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CELLULAR INFILTRATION AND LEUKOTRIENE SYNTHESIS IN BROWN-NORWAY RAT LUNG FOLLOWING ALLERGEN CHALLENGE

by

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ABSTRACT

Although leukotrienes (LTs) are implicated in the pathogenesis of asthma, it is still unclear which types of cells are of primary importance in their formation in asthmatic lungs. To elucidate the mechanism for the increased LT formation in asthma, we investigated the pulmonary cellular infiltration and LT synthesis by dispersed lung cells from BN rats following allergen challenge.

To enable us to do these studies we have developed improved HPLC methods for the analysis of complex mixtures of eicosanoids in biological samples using binary gradients containing trifluoroacetic acid. Samples containing PGB_2 (the internal standard), cysteinyl-LTs, LTB_4, hydroxyeicosatetraenoic acids (HETEs) and the cyclooxygenase product 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHTrE) can be analyzed in as little as 20 min. Mixtures which also contain more polar eicosanoids such as lipoxins and omega-oxidation products of LTB_4 can be analyzed in 40 min.

Ovalbumin (OVA) challenge of sensitized BN rats resulted in a significant influx of neutrophils into the lungs and a significant increase in the synthesis of 5-lipoxygenase products, in particular LTB_4, by lung cells 6 h after challenge. However, there was little change in the production of cysteinyl-LTs compared with saline challenge. There was a significant eosinophilic infiltration in the lungs 24 h after OVA challenge, but cysteinyl-LT production by lung cells was unaltered at this time suggesting that eosinophils from BN rats are unlikely to be a major site for the formation of these compounds. This was confirmed in experiments with partially purified eosinophils obtained from Sephadex-treated rats. In contrast, cysteinyl-LTs were synthesized in appreciable amounts by alveolar macrophages from BN rats. Administration of rabbit anti-rat PMNL serum abolished the influx of neutrophils into the lungs and appeared to reduce the LTB_4 synthesis by lung cells. There was a positive correlation between the numbers of neutrophils in the lung and LTB_4 production by lung cells. In contrast, macrophages were positively associated with cysteinyl-LT production. Exogenous LTB_4 and 5-oxo-ETE induced infiltration of eosinophils into the lungs and therefore may be responsible for the late phase eosinophilic infiltration in BN rat lungs following allergen challenge.

RÉSUMÉ

Bien qu'une production accrue de différentes leucotriènes (LTs) ait été détectée chez des sujets asthmatiques ainsi que dans un modèle animal d'asthme, les cellules à l'origine de cette production au niveau pulmonaire restent inconnues. Afin d'élucider le mécanisme de formation des LTs dans l'asthme, nous avons examiné l'infiltration cellulaire ainsi que la synthèse des LTs dans les poumons de rats BN suite à une provocation antigénique.

Pour ce faire, nous avons développé et perfectionné des méthodes de HPLC permettant, par l'utilisation de gradients binaires contenant de l'acide trifluoroacétique (TFA), l'analyse de mélanges complexes d'eicosanoïdes présents dans des échantillons biologiques. Les échantillons contenant la PGB_2 (comme standard interne), des cysteinyl-LTs, la LTB_4, des HETEs (acide hydroxyeicosatétraènoïque), ainsi que l'acide 12-hydroxy-5,8,10-heptadécatriènoïque (12-HHTrE), produit de la cyclooxygénase, peuvent être analysés en 20 minutes seulement. Les échantillons contenant des eicosanoïdes plus polaires, comme les lipoxines (LXs) et les produits de l'oméga-oxydation de la LTB_4, peuvent être analysés en 40 minutes seulement.

Des rats, préalablement sensibilisés à l'ovalbumine et soumis à une provocation avec cet antigène, développent, 6 heures plus tard, une infiltration marquée de neutrophiles et d' éosinophiles ainsi qu'une production élevée des produits de la 5-lipooxygénase, en particulier LTB_4, au niveau pulmonaire. Par contre, cette provocation à l'ovalbumine n'induit pas d'augmentation de cysteinyl-LTs comparativement à une provocation contrôle avec une solution saline. Dès lors, l'apparition d'une éosinophilie sans élévation des cLTs suggère que les éosinophiles de rats BN ne sont pas à l'origine de la formation de ces produits. Cette hypothèse est confirmée par des expériences in vitro faites avec des éosinophiles partiellement purifiés, obtenus chez rats traités au Sephadex. A l'inverse, les macrophages alvéolaires de rats BN sont une source majeure de cysteinyl-LTs. L'administration de sérum de lapin anti-PMNL de rat permet l'élimination de l'infiltration par les neutrophiles dans le poumon ainsi que la réduction de la formation de LTB_4. De plus, il existe une corrélation positive entre le nombre de neutrophiles et la production de LTB_4 dans le poumon, alors que le nombre de macrophages est associé de façon positive à la production de cysteinyl-LTs. L'administration de LTB_4 et de 5-oxo-ETE exogènes provoque une infiltration d'éosinophiles dans le poumon, suggérant que ces produits sont peut-être responsables de l'infiltration des éosinophiles dans le poumon de rats BN à la suite d'une provocation antigénique.

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ABBREVIATIONS

AA	arachidonic acid (5,8,11,14-eicosatetraenoic acid)
ANOVA	analysis of variance
APAAP	Alkaline phosphatase antialkaline phosphatase
BN rat	Brown-Norway rat
Cysteinyl-LTs (cLTs)	cysteinyl-leukotrienes
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
dho-LTB_4	10,11-dihydro-LTB_4
5,6-diHETEs	5*S*,6*R*- and 5*S*,6*S*-dihydroxy-eicosatetraenoic acid
5,15-diHETE	5,15-dihydroxy-6,8,11,14-eicosatetraenoic acid
8,15-diHETE	8,15-dihydroxy-5,8,10,14-eicosatetraenoic acid
12e-6t-B_4	12-*epi*-6-*trans*-LTB_4
EDTA	ethylenediaminetetraacetic acid
EPR	early phase response
ETYA	5,8,11,14-eicosatetraynoic acid
FLAP	5-lipoxygenase activating protein
GC	gas chromatography
5-HETE (5h)	5-hydroxy-6,8,11,14-eicosatetraenoic acid
12-HETE (12h)	12-hydroxy-5,8,11,13-eicosatetraenoic acid
15-HETE (15h)	15-hydroxy-5,8,10,14-eicosatetraenoic acid;

12-HHTrE	12-hydroxy-6,8,10-heptadecatrienoic acid
13-HODE	13-hydroxy-9,11-octadecadienoic acid
5-HPETE	5-hydroperoxy-6,8,11,14-eicosatetraenoic acid
12-HPETE	12-hydroperoxy-5,8,10,14-eicosatetraenoic acid
15-HPETE	15-hydroperoxy-5,8,11,13-eicosatetraenoic acid
3h-LTB_4	3-hydroxy-LTB_4
20h-LTB_4	20-hydroxy-LTB_4
HPLC	high pressure liquid chromatography
IL	interleukin
5-LO	5-lipoxygenase
LPR	late phase response
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
LXA_4	lipoxin A_4
LXB_4	lipoxin B_4
MBP	major basic protein
MS	mass spectrometry

NP	normal-phase
N-acetyl-LTE_4	N-*acetyl*-leukotriene E_4
NSAID	non steroidal anti-inflammatory drugs
OVA	ovalbumin
ODS	octadecylsilyl
5-oxo-ETE	5-oxo-6,8,11,14-eicosatetraenoic acid
PMNL	polymorphonuclear leukocyte
PAF	platelet activating factor
RP	reversed-phase
5*S*,12*S*-diHETE	5*S*,12*S*,dihydroxy-6,8,10,14-eicosatetraenoic acid
Sal	saline
SRS-A	slow reacting substance of anaphylaxis
6t-B_4	6-*trans*-LTB_4
TFA	trifluoroacetic acid
UV	ultraviolet

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1. INTRODUCTION

1.1. ARACHIDONIC ACID METABOLISM IN THE LUNG

Oxidative metabolites of arachidonic acid are among the most ubiquitous mediators produced in the human body. Unlike mediators such as histamine, which are preformed and stored in granules of mast cells, oxygenated metabolites of arachidonic acid, which are generally termed eicosanoids, are newly synthesized upon cell activation. The lung consists of such a diversity of cell types that it can produce almost all major classes of eicosanoids, including prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), monohydroxy-eicosatetraenoic acids (HETEs) and lipoxins (LXs) (1,2). The major eicosanoid pathways in the lung are summarized in Fig. 1. The two most important are the cyclooxygenase pathway, which is responsible for the formation of prostaglandins and thromboxanes, and the 5-lipoxygenase pathway, which produces leukotrienes and 5-HETE. The availability of arachidonic acid to oxygenation pathways is a rate-limiting step and is tightly controlled in most cells by the activity of cytosolic phospholipase A_2 ($cPLA_2$) that specifically releases arachidonic acid from the *sn*-2 position of glycerophospholipids (3).

1.1.1. Lipid mediators of asthma: from SRS-A to leukotrienes

The discovery of eicosanoids as mediators of asthma dates back to more than half a century ago. A biological activity termed "slow reacting substance of anaphylaxis" (SRS-A) was initially described in asthmatics (4). This activity was subsequently shown

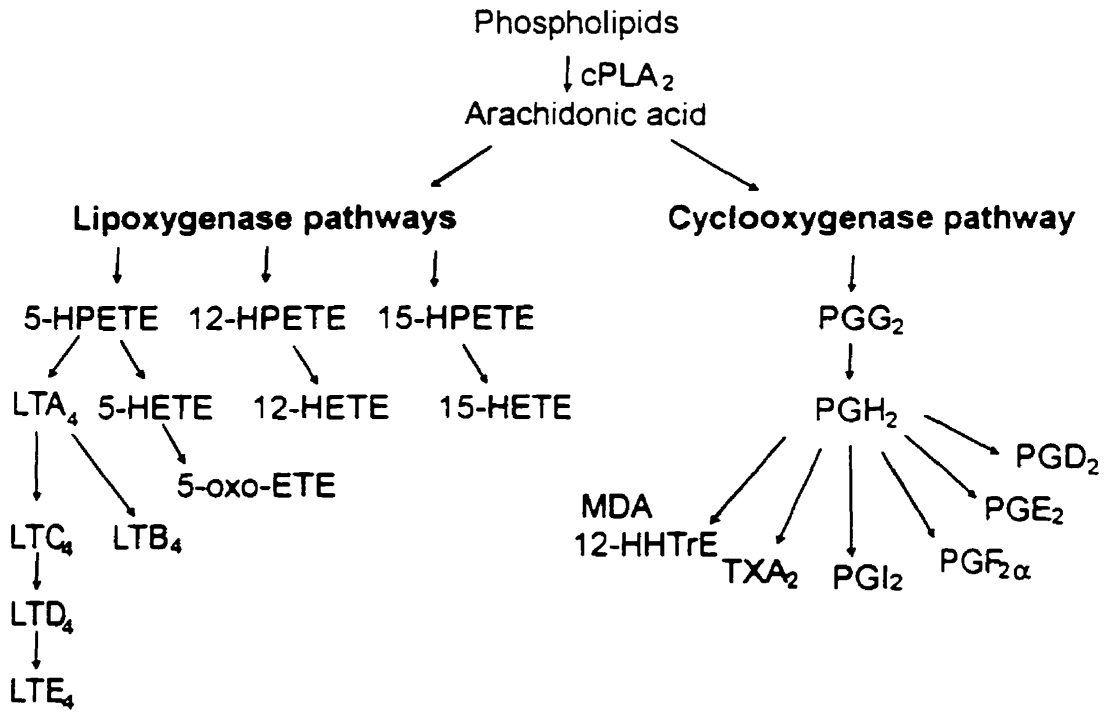

Figure 1. The major pathways for arachidonic acid metabolism in the lung. The first step in the synthesis of oxygenation products is the release of unesterified arachidonic acid (5,8,11,14-eicosatetraenoic acid). Availability of free arachidonic acid is regulated by the activity of phospholipases, in particular, cytosolic phospholipase A_2, which selectively hydrolyzes arachidonic acid from storage sites in cell membrane phospholipids. After release, arachidonic acid can be oxygenated by four major pathways, including three lipoxygenases and cyclooxygenase, each with a distinct enzymatic mechanism. Products of these enzymatic pathways include leukotrienes, HETEs, prostaglandins and other eicosanoids.

to produced by isolated perfused guinea pig lungs treated with either phospholipase A_2 or antigen (4). SRS-A induced smooth muscle contraction, which was slower in onset but more sustained than histamine, by a mechanism independent of histamine receptors (4,5). Although SRS-A was implicated in immediate hypersensitivity reactions (6), little was known of its structure until the late 1970s, mainly because of the small quantities available and limited analytical techniques. It was in 1977 that arachidonic acid was identified as a precursor of SRS-A (7,8). Meanwhile, Borgeat and Samuelsson (9) reported that rabbit leukocytes convert arachidonic acid to 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE). Several years later, it was shown that oxygenation at the 5-position of arachidonic acid was the first in a series of reactions culminating in the formation of the 5, 12-dihydroxy conjugated triene LTB_4 (10). Borgeat et al. (11) subsequently showed that LTB_4 was synthesized from an unstable intermediate, LTA_4, which showed maximal absorbance at 280 nm, reminiscent of the absorption spectrum of SRS-A (6,12). Murphy et al. (13) identified the parent SRS generated by calcium ionophore-activated mouse mastocytoma cells as 5-hydroxy-6*S*-glutathionyl-7,9,11,14-eicosatetraenoic acid (LTC_4). The 6-sulphido-cysteinyl-glycine metabolite (LTD_4) of LTC_4 was also shown to be a potent spasmogen (14,15). Rat SRS-A was subsequently found to be a mixture of LTC_4, LTD_4, and a 6-sulphido-cysteine metabolite (LTE_4) (16).

1.1.2. Lipoxygenase pathways

Arachidonic acid may be oxygenated at different positions by specific lipoxygenases, initiating the formation of LTs, HETEs, and lipoxins (LXs). Lipoxygenases

are a group of iron-containing dioxygenases that catalyze insertion of one oxygen molecule into polyunsaturated fatty acids containing a 1,4-*cis*, *cis*-diene structure. The initial catalytic step is stereospecific removal of a hydrogen atom followed by antarafacial addition of molecular oxygen, producing an S-hydroperoxy eicosanoid. Mammalian lipoxygenases possess regional specificity, inserting oxygen at carbons 5, 12, or 15 of arachidonic acid and have been designated as arachidonate 5-, 12-, and 15-lipoxygenases (1).

1.1.2.1. 5-Lipoxygenase pathway

a) 5-Lipoxygenase

5-Lipoxygenase catalyzes the dioxygenation of arachidonic acid at C-5, initiating the synthesis of leukotrienes. This enzyme first converts arachidonic acid to 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) (17). The 5-HPETE is unstable in aqueous medium and undergoes degradation (either spontanous or catalyzed by glutathione peroxidase) to 5-HETE or further enzymatic conversion by 5-lipoxygenase to the unstable intermediate 5,6-*trans*-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTA_4) (17-19).

5-Lipoxygenase was first found in PMNL and was purified to homogeneity from both human PMNL and rat basophilic leukemia cells. Other cells that express 5-lipoxygenase activity include monocytes, macrophages, basophils, and mast cells (2). The cDNA for human 5-lipoxygenase was cloned from human placenta and dimethyl sulfoxide-differentiated HL-60 cells (20). Human 5-lipoxygenase is nearly identical to rat

5-lipoxygenase (21).

5-Lipoxygenase may be activated by various stumuli including antigen challenge of appropriately sensitized cells or tissues (14,22), PAF (23), the chemotactic peptide FMLP (N-formyl-L-methionyl-L-leucyl-phenylalanine) (24), or the anaphylatoxin derived from the fifth component of complement C5a (25). The calcium ionophore A23187 is a non-physiological but very potent stimulus for leukotriene production (13). Availability of substrate is not sufficient to stimulate 5-lipoxygenase activity, since incubation of exogenous arachidonic acid with resting cells may not induce the formation of leukotrienes.

Initial studies have identified Ca^{--} and ATP as cofactors for 5-lipoxygenase activity. Subsequently, it was shown that the 5-lipoxygenase in neutrophils has to become associated with membranes before expressing its enzymatic activity (26). This suggested that the translocation of 5-lipoxygenase from cytosol to membrane may place it in apposition to its substrate and possibly other factors within the membrane. MK886, a potent and specific inhibitor of leukotriene biosynthesis, was found to inhibit 5-lipoxygenase activity only in intact human neutrophils, but not in cell-free preparations (26,27). Efforts to unravel this phenomenon led to the identification of 5-lipoxygenase-activating protein (FLAP), which was shown to be required for the translocation of 5-lipoxygenase from cytosol to membrane (27). FLAP is a membrane protein with a molecular mass of about 18 kDa and is the primary target of MK886. The cDNA for FLAP hybridizes to an ~1 kb species of mRNA on Northern analysis and encodes a protein of 161 amino acids with three trans-membrane domains. Osteosarcoma cells transfected

with both 5-lipoxygenase and FLAP synthesized LTs, whereas the cells transfected with either 5-lipoxygenase or FLAP alone did not. Therefore, expression of both 5-lipoxygenase and FLAP is essential for leukotriene synthesis (26,27).

The intracellular localization of 5-lipoxygenase and FLAP have been a focus of recent research. 5-Lipoxygenase was found in the cytosol of resting human leukocytes (28). In contrast, it was localized in the nucleus of resting alveolar macrophages (29), and in cytosol and nucleus of resting rat basophilic leukemia (RBL) cells (30). In activated neutrophils and alveolar macrophages, 5-lipoxygenase was shown to be co-localized with FLAP on the nuclear envelope (31-33). 5-Lipoxygenase and FLAP were also found on the endoplasmic reticulum of activated alveolar macrophages (32).

The translocation of 5-lipoxygenase appears to be cell-type specific. To date, two distinct patterns of 5-lipoxygenase translocation have been described: 1) translocation of cytosolic 5-lipoxygenase to the nuclear envelope in human PMNL (28,31); 2) translocation of nuclear 5-lipoxygenase to the nuclear envelope in alveolar macrophages (30,33). In RBL cells, both patterns of translocation occur (30).

Recently, studies with knockout mice showed that 5-lipoxygenase deficient animals resist the lethal effects of anaphylactic shock induced by PAF (34,35). The inflammatory reaction to arachidonic acid and immune-complex challenge in these leukotriene deficient mice were also markedly reduced (34,35). These findings suggest a critical role for 5-lipoxygenase products in anaphylaxis and inflammation.

b) LTA_4 hydrolase: Synthesis of LTB_4

LTA_4 may undergo either enzymatic hydrolysis to form LTB_4, (5*S*, 12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid) (10) or nonenzymatic hydrolysis to form several stereochemically distinct dihydroxyeicosatetraenoic acids including 6-*trans*-LTB_4, 12-*epi*-6-*trans*-LTB_4, 5*S*,6*R*-diHETE and 5*S*,6*S*-diHETE (11). The enzymatic conversion of LTA_4 to LTB_4 is catalyzed by LTA_4 hydrolase, which is relatively specific for LTA_4, with LTA_3 (formed from 5,8,11-eicosatrienoic acid) and LTA_5 being metabolized more slowly. Thus, LTB_4 formation is markedly reduced in essential fatty acid deficiency (36). Non-enzymatically-formed hydrolysis products of LTA_4 have much less biological activity than LTB_4.

LTA_4 hydrolase was initially localized in the cytosol of PMNL and erythrocytes (37,38), and subsequently detected in plasma (39) as well as in a numbers of tissues (40). In addition to catalyzing the conversion of LTA_4 to LTB_4, LTA_4 hydrolase may also be covalently associated with LTA_4, resulting in irreversible inactivation of the enzyme (38). This phenomenon is known as suicide inactivation. The gene for LTA_4 hydrolase was cloned from human lung and placenta (41), and spleen (42). The cDNA from all three sources were identical. LTA_4 hydrolase mRNA is detected in virtually all tissues tested, although the greatest amounts appear to be present in lungs and leukocytes. The amino acid sequence of the enzyme was partially homologous to several unrelated Zn^{++}-metallopeptidase enzymes including angiotensin-converting enzyme. This may explain the peptidase activity of LTA_4 hydrolase and the inhibition of its activity by captopril (43), which is a well-described inhibitor of angiotensin-converting enzyme and is widely used

in the treatment of primary hypertension.

c) LTC_4 synthase: Synthesis of cysteinyl-LTs

LTA_4 may also be converted to LTC_4 by the addition of glutathione at the 6 position by the microsomal enzyme LTC_4 synthase. LTC_4 synthase is a unique member of the family of glutathione-S-transferases and is distinguished from other cytosolic and microsomal glutathione-S-transferases by its inability to use xenobiotic substrates. Nevertheless, LTC_4 synthase is also widely distributed in tissues, including mast cells, basophils, eosinophils, macrophages, endothelial cells, platelets, and epithelial cells. LTC_4 synthase was initially purified from guinea pig lung (44) and microsomal membranes of myeloid leukemia cell lines (44,45). The gene for this enzyme has recently been cloned (46,47). Surprisingly, there is about 30 % identity between LTC_4 synthase and FLAP in their amino acid sequences and both of them have three membrane-spanning regions. Although immunohistochemical studies of lung tissue with a specific anti-LTC_4 synthase polyclonal antibody showed that LTC_4 synthase may be largely perinuclear in distribution in alveolar macrophages (48), the precise subcellular localization of LTC_4 synthase is still unclear.

The γ-glutamate residue of LTC_4 can be removed by γ-glutamyltranspeptidase to give LTD_4. LTD_4 may be further metabolized by loss of a glycine residue to its 6-cysteinyl analog, LTE_4, by a cysteinyl-glycinyl dipeptidase. LTC_4, LTD_4, and LTE_4 are referred to as the sulphidopeptide- or cysteinyl-leukotrienes because each of them contains a thioether-linked amino acid or peptide at the 6-position (49).

d) Metabolism of LTs and 5-HETE

The leukotrienes and 5-HETE are also subject to a variety of enzymatic alterations that result in their biological inactivation or production of active metabolites.

In human neutrophils, LTB_4 is deactivated mainly by 20-hydroxylation via a cytochrome P-450 enzyme (50) and subsequent oxidation to its ω-carboxy metabolites (51). In rat leukocytes, however, LTB_4 is deactivated by 18- and 19- hydroxylation (52). In addition to ω-oxidation products (51-54), LTB_4 is also converted to β-oxidation products (55) as well as to 12-oxo (56) and 10,11-dihydro metabolites (57). Ethanol in low concentrations was shown to alter the hepatocyte metabolism of LTB_4, resulting in the formation of 3-hydroxy-LTB_4, which retains the chemotactic properties of LTB_4 (54).

Cysteinyl-leukotrienes (cysteinyl-LTs) may also undergo metabolic degradation by β- and ω-oxidation (58-64). In human eosinophils, cysteinyl-LTs are primarily degraded by the peroxidase-hydrogen/peroxide-halide system with the formation of the appropriate diastereomeric sulfoxides and 6-*trans* 5,12-diHETEs (58,59). In rat liver, the cysteinyl-LTs are converted primarily to *N*-acetyl-LTE_4 (65,66). Both LTE_4 and N-acetyl-LTE_4 may be ω-oxidized by rat liver microsomal 20-hydroxylase and 20-hydroxyleukotriene E dehydrogenase in sequence (61,64,67,68). ω-Oxidation and subsequent β-oxidation from the ω-end of the molecule also contribute to the metabolic degradation of LTE_4 in monkeys and humans (63,69). Reincorporation of a glutamic acid residue to LTE_4 by γ-glutamyl transpeptidase was found to produce 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglutamyl 7,9,11,14-eicosatetraenoic acid (LTF_4) (70).

5-HETE, although not itself very potent biologically, is converted by a specific

dehydrogenase to 5-oxo-ETE (71), which is a potent agonist of both neutrophils (72) and eosinophils (73). In addition to products formed directly by 5-lipoxygenase and their metabolites, other eicosanoids can be formed by the combined actions of 5-lipoxygenase with either 12-lipoxygenase or 15-lipoxygenase, resulting in the formation of 5*S*,12*S*-diHETE (74), 5*S*,15*S*-diHETE (75), and lipoxins (76).

1.1.2.2. 12-Lipoxygenase pathway

12-Lipoxygenase was the first lipoxygenase discovered in animal or human tissues. The enzyme was initially described in platelets (77) and then later in leukocytes (78). It catalyzes the removal of a hydrogen atom from C10 and the addition of oxygen to C_{12} of arachidonic acid, producing 12-hydroxyperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE). 12-HPETE is then either reduced to 12-HETE by a peroxidase or converted to hepoxilins A_3 and B_3 by non-enzymatic intramolecular transfer of the hydroxyl moiety from the hydroperoxy group at C_{12} to either C_8 or C_{10} (79,80). 12-HETE can be further metabolized to 12-oxo-5,8,10,14-eicosatetraenoic acid (12-oxo-ETE), 12-oxo-5,8,14-eicosatrienoic acid, 12 (R)-hydroxy-5,8,14-eicosatrienoic acid and 12(S)-hydroxy-5,8,14-eicosatrienoic acid by dehydrogenase and 10,11-reductase enzymes in porcine PMNL (81). 12-Lipoxygenase also has lipoxin synthase activity and can convert LTA_4 to lipoxins A_4 and B_4 (76).

Both platelet and leukocyte 12-lipoxygenases have recently been cloned and were found to be different enzymes (21). Unlike 5-lipoxygenase, neither 12-lipoxygenase enzyme requires prior activation. Platelet 12-lipoxygenase is a cytosolic enzyme which

may translocate to membranes in response to increasing intracellular levels of calcium (82). However, it should be noted that calcium is not required for the expression of 12 lipoxygenase activity (82).

1.1.2.3. 15-Lipoxygenase products

15-Lipoxygenase catalyzes the formation of 15-HPETE which is subsequently reduced to 15-HETE and in some cases may also be transformed by other lipoxygenases to lipoxins (76). The cell types capable of producing 15-HETE include eosinophils, neutrophils, macrophages, vascular smooth muscle cells, fibroblasts, endothelial cells and epithelial cells (2,83,84). Human 15-lipoxygenase from eosinophils and neutrophils is a cytosolic enzyme, but has a higher K_m for arachidonic acid (about 70 μM) than rabbit neutrophil 15-lipoxygenase. As with 12-lipoxygenase, 15-lipoxygenase is found in an active form and no further activation is required for expression of enzyme activity.

1.1.3. Cyclooxygenase pathway

It was in the 1930's that human semen was shown to induce contractile responses on uterine, intestinal and vascular smooth muscle (85). In 1936 von Euler observed that human prostate fluid also had similar biological activity and coined the term "prostaglandin" for this activity (86). With the development of new analytical techniques such as gas-chromatography and mass-spectrometry in the early 1960's, the "prostaglandin" activity was found to belong to a family of arachidonic acid metabolites.

1.1.3.1. Cyclooxygenases 1 and 2

Cyclooxygenase (prostaglandin H synthase, PGG/H synthase) catalyzes the conversion of arachidonic acid (and certain other polyunsaturated fatty acids) to prostaglandin endoperoxides, which are the precursors of a series of biologically active compounds including thromboxane, prostacyclin, and other prostaglandins (87). The cyclooxygenase activity of the same enzyme inserts two molecules of oxygen into arachidonic acid to yield PGG_2, and a peroxidase activity of the enzyme reduces PGG_2 to its 15-hydroxy analogue PGH_2. Cyclooxygenase undergoes self-inactivation by oxidants generated during catalysis. Nonsteroidal anti-inflammatory drugs (NSAID) inhibit the cyclooxygenase but not the hydroperoxidase activity of the enzyme. Aspirin acetylates the enzyme, resulting in irreversible inhibition of enzymatic activity. This action is responsible for both the therapeutic effects and idiosyncratic reactions to the drug.

Cyclooxygenase has been purified from ram seminal vesicles, its richest source. The cDNA for the cyclooxygenase from ram seminal vesicles was cloned in 1989 (88). This enzyme is now referred to as cyclooxygenase-1 (COX-1). Although there were earlier indications for the presence of cyclooxygenase isoforms, it was not until recently that the cDNA of a second form of cyclooxygenase (COX-2) was serendipitously cloned from chick embryo fibroblasts (89) and Swiss 3T3 mouse fibroblasts (90). The amino acid sequences of the two forms of cycloxygenase are about 60% identical (89). COX-1 is constitutively expressed in many cells and tissues (87). COX-2 is selectively induced by proinflammatory cytokines at the site of inflammation (91). The discovery of a second COX enzyme led to the hypothesis that toxicity associated with the clinically useful

NSAIDs is caused by inhibition of COX-1, whereas the anti-inflammatory properties are caused by inhibition of the inducible COX-2 (91,92). Thus, selective inhibition of COX-2 may produce superior anti-inflammatory drugs with fewer side effects than existing NSAIDs (93).

Nevertheless, recent studies with gene knockout mice showed that COX-2 deficient mice showed normal inflammatory responses to either arachidonic acid or phorbol ester challenge (94). In contrast, COX-1 deficient mice have reduced platelet aggregation and a decreased inflammatory response to arachidonic acid (95). The COX-1 deficient mice also showed less indomethacin-induced gastric ulceration than wild-type mice, even though their gastric PGE_2 levels are about 1% those of wild type mice. Therefore, more studies will be needed to elucidate the relative roles of COX-1 and COX-2 in inflammation and toxicity associated with NSAIDs.

COX-2 can also be acetylated by aspirin. Surprisingly, although the acetylation of COX-2 prevents the formation of PG endoperoxides, the acetylated enzyme can still metabolize AA to 15(R)-HETE (96-98), leading to the formation of R-isomers of lipoxins (99).

1.1.3.2. Thromboxane and prostacyclin synthases

PGH_2 is converted predominantly to thromboxane (TX) A_2 by thromboxane synthase in platelets and other cell types (77). TXA_2 is a potent constrictor of vascular and airway smooth muscle and may contribute to airway hyperresponsiveness (100). TXA_2 spontaneously hydrolyzes to the hemiacetal TXB_2, which unlike the unstable parent

compound is not active in causing smooth muscle contraction or platelet aggregation. Another major platelet cycloxygenase product is 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHTrE) (77). Formation of 12-HHTrE is due to loss of malondialdehyde (MDA) from the cyclopentane ring of PGH_2. This step is also catalyzed by thromboxane synthase, which forms TXA_2 and 12-HHTrE in equimolar amounts (101). However, 12-HHTrE has not been reported to have significant biological activity.

Vascular endothelial cells and vascular and nonvascular smooth muscle cells can convert PGH_2 to prostacyclin (PGI_2), an unstable enol-ether that is spontaneously hydrolyzed to 6-keto-$PGF_{1\alpha}$. PGI_2 is a potent dilator of vascular and airway smooth muscles and a potent inhibitor of platelet aggregation (102).

1.1.2.3. Other biologically active prostaglandins

Isomerization of PGH_2 to PGE_2 is catalyzed by a microsomal enzyme that has been partially purified from bovine seminal vesicles. PGE_2 is the predominant arachidonic acid metabolite in a variety of cell types such as macrophages and epithelial cells. PGE_2 dilates airways by a direct effect on the airway smooth muscle and by an inhibitory effect on acetylcholine release (2). Athough PGE_2 is a vasodilator in other vascular beds, it is generally a pulmonary vasoconstrictor and was shown to inhibit mucous glycoprotein release (103).

Conversion of PGH_2 to PGD_2 can be catalyzed by serum albumin but occurs more efficiently by the action of PGD_2 isomerase. PGD_2 is the principal cyclooxygenase product of connective tissue mast cells and can induce bronchoconstriction and airway

hyperreactivity (104). PGD_2 and PGE_2 may be metabolized to compounds reflecting various combinations of 11-keto reduction, dehydrogenation of the 15-hydroxyl group, reduction of the Δ^{13} double bond, and β-oxidation.

$PGF_{2\alpha}$ may be formed by enzymatic or nonenzymatic reduction of PGH_2. Both PGH_2 and $PGF_{2\alpha}$ constrict airway smooth muscle. $PGF_{2\alpha}$ may also directly stimulate airway sensory nerve endings and cause heightened reflex bronchoconstriction by sensitizing airway nerve endings. The plasma levels of the $PGF_{2\alpha}$ metabolite 13, 14-dihydro-15-oxo-$PGF_{2\alpha}$ have been reported to be elevated in acute asthma (105).

1.1.4. Transcellular formation of eicosanoids

Eicosanoid intermediates such as LTA_4 and PGH_2 can be released into the extracellular environment and then further metabolized by other types of cells. As a consequence, the control of leukotriene synthesis is not exclusively restricted to cells expressing 5-lipoxygenase, but can also be exerted by other cell types equipped with LTA_4-metabolizing enzymes. For example, LTA_4 can be released to the extracellular environment by activated granulocytes and converted to LTB_4 by surrounding erythrocytes, endothelial cells, and lymphocytes, all of which possess LTA_4 hydrolase activity (106). Similarly, endothelial cells (107), platelets (108,109), and smooth muscle cells (110) have LTC_4 synthase and can convert released LTA_4 to cysteinyl-leukotrienes.

Transcellular interactions between platelets and granulocytes can also produce unique lipoxygenase products that neither cell type alone can produce. For example, 5*S*,12*S*-diHETE is formed via the combined actions of platelet 12-lipoxygenase and

granulocyte 5-lipoxygenase (74,111). In addition, 12S,20-diHETE can be synthesized via neutrophil specific ω-oxidation of platelet-derived 12-HETE (112).

Lipoxins are formed via the combined actions of 5-lipoxygenase and either 12-lipoxygenase or 15-lipoxygenase. They can therefore be produced by transcellular interactions between platelets and granulocytes (113).

1.2. ASTHMA

Asthma is a clinical syndrome characterized by dyspnea and wheeze. It occurs in 3%-8% of the population and is a common human disease throughout the world. 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. Currently asthma is defined as a lung disease characterized by: i) reversible airway obstruction, ii) airway inflammation, and iii) increased airway responsiveness to a variety of stimuli. Asthma, particularly in its chronic form, is an inflammatory disease of the airways.

1.2.1. Classification and pathogenesis of Asthma

Etiologic or pathologic classification of asthma is difficult. However, this disease is generally divided into two forms. An allergic form is responsible for most cases of childhood asthma and is due to an immunologically-mediated hypersensitivity to inhaled antigens. An intrinsic form occurs in adults and shows no evidence of immediate hypersensitivity to specific antigens. It is noteworthy that in spite of different initiating

stimuli, the lung pathology and therapeutic response to medications, in particular antiinflammatory steroids, are quite similar in different types of asthma. Considering this similarity, this discussion will focus on allergic asthma.

Although advances in pathobiology and immunology have yet to delineate the pathogenesis and the 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 (114,115). Recently, it has been appreciated that the immune response and airway inflammation in asthma may be primarily orchestrated by antigen-activated lymphocytes and Th2 lymphocyte-derived cytokines (116) (Fig 2).

In a genetically appropriate host, exposure to an allergen leads to the production of specific immunoglobulin E (IgE) as a result of complex interactions between antigen-presenting cells, T lymphocytes, and B lymphocytes. This initial response is referred to as "sensitization". The IgE then associates with high affinity Fc receptors on mast cells and basophils, as well as 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 IgE on the surface of mast cells. This causes mast cell degranulation and release of mediators, resulting in the immediate asthmatic response (also called 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 transient nature of the EPR suggests that mediator-induced bronchospasm may be the major contributory factor rather than inflammatory events. Of the mast cell mediators, histamine, leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and platelet-activating-factor

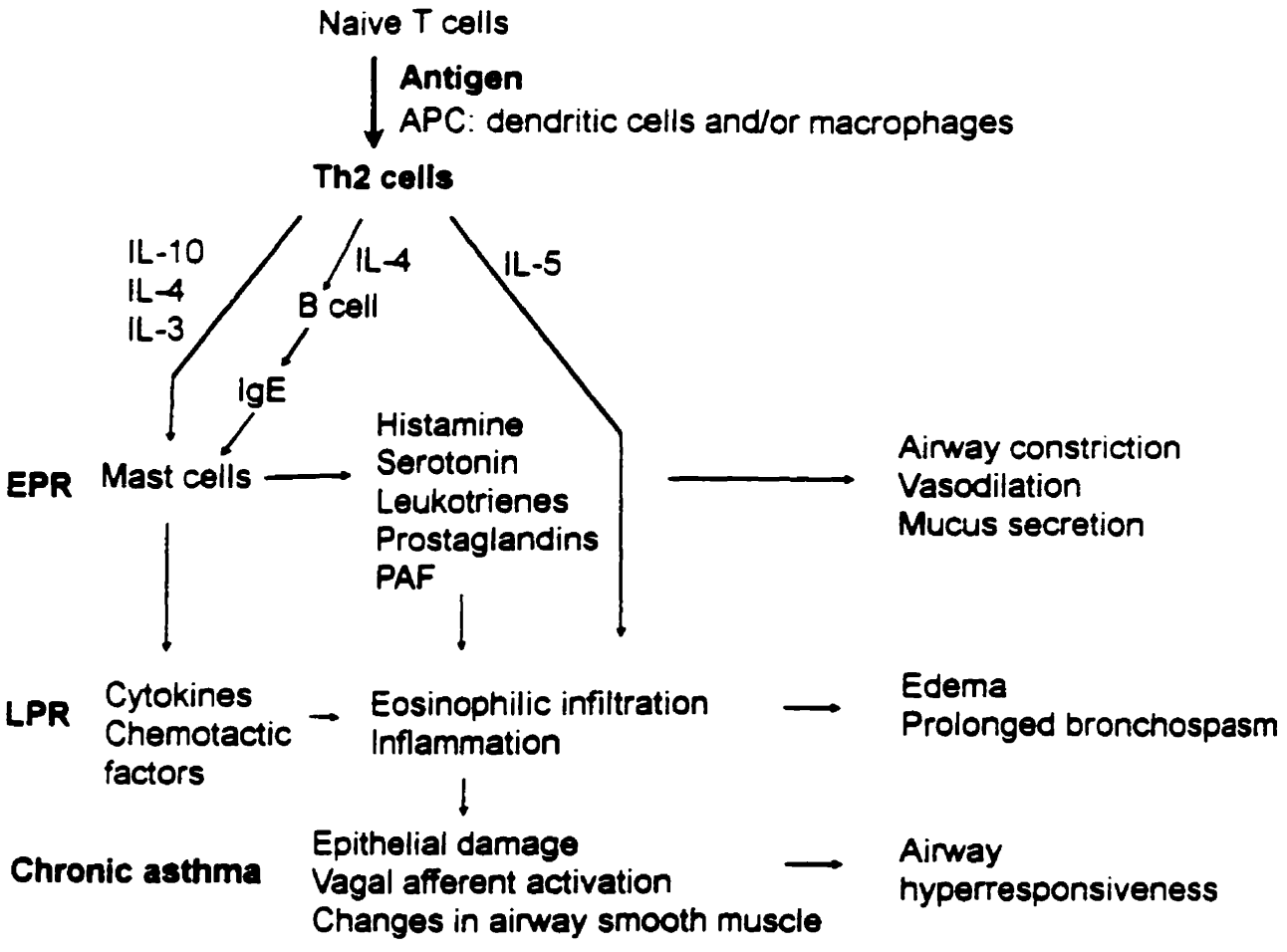

Figure 2. Proposed mechanism of allergic asthma. It is proposed that asthma is the result of an immune inflammatory response to allergen. The proliferation and activation of T lymphocytes, which are stimulated by antigen processed by the antigen processing cells (APC), may be the most important early events in the development of allergic asthma. Cytokines released from activated Th2-type lymphocytes then regulate IgE production, mast cell maturation, and eosinophil differentiation. The early phase response (EPR) of asthma is induced by the preformed and newly formed mediators released by sensitized mast cells and/or basophils located on the airway surface or in the submucosa upon IgE-mediated activation. The subsequent infiltration of eosinophils and their activation in the airways result in an inflammatory reaction and the late phase response (LPR). The release of a variety of toxic products and cytokines by chronically activated eosinophils, other infiltrating cells and resident lung cells eventually leads to epithelial damage and airway hyperresponsiveness to allergen and other non-immunological stimuli.

(PAF) are potent airway constrictors. 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-lymphocytes and macrophages induce an influx of inflammatory cells primarily consisting of eosinophils. This inflammatory reaction appeared to be involved in a second phase of airway narrowing, known as the late phase response (LPR). The late phase response peaks four to eight hours after antigen exposure and may last several hours (117). Although in some individuals resolution may take considerably longer, the LPR is, however, restricted to a subset of individuals and often occurs in isolation in the absence of an EPR. Persistent cellular infiltration and the release of toxic products may eventually result in epithelial damage, airway hyperresponsiveness and chronic asthma.

Although the EPR is thought to result from the direct effects of mast cell-derived mediators on airway smooth muscle, mucous glands, and blood vessels, the mechanism for the LPR is less clear. The LPR has attracted much attention in recent years because it is a very important feature of chronic asthma. Unlike the EPR, the LPR is only partially reversed by sympathomimetic drugs and is usually more completely blocked by corticosteroids.

In some respects, asthma closely resembles the immune response to certain parasitic infections, in which significantly increased serum levels of IgE and tissue eosinophilia are common (118,119). It has been hypothesized that the complex

eosinophilic inflammatory response that evolved to defend against parasites is somehow activated inappropriately in asthmatic patients and results in damage to the respiratory epithelium, making the airway smooth muscle hyperresponsive not only to the specific allergen but also to the various other nonspecific triggers of asthma (120).

1.2.2. Pathophysiology of Asthma

Pathologically, asthma is characterized by airway narrowing, hypersecretion of mucus, edema, inflammatory cell infiltration, and thickening of the basement membrane underlying the airway epithelium. Studies of patients dying from status asthmaticus revealed marked inflammation of the bronchial tree (121,122). Significant changes of airways were also seen in biopsies of patients with mild to moderately severe asthma (123,124). Typically, there was 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 (123).

Bronchoalveolar lavage (BAL) fluid from asthmatic subjects, challenged with aeroallergens or even in the absence of bronchial provocation, contains increased numbers of mast cells, epithelial cells, eosinophils, and neutrophils compared to that obtained from normal controls (125-128). In addition to the cellular changes in BAL fluid in asthma,

elevated levels of eosinophil-derived major basic protein (MBP), histamine, PGD_2, and cysteinyl-leukotrienes have been reported (129-131). Most of these chemical mediators are released locally and have been implicated in bronchospasm, epithelial damage, mucus secretion, and microvascular hyperpermeability.

1.2.2.1. Epithelial cells

The airway epithelium serves as a primary barrier for exogenous irritants such as allergens. Therefore, structural changes in the epithelium, such as shedding, may greatly facilitate the passage of irritants into the deeper mucosa. Extensive damage to the epithelium, swollen ciliated epithelial cells and widened intercellular spaces have been observed at all levels of the respiratory tree in asthmatics (121,132). There were increased numbers of bronchial epithelial cells recovered by bronchoalveolar lavage from asthmatic subjects compared with normal subjects (125,127). A positive correlation between epithelial cell counts and the extent of airway hyperresponsiveness has also been documented (127). Intense infiltration of eosinophils and deposition of eosinophil products such as major basic protein have been found in and around the bronchial epithelium and may be responsible for the epithelial damage (133-135).

The epithelial basement membrane has usually, but not always, been described as thickened in patients with asthma (121,123). Although the thickness of the basement membrane has been shown to be unchanged in asthma by using transmission electron microscopy, a dense deposition of collagen fibrils in the subepithelial region was observed (136). The fibrosis beneath the basement membrane in small airways is probably an

important contributing factor to the development of permanent airway obstruction (136).

Epithelial damage may also lead to changes in airway responsiveness. In isolated airway preparations, removal of the epithelium augments airway responsiveness to smooth muscle constrictors (137,138). Part of this effect is probably related to loss of a diffusion barrier. It is also possible that the epithelium can generate bronchidilators such as PGE_2 and nitric oxide (NO, endothelium-derived relaxing factor), in response to stimuli and that this capacity is lost if there is epithelial shedding (137). These findings support the view that the epithelium may be a major component of the regulatory processes that control bronchomotor tone.

Epithelial damage may result in exposure of nerve endings to irritant factors, penetration of allergen particles into the submucosa where mediator-secreting cells reside, and decreased production of airway relaxant factors and neutral endopeptidases. It is therefore believed that epithelial damage and shedding may be an important feature of asthma.

1.2.2.2. Mast cells

In nonasthmatic subjects, mast cells recovered in BAL fluid constitute 0.04 to 0.6% of the total nucleated cells, whereas in asthmatics these numbers can be increased three-fold to five-fold (125,127,139). Moreover, the mast cells in the airway mucosae of asthmatic patients show evidence of degranulation, suggesting that they are chronically activated (123,127,140). The presence of activated mast cells in the airways, even in patients with mild asthma, suggests the involvement of these cells in the development of

asthma. Although asthmatic patients show great individual variability in the numbers of mast cells in their airway mucosa, extensive studies have provided ample evidence that IgE-mediated release of mediators from mast cells may be responsible for the immediate bronchoconstriction provoked by allergen (125,129).

Mast cells may be activated by allergen via high affinity receptors for IgE as well as by various non-immunological stimuli as shown in Fig. 3. Mast cell-derived mediators such as histamine and leukotrienes are able to evoke smooth muscle contraction, mucus hypersecretion, and increased vascular permeability with consequent edema formation (104).

Purified human lung mast cells produce primarily PGD_2 and LTC_4 in response to either IgE-dependent stimuli or the calcium ionophore A23187 (22,141). However, there are marked differences in 5-lipoxygenase activity between rodent mucosal-type and connective tissue-type mast cells (142,143). Among mast cell populations, those with the morphological and cell surface marker characteristics of T-cell-dependent, mouse-bone-marrow-derived mast cells (putative mucosal mast cells) elaborate large amount of leukotrienes. In contrast, rat serosal (connective tissue) mast cells generate PGD_2 and appear to lack 5-lipoxygenase (144). Lung mast cells (mucosal mast cells) also differ from connective tissue mast cells such as those in the skin and peritoneal cavity by their resistance to activation by some non-IgE stimuli including compound 48/80, tachykinins, basic polyamines, and opiates. In addition to these difference, lung mast cells are sensitive to the inhibitory effects of cromolyn sodium, whereas those in the skin are not (104,143).

Whereas both lung mast cells and blood basophils release LTC_4 in response to

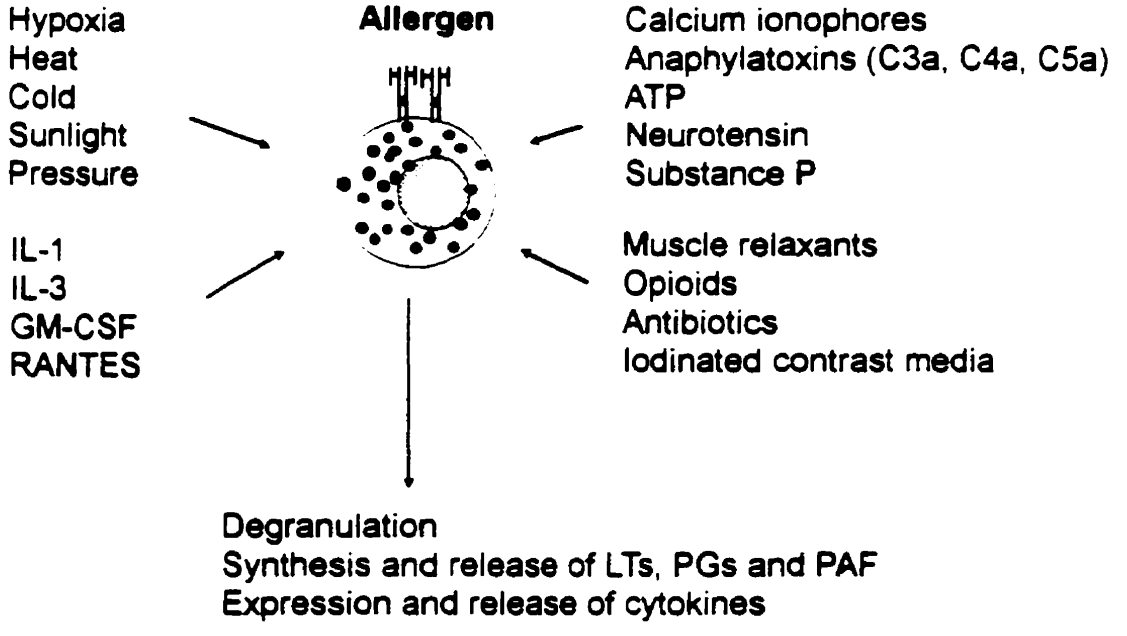

Figure 3. The immunological and non-immunological stimuli of mast cell activation. Allergens such as dust mite, flower powder, and some parasite antigens are important immunological stimuli of mast cells. They can bind to specific IgE molecules conjugated to IgE receptors on the cell surfaces of sensitized mast cells and trigger degranulation and the release of mast cell mediators. A number of physical stimuli, chemicals, biological mediators and some cytokines may activate mast cells by non-immunological mechanism.

IgE-mediated stimulation, basophils seem to produce much less PGD_2 than lung mast cells (22).

Direct evidence of mast cell activation *in vivo* in asthmatic patients is provided by the identification of a number of individual mediators in blood and BAL fluid. After local challenge of asthmatic airways with allergen through a bronchoscope, there was a 150-fold increase in the concentration of PGD_2 in BAL fluid (105,145). Increased concentrations of LTC_4 and its metabolite LTE_4, were also found in BAL fluid following allergen provocation (146,147). Elevated levels of LTE_4 in the urine were associated with both the early reaction following allergen challenge and the natural exacerbations of this disease (148,149).

Although the role of mast cells in the acute asthmatic response is well documented, the role of mast cells in the chronic inflammatory response within the airways is less clear. For many years it was believed that late-phase reactions were also the direct result of mast cell activation, due to the release of chemotactic factors leading to a subsequent infiltration of neutrophils, eosinophils, and other inflammatory cells. It was soon recognized that this view is over-simplified. The complex and heterogenous pathophysiology of bronchial asthma cannot be fully explained by the activation of mast cells alone. Usually less than 2.5% of the cells recovered from the airway lumen of asthmatic patients by BAL are mast cells (127), and paradoxically, the highest levels of mast cells and histamine are recovered from patients with interstitial pulmonary diseases (150). The observation that albuterol, which is a very potent inhibitor of mast cell degranulation, had little effect on allergic inflammation induced by allergen challenge also

casts doubt on the role of mast cell degranulation in chronic inflammation in asthma (151). Therefore, it is still unclear whether the activation of mast cells is a prerequisite for late-phase reactions and airway hyperresponsiveness.

It has been demonstrated lately that murine mast cells and human lung mast cells may generate a variety of cytokines including TNFα , IL-1, IL-4, IL-5, IL-6, and IL-8 (152-154). These early-response cytokines can then regulate immunoglobulin isotype switching to IgE, activate eosinophils, promote mast cell development, and initiate cytokine cascades that facilitate leukocyte recruitment through the induction of adhesion molecule expression as well as chemotactic mediator release from multiple cell types. These new findings suggest that mast cells may contribute to chronic asthma by producing inflammatory cytokines. However, the role of mast cells-derived cytokines in airway inflammation and hyperresponsiveness is not well understood yet.

1.2.2.3. Eosinophils

It has been known for a long time that eosinophils are present in increased numbers in blood, sputum, and airways of asthmatic patients (155). However, it was initially thought that eosinophils played a protective role in allergic responses due to their ability to metabolize histamine, inactivate lipid mediators and suppress histamine release (156). It is now well recognized that eosinophils appear to play a proinflammatory role in allergic asthma by secreting preformed and newly generated mediators (115).

The specific eosinophil granules contain preformed protein mediators capable of causing cellular and tissue damage in the airways. Major basic protein (MBP) and

eosinophil peroxidase (EPO) may be the most important products in the granules since they induce epithelial damage and mast cell degranulation (134). Eosinophils and eosinophil granule proteins, particularly MBP, have been localized at the site of epithelial injury in asthmatics (133,135).

Human eosinophils can also synthesize and release a wide range of newly generated lipid mediators, including platelet-activating factor (157), cysteinyl-leukotrienes (158), and 15-lipoxygenase products (83) upon stimulation. Purified human eosinophils synthesize substantial amounts of LTC_4, but not LTD_4, LTE_4, or LTB_4 in response to calcium ionophore (159), opsonized zymosan or fMLP (160,161). These cells can also be activated by IgE due to the presence of a low-affinity receptor for this immunoglobulin (162). Furthermore, the production of LTC_4 by eosinophils from normal subjects can be doubled by preincubation of cells with GM-CSF and IL-5 (163). This may explain why eosinophils from asthmatic patients produce more leukotrienes than those from normal subjects (164). Eosinophils from asthmatic patients were also found to produce more PAF than those from normal subjects (165).

Peripheral blood eosinophils appear as a heterogeneous population in asthma. A significantly higher proportion of hypodense eosinophils is observed in the blood of patients with allergic asthma than in controls (166). Although the mechanisms for the development of hypodense eosinophils are not fully understood, a number of factors may contribute to this process. Firstly, eosinophils become more dense as they mature in bone marrow. Thus, release of more immature eosinophils from bone marrow in response to allergen challenge may result in the occurance of hypodense eosinophils in the circulation

(115,167). Secondly, activation and degranulation may also reduce eosinophil density and contribute to the formation of hypodense eosinophils. Finally, some hypodense eosinophils may represent a population of "upregulated" cells with increased inflammatory potential. A number of cell-derived factors such as GM-CSF and IL-5 have been found to decrease the density of eosinophils and to increase the production of LTC_4 by these hypodense cells (168-170). Increased percentages of hypodense eosinophils were found in patients with severe asthma (171), suggesting a possible connection between hypodense cells and the development of asthmatic symptoms. Hypodense eosinophils may be an eosinophil subpopulation "programmed" to cause tissue injury when activated.

The degree of eosinophilic infiltration was also found to be positively correlated with the severity of asthma and with the degree of bronchial hyperresponsiveness (135,172). In asthmatic patients, there is a transient blood eosinopenia at 6 hours postchallenge with allergen that is followed by a progressive eosinophilia occurring up to 24 hours postchallenge (172). The finding that circulating eosinophil precursors increase during the late asthmatic reaction, and that their numbers fluctuate in relation to seasonal exposure in atopic subjects (167), suggests a role for allergen in stimulating eosinophil production by the bone marrow. The early response cytokines released from activated lymphocytes and mast cells, including GM-CSF, IL-3, and IL-5, have been shown to stimulate eosinophilopoiesis and to support colony growth and maturation (173-176). These cytokines may therefore liberate eosinophils from the bone marrow and prime them for survival and augmented proinflammatory functions following allergen challenge (Fig.4) (169,177,178).

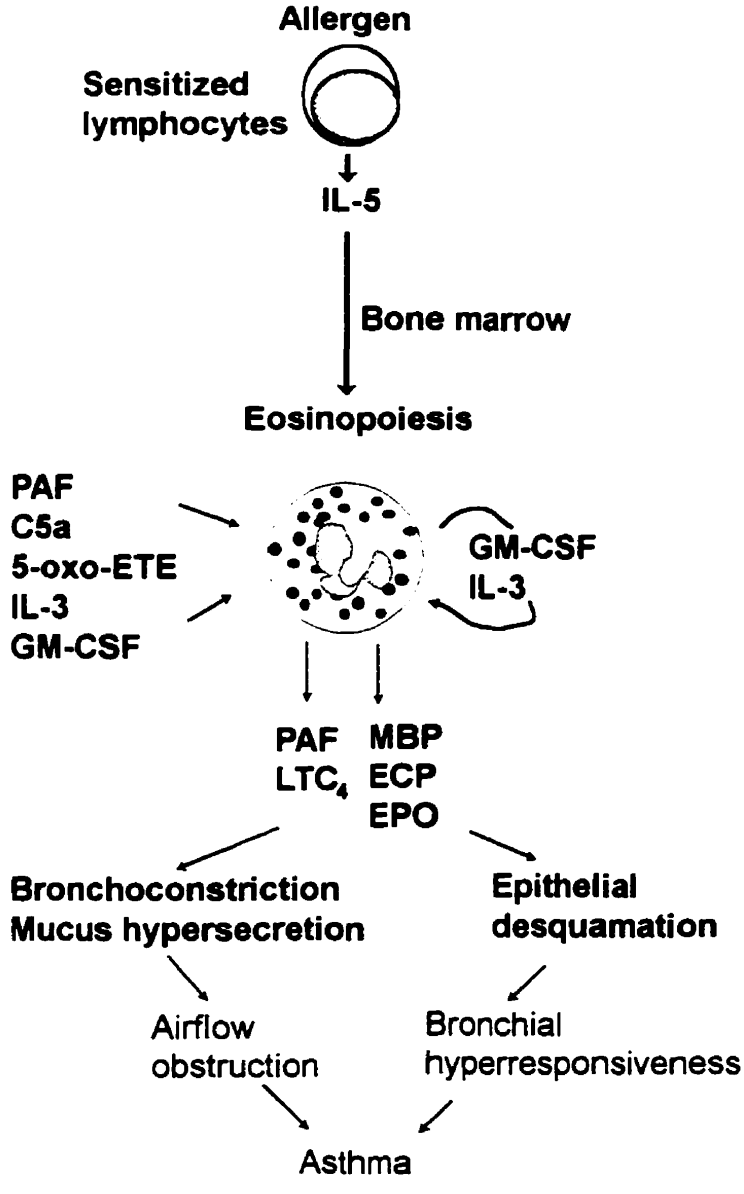

Figure 4. Schematic illustration of the possible role of eosinophils in allergic asthma. (IL-5, interleukin-5; PAF, platelet activating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; MBP, major basic protein; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase).

1.2.2.4. Neutrophils

Allergen challenge of asthmatic patients also induces neutrophil infiltration in airways (179). Neutrophils appear in the lumen after allergen challenge and persist in bronchial secretions in chronic asthma (127). A few patients who had died suddenly of acute asthma had neutrophilic rather than eosinophilic infiltration (122). It was also reported that neutrophils were associated with the transient bronchial hyperresponsiveness induced by either ozone or allergen (180).

Human neutrophils produce large amounts of LTB_4 and 5-HETE in response to calcium ionophore or other agonists (181). LTB_4 is one of the most potent chemotactic factors for human neutrophils (182) whereas the 5-HETE metabolite 5-oxo-ETE is a potent chemoattractant for both human eosinophils and neutrophils (72,73,183). Inhalation of LTB_4 induced neutrophil infiltration into the airways and airway smooth muscle hyperresponsiveness in dogs (184). Increased levels of LTB_4 were detected in BAL fluid from asthmatic patients (130).

However, the importance of neutrophils in the development of airway hyperresponsiveness is still unclear and appears to be species specific. For example, neutrophil depletion by treatment with hydroxyurea or nitrogen mustard inhibits airway hyperresponsiveness induced by ozone exposure in dogs (185) and late phase responses in rabbits (186). However, neutrophil depletion by rabbit anti-guinea pig neutrophil serum does not inhibit allergen-induced airway hyperresponsiveness in conscious guinea pigs (187).

1.2.2.5. Macrophages and monocytes

Macrophages are antigen-presenting-cells and present foreign antigen to lymphocytes. Based on their anatomic distribution, lung macrophages have been classified as either alveolar or interstitial macrophages. The term "alveolar" refers to the macrophages retrieved by bronchoalveolar lavage. Alveolar macrophages are the predominant leukocytes found within the airspace under homeostatic conditions and can respond to a number of stimuli. Althought most studies of BAL fluids have found that the numbers of mononuclear phagocytes were not increased in asthmatic patients, compared to control subjects (128,188), increased numbers of macrophages were observed in biopsies of some patients with asthma (189). The macrophages had phenotypic characteristics of peripheral blood monocytes, suggesting that they had migrated recently into the lung.

Macrophages possess a low-afffinity IgE receptor, as opposed to the high affinity IgE receptor on basophils and mast cells, and can therefore be activated by IgE-dependent mechanisms (162,190). This receptor is referred to as FCεRII and differs in both structure and function from the FCεRI on mast cells and basophils. IgE-antigen complexes activate macrophages and trigger the secretion of cysteinyl-leukotrienes (191,192), LTB_4, prostaglandins (193) and PAF (194). Oxygen-derived free radicals released by macrophages also have proinflammatory effects and may contribute to the inflammatory airway reaction observed in patients with asthma.

Human alveolar macrophages generate substantial quantities of LTB_4 but relatively little LTC_4 in response to calcium ionophore (195). In contrast, immune complexes

formed from IgE and antigen stimulate the immediate and prolonged release of LTC_4 from rat alveolar macrophages (192). Human monocytes can generate comparable quantities of both LTB_4 and LTC_4 in response to transmembrane stimuli or calcium ionophore, with the ratio between the two products depending on the agonist (196).

Alveolar macrophages may also regulate the function of other inflammatory cells. Conditioned medium from alveolar macrophages from asthmatic patients was shown to prime eosinophils for production of LTC_4 (197). The activity derived from alveolar macrophages from asthmatics could be neutralized by incubation with specific antibodies to human GM-CSF, suggesting that the active component could be GM-CSF. This is supported by the observation that recombinant GM-CSF primes eosinophils for enhanced LTC_4 generation following stimulation with A23187.

Macrophages not only have the ability to produce early-response cytokines in response to non-specific stimuli, such as endotoxin, but also can be activated by specific antigen stimulation via IgE-mediated pathways (153). In addition to producing early-response cytokines, alveolar macrophages are also able to generate large amounts of neutrophil and eosinophil-specific chemokines such as IL-8 and MIP-1α (153).

Mononuclear phagocytes are well equipped to play a central role in the amplification of the inflammatory response by virtue of their distribution in the airway, by their ability to be activated by IgE-mediated stimuli, and by their capacity to generate a variety of proinflammatory mediators and cytokines.

1.2.2.6. Platelets

The role of platelets in asthma remains controversial. Platelets have granules containing a variety of inflammatory mediators (198) and may also generate a number of arachidonic acid metabolites including the potent bronchoconstrictor TXA_2 in response to various extracellular stimuli including PAF (100). Although platelets do not have 5-lipoxygenase, they contain LTC_4 synthase and can convert granulocyte-derived LTA_4 to cysteinyl-leukotrienes (108, 109). Platelets isolated from patients with allergic asthma were found to produce cytotoxic mediators in response to specific allergens or IgE-specific ligands (such as anti-IgE antibody) (198). Platelets have low affinity IgE receptors (FcεRII) on their membrane surface and may therefore be specifically sensitized in allergic asthma in a comparable fashion to mast cells, basophils, eosinophils and macrophages. In light of these observations, platelets could be an active cellular partner in the development of asthma.

1.2.2.7. Lymphocytes

Lymphocytes are common cells in normal airways. Although the role of B-lymphocytes in the synthesis of IgE is well estabolished, it is only recently that a role for T-lymphocytes in asthma has been recognized. T cells in blood circulation and tissues express either CD4 or CD8 on their surface. In general, CD4 positive T-cells function as helper or regulatory cells while CD8 postive T-cells act as cytotoxic or suppressor cells in immune responses. More recently based on differences in their pattern of cytokine production, the CD4 postive T-cells has been subdivided into Th1 and Th2 subtypes. The

Th1 subtype T cells elaborate IL-2 and IFN-γ while Th2 lymphocytes produce IL-3, Il-4, IL-5, IL-10 and GM-CSF IL-4 and IFN-γ are involved in the regulation of IgE production by B lymphocytes (199). IL-5, GM-CSF, and IL-3 control eosinophil production and function and regulate mast cell differentiation. Lymphocyte-derived cytokines including IL-5, IL-8 and MIP also have potent chemotactic activity for eosinophils, neutrophils, basophils and monocytes and can stimulate degranulation of these cells (153).

Lymphocytes with memory for specific allergens have been found in asthmatic patients. These cells probably control both the level of IgE and the chronic inflammation (200). T lymphocytes are found not only in increased number but also in activation state in the lamina propria in patients with asthma (135,201). More recently, activated CD4-positive lymphocytes and increased Th2-type cytokine mRNA were identified in mucosal bronchial biopsies and BAL of patient with atopic asthma (202-204). Lymphocytes from the peripheral blood of patients with status asthmaticus also exhibited significantly increased expression of mRNA for Th2-type cytokines and T lymphocyte activation markers including IL-2 receptors (IL2R), class II HLA-DR, and "very late activation"antigen (VLA-4), compared with control subjects (116,205-207). A positive correlation was observed between the degree of airway obstruction and both the percentages of peripheral blood T cells expressing IL-2R and the serum concentrations of soluble IL-2R (208). Corticosteroid treatment decreased the number of CD4/CD25 positive cells and also caused a dramatic fall in the number of IL-5 bearing cells (209). Since CD25 is a marker of lymphocyte activation and IL-5 is a product of activated Th2

lymphocytes (210), these data suggest that the Th2 subpopulation of lymphocytes is an important component of asthma and that IL-5 may be a critical cytokine in this disease.

Lymphocytes are highly antigen-specific and control IgE-mediated allergic reaction. They also produce a number of regulatory cytokines and orchestrate mast cell activation and eosinophil infiltration, resulting in bronchial constriction and airway inflammation (205, 206, 210). Thus, lymphocytes may play an important role in asthma and might be one of the principal target cells for immunotherapy.

1.2.2.8. Dendritic cells

It should be noted that antigen-specific lymphocyte proliferation requires the presence of accessory cells. In this respect, alveolar macrophages are relatively inefficient antigen-presenting cells (194). Studies of cutaneous hypersensitivity suggest an initiating role for dendritic cells, and these efficient accessory cells are also present in the lung interstitium (211,212). Bellini et al. (213) reported increased dendritic cells in bronchial specimens from atopic asthmatics compared with atopic nonasthmatics. They found that the dendritic cells from asthmatics stimulated the proliferation and activation of peripheral blood CD_4 positive memory T lymphocytes in the presence of specific allergen. These activated lymphocytes then released IL-4 and IL-5, but not IL-2 or interferon gamma, suggesting expression of Th2-type lymphocytes. The antigen-presenting activity of dendritic cells could be potentiated by GM-CSF. Autologous peripheral monocytes also induced proliferation of lymphocytes but their antigen-presenting capability did not differ between atopic nonasthmatics and atopic asthmatics, suggesting that the increased antigen-

presenting activity from asthmatic airways was due to increased numbers of dendritic cells.

1.3. THE ROLE OF LEUKOTRIENES IN ASTHMA

Since the initial description of slow-reacting substance of anaphylaxis (SRS-A), evidence has accumulated to support a role for LTs including SRS-A and LTB_4 in allergic asthma. The biological activities of LTs, and the involvement of the cells that produce them (mast cells, basophils, macrophages, and eosinophils) in asthma provide circumstantial evidence for the concept that leukotrienes are mediators of asthma.

1.3.1. Biological activities of leukotrienes

Leukotrienes have distinct biological activities. The cysteinyl-LTs primarily affect smooth muscle cells as well as other contractile cells, whereas the primary targets of LTB_4 are leukocytes (Fig 5).

1.3.1.1. Effects on airway smooth muscles

The bronchospastic action of cysteinyl-LTs has been studied extensively in animal preparations and in human airways (14,214,215). LTC_4, LTD_4, and LTE_4 in various ratios account for the previously observed tracheocontractile activity of SRS-A. *In vitro* studies demonstrate the potent contractile effects of cysteinyl-LTs on airway smooth muscle, particularly in humans (215) and guinea pigs (14). Cysteinyl-LTs also contract airway smooth muscle from monkeys (216), rats, cats, and dogs (217,218) but are somewhat less

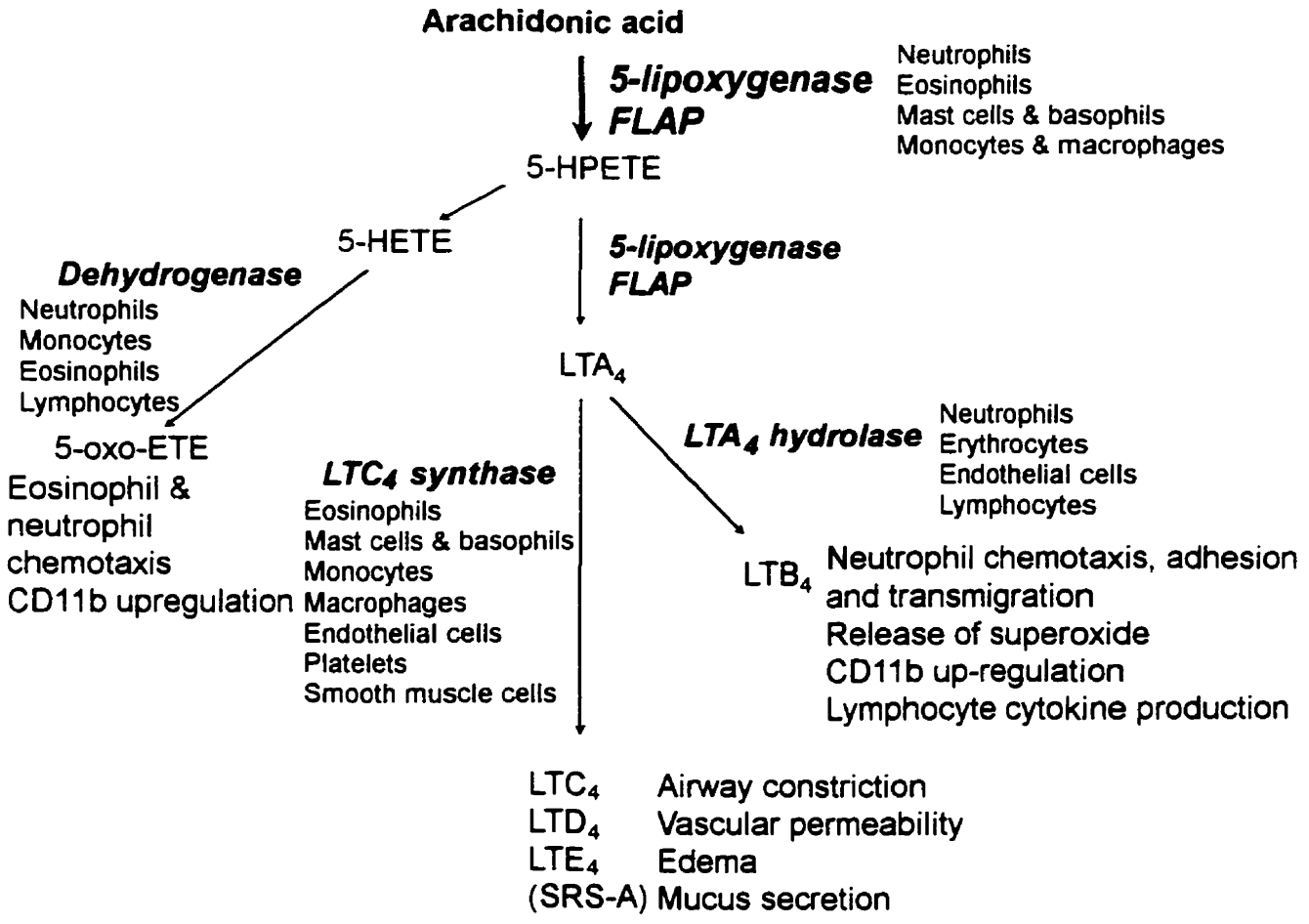

Figure 5. The cellular sources and biological activities of the major 5-lipoxygenase products. Arachidonic acid is metabolized by 5-lipoxygenase in a variety of leukocytes and resident lung cells such as mast cells and macrophages. Due to their distinct biological functions, cysteinyl-LTs (including LTC_4, LTD_4 and LTE_4) are strongly implicated as principal factors in elicitation of asthmatic symptoms, whereas LTB_4 is believed to be involved in diverse inflammatory reactions in the lung. 5-Oxo-ETE is a novel arachidonic acid metabolite that has potent chemotactic effects on both human eosinophils and neutrophils.

active in these species. In general LTC_4 and LTD_4 have very similar activities on human bronchial smooth muscle and are on a molar basis between 100 and 1000 times more potent than histamine (215). Nanomolar concentrations of LTC_4 and LTD_4 contract smooth muscle from lobar and segmental bronchi *in vitro* as well as the smaller airways of pulmonary parenchymal tissues, suggesting that cLTs affect both central and peripheral airways. Inhalation of LTC_4 or LTD_4 induces airway obstruction *in vivo* in both normal volunteers and asthmatic patients (219,220). LTC_4 and LTD_4 are more potent bronchoconstrictors than LTE_4, whereas LTE_4 causes longer lasting contractions in asthmatics (221,222).

1.3.1.2. Chemotaxis and chemokinesis

In contrast to the contractile activities of cysteinyl-LTs, LTB_4 is a potent chemotactic and chemokinetic factor for human neutrophils (182), but is less effective for human eosinophils (157). Its other in vitro activities includes aggregation of neutrophils (182), release of lysosomal enzymes (223) and oxygen radicals (224) from neutrophils, enhanced surface expression of integrins (CD11b) on human neutrophils and eosinophils (225), and augmentation of neutrophil adherence to endothelial cell monolayers (226). *In vivo*, LTB_4 stimulates the emigration of leukocytes from the bloodstream, resulting in neutropenia (227-229). The initial response to LTB_4 is increased adherence of leukocytes, primarily neutrophils, to the endothelium of venules, which is followed by infiltration into the tissue (227).

The most potent chemotactic agent for neutrophils within the lipoxygenase series

TABLE 1

Chemotactic activities of leukotrienes and other eicosanoids

Eicosanoids	EC_{50}	Target cells	Ref
LTB_4	10^{-9}	N, M	(182)
5-HETE	10^{-6}	N	(240)
5-oxo-ETE	10^{-8}	E, N	(72,73,183, 334)
5-oxo-15-HETE	10^{-7}	E, N	(241)
8S,15S-diHETE	10^{-6}	E, N	(242)
cysteinyl-LTs	10^{-6}	E	(73,235)
12R-HETE	10^{-6}	E, N, M	(243)

of products so far studies in *vitro* and *in vivo* is LTB_4 (230,231) (Table I). It is almost as active as fMLP at equimolar concentrations, and the response is stereospecific since the non-enzymatically-formed stereoisomers of LTB_4 are much less active (230,232,233). The ω-oxidation products of LTB_4 are also less active than LTB_4 itself (233,234). Most recently, 5-oxo-ETE, a metabolite of 5-HETE, was found to be the most potent lipid mediator in stimulating migration of human eosinophils (73).

Although not very potent, cysteinyl-LTs were shown to have effects on neutrophil and eosinophil chemotaxis in *in vitro* assays (73,235). This observation is consistent with

the results of a number of *in vivo* studies. In guinea pigs, instillation of LTD_4 into the conjunctival sac causes a modest eosinophilic infiltrate (236), which is greatly potentiated by concomitant application of LTB_4 (237). Although inhalation of 1μM LTC_4 by aerosol failed to induce pulmonary eosinophilia in guinea pigs, higher dose of LTC_4 and LTD_4 induced significant eosinophilia in the same species of animals (238). It was also reported that inhalation of LTE_4 resulted in eosinophilic infiltration in the mucosa of airways in asthmatic patients (239). Other eicosanoids that have significant chemotactic activities are listed in Table I. It should be pointed out that responses in leukocyte migration assays depend, to some extent, upon the technique used and in some cases are species-specific. Responses can also vary considerably between individuals, depending on the activation states of the leukocytes (235).

1.3.1.3. Vascular permeability

Cysteinyl-LTs increase microvascular permeability in the airways and elsewhere, by an apparently direct action on the endothelial lining of postcapillary venules (244,245). The leakage of plasma induced by LTC_4 and its metabolites (LTD_4, and LTE_4) is generally preceded by arteriolar constriction. LTB_4 indirectly increases microvascular permeability due to its effects on leukocytes (246).

1.3.1.4. Mucus Secretion.

The excessive production of mucus is an important feature of bronchial asthma and a major contributing factor to mortality in asthmatics. LTB_4, LTC_4 and LTD_4 have been

shown to stimulate the secretion of mucus from human airways *in vitro* (247). Intravenous administration of 5-, 12-, and 15-HETE, LTD_4 and LTE_4 significantly increase tracheal mucus layer thickness in a dose-dependent manner in rats (248). All of these data suggest a role for LTs and other eicosanoids in regulating mucus secretion.

1.3.1.5. Cell proliferation and differentiation.

Both LTB_4 and cysteinyl-LTs act as growth or differentiation factors for a number of cell types in vitro. Human bone marrow cells can generate substantial quantities of LTB_4, which has been shown to stimulate myelopoiesis in vitro (249,250). In picomolar concentrations, LTB_4 stimulates the differentiation of competent suppressor (CD8) T lymphocytes from precursors lacking the CD8 marker (235,251). LTB_4 also stimulates interferon gamma and interleukin-2 production by T cells (252) and potentiates IL-4 induced B cell proliferation (253,254). LTC_4 and LTD_4 may also stimulate the proliferation of fibroblasts (255) and epithelial cells (256).

1.3.2. Leukotriene Receptors

Once leukotrienes are generated, they exert their effects through binding with specific receptors. Although none of the leukotriene receptors have yet been cloned, several lines of evidence strongly suggest the existence of distinct receptors for LTB_4 and the cysteinyl-LTs.

Cellular receptors for LTB_4 were first postulated on the basis of functional and radioligand binding studies (257,258). The presence of specific LTB_4 receptors was

supported by the findings that the k_d and selectivity of the binding sites were in good agreement with the EC_{50} and structural specificity for the biological activity of LTB_4 and related compounds (259). The LTB_4 receptor appears to be coupled to PLC via a pertussis toxin-sensitive G protein (259,260). There is no evidence for the existence of subtypes of receptors for LTB_4.

There appear to be at least two subtypes of receptors for cysteinyl-LTs (261,262). The evidence that there are LTD_4 receptors on responding airways is compelling, since not only have ligand specificity and stereospecificity been demonstrated (263,264), but also the affinities of these receptors for their ligands are regulated by GTP, implying an association with a GTP-binding protein (265). Furthermore, the receptor can be solubilized from microsomes from guinea pig lung, and its high selectivity for the ligand is maintained (266).

In contrast, a receptor with almost equal affinity for LTC_4 and LTD_4 and much less affinity for LTE_4 was described in rat lung (274). The lack of antagonism of LTC_4-mediated bronchoconstriction in vitro by the putative LTD_4 receptor antagonist FPL55712 and the inhibition of this response by the calcium-channel blocker diltiazem provide physiologic evidence suggesting that there may be unique LTC_4 receptors that differ from LTD_4 receptors (272,273). Some cells, however, including guinea pig ileal smooth muscle, bullfrog lung, smooth-muscle cell lines, human PMN leukocytes, and bovine aortic endothelium have highly selective binding sites for LTC_4 as compared with LTD_4, LTE_4, and less closely related ligands (263,268-271). Nevertheless, a distinct receptor for LTC_4 cannot be demonstrated in human lung on the basis of ligand-binding studies. In addition,

structure-activity studies on bronchi isolated from normal and asthmatic subjects suggest that all the biologic effects attributed to LTC_4 can be accounted for by its bioconversion to LTD_4 (267).

Recently, the International Union of Pharmacologists (IUPHAR) (275) proposed a simple system of nomenclature within which receptors for LTB_4 are termed BLT receptors, and those for the cysteinyl-LTs are termed CysLT receptors. This classification system is based on functional data, obtained mainly with antagonists. Although there are few synthetic agonists for either BLT or CysLT receptors, there are many antagonists, both at BLT and CysLT receptors. Virtually all of the CysLT receptor antagonists block one subtype, termed arbitrarily, CysLT1. At present it is assumed that all CysLT receptors that are not of the CysLT1 subtype are CysLT2 receptors. However, future experiments may reveal the existence of additional LT receptor subtype. So far there are no reports of successful cloning of either BLT or CysLT receptors.

1.3.3. Presence of leukotrienes in asthma

More direct evidence that LTs are mediators of asthma has come from studies of their production during allergen challenge. LTs have been found in the sputum (130), bronchoalveolar lavage fluid (276,277), plasma (278), and urine (100,148,150) of asthmatic patients. LTC_4 and 20h-LTB_4 were found in the sputum of patients with asthma but not in that of patients with other lung diseases (130). Ferreri et al. (279) found LTC_4 in the nasal secretions of aspirin-challenged aspirin-sensitive patients but not in control. Increased urinary LTE_4 levels have been reported during acute severe asthma and at 3

hours following antigen challenge of asthmatic subjects (148,150,280). These results indicated that leukotrienes are released *in vivo* in asthmatic patients following allergen challenge.

1.3.4. Leukotriene synthesis inhibitors and receptor antagonists

Leukotriene-mediated events can be blocked either by using specific 5-LO inhibitors to prevent synthesis or by specific antagonists to LTB_4 or the cysteinyl-LTs.

1.3.4.1. Therapeutic effects of LT synthesis inhibitors in asthma

Efforts have been made over the last decade to develop novel 5-lipoxygenase inhibitors as potential therapeutic agents for the treatment of asthma (281,282). Inhibition of 5-lipoxygenase would reduce the formation of both cysteinyl-LTs and LTB_4 which could attenuate cysteinyl-LT-induced bronchoconstriction and LTB_4-mediated airway inflammation, two essential features of asthma. Early attempts to develop 5-lipoxygenase inhibitors on the basis of substrate analogues such as 5,8,11,14-eicosatetraynoic acid (ETYA) were not very successful due to lack of specificity (283). In addition to this, some of the earlier 5-LO inhibitors were toxic when administered *in vivo*. An example of a new class of leukotriene synthesis inhibitors is MK-886. This compound has no direct activity on the purified enzyme, but inhibits leukotriene generation in intact cells by binding to 5-lipoxygenase activating protein (FLAP) (26). A clinical study with the FLAP inhibitor, MK886, showed a reduction in the early asthmatic response after allergen challenge after two oral doses, with a concomitant reduction in *ex vivo* leukotriene

generation (284). Although the inhibition of the early asthmatic response was not statistically significant, there was a correlation between the inhibition of urinary LTE_4 excretion and the attenuation of the early asthmatic response (284). This suggests that the lack of a significant clinical effect may be related to an insufficient degree of inhibition of 5-LO in the lung (281). A new 5-lipoxygenase inhibitor Zileuton (A-64077) was shown to significantly inhibit airway narrowing in asthmatics induced by cold, dry air (285,286).

1.3.4.2. Therapeutic effects of LT receptor antoganists in asthma

Administration of CysLT antagonists was shown to result in bronchodilation in asthmatics but not in healthy individuals (284,287), suggesting that cLTs contribute to resting airway tone in asthma. This effect is additive to that of albuterol. These agents have also been shown to inhibit the acute asthmatic response to exercise (150,288), allergen (289), and isocapnic hyperventilation (285). In addition, ICI 204,219 inhibited the allergen-induced late asthmatic response and the increased airway hyperresponsiveness that followed allergen challenge (290). Preliminary data also suggest that administration of CysLT antagonists leads to a reduction in the severity of clinical asthma (291). Compared to placebo, treatment with the CysLT antagonist MK-571 led to a mean 8-14% improvement in FEV_1, 30% decrease in morning and evening symptom scores, and an approximately 30% decrease in usage of albuterol (291). Although ICI 204,219 is more potent in antagonizing both allergen- and leukotriene-induced bronchoconstriction than MK571, it is not more effective than MK571 in blunting exercise-induced or isocapnic

hyperventilation-induced bronchoconstriction (285,291).

A number of LTB_4 receptor antagonists have also been developed (292-294). Although LTB_4 receptor antagonists were shown to inhibit allergen- or LTB_4 induced leukocyte infiltration in animal models of asthma (292,294), the potential therapeutic role of such antagonists have yet to be evaluated in clinical studies.

1.4. ANIMAL MODELS OF ASTHMA

Practical difficulties and ethical issues associated with conducting experiments in humans have forced researchers to turn to animal models to gain insight into the mechanisms of airway responses in asthma. Experimental models of both early and late asthmatic responses have been established in a variety of laboratory animals. Induction of bronchospasm by acute antigen challenge has been the basis of most animal models of asthma including guinea pigs (295-297), rats (298-301), dogs (302-304), rabbits (305), sheep (306), and monkeys (307,308). The most common antigens employed are ovalbumin in guinea pigs and rats, and *Ascaris suum* in dogs, sheep and monkeys. Guinea pigs and rats require active sensitization against ovalbumin, whereas dogs, monkeys, and sheep are often nativelly allergic to *Ascaris suum* as a result of prior infection with roundworms, which cross-react with *Ascaris suum*.

The use of sensitized guinea-pigs as animal model of human asthma is well described in the literature (295-297). Guinea pigs are easily sensitized to ovalbumin or other foreign proteins. The lung is the primary target organ in the anaphylactic reaction following systemic challenge in this animal model. Although a variety of mediators are

involved in the pulmonary response to anaphylaxis, histamine and the arachidonic acid metabolite TXA_2 play very important roles in inducing airway narrowing in guinea pigs (309-312). In contrast to humans, the predominant homocytotropic antibody in this species is usually IgG (313), rather than IgE.

In this regard, rat anaphylaxis may resemble asthma in humans because the antibodies produced against antigen are of the IgE class (298,314). Compared with guinea pigs, most strains of rats are more difficult to sensitize to foreign proteins. Systemic anaphylaxis can be produced by appropriate challenge of rats sensitized to foreign proteins using adjuvants such as *Bordetella pertussis*. Antigen-induced bronchoconstriction in the rat has been the subject of numerous studies and some inbred strains that have been developed show a more homogeneous asthma-like response during antigen challenge than is found in the more common laboratory strains (300,315). The highly inbred Brown-Norway (BN) rat is probably one of the most widely used animal models of asthma (300,316-320). Upon active sensitization against ovalbumin, BN rats produce high levels of antigen-specific IgE in the serum (321) and develop both early and late phase responses in high prevalence on inhalation challenge (300). Increased excretion of cysteinyl-LTs in the bile has been demonstrated after allergen challenge (316) and was associated with both early and late phase responses (317). The LTD_4 receptor antagonist MK-571 was shown to inhibit both EPR and LPR in BN rats (322), implicating cysteinyl-LTs as important mediators in this model. Therefore, the BN rat model resembles human asthma and is very useful for the study of both the EPR and the LPR.

In sheep, antigen challenge results in an EPR in most animals, and a LPR in some

animals (323). In this animal model, leukotrienes appear to play a critical role in the development of the LPR (306).

The dog was said to be the only mammal to share the ability of humans to develop respiratory pollenosis (302). This finding, together with its apparent IgE-mediated nature, has stimulated the evaluation of canine bronchial anaphylaxis as a model of human asthma. *Ascaris* antigen-induced bronchoconstriction in monkeys has been used for the preclinical evaluation of numerous potential anti-asthma drugs. Although intuitively we tend to relate studies on monkeys rather than nonprimate species to humans, there is little evidence to suggest any great advantage in using monkeys rather than more readily available laboratory animals as a model of human asthma.

To date, animal models have made important contributions towards the understanding of the pathogenesis of asthma. However, it is apparent that inflammatory cells, generation of mediators, and airway responses may be markedly different between species. Therefore, it should be kept in mind that different animal models may be useful for the investigation of different aspects of asthma.

1.5. METHODS FOR THE ANALYSIS OF LEUKOTRIENE FORMATION IN BIOLOGICAL SAMPLES

Because of its cellular diversity, the lung is capable of synthesizing a large number of different eicosanoids. Although each differs in the number and nature of its functional groups, such as hydroxyl or keto groups, and double bonds, as well as chain length, these numerous metabolites are often similar in their physicochemical properties and therefore

are difficult to separate, purify and analyze. A number of different methods have been developed for the analysis of leukotrienes and other eicosanoids in biological samples, including bioassay, radioimmunoassay (RIA), enzyme-linked-immunoassay (EIA), high pressure liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), and gas chromatograpy-mass spectrometry (GC-MS).

Bioassay is very useful for the measurment of biologically active compounds of unknown structure or highly unstable substances such as PGI_2 and TXA_2. Amounts in the low nanogram range of a compound can be estimated depending on its potency. Initial studies of SRS-A (cysteinyl-LTs) employed bioassay based on contraction of isolated guinea pig ileum. While this method is very sensitive, inexpensive and simple to perform, lack of specificity is a big probelm. For example, it cannot be used to distinguish between the various components of SRS-A. It is more difficult to interpret assay results if confusion with other bioactive compounds exist. This problem is usually dealt with by the use of antagonists to eliminate contributions by potentially interfering compounds. Furthermore, bioassay can not be used to analyze biologically inactive eicosanoids.

RIA is a well-accepted method for the quantitative analysis of eicosanoids. It is very sensitive and has a high sample capacity. It is also comparatively inexpensive and easy to perform. EIA is similar to RIA but is a non-isotopic method which uses an enzyme as a label and is simpler to perform than RIA. However, RIA and EIA permit detection of usually only one product at a time and may be limited in providing unequivocal information due to antigen cross-reactivity.

GC is a useful method for separating and analyzing many products at a time. GC

alone is more sensitive than HPLC, but provides no structural information on compounds. In contrast, MS is considered as a reference method because it is highly sensitive and specific. Quantitative analysis by MS is based on the formation of ions and their separation in a magnetic or electrostatic field. MS can be used for both structure elucidation and quantitation of extremely low concentrations of eicosanoids derived from biological sources. Coupled with GC, MS provides a unique method for the identification and quantitation of various eicosanoids in a single sample. However, this method requires costly apparatus, highly skilled personnel and, compared with RIA and EIA, relatively few samples can be analyzed.

HPLC is a very useful method for the analysis of leukotrienes and other eicosanoids in biological samples and has been instrumental in the identification of many of these compounds. Although it is not as sensitive as immunological methods or GC-MS, it is relatively simple, and permits the simultaneous analysis of a large number of eicosanoids in a single sample (324-327). Most lipoxygenase products can be detected on the basis of UV absorbance due to the presence of conjugated double bonds. The presence of a conjugated triene system in leukotrienes results in characteristic UV absorbance spectra with three UV maxima at approximately 260, 270, and 280 nm (LTB_4 and related compounds) or 270, 280, 290 nm (cysteinyl-LTs). Other major lipoxygenase products such as HETEs contain a conjugated diene chromophore and have absorption maxima at about 237 nm. With the exception of 12-HHTrE, none of the major products of the cyclooxygenase pathway posseses conjugated double bonds. Lipoxins (LXs) are another class of eicosanoids which contain a conjugated tetraene system, resulting in absorption

maxima at approximately 287, 301 and 316 nm (328). The characteristic UV absorbance spectra of LTs, HETEs and LXs permit their detection and quantitation with the use of a UV detector. This provides the specificity to minimize interference from unrelated compounds.

HPLC is the most widely used method for the simultaneous analysis of a wide range of eicosanoids in a single biological sample. The retention times (t_Rs) of most eicosanoids can be reasonably well predicted from the numbers of hydroxyl groups, oxo groups, and double bonds they possess. There are important selectivity differences depending, for example on the relative amounts of methanol and acetonitrile in the mobile phase (325,326). However, these differences do not usually result in large changes in retention time from one mobile phase to another. In contrast, the cysteinyl-leukotrienes, behave quite differently from other eicosanoids because they contain positively charged amino groups in addition to the negatively charged carboxyl groups which are common to all eicosanoids. This results in dramatic changes in selectivity with different acidic modifiers (e.g. acetic acid, trifluoroacetic acid (TFA), or phosphoric acid) of the mobile phase as well as with different stationary phases (324,325,327,329).

Precolumn extraction and RP-HPLC of eicosanoids have been extensively explored in our laboratory (325,326,330). A variety of techniques have been developed which have formed the basis for further development of sensitive methods for the analysis of leukotrienes and related compounds in our current studies.

1.6. OBJECTIVES OF THE PRESENT STUDIES

Although the pathogenesis of asthma remains unclear, inflammatory cellular infiltration with subsequent activation and release of bioactive mediators such as eicosanoids from inflammatory and resident lung cells has been implicated. However, due to the cellular diversity of the lung, it is not clear which cell types are primarily responsible for the synthesis of LTs by this tissue. Based on the observations of the increased synthesis of LTs in asthmatic patients and animal model of asthma, we hypothesized that the increased production of LTs in asthmatic patients may be due to the infiltration of inflammatory cells into the lung and/or to the induction of enzymatic activities in the lung after allergen challenge. The objectives of our study were to investigate the infiltration of inflammatory cells into the lung following allergen challenge and to determine whether changes in the numbers of inflammatory cells are correlated with changes in the synthesis of eicosanoids.

We chose the Brown-Norway (BN) rat as an animal model in our studies. The approaches employed to elucidate the mechanisms for increased LT production in the lung following allergen challenge were as follows:

1) **Development of a sensitive method for the analysis of leukotrienes and other eicosanoids**

Based on the numerous techniques developed over the years for the separation and measurement of eicosanoids by RP-HPLC in our laboratory, we developed specific, sensitive, and reproducible HPLC methods for the measurement of a large array of

eicosanoids in biological samples. These methods were used for the analysis of eicosanoids in the present study.

2) Isolation of lung cells from BN rats

We adapted an enzymatic digestion technique from Pele et al (331) to recover large numbers of dissociated lung cells from BN rats for the study of leukotriene synthesis. This technique was essential for our studies on cellular infiltration and leukotriene production by lung cells.

3) Purification of individual types of cells from lung tissue

To elucidate the cellular localization of leukotriene production in rat lungs, we attempted to isolate and purify different types of lung cells using density gradient centrifugation, centrifugal elutriation, and selective adherence of cells to plastic surfaces.

4) Investigation of cellular infiltration and leukotriene synthesis by lung cells following allergen challenge

To understand the underlying mechanism for altered LT synthesis during early- and late-phase responses following allergen challenge, we analyzed leukotriene production by lung cells at different times after antigen challenge and examined the relationship between cellular infiltration and leukotriene synthesis.

5) Effects of eosinophilia and neutropenia on leukotriene synthesis in the lung

Advantage was taken of available techniques to induce either eosinophilia by intravenous injection of Sephadex particles or neutropenia using rabbit anti-rat PMNL serum. These experimental models provided an excellent tool for the evaluation of the contributions of eosinophils and neutrophils to leukotriene production in the lung following allergen challenge.

6) Effects of eicosanoids on leukocyte infiltration in airways and lungs of BN rats

To understand the role of eicosanoids in cellular infiltration and late-phase responses, we examined the effects of eicosanoids on eosinophil infiltration into the lungs.

2. MATERIALS AND METHODS

2.1. MATERIALS AND ANIMALS

2.1.1. Chemicals and reagents

TABLE 2

Chemicals and reagents

Substance	Source
Arachidonic acid	Nuchek Prep Inc. (Elysian, MN)
PGB_2	Sigma Chemicals Co. (St. Louis, MO)
Calcium ionophore A23187	Calbiochem-Behring (LaJolla, CA)
λ-Carrageenin	Sigma Chemical Co.
Bovine serum albumin	Sigma Chemical Co.
Non-specific esterase kit	Sigma Chemical Co.
DNase (DN25)	Sigma Chemical Co.
Heparin (Hepalean)	Organon Teknika (Toronto, Canada)
LXA_4 and LXB_4	Cayman Chemical Company
LTC_4, LTD_4, LTE_4, *N*-acetyl-LTE_4	Dr. A.W. Ford-Hutchinson, Merck-Frosst
Ficoll-Paque	Pharmacia Fine Chemicals (Dorval, Que.)
Percoll	Pharmacia Fine Chemicals (Dorval, Que.)
Sodium pentobarbital (Somnotal)	MTC Pharmaceuticals (Cambridge, Onterio)
Protease (Type XXIV)	Sigma Chemical Company
Soybean lipoxygenase (Type 1)	Sigma Chemical Company
Sephadex G200	Sigma Chemical Company

Chemicals were obtained from the sources shown in Table 2. All other chemicals were analytical reagent grade and were obtained through local suppliers.

2.1.2. Preparation of eicosanoid standards.

Immediately prior to use, arachidonic acid was purified by reversed-phase-high-pressure liquid chromatography (RP-HPLC) on an analytical octadecylsilyl silica (ODS) column (Spherisorb ODS-2, 4.6 x 250 mm; Phenomenex) using water/acetonitrile/acetic acid (25:75:0.02) as mobile phase. Cysteinyl leukotrienes (including LTC_4, LTD_4, LTE_4 and *N*-acetyl LTE_4) were kept in water at -80°C in plastic vials. LTB_4 was prepared by incubation of arachidonic acid (100 μM) with porcine PMNL in the presence of 5,8,11,14-eicosatetraynoic acid (ETYA) (5 μM) and the calcium ionophore A23187 (5 μM) (332).

5-Hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) was prepared by incubating arachidonic acid and A23187 with porcine leukocytes in the presence of ETYA (81). 15-HETE was prepared using soybean lipoxygenase (333). 12-HETE and 12-hydroxy-5,8,10-heptadecatrienenoic acid (12-HHTrE) were prepared by incubation of arachidonic acid with human blood platelets (77). 5-Oxo-ETE was either biosynthesized in our laboratory as described previously (72) or purchased from Cascade Biochem Ltd., Reading, UK.

8(*S*),15(*S*)-Dihydroxy-ETE (8,15-diHETE) was prepared by double lipoxygenation by incubating arachidonic acid (100 μM) with a relatively high concentration (25 nM) of soybean lipoxygenase (75,335,336). 5(*S*),15(*S*)-dihydroxy-ETE (5,15-diHETE) was prepared by incubating 5-HETE with soybean lipoxygenase (335). The hydroperoxy metabolites were reduced with sodium borohydride in methanol for 20 min at 0°C

followed by 20 min at room temperature. 13-HODE was synthesized by incubation of linoleic acid with soybean lipoxygenase and reduction of the hydroperoxy product with sodium borohydride (333). All of the products prepared in our laboratory were extracted using cartridges containing ODS silica (C_{18} Sep-Paks, Waters Associates) as previously described (330) and were purified by either reversed-phase or normal-phase HPLC.

2.1.3. Solvents

All solvents used for HPLC were HPLC grade and were purchased from Fisher Scientific Co., Fairlawn, NJ or BDH Chemicals, Montreal. Solvents used for other purposes were reagent grade. Water was obtained from a Milli-Q water purification system (Waters-Millipore).

2.1.4. Animals

Specific pathogen-free male Brown Norway (BN) rats (7-9 weeks old, weight range 180-220 g) were purchased from Harlan-Sprague-Dawley, Inc. (Walkerville MD). Male Sprague-Dawley (SD) rats (200-250 g) and New Zealand white rabbits (2.0-2.2 kg) were obtained from Charles River, Inc. and La Ferme Lapro Inc., respectively. The animals were kept in conventional housing facilities, and allowed to eat and drink *ad libitum*.

2.2. *ANALYSIS OF LEUKOTRIENES, LIPOXINS AND OTHER EICOSANOIDS BY RP-HPLC*

2.2.1. HPLC conditions

a) Precolumn extraction and HPLC procedures

Precolumn extraction/RP-HPLC analysis of eicosanoids was performed as previously described (81,326) using a six-port switching valve coupled to a precolumn, an analytical column, a solvent delivery system for the analytical column, and a pump to load the sample onto the precolumn via an automatic injector. The sample was first pumped from a WISP automatic injector (Waters-Millipore) via a WAVS automated switching valve (Waters-Millipore) in the "load" position onto a precolumn cartridge (μBondapak C_{18} Guard-Pak cartridge, Waters-Millipore), which had been preequilibrated with precolumn solvent (15 ml of either 15% or 30% methanol in water containing 2.5 mM phosphoric acid). After the sample was loaded onto the precolumn, the latter was washed with 18 ml of precolumn solvent. The six-port WAVS automated switching valve was then switched to the "inject" position, placing the precolumn in line with an HPLC pump (model 600, Waters-Millipore), and an analytical ODS-silica HPLC column (Spherisorb ODS-2, either 4.6 x 250 mm or 3.2 x 250 mm; Phenomenex), which was also attached to the WAVS switching valve. Leukotrienes and other eicosanoids retained on the precolumn cartridge were then separated by the mobile phase used for the analytical column. The uv spectrum of the column eluate was recorded between 220 and 330 nm using a Waters 991 photodiode array detector. Eicosanoids in biological samples were identified by comparison of their relative retention times and uv spectra with those of

authentic standards.

b) Mobile phases for RP-HPLC

Water obtained from a Milli-Q water purification system (Waters Millipore) was further purified by passing it through a Millipore Norganic cartridge (Millipore Corp., Bedford). Stock solutions of aqueous 2% TFA were purified by passing them through C_{18}

TABLE 3

Composition of mobile phases for RP-HPLC

Solvent	Volume (%)			
	Water	Acetonitrile	Methanol	TFA[b]
A	70		30	
B	20	80		0.005
C	20	55	25	0.009
D	70		30	0.04
E	20	60	20	0.05
F	25	55	20	0.04

[a]The small volume of aqueous TFA added in the mobile phases was not taken into consideration when calculating the composition of the mobile phases.

[b]A stock solution containing 2% TFA in water was used for the preparation of mobile phases.

Sep-Paks prior to use. Organic solvents and phosphoric acid were HPLC grade from BDH and Fisher Scientific. The compositions of the solvents used to prepare the mobile phases used in this study are shown in Table 3.

2.2.2. Incubation conditions

Cells (5×10^6/ml) and lung fragments were suspended in HBSS containing calcium (1.1 mM) and magnesium (1.2 mM). After preincubation at 37° C for 5 min, the cells were stimulated with 1 μM A23187. The reactions were terminated by adding cold methanol to give a final methanol concentration of 30%. PGB_2 (25 ng) was also added into each sample as an internal standard to correct for recovery. The samples were stored at -80 °C before HPLC analysis. Prior to analysis, samples were centrifuged at 2200 x g for 10 minutes to remove precipitates. The procedures used for the analysis of eicosanoids by precolumn extraction and RP-HPLC were as described above.

2.3. PREPARATION OF CELLS FOR EICOSANOID PRODUCTION

The following buffers were prepared as described (331). Buffer I consisted of 0.12 M NaCl, 0.047 M KCl, 1.2 M KH_2PO_4, 0.025 M $NaHCO_3$, and 10 mM glucose. Buffer II was identical to Buffer I plus 1 mM EDTA. Buffer III was identical to Buffer I plus 10 mM HEPES and 2.4 mM $MgSO_4$-$7H_2O$ for DNase activity. Buffer IV was the same as Buffer III except that $NaHCO_3$ was omitted and 25 mM HEPES and 1.1 mM $CaCl_2$ were added. After bubbling with 95% O_2-5% CO_2, the pH of each individual buffer was adjusted to 7.4 with NaOH.

Tyrode's buffer and Hank's balanced salt solution (HBSS) were prepared for fractionating lung cells using elutriation and Percoll density gradient centrifugation. Tyrode's plus gelatin (TG) contained 137 mM NaCl, 2.6 mM KCl, 0.35 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.5 mM glucose, and 1g gelatin/L. The pH was adjusted to 7.4 with HCl. TGMD was identical to TG plus 1 mM $MgCl_2$ and DNase (DN25, 15 mg/ml) (337).

2.3.1. Preparation of lung tissue fragments for eicosanoid production

The production of eicosanoids by lung fragments from male Sprague-Dawley and BN rats was examined in the preliminary study. Rats (200-300g) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). After injection of heparin (1000 units/kg) via the sublingual vein, the thoracic cavity was opened and the heart and lungs were removed *in toto*. The lungs were immediately perfused with Krebs buffer (Buffer I, prewarmed at 37 °C) containing 2 % bovine serum albumin through a cannula introduced into the pulmonary artery via the right ventricle. During the perfusion, the lungs were inflated several times to dilate alveoli and facilitate removal of blood. When the lungs were completely cleared of blood, the heart and blood vessels were dissected away. The lungs were further rinsed in buffer I and chopped manually into fragments of approximately 10-20 mg each. The fragments were washed twice in Buffer I and resuspended in Buffer IV (200 mg/ml). After preincubation for 5 min, the fragments were incubated at 37 °C with agonist or vehicle (dimethyl sulfoxide, DMSO) for either 15 or 45 min. The reaction was terminated by adding cold methanol (final concentration: 30% methanol) and 25 ng PGB_2 as an internal standard. The samples were then stored at -80°C prior to analysis by

precolumn extraction/RP-HPLC, which was performed within one week.

2.3.2. Preparation of rat lung cells from protease-digested lungs

2.3.2.1. Preparation of suspensions of lung cells

Crude lung cells were prepared from male rats by enzymatic digestion (331). The lungs were isolated from rats as described above. After removal of blood, the lungs were perfused with 100 ml of Buffer II containing 0.06% protease (Type XXIV, Sigma) via the trachea at a flow rate of 10 ml/min for 15 min at 37 °C. The trachea and external bronchi were cut from the lungs and large bronchi and blood vessels were then removed using forceps. The gelatinous lung tissue was subsequently dispersed by six in-and-out flushings, using a 60 ml syringe, with 50 ml of Buffer III containing 50 Kunitz units of DNase (DN25, Sigma). The dissociated lung cells were then successivelly filtered through 260 μm and 100 μm pore size nylon mesh and washed twice by centrifugation at 250 x *g* for 10 min. The few contaminating erythrocytes were removed by hypotonic lysis with distilled water.

2.3.2.2. Preparation of lung cell fractions by centrifugal elutriation

We attempted to purify rat lung mast cells by centrifugal elutriation. Crude lung cells were fractionated using a JE-6 elutriator rotor driven by a J4.2 B centrifuge (Beckman Instruments, Palo Alto, CA). Before each run, the system was washed with 200 ml of 70% ethanol, 500 ml of distilled water, and 200 ml of TGMD medium. Lung cell suspensions (100-300 x 10^6 nucleated cells) were loaded into a 75-ml pulse suppressor

tube via a 20-ml syringe fitted with a 3-way valve. The cells were then pumped into the standard elutriation chamber by a peristaltic pump (LKB, Broma). The cells were fractionated by either maintaining a constant rotor speed and sequentially increasing buffer flow rate (method 1) (337) or maintaining a constant buffer flow rate and sequentially decreasing rotor speed (method 2) (337,338). In method 1, the rotor speed was maintained at 1830 rpm (275 x *g*) and the cells were loaded at a flow rate of 11 ml/min. The rate was then incrementally increased to 14, 18, 20, 26, and 30 ml/min. After each adjustment in flow rate, three 50 ml fractions were collected. An additional 90 ml collection was obtained at the end of each run with the centrifuge turned off. Generally, the initial flows of 11 and 12 ml/min allowed selective depletion of red blood cells and other small diameter cells during loading. In method 2, the cells were loaded at a flow rate of 28 ml/min and a rotor speed of 3950 rpm. The flow rate was then increased to and maintained at 30 ml/min and the cells were fractionated by incrementally decreasing the rotor speed (3700, 3500, 2900 and 0 rpm). Fractions were collected as described for method 1. The cells in all collected fractions were washed (150 x *g* for 10 min) and analyzed for mast cell content and eicosanoid biosynthesis.

2.3.2.3. Preparation of lung cell fractions by density gradient centrifugation

Lung cells recovered from enzymatic digestion were also fractionated by density gradient centrifugation. The dispersed cells were resuspended in HBSS and carefully loaded over 5 ml of Ficoll-Paque (density, 1.077 g/ml, Pharmacia, Dorval, Que.). After centrifugation at 250 x *g* for 30 min, the cells at the interface were carefully collected by

aspiration and the pellet was resupended in Ca^{++}/Mg^{++}-free HBSS. The cells in the pellet fraction were further fractionated by Percoll density gradient centrifugation.

Percoll, composed of colloidal silica coated with polyvinyl pyrrolidine (Pharmacia, Dorval, Que.) was prepared as follows: a 100% solution of Percoll was made by mixing 9 vols. of Percoll (density, 1.23 g/ml) with 1 vol. of a 10 x concentrated solution of HSBB. Progressive dilutions were then made with 1 x HBSS to give mixtures containing 80, 70, 60, 50 and 40% Percoll. The densities of these solutions were measured with a DMA 48 Densitometer (AP PAAR, Austria) and were shown to be 1.110, 1.093, 1.080, 1.067, and 1.057 g/ml, respectively,. The osmolalities of the Percoll solutions were adjusted to be 320-330 mOsm by using an Osmette™ microosmometer (Precision Systems Inc., Natick, MA). The cell suspension was loaded on top of the density gradient which consisted of 0.8 ml aliquots of the 80%, 70%, 60%, 50% and 40% Percoll suspension. The samples were then centrifuged at 400 x *g* for 20 min, and the cells at each interface were collected.

2.3.2.4. Partial purification of pulmonary eosinophils

Isolated lung cells, prepared as described in section 2.3.2.1, were fractionated by density gradient centrifugation with Percoll (339) using layers of the following densities: A, 1.051 g/ml; B, 1.059 g/ml; C, 1.067 g/ml; D, 1.078 g/ml; E, 1.087 g/ml; F: 1.096 g/ml. After centrifugation at 1200 x *g* for 20 min, fractions D and E contained the greatest numbers of eosinophils. Various fractions of cells were collected separately, and washed by centrifugation at 250 x *g* for 10 min with Ca^{++}/Mg^{++}-free HBSS to remove any

remaining Percoll. The pellets were suspended in HBSS containing calcium (1.1 mM) and magnesium (1.2 mM) and incubated with A23187 as described in section 2.2.2 for the measurement of eicosanoid synthesis.

2.3.3. Preparation of cells from other sources

2.3.3.1. Alveolar macrophages

Rat bronchoalveolar fluid was obtained from BN rats by bronchoalveolar lavage (BAL) with 4 x 4 ml of HBSS via a tracheal cannula. The alveolar macrophages were purified according to their property of adherence to the plastic surfaces of tissue culture plates.

2.3.3.2. Pleural PMNL

Elicited pleural PMNL were prepared from pleural exudates obtained 4 h after the injection of λ-carrageenin (1 mg in 0.1 ml of sterile saline) into the pleural cavities of male BN rats. In brief, cells obtained from the pleural cavities were layered over Ficoll-Paque in a ratio of 3:2 followed by centrifuge at 500 x *g* for 30 min. The pellet was resuspended in HBSS and the remaining red blood cells were removed by hypotonic lysis. After one more washing in normal saline, the PMNL were resuspended in HBSS.

2.3.3.3. Circulating mononuclear cells

Peripheral blood was collected from anesthetized BN rats by cardiac puncture. The red blood cells were sedimented by mixing the blood with 6% Dextran T-500 and allowed

the mixture to stand at room temperature for 40 min. The leukocytes fraction, which contains about 80% lymphocytes, was then layered over Ficoll-Paque followed by centrifuge at 500 x *g* for 30 min as described above. The mononuclear cell layer at the interface usually contains more than 95% lymphocytes, the remaining cells being principally monocytes.

2.3.3.4. Peritoneal mast cells

Sprague-Dawley rats were anaesthetized as described above. Ca^{++}/Mg^{++}-free HBSS (15 ml) containing 50 mM HEPES and 0.1% bovine serum albumin was injected into the abdominal cavity of each rat. After gentle massage for 2 min, the abdominal wall was opened by midline incision. The fluid containing peritoneal cells was aspirated with a plastic transfer pipette into a tube and centrifuged at 300 x *g* for 10 min. The sedimented cells were resuspended in HBSS and filtered through nylon mesh (100 μm pore size) to remove tissue debris.

The filtrate was centrifugated at 300 x *g* for 10 min and then fractionated on Percoll. Percoll-HBSS was prepared by mixing 6.3 ml of Percoll (Pharmacia, Dorval, Que.) with 0.7 ml of 10 x concentrated Ca^{++}/Mg^{++}-free HBSS containing 200 mM HEPES and 0.4 ml of distilled water. The mixture was adjusted to a pH of 7.0. The osmolality and density were adjusted to 330 mOsm and 1.17 g/ml, respectively, using distilled water and 10 x concentrated HBSS. A continuous gradient of Percoll was generated in a 10 ml cellulose nitrate tube by centrifuging the Percoll-HBSS at 17,000 x *g* for 20 min at 4 °C in a Beckman fixed angle rotor. For each density gradient tube, peritoneal cells from 2-4

rats were layered on the top of the Percoll gradient and centrifuged at 450 x *g* for 15 min. After centrifugation, leukocytes and macrophages formed a rather compact layer at the interface with the gradient, whereas erythrocytes were situated 1 to 2 mm below the interface. After removal of the upper bands, the remaining Percoll-HBSS containing the isolated mast cells (usually more than 90% purity) was aspirated and washed twice with HBSS by centrifugation at 250 x *g* for 10 min.

2.3.3.5. Preparation of human PMNL, eosinophils and platelets

Peripheral blood was mixed with 6% Dextran T-500 (Pharmacia, Dorval, Quebec) (5:1) which accelerates the sedimentation of red blood cells. After 45 min, the supernatant was removed and centrifuged at 250 x *g* for 10 min to pellet leukocytes.

PMNL were prepared by layering a leukocyte suspension over Ficoll-Paque in a ratio of 3:2 followed by centrifugation at 500 x *g* for 30 min at 4 °C. The pellet was resuspended in HBSS and the remaining red blood cells were removed by hypotonic lysis with distilled water. After washing in normal saline, the cells were resuspended in HBSS.

Human eosinophils were purified by the method of Koenderman et al. (340). In brief, PMNL (75 x 10^6 cells/ml in HBSS) were incubated with 10 nM fMLP (Sigma Chemical Co.) for 10 min at 37 °C. The cell suspension was then layered carefully over a discontinuous gradient of isotonic Percoll of densities 1.082 g/ml (4 ml) and 1.100 g/ml (1 ml). The latter was used to prevent contamination of the eosinophils with cell debris and remaining erythrocytes, which were present as a pellet at the bottom of the tube. The tubes were then centrifuged at 300 x *g* for 15 min at room temperature. The eosinophils

present at the interface between the two Percoll layers were collected, washed and resuspended in HBSS. The eosinophil preparation comprised greater than 90% eosinophils, most of the remaining cells being neutrophils.

Human platelets were prepared using citrate dextrose as anticoagulant (112). Blood was centrifuged at 250 x *g* for 10 min to obtain platelet-rich-plasma (PRP). PRP was then centrifuged at 400 x *g* for 10 min. After removing the platelet-poor-plasma (PPP), the platelet pellet was washed and resuspended in HBSS.

2.3.4. Cell viability, staining and differential counts

Cell viability was analyzed by using the trypan blue exclusion test, whereas cell numbers were counted in a haemocytometer. Differential cell counts were done on cytospin slides stained with either Diff-Quik or Alcian blue, or using a kit for non-specific esterase (Sigma Chemical Company) as described below.

Cytospin slides were prepared in duplicate for all fractions of interest. In brief, aliquots containing 5 x 10^4 cells (50 µl of a suspension of 10^6 cells/ml) were cytocentrifuged onto clean slides using a Cytospin (Shandon, Sewickely, PA) at 200 x *g* for 5 min at 22 °C. Slides were allowed to air dry before fixation and staining.

2.3.4.1. Diff-Quik staining of leukocytes

Diff-Quik Stain kit (Baxter Scientific Products Division, McGaw Park, IL) consists of Fixative and Solutions I and II. It is a modified version of the Wright Staining technique. Cytospin slides or blood smears prepared for staining were first fixed in

Fixative solution (5 times, one second each time). The slides were then dipped in Solution I (containing Eosin Y, 5 x 1 sec) followed by Solution II (a buffered solution of thiazine dyes consisting of methylene blue and Azure A) (5 x 1 sec). After rinsing the slides in distilled water, leukocytes can be differentiated under a microscope equipped with an oil immersion lens.

2.3.4.2. Non-specific esterase staining of monocytes and macrophages

Non-specific esterase-containing cells were stained using a kit from Sigma with α-naphthyl acetate as the substrate according to the procedure provided by the supplier. In brief, a citrate-acetone-formaldehyde (CAF) fixation solution was prepared from 25 ml of citrate solution (18 mM citric acid, 12 mM sodium chloride and surfactant, pH 3.6), 65 ml of acetone, and 8 ml of 37% formaldehyde immediately prior to use. The staining solution was prepared by adding a mixture of sodium nitrite solution (1 ml, 0.1 M) and fast blue solution (1 ml, 15 mg/ml) to a Coplin jar containing 40 ml of prewarmed distilled water. The Trizmal buffer concentrate (5 ml) and 1 ml of α-naphthyl acetate solution were added immediately before staining. The Coplin jar was wrapped with aluminum foil to protect the staining solution from light.

The blood smears or cytospin slides were first fixed in CAF solution in a Coplin jar at room temperature for 30 sec. The slides were then rinsed in warm distilled water in a Coplin jar for 1 min before incubated in the staining solution at 37 °C for 30 minutes. The slides were subsequently rinsed in warm distilled water for 5 min and counterstained in haematoxylin solution for 2 min. After rinsing in tap water and air drying, the slides

were mounted with a drop of Permount (Fisher Scientific) and analyzed by microscopy

2.3.4.3. Alcian blue staining of mast cells

Alcian blue dye (Fisher Scientific Company) stains heparin within mast cells and basophils in the presence of lanthanum ions at low pH. In brief, 0.1 ml of a cell suspension was diluted with 0.1% EDTA solution (0.4 ml) and mixed with 0.45 ml of alcian blue solution which contained 0.076% cetyl pyridinum chloride, 0.7% lanthanum chloride, 0.9% sodium chloride, 0.21% Tween 20 and 0.143% alcian blue. After addition of 0.05 ml of 1 N HCl into the mixture, the mast cells were counted.

2.4. MEASUREMENT OF LUNG RESISTANCE

In a preliminary study, airway responses to OVA were measured by monitoring transpulmonary pressure (Ptp) and respiratory air flow (V) of BN rats for periods of 1 min at various times up to 8 hr as described previously (317). This experiment was performed by Lijing Xu at Dr. James G. Martin's laboratory. In brief, sensitized rats were anaesthetized intraperitoneally with urethane (1g/kg, 50 wt/vol). After blind orotracheal intubation (6 cm of PE 240 polyethylene catheter), the rats were placed into a Plexiglas box (volume, 265 ml). A fleisch no. O pneumotachograph coupled to a piezoresistive differential pressure transducer (Micro-Switch 163PC01D36; Honeywell, Scarborough, Ont., Canada) was attached to the other end of the box to measure airflow. Transpulmonary pressure (Ptp) was measured using a water-filled catheter placed in the lower third of the esophagus connected to one port of a differential transducer (Sanborn

268 BC, Sanborn, Watham, MA); the other port was connected to the Plexiglas box. The esophageal catheter consisted of a polyethylene tube (PE-240, 10 cm long), with the terminal tip (6 cm) of a smaller-bore tube (PE-160) placed in the esophagus. The pressure and flow signals were amplified, passed through eight-pole Bessel filters (Model 902LPF; Frequency Devices, Haverhill, MA), with their cutoff frequencies set at 100 Hz, and recorded by a 12-bit analog-to-digital converter at a rate of 200 Hz. The data were stored on the computer. A commercial software package (RHT Infodat Inc., Montreal, Canada) was used to determine pulmonary resistance (R_L) as previously described (228). The rats were allowed to stabilize for a period of 30 min prior to challenge with either aerosolized OVA or saline. The administration of OVA by instillation was also evaluated and compared to administration by aerosol.

2.5. MEASUREMENT OF INFLAMMATORY CELLS AND EICOSANOID PRODUCTION BY LUNG CELLS FROM SENSITIZED BN RATS

2.5.1. Study design

To investigate the cellular infiltration and eicosanoid synthesis by lung cells following allergen challenge, the following groups of rats were studied:

1). Non-sensitized unchallenged rats were used to examine the synthesis of eicosanoids by normal lung cells.

2). Rats actively sensitized against ovalbumin were used to study the effect of sensitization on eicosanoid synthesis by lung cells.

3). Sensitized rats were challenged with ovalbumin and sacrificed after various

times to study the time course of the effect of antigen challenge on eicosanoid production by lung cells. Airway resistance was monitored after antigen challenge in this preliminary study

4). Based on the results of the above preliminary experiments, we further studied the effects of challenge with allergen or vehicle on cellular infiltration and eicosanoid synthesis in the lung before, and 6 or 24 h after challenge. We also examined the relationships between the numbers of inflammatory leukocytes and leukotriene production in this study.

A diagram illustrating the protocol used in the above studies is shown in Fig 6.

2.5.2. Sensitization of Brown-Norway rats

Active sensitization against ovalbumin (OVA) was performed by subcutaneous injection of sterile normal saline (1 ml) containing 1 mg OVA and 200 mg aluminum hydroxide. At the same time 1 ml of Bordetella pertussis vaccine containing 6×10^9 heat-killed bacilli was given intraperitoneally as adjuvant (228). Animals were studied 14-21 days after sensitization.

2.5.3. Antigen challenge of sensitized rats

Sensitized rats were anesthetized with pentobarbital (60 mg/kg, i.p.). Endotracheal intubation was then performed using a 6 cm length of PE-240 polyethylene tubing. The animals were placed in the left lateral decubitus position with the end of the endotracheal tube inside a small plexiglass box (volume, 265 ml) and challenged by inhalation of either

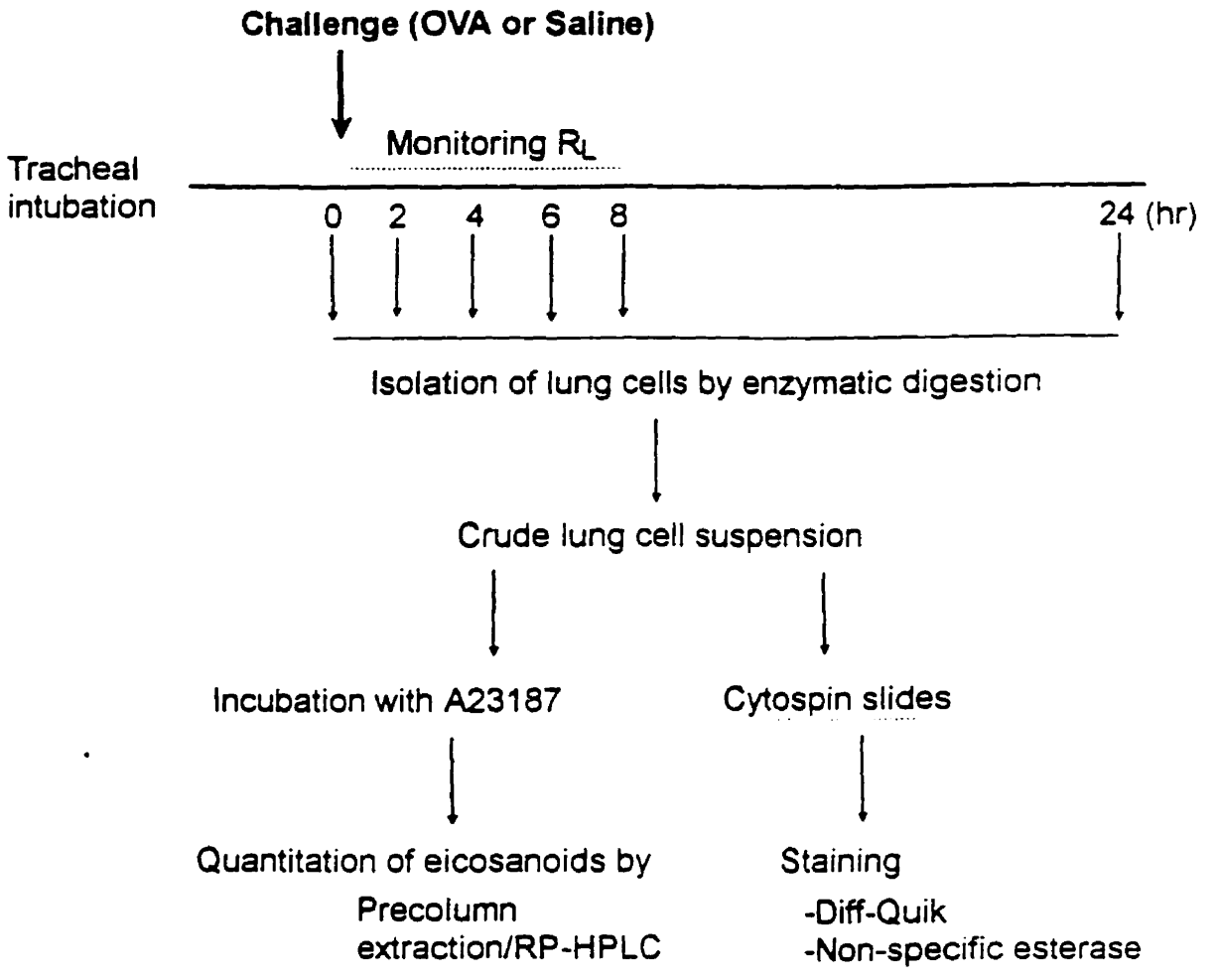

Figure 6. Protocol for the analysis of leukotriene synthesis by lung cells following antigen challenge. Sensitized BN rats were challenged with ovalbumin (OVA). Lung cells were isolated by enzymatic digestion from the rats at various times after challenge. Leukotriene synthesis by lung cells was analyzed by RP-HPLC. The differential counts of inflammatory cells in the lung, which were performed only before and 6, or 24 h after challenge with OVA or saline, were determined on cytospin slides stained with Diff-Quik and non-specific esterase. Airway resistance (R_L) was monitored for up to 8 h following OVA challenge in preliminary experiments.

aerosolized OVA (5% OVA wt/vol in normal saline) for 5 min with a nebulizer output of 0.18 ml/min or vehicle (saline), respectively

2.5.4. Measurement of cellular infiltration and eicosanoid synthesis by lung cells following allergen challenge

Lung cells were isolated as described above by enzymatic digestion. Neutrophils and eosinophils were differentiated on cytospin slides stained with Diff-Quik as described above. Macrophages were counted using a non-specific esterase stain. Eicosanoid synthesis by lung cells was analyzed as described in Section 2.2.

2.6. INDUCTION OF PULMONARY EOSINOPHILIA

To characterize eicosanoid synthesis by rat eosinophils, we attempted to purify these cells from rat lung tissue. Sephadex particles (G200; 40-120 μm diameter; Sigma) were injected intravenously on days 0, 2 and 5 to induce eosinophilia (341). On days 7-9, the lung cells were obtained by enzymatic digestion and used for the purification of pulmonary eosinophils as described in Section 2.3.2.1.

2.7. EFFECTS OF LEUKOCYTE DEPLETION ON PULMONARY CELLULAR INFILTRATION AND EICOSANOID SYNTHESIS BY LUNG CELLS FOLLOWING ALLERGEN CHALLENGE

The study design is illustrated in Fig 7. BN rats were sensitized to OVA as described above. On day 14 after sensitization, about 0.5 ml of blood was taken from each

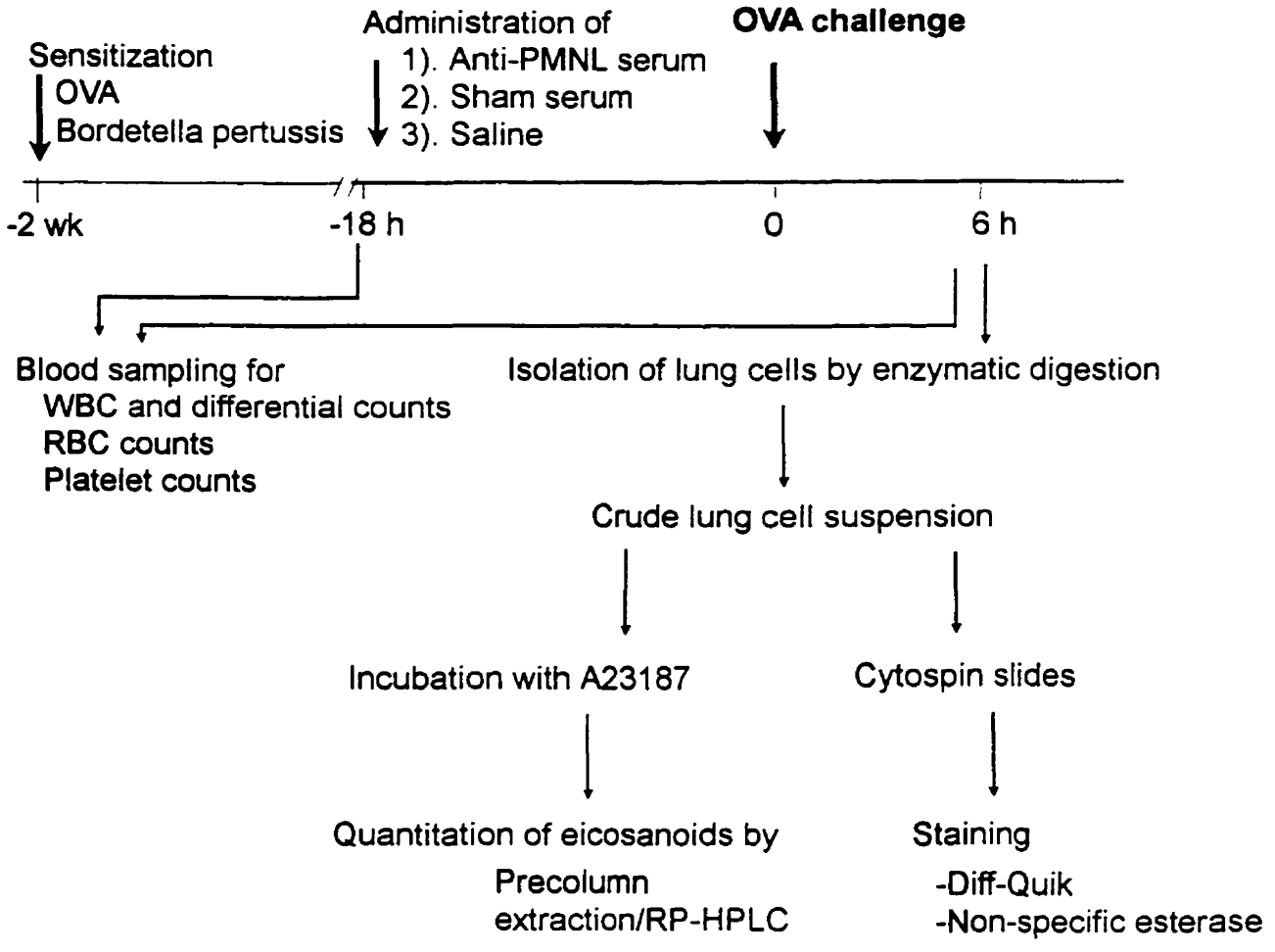

Figure 7. Protocol for the analysis of the effects of leukocyte depletion on pulmonary cellular infiltration and eicosanoid formation by lung cells following antigen challenge. Sensitized BN rats were randomly divided into three groups which were pretreated with 1) anti-PMNL serum; 2) sham serum; or 3). saline 18 h before allergen challenge. Blood samples were taken 18 h before and 6 h after OA challenge for the examination of total WBC, RBC, platelet counts and leukocyte differential counts. Lung cells were then isolated from rats by enzymatic digestion. Leukotriene synthesis by lung cells was analyzed by RP-HPLC. The differential counts of inflammatory cells in the lung were determined on cytospin slides stained with Diff-Quik and non-specific esterase.

rat by retro-orbital plexus puncture under anesthesia with sodium pentobarbital. Total white blood cell (WBC), red blood cell (RBC) and platelet counts were determined using an automated Coulter counter. Differential leukocyte counts were performed on blood smears stained with Diff-Quik. The rats were then randomly divided into three groups which were treated as follows:

1) Anti-PMNL group: rabbit anti-rat PMNL serum (Accurate Chemical and Scientific Corp., Westbury, NY) was injected intraperitoneally into rats at a dose of 1 ml/200 g body weight to induce leukocyte depletion.

2) Sham serum group: the same as the anti-PMNL group, except that serum from non-immunized rabbits was used instead of anti-PMNL serum.

3) Control group: rats were treated with saline instead of serum.

Eighteen hours after administration of serum or saline, the rats were anesthetized and intubated endotracheally using a 6 cm length of PE-240 polyethylene tubing. The animals were challenged as described above by inhalation of OVA. Blood samples were taken from each rat 6 h after OVA challenge. Cellular infiltration into airways and lung tissue was assessed by light microscopic examination of cells recovered from airways by bronchoalveolar lavage and from lung tissue by enzymatic digestion, respectively, as detailed above. Eicosanoid synthesis by lung cells was measured by precolumn extraction/RP-HPLC as described above (section 2.2.).

2.8. *EFFECTS OF EICOSANOIDS ON LEUKOCYTE MIGRATION INTO THE LUNGS*

2.8.1. *Effects of eicosanoids on rat and rabbit neutrophil migration in vitro*

Carrageenin-elicited rat neutrophils were prepared as described above whereas neutrophils from New Zealand White rabbits were isolated by treatment of the peripheral blood with Dextran T-500 followed by centrifugation of the upper layer over Ficoll-Paque. The neutrophils were suspended in RPMI containing $CaCl_2$ (1mM), $MgCl_2$ (1mM) and 1% BSA. The migration of neutrophils toward different eicosanoids was measured by the modified Boyden chamber technique using 48-well microchemotaxis chambers (Neuro Probe Inc., Cabin John, MD) and Sartorius cellulose nitrate filters (5μm pore size, 140 μm thickness) (Neuro Probe Inc.) (72). The substances to be tested were prepared immediately before use. Eicosanoid stock solutions were evaporated to dryness in Eppendorf vials under a stream of nitrogen, and the residue was reconstituted in a small volume of DMSO and diluted in RPMI buffer containing 0.3% BSA (the final concentration of DMSO was 0.1%). Various concentrations of agents were added to the bottom wells in volumes of 30 μl, whereas aliquots (150,000 cells in 55 μl) of the neutrophil suspension were added to each of the top wells. Each agonist concentration and vehicle control were assayed in triplicate. The chambers were then incubated for 1 h at 37 °C in 5% CO_2 and humidified air. The filters were then removed, fixed and stained as described (72). The cells in five random high power fields (HPFs) (x 400) that had migrated through to the bottom of the filter were counted under oil immersion. The average number of cells per HPF for each well was determined, and a mean value for

triplicate wells was calculated.

2.8.2. Effects of eicosanoids on leukocyte migration in vivo

Eicosanoids were evaluated for *in vivo* chemotactic activity. Immediately prior to use, LTB_4, LTC_4, LTD_4, LTE_4 and 5-oxo-ETE were quantitated by RP-HPLC as described in Section 2.2.1. The eicosanoids were then prepared in sterile saline containing 0.3 % ethanol unless specified otherwise.

a) Effects of 5-oxo-ETE and LTB_4 on leukocyte kinetics in rabbit peripheral blood

A protocol was adapted from Marleau et al (229) for this study. Rabbits were placed in restraining cages and allowed to stabilize for 30 min. The substance to be tested (3-5 μg/kg in normal saline containing 0.01% BSA) was injected via an ear vein. Blood samples were taken from an ear artery at 60, 50, 10, 5, and 1 min before, and 0.5, 2, 5, 10, 30, 60, and 120 min after administration of agonist. Total white blood cell (WBC), red blood cell (RBC) and platelet counts were determined using an automated Coulter counter. Differential leukocyte counts were performed on blood smears stained with Diff-Quik.

b) Effects of eicosanoids on leukocyte migration into the pleural cavities of rats

Agonists (0.5-5 μg in 90 ul) were injected directly into the pleural cavities of Wistar and BN rats through a 27-gauge 1/4 inch needle after anesthesia with pentobarbital. Control rats received the same volume of vehicle. The animals were then

killed with ether 4 or 24 h after administration of the agonist. The chest was opened by lateral incision just above the diaphragm and along both sides of the rib cage. The pleural cavities were washed three time with 3 ml of saline containing heparin (10 units/ml). The washes were pooled and cells in the combined washes were recovered by centrifugation. Total cell numbers and differential cell counts were determined as described above.

c) Effects of eicosanoids on leukocyte migration into the lungs and airways of BN rats

BN rats were anesthetized with pentobarbitone (60 mg/kg, i.p.) and agonist (5 μg in 100 μl) or vehicle (100 μl) was then insufflated directly into the airways with a microsyringe. After 24 h, the rats were anesthetized again as described above and cannulated with polyethylene tubing by orotracheal intubation. The bronchoalveolar lavage was performed with HBSS containing 0.1% bovine serum albumin (4 x 4 ml). The combined BAL fluid was centrifuged at 150 x *g* for 10 min. The pellet was washed and resuspended in 1 ml of HBSS. The total cell counts and viability were determined. Cytospin slides were prepared for Diff-Quik staining and immunocytochemistry. Immediately after bronchoalveolar lavage, the lungs were removed from the animals and washed twice in 10 ml of HBSS. A part of hilar area including the central airways was dissected from each lung and washed in HBSS. Both cytospin slides and lung sections were stored at -80 °C until immunostaining for eosinophils by Dr. Q. Hamid. In brief, the samples were immunostained with BMK13, a mouse anti-human monoclonal antibody to eosinophil major basic protein (MBP) generously provided by Dr. R. Moqbel (University of Alberta, Edmontan, Alberta). This antibody was shown by Dr. Hamid to cross-react

with rat MBP. The MBP-positive cells were detected by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method and counted under a microscope by a blinded observer (202). A minimum of 500 BAL cells was counted and the percentage of cells expressing MBP immunoreactivity was evaluated. MBP positive cells in tissue section were expressed as the number of positive cells per mm airway basement membrane.

2.9. STATISTICAL ANALYSIS OF DATA

Results in both text and figures are expressed as means ± SEM unless otherwise specified. The inflammatory cells in the lung were analyzed as percentages of total isolated lung cells. The amounts of leukotrienes and other eicosanoids produced by cells were expressed as ng/10^7 cells. Means of several groups were compared using the Bonferroni *t* test, preceded by Kruskall-Wallis analysis of variance using NCSS (Number Cruncher Statistical System 5.02). Differential cell counts in the blood before and after administration of serum or saline were analyzed by paired T-tests. Pearson's correlation was determined for analyzing the relationship between the numbers of individual cell types and leukotriene formation by isolated lung cells. Differences were considered to be statistically significant when the p value was less than 0.05.

3. RESULTS

3.1. ANALYSIS OF LEUKOTRIENES, LIPOXINS, AND MONOOXYGENATED METABOLITES OF ARACHIDONIC ACID BY REVERSED-PHASE HPLC

Because of the complex profiles of eicosanoids formed by many types of cells, HPLC has proved to be an invaluable technique for their purification and analysis. Precolumn extraction coupled to reversed-phase-HPLC is a rapid and sensitive method for the quantitative analysis of these compounds. In the present study, we have developed improved methods for the analysis of complex mixtures of eicosanoids by RP-HPLC using binary gradients containing trifluoroacetic acid (TFA), which allows considerable manipulation of the retention times of the cysteinyl-LTs relative to those of other eicosanoids.

3.1.1. Conditions for the Precolumn Extraction of Eicosanoids.

Excellent recoveries of a variety of lipoxygenase products, including 20h-LTB_4, LTB_4, LTC_4, and 15-HETE, can be obtained when biological samples are loaded onto a precolumn at neutral pH in medium containing between 15 and 20% methanol (326). However, in the present study we observed that repeated analysis of biological samples extracted in this manner sometimes led to deteriorations in peak shape and selectivity, possibly due to the presence of proteins in the samples. We found that this problem could be alleviated by raising the concentration of methanol in the sample to 30% and freezing at -80°C, followed by removal of precipitated material by centrifugation.

TABLE 4

Recoveries of eicosanoids after precolumn extraction with 30% methanol*

Eicosanoids	Recovery (%)	
	Neutral pH (7.4)	Acidic pH (2.8)
20h-LTB_4	94±2	86±9
LXA_4	95±12	77±4
LTB_4	91±2	102±3
LTC_4	101±13	105±7

*The precolumn was first washed with 30% methanol in water containing 2.5 mM H_3PO_4 (15 ml). Standards were then loaded onto the precolumn in 30% methanol at either neutral (7.4) or acidic (2.8) pH in a volume of 4 ml and the precolumn was then washed with a further 18 ml of 2.5 mM H_3PO_4 in 30% methanol. Recoveries were calculated by comparing the peak areas of standards with those obtained from an identical sample which had been injected directly via a conventional loop injector. All values are means ± SD of triplicate determinations.

Since it was possible that increasing the concentration of methanol in the loading medium could result in reduced retention of the more polar eicosanoids by the precolumn, we evaluated the recoveries of 20-hydroxy-LTB_4, LXA_4 LTB_4 and LTC_4 using these conditions (Table 4). A mixture containing the above compounds was injected onto an HPLC column using either a conventional injector with a sample loop (sample volume, 50 µl) or via a precolumn connected to an automatic injector (sample in 4 ml of 30% methanol at neutral or acidic pH). In the latter case, the precolumn was first equilibrated with 30% methanol containing 2.5 mM H_3PO_4. The components were separated by RP-HPLC on a 3.2 x 250 mm Spherisorb ODS-2 column as described below. Recoveries for the precolumn extraction procedure were calculated by comparison of the peak areas of standards obtained after precolumn extraction with those obtained by direct injection using a conventional injector with a sample loop. Since only 90% of the sample was injected with the WISP, the results obtained with precolumn extraction procedure were corrected for the volumes injected. As shown in Table 4, the recoveries of all of the standards investigated were excellent irrespective of whether the sample was loaded onto the precolumn at neutral or acidic pH.

3.1.2. Analysis of eicosanoids on a 4.6 mm ID Spherisorb ODS-2 column.

We developed two methods for the analysis of lipoxygenase products using a 4.6 x 250 mm Spherisorb ODS-2 column. The first was designed primarily for the analysis of cysteinyl-LTs and some of the less polar eicosanoids, whereas the second could be used for a wide range of lipoxygenase products.

The first of the above methods utilizes a mobile phase consisting of a linear gradient between solvents A (methanol/water (30:70)) and B (acetonitrile/water/TFA (80:20:0.005)) as follows: 0 min, 60% B; 30 min, 100% B with a flow rate of 1.5 ml/min. The separation of a mixture of standards (15-25 ng of each), first loaded onto a precolumn as described above, is shown in Fig. 8A. Using the above conditions, there was baseline separation for all of the standards injected (LTB_4, LTC_4, LTD_4 and LTE_4, PGB_2, 5-HETE, 12-HETE, 15-HETE and 12-HHTrE). Raising the concentration of TFA reduced the retention times of cysteinyl-LTs but did not affect those of other eicosanoids (cf. Ref. 326). For example, with 0.01% TFA in solvent B, all of the above standards can be separated within 20 minutes. The separation of uv-absorbing eicosanoids produced when a mixture of rat lung cells was incubated with A23187 (1 μM) for 45 min is shown in Fig. 8B. The major eicosanoid products detected were LTB_4, LTC_4, 12-HHTrE, 5-HETE and 12-HETE, all of which were very well resolved from one another.

Although the conditions described above give excellent resolution of cysteinyl-LTs and HETEs, the separation of a number of polar eicosanoids is not very good because of their short retention times. For example, the 6-trans isomers of LTB_4 are not well resolved from LTB_4, and lipoxins and ω-oxidation products of LTB_4 are not well separated. To analyze these compounds, methanol can be added to solvent B and the concentration of acetonitrile can be reduced to 55% to give a mobile phase consisting of a linear gradient between solvents A and C (water/methanol/acetonitrile/TFA, 20:25:55:0.009) as follows: 0 min, 50% C; 40 min, 100% C. These conditions can be used to analyze mixtures of eicosanoids containing LTB_4 and its isomers and metabolites, lipoxins, cysteinyl-LTs,

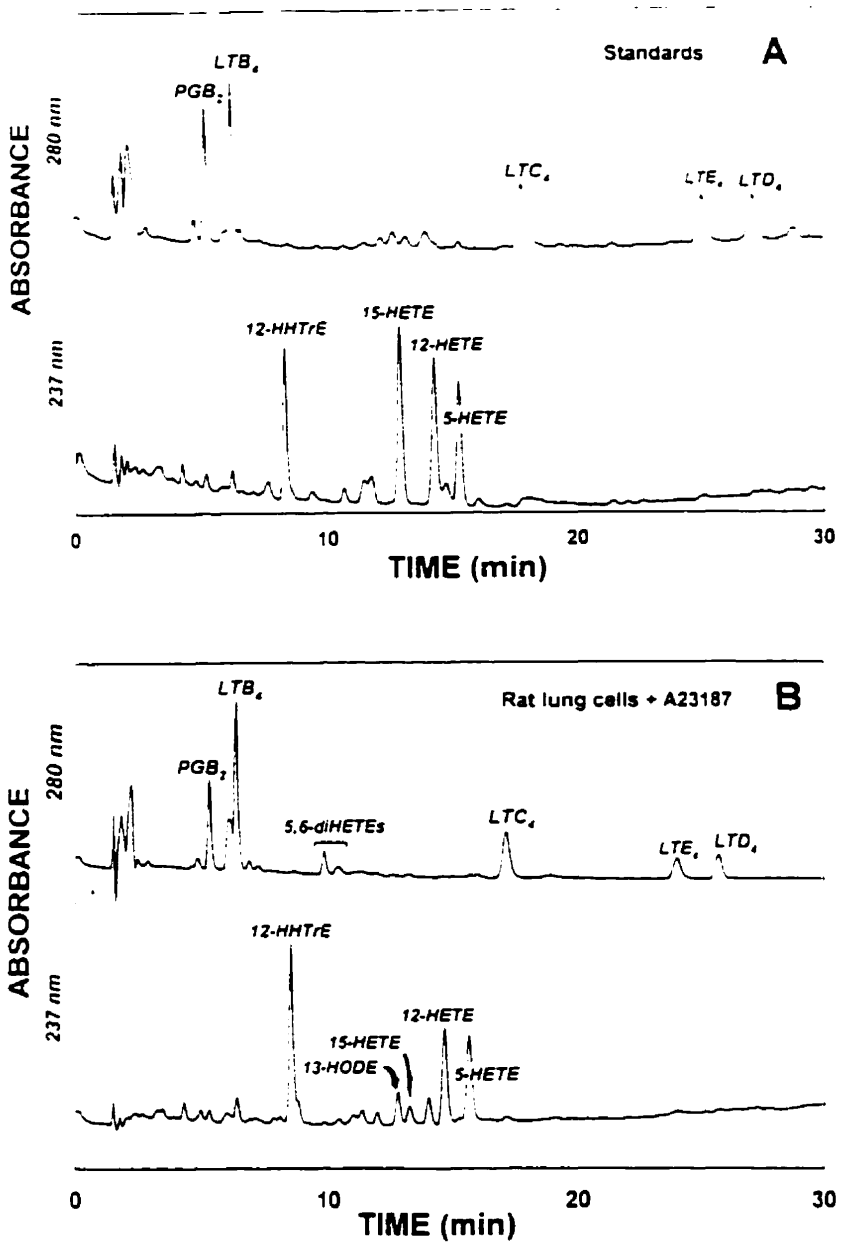

Figure 8. Reversed-phase high pressure liquid chromatograms of standards (A) and products formed by isolated rat lung cells (B). The lung cells (4 ml; 5 x 10^6 cells/ml) were incubated with A23187 (1 μM) for 45 min at 37 °C. The reaction was terminated by addition of 1.7 ml methanol (final concentration: 30% methanol). PGB_2 (25 ng) was added as an internal standard. The standards and samples were directly injected for analysis by precolumn extraction/RP-HPLC as described in Materials and Methods. The stationary phase was a 4.6 mm ID Spherisorb ODS-2 column (4.6 x 250 mm), whereas the mobile phase consisted of a linear gradient between solvents A (water/methanol, (70:30)) and B (water/acetonitrile/TFA, (20:80:0.005)) as follows: 0 min, 60% B; 30 min, 100% B. The flow rate was 1.5 ml/min. The uv spectra of the column eluate was continuously recorded between 220 and 330 nm using a Waters photodiode array detector. The eicosanoids were identified by their retention times and specific uv spectra.

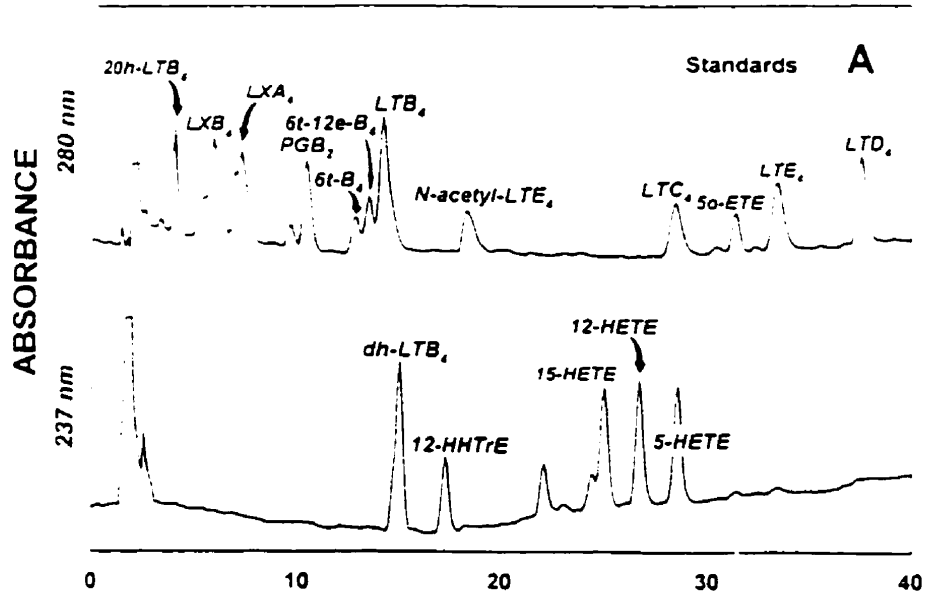

Fig. 9. RP-HPLC of standards (A) and eicosanoids formed by isolated rat lung cells (B) or rat pleural PMNL (C). The isolated lung cells (4 ml; 5 x 10^6 cells/ml) were incubated with A23187 (1 μM) for 15 min, whereas the pleural PMNL (1 ml; 30 x 10^6 cells/ml) were incubated with arachidonic acid (10 μM) and A23187 (5 μM) for 30 min at 37 °C. The eicosanoids were analyzed by precolumn extraction/RP-HPLC on a 4.6 mm ID Spherisorb ODS-2 column (4.6 x 250 mm) with a linear gradient between solvents A (water/methanol, (70:30)) and C (water/methanol/acetonitrile/TFA, (20:25:55:0.009)) as follows: 0 min, 50% B; 40 min, 100% B. The flow rate was 1.5 ml/min. This slightly modified mobile phase could be used to separate LTB_4 isomers and metabolites, LXA_4, LXB_4 and 5-oxo-ETE as well as the other eicosanoids shown in Fig. 8.

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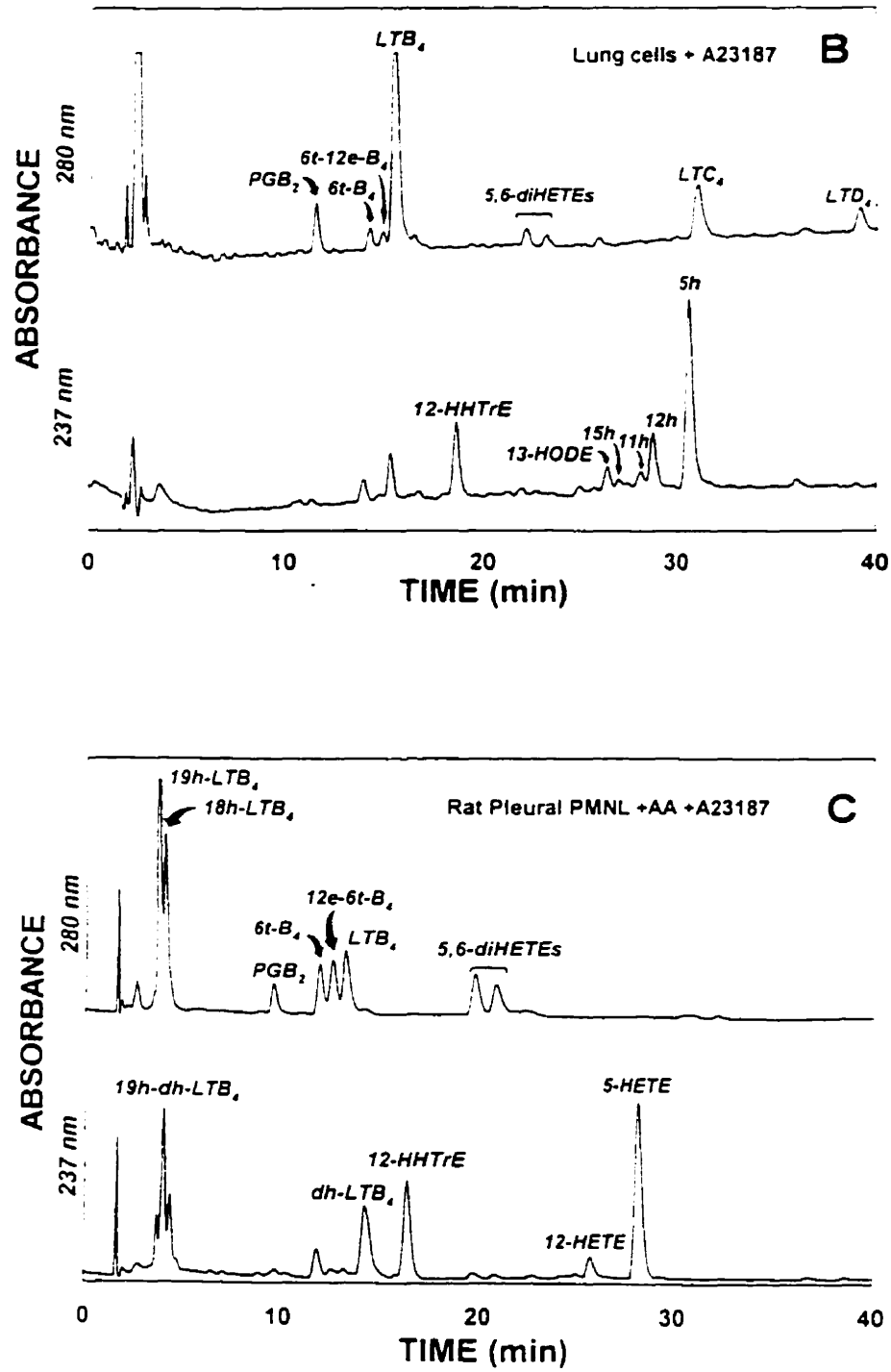
B
Lung cells + A23187
LTB4
6t-12e-B4
PGB2
6t-B4
5,6-diHETEs
LTC4
LTD4
5h
12-HHTrE
15h
11h
12h
13-HODE
280 nm
237 nm
ABSORBANCE
0
10
20
30
40
TIME (min)
C
19h-LTB4
18h-LTB4
Rat Pleural PMNL +AA +A23187
12e-6t-B4
6t-B4
LTB4
PGB2
5,6-diHETEs
19h-dh-LTB4
5-HETE
12-HHTrE
dh-LTB4
12-HETE
280 nm
237 nm
ABSORBANCE
0
10
20
30
40
TIME (min)

Fig. 9. B and C

5-oxo-ETE, 12-HHTrE and HETEs (Fig. 9A). The separation of eicosanoids formed by incubation of rat lung cells with A23187 (1 μM) for 15 min is shown in Fig. 9B. The profile of eicosanoids formed under these conditions is similar to that shown in Fig. 8B, except that the retention times are longer and LTC_4 has a retention time similar to that of 5-HETE. Lung cells did not synthesize appreciable amounts of lipoxins, 20-hydroxy-LTB_4 or 5-oxo-ETE in response to calcium ionophore. Since LTE_4 is formed from LTD_4, only very small amounts are present after incubation of lung cells with A23187 for 15 min (Fig. 9B) compared to 45 min (Fig. 8B).

Figure 9C shows a chromatogram of the products obtained after incubation of carrageenin-elicited rat neutrophils with A23187 (5 μM) and arachidonic acid (10 μM) for 30 min. These cells did not synthesize detectable amounts of cysteinyl-LTs, but extensively metabolized LTB_4 by reduction of the 10,11-double bond to give 10,11-dihydro-LTB_4 (dh-LTB_4) and by ω-oxidation (52). Unlike human neutrophils, which metabolize LTB_4 via a 20-hydroxylase, rat neutrophils convert LTB_4 and 10,11-dihydro-LTB_4 to 18, and 19-hydroxy metabolites (52).

3.1.3. Analysis of eicosanoids by HPLC on a 3.2 mm ID Spherisorb ODS-2 Column

When the diameter of an HPLC column is reduced, the flow rate can be lowered, resulting in a reduction in the volume in which solutes are eluted from the column. This both increases the sensitivity and has the economic benefit of reduced solvent consumption. We therefore investigated various conditions for the analysis of eicosanoids using a column with an ID of 3.2 mm which has one-half the cross sectional area of the

column described in the Section above (Section 3.1.2.). By slightly modifying solvents A and C and raising the concentration of TFA in the mobile phase, excellent separation of all of the eicosanoids described above was obtained using a 3.2 x 250 mm column of Spherisorb ODS-2. As shown in Fig. 10, complete separation of LTB_4, cysteinyl-LTs, HETEs, and 5-oxo-ETE was obtained using a linear gradient between solvents D (water/methanol/TFA (70:30:0.04)) and E (water/methanol/acetonitrile/TFA (20:20:60:0.05)) as follows: 0 min, 75% E; 20 min, 95%E. The flow rate was 0.75 ml/min. The retention times of the cysteinyl-LTs relative to those of other eicosanoids are shorter with the 3.2 mm ID column compared to the 4.6 mm ID column, since they elute between 12-HHTrE and 15-HETE. In contrast, all of the cysteinyl-LTs elute after 15-HETE with the 4.6 mm ID column (Figs. 8 and 9). It should be noted that LTE_4 has a longer t_R than LTD_4 with the 3.2 mm ID column in contrast to the chromatograms shown in Figs. 8 and 9 in which case the reverse is true.

The advantage of the conditions described above is that the analysis is very rapid, the time required being only 20 min. If it is necessary to separate a more complex mixture of lipoxygenase products, a weaker mobile phase can be used, consisting of a linear gradient between solvents D and F (water/methanol/acetonitrile/TFA (25:20:55:0.04)) as follows 0 min, 50% F; 40 min, 100% F. With a flow rate of 0.75 ml/min mixtures containing almost all of the major lipoxygenase products can be resolved in 40 min (Fig. 11A). Thus excellent separation of LTB_4, its 6-trans isomers, 20-hydroxy-LTB_4, cysteinyl-LTs, HETEs, 5-oxo-ETE, and 13-HODE was obtained. Analysis of eicosanoids released from lung cells incubated with A23187 (1 μM) for 15 min using the above conditions is

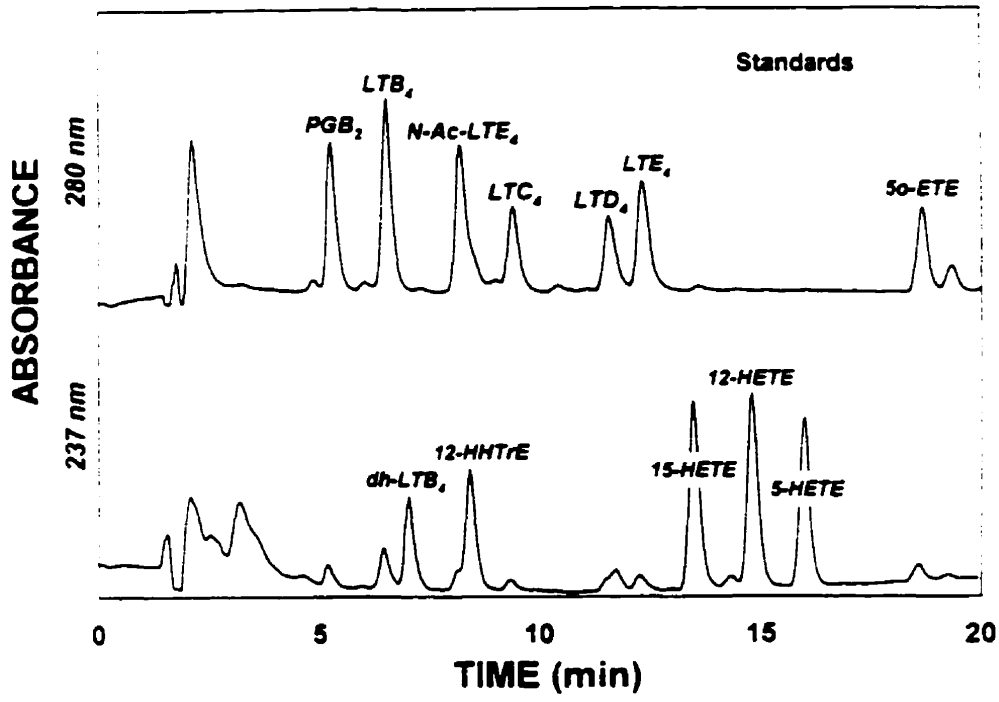

Fig. 10. RP-HPLC analysis of a mixture of standards on a midbore Spherisorb ODS-2 column (3.2 x 250 mm). The mobile phase consisted of a linear gradient between solvents D (water/methanol/TFA (70:30:0.04)) and E (water/acetonitrile/methanol/TFA (20:60:20:0.05)) as follows: 0 min, 75% E; 20 min, 95% E. The flow rate was 0.75 ml/min.

shown in Fig. 11B. This chromatogram closely resembles that obtained for a similar sample analyzed using the 4.6 mm-diameter column (Fig. 9B) except that the retention times of the cysteinyl-LTs are considerably shorter. The HPLC profile of eicosanoids synthesized by mixed human leukocytes (1ml, 30 × 10^6 cells/ml) containing platelets (60 × 10^6/ml) stimulated with 10 μM arachidonic acid and 5 μM A23187 is shown in Fig. 11C. This mixture of cells converts arachidonic acid to a large variety of products including LTB_4, its 6-trans isomers, 20-hydroxy-LTB_4, cysteinyl-LTs, HETEs, 5-oxo-ETE, and 13-HODE, due in part to transcellular metabolism. For example, platelets can convert neutrophil-derived LTA_4 and 5-HETE to LTC_4 (108,111) and 5*S*,12*S*-diHETE (74), respectively, since they contain both LTC_4 synthase and 12-lipoxygenase.

The system described above is also very useful for the analysis eicosanoids in other biological samples. For example, rat peritoneal cells consist mainly of macrophages, eosinophils and mast cells and each of these cell types is capable of producing eicosanoids. The profile of arachidonic acid metabolites synthesized by these cells in response to stimulation with 1 μM A23187 is shown in Fig. 12. These cells not only synthesized significant amounts of LTB_4, cysteinyl-LTs, 5-HETE, 13-HODE, and 12-HHTrE, but also produced appreciable amounts of 12-HETE, 6-trans isomers of LTB_4 and 5,6-diHETEs.

3.1.4. Regeneration of Column after Sample Analysis

A problem which may be encountered in the analysis of cysteinyl-LTs by HPLC is that the reproducibility deteriorates with column use, making accurate and sensitive

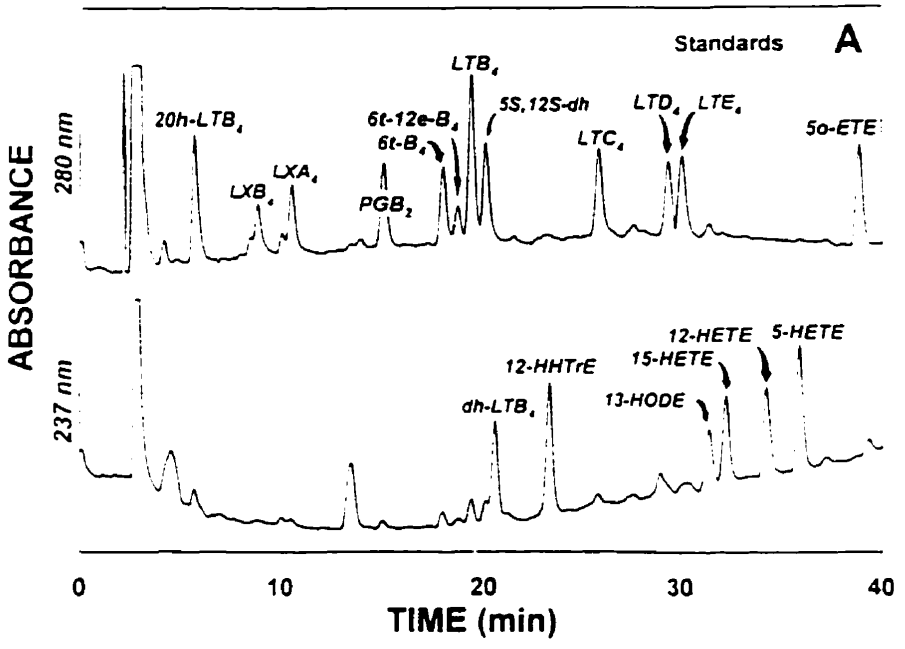

Fig. 11. RP-HPLC analysis of standards (A) and eicosanoids formed by isolated rat lung cells (B) or a mixture of human leukocytes and platelets (C). The isolated lung cells (2 ml, 5 x 10^6 cells/ml) were incubated with 1 μM A23187 for 15 min, whereas the mixed human leukocytes (1 ml; 30 x 10^6 cells/ml) containing 60 x 10^6 platelets were incubated with arachidonic acid (10 μM) and A23187 (5 μM) for 30 min at 37 °C. The products were analyzed on a midbore Spherisorb column (3.2 x 250 mm). The mobile phase consisted of a linear gradient between solvents D (water/methanol/TFA, (70:30:0.04)) and F (water/methanol/acetonitrile/TFA, (25:20:55:0.04)) as follows: 0 min, 50% F; 40 min, 100% F. The flow rate was 0.75 ml/min.

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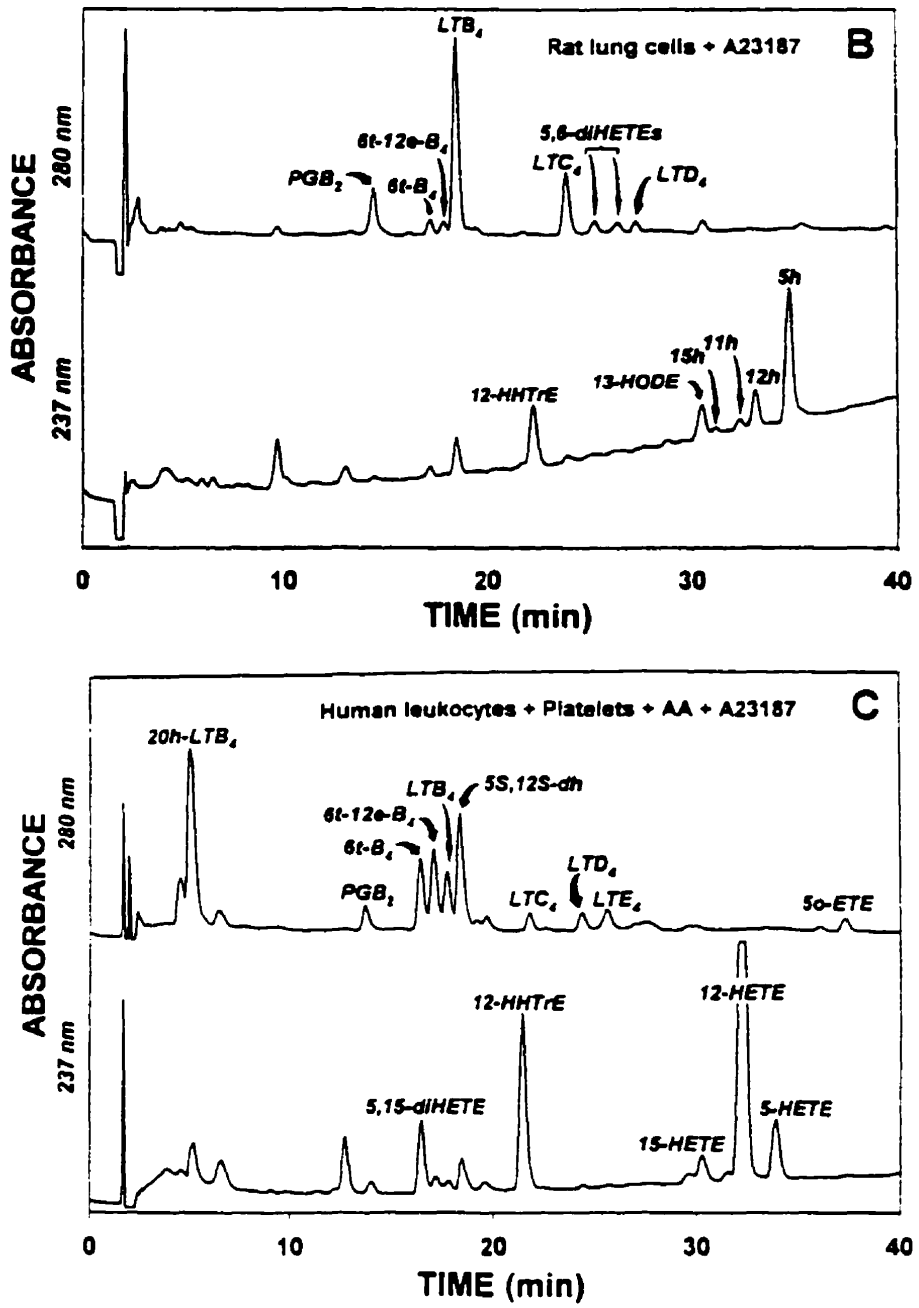
B
Rat lung cells + A23187
LTB4
6t-12e-B4
PGB2
6t-B4
5,6-diHETEs
LTC4
LTD4
5h
11h
15h
12h
13-HODE
12-HHTrE
280 nm
237 nm
ABSORBANCE
0
10
20
30
40
TIME (min)
C
Human leukocytes + Platelets + AA + A23187
20h-LTB4
LTB4
5S,12S-dh
6t-12e-B4
6t-B4
PGB1
LTC4
LTD4
LTE4
5o-ETE
12-HHTrE
12-HETE
5,15-diHETE
15-HETE
5-HETE
280 nm
237 nm
ABSORBANCE
0
10
20
30
40
TIME (min)

Fig. 11B & C.

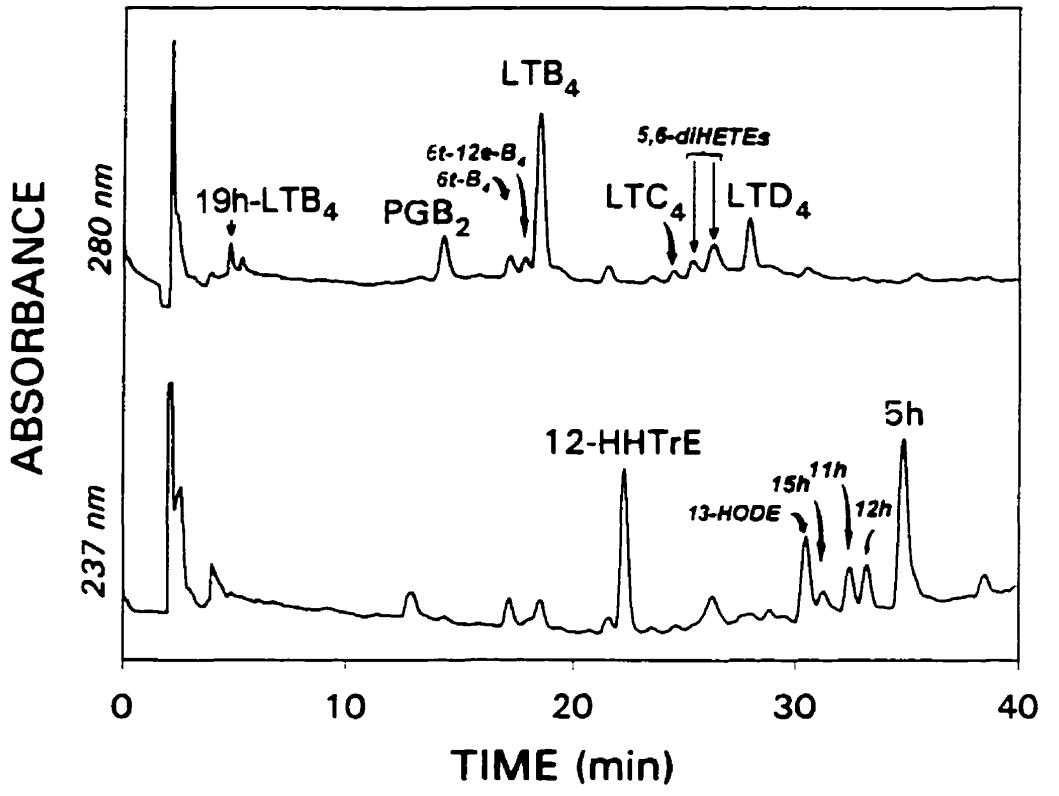

Fig. 12. RP-HPLC of eicosanoids formed by rat peritoneal cells. A mixture of peritoneal cells (1 ml, 5 x 10^6 cells/ml) consisting of macrophages (77%), mast cells (6%) and eosinophils (14%) were incubated with 1 μM A23187 for 20 min. The eicosanoids were analyzed as described in Figure 11.

measurements of these compounds difficult. We observed that with mobile phases containing low concentrations of TFA such as those used with the 4.6 mm ID column (solvents B and C), the retention times of cysteinyl-LTs gradually decreased with successive column use, up to the point where LTC_4, LTD_4 and LTE_4 were no longer resolved from one another. One possible explanation for this is that materials such as peptides and proteins could accumulate on the column because they may require a higher concentration of TFA to be completely eluted. Concentrations of TFA higher than those used in the present study are widely used for the separation of peptides by reversed-phase HPLC (342). Thus, washing the HPLC column with a mobile phase containing a higher concentration of TFA seemed like a potentially useful approach to regenerate columns used for the analysis of cysteinyl-LTs. We found that purging the column with acetonitrile/water/TFA (80/20/0.1) for 5 min after each chromatography dramatically improved reproducibility in the analysis of cysteinyl-LTs. Even if this treatment is not used routinely, it can be used to regenerate columns which have lost their capacity to resolve these compounds. In contrast, deteriorating resolution of cysteinyl-LTs was not a serious problem for analyses using the 3.2 mm ID column, presumably because the concentration of TFA in the mobile phases employed was higher (0.04% in solvents D and F and 0.05% in solvent E; see Table 3, P.60). However, the resolution of some of the other eicosanoids deteriorated with increasing column use. We found that this problem could be alleviated by treating the column with methanol/water (50:50) at a flow rate of 0.75 ml/min for 5 min after each chromatography.

3.2. CHARACTERIZATION OF EICOSANOID SYNTHESIS BY LUNG TISSUES AND ISOLATED LUNG CELLS FROM RATS

Using the analytical methods described in the previous section, we determined the amounts of leukotrienes and other lipoxygenase products synthesized by tissue fragments and isolated cells from rat lungs. The HPLC conditions described in Figure 8 were used for our preliminary studies. This method gave baseline separation within 30 min of all of the eicosanoids of interest, including LTB_4, LTC_4, LTD_4, LTE_4, the monohydroxy eicosanoids 15-HETE, 12-HETE, and 5-HETE, as well as the cyclooxygenase product 12-HHTrE. It is quite sensitive and can be used to measure nanogram levels of leukotrienes and other lipoxygenase products.

3.2.1. Eicosanoid synthesis by lung fragments

In a pilot study, we determined the amounts of lipoxygenase products synthesized by lung fragments from Sprague-Dawley rats. Stimulation of SD rat lung fragments with A23187 (10 μM) induced the release of LTB_4, cysteinyl-LTs, HETEs and 12-HHTrE. The weight of lung tissue per rat was 2.7 ± 0.5 g. The amount of LTB_4 formed per gram tissue was about 1.5 times greater than the total amount of cysteinyl-LTs formed (i.e., 7.2 ± 0.4 ng/200 mg lung tissue *vs* 4.5 ± 1.0; n=5) (Fig. 13). Lung fragments produced much more 12-HETE than 5-HETE, 15-HETE or 12-HHTrE, possibly due to the synthesis of this compound by alveolar macrophages (cf. Fig 28, below). Lower concentrations of A23187 (2.5-5 μM) elicited the release of similar amounts of the above eicosanoids. However, concentrations of 1 μM or lower of A23187 were not sufficient to stimulate the

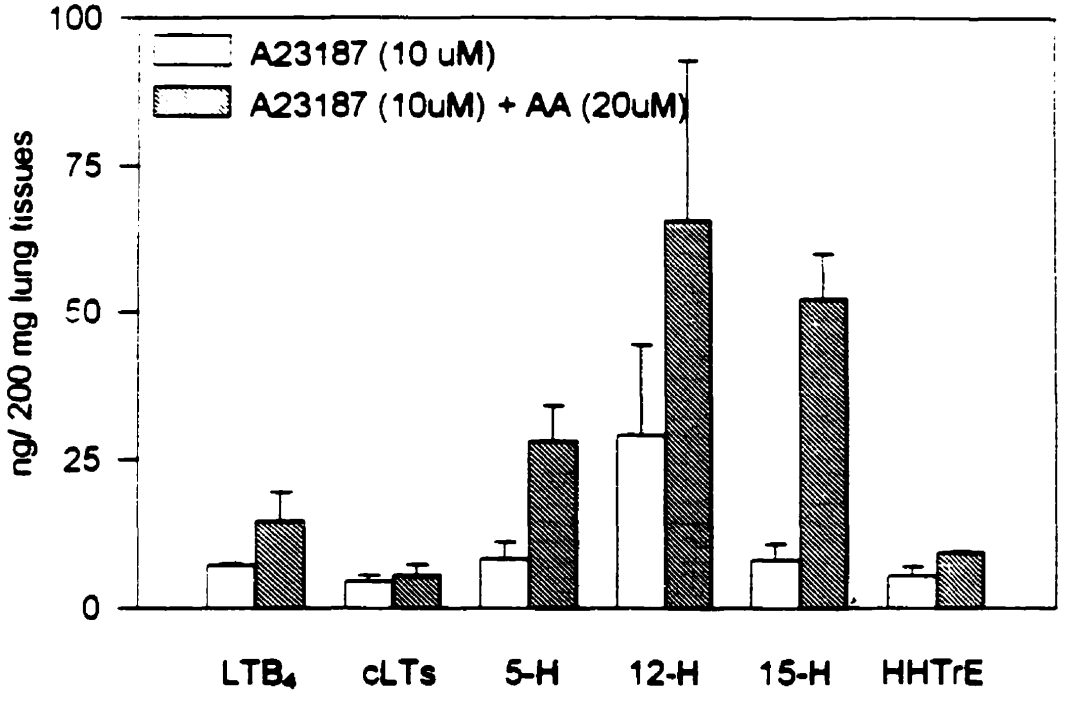

Fig. 13. Eicosanoid formation by lung fragments. SD rat lung fragments (200 mg/ml) were incubated with either A23187 (10 μM) or A23187 (10 μM) and exogenous arachidonic acid (20 μM) at 37 °C for 30 min. Eicosanoids were analyzed by RP-HPLC as described in Fig. 8. Each bar represents the mean ± SEM (n=5).

production of eicosanoids by lung fragments (data not shown). When the tissue was incubated in buffer with vehicle (DMSO) alone, only trace amounts of 12-HHTrE and none of the lipoxygenase products were detected.

Addition of exogenous arachidonic acid (20 μM) potentiated ionophore-induced formation of LTB_4, HETEs and 12-HHTrE by lung fragments, but had no significant effects on ionophore induced cysteinyl-LT formation (Fig. 14). The differential effects of exogenously added substrate on LTB_4 and cysteinyl-LT formation may suggest either limited LTC_4 synthase activity or an abundance of LTA_4 hydrolase activity in the lung. AA alone did not induce the production of LTs by lung tissue (data not shown).

Lung fragments from Brown Norway rats produced the same profiles of eicosanoids in response to calcium inophore stimulation (data not shown). There were no significant differences in the amounts of eicosanoids formed by lung fragments between Sprague-Dawley and Brown Norway rats.

3.2.2. Eicosanoid synthesis by isolated lung cells

To analyze leukotriene synthesis by lung cells, we adapted an enzymatic digestion technique from Pele et al (331) to disperse and prepare a suspension of isolated lung cells. The number of cells recovered after enzymatic digestion and mechanical dispersion was between 100×10^6 and 170×10^6 cells per rat. Viability of the cells, as asseesed by their ability to exclude trypan blue, was usually over 80%. The cells were highly heterogeneous in apperance and size. The cell suspensions consisted of approximately 15-20% macrophages, 2-5% eosinophils, 1-3% neutrophils, and 0.5% mast cells. Other cell types

which were present, but were not quantitated include epithelial cells, endothelial cells, lymphocytes and occasionally red blood cells.

Compared with lung tissue fragments, we found that lower concentrations of calcium ionophore are required for stimulation of eicosanoid production by lung cell suspensions. Incubation of isolated mixtures of lung cells from BN rats with A23187 (1 μM) at 37 °C for 15 min induced significant formation of all of the major lipoxygenase products (Fig. 14). Neither higher concentrations of calcium ionophore (e.g. 5 μM) nor longer incubation times induced increased formation of leukotrienes and other eicosanoids. However, longer incubation times usually resulted in an increase in the ratio of LTD_4 and LTE_4 to LTC_4 due to increased conversion of LTC_4 to these metabolites. Similar to lung fragments, isolated lung cells only produced small amounts of 12-HHTrE when incubated in the presence of the vehicle (DMSO) alone (data not shown). Incubation of lung cells with exogenous AA (10-50 μM) alone or PAF (1 μM) did not result in the formation of detectable amounts of cysteinyl-LTs (data not shown).

The major lipoxygenase products detected after incubation of A23187 with lung fragments or isolated lung cells from BN rats were LTB_4, cysteinyl-LTs, 5-HETE and 12-HETE. Although there were no qualitative differences in the profile of eicosanoids formed by lung cells and fragments, the latter appeared to produce relatively more 12-HETE and 15-HETE than isolated lung cells. Since alveolar macrophages and epithelial cells are major sources of 12-HETE and 15-HETE in the lung (343-345), the localization of these cells on the surface of airways may have facilitated their interaction with A23187 when fragments were incubated with this agent.

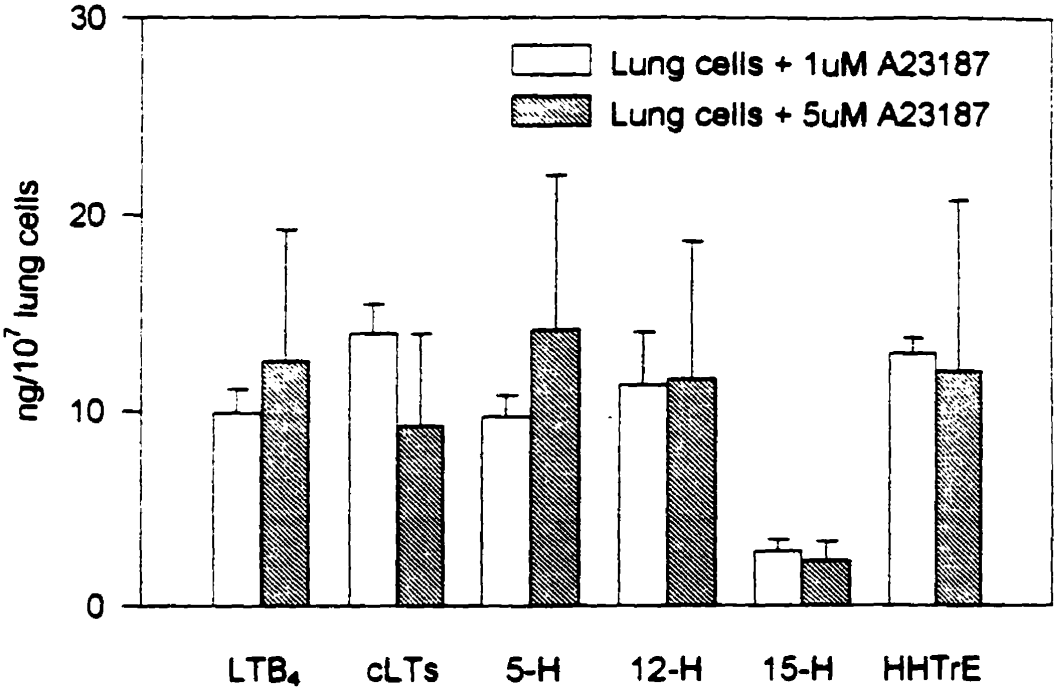

Fig. 14. Eicosanoid formation by BN-rat lung cells. Isolated lung cells (4 ml, 5 x 10^6 /ml) were incubated with either 1 or 5 μM of A23187 at 37 °C for 15 min. The metabolites were analyzed by RP-HPLC as described in the legend to Fig. 8. Each bar represents the mean ± SEM (n=6).

3.2.3. Eicosanoid synthesis by lung cell fractions obtained by centrifugal elutriation

Since mast cells have been reported to be a major source of cysteinyl-LTs in human lung (337), it should be of great interest to investigate the contribution of mast cells to cysteinyl-LT formation in rat lungs. One of our initial objectives was to identify the cells responsible for the formation of cysteinyl-LTs in this tissue. In pursuit of this goal, we attempted to isolate lung mast cells by centrifugal elutriation and density gradient centrifugation.

In spite of great efforts, we were unable to purify mast cells from rat lungs, mainly because in the rat mast cells accounted for only about 0.5% of the cells in the isolated suspension of lung cells. In contrast, human lung tissue contains about 3-5% mast cells. Therefore, we were only able to obtain lung cell fractions enriched with mast cells by only a few fold by using centrifugal elutriation. As shown in Fig. 15, Fraction 4 was enriched with mast cells and produced more leukotrienes and 12-HHTrE than crude lung cells. Since the numbers of other types of lung cells in this fraction were not determined in this preliminary studies, other lung cells in fraction 4 could also account for the increased formation of cysteinyl-LTs and other 5-lipoxygenase products.

We also tried to fractionate the crude lung cells by discontinuous Percoll gradient centrifugation. However, due to the presence of such a large number of different types of cells in the lung, we were unable to selectively purify any specific cell type which appeared to be primarily responsible for leukotriene synthesis in the rat lung using this technique. The synthesis of eicosanoids by purified alveolar macrophages and partially purified pulmonary eosinophils is discussed below in Section 3.5.

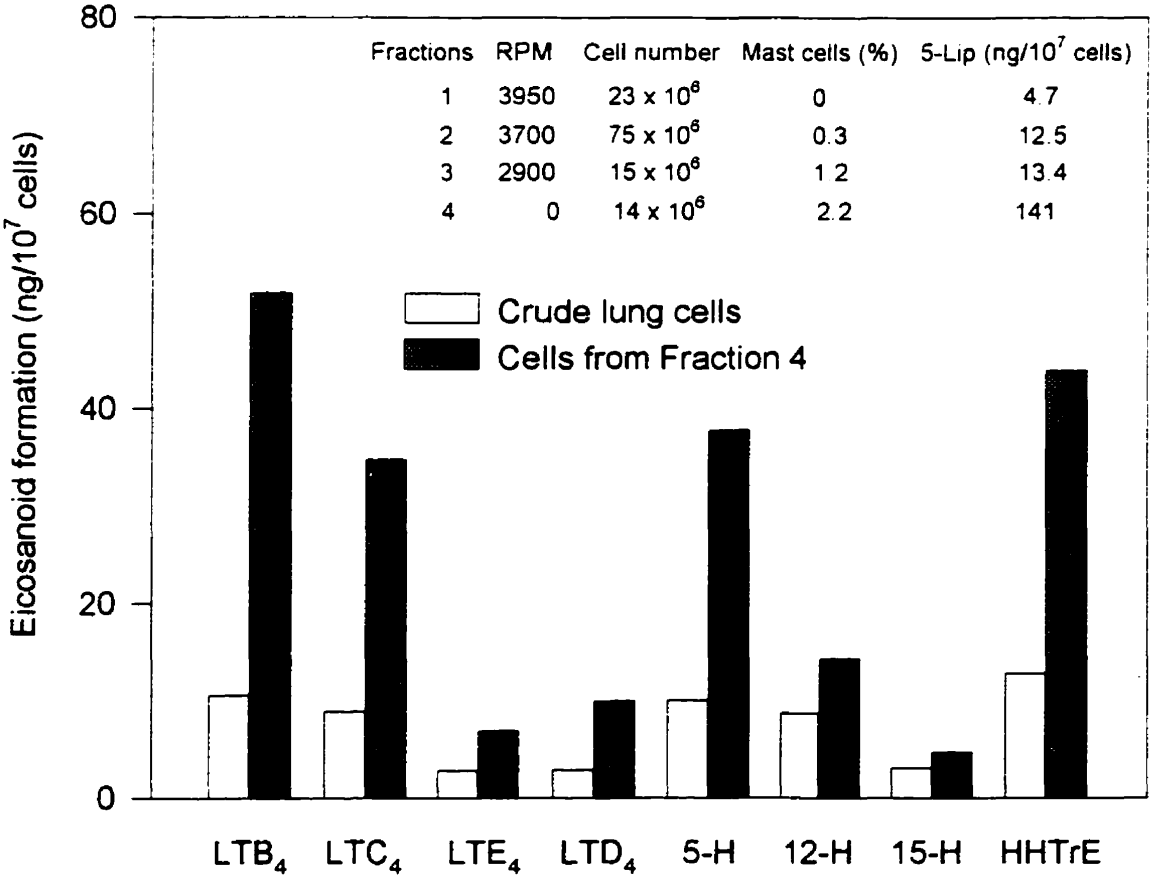

Fig. 15. Eicosanoid synthesis by crude lung cells and a fraction enriched with mast cells. Lung cells from 3 SD rats were pooled together and subjected to cell elutriation as described in Materials and Methods. Fraction 4 (2.2% mast cells) contained about 4 fold more mast cells than the crude lung cell suspension (0.5% mast cells). The cells were incubated with A23187 (1 μM) at 37°C for 45 min and eicosanoids analyzed by RP-HPLC as described in Fig. 8. The amounts of 5-lipoxygenase products (including LTB_4, cLTs and 5-HETE) and the cyclooxygenase product 12-HHTrE formed by fraction 4 appeared to be greater than those formed by crude lung cells. The data is representative of five independent experiments.

3.3. *AIRWAY RESPONSES, CELLULAR INFILTRATION, AND EICOSANOID SYNTHESIS BY LUNG CELLS FOLLOWING ALLERGEN CHALLENGE*

In order to understand the mechanisms of altered leukotriene synthesis by asthmatic lung after antigen challenge, we studied the effects of antigen challenge on the infiltration of inflammatory cells and eicosanoid synthesis by lung cells in BN rats. In preliminary studies, we examined the airway resistance, cell recovery after enzymatic digestion and eicosanoid synthesis by lung cells at various times after antigen challenge. Based on the findings of these preliminary studies, we further investigated the relationship between cellular infiltration in the lung and eicosanoid synthesis by lung cells before challenge, and 6 or 24 h after challenge with either OVA or vehicle (saline).

3.3.1. Airway responses to ovalbumin challenge

In a preliminary experiment, airway resistance was monitored in three groups of BN rats until the animals were sacrificed at 4, 6, and 8h after OVA challenge. A additional group of control rats were challenged with saline and the airway resistance was monitored for 6 h. Compared with saline challenge, OVA challenge resulted in variable airway responses in all of the three groups (Fig. 16). Four of the five rats in 4h group exhibited early responses and three rats showed late responses commencing about 2h after OVA challenge. Two of the four rats in 6h group exhibited significant early responses and three of these rats had late responses between 2 to 6h after OVA challenge. In the 8h group, all of the four rats exhibited early responses, which occurred as early as 5 min after challenge. Three of these rats also had late responses and the R_L was elevated 4 to

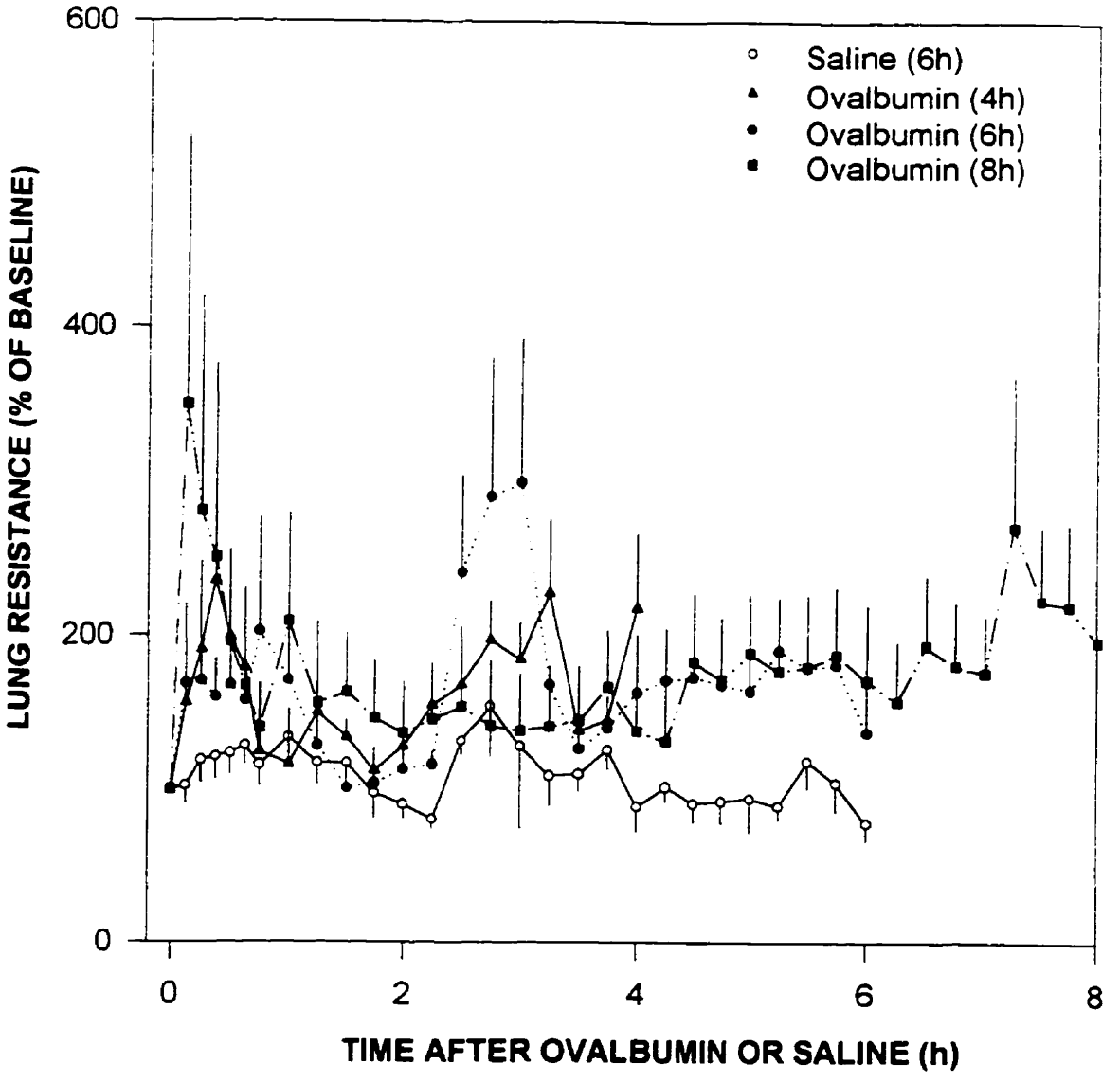

Fig. 16. Changes in lung resistance of rats after challenge with either ovalbumin or saline (n=7). The rats receiving ovalbumin were divided into 4h (n=4), 6h (n=5), and 8h (n=4) groups, depending on the time of sacrifice. All rats were challenged by aerosol and analyzed for changes in lung resistance before, and at 5, 10, and 15 min, and every 15 min thereafter for a total period of up to 8 h. The values for all data points are means ± SEM.

8h after OVA challenge until the animals were sacrificed.

3.3.2. Recovery and viability of lung cells obtained following allergen challenge

In a preliminary study, we determined the cell recovery after enzymatic digestion of lungs taken from rats before and 2, 4, 6, and 24 h after OVA challenge. As shown in Fig 17, about 150 x 10^6 cells were obtained from sensitized rats which had not been challenged. Antigen challenge of sensitized rats did not result in significant changes in cell recovery. However, it appeared that the longer the time after antigen challenge the lower the number of cells recovered. Nevertheless, there was no correlation between cell recovery and early or late phase responses (data not shown).

Consistent with reports by others (318,320,346), our studies showed that OVA sensitization alone did not affect the total numbers of cells recovered from the lung (data not shown).

The continuous presence of a catheter in the airways to enable measurement of lung resistance could have affected the airways and influenced cell recovery. We therefore also examined cell recovery after allergen challenge without monitoring airway resistance. The numbers of lung cells isolated from rats before challenge and 6, or 24 h after OVA or saline challenge are shown in Fig 18. Although there was some variability in cell recovery among individual rats, no significant differences between any of the groups were observed in the total numbers of cells recovered.

The viability of cells recovered from lungs by enzymatic digestion was consistently about 85% and was not significantly different among the different groups

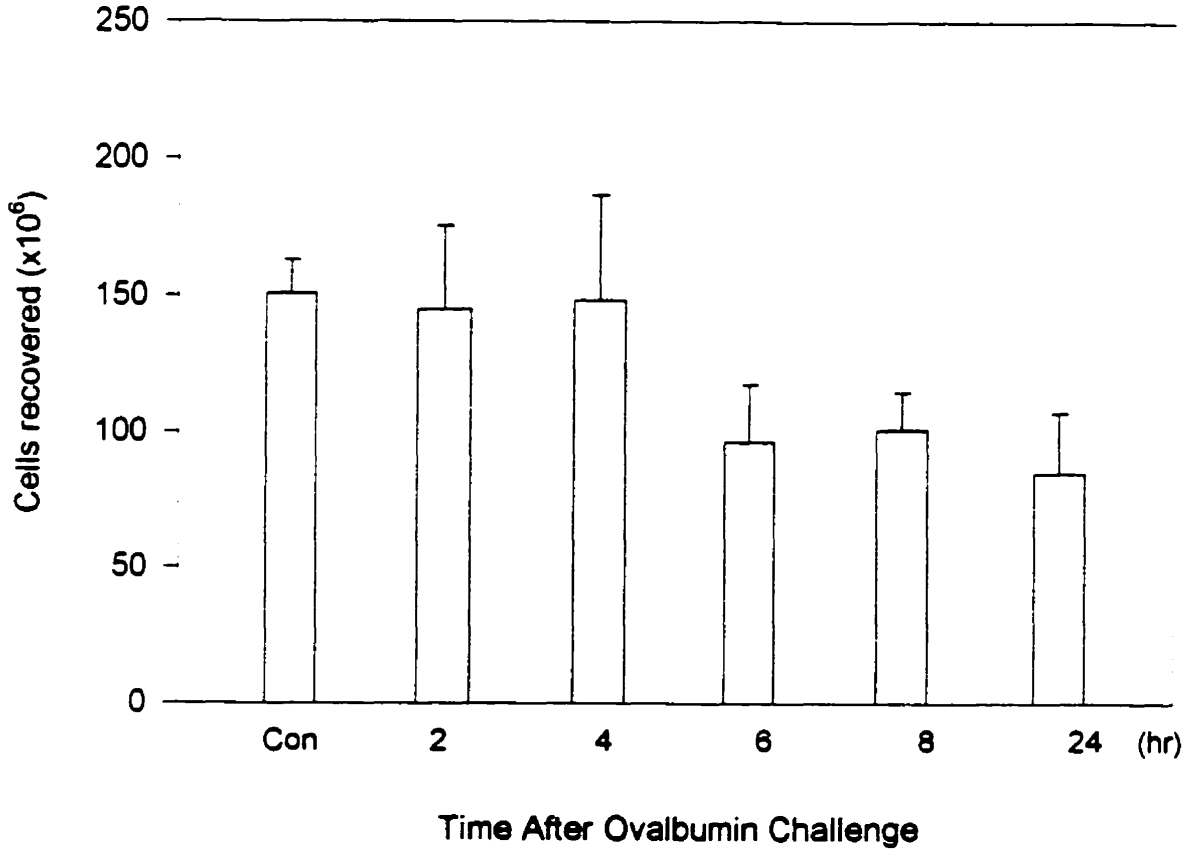

Fig. 17. Numbers of cells recovered after enzymatic digestion of lungs from sensitized BN rats at various times after allergen challenge. Lung cells were obtained by perfusion of lung tissues with 0.006% protease via the trachea for 15 min. The control (CON) group shows the numbers of cells isolated from sensitized rats which had not been challenged. Other time points represent the numbers of cells recovered at various times following ovalbumin challenge. Each point represents the mean ± SEM of data from at least five animals. Airway resistance was monitored in this preliminary study for up to 8 h after antigen challenge.

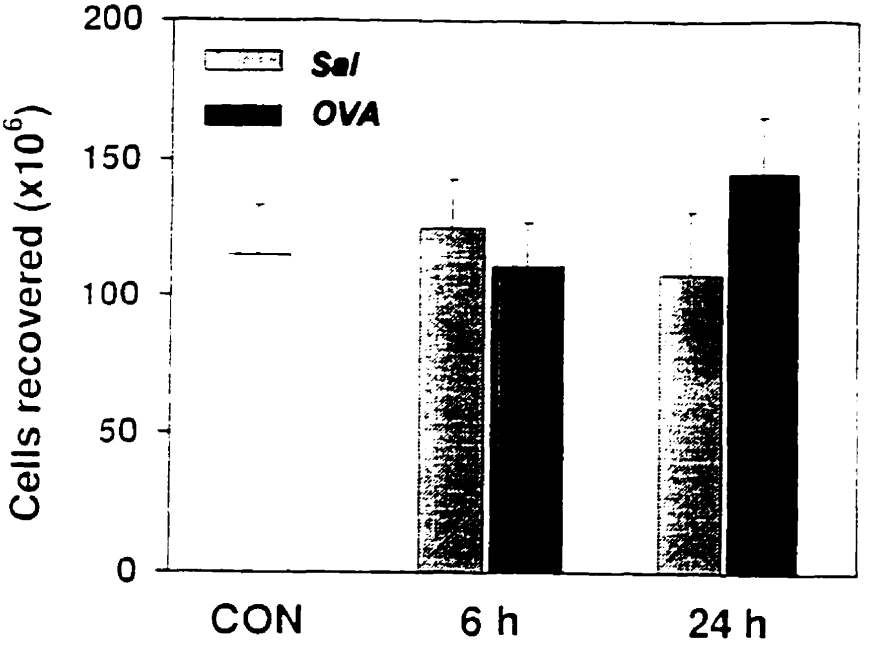

Fig. 18. Numbers of cells recovered after enzymatic digestion of lungs following challenge of sensitized BN rats with either OVA or saline. Lung cells were obtained by perfusion with 0.006% protease via the trachea for 15 min. CON (open bar): the numbers of cells isolated from sensitized rats which had not been challenged; 6 h and 24 h: the numbers of cells recovered from rat lungs at these times following challenge with saline (hatched bars) or OVA (solid bars). Each point represents the mean ± SEM of data from at least five animals

(Table 5). The procedure used for the preparation of lung cells in the present study has been noted to give good results for a variety of different types of lung cells (331).

TABLE 5

Viability of unfractionated lung cells from unchallenged, saline-challenged, and ovalbumin-challenged sensitized BN rats

Group	Viability
Control (unchallenged)	84.3 ± 1.6
Saline-challenged (6 h)	85.6 ± 2.6
Saline-challenged (24 h)	87.6 ± 3.0
OVA-challenged (6 h)	84.5 ± 3.2
OVA-challenged (24 h)	84.8 ± 2.2

3.3.3. Infiltration of inflammatory cells into the lungs following allergen challenge

To delineate the degree of cellular infiltration in the lungs of BN rats following allergen challenge, we analysed the numbers of inflammatory leukocytes in suspensions of crude lung cells isolated from rats before, and 6 or 24 h after OVA or saline challenge. The differential counts of neutrophils, eosinophils and macrophages were determined for each of the rats on cytospin slides using Diff-Quik and non-specific esterase stains. As shown in Fig 19, inhalation of both OVA and saline induced significant increases in the

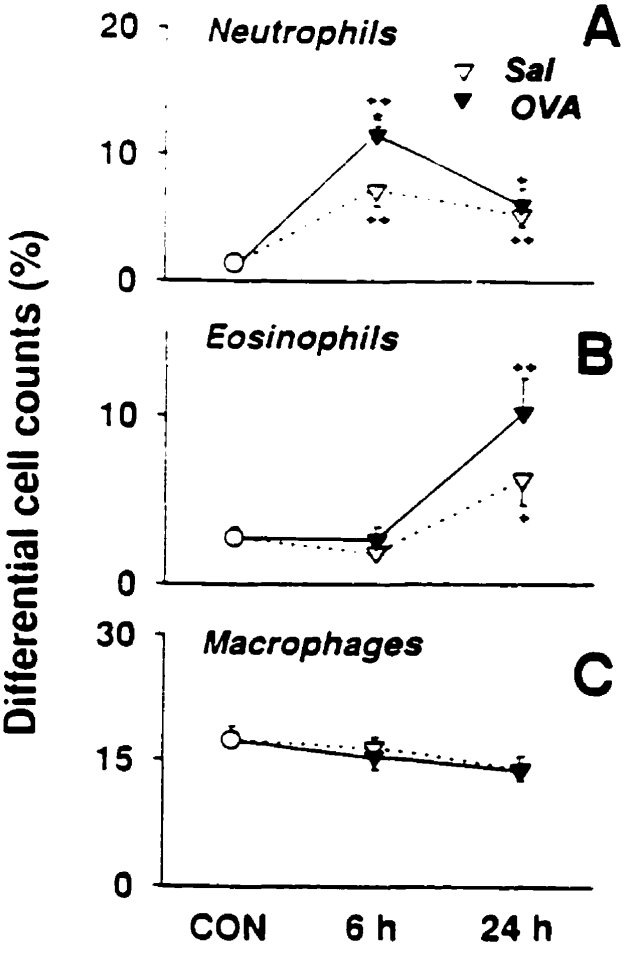

Fig. 19. Recovery of neutrophils (**A**), eosinophils (**B**) and macrophages (**C**) from lungs before (CON; O) and 6 or 24 h after challenge with either saline (∇) or OVA (▾). Differential cell counts are expressed as percentages of total lung cells. Each data point represents the mean ± SEM of determinations on five to seven animals. Statistical significance was evaluated using ANOVA (NCSS, 5.02). *, $p < 0.05$ when compared with saline-challenged groups. +, $p < 0.05$ and ++, $p < 0.001$ when compared with unchallenged controls. Lung resistance was not measured in any of the rats used in this study.

numbers of neutrophils in the lung 6 h after challenge compared with unchallenged controls ($p< 0.001$). The increase in neutrophils induced by OVA (8 times unchallenged controls) was significantly greater than that induced by saline (5 times unchallenged controls). Twenty-four hours after challenge, the neutrophil counts decreased but remained elevated compared with unchallenged controls ($p <0.05$). In contrast, a significant increase in pulmonary eosinophils was seen 24 h after OVA challenge, but not after 6 h. However, since saline also induced a certain degree of eosinophil influx, there was no significant difference in the percentage of eosinophils in the lung tissue between the OVA- and saline- challenged groups after 24 h. The percentage of macrophages tended to decrease after both OVA and saline challenge, presumably due to the increases in the numbers of neutrophils and eosinophils.

3.3.4. Eicosanoid synthesis by lung cells following allergen challenge

To investigate the effects of antigen challenge on eicosanoid synthesis by lung cells, we examined: (i) eicosanoid synthesis by lung cells from unchallenged sensitized BN rats; (ii) eicosanoid synthesis by lung cells at various times following allergen challenge; and (iii) eicosanoid synthesis by lung cells following OVA or saline challenge.

i) Eicosanoid synthesis by lung cells from sensitized BN rats

To determine whether sensitization alone affects eicosanoid synthesis by lung cells, we examined the synthesis of eicosanoids by lung cells isolated from normal control and sensitized BN rats, which had not been challenged with OVA. Lung cell suspensions were

incubated with calcium ionophore at 37 ^{0}C for 45 min. The eicosanoids formed by the cells were analysed by precolumn extraction and RP-HPLC as discussed above. As shown in Table 6, The amounts of leukotrienes and other eicosanoids synthesized by lung cells from sensitized and control BN rats were almost equal to one another, suggesting that OVA sensitization *alone* has no effects on eicosanoid formation by these cells.

TABLE 6

Eicosanoid synthesis by lung cells from normal and OVA-sensitized BN rats*

	LTB_4	cLTs	5-H	12-H	15-H	HHTrE
CON	8.2±0.8	6.4±3.8	12.0±3.7	13.9±1.6	3.7±0.6	6.2±1.0
Sensitization	9.3±1.9	5.2±2.9	10.7±2.7	11.2±3.9	2.4±0.7	8.9±2.4

*Sensitized rats were actively sensitized against OVA while the rats in the control (CON) group were normal rats without any pretreatment. The eicosanoids produced by lung cells are expressed as ng/10^7 cells. All data are means ± SD (n=4).

Sensitized BN rats can be challenged either with aerosolized OVA or by instillation of OVA directly into the airways. To choose an antigen challenge protocol for our study, we randomly divided sensitized BN rats into two groups in a pilot experiment and compared the effects of aerosolization and instillation of antigen on eicosanoid synthesis by isolated lung cells. As shown in Fig 20, there were no significant

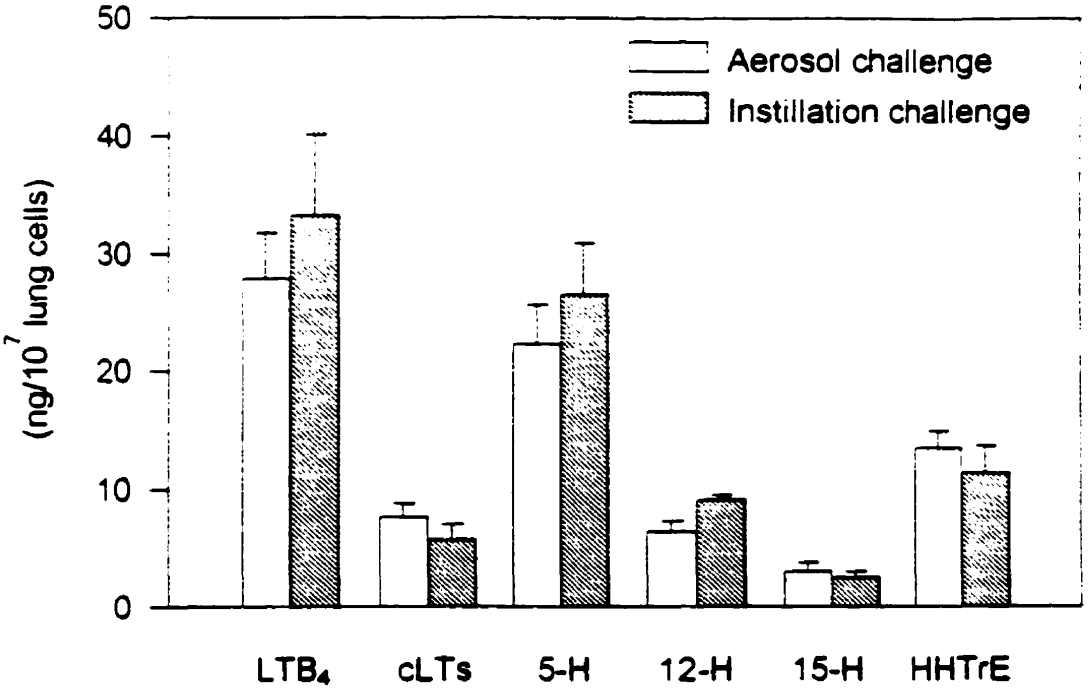

Fig. 20. Eicosanoid synthesis by lung cells from allergen-challenged BN rats. Sensitized BN rats were challenged either with OVA aerosol or by instillation of OVA directly into the airways. The animals were sacrificed 6 h after challenge and lung cells were isolated by enzymatic digestion. The suspensions of crude lung cells (4 ml, 5 x 10^6 /ml) were then incubated with A23187 (1 μM) at 37 °C for 45 min. The eicosanoids released by these cells were analyzed by RP-HPLC as described in Fig. 8. Each bar represents the mean ± SEM (n=5).

differences in eicosanoid synthesis by isolated lung cells between the two groups of rats. Therefore, BN rats were challenged with OVA aerosol for all of the studies described below.

ii). Eicosanoid synthesis by lung cells at various times after OVA challenge

We first examined eicosanoid formation by isolated lung cells at various times following antigen challenge. As discussed above airway resistance was measured in this preliminary study until the time of sacrifice in 4, 6 and 8 h groups (Fig. 16). Compared with the cells isolated from sensitized unchallenged rats (control), OVA challenge of sensitized BN rats resulted in increases in the formation of 5-lipoxygenase products, with maximal increases for cysteinyl-LTs (3.8-fold; $p< 0.05$), LTB_4 (4.1-fold; $p< 0.01$), and 5-HETE (2-fold; $p=0.058$) occurring 6 hours after challenge (Fig. 21A). A more modest 1.8-fold increase (not significant) in the cyclooxygenase product 12-HHTrE was also observed 6 hours after challenge (Fig. 21B). In contrast, no changes in the synthesis of 15-HETE were observed, whereas the amounts of 12-HETE were reduced between 4 and 8 hours after challenge ($p< 0.05$) (Fig. 21C). Taken together, these preliminary results suggest that lung cells isolated 6 h after antigen challenge have significantly increased capacities to synthesize 5-lipoxygenase products.

Although OVA challenge resulted in increased airway responses and leukotriene formation by lung cells (Figs. 16 and 21), there was no correlation between either early or late phase responses and 5-lipoxygenase activity in the lung (Table 7). There are two possible explanations for these findings. Firstly, the capacity of isolated lung cells to

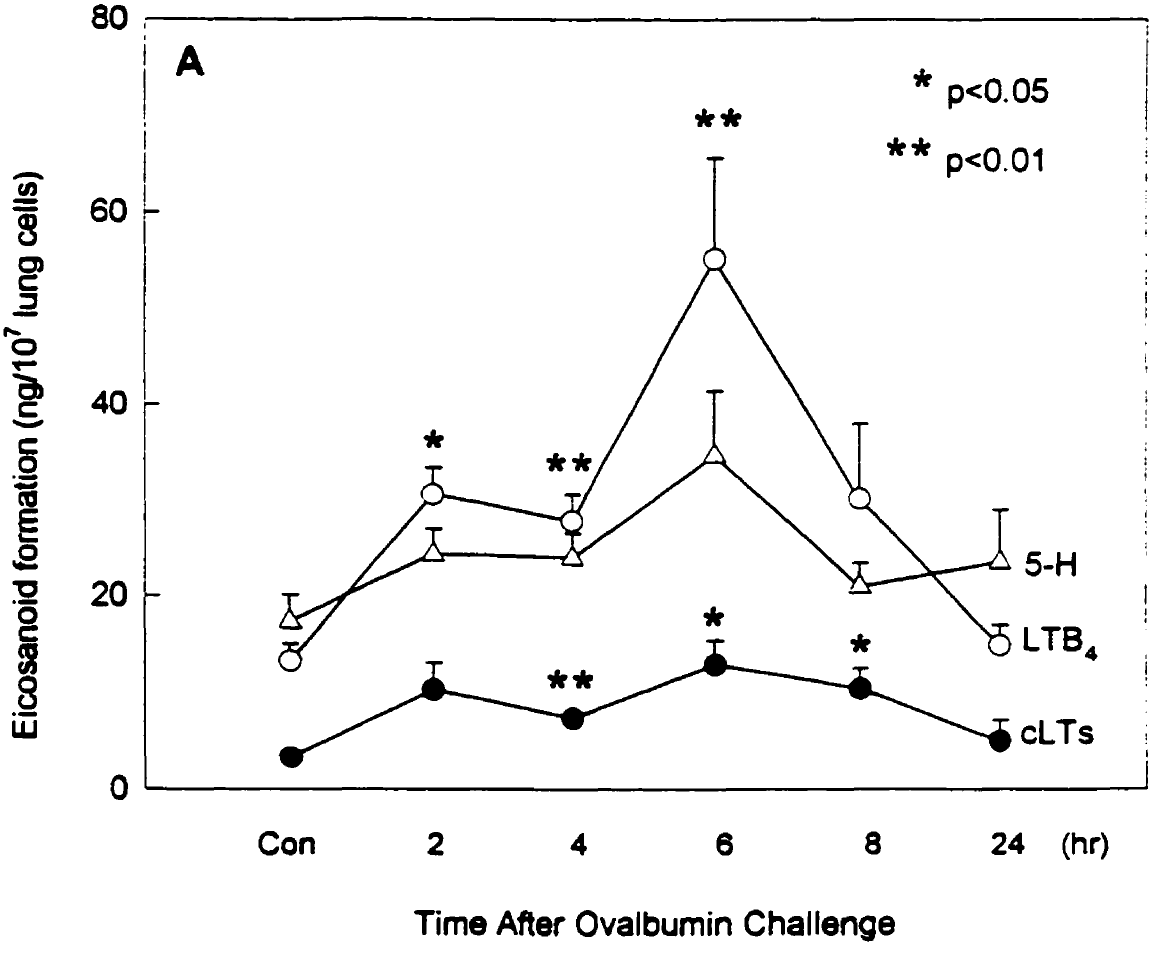

Fig. 21. Eicosanoid synthesis by lung cells at various times after allergen challenge. Lung cells obtained from sensitized rats without OVA challenge were used as control (CON). Isolated lung cell suspensions (4 ml, 5 x 10^6 /ml) were incubated with A23187 (1 μM) at 37 °C for 45 min. The eicosanoids released by these cells were analyzed by RP-HPLC as described in Fig. 8. Each bar represents the mean ± SEM (n=4-5).

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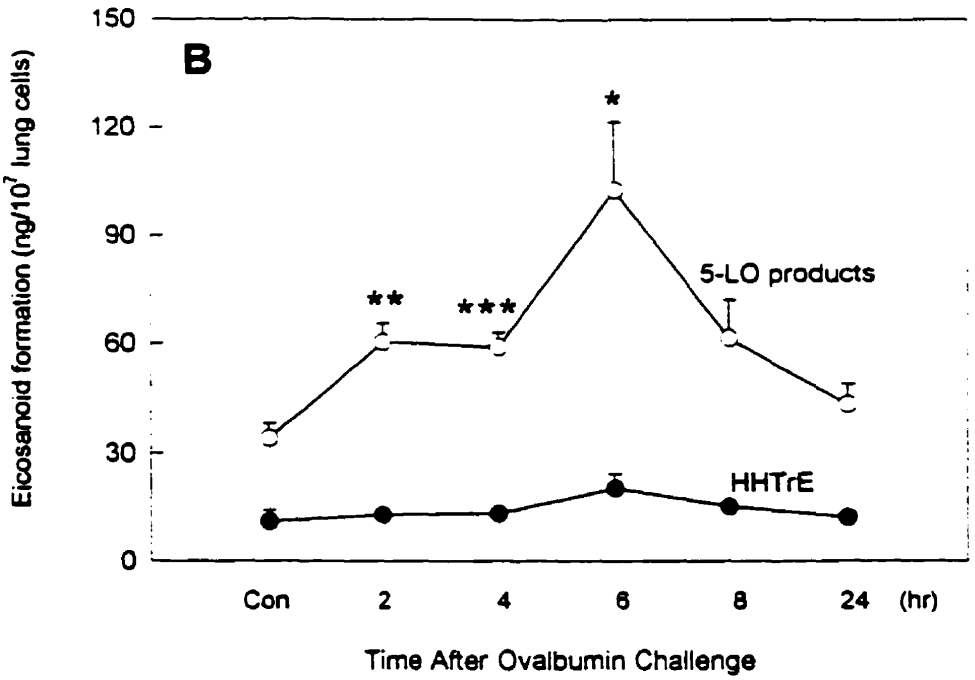
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Eicosanoid formation (ng/10^7 lung cells)
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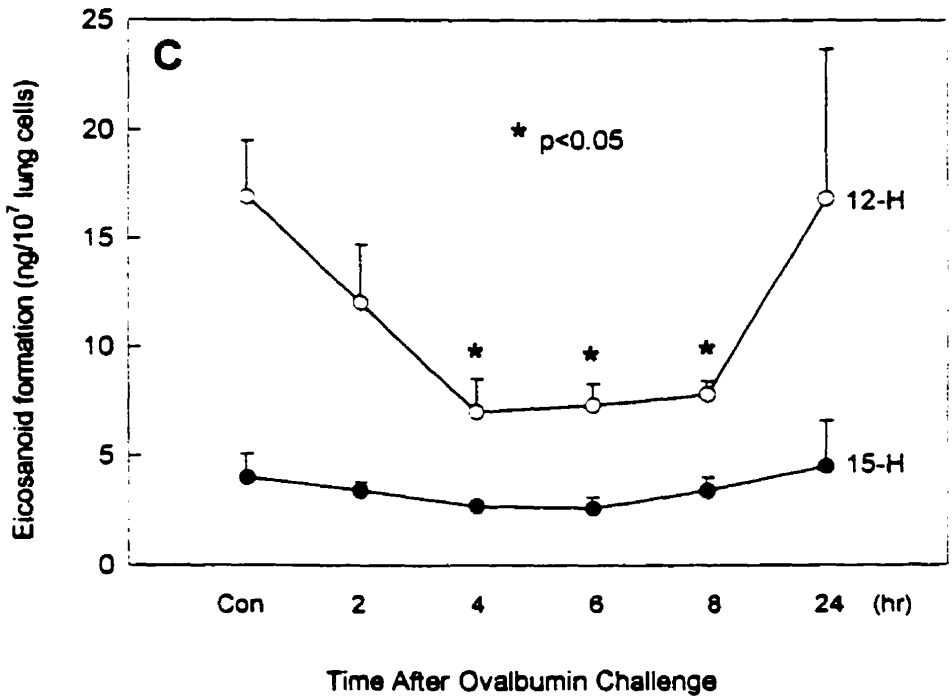
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Time After Ovalbumin Challenge

Fig. 21. B & C

synthesize leukotrienes in response to calcium ionophore rather than the *in vivo* release of leukotrienes by the lung was measured in this study. Therefore, the 5-lipoxygenase activities detected in this experiment may not represent the *in vivo* production of leukotrienes by the lung following OVA challenge. Secondly, the large differences in the timings and patterns of airway responses to OVA challenge among animals suggest that other mediators and pathophysiological mechanisms may also play an important role in the airway responses following allergen challenge.

TABLE 7

Airway responses and the formation of 5-lipoxygenase products by lung cells following OVA challenge

Groups	EPR (% of the baseline)	LPR (area above baseline)	5-Lip ($ng/10^7$ cells)
Saline 6h	145.9 ± 13.9	1.3 ± 0.9	45.5 ± 7.9
OVA 4h	254.2 ± 50.1*	7.8 ± 3.9*	58.9 ± 3.9
OVA 6h	238.3 ± 76.2	9.7 ± 2.0*	102.5 ± 18.9*
OVA 8h	350 ± 175*	19.± 6.7*	61.3 ± 10.6

The early phase response (EPR) and the late phase response (LPR) were calculated as described previously (228). 5-Lipoxygenase activity (5-Lip) indicates the formation of cysteinyl-LTs, LTB_4 and 5-HETE by lung cells isolated from BN rats following challenge with either saline or OVA. Data represents the mean ± SEM (n=4-7). * $p < 0.05$ (*vs* Saline).

iii). Eicosanoid synthesis by lung cells after OVA or saline challenge

In the preliminary studies discussed above, we monitored the airway resistance after allergen challenge. In a second study we eliminated the possible complication of the presence of a catheter in the airways and investigated eicosanoid synthesis by lung cells before, and 6 or 24 h after OVA or saline challenge. The HPLC profiles of the products formed by lung cells isolated from rats 6 h after challenge with either saline or OVA are shown in Figs. 22A and 22B, respectively. The major eicosanoids detected were LTB_4, 5-HETE, 12-HETE, and 12-HHTrE. Smaller amounts of LTC_4, LTD_4, LTE_4, 15-HETE and 5,6-diHETE were also present. Production of LTB_4 was much greater in cells from the OVA-challenged group compared to the saline-challenged group.

Products of the 5-lipoxygenase pathway. Isolated lung cells from OVA-sensitized control rats which were not challenged with either OVA or saline synthesized 13.9 ± 2.1 ng LTB_4, 10.9 ± 1.4 ng cysteinyl-LTs and 18.5 ± 2.2 ng 5-HETE per 10^7 cells. OVA challenge resulted in a 2.4-fold increase in the formation of LTB_4 ($p<0.001$) by lung cells isolated 6 h after challenge (Fig. 23A). There was also a significant increase in the synthesis of 5-HETE but relatively little difference in the synthesis of cLTs (Fig. 23B). In spite of significant infiltration of eosinophils into the lung 24 h after OVA challenge, cells isolated at this time did not synthesize significantly greater amounts of cLTs or other eicosanoids than cells from controls or saline challenged rats (Fig. 23B). Saline challenge resulted in a significant increase in the synthesis of 5-HETE 24 h after challenge compared with unchallenged controls but had no significant effect on the synthesis of either LTB_4 or cysteinyl-LTs by lung cells.

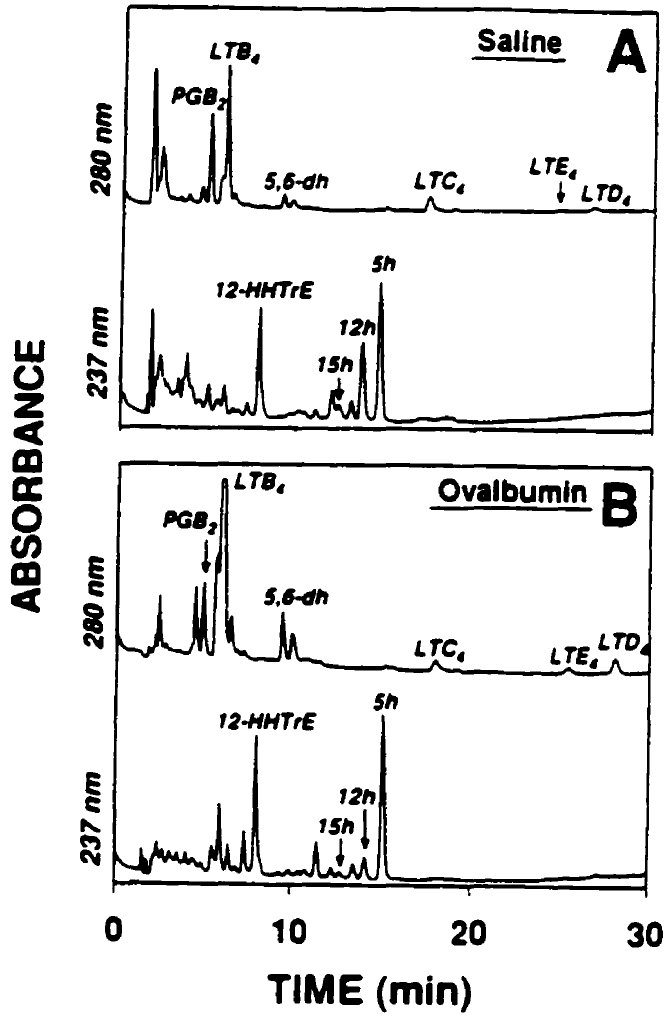

Figure 22. HPLC profile of eicosanoids formed by lung cells from OVA- or saline-challenged rats. The cells were isolated by enzymatic digestion of lungs 6 h after challenge with either saline (**A**) or ovalbumin (**B**). Crude lung cells (4 ml; 5 x 10^6 cells/ml) were incubated with A23187 (1 μM) for 15 min. Eicosanoids were analyzed using a Spherisorb ODS-2 column as described in Materials and Methods. The scales on the ordinates are identical for both chromatograms.

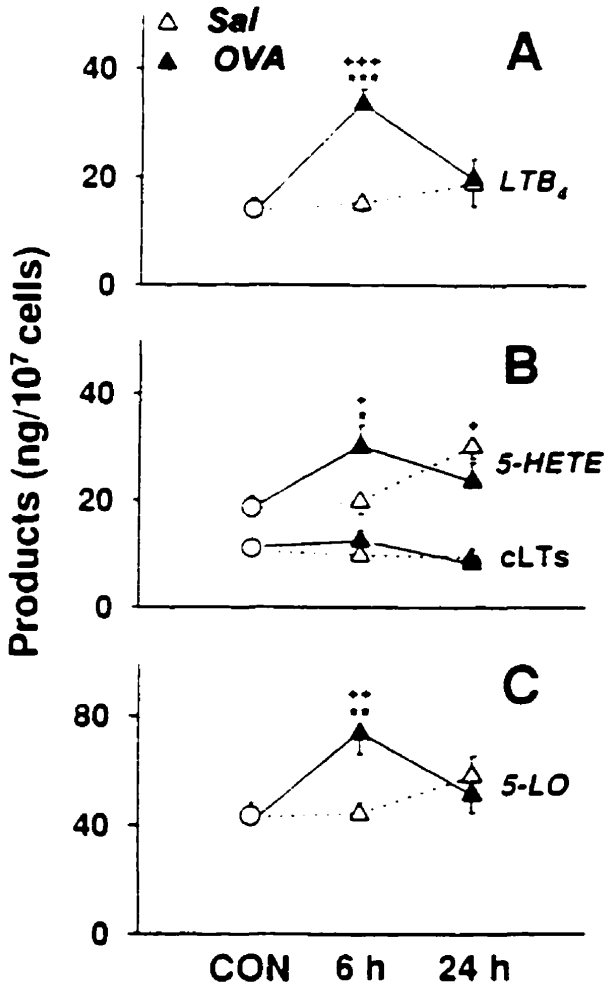

Figure 23. Formation of 5-lipoxygenase products by lung cells obtained from sensitized BN rats before (CON, ○) and 6 or 24 h after challenge with either saline (Δ) or OVA (▲). The amounts of metabolites were determined by precolumn extraction/RP-HPLC using PGB_2 as an internal standard. **A:** LTB_4; **B:** 5-HETE and cLTs (LTC_4 + LTD_4 + LTE_4); **C:** Total amounts of 5-lipoxygenase products (cLTs + LTB_4 + 5-HETE). Each point represents the mean ± SEM. *, $p < 0.05$; **, $p < 0.005$, and ***$p < 0.001$ when compared with saline-challenged groups. +, $p < 0.05$; ++, $p < 0.005$; and +++, $p < 0.001$ when compared with unchallenged controls (ANOVA, n = 5-7 for each data point).

The total amounts of the major 5-lipoxygenase products (i.e. LTB_4 + cLTs + 5-HETE) formed by lung cells from control rats and rats 6 h after challenge with saline were 43.2 ± 4.8 and 44.2 ± 4.1 ng/10^7 cells, respectively. OVA challenge resulted in a 1.7-fold increase in the total amounts of 5-lipoxygenase products synthesized by lung cells obtained from rats 6 h after challenge ($p< 0.005$ vs saline challenge) (Fig. 23C).

Products of the 12-lipoxygenase, 15-lipoxygenase and cyclooxygenase pathways. No significant differences were observed in the synthesis of either the 15-lipoxygenase product 15-HETE or the 12-lipoxygenase product 12-HETE by lung cells from rats of the 5 different groups (Fig. 24). In contrast to human and guinea pig lung cells, which synthesize large amounts of 15-HETE (347), BN rat lung cells synthesized only very small amounts of this compound. The production of the cyclooxygenase product 12-HHTrE by lung cells obtained 6 h after OVA challenge was significantly increased compared to unchallenged controls ($p< 0.05$; Fig. 24B). However, saline challenge induced a similar increase in the formation of 12-HHTrE by lung cells at 24 h after challenge.

3.3.5. Relationship between cellular infiltration and eicosanoid synthesis

The production of LTB_4 by suspensions of rat lung cells was positively correlated with the percentage of neutrophils in the cell suspension ($R=0.59$, $p<0.01$; Fig. 25A), but not with the percentage of eosinophils (Fig. 25B). Interestingly, there was an inverse correlation between the synthesis of cysteinyl-LTs and the percentage of eosinophils in the cell suspension ($R=-0.42$; $p <0.05$; Fig 25D), suggesting that BN rat eosinophils may

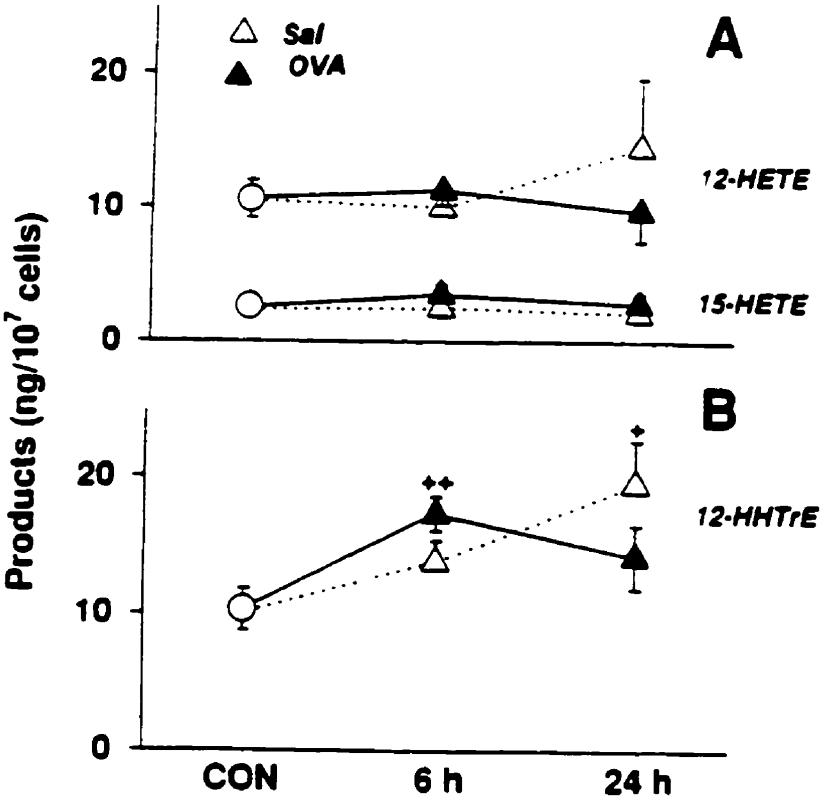

Figure 24. Formation of products of the 12-lipoxygenase, 15-lipoxygenase, and cyclooxygenase pathways by unfractionated lung cells from control rats (CON, O) and rats challenged with either saline (Δ) or OVA (▲). **A:** 12-HETE and 15-HETE; **B:** 12-HHTrE. Each data point represents the mean ± SEM of determinations on cells from at least five animals. +, $p < 0.05$ and ++, $p < 0.01$ vs unchallenged controls.

not be a major source of cLTs. There was no relationship between the production of cysteinyl-LTs and the percentage of neutrophils in the lung cell suspensions (Fig. 25C).

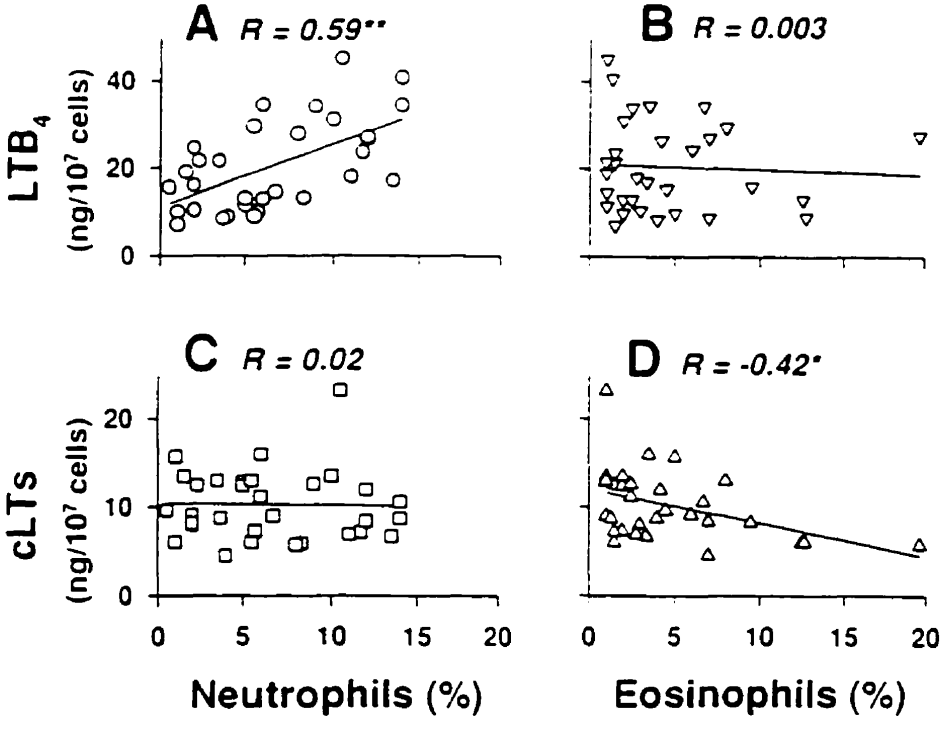

Figure 25. Relationship between cellular infiltration into the lungs and leukotriene formation by unfractionated lung cells. Each data point represents a determination (performed in duplicate) from a single rat. The amount of LTB_4 produced by lung cells was plotted against the percentage of neutrophils (**A**; O) and eosinophils (**B**; ∇) in the cell suspension. The relationship between the production of cLTs and the percentages of neutrophils (**C**; □) and eosinophils (**D**; Δ) are also shown. *, $p < 0.05$; **, $p < 0.01$ (Pearson's correlation). Data from both saline- and antigen- challenged animals are shown.

3.4. FORMATION OF AN UNIDENTIFIED ARACHIDONIC ACID METABOLITE BY LUNG CELLS FROM OVA-CHALLENGED BN RATS

Lung cells from antigen challenged BN rats not only synthesized more LTB_4 than controls but also produced appreciable amounts of an unidentified arachidonic acid metabolite (Fig. 26, Table 8). The unidentified metabolite has a shorter retention time than LTB_4 but shows a uv spectrum similar to that of LTB_4 (Fig. 26). Its synthesis increased by about the same extent as LTB_4 in allergen challenged rats (Table 8). Interestingly, longer incubation appeared to result in decreased formation of LTB_4 but unchanged or increased production of the unidentified compound by lung cells. However, incubation of lung cells with exogenous LTB_4 (1-10 μg) did not result in formation of a detectable amount of this metabolite, suggesting that this compound may not be a physiological metabolite of LTB_4 in the lung.

Among the well described eicosanoids, 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid (8,15-diHETE) and 3-hydroxy-LTB_4(3h-LTB_4), which is produced by hepatocytes in the presence of ethanol (54), also have similar uv spectra to that of LTB_4. We examined the retention times of 8S,15S-diHETE, 3h-LTB_4 and the unknown metabolite with different HPLC systems and found that the retention times of the above two known eicosanoids are different from that of the unknown metabolite. For example, when these compounds were co-chromatographed using a 3.2 x 250 mm Spherisorb ODS-2 column with a mobile phase consisting of a linear gradient between 45% and 50% acetonitrile for 30 min, the retention times of these eicosanoids were as follows: 3(R)h-LTB_4 and 3(S)h-LTB_4, 10.8 min; the unidentified metabolite, 11.91 min; PGB_2, 14.68 min;

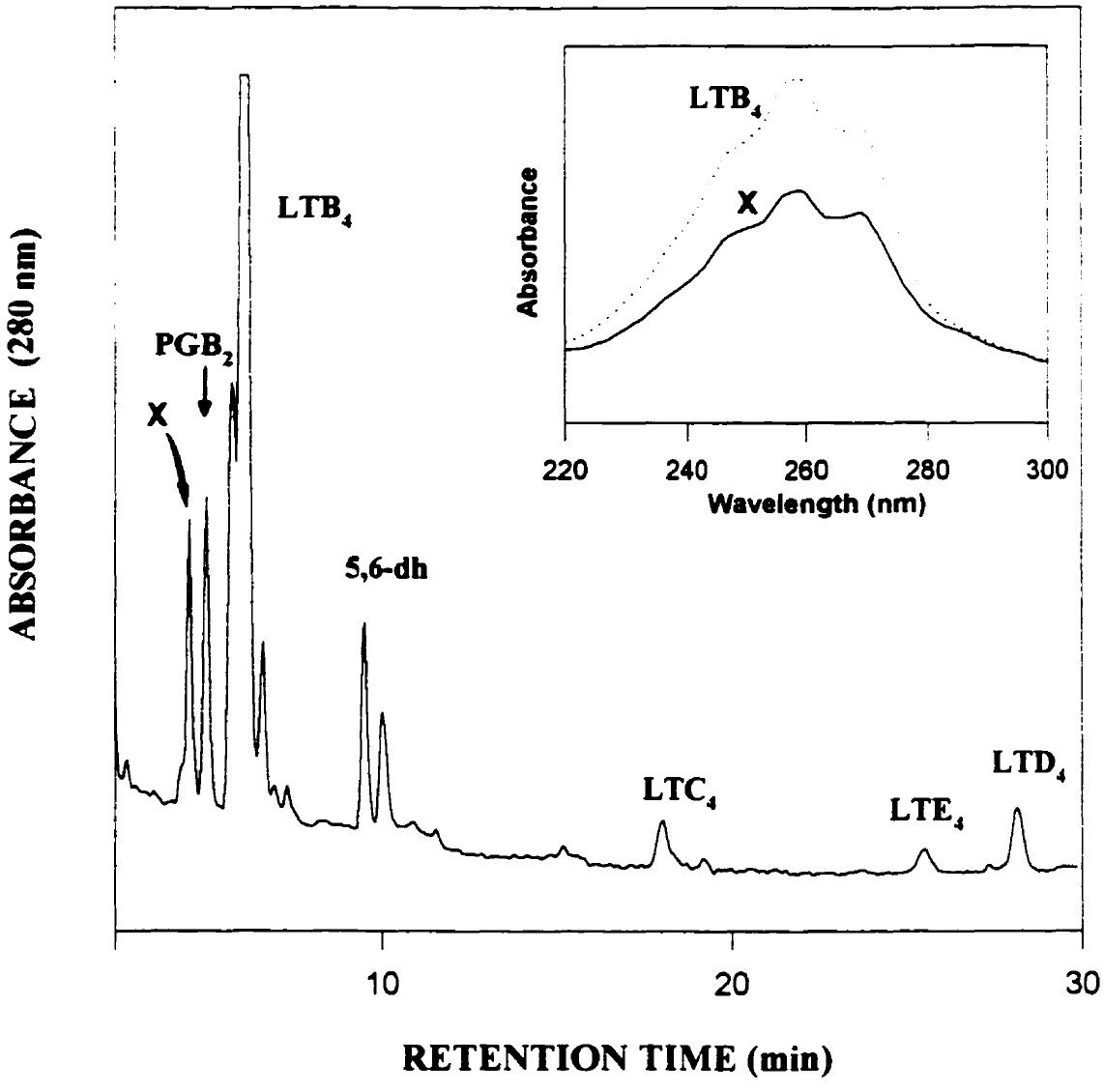

Fig 26. HPLC and uv spectrum of an unidentified arachidonic acid metabolite (X) produced by lung cells from OVA challenged BN rats. The lung cells were isolated 6 h after OVA challenge and the eicosanoids produced by these cells were analysed as described in Figure 8. The unidentified metabolite is indicated by a curved arrow and its uv spectrum, along with that of LTB_4, is shown in the inset.

8S,15S-di-HETE, 15.60 min; LTB_4, 16.89 min.

We also examined the formation of the unidentified metabolite by other types of cells. Rat pleural cavity and peritoneal cavity leukocytes were found to produce appreciable amounts of this compound (about 5 ng/10^7 cells, 2-10% of LTB_4). In contrast, human leukocytes, eosinophils and porcine PMNL did not synthesized detectable amounts (data not shown). Since we were unable to obtained enough of this unidentified metabolite for further structural studies, its nature and source are still unknown.

TABLE 8

Comparison of the formation of the unidentified metabolite (X) and LTB_4 by lung cells isolated from BN rats at various times after OVA challenge*

Incubation Time	Eicosanoid	Con	2	4	6	8	24 (h)
15 min	X	1.2±0.2	2.6±0.4*	2.6±0.2*	6.3±1.1**	3.7±0.8*	2.4±0.3
	LTB_4	13±1.8	31±2.8*	28±2.8**	55±11**	30±7.9	15±2.1
45 min	X	1.2±0.1	3.8±0.6*	2.8±0.4*	7.3±1.0**	3.1±1*	—
	LTB_4	9.8±0.6	24±6.2	20±0.5*	39±5.9*	20±4.3	—

*Lung cells were isolated from BN rats at various times after antigen challenge. The cell suspensions were incubated with A23187 at 37 °C for 15 or 45 min. Analysis of the eicosanoids formed by lung cells were as described in Fig. 21. All data are means ± SEM (n=5). *: $p<0.05$; **: $p<0.01$ (*vs* Con).

3.5. EICOSANOID SYNTHESIS BY PARTIALLY PURIFIED PULMONARY EOSINOPHILS, ALVEOLAR MACROPHAGES AND NEUTROPHILS

3.5.1. Eicosanoid synthesis by partially purified eosinophils from Sephadex-treated BN rats

In an attempt to characterize eicosanoid synthesis by rat pulmonary eosinophils, eosinophilia was induced in rat lungs by i.v. injection of Sephadex particles. As shown in the inset to Fig 27, the percentage of eosinophils in the lungs of Sephadex-treated rats was about 4 times that in controls ($p<0.001$). There was also a similar increase in the percentage of eosinophils in BAL cells from Sephadex-treated animals (data not shown). The eosinophil-rich lung cells synthesized more 5-HETE ($p<0.05$), 12-HETE ($p<0.05$), 15-HETE ($p<0.001$) and 12-HHTrE ($p<0.05$) than cells from control rats, whereas the amounts of LTB_4 and cLTs were not significantly different (Fig 27).

We attempted to purify eosinophils from suspensions of lung cells from Sephadex-treated rats by Percoll density gradient centrifugation. However, because of the high degree of heterogeneity in the density of pulmonary eosinophils, it was not possible to obtain a high degree of purity of these cells. However, we fractionated isolated lung cells over Percoll gradients and tested the capacity of the resulting fractions to synthesize LTs and other eicosanoids. As shown in Fig 28, fractions of lung cells enriched in eosinophils (>34% eosinophils) synthesized LTB_4, 5-HETE, 12-HETE and 12-HHTrE, but negligible amounts of cysteinyl-LTs.

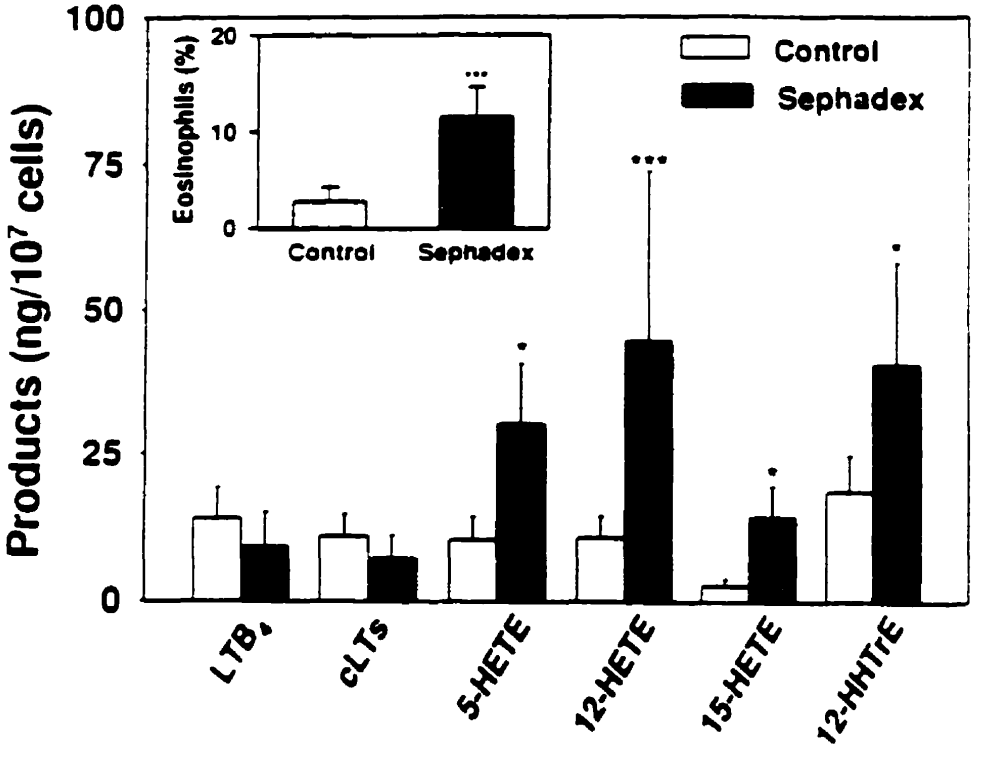

Figure 27. Synthesis of eicosanoids by lung cells from BN rats either treated with Sephadex (solid bars) or untreated (control; open bars). The inset shows the percentage of eosinophils in suspensions of unfractionated lung cells from the two groups of rats. Values are means ± SEM of determinations on 5 to 7 animals. Statistical significance was evaluated using Student's t-test for unpaired data. *, $p < 0.05$; ***, $p < 0.001$ when compared with the control group. Neither the Sephadex-treated nor the control rats in this experiment had been sensitized to ovalbumin.

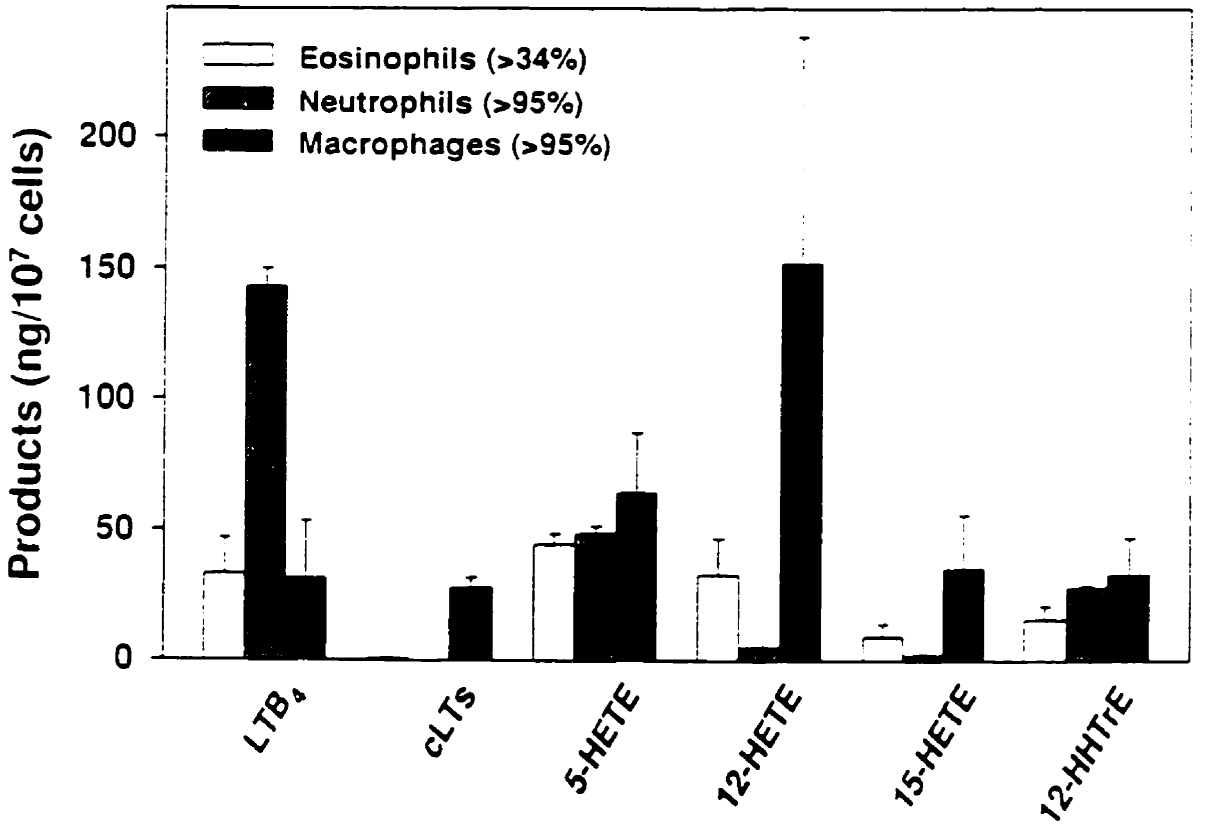

Figure 28. Comparison of eicosanoid synthesis by partially purified rat eosinophils (open bars; n = 4), neutrophils (hatched bars; n = 9) and macrophages (solid bars; n = 5). Cells (5x 10^6/ml) were incubated with A23187 (1 μM) at 37 °C for 30 min. Eicosanoids were analyzed by precolumn extraction/RP-HPLC as described in Fig 8. Data points for eosinophils are results from lung cell fractions enriched in eosinophils (43 ± 4 % pure) obtained by density gradient centrifugation. Data points for neutrophils (97 ± 1 % pure) and macrophages (95 ± 1 % pure) were obtained with highly purified cells.

3.5.2. Eicosanoid synthesis by alveolar macrophages and pleural neutrophils from BN rats

The major lipoxygenase product synthesized by neutrophils from BN rats was LTB_4 (Fig. 28), suggesting that these cells may be a major site for the production of this substance by lungs from this species. These cells did not synthesize detectable amounts of cysteinyl-LTs. Lymphocytes from BN rats did not synthesize any detectable lipoxygenase products in response to A23187 (data not shown). In contrast to eosinophil-enriched fractions and neutrophils, alveolar macrophages synthesized appreciable amounts of cysteinyl-LTs as well as other lipoxygenase products (Fig 28) and may be an important source of these metabolites in lungs from allergic rats.

3.5.3. Correlation between LT synthesis and the percentages of eosinophils in various lung cell fractions

It was difficult to clearly define the profile of eicosanoids synthesized by BN rat eosinophils since we were unable to obtain highly purified preparations of these cells. However, we examined the relationship between the percentages of eosinophils and the synthesis of leukotrienes in various fractions obtained after density gradient centrifugation using Percoll (Fig. 29). The production of cysteiny-LTs appeared to be lowest in suspensions of lung cells containing the highest percentages of eosinophils ($R = -0.4$) (Fig. 29), but this relationship was not statistically significant. In contrast, LTB_4 production was positively correlated with the percentage of eosinophils (Fig. 29B; $p<0.05$). Since rat neutrophils tended to be copurified with eosinophils, we cannot rule

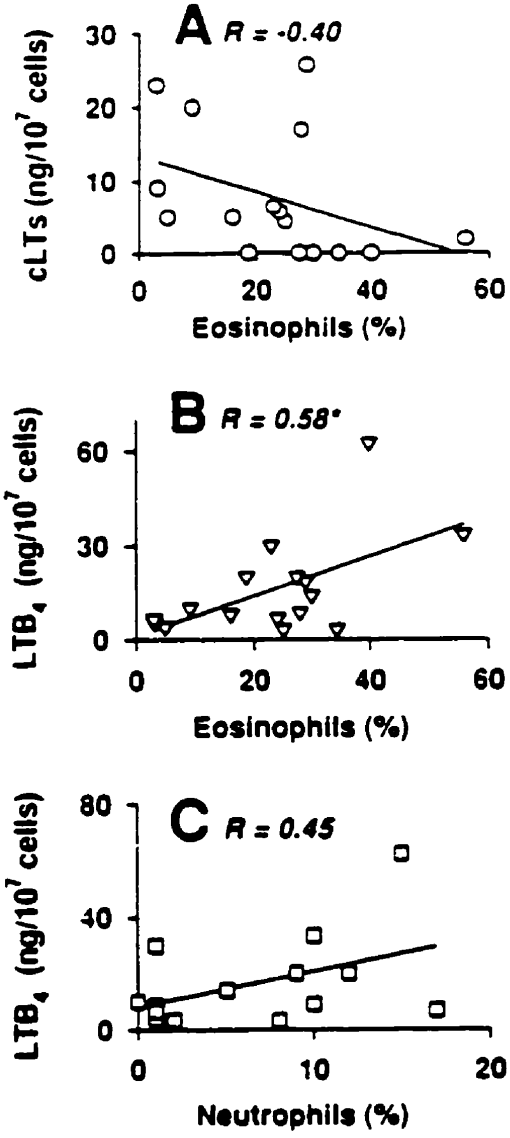

Figure 29. Relationship between the percentages of eosinophils and neutrophils in cell fractions and leukotriene synthesis. Various lung cell fractions were obtained from Sephadex-treated rats by density gradient centrifugation over Percoll. Each data point represents a determination on a single gradient fraction. There appears to be an inverse correlation between cLT production and the percentages of eosinophils in lung cell fractions (**A**; O), but this was not significant. LTB_4 production was positively associated with the percentages of eosinophils (**B**; ∇)(*, $p < 0.05$ (Pearson's correlation)) in cell fractions, but not significantly correlated with the percentages of neutrophils (**C**; □).

out the possibility that neutrophils were partly responsible for the apparent positive correlation between eosinophil numbers and LTB_4 production. However, the lack of a positive correlation between neutrophil numbers and LTB_4 production would suggest that eosinophils were primarily responsible for this phenomenon. These results differ from those shown in Fig. 25, which shows that there is a positive correlation between LTB_4 production and the percentages of neutrophils, but not eosinophils, in unfractionated lung cells from antigen-challenged rats. This is possibly because the two sets of data are from different cell populations. The data shown in Fig. 29 were from fractions enriched in eosinophils obtained by centrifugation of lung cells over Percoll, whereas Fig. 25 shows data from experiments with unfractionated lung cells. There may also be differences in cells from Sephadex- and antigen- treated rats due to differences in the *in vivo* environment (*e.g.* cytokine concentration) of the granulocytes prior to preparation of the lung cells.

3.6. EFFECTS OF LEUKOCYTE DEPLETION ON PULMONARY CELLULAR INFILTRATION AND EICOSANOID SYNTHESIS BY LUNG CELLS

In order to understand the role of infiltrating leukocytes in eicosanoid synthesis in the lung after allergen challenge, we investigated the effects of rabbit anti-rat PMNL serum on cellular infiltration and leukotriene synthesis. In a preliminary study, we first examined the effects of antiserum pretreatment on the numbers of white blood cells, red blood cells and platelets in peripheral blood in BN rats. Consistent with a report in the literature (348), we found that treatment of BN rats with rabbit anti-rat PMNL serum resulted in decreased neutrophil numbers within 2h, with a maximal effect at 18h. The neutropenia persisted for 48 h (data not shown). Since allergen challenge is known to induce neutrophilia in blood and neutrophil infiltration in the lung, we therefore decided to determine whether anti-PMNL serum could block allergen induced-neutrophil infiltration into the lung and, if so, whether eicosanoid synthesis by lung cells was also affected.

Study design: Eighteen sensitized BN rats were randomly divided into three groups and pretreated with anti-PMNL serum, non-immune serum and saline. A pre-treatment blood sample was collected from each rat prior to peritoneal injection of serum or saline. The rats were challenged 18 h later by OVA aerosol. Blood samples, bronchoalveolar lavage fluid and lung cells were obtained 6 h after OVA challenge. The differential counts of leukocytes and eicosanoid production by lung cells were analysed as described in Materials and Methods.

3.6.1. Effects of rabbit anti-rat PMNL serum on leukocyte numbers in peripheral blood

We determined the numbers of leukocytes in blood from rats before pretreatment as well as after serum treatment and OVA challenge. As shown in Table 9, treatment of rats with either saline or non-immune serum followed by OVA had no effect on the numbers of total leukocytes, RBC or platelets in the blood. In contrast, treatment of BN rats with anti-PMNL serum followed by OVA challenge resulted in decreased numbers of leukocytes (WBC). The leukocyte counts were reduced from 11.8 ± 0.5 to 3.7 ± 1.2 $x10^6$/ml blood ($p< 0.01$). No changes in the numbers of red blood cells or platelets were observed. Therefore, the rabbit anti-rat PMNL serum appeared to selectively deplete circulating white blood cells in antigen-challenged BN rats.

The differential counts of leukocytes are shown in Fig 30. Neutrophils accounted for about 5-10% of total leukocytes in the blood prior to serum pretreatment and OVA challenge (Fig. 30A). More than 80% of the leukocytes were lymphocytes (Fig. 30B). OVA challenge of sensitized rats increased the numbers of neutrophils by about 400% ($p<0.01$) and decreased the numbers of lymphocytes by about 40% ($p<0.05$) in rats treated with either non-immune serum or saline. The numbers of neutrophils in anti-PMNL serum/OVA-treated rats were significantly lower than those in rats treated with saline or non-immune serum and OVA ($p <0.005$) as well as in rats prior to treatment ($p< 0.05$). Thus, anti-PMNL serum blocked OVA-induced neutrophilia in blood. There were no significant differences between the numbers of lymphocytes among the three groups 6 h after OVA challenge. There were only small numbers of eosinophils and monocytes in the blood of BN rats before serum pretreatment and 6h after OVA challenge, and we were

TABLE 9

Effects of treatment with anti-PMNL serum and OVA on the numbers of blood cells in sensitized BN rats*

	Treatment**	Saline	Non-immune	Anti-PMNL
Weight (g)		227 ± 10	228 ± 14	226 ± 8
WBC (10^6/ml)	Before	11.7 ± 1.8	12.4 ± 3.4	11.8 ± 1.1
	After	11.6 ± 3.0	9.4 ± 1.4	3.7 ± 3.0**
RBC (10^9/ml)	Before	7.7 ± 0.3	7.7 ± 0.4	7.6 ± 0.9
	After	7.7 ± 0.4	7.7 ± 0.2	7.2 ± 0.9
PLT (10^6/ml)	Before	711 ± 73	688 ± 25	692 ± 79
	After	833 ± 74	771 ± 106	546 ± 138

*Rabbit anti-PMNL serum, non-immune serum or saline were injected intraperitoneally into sensitized rats 18 h before OVA challenge. Blood samples were collected prior to injection of serum or saline and 6 h after OVA challenge. The results are expressed as mean ± SEM (n=6). The numbers of leukocytes (WBC) in blood of rats pretreated with anti-rat PMNL serum were significantly lower than those of rats pretreated with non-immune serum or saline ($P < 0.01$). **Before, pretreatment with saline or serum; After, after pretreatment with saline or serum, followed by OVA.

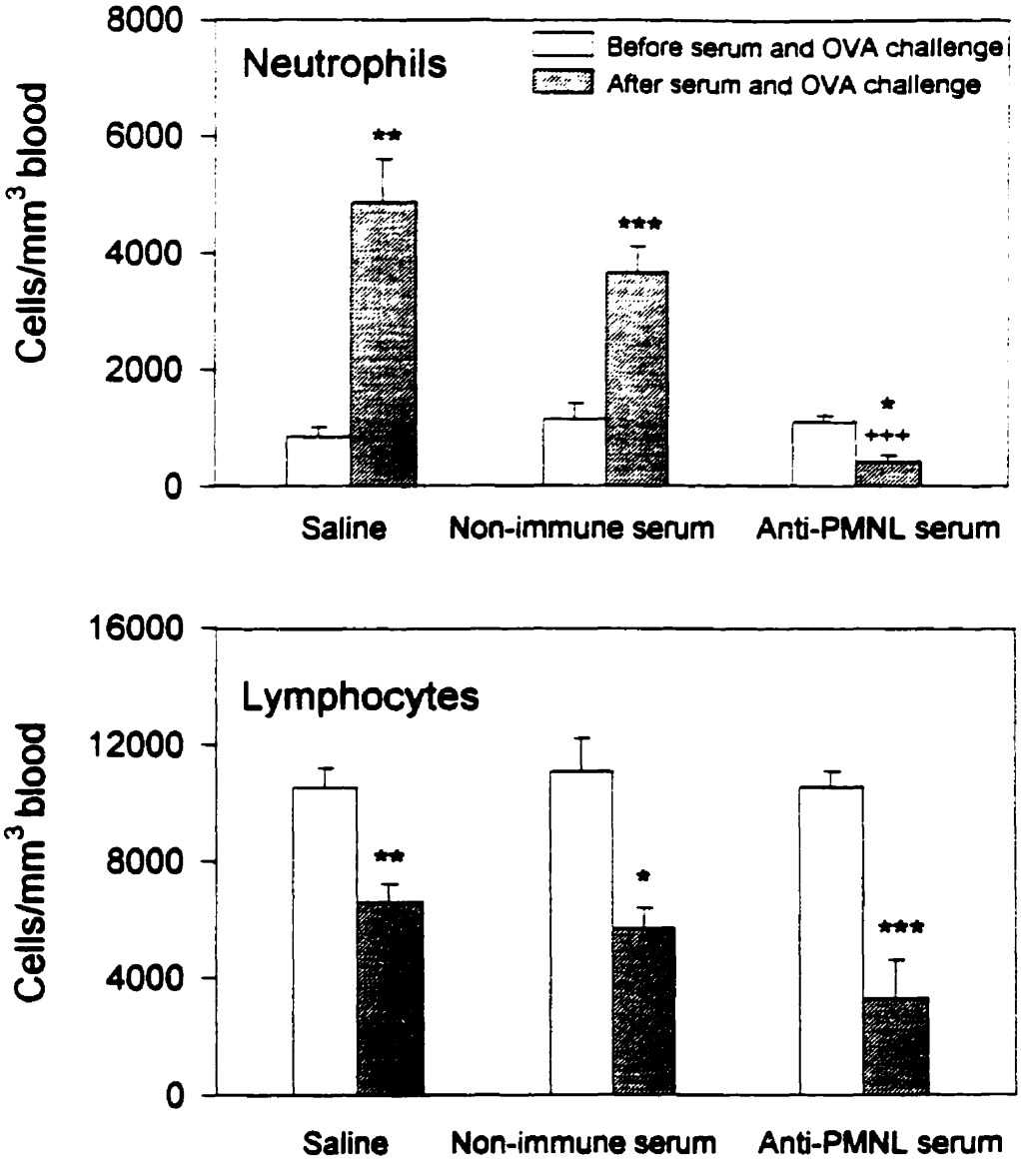

Fig. 30. Numbers of neutrophils (A) and lymphocytes (B) in the blood of rats 18h before serum and OVA as well as 6h after OVA challenge. All of the rats were pretreated with either serum or saline 18h before OVA challenge. Blood samples were collected before pretreatment and 6h after OVA challenge. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (vs. before serum & OVA challenge); +++, $p < 0.005$ (*vs.* saline and non-immune serum group).

unable to see any significant changes in the numbers of these cells in the three groups.

3.6.2. Effects of leukocyte depletion on cellular infiltration into the lung after OVA Challenge

The numbers of cells recovered from BAL fluids and lung tissues after enzymatic digestion are shown in Table 10. Although there appeared to be slightly decreased numbers of cells in BAL fluids of rats pretreated with anti-PMNL serum, no significant differences in the numbers of cells recovered from BAL and lungs were observed among the three groups. However, administration of anti-PMNL serum resulted in decreased numbers of neutrophils in BAL ($p< 0.005$) and lungs ($p< 0.005$) compared with rats pretreated with either saline or non-immune serum (Fig. 31). There were no significant differences in the percentages of eosinophils in BAL fluid and lung tissue between any two groups. However, the numbers of lymphocytes increased significantly in both BAL and lungs of rats pretreated with anti-PMNL serum. The percentages of macrophages also increased significantly in BAL in the serum-treated group possibly due to the depletion of neutrophils. These results suggest that pretreatment of BN rats with rabbit anti-rat PMNL serum selectively inhibited neutrophil infiltration into lungs 6 h after allergen challenge.

TABLE 10

The total numbers of cells recovered from BAL fluid and lungs after enzymatic digestion*

	Cell recovery per rat (÷ 10^6)	
	BAL	Lungs
Saline	4.3 ± 1.1	75 ± 10
Non-immune serum	3.0 ± 0.4	64 ± 3.9
Anti-PMNL serum	2.7 ± 1.0	60 ± 4.7

*Cells were recovered from the airways by BAL and from lung tissue after enzymatic digestion. The data are means ± SEM of cell numbers from individual rats in the same group. There were no significant differences between any two groups in total cell numbers recovered from BAL fluid or enzymatically digested lungs.

3.6.3. Effects of anti-PMNL serum on eicosanoid synthesis by lung cells after OVA challenge

Eicosanoid synthesis by lung cells was analyzed on a 3.2 mm ID spherisorb ODS-2 column. Under the conditions used, almost all of the major lipoxygenase products, lipoxins and other monooxygenated metabolites of arachidonic acid could be resolved within 40 min as shown in Fig. 11A. Typical chromatograms of eicosanoids produced by

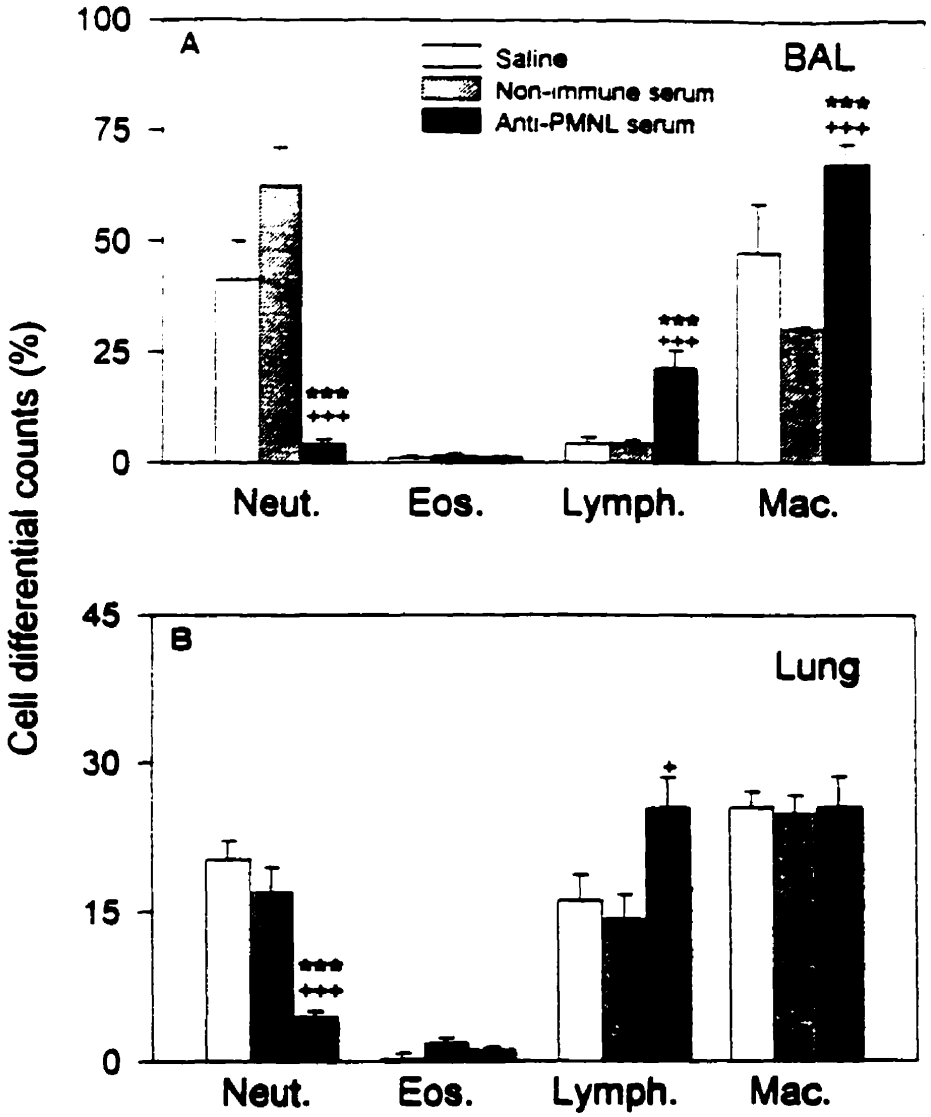

Fig. 31. Levels of inflammatory cells in BAL (A) and lungs (B) 6h after OVA challenge in rats pretreated with serum or saline. ***, $p < 0.005$ (*vs.* saline group); +, $p < 0.05$; +++, $p < 0.005$ (*vs.* non-immune serum group).

lung cells isolated from the three different groups of rats 6h after OVA challenge are shown in Fig 31. The major lipoxygenase products detected after incubation of isolated lung cells with A23187 were LTB_4, cysteinyl-LTs, HETEs and the cyclooxygenase metabolite 12-HHTrE (Fig. 32A, B & C). We did not detect any lipoxins using these experimental conditions. Compared with lung cells from rats pretreated with saline (Fig. 32A) or non-immune serum (Fig. 32B), the cells from anti-PMNL serum-pretreated rats appeared to produce smaller amounts of LTB_4, cysteinyl-LTs and 5-HETE (Fig. 32C) .

Products of the 5-lipoxygenase pathway. Lung cells isolated 6 h after OVA challenge of saline-pretreated rats synthesized 36.0 ± 3.3 ng LTB_4, 25.9 ± 3.3 ng cLTs and 41.4 ± 2.3 ng 5-HETE per 10^7 cells. 5-Oxo-ETE was not detectable using our experimental conditions unless exogenous arachidonic acid or 5-HETE were added to the incubation (data not shown). As shown in Fig. 33A, there appeared to be no significant differences in leukotriene production between rats pretreated with anti-PMNL serum, non-immune serum and saline. However, one of the rats pretreated with anti-PMNL serum had extremely high 5-lipoxygenase activity (three times higher than the average value for the remaining rats in this group). This rat also had the highest percentages of neutrophils and macrophages in this group (see Figs. 34 and 35 below). When the data of the outlier was not included, the remaining rats in the anti-PMNL serum-treated group synthesized significantly less LTB_4 than rats pretreated with either saline ($p < 0.005$) or non-immune serum ($p < 0.05$). Excluding the outlier, the synthesis of cysteinyl-LTs and 5-HETE was also lower in the anti-PMNL serum-treated rats than in the saline-treated rats ($p < 0.05$ and $p < 0.01$, respectively). When the outlier was excluded, the total amounts of

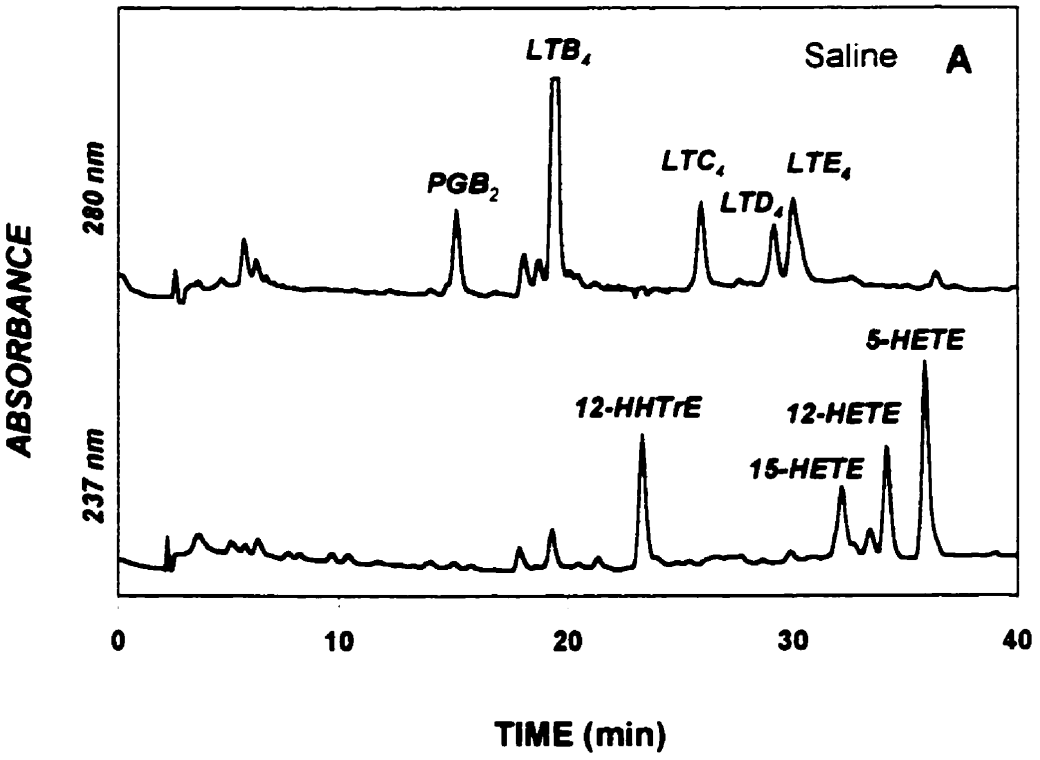

Fig. 32. Reversed-phase HPLC profiles of eicosanoids formed by lung cells isolated after OVA challenge of a saline-pretreated rat (A), a non-immune serum pretreated rat (B), and a rabbit anti-rat PMNL serum-pretreated rat (C). The isolated lung cells (2 ml, 5 x 10^6 cells/ml) were incubated with 1 μM A23187 for 45 min. The products were analyzed on a midbore Spherisorb ODS-2 column (3.2 x 250 mm). The mobile phase consisted of a linear gradient between solvents D (water/methanol/TFA, (70:30:0.04)) and F (water/acetonitrile/methanol/TFA, (25:55:20:0.04)) as follows: 0 min, 50% F; 40 min, 100% F. The flow rate was 0.75 ml/min.

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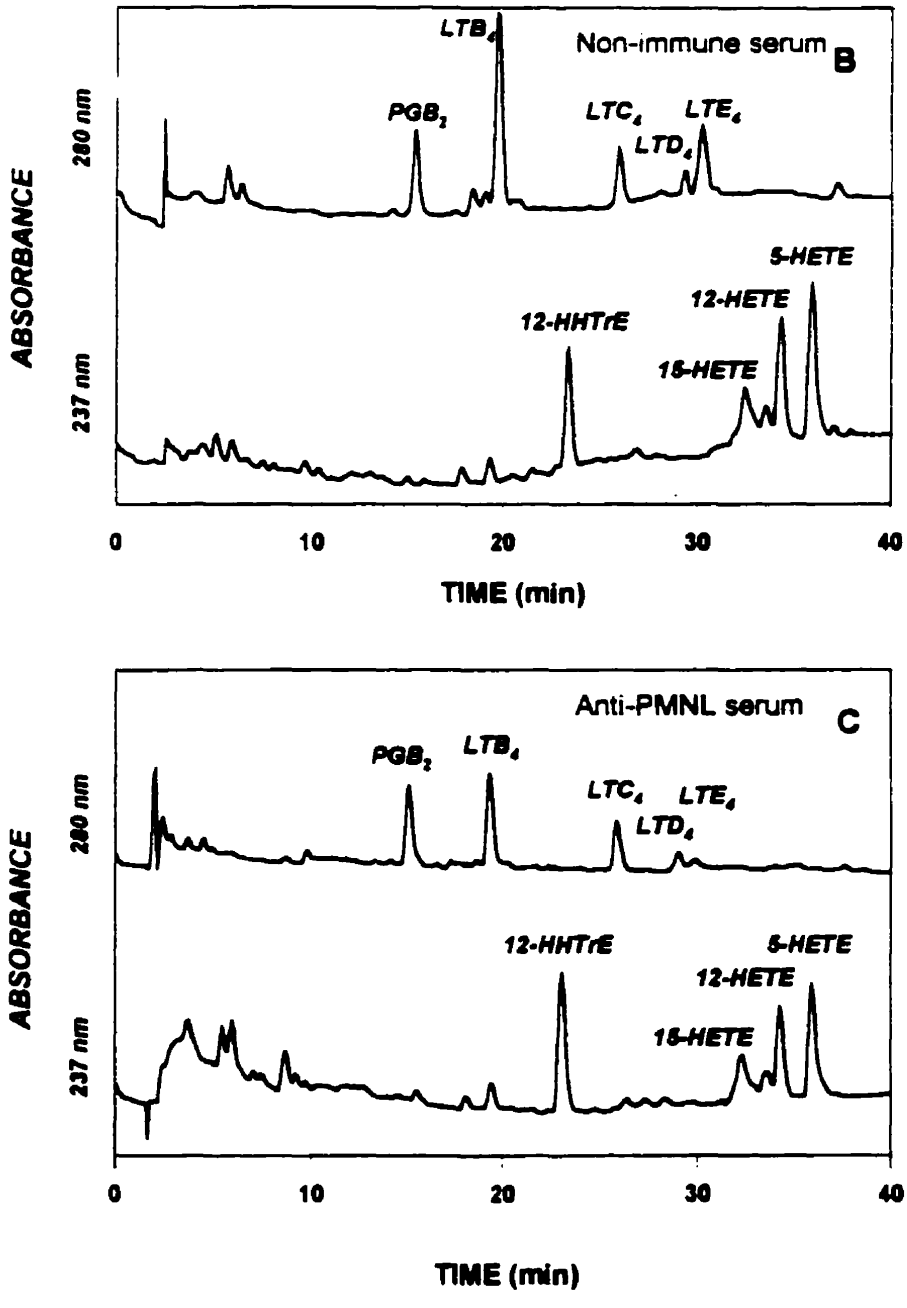
Non-immune serum
B
LTB4
PGB2
LTC4
LTE4
LTD4
5-HETE
12-HETE
12-HHTrE
15-HETE
280 nm
237 nm
ABSORBANCE
0
10
20
30
40
TIME (min)
Anti-PMNL serum
C
PGB2
LTB4
LTC4
LTE4
LTD4
12-HHTrE
5-HETE
12-HETE
15-HETE
280 nm
237 nm
ABSORBANCE
0
10
20
30
40
TIME (min)

Fig. 32 B & C

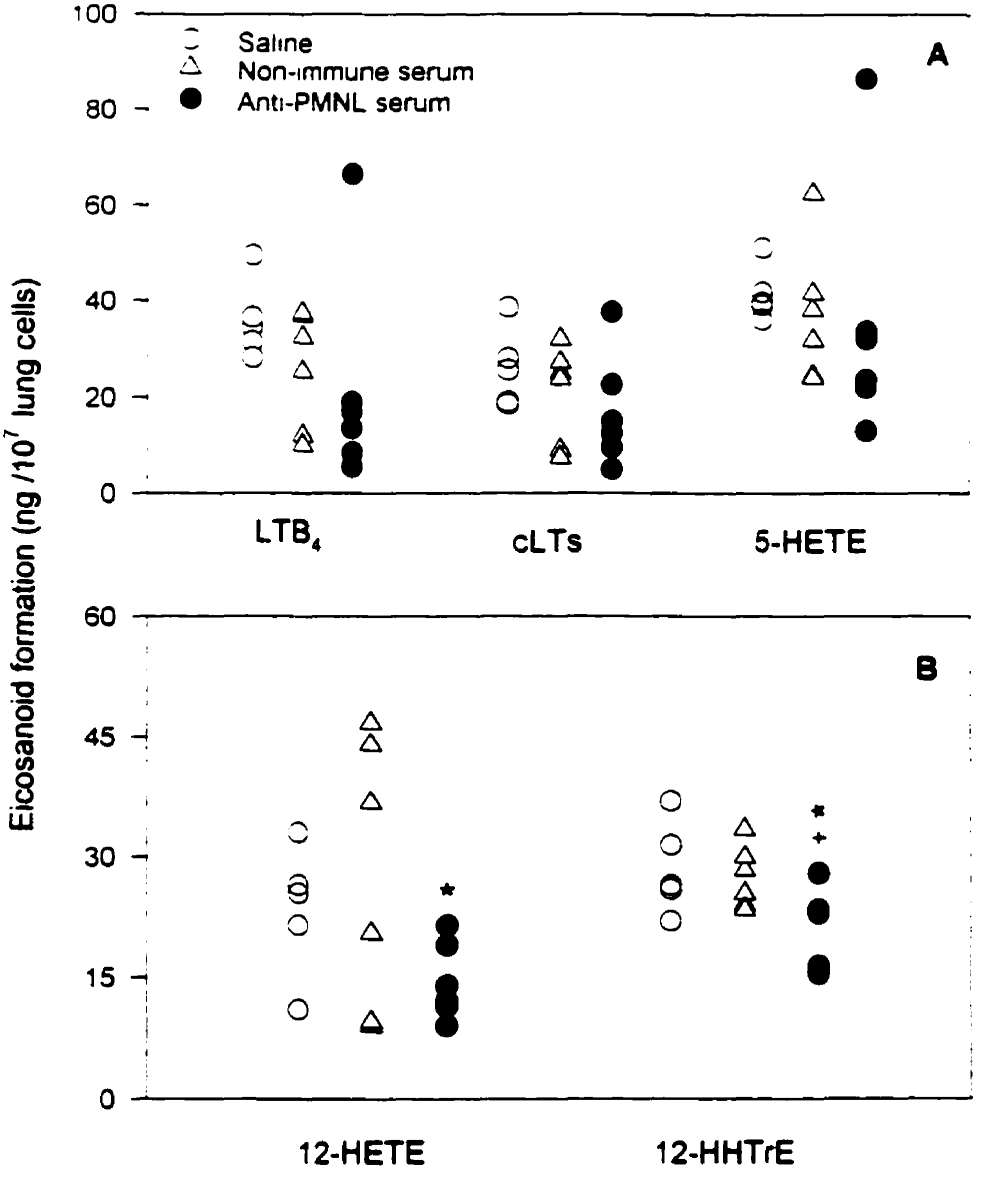

Fig. 33. The formation of 5-lipoxygenase products (A) and other eicosanoids (B) by lung cells obtained from rats 6h after challenge with OVA after pretreatment with saline, non-immune serum, or anti-PMNL serum. *: $p < 0.05$ *vs.* saline group; +: $p < 0.05$ *vs.* non-immune serum group.

major 5-lipoxygenase products (i.e. LTB_4 + cysteinyl-LTs + 5-HETE) formed by lung cells from rats pretreated with anti-PMNL serum were 50% lower than those from rats pretreated with saline ($p< 0.001$) and 30% lower than non-immune serum (not significant, $p= 0.068$). These results suggest that depletion of neutrophils may result in decreased formation of leukotrienes by lung cells following allergen challenge.

Products of the 12-lipoxygenase and cyclooxygenase pathways. Anti-PMNL serum pretreatment also seemed to decrease the formation of the 12-lipoxygenase product 12-HETE by lung cells ($p< 0.05$ vs. saline; Fig. 33B). The production of the cyclooxygenase product 12-HHTrE by lung cells obtained 6h after OVA challenge was also decreased significantly in rats pretreated with anti-PMNL serum ($p <0.05$ vs. both the saline and non-immune group). These results suggest the possible contribution of pulmonary neutrophils to the formation of 12-HETE and 12-HHTrE in allergic lungs.

3.6.4. Relationship between leukocytes and eicosanoid synthesis

The relationships between the synthesis of eicosanoids and the numbers of neutrophils and macrophages in the lung cell suspensions are shown in Figs. 34 and 35. There appeared to be a positive correlation between the percentages of neutrophils in the suspensions of mixed lung cells and the production of LTB_4, but this was only of borderline significance ($p=0.05$) due to the presence of the outlier referred to above (Fig. 34). If the value for the outlier is not included in the analysis, there is a highly significant positive correlation between the percentage of neutrophils in the suspensions of mixed lung cells and the production of LTB_4 ($p< 0.01$), cysteinyl-LTs ($p< 0.05$) and 5-HETE ($p<$

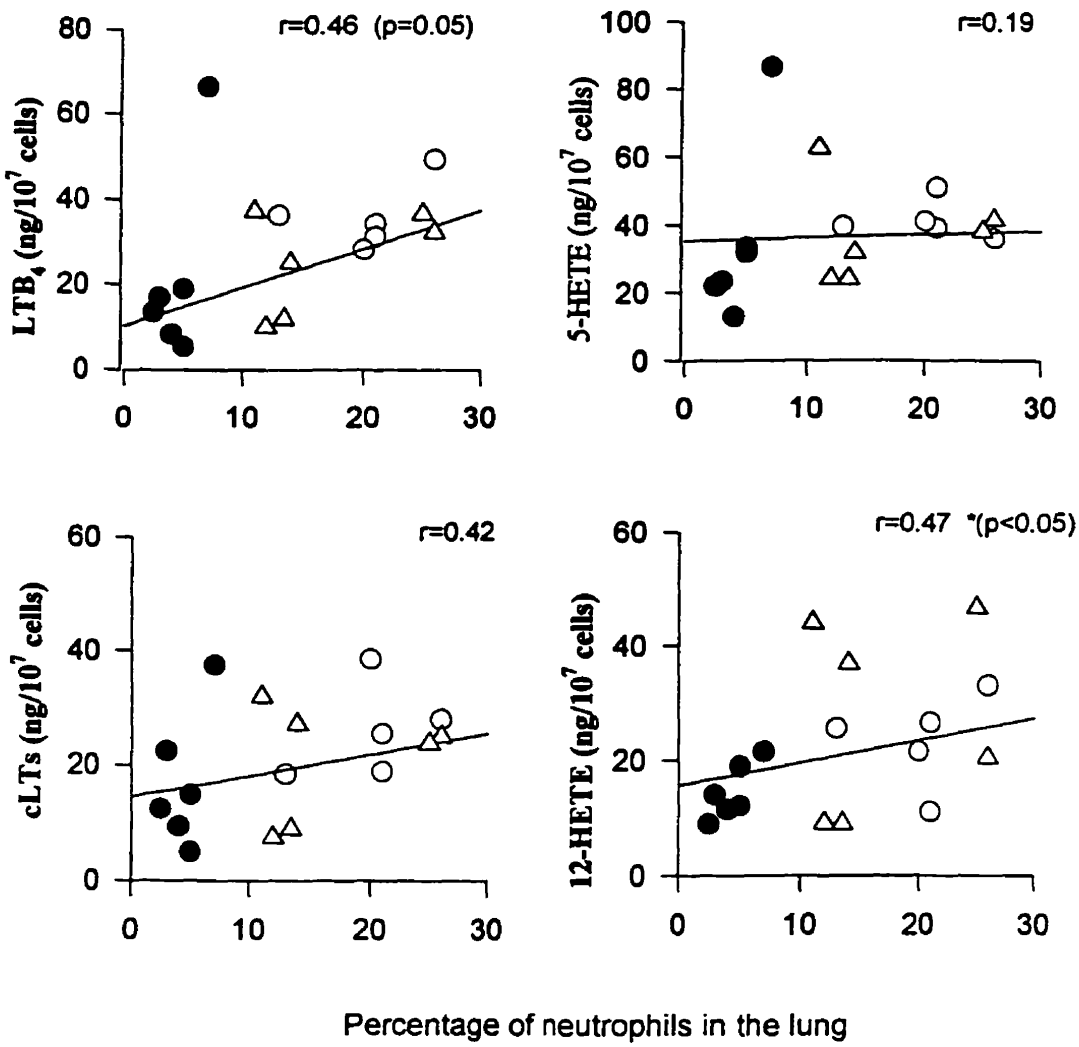

Fig. 34. Relationship between the percentage of neutrophils and eicosanoid formation by lung cells. Data shown in the plots include all values from the three experimental groups: saline control (open circles), non-immune serum (open triangles), and anti-PMNL serum (filled circles). Each data point represents a determination (performed in duplicate) on cells from a single rat. The amounts of LTB_4, cLTs, 5-HETE and 12-HETE produced by lung cells were plotted against the percentages of neutrophils. There was considered to be a positive correlation when the p value was less than 0.05 (Pearson's correlation).

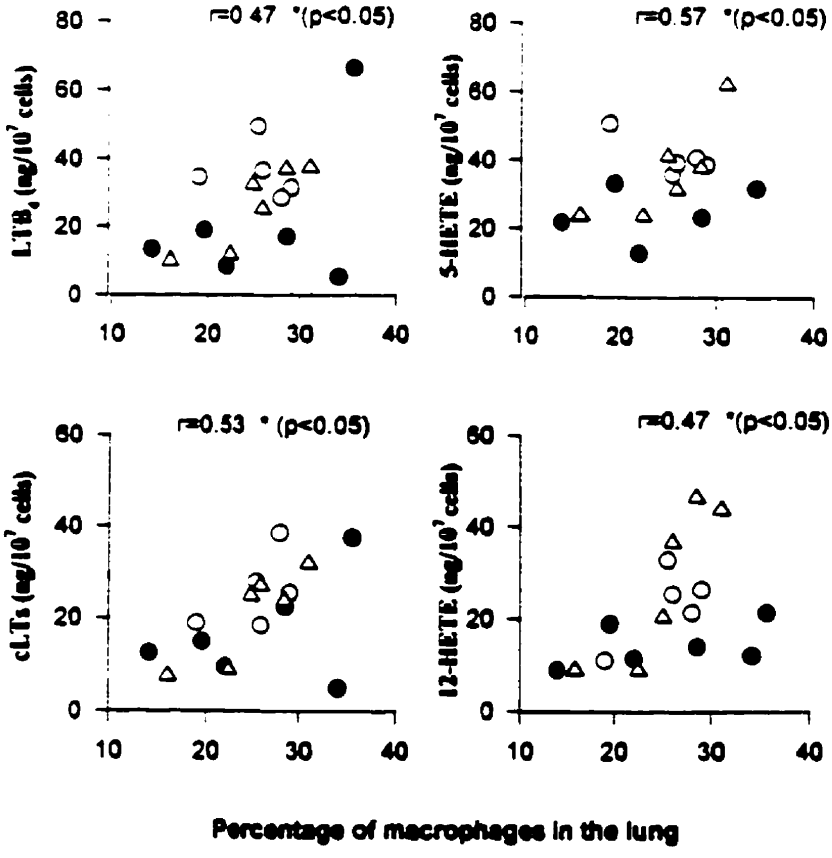

Fig. 35. Relationship between the percentage of macrophages and eicosanoid formation by lung cells. Data shown in the plots include all values from the three experimental groups: saline control (open circles), normal serum (open triangles), and anti-PMNL serum (filled circles). Each data point represents a determination (performed in duplicate) on cells from a single rat. The amounts of LTB_4, cLTs, 5-HETE and 12-HETE produced by isolated lung cells were plotted against the percentages of macrophages. There was considered to be a positive correlation when the p value was less than 0.05 (Pearson's correlation).

0.05) (Table 11). The production of 12-HETE also appeared to be positively correlated with the numbers of neutrophils in the lung cell suspensions ($p< 0.05$) irrespective of the presence or absence of the outlier. In contrast, all of the 5-lipoxygenase products as well as 12-HETE were positively correlated with the numbers of macrophages in the lung cell suspensions (Fig 35). When the outlier is removed the macrophages are only positively associated with the formation of 12-HETE by lung cells (Table 11). Therefore, although the presence of the outlier has complicated the interpretation of the results, our findings suggest that neutrophils may contribute to leukotriene formation in the lungs of BN rats following allergen challenge.

TABLE 11

The correlation between the percentages of leukocytes and eicosanoid formation by lung cells*

Eicosanoids	*vs.* Neutrophils		vs. Macrophages	
	Include outlier	Exclude outlier	Include outlier	Exclude outlier
LTB_4	0.46 ($p = 0.05$)	0.76 ($p < 0.01$)	0.47 ($p < 0.05$)	0.27
cLTs	0.42	0.56 ($p < 0.05$)	0.55 ($p < 0.05$)	0.39
5-HETE	0.19	0.51 ($p < 0.05$)	0.53 ($p < 0.05$)	0.42
12-HETE	0.47 ($p < 0.05$)	0.48 ($p < 0.05$)	0.47 ($p < 0.05$)	0.53 ($p<0.05$)

Data from Figs 34 and 35 were analyzed to test whether excluding the outlier would affect the correlation between the percentages of neutrophils and macrophages in the lung cell suspensions and the formation of eicosanoids by lung cells. There was considered to be a positive correlation when the p value was less than 0.05 (Pearson's correlation).

3.7. ROLE OF EICOSANOIDS IN LEUKOCYTE MIGRATION AND CELLULAR INFILTRATION INTO THE LUNGS

Allergen challenge resulted in significant neutrophil infiltration and increased LTB_4 synthesis by lung cells at 6 h, followed by an influx of eosinophils which was observed 24h after challenge. This finding would be consistent with a role for LTB_4 in the eosinophilic infiltration which occurs in response to OVA in sensitized rats as reported by others (349). Other eicosanoids produced by inflammatory cells such as cysteinyl-LTs and 5-oxo-ETE have also been reported to have chemotactic effects on eosinophils and neutrophils. We therefore investigated the effects of LTB_4 and the other eicosanoids mentioned above on granulocyte migration both *in vitro* and *in vivo* in rats and rabbits.

3.7.1 Effects of eicosanoids on rabbit neutrophil migration

Due to the difficulty in isolating adequate numbers of normal neutrophils from rat peripheral blood, we evaluated the effects of eicosanoids on rabbit neutrophil migration using an *in vitro* Boyden chamber assay. In agreement with previous reports (223), LTB_4 was found to be a potent stimulus of rabbit neutrophil migration (Fig. 36). In contrast to fMLP and LTB_4, 5-oxo-ETE (Fig. 36) and LTC_4, LTD_4 and LTE_4 (data not shown) showed no significant chemotactic effects.

LTB_4 and 5-oxo-ETE were further tested for chemotactic activity in an *in vivo* assay. Due to the convenience of collecting multiple blood samples from rabbits for the analysis of the dynamic changes in leukocyte numbers in blood, we explored a method described by Marleau et al. (229) for the evaluation of *in vivo* leukocyte activation.

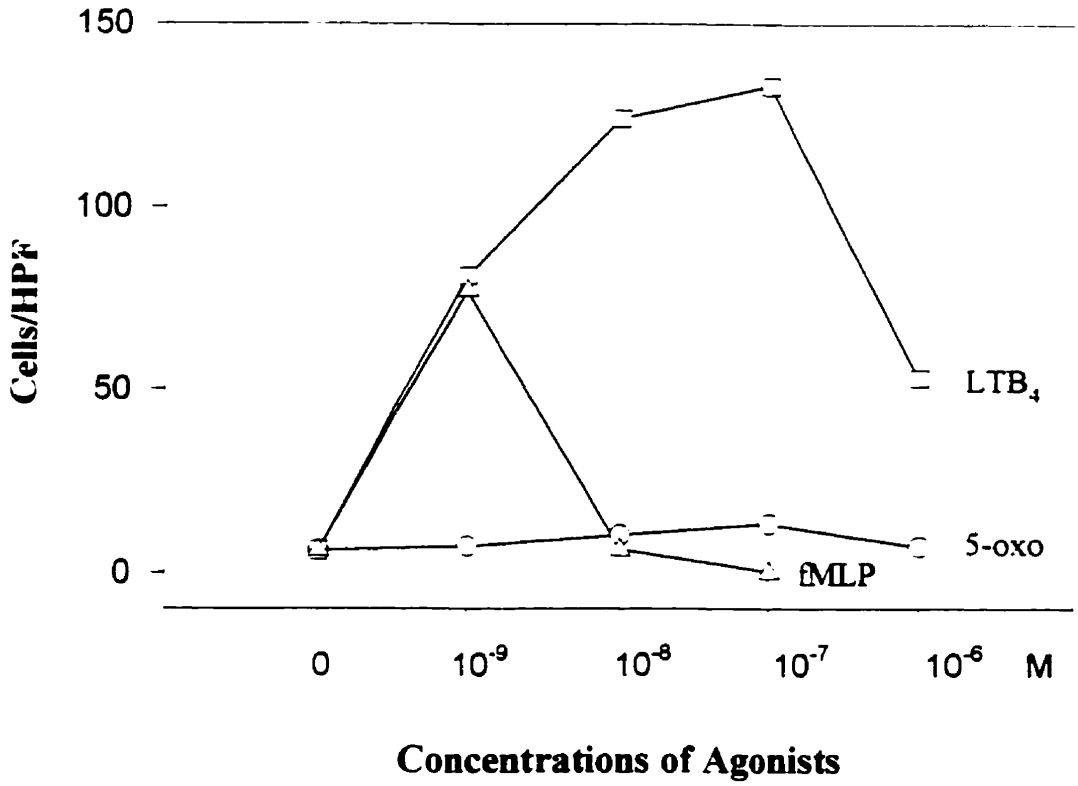

Fig 36. Chemotactic effects of LTB_4 and 5-oxo-ETE on rabbit neutrophils. Neutrophils were placed in the upper chambers of modified Boyden chambers, whereas various concentrations of LTB_4 (□), 5-oxo-ETE (○) and fMLP (△) were placed in the lower chambers. The numbers of neutrophils which penetrated to the bottoms of the filters were counted. The data are representive of at least three independent experiments. HPF, high power (x 400) field.

Consistent with the findings of others (229,350), intravenous injection of LTB_4 (3 μg/kg) induced an early reversible neutropenia followed by a later but sustained neutrocytosis in rabbits (Fig. 37A). However, 5-oxo-ETE (3 μg/kg) had no significant effects on blood neutrophil levels (Fig 37B). Similarly, 12(R)- and 12-(S)-HETE (3-6 μg/kg) also had no apparent effects on circulating neutrophils (data not shown).

3.7.2. Effects of eicosanoids on cellular infiltration into the pleural cavities of rats

The abilities of LTB_4, LTE_4 and 5-oxo-ETE to induce infiltration of inflammatory cells into the pleural cavities of rats were then evaluated. Injection of LTB_4 induced eosinophil infiltration into the pleural cavities of both Wistar- (Table 12) and BN- (Table13) rats. However, we observed considerable varibility in the responses of neutrophils from different rats to 5-oxo-ETE (Tables 12 & 13) and LTE_4 (Table 13). As shown in Table 12, LTB_4 was found to specifically induce eosinophil infiltration into the pleural cavities of rats after 24h. In contrast, 5-oxo-ETE tended to increase the total numbers of cells recovered from the pleural cavities of both Wistar and BN rats, although this was not statistically significant. Although it did not change the proportions of the three types of inflammatory cells in Wistar rats (Table 13), 5-oxo-ETE significantly decreased the levels of neutrophils in the pleural cavities of BN rats ($p < 0.05$ *vs.* vehicle control). LTE_4 also appeared to induce infiltration of eosinophils and neutrophils into the pleural cavities of BN rats but this was not significant (Table 13).

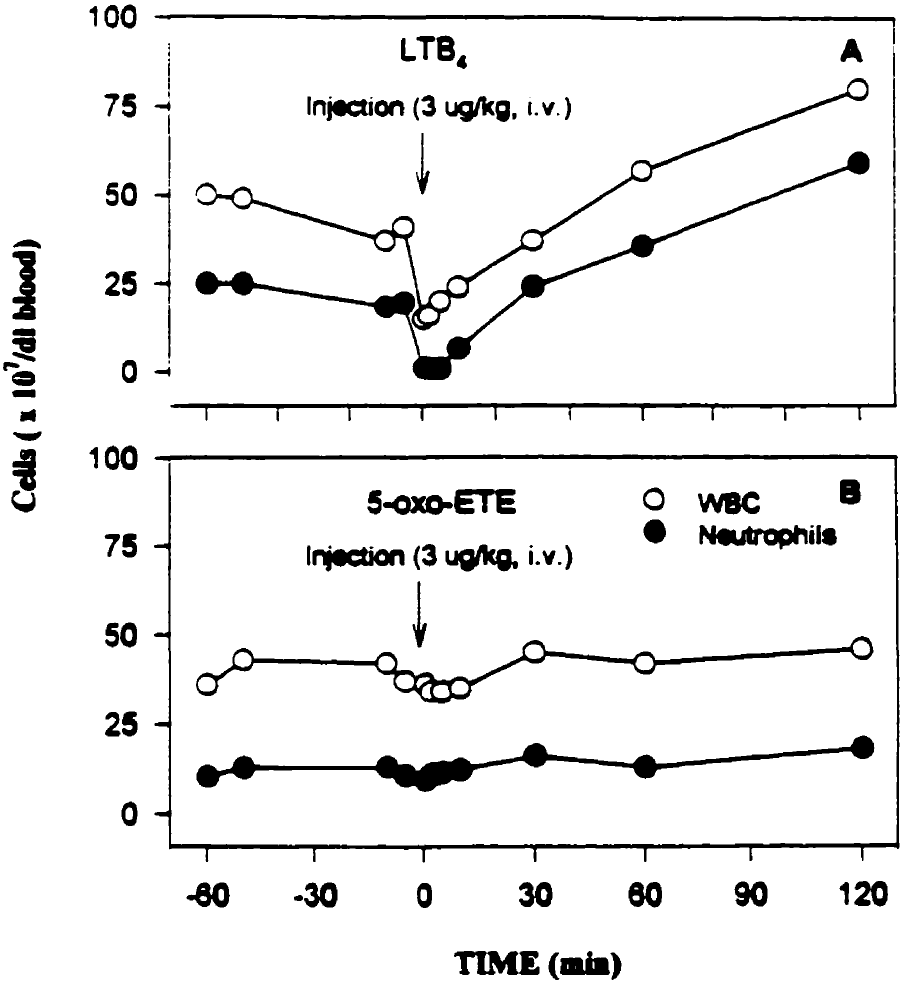

Fig 37. Dynamic changes in the numbers of blood leukocytes (open circles) and neutrophils (filled circles) in rabbits after intravenous injection of LTB_4 (A) and 5-oxo-ETE (B). Both LTB_4 (3 μg/kg) and 5-oxo-ETE (6 μg/kg) were injected at time 0 (*arrow*). The data are representive of three independent experiments.

TABLE 12

Cellular infiltration into the pleural cavities of Wistar rats 24h after administration of LTB_4 and 5-Oxo-ETE*

	Total cells	Eosinophils (%)	Neutrophils (%)	Mon/Lym (%)
Vehicle	6.0 ± 1.1	9.8 ± 0.6	13.2 ± 1.9	71.6 ± 2.0
LTB_4	4.0 ± 0.4	30.1 ± 4.1*	14.8 ± 2.1	52.0 ± 6.7
5-oxo-ETE	13 ± 5.4	8.6 ± 1.3	15.8 ± 1.4	65.8 ± 1.2

*5 μg of agonist was injected into the pleural cavities of rats. The pleural cavities were then washed three times with 3 ml of saline 24 h later. Differential cell counts were determined on cytospin slides stained with Diff-Quik. Each value represents the mean ± SEM of data from at least four animals. *$p < 0.05$ compared with vehicle.

TABLE 13

Cellular Infiltration into the Pleural Cavities of BN Rats 24h after Administration of LTB_4, 5-oxo-ETE and LTE_4

	Total cells (x 10^6)	Eosinophils (%)	Neutrophils (%)	Lymph (%)	Mon/Mac. (%)
Vehicle	1.4 ± 0.4	3.1 ± 0.6	18.8 ± 4.1	8.5 ± 3.6	65.9 ± 4.0
LTB_4	1.8 ± 0.6	7.8 ± 0.7*	12.6 ± 4.8	8.3 ± 1.6	64.5 ± 5.6
5-oxo-ETE	2.4 ± 0.3	3.4 ± 2.1	5.6 ± 2.0*	4.4 ± 2.4	84.4 ± 6.2
LTE_4	3.3 ± 0.5	7.4 ± 2.8	29.6 ± 7.8	4.3 ± 0.8	56.3 ± 8.0

*5 μg of agonist was injected into the pleural cavities of rats. The pleural cavities were then washed with 3 ml of saline for three times 24 h later. Differential cell counts were determined on cytospin slides stained with Diff-Quik. Each value represents the mean ± SEM of data from at least four animals. *$p < 0.05$ compared with vehicle control.

3.7.3. Effects of Eicosanoids on the Infiltration of Eosinophils into the Lungs of BN Rats

To determine which 5-lipoxygenase products are capable of inducing eosinophil infiltration into the lungs, The effects of leukotrienes B_4, D_4, and E_4, as well as 5-oxo-ETE on eosinophil levels in lung tissues and airways were investigated. The eicosanoids to be tested were instilled into airways via an endotracheal catheter. BAL fluid and lung tissues were collected 24h after administration of agonists. The eosinophils on cytospin slides of BAL cells or lung tissue sections were identified by immunocytochemistry using an eosinophil-specific anti-major basic protein monoclonal antibody and alkaline phosphatase anti-alkaline phosphatase (APAAP) as described in Materials and Methods. As shown in Fig 38, LTB_4 ($p < 0.01$) and 5-oxo-ETE ($p < 0.05$) induced increased eosinophil numbers in lung tissue. This also appeared to be true for BAL fluid, but in this case there was considerable varibility and the difference were not significant. Although LTE_4 tended to induce varible eosinophil and neutrophil infiltration (statistically not significant) in the pleural cavities of BN rats (Table 12), neither LTE_4 nor LTD_4 caused eosinophilic influx into the airways or lung tissue (Fig 38). Since LTB_4 is the predominant 5-lipoxygenase metabolite produced by lung cells following allergen challenge, it may be the major eicosanoid responsible for the eosinophilic infiltration at 24h after OVA challenge in BN rats.

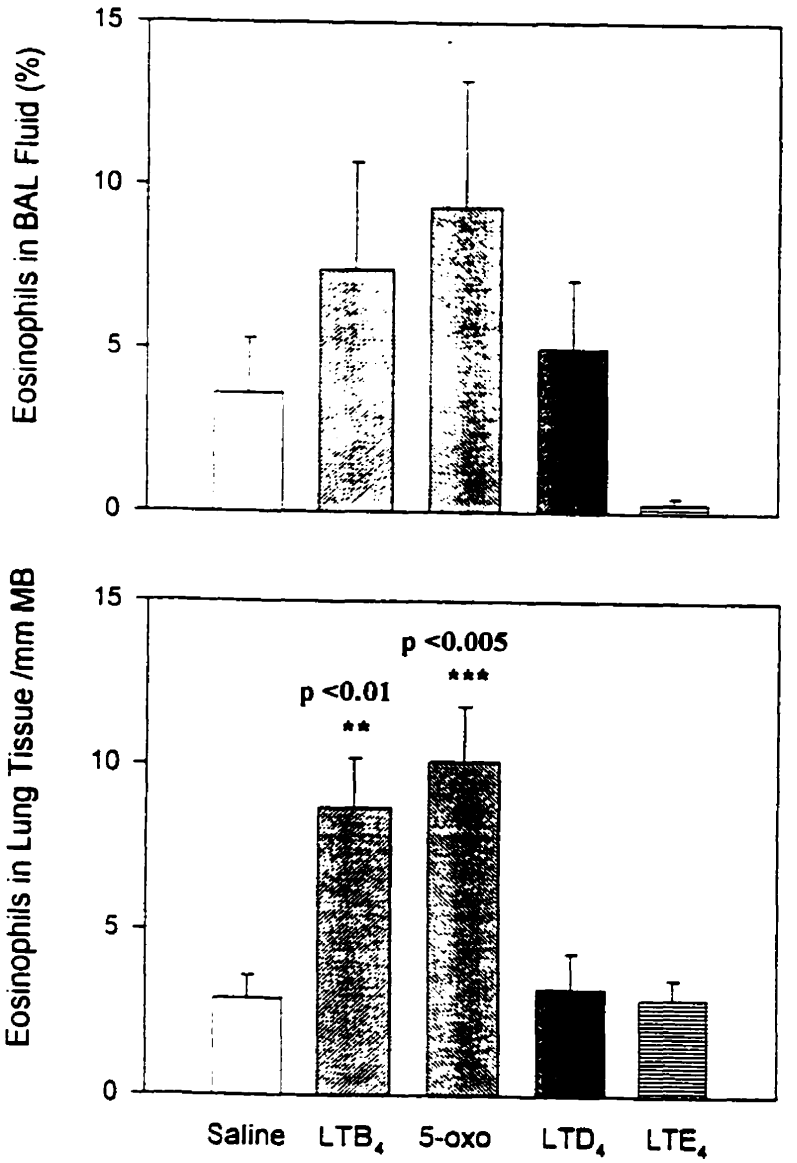

Fig. 38. Eosinophil counts in BAL fluid and lung tissues after insufflation of eicosanoids into the airways of BN rats. Eosinophilic infiltration after insufflation of the agonists (5 µg/rat) was assessed by immunocytochemistry using BMK13, an anti-human eosinophil major basic protein mAb. The antigen-positive cells were visualized using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method (202). The data represent means ± SEM. **: $p < 0.01$; ***$p < 0.005$ compared with saline controls.

4. DISCUSSIONS

4.1. ANALYSIS OF EICOSANOIDS BY REVERSED-PHASE HPLC

A variety of techniques have been reported for the analysis of leukotrienes and other lipoxygenase products by RP-HPLC. However, a number of problems have been encountered during analysis of cysteinyl-LTs in biological samples because their chromatographic behaviour is quite different from that of other eicosanoids due to the presence of amino groups. To circumvent some of these problems, we developed various binary mobile phase systems which give very good resolution of cysteinyl leukotrienes and other eicosanoids not containing amino acids and avoid the baseline fluctuations encountered with ternary systems (325,327).

Analysis of mixtures of eicosanoids not containing cysteinyl-LTs by reversed-phase HPLC on ODS-silica columns is relatively straightforward, since the t_R's and peak shapes are relatively independent of the particular brand of ODS-silica used as well as the nature of the acidic modifier (trifluoroacetic acid, acetic acid, phosphoric acid, etc.). In this case, a major consideration is the relative amounts of methanol and acetonitrile in the mobile phase, since there are substantial selectivity differences between these two solvents (326).

The HPLC systems developed in the present study were designed for the analysis of mixtures of cysteinyl-LTs and other eicosanoids which do not contain amino groups. For the analysis of cysteinyl-LTs, both the precise nature of the ODS-silica and the acidic modifier present in the mobile phase are critical because of the positively charged amino

groups of these compounds. It should be pointed out that the LTE_4 metabolite N-acetyl-LTE_4 (65) exhibits chromatographic behaviour similar to the nonamino acid-containing eicosanoids since its amino group is acetylated.

In addition to the dramatic changes in selectivity between cysteinyl-LTs and other eicosanoids induced by altering the concentration of TFA, there are also changes in selectivity between LTD_4 and LTE_4. As previously reported (326), when the mobile phase contains very low concentrations of TFA, the t_R of LTD_4 is greater than that of LTE_4 (Fig. 8 and 9). However, with mobile phases containing higher concentrations of TFA (Fig. 10 and 11), LTE_4 has a slightly longer t_R than LTD_4 (cf. Ref. 326).

We have analyzed mixtures of cysteinyl-containing leukotrienes on many different stationary phases and have found that, in general, TFA affects their t_R's in a manner similar to that reported previously (326) (data not shown). However, there were considerable differences between stationary phases in the degree of retention of cysteinyl-LTs and the sensitivity to changes in the concentration of TFA. In addition, certain stationary phases, such as Ultrasphere ODS, give rather poor peak shapes with these compounds. Compared to other stationary phases, Spherisorb ODS-2 exhibits a high degree of retention of cysteinyl-LTs at low concentrations of TFA. The t_R's of these compounds are quite sensitive to changes in the concentration of TFA, allowing them to be readily manipulated, and excellent peak shapes are obtained. It should be noted that the separations obtained using the mobile phases reported here with types of ODS-silica other than Spherisorb ODS-2 may be quite different, depending on the degrees of end-capping and carbon loading.

The chromatograms shown in Figs. 8 & 9 show that complex mixtures of leukotrienes and other lipoxygenase products can be analyzed on Spherisorb ODS-2 columns using mobile phases consisting of binary gradients containing low concentrations of TFA. The addition of acidic modifiers such as TFA (325,326), heptafluorobutyric acid (329,351), or phosphoric acid (326,327) to mobile phases has been shown to be very useful for the chromatography of cysteinyl-LTs, since sharp peaks are obtained without the use of EDTA (324) to complex divalent cations. TFA is particularly useful, since it is volatile and can therefore be readily removed from column fractions. However, it is important to note that since TFA is a relatively strong acid, the acidity of the fraction will increase as the solvent is evaporated, resulting in a very low pH just prior to complete removal of the solvent. This can cause degradation of cysteinyl-LTs and low recoveries. To circumvent this problem, ammonium hydroxide can be added to the column fractions prior to evaporation, resulting in excellent recoveries of cysteinyl-LTs after evaporation of the column solvent.

Although our group (325) and others (327) have shown that ternary gradients give excellent resolution of a large number of eicosanoids, with cysteinyl-LTs appearing at the end of the gradient, the baselines obtained using these conditions may contain spurious peaks which can interfere with the analysis. This is presumably due to the accumulation of various contaminants on the column during the first phase of the chromatography, which utilizes either low concentrations of TFA (325) or phosphoric acid (327). These contaminants are rapidly eluted from the column early in the second phase of the gradient when the concentration of TFA or the pH is increased. These problems are largely

circumvented by the use of the binary gradients described in the present study

The chromatograms in Figs. 10, 11 and 12 were obtained with a column of Spherisorb ODS-2 with an internal diameter less than that used for Figs. 8 and 9. Reducing the internal diameter of the column from 4.6 to 3.2 mm has the advantages of improved sensitivity and a reduction in solvent consumption by half, since the flow rates can be reduced by 50% compared to the wider bore column. Since the cysteinyl-LTs have t_R's between 12-HHTrE and 15-HETE, analysis can be completed in as little as 20 min (Fig. 9) or somewhat longer if it is necessary to analyze more polar eicosanoids such as hydroxylated metabolites of LTB_4 or lipoxins (Figs. 11 and 12).

As discussed above, we have developed several methods for the analysis of complex mixtures of eicosanoids in biological samples (Figs. 8-12). The optimal conditions for a particular application will vary, depending on the metabolites to be measured. For example, the conditions shown in Figs. 8 are very useful for the analysis of cysteinyl-LTs since they are well separated and have t_R's longer than those of non-amino acid-containing eicosanoids. On the other hand, if it is necessary to analyze a larger number of eicosanoids, the conditions illustrated in either Figs. 9 or 11 can be used.

4.2. EICOSANOID SYNTHESIS BY LUNG TISSUE AND ISOLATED LUNG CELLS

The calcium ionophore A23187 is a very useful tool to measure the capacity of cells to synthesize eicosanoids. It is not cell-specific and dramatically elevates cytosolic calcium levels, resulting in activation of both 5-lipoxygenase and $cPLA_2$. Because it is a

strong stimulus, the amounts of products released can readily be analyzed by HPLC When added to lung cells, A23187 stimulates the formation of a variety of lipoxygenase and cyclooxygenase products.

Both rat lung fragments and isolated lung cells were shown to produce a complex mixture of leukotrienes, HETEs and the cyclooxygenase product 12-HHTrE in response to calcium ionophore stimulation. However, there were appreciable differences in the profiles of products formed by lung fragments and isolated lung cells. It should be noted that although prostaglandins and TXA_2 can also be formed by the lung and may play an active role in asthma (2), these metabolites are not detectable with the HPLC assay used for this study becasue they absorb uv light only at very low wavelength.

Lung fragments seemed to produce more HETEs than LTs, particularly in the presence of exogenous arachidonic acid, whereas isolated lung cells released about equal amounts of leukotrienes and HETEs (Figs. 13 & 14). These differences might be the result of differential enzymatic activation in these two preparations. For example, in lung fragments the cells residing on airway surfaces, including epithelial cells, mast cells, macrophages and eosinophils, would be well-preserved and would be more accessible to the medium containing calcium ionophore. In contrast, all the cells in lung cell suspensions had equal probability of interaction with the stimulus. It should also be noted that in the case of isolated cell suspensions, we could not exclude the possibility of loss of eicosanoid synthetic activity due to preferential protease digestion of some types of cells. Nevertheless, considering the complexity of the cellular composition of the lungs, the use of isolated lung cells made it much easier to investigate the relationship between

eicosanoid synthesis and the presence of different types of inflammatory cells

Although 15-HETE is a major product of arachidonic acid metabolism in human lung (145,343,347), it is only a minor product formed by rat lung in response to calcium ionophore stimulation (Fig. 14). In humans, 15-lipoxygenase is expressed primarily in eosinophils and reticulocytes as well as in epithelial cells in nasal, tracheal, and bronchial epithelium (2). In contrast, very small amounts of 15-HETE were synthesized by partially purified rat eosinophils (Fig. 28). Therefore, it would appear that in the rat these cells have a lower level of 15-lipoxygenase activity than in humans. Consistent with the low level of 15-lipoxygenase activity, we did not observe lipoxin formation by lung cells from BN rats. Unlike human neutrophils, rat neutrophils synthesize little 5-oxo-ETE. Similarly, lung cells produced only very small amounts of this substance unless they were incubated with PMA and arachidonic acid as well as A23187. PMA and arachidonic acid have been shown to stimulate the production of 5-oxo-ETE in human neutrophils (384).

4.3. CELLULAR INFILTRATION AND LEUKOTRIENE SYNTHESIS BY LUNG CELLS FOLLOWING ALLERGEN CHALLENGE

BN rats respond to sensitization with the development of increased titers of specific IgE antibody (320). In a preliminary study, we found that allergen sensitization itself did not result in significant changes in either total cell numbers or in the proportions of different cell types. This observation is consistent with reports that sensitization *per se* was not accompanied by significant changes in the cellular distribution and the relative proportions of cells recovered in BAL fluid from BN rats (318,346,352). Likewise,

allergen challenge of nonsensitized rats also caused no increase in cell recovery from BAL fluid (318).

Lung cells isolated from unchallenged ovalbumin-sensitized rats synthesized equal amounts of eicosanoids compared with the cells from control unsensitized rats (Table 6), suggesting that sensitization alone has no effects on eicosanoid formation by lung cells.

Allergen challenge has been reported to induce a neutrophilic influx into the lungs of humans (127,179,353,354), sheep (355), guinea pigs (356) and BN rats (346). This is confirmed by the present results, which show that ovalbumin challenge of sensitized BN rats resulted in a significant increase in the number of pulmonary neutrophils 6h after challenge (Fig. 18). In contrast, no significant changes occurred in eosinophil numbers after 6h (Fig. 18), consistent with a previous study indicating that eosinophil levels in BN rat lungs did not change significantly 8 hours after antigen challenge (346). However, it was reported in the above study that there was a reduction in eosinophils in lung parenchyma and small airways at this time in rats which exhibited a late response (346). This possible discrepancy may be due to methodological differences between the two studies. In the present investigation, lung cells (ca. 100 million cells/rat) were prepared by perfusion of lungs with a low concentration of protease via the trachea (cf. 334), whereas in the previous study, lung cells were prepared by mincing and digestion with collagenase of large airways and small airways/parenchyma separately. In the latter case, the total recovery of lung cells was lower (between 10 and 30 million cells/rat). Another difference was that in the previous study, the airways were suctioned, possibly resulting in losses of inflammatory cells.

Ovalbumin challenge of sensitized BN rats not only induced an increased neutrophil infiltration but also resulted in increased formation of LTB_4, 5-HETE and 12-HHTrE by lung cells 6h after challenge (Figs. 18, 21, 23 and 24). This observation provides direct evidence of increased 5-lipoxygenase and cyclooxygenase activities in the lung following allergen challenge. Although neutrophils from asthmatic patients were previously shown to have greater 5-lipoxygenase activity than neutrophils from normal subjects (357), there were no prior reports in the literature on the increased production of leukotrienes by isolated lung cells after allergen challenge.

Although inhalation of saline also resulted in an influx of neutrophils into the lung, albeit to a lesser extent than with antigen, LTB_4 production by lung cells was not significantly increased. This could be due in part to our inability to detect LTB_4 production associated with increased neutrophil numbers against a background of LTB_4 production by other lung cells such as macrophages. Another explanation might be that the neutrophils in lungs from antigen-challenged rats were activated compared to neutrophils from saline-treated rats. This could be due to priming of the neutrophils from ovalbumin-treated animals by cytokines such as GM-CSF, which has been shown to be increased in lungs after antigen challenge (358). GM-CSF could stimulate leukotriene formation at several levels, including activation of the cytosolic form of phospholipase A_2 (359) and increased expression of 5-lipoxygenase (360) and its activating protein FLAP (361). Granulocytes in BAL fluid from a subpopulation of antigen-challenged sheep which displayed late responses have also been shown to exhibit enhanced LTB_4 production compared to granulocytes from antigen-challenged sheep which exhibited only

acute phase responses (362) The present study shows that, as is the case for other strains of rats (363), neutrophils from BN rats synthesize large amounts of LTB_4 upon stimulation. Because of their presence in increased levels, along with their possible activation due to antigen challenge, these cells may be primarily responsible for the increased production of LTB_4 induced by OVA.

The capacity of lung cells to synthesize leukotrienes was shown to change with time following ovalbumin challenge (Fig 21). 5-Lipoxygenase activity increased gradually over the first 4 hours, reached maximal levels at 6h, and then decreased to the level of control by 24h after challenge. The increase in 5-lipoxygenase activity at 6h was significant compared with either unchallenged or saline-challenged controls (Fig 23). It also seemed to be well correlated with the timing of neutrophil infiltration in the lung of BN rats, suggesting that these cells may be the source of this increased enzyme activity. The observed decrease in 12-HETE formation (Fig 21) between 4 and 8 h after ovalbumin challenge is more difficult to understand.

Consistent with other reports (320,349,352), we observed eosinophil infiltration into the lungs 24h after challenge with OVA (Fig. 18). In spite of this, there were no detectable increases in the ability of rat lung cells to synthesize any of the eicosanoids we measured. In fact there was an inverse correlation between the percentage of eosinophils in isolated lung cells and the production of cysteinyl-LTs, suggesting that BN rat eosinophils, unlike human eosinophils (158,159), are unlikely to be a major site for the synthesis of these substances in the lungs of this species.

It has been shown that antigen challenge of sensitized BN rats results in increased

excretion of cysteinyl-LTs in the bile (317). There was a positive correlation between the severity of the late response in these animals and the amounts of *N*-acetyl-LTE_4 excreted in the bile at a time corresponding to the late response. However, in the present study we were unable to detect a significant increase in the capacity of lung cells to synthesize cysteinyl-LTs at any time following OVA challenge. This may be due in part to variability in the intensity and timing of the late response in these animals. In addition, it is unlikely that all of the rats in the present study would have undergone a late response, since this is normally observed only in 70-75% of antigen-challenged BN rats (300,317). Although we attempted in preliminary studies to monitor airway responses prior to sacrifice of the rats and preparation of lung cells, this proved to be impractical because of the large differences in the timing and pattern of the late responses among animals. Another possible explanation for this could be that the cell isolation procedure attenuates the function of the 5-lipoxygenase pathway in the particular cell types responsible for the synthesis of cysteinyl-LTs during the late response. For example, it was reported that mast cells freshly purified from human lung synthesized small amounts of cysteinyl-LTs compared with mast cells co-cultured for seven days with murine 3T3 cells (142). This raises the possibility that assessment of eicosanoid synthesis immediately after enzymatic dispersion of cells from the lungs may underestimate the potential for cysteinyl-LT synthesis by certain types of cells. On the other hand, it is also possible that the low synthesis of cysteinyl-LTs by lung cells after isolation is more typical of the *in vivo* situation. Although we cannot exclude these possibilities, the fact that we were able to detect increased LTB_4 synthesis in lung cells 6h after antigen challenge indicates that

it is possible to detect increased synthesis of products of the 5-lipoxygenase pathway in at least some types of cells. Alternatively, the increased release of cysteinyl-LTs in bile during the late phase response may be more a feature of IgE-mediated cell activation than an increased expression of the enzymes responsible for the synthesis of cysteinyl-LTs in the lung. The fact that the requirement for IgE is bypassed when the cells are stimulated with A23187 may explain the lack of increase in cLT synthesis by lung cells following antigen challenge.

4.4. LEUKOTRIENE SYNTHESIS BY PULMONARY EOSINOPHILS, MACROPHAGES AND NEUTROPHILS

To determine the nature of eicosanoids synthesized by BN rat pulmonary eosinophils, we attempted to purify these cells from the lungs of rats treated with Sephadex particles, which have been shown to induce pulmonary eosinophilia (341). In spite of a considerable elevation in eosinophil levels, unfractionated lung cells from Sephadex-treated rats did not synthesize more cysteinyl-LTs than cells from control rats (Fig. 27). Because of the high degree of heterogeneity in the density of BN rat pulmonary eosinophils after enzymatic digestion of the lungs, it was not possible to achieve a maximal purity of greater than 56% using density gradient centrifugation. However, the fractions which contained the highest proportions of eosinophils did not synthesize appreciable amounts of cysteinyl-LTs (Fig. 28). Furthermore, consistent with our results from OVA-challenged rats, the fractions with the lowest numbers of eosinophils appeared to produce greater amounts of cysteinyl-LTs than the fractions with the highest numbers

of these cells.

We also attempted to purify eosinophils from BAL from Sephadex-treated rats but were unsuccessful. Unlike BAL from Sephadex-treated guinea pigs, which has been reported to contain over 80 x 10^6 cells per animal, of which over 40% are eosinophils (364), BAL from Sephadex-treated rats contained only between 1 and 5 x 10^6 cells/rat, of which only 10% were eosinophils. We were unable to obtain sufficient numbers of these cells in high purity from rat BAL because of the low initial cell numbers and losses incurred during the purification procedures. Although we could find no evidence for the synthesis of appreciable amounts of cysteinyl-LTs from partially purified BAL eosinophils, the low numbers of cells available made it difficult draw a definitive conclusion about the profile of lipoxygenase products synthesized by these cells.

Our results on the synthesis of lipoxygenase products by lung cell fractions enriched in eosinophils as well as the lack of correlation between the production of cysteinyl-LTs by unfractionated lung cells and eosinophil numbers strongly suggest that rat eosinophils do not synthesize appreciable amounts of cysteinyl-LTs. In this respect, they appear to be similar to guinea pig eosinophils, which convert LTA_4 to LTB_4 rather than to LTC_4 and release thromboxane B_2 and LTB_4 upon stimulation (339,365). These cells may lack LTA_4 glutathione S-transferase (LTC_4 synthase) which is required for the formation of LTC_4 (339). Human (158) and rhesus monkey (366) eosinophils, on the other hand, form only very small amounts of LTB_4, but are very active in synthesizing LTC_4. Murine eosinophils were shown to release LTC_4 in response to calcium ionophore (367). However, it is not known whether murine eosinophils synthesize LTB_4 and other

eicosanoids. There are species differences not only in the production of leukotrienes by eosinophils but also in functional response of these cells to LTB_4. For example, LTB_4 is a potent chemotactic agent for guinea pig eosinophils, but exhibits only weak chemotactic activity for human and monkey eosinophils (366).

In contrast to eosinophils, BN rat alveolar macrophages synthesized substantial amounts of cysteinyl-LTs upon stimulation with A23187 (Fig. 28). Thus macrophages may be an important source of cysteinyl-LTs in the lungs of this species. Our results are in agreement with previous data showing that alveolar and pulmonary interstitial macrophages from Sprague-Dawley rats are quite active in the synthesis of 5-lipoxygenase products (368). In the latter study it was found that the ratio of LTB_4 to LTC_4 was about 10:1, whereas we found that significant amounts of cysteinyl-LTs (i.e. LTC_4 + LTD_4 + LTE_4) were produced by BN rat alveolar macrophages and the ratio of LTB_4 to LTC_4 was about 2:1. More recently, it was reported that GM-CSF increases the capacity of alveolar macrophages, but not peritoneal macrophages or peripheral blood monocytes, to generate leukotrienes (369). In light of these observations and the fact that alveolar macrophages are the predominant leukocytes in the airways and have the ability to release mediators and cytokines (153,370) in response to IgE-mediated stimulation, it is quite possible that alveolar macrophages may play an important role in airway inflammation of allergic asthma.

Another potentially important site for the synthesis of cysteinyl-LTs in the lung is the mast cell. In human and guinea pig, mast cells account for between 3 and 5 % of total lung cells (331,371). The major eicosanoids produced by purified human mast cells

in response to IgE-dependent stimuli are PGD_2 and LTC_4 (141). However, we found that cell suspensions from BN rat lungs usually contained only about 0.5% mast cells, suggesting that these cells may not be as important a site for the synthesis of cysteinyl-LTs in rats as in humans.

In agreement with our findings, Bachelet et al. (372) found that in tracheal mucosa fewer mast cells were found in rats than in guinea pigs (3.5 ± 0.2 *vs.* 12.1 ± 0.3 mast cells/mm length of trachea). Although there were about the same numbers of mast cells in larger bronchi from both species, the smaller bronchi in guinea pigs contained about 5 time more mast cells than in rats. In addition, mast cells were never observed in the interalveolar septa in rats, but in contrast, they were present at this site in guinea pigs. However, Tainsh et al. (373) reported that the crude mixture of rat lung cells obtained after incubating chopped lung tissue with collagenase contained 2.8% mast cells. Since information on the numbers of total lung cells and mast cells was not presented in this report, it is difficult to compare these data with ours. However, as discussed above collagenase digestion usually resulted in a much lower recovery of lung cells than the protease perfusion method used in our study. It is possible that collagenase treatment results in good recoveries of airway mast cells but poor recoveries of other types of lung cells, thus increasing the apparent percentage of mast cells in crude lung cell preparations.

It should be noted that rat mast cells may release considerably more cysteinyl-LTs per cell than other types of cells. Thus, even a relatively small number of mast cells could contribute significantly to the formation of cysteinyl-LTs in the lung. There are no reports in the literature on cLT synthesis by rat lung mast cells.

In addition to eosinophils, neutrophils, macrophages and mast cells, airway epithelial cells may be an important site for eicosanoid synthesis in the lung since they possess cyclooxygenase, 5-lipoxygenase and 15-lipoxygenase activities (2). Clara cells, type II alveolar epithelial cells, and Kurloff cells also possess cyclooxygenase, but not 5- or 15-lipoxygenase activity (364). Consistent with the finding that human lymphocytes do not express 5-lipoxygenase activity under normal conditions (374), we could not detect any 5-lipoxygenase products when purified BN rat lymphocytes were incubated with A23187. It should be noted although lymphocytes display little or no 5-lipoxygenase activity, most of them possess LTA_4 hydrolase activity and can thus convert LTA_4 derived from myeloid cells into LTB_4 (364). Transcellular metabolism of LTA_4 could therefore have contributed to the synthesis of LTB_4 by our preparations of rat lung cells. However, the contribution of this phenomenon is difficult to assess under the conditions we used, since we did not evaluate the numbers of lymphocytes in our preparations.

Transcellular metabolism may also be important for the formation of cysteinyl-LTs and could be related to how close the participating cells are to one another in the intact lungs. These anatomical relationships would not be present in the suspended cells and LTC_4 synthesis due to these local associations between cells would not be seen with lung cell suspensions.

4.5. EFFECTS OF LEUKOCYTE DEPLETION ON CELLULAR INFILTRATION AND EICOSANOID SYNTHESIS IN BN RAT LUNGS AFTER ALLERGEN CHALLENGE

Allergen challenge of BN rats resulted in significant increases in both the number of pulmonary neutrophils and the ability of suspensions of lung cells to synthesize LTB_4 6 h after challenge. We therefore evaluated the role of infiltrating neutrophils in the production of leukotrienes by challenging PMNL-depleted BN rats with OVA..

OVA inhalation induced marked neutrophilia in peripheral blood (Fig. 30) and significant influx of neutrophils into airways and lungs 6 h after challenge (Figs. 18 and 31). These changes in neutrophil numbers in response to allergen challenge were significantly inhibited by pretreatment of the rats with rabbit anti-rat PMNL serum (Fig. 30). Although lung cells from PMNL-depleted rats appeared to synthesize less 12-HETE and 12-HHTrE than those from controls rats, there appeared to be no significant differences in leukotriene production among rats pretreated with anti-PMNL-serum, non-immune serum and saline. However, lung cells from one of the rats pretreated with anti-PMNL serum had extremely high 5-lipoxygenase activity and highest numbers of macrophages and neutrophils. When this outlier is removed, the lung cells from rats pretreated with anti-PMNL serum synthesized significantly less LTB_4 than those from rats pretreated with either non-immue serum or saline. Excluding the outlier, the synthesis of cysteinyl-LTs and 5-HETE were also lower in the anti-PMNL serum-treated rats than in the saline-treated rats. In addition, if the value for the outlier is excluded, there is a highly significant positive correlation between the percentage of neutrophils in lung cell

suspensions and the production of LTB_4, cysteinyl-LTs and 5-HETE by lung cells (Table 11). This is consistent with our prior observations, that the numbers of neutrophils in the lungs 6h after OVA challenge were positively correlated with the amounts of LTB_4 formed by lung cells (Fig. 25). Therefore, infiltrating neutrophils may make significant contribution to the formation of 5-lipoxygenase products, in particular LTB_4, in the lung after allergen challenge.

Since rat neutrophils produce little or no cysteinyl-LTs and 12-HETE (Fig. 28), our findings also raise the possibility that neutrophils contributed indirectly to the synthesis of cysteinyl-LTs and 12-HETE by the lung. In the case of cysteinyl-LTs, this could be due to release of LTA_4 by neutrophils followed by metabolism of this intermediate to cysteinyl-LTs by other cells by the mechanism of transcellular metabolism (106). Alternatively, in the case of 12-HETE, the neutrophil-derived arachidonic acid could be converted to 12-HETE by other cells.

Rat neutrophils can also produce 12-HHTrE since they posses COX and TXA_2 synthase (52,363). Consistent with this, the neutrophil-depleted lung cells synthesized smaller amounts of 12-HHTrE than controls. In another study, pretreatment of rats with anti-PMNL serum was shown to result in similar decreases in the production of TXB_2 and LTB_4 as well as 12-HETE by glomeruli in a model of antibody-induced mesangial cell injury (348). However, there were no data in the discussed study to indicate which types of leukocytes were depleted. Another possible explanation for the reduced synthesis of 12-HHTrE is that the anti-PMNL serum used in our study could also affect cells other than neutrophils.

Although neutrophil accumulation in BAL fluid was associated with airway hyperresponsiveness (180), it is very difficult to address the relative contribution of neutrophils to altered airway function. In the last few years, efforts have been made to investigate the role of neutrophils in airway responses to allergen challenge by depleting neutrophils with cytotoxic drugs or anti-neutrophil serum. In rabbits, depletion of polymorphonuclear leukocytes by treatment of the animals with nitrogen mustard, although not affecting the early phase response, prevented the late phase response and the development of hyperresponsiveness (186). Neutrophil depletion by hydroxyurea treatment was also shown to inhibit airway hyperresponsiveness induced by ozone exposure in dogs (185). In contrast, Hutson et al. (187) showed that neutrophil depletion by rabbit anti-guinea pig neutrophil serum did not inhibit allergen-induced late-phase asthmatic responses in conscious sensitized guinea pigs. Although all of the three studies addressed the role of neutrophils in altered airway function, they were done in different species and with different models of neutrophil depletion. It should also be noted that much of these data are descriptive and circumstantial and do not necessarily establish a cause-and-effect relationship between specific inflammatory cell type and airway response. For example, in studies in which effects are noted with depletion, the cytotoxic effects of hydroxyurea and nitrogen mustard rather than depletion itself may cause the observed changes. In addition, the specificity of these cytotoxic drugs on different type of leukocytes in animals are not well understood. In the case of the negative results, it is possible that the guinea pigs were not sufficiently depleted of neutrophils. Therefore, it seems evident that further studies are necessary to clarify the relationship between the accumulation of neutrophils

in the lungs and altered airway function. In our neutrophil depletion studies, however, we demonstrated that infiltrating neutrophils may contribute to the formation of LTB_4 and other eicosanoids in the lung. Considering the complexity of the possible interactions between different types of lung cells via mediators and cytokines and the chronic nature of asthma, it is possible that neutrophils may play an active role in airway inflammation and hyperresponsiveness.

4.6. POSSIBLE ROLE OF NEUTROPHILS IN ASTHMA

The role of neutrophils in allergic asthma has been questioned for a long time. There is ample evidence in the literature in support of an important role for these cells in allergic asthma. Physiologically, human neutrophils are equipped to play such a role. These cells release large amounts of proinflammatory LTB_4 and 5-HETE (11), destructive proteases (375), and regulatory chemokines including IL-8, MIP-1α and MIP-1β (153) *in vitro* in response to a variety of stimuli. Neutrophils are usually the first line of defense against inflammatory stimuli *in vivo* and the activation of neutrophils is associated with tissue injury in many inflammatory conditions (375). A number of studies indicate that peripheral blood neutrophils become activated after allergen- and exercise-induced asthmatic responses (172). Allergen challenge of asthmatic patients induces neutrophil infiltration in airways (179). Neutrophils also persist in bronchial secretions in chronic asthma (127) and in the airways of patients who died suddenly of acute asthma (122). In addition, neutrophils predominate more frequently than eosinophils as the major inflammatory cell in sputum from patients with asthmna in acute exacerbation (376).

Neutrophils from asthmatic patients (357) and allergic sheep (362) also synthesized more LTB_4 than neutrophils from normal controls.

Among neutrophil-derived mediators, LTB_4 is a very important proinflammatory eicosanoid. It has potent chemotactic effects on human neutrophils (182) and may induce neutrophil adhesion to endothelial cell monolayers (227). LTB_4 also stimulates the synthesis and release of the chemokine IL-8 by neutrophils (377), promoting neutrophil-mediated inflammatory reactions by an autocrine mechanism. Furthermore, neutrophil-derived chemokines such as IL-8, MIP-1α and MIP-1β can also initiate the activation of monocytes, lymphocytes and eosinophils (153,378). Increased levels of LTB_4 were found in BAL fluid (130,276) and arterial blood of asthmatic pateints (379). Increased levels of IL-8 were also shown in BAL fluid of patients with asthma (376,380,381). In addition, there was a positive correlation between the levels of IL-8 and the percentages of neutrophils in BAL of asthmatic patients (376,380). It was recently reported that the LTB_4 antagonist CP-105,696 inhibited not only the increases of IL-6 and IL-8 in BAL but also the airway responsiveness to methacholine which was observed after multiple antigen challenge in a primate experimental model of asthma (382). Therefore, infiltrating neutrophils capable of producing LTB_4 and chemokines may not only participate directly but also amplify or perpetuate the acute inflammatory response by recruiting additional PMNL into the lungs following allergen challenge.

However, there are also data which do not support a direct involvement of neutrophils in asthma. For example, a high percentage of neutrophils were also found in unstimulated healthy subjects or saline challenged-control groups (127,383). Nevertheless,

the neutrophils in healthy subjects and saline-challenged controls may be different from those present in asthmatic patients. As discussed in Section 4.3., neutrophils from asthmatic patients are activated for the production of LTB_4 probably due to the priming by cytokines such as GM-CSF (358-361). Therefore, the presence of neutrophils in healthy subjects or saline-challenged controls does not necessarily argue against a role for neutrophils in asthma.

In the current study, we showed that ovalbumin challenge results in increased neutrophil infiltration and LTB_4 synthesis by lung cells 6 h after challenge. The increases in both neutrophil infiltration and LTB_4 production appeared to be inhibited by neutrophil depletion. We also observed an eosinophilic influx into the lungs 24 h after OVA challenge. Since instillation of exogenous LTB_4 induced increased numbers of eosinophils in airways and lung tissue, the neutrophil infiltration and associated LTB_4 formation by lung cells 6 h after allergen challenge may be responsible for the late phase eosinophilic influx in the lung 24 h after challenge. It should be noted that the rabbit anti-rat PMNL serum may be useful for exploring this temporal relationship if it selectively depletes neutrophils in BN rats. However, we have not attempted to examine this possibility in depletion experiments because the serum itself may also deplete eosinophils. Since the lung consists of leukocytes and resident lung cells, the early infiltration of neutrophils into the lung following allergen challenge and the action of neutrophil-derived LTB_4 on other lung cells and airway tissues may be an integrated part of the airway inflammatory cascade. Therefore, our results support the notion that neutrophils may play an important role in airway hyperresponsiveness and asthma, at least in the BN rat..

4.7. EFFECTS OF LTB_4 AND 5-OXO-ETE ON PULMONARY EOSINOPHIL INFILTRATION IN BN RATS

It was reported that inhalation of LTB_4 induced increased levels of eosinophils in BAL from BN rats (294,349). Two structurally unrelated LTB_4 antagonists, U-75302 and Ly255283, not only completely blocked LTB_4-induced airway eosinophilia but also significantly inhibited allergen-induced eosinophilia in BAL of both BN rats and guinea pigs (292,294,349). This property of these two compounds is shared by the leukotriene synthesis inhibitor U-66858 and several analogs of U-75302 but not by the cyclooxygenase inhibitor, indomethacin, suggesting a role for LTB_4 in antigen-induced pulmonary eosinophilia in BN rats and guinea pigs (349). Our results further extend these findings and provide evidence for the contribution of neutrophils to the formation of LTB_4 in the lung which may be at least partially responsible for the subsequent eosinophilic infiltration after allergen challenge.

5-Oxo-ETE is a newly discovered potent chemotactic agent for human neutrophils and eosinophils (73,183). It was shown to be synthesized by human PMNL in the presence of PMA and arachidonic acid (384). 5-Oxo-ETE may be more important as an eosinophil chemoattractant in human asthma, since human eosinophils are not very responsive to LTB_4. In this study, we show that instillation of this compound into the airways of BN rats induced increased numbers of eosinophils in lung tissue (Fig. 38). Although isolated lung cells produced very small amounts of 5-oxo-ETE, it is unclear if this compound is produced in vivo by the lung after allergen challenge in the rat. If so, 5-oxo-ETE may also play an active role in pulmonary inflammation in this species.

5. SUMMARY AND CONCLUSIONS

Allergen challenge of BN rats results in increased excretion of cysteinyl-LTs in bile. It is unclear whether this reflects an increased capacity of lung cells to synthesize 5-lipoxygenase products, and if so, which cells are primarily responsible for this phenomenon. In the current study, we have examined the effects of allergen challenge on cellular infiltration and the capacity of isolated lung cells to synthesize leukotrienes in BN rats. The relationships between different types of inflammatory cells and eicosanoid synthesis were also investigated to gain a better understanding of the contribution of individual cell types to eicosanoid formation in the lung.

We developed improved HPLC methods for the analysis of complex mixtures of eicosanoids by HPLC using binary gradients containing trifluoroacetic acid (TFA), which allows considerable manipulation of the retention times of the cysteinyl-leukotrienes LTC_4, LTD_4, and LTE_4 relative to those of other eicosanoids. With a gradient between 0.003% and 0.005% TFA and a 4.6 mm ID column of Spherisorb ODS-2, cysteinyl-LTs are very well resolved from one another and are separated as a group with retention times longer than those of all other major eicosanoids. These conditions can be used for the analysis of prostaglandin B_2 (PGB_2), LTB_4, HETEs, and cysteinyl-LTs in only 30 min. Slightly longer analysis times must be used for the separation of more polar eicosanoids such as hydroxy metabolites of LTB_4 and lipoxins. We have also developed methods for the analysis of eicosanoids using a midbore (3.2 mm ID) column containing Spherisorb ODS-2, which improves sensitivity and reduces solvent consumption. In this case higher

concentrations (0.04 to 0.05%) of TFA are required, resulting in retention times for cysteinyl-LTs between those of the cyclooxygenase product 12-HHTrE and the HETEs. This approach permits analysis of PGB_2, LTB_4, HETEs, 12-HHTrE, and cysteinyl-LTs in only 20 min. Samples which also contain hydroxy-LTB_4 and lipoxins can be analyzed in 40 min. The above techniques are highly reproducible and give baselines which are free of interfering peaks.

Ovalbumin challenge of sensitized BN rats resulted in a significant influx of neutrophils into the lungs and a significant increase in the synthesis of 5-lipoxygenase products, in particular LTB_4, by lung cells after 6 h. There was a positive correlation between the percentages of neutrophils in unfractionated lung cells and the amounts of LTB_4 produced by these cells. OVA challenge had little or no effect on the production of cysteinyl-LTs and 12-HHTrE by lung cells when compared with saline challenge. There was a significant increase in the infiltration of eosinophils into the lungs 24 h after OVA challenge but no increase in the production of cysteinyl-LTs by lung cells at this time, suggesting that eosinophils from BN rats are unlikely to be the major site for the production of these substances. This was confirmed in experiments with partially purified eosinophils obtained from Sephadex-treated rats. In contrast, cysteinyl-LTs were synthesized in appreciable amounts by alveolar macrophages from BN rats.

Pretreatment of sensitized BN rats with rabbit anti-rat PMNL serum induced significant neutropenia and abolished the influx of neutrophils into the airways and lungs after ovalbumin challenge. There was a positive correlation between the numbers of neutrophils in the lung and LTB_4 production by lung cells. We have also shown that

macrophage numbers were positively correlated with cysteinyl-LT production by lung cells. Instillation of exogenous LTB_4 and 5-oxo-ETE induced infiltration of eosinophils into the lungs and possibly the airways. This would be consistent with the concept that the increased LTB_4 production by infiltrating neutrophils 6 h after ovalbumin challenge may be responsible for the subsequent infiltration of eosinophils.

We conclude that allergen challenge of sensitized BN rats results in an increased capacity of lung cells to synthesize 5-lipoxygenase products, in particular LTB_4. In spite of the significant infiltration of eosinophils into the lung 24 h after challenge, the capacity of lung cells to synthesize cysteinyl-LTs was not altered. The production of LTB_4 was positively correlated with neutrophil numbers in the lung whereas cLT formation was associated with alveolar macrophages but not eosinophils. Therefore, macrophages may be an important site for the synthesis of cysteinyl-LTs in BN rats. The increased LTB_4 production 6 h after ovalbumin challenge and the property of this eicosanoid to induce eosinophilic influx in the lung and airways would be consistent with an important role for LTB_4 in allergen-induced eosinophil infiltration in the BN rat.

6. CLAIMS TO ORIGINAL RESEARCH

1. We have developed improved HPLC methods for the analysis of complex mixtures of leukotrienes, lipoxins, and monooxygenated metabolites of arachidonic acid.
2. We have found that ovalbumin challenge of sensitized BN rats resulted in a significant influx of neutrophils into the lungs and a significant increase in the synthesis of 5-lipoxygenase products, in particular LTB_4, by lung cells after 6 h. We originally demonstrated a positive correlation between neutrophil infiltration and LTB_4 production in the lung following allergen challenge.
3. We were the first to demonstrate that there was no correlation between the production of cysteinyl-LTs by lung cells and the numbers of pulmonary eosinophils in BN rats. This suggests that BN rat eosinophils, unlike human eosinophils, are not a major site for the production of cysteinyl-LTs in this species. We also found that alveolar macrophages from BN rats synthesize significant amounts of cysteinyl-LTs and may contribute to the formation of these eicosanoids and airway hyperresponsiveness in BN rats.
4. We detected an unidentified arachidonic acid metabolite produced by lung cells from ovalbumin-challenged BN rats. No such metabolite has been reported in asthmatic patients or animal models of asthma.
5. We found that instillation of exogenous 5-oxo-ETE and LTB_4 induced infiltration of eosinophils into the lungs. This finding suggests that 5-oxo-ETE and LTB_4 may contribute to the late eosinophilic infiltration after ovalbumin challenge.

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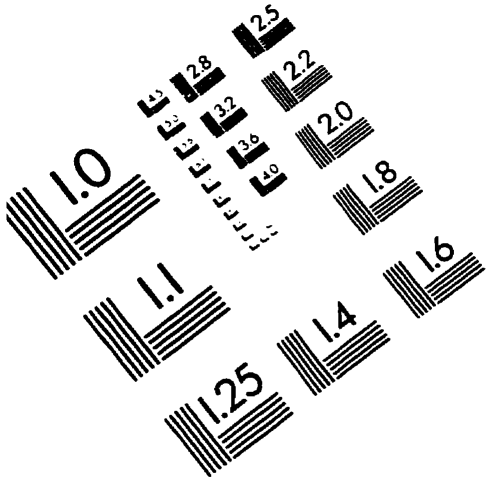
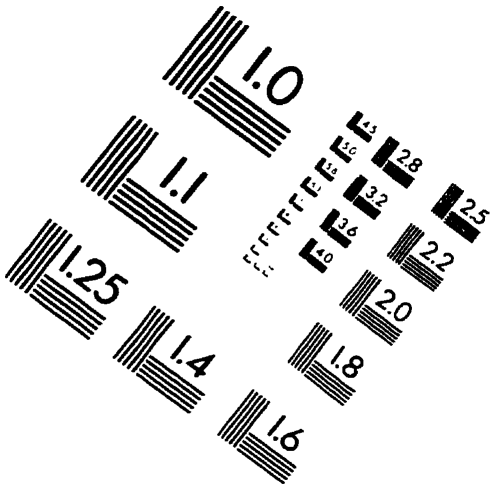
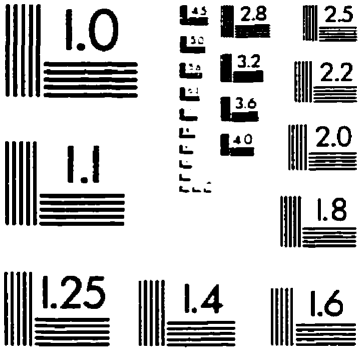
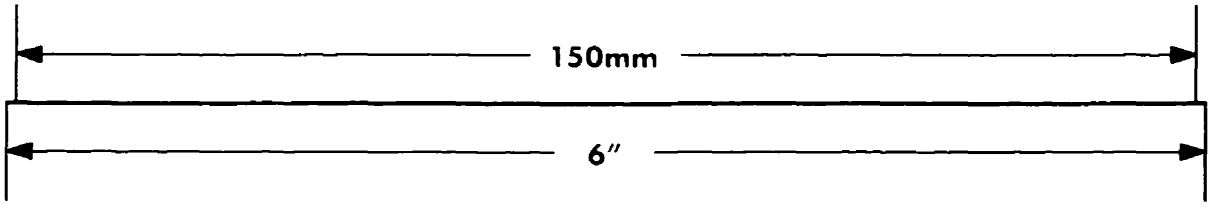
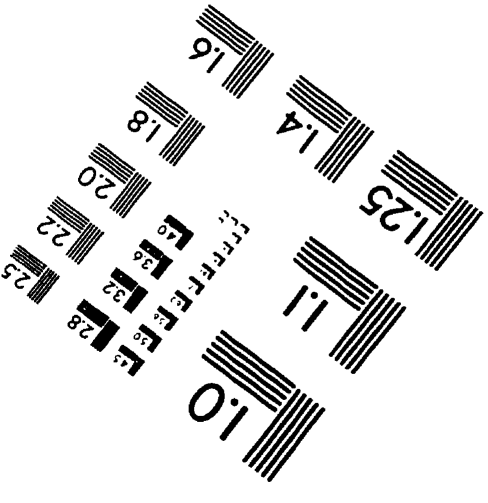
IMAGE EVALUATION
TEST TARGET (QA-3)

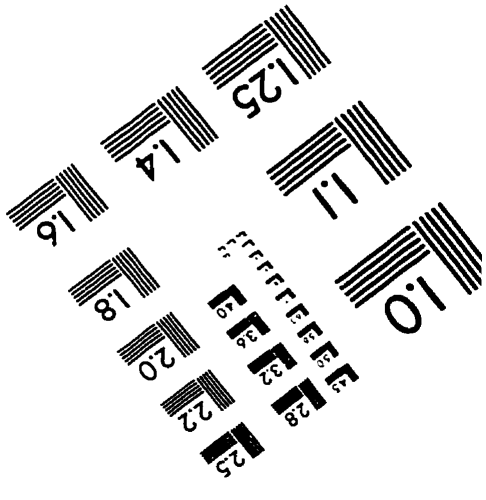












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