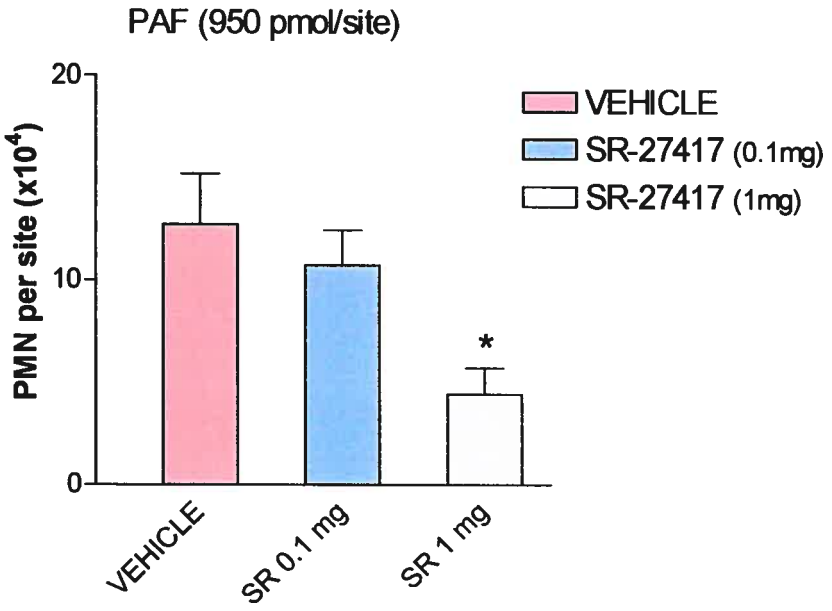


Figure 3.3. Dose-dependent inhibition of PAF-elicited PMN accumulation by pretreatment of rats with SR-27417.
Data represent the mean ± SEM of 4-8 sites obtained from 2-4 rats.

According to the results of these preliminary experiments, we conducted subsequent studies using the selective PAF receptor antagonists UK-74,505 at a dose of 5mg/kg and SR-27417 at a dose of 1mg/kg. The selective LTB_4 receptor antagonist CP-105,696 was used at doses of 10mg/kg and 30mg/kg.

3.2. Effects of PAF and LTB_4 antagonists on PMN accumulation elicited by a variety of chemoattractant agonists

To delineate the role of PAF and LTB_4 in the inflammatory response induced by PAF and LTB_4, we have used selective and long-acting PAF antagonists (UK-74,505 and SR-27417) and a potent and long-acting LTB_4 antagonist CP-105,696. A second chemically unrelated LTB_4 antagonist such as LY 293111 or BIIL 284 was unavailable in sufficient quantities for *in vivo* administration in rats at the time of study.

Results are presented as a percentage of PMN accumulation compared to the vehicle-treated group. The (n) number is specified in figure legends.

3.2.1. Effect of UK-74,505 and/or CP-105,696 on inflammatory mediator-induced PMN accumulation in rat skin

Experiments were undertaken to study the effect of UK-74,505 at a dose of 5 mg/kg and/or CP-105,696 at a dose of 30 mg/kg on PMN accumulation induced by PAF (1.9 nmol/site, a dose higher than tested previously but giving more reproducible PMN accumulation) and LTB_4 (500 pmol/site). PMN accumulation, in vehicle-treated rats, elicited by PAF (1.9 nmol) was 47812 ± 8105 PMN per site and 157955 ± 28385 PMN per site when LTB_4 (500 pmol) was used as an i.d. agonist. UK-74,505 significantly reduced PAF-induced PMN accumulation by 51% *($P < 0.05$)*, whereas CP-105,969 tended to reduce PMN numbers in the skin (38%, *$P > 0.05$*) **(figure 3.4A)**. When CP-105,696 and UK-74,505 were co-administered, an additive inhibitory effect on PAF-elicited PMN accumulation (73%, *$P < 0.01$*) was observed.

In contrast, while CP-105,696 potently reduced PMN accumulation induced by LTB_4 (79%, *$P < 0.001$*), UK-74,505 tended to reduce LTB_4-elicited PMN accumulation (39%, $P > 0.05$). When both antagonists were administered to the rats, no additive inhibitory on LTB_4-elicited PMN accumulation was observed (78%, *$P < 0.001$)* **(figure 3.4B)**.

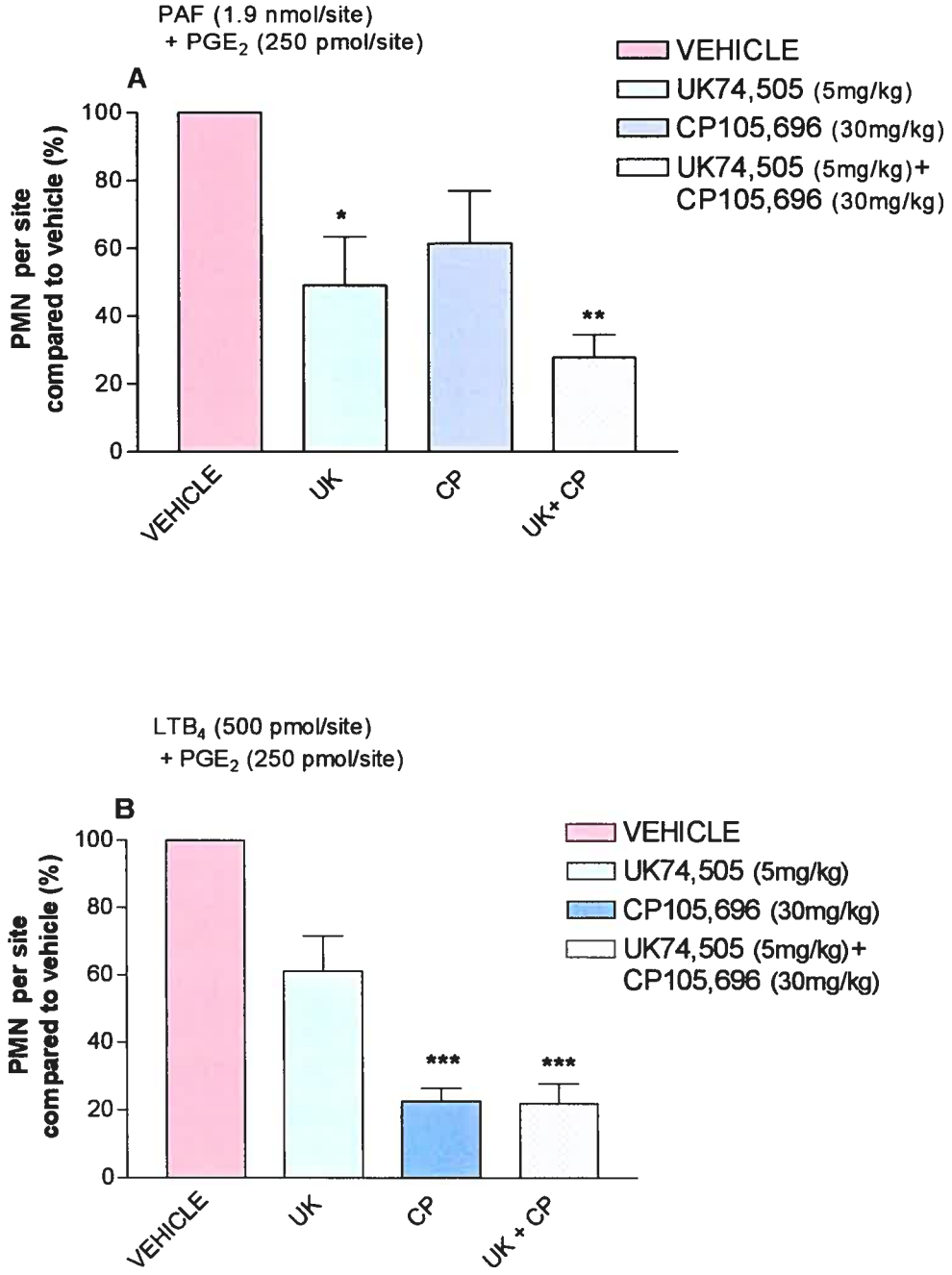


Figure 3.4. Effect of UK-74,505 and/or CP-105,696 on PAF-elicited PMN accumulation (A) & LTB_4-elicited PMN accumulation (B) in rat dorsal skin
Data represent the mean ± SEM of 8-12 sites obtained from 4-6 rats.

3.2.2. Effect of SR-27417 and/or CP-105,696 on inflammatory mediator-induced PMN accumulation in rat skin

For these studies, we initially used SR-27417 at a dose of 1 mg/kg which, according to our preliminary experiments, was shown to inhibit PAF-induced PMN accumulation in rat skin by 65%. However, we have found that this dose inhibited the effect of PAF by 94 % in subsequent experiments; it may be partly due to the fact that we undertook studies in slightly lower weight rats (100 vs 125 gm) in addition of using a new batch of the drug. Hence, subsequent studies were done using a 0.3 mg/kg dose of SR-27417.

The first series of experiments were done using of SR-27417 at a dose of 1 mg/kg and/or CP-105,696 at a dose of 10 mg/kg as a pretreatment in rats locally injected with LTB_4 (500 pmol/site) and PAF (1.9 nmol/site).

Our results show that SR-27417 (1 mg/kg) inhibited PAF-elicited PMN accumulation by 94% *($P < 0.01$)* whereas CP-105,696, at dose of 10 mg/kg, tended to reduce the PAF-induced PMN accumulation by 54% *($P > 0.05$)* **(figure 3.5A)**. When both drugs are co-administered, the PMN accumulation induced by PAF was inhibited by 96% *($P < 0.001$)* **(figure 3.5A)**

When LTB_4 was used as an i.d. agonist, we observed that CP-105,696 significantly inhibited PMN accumulation (84%, *$P < 0.001$*) **(figure 3.5B).** In contrast, SR-27417 did not affect the LTB_4-induced PMN accumulation. When SR-27417 was administered together with CP-105,696, PMN accumulation was reduced by 90% *($P < 0.01$)* **(figure 3.5B)**.

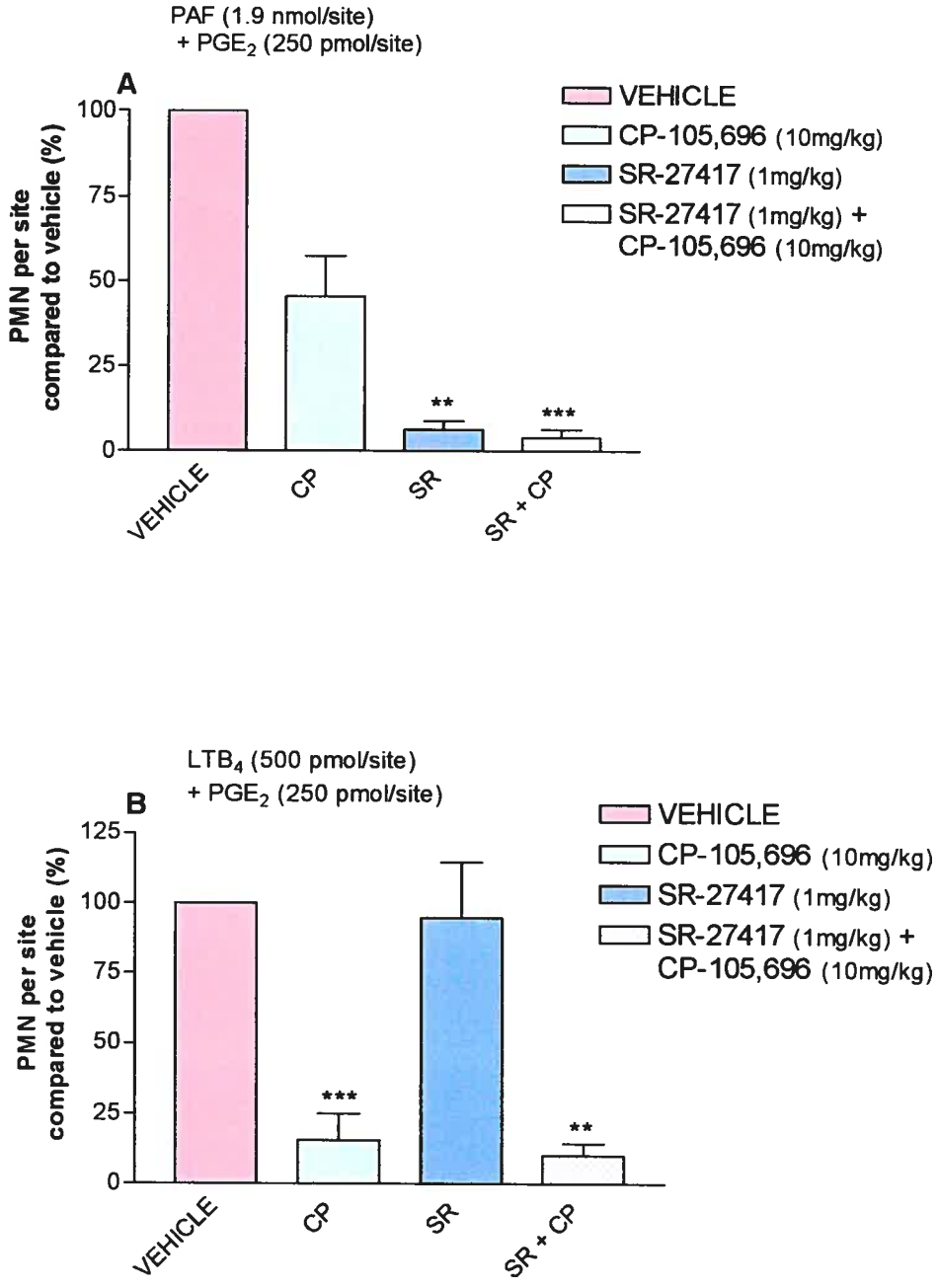


Figure 3.5. Effect of SR-27417 (1 mg/kg) and/or CP-105,696 (10 mg/kg) on PAF-elicited PMN accumulation (A) & LTB_4-elicited PMN accumulation (B) in rat dorsal skin

Data represent the mean ± SEM of 8-12 sites obtained from 4-6 rats.

In another series of experiments, we studied the effect of SR-27417 at a lower dose of 0.3 mg/kg and of CP-105,696 on PMN accumulation elicited by different agonists, including LTB_4 (500 pmol/site), PAF (1.9 nmol/site) and ZAP (3%).

SR-27417, at a dose of 0.3 mg/kg, significantly reduced PMN accumulation induced by PAF 53% *($P < 0.05$),* whereas CP-105,969 (10 mg/kg) did not significantly reduce PAF-elicited PMN accumulation (29%, *$P > 0.05$*) **(figure 3.6A)**. When CP-105,696 (10 mg/kg) and SR-27417 (0.3 mg/kg) were co-administered, an additive inhibitory effect on PMN accumulation induced by PAF was observed (75%, *$P < 0.001$)*.

When LTB_4 was used as an i.d. agonist, our results showed that CP-105,696 significantly inhibited PMN accumulation (79%, *$P < 0.01$*) **(figure 3.6B)**. In contrast, SR-27417 did not affect LTB_4-induced PMN accumulation. Furthermore, co-administration of the two drugs did not further inhibit LTB_4–elicited PMN (82%, *$P < 0.01$*).

PMN accumulation elicited by ZAP (3%) was 42762 ± 8269 PMN per site in vehicle-treated rats. The effect of SR-27417 (0.3 mg/kg) and/or CP-105,696 (10 mg/kg) on PMN accumulation induced by ZAP (3%) was investigated. As shown in **figure 3.7,** SR-27417 at a dose of 0.3 mg/kg tended to reduce PMN accumulation induced by ZAP (30%, *$P > 0.05$*), whereas CP-105,969 (10 mg/kg) tended to reduce ZAP-elicited PMN accumulation (55% *$P > 0.05$*). When rats were treated with both SR-27417 and CP-105,696, PMN accumulation induced by ZAP was further reduced by 63% *($P < 0.05$)*

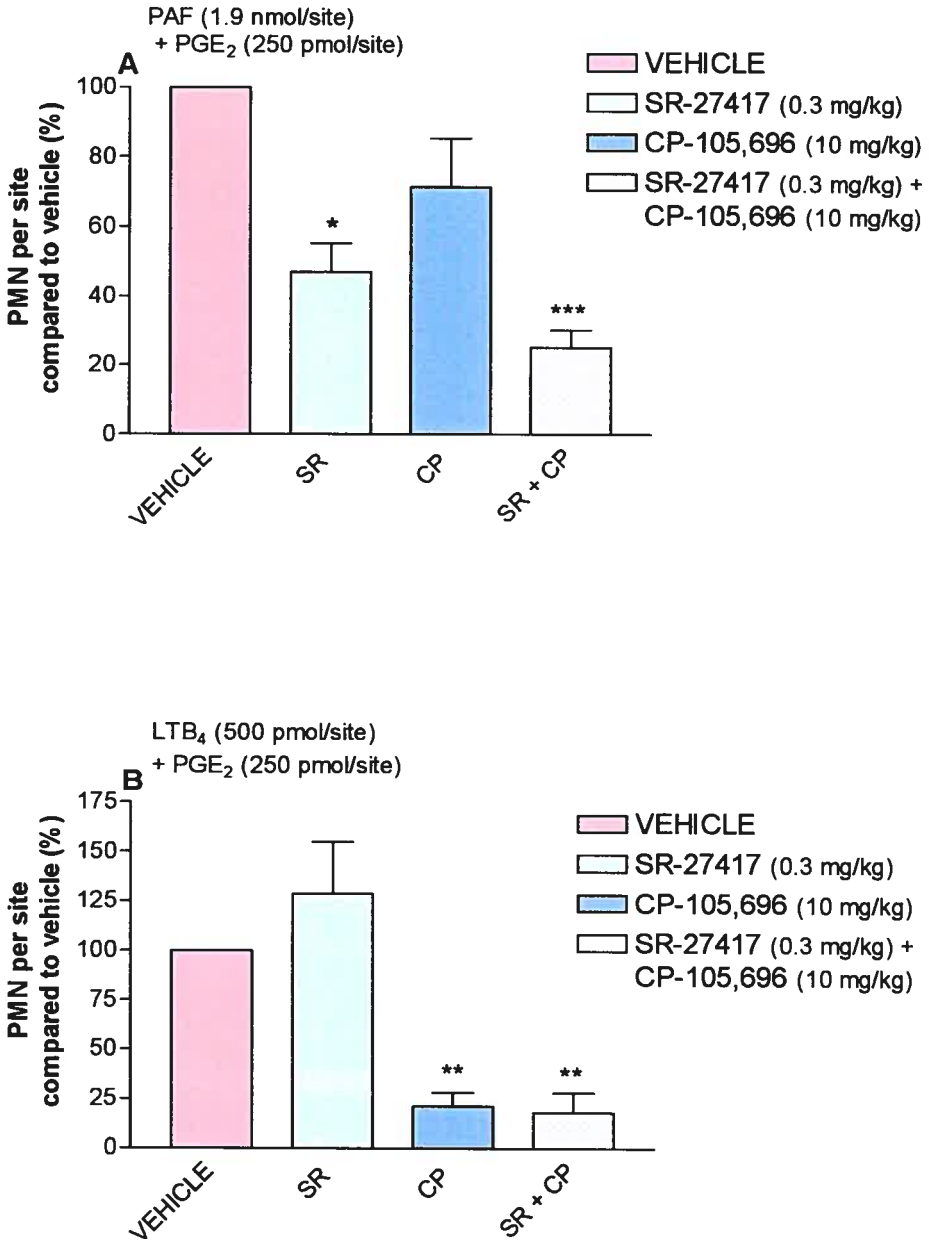


Figure 3.6. Effect of SR-27417 (0.3 mg/kg) and/or CP-105,696 (10 mg/kg) on PAF-elicited PMN accumulation (A) & LTB_4-elicited PMN accumulation (B) in rat dorsal skin

Data represent the mean ± SEM of 8-12 sites obtained from 4-6 rats.

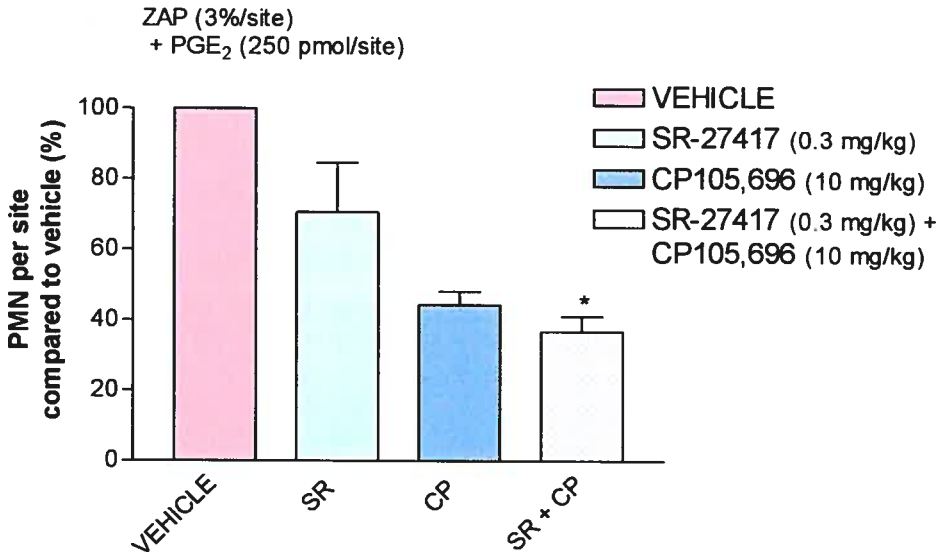


Figure 3.7. Effect of SR-27417 (0.3 mg/kg) and/or CP-105,696 (10 mg/kg) on ZAP-elicited PMN accumulation in rat dorsal skin
Data represent the mean ± SEM of 4-6 sites obtained from 2-3 rats.

3.2.3. Effect of UK-74,505 and/or CP-105,696 on PMN accumulation induced by chemically unrelated chemoattractants (TNF-α, IL-8 and ZAP)

Intradermal PMN accumulation triggered by i.d injections of chemically unrelated chemoattractants including TNF-α, IL-8 and ZAP was studied using selective PAF (UK-74,505) and LTB_4 (CP-105,696) antagonists.

PMN accumulation in response to TNF-α (50 pmol/site) was 93582 ± 8121 PMN per site in vehicle-treated rats. Oral administration of CP-105,696 at a dose of 10 mg/kg significantly reduced the accumulation induced by TNF-α by 42% *($P < 0.05$)*, while UK-74,505 (5 mg/kg) tended to reduce the PMN accumulation by 15 % *($P > 0.05$)* **(figure 3.8A)**. When UK-74,505 was co-administered with CP-105,696, no significant effect on TNF-α-elicited PMN accumulation was observed.

PMN accumulation induced by IL-8 (50 pmol/site) was 119995 ± 14054 PMN per site. In this experiment, neither CP-105,696 (10 mg/kg) nor UK-74,505 (5 mg/kg) significantly inhibited PMN accumulation induced by IL-8, (around 25%, $P > 0.05$) **(figure 3.8B)**. Co-administration of CP-105,696 (10mg/kg) and UK-74,505 (5 mg/kg) also failed to inhibit the PMN accumulation induced by IL-8.

The effect of UK-74,505 (5 mg/kg) and/or CP-105,696 (10 mg/kg) on PMN accumulation induced by ZAP (3%) was investigated. As shown in **figure 3.9,** UK-74,696 tended to reduce PMN accumulation induced by ZAP (55%, *$P > 0.05$*), whereas CP-105,696 (10 mg/kg) tended to reduce ZAP accumulation (41% *$P > 0.05$*). In the animals treated with UK-74,505 (5 mg/kg) and CP-105,696 (10 mg/kg) PMN accumulation induced by ZAP was inhibited by 49% *($P < 0.05$).*

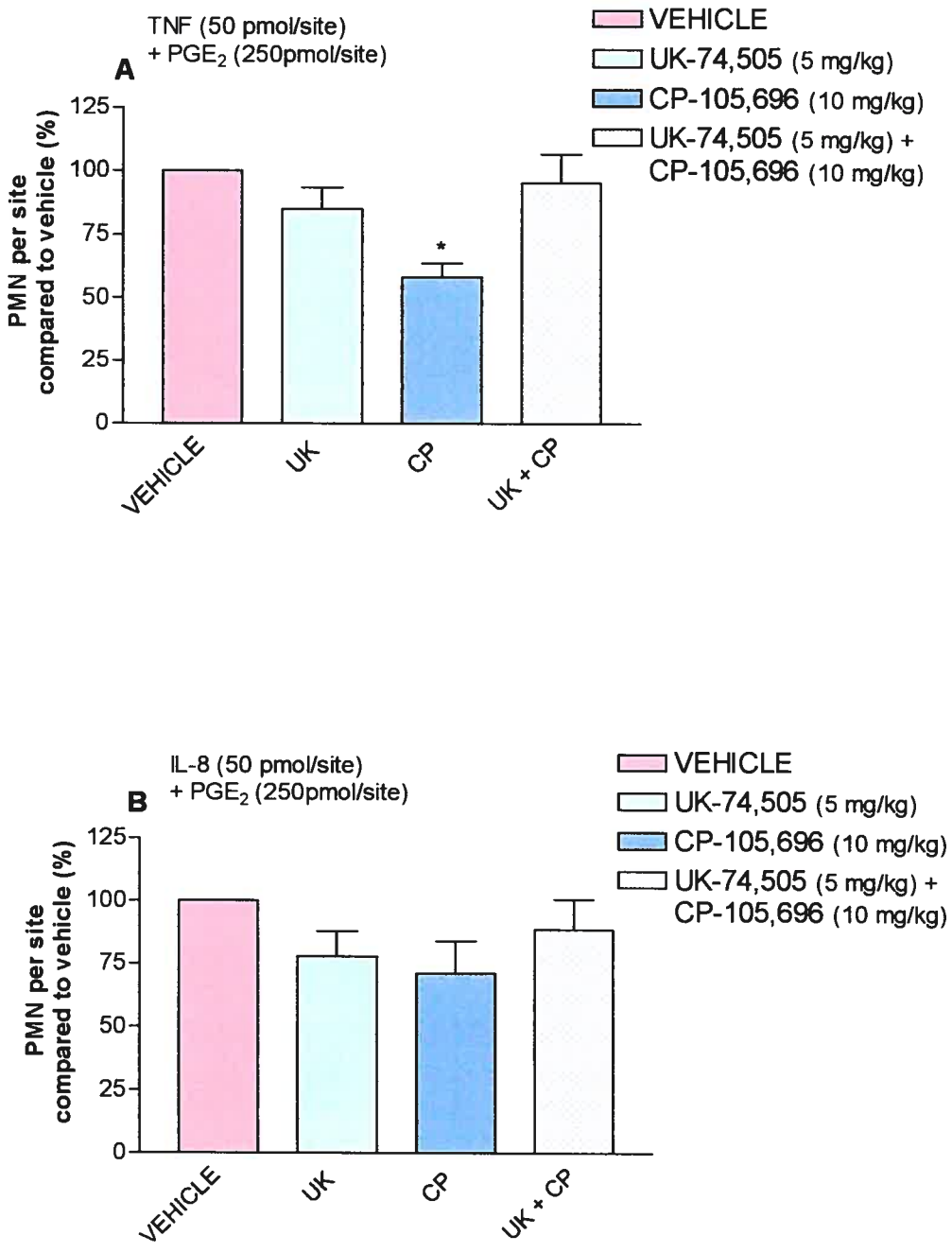


Figure 3.8. Effect of UK-74,505 and/or CP-105,696 on TNF-α-elicited PMN accumulation (A) & IL-8-elicited PMN accumulation (B) in rat dorsal skin
Data represent the mean ± SEM of 12-20 sites obtained from 6-10 rats.

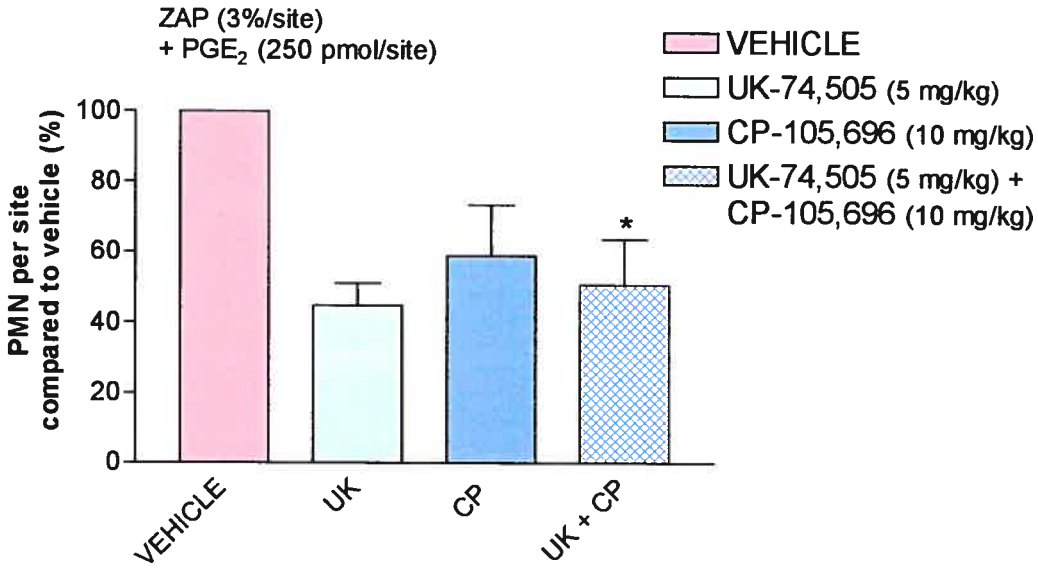


Figure 3.9. Effect of UK-74,505 and/or CP-105,696 on ZAP (3%)-elicited PMN accumulation in rat dorsal skin
Data represent the mean ± SEM of 12 sites obtained from 6 rats.

3.3. Effects of PAF and LTB_4 antagonists on oedema formation elicited by different agonists

Intradermally injected PAF induced oedema formation in rat skin. Moreover PAF and PGE_2 act synergistically to induce oedema formation, as previously reported by Wedmore and Williams (1981). Preliminary experiments were performed to determine the optimal dose of SR-27417 to reduce PAF-induced plasma extravasation in rat skin by 40-50 %. The optimal dose was found to be 0.3 mg/kg SR-27417.

Results were calculated by determining µl plasma extravasated from the 8-12 sites from 4-6 rats per group.

3.3.1. Effect of SR-27417 and/or CP-105,696 on oedema formation elicited by PAF

As shown in **figure 3.10**, SR-27417 (0.3 mg/kg) significantly reduced oedema formation induced by PAF by 41% *($P < 0.001$),* whereas CP-105,696, when administered orally at a dose of 10 mg/kg, tended to reduce oedema formation induced by PAF by 14% *($P > 0.05$)*. The animals pretreated orally with SR-27417 and CP-105,696 showed further inhibition of the PAF-induced oedema formation (50%, *$P < 0.001$).*

3.3.2. Effect of SR-27417 and/or CP-105,696 on oedema formation elicited by LTD_4 and substance P

To elucidate the role of PAF in contributing to the exudation of plasma elicited by others inflammatory mediators over a 30 min test period, we have investigated the effect of the selective PAF antagonist (SR-27417) on oedema formation. When LTD_4 was used as an agonist, pretreatment of rats with SR-27417 did not significantly reduce the oedema formation (24%, *$P > 0.05$)*. Similarly, there is

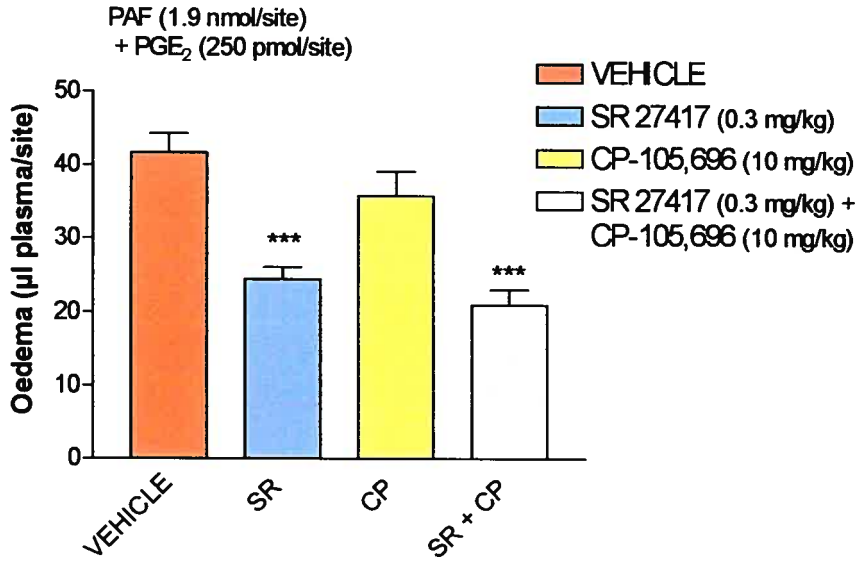


Figure 3.10. Effect of SR-27417 (0.3 mg/kg) and/or CP-105,696 (10 mg/kg) on PAF-elicited oedema formation

Data represent the mean ± SEM of 8-12 sites obtained from 4-6 rats.

no inhibitory effect of CP-105,696 (at a dose of 10 mg/kg) on the LTD_4-induced oedema **(Figure 3.11A)**. However co-administration of SR-27417 (0.3mg/kg) and CP-105,696 (10mg/kg) significantly reduced LTD_4-induced oedema (36%, $P < 0.01$).

Substance P (100 pmol/site), co-administered with PGE_2 (250 pmol/site), induced a small, but measurable, oedema in rat skin over a 30 min test period. As observed with LTD_4, the oedema induced by substance P was not significantly inhibited in rats pretreated orally with 0.3 mg/kg SR-27417 (28%, $P > 0.05$) **(figure 3.11B)**. In addition, CP-105,696 (at a dose of 10 mg/kg) did not affect the plasma leakage induced by substance P. Co-administration of the two drugs did not further reduce skin oedema elicited by substance P.

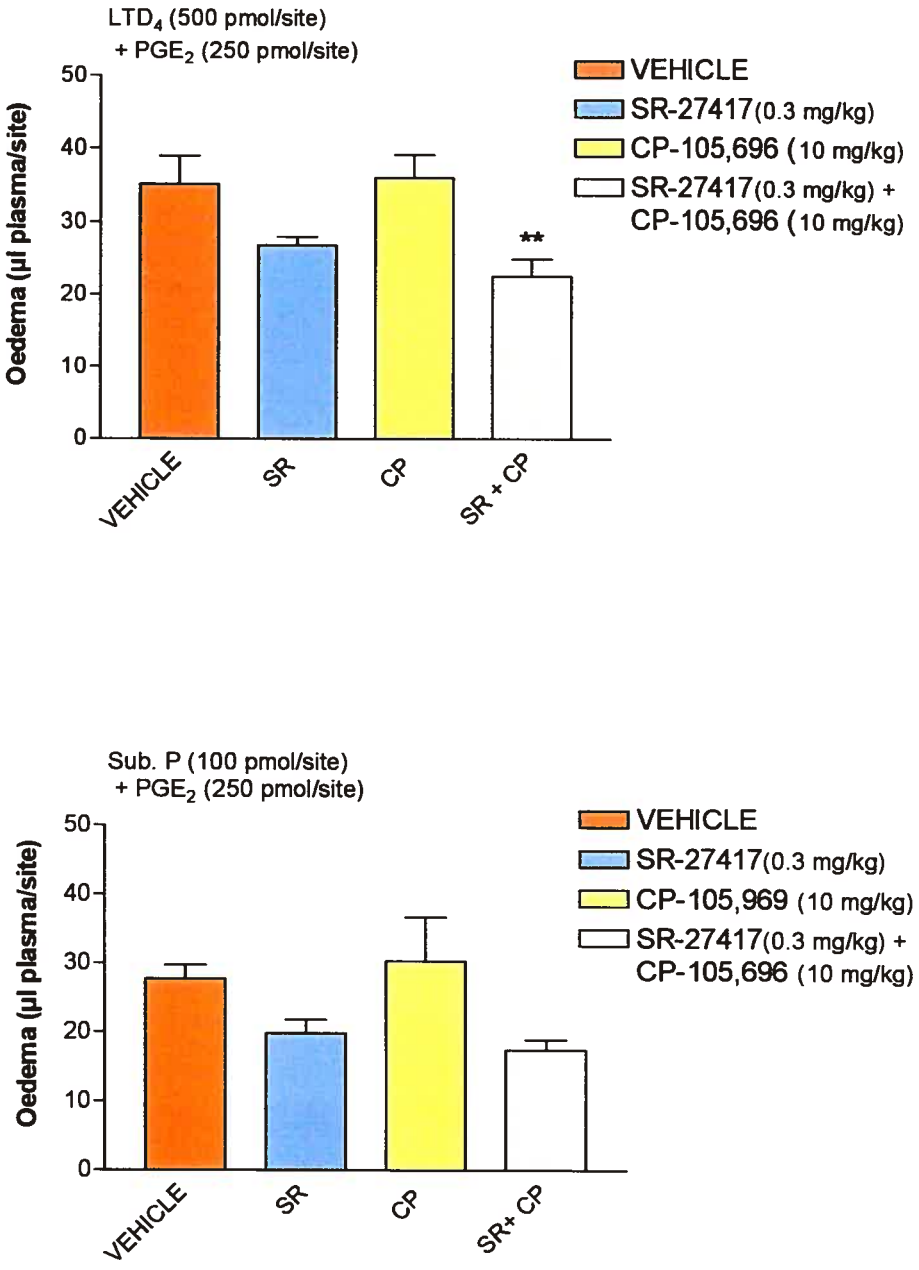


Figure 3.11. Effect of SR 27417 (0.3 mg/kg) and/or CP-105,696 (10 mg/kg) on LTD_4-elicited (A) & substance P-elicited (B) oedema formation
Data represent the mean ± SEM of 8-12 sites obtained from 4-6 rats.

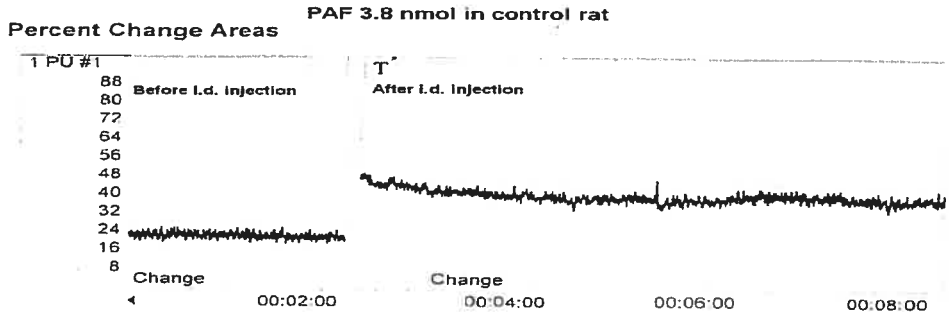
3.4. Effect of a PAF antagonist on the microcirculatory blood flow in rat skin

Local microcirculatory skin blood flow before the injection of either the agonist or vehicle was very similar, around 24 (arbitrary scale) as shown in **figure 3.12** and **3.13.** The intradermal injection of the vehicle (HBSS BSA 0.1%) induced a slight increase (20%) in skin blood flow. In rats, this trauma-induced increase in blood flow was transient and lasted for less than 3 min.

Figure 3.12 and **3.13** are representative examples of the vasodilator response to LTB_4 and PAF. Injection of LTB_4 i.d. at dose of 1000 pmol/site induced a stable vasodilatory response which lasted for 3-6 min, as observed by an increase in skin blood flow. Similar vasodilatory effect occurred when PAF (3.8 pmol/site) was injected i.d.

SR-27417 pretreatment inhibited blood flow elevation elicited by intradermally injected PAF (**figure 3.12**). The vasodilator effect of PAF (3.8 nmol) was reduced by ~ 48% at a 0.3 mg/kg dose of SR-27417 and by 58 % at a 1 mg/kg dose.

Figure 3.13 shows the effect of SR-27417 on LTB_4 (1000 pmol/site) induced elevation in blood flow. Interestingly, SR-27417 at a dose of 0.3 mg/kg also reduced the skin blood induced by LTB_4 by 35 %.



Mean value channel 1 : PU #1

Item	Area 1	Area 2
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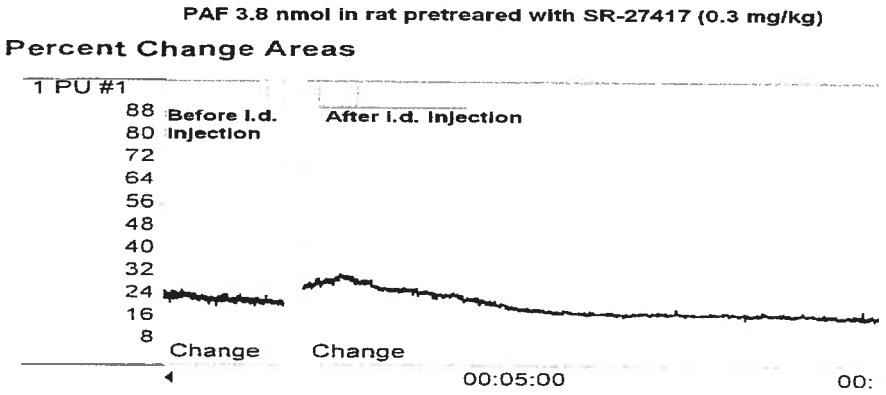

Mean value channel 1 : PU #1

Item	Area 1	Area 2
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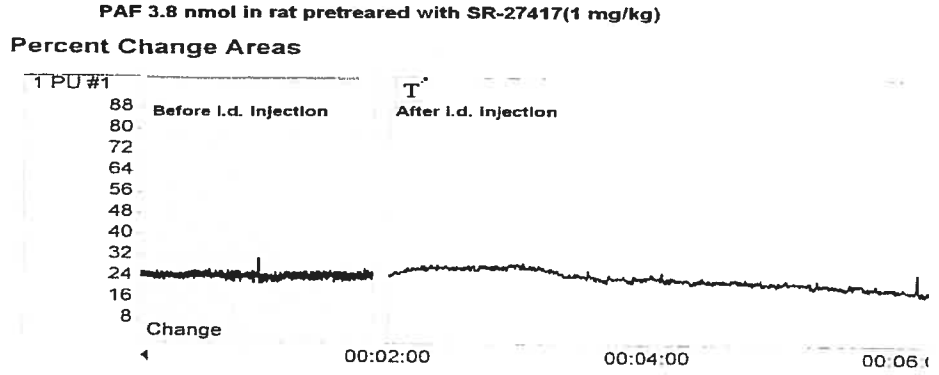

Mean value channel 1 : PU #1

Item	Area 1	Area 2
Mean value	24,30	16,28

Figure 3.12. Representative tracing of the vasodilator responses to PAF and the inhibitory effect of SR-27417

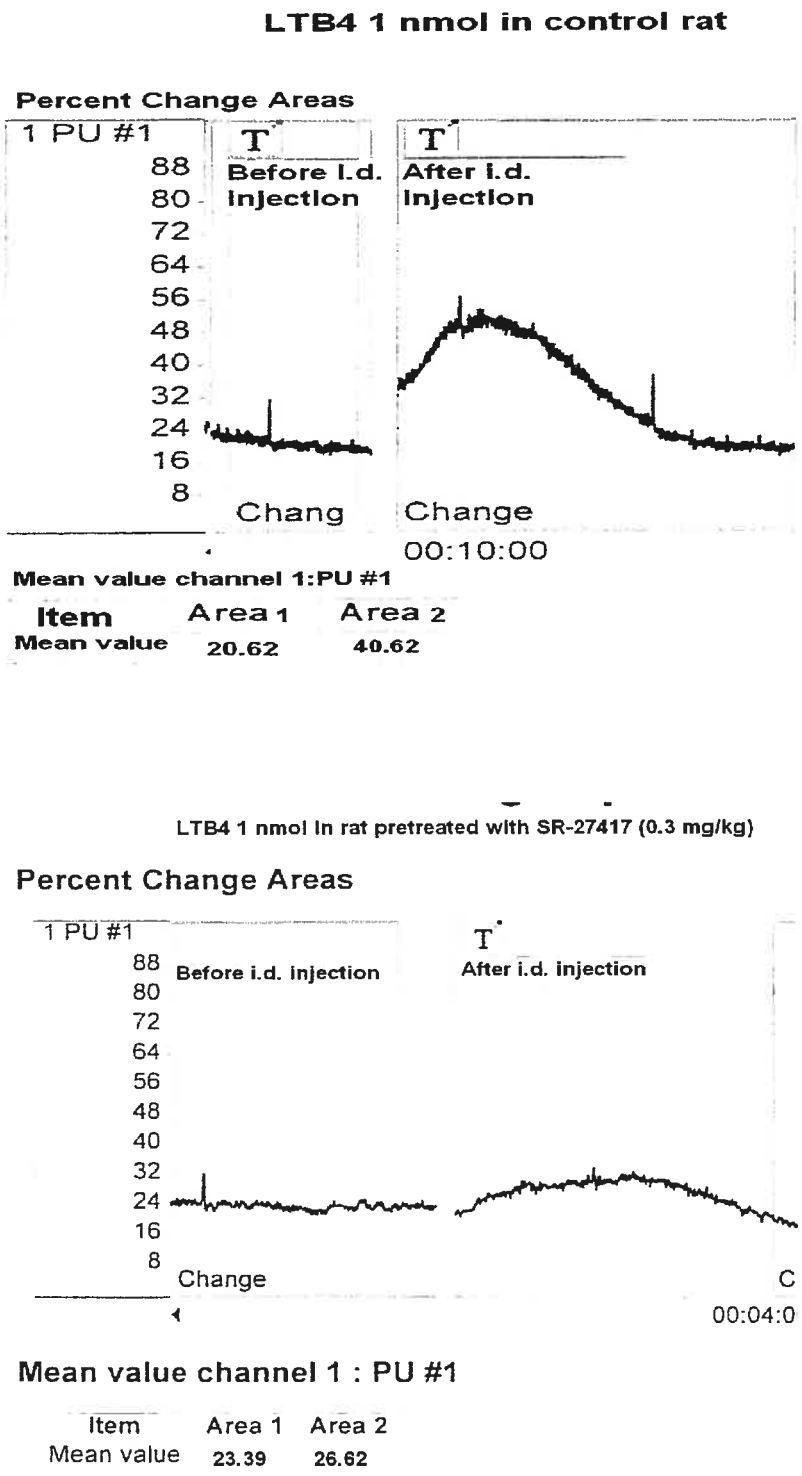

Figure 3.13. Representative tracing of the vasodilator response to LTB_4 and the effect of SR-27417

CHAPTER IV: GENERAL DISCUSSION

4.1. Problem situation

The local release of chemotactic and stimulatory factors triggers the molecular events that drive the migration of PMNs to the site of inflammation. These factors include chemokines, cytokines, complement-derived protein fragments, lipid mediators and bacterial products such as formylated peptides which coordinate the cytoskeletal rearrangements and adhesion changes essential for effective cell motility.

Lipid mediators play a pivotal role in regulating PMN extravasation at the blood endothelium interface, by regulating, for instance, vascular permeability, vasodilation, and/or endothelial cell hyperadhesiveness for neutrophils (Marleau et al., 1999). Bioactive lipid mediators including, LTs and PAF, are produced by the invading PMN themselves.

We have proposed that lipids mediators, particularly LTB_4, LTD_4 and PAF participate in PMN activation by interacting with PMN themselves (autocrine activation) and/or with the endothelium to stimulate cellular events that promote and facilitate the transmigration process of PMNs. Blocking of the activity of one of these mediators has been shown to exert a significant, but only partial, effect on transmigration. We propose that the concommittant blockade of the activity of PAF and LTB_4 may have a synergistic or additive effect and a more profound impact on PMN migration. Different experimental approaches were developed to confirm our hypothesis and delineate the role of PAF and LTB_4 in neutrophil trafficking.

4.2. Methodology considerations

The experimental model used in our study is dermal inflammation in the male Sprague-Dawley rat (Charles River, Québec). The model is very convenient for i.d. injections of the chemotatic factors and the study of PMN migration to the site of inflammation. One of the advantages is that we can perform many injections in the rat dorsal skin and consequently, we can test various agonists in the same animal.

Rabbits could have been useful for this purpose but the rat is an economical model. In addition, the LTB_4 antagonist (CP-105,696) used in our protocol is active in rats and not in rabbits (Showell et al., 1995).

Rats were injected with G-CSF (filgrastim or Neupogen^{MD}) at a dose 5 μg/kg for 9-11 days before doing the experiment. G-CSF was injected to increase the number of circulating PMN in the rats. When we started our studies we did not use G-CSF and we observed that the number of circulating PMN was very low. We then followed the kinetics of blood PMN accumulation using G-CSF and found that there was a 5 to 6-fold increase in blood PMN numbers, most of which were presumably released from the bone marrow following G-CSF treatment (Valente et al., 2002). The increase in circulating PMNs in response to G-CSF injection in rats was associated with a proportional increase in the local accumulation of PMN to chemoattractants, allowing us to assess the effect of drugs on agonist-induced PMN accumulation in a quantitative manner.

To study the role of PAF and LTB_4 in regulating neutrophil trafficking in response to locally injected chemoattractants, we have used two selective PAF antagonists, UK-74,505 and SR-27417 and one LTB_4 antagonist, CP-105,696 (the only selective LTB_4 antagonist available for *in vivo* studies). BIIL 284, a potent and selective LTB_4 antagonist that has recently been made available by Boehringer Ingelheim, Germany will be used in future studies.

Drugs were given orally to the rats, as this mode of administration is noninvasive in contrast to i.v. administration. The optimal doses of the drugs were chosen according to our preliminary experiments. For instance, CP-105,696 has been administered orally at a dose 10 and 30 mg/kg in a suspension of 0.5% CMC 16 h prior to the i.d. injections of agonists , because of the extremely long half-life of the drug in this species ($t_{½}$ = 16-18 h), (Showell et al., 1995). In contrast, UK-74,505 and SR-27417 were administered orally 2 h before the i.d injections of agonists due to the short half-life of these drugs. SR-27417 for example, was absorbed optimally 3 h

after oral administration in mice and rabbits (Herbert et al., 1991; Herbert et al., 1992). These drugs were given at relatively lower doses than for CP-105,696 (up to 1 mg/kg for SR-27417 and 5 mg/kg for UK-74,505). The apparent lower potency of CP-105,696 may be attributed, at least in part, to the high level of protein binding of the compound in whole blood (Showell et al., 1998). High plasma protein binding is also likely to contribute to the remarkably long plasma half-life of CP-105,696, seen in both animals and human phase I clinical studies (Liston et al., 1998; Showell et al., 1998).

In order to confirm our observations, we have used two selective, chemically unrelated PAF antagonists; UK-74,505 and SR-27417. The potency of the two drugs is slightly different. SR-27417 has been shown to be a highly potent, specific and competitive PAF receptor antagonist that is about 120 times more potent than WEB-2086, another PAF receptor antagonist, *in vivo*. In contrast, UK-74,505 was 10-30 times more potent than WEB-2086 as an inhibitor of PAF-induced hypotension in rats (Herbert et al., 1992; Parry et al., 1994).

A skin bioassay model has been used to determine PMN accumulation in response to intradermally injected agonists, including chemokines, cytokines and LPS as described previously (Marleau et al., 1996; Marleau et al., 1999). Different methods can be used to assess PMN accumulation in a quantitative manner. One of these methods requires the use of radioactive *labeled PMNs*, harvested from a donor animal, and radiolabeled before injection into a recipient animal. For example, ^{51}Cr-labeled PMNs and ^{111}In-labeled PMNs were used to determine PMN accumulation in different species like rabbits (Marleau et al., 1999), and rats (Sanz et al., 1995). Alternatively, we may use an *enzymatic marker*, such as the MPO content of PMNs to quantify PMN accumulation. In this proposal, we have assessed PMN accumulation using the MPO assay to minimize the number of animals to be used (no donor animals for cell labeling). In addition, the MPO assay is a simple and reproducible technique (Kitahara et al., 1979), economic when compared to the use of

radioisotopes, and that limits radioactive waste products. Moreover, PMN-labeling with $^{51}CrO_4$ has been found to be quite variable and unreliable (Stark et al., 1992).

Local oedema formation elicited by locally injected chemoattractants was measured by the Evans blue assay. Briefly, Evans blue injected i.v. binds to plasma albumin, and extravasate with albumin at the sites of increased vascular permeability, appearing as a blue dot on the dorsal skin of the rat. Evans blue is extracted from the skin sites with DMF, and quantified by measuring the absorbance at 630 nm (with reference wavelength at 450 nm) (Takano et al., 1998). Work in different laboratories has assessed local skin oedema in different species including rats, rabbits and guinea-pigs by the i.v. injection of human serum albumin (HAS) labeled with ^{125}I (Sanz et al., 1995; Norman et al., 1997; Teixeira et al., 1994), or labeled with ^{99m}Tc (in domestic pigs) (Chan and Ford-Hutchinson, 1985). Alternatively, other groups have used Evans blue extravasation to assess, either qualitatively or quantitatively, skin oedema (Flower et al., 1976; Souza et al., 2000; Souza et al., 2002; Souza et al., 2003). In our studies, we have used Evans blue as a marker of protein extravasation in order to limit radioactive waste products and also because the radioactive materials are much more expensive.

Intradermally injected chemotactic mediators increase vascular permeability as a result of activation and accumulation of PMN (Wedmore and Williams, 1981; Bjork et al., 1982). This effect usually is potentiated by PGE_2. All agonists were co-injected with PGE_2 (250 pmol), used as a vasodilator in order to enhance PMN extravasation to the skin (Movat et al., 1984; Wedmore and Williams, 1981), which may be relatively low in rat species compared with other species such as rabbits (Marleau S. unpublished observations).

4.3. Role of LTB_4 in PMN accumulation at cutaneous sites

The intradermal injection of LTB_4 into guinea pig dermis has previously been shown to induce time- and concentration-dependent infiltration of neutrophils (Fretland et al., 1989; Pettipher et al., 1993; Showell et al., 1995). This observation has been confirmed in the rat model. In addition, LTB_4- induced PMN accumulation was reduced in a dose-dependent manner by the selective LTB_4 antagonist, CP-105,696 **(figure 3.1)**.

In the present studies, CP-105,696 was used as a pharmacologic tool to investigate the role of LTB_4 in PMN accumulation and oedema formation elicited by a variety of inflammatory mediators including LTB_4. Our results showed that the oral administration CP-105,696, at doses of 10 and 30 mg/kg, significantly and dose-dependently inhibited LTB_4-elicited PMN accumulation and tended to reduce PAF-induced PMN accumulation (by ~30%) as shown in **figures 3.4** and **3.6.**

Previous observations supported a role for 5-LO products in the local accumulation of neutrophils in dermal inflammation (Marleau et al., 1999). For instance, PMN accumulation in rabbit skin was significantly reduced by a pretreatment of animals with MK-0591 (a 5-LO product synthesis inhibitor). Goldman et al. (1990) also showed that an i.v. pre-treatment of rabbits with the LO inhibitor diethylcarbamazine prevented PMN accumulation into skin blisters filled with either LTB_4 or with plasma collected from the ischemic hindlimbs of rabbits which contained elevated levels of LTB_4.

In addition, a role for LTB_4 in stimulating neutrophil chemotaxis in different pathologic contexts is supported by a number of studies. For example, Turner et al. (1996) found that the *in vivo* treatment of monkeys with CP-105,696 significantly reduces the increase number of BAL neutrophils after *Ascaris suum* antigen challenge. Griffiths et al. (1995) also studied the role of LTB_4 in the progression of collagen-induced arthritis and showed a striking efficacy of CP-105,696 in a murine

model. Showell et al. (1995) reported that CP-105,696 inhibited PMN (and eosinophil) infiltration in guinea pig skin induced by LTB_4, arachidonic acid and by the monooxygenase product 12(R)-HETE (which also acts at the BLTR) in a dose-dependent manner, with an ED_{50} of 0.3 ± 0.1 mg/kg p.o.

In order to delineate the role of LTB_4 in mediating or regulating PMN accumulation elicited by various chemically unrelated chemotactic stimuli including TNF-α, IL-8 and ZAP, we pretreated the animals with the CP-105,696 compound. Our results showed that CP-105,696 significantly reduced PMN accumulation elicited by TNF-α by *42% ($P < 0.05$)* and tended to reduce ZAP-induced accumulation, but did not significantly reduced the accumulation induced by IL-8 **(figures 3.8** and **3.9).** All these studies were performed in a single animal species, the rat, and it cannot be excluded that similar experiments performed in other species may yield slightly different results. For example, in previous studies, Dr. Marleau (1999) has shown that IL-8-induced PMN migation in rabbit can be significantly reduced by treatment with a leukotriene biosynthesis inhibitor or following desensitization of LTB_4 receptors, suggesting that in the rabbit, in contrast to the rat, LTB_4 seems to play a role in IL-8-induced PMN migration. TNF-α may induce neutrophil accumulation in part via the release of LTB_4. Indeed, previous studies showed that in a model of ovalbumin-sensitized mice, the i.p. administration of TNF-α induced neutrophil migration in a LTB_4-dependent manner, inasmuch as PMN accumulation was inhibited by 86% after pretreatment of the mice with MK-886 (a LTB_4 synthesis inhibitor) (Canetti et al., 2001).

Our results support an important role for LTB_4 in mediating neutrophil accumulation in dermal inflammation, in agreement with observations in other inflammatory models such as in a model of transient ischemia and reperfusion injury of the superior mesenteric artery in rats, which showed that post-ischemic treatment with CP-105,696 inhibited neutrophil accumulation in the intestine and mesentery, suggesting that LTB_4 may play an important role in mediating neutrophil-dependent local ischemia and reperfusion injury (Souza et al., 2000). However, the relative

importance of LTB_4 contribution to the tissue injury in this model is still uncertain (Souza et al., 2002).

4.4. Role of PAF in PMN accumulation and oedema formation at cutaneous sites

In the present study, the long-acting and selective PAF receptor antagonist, UK-74,505 (Alabaster et al., 1991) was employed to investigate the possible contribution of endogenous PAF to PMN accumulation in rat skin in response to soluble mediators.

Oral administration of UK-74,505 reduced PMN accumulation elicited by intradermally–injected PAF (51% $P < 0.05$), and tended to reduce LTB_4 and ZAP-induced PMN accumulation **(figures 3.4** and **3.9).** However, this PAF antagonist did not prevent PMN accumulation induced by TNF-α and IL-8 **(figure 3.8)**.

In agreement with our results, Sanz et al. (1994) reported that i.v. UK-74,505 blocked neutrophil and eosinophil accumulation and oedema formation induced by PAF in guinea pig skin but had no significant effect on the responses elicited by LTB_4 or ZAP.

In a previous study, it has been shown that while PAF caused little or no neutrophil accumulation in rabbit skin, it caused marked oedema formation, particularly when co-injected with PGE_2 (Norman et al., 1997). Interestingly, only oedema was reduced by pretreatment of the animals with UK-74,505. These findings are not consistent with our data and with data from other studies showing that the PAF antagonist, L-659,989, reduced neutrophil accumulation in reversed passive Arthus (RPA) reaction in rabbit skin (Hellewell, 1990). Norman et al. (1997) explained this finding by suggesting that PAF may be rapidly metabolized locally, and/or that the lack of effect of the antagonist on PMN accumulation may be due to

failure of the drug to reach the site of action at the appropriate time rather than a lack of bioactivity.

To further delineate the role of PAF in PMN accumulation and oedema formation in rat skin in response to different inflammatory stimuli, we also tested the effect of the potent and selective PAF antagonist SR-27417 on PMN accumulation and oedema formation elicited by PAF and other mediators. Interestingly, the results of our study confirmed our previous results with UK-74,505, inasmuch as orally administered SR-27417, at a dose of 0.3 mg/kg, significantly reduced PMN accumulation elicited by PAF by 53% *($P < 0.05$)* **(figure 3.6)** and tended to reduce ZAP- elicited PMN accumulation **(figure 3.7)**. No significant effect was observed on LTB_4-induced PMN accumulation. Yet, inhibition of PMN accumulation by the LTB_4 receptor antagonist alone inhibited up to 78-79% of LTB_4-induced PMN accumulation, which may have been too high to observe either an additive or a synergistic effect of both LTB_4- and PAF-receptor antagonists on PMN accumulation. Additional studies, using a lower dose of the CP-105,696 antagonist, may be necessary to resolve this issue.

Acute oedema being one of the most important initial feature of inflammation, we therefore examined the role of PAF in mediating agonist-induced oedema in rat skin by studying the effect of SR-27417 in protecting rats from agonists-induced oedema. As expected, SR-27417 (0.3 mg/kg) significantly reduced oedema formation elicited by PAF (41% *$P < 0.001$*) **(figure 3.10)**. SR-27417 was highly selective for PAF since it did not significantly altered oedema formation evoked by a range of other inflammatory mediators such as LTD_4 and substance P **(figure 3.11)**. In agreement with our data, Herbert et al. (1992) concluded that SR-27417 prevented the increase in vascular permeability associated with the local allergic reaction, confirming the role of PAF in this acute inflammatory reaction. Additional previous studies showed that SR-27417 has a modest inhibitory effect on the late asthmatic response, suggesting that PAF has a small role in allergic inflammation (Evans et al., 1997). In addition, a number of studies have implicated PAF as an important

mediator in an immune complex-mediated inflammation (RPA reaction) (Williams et al., 1986; Pons et al., 1993; Rossi et al., 1992). PAF also appears to play an important role in intestinal ischemia and reperfusion (I/R)-induced tissue injury, as shown in a model of intestinal I/R (Souza et al., 2003). In that model, use of either PAF receptor-deficient mice or PAF receptor antagonist attenuated tissue injury, suggesting a role for PAF in that model.

In contrast, other recent studies showed that the PAF receptor do not play a major role in lung injury secondary to pulmonary infection with gram negative bacteria *(Klebsiella pneumoniae)* in mice, inasmuch as inoculation of this bacteria induced an increase in the number of infiltrating neutrophils and in the generation of pro-inflammatory cytokines in the lung of infected mice that was not prevented by administration of the PAF receptor antagonist UK-74,505 suggesting that PAF, by acting on its receptor, do not play a major role in the local production of chemokines and recruitment of the leukocytes (Soares et al., 2002).

To summarize our observations on the role of PAF in mediating local oedema in response to different agonists, we could not show the role for PAF in contributing to the local oedema evoked by substance P and LTD_4. However, a number of studies have shown a role of PAF in different pathologic conditions, suggesting that additional studies should be performed with additional substances known to increase vascular permeability such as bradykinin, histamine and serotonin. It may also be that the contribution of PAF to local oedema in response to agonists may be related to the PMN extravasation process, and could be more apparent with agonists such as LTB_4. Unfortunately, we have not been able to elicit significant local oedema with up to 3 nmol/site of LTB_4 injected intradermally in rat skin.

4.5. Role of LTB_4 and PAF in PMN accumulation and oedema formation at cutaneous sites

The combination of PAF and LTB_4 antagonists has been employed to assess the co-operative effect of PAF and LTB_4 in PMN accumulation and oedema formation in dermal inflammation induced by a variety of agonists. Our results revealed that the co-administration of CP-105,696 and UK-74,505 had an additive inhibitory effect on PMN accumulation induced by PAF but not by LTB_4 **(figure 3.4)**. These results were confirmed by the co-administration of SR-27417 (0.3 mg/kg) and CP-105,696 **(figure 3.6)**. Interestingly, both agonists are synthesized within PMN and exert autocrine and paracrine effects on neutrophils and/or neighboring cells such as endothelial cells (McDonald et al., 1994). Our results suggest that both mediators exert additives effect in regulating PMN extravasation at the blood-endothelium interface, more particularly when PAF is used to elicit PMN accumulation. Our results are less conclusive for LTB_4-elicited PMN accumulation, but as mentioned before, it could be due to a bias in our experimental protocol (too high dose of CP-105,696). Additional studies are undergoing in our laboratories, using a lower dose of the CP compound as well as an additional selective LTB_4 receptor antagonist.

Other recent studies demonstrated a role of lipid mediators in hypoxia-induced microvascular inflammatory responses using PAF and LTB_4 antagonists. In contrast, no additive effect was observed in leukocyte emigration and the increase in vascular permeability when both PAF receptor antagonist (WEB-2086) and the LTB_4 receptor antagonist (LTB_4-dimethyl amide) were administered simultaneously (Casillan et al., 2003).

Administration of SR-27417 and CP-105,696 significantly attenuated PAF-induced PMN accumulation and vascular permeability, and both agents had a stronger effect on PMN accumulation **(figure 3.6A)** than on vascular permeability **(figure 3.10)**. This pattern may indicate that mediators other than PAF and LTB_4 are largely responsible for change in vascular permeability and/or that such permeability

changes may result from leukocyte-dependent and leukocyte-independent mechanisms.

To our knowledge, it is the first time that an additive effect for PAF and LTB_4 in inducing PMN accumulation is shown *in vivo*.

In summary, our findings support the previously proposed hypothesis that in activated PMN, PAF and/or LTB_4 biosynthesis represents an important amplification mechanism regulating PMN and plasma extravasation at the blood-endothelium interface as summarized in **figure 4.1** (Marleau et al., 1999). The mechanism of action PAF and/or LTB_4 depends on PMN chemoattractant receptor engagement which results in the activation of the 5-LO pathway. This involves a rapid Ca^{2+}-dependent redistribution of the enzyme from its location in resting cell to a nuclear membrane compartment after agonist activation. This process of translocation could reasonably be assumed to bring the enzyme to its membrane-derived substrate. cPLA2 acts to release arachidonate from the nuclear envelope phospholipids (Peters-Golden and Brock, 2000). Arachidonate binds to FLAP, an integral nuclear envelope protein, to facilitate its processing by 5-LO. This leads to LTA_4 biosynthesis which can be either metabolized to LTB_4 or released and used by other cells such as PMN, endothelial cells and platelets to form lipoxgynase products independently of the nature of chemoattractant involved **(figure 4.1)**. In agreement with such hypothesis, it has been reported that PMN responsitivity to chemoattractants is necessary for the emigration process *in vivo* (Nourshargh and Williams, 1990) and that PMN agonists (C5a, IL-8, FMLP, PAF and LTB_4 itself) stimulate LTB_4 biosynthesis in PMN (McColl et al., 1991; McDonald et al., 1992; Marleau et al., 1999). Alternatively, PMN agonists may also lead to PAF biosynthesis and secretion (Sisson et al., 1987; Bauldry et al., 1991; Watanabe et al., 2003). The LTB_4 and/or PAF generated may then ligate their receptors either on PMN (autocrine manner) or on endothelial cells, and trigger events that are important for transendothelial migration such as regulation of PMN-endothelium adhesive interaction or possibly endothelial cells retraction (Marleau et al., 1999). In this scenario, activation of 5-LO and ligation of PAF and

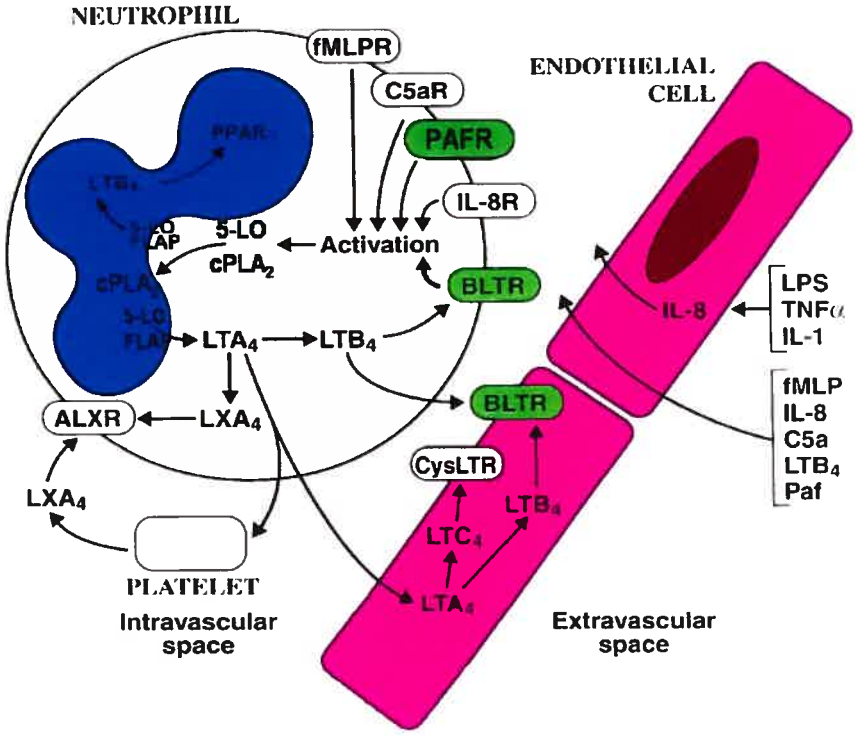

Figure 4.1. Hypothetical scheme of events for the involvement of 5-LO products and PAF in the regulation of PMN trafficking to inflammatory sites
R, receptor; BLTR, LTB_4 receptor; ALXR, LXA_4 receptor; CysLTR, cysteinyl LT receptor; $cPLA_2$, cytosolic phospholipase A_2; 12-LO, 12-lipoxygenase; 15-LO, 15-lipoxygenase; FLAP, 5-LO activating protein; A_4-H, LTA_4 hydrolase; C_4-S, LTC_4 synthase.

From **Marleau et al.** (1999) *Journal of Immunology*, 163: 3349–3458.

LTB_4 receptors represent common events in the mechanism of PMN emigration elicited by soluble chemoattractants. Consequently inhibition of 5-LO activation by blocking of the LTB_4 and/or PAF receptors would be expected to down-regulate PMN chemotaxis to most PMN agonists, in agreement with the data reported herein.

The results obtained in this work support a role for PAF and/or LTB_4 in regulating PMN extravasation elicited by a number of inflammatory mediators at the blood-endothelial interface. In addition, PAF and LTB_4 exert a co-operative effect in inducing PMN accumulation *in vivo*. These observations could be of potential value for future anti-inflammatory drug development.

4.6. Perspectives

We believe that the results obtained in these studies have provided a better understanding of the biological significance of lipid mediators, particularly PAF and LTB_4, in neutrophil trafficking. Future studies, using a different selective LTB_4 receptor antagonist, will help to clarify unresolved issues.

In addition, further studies are required to delineate the role of PAF and LTB_4 in mediating microcirculatory skin blood flow changes, elicited by a variety of agonists, using selective and highly potent PAF and LTB_4 antagonists.

4.7. Conclusion

In conclusion, this study provides novel information regarding the role of lipid inflammatory mediators in the regulation of neutrophil trafficking to inflammatory lesions.

We have been able to document the following findings:

1. LTB_4 is involved in PMN emigration and plasma extravasation elicited by LTB_4 and other soluble agonists (PAF, ZAP and TNF-α).
2. PAF is involved in PMN emigration and plasma extravasation elicited by PAF and other soluble agonists (ZAP).
3. PAF and LTB_4 may act co-operatively at the blood-endothelial interface to regulate PMN extravasation, inasmuch as PAF and LTB_4 receptor anatgonists exerted an additive effect in reducing PMN accumulation at dermal inflammatory sites in rats.
4. The chemotactic effect of PAF appears to involve, at least in part, 5-LO activation and BLT receptor signaling on circulating neutrophils and/or endothelial cells.

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