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5-OXO-ETE AND ITS EFFECT ON EOSINOPHIL RECRUITMENT IN THE BROWN NORWAY RAT LUNG

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August 1998

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of Masters of Science

ABSTRACT

The 5-lipoxygenase product 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is a potent eosinophil chemoattractant *in vitro*. To determine whether it is active *in vivo*, 5-oxo-ETE was administered intratracheally to BN rats and pulmonary eosinophils were immunostained with an antibody to major basic protein. 5-Oxo-ETE induced a dramatic increase in eosinophils, which reached maximal levels (5 times control) between 15 and 24 h following administration, and thereafter declined. LTB_4 had a similar effect to 5-oxo-ETE but appeared to be somewhat less effective. In contrast, LTD_4 and LTE_4 were inactive. 5-Oxo-ETE-induced eosinophilia was inhibited by 75% following pretreatment of the animals with antibodies to integrins VLA-4 or LFA-1, but was not significantly inhibited by an antibody to Mac-1, nor after pretreatment with receptor antagonists to LTB_4 (LY255283) or PAF (WEB 2170). These observations raise the possibility that 5-oxo-ETE may be an important physiological mediator in inflammatory diseases characterized with eosinophil recruitment, such as asthma.

RESUME

L'acide 5-oxo-6, 8, 11, 14-eicosatétraenoique (5-oxo-ETE) est un facteur chimiotactique très puissant pour les eosinophiles *in vitro*. Pour déterminer s'il est actif *in vivo*, 5-oxo-ETE a été administré par insufflation de la trachée chez des rats. Les eosinophiles pulmonaires ont été immuno-marqués avec un anticorps contre la protéine basique majeure et comptés. 5-oxo-ETE a induit une augmentation dramatique d'eosinophiles qui a atteint des niveaux maximales (5x contrôle) entre 15 et 24 hres suivant l'administration, pour ensuite décliner. Le LTB_4 a eu un effet similaire au 5-oxo-ETE, mais le 5-oxo-ETE a induit une réponse maximale significativement plus élevée. Au contraire, le LTD_4 et le LTE_4 étaient inactifs. L'augmentation d'eosinophiles induite par le 5-oxo-ETE a été inhibée d'environ 75% à la suite d'un pré-traitement des animaux avec les anticorps contre les intégrines VLA-4 ou LFA-1, mais n'a pas été inhibée de façon significative par l'anticorps contre Mac-1 ni par les antagonistes des récepteurs du LTB_4 (LY255283) ou de PAF (WEB 2170). Ces observations soulèvent la possibilité que le 5-oxo-ETE puisse être un médiateur physiologique important dans la réponse inflammatoire de l'asthme.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank a number of colleagues and friends whose support and advice have directly influenced the content of the present study.

My largest debt of gratitude goes to Dr. W.S. Powell, my research director, for welcoming me into his group and giving me the opportunity to participate in an exciting and very interesting research project. I am especially grateful for his close supervision, invaluable support and instructive advice throughout my studies. His dedication to research excellence and innovative thinking are qualities I will strive for in my future research career.

I am also very grateful to Dr. J.G. Martin for giving me the opportunity to pursue my graduate studies within a very stimulating environment of dedicated and excellent researchers. I cannot thank him enough for his support and encouragement.

I would like to thank all the Meakins Christie directors who have offered their support and advice during my studies. In particular, I would like to thank Dr. Q. Hamid for his substantial contribution in this study, his helpful advice and for always being there when help was needed. I also appreciated his patience and tolerance with me every time I presented him with yet another tissue slide to be scored. He always managed to keep his sense of humor and I am especially grateful to him for that.

My work mates, Sylvie Gravel, Jian-Hua Zhang, Mary Zacour, Petra Turkewitsch, Florence Tao and Barbara Tolloczko were remarkable. I thank them for their help, the scientific exchanges and, most importantly, for their moral support and friendship. I would also like to thank Rosa Pantano and Christine Mallette for their help with handling rats, as well as, Ms. Liz Milne for her secretarial assistance. My warm thanks are also extended to Dr. G. Hatzakis for his wizardry with Microsoft Power Point and his invaluable help with the preparation of figures presented in this thesis.

Finally, I would like to acknowledge the loving support of my family.I want to thank my dear parents, Sofia and Peter, as well as Dino, Perry and Debby for their patience and understanding during the course of my study and preparation of this thesis.

PREFACE

During my MSc studies at the Meakins Christie Laboratories I have contributed to the following manuscripts and abstracts:

A) Manuscripts

1. Stamatiou P, Hamid Q, Taha R, Yu W, Issekutz TB, Rokach J, Khanapure SP, Powell WS. 5-Oxo-6,8,11,14-eicosatetraenoic acid stimulates eosinophil infiltration into the lungs of Brown Norway rats in an integrin-dependent manner. Journal of Clinical Investigation 1998;102 (12):2165-2172.
2. Powell WS, Gravel S, Stamatiou P, Macleod RJ. Effects of 5-oxo-ETE on neutrophils, eosinophils and intestinal epithelial cells. Advances in Prostaglandin, Thromboxane, and Leukotriene Research. 1995;23:431-433.

B) Abstracts

1) Stamatiou P, Hamid Q, Song Y, Yu W, Powell WS. 5-Oxo-ETE effects on eosinophil recruitment in the lung. 1997 American Thoracic Society - San Francisco, CA.
2) Stamatiou P, Hamid Q, Song Y, Yu W, Powell WS. 5-Oxo-ETE effects on eosinophil recruitment in the lung. 1996 ASMB/ASIP/AAI Joint Meeting - New Orleans, LA.
3) Powell WS, Gravel S, Stamatiou P, Macleod RJ. Effects of 5-oxo-ETE on neutrophils, eosinophils and intestinal epithelial cells. International Conference on Prostaglandins and Related Compounds - Florence, Italy 1994.

May the reader take note that I have chosen to present this thesis in a traditional format. Furthermore, I have chosen the option of presenting the data on a full, unnumbered page with figure legends on the same page. As concerns my contribution to the work presented herein, I am responsible for all the experimental work described except for the scoring of the tissue and the experiment represented in Figure 3.

TABLE OF CONTENTS

ABBREVIATIONS

AA	arachidonic acid
ANOVA	analysis of variance
APAAP	alkaline phosphatase anti-alkaline phosphatase
BAL	bronchoalveolar lavage
B cell	B lymphocyte
BN rat	Brown Norway rat
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
Cys-LTs	cysteinyl leukotrienes
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CR4	complement receptor-4
ECF-A	eosinophil chemotactic factor of anaphylaxis
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
E-selectin	endothelial selectin
EPO	eosinophil peroxidase
EPR	early phase response
FLAP	5-lipoxygenase activating protein
FMLP	N-Formyl-Met-Leu-Phen
GM-CSF	granulocyte macrophage colony stimulating factor
HETE	5-hydroxy-6,8,11,14-eicosatetraenoic acid
5-HETE	5-hydroxy-6,8,11,14-eicosatetraenoic acid
5-HPETE	5-hydroperoxy-6,8,11,14-eicosatetraenoic acid
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ

Ig	immunoglobulin
IL	interleukin
LAM-1	leukocyte adhesion molecule-1 (L-selectin)
LFA-1	lymphocyte function related antigen-1 (CD11a/CD18)
5-LO	5-lipoxygenase
LPR	late phase response
L-selectin	leukocyte-selectin
LTs	leukotrienes
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
LXs	lipoxins
mAb	monoclonal antibody
Mac-1	macrophage-1 (CD11b/CD18)
MadCAM-1	mucosal addressin cell adhesion molecule-1
MBP	major basic protein
MCP	monocyte chemotactic peptide
MIP-1α	macrophage inflammatory protein-1α
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NK cells	natural killer cells
OVA	ovalbumin
5-oxo-ETE	5-oxo-6,8,11,14-eicosatetraenoic acid
PAF	platelet activating factor
PECAM-1	platelet endothelial cell adhesion molecule-1
PGD_2	prostaglandin D_2
PGs	prostaglandins
PLA_2	phospholipase A_2

PMA	phorbol myristate acetate
PMNL	polymorphonuclear leukocytes
P-selectin	platelet-selectin
RANTES	regulated upon activation in normal T cells expressed and secreted
SLe^x	sialyl Lewis X blood antigen
SRS-A	slow reacting substance of anaphylaxis
T cell	T lymphocyte
Th1	T helper type 1
Th2	T helper type 2
TNF-α	tumor necrosis factor-α
TXA_2	thromboxane A_2
TXs	thromboxanes
VCAM-1	vascular adhesion molecule-1
VLA-4	very late antigen-4 (CD1149d/CD29)

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction - Aim of Study

The discovery and characterization of biologically active metabolites of arachidonic acid (AA), which has now spanned almost seven decades of industrious research, has produced many new insights into the role of lipid mediators in the body. In the beginning of 1930's two seemingly unrelated discoveries were reported. Kurzrok and Lieb performed studies on the action of semen on the uterus and found both contraction and relaxation of tissue specimens. At the same time Harkavy (1) found that alcohol-soluble extracts of sputum from allergic asthmatic patients contained an agent which provoked spasm of cat and rabbit intestines *in vitro*. These two discoveries might be regarded as the very beginning of research on AA metabolism which ultimately lead to the discovery of prostaglandins (PGs), thromboxanes (TXs), lipoxins (LXs) and leukotrienes (LTs).

Oxidative metabolites of AA, which are generally termed eicosanoids, are among the most ubiquitous mediators produced in the human body by various cells following their activation. Consequently, there are virtually no organs which are not affected by eicosanoids in one way or the other. For example the lung, which consists of a diversity of cell types, can produce and is affected by almost all classes of eicosanoids, including PGs, TXs, LXs, LTs, and monohydroxy-eicosateraenoic acids (HETEs) (2,3). The two major eicosanoid pathways are the cyclooxygenase (COX) pathway, which is responsible for PG and TX formation, and the 5-lipoxygenase (5-LO) pathway, which produces LTs and 5-HETE. The focus of this thesis will be on the latter pathway.

The discovery of the 5-LO pathway was the result of the combination of two separate lines of research, namely the metabolism of AA by polymorphonuclear leukocytes (PMNL) and the characterization of an unknown mediator of anaphylaxis. Although the latter compound, termed "slow-reacting substance of anaphylaxis" (SRS-A), was implicated in immediate hypersensitivity reactions (4), little was known of its structure. It was not until the late 1970s that AA was identified as a precursor of SRS-A (5).

Meanwhile, Borgeat and Sammuelson reported that rabbit leukocytes can convert AA to 5-HETE (6) and to LTB_4 (7). They subsequently showed that LTB_4 is synthesized from an unstable intermediate, LTA_4, which displayed an absorption spectrum reminiscent to that of SRS-A. It is now known that the biological effects first attributed to SRS-A were due to the combined activities of the cysteinyl leukotrienes (cys-LTs), LTC_4, LTD_4 and LTE_4, whose precursor is also LTA_4 (8). Since then, further investigations on the major 5-LO metabolites such as, 5-HETE, LTB_4, and the cys-LTs, have shown that they are all biologically active, albeit 5-HETE less so than the LTs. In general they are important mediators in cells of the immune system, allergic reactions, and inflammation. They have potent effects in the lung and appear to be important mediators in asthma.

Recently, Powell et al., identified a highly specific dehydrogenase in neutrophils which converts the 5-LO product 5-HETE to the novel eicosanoid 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) (9). *In vitro* studies demonstrate that 5-oxo-ETE is a potent agonist for both neutrophils and eosinophils. Experiments testing the effects of various lipid mediators and their chemotactic activity on human eosinophils, showed that 5-oxo-ETE is the most potent eosinophil chemoattractant among this class of substances (10) . In view of its novelty and its potent *in vitro* effects on eosinophils it was important to further characterize 5-oxo-ETE biologically. The aim of this study was to investigate the *in vivo* effects of this recently discovered lipid eosinophil chemoattractant.

There is an abundance of data in the literature to support a role for 5-LO products in eosinophil infiltration *in vivo*, suggesting that members of this class may act along with other mediators, such as cytokines and chemokines, in regulating eosinophil migration. The most important finding of the present study is that 5-oxo-ETE is the most effective 5-LO metabolite in inducing eosinophil recruitment *in vivo*. This is the first report that 5-oxo-ETE has biological effects *in vivo* and raises the possibility that this compound may be an important physiological mediator of eosinophil infiltration in diseases in which these cells are a distinctive feature, such as asthma.

To fulfill the aim of this thesis, background information essential to the understanding of issues involved in both the rationale and the interpretation of the current study will first be presented in Chapter 1. In particular, this chapter will deal with 5-LO products (their biosynthesis and biology) as well as mechanisms involved in eosinophil recruitment into tissue and more specifically into the airways. Chapters 2 (Materials and Methods) and 3 (Results) will deal with the present investigation: 5-oxo-ETE and its effect on eosinophil recruitment *in vivo*. Finally Chapter 4 will present a critical discussion on the present experimental findings in terms of other relevant investigations and conclude with some directions for future studies.

1.2 Biosynthesis of PAF and 5-Lipoxygenase Products

The rate-determining step in the formation of eicosanoids is the release of AA from membrane lipids by phospholipase A_2 (PLA_2). Once AA has been released, it is rapidly converted to oxygenated products in most cells by various intracellular enzymes. The specific products which are synthesized depends on which enzymes are present in the type of cell in question. The focus of this thesis will be on the synthesis of 5-LO products and platelet activating factor (PAF) which are believed to play a role in in the inflammatory process and leukocyte recruitment into tissue (Fig.1).

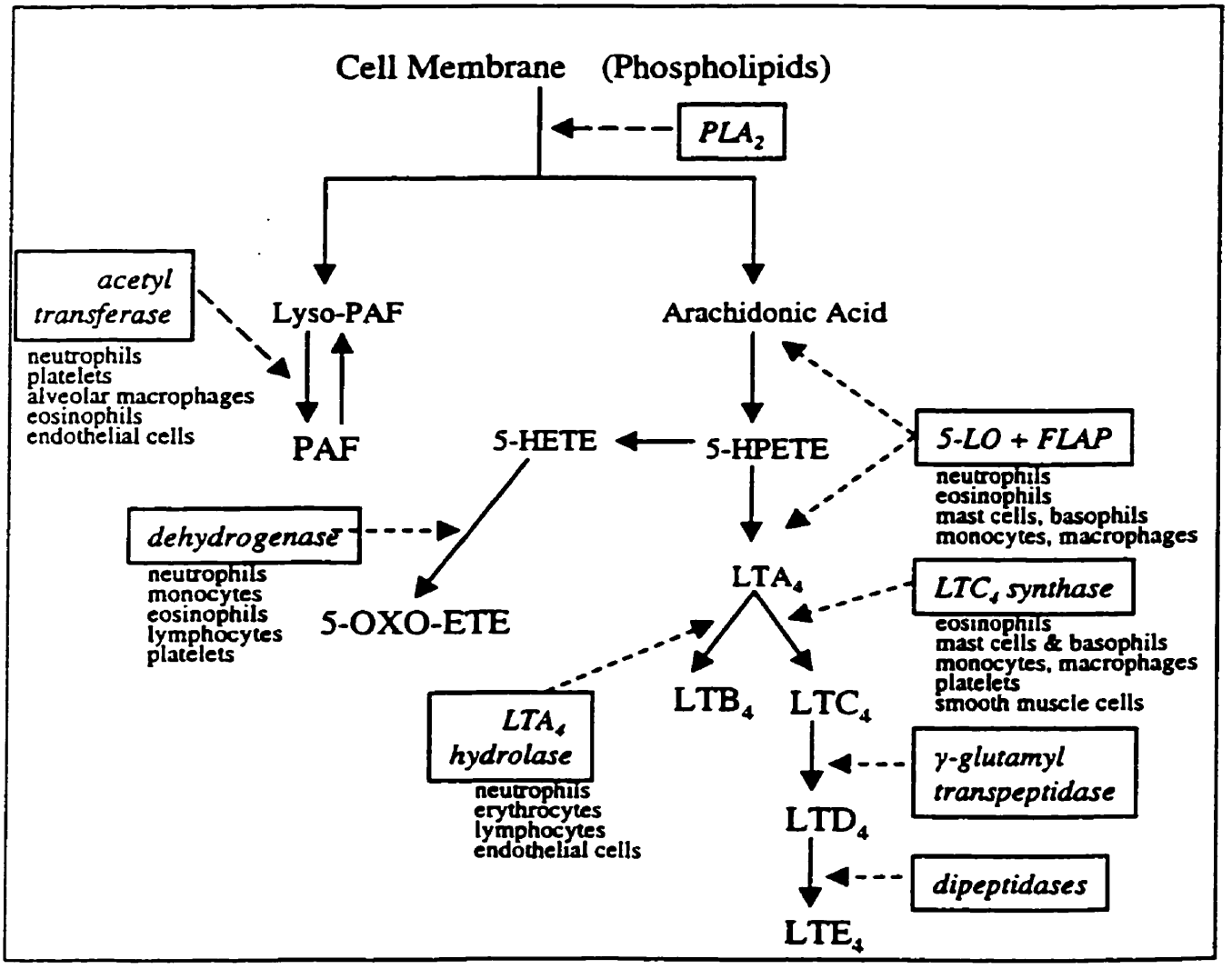

Fig. 1. Simplified scheme of the biosynthesis of PAF and 5-LO products. Enzymes are in boxes.

1.2.1 Formation of PAF

AA is stored in cell membranes esterified in the sn-2 position of various diacyl and alkyl-acyl phospholipids. The most abundant AA and PAF precursor in leukocytes is 1-palmityl-2-arachidonoyl-phosphatidylcholine. The release of AA and PAF is initiated by stimuli which raise intracellular calcium levels and in turn stimulate cytosolic PLA_2 ($cPLA_2$) translocation to the membrane. The enzyme $cPLA_2$ catalyzes the hydrolysis of the ester linkage in the sn-2 position of the glycerol to produce AA and lyso-PAF. The free AA can then be metabolized by the 5-LO pathway. The lyso-PAF is in turn acetylated by a rate limiting acetyltransferase enzyme to produce the active PAF (11). The half-life of PAF is very short (less than 1 min) and it is metabolized by removal of the acetyl group to produce its inactive precursor, lyso-PAF. This two step enzymatic process, known as the 'remodeling' pathway of PAF synthesis, predominates in inflammatory cells (12). PAF is synthesized by a variety of inflammatory cells including neutrophils, platelets, alveolar macrophages, eosinophils and vascular endothelial cells. In some animal species, but not humans, PAF can be synthesized and released by mast cells (13).

1.2.2 Formation of Leukotrienes

5-LO, in conjuction with other enzymes, produces a series of biologically active eicosanoids including the LTs, HETE and 5-oxo-ETE. In the biosynthesis of LTs, free AA is presented by an integral perinuclear membrane protein, 5-lipoxygenase-activating protein (FLAP) (14), to 5-LO, which translocates to the nuclear membrane upon activation. The 5-LO then catalyzes the first two steps of the pathway (5): oxygenation of AA to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and the conversion of the latter to the unstable epoxide intermediate LTA_4 (15). The fate of LTA_4 is determined either by cytosolic metabolism via LTA_4 hydrolase, to LTB_4 (16), or conversion by the integral membrane protein LTC_4 synthase (17) to LTC_4 by addition of glutathione. The subsequent conversion of LTC_4 to LTD_4, a cysteinyl glycinyl derivative, is via the action of γ-glutamyl transpeptidase. LTD_4 is further metabolized to the cysteinyl derivative, LTE_4, by the action of a dipeptidase. LTs are rapidly metabolized and removed from the circulation. Cys-LTs undergo oxidation, resulting in biliary and urinary elimination of

biologically less active and inactive metabolites. LTE_4, which is usually less active biologically than LTC_4 and LTD_4, is an important urinary metabolite that can be used to monitor the production of cys-LTs in man (18).

The location of LT synthesis is determined by the cellular distribution of the enzymes controlling each step of the pathway. The 5-LO enzyme is present in neutrophils (19), eosinophils (20), monocytes (21), macrophages (22), mast cells (23) and keratinocytes (24). However, a number of cell types, such as lymphocytes (25), erythrocytes (26), platelets (27) and endothelial cells (28) do not contain 5-LO, and are thus incapable of generating LTs from endogenous or exogenous AA. Some of these cells contain LTA_4 hydrolase and/or LTC_4 synthase activity and may thus convert LTA_4 into LTB_4 and LTC_4, respectively. LTA_4 hydrolase has been found in human erythrocytes, inflammatory cells, and airway epithelial cells. Furthermore, LTC_4 synthase has been identified in mast cells, eosinophils, and platelets. Thus, because these enzymes are distributed among different cell types, various inflammatory cells, in concert with noninflammatory cells, such as endothelial cells or epithelial cells, can participate in the transcellular synthesis of LTs (29,30). Moreover, the preferential generation of LTB_4 and LTC_4 is cell- and species-specific. This can be illustrated by preferential production of LTB_4 in human alveolar macrophages (31), whereas rat alveolar macrophages generate LTC_4 (32). The predominant 5-LO product of guinea pig eosinophils is LTB_4 , whereas human eosinophils is LTC_4 (33), and cytokines such as interleukin-3 (IL-3), IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), are capable of enhancing its synthesis (34,35).

1.2.3 Formation of 5-Oxo-ETE

As stated earlier, 5-LO first converts AA to 5-HPETE. This hydroperoxy acid can then be reduced by a peroxidase to 5-HETE which is not very active biologically. Several years ago Powell et al., discovered a novel microsomal dehydrogenase in human neutrophils which oxidizes 5-HETE to 5-oxo-ETE resulting in a 100 fold increase in biological potency (9) (Fig 2). The human 5-hydroxyeicosanoid dehydrogenase is highly specific for eicosanoids with 5-hydroxyl groups, followed by a 6-trans double bond. Its activity is localized in the microsomal fraction and it requires $NADP^+$ as a cofactor. This enzyme has been identified in human neutrophils (9), eosinophils (36), monocytes, lymphocytes (37) and platelets (38). Since platelets lack 5-LO they can produce 5-oxo-ETE by transcellular metabolism from neutrophil derived LTA_4. The preferred substrate for this enzyme is 5-HETE, which is likely to be its most important physiological substrate, since it is synthesized in relatively large quantities by stimulated human neutrophils and other inflammatory cells (6). Furtheremore, Powell et al., have found that 5-oxo-ETE is metabolized by at least four different pathways: ω-oxidation to 5-oxo-20-HETE (neutrophils), reduction to 6,7-dihydro product (neutrophils) and conversion to 12-hydroxy (platelets) and 15-hydroxy (neutrophils) metabolites by 12- and 15-LO, respectively. ω-Oxidation of 5-oxo-ETE is presumably catalyzed by LTB_4 20-hydroxylase which is highly active in neutrophils. Reduction of the 6,7-double bond of 5-oxo-ETE appears to be catalyzed by a novel NADPH-dependent cytosolic Δ^6 reducatse in neutrophils (133).

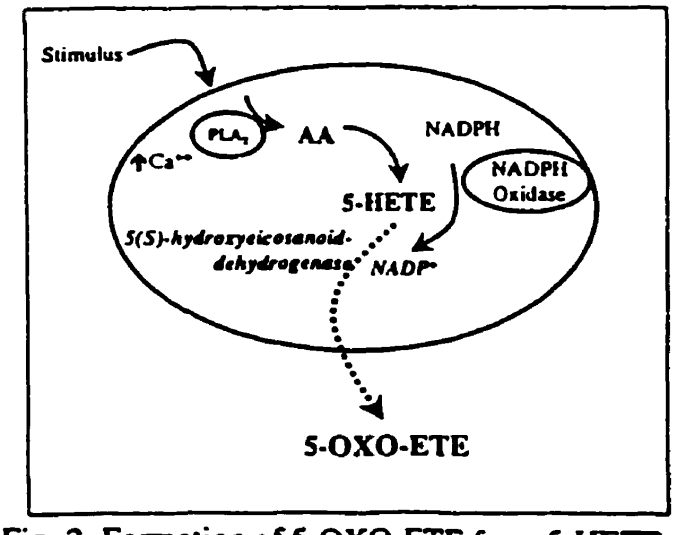

Fig. 2. Formation of 5-OXO-ETE from 5-HETE

1.3 Biological Effects of PAF and 5-Lipoxygenase Products

1.3.1 PAF

PAF appears to have a wide variety of actions (13), both direct and indirect, mediated by other substances, such as LTs. It recruits and activates inflammatory cells and induces vascular permeability change (Fig. 3). *In vitro* studies have shown it to be chemotactic for human neutrophils and eosinophils (39). It causes eosinophil degranulation (40) and release of mediators such as oxygen free radicals (41) and 5-LO products (42). These studies have also shown that PAF can upregulate expression of the β2 integrins, lymphocyte function related antigen-1 (LFA-1; CD11a/CD18) and macrophage-1 (Mac-1; CD11b/CD18) on inflammatory cells thereby stimulating their adherence to vascular endothelium (43). In addition PAF, in conjuction with certain cytokines, is capable of both priming and enhancing the inflammatory response in cells such as lymphocytes and monocytes (13). Furthermore, in animal models PAF can cause smooth muscle hyperplasia and fibroblast proliferation (44).

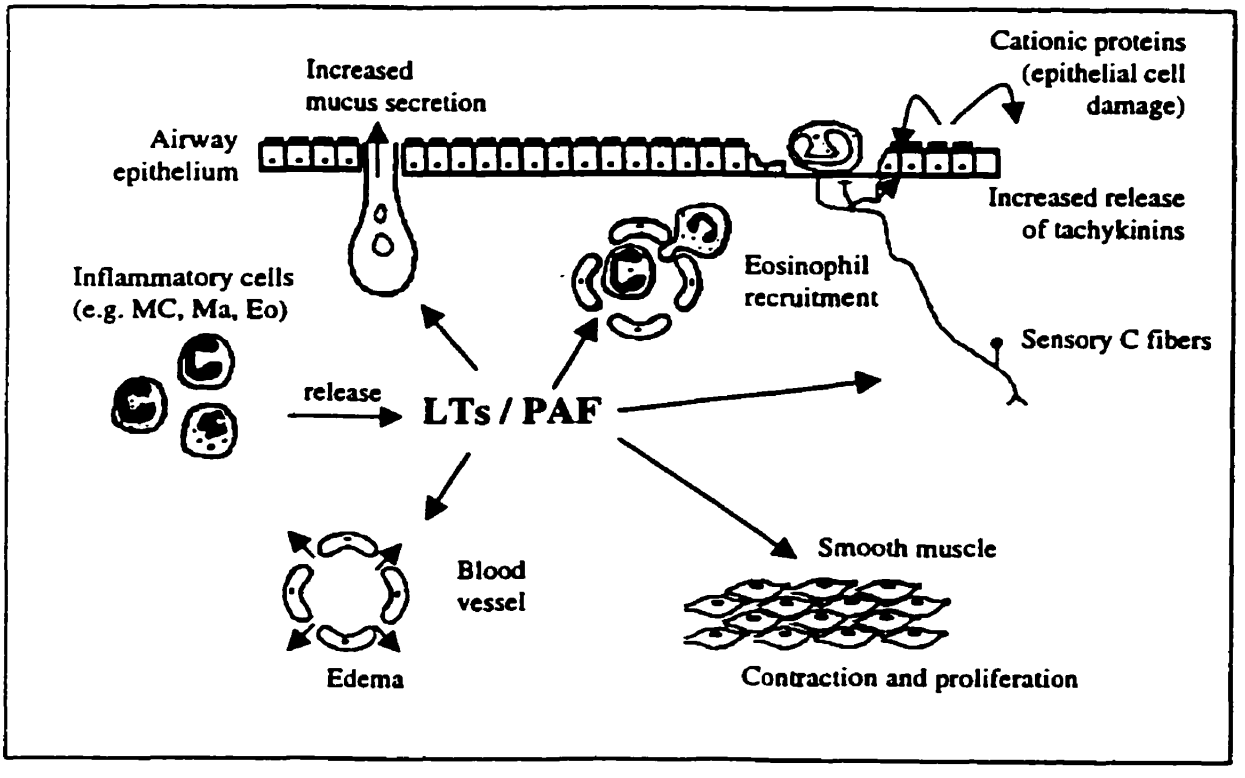

Fig. 3. Potential sites of action and effects of LTs and PAF with relevance to a pathophysiologic role in the lung.
(Eo : Eosinophil; Ma : Macrophage; MC : Mast Cell)

It is of particular interest that PAF elicits a neutrophil-rich infiltrate following its inhalation (45) or injection into the skin of normal volunteers (46), whereas local administration of PAF to the skin of atopic volunteers results in a selective eosinophilic infiltration very reminiscent to that induced by antigen in the same subjects (47) . In animal models, nebulized PAF causes bronchoconstriction (except in the rat) (49), hyperresponsiveness and an inflammatory cell infiltrate (44, 48), which in some animal models consists mainly of eosinophils (49). In some preparations, the effects of PAF, particularly the bronchoconstriction, seem to be mediated by cys-LTs and TXA_2 (50). In man, the acute bronchoconstriction is associated with a release of cys-LTs as shown by a rise in urinary LTE_4. At least part of PAF-induced bronchoconstriction can be blocked with specific cys-LT_1 antagonists (50, 51). Whether the remaining portion of PAF-induced bronchoconstriction is due to the direct effects of PAF or some other mediator is not clear.

PAF appears to be one of the most potent agents for inducing increased vascular permeability in all species tested, including man (52). It is able to elicit edema in the bronchi (53) which can be abolished by PAF receptor antagonists. PAF is thought to increase vascular permeability via contraction of endothelial cells presumably as a result of interaction with high affinity PAF receptors on these cells (54). The role of PAF in inducing edema after allergen or other mediators is still not clear. PAF antagonists do not inhibit plasma extravasation after acute allergen exposure in the guinea pig (55), but partially inhibit the extravasation induced by bradykinin (56). The role of PAF in chronic inflammation of the airways is not yet clear.

A number of groups have reported that high affinity binding sites exist for PAF and these have been demonstrated on platelets, neutrophils, eosinophils, macrophages (57,58) and lung tissue (59). The PAF receptor has now been cloned in both guinea-pig lung (60), and human neutrophils (61) and shares significant amino acid homology between the two species. It is a G-protein-linked surface receptor and in cells such as leukocytes it exists in both high and low affinity states. The existence of receptor subtypes is suggested by pharmacological studies with PAF antagonists that have shown a more than 10-fold difference in potency in different cell types from the same species (62). Functional studies

suggest that there may be intracellular PAF receptors (63,64). In rat cerebral cortex at least three distinct intracellular binding sites have been identified, including sites within microsomal fractions suggesting that PAF may be involved in gene expression (64,65). Futur investigations will provide a better understanding of the potential significance of PAF in gene activation / transcription.

1.3.2 Leukotriene B4

LTB_4 is a potent chemoattractant that is primarily involved in inflammation, immune responses and host defence against infection. The human neutrophil is a major site of LTB_4 synthesis and metabolism, and is also the major target for LTB_4 action (66).

LTB_4 causes chemokinesis, chemotaxis and aggregation of PMNL of several species, including man (67,68). It also mobilizes cytosolic calcium and stimulates leukocyte degranulation (66). In addition, LTB_4 can mediate neutrophil adherence to endothelial cell monolayers (69) and enhance surface expression of integrins (i.e. Mac-1; CD11b/CD18) on human neutrophils and eosinophils (70). *In vivo* effects of LTB_4 as a leukocyte chemoattractant have been documented in several animal models of inflammation (71,72). Instillation of LTB_4 into the bronchi of human subjects result in the recovery of increased numbers of neutrophils in bronchoalveolar lavage (BAL) fluid (73). It appears that the increased adherence of leukocytes to LTB_4 is due to an increased adhesiveness of endothelial cells for neutrophils which then may be followed by infiltration into the tissue (74). Unlike PAF, the increased vascular permeability observed with LTB_4 is a consequence of the activation of leukocyte adherence and emigration, and not due to a direct action on the small venules (75). LTB_4 is also a potent chemoattractant for guinea pig and Brown Norway (BN) rat eosinophils. Furthermore, antigen-induced eosinophilia is inhibited, both in BN rats and in guinea pigs, by the LTB_4 antagonists U-753032 and LY255283 (76). This potent LTB_4-mediated eosinophil recruitment is not seen with human eosinophils, indicating species differences. The major AA metabolite formed by guinea pig eosinophils is LTB_4, whereas the major product formed by human eosinophils is LTC_4 (77). Nevertheless, the proinflammatory effects elicited by LTB_4 vis-a-vis the

neutrophil make this LT a possible candidate as one of the mediators of the inflammatory process in diseases (Fig. 3).

Immunomodulation exerted by LTB_4 has also been suggested, through actions on T and B cells. Generation of LTB_4 by nonlymphoid cells at the inner cortex of the thymus may alter the balance of immature thymocytes ($CD4^+$ $CD8^+$) (78). LTB_4 enhances the proliferation of suppressor-cytotoxic T-cells (CD8) and inhibits the proliferation of helper-T-cells (CD4) (79). LTB_4 can also evoke T cell migration across basement membrane-like Matrigel as a result of concerted stimulation of T cell chemotaxis (maximal effective concentrations of 10-100 nM) and secretion of locally expressed metalloproteinases (80). Selective effects of LTB_4 on cytokines include inhibition of secretion of interferon-γ (IFN-γ) by CD8 cells and the stimulation of IFN-γ by CD4 cells (81). Exogenous LTB_4 promotes the synthesis of IL-2, IL-4, and IL-5 by human T cells (82, 83, 84). It stimulates synthesis of IL-6 by monocytes (85), and IL-8 by human neutrophils (86). LTB_4 also acts indirectly by stimulating monocytes to produce PGs and cytokines, which in turn leads to the production of IL-1 (87). Several B-lymphocytic functions including B-cell differentiation, activation, immunoglobulin (Ig) G and IgM synthesis, and cellular replication are also enhanced by LTB_4 (88), due to an augmentation of the effects of IL-4 and IL-2 on these cells. LTB_4 has also been reported to augment natural killer (NK) cell activity (89,90) . However, this aspect of LTB_4 activity has been questioned by reports from other groups who failed to show a direct effect of LTB_4 on these cells (91).

LTB_4 also possesses marked myotropic activity on the guinea-pig lung strip (92), which was shown to be mediated by release of COX products, most likely TXA_2 (93,94). Moreover LTB_4 has also been implicated in pain responses, such as nerve growth factor-induced thermal hyperalgesia in the rat (95).

Receptors for LTB_4 have been demonstrated in leukocytes of different species (96,97). Both high and low affinity binding sites for LTB_4 were observed in human neutrophils (98). Occupancy of the high affinity receptors is believed to mediate Ca^{2+} mobilization and chemotaxis, whereas occupancy of the low affinity receptor appears to result in degranulation (99). In addition LTB_4 activates inflammatory cells by binding to its cell surface receptor, but it can also bind and activate an intranuclear transcription factor ,

PPARα (100) resulting in the activation of genes that terminate the inflammatory processes. Recently, an LTB_4 receptor that is highly expressed in human leukocytes was cloned and expressed (101). It is a member of the seven-transmembrane receptor family and is sensitive to pertussis toxin.

1.3.3 Cysteinyl Leukotrienes

As mentioned earlier, cys-LTs accounted for the activity of SRS-A initially observed during antigenic challenge of sensitized lungs, hence suggesting a role of these compounds in allergic diseases (102) (Fig. 3). The observed *in vivo* formation of cys-LTs following allergen challenge of allergic patients provided strong evidence for this concept (103).

The major sites of cys-LT action are the airways where they are thought to act as mediators of allergic asthma (104). Both large and small airways of normal and asthmatic patients are constricted by cys-LTs (105,106,107). Inhaled LTC_4 and LTD_4 are 1000 - 5000 times more potent than histamine. Although the bronchoconstrictor effects of LTE_4 are less than those of LTC_4 and LTD_4, they are reported to be longer lasting (108). LTC_4 and LTD_4 constrict sensitized guinea pig lungs partially due to the generation of TXA_2 (109), whereas they appear to act principally by a direct effect on human lung tissue (110). It has also been suggested that cys-LTS participate in the neurally-evoked tachykinergic contraction of guinea pig airways by amplifying action potential-dependent release of tachykinins from airway afferent nerve fibers (111).

LTC_4 and LTD_4, unlike LTB_4, can induce increases in vascular permeability by an apparently direct action on the endothelial lining of postcapillary venules (112). They are potent stimulants of mucous glycoprotein secretion from human airways *in vitro* (113). *In vivo*, they enhance secretion of mucus (114) and stimulate secretion of chloride across the epithelium in dog trachea (115). Maximal airway narrowing induced by methacholine is augmented by LTD_4 in normal subjects, an effect attributed to induction of airway edema (116).

Moreover, the cys-LTs have also been implicated in eosinophil recruitment that is characteristic of atopic diseases. Bronchial mucosal biopsies, studied 4 h after LTE_4

inhalation by asthmatic subjects, showed an increase in the number of eosinophils and to a lesser extent of neutrophils (117). Recruitment of eosinophils is stimulated by LTC_4 or LTD_4 aerosolization of guinea pigs and attenuated by the cys-LT_1 receptor antagonist MK-571. Pretreatment of sensitized guinea pigs with the cys-LT_1 receptor antagonist MK-571 significantly inhibited the ovalbumin (OVA)-induced migration of eosinophils (118). Underwood and associates evaluated the ability of pranlukast, another cys-LT_1 receptor antagonist, to antagonize LTD_4-induced microvascular leakage, eosinophil influx, and bronchoconstriction in guinea pig airways. Pranlukast significantly inhibited both the eosinophilia and the bronchoconstriction. It also antagonized antigen-induced bronchoconstriction and eosinophil influx in OVA-sensitized guinea pigs. However, the mechanism of this effect is not clear, since LTD_4 is not a very potent chemoattractant for guinea pig eosinophils *in vitro* and pretreatment with an anti-IL-5 antibody antagonized the LTD_4-induced eosinophilia in this animal (119).

Human airway tissue contains two types of cys-LT receptors, those blocked by known antagonists (cys-LT_1 receptors) and those that are resistant to blockade (cys-LT_2 receptors). A cys-LT_2 receptor has also been identified in human pulmonary vasculature. In human airway smooth muscle, LTC_4, LTD_4 and LTE_4 all activate a cys-LT_1 receptor (120) which appears to be G-protein-coupled and leads to calcium mobilization upon activation (121). Guinea pig trachea, which has been extensively studied, has been reported to have three receptors, including one LTC_4 receptor (122,123) and two LTD_4 receptors (124). A major goal in further classifying cys-LT receptors will be to identify specific receptor subtypes that are responsible for the various pharmacologic effects of the cys-LTs other than bronchoconstriction.

1.3.4 5-OXO-ETE

5-Oxo-ETE has biological activities which are 100 times more potent than its precursor 5-HETE (9). It is a potent stimulus of neutrophils, eosinophils, monocytes and intestinal epithelial cells. It induces Ca^{2+} mobilization, chemotaxis, adhesion, Mac-1 (CD11b/CD18) expression and actin polymerization in neutrophils (125). Pretreatment of these cells with tumor necrosis factor-α (TNF-α) (126) or GM-CSF (127) greatly potentiates the effects of 5-oxo-ETE on their degranulation response and superoxide production.

Although 5-oxo-ETE is active in stimulating neutrophils its more potent effects are on the eosinophil. 5-Oxo-ETE is over two-fold more effective than PAF and over 30 times more effective than LTB_4, $-C_4$, $-D_4$ and $-E_4$ as a chemotactic agent for human eosinophils *in vitro* (10) . 5-Oxo-ETE is also more active than LTB_4 and PAF in inducing actin polymerization and L-selectin shedding by eosinophils. It can also stimulate Mac-1 (CD11b/CD18) expression and Ca^{2+} mobilization in these cells *in vitro*. 5-Oxo-ETE has also been reported to induce both superoxide production and degranulation (128) in human eosinophils, the latter response being potentiated by pretreatment with GM-CSF.

In addition to its effects on eosinophils and neutrophils, 5-oxo-ETE also induces actin polymerization and migration of human monocytes and enhances their responsiveness to the chemokines monocyte chemotactic peptide (MCP)-1 and MCP-3 (129). 5-Oxo-ETE is also an extremely potent stimulator of Cl^- / K^+ -dependent volume reduction in guinea pig jejunal crypt epithelial cells (130).

More importantly, this potent lipid mediator appears to act via its own receptor. Structure activity studies demonstrate that neutrophils possess a highly specific recognition mechanism for 5-oxo-ETE. This compound can cross-desensitize neutrophils to itself but not to other agonists (131,132). Furthermore, this 5-oxo-ETE-specific receptor appears to be G-protein-coupled and pertussis toxin-sensitive (133) and is present on the eosinophil as well (134). The following figure summarizes the biological actions mediated by 5-oxo-ETE (Fig 4).

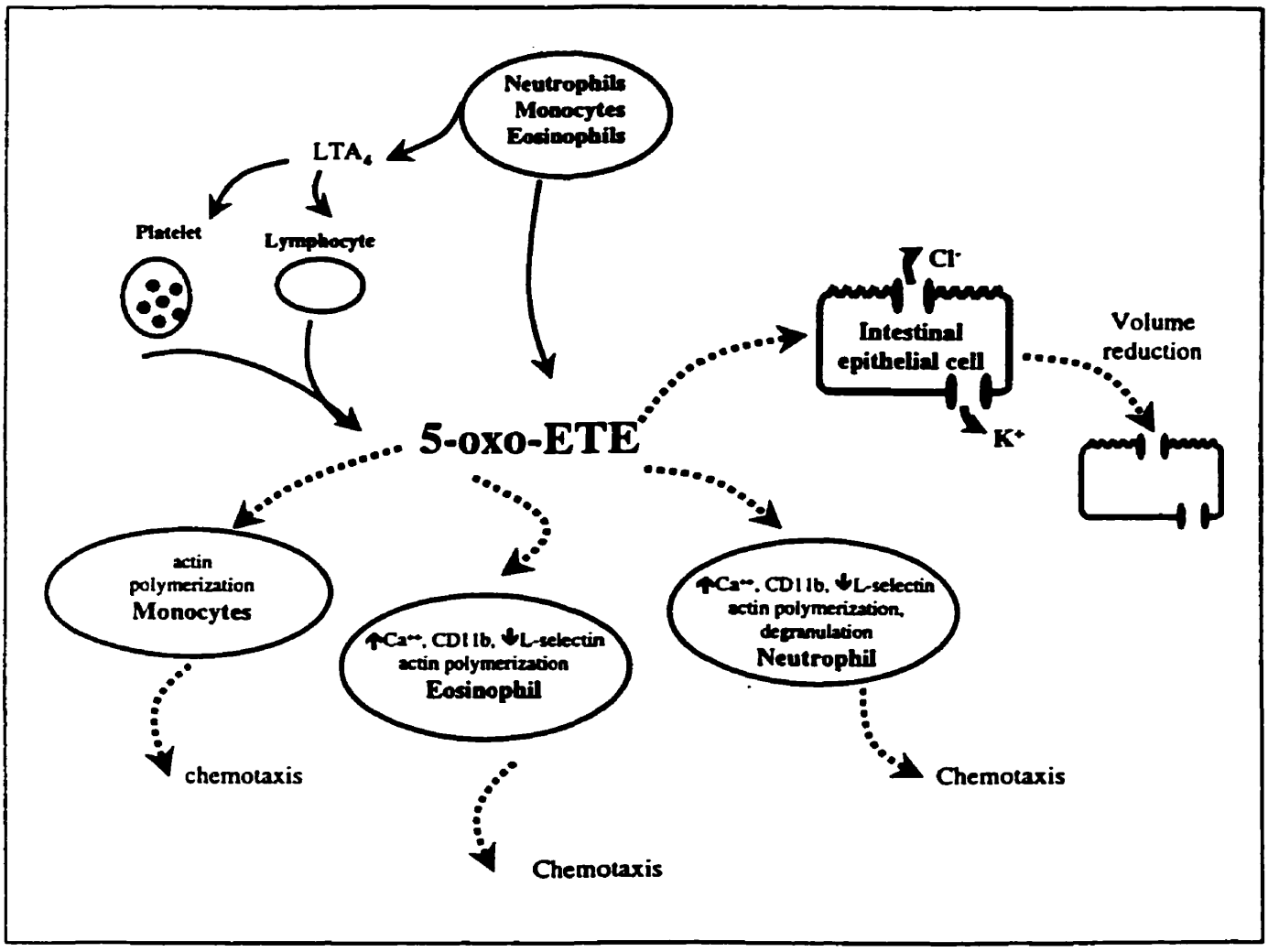

Fig. 4. Sources and biological activities of 5-oxo-ETE.

1.4 Evidence of a Role for PAF and 5-LO Products in Asthma

Although eicosanoids are capable of eliciting biological effects on different cells and tissues, it does not necessarily mean that they are important physiologically. Their production should be identified in physiological fluids or tissues. For example, increased urinary LTE_4 has been demonstrated following allergen challenge, during acute and aspirin-induced asthma (135). Raised levels of LTs (e.g. LTB_4 and cys-LTs), particularly LTE_4 have been found in BAL fluid of asthmatics (136), with further increases after endobronchial allergen challenge (137).

To further substantiate a role for eicosanoids in a disease such as asthma another criteria needs to be fullfiled. The alleviation of the asthmatic attack or symptoms, by eicosanoid biosynthesis inhibitors or by blockage of the biological actions of these substances by specific receptor antagonists, has to be established. Great efforts have been made to develop such drugs directed against LT activity and they fall into four main classes (Fig. 5). The synthesis of all 5-LO products can be blocked by inhibitors either of 5-LO itself or of FLAP, while blockade of LT actions can be obtained with specific antagonists acting at the cys-LT_1 receptor or at the LTB_4 receptor. In addition, PAF-mediated actions can also be inhibited by PAF receptor antagonists.

1.4.1 Synthesis Inhibitors

Among the LT synthesis inhibitors described in the literature, FLAP inhibitors include MK-886 and MK-0591, while the leading 5-LO inhibitor is zileuton (A-64077). The FLAP inhibitors, such as MK-886 and MK-0591, have no direct activity on 5-LO but antagonize FLAP thus preventing the translocation of the enzyme to the membrane (138). A clinical study with astmatics after 2 oral doses of MK-886 showed a reduction in the early-phase asthmatic airway response (see section 1.5.1) after allergen challenge, with a concomitant reduction in *ex vivo* LT generation (139). Moreover, there was a correlation between the inhibition of urinary LTE_4 excretion and attenuation of the early-phase response.

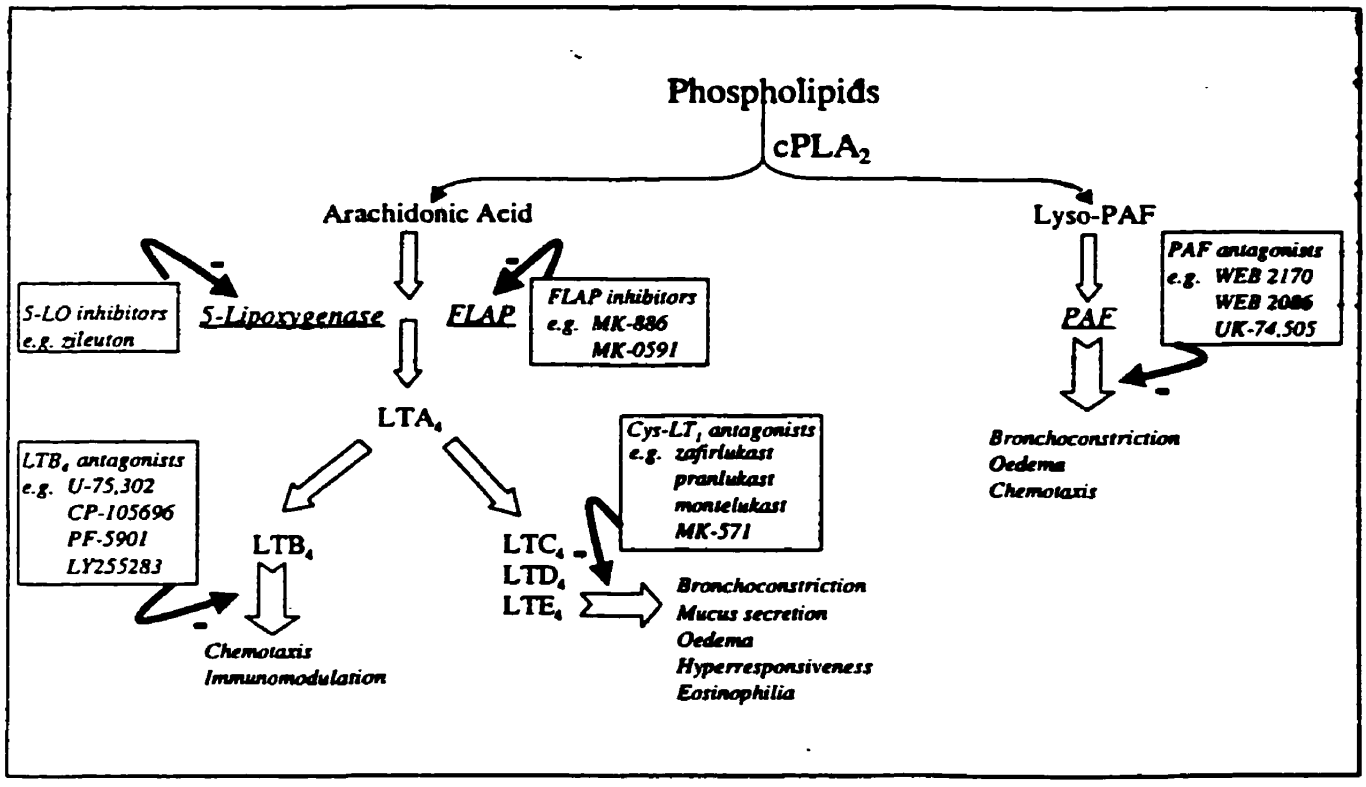

Fig. 5. Synthesis inhibitors and receptor antagonists of 5-LO products and PAF. Examples of inhibitor and antagonist drugs shown in boxes.

A more recent FLAP inhibitor, MK-0591, which almost completely abolishes endogenous LT production (assessed by urinary LTE_4 levels and *ex vivo* blood LTB_4 production), blocks the early-phase response to allergen by 79 % and the late-phase asthmatic airway response (associated with leukocyte influx (see section 1.5.1)) only by 39 % (140). This lack of significant clinical effect with the above inhibitors may be due to an insufficient degree of inhibition of 5-LO in the lung (141). The 5-LO inhibitor zileuton, which acts partly through an iron-catalysed redox mechanism, blocks the increase in BAL eosinophis and bronchial responsiveness induced by antigen challenge in sheep (142). A recent study by Namovic et al., (143) reported that zileuton effectively inhibited influx of eosinophils into the lungs of Sephadex treated BN rats. In addition, this 5-LO inhibitor was shown to inhibit airway narrowing in asthmatics induced by cold, dry air (144). Subjects (n = 12) with nocturnal asthma treated for one week with zileuton showed reduced BAL fluid LTB_4 and urinary LTE_4 levels and this was accompanied by significant reductions in BAL and peripheral blood eosinophil counts compared with placebo (145). Zileuton has now been approved for use in human asthmatics.

1.4.2 Receptor Antagonists

The cys-LT_1 antagonists developed in the 1990's have much greater potency than earlier compounds. These include montelukast (MK-476; Singulair), pranlukast (ONO-1078), zafirlukast (ICI 204,219; Accolate) and MK-571 and are all now in Phase III clinical trials whereas, montelukast and zafirlukast are already in clinical use. The LTD_4 antagonist MK-571 attenuated the recruitment of eosinophils into sensitized guinea pig lungs following LTC_4, LTD_4 or OVA administration (118). Moreover, MK-571 strongly inhibited the early-phase response caused by antigen challenge in sensitized BN rats and completely suppressed the late-phase response (146). Pranlukast, a selective cys-LT_1-receptor antagonist, also has been shown to reduce markedly (83-89%) LTD_4-induced eosinophilic influx in guinea pig trachea, main bronchi and small airways (147). Recent studies in man have shown that pranlukast inhibits allergen-induced immediate bronchoconstriction in subjects who have asthma (148) and aspirin-induced asthma (149), and significantly reduces airway hyperresponsiveness (150). Zafirlukast is an orally active

LTD_4 antagonist and has been reported to attenuate allergen-induced migration of inflammatory cells in guinea pigs (151). At 48 h after challenge basophil and lymphocyte counts and histamine concentration were reduced by zafirlukast while eosinophil and macrophage numbers were unaffected (152). Although zafirlukast is more potent in antagonizing both allergen- and LT-induced bronchoconstriction than MK-571, it is not more effective than MK-571 in blunting exercise-induced or isocapnic hyperventillation-induced bronchoconstriction (153,154).

Apart from the cys-LT_1 antagonists there are also a number of LTB_4 and PAF antagonists that have been developed and have been used to better determine their physiological roles. Sensitized guinea pigs treated with the selective LTB_4 antagonist U-75302 prior to antigen challenge showed a dramatic reduction of peribronchial eosinophil infiltration (155,156). LY255283 (Fig 6), an effective LTB_4 receptor antagonist, is a potent inhibitor of LTB_4-induced aggregation of guinea pig neutrophils (157).

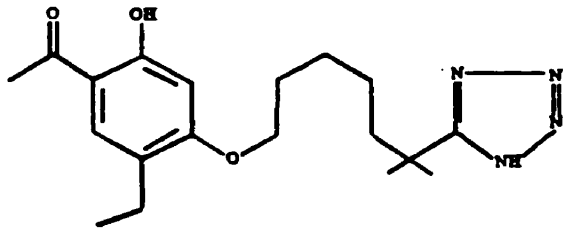

Fig. 6. Structure of LY255283

In rabbits, LY255283 reduced the transient neutropenia following intravenous. administration of LTB_4 (158). In BN rats, it inhibited antigen- as well as LTB_4-induced pulmonary eosinophil influx (76) . This has also been observed by other groups using the guinea pig as a model (159). In contrast the LTB_4 antagonist, PF-10042, did not block antigen-induced pulmonary eosinophilia in guinea pigs, whereas the 5-LO inhibitor PF-5901 did (160). Although LTB_4 receptor antagonists have been shown to inhibit allergen-

or LTB_4-induced leukocyte infiltration in animal models of asthma, the potential therapeutic role of such antagonists has yet to be evaluated in clinical studies.

In addition to LTs, there is also a considerable interest in elucidating the physiological role of PAF. The results of investigations with several potent synthetic PAF antagonists with different chemical structures have been reported (161,162,163). Even though these drugs have been shown to block PAF-induced bronchoconstriction they appear to have no effect on either the early or late response to allergen challenge in man. It could be suggested that insufficient drug was present to antagonize the effects of PAF but, as discussed previously, studies on the effects of PAF inhalation would suggest this is not the case. In animal studies PAF antagonists have been reported to inhibit eosinophil recruitment into tissues. Two structurally related PAF antagonists, WEB 2086 and WEB 2170 (Fig 7), are able to inhibit eosinophil accumulation caused by antigen in rat pleural cavity (164).

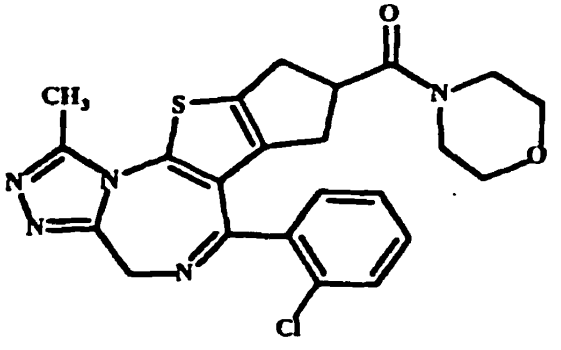

Fig. 7. Structure of WEB 2170

In addition, OVA-sensitized guinea pigs showed an attenuation of eosinophil accumulation following treatment with WEB 2170 (49). A recent study showed that treatment with WEB 2170 significantly inhibited eosinophil migration induced by *Mycobacterium bovis bacillus Calmette-Guerin* in the mouse pleural cavity (165). Eventhough PAF appears to mediate eosinophil recruitment in various animal species, its actions are indirect in man. In fact there are several reports showing that PAF effects can be blocked by cys-LT_1

antagonists or even LT synthesis inhibitors such as MK-886. This is a recurrent argument against PAF antagonists that have performed poorly in clinical trials.

In conclusion, there is both indirect and direct evidence that 5-LO products may play a role in the pathogenesis of asthma. The present LT antagonists and synthesis inhibitors are likely to be effective in some patients with specific forms of asthma, such as aspirin-sensitive asthma. In terms of allergic asthma, LT antgonists and synthesis inhibitors block only ~50% of the late-phase asthmatic airway response to allergen. It is not clear whether an even more potent LT antagonist or one with a longer duration of action would have a greater effect, or whether mechanisms such as edema formation and cellular infiltration involving other mediators are responsible for the residual airway narrowing. Clearly, the development of more sprcific inhibitors will be able to answer these questions.

1.4.3 Mouse Knockouts

An alternative approach for studying the contribution of 5-LO products to the inflammatory process is through the use of targeted gene disruption (166,167). Chen and coworkers (168) exploited this technique to produce homozygous 5-LO deficient mice (5-LO -/-) which, although appeared normal and healthy, demonstrated alterations in certain inflammatory responses. Experiments with these 5-LO -/- mice suggested that involvement of 5-LO products, in certain responses, appears to be stimulus selective. For example, these knock-out mice showed defects in the peritoneal responses of PMNL to immune complexes but not to glycogen. Furthermore, there was a deficient response in the ear edema evoked by AA but not by PMA, and there was an inadequate reaction to PAF-induced shock but not that to endotoxin. The defect seen in the PAF model of shock supports previously held views on the interactions of LTs and PAF (169). More recently these mice were used to study their capacity to reproduce some of the hallmark signs of asthma (airway hyperresponsiveness and eosinophilia).

Mice were sensitized to OVA followed by repeated aerosol challenge and studied 24 hr after the last challenge (170). The 5-LO -/- mice had a diminished airway eosinophilia and IgE production as well as an airway reactivity which was similar to that of the unsensitized controls. It is known that mice do not respond to intravenous infusion of cys-LTs (171) hence, the reduced tissue and airway eosinophilia in the 5-LO -/- mice could be due to absent LTB_4 or 5-oxo-ETE synthesis (10) during OVA or IgE stimulation of mast cells or other inflammatory cells. The development of genetically modified mouse models over-expressing, mutant expressing or null for various enzymes or receptors in the eicosanoid pathway holds promise for exciting new *in vivo* studies.

1.5 Asthma

Asthma is a clinical syndrome characterized by intermittent airway obstruction, airway hyperresponsiveness and chronic airway inflammation. According to the U.S. National Center of Health Statistics the prevalence of asthma has risen steadily, doubling in the past 20 years (172), and it now affects about 10 % of the population. There has been significant progress in the understanding of the immunobiology of asthma in the last decade. One of the most important advances is probably the finding that airway inflammation is a key feature of asthma.

1.5.1 Classification and Pathogenesis of Asthma

Etiologic or pathologic classification of asthma is difficult. Nevertheless this disease can be generally divided into three types: allergic (atopic; extrinsic), non-allergic (nonatopic; intrinsic) and occupational asthma. Airway inflammation is characteristic of all three types of asthma. It is seen in atopic asthma (IgE mediated) as well as in nonatopic asthma (non-IgE-mediated) and occupational asthmatics who do not always demonstrate an IgE response (173,174). These and other studies suggest that inflammation may play an important role in the pathogenesis of the disease regardless of the nature of identifiable provoking agent. IgE-mediated mechanisms are clearly important in allergen-induced short-term exacerbations of asthma in atopic individuals but their role in the pathogenesis of chronic disease is less certain. Nevertheless, many studies on the pathogenesis of asthma have been gathered from patients with atopic asthma because their disease can be conveniently provoked by allergen challenge.

Although advances in pathobiology and immunology have yet to delineate the pathogenesis and genetic basis of allergic asthma, it is generally believed that asthma is the result of an immune response to allergens including innocuous organisms such as pollens or free-living mites (175,176) . Recently it has been appreciated that the immune response and airway inflammation in asthma may be primarily orchestrated by antigen-activated lymphocytes and Th2 type cytokines (i.e. IL-4, -5, -6, -10, -13) (177).

1.5.1. a. *Orchestration of airway inflammation*

In the genetically appropriate host (i.e. atopic asthmatics), exposure to an allergen leads to the production of specific IgE as a result of complex and cognate interactions between antigen presenting cells (i.e. dendritic cells and alveolar macrophages in the epithelium and submucosa of the airways), T cells and B cells (178, 179, 180, 181, 182, 183). This initial response is referred to as 'sensitization'. The specific IgE produced by the B cell will bind to high affinity Fc receptors on effector cells such as mast cells, and basophils, as well as the low affinity Fc receptors on macrophages, eosinophils and platelets. Later, when a sensitized individual is re-exposed to a relevant antigen, the antigen binds and crosslinks to IgE on the surface of the mast cell (184). This causes the mast cell to degranulate and release mediators that may induce constriction of the airways. This is known as the early phase response (EPR). This reaction will normally occur within minutes of provocation, peaking between 5 and 20 min, and resolving within 60 min. The mast cell-mediators and potent airway constrictors responsible for this response are histamine, LTC_4, LTD_4 and PAF. These mediators also augment mucus secretion and vascular leakage, leading to further airway obstruction. Alveolar macrophages may also be activated by an IgE-dependent mechanism and release mediators such as PAF, eicosanoids and cytokines. Subsequently, the cytokines and chemotactic mediators released by activated mast cells, T cells and macrophages induce an influx of inflammatory cells, most notably eosinophils. This inflammatory reaction appears to be involved in a second phase of longer-lasting decline in pulmonary function known as the late phase response (LPR) (185, 186). The LPR begins around 3 h and peaks around 8 h after antigen exposure and may last for days. Approximately 50% of the patients that develop an EPR also develop a LPR and moreover there are patients that develop a LPR in absence of an EPR. The LPR in the airways is characterized by an initial influx of neutrophils which is then followed by a large influx of eosinophils and T-cells. Monocytes and macrophages are also recruited during this phase but appear to play a secondary role in the inflammatory cascade of events. Persistent cellular infiltration and the release of toxic products may eventually result in epithelial damage, airway hyperresponsiveness and chronic asthma. Although chronic asthma differs from the responses seen after allergen provocation, the LPR seen

after challenge bears similarities to the clinical disease. One of the major similarities is the increased recruitment of activated eosinophils into the lung. Pathological processes that result in lung eosinophilia may involve antigen-induced T cell activation through macrophages or other antigen presenting cells, T cell cytokine release, specific sensitization of mast cells, and release of mediators by macrophages (187).

1.5.2 Pathophysiology of asthma

Pathophysiologically, asthma is characterized by airway narrowing; hypersecretion of mucus; edema of airway mucosa; cellular, especially eosinophilic, infiltration of the airway wall, and desquamation of the airways epithelium. Autopsy of airway samples from patients dying from status asthmaticus, even those who died of nonasthma causes, as well as biopsies from asthmatics, even some of mild disease, all show significant changes of the airways with a marked inflammation of the bronchial tree (188,189). Typically, there is plugging of the lumen with mucus, epithelial cells, activated lymphocytes and eosinophils; shedding of the ciliated epithelium; deposition of collagen beneath the basement membrane; smooth muscle hypertrophy; and an intense eosinophilic infiltrate in the mucosa and submucosa accompanied by a low degree of infiltration of mononuclear cells and neutrophils. An association between the degree of inflammation and nonspecific bronchial hyperresponsiveness has also been described (190).

BAL fluid from asthmatic subjects, challenged with aeroallergen or even in the absence of bronchial provocation, contains increased numbers of mast cells, neutrophils, eosinophils and lymphocytes (191,192). In addition to these cellular changes increased eosinophilic degranulation and elevated levels of histamine, PGD_2 and cys-LTs have been reported (138, 193). Most of these chemical mediators are released locally and have been implicated in bronchospasm, epithelial damage, mucus secretion and microvascular hyperpermeability.

A number of studies speak to the importance of the eosinophil in the asthmatic process. Among the most striking is the positive correlation between the levels of blood and airway eosinophilia and the severity of asthma (194). Blood and infiltrating pulmonary eosinophils are often primed or activated in asthmatics, and their increase in numbers

correlate with the presence of activated T cells (195), elevated levels of eosinophilic constituents in the BAL fluid (196), the degree of airways epithelial damage and the level of airways hyperreactivity to inhaled spasmogens (197).

The role of the eosinophil in the asthmatic process is believed to be governed by mediators released - LTs, PAF and PGs - which produce an intense inflammatory reaction involving bronchoconstriction, vascular congestion, and edema formation. In addition to their ability to evoke prolonged contraction of the airway smooth muscle and mucosal edema, the LTs may also account for some of the other pathophysiologic features of asthma such as increased mucus production and impaired mucociliary transport. This intense local event can then be followed by a more chronic one. The chemotactic factors elaborated (i.e. LTB_4, PAF etc.) bring eosinophils and other leukocytes to the site of the reaction. These infiltrating cells, as well as resident macrophages and the airway epithelium iself, potentially are an additional source of mediators to enhance both the immediate and cellular phase.

Furthermore, the eosinophilic granular proteins (see section 1.6.1) are capable of destroying the airway epithelium, which is then sloughed into the bronchial lumen. Besides resulting in a loss of barrier and secretory function, such damage elicits the production of chemotactic cytokines, leading to further inflammation. In theory it also can expose sensory nerve endings, thus initiating neurogenic inflammatory pathways. That, in turn, could convert a primary local event into a generalized reaction via a reflex mechanism.

Hence, the view that airway inflammation, and more particularly eosinophil accumulation into the airways, is a major component of the asthmatic process has led to an intense investigation of the mechanisms involved in eosinophil recruitment into the lung.

1.6 Eosinophilia

Eosinophils normally account for only 1 to 3 % of peripheral blood leukocytes, and their presence in tissues is primarily limited to the gastrointestinal mucosa (198). However, in certain disease states, eosinophils can selectively accumulate in the peripheral blood or any tissue in the body. The most common cause of eosinophilia worldwide is helminthic infections, and the most common cause in industrialized nations is atopic disease (i.e. allergic disease of the eye, lung, nose, skin). Since tissue eosinophilia is a hallmark of atopic disease and eosinophils are a major effector cell in these disorders, allergic diseases serve as a prototype of understanding the pathogenesis and processes involved in eosinophilia.

1.6.1 Pathogenesis of Eosinophilia in tissue

Once eosinophils arrive at an inflammatory focus, they may undergo apoptosis with rapid clearance by macrophages, but if they are stimulated by IL-3, IL-5, or GM-CSF, they survive for prolonged periods and have increased responsiveness to other activating agents. Eosinophils activated in this way express increased levels of receptors for cytokines, Igs and complement.

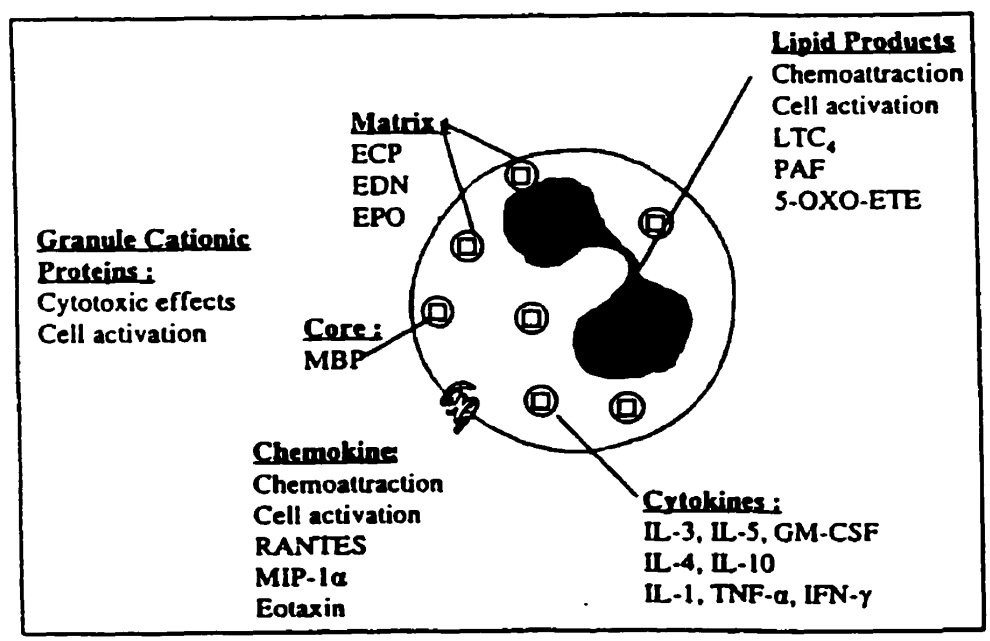

Fig. 8. The granules of eosinophils contain a crystalloid core composed of MBP, and matrix composed of ECP, EDN and EPO. Eosinophils also produce a variety of cytokines, some of which are stored in granules, and lipid mediators that are generated after cellular activation.

Eosinophils produce unique toxic inflammatory mediators, which are stored in granules and synthesized after cellular activation (Fig. 8). The granules contain a crystalloid core composed of major basic protein (MBP) and a matrix composed of eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO). These cationic proteins share certain proinflammatory properties but differ in other ways. For example, at concentrations similar to those in BAL fluids from asthmatics with eosinophilia MBP, ECP and EPO have cytotoxic effects on respiratory epithelium (199,200). In addition, ECP and EDN are ribonucleases (201,202). ECP can cause voltage-insensitive, ion-nonselective toxic pores in the membranes of target cells, and these pores may facilitate the entry of other toxic molecules (203). MBP directly increases smooth muscle reactivity by causing the dysfunction of vagal muscarinic M2 receptors (204). Alternatively or in addition, MBP and EPO may directly stimulate the respiratory epithelium on contact which, in turn, causes smooth muscle contraction and increased sensitivity of the muscle to methacholine (205); recent studies suggest that this may be the result of bradykinin generation (206). Moreover, MBP may also trigger the degranulation of mast cells and basophils.

Further damage is caused by hydrogen peroxide and halide acids, which are generated by eosinophil peroxidase, and by superoxide, which is generated by the respiratory-burst-oxidase pathway in eosinophils. Eosinophils also generate large amounts of cys-LTs. These lipid mediators increase vascular permeability and mucus secretion and are potent stimulators of smooth muscle contraction (103) . They can also promote migration of eosinophils *in vitro* (207) as well as into the lungs of asthmatics (117).

In addition, activated eosinophils produce a wide range of inflammatory cytokines (e.g. TNF-α, GM-CSF, IL-3, IL-4, IL-5) that have the potential to modulate multiple aspects of the immune response, regulate eosinophil effector function and perpetuate tissue eosinophilia (208, 209). Furthermore, eosinophils amplify the inflammatory cascade by producing their own chemoattractants (e.g. RANTES [regulated upon activation normal T-cell expressed and secreted], eotaxin and PAF), which accelerate the recruitment of eosinophils into the inflammatory focus.

This functional role for recruited eosinophils and their products in the pathogenesis of atopic diseases has led to intense investigations into the mechanisms regulating eosinophilia. Identification of key molecules and cells that selectively regulate eosinophil recruitment has been a major focus for the last five years in the study of asthma.

1.6.2 Mechanisms involved in Eosinophilia

Eosinophilia occurs as a result of four processes (Fig. 9): 1) differentiation of progenitor cells and proliferation of eosinophils in bone marrow; 2) interaction between eosinophils and endothelial cells that involve rolling, adhesion, and migration of eosinophils; 3) chemoattraction directing eosinophils to a specific location; and 4) activation and prolonged survival within tissue.

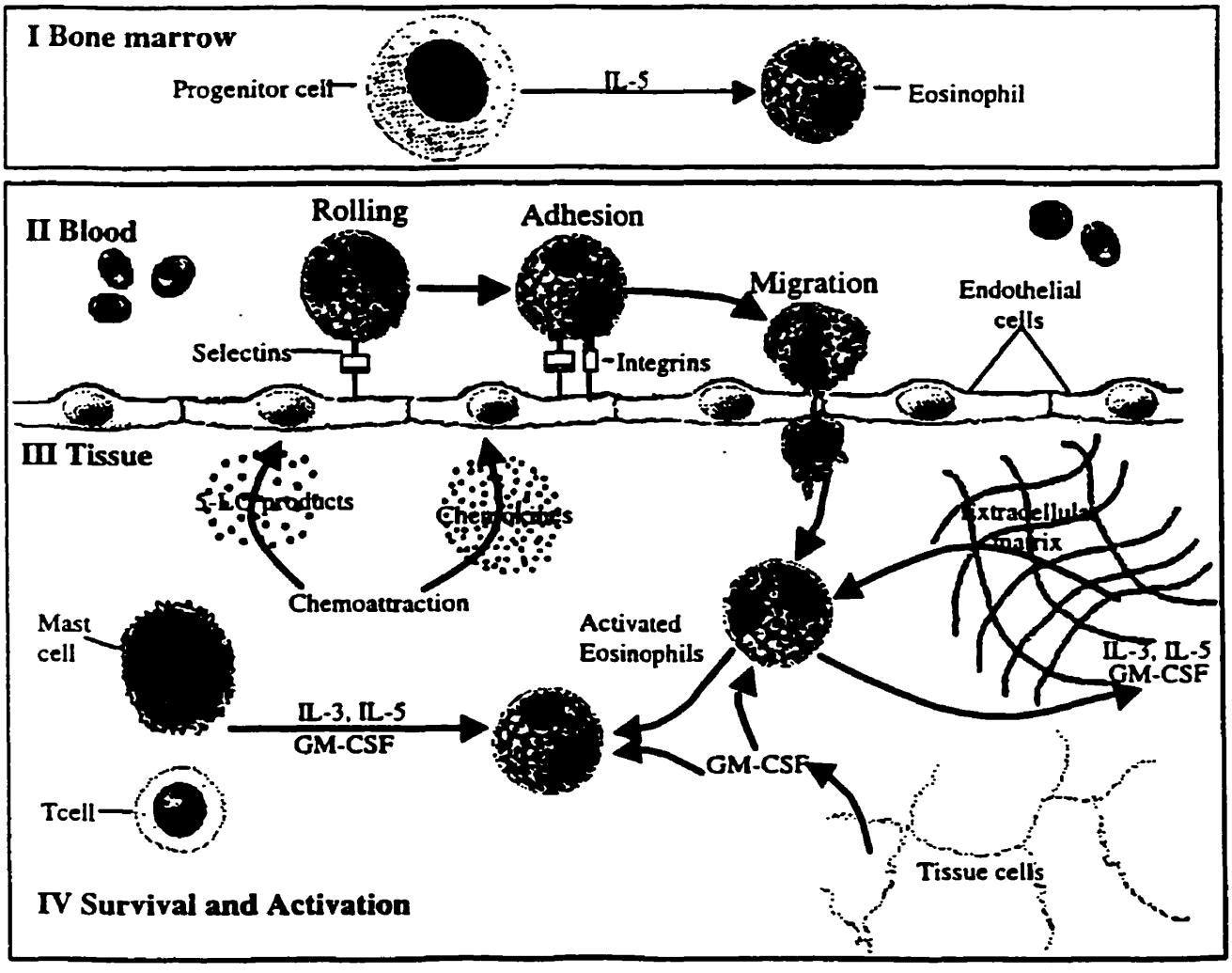

Fig. 9. Processes involved in Eosinophilia

It is interesting to note that the three cytokines, IL-3, IL-5 and GM-CSF, whose overproduction occurs in humans with eosinophilia such as asthmatics (177, 210), are implicated in most, if not all of the stages mentioned above. Only eosinophils and basophils have receptors for IL-3, IL-5 and GM-CSF on both the precursor cells in bone marrow and the circulating cells. Of the three cytokines, IL-5 (also known as eosinophil-differentiation factor) is the most specific for the eosinophil lineage. It is responsible for selective differentiation of eosinophils (211), and it stimulates their release from bone marrow into the peripheral circulation (212). The critical role of IL-5 in the production of eosinophils is best demonstrated by genetic manipulation in mice. Overproduction of IL-5 in transgenic mice results in profound eosinophilia (211) , and deletion of the IL-5 gene causes a marked reduction of eosinophils in the blood and lungs after an allergen challenge (213).

IL-5, IL-3 and GM-CSF are the three main cytokines involved in the recruitment and activation of eosinophils. IL-5 is a selective eosinophil chemoattractant and increases the expression of Mac-1(CD11b) on human eosinophils (214) . The role of all three cytokines in the regulation of eosinophil infiltration has been documented *in vivo* in the skin of patients with atopy (215) and in mucosal eosinophilic diseases (e.g asthma) involving the gastrointestinal (216,217) or respiratory tract (218,219). Furthermore, *in vitro* and explant studies of allergic sinus tissue have shown that IL-3, IL-5 and GM-CSF delay eosinophil apoptosis for at least 12 to 14 days (220). In contrast, eosinophils survive for less than 48 h in the absence of these cytokines (221). Tissue eosinophils can also regulate their own survival through an autocrine pathway (208).

The remaining part of this thesis will focus on the processes by which eosinophils migrate from the blood into target tissues.

1.7 Recruitment of eosinophils

1.7.1 Adhesion Molecules

Recruitment of eosinophils and other inflammatory cells from the blood into the lung is regulated by inflammatory mediators produced in the airways and subsequently released into the circulation, such as PAF, (222) IL-5 (223) and eotaxin (224). This traffic is mediated by adhesion molecules which in turn are upregulated by certain cytokines.

Adhesion molecules are glycoproteins expressed on cell surfaces which mediate the contact between two cells or between the cell and extracellular matrix (225). They are important in the migration of leukocytes from the blood into the tissues during inflammation. They may also serve as signalling molecules (226), thereby influencing several eosinophil functions such as degranulation (227), secretion of LTC_4 (228) and generation of superoxide (229). Moreover, there is evidence that adhesion molecules, such as VLA4, are involved in the interactions between human hematopoietic progenitor and stromal cells in the bone marrow (230), and may also be involved in the proliferation of progenitor $CD34^+$ cells (231). The focus herein will be on adhesion molecules and cellular recruitment.

Adhesion molecules are subdivided into several families based on common characteristics. In general the adhesion process leading to recruitment of inflammatory cells is mediated by three major groups of receptors: the selectins, the integrin family (232,233) and the Ig superfamily (234).

Selectins have a common molecular structure comprising of several domains one of which is the N-terminal lectin domain, essential for cell adhesion. The selectin family consists of three proteins; E-(endothelial), P-(platelet) and L-(leukocyte) selectin. E-selectin and P-selectin are expressed on activated endothelium (232,235,236,237). Maximal E-selectin expression on endothelium is found within hours (2 to 6 h) after stimulation by the cytokines IL-1 and TNF-α (238) and then subsequently declines to basal levels within 24 h. P-selectin, which is stored in Weibel-Palade bodies, is rapidly mobilized (within minutes) to the surface of endothelial cells upon activation with thrombin (239), histamine (240), LTC_4 or PAF released from activated mast cells. Unlike E- and P-selectn, L-selectin is expressed constitutively on all leukocytes (241). Upon

leukocyte activation, L-selectin is rapidly shed from the cell surface and this may be en important event in the process of leukocyte emigration (242). The most studied ligands for the selectins are sialyl Lewis X blood antigen (SLe^x) and other fucose-containing carbohydrate determinants (235, 243). Moreover, there appears to be an interaction between L-selectin and the E- and P-selectin mediated adhesion pathways (235).

The integrin family comprises of receptor molecules which are α and β-heterodimers and are divided into different subfamilies according to the β subunit expression (232, 244). The $\beta 2$ subfamily is found exclussively on leukocytes and is composed of three distinct, but related, α-chain polypeptides: CD11a (α_L), CD11b (α_M) and CD11c (α_X), which are expressed on the cell surface in non-covalent association with a common $\beta 2$ subunit, CD18 (245). These three α/β heterodimers are often referred to by their earlier names, LFA-1 (lymphocyte function related antigen-1), Mac-1 (macrophage - 1) and CR4 (complement receptor-4) respectively. The whole complex is referred to as CD11/CD18. Peripheral blood eosinophils, neutrophils, monocytes and NK cells express all three $\beta 2$ integrins, whereas lymphocytes express primarily LFA-1. The members of the $\beta 1$ integrin subfamily, which are sometimes referred to as very late activation antigens (VLA), are found on many different cell types and function primarily as receptors for extracellular matrix proteins such as collagen, laminin, and fibronectin (226). In contrast, VLA-4 ($\alpha 4\beta 1$; CD49d/CD29), in addition to functioning as an extracellular matrix receptor for fibronectin (246), also mediates cell-cell interactions by interacting with another adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1) (247). VLA-4 is present on resting lymphocytes, monocytes and eosinophils, but not on neutrophils (233). Eosinophils bind via LFA-1 and Mac-1 to intercellular adhesion molecule-1 (ICAM-1) (43), and since they also express VLA-4, they additionally bind to VCAM-1 (248) (see below).

The Ig superfamily is a large family of adhesion molecules whose structure is characterized by repeated domains similar to those found in Igs. For endothelial-leukocyte interactions, the most important members of this family ICAM-1, ICAM-2 and VCAM-1 (232). ICAM-1 binds to both LFA-1 and Mac-1, whereas, ICAM-2, binds only LFA-1 (249). Expression of ICAM-1 and VCAM-1 on endothelial cells is increased after

stimulation with tumor necrosis factor-α (TNF-α) or IL-1 (238). Additionally, expression of ICAM-1 is also increased by interferon-γ (IFN-γ) (238), whereas two major cytokines in asthma, IL-4 and IL-13, upregulate VCAM-1 expression (250,251). In contrast ICAM-2 is constitutively expressed as an endothelial cell marker (249). *In vitro* expression of ICAM-1 and VCAM-1 on cultured endothelial cells is protein synthesis dependent. ICAM-1 expression peaks after 12 h and is maintained for at least a further 36 h whereas VCAM-1 expression peaks after 6 to 10 h of cytokine treatment. In asthmatic patients, expression of both ICAM-1 and VCAM-1 is increased (252). Furthermore, *in situ* expression of ICAM-1 and VCAM-1 in bronchial tissues from asthmatics experiencing air flow limitation have demonstrated an increased expression of these molecules in the apical membrane of the endothelium as well as in intracellular organelles (253). This may suggest an additional *de novo* synthesis of adhesion molecules prior to a spontaneous asthmatic attack.

1.7.1.a. *Adhesion and Migration*

During inflammation mediators (i.e. thrombin, histamine, PAF, LTC_4 , cytokines and chemokines) are released locally by mast cells, macrophages, T cells and airway epithelium. These inflammatory mediators act as chemoattractants and/or cell adhesion molecule-inducing agents thereby inducing infiltration of blood leukocytes into the inflamed tissue. Extravasation of leukocytes to sites of inflammation is thought to consist of at least three different processes. Firstly, circulating leukocytes undergo margination whereby they begin to roll along the endothelium of postcapillary venules adjacent to the extravascular site of lung inflammation. This process is mediated by the contact of L-selectin on leukocytes with diverse carbohydrate-containing structures (243), as well as P-selectin and E-selectin on activated endothelial cells (237). Tethering and rolling of leukocytes through selectins prolongs leukocyte contact with the vascular endothelium and in turn enhances their exposure to chemoattractants such as PAF, eotaxin or IL-8. This exposure to chemoattractants allows the adherent leukocyte to undergo changes including upregulation of intracellular Ca^{2+}, polarization in shape (i.e. reorganization of actin cytoskeleton), priming for enhanced activation, shedding of L-selectin and the induction of integrin adhesive functions (254). The rolling of circulating eosinophils on the endothelium is mediated primarily by P-selectin, whereas neutrophil rolling is mediated primarily by E-selectin (255,256). Interestingly, one particular antibody, LAM1-11, which recognizes a specific epitope on L-selectin, inhibits adhesion of eosinophils but not neutrophils, lymphocytes or monocytes under nonstatic conditions (257). Moreover, a reduced expression of L-selectin has been seen with eosinophils recovered from BAL fluid following allergen challenge, or activated *in vitro* (258,259). It is important to note here that in the lung there are two microvascular beds to consider, the large pulmonary circulation that is intimately associated with leukocytes to form the so-called marginating pool, and the smaller bronchial circulation that supplies the airways. Hence, the phenomenon of leukocyte rolling and tethering mediated by selectins is more predominant in post-capillary venules of the bronchial circulation. It would appear that such a mechanism of cell accumulation is not as important in the pulmonary circulation where the cells have to migrate through capillaries whose mean diameter is 5.5 to 6 μm compared to

12-17 μm for eosinophils and 7-8 μm for neutrophils (260). Thus leukocytes go through a stage of retention in the lung rather than tethering (Fig. 10) and would have to deform to pass through a capillary, a process that is likely to slow its progress even in the absence of selectin interactions. Hence, this possible sequestration of eosinophils in capillaries may be analogous to the tethering of leukocytes by selectins in post-capillary venules.

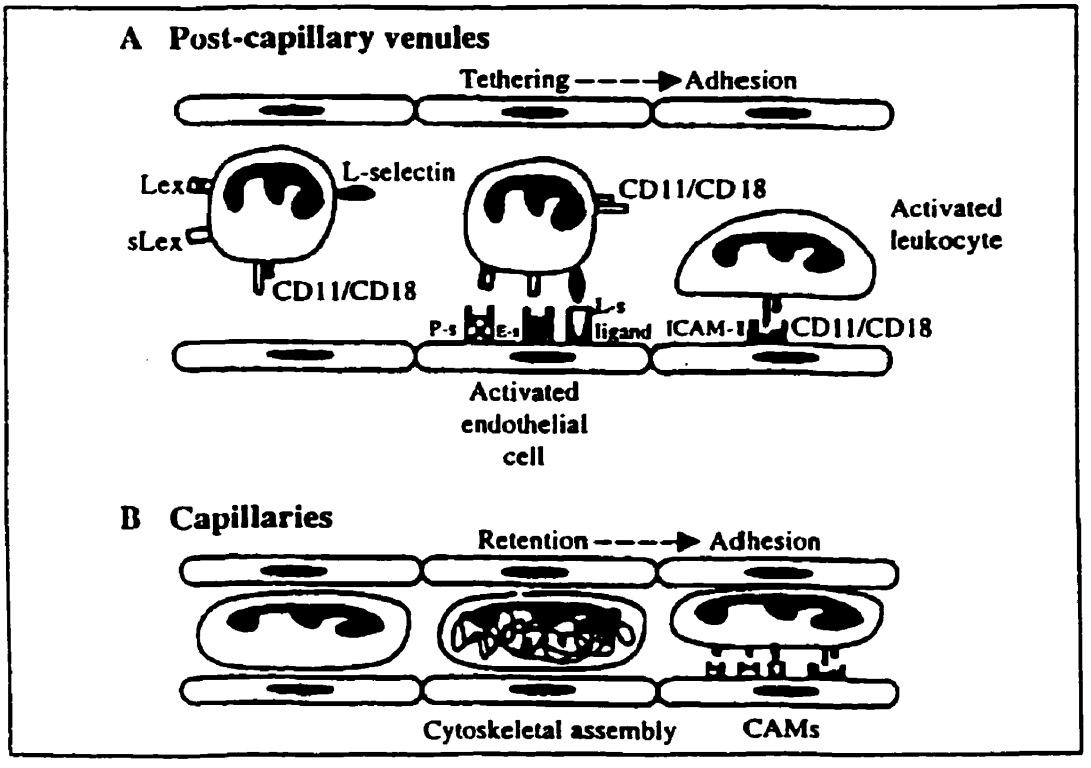

Fig. 10.Proposed mechanisms of leukocyte accumulation in (A) bronchial and (B) pulmonary circulation. The VLA-4/VCAM-1 adhesion pathway should also be considered. P-s : P-selectin; E-s : E-selectin; L-s : L-selectin.

The second step is firm adhesion and is mediated by leukocyte β2 (i.e. Mac-1, LFA-1) and β1 (i.e. VLA-4) integrins interacting with the endothelial adhesion molecules ICAM-1 and VCAM-1 respectively (261). This step in the inflammatory adhesion mechanism requires the activation of the leukocyte integrins by chemoattractants, such as PAF, cytokines or chemokines (see section 1.7.2). During activation of eosinophils, the integrins not only show an increased expression on the cell surface, but also experience a conformational change in the integrin heterodimer, leading to enhanced avidity (262) for their ligand. Recently, Weber et al, (263) demonstrated differential regulation of β1 and β2 integrin avidity by chemoattractants (RANTES, MCP-3, C5a and PMA) in human eosinophils *in vitro*. Activation of VLA-4 was transient and dependent on the actin cytoskeleton, whereas more prolonged activation with conformational changes appeared to be crucial for Mac-1. The adhesive properties of eosinophils are increased after stimulation with PAF, IL-3, IL-5 and GM-CSF (264), due to increased membrane expression of CD18 and CD11b (265). Recently, Sung et al (266), using a micropipette single cell adhesion assay able to measure the strength of adhesion forces, demonstrated that after incubating eosinophils with GM-CSF, the mean adhesion strength of eosinophils to the fibronectin connecting segment-1 (CS-1), and VCAM-1 increased significantly, compared to controls. This increased binding of eosinophils to VCAM-1 or CS-1 was not due to alterations in VLA-4 receptor number (assessed by FACS analysis) or alterations in VLA-4 receptor distribution (assessed by confocal microscopy), suggesting that endothelial-derived cytokines, such as GM-CSF, have the potential to alter the functional state of eosinophil-expressed VLA-4 from a low affinity to a high affinity state. Moreover, eosinophils from asthmatics show increased adhesion to VCAM-1 and ICAM-1 when compared to normal controls (267) and elevated levels of soluble ICAM-1 and VCAM-1 can be found in the blood and BAL fluid of patients with acute asthma (268,269).

The final step of cell recruitment is the transmigration of the leukocyte between two endothelial cells into the tissue. This process involves reversible adhesion, i.e. cyclic modulation of integrin receptor avidity. The molecular basis of transmigration is not clear, although LFA-1:ICAM-1 interaction appears to be critical (270). In addition this process is controlled by platelet endothelial cell adhesion molecule-1 (PECAM-1) (271) as well as

by endothelial cell-derived chemoatractants (272). Many *in vitro* studies have shown that transendothelial migration of eosinophils is predominantly mediated by the β2 integrins (273,274, 275,276). However, depending on the cytokine/chemotactic stimulus, transendothelial migration can also be mediated by the β1 integrin , VLA-4 (277) (275). *In vivo* studies using two different animal models suggest that the migration of eosinophils into the respiratory mucosa predominantly involves the VLA-4/VCAM-1 interaction (278,279).

Expression of cell adhesion molecules is regulated at multiple levels. First of all, the different cytokines have a selectivity for the induction of certain adhesion proteins, such as IL-4 (277) and IL-13 (251) for VCAM-1 . This selectivity is even dependent on the cell type, since IFN-γ is much more potent in inducing ICAM-1 on epithelial cells than on endothelial cells (280). Moreover, combinations of the different cytokines produce additive, synergistic or antagonistic effects (281). In addition, cytokines activate the airway epithelium to secrete chemokines, such as RANTES and eotaxin, which further aid in the recruitment of leukocytes, namely eosinophils, into the airways (see section 1.7.2). These multiple levels of regulation of cell adhesion molecule expression and function together with leukocyte-specific stimuli can create a unique sequence of events related to a certain inflammatory situation (261,282,283,284).

1.7.1.b. *Animal Models*

Although human studies provide evidence for important associations between eosinophil recruitment into tissue and increased expression of cell adhesion molecules and chemotactic agents, such studies are limited in scope due to practical difficulties and ethical issues associated. For this reason, models of eosinophil recruitment in rats, guinea pigs, mice, sheep and monkeys have been used.

The functional relevance of ICAM-1 in eosinophil recruitment was first shown in a primate model of allergic asthma induced by multiple inhalations of antigen (285). Treatment with a monoclonal antibody (mAb) against ICAM-1 attenuated airway eosinophilia and hyperresponsiveness while anti-Mac-1 mAb treatment reduced the levels of ECP in the BAL fluid but didn't inhibit the airway eosinophilia. In sensitized mice,

however, mAbs against ICAM-1 and LFA-1 did not inhibit eosinophil infiltration into the trachea after OVA challenge, whereas anti-VLA-4 and anti-VCAM-1 treatmant did (279). Local administration of PAF, C5a or LTB_4 in the skin of guinea-pigs induced eosinophil infiltration, which was potently inhibited by mAbs to VLA-4 (286).This decrease of eosinophil recruitment was also seen in the BAL fluid and lungs of allergic guinea pigs treated with anti-VLA-4 mAb prior to challenge (287). Aerosolized anti-VLA-4 mAb inhibited allergen-induced BAL eosinophilia and hyperresponsiveness in rabbits sensitized to house dust mite (288), as did intravenous treatment with an anti-CD18 antibody (289). A mAb to VLA-4 did not inhibit eosinophil infiltration in allergic sheep after allergen exposure (290), whereas it did in OVA-sensitized guinea pigs (278).

Another animal model that has been used in studying the adhesion pathways of eosinophil recruitment into the lungs is the rat. Rat models are becoming more useful as many immunological reagents including mAbs to cell adhesion molecules and cytokines have become available. The BN rat is one of the most widely used animal models of asthma because it develops an asthma-like response with a lot of similarities to that in humans (291,292,293,294). Recently, Richards and others have demonstrated that *in vivo* treatment of sensitized BN rats with mAbs against ICAM-1 (1A29) (295) or VLA-4 (TA-2) (296,297) prior to OVA challenge significantly inhibited the eosinophil recruitment into the airway lumen and alveolar spaces. There are also other reports that demonstrate that although treatment with anti-VLA-4 mAb can abbrogate the hypperresponsiveness after allergen challenge, it has no effect on eosinophil recruitment into airways of allergic BN rats (298). This lack of a consistent effect of antibody treatments on leukocyte numbers in the lung suggests that these antibodies may be affecting biological processes other than cell adhesion. There is evidence that adhesion molecules, particularly integrin receptors, have transmembrane signalling properties that mediate cell activation. Integrins LFA-1 and VLA-4 may act as costimulatory molecules in T-cell proliferation and activation (226). Anti-VLA-4 mAb treatment can attenuate PAF-induced EPO release from eosinophils (290). In addition, Mac-1, can mediate degranulation of eosinophils caused by GM-CSF or PAF *in vitro* (227). Furthermore, it is important to note here that mAbs against VLA-4 are antibodies against the $\alpha 4$ subunit of this integrin. The $\alpha 4$-integrin subunit, apart from

its association with the β1 chain, can also associate with β7 and is found on NK cells, eosinophils (weakly expressed), most newborn and some adult blood T and B cells, and most lymph node T and B cells (299,300). Like α4β1, α4β7 binds to VCAM-1 and fibronectin (301,302), but it can also bind to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), a homing receptor found most abundantly in gut lymphoid tissue (303). These recently discovered overlapping functions and structural components of α4 integrins and their counter-receptors have complicated the molecular interpretation of *in vivo* studies utilizing blocking mAbs to the α4-integrin subunit. Clearly, *in vivo* mAb-treatment against adhesion molecules has its limitations, however, the increased availability of mAbs to all components of adhesion pathways will allow clarification of these issues in the future.

Gene disruption, is another technique by which the functional relevance of a molecule to eosinophil recruitment can be studied. Gonzalo et al, studied the role of adhesion receptors ICAM-1, VCAM-1, P-selectin, and L-selectin in OVA-induced pulmonary eosinophilia by using mice lacking these adhesion molecules (304). They demonstrated that eosinophil migration into the lung tissue and BAL fluid is abolished in the absence of ICAM-1 or VCAM-1. Since the lack of adhesion receptors may result in impaired eosinophil differentiation, these deficient mice were injected intravenously with IL-5. No subsequent differences in the numbers of circulating eosinophils were detected among these IL-5-injected mutant and wild type mice.

Taken jointly, these data suggest that, although animal studies of eosinophil recruitment do not completely represent the complexity of tissue eosinophilia, they may delineate particular pathways or basic mechanisms contributing to this phenomenon. However, great care must be taken in extrapolating the information obtained from the analysis of animal models to humans. Firstly, there appears to be important species differences in the biochemistry and immunology of eosinophils and secondly, the notion that a disrupted gene or a mAb for an adhesion molecule will only inhibit effects of the adhesion pathway is too simplistic.

1.7.2 Chemoattraction

The migration of eosinophils into the lung is initiated by local chemoattractant molecules, which are likely to be responsible for both physiologic homing, and the recruitment of eosinophils into inflamed tissues. Numerous chemotactic substances act on eosinophils, including the previously discussed lipid mediators such as, PAF, LTB_4 (39), cys-LTs (117) and 5-oxo-ETE (10), as well as complement components (C5a) (39), cytokines (e.g. IL-5) (223), and chemokines (chemotactic cytokines) (305). Although all of these substances mediate the recruitment of eosinophils, most are not selective for eosinophils. Within the chemokine family eotaxin and eotaxin-2 are relatively specific for eosinophils (306,307). Unlike many other chemokines, the eotaxins mediate their effects through only one receptor, the CCR-3 receptor (308) which is found on eosinophils.

Chemokines are a superfamily of small peptides chemoattractants (8-14 kDa). They are subdivided into families on the basis of the relative position of their cysteine residues (309). There are at least four families of chemokines, but only two have been extensively characterized. The α-chemokines, or CXC chemokines (one amino acid (X) separates the first two cysteine residues), in general induce neutrophil or lymphocyte but not monocyte locomotion. The β-chemokines, or C-C chemokines (first two cysteine residues are adjacent to each other), in general do not act on neutrophils but attract monocytes, eosinophils, basophils and lymphocytes with variable selectivity. Two chemokines that do not fit into this classification, lymphotactin (310), with only two cysteines, and fractalkine (311), a membrane bound glycoprotein in which the first two cysteine residues are separated by three amino acids (CXXXC), may represent additional families.

C-C chemokines such as RANTES, MCP-3, MCP-4, macrophage inflammatory peptide-1α (MIP-1α) and the eotaxins, in association with cytokines IL-3, IL-4, IL-5, GM-CSF (312,313,314,315,316) and 5-LO products (317,318) may play key roles in regulating eosinophil recruitment into sites of allergic inflammation.

RANTES and MCP-3 are more potent than MIP-1α for eosinophil chemotaxis (314, 319), however, they do not selectively regulate eosinophil trafficking. The role of MIP-1α was identified in a murine model of allergic eosinophilic inflammation (320).

When antigen-sensitized and airway-challenged mice were passively immunized with anti-MIP-1α antibodies, the eosinophil infiltration into the airways was decreased by about 50 %. Expression of MCP-3 and RANTES mRNAs has been correlated with the level of eosinophil infiltration induced by allergen challenge in the skin of patients with atopy (321). In a more recent investigation, when RANTES was injected intradermally in both allergic and nonallergic subjects it induced an eosinophilic recruitment which was maximal 6 h and 24 h later respectively (322). The recently described chemokine, MCP-4, is a chemoattractant of high efficacy for eosinophils, matching *in vitro* the effectiveness of RANTES, MCP-3 and eotaxin (323). Moreover all three chemokines, RANTES, MCP-3 and MCP-4, mediate their effects on the eosinophil through the CCR-3 receptor (308).

Eotaxin, first discovered in guinea pigs and subsequently cloned in mice and humans, has the particularity of being a potent and specific eosinophil chemoattractant *in vitro* and *in vivo* (224, 324) . Increased expression of this chemokine has been seen in the BAL fluid and airways of asthmatics and has been suggested to contribute to the airway eosinophilia seen in asthma (325). Significant lung eosinophilia occurs 20 h after delivery of aerosolised eotaxin (326). Eotaxin expression can be induced locally in tissues after transplantation of IL-4-secreting tumour cells (315), suggesting that eotaxin may be involved in immune responses regulated by IL-4. Investigations in guinea pigs and mice suggest that eotaxin may act cooperatively with IL-5 to promote the recruitment of eosinophils into tissues (212, 327). The relationship between IL-5 and eotaxin in the regulation of eosinophil homing as well as its recruitment into blood and tissue was investigated in IL-5 -/- mice (328). This study suggests that there is an essential requirement for IL-5 in eotaxin-induced recruitment of eosinophils to mucosal tissues.

There is evidence that 5-LO products may be involved in the response of eosinophils to chemokines. Recently Harris et al., (317) demonstrated that specific 5-LO inhibitors zileuton and ABT-761 attenuated the eotaxin-induced eosinophil recruitment into the mouse peritoneal lavage fluid by approximately 70%. This data suggests that eotaxin may either be activating eosinophils to release 5-LO metabolites, such as 5-oxo-ETE, or making them more responsive to these metabolites. Interestingly, a similar degree of inhibition in eosinophil recruitment was seen when mice were treated with the 5-LO

inhibitors prior to IL-5 and eotaxin injections. The fact that the 5-LO inhibitors could attenuate the combined effects of IL-5 and eotaxin suggests that their effect may involve 5-LO products. In a recent abstract, Stafford and Alam (318) also presented data implicating 5-LO products in the chemokine-mediated effect. In this report inhibition of 5-LO attenuated RANTES-induced eosinophil chemotaxis *in vitro*.

In summary, the clinical and experimental investigations suggest that migration of eosinophils into tissues involves the cooperation of different classes of mediators. These would include 5-LO products, eotaxin and cytokines. The combined effects of various mediators in the influx of cells is probably more reflective of human disease than is normally proposed from cellular or animal experimentation where single mediators are assumed to be working.

1.8 Hypotheses

It is clear that the cellular and molecular events regulating eosinophil recruitment to sites of allergic inflammation are complex processes that involve a co-ordinate network of inflammatory cells, vascular adhesion molecules, cytokines, chemokines and lipid mediators.

An important area of research is now concerned with the identification of specific chemoattractant molecules that issue the chemotactic and chemokinetic stimuli which localise eosinophils to the foci of tissue inflammation, where they elicit their effector function. Studies using receptor antagonists and synthesis inhibitors have revealed that 5-LO products may be such candidates. Recent *in vitro* studies have demonstrated that the 5-LO product, 5-oxo-ETE, is a potent eosinophil chemoattractant and also stimulates calcium mobilization, degranulation, superoxide formation, actin polymerization, Mac-1 expression and L-selectin shedding in these cells. In view of these potent *in vitro* effects on eosinophils, the objective of this study was to determine whether 5-oxo-ETE is also active *in vivo*.

To fulfill this objective the BN rat was used to test the following hypotheses:

1) **5-Oxo-ETE induces eosinophil recruitment in *in vivo*.** The effects of intratracheally administered 5-oxo-ETE on eosinophil infiltration in the lungs of BN rats was investigated and compared to those of other 5-LO products, such as LTB_4, LTC_4 and LTD_4.

2) **5-Oxo-ETE-induced eosinophil recruitment is independent of actions of other lipid mediators such as PAF and LTB_4.** To test this hypothesis animals were treated with receptor antagonists to PAF and LTB_4 before and after 5-oxo-ETE intratracheal insufflation.

3) **5-Oxo-ETE-induced eosinophil recruitment is dependent on integrins.** This hypothesis was tested by injecting the animals intravenously with mAbs against β1 and β2 integrins prior to 5-oxo-ETE treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1. Chemicals

5-Oxo-ETE was prepared by Drs. J. Rokach and S. Khanapure; Florida Institute of Technology (329). LTB_4 was synthesized by Syvie Gravel in Dr. Powell's laboratory by incubation of AA (Nuchek Prep. Inc., Elysian, MN) with porcine neutrophils in the presence of 5, 8, 11, 14-eicosatetraynoic acid and the calcium ionophore A23187 (Calbiochem, LaJolla, CA). LTD_4 and LTE_4 were obtained from the Cayman Chemical Co., (Ann Arbor, MI) and PAF (1-palmityl-2-acetyl-sn-glycero-3-phosphocholine) was purchased from Sigma. The anesthetics used were the muscle relaxant xylazine and the sedative sodium pentobarbital which were purchased from Chemagro Limited, Etobicoke, Ontario, Canada and MTC Pharmaceuticals, Cambridge, Ontario, Canada, respectively.

2.1.2. Antagonists

The LTB_4 antagonist, LY255283, (5-ethyl-2-hydroxy-4-(6-methyl-6-(1H-tetrazol-5-yl)heptyloxy) acetophenone), was a kind gift from Eli Lilly Co., Indianapolis, Indiana. The potent long-acting PAF antagonist, WEB 2170, (6-(2-chlorophenyl)-8-9-dihydro-1-methyl-8-(4-morpholinyl-carbonyl)-4H,7H-cyclopental (4,5) thieno (3,2,f) (1,2,4)-triazolo-(4,3-a) (1,4)daizepine) (330) was provided by Boehringer-Ingelheim, USA. These receptor antagonists have been previously used in studies with BN rats and other animals (76, 331) .

2.1.3. Monoclonal Antibodies

Mouse anti-rat monoclonal antibodies (mAbs) to VLA-4 (TA-2; IgG1) 332, LFA-1 (TA-3; IgG1) (333), and Mac-1 (OX-42; IgG2a) (333) were obtained from Dr. T Issekutz; Dalhousie University. These antibodies were prepared by immunizing BALB/c mice with rat leukocytes and characterized as previously described. The anti-rat VLA-4 mAb (TA-2) reacts with all rat leukocytes and blocks their adhesion to rat microvascular endothelial cells stimulated with IFN-γ, IL-1, TNF-α and lipopolysaccharide (332) *in vitro*. Experiments with fluorescence microscopy demonstrated that this mAb reacts with VLA-4 on eosinophils of BN rats. The mAbs to LFA-1 (TA-3) and Mac-1 (OX-42) also

blocked the adhesive function of rat leukocytes by interacting with their respective integrins (333). An isotype-matched mAb (3h11-B9; IgG1) (334) directed to an irrelevant cell surface protein, pertussis toxin, was used as a control. Various studies in the past have examined the effects of these mAbs on leukocyte recruitment in the BN rat lung (298, 335).

The airway-infiltrating eosinophils were quantitated by immunocytochemistry using a mAb to MBP that has previously been shown to detect rat eosinophils (336). This mAb was a kind gift from Dr. R. Moqbel, University of Alberta, Edmonton, Canada.

2.1.4. Animals

Experiments were performed on 180 ($n \geq 5$) male highly inbred (> 58 generations) BN rats (Rij substrain; 6 - 8 weeks old; 180 - 220 grams) obtained from Harlan Sprague Dawley (Indianapolis, IN). They were housed in groups of four-to-five with food and water available *ad libitum*. Animal housing was carried out in accordance with McGill University's Policy on the Handling and Treatment of Laboratory animals and the Canadian Council on Animals Care guidelines. A period of five-to-six days of acclimatization was allowed prior to experimentation.

2.2 Methods

2.2.1. Agonist Administration

Rats were anesthetized by intraperitoneal injection of xylazine (7 mg/kg) and pentobarbital (50 mg/ kg). Endotracheal intubation was performed with a 6 cm length of polyethylene tubing (PE-240). Agonists (5 μg unless otherwise indicated) in 100 μl of saline containing 0.5% ethanol were administered by insufflation using a 1 ml syringe containing the agonist and 1 ml of air to force the agonist into the lungs. This method of agonist administration should enable a better distribution into the airways. Alternatively, agonists in 100 μl of saline containing 0.5% ethanol were instilled directly into the trachea without the use of air. Control animals recieved vehicle (saline containing 0.5% ethanol) alone. After administration of the agonist, the animals awakened spontaneously and were extubated. At various time intervals (6, 15, 24 and 36 h) the animals were anesthetized and the lungs removed for immunocytochemistry as described below.

2.2.2. Receptor Antagonist Treatment

To explore further the role of 5-oxo-ETE in the induction of airway eosinophilia in the BN rat, antagonists to LTB_4 (LY255283) and PAF (WEB 2170) were tested for the inability to prevent eosinophilic influx following insufflation of 5-oxo-ETE. Preliminary tests with our BN rat model revealed that treatment with 20 mg/kg (and not 10 mg/kg) of LY255283 attenuated LTB_4-induced airway eosinophilia. The PAF antagonist, WEB 2170, has not been characterized in BN rats. However, our preliminary experiments demonstrated that 30 mg/kg of WEB 2170 was sufficient to significantly reduce airway eosinophilia in BN rats following insufflation with PAF. Hence, the subsequent groups of animals were treated with 20 mg/kg of LY255283 and 30 mg/kg of WEB 2170.

Seven groups ($n \geq 5$), were orally gavaged (intragastric administration) with either LY255283, WEB 2170 or vehicle (carboxymethylcellulose 0.2% w/v H_2O) (143). The animals were then anesthetized, intubated and subsequently insufflated with 5 μg of either LTB_4, PAF (reconstituted in PBS with 0.125% BSA) or 5-oxo-ETE 1 h after drug treatment. At some later time the animals awakened spontaneously and 7 h after agonist insufflation they were gavaged once more with either LY255283, WEB 2170 or vehicle.

The lungs were excised 15 h after agonist insufflation and prepared for immunocytochemistry

2.2.3. Monoclonal Antibody Treatment

Monoclonal antibodies to the integrins VLA-4 (β1 integrin family), LFA-1 and Mac-1 (β2 family) were used to study the adhesion mechanisms involved in 5-oxo-ETE-induced eosinophil recruitment. Seven groups of 5 rats each were injected intravenously (i.v.) with either anti-integrin or control mAbs. Stock mAb was diluted with saline, then filter sterilized through a 0.22 μm filter (Millipore Co., Bedford MA). A concentration of 1.4 mg of mAb in 0.5 ml sterile saline was injected 30 min prior to agonist insufflation. These amounts of antibodies have been previously shown to maximally block leukocyte migration *in vivo* and provide plasma levels that are several times higher than required to saturate integrins on blood leukocytes (332, 333, 334).

The animals were first anesthetized and then intubated in preparation for agonist insufflation 30 min after mAb treatment. The first three groups of animals served as controls. Group 1 was the negative control where animals were injected and insufflated with vehicle (saline (0.5 ml)) and saline with 0.5% ethanol (100 μl) respectively). Rats in the positive control group 2, were injected with vehicle and insufflated with 5-oxo-ETE (5 μg). The third group served as the control for the mAb treatment. In this group, animals were treated with the isotype matched mAb (3H11-B9) and then insufflated with 5-oxo-ETE. The remaining four groups of animals were the test groups. Groups 4, 5 and 6 were treated with mAbs to the integrins VLA-4, Mac-1 and LFA-1 respectively. Group 7 was treated with a combination of mAbs to both β2 integrins, Mac-1 and LFA-1. All these test groups were insufflated with 5-oxo-ETE (5 μg). The lungs were excised 15 h following agonist insufflation and prepared for immunocytochemistry.

2.2.4. Histology

Following induction of anesthesia, rats were exsanguinated via the abdominal aorta. The lobes of the lungs were dissected around the hilum (0.5 x 1 cm), immediately placed in phosphate-buffered saline and frozen within 30 min. The fresh tissue was placed in OCT embedding medium and snap frozen in isopentane precooled in liquid nitrogen and stored at -80 °C. Sections of 6 μm were cut in a cryostat and three consecutive sections were placed on microscope slides. They were air dried for 1 h and fixed in acetone/methanol (1:1) for 5 min and further air dried for 1 h. Slides were then wrapped back to back in pairs in aluminum foil and stored at -20 °C prior to immunostaining.

It is important to note here that many investigators in the past have quantitated eosinophils in lung tissue following enzymatic dispersion by tissue mincing and digestion with collagenase. However enzymatic dispersion and cell counting may be subject to non-specific cell loss or variation due to heterogeneous involvement of lung tissue. These limitations were overcome in the present study by using whole tissue and quantitating eosinophils following immunostaining for MBP.

2.2.5. Immunocytochemistry

Eosinophils in lung sections were quantitated by Drs. Q. Hamid and R. Taha. Slides were allowed to defrost and then stained with a mAb to MBP using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method as previously described (337). To prevent nonspecific binding of the second and third antibodies the sections were treated with 20% normal rat serum. Slides were coded and read in a blind fashion at 200x magnification. The numbers of positively-stained cells were counted in the walls of the airways identified in a zone 115 μm deep (as defined by a squared eyepiece graticule) along the length of the epithelial basement membrane. A calibrated computerized graphics tablet (IBM) was employed to determine the length of the basement membrane. Cell counts are expressed as the mean numbers of immunoreactive cells per unit length (1 mm) of basement membrane of the airway.

2.2.6. Data Analysis

One way ANOVA using Dunnett's test as a multiple comparison method was used to determine whether there were statistically significant differences among groups of animals. For comparison of two groups Student's t-test was used using the Bonferroni correction. All data are presented as means ± SE (n = 5, unless indicated otherwise). Differences were considered to be statistically significant for p values of less than 0.05.

CHAPTER 3

EXPERIMENTAL RESULTS

3.1. 5-Oxo-ETE is a strong inducer of pulmonary eosinophilia.

Powell et al., (10) have demonstrated that 5-oxo-ETE is a very potent chemotactic agent for human eosinophils *in vitro*. To test this effect *in vivo*, BN rats (n = 5) were treated via tracheal insufflation with either vehicle or 5-oxo-ETE (5 μg). The lungs were removed after 15 h and sections were immunostained for the eosinophil marker, MBP. Preliminary experiments indicated that 15 h were sufficient to detect eosinophil recruitment into tissue. Representative slides of the rat lung sections are shown in Figure 1. Consistent with its *in vitro* effect, 5-oxo-ETE induced a dramatic increase in the numbers of eosinophils detected in the BN rat lung as illustrated by Figures 1A and 1B. The MBP-positive cells were found principally around the airways among other inflammatory cells. There were also occasional eosinophils around blood vessels and in the parenchyma (Figure 1B). Lung sections from control rats treated with vehicle contained significantly fewer numbers of eosinophils (Figure 1C). Figure 1D represents a control for background and non-specific staining in which lung sections from 5-oxo-ETE-treated rats were processed identically with the exception of the mAb to MBP.

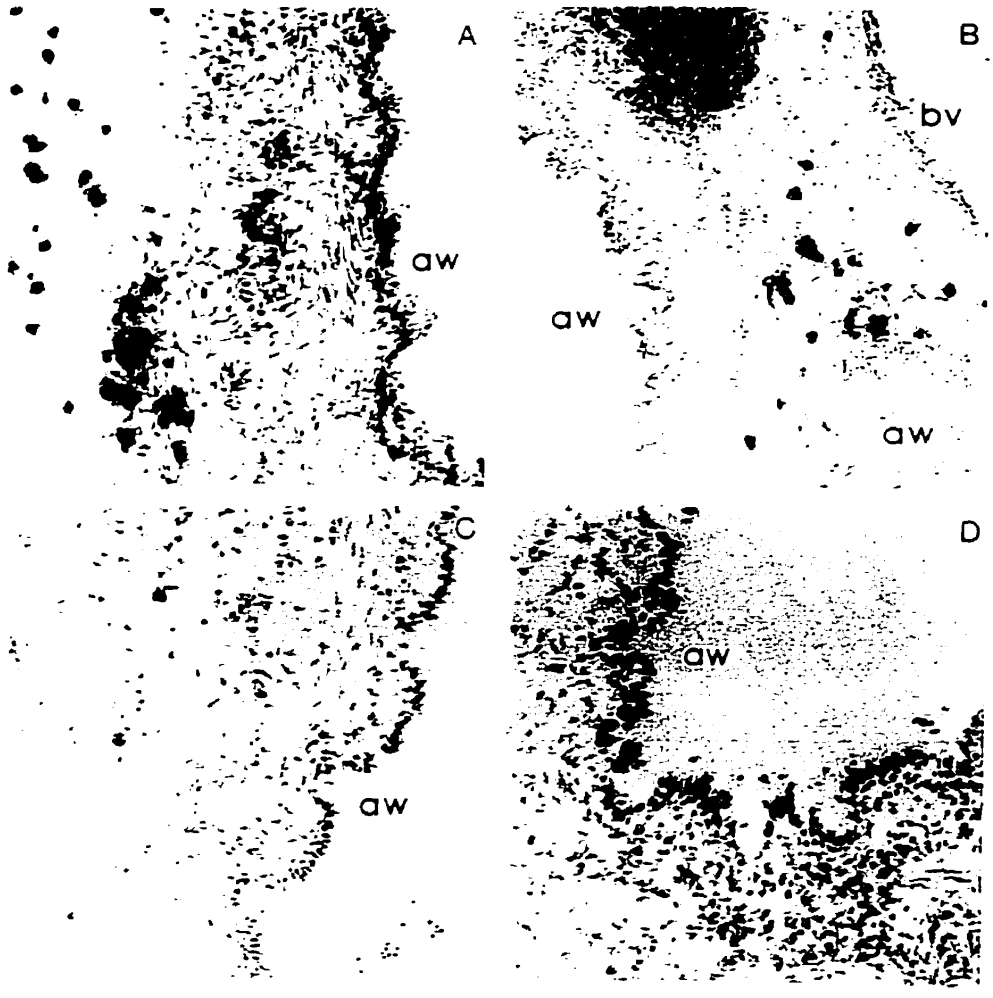

Figure 1. ***Immunochemical staining of lung sections (cryostat) using the alkaline phosphatase-anti-alkaline phosphatase method.*** The location of airways (aw) and blood vessels (bv) are indicated. **A**: example of an MBP immunostained section from an animal that received 5-oxo-ETE (5 μg) 15 h prior to removal of the lungs (x 400). **B**: a lower power (x 200) view of an MBP immunostained lung section from an animal that received 5 μg of 5-oxo-ETE 15 h before removal of the lungs. **C**: an example of a section immunostained for MBP from the lungs of an animal that received saline 15 h prior to removal of the lungs (x 400). **D**: negative control in which a lung section from a rat treated with 5-oxo-ETE for 15 h was processed in a manner identical to the sections shown in A, B, and C, except that PBS was substituted for the mAb to MBP (x 400).

3.2. 5-Oxo-ETE induces eosinophil infiltration in a time-dependent manner.

The BN rat is known to develop a neutrophilic infiltration in the airway 8 h after a single allergen challenge, followed by an accumulation of eosinophils 24 to 32 h after the challenge (338,339). Even though our study did not involve an allergic model, the time range mentioned above served as a guideline none-the-less. To investigate the time-course for 5-oxo-ETE-mediated eosinophil recruitment, rats ($n = 5$) were insufflated with either vehicle or 5-oxo-ETE (5 μg), the lungs were removed following various times (6, 15, 24 and 36 h) and sections were immunostained for MBP. At 6 h following 5-oxo-ETE treatment there was an increase in the number of pulmonary eosinophils which was about 3 times higher than that following treatment with control (vehicle) ($p < 0.05$) (Figure 2). This effect was maximal between 15 h ($p < 0.005$) and 24 h ($p < 0.01$), with the numbers of eosinophils increasing to about 5 times control values. By 36 h the numbers of infiltrating eosinophils had decreased to about 3 times control values ($p < 0.05$), similar to what was observed at the initial time point of 6 h. Subsequent experiments were therefore performed on rats 15 or 24 h after treatment with 5-oxo-ETE.

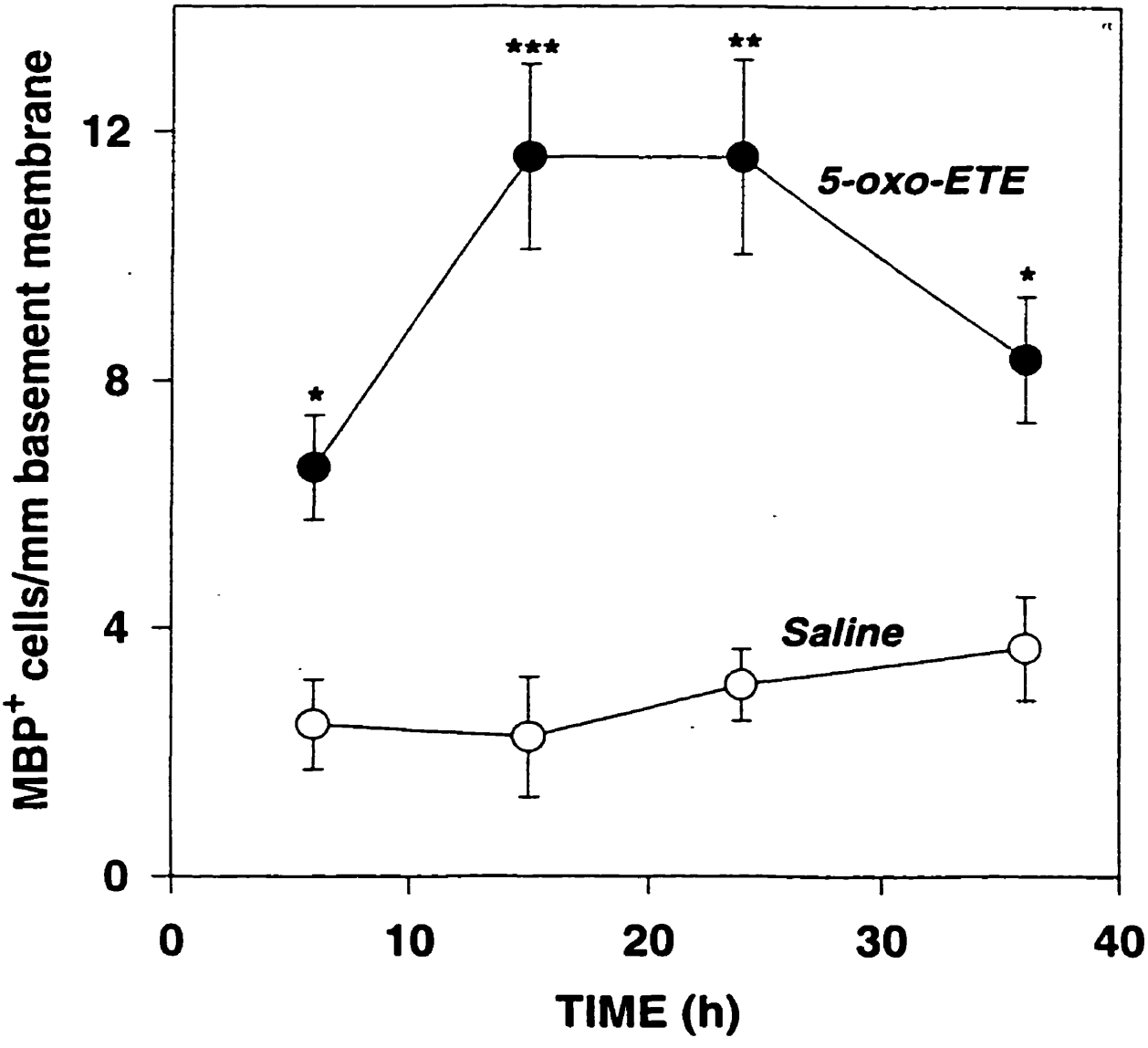

Figure 2. ***Time course for 5-oxo-ETE-induced eosinophil recruitment.*** BN rats were insufflated with 5-oxo-ETE (5 μg; n = 5) or vehicle (100 μl of 0.5% ethanol in saline; n = 8). The lungs were removed 6, 15, 24 or 36 h later and stained for eosinophils using an antibody to MBP. The results are expressed as the numbers of positive cells per mm of basement membrane of the airway and are means ± SE (n = 5). Differences between treated and control rats at different time points were evaluated using Student's t-test followed by a Bonferroni correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

3.3. Comparison of effects of 5-oxo-ETE to those of other 5-LO products.

The effects of 5-oxo-ETE were compared to those of various LTs, including LTB_4, LTD_4, and LTE_4. This experiment was performed by Dr. W. Yu in Dr. Powell's laboratory. Five groups of rats (n = 5) were treated by intratracheal instillation of either vehicle or one of the four agonists (5 μg). The lungs were removed and sections were stained for MBP 24 h later. Of the 5-LO products tested, only 5-oxo-ETE ($p < 0.01$) and LTB_4 ($p < 0.05$) induced pulmonary eosinophil infiltration, with the numbers of eosinophils in lung sections being about 3.5 and 3 times, respectively, higher than in lung sections from control animals (Figure 3). In contrast, eosinophil numbers in lungs from rats treated with LTD_4 and LTE_4 were the same as in control lungs.

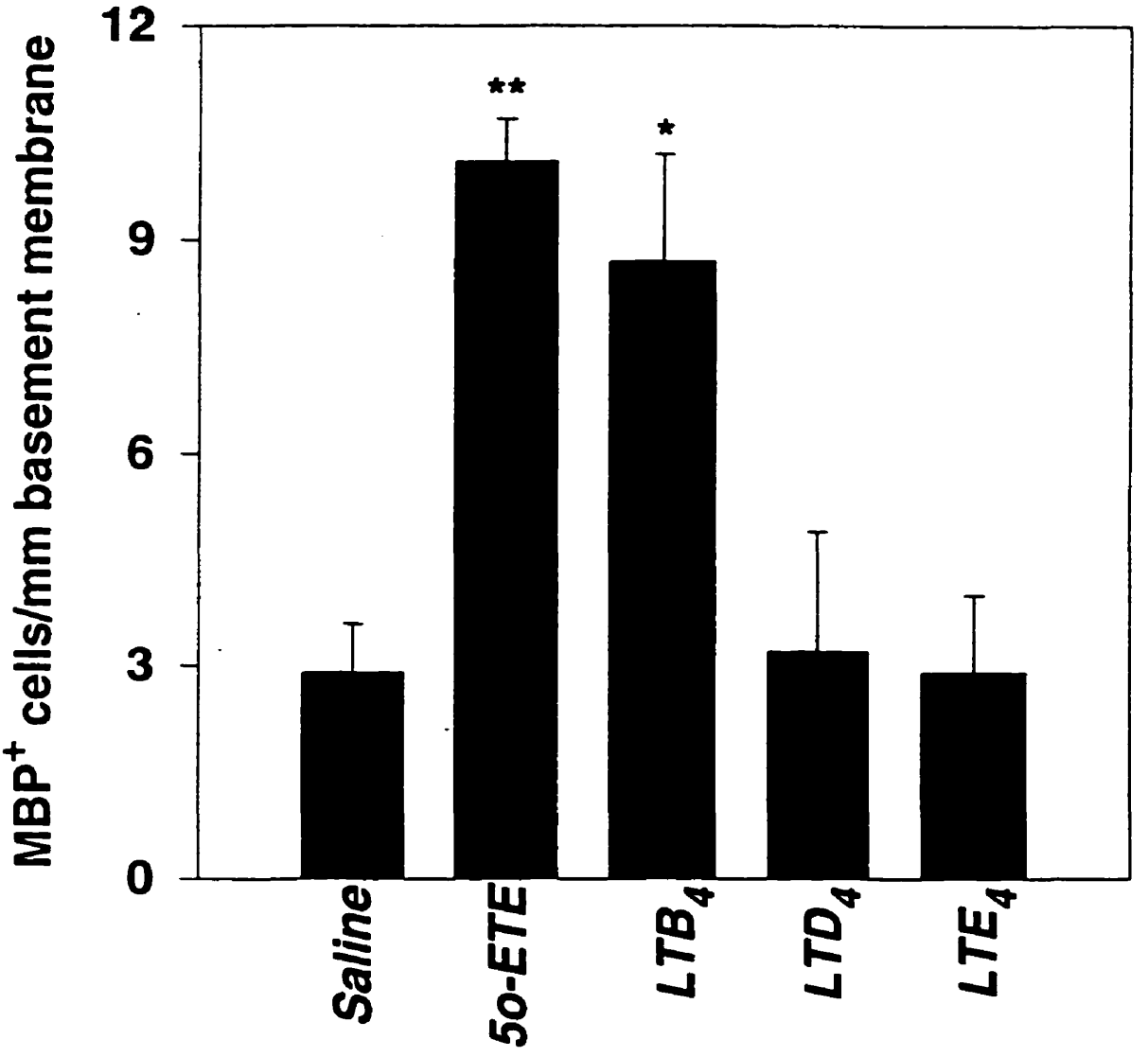

Figure 3. ***Comparison of the effects of 5-oxo-ETE to those of leukotrienes on pulmonary eosinophil recruitment.*** Various eicosanoids (5 μg) or vehicle (100 μl of 0.5% ethanol in saline) were administered to BN rats by tracheal instillation and the lungs were removed 24 h later and sections stained for eosinophils using an antibody to MBP. The results are expressed as the numbers of positive cells per mm of airway basement membrane and are means ± SE (n = 5). Differences among groups were evaluated by one-way ANOVA with Dunnett's test as a multiple comparison method. *, $p < 0.05$; **, $p < 0.01$.

3.4. Comparison of the dose-response for 5-oxo-ETE to that for LTB_4.

To further investigate the effects of 5-oxo-ETE and LTB_4 on eosinophil recruitment into the rat lung their dose-response relationships were investigated and compared. BN rats were insufflated intratracheally with either vehicle (n = 8) or various amounts of 5-oxo-ETE or LTB_4 (2, 5, and 10 μg; n = 5). After 15 h, the lungs were removed and the numbers of eosinophils counted in lung sections after immunostaining for MBP. Both agonists induced a dose-dependent increase in pulmonary eosinophils, which was maximal at a dose of 5 μg in each case (Figure 4). The dose response curves for the two eicosanoids were similar to one another, except that the maximal response to 5-oxo-ETE, observed at 5 and 10 μg, was nearly twice that to LTB_4 ($p < 0.05$). Rats treated with the smallest amounts (2 μg) of 5-oxo-ETE ($p < 0.01$) and LTB_4 ($p < 0.05$) showed increased numbers of pulmonary eosinophils which were about 2.6 and 2.3 times greater than control respectively. At the maximal doses of 5 or 10 μg, these increases in eosinophil numbers were about 5.5 times (5-oxo-ETE) and 3.5 times (LTB_4) the control levels ($p < 0.005$).

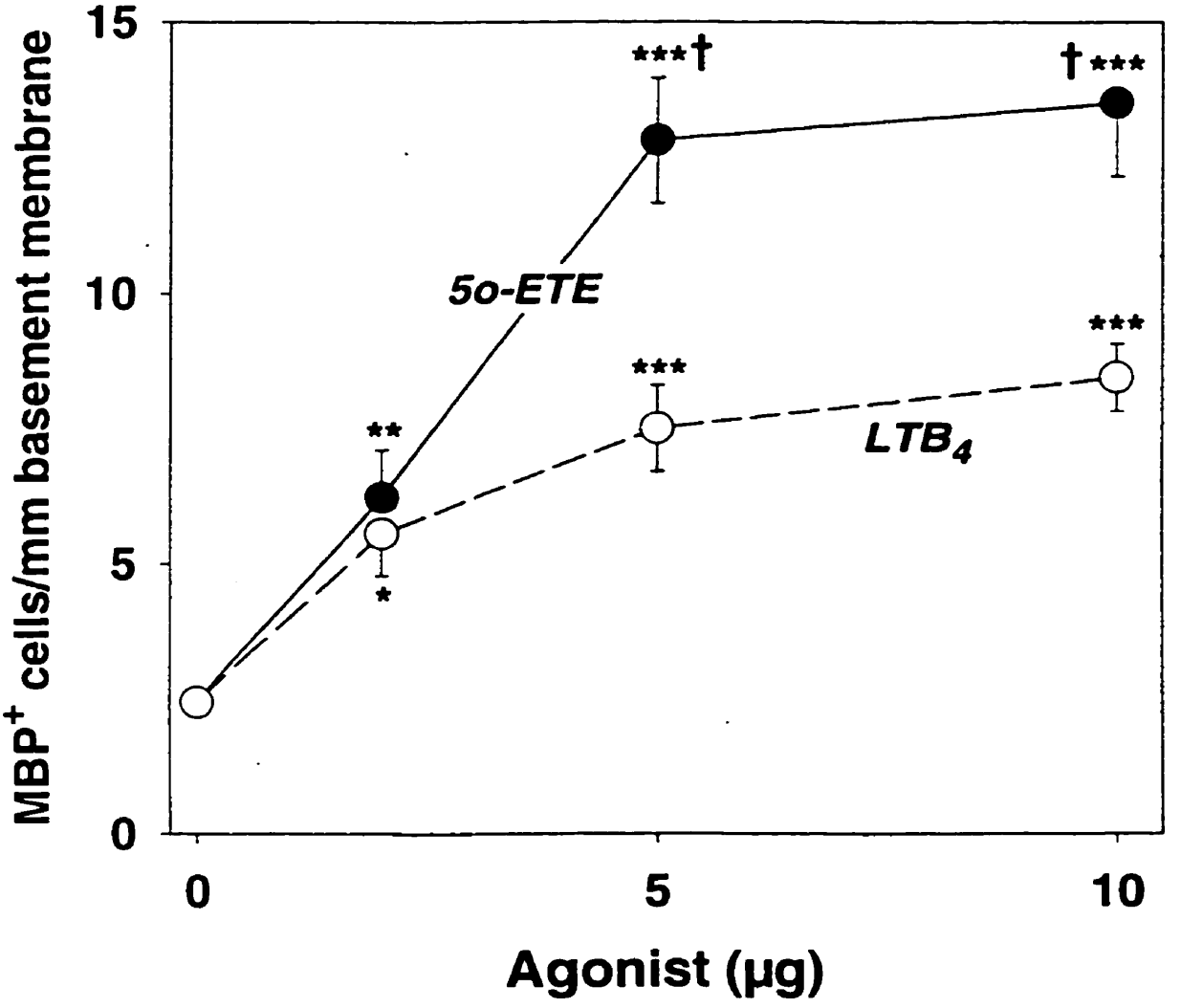

Figure 4. ***Dose-response for the effects of 5-oxo-ETE and LTB4 on eosinophil infiltration***. 5-Oxo-ETE, LTB_4 (2, 5 or 10 µg) or vehicle (100 µl of 0.5% ethanol in saline) were administered to BN rats by tracheal insufflation. The lungs were removed 15 h later and sections were stained for eosinophils using an antibody to major basic protein. The results are expressed as the numbers of positive cells per mm of airway basement membrane and are means ± SE (n = 5). Differences between groups were evaluated using Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ when comparing a given dose of the test group to the vehicle-treated control. †, $p < 0.05$ when comparing the groups treated with identical doses of 5-oxo-ETE and LTB4.

3.5. Effects of LTB_4 and PAF antagonists on 5-oxo-ETE-induced eosinophil infiltration.

To determine whether the effects of 5-oxo-ETE on eosinophil recruitment could be mediated by LTB_4 or PAF, BN rats (n = 5) were pretreated via oral gavage with receptor antagonists to LTB_4 (LY255283) or PAF (WEB 2170) 1 h before and 7 h following insufflation with agonist (5 μg). The lungs were excised 15 h after agonist insufflation and sections were stained for MBP. Treatment of animals with vehicle followed by insufflation with 5-oxo-ETE resulted in a nearly 6-fold increase in the numbers of lung eosinophils compared to the negative control treated with vehicles for the receptor antagonist and 5-oxo-ETE (Figure5). Similarly, animals treated with vehicle followed by either LTB_4 or PAF also demonstrated increased numbers of pulmonary eosinophils which were nearly 4 and 6-fold, respectively, higher than the vehicle-treated controls. Treatment with LY255283 (20 mg/kg) inhibited the LTB_4-mediated effect on lung eosinophilia by 72 % ($p < 0.005$), whereas WEB 2170 (30 mg/kg) treatment supressed PAF-mediated eosinophil recruitment by 78 % ($p < 0.0001$). However, neither of these receptor antagonists had any effect on 5-oxo-ETE-induced eosinophil infiltration. Representative slides of animals treated with LY255283 and insufflated with either LTB_4 or 5-oxo-ETE are depicted in Figure 6.

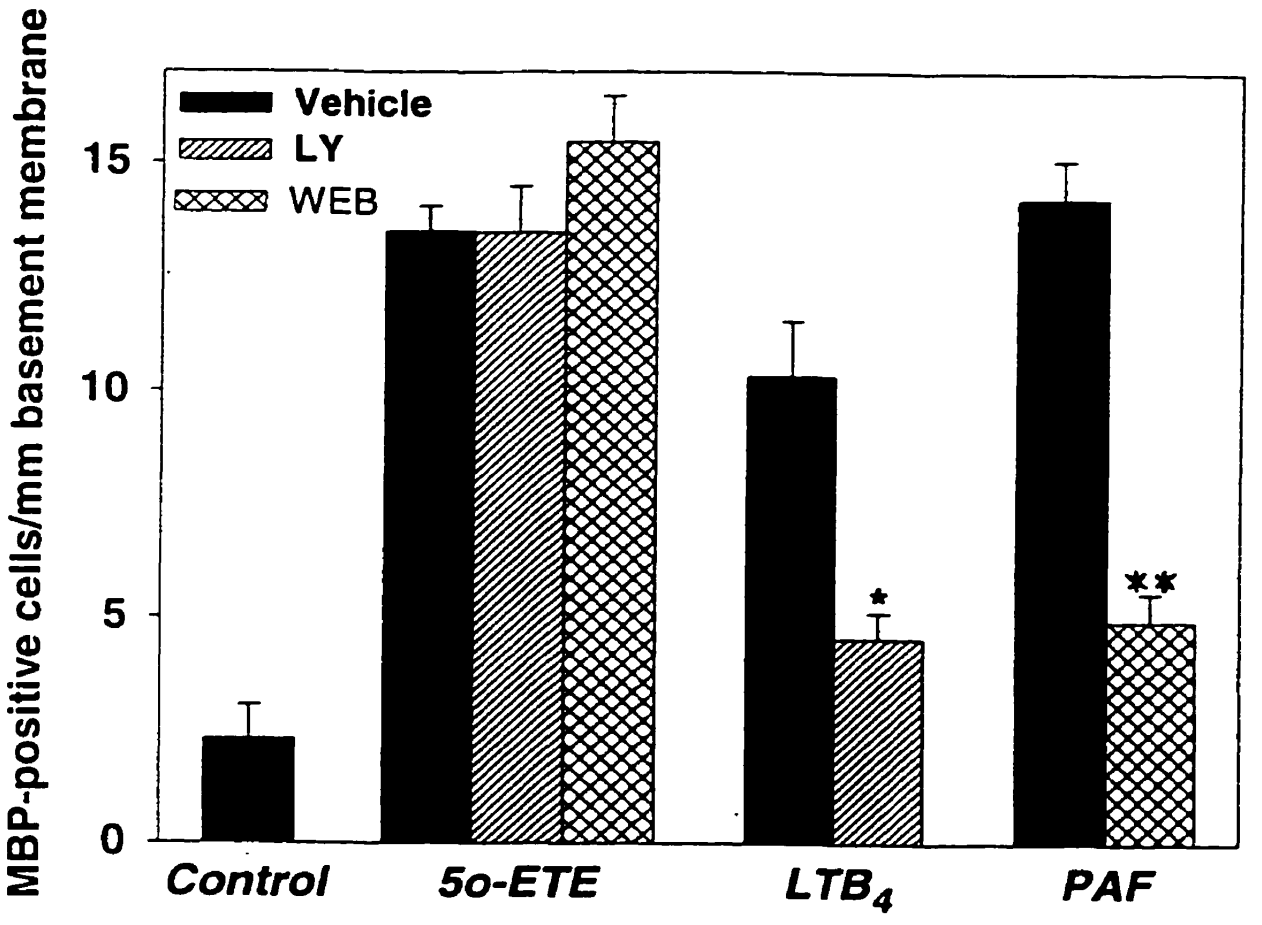

Figure 5. ***Effects of receptor antagonists LY255283 and WEB 2170 on 5-oxo-ETE-induced eosinophil recruitment.*** LY255283 (20 mg/kg) or WEB 2170 (30 mg/kg) in a volume of 0.5 ml were administered by oral gavage 1 h before and 7 h after agonist treatment. The control group was treated with 0.5 ml of the antagonist vehicle (0.2 % carboxymethylcellulose) and insufflated intratracheally with 100 ml of the agonist vehicle (0.5% ethanol in saline). The agonists 5-oxo-ETE (5 μg), LTB_4 (5 μg) and PAF (5 μg) were administered by intratracheal insufflation. The lungs were removed 15 h after agonist treatment (8 h following the second oral gavage with antagonist) and sections were stained for MBP. The results are expressed as the numbers of positive cells per mm of airway basement membrane and are means ± SE (n = 5). Differences among groups were evaluated by one-way ANOVA with Dunnett's test as a multiple comparison method. *, $p < 0.01$; **, $p < 0.005$.

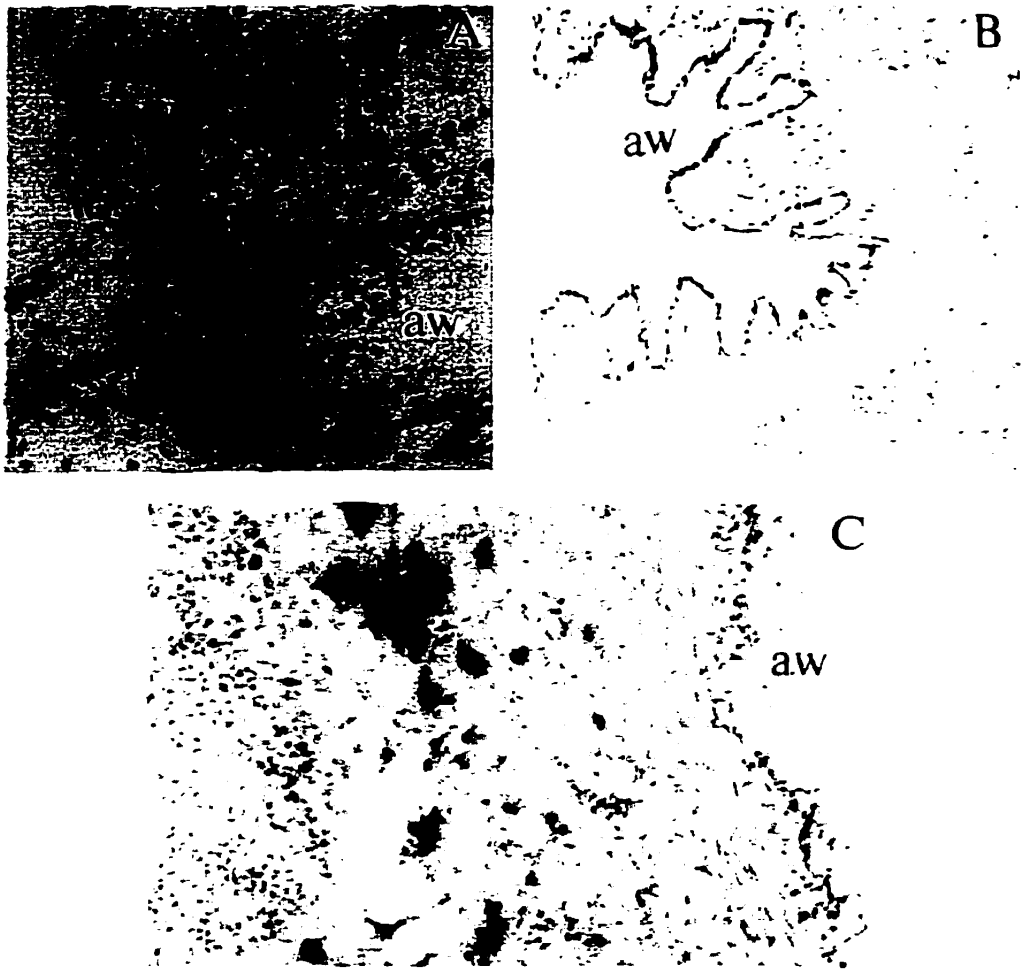

Figure 6. ***Immunocytochemical assessment of the effects of LY255283 on LTB_4 - and 5-oxo-ETE-induced pulmonary eosinophilia.*** The location of airways (aw) are indicated. Lung tissue was fixed, sectioned, and stained for MBP 15 h following agonist insufflation. **A:** example of an MBP-stained section from an animal treated with vehicle (0.5 ml of 0.2% carboxymethylcellulose) 1 h before and 7 h following LTB_4 (5 μg) insufflation. A significant number of esinophil recruitment into the airways (x 200). **B:** example of an MBP immunostained section from an animal treated with 20 mg/kg LY255283 1h before and 7 h after LTB_4 (5 μg) insufflation. Few eosinophils are present around the airways (x 200). **C:** example of an MBP immunostained section from an animal treated with 20 mg/kg LY255283 1h before and 7 h after 5-oxo-ETE (5 μg) insufflation. Large numbers of eosinophils have infiltrated the airways (x 400).

3.6. Effects of anti-integrin antibodies on 5-oxo-ETE-induced eosinophil infiltration.

To determine whether the 5-oxo-ETE-induced eosinophilia was dependent on eosinophil integrins, rats (n = 5) were pretreated with mAbs to VLA-4, LFA-1, and Mac-1, 30 min prior to intratracheal insufflation with 5-oxo-ETE (5 μg). The amounts of antibodies employed have previously been shown to be sufficient to saturate integrins on blood leukocytes (332, 333, 334). The lungs were removed 15 h later and sections stained for MBP. The positive control group, where animals were pretreated with vehicle and insufflated with 5-oxo-ETE, had similar numbers of pulmonary eosinophils as the positive controls in the other experiments described above, displaying a nearly 8-fold increase in the numbers of lung eosinophils when compared to the negative control group ($p < 0.005$) (Fig. 7). Pretreatment of rats with the isotype-matched control mAb 3H11-B9 had little effect on 5-oxo-ETE-induced eosinophil infiltration. When the rats were pretreated with a mAb to VLA-4 the response to 5-oxo-ETE was reduced by 70% ($p < 0.01$) compared to that of animals pretreated with the isotype matched mAb. Anti-LFA-1 had a similar effect, inhibiting the response to 5-oxo-ETE by 77% , ($p < 0.005$) whereas anti-Mac-1 had a smaller effect, reducing pulmonary eosinophils by only 30% (not significant). The combined effect of anti-LFA-1 and anti-Mac-1 (80% inhibition; $p < 0.005$) was similar to that of anti-LFA-1 alone.

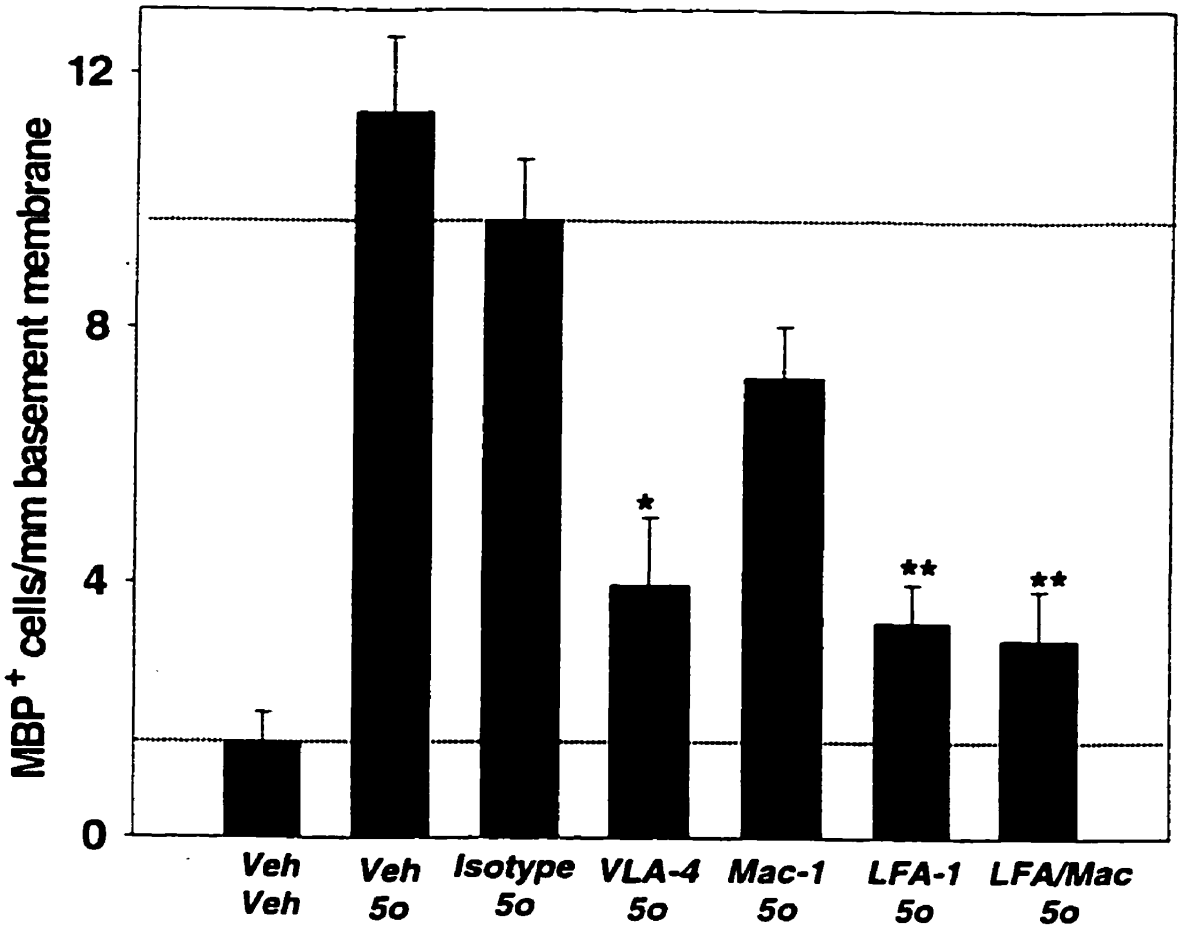

Figure 7. ***Effects of monoclonal antibodies to integrins on 5-oxo-ETE-induced eosinophil recruitment.*** BN rats were injected intravenously with either vehicle (0.5 ml of saline) or saturating amounts of antibodies to VLA-4, Mac-1, LFA-1, or a combination of LFA-1 and Mac-1. A control group of animals was pretreated with an irrelevant IgG1 antibody. After 30 min, either 5-oxo-ETE (5 μg) or vehicle (100 μl of 0.5% ethanol in saline) were administered by intratracheal insufflation. The lungs were removed 24 h later and sections were stained for eosinophils using an antibody to MBP. The results are expressed as the numbers of positive cells per mm of airway basement membrane and are means ± SE (n = 5). Differences among groups were evaluated by one-way ANOVA with Dunnett's test as a multiple comparison method. *, $p < 0.01$; **, $p < 0.005$.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1. Discussion

Evidence for the contribution of the eosinophil and eosinophil-derived mediators to the pathophysiology of various disorders, such as asthma, is increasing. While eosinophils may be present to varying extents with other cell types, including neutrophils, lymphocytes, and monocytes, the mechanisms whereby eosinophils, which constitute a small minority of circulating blood leukocytes, are recruited in large numbers into tissue sites is not completely understood. Eosinophil recruitment into sites of inflammation involves adhesion and transmigration through the endothelial barrier, a process that can be regulated by differential expression of adhesion molecules. This process, in turn, is coupled to local production of chemotactic factors that promote tissue migration of eosinophils.

The original recognition that diffusates of sensitized guinea pig and human lungs, following challenge with specific antigen, contained an activity (eosinophilic chemotactic activity of anaphylaxis [ECF-A]) that elicited chemotaxis of eosinophils (340), but not neutrophils, stimulated the search for chemoattractants that would account for the selective recruitment of eosinophils to sites of allergic reactions. Since then, experimental data has demonstrated that a variety of mediators display chemoattractant properties for eosinophils and may be involved in the infiltration of these cells into tissue. A number of eosinophil attractants have been described in the past including C5a (341) and PAF (39) which, although not specific, have been shown to be potent and efficacious eosinophil chemotaxins *in vitro*. Recently, a number of C-C chemokines, notably eotaxin, have been shown to be potent eosinophil chemoattractants and certainly are critical mediators in the accumulation of these cells in tissues. However, it would seem unlikely that a single mediator is responsible for this phenomenon. For example, disruption of the eotaxin gene in mice (342), or administration of antibodies to eotaxin (304) only partially inhibited the recruitment of eosinophils into the airways after antigen challenge of sensitized mice. Partial inhibition of antigen-induced pulmonary eosinophilia was also observed following treatment of mice with antibodies against either MIP-1α or RANTES (343). The absence of a single agent that functions solely as the predominant eosinophilic chemoattractant is in accord with the growing evidence that several chemoattractants may be responsible for

this preferential accumulation of eosinophils in tissues. These chemoattractant molecules may act in concert to stimulate eosinophil migration either in series or in parallel.

Apart from the polypeptide-like chemotaxins mentioned above, lipid mediators also appear to be important physiological mediators of eosinophil migration in various species both *in vitro* and *in vivo*. For example, a study by Sehmi et al, (349) identified LTB4 and 8(S),15(S)-diHETE (15-HETE dioxygenated product) as the ECF-A of guinea pigs, but neither of these was specific for eosinophils in comparison with neutrophils. Schwenk et al, (344) reported that incubation of soybean lipoxygenase with AA led to the formation of the oxygenated derivative of 5,15-diHETE (15-LO product), 5-oxo-15-HETE. They reported that this eicosanoid is a more potent chemotactic lipid for human eosinophils than either LTB_4 or 8(S),15(S)-diHETE. More recently, a comparison of eosinophil chemotactic activity of several structurally related eicosanoids led to the conclusion that maximal potency and efficacy of eosinophil-chemotactic activity is present in 5-oxo-ETE (10).

Further evidence for a role of eicosanoids in eosinophil recruitment comes from studies using synthesis inhibitors and receptor antagonists. 5-LO inhibitors have been shown to inhibit the infiltration of these cells into the airways in humans (145, 345) as well as other species (142, 346) , and this may contribute to the beneficial effects (347) of these agents in asthmatics. Moreover, antigen-induced eosinophilia was dramatically reduced in mice lacking the normal 5-LO gene compared to control antigen-challenged mice (170) . Antigen-induced pulmonary eosinophilia was blocked by the 5-LO inhibitor PF 5901, but not by the LTB_4 antagonist PF 10042 in guinea pigs (160) . Sephadex-induced pulmonary eosinophilia was strongly inhibited in the BN rat by Zileuton, another 5-LO inhibitor (143). Neither the LTB_4 antagonist CGS-25019c nor the cys-LT receptor antagonist MK-476 (Montelukast), except at high doses when it also inhibited the formation of LTs, were capable of reproducing the effect of Zileuton on eosinophil infiltration (143). These results raise the possibility that a 5-LO product other than LTB_4 or the cys-LTs is involved in eosinophil recruitment in these animals. It is possible that 5-oxo-ETE could fulfill such a role. Powell (10) and others (128, 348 have shown that 5-oxo-ETE is a potent chemoattractant for human eosinophils *in vitro*. However, it was not previously known

whether this AA metabolite is also active *in vivo*, or indeed whether it is active on eosinophils from species other than humans. To answer this question we chose the BN rat as the experimental animal because it is has been used as a model of human asthma (291) and displays marked accumulation of eosinophils in the lungs in response to antigen challenge (76).

Our results clearly show that intratracheally administered 5-oxo-ETE induces the accumulation of eosinophils in the lungs of BN rats in a time-and dose- dependent manner. Between 15 and 24 h following treatment of rats with 5-oxo-ETE, the levels of eosinophils in the lungs were approximately 5 to 6 times that in control, vehicle-treated animals. This is the first report that 5-oxo-ETE has biological effects *in vivo*. The effect of 5-oxo-ETE on eosinophil infiltration was highly reproducible, although there was some variability in the fold increase over control among the different experiments. This was due in large part to the variability in the mean numbers of eosinophils in control lungs in the different groups, which varied between 1.5 and 3.1 cells/mm basement membrane, presumably due to biological variability among the different rats. In comparison the mean numbers of eosinophils in the lungs of 5-oxo-ETE-treated animals varied between 10.1 and 13.2 among experiments.

Other 5-LO products have also been reported to be active as eosinophil chemoattractants in humans and other species. LTB_4 is not very effective in stimulating human eosinophil chemotaxis (10) but is a potent chemoattractant for guinea pig eosinophils, both *in vitro* (349,350) and *in vivo* (76), and also stimulates the accumulation of these cells following administration to BN rats by aerosol (76). Moreover, the LTB_4 antagonist LY255283 was found to partially block antigen-induced pulmonary eosinophilia in both BN rats and guinea pigs (76). *In vitro* binding and chemotaxis studies (76) characterizing the antagonist LY255283 have shown that it produces a potent and dose-related inhibition of chemotaxis (ID_{50} 2.4 μM) of guinea pig eosinophils in the presence of LTB_4 with an 80% inhibition at 10 μM. However, although this antagonist is a good inhibitor of the actions of LTB_4, it has been demonstrated that at higher concentrations it can also partially inhibit 5-oxo-ETE-induced responses (131). There is also evidence that cys-LTs have chemotactic effects on human eosinophils *in vitro* (207),

although Powell et al., have found them to be far less active than 5-oxo-ETE in this respect (10). LTE_4 has been reported to cause eosinophil infiltration into the airways of human asthmatics (117), whereas *in vivo* administration of LTD_4 was shown to induce the accumulation of these cells in guinea pig conjunctiva (351). Furthermore, the cys-LT1 antagonist MK-571 partially inhibited antigen-induced eosinophil infiltration in guinea pig conjunctiva (351). It is not clear whether the effects of cys-LTs on eosinophil accumulation in the above studies were due to a direct action on eosinophils, or whether these agents stimulated the release of other mediators such as PAF or 5-oxo-ETE. Cys-LTs are known to stimulate the release of eicosanoids (352) and PAF (353) from various tissues and cells, and it is thus quite possible that their *in vivo* chemotactic effects could be mediated by an indirect mechanism.

A comparison of 5-oxo-ETE to other lipid mediators as an eosinophil chemoattractant suggests that in humans it may be the most significant eosinophil chemoattractant among this class of substances. Powell (10) and others (128, 348) have demonstrated that 5-oxo-ETE is considerably more active than 5-oxo-15-HETE, PAF or any of the LTs in stimulating chemotaxis of human eosinophils *in vitro*. The present study suggests that in rats *in vivo* it is also much more effective than the cys-LTs and may be somewhat more effective than LTB_4 in inducing pulmonary eosinophilia. 5-Oxo-ETE and PAF, unlike with human eosinophils, are equipotent in stimulating pulmonary eosinophil infiltration in the BN rat.

As discussed above, there is ample evidence in the literature suggesting that there are interactions between LTs and PAF (169) and imply that some of their effects may be indirect. LTs are known to stimulate the release of eicosanoids (352) and PAF (353) from various tissues and cells. PAF, in turn, can mediate some of its effects through the subsequent release of 5-LO products. At least part of PAF-induced bronchoconstriction can be blocked by specific cys-LT antagonists (51). Moreover, Chen et al., in studying the mechanism of PAF-induced shock, have shown that 5-LO knockout mice are much more resistant to the lethal effects of PAF than control mice (168) . The data presented herein clearly demonstrates that the chemoattractant effects of 5-oxo-ETE are not mediated by subsequent release of LTB_4 or PAF. Concentrations of the antagonists LY255283 and

WEB 2170, that were able to significantly attenuate the LTB_4- and PAF-mediated effects respectively, had absolutely no effect on eosinophil recruitment induced by 5-oxo-ETE. Moreover, it would seem unlikely that 5-oxo-ETE acts via cys-LT_1 receptors, since LTD_4 and LTE_4 did not appear to induce eosinophil infiltration in the present study. This lends further support to previous *in vitro* experiments which suggest that 5-oxo-ETE acts through a putative specific receptor distinct from the LTB_4 or PAF receptors. The development of antagonists and inhibitors for 5-oxo-ETE would help to answer this question.

Blocking certain adhesion receptors with mAbs effectively supresses the effects of lipid mediators on leukocyte adhesion and recruitment. For example, in an animal model, mAbs against β2 integrins, ICAM-1 and E-selectin, but not against P-selectin, have all been shown to significantly attenuate LTB_4- and PAF-induced leukocyte endothelial adherence and transendothelial migration (354). Thus there is an interaction between lipid mediators and the specific cell adhesion molecules involved in the process of tissue cell recruitment. Our findings that mAbs to LFA-1 and VLA-4 strongly inhibit 5-oxo-ETE-induced pulmonary eosinophilia indicate that these integrins may be required for this response. Furthermore, due to the mode of administration (intravenous injection) of the mAbs, these results suggest that the response to 5-oxo-ETE is due to the entry of circulating eosinophils into the lung. However, it is not clear whether these mAbs to the integrins are inhibiting 5-oxo-ETE-induced effects on the endothelium or in the bone marrow, since these adhesion molecules have also been implicated in the interactions between progenitor cells and stromal cells (230,231). This could be answered in future experiments where progenitor cells and mature eosinophils are quantitated in the bone marrow and peripheral blood following 5-oxo-ETE treatment. Nevertheless, the importance of LFA-1 and VLA-4 is in agreement with previous findings showing that these integrins are required for allergen-induced infiltration of eosinophils into the lungs of sensitized BN rats (296, 298) . In contrast, Mac-1 appears to play a relatively minor role in 5-oxo-ETE-induced eosinophil infiltration, in spite of the fact that our laboratory has recently found that this compound stimulates the surface expression of Mac-1 on both human neutrophils (133) and eosinophils *in vitro*. The modest effect of anti-Mac-1 on 5-

oxo-ETE-induced eosinophil recruitment in rats is in accord with recent findings with neutrophils showing that migration of these cells into the peritoneal cavity is not impaired in Mac-1-deficient mice (355) Thus, although Mac-1 may be important for certain aspects of neutrophil (355) and eosinophil activation and adherence, it may not be a critical requirement for the infiltration of these cells into tissues. Integrins involvement in cellular activation has been confirmed by several studies (226, 227, 228, 229). The integrin Mac-1 mediates human eosinophil degranulation and respiratory burst caused by GM-CSF and PAF *in vitro* (227).

There are several studies (290, 298) that can demonstrate that mAbs to the α4-subunit integrin can block lung pathology without blocking leukocyte recruitment. This raises important issues about the mechanism of action of these mAbs which have been selected on the basis of blockade of adhesive function *in vitro*. There is evidence that adhesion molecules, particularly integrin receptors, have transmembrane signaling properties that mediate cell activation. Several reports suggest that LFA-1 and VLA-4 integrins may act as costimulatory molecules in eosinophil and T cell activation (226, 229). Another study has shown a reduction of PAF-induced EPO release following treatment with anti-VLA-4 mAb (290). A recent study by Munoz et al (228) demonstrates that inhibition of eosinophil binding to fibronectin-coated plates via pretreatment with mAb against VLA-4 inhibits the secretion of LTC_4 and luminal narrowing of explanted human bronchi *in vitro* that is normally seen following PAF stimulation. Clearly, pretreatment with mAbs against adhesion molecules has the potential to interfere with cellular mechanisms that are not directly related to cell recruitment.

Another problem is the use of mAbs against integrin subunits. An example of this is the TA-2 mAb against the α4 subunit. This subunit can associate with β1 or β7 chain to form integrin α4β1 or α4β7 respectively. Both integrins are found on eosinophils and both can bind VCAM-1 and fibronectin. The integrin α4β7 can additionally bind MadCAM-1 found in the gut lymphoid tissue (303). These overlapping functions and structural components of α4 integrins and their counter-receptors further complicate the interpretation of *in vivo* studies utilizing blocking mAbs to the α4-integrin subunit.

Increased availability of mAbs to all components of adhesion molecules and/or pathways will clarify these issues in the future.

As reviewed in the previous sections, it is clear that 5-oxo-ETE is a potent activator of eosinophils *in vitro*. Moreover, this compound induces rapid changes in the expression of adhesion molecules on human eosinophils, including upregulation of Mac-1 and shedding of L-selectin. This would suggest that the *in vivo* effects of this substance reported in the present study are due to direct effects of 5-oxo-ETE on eosinophils. However, although increased numbers of eosinophils were apparent at the earliest time point investigated (6 h), the time required to reach the maximal response to 5-oxo-ETE was rather long (15 h). Therefore, other mechanisms such as interactions with cytokines or chemokines cannot be ruled out. In fact, GM-CSF has been shown to potentiate 5-oxo-ETE-induced degranulation in eosinophils (128). Similarly, IL-5 has been shown to enhance the chemotactic responses of eosinophils to LTB_4 and PAF (223) . Further, pretreatment with a mAb against IL-5 was demonstrated to antagonize LTD4-induced eosinophilia in guinea pig lungs (119). Cooperation between cytokines, such as IL-5 and GM-CSF, and 5-oxo-ETE, in inducing eosinophil recruitment into the airways, may be of physiological relevance in asthma.

It is known that cell activation by cytokines may potentiate release of eicosanoids and vice versa. Cytokines such as IL-3, IL-5 and GM-CSF prime eosinophils for increased LTC_4 production *in vitro* (356,357). Moreover, 5-LO products have also been implicated in cytokine synthesis. For example, LTB_4 promotes the synthesis of IL-2, IL-4, and IL-5 by human T cells (82, 83, 84) of IL-6 by monocytes (85) and of IL-8 by human neutrophils (86). In human blood mononuclear cells, a 5-LO inhibitor blocks, not only the synthesis of LTB_4 , but also the expression of IL-2 and IL-6 (358). Although the involvement of cytokines in 5-oxo-ETE-induced eosinophilia in the BN rat was not investigated in this study, the *in vitro* data by other investigators suggests that there may be synergy between cytokines and 5-oxo-ETE and this should be investigated in the future.

As suggested earlier, there may also be some interactions between 5-LO products and chemokines, such as eotaxin, RANTES, MCP-3 and MIP-1α in regulating eosinophil

migration *in vivo*. There is evidence that the metabolism of AA is implicated in monocyte chemotaxis. For example, a synergistc interaction exists between PAF and C-C chemokines for both of $cPLA_2$ activation and chemotaxis (359,360). Eotaxin and other chemokines have been shown to stimulate the release of eicosanoids from basophils (307). Moreover, 5-oxo-ETE was reported to enhance migration of monocytes in response to the chemokines MCP-1 and MCP-3. (129). The synergistic interaction between 5-oxo-ETEs and C-C chemokines may be relevant in the regulation of eosinophil accumulation at sites of allergic and inflammatory reactions. The recent study (317) demonstrating that mice treated with zileuton prior to eotaxin administration showed a significant attenuation of eosinophil accumulation in the peritoneal cavity suggests that eotaxin may be acting in series with 5-LO products in the recruitment of eosinophils. Inhibition of 5-LO also attenuated *in vitro* chemotaxis of eosinophils induced by RANTES (318). Chemokines, such as eotaxin, may be activating eosinophils to release these products or make eosinophils more reactive to them.

In summary, the data suggests that eosinophil recruitment into tissue involves the cooperation of different classes of mediators, such as 5-LO products, chemokines and cytokines. This in fact, would be more reflective of human diseases characterized with eosinophil recruitment, where many cells and mediators with overlapping effects have been implicated.

In conclusion, 5-oxo-ETE is a potent activator of human eosinophils *in vitro* and induces their accumulation in the lungs of BN rats *in vivo*. These results raise the possibility that this compound may be an important physiological mediator of eosinophil infiltration in asthma and other diseases in which these cells are a distinctive feature. This hypothesis could be addressed by the development of specific 5-oxo-ETE antagonists.

4.2. Claims to Original Research

1. This thesis presents the first published data concerning the *in vivo* effects of 5-oxo-ETE in any species.

2. The most important contribution of this work is the discovery that 5-oxo-ETE, induces pulmonary eosinophil infiltration in the BN rat, raising the possibility it may be an important physiological mediator of inflammation.

3. The *in vivo* effects of 5-oxo-ETE appear to be specific and are not mediated by LTB_4 or PAF receptors, suggesting that it may interact with its own receptor on inflammatory cells.

4. 5-Oxo-ETE-induced eosinophil trafficking in the rat lung is dependent on the integrins VLA-4 and LFA-1. Although these integrins have been previously implicated in airway eosinophil recruitment, this is the first demonstration that they are required for the response to 5-oxo-ETE.

4.3 Conclusion and Future Considerations

As discussed earlier, experimental evidence suggests that lipid mediators may play a key role in eosinophil migration into inflammatory sites. The results presented herein suggest that 5-oxo-ETE could be a significant eosinophil chemoattractant within this class of compounds. Indeed 5-oxo-ETE is not only a potent chemotactic agent of human eosinophils *in vitro*, but also induces their accumulation in the lungs of BN rats *in vivo*. Intratracheal administration of 5-oxo-ETE into the lungs of BN rats induces accumulation of pulmonary eosinophils in a time- and dose-dependent manner. Furthermore, 5-oxo-ETE appears to be a lot more effective than LTD_4 and E_4 and somewhat more effective than LTB_4 in inducing eosinophil recruitment in the BN rat lung. Moreover, this 5-oxo-ETE-induced effect is dependent on VLA-4 and LFA-1 integrins and independent of LTB_4 or PAF receptor-mediated mechanisms. All these results support the hypothesis that 5-oxo-ETE may be a very important physiological mediator of inflammation, although more experimental evidence is needed to confirm this.

A very important issue that needs to be addressed is whether 5-oxo-ETE is involved in any physiological inflammatory processes such as asthma. Three main lines of evidence would support a role for 5-oxo-ETE in asthma. First, it should be identified in the lungs or BAL fluid of allergic animal models, as well as asthmatics. Second, it should mimic some of the characteristic features of asthma in humans. Third, blocking the formation of this compound or antagonizing its effect should have a beneficial role in asthmatic patients. A tool that can be used to detect 5-oxo-ETE in BAL fluid of astmatics is electrospray mass spectrometry. In fact, Hall et al., (361) have recently demonstrated that this analytical tool can be used for detection of 5-HETE, 5-HPETE and 5-oxo-ETE, formed by activation of AA. This method could potentially be used to quantitate 5-oxo-ETE in BAL fluid of asthmatics. Next, the effects of aerosolized 5-oxo-ETE into asthmatic lungs could be investigated, as has been done for LTE_4 (117), to see whether it can reproduce the effects seen in the BN rat lung. In order to investigate the outcome of inhibiting the formation or antagonizing the effects of this compound both the enzyme responsible for its synthesis as well as its putative receptor need to be further characterized. It may be possible to develop inhibitors to the dehydrogenase responsible

for the formation of 5-oxo-ETE. By characterizing the 5-oxo-ETE receptor it may be possible to synthesize an antagonist and test its effects of eosinophil recruitment in asthmatic airways.

Before going on to confirm the effect of 5-oxo-ETE in man, it will be necessary to accumulate as much information as possible on the effects of this putative mediator on cells, tissues and *in vivo* animal models. The *in vivo* findings presented here, as well as prior *in vitro* findings, suggest that 5-oxo-ETE could promote the adherence of eosinophils to endothelial cells and/or transendothelial migration. The effect of 5-oxo-ETE on eosinophil adhesion and migration could be tested using endothelial cells grown on culture plates or on Transwell inserts and compared to those of other agonists. The requirement for different eosinophil integrins in these processes could then be determined by using mAbs to block them. As discussed earlier, various cytokines and chemokines also activate eosinophils and it is important to investigate how they may interact with 5-oxo-ETE in terms of eosinophil chemotaxis, adherence, and transendothelial migration.

In conclusion, although the findings presented here suggest that 5-oxo-ETE may be a potent eosinophil-chemoattractant, further evidence is clearly required to demonstrate that it has a physiological role in recruitment of this cell type in asthma. This information may be of major importance in understanding the complex relationship between eosinophil recruitment and the pathogenesis of asthma and in turn help develop therapeutic strategies to prevent the pathological eosinophil influx into the asthmatic lung.

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